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"Interdisciplinary Approaches from Lab. to Clinical Breakthroughs"

June 13, 2025
Faculty of Pharmacy, Sanata Dharma University
Yogyakarta, Indonesia

Proceeding

PROCEEDING

OF THE INTERNATIONAL CONFERENCE ON SUSTAINABLE NATURAL PRODUCTS IN HEALTHCARE (ICSNPH):

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PREFACE

Recently, interest in natural products within the healthcare sector has grown significantly, both as complementary therapies and alternatives to conventional treatments. Intensive research into natural materials derived from plants, animals, and microorganisms has revealed their immense potential in creating safer and more sustainable therapeutic solutions. Natural products such as plant extracts, bioactive compounds, and natural biopolymers garner greater attention due to their effective pharmacological properties and lower risk of side effects. These advancements extend beyond laboratory settings and are beginning to be implemented in clinical practice to develop more environmentally friendly and cost-effective medications and healthcare products.

Despite this promising potential, the development of natural products faces considerable challenges. Clinical application requires a strong synergy among various disciplines—including biotechnology, pharmacy, chemistry, biology, and health sciences. Most research remains in the laboratory phase, supported by a robust scientific foundation, yet struggles to transition into drug development, clinical trials, and mass production. This process demands close collaboration between scientists, clinicians, regulators, and the pharmaceutical industry to expedite practical implementation.

The healthcare system's resilience and accessibility to healthcare services are foundational pillars of health transformation, as the Indonesian Health Law mandates. In addition to focusing on treatment, the law emphasizes accessible healthcare services, including the development of promotive and preventive health services. Within this context, Pharmacy Practice is vital in supporting healthcare accessibility through education and pharmaceutical services, prioritizing the safe and effective use of natural medicinal products. Pharmacists serve not only as a source of information for the community but also as strategic players in developing and implementing natural products in therapy.

As part of the effort to enhance healthcare quality, the role of Pharmacy Practice in developing natural medicines and providing pharmaceutical services is becoming increasingly important. Through involvement in clinical trials, formulation, and quality assurance of natural products, pharmaceutical practitioners can ensure these products meet safety and efficacy standards while educating the public about their benefits and proper use. This aligns with Law No. 17 of 2023 on Health, which underscores the importance of promotive and preventive programs to reduce disease risks and improve community quality of life.

This seminar aims to serve as a platform for innovative ideas and breakthroughs in developing natural products as part of national health independence, in line with the Indonesian Ministry of Health's roadmap. The Ministry has published the Indonesian Herbal Medicines Formulary (2016) to support research and regulation of herbal products. By synergizing research results and regulatory frameworks, we can accelerate the development of natural products toward clinical implementation.

Through this background, this international conference aims to become a forum for scientists, researchers, practitioners, and industry professionals to share the latest knowledge about sustainable natural products and their applications in healthcare. With cross-disciplinary collaboration, this conference is expected to accelerate clinical breakthroughs from basic research to clinical applications. Participants will have the opportunity to discuss various aspects of research, from the formulation of innovative natural products and clinical trials to regulatory and industrialization challenges.

Conference Committee

CHAIRPERSON REMARKS

On behalf of the organizing committee, I extend my sincere appreciation to all attendees who joined us in the International Conference on Sustainable Natural Products in Healthcare (ICSNPH) from various parts of the world, both in person and virtually. This conference was held by the Faculty of Pharmacy, Sanata Dharma University.

The theme of ICSNPH, **"Interdisciplinary Approaches from Lab. to Clinical Breakthroughs,"** underscored the crucial need for collaboration across disciplines in translating research on natural products into practical health solutions. This theme served as a call to action for researchers, clinicians, and practitioners to work together—merging insights from traditional knowledge, laboratory investigations, and clinical applications—to address the complex health challenges of our time.

ICSNPH was conceived as a platform where knowledge meets action. From traditional remedies to advanced biomedical innovations, the conference explored how natural products can shape the future of healthcare in ways that are **scientifically rigorous, ethically sound, and environmentally sustainable**. In the face of escalating global health and environmental concerns, we emphasized the importance of developing natural product-based solutions that are inclusive, safe, and resilient.

The conference featured a dynamic and diverse program, including **100 abstracts**—comprising oral presentations and poster sessions—submitted by researchers from various institutions and fields. Among them, there are 20 titles that are submitted for this proceeding. These contributions reflected the interest of current research in pharmaceutical sciences, biomedical sciences, clinical pharmacology and public health, social behavioral pharmacy, and others.

We were honored to host distinguished keynote and invited speakers who generously shared their expertise and insights. Their participation, along with the valuable contributions of our presenters, reviewers, session chairs, and moderators, ensured the scientific richness and success of the conference. I would like to extend my deepest gratitude to the organizing committee and all volunteers who worked tirelessly to bring this event to success. We hope the discussions and connections fostered during the event will continue to influence research, practice, and policy for years to come, advancing the role of natural products in creating a healthier and more sustainable world.

Thank you once again to all who made ICSNPH a success.

Agustina Setiawati, Ph.D.

Chairperson, ICSNPH

DEAN REMARKS

Greetings to all,

It is with great pleasure and gratitude that I extend my warmest welcome to all readers of this proceeding of the International Conference on Sustainable Natural Product in Healthcare (ICSNPH), organized by the Faculty of Pharmacy, Sanata Dharma University. This publication marks a significant milestone in our ongoing commitment to fostering scientific excellence and academic collaboration. The articles compiled in this proceeding reflect the dynamic and diverse research activities in the field of pharmacy, covering essential areas such as public health and clinical sciences, pharmaceutical sciences, biomedical sciences, Social Behavioral Administrative and Health Education, Nursing, Midwifery, and Others

We believe that research is not only a cornerstone of academic growth but also a driving force behind innovations that benefit society. The findings presented here showcase the dedication of researchers to advancing pharmaceutical sciences, addressing public health challenges, and supporting evidence-based practices in healthcare.

We hope that this proceeding will serve as a valuable reference for researchers, practitioners, and students alike, inspiring further investigation, knowledge exchange, and interdisciplinary collaboration. May it also contribute meaningfully to the development of pharmacy as a science and as a profession. On behalf of the Faculty of Pharmacy, I would like to express my sincere appreciation to all authors, reviewers, editors, and organizing committee members whose hard work and commitment have made this publication possible.

Thank you, and I wish you a fruitful and inspiring reading experience.

Kind regards,

Dr. apt. Dewi Setyaningsih
Dean, Faculty of Pharmacy
Sanata Dharma University

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Effect of variations in *Spatholobus littoralis* Hassk Stem Extraction on the Sun Protection Factor (SPF) Value and Phenolic Content

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ABSTRACT

Spatholobus littoralis Hassk stem contains phenolic compounds that can protect the skin from UV exposure due to the presence of chromophore groups. The duration and ratio of simplicia: solvent affect the amount of phenolic content in the extract, so the right maceration time and ratio of simplicia: solvent will produce optimal phenolic compounds. This study aimed to see the effect of the ratio of simplicia: solvent and the duration of extraction on the total phenolic content and sunscreen activity measured in the Sun Protection Factor (SPF) value o *Spatholobus littoralis* stem extract. *Spatholobus littoralis* stem powder was extracted by the maceration method using 96% ethanol solvent with a ratio of simplicia: solvent 1:10 w/v, 1:20 w/v, 1:30 w/v, with an extraction time of 24 hours, 48 hours, and 72 hours followed by testing sunscreen activity in vitro using UV-Vis (Ultraviolet-Visible) spectrophotometry to determine the SPF value and the Folin-Ciocalteu method was used for quantitative analysis of total phenolics. Total phenolic content is expressed as the equivalent mass value of gallic acid per g of extract. The results showed that the maceration time and the ratio of the simplicia to the solvent did not significantly affect the SPF value at concentrations of 500 ppm, 1000 ppm, and 5000 ppm. On the other hand, the length of maceration time and the ratio of the simplicia to the solvent significantly affected the total phenolic content of the ethanol extract of the *Spatholobus littoralis* stem.

Keywords: Bajakah Tampala; Phenolic; *Sun Protection Factor* (SPF)

INTRODUCTION

Ultraviolet rays can be beneficial for the skin, however, excessive exposure cause skin damage, such as redness, wrinkles, and cancer. Exposure to UV rays accelerates skin damage, where UVB (290–320 nm) with high energy can cause sunburn and erythema, while UVA (320–400 nm) with lower energy causes pigmentation and premature aging (Hapsah et al., 2018). Regular use of sunscreen can help prevent skin damage or skin cancer. The effectiveness of sunscreen is measured by the Sun Protection Factor (SPF), which indicates the level of protection against UV rays, the higher the SPF, the greater the protection provided (Avianka et al., 2022) (Isfardiyana and Safitri, 2014). The SPF value ranges from 2 - 60, this number indicates how long the preparation or product is able to protect or block UV rays that cause sunburn. The higher the SPF, the better the protection provided to the skin against UV rays.

The polyphenol content in *Spatholobus littoralis* Hassk. functions as a natural sunscreen that absorbs UV rays, thus protecting the skin from damage (Mariska et al., 2022; Nursyafitri et al., 2021). *Spatholobus littoralis* plants contain phenolic compounds of 12.33 mg GAE/g. These compounds are believed to help protect the skin from exposure to ultraviolet rays (Ayuchecaria et al., 2020). Ethanol solvents have a wide range of extracting abilities, including polar and nonpolar compounds (Endarini, 2019) so that they can dissolve the compounds contained in the *Spatholobus littoralis* stem, especially phenolic compounds including flavonoids, tannins, and other aromatic compound derivatives (Dhurhania & Novianto, 2019). *Spatholobus littoralis* also has antioxidant activity that can ward off free radicals, prevent inflammation, and protect body cells because the presence of phenolic compounds as the largest natural antioxidants, can delay and inhibit oxidation reactions (Mariska et al., 2022). Phenolic compounds, with hydroxyl groups on their aromatic rings, have the potential to be antioxidants because they can donate hydrogen to stabilize free radicals.

This research was conducted using the maceration method because this method does not require heating so it can extract unstable chemical content. This method is suitable for use on both small and large scales. This maceration method can also avoid damage to compounds that are thermolabile (Theodora et al., 2019). Maceration is influenced by several factors such as time, temperature, type of solvent, particle size, and the ratio of simplicia to solvent (Chairunnisa et al., 2019). According to several studies, the extraction time greatly affects the resulting compounds; ideal compounds are produced with the right maceration time, while too short a maceration time can cause not all compounds to dissolve in the solvent used. In Amelinda's study (2018), the extraction of temulawak rhizomes using an 80% ethanol solvent with variations in maceration time yielded the most phenolic compounds at a maceration time of 24 hours. This indicates that the length of maceration time for the extract significantly affects the total phenolic content. Ethanol extract of bajakah tampala stems has the potential as an active ingredient in natural sunscreen because it contains phenolic compounds, the quality of which is influenced by the ratio of simplicia and ethanol solvent (Diachanty et al., 2017). Increasing the ratio of material to solvent can increase the average total phenolic produced. The more solvent used, the higher the ability of the solvent to dissolve the material, so that more components in the material can be extracted. In a study conducted by Handayani et al. (2016), it was shown that increasing the ratio of material to solvent, such as in soursop leaves: ethanol (1:5 w/v to 1:15 w/v), increased the total phenolic, with the best ratio of 1:15 w/v producing 15,521.27 ppm.

This study aims to determine the effect of maceration time (1 day, 2 days, 3 days) and the ratio of Spatholobus littoralis stem powder to ethanol solvent (1:10 w/v, 1:20 w/v, 1:30 w/v) on the SPF value and total phenolic content of the extract.

METHODS

1. Materials

Spatholobus littoralis stem powder (010324) obtained from Teweh Tengah sub-district, North Barito Regency, Central Kalimantan, ethanol p.a (Emsure®), ethanol 96% (Merck®), methanol p.a (Emsure®), n-butanol (Emsure®), acetic acid (Emsure®), FeCl3 (Merck®), TLC plate (Merck®), Folin-Ciocalteu reagent (Merck®), gallic acid (Merck®), aquadest, quercetin (Merck®), sodium carbonate (Na2CO3) (Merck®), and aluminum foil

2. Instrument

Oven (Memmert®), spektrofotometer UV-Vis (Shimadzu®), *shaker* (Optima®), analytical balance (Ohaus PA224C®), sieve number 60 mesh, UV detector, *chamber*, pollinator tool (Retsch 100®), stirring rod,, fume hood (Biobase Fume Hood®), *vacuum rotary evaporator* (Buchi R300®), Buchner funnel, *vortex* (Janke & Kunkel®), micropipette (Socorex®), *white tips*, sentrifuge tube, cuvette (Hellma Analytics), *stirrer*, *waterbath* (Labo-tech, Haracus®), glassware (Pyrex-Germany® dan Iwaki®).

3. Research Procedures

3.1 Plant Material Collection

The plant used is Spatholobus littoralis which originates from the lowland area in Teweh Tengah District, North Barito Regency, Central Kalimantan Province which has been determined in the medicinal garden laboratory of the Faculty of Pharmacy with a determination letter number 04/LKTO/Far.USD/X/2024. The process of simplicia to dry powder is carried out immediately after collection at the place of origin on the same day to maintain the physical and chemical quality of the material during the storage process (Sirait and Enriyani, 2021)

3.2 Extract Preparation

The extraction of *Spatholobus littoralis* stems in this study was carried out using the maceration method. *Spatholobus littoralis* stem powder weighing 30 g was macerated using 96% ethanol solvent with each volume according to the ratio of simplicia: solvent, namely 1:10 w/v; 1:20 w/v; 1:30 w/v. The maceration process was carried out with three time variations, namely 1 day, 2 days and 3 days while stirring using a shaker. The maceration was then separated from the dregs. The maceration results were then filtered using a Buchner funnel. The resulting maceration was concentrated using a rotary vacuum evaporator. The extract was evaporated using a waterbath until it became a thick extract, then the extract was dried in an oven until a constant weight was obtained. Furthermore, calculations were carried out to determine the extract yield (Ayucheria, 2020)

3.3 Qualitative Test of Phenol

The TLC plate of silica gel 60 F254 was used as the stationary phase and activated in an oven at 100°C for 60 minutes. Ethanol extracts with a ratio of 1:10 w/v, 1:20 w/v, and 1:30 w/v and a time of 1 day, 2 days, and 3 days were weighed as much as 100 mg each, then dissolved in 1 mL of ethanol p.a. As a comparison, gallic acid was weighed as much as 10 mg and dissolved in 0.5 mL of ethanol p.a. then diluted with distilled water to a volume of 100 mL, while quercetin was weighed as much as 25 mg and diluted with ethanol p.a. to a volume of 25 mL. The extract solution and reference solution were spotted on the TLC plate, then eluted using the mobile phase n-butanol:acetic acid:water (2:1:2 v/v/v). After that, the plate was observed under UV light at wavelengths of 254 nm and 365 nm, sprayed with $FeCl_3$ reagent. Deep blue or black spots indicate a positive result.

3.4 Quantitative Phenolic Test

1) Determination of Operation Time

   The intermediate gallic acid solution with a concentration of 500 ppm was taken as much as 0.4 mL, 1.2 mL, and 2 mL respectively, then put into a 10 mL measuring flask and diluted using methanol until it reached a volume of 10 mL. From this solution, a series of gallic acid solutions were produced with concentrations of 20 ppm, 60 ppm, and 100 ppm. A total of 0.5 mL of each

concentration solution was transferred into a test tube, then 5 mL of Folin- Ciocalteu reagent was added. This mixture was vortexed for 30 seconds and left for 3 minutes. Next, 4 mL of 1 M Na_2CO_3 solution was added, and the mixture was vortexed again for 30 seconds. Absorbance measurements were carried out at intervals of every 5 minutes over a time span of 0–60 minutes at the theoretical maximum absorption wavelength.

2) Determination of Maximum Absorption Wavelength

Gallic acid series solutions with concentrations of 20 ppm, 60 ppm, and 100 ppm were each pipetted as much as 0.5 mL and put into a test tube. Then 5 mL of Folin-Ciocalteu reagent was added, the mixture was vortexed, and left for 3 minutes. After that, 4 mL of 1 M Na_2CO_3 solution was added, then vortexed again for 30 seconds. The solution was left for the specified OT, then its absorbance was measured in the wavelength range of 600–800 nm.

3) Preparation of Gallic Acid Standard Curve

A series of gallic acid concentration solutions at concentrations of 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm were pipetted as much as 0.4 mL, 0.8 mL, 1.2 mL, 1.6 mL, and 2 mL, respectively, then put into a 10 mL measuring flask and methanol was added to the boundary mark. A total of 0.5 mL of each gallic acid series solution was taken, 5 mL of Folin-Ciocalteu reagent (1:10 v/v) was added, then vortexed for 30 seconds and left for 3 minutes. Next, 4 mL of 1 M Na_2CO_3 solution was added and the mixture was vortexed again for 30 seconds. The absorbance of the solution was measured using a UV-Vis spectrophotometer at a wavelength of 600–800 nm.

4) Measurement of Total Phenolic Content

Spatholobus littoralis stem extract solution with a ratio of 1:10 w/v, 1:20 w/v, 1:30 w/v and maceration time of 1 day, 2 days and 3 days was weighed as much as 50 mg each, then dripped with DSMO and heated on a waterbath until the entire extract dissolved. The liquid extract was transferred into a 10 mL measuring flask and diluted using methanol solvent. The 5000 ppm extract solution was taken as much as 1 mL and put into a 10 mL measuring flask then added solvent to the boundary mark to produce a 500 ppm concentration extract solution. The 500 ppm concentration extract solution was pipetted as much as 0.5 mL and added 5 mL of Folin Ciocalteu reagent (1:10 v/v) then vortexed for 30 seconds and left for 3 minutes. Then 4 mL of 1 M Na_2CO_3 was added and stirred for 30 seconds. Absorbance was read using a UV-Vis spectrophotometer at the maximum wavelength and operating time obtained.

4. Measurement of Sunscreen Activity

A total of 50 mg of ethanol extract of *Spatholobus littoralis* stem with a ratio of 1:10 w/v; 1:20 w/v; 1:30 w/v maceration time of 1 day; 2 days; and 3 days was weighed and then dissolved in DMSO, then heated until dissolved. The dissolved extract was then transferred into a 10 mL measuring flask and diluted with methanol solvent to the limit mark. An extract solution with a concentration of 5000 ppm was made and diluted again to obtain a solution with a concentration of 1000 ppm and 500 ppm. Measurement of sunscreen activity was carried out using a UV-Vis spectrophotometer at a wavelength of 290-320 nm after being calibrated with a solvent as a blank. The SPF value was calculated using the Mansur equation, using sample absorbance data and appropriate correction factors. (Dutra, 2004; Sari, 2020).

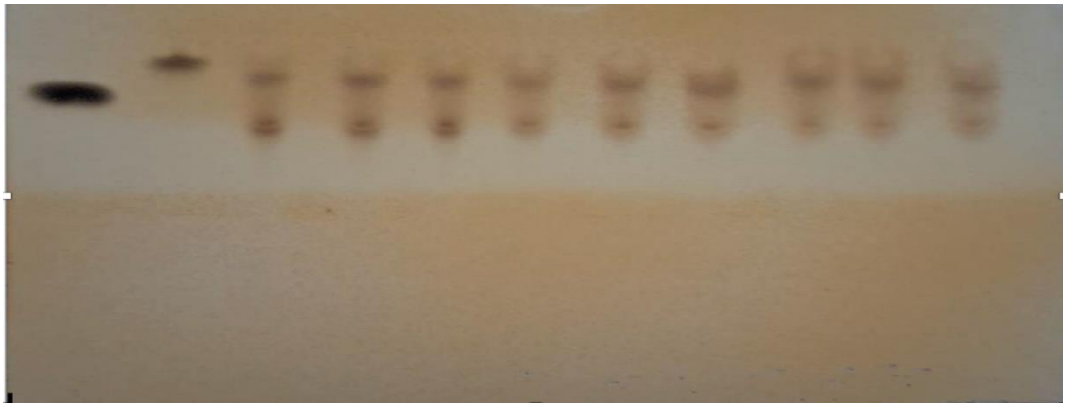
RESULTS AND DISCUSSION

1. *Spatholobus littoralis* Stem Extraction

The extraction of *Spatholobus littoralis* stems in this study was carried out using the maceration method with 96% ethanol solvent because it can dissolve phenol compounds. Based on the results of the study, the highest yield was achieved at a ratio of 1:20 for a maceration time of 2 days, the same as a ratio of 1:30 for a maceration time of 1 day, which was 2.62% w/v. The yield value is influenced by the type of solvent, extraction time, and size of the simplicia. The results of this study are not in line with the study of Amelinda (2018) who conducted maceration of Temulawak rhizomes with 80% ethanol solvent, obtained the highest yield from the 36-hour maceration time treatment and the lowest extract yield was obtained from the 18-hour maceration time treatment. The results of the study showed that the length of extract maceration did not always affect the yield results. In some plants, longer maceration times actually increased the yield results. This is due to the possibility that the sample was in contact with the solvent for longer, which resulted in an increase in the amount of material extracted until the right time limit. Variations in plant types and extracted metabolites may be the cause of the results of this study being different from the results of the previous studies. In addition, another factor that causes inconsistent results is the chemical nature of metabolites that can affect how efficiently the compound is extracted during long maceration times. Some compounds can degrade or lose their activity if left in the solvent for too long, reducing the final yield produced. In addition, the suitability of the chemical properties or structures between the solvent and the substance to be extracted determines the solubility of the substance in the solvent, in accordance with the principle of like dissolves like (Yasa et al., 2019). In this study, the yield value obtained was below 10%. Several factors that affect the yield value include the length of the extraction process, the type of solvent and its polarity level, and the size of the simplicia.

2. Qualitative Phenol Test

This study used Thin Layer Chromatography (TLC) to identify compounds in *Spatholobus littoralis* stem extract through component separation. Before testing, the chamber was saturated with filter paper eluted using a mobile phase of n-butanol: acetic acid: water (2:1:2 v/v/v). The 60F254 silica gel plate was activated at 100°C for 60 minutes to remove water content and activate silanol groups. Samples in the form of ethanol extracts with solvent ratios of 1:10 w/v, 1:20 w/v, and 1:30 w/v maceration times of 1 day, 2 days and 3 days, standard solutions of gallic acid and quercetin, were spotted on the TLC plate as much as 3 µL. After elution, the plate was dried then observed under UV light (254 nm and 365 nm), and sprayed with $FeCl_3$ reagent. The observation results showed the presence of blackish blue spots indicating the presence of phenolic compounds, in accordance with the reaction of $FeCl_3$ with hydroxyl groups. The Rf value is calculated from the distance of the spot to the solvent, with an ideal value between 0.2-0.8. In gallic acid, the Rf value is 0.81, while quercetin is 0.90 which exceeds the theoretical range. However, in another study, the Rf value of quercetin was obtained at 0.975 (**figure 1**). The three samples showed spots with Rf values close to gallic acid, indicating the presence of phenolic compounds, especially gallic acid (Ayu et al., 2019; Maryam et al., 2020; Islamiyati and Saputri, 2018).



A B F1 F2 F3 G1 G2 G3 H1 H2 H3

Figure 1. Chromatogram of ethanol extract of bajakah tampala stem variation of ratio simplicia: solvent detection of FCl_3/visible

Information:

A= gallic acid F1,F2,F3 = 1 day maceration 1:10 w/v , 1: 20 w/v, 1: 30 w/v

B= quercetin G1,G2,G3 = 2 day maceration 1:10 w/v , 1: 20 w/v, 1: 30 w/v

H1,H2,H3 = 3 day maceration 1:10 w/v , 1: 20 w/v, 1: 30 w/v

3. Quantitative Phenolic Test

3.1 Determination of Operating Time

Determination of operating time (OT) is carried out to ensure optimal measurement with a stable absorbance value, which indicates that the system is in a consistent and accurate condition. OT measurements are carried out to find the optimal reaction time, which is characterized by stable absorbance, this ensures that the system is in a consistent and accurate condition to maximize measurement (Rumoroy et al., 2019). This test uses gallic acid concentrations of 20 ppm, 60 ppm, and 100 ppm, which are reacted with Folin-Ciocalteu reagent and sodium carbonate. The reaction produces a color change from yellow to blue, indicating the presence of phenol (Febriyanto et al., 2021). The purpose of using this concentration variation is to determine whether the difference in concentration affects the difference in time required to achieve stability. The measurement results show that the absorbance values for all tested concentrations began to stabilize at 35 minutes. This stability of absorbance indicates that the reaction has reached equilibrium at that time. Therefore, the 35 minute is the ideal time to measure absorbance. At this minute, the change in absorbance value is very small or no longer occurs, indicating that the reaction has been completed and there are no more significant changes that affect the measurement results. Susanti et al. (2022) also found stability of absorbance after 35 minutes of incubation with Folin-Ciocalteu reagent, while Margata et al. (2024) reported the stability of absorbance of standard gallic acid solution against time after adding Folin-Ciocalteu reagent in the range of 35 to 40 minutes. Both of these studies support the results of the study which showed that the optimal operating time is 35 minutes, where the reaction reaches stability.

3.2 Determination of Maximum Absorption Wavelength

Determination of the maximum absorption wavelength (λ maximum) is carried out to determine the maximum absorption wavelength to ensure that the sample absorbance reaches the maximum value at the theoretical wavelength. This is necessary so that the reaction between gallic acid and Folin-Ciocalteu produces maximum absorbance which is then used to measure the total phenol content in the

sample (Anngela et al., 2021). Measurements at the maximum wavelength will show significant changes in absorbance at each concentration level (Suharyanto and Prima, 2020). In this study, the determination of the maximum absorption wavelength was carried out using gallic acid solutions at concentrations of 20 ppm, 60 ppm, and 100 ppm. Based on the measurement results, the maximum λ was obtained at a wavelength of 760.5 nm for the three concentrations of gallic acid, with slight variations up to 761 nm in several repetitions. Previous studies have shown that the maximum absorption wavelength of gallic acid is in the range of 760 nm, where phosphomolybdate and phosphotungstate react with phenolic compounds to produce a blue chromophore that absorbs light maximally at that wavelength (Qayyum et al. 2016). In addition, Molole et al. (2022) also found the optimum wavelength for the Folin-Ciocalteu test at 760 nm. Research by Dhurhania and Novianto (2018) and Luhurningtyas et al. (2021) also reported that the maximum wavelength of gallic acid using the Folin-Ciocalteu method was in the range of 760.5 nm. With a maximum wavelength difference tolerance of ±3 nm, the measurement results at 760.5 nm are considered valid and accepted (Indonesian Ministry of Health, 2020).

4. Gallic Acid Standard Curve

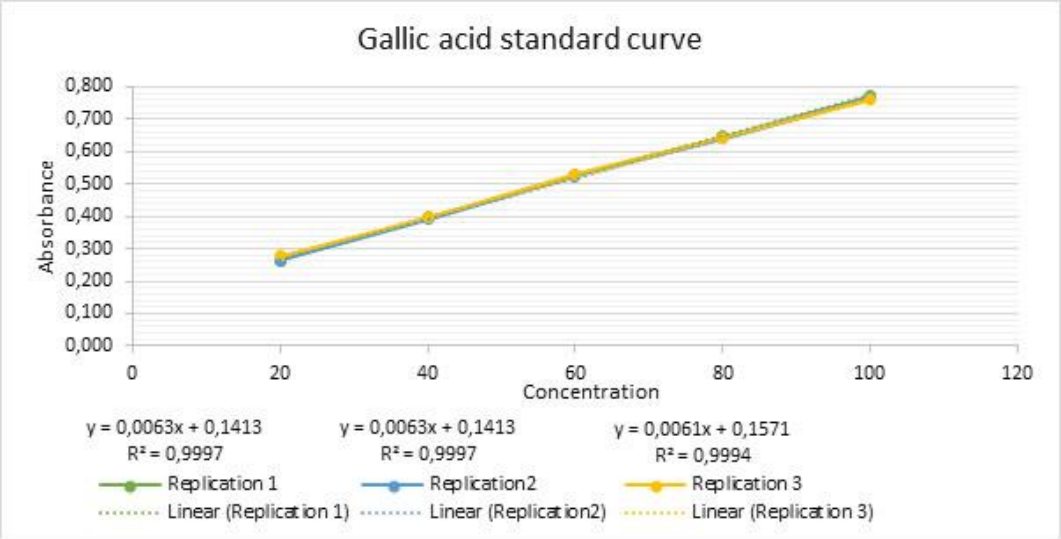


Figure 2. Gallic acid standard curve

A standard curve is a graph made by describing the relationship between absorbance values and the concentration of standard solutions that vary at maximum wavelengths. This graph shows the quantitative relationship between absorbance and compound concentration, which allows the determination of compound concentration in a sample based on the measured absorbance (Tulandi et al., 2015). The determination of the standard curve was carried out three times with concentrations of 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm to calculate the total phenolic content. For each concentration, the maximum wavelength was used to measure absorbance. **Figure 2** shows a graph of the standard curve equation of gallic acid, where all three replications show linear results with an R2 value close to 1. The equation used in the calculation of the determination of phenolic content in the ethanol extract of *Spatholobus littoralis* stems is the equation from replication 2 with the equation y = 0.0063x + 0.1413 and an R2 value of 0.9997. This is because the R2 value in replication 2 is closest to 1. This is in accordance with the standards explained by Sugito and Marliyana (2021), where an R2 value close to 1 indicates a very strong relationship between the measured variable (absorbance) and the determined variable (concentration).

4.1 Measurement of Total Phenolic Content

Measurement of total phenolic content is carried out using a standard curve made from the relationship between absorbance values and the concentration of standard gallic acid compounds. The measured absorbance values are then entered into the standard curve equation. Using this equation, the total phenolic concentration in the extract can be calculated. **Table 1** shows the results of measuring the total phenolic content of ethanol extract of *Spatholobus littoralis* stems with variations in the ratio of 1:10 w/v, 1:20 w/v, 1:30 w/v during maceration times of 1 day, 2 days and 3 days. The results showed that the maceration ratio of 1:10 w/v for 1 day produced the highest average phenolic content of 159.38 ± 28.68 mg GAE/g among all repetitions. While the maceration time ratio of 1:30 for 3 days produced an average phenolic content of 49.74 ± 7.48 mg GAE/g which was the lowest value among all repetitions, this shows that the solvent ratio has a significant effect on the total phenolic content of *Spatholobus littoralis* stem extract at the same maceration time. In 1-day maseration the ratio of 1:10 w/v produced the highest phenolic content (159.38 mg GAE/g) compared to the ratio of 1:20 w/v (104.56 mg GAE/g) and 1:30 w/v (62.76 mg GAE/g). A smaller solvent ratio is more effective because it allows phenolic compounds to be more concentrated in a smaller amount of solvent, supporting the theory of extraction effectiveness under these conditions (Rifai et al., 2018). Statistical analysis showed that the data were normally distributed ($p>0.05$) but had non-homogeneous variance ($p = 0.013$). Two-way ANOVA test showed significant differences in total phenolic content between treatment groups ($p <0.001$), with Post Hoc Bonferroni confirming significant differences between groups. It can be concluded that the simplicia: solvent ratio and maceration time have a significant effect on the total phenolic content, with a ratio of 1:10 w/v and a maceration time of 1 day being the most optimal (Listiawati et al., 2022; Purbasari et al., 2023).

Table 1: Total phenolic content of ethanol extract of *Spatholobus littoralis* stems with variations in maceration time and ratio of simplicia: solvent

Maceration time	Ratio of simplicia: solvent	Total phenolic content (mg GAE/g)
1 day	1: 10	159,38 ± 26,86
	1:20	104,56 ± 23,16
	1:30	62,76 ± 7,70
2 day	1:10	89,22 ± 10,65
	1:20	60,01 ± 6,15
	1:30	67,41 ± 2,40
3 day	1:10	101,28 ± 1,74
	1:20	58,53 ± 5,72
	1;30	49,74 ± 7,48

4.2 Measurement of Sunscreen Activity

SPF measurements were carried out at concentrations of 500 ppm, 1000 ppm, and 5000 ppm with a UV-Vis spectrophotometer at a wavelength of 290-320 nm. Sun Protection Factor (SPF) is a measure of how well a compound protects the skin from exposure to ultraviolet (UV) rays. Testing was carried out using UV-Vis spectrophotometry with an interval of 5 nm. Each extract concentration was tested in three replications with variations in the ratio of simplicia: solvent and maceration time. Absorbance measurements were carried out at wavelengths of 290 nm to 320 nm. Determination of the SPF value was carried out using the Mansur Equation which involves calculations based on the erythema effect (EE) and light intensity (I) values that have been set according to international standards. The absorbance

value resulting from the UV-Vis spectrophotometry measurement was multiplied by a correction factor (CF = 10) to obtain the SPF value. There are three levels of SPF protection, namely low sun protection (SPF value 2 to below 12), moderate sun protection (SPF value 12 to below 30), and high sun protection (SPF value ≥ 30) (Sianipar et al., 2023).

Table 2: SPF value of ethanol extract of *Spatholobus littoralis* stem with variation time maceration and ratio simplicia: solvent SPF

Ratio of simplicia: solvent	Concentration extract (ppm)	Maceration time		
		1 Day	2 Day	3 Day
1: 10	500	7.59 ± 1.21	7.30 ±0.18	9.68 ± 0.65
	1000	13.96 ± 1.20	15.22 ± 0.49	18.78 ± 1.34
	5000	38. 23 ± 0.17	36.59 ±1.12	37.21 ± 0.70
1: 20	500	7.91 ± 0.67	6.75 ± 0.49	10.51 ± 2.32
	1000	14.71 ± 0.84	13.41 ± 1.24	18.75 ± 0.68
	5000	35.58 ± 1.49	37.39 ± 1.45	37.43 ± 0.81
1: 30	500	8.03 ± 1.29	7.20 ± 0.30	7.96 ± 1.18
	1000	14.53 ± 0.84	14.39 ± 0.58	15. 65 ± 1.51
	5000	32.97 ± 5.48	35.32 ± 0.26	35.65 ± 0.58

Table 2 shows that the difference in the ratio of simplicia: solvent and maceration time produces extracts that provide different SPF values. The use of 1 day maceration time with a ratio of 1:10 w/v produces a significant SPF value along with the increase in extract concentration. At a concentration of 500 ppm, the SPF value ranged from 6.75 to 9.58 which is included in the low protection category. However, at a concentration of 5000 ppm, the SPF value jumped with a range of 32.97 to 38.23 which is included in the high protection category. A similar pattern was seen at ethanol maceration times of 2 days and 3 days with a simplicia: solvent ratio of 1:10 w/v although the average SPF value at a concentration of 5000 ppm was lower, namely 36.59 ± 1.12 and at a maceration time of 3 days produced an average SPF value of 37.21 ± 0.69. Overall, higher concentrations produce higher SPF values, and the ratio solvent 1:10 w/v maceration time 1 day produced the highest average SPF value at an extract concentration of 5000 ppm. When examined from the perspective of extract concentration, there is a consistent tendency where increasing concentration is directly proportional to increasing SPF value. At a concentration of 500 ppm, all extracts provide SPF values in the low protection category. However, at a concentration of 1000 ppm, the SPF value increases to the medium category indicating stronger UV protection. At the longest time, which is 5000 ppm, all extracts produce SPF values in the high category. The average SPF value ranges from 32.97 to 38.23 indicating very high UV protection. This shows that at high concentrations, *Spatholobus littoralis* stem extract has great potential as a sunscreen agent. The results of this study indicate that the SPF value produced from the ethanol extract of *Spatholobus littoralis* stems does not correlate with its phenolic content. Although the highest phenolic content was found in the ethanol extract with a ratio of 1:10 w/v during 1-day maceration (159.38 mg GAE/g) with the highest SPF value (38.23) at a ratio of 1:10 w/v for 1 day, the lowest phenol content at a ratio of 1:30 w/v during 3-day maceration (49.75 mg GAE/g) the SPF value (35.65) is not the lowest. The lowest SPF value was found at a ratio of 1:30 w/v during 1-day maceration. The SPF value did not show any significant difference between the three time variations. This shows that compounds that have the potential to be sunscreens are not limited to phenolic compounds alone. Previous studies have also revealed that other compounds,

such as vitamin C and vitamin E, also play a role in UV protection activity. Although all extracts positively contain phenolic compounds, it is likely that the amount and type of secondary metabolites from each extract are different, so the results obtained are also different. In addition, compounds with certain chemical structures, such as compounds with aromatic rings and chromophore groups, can increase the efficiency of UV protection. The involvement of these various types of compounds explains why even though the phenolic content is higher in certain extracts, the resulting SPF value is not always in line with that content (Fatmawati et al., 2021). The results of the analysis showed that the SPF value tended to increase with increasing concentration, supported by phenolic compounds in the extract that have photoprotective properties. Phenol is able to absorb UV-A and UV-B rays through conjugation bonds, which allows resonance to occur and increases the effectiveness of skin protection from UV radiation. The results of the statistical analysis showed that variations in maceration time and the ratio of simplicia: solvent did not have a significant effect on the SPF value of *Spatholobus littoralis* stem extract. This shows that changes in the variation of the simplicia: solvent ratio and maceration time in the range used in this study did not cause statistically significant changes in the resulting SPF value. This shows that the phenolic content in the extract contributes to the SPF value, but variations in the ratio of simplicia: solvent and maceration time do not significantly affect the SPF value results. Other studies support these findings, showing that the effectiveness of plant extracts can vary depending on the concentration and formulation, with limited influence on certain parameters. Thus, *Spatholobus littoralis* stem extract has the potential as a sunscreen ingredient, but further formulation is needed to increase its effectiveness (Rahmayani et al., 2013).

CONCLUSIONS

Based on the research results, variations in the ratio of simplicia: ethanol solvent and maceration time in the extraction of *Spatholobus littoralis* stems affect the total phenolic content produced. However, variations in the ratio of simplicia: solvent and maceration time do not significantly affect the sunscreen activity (SPF value) of *Spatholobus littoralis* stem extract.

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CONFLICT OF INTEREST

This research non conflict interest.

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Comparison of Ketamine versus Fentanyl as An Analgesic for Post-Surgical Patients: A Systematic Review

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ABSTRACT

Post-operative pain is an unpleasant experience for patients after undergoing surgery. Anesthesia practice often relies on the use of opioids before and after surgery to manage post-operative pain. However, surgery involving high doses of opioids can increase the risk of post-operative complications. One of the drugs that is often used as an alternative to treat post-surgical pain is ketamine. This review aimed to compare the analgesic effect of ketamine vs fentanyl to outcomes (pain intensity, pain presence, time to onset of analgesia, and any adverse events). We conducted a systematic review of randomized controlled trials (RCTs) studies. Literature was searched electronically through Pubmed, Google Scholar, and Scielo and we reported according to PRISMA statement guideline. A total three studies included in this systematic review. There was a significant difference between systolic and diastolic blood pressure in patients induced with ketamine and patients induced with fentanyl at 3 and 5 minutes. The average systolic and diastolic blood pressure values of patients induced with fentanyl were lower than those of patients induced with ketamine. The group of patients induced with fentanyl could achieve lower systolic and diastolic blood pressure than patients induced with ketamine. The ketamine group had a better total QoR-40 score than the fentanyl group; this is often due to the typical side effects of fentanyl. Overall, the fentanyl group had a higher Post-Sedation Symptom Inventory (PSSI) score than the ketamine group. The ketamine group had a higher pain value according to the Hannallah scale and Pediatric Anesthesia Emergence Delirium (PAED) than the fentanyl group, but it was not significantly different in pediatrics. In conclusion, ketamine provides a safe and effective alternative to opioids (fentanyl) for analgesia during surgical procedures if lower hypotensive side effects and a better level of post-operative comfort are desired in adult patients.

Keywords:
Analgesic; fentanyl; ketamine; post-surgical

INTRODUCTION

Post-operative pain remains a clinical challenge for healthcare professionals to overcome the patient's pain after undergoing surgery. Indeed, that problem still has not been addressed well yet (Nicholas et al., 2019; Paladini *et al.*, 2023). In addition, the pain after surgery requires time for recovery,

and the pain during this period induces patient discomfort and affects overall well-being. Post-operative pain is caused by tissue injury associated with the surgical procedure (S. Liu & Kelliher, 2022). However, the pain could become chronic or persistent and poorly controlled after several episodes (Gan, 2017).

Postoperative pain management is essential to ease patient conditions. Anesthesia treatment procedure often applies opioid medication before and after surgery to manage postoperative pain. However, surgery involving high doses of opioids can increase the likelihood of postoperative complications occurring. Opioid prescription needs further consideration to prescribe it appropriately due to worries about potential complications, the inability to assess patient pain, and the neglect of psychological aspects. As a result, emergency physicians have limited analgesic options (Bounes et al., 2010; Saunders *et al.,* 2010; Strang *et al.*, 2020). The Opioid-Free Anesthesia (OFA) protocol has spread worldwide. The OFA avoids opioid used during anesthesia to prevent both short-term and long-term opioid side effects, while assuring effective analgesic control and enhancing postoperative recovery (Léger et al., 2021). Fentanyl is one of the most well-known and widely used synthetic opioids for pain control. Fentanyl can cause decreased hemodynamics during induction, which is caused by bradycardia, vasodilation, and decreased sympathetic reflexes (Hontoir et al., 2016). Therefore, in the perioperative and outpatient settings, experts have explored alternative uses of non-opioid analgesics (Eidan *et al*., 2020).

One of the drugs that is often used as an alternative to treat post-surgical pain is ketamine. Ketamine is non-opioids and works as a non-competitive N-methyl-D-aspartate (NMDA) antagonist. The use of ketamine as a general anesthetic and analgesic has been going on for the past three decades. Administration of ketamine in sub-anesthetic doses (0.1–0.5 mg/kg via intravenous infusion) is one option that could replace opioids as analgesics. Ketamine provides minimal side effects, such as hallucinations, nightmares, respiratory disorders, and nausea-vomiting, without causing sedation or changes in hemodynamic and respiratory function. In short surgical procedures, ketamine has been shown to be better at maintaining hemodynamic stability with minimal side effects compared to fentanyl (Pratama *et al.*, 2020; Wiryana *et al.*, 2017). The advantages of ketamine include not suppressing cardiovascular function and reducing ventilatory depression when compared to opioids. However, the use of ketamine can also cause malaise. In addition, ketamine has a rapid onset of action and maximum effect in a relatively short time. The price of ketamine is also relatively more affordable (Silalahi *et al.*, 2014). Thus, the aim of this study as a systematic review was to determine the comparison of the effectiveness and safety of ketamine and fentanyl as an analgesic for post-surgical patients.

METHODS

1. Search Strategy and Data Resources

A search of randomized controlled trials (RCTs) was conducted electronically through PUBMED, Google Scholar, and Scielo. RCTs comparing the analgesic effect of ketamine or fentanyl and outcomes (pain intensity, pain presence, time to onset analgesia, any adverse event). The study applied standard PICO for systematic reviews. An initial search using keywords of 'Human*' AND 'Surgery' AND 'Ketamine' AND 'Fentanyl' AND 'Ketamine' to determine additional keywords and search terms. The search query then subsequently applied. The reference lists of all identified articles were manually reviewed to find any additional relevant articles. All results were displayed in Figure 1. according to PRISMA statement guideline.

2. Inclusion and Exclusion Criteria

The population of this study is patients, men and women, who were undergoing surgery and got pain relievers after surgery. All the studies that met the eligibility requirements adhered to the following criteria:

a) Compared Fentanyl IV and Ketamine IV;
b) Observational studies;
c) Research on human patients;
d) Outcome therapy in post-operative patients;
e) Full-text publications were available.

Studies were excluded based on the following criteria:

a) Irrelevant Data; and
b) Not a comparator of interest;
c) Preterm infants patient.

3. Data collection and analysis

Data were manually extracted according to study design, the intravenous route, full-text avaibility, and results regarding to the post-operative outcome. The three reviewer extracted and rechecked the data. Quality assessment has done for all included studies. Data were presented in detailed, including study design, sample, and results for each study. The results of a difference of fentanyl vs ketamine was reported in terms of mean±SD or median (range) and p-value for statistical analysis. Endpoint of this review was the effectiveness of analgesic, which could be measured through blood pressure, heart rate, and pain severity dimensions. Later, we discussed pain severity dimension as a qualitative response of pain.

RESULTS AND DISCUSSION

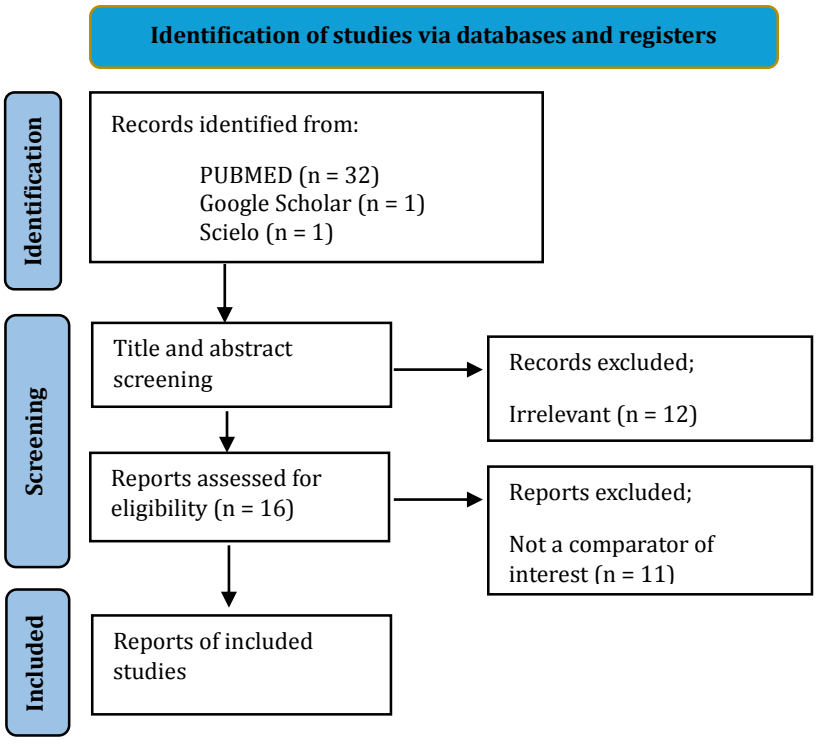


Figure 1. PRISMA flow diagram for preferred reporting studies

A total of 34 studies was screening and only 3 studies has been included in this review after removal the irrelevant studies, unavailable full text articles, no comparator medicines, and not an intravenous medicine. The process used to identify the eligible study was presents in **Figure 1.**

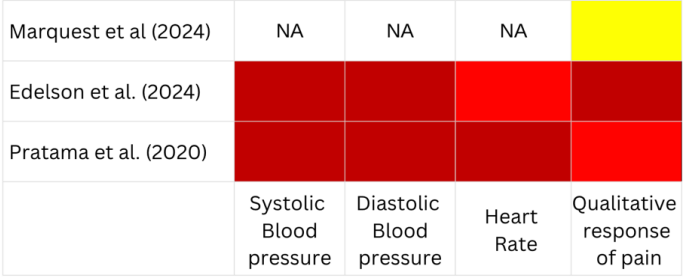
Author(s) (year)	Random sequence generation	Allocation concealment	Binding of participant and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias	Quality of Study
Pratama et al. (2020)	(+)	(+)	(?)	(?)	(+)	(+)	(?)	Fair
Edelson et al. (2024)	(+)	(+)	(-)	(?)	(+)	(+)	(?)	Fair
Marques et al. (2024)	(+)	(+)	(+)	(+)	(-)	(+)	(?)	Good

(+) Low risk of bias
(-) High risk of bias
(?) Unclear risk of bias

Figure 2. Risk of bias of study

Figure 2 displayed study quality assessment. Eligible studies were evaluated for quality using a standardised quality assessment tool, the Cochrane bias assessment tool. Quality assessment using this tool was carried out independently by three reviewers (IPDNH, LWTP, BNS), with any disagreements resolved through discussion. Three reviewers appraised the quality assessment independently. Any discrepancies were resolved through discussion between reviewers. Studies were considered to be of good quality if all criteria indicated a low risk of bias as determined by the assessor, fair if the study had one high bias risk or two uncertain bias criteria, and poor if two or more criteria had high or uncertain bias risks.

Because the previous studies did not measure the same outcome parameters, then we displayed the results for each study. **Table 1.** presents the study characteristics and the specific outcomes. However, we analyzed further the outcomes based on their significant results (**Figure 3.**). We divided them into four categories, including systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and qualitative response of pain. Since adjusting to the Edelson et al. (2024) study, the p value of SBP, DBP, and HR in Pratama et al. (2020) was chosen at 5 minutes. The QoR-40 (Quality of Recovery-40) response, PSSI (Post-Sedation Symptom Inventory) Questionnaire, Pain values according to the Hannallah scale, and PAED (Pediatric Anesthesia Emergence Delirium) were classified as qualitative response of pain.



NA: not available

Figure 3. Summary of measured outcome in included studies

Table 1: A Study Characteristics

Author (year); country	Aim/objectives, as described by the author(s)	Study design	Sample	Results			
Pratama *et al.* (2020); Indonesia	To determine the quality of recovery score after general anesthesia and changes in blood pressure and pulse with ketamine compared to fentanyl in odontectomy surgery as assessed by QoR-40.	Prospective cross-sectional study	The study involved a total of 30 patients (adults aged 18 to 65 years) who were undergoing odontectomy surgery with general anesthesia. Total of 30 patients were randomized into the two groups. Ketamine group = 15 patients Fentanyl group =15 patients	**Time**	**Ketamine**	**Fentanyl**	**P value**
				Systolic Blood Pressure (Mean±SD mmHg)			
				Baseline	119.80±9.09	120.73±9.06	0.780
				1 minute	116.60±11.56	104.00±9.94	<0.01*
				3 minutes	116.73±7.50	99.40±7.59	<0.01*
				5 minutes	118.73±8.88	103.00±6.16	<0.01*
				10 minutes	119.67±5.90	116.00±9.99	0.23
				Diastolic Blood Pressure (Mean±SD mmHg)			
				Baseline	56.33±5.19	56.33±5.47	1.000
				1 minute	52.60±5.24	49.20±5.38	0.090
				3 minutes	52.93±4.22	48.27±3.45	<0.01*
				5 minutes	54.80±4.18	48.13±4.22	<0.01*
				10 minutes	54.20±4.18	54.00±5.20	0.91
				Heart Rate (Mean±SD beats/minute)			
				Baseline	86.13±6.010	84.13±7.259	0.418
				1 minute	88.00±6.887	75.80±7.466	<0.01*
				3 minutes	83.13±6.232	71.73±7.285	<0.01*
				5 minutes	89.27±7.353	71.87±7.827	<0.01*
				10 minutes	88.47±4.611	85.87±6.967	0.24
				QoR-40 Score			
				Variabel	**Ketamine**	**Fentanyl**	**P value**
				Physical Comfort (Mean±SD)	54.60±1.99	51.47±1.55	<0.01*
				Emotional Status (Mean±SD)	41.40±1.20	40.73±1.10	0.57
				Psychological Support (Mean±SD)	32.20±1.37	31.67±1.35	0.33
				Physical Independence (Mean±SD)	21.80±1.21	21.73±1.10	0.81
				Pain (Mean±SD)	31.47±1.19	31.00±1.00	0.27
				Total Score	**181.07±5.32**	**176.60±2.59**	<0.01*
Edelson *et al* (2024); America	To compare ketamine with standard moderate sedation (SMS) in endoscopic procedures in adults by assessing the level of effectiveness and patient comfort and determining the appropriate dose of ketamine for endoscopic procedures	Prospective cross-sectional study	The study involved a total of 66 patients (adults aged 18 to 65 years) who were undergoing endoscopic procedures. Total of 66 patients were randomized into the two groups. Ketamine group = 33 patients Fentanyl group = 33 patients	**Vital Signs**	**Ketamine**	**Fentanyl**	**P value**
				Lowest systolic blood pressure (mmHg) (median (range))	129 (105-160)	115 (83-153)	<0.01*
				Lowest diastolic blood pressure (mm/Hg) (median (range))	75 (52-98)	68 (51-88)	<0.01*
				Highest heart rate (beats per minute) (median (range))	92 (58-154)	80 (46-106)	<0.01*
				PSSI (Post-Sedation Symptom Inventory) Questionnaire			
				Variabel	**Ketamine**	**Fentanyl**	**P value**
				Satisfaction with sedation delivery (median; range)	2; 1-7	2,5; 1-7	<0.01*
				Satisfaction with procedural recall (median; range)	1; 1-7	1,5; 1-7	<0.01*
				Satisfaction with sedation side effects (median; range)	1,3; 1-7	1,8; 1-7	<0.01*
				Overall average satisfaction (median; range)	**1,3; 1-7**	**1,8; 1-7**	**<0.01***
Marques *et al*, (2024); Brazil	To compare the analgesic and adverse effects of using ketamine and fentanyl in pediatric patients undergoing orthopedic procedures	Prospective cross-sectional study	The study involved a total of 50 patients (children aged between 24 and 192 months) who were undergoing orthopedic procedures. Total of 50 patients were randomized into the two groups. Ketamine group = 25 patients Fentanyl group = 25 patients	**Pain values according to the Hannallah scale (Mean±SD)**			
				Time	**Ketamine**	**Fentanyl**	**P value**
				Pain 10 minutes	1.4 ± 2.7	0.6 ± 1.5	0.30
				Pain 20 minutes	1.0 ± 2.0	0.5 ± 1.7	0.11
				Pain 30 minutes	0.9 ± 2.0	0.6 ± 1.8	0.12
				PAED (Pediatric Anesthesia Emergence Delirium) (Mean±SD)			
				PAED 10 minutes	4.0 ± 4.9	3.5 ± 5.8	0.65
				PAED 20 minutes	4.0 ± 4.6	3.5 ± 5.7	0.38
				PAED 30 minutes	3.2 ± 3.8	3 ± 5.4	0.32

2. Blood pressure and heart rate

Controlled blood pressure during anesthesia is important to ensure patient safety and prevent serious complications. High blood pressure could increase the risk of bleeding, organ damage, or cardiac stress, while low blood pressure can interfere with blood flow to essential organs such as the brain, heart, and kidneys, potentially causing permanent damage (Guarracino & Bertini, 2022; Yucepur, 2023). During anesthesia procedures, ketamine and fentanyl affect blood pressure in different ways. Therefore, careful monitoring and appropriate therapy adjustments are needed. By optimally controlling blood pressure, anesthesiologists can maintain patient hemodynamic stability, improve tissue perfusion, and minimize the risk of intraoperative and postoperative complications (Brienza *et al.*, 2019).

Only two studies that observed blood pressure and heart rate. Research conducted by Pratama *et al.* (2020) found that there was a significant difference between systolic and diastolic blood pressure in

patients induced with ketamine and patients induced with fentanyl at 3 and 5 minutes. The average systolic and diastolic blood pressure values of patients induced with fentanyl were lower than those of patients induced with ketamine. This aligns with the study carried out by Edelson *et al.* (2024), which compared the use of ketamine and fentanyl in anesthesia for endoscopic surgery. The study found that the group of patients induced with fentanyl could achieve lower systolic and diastolic blood pressure than patients induced with ketamine.

Our further analysis showed that the two studies had similar findings, with the ketamine group having a higher SBP and DBP but still at a normal value. We found that HR is more significant in Pratama *et al.* (2020), whereas ketamine produces higher HR than fentanyl. Nevertheless, once again, the HR is still in the normal range.

Fentanyl has the potential to cause hypotension during anesthesia, mainly through its depressant mechanism on the sympathetic nervous system (Krauss *et al.*, 2011). As a potent opioid, fentanyl works by binding to μ-opioid receptors in the brain and spinal cord, which reduces sympathetic tone and causes peripheral vasodilation. This decrease in systemic vascular resistance significantly lowers blood pressure, especially in patients with limited cardiovascular reserve (Baumgartner *et al.*, 2009). In addition, fentanyl induce bradycardia by enhancing parasympathetic tone through vagal effects, which in turn reduces cardiac output (Kim *et al.*, 2012). This combination of vasodilation and bradycardia can worsen hypotension, especially in patients who are hypovolemic, dehydrated, or have myocardial dysfunction. Therefore, blood pressure monitoring and prompt treatment, such as intravenous fluids or vasopressors, are essential to prevent complications from hypotension during anesthesia (Ejaimi *et al.*, 2021; Morris *et al.*, 2005). In contrast, ketamine has a lower potential for causing hypotension than fentanyl during anesthesia, due to its mechanism of action that stimulates the sympathetic nervous system. Ketamine acts as an antagonist of the NMDA (N-methyl-D-aspartate) receptor in the brain, which produces dissociative effects while stimulating the release of catecholamines such as adrenaline and noradrenaline (Juang *et al.*, 1980; Ko *et al.*, 2008; G. liang Liu *et al.*, 2020). This sympathetic stimulation increases heart rate and myocardial contractility, ultimately helping to maintain or even increase blood pressure (Goddard *et al.*, 2021). However, in patients with low catecholamine stores, such as those in shock or sepsis who are already depleted, this response may not occur, and ketamine may still cause hypotension under certain conditions (Natoli, 2021). In general, ketamine's ability to support blood pressure makes it more hemodynamically stable than fentanyl, which tends to cause hypotension through sympathetic tone depression and peripheral vasodilation. Therefore, ketamine is a safer option for patients at risk for hypotension during anesthesia.

3. Qualitative response of pain

Qualitative response of pain was including patient comfort and satisfaction after anesthesia. Those response are greatly influenced by the choice of anesthetic drugs, such as ketamine or fentanyl, used during the procedure. Ketamine, with its strong analgesic properties, not only helps reduce postoperative pain but also provides a stable sedation effect, so that patients tend to be more comfortable when entering the recovery phase (Zhang *et al.*, 2023). Meanwhile, fentanyl, as a potent opioid, is effective in controlling acute postoperative pain at a relatively low dose, thereby reducing the risk of side effects such as nausea or vomiting (Shin *et al.*, 2019). The appropriate use of ketamine or fentanyl, combined with multimodal pain management, ensures that patients can wake up from anesthesia with more calm, minimal discomfort, and without significant disorientation. This approach not only improves physical

comfort but also patient satisfaction with the quality of care received, creating a more positive postoperative experience.

A study conducted by Pratama et al. (2020) found that there was a notable difference in the QoR-40 Score between the group of patients induced with ketamine and the group of patients induced with fentanyl. The QoR-40 Score is a standardized assessment tool used to measure the quality of patient recovery after undergoing anesthesia and surgery. QoR-40 not only helps identify problems that can affect recovery, such as pain, nausea, or anxiety, but also provides important insights for the medical team to improve perioperative care (Gornall *et al*., 2013). The ketamine group had a better total QoR-40 score than the fentanyl group, this is often due to the typical side effects of fentanyl, such as nausea, vomiting, or excessive postoperative drowsiness, which can interfere with the patient's physical comfort and function during the recovery phase (Miyoshi *et al.*, 2021). In contrast, ketamine, with its unique analgesic properties and proven antidepressant effects, is able to provide good pain control while supporting the patient's emotional and psychological well-being, thus contributing to a higher QoR-40 score (Niesters *et al.*, 2014). In addition, ketamine has minimal impact on respiratory function and is often more tolerable for patients, especially in the context of rapid and comfortable recovery (Irwin *et al.*, 2023). These findings highlight the importance of selecting an anesthetic agent that is not only clinically effective but also supports the quality of recovery holistically.

Patient satisfaction assessment conducted in the study by Edelson *et al.* (2024) using the PSSI (Patient Satisfaction with Sedation Instrument) found that there was a significant difference in overall patient satisfaction levels in the ketamine-induced patient group with the fentanyl-induced patient group. The PSSI (Patient Satisfaction with Sedation Instrument) aims to evaluate patient symptoms and experiences after receiving sedation during medical procedures, such as endoscopy or surgery. The scores from this questionnaire help the medical team understand the extent to which patients experience side effects of sedation and the quality of their recovery (Revicki *et al.*, 2006). Overall, the fentanyl group had a higher PSSI score than the ketamine group. The higher PSSI score in fentanyl-induced patients compared to ketamine indicates that patients with fentanyl tend to experience more post-sedation symptoms, such as nausea, vomiting, excessive sleepiness, or dizziness (Shin *et al.*, 2019). These side effects are common because of the nature of fentanyl as an opioid that often affects the gastrointestinal system and can prolong recovery time (Khansari *et al.*, 2013). In contrast, ketamine, with its analgesic effects and mild central nervous system stimulant properties, tends to provide a better post-sedation experience, with a lower risk of side effects, such as dysphoria or pain (Niesters *et al.*, 2014). This difference highlights that although fentanyl is effective for pain control, its use may increase post-sedation discomfort, affecting the quality of patient recovery. Therefore, the choice of anesthetic agent should consider the balance between pain control and its impact on the patient's overall recovery experience.

Research related to post-sedation comfort in children was conducted by Marques *et al.* (2024) using two parameters, namely Pain values based on the Hannallah scale and Pediatric Anesthesia Emergence Delirium (PAED) assessment. The study showed that there was no significant difference in the Hannallah scale and PAED values 30 minutes after sedation, but overall the ketamine group had a higher Hannallah scale and PAED than the fentanyl group. The Hannallah Scale is an assessment tool used to measure the level of pain in children after surgery, especially in patients who have difficulty expressing their pain verbally. This scale is based on observations of the child's behavior, such as facial expressions, crying, response to stimuli, and other behaviors that indicate discomfort or pain (Royse *et al.,* 2010). Pediatric Anesthesia Emergence Delirium (PAED) is a condition in which children experience

confusion, discomfort, or restless behavior after waking up from anesthesia, especially after procedures involving general anesthesia. The PAED assessment scale is usually used to measure the severity of this delirium and assist in its management (Sudhakar Russell *et al.,* 2022). Hannallah Scale and PAED scores in patients given fentanyl tended to be lower than those given ketamine, indicating that patients receiving fentanyl experienced lower levels of post-anesthetic delirium or agitation. Fentanyl, although effective in controlling pain, has a sedative effect that can help calm patients, reducing symptoms of agitation or confusion that often occur after anesthesia (Lee *et al.*, 2019). In contrast, ketamine, although also effective as an analgesic, has a dissociative effect that can cause anxiety, confusion, or restless behavior in some patients after awakening from anesthesia (Glickman, 1995).

All included studies have observed the patient's response to pain post-operation. Even though each study applied different methods of pain response measurement, our study was analyzed according to the significance (p-value). Figure 2. showed there was an inconsistency of qualitative pain response between fentanyl vs ketamine among three studies. Therefore, the choice between fentanyl or ketamine in anesthesia should be adjusted to the clinical condition and individual patient response to minimize symptoms of pain and ensure a more comfortable recovery.

CONCLUSIONS

Ketamine provides a safe and effective alternative to fentanyl for analgesia undergoing surgical procedures if lower hypotensive side effects and a better level of post-operative comfort are desired in adult patients.

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CONFLICT OF INTEREST

There is no conflict interest(s) to declare.

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Bridging The Gap: Innovations and Challenges in Natural Product Drug Discovery

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ABSTRACT

Natural products have historically played a crucial role in drug discovery. They have led to groundbreaking therapies such as paclitaxel, artemisinin, and reserpine (Atanasov *et al.*, 2015; Nasim *et al*., 2022). However, their transition from laboratory discovery to clinical application remains a significant challenge, including structural complexity, limited scalability, and regulatory hurdles (Fernandez-Moure, 2016). This review explores innovative interdisciplinary strategies to overcome translational barriers in natural product-based drug discovery, integrating metabolomics, genomics, computational tools, and synthetic biology to accelerate drug development (Salem *et al*., 2020). Case studies from biodiversity-rich nations, Indonesia and Madagascar, illustrate how local innovations help to bridge the gap between discovery and application. In Indonesia, platforms such as the RAMES Library and the National Genome Project enable rapid metabolomic and genomic screening of native medicinal plants. In Madagascar, synthetic biology approaches applied to *Catharanthus roseus* and *Rauvolfia serpentina* illustrate how endemic biodiversity can be leveraged through virus-induced gene silencing and in vitro biosynthesis techniques. Furthermore, we discuss sustainability challenges, biotechnological advancements for scalable production, and AI-driven methodologies aimed at optimizing clinical trials design (Chopra *et al*., 2023). Regulatory challenges across different jurisdictions, including the FDA (**U.S. Food and Drug Administration)** (U.S), **EMA (European Medicines Agency)** (Europe), and **CFDA (China Food and Drug Administration)** (China), are discussed to provide a global perspective on natural product commercialization (Tresina *et al*., 2021). The review underscores the need for international collaboration, open-access data sharing, and innovative clinical trial methodologies to enhance efficiency and reduce drug development timelines. By integrating advanced interdisciplinary technologies with sustainability and ethics, the full therapeutic potential of natural products can be unlocked, transforming them into impactful, accessible medicines.

Keywords: Natural products; Drug Discovery, Clinical Translation, Indonesia, Madagascar, Therapeutics

INTRODUCTION

Many effective modern drugs originate from natural sources such as plants, marine organisms, and microorganisms (Najmi *et al*., 2022). Advances in **metabolomics** (the comprehensive analysis of small molecule metabolites in biological systems), **genomics** (the study of an organism's complete set of genes, including their interactions and influences), **computational chemistry**, and **bioinformatics** (the application of computer science to the analysis of biological data, including genomics and proteomics) have revolutionized the discovery process, allowing researchers to identify and develop novel bioactive compounds with increased efficiency (Salem *et al*., 2020). These tools facilitate the discovery of complex secondary metabolites and allow for a deeper understanding of the biosynthetic pathways that produce them.

Despite these technological breakthroughs, significant obstacles remain in translating natural product discoveries into clinically approved therapies. Structural complexity, inconsistent compound yields, and the ecological impact of harvesting rare species often hinder development. Additionally, regulatory processes are not uniformly equipped to evaluate the multifaceted nature of natural compounds, delaying approval and limiting access (Fernandez-Moure, 2016). This review explores how interdisciplinary approaches are helping to bridge these translational gaps. We examine how metabolomics, genomics, synthetic biology, and artificial intelligence are being leveraged to streamline discovery and development. Furthermore, we investigate how biodiversity-rich regions such as Indonesia and Madagascar are applying these innovations within local contexts, offering valuable case studies in sustainable bioprospecting and technological integration. Finally, we address the pressing need for ethical frameworks, international collaboration, and equitable benefit-sharing to ensure that natural product research contributes meaningfully to global health.

1. Interdisciplinary Approaches

Drug discovery involving natural products is inherently complex and increasingly reliant on interdisciplinary collaboration. As emphasized by Araújo et al., 2018, breakthroughs arise not from isolated disciplines but through synergistic efforts between chemistry, pharmacology, biotechnology, informatics, and systems biology. This integrated perspective is essential to address the intricate molecular structures, biosynthetic pathways, and pharmacological profiles typical of natural compounds.

Natural product research demands diverse scientific inputs. Chemists elucidate compound structures, pharmacologists evaluate bioactivity, and bioinformaticians manage massive datasets derived from genomic and metabolomic profiling. Synthetic biologists, in turn, engineer biosynthetic pathways for scalable production. Together, these experts contribute to a cohesive strategy that enhances efficiency and innovation.

The following subsections highlight three key areas of interdisciplinary advancement: the integration of metabolomics and genomics, the use of computational tools and artificial intelligence, and the role of synthetic biology and biotechnology in achieving sustainable drug production.

These interdisciplinary collaborations are not merely parallel contributions but dynamic interactions that drive innovation across natural product drug discovery. In Indonesia, the RAMES Library exemplifies such synergy: botanists collect plant samples, chemists perform metabolite profiling, and data engineers structure searchable databases, enabling high-throughput screening (Armas et al., 2024). Similarly, in Madagascar, the successful use of virus-induced gene silencing (VIGS) in *Catharanthus roseus* for studying alkaloid biosynthesis reflects coordinated efforts among geneticists, pharmacologists,

and synthetic biologists (Corbin et al., 2017). These integrative efforts ensure not only research efficiency but also clinical relevance and scalability, particularly crucial in biodiversity-rich regions (Atanasov et al., 2015; Fernandez-Moure, 2016).

1.1 Metabolomics and Genomics

Metabolomics and genomic sequencing play a crucial role in modern natural product discovery (Gaudêncio *et al*., 2023). Metabolomics maps the chemical makeup of plants and microbes, helping identify active compounds for drugs. Genomics complements this approach by identifying biosynthetic gene clusters (BGCs) that encode the enzymatic machinery necessary for natural product biosynthesis (Thomford *et al*., 2018). The synergy between these fields allows for the targeted exploration of bioactive molecules and their biosynthetic pathways, as demonstrated in the discovery of new antibiotic classes through genome mining of Actinobacteria (Simoben *et al*., 2023).

In an interdisciplinary context, both metabolomics and genomics have been increasingly applied in biodiverse regions such as Indonesia and Madagascar, offering valuable case studies for natural product-based drug discovery. Specifically, in Indonesia, metabolomics has been extensively utilized to analyze bioactive compounds from medicinal plants, supporting research in health, food, and pharmaceuticals. A major initiative includes the establishment of a plant metabolomic library comprising 501 samples from 296 species, using the RApid Metabolome Extraction and Storage (RAMES) technology, aimed at preserving plant diversity and facilitating rapid secondary metabolite analysis for future drug discovery(Armas *et al*., 2024). Research centers such as Universitas Nasional Jakarta (UNAS) and field sites across Java, Kalimantan, and Sumatra play key roles in these efforts.

In parallel, genomic strategies have been employed to identify genes responsible for the biosynthesis of bioactive compounds. The Indonesian National Genome Project, launched in mid-2023, is a prominent initiative focusing on sequencing local medicinal plants and microbes to accelerate bioactive compound discovery. Furthermore, genomic studies contribute to a deeper understanding of species traditionally used in **jamu** (Indonesian herbal medicine), with significant activities centered in Jakarta's RSCM hospital and BRIN laboratories in Bogor, Jakarta, and Bandung.

Meanwhile, in Madagascar, metabolomics is increasingly applied to study secondary metabolites derived from endemic fungi and plants. For instance, a study investigating **Aspergillus niger** isolated from Madagascar shrimps utilized LC-MS and NMR analyses to explore antifungal and anticancer potential (Fernand *et al*., 2017). This research was primarily conducted in coastal regions associated with shrimp farming, as well as in laboratories located in Antananarivo.

Although genomics research in Madagascar is still in an early developmental stage, significant progress is being made. A notable example is the mid-pass whole-genome sequencing of Malagasy populations, which not only provides insights into human genetic diversity but also offers opportunities to explore ethnobotanical species for future drug development (Hamid *et al*., 2024). Genomic investigations are also being initiated for endemic medicinal plants such as *Centella asiatica*, aiming to uncover genes related to pharmacological activities.

Despite infrastructure and funding limitations, these countries exemplify the power of combining chemical and genetic data to identify novel therapeutic leads. International partnerships are essential to fully capitalize on this potential.

1.2 Computational Tools and AI

Artificial intelligence (AI) and machine learning have significantly accelerated drug discovery by enabling in silico screening, drug-target interaction modeling, and structure-activity relationship (SAR) analysis (Zheng *et al.,* 2018). AI-driven models streamline drug discovery by predicting bioactivity, optimizing lead compounds, and designing novel bioactive molecules, reducing dependency on conventional trial-and-error approaches (Chunarkar-Patil *et al.,* 2024). AI-enhanced clinical trials increase efficiency by refining patient recruitment strategies, adaptive trial designs, and real-time data analysis for better decision-making (Chopra *et al.,* 2023).

In Indonesia, the country's extensive biodiversity has catalyzed numerous initiatives employing computational tools and AI to accelerate drug discovery. Researchers have conducted virtual screening studies of Indonesian herbal compounds for potential COVID-19 therapies, utilizing machine learning algorithms such as support vector machines, multilayer perceptrons, and random forests, combined with pharmacophore modeling and molecular docking techniques. This approach successfully identified compounds such as hesperidin and kaempferol derivatives as promising SARS-CoV-2 inhibitors (Kowalczyk, 2024).

In addition, the National Research and Innovation Agency (BRIN) has also launched platforms that integrate machine learning with large-scale chemical data for antiviral drug discovery. Similarly, AI has been instrumental in modernizing the development of *jamu* (traditional Indonesian herbal medicine). The integration of computational methods, including quantitative structure-activity relationship (QSAR) modeling, pharmacophore modeling, and molecular docking, has enhanced the efficacy and safety profiles of *jamu* formulations, demonstrating the value of AI in revitalizing traditional herbal drug discovery (Rustandi *et al.*, 2023).

Madagascar is in the early stages of AI implementation but shows promise. Collaborations with global institutions are developing digital repositories of endemic species and utilizing in silico tools for compound evaluation. These steps mark significant progress in transforming Madagascar's biodiversity into a bioinformatics resource for drug discovery.

1.3 Synthetic Biology and Biotechnology

Synthetic biology, which involves the design and construction of new biological parts, devices, and systems, or the redesign of existing biological systems for useful purposes, offers sustainable alternatives to traditional extraction methods. This is achieved through microbial fermentation and plant cell culture-based biosynthesis, ensuring consistent and scalable drug production (Zhang *et al*., 2016). Engineered biosynthetic pathways allow for the scalable production of complex natural products, reducing dependence on wild harvesting and mitigating ecological impacts (Atanasov *et al*., 2015). For example, microbial fermentation has been successfully employed to produce the anti-malarial drug artemisinin, providing a sustainable alternative to traditional extraction methods (Nasim *et al*., 2022). While synthetic biology focuses on creating new biological systems, biotechnology applies these systems to develop products, including medicines.

In drug development, synthetic biology enables the design of microorganisms, such as bacteria or yeast, to produce bioactive compounds that can be further developed into drugs. These microorganisms are engineered to produce substances that treat diseases, making drug production more efficient and sustainable. Rather than relying on extracting drugs from plants, which require significant land and resources, biotechnology facilitates drug production in laboratory settings using microorganisms or plant cells, greatly reducing resource consumption and waste. Moreover, synthetic biology and biotechnology

make drug production more cost-effective, as microorganisms grow quickly and require fewer resources compared to traditional methods.

Indonesia has initiated several biotechnology programs to enhance the yield and efficacy of traditional medicinal plants. For instance, *Glycyrrhiza glabra* (licorice), commonly used in traditional medicine, is being genetically modified to increase its active compounds, which may help treat diseases such as cancer and inflammation (Esmaeili *et al*., 2020). Additionally, microbial fermentation processes are being employed to produce antibiotics and anti-inflammatory drugs from microorganisms like bacteria and yeast. Bioengineering in Indonesia is also advancing, with scientists designing microorganisms to produce bioactive compounds from waste materials and agricultural residues, further promoting sustainable drug development.

In Madagascar, although the use of synthetic biology and biotechnology is still emerging, efforts are underway to explore these technologies for improving drug development while preserving natural resources. Madagascar's traditional medicine utilizes plants like *Rauvolfia serpentina* for treating hypertension. Recent biotechnological efforts have demonstrated enhanced reserpine production through in vitro regeneration systems, significantly increasing active compound yields (Mukherjee *et al.*, 2020). Additionally, Corbin *et al*., (2017) successfully employed virus-induced gene silencing (VIGS) in *R. serpentina* to investigate and modulate alkaloid biosynthesis pathways, advancing the genetic understanding of medicinal traits in this species.

Catharanthus roseus, also endemic to Madagascar, is one of the most pharmaceutically significant plants globally due to its production of over 100 monoterpenoid indole alkaloids (MIAs), including vinblastine and vincristine. These complex molecules are difficult to produce synthetically and are present at very low concentrations in the plant, presenting scalability challenges. Recent breakthroughs in phytochemical genomics and functional genomics, through consortia such as PhytoMetaSyn (Phytochemical Metabolic Synthetic Biology) (Canada) and SmartCell (Smart Cell Factory) (European Union), have enabled the identification of previously unknown steps in MIA biosynthesis using transcriptomic and metabolomic datasets (Dugé de Bernonville *et al.*, 2015). Furthermore, the discovery of serpentine synthase, a cytochrome P450 enzyme involved in the biosynthesis of β-carboline alkaloids, was achieved through an improved virus-induced gene silencing (VIGS) method in *C. roseus*, showcasing how modern tools can uncover and engineer complex biosynthetic networks (Yamamoto *et al*., 2021). These advances make *C. roseus* a compelling model for synthetic biology applications in Madagascar's biodiversity landscape.

Besides, Madagascar is engaging in international collaborations to develop microbial systems that mimic plant biosynthetic pathways, offering scalable alternatives to overharvesting. These include the exploration of eco-friendly biotechnological processes such as plant cell culture and microbial fermentation to sustainably produce natural bioactives. La Mesa *et al*. (2021) emphasize the importance of integrating socio-cultural traditions with modern biotechnological platforms to unlock the full therapeutic potential of Madagascar's biodiversity.

Together, these interdisciplinary strategies enhance both the scientific rigor and sustainability of natural product drug discovery, particularly in biodiversity-rich regions. The next section examines how these innovations are being integrated into long-term sustainability frameworks.

1.4 Case Studies: Successful Clinical Translation of Natural Products

1.4.1 Case Study 1: Artemisinin

Artemisinin, derived from the plant *Artemisia annua*, is a landmark example of natural product translation. Initially used in traditional Chinese medicine to treat fevers, its antimalarial properties were formally recognized in the 1970s. The isolation and structural elucidation of artemisinin enabled further chemical modification, resulting in derivatives such as artesunate and artemether.

One of the key success factors in its clinical development was the collaborative effort between traditional knowledge holders, synthetic chemists, and global health institutions. The World Health Organization endorsed artemisinin-based combination therapies (ACTs), which significantly reduced malaria mortality. A major innovation was the development of semi-synthetic artemisinin using engineered yeast, which addressed scalability and supply-chain limitations (Kung *et al*., 2018).

1.4.2 Case Study 2: Paclitaxel

Paclitaxel, originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*), represents a hallmark example of clinical translation for a complex natural product. Discovered through a U.S. National Cancer Institute screening program, paclitaxel exhibited a novel mechanism of action, stabilization of microtubules, which disrupted mitosis in rapidly dividing cancer cells. Despite its therapeutic potential, clinical translation was initially hampered by its low natural yield and unsustainable harvesting practices.

A critical advancement was the development of a semi-synthetic production process using precursor compounds from cultivated *Taxus* species, ensuring a scalable and more sustainable supply chain. Paclitaxel was approved by the FDA in 1992 for ovarian cancer and subsequently for breast, lung, and pancreatic cancers.

Key success factors included robust pharmacological validation, public-private collaboration, and regulatory adaptability. Furthermore, innovations in drug formulation, particularly the development of albumin-bound paclitaxel nanoparticles (Abraxane), enhanced solubility, reduced toxicity, and broadened clinical applicability. These developments helped paclitaxel become one of the most widely used chemotherapeutics globally (Sofias *et al*., 2017; Yang *et al*., 2020).

To provide a comparative overview of the innovation ecosystems in Indonesia and Madagascar, **Table 1** summarizes their respective strengths, challenges, and ongoing developments in natural product drug discovery.

Table 1: Comparative Summary of Innovation Ecosystems in Indonesia and Madagascar

Category	Indonesia	Madagascar
Biodiversity	Rich in plants and marine life across Java, Sumatra, Papua	High plant endemism, especially alkaloid-rich species
Traditional Medicine	Integrated system (e.g., *jamu*) with regulatory recognition	Widespread use, mostly undocumented
R&D Infrastructure	Moderate, improving with national genome & AI platforms (e.g., BRIN)	Limited infrastructure; depends heavily on international collaboration
Key Innovations	RAMES Library, AI-driven COVID-19 screening, synthetic biology (Glycyrrhiza)	VIGS in *Catharanthus*, microbial metabolomics from coastal fungi
Clinical Trials Capacity	Growing, but still limited	Nascent; mostly reliant on global partners

Category	Indonesia	Madagascar
Bioprospecting Ethics	Nagoya Protocol enforced; active benefit-sharing discussion	Frameworks emerging; community participation increasing
Global Collaborations	Moderate, rising in genomics and pharmacology	Strong dependency on foreign partnerships (EU, Canada)

2. Sustainability in Natural Product Drug Discovery

The increasing demand for natural products has raised concerns about overharvesting and ecosystem degradation, particularly in biodiversity-rich regions like Indonesia and Madagascar. Traditional extraction methods, often reliant on wild harvesting, pose serious ecological threats, including habitat destruction and depletion of endangered species (Suwardi and Navia, 2023)

To address these challenges, both countries are increasingly turning to alternative production strategies that reduce the environmental impact of drug discovery. In Indonesia, biotechnological methods, such as plant cell culture and microbial fermentation, are being employed to produce bioactive compounds in controlled environments. These methods not only mitigate the risks associated with overharvesting but also enable scalable production. For instance, the microbial synthesis of artemisinin provides a scalable and environmentally friendly alternative to traditional extraction from *Artemisia annua* (Nasim *et al*., 2022). Additionally, the genetic enhancement of medicinal plants, such as *Glycyrrhiza glabra*, has shown promise in increasing the yield of therapeutic compounds without extensive land use (Esmaeili *et al.*, 2020).

Similarly, Madagascar is working to integrate biotechnological solutions into its natural product research. The overharvesting of endemic plants like *Rauvolfia serpentina* has prompted a shift toward synthetic biology and tissue culture methods. Collaborative projects with international institutions aim to establish microbial fermentation systems and plant cell cultures capable of producing pharmacologically active compounds found in endangered species. These initiatives reduce the ecological footprint of drug development while preserving biodiversity (Mesa *et al*., 2021).

Microbial fermentation, semi-synthetic modifications, and engineered biosynthesis are promising alternatives to traditional drug production methods, with the potential to scale production while reducing environmental impacts (Salem *et al*., 2020). Moreover, ethical bioprospecting practices are critical for ensuring that the benefits of drug development are shared equitably with local communities, including indigenous groups, who possess valuable knowledge of medicinal plants. Benefit-sharing agreements are essential to preserve biodiversity while supporting global drug development initiatives (Pirintsos *et al.*, 2022).

Both Indonesia and Madagascar stand to benefit from international collaborations and knowledge exchange to advance sustainable drug discovery. Indonesia's biotechnology research, which focuses on bioengineering, microbial fermentation, and plant-based drug production, offers a model for other biodiversity-rich regions. Madagascar's efforts to utilize synthetic biology and eco-friendly biotechnological processes reflect a commitment to preserving its unique biodiversity while contributing to sustainable drug production.

3. Clinical Translation of Natural Products

3.1 From Lab to Clinic: The Pathway of Natural Products

To better understand how natural compounds move from discovery to clinical use, the drug development process can be summarized into six major stages, as illustrated in the **figure 1.**

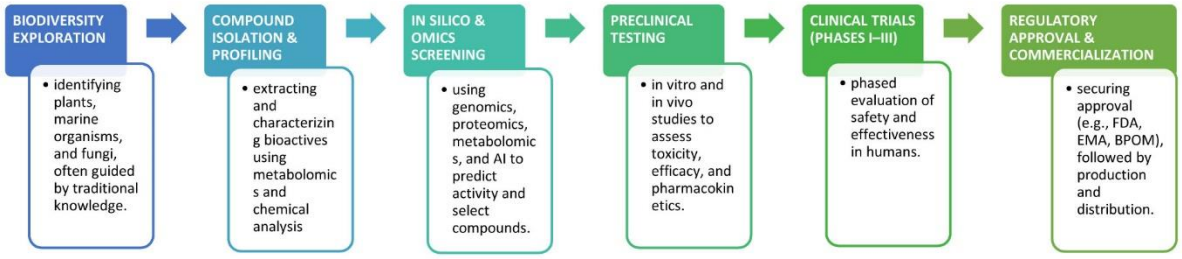

Figure 1. Simplified Pipeline for Natural Product Drug Discovery and Clinical Translation.
The pathway from natural source to approved drug includes six key stages: (1) Biodiversity exploration guided by traditional knowledge, (2) Extraction and chemical profiling of bioactive compounds, (3) Screening using in silico tools, genomics, and metabolomics, (4) Preclinical testing in vitro and in vivo, (5) Clinical trials in humans (Phases I–III), and (6) Regulatory approval and commercialization. These steps often overlap and require interdisciplinary input to overcome scalability, safety, and regulatory challenges.

However, despite this seemingly linear progression, the clinical translation of natural products faces several critical hurdles that must be addressed to ensure their successful development as therapeutic agents (Fernandez-Moure, 2016). This process spans multiple stages, starting with laboratory discovery, followed by rigorous preclinical evaluations, toxicity assessments, and multi-phase clinical trials to confirm safety and efficacy (Deore *et al*., 2019).

Despite promising preclinical data, many natural product candidates fail to progress due to issues of scalability, bioavailability, or regulatory compliance.

Both Indonesia and Madagascar offer distinct case studies in the clinical translation of natural products. In Indonesia, for example, the pharmaceutical industry has increasingly recognized the potential of natural products, especially those derived from the country's abundant plant and marine life. The path to clinical application begins with the collection of plant samples from biodiverse regions like the rainforests of Kalimantan and Papua, which are rich in medicinal plants. Indonesian researchers focus on identifying bioactive compounds through chemical analysis, followed by in vitro and in vivo testing to evaluate their pharmacological properties. For example, *Andrographis paniculata* (known as "sambiloto") has been extensively studied for its anti-inflammatory and antiviral properties. Research is advancing toward clinical applications in treating infections like dengue fever (Kaushik *et al*., 2021).

Indonesia's efforts face infrastructure limitations, regulatory hurdles, and funding constraints. Despite these obstacles, the Indonesian government has taken steps to improve the regulatory environment for natural product-based drugs. For instance, Indonesian authorities have recognized the importance of integrating traditional medicine with biotechnology and have implemented regulations supporting clinical studies focused on locally sourced natural products.

In Madagascar, the clinical translation of natural products is a rapidly growing area of interest, although it remains in the early stages compared to more industrialized countries. Madagascar's unique flora, including plants such as *Rauvolfia serpentina* and *Pachypodium lamerei*, has been used for centuries in traditional medicine. Researchers are now exploring these plants for their potential in treating diseases such as hypertension and cancer. For example, the alkaloid reserpine, derived from *Rauvolfia serpentina*,

is already used in pharmaceutical treatments for high blood pressure, and ongoing studies are focusing on enhancing its efficacy and safety through modern biotechnological methods (Mukherjee *et al*., 2020).

Successful clinical translation depends not only on robust scientific evidence but also on enabling ecosystems that include trained personnel, modern trial facilities, and regulatory clarity. Building such ecosystems in biodiverse, resource-constrained regions is essential to realizing the full potential of their natural resources.

3.2 Regulatory and Translational Challenges

The clinical translation of natural products into approved therapeutic agents faces significant regulatory and translational challenges that can impede their timely development. These vary by region and include complex regulatory frameworks, lack of standardization, and insufficient clinical trial infrastructure. Regulatory bodies such as the FDA (U.S. Food and Drug Administration) in the United States, the EMA (European Medicines Agency) in Europe, and the CFDA (China Food and Drug Administration) in China operate under distinct approval pathways, emphasizing the importance of harmonizing global standards (Thomford *et al*., 2018; Fernandez-Moure, 2016).

Regulatory Challenges

Navigating the regulatory approval process is a primary obstacle in the clinical translation of natural products. Regulatory bodies are tasked with ensuring the safety, efficacy, and quality of medicinal products, but natural products present unique challenges due to their complexity and variability. Unlike synthetic drugs, which are typically composed of a single, well-defined molecule, natural products are mixtures of various compounds that can vary based on factors such as plant species, harvest conditions, and extraction methods. This variability makes it difficult to establish consistent manufacturing processes and quality control standards necessary for regulatory approval.

In Indonesia, the regulatory process is overseen by the National Agency of Drug and Food Control (BPOM). Although BPOM has made progress in recognizing the therapeutic potential of natural products, the approval process remains time-consuming and complex, particularly for traditional medicines. The variability of natural products presents significant challenges in developing consistent and standardized manufacturing processes, which are crucial for regulatory compliance. Additionally, BPOM faces resource and infrastructure limitations, which can slow down the clinical development of herbal medicines (Sulastri, 2025).

In Madagascar, regulatory bodies are working to adapt existing frameworks to accommodate natural product-based drugs. However, the country's regulatory infrastructure is still in development, and the complexity and variability of natural products pose significant hurdles. The lack of standardized systems for evaluating these products makes it difficult to gain clinical approval, while limited capacity restricts the ability to conduct large-scale trials that meet international standards. Ongoing bioprospecting initiatives reflect both the potential and the regulatory challenges of integrating Madagascar's botanical diversity into pharmaceutical pipelines (Rasoanaivo, 2011).

Translational Challenges

Beyond regulatory barriers, the translation of natural products from the laboratory to the clinic is complicated by logistical and infrastructural challenges. Both Indonesia and Madagascar face significant limitations in infrastructure for conducting high-quality clinical trials. These

challenges include the lack of modern clinical trial facilities, insufficient trained personnel, and limited capacity to scale up production for large-scale studies. Clinical trials require robust data collection and monitoring systems, which are often lacking in developing countries (Low *et al*., 2024); (Rasoanaivo, 2011).

In Indonesia, although there is growing interest in the clinical development of natural products, challenges such as insufficient funding for research and development and limited resources for large-scale trials persist. These constraints hinder the ability to translate promising natural compounds into clinically viable treatments. However, efforts to strengthen public-private partnerships and foster collaborations with international research institutions are beginning to address some of these issues (Ferdiana *et al*., 2021).

Madagascar, as a low-resource country, faces even more pronounced translational challenges. The absence of advanced laboratories and clinical trial facilities restricts the scope of research. Additionally, there is a shortage of local expertise in drug development, making it difficult to bridge the gap between basic research and clinical applications. International collaborations and capacity-building initiatives are vital for improving Madagascar's ability to conduct translational research. These partnerships also aim to help establish the infrastructure needed for clinical trials and large-scale production of natural products for medicinal use (Olatunji *et al*., 2023).

Ethical and Sustainability Issues

Both Indonesia and Madagascar face ethical challenges regarding the use of traditional knowledge in the commercialization of natural products. Bioprospecting, the exploration of natural resources for commercially valuable compounds, must be conducted with respect for indigenous knowledge and ensure that local communities benefit from the commercialization of their resources. While both countries have made progress in developing frameworks for fair benefit-sharing agreements and sustainable bioprospecting practices, there is still much work to be done to ensure these agreements are properly implemented and that local communities are adequately compensated for their contributions (Rakotondrabe & Girard, 2021).

The sustainability of bioprospecting efforts is also a growing concern. Overharvesting of natural resources can lead to environmental degradation, threatening the very biodiversity that underpins the development of natural product-based drugs. Both Indonesia and Madagascar need stronger policies to ensure the responsible use of natural resources and the preservation of biodiversity (Neimark & Wilson, 2015; Sulistyawan *et al.*, 2019)

To realize the full promise of natural products in clinical use, countries like Indonesia and Madagascar must align innovation scientific rigor with ethical responsibility, infrastructural investment, international cooperation, and sustainable development strategies.

4. Emerging Innovations in Natural Product-Based Drug Discovery

Innovations in computational biology, high-throughput screening, and synthetic biology are dramatically reshaping the landscape of natural product drug discovery. These technologies reduce the time and cost traditionally required for identifying and optimizing drug candidates, making them especially valuable in biodiverse yet resource-limited contexts such as Indonesia and Madagascar.

4.1 Artificial Intelligence and Machine Learning

Artificial intelligence (AI) and machine learning (ML) are increasingly being utilized to predict bioactivity, optimize compound structures, and accelerate drug candidate selection. Platforms such as DeepChem apply one-shot learning and other ML techniques to forecast compound toxicity and pharmacokinetic properties, significantly expediting the early stages of drug discovery (Altae-Tran *et a*l., 2017). In parallel, AlphaFold2, developed by DeepMind, has revolutionized protein structure prediction, offering highly accurate 3D models that inform molecular docking, target validation, and structure-based drug design (Jumper *et al*., 2021).

In Indonesia, AI is applied to screen compound libraries derived from native plants such as *Andrographis paniculata* and *Curcuma longa*. These tools help predict antiviral and anti-inflammatory properties, guiding experimental validation (Erlina *et al*., 2022; Khalid *et al*., 2023). Madagascar is beginning to adopt AI technologies in partnership with international collaborators, using them to explore the bioactive potential of endemic plants like *Rauvolfia serpentina*.

4.2 High-Throughput Screening (HTS)

High-throughput screening allows researchers to quickly evaluate thousands of compounds for biological activity using automated assays. HTS has proven invaluable for natural products, which often exist as complex mixtures requiring rapid dereplication (Ayon, 2023).

In Indonesia, HTS has identified novel anti-inflammatory agents from rainforest flora and marine microorganisms (Waluyo *et al*., 2021). Madagascar is piloting HTS platforms to test alkaloid-rich extracts against diseases such as cancer and hypertension. These approaches enable faster prioritization of lead candidates for preclinical testing.

4.3 Synthetic Biology and Metabolic Engineering

Synthetic biology enables the scalable and sustainable production of complex natural products through engineered microbial platforms. This is especially useful for rare or slow-growing species that cannot support industrial demand (Zhang *et al*., 2016)

Indonesia has initiated projects to engineer microbes for producing compounds from traditional medicinal plants. For example, bioengineering strategies are being used to enhance yields of curcumin and related metabolites from *Curcuma longa* (Utomo *et al*., 2024). Madagascar is similarly exploring microbial production of alkaloids from *R. serpentina*, aiming to reduce pressure on wild populations (Rasoanaivo, 2011; Srivastava *et al.*, 2021).

4.4 Integrated Omics and Bioinformatics Tools

The integration of genomics, proteomics, and metabolomics, supported by open-access data platforms, offers a systems-level understanding of bioactive compound biosynthesis and activity. These tools facilitate target discovery, pathway elucidation, and compound optimization (Gaudêncio *et al*., 2023).

Both Indonesia and Madagascar are beginning to leverage omics-driven approaches to catalog and analyze their unique biodiversity. International collaborations are helping build capacity in data analysis and database integration, positioning these countries to contribute to global discovery networks.

To summarize how these technologies converge in sustainable drug discovery, **Figure 2** outlines key traits of an integrated, modern pipeline.

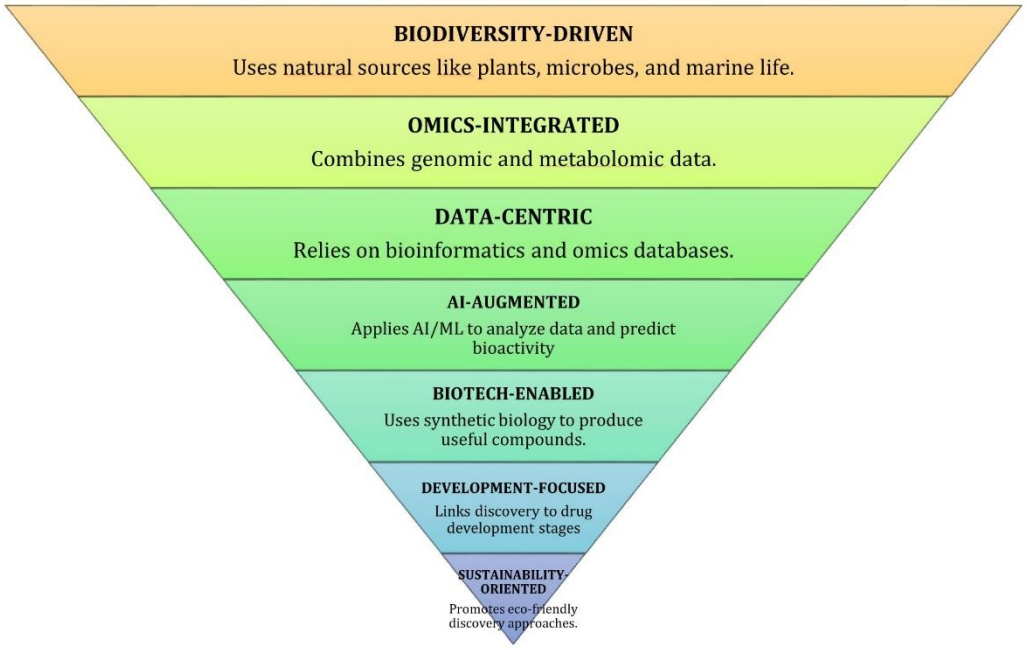

Figure 2. Integrated Innovation Pipeline for Natural Product Drug Discovery.
The process begins with biodiversity sourcing and progresses through omics integration, data analysis, AI and synthetic biology, leading to drug development and sustainability. This pipeline reflects how modern tools streamline natural product translation from discovery to clinical use.

4.5 Outlook and Implementation Challenges

While these technologies hold transformative potential, several barriers remain. Both countries face limitations in funding, infrastructure, and trained personnel. To fully benefit from emerging innovations, continued investment in capacity-building, cross-border collaboration, and digital infrastructure is essential.

Despite these challenges, the increasing adoption of AI, HTS, synthetic biology, and integrated omics is a promising step toward modernizing natural product-based drug discovery in Indonesia and Madagascar. These innovations not only accelerate the discovery pipeline but also promote more sustainable, data-driven, and globally competitive research environments (**Table 2**.).

Table 2: Key Innovations in Natural Product Drug Discovery in Indonesia and Madagascar

Innovation	Function	Indonesia Example	Madagascar Example
Artificial Intelligence & Machine Learning	Predict bioactivity, toxicity, protein structures	AI-assisted screening of phytochemicals from *Andrographis paniculata* and *Curcuma longa* (Khalid et al., 2023).	In silico prediction of anticancer activity of *Rauvolfia serpentina* alkaloids (Mukherjee et al., 2020).
High-Throughput Screening (HTS)	Rapid testing of compound libraries for activity	HTS applied to rainforest flora and marine microbes for anti-malarial compounds (Waluyo *et al*., 2021).	Pilot screening of secondary metabolites from endemic fungi using LC-MS/NMR metabolomics (Fernand et al., 2017).
Synthetic Biology & Metabolic Engineering	Microbial production of complex natural products	Engineered microbes producing curcumin analogs for enhanced activity (Utomo et al., 2024).	Microbial synthesis pathways for *R. serpentina* compounds using gene clusters (Mukherjee et al., 2020).

Innovation	Function	Indonesia Example	Madagascar Example
Integrated Omics & Bioinformatics	System-level analysis for target and pathway discovery	Establishment of Indonesia's metabolomics reference library (Nasim et al., 2022).	Collaborative genomic and metabolomic profiling of endemic species (Fernand et al., 2017).

5. Collaboration and Open-Access Data Sharing in Drug Discovery Research

Collaboration and open-access data sharing are essential to advancing natural product-based drug discovery, particularly in biodiversity-rich countries like Indonesia and Madagascar. These approaches foster interdisciplinary partnerships, improve access to global scientific resources, and enable more efficient translation from traditional knowledge to clinical innovation.

5.1 Importance of Collaborative Frameworks

Interdisciplinary collaboration, between academic institutions, government agencies, industry stakeholders, and indigenous communities, is critical to bridging the gap between discovery and application. In Indonesia, such partnerships have accelerated research on medicinal plants like *Andrographis paniculata* and *Curcuma longa*, supporting their evaluation in preclinical and clinical settings (Khalid *et al*., 2023). Government-supported initiatives have enabled joint projects involving local universities and pharmaceutical companies.

Madagascar has benefited substantially from international collaborations. Partnerships with European and North American institutions have supported botanical surveys, compound isolation, and bioprospecting ethics. Notably, joint studies have strengthened scientific validation of traditional uses, emphasizing the integration of indigenous knowledge into modern pharmacological research (Maggi, 2016; Rasoanaivo, 2011).

5.2 Role of Open-Access Data Platforms

Open-access platforms facilitate global sharing of chemical, genomic, and bioactivity data, reducing redundancy and improving reproducibility. Tools such as the Global Natural Products Social (GNPS) molecular networking platform and the Dictionary of Natural Products (DNP) provide centralized access to compound spectra, metabolic profiles, and pharmacological annotations (Atanasov *et al*., 2015).

Researchers in Indonesia increasingly use such platforms to catalog and compare bioactive compounds. In Madagascar, open-access tools help link endemic species with global databases, supporting computational screening and dereplication. These platforms are particularly important in resource-limited settings where proprietary software may be inaccessible.

5.3 Ethical Bioprospecting and Benefit Sharing

Ethical bioprospecting ensures that indigenous knowledge and biodiversity are not exploited but valued as vital assets. Both Indonesia and Madagascar have begun implementing frameworks to ensure that local communities are acknowledged and compensated for their contributions to drug discovery.

For example, bioprospecting agreements often include commitments to share royalties, provide employment, and invest in community development. These practices align with the Nagoya Protocol and ensure that conservation and equity are embedded in research and commercialization (Maggi, 2016; Rasoanaivo, 2011).

5.4 Strengthening Research Capacity through Collaborative Networks

Regional and international networks are vital for translating natural products into clinical therapies, addressing common bottlenecks, clinical trial design, GMP manufacturing, and regulatory navigation, by pooling expertise and shared infrastructure (Fernandez-Moure, 2016). For example, workshop series and data-sharing platforms under the International Cooperative Biodiversity Groups (ICBG) helped Indonesia develop advanced bioinformatics pipelines and update its national pharmacopoeia (Ferdiana *et al*., 2021; Low *et al*., 2024). In Madagascar, consortium-funded training programs and lab upgrades have enabled high-throughput screening and early-phase trial planning for endemic plant compounds (Rasoanaivo, 2011).

CONCLUSION

In an era of rising antimicrobial resistance and novel health threats, natural products remain an invaluable resource for drug discovery, offering structural diversity that synthetic libraries cannot match. Yet the path from bench to bedside is fraught with challenges, from molecular complexity and regulatory constraints to sustainability and scalability.

This review demonstrates how interdisciplinary strategies, spanning metabolomics, genomics, synthetic biology, AI, and computational modeling are closing these translational gaps. Biodiversity-rich nations like Indonesia and Madagascar illustrate what is possible: Indonesia's integrated metabolomics and genomic pipelines have already produced promising antiviral leads, while Madagascar's consortium-backed alkaloid screens have doubled screening success rates.

Key innovations such as high-throughput screening, microbial biosynthesis, and digital collaboration platforms are revolutionizing discovery, and open-access databases coupled with ethical bioprospecting ensure transparency and fair benefit-sharing. Crucially, sustained capacity-building and forward-looking policy reforms are the foundation for a robust, equitable natural-product ecosystem.

To realize the full promise of nature's pharmacopeia, several key actions should be prioritized. These include investing in regional omics and synthetic biology platforms to enhance local discovery and scale-up capabilities; harmonizing regulatory pathways across jurisdictions to streamline the approval of natural product-based therapeutics; establishing regional innovation hubs in biodiversity-rich areas to accelerate the translation of discoveries into clinical applications; and ensuring fair benefit-sharing and meaningful community engagement through enforceable bioprospecting and access policies. By embracing these strategies, the global scientific community can fast-track the development of life-saving therapies while preserving the ecological and cultural heritage that underpins these discoveries.

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
AI	Artificial Intelligence
SAR	Structure–Activity Relationship
BGCs	Biosynthetic Gene Clusters
HTS	High-Throughput Screening
QSAR	Quantitative Structure–Activity Relationship
VIGS	Virus-Induced Gene Silencing
ACTs	Artemisinin-based Combination Therapies
FDA	Food and Drug Administration
EMA	European Medicines Agency
CFDA	China Food and Drug Administration
GMP	Good Manufacturing Practice
RAMES	RApid Metabolome Extraction and Storage
UNAS	Universitas Nasional Jakarta
BRIN	Badan Riset dan Inovasi Nasional
MIAs	Monoterpenoid Indole Alkaloids
GNPS	Global Natural Products Social
DNP	Dictionary of Natural Products
ICBG	International Cooperative Biodiversity Groups
BPOM	Badan Pengawas Obat dan Makanan
PhytoMetaSyn	Phytochemical Metabolic Synthetic Biology
SmartCell	Smart Cell Factory
AlphaFold2	–
LC-MS	Liquid Chromatography–Mass Spectrometry
NMR	Nuclear Magnetic Resonance
RSCM	Rumah Sakit Cipto Mangunkusumo
3D	Three-Dimensional

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Sunscreen Potential of a Gel Formulated with Kale (*Brassica oleracea var. sabellica*) Leaves Extract: Formulation and Evaluation Study

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ABSTRACT

Ultraviolet radiation from sunlight can cause skin redness and hyperpigmentation. Kale (*Brassica oleracea var. sabellica*) is known for its high flavonoid content, making it a promising candidate for natural sunscreen formulation. This study aimed to develop a kale leaf extract gel with protective activity against UV radiation, as indicated by the Sun Protection Factor (SPF). Kale leaf extract was formulated into a gel preparation at concentrations of F1 (1%), F2 (2%), and F3 (4%). The gel was evaluated for organoleptic properties, homogeneity, pH, viscosity, spreadability, and adhesion. Sunscreen activity was assessed using a UV-Vis spectrophotometer, measuring SPF, percentage of erythema, and percentage of pigmentation. The formulated gel showed acceptable physical characteristics, including thick consistency, yellow-brown color, typical kale aroma, and homogeneous texture. The viscosity values ranged from 8888-9443 mPa.s, with a pH of 4.5. The spreadability was measured in the range of 5.24-6.55 cm, and the adhesion was recorded at 2.5 seconds. The highest sunscreen activity was observed in F3 (4%), which achieved an SPF of 0.28, with erythema and pigmentation protection values of 79.1% and 79.2%, respectively, categorizing it as low protection. Although the gel product has a low SPF, kale leaf extract showed potential photoprotective activity.

Keywords:
Gel Formulation; Kale (*Brassica oleracea var. sabellica*); Sun Protection Factor; Sunscreen

INTRODUCTION

Excessive exposure to ultraviolet (UV) light can have adverse effects on the skin, including darkening, burning, premature aging, and an increased risk of skin cancer (Manikrao Donglikar and Laxman Deore, 2016). Applying sunscreen is one method of shielding oneself from UV light (Rachmawati et al., 2021). There are two forms of sunscreens: chemical sunscreens, which absorb UV rays, and physical sunscreens, which reflect them. Sunscreens are cosmetic items used to shield the skin from UV radiation (Usman and Muin, 2021). Sunscreen convenience is a crucial factor, especially in Indonesia's tropical climate, characterized by high temperatures and humidity.

High water-content gel preparations provide the skin with a cool, light feeling (Bokti and Saputri, 2018). Furthermore, gel formulations offer benefits in terms of equal distribution, absorption, pore-

clearing avoidance, cooling, ease of water rinsing, and effective active ingredient delivery (Sani et al., 2021). Gels are generally composed of active ingredients and supporting ingredients such as moisturizers, gelling agents, and preservatives (Bokti and Saputri, 2018). The effectiveness of sunscreens can be tested in the laboratory using a UV-Vis spectrophotometer to determine the Sun Protection Factor (SPF) value, erythema transmission percentage (%Te), and pigmentation transmission percentage (%Tp). SPF describes the ability of a sunscreen to prolong the time for redness to appear on protected skin compared to unprotected skin (Nopiyanti and Aisiyah, 2020), while %Te and %Tp indicate how much UV radiation can penetrate the sunscreen layer and potentially cause redness or darkening of the skin (Mutmainah et al., 2020).

Some persons may experience allergies and discomfort when using synthetic sunscreens (Beladini et al., 2021). Consequently, using natural substances is a desirable substitute to lessen these possible negative effects. Kale (*Brassica oleracea var. sabellica*) leaves are rich in antioxidant chemicals such as vitamins C and E, flavonoids, and carotenoids (Khalid et al., 2023), and they are known to contain more flavonoids than other vegetable varieties (Mageney et al., 2017). Antioxidant compounds play a role in protecting the body from cell damage due to free radicals, one of which comes from UV exposure (Mudjiran and Karneli, 2024). Based on this background, this study aims to develop a sunscreen gel formulation that utilizes kale leaf extract in three concentration variations (1%, 2%, and 4%). Furthermore, this study will compare the sunscreen effectiveness between kale leaf extract and kale leaf extract gel preparation based on the SPF, %Te, and %Tp values obtained from the test results.

METHODS

1. Materials

This research used Kale leaves (*Brassica oleracea var. sabellica*) sourced from the Bumiaji plantation in Malang, which were identified and verified by botanists at Materia Medica in Batu, East Java. All pharmaceutical-grade raw materials in the form of Hydroxypropyl methylcellulose (Sigma-Aldrich), methylparaben (Sigma-Aldrich), propylene glycol (Smart-Lab), and ethanol 96% (Smart-Lab).

2. Instrumentations

Shimadzu Spectrophotometer UV-Visible type 1780, pH meter (Benchtop), rotary evaporator (SCI100-Pro), viscometer (Brookfield DV1), analytical balance (Ohaus AX223/E), mortar, stamper, and laboratory glassware (Pyrex).

3. Extract Preparation

A total of 400 grams of kale leaves simplicia was macerated in ethanol 96% at a ratio of 1:5 for four days. The resulting macerate was then filtered and concentrated using a rotary evaporator at 60°C to produce a thick extract.

4. Phytochemical Tests for Extracts

Phytochemical analyses in this study were performed to examine the secondary metabolite compounds in kale leaf extract using established techniques (Nortjie et al., 2022). The purpose of these tests was to identify alkaloids, flavonoids, saponins, tannins, terpenoids, and steroids.

5. Determination of Sun Protection Activity of Kale Leaves Extract

The sun protection factor (SPF) of kale leaf extract was determined using an in vitro method with a UV-Vis spectrophotometer. The extract was dissolved in 96% ethanol (analytical grade), and solutions with concentrations ranging from 100 to 600 ppm were prepared. Absorbance measurements were taken at 5 nm intervals within the 290–320 nm wavelength range, with each measurement repeated three times. The recorded absorbance values were then used to calculate the SPF using a mathematical formula developed by (Mansur et al., 1986).

$$SPF = CF \sum_{290}^{320} EE\ (\lambda) \text{ x I } (\lambda)\ x\ Abs\ (\lambda)$$

Where: EE represents the erythema spectrum; I is the light intensity spectrum; Abs is the absorbance of the sunscreen sample; and CF is the correction factor (=10).

6. Preparation of Gel Formulation

Hydroxypropyl methylcellulose (HPMC) was hydrated for one hour using hot distilled water maintained at a temperature of 80°C and stirred to form a gel base. Propylene glycol and methylparaben were mixed until a homogeneous solution was obtained. This solution was then used to dissolve the kale leaf extract, which was subsequently incorporated into the gel base to produce a uniform preparation. Three gel formulations containing kale leaf extract were developed, with the composition details presented in **Table 1**. The gel formulations were evaluated based on organoleptic, homogeneity, pH, viscosity, spreadability, and adhesiveness.

Table 1: Gel formulation composition

Ingredients	Formula 1 (%)	Formula 2 (%)	Formula 3 (%)
Kale leaves extract	1%	2%	4%
Hydroxypropyl methylcellulose (HPMC)	1,5%	1,5%	1,5%
Propylene glycol	5%	5%	5%
Methylparaben	0,1%	0,1%	0,1%
Aquadest	add 100%	add 100%	add 100%

7. Sun Protection Activity Test of Kale Leaves Extract Gel

The sun protection potential of the kale leaves extract gel formulation was assessed through in vitro analysis, which included the determination of sun protection factor (SPF), erythema transmission percentage (%Te), and pigmentation transmission percentage (%Tp). For spectrophotometric evaluation, the gel formulation was dissolved in analytical-grade ethanol, and its absorbance was measured using a UV-visible spectrophotometer, with ethanol serving as the blank. SPF values were calculated using the Mansur equation, a widely accepted method for in vitro SPF determination. Transmittance was assessed by measuring the absorbance of the sample across wavelengths ranging from 292.5 nm to 317.5 nm for erythema and from 322.5 nm to 372.5 nm for pigmentation, at 5 nm intervals. Each measurement was performed in triplicate. The percentage of transmitted erythema (%TE) and pigmentation (%TP) were subsequently calculated using a previously established equation (Cumpelik, 1972).

$$\%Te = \frac{\text{Ee}}{\Sigma Fe} = \frac{\Sigma \text{TxFe}}{\Sigma \text{Fe}}$$

Where *T* denotes the percentage of transmittance; *Fe* represents the erythema flux, a constant value; *ΣFe* refers to the total erythema flux; and *Σ(T × Fe)* indicates the cumulative erythema flux attenuated by the photoprotective formulation.

$$\%Tp = \frac{\mathrm{Ep}}{\Sigma Fp} = \frac{\Sigma \mathrm{TxFp}}{\Sigma \mathrm{Fp}}$$

Where *T* denotes the transmittance percentage; *Fp* represents the pigmentation flux, a constant value; *ΣFp* corresponds to the total pigmentation flux; and *Σ(T × Fp)* indicates the portion of pigmentation flux blocked by the photoprotective product. The values for erythema and pigmentation flux were obtained from *Cosmetics Science and Technology* (Balsam and Sagarin, 1972).

RESULTS AND DISCUSSION

1. Phytochemical Screening

Phytochemical screening of kale (*Brassica oleracea var. sabellica*) leaves extract indicated the presence of several bioactive compounds, including flavonoids, alkaloids, tannins, and triterpenoids (**Table 2**). The presence of flavonoids and carotenoids has been reported to contribute significantly to the antioxidant activity of kale, which may play a role in inhibiting skin aging processes (Handajani, 2019). Flavonoids, in particular, have demonstrated the capacity to absorb ultraviolet (UV) radiation effectively, thus presenting potential as active ingredients in sunscreen formulations. Their photoprotective properties are attributed to the presence of chromophore groups—conjugated aromatic systems typically responsible for the yellow pigmentation in plants. These chromophores enable strong absorption within the UV spectrum, including both UVA and UVB wavelengths (Abdiana and Anggraini, 2017). Moreover, flavonoids and other polyphenolic compounds exhibit antioxidant properties when applied topically, enhancing the concentration of antioxidants in both the epidermal and dermal layers of the skin. In addition to providing a protective barrier against UV penetration, these antioxidants may also mitigate the cytotoxic effects of UV-induced oxidative stress (Boo, 2020).

Table 2: Phytochemical screening results

Phytochemical screening	Result
Alkaloids	+
Flavonoids	+
Saponins	-
Tannins	+
Terpenoids	+
Steroids	-

2. SPF Determination of Kale Leaves Extract

Among all tested concentrations, the kale leaves extract at 600 ppm demonstrated the highest sun protection factor (SPF), with a value of 16.6. The results of the SPF analysis indicated a positive correlation between extract concentration and sunscreen activity, as reflected by increased absorbance values (**Figure 1**). These findings suggest that higher concentrations of kale leaf extract enhance the formulation's ability to absorb ultraviolet (UV) radiation.

Figure 1. Kale Leaves Extract Gel Formulation

According to (Ruslan et al., 2019), sunscreen products with an SPF value ≥15 are generally considered effective in protecting against long-term skin damage, including the risk of skin cancer associated with prolonged UV exposure. Furthermore, as noted by (Utami, 2021), the SPF value not only reflects a formulation's UV-absorbing capacity but also serves as an indicator of the duration for which skin can be exposed to sunlight without exhibiting signs of irritation.

3. Evaluation of Gel Formulation

In the present study, a gel formulation was successfully developed using varying concentrations of kale leaf extract. The organoleptic assessment demonstrated that the gel's color changed in response to the amount of extract incorporated. Specifically, the gel color transitioned from yellow to brownish shades, corresponding to increasing extract concentrations (**Figure 2**).

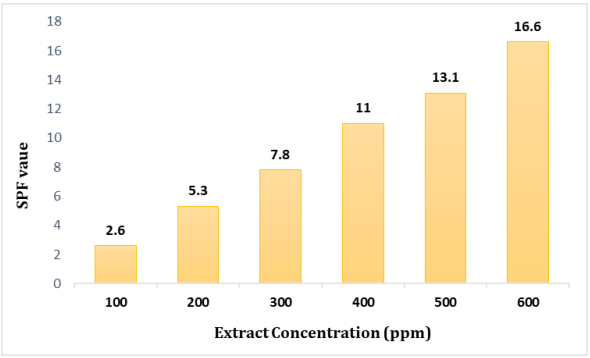

Figure 2. Sun Protection Factor (SPF) Value of Kale Leaves Extract at various concentrations

Physicochemical evaluation of the gel formulations revealed acceptable characteristics for topical application. The gels exhibited a thick consistency, yellowish-brown coloration, a distinctive kale leaves aroma, and a homogeneous texture. Viscosity measurements ranged from 8888 to 9443 mPa·s, indicating a stable and spreadable consistency. Spreadability values were recorded between 5.24 and 6.55 cm, while adhesiveness was measured at 2.5 seconds (**Table 3**).

Table 3: Evaluation of gel formulation

Parameter	Formula 1	Formula 2	Formula 3
Color	Yellow	Brownish yellow	Light brown
Consistency	Thick gel	Thick gel	Thick gel
Odor	Typical kale aroma	Typical kale aroma	Typical kale aroma
pH*	4.52±0.09	4.57±0.01	4.57±0.14
Homogeneity	Homogeneous	Homogeneous	Homogeneous
Viscosity (mPa.s)*	8888±0.05	9170±0.07	9443±0.04
Spreadability (cm)*	5,24±0.02	6,10±0.07	6,24±0.02
Adhesion (seconds)*	2.60±0.03	2.54±0.05	2.57±0.05

*numeric data presented as mean±SD, standard deviation

These findings align with the observations of (Yuniarsih et al., 2021), who reported that enhanced consistency and texture contribute to improved spreadability in cosmetic products. Furthermore, due to the hydrophilic nature of the external phase in water-in-oil emulsions, the emulsified oils mix readily with skin secretions and water, facilitating rapid absorption through the skin. This property enhances the ease of application and overall sensory comfort of the gel (Novitasari and Amboro, 2021). The pH of the kale extract gel formulations was approximately 4.5, falling within the acceptable pH range for skin applications, which is 4.5–6.5. Maintaining pH within this range is crucial, as deviations may lead to skin irritation (Ilmaknun and Endriyatno, 2024).

4. Sunscreen Activity of Kale Leaves Extract Gel Formulation

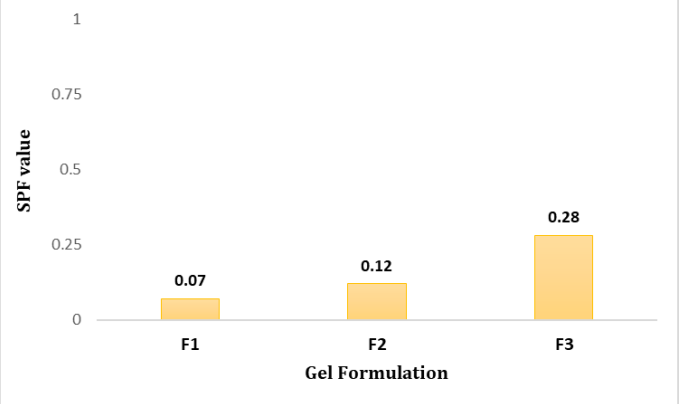

Figure 3. Sun Protection Factor (SPF) Value of Kale Leaves Extract Gel with Different Formulas

The results of this study indicate that the sunscreen activity of kale leaf extract decreased following its formulation into a gel dosage form. The Sun Protection Factor (SPF) value of the formulated gel has been presented (**Figure 3**), while the percentage of erythema inhibition (%Te) and pigmentation inhibition (%Tp) have been shown (**Figure 4**).

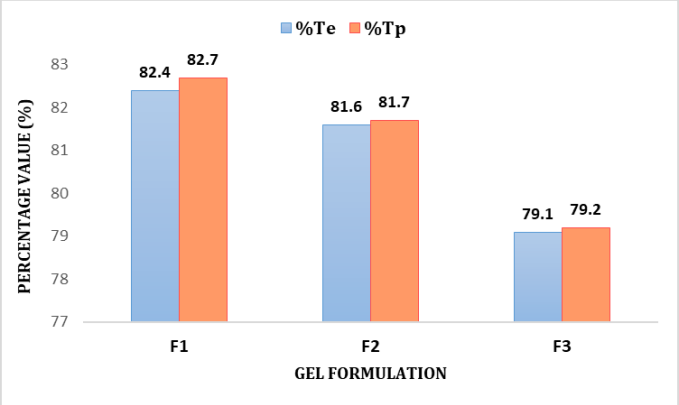

Figure 4. Results of The Percentage of Erythema (%Te) and Percentage of Pigmentation (%Tp) of Kale Leaves Extract Gel with Different Formulas

Variations in SPF values may be attributed to several formulation-related factors, including the choice of solvent, interactions among excipients and active compounds, pH of the system, and rheological characteristics of the gel matrix. Variations in formulation parameters can modulate ultraviolet (UV) absorbance at specific wavelengths, thereby altering the maximum absorbance profile of the sunscreen product. Moreover, the presence of excipients and other non-active constituents may introduce

supplementary absorption bands that overlap with or disrupt the absorption spectrum of the active UV filters, potentially leading to deviations in the measured SPF (Dimitrovska Cvetkovska et al., 2017). Despite the observed reduction in SPF, the formulation retains its functional quality. The SPF values obtained from the kale extract gel formulation serve as predictive indicators of photoprotective efficacy. Although in vitro SPF evaluation methods demonstrate a degree of correlation with in vivo assessments, further validation through clinical studies involving human volunteers is recommended for more conclusive results (Nadia et al., 2023).

CONCLUSIONS

The formulation of a gel-based sunscreen incorporating kale (*Brassica oleracea var. sabellica*) leaves extract and the subsequent evaluation of its photoprotective efficacy have been successfully conducted. In vitro studies revealed that the extract provides broad-spectrum protection against ultraviolet (UV) radiation, effectively mitigating the risk of erythema and hyperpigmentation. However, a reduction in the Sun Protection Factor (SPF) was observed following the incorporation of the extract into the gel formulation, which is presumed to result from the interaction of the active compound with various excipients used during the formulation process. Despite this decrease, the SPF value remained within the high protection category as defined by standard classification systems. These results underscore the potential of kale leaf extract as a viable natural active ingredient in sunscreen formulations, particularly when supported by formulation optimization to preserve and enhance its photoprotective properties.

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CONFLICT OF INTEREST

The authors hereby declare that there are no conflicts of interest related to the publication of this research.

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Comparison of Maceration and Soxhlet Towards Phenolic-Flavonoid Content and Antioxidant Activity of Leaves Methanolic Extract of *Alphitonia Incana*

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ABSTRACT

Alphitonia incana (Roxb.) Teijsm. & Binn. ex Kurz is a plant that can grow on mountain slopes on the island of Borneo and is locally known as Balik Angin. The leaves of this plant have been proven to contain phenolic and flavonoid compounds, so it has the potential to be a source of natural antioxidants. This study aimed to compare the maceration and Soxhlet extraction of Balik Angin leaves in producing antioxidant activity and total phenolic-flavonoid contents. The leaves were extracted using methanol, consisting of two different extractions (maceration and Soxhlet). Quantitative analysis was determined spectrophotometrically for total phenolic contents, total flavonoid contents, and measurement of antioxidant activity was carried out by 2,2-diphenyl-1-picrylhydrazyl (DPPH), Cupric Ion Reducing Antioxidant Capacity (CUPRAC), and Ferric Reducing Antioxidant Power (FRAP) methods. The results showed that the total phenolic contents of the Soxhlet extract (708.807±0.399 µg GAE/mg) were found higher as compared to the macerated extract (631.131±0.529 µg GAE/mg), however, the total flavonoid content of the macerated extract (51.433±0,248 µg QE/mg) showed a higher value than the Soxhlet extract (44.946±0,518 µg QE/mg). The Soxhlet extract has a more powerful DPPH scavenging (IC_{50} 9.983 ppm) and reducing cupric ions (EC_{50} 8.527 ppm) activity than the macerated extract (IC_{50} 13.703 ppm and EC_{50} 11.202 ppm), which were both included in a very powerful antioxidant category. The ferric ion reduction power of the macerated extract was 1,739.2 mgAAE/g extract, while the Soxhlet extract was 1,846.5 mgAAE/g extract. The conclusion is that the Soxhlet extraction of Balik Angin leaves methanol extract produces better antioxidant activity compared to the maceration and is correlated with their high phenolic contents, but no significant difference in statistical analysis.

Keywords: *Alphitonia incana*; Antioxidant; Maceration; Phenolic-flavonoid; Soxhlet

INTRODUCTION

Nowadays, there is an increasing number of people with degenerative diseases in the world community, such as coronary heart disease, hypertension, stroke, and cancer. The increase in degenerative diseases can be caused by several factors, namely the decline in environmental quality

due to pollution, fatty and fast food, high cholesterol, and an unhealthy lifestyle, namely smoking. These factors play an important role in the formation of free radicals in the body. Free radicals attack unsaturated lipid compounds that have electron-rich groups. These free radicals combine with lipids to produce lipid peroxides. Free radicals are most effectively neutralized by giving antioxidants (Gulcin, 2025). Antioxidants are compounds that can stabilize free radicals. Antioxidants are needed so that oxidative reactions caused by free radicals do not occur. Antioxidant compounds can donate electrons to unstable free radicals, so that antioxidants neutralize these free radicals and do not interfere with body metabolism (Tumilaar *et al*., 2024).

Natural antioxidants are in demand by the public because natural antioxidants are safer than synthetic antioxidants. Synthetic antioxidants have carcinogenic properties, so that in the long term they can turn into toxins in the body, thus, natural antioxidants are needed as an alternative treatment (Ki *et al*., 2024). One of the Alphitonia species that has antioxidant properties is *Alphitonia incana* (Roxb.) Teijsm. & Binn. ex Kurz. The plant that can grow on mountain slopes on the island of Borneo is locally known as Balik Angin. The Balik Angin plant has Latin synonyms including *A. excelsa*, *A. moluccana*, and *A. philippinensis*. Balik angin plants are potentially a natural antioxidant and contain flavonoids of the hydroxyauron group, which is alphitonin (Nurjanah *et al*., 2022; Ramadhan *et al*., 2025).

An ethnobotanical study states that Balik Angin leaves are used by the people of Borneo, especially the Dayak tribe as traditional medicine. The leaves are usually used daily as a natural bath soap for skin care and are used to treat skin diseases (Wardah & Sundari, 2019; Ramadhan *et al*., 2023). The leaf of the Balik angin has the potential to be an alternative natural antioxidant, based on screening for its antioxidant activity. Research on the *Alphitonia philipphinensis* showed that the part of the leaf that was extracted with methanol using a Soxhlet had an IC_{50} of 32 g/mL which is included in the category of very strong antioxidants compared to the fruit, skin, and stems The methanol extract of the leaves also contains higher levels of phenols and flavonoids than other parts of the Balik Angin plant (Ahmed *et al*., 2019). Another study stated that the maceration results of the methanolic extract of the leaves of the Balik angin contain phenols, saponins, phytosteroids, flavonoids, and tannins, but the antioxidant activity is unknown (Ramadhan *et al*., 2023). This underlies the selection of the Balik Angin leaf parts used in this study, to explore its potential as antioxidants.

Factors that affect the quality of the extract is the method that is used in the extraction process. Maceration and soxhletation are two extraction methods that are often used because they have their respective advantages in terms of the withdrawal of phytochemical compounds (Anisa *et al*., 2025). The cold extraction method chosen is maceration, which has several advantages: it likely avoids the risk of damage to thermolabile compounds in plants, the equipment used is simple, and the solvent used is less than the percolation cold extraction method (Sayakti *et al*., 2022).The Soxhlet method is a widely used extraction technique for extracting bioactive compounds, including antioxidants, from plant materials and other natural sources. This method has several advantages that make it popular in extraction research, including: Soxhlet utilizes the principle of repeated percolation, so that it can produce more efficient extraction; the temperature in the Soxhlet apparatus can be kept constant, to avoid degradation of compounds that are easily degraded due to high temperatures; Soxhlet allows the use of various types of solvents (polar and non-polar), so that they can be adjusted to the properties of the compounds to be extracted; and the extraction of bioactive compounds can be carried out in a more consistent manner and free from excess solvent contamination (Awad *et al*., 2021; Hajji Nabih *et al*., 2023; Zhang *et al*., 2023). Based on this background, it is necessary to compare the extraction method which are maceration and Soxhlet of Balik Angin leaves with methanol solvent, in producing antioxidant activity using the DPPH

(2,2-diphenyl-1-picrylhydrazil), Cupric Ion Reducing Antioxidant Capacity (CUPRAC), and Ferric Reducing Antioxidant Power (FRAP) methods, and also to determine the total phenolic-flavonoid contents.

METHODS

1. Chemicals and Instruments

All chemicals, solvents, and reagents used in the experiments were purchased from Merck KGaA were of analytical grade, except standards quercetin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl, and ascorbic acid from Sigma Aldrich Co. All spectrophotometric measurements were made with a pair of matched quartz cuvettes using a PG Instruments-T60 UV-Vis spectrophotometer.

2. Sample Preparation and Extraction

Samples of Balik angin leaves were obtained from Mount Tahura, Banjar Regence, South Kalimantan. The sample used in the form of Balik angin leaves is fresh green and still on the tree. The samples of the leaf plant were carried out by wet sorting, by washing them first with clean running water to remove dirt adhering to the sample. The leaves were dried in a room free from direct sunlight exposure. The dried leaf samples were ground into powder and then sieved using a 40 mesh sieve (Cock, 2020; Fuentes *et al*., 2020).

3. Maceration Extraction Preparation

Leaves powder was weighed (50 g) and put into a maceration vessel, then 500 mL of methanol was added to the sample. Samples were extracted for 24 hours at room temperature with light stirring. The extract was filtered and the residue was extracted again with methanol and filtered again 2 times. The extract was filtered using filter paper while in a vacuum, then dried using a rotary evaporator and a water bath at 50°C until a constant weight was obtained (Ramadhan *et al*., 2024).

4. Soxhlet extraction Preparation

Leaves powder (35 g) was wrapped in filter paper and tied on both sides then placed in a thimble and extracted with 250 mL of methanol using a soxhlet apparatus until the cycle was colorless at <60°C. The solvent was evaporated at a temperature of 50°C using a rotary evaporator and a water bath. The viscous extract that had been allowed to stand was dried until a constant mass was obtained and stored at 4°C in the dark (Ramadhan *et al*., 2024).

5. Phytochemical Screening

a. Phenol Test

The sample was weighed as much as 0.1 g dissolved with 2 mL of the solvent and put into a test tube. Added 2 drops of 10% $FeCl_3$ solution into the reaction tube. Positive test results contain phenolic compounds if they produce green, red, purple, blue or black colors (Ramadhan *et al*., 2020).

b. Flavonoid test

The sample was weighed as much as 0.1 g dissolved with 2 mL of the solvent and put into a test tube. Added 2 mg of Mg powder and 1 mL of concentrated HCl. A positive sample contains flavonoids if it causes a red, yellow, or orange color change (Ramadhan *et al*., 2024).

c. Alkaloid test

The sample is weighed as much as 0.1 g dissolved with 2 mL of solvent and put into a test tube. Added 3-5 drops of H_2SO_4 and then shake until it form two layers, the top layer formed from the reaction of the sample and the H_2SO_4 tested with 3 reagents namely Dragendorff, Mayer, and Wagner. The sample tested positive for alkaloids with the formation of a red orange precipitate with Dragendorff's reagent, a white precipitate with Mayer's reagent and a brown precipitate with Wagner's reagent (Ramadhan *et al*., 2024).

d. Tannin test

A sample of 0.5 g was dissolved with 2 mL of the solvent, then added 1% gelatin solution mixed with NaCl. Positive samples contain tannins if a white precipitate is formed (Muthia *et al*., 2023).

e. Saponin test

The sample was weighed as much as 0.1 g, added with 10 mL of distilled water. The sample was then shaken for 10 seconds and then left for 10 minutes. 1 mL of 2 N HCl was added to the test tube containing the sample. The sample is declared positive if the foam formed is stable after the addition of HCl (Ramadhan *et al*., 2024).

f. Steroid and triterpenoid test

The sample was weighed as much as 0.1 g, added 2-3 mL of chloroform and 10 drops of anhydrous acetic acid and 2-3 drops of concentrated H_2SO_4 (Lieberman Burchard's reagent) on a drip plate. A positive steroid test will give a blue to green color, while a red or purple color indicates that the positive extract contains triterpenoids (Ramadhan *et al*., 2024).

6. The total phenolic contents (TPCs)

The TPCs of the extracts were estimated by a colorimetric assay (Folin-Ciocalteu method) with a gallic acid standard solution. Exactly 0.6 mL of each appropriate dilutions of the extracts or gallic acid (60, 70, 80, 90, and 100 ppm) was mixed with 1 mL of Folin-Ciocalteu reagent (previously diluted with aquadest 1:10), 2 mL of 10% Na_2CO_3 was added after 5 min. The absorbance value was measured spectrophotometrically at 734 nm after allowed to stand in the dark for 70 minutes. TPCs were expressed as µg gallic acid equivalent in 1 mg of extract (Yuliani *et al*., 2022).

7. The total flavonoid contents (TFCs)

The aluminium colorimetric method was used to determine TFCs based on the method described by Chang et al.14 with some modifications. Exactly 0.5 mL of each extracts and the dilutions of the quercetin calibration curve (40, 60, 80, 100, and 120 ppm) were prepared in methanol. Each of the dilutions was added to a tube containing 0.5 mL of 10% $AlCl_3.6H_2O$ solution and mixed with 4 mL of acetic acid glacial 5%. All samples were analyzed in triplicate and the absorbance was measured immediately at 415 nm after 20 minutes of incubation. Quercetin was used to calculate the standard curve and the results were expressed as µg quercetin per 1 mg extract (Yuliani *et al*., 2022).

8. 2,2-diphenyl-1-picrylhydrazil (DPPH) Scavenging Activity

The quantitative assay by measuring the absorbance of dilute solutions (4 mL each serial concentrations of both fractions) is mixed with DPPH 0.4 mM (1 mL) and allowed to stand for 30 min for any reaction to take place. For the blank, mix 4 mL of MeOH and 1 mL of DPPH solution. The UV-

Vis absorbance of these solutions is recorded at 515 nm. The same procedure is followed for the standard (quercetin). All samples and the control were made in triplicate. Finally, calculate the percentage inhibition of DPPH absorption by each dilution to find IC_{50} value. The DPPH value expressed as percent inhibition (% inhibition) is calculated based on the following equation:

$$\text{Percent inhibition} = \frac{\text{abs of control} - \text{abs of sample}}{\text{abs of control}} \text{ x } 100\%$$

The relationship between the concentration of the test sample or comparison and the percentage of inhibition was obtained from the linear regression equation y = bx + a. The equation is to determine the IC_{50} value with a y value of 50 and an x value as IC_{50}(Ramadhan & Forestryana, 2021; Purnama *et al*., 2022).

9. Cupric Ion Reducing Antioxidant Capacity (CUPRAC) assay

The antioxidant activity of Balik Angin leaves extract and quercetin (comparative standard) 1 mL of each sample solution separately with a concentration series of 3 ppm, 6 ppm, 9 ppm, 12 ppm and 15 ppm for each extract and 1-5 ppm for quercetin, were taken and each put into a vial. 1 mL of 0.01 M $CuCl_2 \cdot 2H_2O$ solution, 1 mL of 0.0075 M Neocuproine Ethanolic Solution, 1 mL of 1 M NH_4Ac Buffer, and 0.1 mL of distilled water were then added. All sample series were incubated in a dark room for 30 minutes. Following this, the absorbance was measured using a UV-Vis Spectrophotometer at a predetermined maximum wavelength of 450 nm. This process was replicated and carried out three times. The determination of EC_{50} was carried out after obtaining the sample absorbance value. The percent capacity value is calculated using the formula: % Capacity = (1 – Antilog of Absorbance) x 100%. A standard curve for the relationship between %capacity and concentration is created to calculate the EC_{50} value using the line equation y = bx + a obtained from the standard curve. This equation can be used to determine the EC_{50} value of the sample by entering the y value of 50 and the x value as the concentration, which will be obtained as EC_{50} (Ramadhan *et al*., 2022).

10. Ferric Reducing Antioxidant Power (FRAP) assay

The standard curve of ascorbic acid was made with correlated concentration series (60-100 ppm) and absorbances. Each ascorbic acid concentration was taken as 1 mL and was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50˚C for 20 minutes. After incubation, 1 mL of TCA was added and homogenised for ten minutes, then centrifuged at 3000 rpm for ten minutes. One millilitre of the top layer was taken from the solution and added with 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$, and incubated again for a further ten minutes. Absorbance was measured at the maximum wavelength of 725 nm with a UV-Vis spectrophotometer. The antioxidant activity test of each Balik Angin leaf extract was carried out by making the concentration of each extract 100 ppm and pipetting 1 mL of the extract, which was added with the same reagent as the treatment in the ascorbic acid standard curve. The absorbance of the solution was measured at the maximum wavelength using a UV-Vis spectrophotometer. Determination of antioxidant power was calculated after obtaining the linear regression equation from the ascorbic acid standard curve (y = bx + a) to count the x-axis as sample concentration (Ramadhan *et al*., 2024). Ahmed *et al*. (2019) stated that the antioxidant power value is shown as milligram Ascorbic Acid Equivalent per gram (mg AAE/g) extract and was calculated using the following formula:

$$\text{AAE} = \frac{Sample\ Concentration\ x\ Volume\ x\ Dilution\ Factor}{Sample\ Weight}$$

RESULTS AND DISCUSSION

The extraction of Balik angin leaves was carried out by two methods, which are maceration and soxhlet extraction. The extraction method greatly affects the extraction results, this is because the extraction method affects the concentration or therapeutic effect of simplisia because some simplisia are not stable and thermolabile (can be damaged by heating). This research uses maceration and soxhletation methods because this method is the most common method used. The temperature difference between maceration and soxhlet extraction methods can affect the level of solubility of flavonoids and phenols contained in the leaf of the Balik angin, where high temperatures will increase the solubility of phenol using the solvent. An increase in temperature can cause a greater diffusion process (Hasnaeni *et al*., 2019; Ramadhan *et al*., 2024).

The reason for choosing the maceration and soxhlet extraction method is because it has many advantages compared to other extraction methods. nature does not decompose. Cold extraction allows many compounds to be extracted. The hot extraction method (soxhlet) is the best extraction method to get a lot of extract results and also less solvent is used, the sample is extracted perfectly because it is done repeatedly (Nurhasnawati *et al*., 2017). This is the basis for phytochemical screening tests to conduct investigations on compounds including phenols, flavonoids, alkaloids, tannins, saponins, steroids, and triterpenoids. The test results are shown in **Table 1**.

Tabel 1: Comparison of Phytochemical Screening Results of Balik angin leaves Methanol Extract.

Secondary metabolite class	Leaves Methanol Extract	
	Maceration	Soxhlet
Phenol	+	+
Flavonoids	+	+
Alkaloids	-	-
Tannins	+	+
Saponins	+	+
Steroids	+	+
Triterpenoids	-	-

The results of this study showed that both Balik Angin leaves methanol extract containing phenolic, flavonoid, tannin, saponin, and steroid. These results are in line with research by Ramadhan *et al*. (2023), which states that the results of maceration of Balik Angin leaves with methanol can extract phenols and flavonoids from the sample matrix. Polyphenolic phytochemicals are non-nutritional plant compounds that have been used to prevent and treat diseases triggered by oxidative stress (Abu Bakar *et al*., 2015). Flavonoids are the most common and widely distributed group of plant phenolic compounds that act as natural antioxidants (Hasibuan & Mardiana, 2018). Flavonoids possess many biochemical properties, but the best-described property of almost every group of flavonoids is their capacity to act as antioxidants (Forestryana *et al*., 2024).

Determination of total phenolic content (TPC) of methanol extract of Balik Angin Leaf using the Folin-Ciocalteu method which has the principle of reduction and oxidation. Determination of total phenolic content using gallic acid as a standard solution because this compound was one of the natural phenolic compounds which have been shown to be contained in many plants. Gallic acid is used as a standard solution because it is one of the most stable natural phenols and gallic acid is a phenolic compound derived from hydroxybenzoic acid which is a simple phenolic acid. Gallic acid or phenolic compounds react with Folin-Ciocalteu reagent to form a yellow color and adding Na_2CO_3 solution

creates an alkaline atmosphere so that it produces a blue color. The addition of Na_2CO_3 is due to the phenolic compounds reacting with the Folin-Ciocalteu reagent only in an alkaline environment so that protons dissociate in phenolic compounds into phenolic ions (Ramadhan *et al*., 2021; Yuliani *et al*., 2022). The total phenol content was obtained from the regression equation of y = 0.0109x – 0.2316. Meanwhile in the determination of Total Flavonoid Contents (TFC) on methanol extract of Balik Angin leaves was performed by using quercetin as a standard solution at a wavelength of 415 nm. Quercetin is used as a standard solution because it is a flavonol group that can form complexes with AlCl3 and glacial acetic acid reagents to form a yellow solution (Marjoni *et al*., 2018) The total flavonoid level was calculated according to the equation of the calibration curve for quercetin (y = 0,0062x - 0,002).

The reported study by Ahmed *et al*. (2019) showed the TPC which is 25.23±0.15 µg GAE/mg extract and TFC which is 9.84±0.06 µg QE/mg extract of Balik Angin leaves methanol extract was lower than the result in the present study. These results highlighting chemical differences between various extracts obtained according to the different growing regions (Yahyaoui *et al*., 2017). The TFC of macerated extract in the present study also showed a higher value compared to the extract from Soxhlet extraction (**Table 2**).

Tabel 2: Total phenolic-flavanoid contents of the Balik angin leaves methanol extract.

Extraction method	TPCs (µg GAE/mg)	TFCs (µg QE/mg)
Maceration	631.131 ± 0.529	51.433 ± 0.248
Soxhlet	708.807 ± 0.399	44.946 ± 0.518

While Soxhlet extraction is generally effective for isolating compounds, including antioxidants, some studies have found that maceration can yield higher total flavonoid content (TFC) in certain cases. This discrepancy may be due to factors like the specific plant material, solvent used, and the temperature sensitivity of certain antioxidant compounds. Based on the statement, the lowest TFC observed with Soxhlet method could be explained bythe effect of heat on the stability of flavonoid and their biological activity. Depending on thestructure, the sensitivity of flavonoid to heat treatment can be varied. A decrease in total flavonoids at highertemperature could be due to its degradation. Most flavonoids in fruits and vegetables containC-glycoside bonds which exist as dimers and oligomers, industrial processing such as heatingor boiling results in the formation of monomers by the hydrolysis of C-glycosides bond (Chuah *et al*., 2020).

This is in accordance with previous research by Rudiana *et al.* (2023), which states that the maceration method is able to provide higher flavonoid levels compared to soxhlet extraction. Maceration can increase the contact time between sample particles and solvents through a stirring process at room temperature, so that the use of the maceration method can also protect flavonoid compounds that are vulnerable to high temperatures. This phytochemical study showed that the macerated extract was richer in flavonoid compounds than the extract obtained by soxhlet, indicating that extraction methods had a significant impact on the extraction of bioactive molecules. The cold maceration technique is one of the most widely used methods to extract a group of fragile molecules but it is time-consuming. However, the soxhlet extraction method requires a high temperature to extract bioactive substances, which could be influences on the quality of extract causing thermal degradation, because some thermolabile compounds may decompose during the extraction process. In fact, some flavonoid compounds are thermosensitive especially flavan-3-ol and its derivatives as well as anthocyanins, which need to be extracted under moderate temperatures (Yahyaoui *et al*., 2017).

Flavonoids are responsible for the radical scavenging effects of most medicinal plants through scavenging or chelating process *in vivo* as well as *in vitro* (Adawia, 2017). Various methods have been adopted to evaluate the antioxidant activities of phenol-flavonoids. The most common antioxidant activity test uses the DPPH method (2,2-diphenyl-1-picrylhydrazyl). DPPH is a stable radical that is widely used to determine the antioxidant activity of plant extracts. This DPPH method can be used on solid samples or in solution form and is not specific for certain antioxidants. The DPPH free radical scavenging assay is a widely used method that depends on the hydrogen donating ability of the tested compound in which the stable DPPH free radical is converted to 2,2-diphenyl-1-picrylhydrazine. This reaction which is accompanied by a change in color from deep violet to light-yellow is the preferred method in this research. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant (Alisi *et al*., 2018; Mirfat *et al*., 2016).

The DPPH scavenging activity also has been testing quantitatively for measuring the ability of the corresponding extracts and positive control (quercetin) to quench DPPH free radicals by providing hydrogen atoms or by electron donation conceivably via a free-radical attack on the DPPH molecules. The IC_{50} values reflected the sample concentration that could produce 50% DPPH radical scavenging activity. The antioxidant properties are inversely proportional to the IC_{50} values. Highly active antioxidant activity in preventing free radical if their score were lower than 50 ppm (Yunitrianti *et al*., 2020). Quercetin in this study was used as a comparison because quercetin is a flavonoid of the flavonol group which has a keto group at C-4 and has a hydroxyl group on the C-3 or C-5 atom adjacent to flavones and flavonols (Megawati *et al*., 2021). The stems of Alphitonia philippinensis were also shown to have flavonoid glycosides which are derivatives of quercetin and isorhamnetin (Al Omar *et al*., 2022). The measurement curve of antioxidant activity of quercetin as positive control shows the regression equation obtained is linear y = 24.092x-21.786 which is used to calculate the IC_{50} value. The results of the quercetin antioxidant activity test are included in the very strong antioxidant activity group with a value of IC50 2.9797 ppm.

Antioxidant activity was also evaluated using the CUPRAC and FRAP method. The principle of the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) method is to measure the ability of an antioxidant to reduce Cu^{2+} to Cu^{+} which is indicated by a change in colour from blue to yellow. Meanwhile, the FRAP method shows the ability of antioxidant to reduce Fe^{3+} and becomes Fe^{2+} with a change in colour from green to bluish-green. Both methods were used to measure and show that the antioxidant ability is analogous to the reducing ability of a compound (Munteanu & Apetrei, 2021). The CUPRAC method uses comparison samples of quercetin because it has very strong antioxidant activity, with four to five times the effectiveness compared to vitamin C and vitamin E, and two times higher than other flavonoid derivatives (Banjarnahor & Artanti, 2014). It is proven that quercetin has a high antioxidant capacity in reducing cupric ions and includes a very strong category (EC_{50} of 1.636 ppm). Quercetin and ascorbic acid play a role in redox reactions as oxidants that can chelate metal ions (Mendoza-Wilson *et al*., 2022). The chelation process will reduce the catalytic activity of metal ions so that it can reduce the formation of OH radicals and will automatically reduce the process of DNA damage and the process of fat peroxidation (He *et al*., 2017).

The results of DPPH scavenging activity on **Table 3** showed the linear regression equation of the methanol extract of the leaves Balik angin with the maceration method are y = 3.6392x + 0.1296, so that the IC_{50} value of 13.704 ppm which is included in the very strong antioxidant activity.

Tabel 3: Comparison of CUPRAC and DPPH scavenging activity Results

Samples	DPPH scavenging activity		CUPRAC activity		Category
	Regression equation	IC_{50} (ppm)	Regression equation	EC_{50} (ppm)	
Quercetin	y = 24.092x - 21.786	2.980	y = 7.6783x + 37.438	1.636	very strong
Macerated extract	y = 3.6392x + 0.1296	13.704	y = 3.3185x + 12.826	11.202	very strong
Soxhlet extract	y = 4.9626x + 0.4541	9.984	y = 2.235x + 30.94	8.527	very strong

The results of the concentration of the methanol extract of the Balik angin leaf extract extracted by the soxhlet method and the % inhibition by linear regression equation y = 4.9626x + 0.4541, so that the IC_{50} value of the methanol extract of the leaves of Balik angin extract which was extracted by the soxhlet method had a value of IC_{50} 9.984 ppm. IC_{50} value obtained from the antioxidant activity test is classified as a very strong category. The present results show all samples and positive control including a very strong category of antioxidant activity even though there was a difference in IC_{50} score between the extracts and quercetin. The test results of the antioxidant activity of quercetin belong to the group of very strong antioxidant activity. Quercetin as positive control has shown the highest DPPH scavenging activity than both extracts, but the soxhlet extract of Balik Angin leaves has shown a more powerful DPPH scavenging activity than the macerated extract. The results on **Table 3** also showed that extraction of Balik Angin leaves through soxhlet produced stronger antioxidant capacity in reducing cupric ions compared to macerated extract. Balik Angin leaves methanol extract extracted by soxhlet showed antioxidant capacity with EC_{50} values of 8.527 ppm compared to that extracted by maceration with EC_{50} values of 11.202 ppm. These antioxidant activities were contributed by the number of secondary metabolite content (TPCs) which is quite high in the soxhlet extract compared to macerated extract.

These research results have the potential to develop into a traditional medicine with antioxidant properties. The results showed that both Balik Angin leaves' methanol extract demonstrated a significantly higher DPPH scavenging activity than previous research by Ahmed *et al*. (2019), which reported an IC_{50} of 32 ppm. Besides, the results also showed a better antioxidant capacity in reducing cupric ions compared to Balik Angin leaves aqueous extract extracted by infusion (EC_{50} of 17.76 ppm) and maceration (EC_{50} of 44.71 ppm) (Ramadhan *et al*., 2024). Many factors affect the content of compounds extracted and their bioactivity in the extraction process of plant material. These factors include the type of solvent, solvent concentration, extraction method, and the temperature used for extraction (Senja *et al*., 2014).

The antioxidant power of the FRAP assay was calculated using the regression equation of the ascorbic acid standard, which was y = 0.0101x – 0.3805 (**Figure 1**), and continued with the determination of mg AAE/g extract, resulting in the value of antioxidant power.

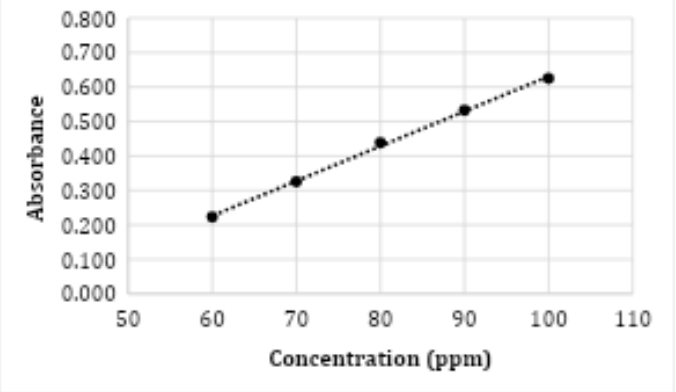

Figure 1. Curve of the regression equation of ascorbic acid

The ferric ion reduction power of the macerated extract was 1,739.2 mgAAE/g extract, while the Soxhlet extract was 1,846.5 mgAAE/g extract. The results of the FRAP assay also prove that the Soxhlet method can attract more antioxidant compounds in Balik Angin leaves because they have higher antioxidant power compared to the macerated extract of Balik Angin leaves. Based on these results, the antioxidant activity of Balik Angin (*Alphitonia incana* (Roxb.) Teijsm. & Binn. Ex Kurz) leaves methanol extract from the maceration and soxhlet method, has a higher Ascorbic Acid Equivalent (AAE) value compared to previous research of Ramadhan *et al*. (2024) that reported the Balik Angin leaves aqueous extract from the maceration and infusion method with antioxidant power of 635 ± 0.5 mg AAE/g extract and 726 ± 0.8 mg AAE/g extract, respectively. This research's results also showed a higher antioxidant power than the Alphitonia philippinensis, which was extracted using methanol solvent using the Soxhlet method, that was only obtained 9.36 ± 0.05 mg AAE/g extract (Ahmed *et al*., 2019). Reviewed by Mungwari *et al*. (2019) reported that the Soxhlet method is indeed very effective in extracting active compounds, including antioxidants, from various natural materials. This method uses solvents that are heated and condensed repeatedly to extract compounds from the natural material matrix. This Soxhlet extraction process is usually efficient in extracting compounds that are soluble in certain solvents.

The data from the antioxidant assay of both leaf methanol extracts were analyzed using the normality test with the Shapiro-Wilk Test and showed a significance value of maceration method extraction of 0.310, a significance value of soxhlet method extraction of 0.215, and a significance value of quercetin standard of 0.072. The data from the normality test results showed a sig value > 0.05. The data were then analyzed for a homogeneity test using Levene's Test, with a significance result of 0.000. The homogeneity test data is not met with a value of sig < 0.05. The data was then tested for comparison with the Kruskal-Wallis test. The results of the data obtained from the Kruskal-Wallis test are a sig value of 0.170 > 0.05. Based on the results above, it can be said that there is no significant difference between the results of the methanolic extracts of the Balik Angin leaves extracted by maceration and the Soxhlet method.

CONCLUSIONS

This study concludes that the Soxhlet method is very effective for the extraction of antioxidant compounds because of its high efficiency, the ability to control temperature, and solvents. This is indicated by the Soxhlet extraction of Balik Angin leaf methanol extract, producing better antioxidant activity compared to maceration, both in antioxidant assay using the DPPH, CUPRAC, and FRAP methods. This activity is correlated with its high phenolic content, but there is no significant difference in statistical analysis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Effect of Gastric Emptying Time on Analgesic Response of N-Hexane Fraction from Ethanol Extract of *Coleus Atropurpureus* Leaves

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ABSTRACT

Previous studies have shown that *Coleus atropurpureus* leaves have analgesic effects in female mice at a dose of 252 mg/kg BW. However, the effectiveness of analgesic compounds may decrease due to interactions with food in the stomach. This study is a true experimental research with a completely randomized one-way design, aimed at investigating the effect of gastric emptying time on the analgesic response of the n-hexane fraction of ethanol extract from *Coleus atropurpureus* leaves (NHFCL). The test groups included a positive control (aspirin 91 mg/kg BW), a negative control (1% CMC-Na 333.3 mg/kg BW), and a treatment group (NHFCL 252 mg/kg BW). Each group was split into two subgroups, with one receiving the treatment on an empty stomach and the other receiving it 10 minutes after feeding. The pain response was measured by counting writhes induced by intraperitoneal injection of acetic acid, recorded every 5 minutes for 60 minutes, and the percentage of protection was calculated. Data were tested for normality using the Shapiro-Wilk test, followed by One-Way ANOVA and Tamhane's Test. The results showed that gastric emptying time significantly influenced the analgesic effect of NHFCL, with a p-value <0.05 between mice treated before and after feeding.

Keywords:
Analgesic; *Coleus atropurpureus* leaves; gastric emptying time; n-hexane fraction; writhing

INTRODUCTION

Pain is an unpleasant sensory and emotional experience for those who suffer from it and can occur due to tissue damage within the body. Tissue damage that triggers the onset of pain can occur either actually or potentially (Dipiro et al., 2020). Therefore, appropriate management is necessary to reduce or eliminate this pain. In addition to analgesic medications available in both generic and brand-name forms, there are also plants used as traditional analgesics to address pain with a lower risk of side effects. *Coleus atropurpureus* leaves are one of the herbal plants from Indonesia that can be used to treat inflammation and pain (Djunarko et al., 2022). *Coleus atropurpureus* leaves, also known as miana leaves, are known to contain flavonoids that can inhibit the cyclooxygenase enzyme, which is the first pathway for the synthesis of pain mediator prostaglandins, and can protect lipid membranes from tissue damage events (Sukmawati et al., 2022). *Coleus atropurpureus* leaves also contain terpenoid

compounds that can act as anti-inflammatory agents (Djunarko et al., 2022). Terpenoids are known to have analgesic activity because they can inhibit the production of prostaglandin E2 (PGE2) when tested in vitro, thereby preventing the formation of various inflammatory mediators such as IL-1β and TNFα (Rahmadanita et al., 2022).

A method using chemical stimulation was used to assess the analgesic effects. The chemical stimulus used in this study was acetic acid. The chemical stimulation method was chosen because the analgesic compounds to be tested are non-narcotic analgesics. The pain response in mice is indicated by writhing, where the mice will pull their legs back until their abdomen touches the bottom of the container (Lara et al., 2021). The test animals used in this study were female mice. Female mice were selected because they possess corticotropin-releasing factor (CRF) hormones as neurotransmitters that facilitate communication of impulses between nerve cells. Female mice are more sensitive and responsive to pain stimuli because they have stronger CRF receptors that bind to CRF hormones compared to male mice (Muqsith, 2015).

One issue that arises from the use of medications is the interaction between drugs and food. According to research conducted by Moore et al. (2015), it is known that oral analgesics consumed with food can lead to a less effective drug response. The interaction between drugs and food is certainly related to gastric emptying time. Therefore, this study was conducted to investigate the effect of gastric emptying time on the analgesic response induced by the n-hexane fraction of the ethanol extract of *Coleus atropurpureus* leaves.

METHODS

The materials used in this study include *Coleus atropurpureus* leaves obtained from the Ngestiharjo area, female Swiss strain mice aged 2-3 months with a body weight of 20-30 grams obtained from the Toxicology Pharmacology Laboratory of the Faculty of Pharmacy Sanata Dharma University, n-hexane p.a. (Merck) and aquades as solvents, ethanol p.a. (Merck), Liebermann-Burchard reagent (anhydrous acetic acid-H_2SO_4), acetic acid (Merck) as a pain inducer, acetylsalicylic acid as a positive control, and CMC-Na as a negative control, with the mice feed (BR2).

The equipment used in this study includes a tray, black cloth, grinder, blender, a set of glassware (test tubes, beakers, measuring cylinders, dropper pipettes, funnels, volumetric flasks, stirring rods, Erlenmeyer flasks), a 19 mesh 40 sieve, stopwatch, 1 mL injection syringe, needle, analytical balance, vacuum pump, Buchner funnel, porcelain dish, maceration or shaker device (Optima®), water bath (Labo-tech, Haracus®), glass observation box for writhing, fume hood, and Buchi rotary vacuum evaporator (Interface 1-300, Rotavapor R-300, Heating Bath B-300).

The *Coleus atropurpureus* leaves to be used were identified at the Medicinal Plant Laboratory, Faculty of Pharmacy, Sanata Dharma University. The leaves used were fresh *Coleus atropurpureus* leaves aged 2-3 months, with a reddish-purple color and no holes, cultivated and harvested from Ngestiharjo, Kasihan, Bantul, Special Region of Yogyakarta. The harvested leaves underwent a drying process before being processed into dry powder. The dry powder of *Coleus atropurpureus* leaves was extracted using the maceration method with ethanol as the solvent until a thick extract of the leaves was obtained. The thick extract was fractionated using a mixture of n-hexane, ethanol, and water in a ratio of 1:1:2 in a separating funnel. The obtained n-hexane fraction was concentrated to yield a thick n-hexane fraction of *Coleus atropurpureus* leaves. The n-hexane fraction was identified for its terpenoid content by dissolving the fraction in chloroform and then adding Liebermann-Burchard reagent. A

positive result indicating the presence of terpenoid compounds was marked by a color change of the mixture to red or purple and the formation of a brown ring.

All solutions used in the testing were prepared. The mouse feed used in this study was of the BR2 type. The female mice feed in the form of BR2 was first reconstructed into a thick paste by weighing 12 grams of BR2 feed and grinding it with a blender. The ground BR2 feed was dissolved in aquades until a volume of 48 mL of thick paste was obtained.

The test animals used in the research were female Swiss strain mice aged 2-3 months with a body weight of 20-30 grams. The female mice were acclimatized for 7 days before the observation day to reduce stress and allow them to adapt to their surroundings. The test animals were fasted for a minimum of 8-12 hours before observation but were allowed to drink ad libitum. A total of 30 mice were divided into 6 treatment groups. Group 1 was the first negative control (-) using mice that were given 1% CMC-Na at a dose of 333.3 mg/kg body weight orally when the mice's stomachs were empty and had not been fed. Group 2 was the second negative control (-) where the mice were given 1% CMC-Na at a dose of 333.3 mg/kg body weight orally 10 minutes after the reconstructed feed was administered orally to the mice. Group 3 was the first positive control (+) using female mice that were given acetylsalicylic acid orally at a dose of 91 mg/kg body weight when the mice's stomachs were empty and had not been fed. Group 4 was the second positive control (+) where the mice were given acetylsalicylic acid orally at a dose of 91 mg/kg body weight 10 minutes after the reconstructed feed was administered orally to the mice. Group 5 was the first treatment group where the mice were given the n-hexane fraction of *Coleus atropurpureus* leaves orally at a dose of 252 mg/kg body weight when the mice's stomachs were empty and had not been fed. Group 6 was the second treatment group where the mice were given the n-hexane fraction of *Coleus atropurpureus* leaves orally at a dose of 252 mg/kg body weight 10 minutes after the reconstructed feed was administered orally to the mice. The feeding of the test animals was conducted by oral injection of the reconstructed mouse feed with a volume of 0.5 mL/30 grams of body weight of the mice. After 10 minutes from the treatment administration in each group, the mice were induced with 1% acetic acid intraperitoneally as a pain stimulus. The writhing response was observed every 5 minutes for 60 minutes. The observed writhing response included torsion on one side, intermittent muscle contractions in the mice, retraction of the hind legs and head of the mice backward until the mice's abdomen touched the bottom of their enclosure, and the retraction of the head and hind legs of the mice toward the abdomen (Titu, 2023).

The data on the number of writhes obtained were then processed to determine the percentage of writhing protection from the n-hexane fraction of *Coleus atropurpureus* leaves given to female mice against the pain stimulus induced by acetic acid. The percentage of writhing protection was established and calculated to determine the ability of the n-hexane fraction of *Coleus atropurpureus* leaves in alleviating pain. The following is the formula for calculating the percentage of writhing protection according to Sasongko (2016):

$$\text{Percentage of writhing protection} = 100 - (\frac{P}{K} \times 100\%)$$

Where:
P = Cumulative number of writhes of the test animals after the administration of the analgesic substance
K = Cumulative number of writhes of the test animals in the negative control group

The data obtained from the analysis will be tested for normality using the Shapiro-Wilk method. If the data is normally distributed (as evidenced by a p-value > 0.05), the data analysis can proceed with a One-Way ANOVA test to examine the differences or variations among the treatment groups. If the data is homogeneously distributed, a Post-Hoc Bonferroni test can be conducted. However, if the data is not homogeneously distributed, a Post-Hoc Tamhane's test can be performed. Data that is not normally distributed will be analyzed using the Kruskal-Wallis test, followed by a Post-Hoc Mann-Whitney test. A p-value > 0.05 indicates that the results obtained are not significant, and gastric emptying time does not affect the results obtained. A p-value < 0.05 indicates that the results obtained are significant, and gastric emptying time does affect the results obtained.

RESULTS AND DISCUSSION

The results of the phytochemical testing of the n-hexane fraction of *Coleus atropurpureus* leaves using the test tube method with Liebermann-Burchard reagent indicated that the preparation tested positive for terpenoids. Liebermann-Burchard is a reagent that contains concentrated H_2SO_4 and anhydrous acetic acid. The anhydrous acetic acid present in the Liebermann-Burchard reagent reacts with acid compounds, causing the carbon atoms in the anhydride compound to form a carbocation. This carbocation can react with the oxygen atom in the hydroxyl group found in terpenoid compounds (Takaeb and Leo, 2023). Through this reaction, an ester formation from the terpenoid compound occurs. This reaction is referred to as esterification. This reaction causes a color change in the test solution to red and the formation of a reddish-brown ring in the test tube. The occurrence of this reaction can yield a positive result in the phytochemical test, indicating the presence of terpenoid compounds in the tested material.

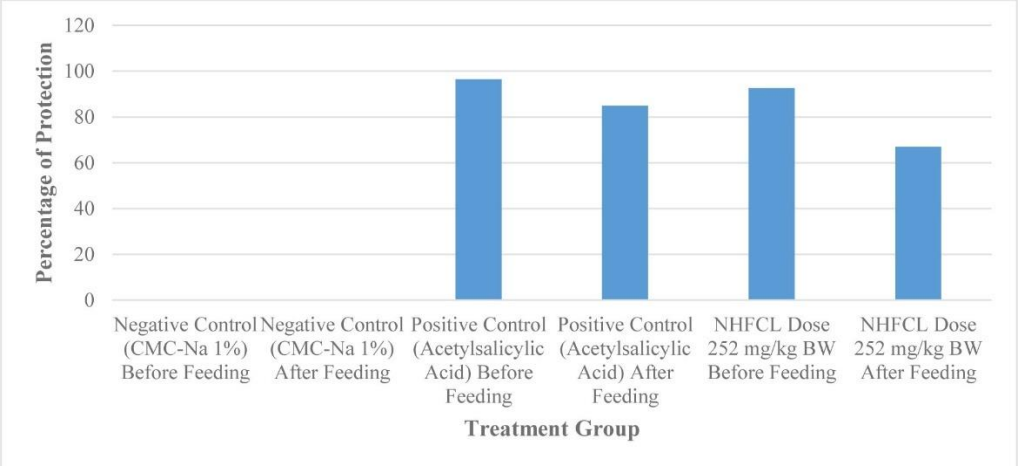

Figure 1. Histogram of Writhing Protection Percentage Data

The preparation was subsequently tested on test animals, and the percentage of writhing protection (**figure 1**) obtained from the analgesic testing was statistically analyzed. The data were tested for normality using the Shapiro-Wilk method and were found to be normally distributed (p > 0.05), allowing the data analysis to proceed with a One-Way ANOVA test to examine the differences or variations among the treatment groups. Since the tested data were not homogeneously distributed, a Post-Hoc Tamhane's test was conducted to observe the differences among the treatment groups.

Table 1: Average Number of Writhes and Percentage of Writhing Protection in Treatment Groups

Treatment Group	Average Number of Writhes (Mean ± SE)	Average Percentage of Protection (Mean ± SE)
Negative Control (CMC-Na 1%) Before Feeding	83,00 ± 10,06	0,00% ± 12,13
Negative Control (CMC-Na 1%) After Feeding	74,40 ± 9,60	0,00% ± 12,90
Positive Control (Acetylsalicylic Acid) Before Feeding	3,00 ± 0,89	96,39% ± 1,08
Positive Control (Acetylsalicylic Acid) After Feeding	11,20 ± 1,39	84,95% ± 1,87
NHFCL Dose 252 mg/kg BW Before Feeding	6,20 ± 0,86	92,53% ± 1,04
NHFCL Dose 252 mg/kg BW After Feeding	24,60 ± 3,16	66,94% ± 4,24

The average number of writhes in the negative control group with 1% CMC-Na before feeding and in the negative control group with 1% CMC-Na after feeding showed the highest values (**Table 1.**). Additionally, the calculated percentage of writhing protection in the negative control group before feeding was 0.00% ± 12.13, and the percentage of writhing protection in the negative control group after feeding was 0.00% ± 12.90. This indicates that CMC-Na, used as a solvent for the n-hexane fraction of *Coleus atropurpureus* leaves, was unable to protect the test animals from the pain induced by acetic acid as a pain stimulus. Therefore, CMC-Na used as a solvent for the n-hexane fraction did not contribute to the analgesic activity observed in this study. The negative control group was divided into a negative control group before feeding and a negative control group after feeding because both control groups served as baselines or references for pain response used for each treatment group. The negative control before feeding will be compared with other treatment groups before feeding, and the negative control after feeding in the test animals will be compared with other treatment groups after feeding.

The positive control in this study used acetylsalicylic acid at a dose of 91 mg/kg body weight. The positive control was divided into two groups: the positive control group with acetylsalicylic acid given to the test animals before feeding and the positive control group with acetylsalicylic acid given to the test animals after feeding. The positive control group before feeding had the smallest average number of writhes among the other treatment groups, which was 3.00 ± 0.89. The percentage of writhing protection produced in the positive control group before feeding was 96.39% ± 1.08, while the positive control group after feeding obtained a percentage of writhing protection of 84.95% ± 1.87. The percentage of writhing protection produced in both positive control groups was greater than 50%, and a compound can be said to have analgesic activity if it can inhibit writhing induced by acetic acid by more than 50% compared to the negative control (Turner and Hebborn, 1971). Therefore, acetylsalicylic acid is stated to have analgesic activity and can serve as a reference group for reducing the number of writhes in the test animals. The statistical test results also showed that both positive control groups differed significantly when compared to the two negative control groups that did not exhibit analgesic activity in the test animals.

The average percentage of writhing protection in the positive control group before feeding compared to the average percentage of writhing protection in the positive control group after feeding also showed a significant difference (**Table 2**). The average percentage of writhing protection in the positive control group before feeding was significantly greater than the average percentage of writhing protection in the positive control group after feeding. This indicates that acetylsalicylic acid administered when the stomach is empty has significantly better analgesic activity compared to acetylsalicylic acid given when the stomach is filled with food. This is consistent with research conducted by Moore et al. (2015), which found that oral analgesics consumed with food can result in a less effective drug response. The presence of food in the stomach can prolong the absorption process of the drug and reduce its effects. Food entering the stomach can trigger drug-food interactions, where food

stimulates the secretion of gastric acid. The acidic environment in the stomach prompts the drug, initially in its intact molecular form, to convert into its ionized form. The form of the drug that can be absorbed by the body is the molecular form. In contrast, the ionized form of the drug is generally more difficult to absorb, thus the presence of food in the stomach reduces the absorption of the drug in its intact molecular form.

Table 2: Tamhane's Post-Hoc Test Results

	NC Before Feeding	NC After Feeding	PC Before Feeding	PC After Feeding	NHFCL Before Feeding	NHFCL After Feeding
NC Before Feeding		NSD	SD	SD	SD	NSD
NC After Feeding	NSD		SD	SD	SD	NSD
PC Before Feeding	SD	SD		SD	NSD	SD
PC After Feeding	SD	SD	SD		NSD	NSD
NHFCL Before Feeding	SD	SD	NSD	NSD		SD
NHFCL After Feeding	NSD	NSD	SD	NSD	SD	

Where:
NHFCL = n-Hexane Fraction of Coleus atropurpureus Leaves; SD = Significantly Different (p-value < 0.05); NSD = Not Significantly Different (p-value > 0.05); NC = Negative Control; PC = Positive Control

The percentage of writhing protection produced in the treatment group NHFCL at a dose of 252 mg/kg body weight before feeding was 92.53% ± 1.04. The n-hexane fraction of *Coleus atropurpureus* leaves administered to the test animals before feeding is stated to provide an analgesic effect. The statistical test results indicate that the analgesic activity of the NHFCL treatment group before feeding significantly differs when compared to the negative control group given before feeding. Meanwhile, the comparison of the NHFCL treatment group before feeding with the positive control group before feeding showed statistically non-significant results. Therefore, it can be stated that the n-hexane fraction of *Coleus atropurpureus* leaves at a dose of 252 mg/kg body weight administered before feeding has analgesic effects that are nearly equivalent to those of acetylsalicylic acid as a positive control given before feeding in the test animals.

The percentage of writhing protection produced in the NHFCL treatment group at a dose of 252 mg/kg body weight after feeding was 66.94% ± 4.24. The n-hexane fraction of *Coleus atropurpureus* leaves administered to the test animals after feeding is still stated to provide an analgesic effect. The statistical test results indicate that the analgesic activity of the NHFCL treatment group after feeding does not significantly differ when compared to the negative control group after feeding (**Table 2**). This suggests that the n-hexane fraction of *Coleus atropurpureus* leaves at a dose of 252 mg/kg body weight administered after feeding does not provide adequate analgesic effects. Due to the administration of food to the test animals before treatment, the analgesic effect produced by the n-hexane fraction of *Coleus atropurpureus* leaves was reduced to nearly equivalent to that of the negative control group. The statistical test results of the NHFCL treatment group after feeding compared to the positive control group after feeding showed non-significant differences, indicating that the NHFCL treatment group after eating still has analgesic effects, although its percentage of writhing protection is only 66.94% ± 4.24.

The treatment groups tested on the test animals were divided into the n-hexane fraction of *Coleus atropurpureus* leaves administered before feeding and the n-hexane fraction of *Coleus atropurpureus*

leaves administered after feeding. Both treatment groups were given the n-hexane fraction at a dose of 252 mg/kg body weight. The percentage of writhing protection produced in the NHFCL treatment group at a dose of 252 mg/kg body weight before feeding was 92.53% ± 1.04. Meanwhile, the percentage of writhing protection produced in the NHFCL treatment group at a dose of 252 mg/kg body weight after feeding was 66.94% ± 4.24. This indicates that both treatment groups were able to provide analgesic effects in the test animals, as the percentage of protection against acetic acid induction was greater than 50% compared to the negative control. The analgesic activity arising from the n-hexane fraction of *Coleus atropurpureus* leaves is due to the terpenoid compounds contained within. The terpenoid compounds in the n-hexane fraction of *Coleus atropurpureus* leaves can inhibit the production of prostaglandin E2 (PGE2) when tested in vitro, thereby preventing the formation of various inflammatory mediators such as IL-1β and TNF-α, and reducing pain events (Rahmadanita et al., 2022).

The statistical test results indicate that the percentage of writhing protection between the treatment group of the NHFCL administered before feeding and the treatment group of the NHFCL administered after feeding differ significantly, as evidenced by a p-value of less than 0.05 between the two treatment groups. This means that both treatment groups are capable of providing analgesic effects; however, gastric emptying time can influence the analgesic effects produced in both treatment groups. The percentage of writhing protection produced in the treatment group of the NHFCL before feeding is significantly greater than that in the treatment group of the NHFCL after feeding. This indicates that the interaction and drug-food interactions in the stomach can reduce the analgesic effects produced by the analgesic agent. The analgesic activity produced by an analgesic agent can be maximized when the analgesic compound is consumed on an empty stomach. The presence of food in the stomach can prolong the absorption process of the drug and reduce its effects. Food entering the stomach can trigger drug-food interactions, where food stimulates the secretion of gastric acid. The acidic environment in the stomach prompts the drug, initially in its intact molecular form, to convert into its ionized form. The form of the drug that can be absorbed by the body is the molecular form, while the ionized form of the drug is generally more difficult to absorb, thus the presence of food in the stomach reduces the absorption of the drug in its intact molecular form. Through this research, it can be concluded that gastric emptying time can enhance the analgesic response of the n-hexane fraction of *Coleus atropurpureus* leaves at a dose of 252 mg/kg body weight administered to female mice, suggesting that this preparation will tend to provide a more maximal effect when consumed on an empty stomach.

CONCLUSIONS

The gastric emptying time affects the analgesic response of the *Coleus atropurpureus* leaves n-hexane fraction. The percentage of writhing protection produced in the NHFCL treatment group at a dose of 252 mg/kg body weight before feeding was 92.53% ± 1.04. Meanwhile, the percentage of writhing protection produced in the NHFCL treatment group at a dose of 252 mg/kg body weight after feeding was 66.94% ± 4.24. The gastric emptying time enhances the analgesic response of the n-hexane fraction from the ethanol extract of *Coleus atropurpureus* leaves in female mice, which can be demonstrated by the significant difference (*p-value* <0,05) in the percentage of protection against pain response between the test animal groups that received the n-hexane fraction of *Coleus atropurpureus* leaves before feeding and those that received the n-hexane fraction after feeding.

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CONFLICT OF INTEREST

No conflicts of interest are declared in this study.

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Unraveling Kojic Acid's Molecular Targets: Implications for Dermatological Therapeutics

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ABSTRACT

Objectives: Melanogenesis is a complex biogenesis involving a synthesis pathway combined with the maturation and transport of melanin inside melanosomes. Kojic acid (KA) is one of the most well-known anti-melanogenesis agents, used to treat hyperpigmentation by inhibiting the tyrosinase enzyme in the melanin synthesis pathway. However, the molecular targets and their molecular cascade have not been fully identified and explained yet. This study is designed to identify KA's molecular targets and its molecular cascade for inhibiting melanogenesis in the skin.

Methodology: Genes related to pigmentation and melanogenesis in the skin, as well as genes affected by KA were collected from various databases. The Venn diagram result of these genes was further utilized to construct a protein-protein interaction network and gene clustering. The interaction between KA and TYR, as well as STAT5A were modeled using molecular docking using AutoDock.

Results: A total of 15 proteins were identified as the targets of KA. During KA treatment, the identified proteins work together in a coordinated manner to support its anti-melanogenesis activity.

Conclusion: This orchestrated action of KA on various protein targets leads to the inhibition of melanin synthesis and ultimately helps in reducing hyperpigmentation and brightening the skin tone.

Keywords: Melanogenesis; molecular pathway; skin; tyrosinase

INTRODUCTION

In Asian culture, having a light skin tone has been recognized as a beauty standard, leads to remarkable growth in the market for skin-lightening products (Qian et al., 2020). Skin tone is affected by intrinsic, such as genetics, skin type, and extrinsic factors, including the level of UV radiation and environmental stress (Costin and Hearing, 2007; Desmedt et al., 2016; Qian et al., 2020). Skin color originates from the stratum basal of the epidermis where melanocytes, melanin-producing cells, are located and transfer to keratinocytes (Costin and Hearing, 2007; Jeon et al., 2021). On average, the human epidermis has 74% eumelanin and 26% pheomelanin, and people with lighter skin have low

eumelanin degrees. Under UV stress, melanocytes produce and release melanin extensively leading to melasma, pigmentation, and post-inflammatory hyperpigmentation, which can cause aesthetic issues (Costin and Hearing, 2007; Diehl, 2014) Melanogenesis, the biosynthesis pathway of melanin, has also been found to enhance skin cancer progression (Slominski et al., 2022). Therefore, there is an urgent need for melanogenesis inhibitors to treat hyperpigmentation, brighten the skin tone, and prevent skin cancer.

Melanogenesis is an exceptionally intricate biogenesis in membrane-bound organelles called melanosomes, as the result of the budding vesicle of the trans-Golgi network and undergoing maturation in the cytoplasm (Costin and Hearing, 2007). Inside the melanosome, the synthesis of melanin commences with the hydroxylation of tyrosine, resulting in the formation of L-3,4-dihydroxyphenylalanine (DOPA) by tyrosinase (TYR). The DOPA then undergoes rapid oxidation, leading to the formation of DOPAquinone (DQ), then DQ undergoes self-cyclization and redox reaction to generate dopachrome (Li et al., 2022). Further, DQ tyrosine-related protein 1 (TRP1) converts dopachrome to 5,6-dihydroxy indole carboxylic acid (DHICA) which then, TRP2 converts to DHICA melanin (Gillbro and Olsson, 2011; Li et al., 2022). Indeed, TYR, TRP1, and TRP2 play crucial roles as key enzymes in the process of melanogenesis, and their expression is regulated by Microphthalmia-associated transcription factor (MITF) and cAMP response element-binding protein (CREB) transcription factor (Lee et al., 2021). Melanogenesis is additionally regulated by protein membrane with potassium-dependent sodium-calcium exchanger activity in trans-Golgi network and mitochondria, called Solute Carrier Family 24 Member 5 (SLC24A5) or Oculocutaneous albinism type 6 (OCA6) (Yousaf et al., 2020). Moreover, the maturation of melanosome depends on the presence of a structural fiber protein called Pmel17. It forms a complex with MART-1 (Melanoma antigen recognized by T cells 1)/MLANA for the deposition of eumelanin during stage I and II melanosomes (Knaust et al., 2020; Serre et al., 2018). During the maturation process, OCA2 and OCA4 help to maintain the pH inside of the melanosome by proton transport (Le et al., 2020). Those complex mechanisms suggest that the skin-lightening agent engages not only on one target as a TYR inhibitor but also possibly works on the other multiple molecular cascades.

Kojic acid (KA), 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (Figure 1), is one of the most popular anti-melanogenesis agents in the cosmetics market. It is a by-product of the metabolic product of fungi from Aspergillus oryzae named koji in Japan which was first isolated in 1907. The biological activity of KA is inhibiting as TYR to treat hyperpigmentation, antibacterial, antimicrobial, and anti-inflammatory agents (Phasha et al., 2022). Additionally, KA also showed NF-κB inhibition in keratinocytes suggesting to induce anti-melanogenic activity. Previous, bioinformatics data showed that KA induces the signaling of intracellular steroid receptors, glucose metabolizing enzyme, interleukin-15 (IL-15), adenylyl cyclase associated protein-1/2 (CAP1/2), and inflammatory mediator synthesizing enzyme; however, the molecular pathway to interfere melanogenesis process remains unclear (Wahab et al., 2021).

This study offered more comprehensive approaches to investigate molecular pathways involved in KA that affect hyperpigmentation in skin tissue. We employed STITCH and STRING to extract the database, as well as to build a protein-protein interaction (PPI) network of the affected genes. The proteins were clustered using CytoHubba, revealing found top 15 proteins that have strong connections to the KA in melanogenesis and hyperpigmentation. When skin tissue was irradiated with UV, KA modulated the Natural Killer Group 2D (NKG2D) receptor, thereby enhancing the killing activity of melanocyte, its target (Wang et al., 2021). Our study predicted that KA inhibits various cytokines such

as IL-2, IL-7, IL-15, and IL-18; leading to downstream effects to inhibit the MITF transcription factor through CREB and STAT5A. This inhibition prevents the expression of genes including TYR, TRP1, TRP2, and PMEL. Within the melanosome, KA hampers the function of TYR, TRP1, and TRP2, which are crucial for melanin synthesis. Interestingly, KA also influences the melanosome maturation process by suppressing PMEL and MLANA during stage I-II of melanosome development, as well as OCA2 and OCA4. The molecular docking of KA to TYR and STAT5A presented a low energy binding, with three and two hydrogen bonds. As a result, KA binding to TYR displayed five Van der Waals interactions, whereas no Van der Waals interactions were observed with STAT5A. Taken together, our study identified multiple molecular cascades involving various targets such as natural killer receptors, inflammatory cytokines, structural proteins, and protein membranes which impact melanosome maturation.

METHODS

1. Data Mining and Collection

Before conducting the analysis, protein/gen involved in the pigmentation and melanogenesis process was gathered from Pubmed (www.ncbi.nlm.nih.gov), OMIM (www.omim.org), and GeneCard (www.genecard.org). Subsequently, the proteins/genes affected by KA, both directly and indirectly, were screened from the STITCH database (www.stitch.embl.de). A Venn diagram (www.interactivenn.net) was then employed to determine the overlap of genes affected by KA in pigmentation and melanogenesis were determined (Heberle et al., 2015).

2. Protein-protein Interaction (PPI) Network and Gene Clustering Construction

The protein-protein interaction (PPI) was elucidated using the www.string-db.org platform, and subsequently, the data was analyzed through Cytoscape 3.9.1 and STRING-DB v11.5 software (Szklarczyk et al., 2015). To identify the top 15 genes, this study employed the MCC and Degree algorithm from the Cyto-Hubba plugin, which was used to classify as hub genes (Chin et al., 2014).

3. Molecular Docking

The STAT5A (7TVB) and Tyr (5M8O) proteins were obtained from rcsb.org. The ligand was taken out of STAT5A and Tyr using BIOVIA Discovery Studio 2021, and the control docking was made using AutodockTools 1.5.7. (www.scripps.edu). In contrast to the ligand, which received Gasteiger charges, the protein was protonated and given Kollman charges. The middle of the grid box's coordinates for STAT5A is at x = 19.712, y = 59.581, and z = 20.389 with several grid points being 40 x 40 x 40 Å; and for Tyr, they are at x = -26.889, y = -28.773, and z = 26.136 with number of grid points was 30 x 30 x 30 Å. The grid box's grid point spacing is 0.375. Using AutoDock 4.2 and the Lamarckian Genetic Algorithm (LGA) for 1000 rounds, docking was carried out. The final intermolecular energy, van der Waals, H-bond, desolvation, electrostatic, final total internal energy, torsional free, and unconstrained system energies were added to determine the free energy of binding. The docking parameter was considered valid if the RMSD value between the initial and post-docking poses was less than or equal to 2.0 Å (Allouche, 2011). The docking poses were visualized using BIOVIA Discovery Studio 2021. The new ligand (kojic acid) was prepared and docked using the same settings as the control dockings using PubChem (https://pubchem.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Kojic acid (KA), a pyranone molecule, has revealed excellent and widespread application in cosmetics (**Figure 1a**). A total of 10,748 genes were identified to be involved in the pigmentation and melanogenesis process, while 33 genes interfered with KA. Using Venn diagram analysis, we determined that KA affected 23 genes related to pigmentation and melanogenesis (**Figure 1b**).

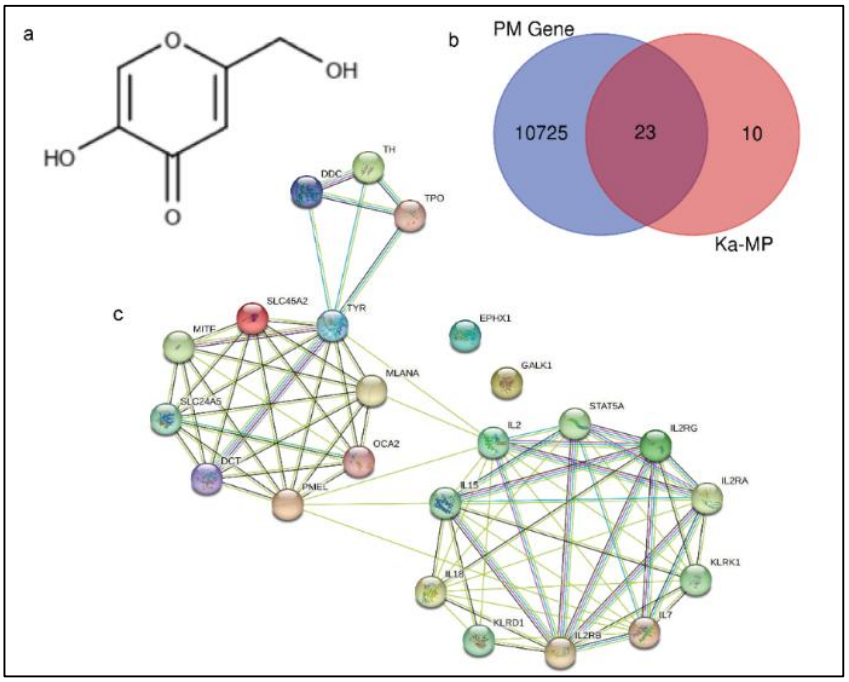

Figure 1. Kojic acid's top target genes and proteins related to skin melanogenesis and pigmentation.
a. Kojic acid's structure, b. Venn diagram of kojic acid and skin melanogenesis and pigmentation interfered genes,
c. Protein-protein interaction (PPI) network of the intersecting genes.

Hence, the protein-protein interactions (PPI) network analysis revealed that EPHX1 and GALK1 had no network with other proteins, while the interaction network involved a total of 31 genes. To delve deeper, Cytoscape was utilized to identify gene clusters on those genes. Employing the MCC and DMNC algorithm, the top 15 genes were categorized into two gene clusters. The two clusters are interconnected at 6 nodes, including PMEL, MLANA, TYR, IL7, IL2, and IL15 using the MCC algorithm (**Figure 2a**). Conversely, based on DMNC algorithms, there were two independent clusters, involving OCA2 and DCT instead of MITF and PMEL (**Figure 2b**). We ranked the 15 top genes based on MCC and DMNC algorithms, and their ranking is revealed in **Table 1**.

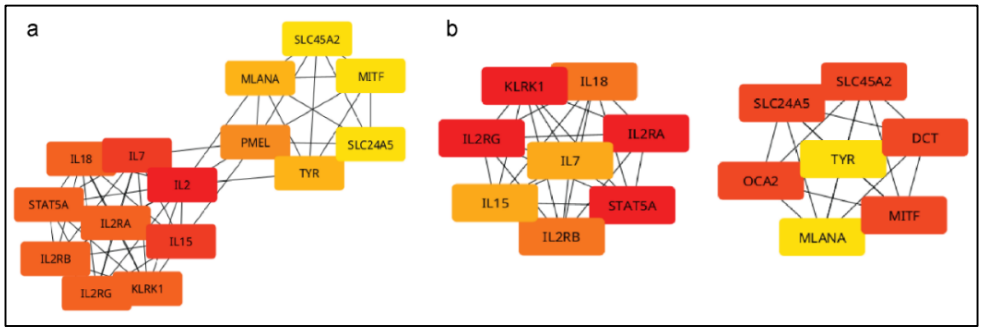

Figure 2. The clustering of the top 15 genes related to skin melanogenesis and pigmentation according to MCC and DMNC algorithm in CytoHubba.

Table : Top 15 proteins network interaction ranked by MCC and DMNC method 1.

No	Gene symbol	Gene/Protein name	Biological function related to melanogenesis and pigmentation	Ref.
1	IL-2	Interleukin-2	Secreted by Th1 and dendritic cells in skin, bind to the receptors and activate JAK1 and JAK3, which then activates STAT5.	(Kirken et al., 1995; Miyazaki et al., 1994)
2	IL-7	Interleukin-7	Secrete by keratinocytes, bind to the receptors, α and γ, to activate JAK1 and JAK3, which then activates STAT5 and MAPK pathway	(Hida et al., 2020; Sermaxhaj et al., 2022)
3	IL-15	Interleukin-15	Secreted by keratinocytes/ neutrophil bind to IL-2/IL-15Rβ/γc heterodimer receptor induces JAK1 activation that phosphorylates STAT3 via the β chain and JAK3 that phosphorylates STAT5.	(Chen et al., 2019)
4	IL-18	Interleukin-18	Secrete by keratinocyte to stimulate melanogenesis of melanocyte by activating the p38/MAPK and PKA pathway to elevate MITF expression.	(Fu et al., 2020)
5	IL2RB	Interleukin-2 receptor Subunit Beta (β)	Bind to inflammatory cytokine IL-2 then induces oligomerization of subtype β and γ receptors.	(Kirken et al., 1995; Miyazaki et al., 1994)
6	KLRK1	Killer Cell Lectin Like Receptor K1, encodes Natural Killer Group 2D (NKG2D).	NKG2D is a transmembrane receptor encoded by the killer cell lectin-like receptor subfamily K member 1 gene (Klrk1). Activation of NKG2D signaling in effector CD8 T cells kills melanocytes by recognizing stressed/damaged cells.	(Plaza-Rojas and Guevara-Patiño, 2021)
7	IL2RG	Interleukin-2 (IL-2) receptor Subunit Gamma (γ)	Bind to inflammatory cytokine IL-2 then induces oligomerization of subtype β and γ receptors.	(Kirken et al., 1995; Miyazaki et al., 1994)
8	STAT5A	Signal transducer and activator of transcription 5A	Transcription factor of tyrosinase (TYR), tyrosinase-related protein 1 (TRP-1), TRP-2	(Fu et al., 2020; Hossain et al., 2021)
9	IL2RA	Interleukin-2 receptor Subunit α	One of IL-2 receptor oligomer	(Kirken et al., 1995; Miyazaki et al., 1994)
10	PMEL	Premelanosome protein	An integral protein that determines the elongated shape characteristic of eumelanosomes and regulates fibril formation which is responsible for the ellipsoidal shape of melanosomes.	(Hida et al., 2020; Knaust et al., 2020)

In our study, we conducted further analysis involving molecular docking of KA and two selected top 15 genes: Signal transducer and activator of transcription 5A (STAT5A) and tyrosinase (TYR). To validate the parameters, we performed re-docking of native ligands for STAT5A and Tyr, resulting in a root mean square deviation (RMSD) of 1.94 and 1.13 Å (<2.0 Å), confirming the reproducibility of the docking parameters. Following the successful validation, the molecular docking of KA to STAT5A and Tyr was carried out. The overlap between the initial and post-docking of control docking was visualized in Figure S1 and the docking result revealed that KA formed a binding interaction with STAT5A, yielding a binding energy of -4.04 kcal/mol. The interaction involved three hydrogen bonds at Ser620, Ser622, and Asn642 residues, along with Van der Waals interaction at Asp621, Glu623, Thr628, and Leu643 residues. Furthermore, the interaction between KA and TYR displayed a weaker hydrogen bond

binding energy of -4.25 kcal/mol, involving 2 hydrogen bonds at Asn378 and Ser294 residues. Van der Waals interactions were observed at His192, His215, His377, Leu379, Leu382, Gly388, Gly389, Gln390, Thr391, Pro395, and Phe400 residues (**Table 2**). These findings suggest that KA could serve as a potential inhibitor for both TYR and STAT5A. The molecular interactions of KA docked within the active site pockets of TYR and STAT5A are visualized in the ribbon diagram. The predicted binding poses in both 2D and 3D diagrams, as depicted in **Figures 3a and 3b**.

Table 2: Top 15 proteins network interaction ranked by MCC and DMNC method 2.

No	Gene symbol	Gene/Protein name	Biological function related to melanogenesis and pigmentation	Ref.
11	TYR	Tyrosinase	Dominant melanin synthesizing enzyme using L-tyrosine, L-DOPA, and 5,6-dihydroxyindole as substrates.	(D'Mello et al., 2016; Solano, 2020)
12	MLANA	Melanoma antigen recognized by T cells 1	MART-1 and Pmel17 play an important role in melanogenesis by forming a complex, that regulates the expression, stability, trafficking, and processing of Pmel17, which in turn directs melanosome maturation.	(Oiso and Kaw, 2011)
13	MITF	Microphthalmia-associated transcription factor (MITF)	Dominant transcription factors in melanogenesis induce the transcription of the melanogenic genes, including TYR, TYRP-1, and TYRP-2.	(Shen et al., 2020)
14	SLC45A2/OCA4	Solute Carrier family 45 Member 2, Oculocutaneous albinism type 4	Regulate melanosome pH by proton exporting for melanosome maturation after OCA2 activity	(Bin et al., 2015; Le et al., 2020)
15	SLC24A5/OCA6	Solute Carrier family 24 Member 5, Oculocutaneous albinism type 6, NCKX5	An antiporter K^+-dependent Na^+ /Ca^{2+} exchanger that participates in melanogenesis. Located in trans-Golgi network or mitochondria.	(Le et al., 2021; Yousaf et al., 2020)
16	OCA2	Oculocutaneous albinism type 2	Melanosome-specific transmembrane protein to maintain chloride across the melanosome and exporting proton to control pH level in mature melanosome.	(Bellono et al., 2014; Yang et al., 2019)
17	DCT/TYRP2	Dopachrome Tautomerase, Tyrosinase related Protein-2	Enzyme thet converts 5,6-dihydroxyindole-2'-carboxylic acid (DHICA) to DHICA melanin in melanosome.	(Shen et al., 2020)

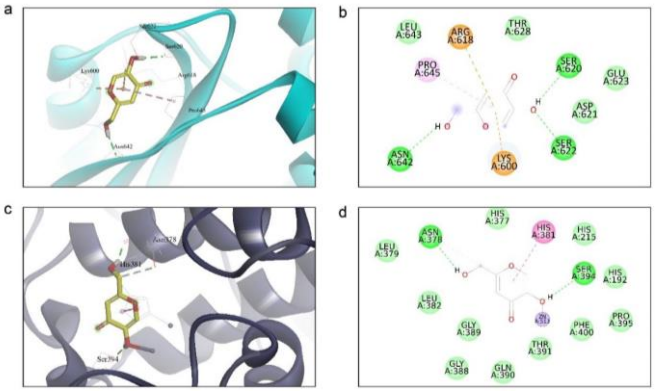

Figure 3. The binding poses of KA in TYR and STAT5A binding pocket in 3D view (a), and 2D view (b); and with TYR, in 3D view (c), and 2D view (d). Yellow, red, and white indicated carbon, oxygen, and hydrogen atoms.

Based on a comprehensive literature review, this study predicted a molecular cascade pathway, illustrated in **Figure 2**. This pathway involves inflammatory cytokines, namely IL-2, IL-7, IL-15, and IL-18; along with the α and γ type receptor of IL-2 (**Table 1, Figure 4**). Upon binding to their respective receptors, these cytokines initiated a molecular cascade inside the cytoplasm, subsequently activating the JAK1/JAK3 pathway. This activation, in turn, facilitates the activation of MITF. As a result, the active form of MITF translocates into the nucleus, where it functions as a transcription factor for various proteins in melanin synthesis, such as TYR, TYRP1, and TYRP2, as well as proteins associated with melanosome maturation, like PMEL. Additionally, the study suggests that KA could disrupt the function of PMEL/MLANA, which are crucial proteins involved in melanin maturation and fiber formation inside the melanosome. KA is also predicted to interfere with proteins responsible for maintaining ionic transport and pH levels essential for melanosome maturation, including OCA2, OCA4, and OCA6 (**Figure 4**). KA was found to inhibit KLRK1 which is activated by stress or UV irradiation in effector CD8 T cells and kills melanocytes (**Figure 4**).

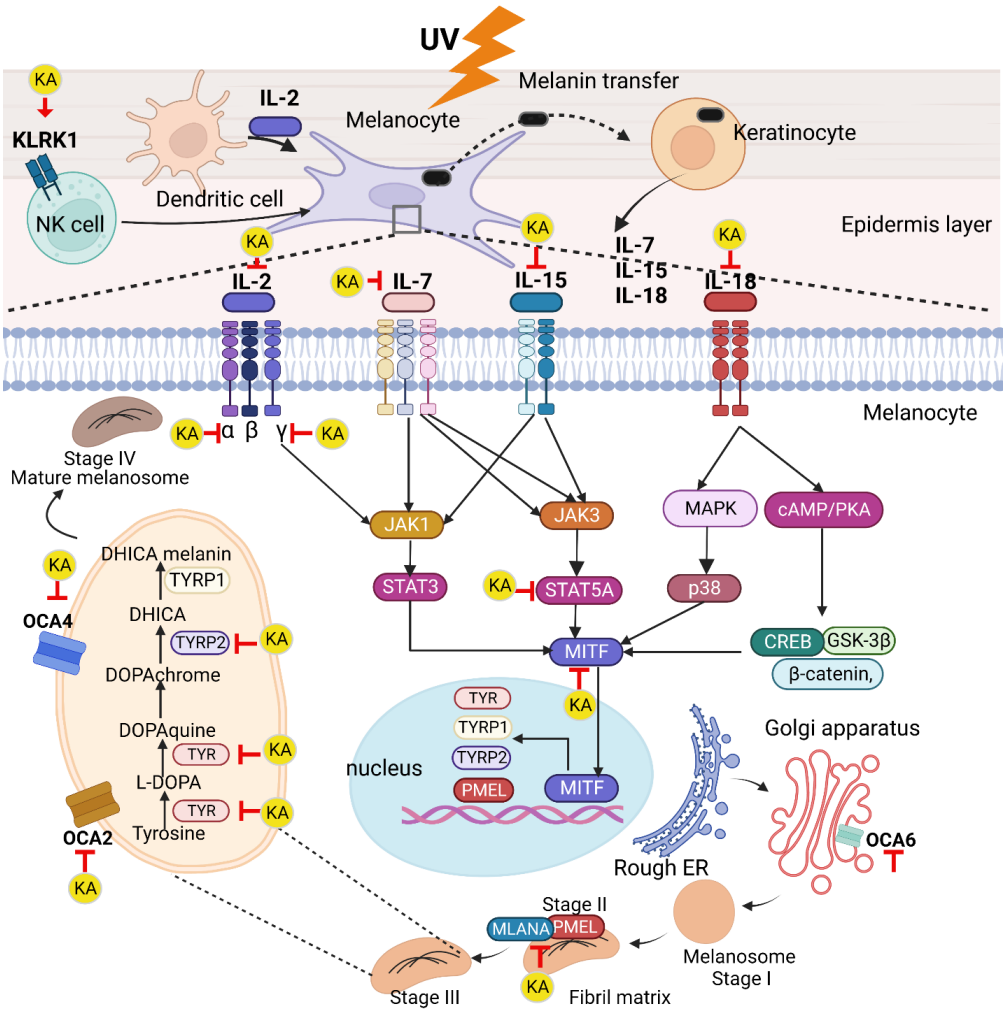

Figure 4: A molecular cascade of kojic acid (KA) in melanogenesis and pigmentation of the skin. NK cells: Neutral Killer cells, IL-2/7/15/18: Interleukin-2/7/15/18, TYR: tyrosinase, DOPA: L-dihydroxyphenylalanine, DOPAchrome:, DHICA: 5,6-dihydroxyindole-2'-carboxylic acid, PMEL: pre melanosome, JAK1/3: Janus Kinase 1/3, MAPK: mitogen-activated protein kinase, JNK: c-Jun NH2-terminal kinase, ERK: Extracellular signal-regulated kinase, MITF: microphthalmia-associated transcription factor (MITF), CREB: cAMP response element-binding protein, STAT5: Signal transducer and activator of transcription 5, TYRP-1/2: tyrosinase related-protein 1/2, OCA2/4/6: Oculocutaneous albinism type 1/4/6; Rough RE: rough endoplasmic reticulum; GSK-3β: Glycogen synthase kinase-3β OCA2: oculocutaneous albinism II. The graph was prepared using BioRender.

This study represents the first report investigating the impact of KA on molecular pathways associated with skin melanogenesis and pigmentation, spanning both upstream and downstream proteins of molecular pathways. Melanogenesis involves the biogenesis of melanin within the melanocyte, which is subsequently transferred to the keratinocyte in the basal layer of the skin's epidermis. While previous studies exclusively focused on TYR and NF-κB inhibition activities, they solely observed the molecular pathway of KA activity [37]. Upon UV exposure reaching the epidermis, cellular stress is induced, leading to the upregulation of the NKG2D receptor, which is encoded by Killer Cell Lectin-like receptor K1 (KLRK1) [38]. It is found in various mammalian cells, including Natural Killer (NK) cells, Natural Killer T (NKT) cells, certain subsets of gamma delta T cells, and a small proportion of CD4+ T cells. When the T cells are active, they are navigated to precisely locate melanocytes and induce their apoptosis, resulting in skin depigmentation [28, 39]. KA has been observed to modulate the activity of NKG2D, suggesting a potential elevation in melanocyte recognition and subsequently apoptotic activity.

Interestingly, KA also exhibited targeting capabilities towards various cytokine-related inflammatory; namely IL-2, IL-7, IL-15, and IL-18. These cytokines subsequently bind to the receptors, initiating a downstream signaling pathway in the cytoplasm. It is widely acknowledged that hyperpigmentation is often linked to various skin inflammation processes. Recent studies have shed light on a significant relationship between secreted inflammatory cytokines and skin pigmentation (Fu et al., 2020; Hossain et al., 2021). Upon exposure to UV stress, dendritic cells secrete IL-2, whereas keratinocytes and fibroblast release IL-7, IL-15, and IL-18 (Chen et al., 2019; Fu et al., 2020). The binding of IL-2 to its heterotrimeric, composed of α, β, and γ, on the melanocyte membrane lead to tyrosine phosphorylation of Janus kinase-1 (JAK1). This activation subsequently upregulates STAT3 activity which in turn triggers MITF expression. In addition to IL-2, the α and γ subunit receptors of IL-2 have also been investigated as potential targets of KA. Subsequently, the downstream effector, active MITF, undergoes translocation to the nucleus and serves its functions as a transcription factor. MITF is responsible for regulating the expression of key enzymes involved in melanin synthesis, such as TYR, TRP1, and TRP2; as well as protein essential for structure integrity of melanosome maturation; PMEL (Gillbro and Olsson, 2011; Slominski et al., 2004).

Another signaling pathway targeted by KA is Janus kinase-3 (JAK3) which is triggered by IL-2, IL-7, and IL-18. Kirken and colleagues (1995) revealed that IL-2 has a stronger affinity for activating JAK3 than JAK1 (Kirken et al., 1995). Once phosphorylated, JAK3 subsequently leads to the activation of STAT5A, which turn triggers MITF expression in a manner similar to the previously discussed cascade. Remarkably, the effector proteins in this pathway including STAT5A and MITF, are directly interfered with KA. Furthermore, the molecular cascade related to IL-7 and IL-15 during pigmentation is switched off by KA. Pigmentation-related cytokine: IL-18, is involved as a KA target and impeded the MAPK pathway. Consequently, p38 phosphorylation decreased and failed to activate MITF. Another molecular cascade of IL-18 inhibited by KA is the cAMP/PKA pathway. Thus, the CREB and β-catenin activation, suppress GSK-3β activation, causing elevated MITF expression.

During melanin biosynthesis, KA comprehensively suppresses most of the enzymes related to the melanin synthesis process TYR, TYRP1, and TYRP2 inside of the melanosome. Else, KA interferes with the melanosome maturation starting from the early stage of the budding trans-Golgi network by retarding the OCA6 protein membrane, the K^+-dependent Na^+ /Ca^{2+} exchanger. Previous study predicted Ca^{2+} transport from the cytoplasm into the melanosome using a Na^+ gradient that is possibly by coupling to a Na^+/H^+ exchanger an ATPase. It caused the pH increase inside the melanosome to provide an ideal environment for melanogenesis in the pH range of 7 to 8 is the optimum condition for tyrosinase (Zeng

et al., 2017). It had shown by the pH of melanocytes derived from Negroid donors was higher than Caucasian donors. The other protein transporters embedded in the melanosome membrane which interfere with KA are OCA2 and OCA4. OCA2 is an anion channel to transport chloride, coupling to proton motive force to maintain the optimal luminal pH for the tyrosinase in melanogenesis (Bellono et al., 2014). A similar function is supported by OCA4 that works to transport sugar from melanosome to cytoplasm with proton transport by the proton pump. As a consequence, melanosome pH elevated and admitted copper to bind to Apoenzyme tyrosinase (Tóth et al., 2017).

During the melanosome maturation, KA interrupted structural fiber proteins: PMEL and MLANA (MART). PMEL or Pmel17 (also known as GP100 or SILV), the protein expression regulated by MITF, forms fibers as a matrix for melanin deposition inside melanosome (Knaust et al., 2020). Pre melanosome (melanosome stage I and II) is determined by the formation of fibril Pmel17, yet it had no TYR and TRP. During the melanin synthesis in melanosome stage III, newly synthesized melanin is deposited along the fiber (Giordano et al., 2009). A study by Hearing and colleagues reported that MART-1 is bound to Pmel17 as a strong complex, and plays a role in regulating Pmel17 stability, trafficking, and processing (Hoashi et al., 2005). However, the detailed function of MART-1 remains questioned.

To sum up, the proteins were identified as the targets of KA, including NKG2D receptor, inflammatory-related interleukins (IL-2, IL-7, IL-15, and IL-18), melanin synthesizing enzyme (TYR, TRP1, TRP2), melanosome maturated protein (OCA2, OCA4, OCA6), and melanosome related structure for maturation (PMEL, MLANA). Those proteins are orchestrated during the KA treatment to support anti-melanogenesis activity. This study briefly gives the first insight into the dermatology field of KA molecular mechanisms in anti-pigmentation treatment.

CONCLUSIONS

In conclusion, our bioinformatics study sheds light on the multiple molecular targets and cascade of kojic acid (KA) in inhibiting melanogenesis, offering insights into its mechanism of action in treating hyperpigmentation and brightening skin tone. Through the analysis of genes related to pigmentation and melanogenesis, we identified 15 proteins targeted by KA, including key enzymes involved in melanin synthesis and melanosome maturation, as well as immune-related proteins and cytokines. Molecular docking simulations further supported the interaction between KA and specific targets, such as TYR and STAT5A. These findings suggest that KA exerts its anti-melanogenic effects through a coordinated action on various molecular pathways involved in melanogenesis regulation. However, it is essential to acknowledge the limitations of our study, including reliance on computational predictions and the need for experimental validation of identified targets. Future research should focus on confirming the interactions between KA and its targets and elucidating the precise mechanisms underlying its therapeutic effects in dermatology. Overall, our study contributes to the understanding of KA's role in melanogenesis inhibition and provides a basis for further exploration of its clinical applications in treating skin pigmentation disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Iodimetric Titration versus Colorimetric Analysis of Ascorbic Acid in Health Supplements: A Pharmacopoeial Compliance Study

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ABSTRACT

Ascorbic acid (vitamin C) is widely recognized for its role in supporting immune function and its potential in protecting against COVID-19 infection. Consequently, global consumption of ascorbic acid-containing health supplements has increased, including in Indonesia, especially during the COVID-19 pandemic. This study aimed to compare the analytical accuracy of two commonly used methods, i.e. iodimetric titration and colorimetric analysis, for determining ascorbic acid concentrations in health supplement of 1000 mg ascorbic acid. Both methods leverage ascorbic acid's reducing properties, which will reduce iodine in iodimetric titration and 2,6-dichloroiondophenol in colorimetric analysis. The results demonstrated that iodimetric titration yielded concentration range between 98.11% and 101.57%, while the colorimetric method produced values ranging from 96.75% to 99,75%. All results complied with the Indonesian Pharmacopoeia standards, which require active substance levels to be within 90.0% to 110.0% of the labeled amount. The findings indicate the iodimetric titration offers accuracy comparable to the colorimetric method and is a reliable alternative for routine quality control of ascorbic acid in health supplements.

Keywords: Accuracy; Indonesian Pharmacopoeia; Reducing agent

INTRODUCTION

A novel flu-like coronavirus (COVID-19) was found at the end of 2019 in China (Cohen & Normile, 2020) and close contact was caused human-to-human transmission (Li et al., 2020). It is urgent to find an alternative solution to prevent and control the virus spread, due to no registered treatment or vaccine. The global surge in demand for immune-boosting supplements, particularly during and after the COVID-19 pandemic, has intensified the need for reliable and accurate methods to quantify ascorbic acid (vitamin C) in health products (Ball, 2006). Ascorbic acid has various pharmacological activities, such as immunomodulator, antioxidant, antimicrobial, antiviral, anti-parasite, and anti-fungal activities (Mousavi et al., 2019). This vitamin was proved to be support immune functions and protect against COVID-19 infection (Hemila, 2003). The immunostimulant activity due to infection occurs through enhancing T-lymphocytes proliferation, which increasing cytokine

production and immunoglobulin synthesis (Naidu, 2003). While the antiviral activity occurs through increased IFN-IL-1α/β production (Kim et al., 2013).

Humans lost the capability to biosynthesize ascorbic acid from glucose in the liver, due to lack of L-glucuno-γ-lactone oxidase (De Tullio, 2012; Fujii, 2021; Sorice et al., 2014). This vitamin is an essential dietary (Bekele & Geleta, 2015) nutrient for the collagen biosynthesis and co-factor in the biosynthesis of cholesterol, catecholamines, L-carnitine, amino acids, and some peptide hormones (Grosso et al., 2013). The requirement of ascorbic acid per day is 25-40 mg for infants, 70 mg for adults, 90 mg for pregnant women, and 110 mg for breastfeeding women. These requirements increase to 300 mg/day in certain circumstances, such as exercise, malignant tumors, and infections (Sorice et al., 2014). Ascorbic acid is easily reduced by heat and oxygen exposure during processing, packaging, and storage of food (De Tullio, 2012). Because of, ascorbic acid highly sensitive to oxidation, light, and heat, making its stability and accurate quantification crucial for ensuring product efficacy and compliance with regulatory standards (Ball, 2006).

There are many methods for ascorbic acid quantification, such as titrimetry (Bekele & Geleta, 2015), voltammetry (Pisoschi et al., 2011), spectrophotometry (Al Majidi & Al Qubury, 2016; Arya et al., 1998; Revanasiddappa & Veena, 2008), high performance liquid chromatography (Frenich et al., 2005; Nour et al., 2015), enzymatic (Casella et al., 1989), etc. In Indonesia, where the supplement market is rapidly expanding, adherence to the Indonesian Pharmacopoeia standards, i.e., 90.0%–110.0% of the labeled content, is essential for consumer safety and product quality assurance (Department of Health, 2020). Iodimetric titration and colorimetric analysis are two commonly used techniques for ascorbic acid determination, yet discrepancies in accuracy, cost-effectiveness, and operational simplicity raise questions about their suitability in different laboratory and manufacturing contexts (Arya et al., 2000). Therefore, a comparative study is urgently needed to assess the analytical performance of these methods and guide the selection of appropriate quality control techniques in line with pharmacopoeial compliance.

This study aimed to compare the analytical accuracy of two commonly used methods, i.e. iodimetric titration and colorimetric analysis, for determining ascorbic acid concentrations in health supplement of 1000 mg ascorbic acid. Both methods leverage ascorbic acid's reducing properties, which will reduce iodine in iodimetric titration and 2,6-dichloroiondophenol in colorimetric analysis. This was interesting to do, due to the colorimetric method is an easy and fast method, but more expensive than the iodometric titration. The iodometric method is simpler and without instruments, so cheaper than the colorimetric method. This paper was focused on quantification of ascorbic acid in health supplements, which marketed in Bandung, Indonesia.

METHODS

1. Materials

Ten different brands of health supplements containing 1000 mg of ascorbic acid, which marketed in Bandung, Indonesia, were tested. Health supplements were selected and purchased from pharmacies with the same batch number. The samples were one solution, seven effervescent tablets, and two effervescent granules.

Ascorbic acid Indonesian pharmacopoeia reference standard (IPRS) was purchased from the National Agency of Drug and Food Control of Republic of Indonesia. All substances were analytical

grade and purchased from Merck, i.e. sulfuric acid, starch, 2,6-dichlorophenol indophenol, sodium citrate, iodine, potassium iodide, arsenic trioxide, sodium bicarbonate, sodium hydroxide, methyl orange, and hydrochloric acid.

2. Sample Preparation

Solution: The solutions were measured and equivalent to 50 mg of ascorbic acid, placed in a 100 ml volumetric flask. Diluted with distilled water to volume (Department of Health, 2020).

Effervescent granules and tablets: Twenty tablets or granules were placed in 1000 ml of volumetric flask containing a mixture of distilled water and 2 N sulfuric acid, at a ratio of 4:1, then shaken mechanically for 30 min until the granules or tablets were disintegrated completely. The mixture was diluted with distilled water to volume. About 10 ml of the solution was transferred into a centrifuge tube, and centrifuged with Hettich EBA 200 centrifuge for 15 min (Department of Health, 2020).

3. Analysis of Ascorbic Acid Concentration

Iodometric titration: Weighed 400 mg of ascorbic acid IPRS with Ohaus Pioneer balance, then dissolved in a mixture of 100 ml of distilled water and 25 ml of 2 N sulfuric acid. The solution was added to 3 ml of starch and titrated immediately with 0.1 N iodine. 1 ml of 0.1N iodine is equivalent to 8.806 mg of ascorbic acid (Department of Health, 2020).

Standardization of 0.1 N iodine: Weighed 150 mg of arsenic trioxide, which had been dried at 105 C for 1 h, dissolved in 20 ml of 1 N sodium hydroxide. Diluted with 40 ml of distilled water, added 2 drops of methyl orange and diluted hydrochloric acid, until the yellow color changed to pink. Added 2 g of sodium bicarbonate, 10 ml of distilled water and 3 ml of starch. The solution was titrated with 0.1 N iodine until a steady blue color. Calculate the normality of the solution. 1 ml of 0.1 N iodine is equivalent to 4.946 mg of arsenic trioxide (Department of Health, 2020).

Colorimetric method: Weighed 100 mg of ascorbic acid IPRS, then dissolved in a 50 ml of volumetric flask. Five various concentrations of ascorbic acid IPRS (0.1, 0.2, 0.4, 0.8, 1.6 mg/ml), each 1 ml, were reacted with 1 ml of 4.37% sodium citrate and 2 ml of 2,6-dichlorophenol indophenol solution, incubated for 30 sec. Absorbance was measured at 518 nm with Analytik Jena – Specord 200 spectrophotometer and a calibration curve was generated (Chang et al., 2016).

Sample preparation for assay: About 4 ml of sample solution equivalent to 2 mg of ascorbic acid was used for iodometric method, and 2 ml of sample solution equivalent to 1 mg of ascorbic acid was used for colorimetry. The assay of ascorbic acid concentration in the sample was determined by the same method for ascorbic acid IPRS (Chang et al., 2016; Department of Health, 2020).

4. Data Analysis

Data are displayed as means and standard deviations from three repetitions. Data were analyzed using SPSS 21. Moreover, statistical analysis was performed using one-way ANOVA, repeated measures ANOVA for parametric analysis, and Kruskal–Wallis and Friedman tests for nonparametric analysis ($p < 0.05$).

RESULTS AND DISCUSSION

1. Sample Preparation

Fruits, vegetables, and animal organ, such as liver and kidney, are the best sources of ascorbic acid (Bidlack, 2000). Ascorbic acid is the immune system booster (Mousavi et al., 2019). It has led to an increased consumption of ascorbic acid by Indonesian people during the COVID-19 pandemic. As a result of an increased market demand, without an increased production, the price of ascorbic acid was increased up to 40% (Liputan6.com, 2020). In this study, 10 health supplements were selected with 1000 mg of ascorbic acid in the form of solutions, effervescent granules and tablets, which often consumed by Indonesian people during and after the COVID-19 pandemic.

2. Analysis of Ascorbic Acid Concentration

Analyzing the 1000 mg ascorbic acid supplements that are marketed in Indonesia was crucial for several scientific, health, and regulatory reasons. In Indonesia, like many countries, supplement labeling must accurately reflect content**.** Analysis helps verify whether the actual ascorbic acid content matches the label claim of 1000 mg**.** These were important, because ascorbic acid overdosing may lead to gastrointestinal distress or kidney stones, but underdosing may render the supplement ineffective for its intended health benefits (Jacob & Sotoudeh, 2002). The National Agency of Drug and Food Control (Badan Pengawas Obat dan Makanan**,** BPOM**)** regulates all marketed supplements in Indonesia. Analytical testing is required to ensure that (a) products meet pharmaceutical-grade specifications, (b) No degradation of ascorbic acid has occurred due to heat, light, or moisture, (c) any adulteration or contamination **(**e.g., with other antioxidants or non-declared drugs) is detected (NADFC, 2022). Ascorbic acid is sensitive to oxidation and can degrade over time. So, analyzing was conducted to assess the product stability during storage and determine the expiration date validity. This was especially relevant in Indonesia's humid and tropical climate, which accelerates degradation (Yin et al., 2022). From the side of consumer safety and public health protection, high-dose ascorbic acid supplements are popular during flu seasons and the COVID-19 pandemic. However, doses above 2000 mg/day may cause adverse effects. Then, it is necessary continuous monitoring protects the public from misleading health claims or unsafe formulations (EFSA et al., 2024).

Accurate quantification of ascorbic acid in health supplements is essential to ensure product quality, regulatory compliance, and consumer safety. Iodimetric titration and colorimetric analysis are among the most commonly employed methods due to their basis in redox chemistry, leveraging the strong reducing property of ascorbic acid (Arya et al., 2000). However, each method has distinct advantages and limitations. Iodimetric titration is known for its simplicity, low cost, and suitability for routine analysis in resource-limited settings, but it can be affected by the presence of interfering substances and requires careful endpoint detection (Elgailani et al., 2017). On the other hand, colorimetric methods offer higher sensitivity and can be automated for high-throughput analysis, though they often require specialized reagents and instruments, and may be more costly (Mohammed et al., 2009). A comparative evaluation is therefore critical to determine which method provides better accuracy, reproducibility, and compliance with pharmacopeial standards under different laboratory conditions. This comparison supports evidence-based decision-making for method selection in pharmaceutical quality control laboratories.

Ascorbic acid is a good reducing agent, so iodometric titration with iodine can be used to ascorbic acid determination. Iodine was reduced to iodide by ascorbic acid. After all ascorbic acid has been

oxidized, the excess iodine was reacted with starch indicator to form the blue-black-iodine complex as the endpoint (Wright, 2002). The colorimetric method is based on the reduction of 2,6-dichloroindophenol by ascorbic acid. The 2,6-dichlorophenolindophenol solution is blue, which change to colorless, due to chromophores disappeared when reduced by ascorbic acid (Nielsen, 2010). Ascorbic acid undergoes oxidized to dehydroascorbic acid. In the colorimetric method, a decrease in the absorbance of 2,6-dichlorophenolindophenol was observed due to reduction by ascorbic acid (Ciuti & Liguri, 2017).

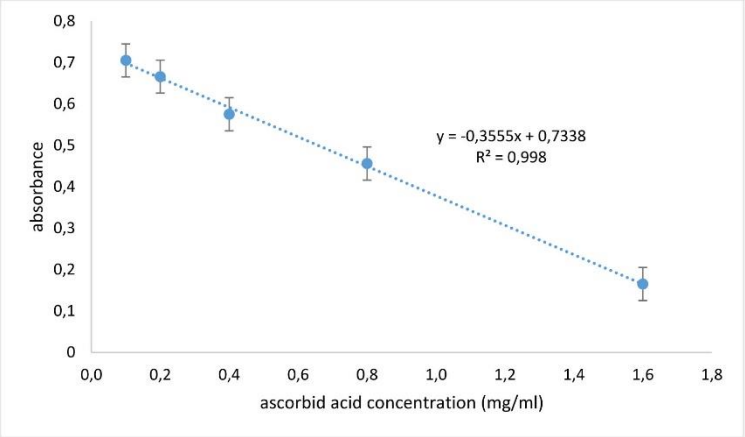

Figure 1. Calibration curve of ascorbic acid IPRS (n = 3)

The linearity of the calibration curve of ascorbic acid IPRS showed the correlation degree between ascorbic acid concentration with detector response. **Figure 1** showed a good correlation between ascorbic acid concentration and detector response (r = 0.998). This value was accorded with literature (Borman & Elder, 2017).

Table 1: Concentration of Ascorbic Acid in the Health Supplements (n = 3)

Sample	Calculated mass of ascorbic acid (mg)	
	Iodometric titration	Colorimetric method
A	984.5 ± 0.02	987.5 ± 0.01
B	998.5 ± 0.03	997.5 ± 0.01
C	981.1 ± 0.01	980.2 ± 0.02
D	985.2 ± 0.02	990.3 ± 0.01
E	1003.5 ± 0.01	999.1 ± 0.02
F	987.9 ± 0.04	987.5 ± 0.03
G	993.4 ± 0.05	984.3 ± 0.02
H	1000.1 ± 0.05	985.4 ± 0.01
I	1015.7 ± 0.02	998.5 ± 0.01
J	1014.1 ± 0.01	997.5 ± 0.02

The iodometric titration and colorimetric method based on oxidation reduction reactions. Ascorbic acid as a reducing agent will reduce iodine and 2,6-dichloroindophenol, so that the mass of ascorbic acid in the sample can be calculated (**Table 1**). All ascorbic acid concentration in the sample met the requirement, i.e. 90-110% of those listed on the label (Department of Health, 2020). The percentage range using iodometric titration was 98.11-101.57%, while using colorimetric method was 96.75-99.75%. Both iodometric and colorimetric methods demonstrated high precision, as evidenced by the low standard deviation values (≤ ±0.05 mg). This indicates that both methods are reproducible and suitable for routine quality control purposes. In the ascorbic acid determination by iodometric titration,

there were three samples with concentrations of more than 1000 mg. This was due to the excess titrant droplets, which can resolve by using a microburette or using a more dilute titrant, i.e. 0.05 N iodine.

Although both methods provided values within the pharmacopoeial limits, iodometric titration consistently reported slightly higher concentrations across most samples. This difference, while minimal, may be attributed to methodological factors**.** Iodometric titration measures ascorbic acid via its direct reducing action on iodine, which can sometimes be influenced by the presence of other reducing agents in complex matrices (Arya et al., 2000). Meanwhile, colorimetric methods, such as those using 2,6-dichlorophenolindophenol (DCPIP) or Fe^{3+}-based complexes, are often more specific but less robust in the presence of sample turbidity or color interference (Ciuti & Liguri, 2017).

Both methods are pharmaceutically acceptable for determining ascorbic acid content in dietary supplements, and can be selected based on context of **i**odometric titration is advantageous for its simplicity, cost-effectiveness, and minimal equipment requirements. It is ideal for routine QC in low-resource settings. While, colorimetric methods offer higher sensitivity and may be more suitable for automated or high-throughput laboratory settings, albeit with greater operational cost and complexity**.**

Given the growing market for immune-boosting supplements in the post-pandemic era, particularly those containing ascorbic acid, selecting an accurate, validated, and fit-for-purpose analytical method is essential for ensuring product integrity and compliance with pharmacopoeial standards (Ball, 2006; Department of Health, 2020).

Statistical analysis showed that there was no difference between the ascorbic acid concentration using iodometric titration and colorimetric method ($p = 0.22$). This showed that these two methods have the same accuracy. Limitation of these methods was the excipient particles, which can interfere the ability to determine the end point of iodometric titration and cause the cloudy solution, which interferes with the absorbance measurement in colorimetric method. This problem was resolved by centrifugation to remove excipient particles.

CONCLUSION

Analyzing 1000 mg ascorbic acid marketed in Indonesia was necessary for regulatory compliance, health safety, scientific integrity, and consumer trust. It ensures that supplements are safe**,** effective, and truthfully labeled; producers adhere to government's standards and international best practices, and the Indonesian public receives health products of consistent quality. This comparative analysis demonstrated that both iodometric and colorimetric methods produce accurate, precise, and pharmacopeially compliant results for the determination of ascorbic acid in health supplements. The choice between methods should consider the operational context, such as equipment availability, throughput demands, and cost constraints.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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From Ethnomedicine to Potential Herbal Product: Standardization of *Uvaria Rufa* Blume Bark

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ABSTRACT

Uvaria rufa Blume, locally known as lelak, is an ethnomedicinal plant traditionally used in East Nusa Tenggara, Indonesia, to treat conditions such as fever, bleeding, and skin allergies. To support its development as a raw material for herbal medicines, standardization is crucial to ensure its safety, efficacy, and quality. This study aimed to establish a standardization profile to ensure the consistent quality of *U. rufa* bark sourced from East Nusa Tenggara. A descriptive experimental method was used to determine specific and non-specific parameters of the bark. Specific parameters included identification of the plant part (bark), macroscopic characteristics (8–10 cm long, dark brown exterior, yellow interior, fibrous texture), microscopic features (parenchyma with stone cells, sclerenchyma, cork tissue), and organoleptic properties (light brown color, slightly astringent, aromatic, fine powder). The water-soluble extractive value was 12.0160% ± 0.0090, and ethanol-soluble was 22.475% ± 0.0111. Non-specific parameters assessed safety and stability: loss on drying (7.26% ± 0.8723), moisture content (9.4% ± 0.2000), total ash content (2.601% ± 0.0002), and heavy metal levels (Pb: 0.0472 ppm, Cd: 0.0014 ppm). All results met the general requirements for herbal standardization, supporting *U. rufa* bark's potential as a standardized herbal medicine ingredient.

Keywords: East Nusa Tenggara; Ethnomedicine; Standardization; *Uvaria rufa* Blume.

INTRODUCTION

Indonesia is a country rich in natural resources, including those derived from plants, animals, and microorganisms. One of these resources is medicinal plants, which have been widely developed and utilized for therapeutic purposes (Setyani et al., 2021; Hermawati et al., 2023). A native Indonesian plant commonly used in traditional medicine is lelak (*Uvaria rufa* Blume) from the Annonaceae family. People in East Nusa Tenggara, in particular, have long used this plant for medicinal purposes.

In traditional medicine, *U. rufa* is known to exhibit pharmacological effects that can help treat various health conditions (Alimboyoguen et al., 2023). Different parts of the *U. rufa* plant have

medicinal applications: the bark and roots are used to reduce fever, decoctions of the fruit are used to treat skin allergies and intestinal ulcers, and decoctions of the stem are traditionally used by Thai communities to treat benign prostatic hyperplasia (BPH) (Buncharoen et al., 2016). Its flavonoid compounds are reported to have the potential to inhibit the formation of advanced glycation end products (AGEs), and its alkaloid content is believed to have anticancer potential (Thang et al., 2014).

Given its therapeutic uses, the development of herbal medicines from natural ingredients holds great potential. However, the development of plant-based medicines requires a standardization process to ensure the quality, safety, and efficacy of the medicinal raw materials (Faramayuda et al., 2021). Standardization is a crucial step in the development of natural medicines to ensure consistency in chemical profiles, pharmacological activity, and quality assurance during production (Setyani et al., 2021).

The potential use of *U. rufa* bark as a medicinal plant highlights the need for standardization. To date, no standardization has been conducted for this material, and it has not yet been included in the official monograph published by the Indonesian Ministry of Health(Andini & Putri, 2021). Therefore, this study aims to conduct standardization of specific and non-specific parameters of *U. rufa* bark simplicia to determine the values of each parameter according to the standardization procedures established by the Ministry of Health of Indonesia (Depkes RI, 2000; Andini & Putri, 2021).

METHODS

1. Materials

Powdered *U. rufa* bark were obtained from Kupang, East Nusa Tenggara, and identified in Phytochemistry Laboratory, Widya Mandira Catholic University. Aquadest, chloroform, 70% ethanol, nitric acid, and chloral hydrate were purchased from Merck (Darmstadt, Germany).

2. Instrumentations

Fume hood, Atomic Absorption Spectrophotometer (AA240 Varian), Oven (Binder), Moisture Analyzer (HC103), Hot Plate (Cimarec Thermo Scientific), Analytical Balance (Pioneer Ohaus), Porcelain Cup, Erlenmeyer Flask (250 mL, Schott Duran), Beaker Glass (100 mL and 250 mL, Herma), Measuring Cylinder (100 mL, Iwaki), Glass Funnel, Microscope Slide, Stirring Rod, and Volumetric Flask (25 mL, Iwaki).

3. Research Prosedures

3.1 Preparation of Simplicia

The preparation of simplicia involved two main steps: drying and powdering. The *U. rufa* bark was first cut into smaller pieces, then dried by sun exposure and air circulation for several days. Once subjectively assessed as dry, the material was sorted and ground into a fine powder to achieve a uniform simplicia consistency. Upon completion, the simplicia was ready for standardization.

3.2 Standardization of Simplicia

Standardization was carried out based on procedures from the Indonesian General Standard Parameters for Medicinal Plant Extracts (Indonesian Ministry of Health, 2000), covering both specific and non-specific parameters.

4. Specific Parameters

4.1 Identity

The identity test involves determining the botanical name, the plant part used, and its common Indonesian name.

4.2 Organoleptic Test

This test was conducted by evaluating the sample's shape, smell, taste, and color using the senses. A small amount of the sample was placed in a porcelain cup, and descriptive observations were recorded.

4.3 Macroscopic Observation

Macroscopic characteristics such as shape, size, and texture were observed with the naked eye. Measurements were taken using a ruler and the findings were documented.

4.4 Microscopic Observation

Microscopic examination was performed using a trinocular microscope connected to a computer. A thin layer of the powdered simplicia was placed on a slide, moistened with chloral hydrate, covered with a cover slip, and examined. Visible structural fragments were identified and documented.

4.5 Water and Ethanol-Soluble Extract Content

Two grams of simplicia were placed into an Erlenmeyer flask containing 100 mL of either water-chloroform (2.5 mL chloroform in 1 L water) or 70% ethanol. The mixture was shaken for 6 hours and then soaked for 18 hours. Twenty milliliters of the filtrate were evaporated at 105 °C to constant weight. The yield was calculated according to Equation 1.

5. Non-Specific Parameters

5.1 Loss on Drying

Two grams of simplicia were weighed and placed into a moisture analyzer (Mettler Toledo HC103), set at 105 °C. The sample was heated until a constant weight was reached. Results were expressed as percentage weight loss and the test was repeated three times (Ma'arif et al., 2023).

5.2 Moisture Content

Using the gravimetric method, 2 g of powder were weighed into a pre-weighed porcelain cup. The sample was dried at 105 °C for 5 hours and reweighed. Drying and weighing were repeated every hour until a constant weight was achieved, defined as less than 0.25% difference between weighings.

5.3 Ash Content

The crucible was first ignited in a furnace at 600 °C to a constant weight. Then, 2 g of sample were placed into the crucible and incinerated in the furnace. After ashing, the crucible was cooled in a desiccator for 15 minutes and reweighed to a constant weight. The test was performed in triplicate.

5.4 Heavy Metal Contamination

Levels of heavy metals including lead (Pb) and cadmium (Cd) were determined using an AAS. Sample preparation was carried out using wet destruction method. Then the preparation of standard solutions of Pb was made in concentrations of 0.2; 0.4; 0.8; 1.2; 2 ppm and Cd was made in

concentrations of 0.2; 0.4; 0.6; 0.8 ppm from a standard solution of 50 ppm of each metal. The absorbance was measured at a wavelength of 217.0 nm (Pb) and 228.8 nm (Cd).

RESULTS AND DISCUSSION

The process of standardizing simplicia is carried out by referring to the standard operating procedures adapted from the Indonesian General Standard Parameters for Medicinal Plant Extracts (Indonesian Ministry of Health, 2000) and the Indonesian Herbal Pharmacopoeia Second Edition 2017. The standardization includes specific parameters and non-specific parameters. Standardization is done to ensure that the quality of simplicia is consistent and effective for natural medicine; this way, the active compounds can be reliably measured across different treatments, keeping the simplicia stable in terms of effectiveness and safety (Ningsih et al., 2022).

1. Specific Parameter Determination Results

1.1 Identity Test

Identity testing of simplicia is very important in the introduction as an initial introduction and part of the plant used by describing the plant's name, here including the name of the simplicia used, the Latin name of the plant, the plant part used, and the Indonesian name of the plant (Indonesian Ministry of Health, 2000; Mustapa et al., 2020). The results of the identity of the plant obtained from the literature are that this plant is known as lelak with the Latin name *Uvaria rufa* Blume with the part of the plant used is the stem bark (Isnayanti, 2020).

1.2 Organoleptic Test

U. rufa bark simplicia have a light brown color, a tasteless taste that tends to be astringent, a typical aromatic odor, and a fine powder (**Figure 1**). The distinctive aroma of plants comes from secondary metabolites of the terpenoid group (Ma'arif et al., 2023). Based on the Indonesian Herbal Pharmacopoeia Edition II 2017, the interpretation of organoleptic test results stating "typical aromatic odor" or others is only descriptive and cannot be considered as a standard of purity of the material concerned.

Figure 1. *U. rufa* bark powder

1.3 Macroscopic Test

Based on **Figure 2.** U. rufa bark has a dark brown color like stem bark in general, with the texture of the bark fibrous, thick, and shaped like pieces. The size of the U. rufa bark has a length of approximately 8-10 cm. In general, the morphology of U. rufa, which is in the Annonaceae family, can be recognized by its characteristics, such as skin that has fibers (Erkens et al., 2022). Another feature

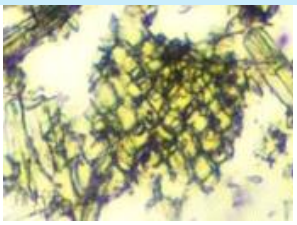
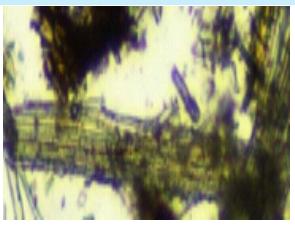
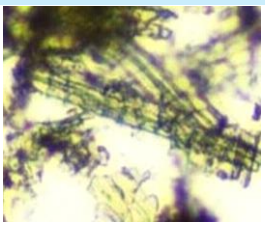
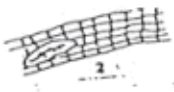
that can be useful in identifying Annonaceae is the thick, hard skin, but it peels off easily in one piece with a distinctive bright yellow inner side of the skin and brown on the outside (Couvreur et al., 2022).

Figure 2. *U. rufa* bark

1.4 Microscopic Test

The results of microscopic observations with a magnification of 10/0.25 (**Table 1**) show that the bark powder from U. rufa contains fragments of cortex parenchyma with stone cells, sclerenchyma tissue, and cork tissue. Plant stem bark is composed of collenchyma and parenchyma (Rahayu and Kurniawan, 2020). In addition, the skin consists of epidermal tissue, cork cambium, cortex, and phloem. Stem bark used as a medicinal material can consist of secondary phloem formed from cambium with or without cork (Hayati et al., 2019).

Table 1. Comparison of microscopic test results with literature

	Picture A	Image B	Picture C
Research Results			
Literature			

(Hayati et al., 2019)

2. Test for Soluble Essence Content in Certain Solvents

Tests of soluble essence content in certain solvents were carried out on U. rufa bark. Testing the content of water-soluble juice and ethanol is shown to know the initial picture of some of the content in it. This test can estimate the amount of active compounds that have polar properties (soluble in water) and semi-polar-nonpolar properties (soluble in ethanol). The test results (**Table 2**) show the water-soluble essence content in the sample amounted to 12.016% ± 0.0090, while the ethanol-soluble juice content amounted to 22.475% ± 0.0111. This shows that the U. rufa bark contains more compounds

soluble in ethanol organic solvents than compounds soluble in water solvents. Ethanol as a solvent has a wide polarity range from semi-polar to non-polar so that it can attract alkaloid compounds that are semi-polar, non-polar flavonoids, and triterpenoids, which are compounds included in terpenoids that have non-polar properties (Putri et al., 2022).

Table 2: Water and ethanol soluble essence test results

Testing parameters	% soluble essence content $\bar{x} \pm SD$
Water Soluble	12.016% ± 0.0090
Ethanol Soluble	22.475% ± 0.0111
Requirements	Not available

Description**:** SD value = Standard deviation of n = 3

3. Non-specific Parameter Determination Results

Non-specific parameter testing resulted (**Table 3**) in a drying shrinkage of 7.26% ± 0.8723 and a moisture content of 9.4% ± 0.2000. Moisture content that has a value below 10% will minimize damage to the simplicia caused by the growth of fungi and molds so that the shelf life becomes durable and improves the quality of *U. rufa* bark (Sagita, 2021). The determination of total ash content (Table 3) shows that the simplicia has an ash content of 2.601% ± 0.0002. In simplicia, ash content can come from soil contamination that is carried in the simplicia; the higher the ash content, the more inorganic content due to contaminants such as soil, sand, or dust. Conversely, the lower the ash content, the simpler it is within reasonable limits, and there are no contaminants, so the purity level of the simplicia as raw material will be higher (Falah and Sa'diyah, 2024).

Table 3: Loss on drying and moisture content test results

Testing parameters	% test result $\bar{x} \pm SD$
Drying Shrinkage	7.26% ± 0.8723
Water Content	9.4 % ± 0.2000
Ash content	2.601 % ± 0.0002

Description: SD value = Standard deviation of n = 3

4. Heavy Metal Contamination

Metal contamination levels in U. rufa bark (**Table 4**) showed Pb metal content of 0.0472 ppm and Cd metal of 0.0014 ppm. These metals can come from the soil, where plants have the ability of phytoextraction, or absorption and accumulation of polluting substances through other plant parts, so that they can capture metal particles (Rusmalina et al., 2023). Naturally, heavy metals can come from rock weathering and atmospheric deposition, but also from anthropogenic sources, including agriculture, livestock, and industry (Patty et al., 2018). Heavy metals can endanger health if they enter the body's metabolic system in amounts that exceed the threshold (Setyaningrum et al., 2018).

Table 4: Results of metal contamination levels in u. rufa bark

Type of Heavy Metal	Level (ppm)	Requirements (BPOM, 2019)	Description
Pb	0.0472	<10 ppm	Qualified
Cd	0.0014	<0.3 ppm	Qualified

CONCLUSIONS

The identification results confirm that the sample is *U. rufa* bark, characterized by macroscopic features (8–10 cm in length, dark brown exterior, yellow interior, fibrous texture), microscopic attributes (parenchyma with stone cells, sclerenchyma, cork tissue), and organoleptic properties (light brown color, slightly astringent, aromatic, fine powder). The water-soluble extractive value was 12.0160% ± 0.0090, whereas the ethanol-soluble value was 22.475% ± 0.0111. Non-specific criteria evaluated for safety and stability include loss on drying (7.26% ± 0.8723), moisture content (9.4% ± 0.2000), total ash content (2.601% ± 0.0002), and heavy metal concentrations (Pb: 0.0472 ppm, Cd: 0.0014 ppm). All outcomes satisfied the overarching criteria for herbal standardization, substantiating *U. rufa* bark's viability as a standardized herbal medicinal material.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest between all parties involved. The authors have full freedom in designing and carrying out this research. The results of this study will be published openly, regardless of the outcome. The authors have minimized any potential bias by taking appropriate measures.

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Optimization of Drying Temperature and Time in Paracetamol Tablets Using Wet Granulation with 3% Gelatin Binder

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ABSTRACT

Tablets are solid dosage forms containing active ingredients with or without additional excipients. A binder is required to produce compact tablets that do not break easily in manufacturing. One commonly used binder is gelatin. Wet granulation is one of the tablet manufacturing methods that require a drying stage with specific temperature and duration settings to obtain tablets that fulfill the quality, like physical properties, and other specifications to be released in the market. This study aims to analyze the effect of granule drying temperature and time on the characteristics of paracetamol tablets using two different levels, and to determine the optimum area between the two factors. The research method used is a pure experimental design with two factors and two levels of treatment. Data were analyzed using the Shapiro-Wilk normality test with a criterion of $p > 0.05$. Normally distributed data were further analyzed using the Design Expert 13 (Free Trial) application to determine the optimal formulation. The results showed that drying temperature and time significantly affected the physical characteristics of paracetamol granules and tablets. Increasing the temperature and drying time led to decreased moisture content, powder flow, angle of repose, compressibility, increased tablet hardness, tablet friability, and disintegration time. Based on the analysis, the optimal temperature range for paracetamol granule preparation was 40-60°C, with a drying duration of 3-6 hours.

Keywords: Paracetamol tablet; wet granulation; gelatin binder; drying temperature; drying time

INTRODUCTION

Tablets are widely used due to their ease of consumption, low production cost, and ability to improve patient compliance. Tablets are solid dosage forms that contain active ingredients, either with or without additional substances such as fillers (Kemenkes RI., 2020). Their manufacturing requires the selection of appropriate excipients and methods to ensure optimal quality. The three primary methods are wet granulation, dry granulation, and direct compression (Zaman & Sopyan, 2020). Wet granulation is commonly used in the pharmaceutical industry as it forms cohesive granules, improves material flow and compressibility by adding binders, and produces high-quality tablets (Safitri et al., 2014). This method is suitable for paracetamol, which has poor flow properties and low compressibility but is resistant to high temperatures (Ardiansyah et al., 2022).

Binders are essential in determining tablet compactness. In this study, gelatin was used due to its strong binding ability, which enables the formation of uniform granules with good compressibility and is effective for materials that are difficult to bind (Sheskey et al., 2017). However, gelatin tends to increase tablet hardness, which may affect disintegration time. Nevertheless, its advantages, such as cost-effectiveness, safety, and stability with active ingredients, make it a good choice (Fadhilah & Saryanti, 2019).

After the granulation process, the drying stage is necessary to reduce moisture content before tablet compression. The ideal temperature ranges from 40–60°C, where temperature and drying time variations affect the granules' moisture content and the tablets' physical properties (Sudarsono et al., 2021). This study optimizes temperature (40°C and 60°C) and time (3 and 6 hours) using a factorial design to determine the best conditions for producing paracetamol tablets with physical characteristics that meet the standards.

The aims of the study:

1. To determine the effect of temperature and drying time of granules on the physical properties of paracetamol granules and tablets with gelatin binder.
2. To determine the optimum area between temperature and drying time in the manufacture of paracetamol granules that produce tablets with tablet hardness, tablet friability, and disintegration time that meet quality requirements.

METHODS

1. Materials

The materials used are paracetamol (Anqiu Lu'an Pharmaceutical Batch No. 2150096, pharmaceutical grade), lactose monohydrate (Brataco Batch No. J 017/23 (23208277), pharmaceutical grade), gelatin (Brataco, pharmaceutical grade), corn starch (Brataco, pharmaceutical grade), magnesium stearate (Eur Phar Batch No. MGSV1230092, pharmaceutical grade), talc (Pingdu Talc Mine Co., LTD Shandong Batch No. YF1-022-201 24 MT, pharmaceutical grade), ethanol (pro analysis), and aquadest.

2. Instruments

The instruments used are analytical balances (OHAUS Pioneer PA213), oven (MEMMERT BE 500), sieves no. 12 dan 14 (Indotest Multi Lab), cube mixer, rotary tablet press (Shanghai Develop Machinery ZP-5B), moisture balance (Kern MLS 50-3C), flowability tester (Erweka AR 401), volumenometer (HY-100B), friability tester (ATMI SURAKARTA), hardness tester (Pharmastest PTB 302), disintegration tester (Develop BJ-2), hotplate magnetic stirrer (Cenco 34532), and UV-VIS spectrophotometer (UVmini-1240 Shimadzu®), Design Expert 13 (Free Trial) software.

3. Granule Preparation

Wet granulation was carried out by weighing the gelatine according to the predetermined amount and then dissolving it in hot water until it was completely dissolved. The paracetamol powder was mixed with lactose filler, and the gelatine solution was gradually added as a wetting agent until a moist and homogeneous granule mass was formed. Some amylum maydis was added to the mixture. The resulting granule mass was sieved using a No. 12 sieve to obtain a uniform size, and then it was dried in an oven at 40ºC and 60ºC with drying times of 3 and 6 hours. **Table 1** represents the formulation, temperature variations, and granule drying time of paracetamol tablets used in this study.

Table 1: Formulation, Temperature, and Granule Drying Time of Paracetamol Tablets

Ingredients	Formula (mg)
Paracetamol	500
Lactose	320
Gelatin 3%	3,6
Corn Starch	20
Magnesium Stearate	6,5
Talc	6,5

Factors	F1	Fa	Fb	Fab
Temperature (°C)	40	60	40	60
Time (hours)	3	3	6	6

Notes:
F1 : Formulation with a low temperature level and a low time level
Fa : Formulation with a high temperature level and a low time level
Fb : Formulation with a low temperature level and a high time level
Fab : Formulation with a high temperature level and a high time level

4. Lubrication Method

The dried granules are sieved using a No. 14 sieve to obtain a uniform size, followed by testing of the granules' physical properties. After testing, talc, magnesium stearate, and the remaining corn starch are added to the granules. The mixture is then stirred until homogeneous.

5. Evaluation of Granule Characteristics

5.1 Moisture Content Test

One gram of dried granules is weighed and placed into a moisture balance device. The device is then closed, allowing it to automatically measure the moisture content in the granules. Once the measurement process is complete, the device is opened, and the reduction in granule moisture content is recorded. The moisture content test meets the requirements if the moisture content value falls within the range of 1-5% (Sudarsono et al., 2021).

5.2 Powder Flow Test

The outlet hole of the funnel on the flowability tester is closed before the granules are added. A total of 100 grams of pre-weighed granules is placed into the funnel of the flowability tester. Once the granules are in the funnel, the outlet hole is opened, and the time required for all the granules to exit the funnel is recorded. The flow time test is considered acceptable if it meets the acceptance parameter, which is no more than 10 seconds for 100 grams of granules (Balfas & Nanda, 2019).

5.3 Angle of Repose Test

The outlet hole of the funnel is closed before adding the granules. A total of 100 grams of granules is poured into the funnel, then the outlet hole is opened to allow the granules to flow into the collection

container. Once the granules form a stable cone, the angle of repose is measured to assess the powder flow properties. The angle of repose test is considered acceptable if it meets the acceptance parameter, which ranges from 25–40° (USP, 2024).

$$\text{Tan } \alpha = \frac{h}{r}\frac{h}{r}$$

Notes:

α : Angle of repose

h : Height of formed granule cone

r : Radius of the cone base

5.4 Compressibility Test

A 100 mL graduated cylinder filled with granules is placed in a volumenometer. The initial volume of the granules is recorded before the test begins. The device is then operated to generate 500 taps, after which the final volume of the granules is recorded. The compressibility value is calculated using the compressibility index formula. The compressibility test is considered acceptable if it meets the acceptance parameter, which ranges from 25–40% (USP, 2024).

$$\text{Compressibility Index} = 100\% \left(\frac{V_o - V_F}{V_o}\right)\left(\frac{V_o - V_F}{V_o}\right)$$

Notes:

V_o : Initial apparent volume

V_F : Final tapped volume

5.5 Tablet Manufacturing

The mixed granules are then compressed into tablets using a single punch tablet machine. After the compression process, the physical properties of the tablets are tested to assess the quality of the final product.

6. Evaluation of Granule Characteristics

6.1 Organoleptic Test

The organoleptic test visually observes the tablets' shape, color, and odor using human senses. This test aims to ensure uniformity in shape without cracks, chipping, or any undesirable characteristics. It also evaluates color stability by detecting spots or uneven color distribution, except in cases where such differences are intentional. Additionally, this test aims to identify any odors that may indicate changes in the tablet (Siregar, 2008).

6.2 Weight Variation Test

A total of 10 tablets are individually weighed with precision. The active ingredient content in each tablet is determined and expressed as a percentage of the labeled amount. The obtained assay results from each tablet are then used to calculate the acceptance value according to the established criteria. The acceptance value for weight uniformity is met if L1 = 15.0 and L2 = 25.0 (Kemenkes RI, 2020).

6.3 Tablet Hardness Test

A total of 10 tablets are randomly selected and placed in a vertical position on the hardness tester. The pressure is adjusted until the tablet is stable and the scale reads zero. Then, the start button is pressed to begin the test, and the device automatically measures and displays the tablet's hardness value. The tablet hardness test is considered to meet the requirements if the obtained value falls within the range of 4–8 kg (Sudarsono et al., 2021).

6.4 Tablet Friability Test

A total of 10 tablets are dedusted and carefully weighed to determine the initial weight. The tablets are then placed into the friability tester and rotated for 4 minutes or 200 revolutions. After the process, the tablets are removed, dedusted, and reweighed to determine the final weight. The friability meets the acceptance criteria if the friability value is less than 1% (USP, 2024). The friability value is calculated based on the weight difference before and after testing.

$$F = 100\% \left(\frac{W_1 - W_2}{W_1}\right)\left(\frac{W_1 - W_2}{W_1}\right)$$

Notes:
F : Friability percentage
W_1 : Initial tablet weight
W_2 : Final tablet weight

6.5 Disintegration Time Test

One tablet is placed in each of the six tubes of the disintegration test basket. The device is operated at 37°C as the test medium for 15 minutes, unless the monograph specifies a different liquid. The basket is lifted once the test duration is reached, and the tablets are observed. All tablets must disintegrate completely. If one or two tablets fail to disintegrate, the test is repeated with 12 additional tablets. Out of 18 tested tablets, at least 16 must fully disintegrate to meet the requirements. The disintegration time test is considered acceptable if the disintegration time is less than 15 minutes (Kemenkes RI, 2020).

7. Data Analysis

Data were analyzed using the Shapiro-Wilk test to assess normality, where a p-value > 0.05 indicates a normal distribution. Next, the factorial design equation was calculated using the model equation ($Y = \beta0 + \beta1.x1 + \beta2.x2 + \beta12.x1.x2$). Normally distributed data were analyzed using two-way ANOVA with the Design Expert 13 software to determine the optimal formula. The collected data were obtained from the granule characteristic evaluation, which included moisture content test, powder flow test, angle of repose test, and compressibility index test, as well as the tablet characteristic evaluation, which included tablet hardness test, friability test, and disintegration time test.

RESULTS AND DISCUSSION

The results of the physical properties testing of paracetamol granules are presented in **Table 2**. The optimal drying conditions for paracetamol granules were determined based on the analysis using Design Expert software with the factorial design method. The relationship between drying time and temperature on the characteristics of paracetamol granules is illustrated in the contour plot in **Figure 1**.

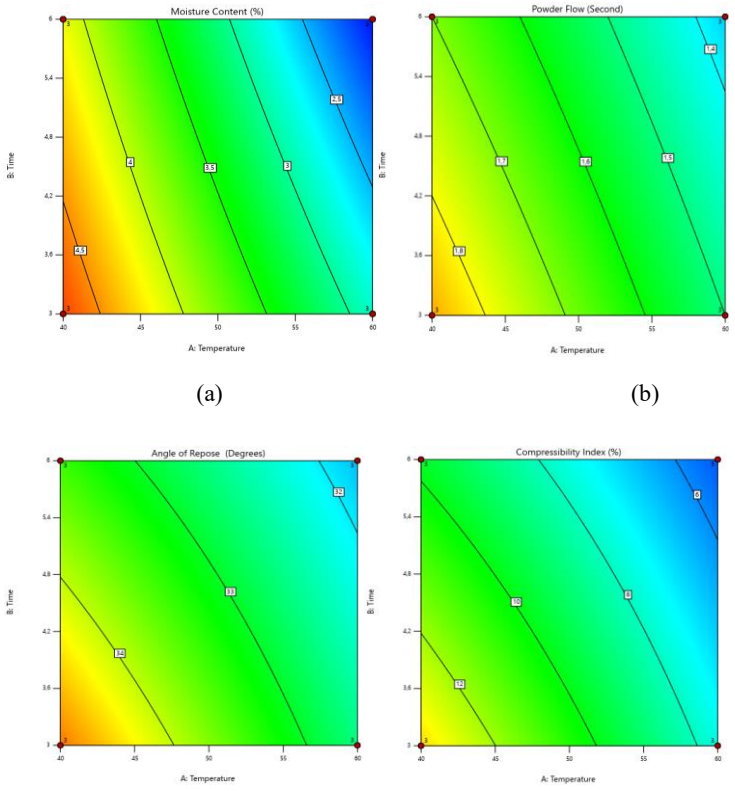


Figure 1. Contour Plot of Granule Test Response
(a) Moisture Content; (b) Powder Flow; (c) Angle of Repose

Table 2: Results of Evaluation of Paracetamol Granule Characteristics

Parameter	F1	Fa	Fb	Fab
Moisture Content (%)	4,72 ± 0,31	2,86 ± 0,43	4,14 ± 0,18	2,02 ± 0,07
Powder Flow (s)	1,9 ± 0,2	1,5 ± 0,1	1,7 ± 0,1	1,4 ± 0,2
Angle of Repose (°)	34,86 ± 0,59	32,62 ± 0,41	33,41 ± 1,43	31,79 ± 0,83
Compressibillity Index	13,48 ± 2,15	7,59 ± 1,55	9,72 ± 0,63	5,38 ± 0,97

Notes:
F1 : Formula with a drying temperature of 40°C for 3 hours
Fa : Formula with a drying temperature of 60°C for 3 hours
Fb : Formula with a drying temperature of 40°C for 6 hours
Fab : Formula with a drying temperature of 60°C for 6 hours

Table 2 shows that all formulas meet the moisture content requirement of 1-5% (Sudarsono et al., 2021). Formulas ab and a have the lowest moisture content (2.02% and 2.86%) compared to formulas 1 and b (4.72% and 4.14%) due to the higher drying temperature of 60ºC. Figure 1a supports this finding with a contour plot, where the red shading indicates high moisture content and the blue shading indicates low moisture content. Higher temperatures accelerate water evaporation. However, excessively high temperatures can increase particle cohesion and hinder granule flowability (Hadi et al., 2014). The factorial equation indicates that moisture content can be predicted using the formula $Y = 3.435 - 0.995(X_1) - 0.355(X_1) - 0.0213(X_1)(X_2)$, where Y represents the moisture content response, X_1 indicates the temperature level, X_2 represents the drying time level, and X_1X_2 is the interaction between temperature and time.

Table 3: Effect of Temperature, Time, and Interaction on Granule Characteristics

Responses	Factor	Effect Value	%Contribution	p-*value*	p-*value* Equation
Moisture Content	Temperature	-1,99	84,34	<0,0001	<0,0001 (Significant)
	Time	-0,71	10,84	<0,0001	
	Interaction	-0,13	0,36	<0,0001	
Powder Flow	Temperature	-0,35	64,57	<0,0001	<0,0001 (Significant)
	Time	-0,15	11,86	<0,0001	
	Interaction	0,05	0,15	<0,0001	
Angle of Repose	Temperature	-1,93	51,11	<0,0001	<0,0001 (Significant)
	Time	-1,14	17,81	<0,0001	
	Interaction	0,31	1,34	<0,0001	
Compressibility Index	Temperature	-5,12	63,41	<0,0001	<0,0001 (Significant)
	Time	-2,99	21,67	<0,0001	
	Interaction	0,77	1,45	<0,0001	

Table 3 shows that temperature, time, and their interaction reduce the moisture content response, as indicated by negative values of 1.99, 0.71, and 0.13, with respective contributions of 84.34%, 10.84%, and 0.36%. Based on the two-way ANOVA test, the p-value for both factors is <0.0001, indicating that both factors significantly affect the moisture content response. **Figure 2** shows that moisture content decreases with increasing temperature, with a sharper decline at the high time level (red line) compared to the low time level (black line). A longer drying time enhances the effect of temperature in reducing moisture content. The absence of line intersection indicates that temperature and time do not significantly interact, with temperature being the dominant factor affecting moisture content.

All formulas meet the flow time requirement of no more than 10 seconds for 100 g of granules (Balfas & Nanda, 2019). Formulas 1 and b have the longest flow times (1.9 seconds and 1.7 seconds) compared to formulas a and ab (1.5 seconds and 1.4 seconds) due to the lower drying temperature of 40°C. **Figure 1b** supports this finding with a contour plot, where the red-shaded area represents longer flow times and the blue-shaded area indicates faster flow times. Granules with high moisture content tend to have poor flowability due to particle adhesion, which increases cohesion. Conversely, lower moisture content improves flow properties by reducing friction between granules and the die wall (Hadi et al., 2014). The factorial equation shows that flow time can be predicted using $Y = 1.625 - 0.175(X_1) - 0.075(X_2) + 0.025(X_1)(X_2)$, where Y represents the flow time response, X_1 indicates the temperature level, X_2 represents the drying time level, and X_1X_2 represents the interaction between temperature and time.

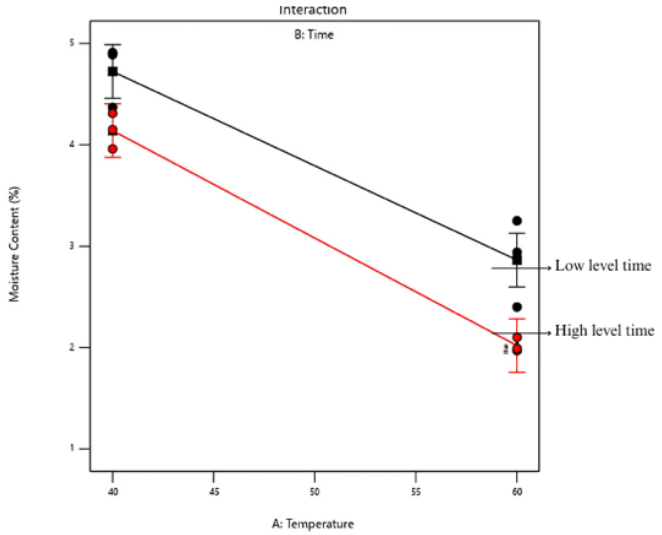


Figure 2. Interaction of Drying Temperature and Time on Moisture Content Response

Table 3 and **Figure 3** shows that temperature and time decrease the flow time response, as indicated by the negative values of -0.35 and -0.15, with contributions of 64.57% and 11.86%, respectively. Their interaction increases flow time by 0.05, contributing 0.15%. Based on the two-way ANOVA test, the p-value for both factors is <0.0001, indicating that these factors significantly affect the flow time response.

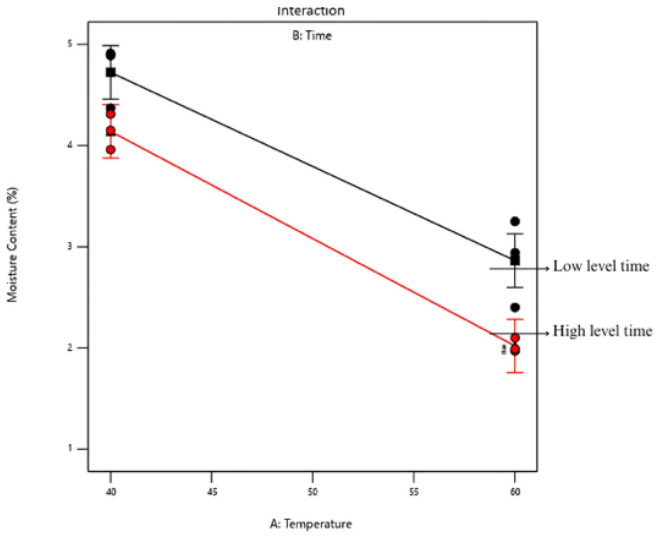


Figure 3. Interaction of Drying Temperature and Time on Powder Flow Response

Figure 4 shows that increasing temperature decreases the angle of repose at both time levels, with the high-time level resulting in a lower angle of repose across the entire temperature range. This indicates that temperature is the dominant factor influencing the angle of repose, while longer drying times produce granules with better flowability. The absence of line intersections suggests that both factors act independently, with temperature as the primary factor.

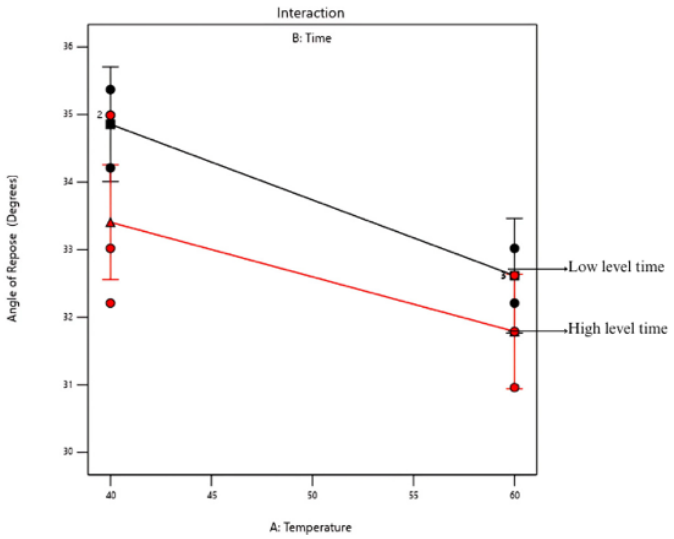


Figure 4. Interaction of Drying Temperature and Time on Angle of Repose Response

Table 2 shows that all formulas meet the compressibility index standard of less than 20% (USP, 2024). The study results indicate that formula 1 has the highest compressibility index (13.48%) compared to formulas a, b, and ab (7.59%, 9.72%, and 5.38%). This is due to the use of low-level temperature and time in formula 1, due to the lower drying temperature of 40°C. Figure 1d supports these findings with a contour plot, where red shading indicates a higher compressibility index and blue shading indicates a lower compressibility index. Granules with fast flow times exhibit uniform particle size and low cohesion, enabling optimal die-filling and producing harder tablets. Moisture content also affects compressibility; an optimal level enhances interparticle bonding, while excessively high moisture increases cohesion and hinders flow, whereas excessively low moisture makes granules brittle and reduces compressibility (Husni et al., 2020). The factorial equation predicts the compressibility index as $Y = 9.043 - 2.5575(X_1) - 1.4925(X_2) + 0.3875(X_1)(X_2)$, where Y represents the compressibility index response, X_1 indicates temperature level, X_2 indicates time level, and X_1X_2 represents the interaction between temperature and time.

The temperature and time reduce the angle of repose, with effects of -1.93 (51.11% contribution) and -1.14 (17.81% contribution), respectively (**Table 3**). The interaction between these factors increases the angle of repose by 0.31, contributing 1.34%. The two-way ANOVA test shows a p-value of <0.0001, indicating that both factors have a significant effect. **Figure 4** shows that increasing temperature reduces the angle of repose at both time levels, with the high-time level resulting in a lower angle of repose across the entire temperature range. This suggests that temperature is the dominant factor influencing the angle of repose, while longer drying times produce granules with better flow properties. The absence of line intersections indicates that both factors operate independently, with temperature being the primary factor.

Table 2 shows that all formulas comply with the compressibility index standard of less than 20% (USP, 2024). The results indicate that formula 1 has the highest compressibility index (13.48%) compared to formulas a, b, and ab (7.59%, 9.72%, and 5.38%). This is attributed to using low-temperature and short drying time in formula 1, which was set at 40°C. Figure 1d supports these findings through a contour plot, where red shading represents a higher compressibility index, while blue shading indicates a lower compressibility index. Granules with faster flow times exhibit uniform particle size and low cohesion, facilitating optimal die-filling and producing harder tablets. Moisture content also influences compressibility; an optimal moisture level enhances interparticle bonding, whereas excessive

moisture increases cohesion and hinders flow, while insufficient moisture results in brittle granules and reduces compressibility (Husni et al., 2020). The factorial equation predicts the compressibility index as $Y = 9.043 - 2.5575(X_1) - 1.4925(X_2) + 0.3875(X_1)(X_2)$, where Y represents the compressibility index response, X_1 indicates the temperature level, X_2 represents the time level, and X_1X_2 denotes the interaction between temperature and time.

Table 3 shows that temperature and time decrease the compressibility index by 5.12 and 2.99, contributing 63.41% and 21.67%, respectively. Their interaction increases the compressibility index by 0.77, contributing 1.45%. The two-way ANOVA test results show a p-value of <0.0001, indicating that both factors significantly affect compressibility. **Figure 5** shows no significant interaction between temperature and time on the compressibility index. The compressibility index decreases as temperature increases at both time levels, with temperature as the dominant factor. Higher drying temperatures and longer drying times result in a lower compressibility index.

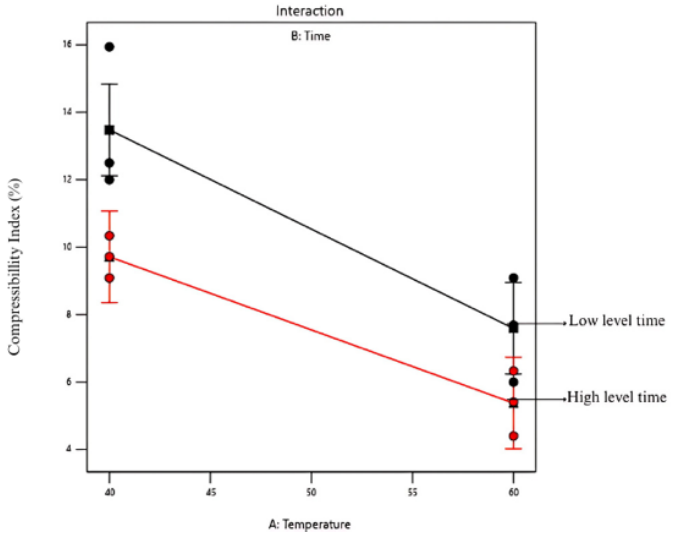
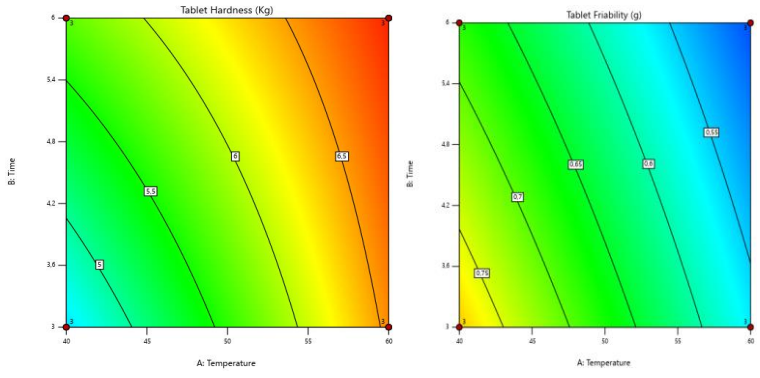


Figure 5. Interaction of Drying Temperature and Time on Compressibility Response

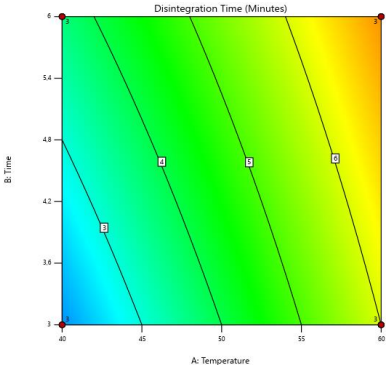
The results of the physical evaluation of paracetamol tablets are presented in **Table 4**. Based on the analysis using Design Expert software with the factorial design method, the optimal drying conditions for the physical properties of granules and paracetamol tablets were determined. The relationship between drying time and temperature on the physical properties of paracetamol granules is illustrated in the contour plot in **Figure 6**.

Table 4 shows that the NP acceptance value meets the weight uniformity requirements with L1 <15, by the Ministry of Health (2020). Weight variation is related to content uniformity, ensuring a consistent drug dose in each tablet. Factors influencing weight uniformity include particle size, shape, flow properties, and moisture content of the granules. Good flow time during tablet compression ensures uniform die filling, improving weight and content uniformity (Nining et al., 2020). All formulations meet the acceptance criteria for weight uniformity within the 4-8 kg (Sudarsono et al., 2021). Formula ab and a exhibited the highest tablet hardness, measuring 6.86 kg and 6.55 kg, respectively, whereas Formula 1 and b had hardness values of 4.60 kg and 5.73 kg. The higher hardness observed in Formulas ab and a was attributed to a drying temperature of 60°C. **Figure 6a** presents a contour plot, with red shading indicating higher hardness and blue shading indicating lower hardness. Increased drying temperature and time reduced the granule moisture content, enhancing particle bonding during compaction and making harder tablets. Conversely, excessive moisture can make tablets fragile, while

excessively low moisture levels reduce cohesion, making tablets prone to cracking and breaking (Hadi et al., 2014). The factorial equation predicts tablet hardness using the formula $Y = 5.935 + 0.77(X_1) + 0.36(X_2) - 0.205(X_1)(X_2)$, where Y represents the tablet hardness response, X_1 denotes the temperature level, X_2 denotes the drying time level, and X_1X_2 represents the interaction between temperature and drying time.



(a) (b)



(c)

Figure 6. Contour Plot of Tablets Test Response
(a) Hardness Tester; (b) Flowability Tester; (c) Disintegration Time

Table 5 shows that temperature and drying time positively influence tablet hardness, with effects of 1.54 (67.52% contribution) and 0.72 (14.66% contribution), respectively. However, their interaction negatively affects tablet hardness, reducing it by 0.41, contributing 4.66%. The two-way ANOVA test resulted in a p-value of <0.0001, indicating that both factors have a significant impact on tablet hardness.

Figure 7 shows that tablet hardness increases with rising temperature at both drying time levels, with temperature being the dominant factor. The absence of line intersections indicates that the interaction between temperature and drying time is not significant, meaning both factors work independently.

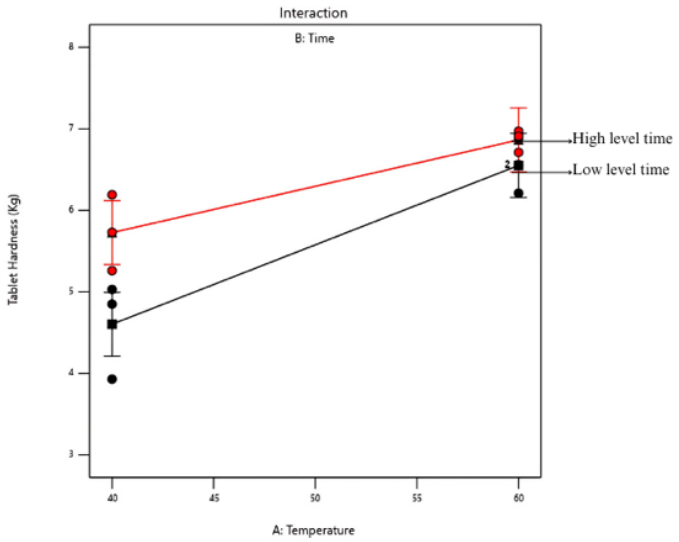


Figure 7. Interaction of Drying Temperature and Time on Tablet Hardness Response

Table 4 confirms that all formulations meet the friability test standard of less than 1% (USP, 2024). Formulations 1 and b exhibit the highest friability, 0.78% and 0.68%, respectively, compared to formulations a and ab, which show 0.56% and 0.50%. This difference is attributed to the lower drying temperature (40°C) used in formulations 1 and b, which hinders interparticle bonding, resulting in more fragile tablets than those in formulations a and ab, which were dried at 60°C. This finding is supported by **Figure 6b**, where red shading indicates high friability, while blue shading represents low friability. Tablet friability is influenced by granule size uniformity and the amount of fines. Uniformly sized granules enhance tablet strength by optimizing particle arrangement during compression. However, excessive fines can increase surface adhesion while weakening interparticle cohesion, making tablets more fragile (Monica et al., 2023). The factorial equation indicates that tablet friability can be predicted using $Y = 0.63 - 0.1(X_1) - 0.04(X_2) + 0.01(X_1)(X_2)$, where Y represents tablet friability response, X_1 denotes temperature level, X_2 denotes time level, and X_1X_2 represents the interaction between temperature and time.

Table 4: Results of Evaluation of Paracetamol Tablet Characteristics

Parameter	F1	Fa	Fb	Fab
Weight Variation (NP)	3,11	6,11	2,23	8,65
Tablet Hardness (kg)	4,60 ± 0,57	6,55 ± 0,96	5,73 ± 0,98	6,86 ± 0,82
Tablet Friability (g)	0,78 ± 0,07	0,56 ± 0,05	0,68 ± 0,05	0,50 ± 0,03
Disintegration Time (min)	2,00 ± 1,00	6,00 ± 1,00	3,67 ± 1,53	7,00 ± 1,00

Notes:
NP : Acceptance value
F1 : Formula with a drying temperature of 40°C for 3 hours
Fa : Formula with a drying temperature of 60°C for 3 hours
Fb : Formula with a drying temperature of 40°C for 6 hours
Fab : Formula with a drying temperature of 60°C for 6 hours

Table 5 shows that temperature and drying time negatively affect tablet friability, reducing the response by 0.2 (73.62% contribution) and 0.08 (12.78% contribution), respectively. Their interaction

has a positive effect, slightly increasing friability by 0.02, contributing 0.74%. The two-way ANOVA test yielded a p-value of <0.0001, indicating that both factors significantly influence tablet friability.

Table 5: Effect of Temperature, Time, and Interaction on tablet Hardness and Friability

Responses	Factor	Effect Value	%Contribution	p-*value*	p-*value* Equation
Tablet Hardness	Temperature	1,54	67,52	<0,0001	<0,0001 (Significant)
	Time	0,72	14,66	<0,0001	
	Interaction	-0,41	4,66	<0,0001	
Tablet Friability	Temperature	-0,2	73,63	<0,0001	<0,0001 (Significant)
	Time	-0,08	12,78	<0,0001	
	Interaction	0,02	0,74	<0,0001	

Figure 8 shows that tablet friability decreases as temperature increases, with both low-level drying time (black line) and high-level drying time (red line) following the same trend, with temperature being the dominant factor. The absence of line intersections indicates that the interaction between temperature and drying time is not significant, meaning both factors work independently.

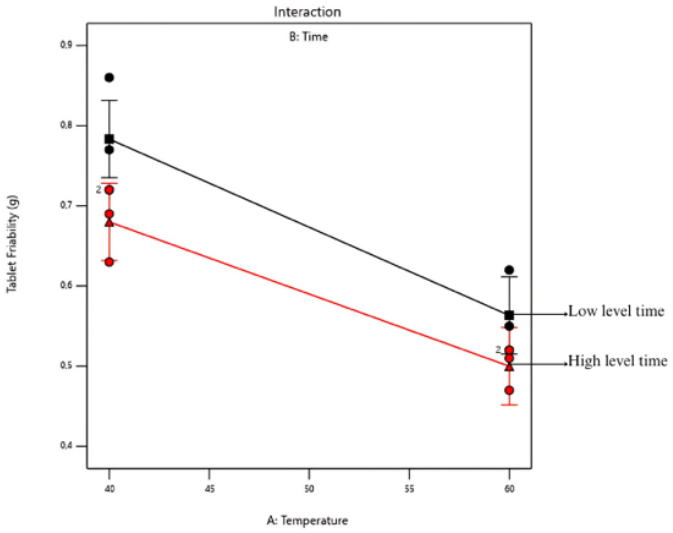


Figure 8. Interaction of Drying Temperature and Time on Tablet Friablity Response

Table 4 indicates that all formulations meet the disintegration time standard of less than 15 minutes. Formulations ab and a have the longest disintegration times, at 7 minutes and 6 minutes, respectively, compared to formulations 1 and b, which disintegrate in 2 minutes and 3.67 minutes, respectively. The ab and a formulation underwent drying at a higher temperature (60°C), resulting in lower moisture content, which strengthened interparticle bonds and produced denser granules and harder tablets. This finding is consistent with **Figure 6c**, where red shading represents faster disintegration times, while blue shading indicates slower disintegration times. Tablets with higher hardness tend to have greater density and lower porosity, which hinders liquid absorption and reduces the effectiveness of disintegrants, ultimately prolonging disintegration time (Kusuma & Apriliani, 2018). The factorial equation predicts tablet disintegration time as follows $Y = 4.6675 + 1.8325(X_1) + 0.6675(X_2) - 0.1675(X_1)(X_2)$, where Y represents disintegration time, X_1 denotes temperature level, X_2 denotes time level, and X_1X_2 represents the interaction between temperature and time.

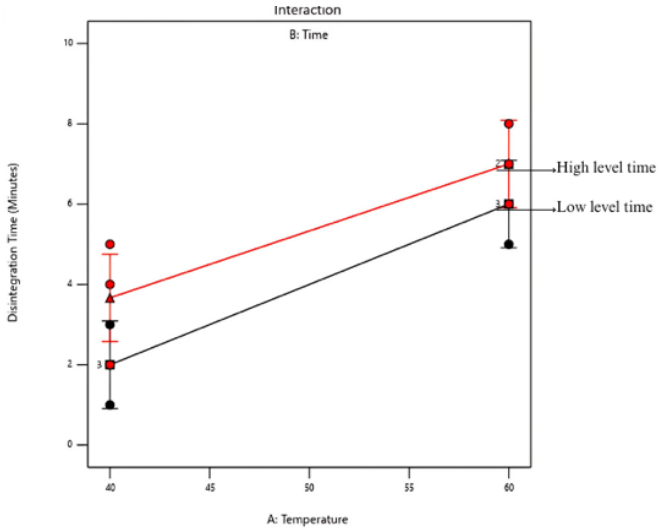


Figure 9. Interaction of Drying Temperature and Time on Disintegration Time Response

Figure 9 shows that disintegration time increases as temperature rises at both drying time levels, with no significant interaction between the two factors. Temperature is the dominant factor, where higher drying temperature and longer drying time result in granules with a longer disintegration time. **Figure 10** presents the Superimposed Contour Plot, determined based on the following acceptance criteria: moisture content of 1-5%, flow time not exceeding 10 seconds, an angle of repose between 25°-40°, compressibility index of 1-20%, tablet hardness ranging from 4-8 kg, maximum tablet friability of 1%, and a disintegration time of no more than 15 minutes. The yellow-shaded area in the figure indicates that a drying temperature of 40-60°C and a drying duration of 4-6 hours meet the acceptance criteria, resulting in tablets of optimal quality. Meanwhile, the gray-shaded area represents combinations of drying temperature and time outside the acceptance range.

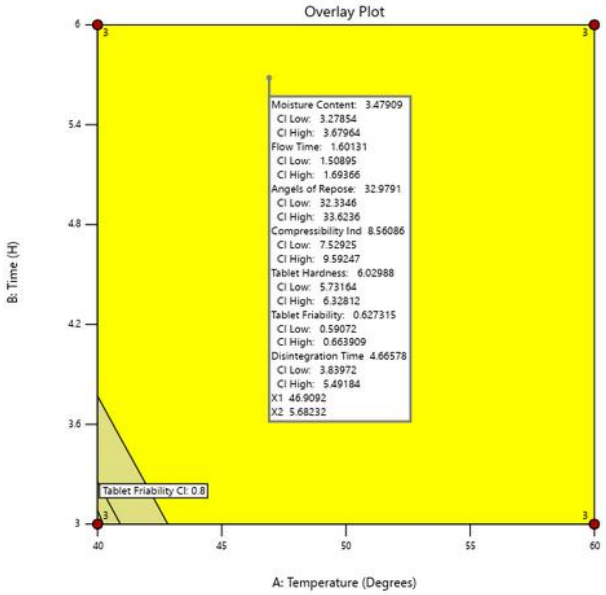


Figure 10. Superimposed Contour Plot

CONCLUSIONS

The temperature and drying time of granules affect the physical properties of granules and paracetamol tablets. Increasing the temperature and drying duration leads to reduced moisture content,

faster flow time, smaller angle of repose, and lower compressibility index. Additionally, these conditions increase tablet hardness, reduce friability, and extend disintegration time. Based on the analysis, the optimal range was identified within a temperature of 40-60°C and a drying time of 3-6 hours, as indicated by the yellow shaded area. Under these conditions, the resulting paracetamol tablets met quality requirements, particularly in terms of hardness, friability, and disintegration time.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Social Behavioral Administrative and Health Education

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ABS-043

Pre-Marketing Analysis of Segmenting, Targeting, and Positioning for Black Pule (*Alstonia spectabilis*) Antimalarial Tablets in South Central Timor Regency

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ABSTRACT

Black pule (*Alstonia spectabilis*) antimalarial tablets have the potential to serve as effective herbal medicines; however, their utilization remains limited. A strategic Segmenting, Targeting, and Positioning (STP) approach is essential to support downstream product development, reach the appropriate market segments, and promote pharmaceutical self-sufficiency in malaria-endemic regions such as South Central Timor District. This study aims to identify market segments, evaluate those with the highest potential, and establish effective product positioning. A descriptive quantitative method was employed, involving 100 respondents aged 18–65 years selected through cluster sampling across three high-malaria-incidence subdistricts. Data were analyzed using a Two-Step Cluster, K-means clustering, and cross-tabulation. The analysis revealed two distinct consumer segments: (1) experienced users of herbal antimalarial remedies and (2) inexperienced but highly interested individuals. The targeting applied was a Differentiated Marketing Strategy, positioning the product as a safe, high-quality, and scientifically validated herbal antimalarial effective in reducing *Plasmodium falciparum* parasitemia. The findings indicate that with proper market segmentation and targeted positioning, black pule tablets hold significant market potential in the South Central Timor District and can enhance consumer trust, awareness, and long-term loyalty.

Keywords: *Alstonia spectabilis*; antimalarial tablet; positioning; segmenting; targeting.

INTRODUCTION

Malaria is still a health problem in many parts of the world. Malaria is an infectious disease caused by plasmodium parasites that multiply in human red blood cells with the main cause being female anopheles (Ocvanirista et al., 2024). Based on the World Health Organization malaria report in 2022 there were 249 million people infected with malaria and as many as 608,000 people were declared dead (WHO, 2023). Meanwhile, Indonesia also still has a number of malaria cases, according to the Ministry of Health, the number of malaria cases has increased in Indonesia in 2020 there were

254,055 cases to 443,530 cases in 2022. Malaria is the main infectious disease that occurs in the eastern region which includes Papua, West Papua and East Nusa Tenggara (NTT) (Taek, 2023).

East Nusa Tenggara is one of the target areas for malaria elimination to achieve the national elimination program by 2030. One of the districts of East Nusa Tenggara (NTT) that has not been free from malaria cases is South Central Timor (TTS) (Inderiati et al., 2022). South Central Timor District is not yet free from malaria cases, namely in 2023 there were 696 positive cases of malaria with 1.53 API (Annual Parasite Incidence). API which is classified as moderate endemicity (Santos et al., 2023).

One of the drugs used to prevent and treat *Plasmodium sp*. is chloroquine. However, the use of chloroquine is starting to be abandoned due to the resistance of *Plasmodium sp*. to this quinine-derived compound. As an alternative, artemisinin and its derivatives, such as artesunate, isolated from Artemisia annua, are now the main options (Taek et al., 2021). However, recent reports indicate the emergence of resistance of *Plasmodium falciparum*, the most dangerous plasmodium species in Indonesia, to artesunate combination therapy with mefloquine, with cases of resistance reported in Korea (Taek, 2020). Therefore, new antimalarial drugs that are effective and affordable by the community are needed.

The black pule plant (*Alstonia spectabilis*), known as *kroti metan* by the Tetun ethnic group, is a traditional plant with potential for development as an antimalarial agent. Previous studies have investigated the antimalarial properties of *Alstonia spectabilis*. Metabolite profiling test of black pule (*Alstonia spectabilis*) extract using *ultra performance liquid chromatography–quadrupole time-of-flight mass spectrometry* (UPLC-QToF-MS/MS) and *gas chromatography* (GC-MS) identified the presence of *macralstonidine*, *pleiocarpamine*, and *villalstonine* compounds with antimalarial potential. *In vitro* testing of the 70% ethanol extract of *Alstonia spectabilis* demonstrated antimalarial activity by inhibiting *Plasmodium* growth with an LC_{50} value of 1.23 µg/mL(Taek et al., 2021). *In vivo* testing also showed that the 70% ethanol extract of black pule (*Alstonia spectabilis)* inhibited the growth of *Plasmodium berghei* in mice at an optimal dose of 0.4914 mg/20g body weight/day (Taek, 2023).

So it is necessary to develop downstream made from black pule to be used as tablets in supporting national pharmaceutical independence through the availability, quality, and affordability of local products (Saputra et al., 2022). To support product downstreaming, product commercialization is necessary through marketing strategies. Companies are required to be more creative in developing marketing strategies and appropriate products so that they can be accepted by consumers and increase purchase intention (Maulina et al., 2025). This, in turn, supports the increase in product value by representing the unique characteristics of flagship products and expanding market share (Tedjalaksana and Trimo, 2022).

An optimal marketing strategy requires structured planning, both internally and externally. Before running a promotion, the company must set a clear target market. Many businesses fail because they are unable to identify and analyze the market properly. Given the diverse consumers, companies must choose the right segment to achieve marketing success (Aliami et al., 2022).

One of the right marketing strategy approaches is Segmenting, Targeting, and Positioning (STP) (Handayani et al., 2023). Segmenting, Targeting, and Positioning (STP) analysis helps understand consumer characteristics and determine effective marketing strategies (Aliami et al., 2022). This strategy provides various benefits for companies to identify market opportunities, allocate resources efficiently, and build a positive brand image, which is important for increasing awareness and influencing purchasing decisions in a competitive market (Sani and Aslami, 2022). Therefore, this study aims to determine the number of segments with similar characteristics based on demographic, psychographic

and behavioral segments, identify segments that have the highest profit potential and determine target market strategies and determine effective positioning for black pule (*Alstonia spectabilis*) antimalarial tablets in South Central Timor District.

METHODS

This research method was designed using a quantitative descriptive method to understand the market for black pule (*Alstonia spectabilis*) antimalarial tablets in South Central Timor. This study used a questionnaire as a data collection tool. The questionnaire was designed to explore information related to the preferences, habits, and beliefs of respondents which will be used as a suitable potential for marketing black pule (*Alstonia spectabilis*) antimalarial tablets. This study was approved by the Ethics Committee for Health Research, Islamic University Hospital Malang with letter number 36/KEPK/RSI U/IX/2024.

1. Materials and Instrumentations

The instruments used in this study include Microsoft Excel and IBM SPSS Statistics version 24. A questionnaire instrument was used to collect data from respondents. Microsoft Excel was used to input the data obtained from the questionnaires. Meanwhile, IBM SPSS Statistics version 24 was used to classify and process the research data obtained from the questionnaires, allowing for the formation of groups based on similarities. The materials used in this study were questionnaire sheets and pens for data collection.

2. Location, Time and Research Sample

This research was conducted in South Central Timor District, East Nusa Tenggara in September 2024. The number of respondents in this study was 100 respondents (**Table 1**) with inclusion criteria for respondents aged 18 years - 65 years, respondents can communicate well and answer the questionnaire, and are willing to fill out the research instrument questionnaire. The exclusion criteria for respondents are that they are not willing to fill out questionnaires for researchers. The location of this study was determined purposively by selecting 3 sub-districts in South Central Timor District which were selected using cluster sampling technique. The three sub-districts were SoE City Sub-district, South Amanuban Sub-district, and Boking Sub-district, which were selected based on the highest malaria incidence areas in 2019. So that the three sub-districts were chosen as data sampling sites with endemicity that is still quite high and based on the most malaria cases. It is expected that the people of South Central Timor District will be suitable for segmenting, targeting and positioning marketing for black pule antimalarial tablets.

Tabel 1: Distribution of Sample Number of Respondents Based on Domicile

District	Number of Respondents	Percentage
Kota SoE	54	54%
Amanuban Selatan	32	32%
Boking	14	14%
Total	100	100%

3. Data Analysis

3.1 Validity test

Validity test is a measuring tool used to prove the accuracy of the items in the research instrument and measure the clarity of the framework in a study. The instrument to be used in research must have been declared valid and reliable. Indicators in each instrument are said to be valid if the value of r count is greater than r table (Utami et al., 2023).

3.2 Reliability Test

Reliability test is the consistency of a series of measurements or a series of measuring instruments. This can be in the form of measurements from the same measuring instrument (test with retest) will give the same results, or for more subjective measurements, whether two raters give similar scores (inter-rater reliability). Reliability is not the same as validity. Reliability means that a reliable measurement will measure consistently, but not necessarily measure what should be measured (Sanaky et al., 2021).

3.3 Segmenting, Targeting, Positioning Analysis

Segmentation data analysis uses Two-step cluster testing to determine the number of segments to be divided based on the similarity of answers to demographic, psychographic, and behavioral segment questions. After knowing the number to be divided, K-Means cluster testing is carried out to divide the number of segments according to the results of the Two-step cluster to determine the number of members in each segment. After that, a crosstabulation analysis was conducted to get a clear interpretation with the percentage results of each segment. The results of this analysis are used to determine segmenting based on demographic segments, namely age, gender, psychographic segments, namely lifestyle and values, and behavioral segments, namely knowledge, use, attitudes, and responses. Furthermore, targeting analysis to evaluate segments and select potential target markets from segmentation results. This research uses a Differentiated Marketing Strategy, in which the company adjusts the marketing program for each segment by meeting different characteristics (Manggu and Beni, 2021). And finally, determining positioning aims to instill product advantages in the minds of consumers in the target segment with a consumer group and benefit-based approach, according to the behavioral characteristics of the intended market.

RESULTS AND DISCUSSION

1. Validity Test Results

The validity test was carried out testing 11 question items consisting of indicators of lifestyle, values, knowledge, responses, use and attitudes towards products. Meanwhile, the age and gender indicators were not tested for validity because only demographic data was objective. Validity testing of the questionnaire can be said to be valid if the r-count> r-table value is 0.361. The value of r table is known from the distribution value of 5% significance with 30 respondents. So that in the validity test in **Table 2** obtained the validity test results that all items are valid so that they can be used in research.

Tabel 2: Validity Testing Results

Indicator	Questions	r-count	r-table	Description
Life Style	Q3	0,800	0,361	Valid
Value	Q4	0,720	0,361	Valid
Knowledge	Q5	0,540	0,361	Valid
Responses	Q6	0,623	0,361	Valid
Usage	Q7	0,501	0,361	Valid
	Q8	0,502	0,361	Valid
	Q9	0,477	0,361	Valid
	Q10	0,515	0,361	Valid
Attitude Toward the Product	Q11	0,722	0,361	Valid
	Q12	0,527	0,361	Valid
	Q13	0,744	0,361	Valid

2. Reliability Test Results

Reliability testing was carried out by testing 11 question items with Cronbach's Alpha on the questionnaire. The reliability test was carried out to determine whether all question items used in the questionnaire were reliable or not. The reliability test results in **Table 3** that all items are reliable. A research instrument is said to be reliable if the Cronbach's Alpha value is> 0.60 (Slamet and Wahyuningsih, 2022).

Tabel 3: Reliability Testing Results

Cronbach's Alpha	N of Item	Requirement	Information
0.825	11	>0.60	High Reliable

3. Segmenting Analysis Results

Segmenting testing is carried out first clustering testing to divide respondents into several small groups that have similar or the same criteria. The clustering method can be used to group data that has similarities between one data and another (Herlinda et al., 2021). Clustering testing is carried out using Two-step cluster to determine the number of segments formed based on the similarity and similarity of criteria. The two-step cluster method is a method that is considered the most suitable for clustering this data so that we can find out the optimal cluster or segment solution based on company performance (Putri and Wulandari, 2023).

The Two-step cluster test results (**Figure 1**) and (**Table 4**) show that two small segments are formed that have similarity criteria and similar characteristics. The quality of the Two-step cluster results generated from the test is included in a fairly good level, so that it can be used further, namely conducting K-Means clusters to divide the similarity of respondents into small groups according to the number of Two-step cluster results, namely two segments to determine the number of members of each segment. The K-Means clustering method is a non-hierarchical data clustering technique that separates data into clusters, groups data with the same features together and groups data with different characteristics into different groups (Amalina et al., 2024).

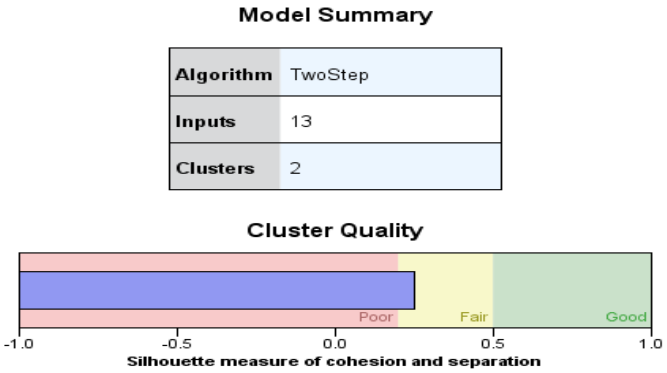

Figure 1. Two-step Cluster Testing Results

Table 4: K-Means Cluster Testing Results

Segment	Number
1	66
2	34
Valid	100
Mising	0

The results of K-Means cluster testing show that there are 66 respondents who are members of the first segment and have the same criteria among respondents, then in the second segment there are 34 respondents who have the same criteria. After knowing the results of the formation of the number of members in each segment, then proceed with conducting a crosstabulation test of the segment to find out more details about the information and percentage of each segment. Then, the highest percentage response for each indicator in each segment was selected, resulting in the **Table 5**.

Table 5: Segmenting Results Segment Characteristics

Characteristic	Segment	
	First Segment	Second Segment
Age	18-25 Years	18-25 Years
Gender	Female	Female
Medicine Preference	Strongly Prefer Herbal Medicine	Prefer Herbal Medicine
Main Reason for Buying Herbal Medicine	Product Quality	Product Safety
Source of Information on Herbal Medicine	Family/Friends	Social Media
	Doctor/Health Professionals	Doctor/Health Professionals
Willingness to Try New Products from Doctors	Very Willing	Willing
Malaria Prevention Measures	Using Antimalarial Medicine	Using Mosquito Repellent
Usage and Frequency of Herbal Antimalarial	Ever Used, 2 Times	Never Used
Source of Herbal Antimalarial Medicine	Pharmacy	Never Used
Experience with Branded Herbal Medicine	Used, But Forgot the Brand	Never Used
Interest in Trying New Herbal Antimalarial	Very Interested	Interested
Belief in No Side Effects of Herbal Medicine	Not Sure	Sure
Belief in Efficacy of Herbal Medicine	Very Sure	Sure

4. Targeting Analysis Results

Targeting or target market is defined as the process of evaluating the attractiveness of segments that have been analyzed and selecting segments that are the main targets of marketing strategies (Robbani, 2024). In this study, the targeting approach used for marketing black pule (*Alstonia spectabilis*) antimalarial tablets is Differentiated Marketing Strategy. This strategy allows companies to customize products and marketing for each segment, thus reaching more customers (Rosyida et al., 2020). This approach also supports different marketing programs according to the characteristics of each customer group (Manggu and Beni, 2021).

Based on **Table 6** The determination of the target market for black pule (*Alstonia spectabilis*) antimalarial tablets did not consider age and gender factors due to disproportionate sample distribution. Therefore, the targeting analysis focused more on psychographic and behavioral approaches to identify potential and relevant market segments. Psychographically, both segments showed a lifestyle preference for herbal medicine over synthetic drugs. The preference for a natural lifestyle towards herbal medicine is a great potential for black pule (*Alstonia spectabilis*) antimalarial tablets when it will be marketed in large quantities. This is because the alignment of consumer preferences can influence purchasing decisions, especially when the products offered match their values and beliefs (Smith et al., 2021).

Table 6: Results of Analysis of Characteristics of Each Segment to Be Targeted

Indicator	Characteristics Segment	
	First Segment	Second Segment
Age	18-24 Years	18-24 Years
Gender	Female	Female
Life Style	Have a strong preference for herbal medicine	Have a preference for herbal medicine
Value	The main consideration in buying herbal medicine is due to the quality of medicinal products	The main consideration in buying herbal medicine is due to drug safety
Knowledge	Get sources of information on herbal medicines through family/friends and doctors/health workers	Get sources of information on herbal medicines through social media and doctors / health workers
Response	Very willing to try new products recommended by doctors/health workers	Willing to try new products recommended by doctors/health workers
Usage	Use antimalarial herbal medicine in preventing malaria, have used antimalarial herbal medicine 2 times by getting it through the pharmacy and have used branded herbal medicine forgot about the brand	Use mosquito repellent to prevent malaria, never use antimalarial herbal medicine and branded antimalarial herbal medicine, never get antimalarial herbal medicine
Attitude Toward the Product	Very interested in new antimalarial herbal medicines, not sure there are no side effects and very sure herbal medicines have efficacy	Interested in new antimalarial herbal medicines, believe there are no side effects and believe herbal medicines have efficacy.

The first segment chooses herbal medicine because of product quality, while the second segment prioritizes safety. The focus on quality and safety is expected to strengthen product positioning because black pule (*Alstonia spectabilis)* antimalarial tablets have met CPOTB standards and passed toxicity tests on the extract, which is a guarantee of quality and safety. The quality and safety of herbal medicines have proven to be important factors in purchasing decisions (Anggreini and Suwitho, 2020). Research

findings by Yuwanda et al (2025) show that consumers tend to choose herbal medicines that are considered safe (Yuwanda et al., 2025).

In terms of knowledge, the first segment obtained information about herbal medicines from family, friends, and health workers. Trust in this source of information can form positive perceptions and encourage the use of herbal products because of the benefits that are felt directly (Adiyasa and Meiyanti, 2021). Marwati and Amidi's (2019) research findings that perception and trust influence herbal medicine decisions (Marwati and Amidi, 2019). Meanwhile, the second segment gets information from social media and health workers, so the promotional strategy for black pule (*Alstonia spectabilis*) antimalarial tablets can be focused on collaboration between digital media and health professionals before being marketed or to be marketed in large quantities. Social media has become an effective tool for expanding access to information and increasing trust in herbal products (Alifiyah et al., 2021). In terms of response to new products, both segments showed willingness to try new products if they received recommendations from health workers. This is an important opportunity for black pule (*Alstonia spectabilis*) antimalarial tablets because it is a new product with support from health workers who can strengthen the adoption of health worker research-based products, especially in the face of the many resistances to synthetic drugs (Wulandini, et al., 2024).

In terms of use, the first segment showed a habit of using antimalarial herbal medicine, getting medicine from pharmacies, and having tried a brand of herbal medicine even though they forgot the name. This reflects the acceptance of herbal medicine and is a strategic target for introducing a new product brand, namely black pule (*Alstonia spectabilis*) antimalarial tablets. Brand strengthening is important because it can increase purchase interest and differentiate the product in the market (Fabella et al., 2023). Distributing through pharmacies is the company's main strategy to reach consumers, increase trust, and accessibility of black pule (*Alstonia spectabilis*) antimalarial tablet products (Dysyandi et al., 2019). So that black pule antimalarial tablets (*Alstonia spectabilis)* will also be marketed through pharmacies for drug distribution. Pharmacies were chosen because they are dominant in pharmaceutical distribution and are easily accessible to the public, supporting optimal health services (Yunarti et al., 2024).

By fulfilling needs according to the characteristics of their preferences and habits, it can improve effective marketing strategies. Marketing strategies are more effective if they are tailored to the preferences and habits of the target segment (Santoso et al., 2024). In contrast, the second segment has never used antimalarial herbal medicine and has never obtained it from a health distribution point. However, this segment has great potential as it is inexperienced in the use of antimalarial herbal medicines and is still open to education.

Although the second segment has measures to prevent malaria using mosquito repellent. This is still an opportunity for black pule (*Alstonia spectabilis*) antimalarial tablets. Although the second segment already uses mosquito repellent, there is still an opportunity for black pule tablets. According to Sari et al (2023), people are reluctant to try other alternatives due to the cost and availability of conventional mosquito repellent (Sari Sir et al., 2023). Therefore, a more affordable pricing strategy and education on the long-term economic benefits can be an effective way to attract them. Wider distribution and community-based approaches as well as online media in the second segment can strengthen market penetration, given that digital marketing can increase trust, loyalty and brand visibility in the community (Merrynda & Andriani, 2023; Manik, et al., 2024).

Based on attitudes towards the product, both segments have an interest in new antimalarial herbal medicines and believe in their efficacy. This makes the potential for black pule (*Alstonia spectabilis*) antimalarial tablets, which include new antimalarial herbal medicines, to enter the market competition and also as an alternative in the treatment of malaria, this is because synthetic antimalarial drugs cause resistance. Although the first segment is still hesitant regarding side effects, while the second segment is more confident of its safety, this still opens up opportunities because herbal medicines generally have minimal to no side effects if used appropriately Experience and trust in herbal medicines are key in forming positive perceptions that encourage consumer loyalty (Marwati and Amidi, 2019). Therefore, communication strategies that emphasize safety and efficacy need to be strengthened to build loyalty to black pule (*Alstonia spectabilis*) antimalarial tablets because trust is a factor that can significantly influence purchasing decisions (Istiyani et al., 2022) (Mubarak and Mufeeth, 2020).

Based on the number of respondents, the first segment consists of 66 members and shows more complete characteristics as a potential market. However, the second segment with 34 members is also being targeted as it still shows great opportunities for development. Both segments have interest in new herbal medicines, trust in their efficacy, and value congruence on quality and safety, making them strategic target markets for marketing black pule (*Alstonia spectabilis*) antimalarial tablets.

5. Positioning Analysis Results

Positioning is a strategic decision to serve a particular segment with a specially designed program according to consumer needs (Haque-fawzi et al., 2022). Strong positioning helps build a positive brand image, increase consumer awareness, and influence purchasing decisions. Companies are aware that products and promotions are not always relevant to all customers, because consumer preferences are constantly changing, thus driving competition to become the first choice for customers (Sani and Aslami, 2022).

Table 7: Targeted Segment Characteristics

Indicator	Characteristic	
	First Segment	Second Segment
Life Style	Has a strong preference for herbal medicine	Have a preference for herbal medicine
Value	The main consideration for buying herbal medicine is the quality of the medicinal product	Main consideration for buying herbal medicine is drug safety
Attitude Toward the Product	Very interested in new antimalarial herbal medicines, not sure about no side effects and very confident in the efficacy of herbal medicines	Interested in new antimalarial herbal medicines, confident in the absence of side effects and confident in the efficacy of herbal medicines

Based on **Table 7**, the characteristics table of the two target segments, both show a strong preference for herbal medicines, especially those that have high quality and are safe to use. Therefore, the right positioning for black pule antimalarial tablets is as a new herbal alternative that effectively reduces parasitemia growth, especially *Plasmodium falciparum*, with guaranteed quality and safety.

Product quality is a major factor in consumer purchasing decisions. Products with high quality will be more easily embedded in the minds of consumers and increase their willingness to pay (Anggreini and Suwitho, 2020). Quality also contributes greatly to consumer satisfaction (Munisih, and Malik, 2019). Black pule tablets are produced in accordance with the standards of the Good Manufacturing Practice of Traditional Medicines (CPOTB). Products that comply with CPOTB standards will meet the safety and quality according to their intended use (Kusnadi and Husni, 2023). In

terms of benefits, research shows that *Alstonia spectabilis* extract has strong antiplasmodial activity (IC50: 1.23 µg/mL) against Plasmodium falciparum (Taek et al., 2021), and has proven effective in vivo in reducing parasitemia in mice, even exceeding chloroquine (Taek et al., 2024). The safety of black pule tablet products in the extract is also guaranteed through toxicity tests, with an LD50 value of 7,008 mg/kgBB - classified as non-toxic (BPOM RI, 2022). The wide effective dose range indicates a high therapeutic index, so this product has a high level of safety and is safe to use.

Communicating the benefits, quality and safety of products through effective channels such as social media is essential for building trust, especially by conveying scientific evidence-based information (Bahri et al., 2023). Transparency in the production process and selection of raw materials can strengthen consumer loyalty. With positioning that emphasizes quality, safety, and scientific evidence, as well as targeted marketing strategies, black pule antimalarial tablets have the potential to gain consumer trust, expand the market, and build a strong product identity in a sustainable manner (Khairi et al., 2025).

CONCLUSIONS

Market segmentation of black pule (*Alstonia spectabilis*) antimalarial tablets in South Central Timor District resulted in two segments, with the targeting strategy applied, namely Differentiated Marketing Strategy, which selected both segments as target markets. Both segments have a strong preference for herbal medicine and prioritize product quality and safety. The first segment has experience in using antimalarial herbal medicines, while the second segment has no experience but shows high interest in new products, so it has the potential to accept and trust the efficacy of black pule (*Alstonia spectabilis*) tablets as an antimalarial treatment. Black pule tablets are positioned as a new alternative herbal antimalarial medicine that can reduce the growth of parasitemia, especially *Plasmodium falciparum*, with guaranteed quality and safety. By conducting proper market segmentation and positioning, it is expected that black pule (*Alstonia spectabilis*) antimalarial tablets have significant market potential in South Central Timor District and can increase consumer trust, awareness and long-term loyalty.

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CONFLICT OF INTEREST

The authors emphasize that there is no conflict of interest between the parties involved in this research. The research was conducted independently, with full freedom of design and execution. The results of the research will be published publicly, regardless of the outcome. To maintain objectivity, the authors have taken the necessary steps to minimize the possibility of bias.

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Optimization of Pectin and Hydroxypropyl Methly Cellulose in Fast Dissolving Sublingual Film Salbutamol Sulfate

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ABSTRACT

Salbutamol sulfate is a β-2 adrenergic agonist that works by relaxing bronchial smooth muscles. This drug is suitable for the sublingual fast-disintegrating film (FDSF) delivery system, which offers advantages such as high flexibility and bypassing first-pass metabolism in the liver. A combination of hydrophilic polymers like pectin and HPMC enhances drug release compared to lipophilic polymers. This study applied a 2-level factorial design method, with pectin (0.20 g at low level and 0.30 g at high level) and HPMC (0.01 g at low level and 0.015 g at high level) to determine the optimal formulation. All formulas met the evaluation criteria, including organoleptic and pH testing. Analysis using Design Expert software 13.0.5.0 identified the optimal formulation at the low level of pectin (0.20 g) and HPMC (0.01 g). This formulation showed the shortest disintegration time (12 seconds) and the highest folding endurance (700 times). Verification using a t-test demonstrated no significant differences between experimental results and predictions ($p>0.05$). In vitro dissolution testing revealed that 98.94%±0.57 of salbutamol sulfate dissolved within 60 seconds.

Keywords: Salbutamol sulfate, FDSF, pectin, HPMC

INTRODUCTION

Salbutamol sulfate is the first selective Short-Acting β-Agonist (SABA) widely used as an anti-asthma drug. It belongs to the class of selective β2-adrenergic agonists and acts as a bronchodilator for acute bronchospasm (Marques, 2022). Salbutamol sulfate is primarily available in oral and inhalation forms. However, the oral route may be less suitable for patients who have difficulty swallowing, especially pediatric and geriatric patients. Oral forms such as tablets are subject to first-pass metabolism in the stomach and small intestine, resulting in low bioavailability of around 40% (Sallam *et al*., 2017).

Fast Dissolving Sublingual Film (FDSF) offers an effective alternative for enhancing the bioavailability of oral medications. FDSF dissolves and is absorbed systemically through blood vessels without passing through the digestive system. This form is ideal for patients who have difficulty swallowing, those with gastrointestinal disorders, and those experiencing acute attacks like asthma, as it provides rapid drug release. Its lightweight, flexible structure, and fast onset of action make it a suitable option for asthma treatment.

FDSF is a solid dosage form that rapidly dissolves in saliva when placed under the tongue. It quickly hydrates, adheres, or dissolves (Fitriany, 2022). The preparation of FDSF involves the solvent casting method, where solvents, excipients, and active ingredients—all water-soluble components—are mixed. A crucial component in this formulation is the film-forming agent. Pectin-based films possess excellent mechanical strength and water solubility (Febrasca, 2020). Pectin, a hydrophilic polymer, is brittle and forms a gel layer upon contact with water. Drug release from the pectin matrix occurs through diffusion and erosion (Armadani, 2020).

In the production of oral films, combining hydrophilic polymers results in faster drug release compared to lipophilic polymers (Ermawati & Prilanti, 2019). This study utilized a combination of pectin and a synthetic polymer, HPMC (Hydroxypropyl Methyl Cellulose). HPMC K4M, a low-viscosity variant (4000 mPa·s in a 2% solution), is transparent, strong, highly water-soluble, and allows for faster initial drug release as a film-forming agent (Newton *et al*., 2016). The combination of pectin and HPMC provides the most optimal film integrity compared to other synthetic polymers, making it the choice for optimization in this study.

The optimization process used a factorial design method, selected for its ability to identify dominant factors influencing the response. The responses measured in this optimization were disintegration time and folding resistance. This study aims to obtain the optimal FDSF formulation of salbutamol sulfate using a combination of pectin and HPMC.

METHODS

1. Instrumentations and Materials

Analytical balance (Adventurer^{TM}, Ohaus USA), oven (Memmert, Germany), pH meter (Elemetron CP-502, Poland), UV spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA), micrometer screw gauge, paddle-type dissolution tester (Logan), Design Expert software version 13.0.5.0, SPSS software version 22.0 for method validation analysis, and glassware.

Salbutamol sulfate (Supriya Lifescience LTD, India), pectin (pectin from citrus peel, Galacturonic acid ≥74.0%, Sigma-Aldrich), HPMC (PT. Alpha Chemical), triethanolamine/TEA (BrataChem), KH_2PO4 (PT. Bintang Gemilang), NaOH (PT. Inalab Utama), and aquadest (BrataChem).

2. Preparation of FDSF Salbutamol Sulfate

Salbutamol sulfate FDSF preparations were made using the solvent casting method. First, pectin polymer was dissolved in distilled water and then added to the HPMC solution to form a homogeneous mixture. Salbutamol sulfate and TEA were each dissolved in distilled water before being added to the polymer mixture. The remaining distilled water was then added to the mixture. The mixture was cast and dried in an oven at 50°C for 15 hours. The resulting dry film was then cut into pieces measuring 2 x 1 cm (Pichayakorn *et al*., 2022).

3. Organoleptic Property

Organoleptic testing was conducted by visually observing the color, shape, odor, taste, and surface properties of the film. The desired characteristics of the film in the FDSF preparation include a smooth and dry surface (Nurhabibah *et al*., 2019).

4. Thickness

Thickness test is conducted using a micrometer screw gauge. The thickness of each film is measured at 5 different points: the center and the four corners of the film. An ideal film thickness is 0.5–1.0 mm (Nurhabibah *et al*., 2019).

5. Surface pH

pH surface test involves immersing the film in a vial containing 5 ml of distilled water and then contacting the electrode with the film's surface. Salbutamol sulfat FDSF preparation has a pH range 6-7 (Liu *et al*., 2017).

6. Folding endurance

The test involves three replicates of the film, each folded repeatedly along the same line. A good film should have a folding endurance value of 300 folds or more (Darusman, 2021).

7. Disintegration time

The test is performed by placing the film in a Petri dish containing 20 ml of phosphate buffer at pH 6.8 and observing the time it takes for the film to disintegrate. The film is considered disintegrated when it begins to break apart and dissolve. An ideal disintegration time for FDSF is less than 1 minute (Zubaydah & Sahumena, 2021).

9. %Drug Content

Content determination was performed by dissolving the FDSF in 100 ml of phosphate buffer solution at pH 6.8. The dissolution process was facilitated using a magnetic stirrer. The salbutamol sulfate content in the preparation was determined using UV-Vis spectrophotometry at the maximum wavelength of salbutamol sulfate, which is 276 nm (Zubaydah & Sahumena, 2021). The requirement for salbutamol sulfate content is not less than 98.5% and not more than 100% (Depkes, 2020).

10. Formula Optimization

The folding endurance and disintegration time of Salbutamol Sulfate were analyzed using design expert software version 13.0.5. The optimum formula was obtained from the overlay of the two responses.

11. Optimum Formula Verification

Predicted responses from the factorial design to the observation results were compared statistically with the One-Sample T-test using a confidence level of 95%. The p-value >0.05 shows no difference between the predicted responses and the observed responses.

12. Salbutamol Sulfate Release Study

Salbutamol sulfate in vitro release test was conducted using a type 2 dissolution test kit (paddle type) with a dissolution medium volume of 100 mL, a speed of 100 rpm, and a temperature of 37.5°C. Each formula was placed into a dissolution chamber containing phosphate buffer medium pH 6.8 and then a sample of 5 mL was taken at the specified time (60 seconds). Samples were then taken and

analyzed by looking at the absorbance through UV-Vis spectrophotometry at the maximum wavelength of salbutamol sulfate which is 276 nm. The test was carried out in 3 replicates (Kanna *et al*., 2022).

RESULTS AND DISCUSSION

1. The Physichocemical Characteristics

Table 1 shows the composition of the FDSF salbutamol sulfate. The prepared films were subjected to different physicochemical tests such as thickness, surface pH, folding endurance, disintegration time, content, and %drug content. The four-film formulas were transparent, odorless, elastic, dry, and smooth surface (**Figure 1**).

Table 1: Formula of Fast Dissolving Sublingual Film Salbutamol Sulfate

Materials	Function	Formula (g)			
		F(1)	F(A)	F(B)	F(AB)
Salbutamol sulfate	Active Ingredient	0.096	0.096	0.096	0.096
Pectin	**Polymer**	**0.20**	**0.30**	**0.20**	**0.30**
HPMC	**Polymer**	**0.01**	**0.01**	**0.015**	**0.015**
TEA	Alkalizing Agent	0.03	0.03	0.03	0.03
Aquadest	Solvent	20 ml	20 ml	20 ml	20 ml

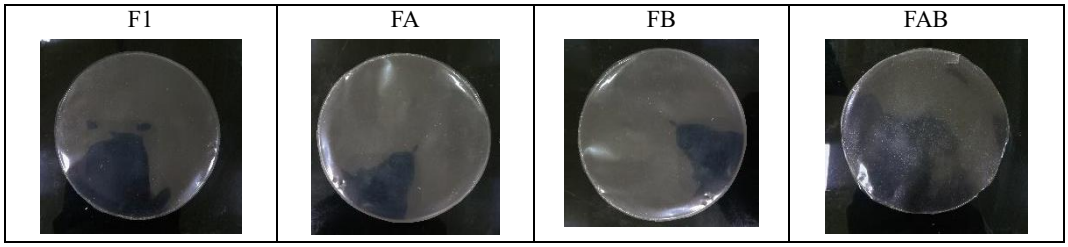

Figure 1. Fast Dissolving Sublingual Film Salbutamol Sulfate

The film thickness for each formula was found to be uniform, indicates that the thickness of all four formulas was uniform, 0.09 mm. Placing the film on a flat surface and drying it evenly can produce a film with uniform thickness.

The pH of the film should meet the ideal pH for sublingual film application because the acidic or alkaline pH may irritate the mucosa. Salbutamol sulfat FDSF preparation has a pH range 6-7 (Liu *et al*., 2017). The four film formula of pH have a range between 6.02-6.41 (**Table 2**).

Table 2: The results of three FDSF Salbutamol Sulfate for Optimization

Formula	Thickness uniformity* (mm)	Surface pH*	Drug Content* (%)
1	0.09	6.41	108.62%
A	0.09	6.02	105.72%
B	0.09	6.27	102.21%
AB	0.09	6.05	93.16%

* Data obtained with replication of 3 times

Persentation of drug content of salbutamol sulfate FDSF preparation ranged from 93.16- 99.95% That it can be said that the resulting content of all formulas has met the requirements of good content in accordance with the Indonesian Pharmacopoeia, namely 90-110%.

Based on **Table 3**, it is shown that all four formulas of salbutamol sulfate FDSF have disintegration times ranging from 12.00-50.67 seconds, meeting the requirement for salbutamol sulfate FDSF disintegration time of <60 seconds. **Table 3** also indicates that the four formulas have folding endurance values ranging from 380 to 700 folds, fulfilling the requirement for salbutamol sulfate FDSF folding endurance of >300 folds (Darusman, 2011). The formula with the shortest disintegration time (12 seconds) and the highest folding endurance (700 folds) is F(1).

Table 3: The result of disintegration time and folding edurance FDSF salbutamol sulfate

Formula	Disintegration Time (seconds) ± SD*	Folding Endurance (folds) ± SD*
1	12,00 ± 2,65	700 ± 43,52
A	28,67 ± 6,11	502 ± 3,61
B	22,00 ± 3,00	647 ± 4,041
AB	50,67 ± 5,13	380 ± 4,58

* Data obtained with replication of 3 times

2. Disintegration Time

The disintegration time test results for the four formulas have met the disintegration time requirements so that the film disintegrates quickly (<60 seconds). The formula with the smallest disintegration time was formula 1 and the longest was formula AB. The order of folding resistance from the lowest to the highest is F1<FB<FA<FAB. Formula 1 had the smallest disintegration time because the amount of pectin and HPMC was at a low level so that the film disintegrated faster. Increasing the concentration of pectin and HPMC as film-forming polymers will also increase the disintegration time of the preparation. Final Equation in terms of Coded Factor:

$$y = +28.33 + 11.33*A + 8.00*B + 3.00*AB.$$

The disintegration time test results were then analyzed using Design Expert software version 13.0.5. The equation shows that the factor coefficients of pectin and HPMC as well as the interaction of pectin and HPMC can significantly improve the disintegration time response (**Figure 2)**.

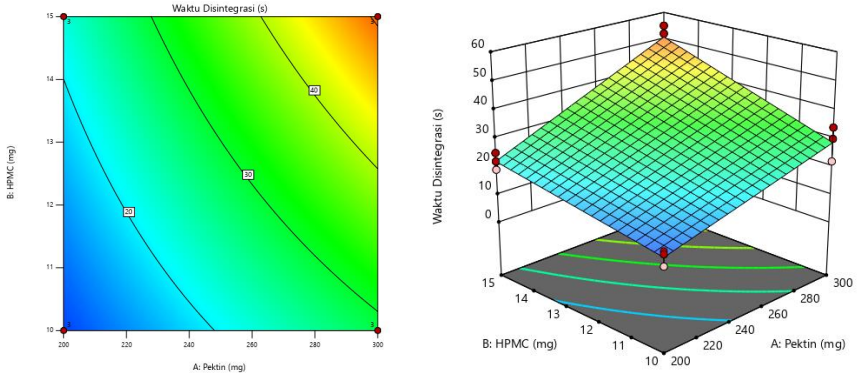

Figure 2. The Result of Countor Plot Disintegration Time

The results of the calculation of the single-factor effect of disintegration time show that pectin is more dominant in determining folding endurance. The use of pectin and HPMC alone resulted in a

positive factor effect. The use of a pectin, HPMC, and combination of pectin and HPMC resulted in a negative factor effect indicating a increase in the disintegration time response (**Table 4**).

Table 4: Effect Factor of Disintegratiom Time

Factor	Disintegration Time Effect
(A) Pectin	+22,67
(B) HPMC	+16
(AB) Interaction	+6

3. Folding Endurance

The folding endurance test results for four formulas have met the folding resistance requirements so that the film has good flexibility. The formula with the highest folding resistance is formula1 and the lowest is formula AB. The order of folding resistance from the lowest to the highest is F1>FB>FA>FAB. Formula AB has the smallest folding resistance because the amount of pectin and HPMC is at a high level so the number of folds is smaller. Increasing the concentration of pectin and HPMC as film forming polymers will lower the folding resistance of the preparation. Final Equation in Terms of Coded Factor:

$$Y = 557.00 - 116.00*A - 43.67*B - 17.33*AB$$

The folding endurance test results were then analysed using Design Expert software version 13.0.5. The equation shows that the factor coefficient of pectin and HPMC can significantly (p value < 0.05) the folding resistance response and then the combination of pectin and HPMC can increase the folding resistance response (**Figure 3**).

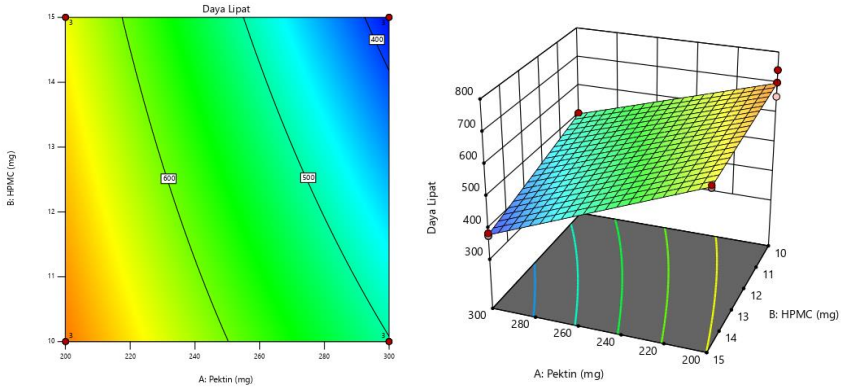

Figure 3. The Result of Countor Plot Folding Endurance

The results of the calculation of the single-factor effect of folding endurance show that pectin is more dominant in determining folding endurance. The use of pectin and HPMC alone resulted in a positive factor effect. The use of a pectin, HPMC, and combination of pectin and HPMC resulted in a negative factor effect indicating a decrease in the folding resistance response (**Table 5**).

Table 5: Effect Factor of Folding Endurance

Factor	Folding Endurance Effect
(A) Pectin	-232.5
(B) HPMC	-118.5
(AB) Interaction	-34.5

4. %Drug Content

The drug content requirements are to contain salbutamol sulfate as much as 90.0% and not more than 110.0% of anhydrous substances (Depkes, 2020). The results show in **Table 6** that all formulas meet the requirements for determining the content of salbutamol sulfate FDSF preparations.

Table 6: %drug content

Formula	%Drug Content
1	108,62%
A	105,72%
B	102,21%
AB	93,16%

5. Formula Optimization

The desired characteristics of the optimum formula include shorter disintegration time and high folding resistance. The results of determining the optimum formula area (overlay plot) can be seen in **Figure 4**.

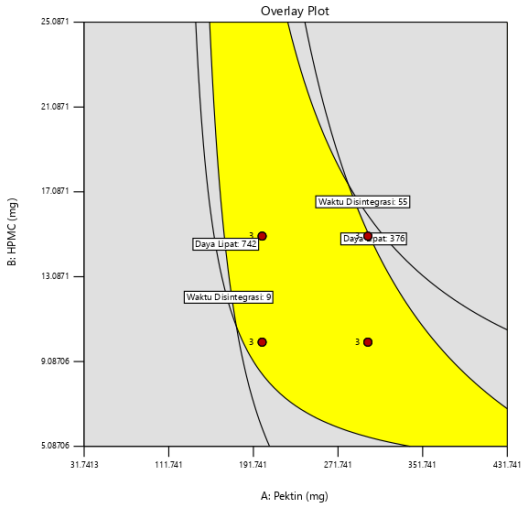

Figure 4. Overlay Plot of Optimum Region Determination

Based on **Table 7**, the optimum formula was chosen with a desirability value of 0.903 with the amount of pectin 0.2 grams and HPMC 0.010 grams. The prediction of the resulting disintegration time is 12 seconds and the prediction of folding resistance is 699 times.

Table 7: Optimum formula solution

Number	Pectin	HPMC	Folding Endurance	Disintegration Time	Desirability	
1	**200,000**	**10,000**	**699,333**	**12,000**	**0,903**	**Selected**
2	200,000	10,054	698,769	12,107	0,902	
3	200,645	10,000	698,060	12,108	0,901	
4	201,095	10,000	697,172	12,183	0,899	
5	200,000	10,126	698,004	12,252	0,899	
6	200,000	10,190	697,328	12,381	0,897	
7	200,000	10,333	695,824	12,666	0,892	
8	200,000	10,496	694,106	12,992	0,886	
9	200,000	12,290	675,212	16,580	0,820	

6. Optimum Formula Verification

Based on the data in **Table 8** the significant results are> 0.05 in both responses, which means that the data from the verification of the optimum formula is not significantly different, so it is almost the same as the prediction results.

Table 8: Optimum formula verification results

Response	Prediction Response Results	Observation Response Results (Average ± SD)*	Significance Results (p)
Disintegration time (seconds)	12	12,67 ± 1,53	0,339
Folding Endurance (times)	700	736 ± 5,57	0,203

* Data obtained with replication of 3 times

7. Salbutamol Sulfate Release Study

The optimum formula obtained is with a low level of pectin (0,20 g) and a low level of HPMC. (0,01 g) The results of the percent dissolution of salbutamol sulfate FDSF preparation can be seen in **Table 9**.

Table 9: Results of Percent Release of Salbutamol Sulfate in vitro

Replication	Absorbance*	Drug Concentration* (ppm)	Drug Content* (%)
1	0,211	29,47	97,94
2	0,213	29,78	98,96
3	0,214	29,93	99,45
Avarage ± SD		98,78% ± 0,77	

* Data obtained with replication of 3 times

CONCLUSIONS

The polymers pectin, HPMC and HPMC pectin interaction had an effect on increasing the disintegration time, but pectin, HPMC and HPMC pectin interaction decreased the folding resistance of the resulting salbutamol sulfate FDSF preparation. Pectin became the dominant factor effect of the response of disintegration time and folding resistance. The optimum salbutamol sulfate FDSF preparation was obtained from the formula with 0.20 g pectin and 0.01 g HPMC. The optimum formula of salbutamol sulfate FDSF preparation had a disintegration time of 12 ± 2.65 seconds and folding resistance of 700 ± 43.52. The results of percent drug release of salbutamol sulfate FDSF in the 1st minute were 98.79% ± 0.57.

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CONFLICT OF INTEREST

There is no conflict of interest.

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Optimization of Pectin and Carboxymethylcellulose Sodium in Fast Dissolving Sublingual Film Salbutamol Sulfate

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ABSTRACT

Salbutamol sulfate is a bronchodilator used to treat asthma, but existing dosage forms have limitations. This study aimed to develop a Fast Dissolving Sublingual Film (FDSF) for improved delivery. The film was prepared using the solvent casting method with a formula based on factorial design, optimizing the amount of pectin and CMC Na. The evaluation of the film included organoleptic, thickness, surface pH, disintegration time, folding resistance, and % drug content. The optimum formula was a combination of 300 mg pectin and 10 mg CMC Na. This combination exhibited rapid disintegration (4.67 ± 1.02 seconds) and high folding endurance (725.33 ± 4.73 folds). The FDSF demonstrated excellent drug release, with 95.19 ± 1.30% of salbutamol sulfate released within 60 seconds. These results suggest that the developed FDSF has the potential to offer a convenient and effective alternative for asthma treatment.

Keywords: FDSF; salbutamol sulfate; pectin; CMC Na

INTRODUCTION

Asthma is a chronic inflammatory disease in which patients experience reversible airway obstruction due to bronchial hyperresponsiveness. It is a health problem in all age groups and is increasingly prevalent in various countries. The World Health Organization (WHO) states that there were 300 million asthma patients worldwide in 2018 and this number may increase to 400 million by 2025 (Firmansyah *et al*., 2023). The prevalence of asthma in Indonesia has reached 4.5% of the population, which amounts to around 11,179,032 individuals (Kementerian Kesehatan RI, 2019). Although the prevalence of asthma has decreased from 4.5% in 2013 to 2.4% in 2018, it remains among the top 10 causes of disease and death in Indonesia (Sutrisna, 2021).

Salbutamol sulfate is a first-line drug in the treatment of asthma This medication is a type of short-acting β2-adrenergic receptor agonist that is intended to relieve bronchospasm occurring in asthma or other chronic obstructive pulmonary diseases. Salbutamol sulfate can be administered in oral, injectable, and inhalation forms. Oral administration in the form of tablets provides only 50% bioavailability, while only 10-20% of the drug dose can reach the lower respiratory tract via inhalation. This highlights the need for recent developments to provide a dosage form that is more effective and preferred by patients as an alternative to the oral form, specifically Fast Dissolving Sublingual Film (FDSF).

Fast Dissolving Sublingual Film (FDSF) is a drug dosage form designed to dissolve quickly when placed under the tongue (sublingual route). FDSF appears as a thin film that dissolves rapidly upon contact with saliva without the need for water or chewing. This route offers advantages such as faster drug absorption and high bioavailability, as the sublingual part of the oral cavity is relatively thinner and has higher blood flow. This dosage form will undoubtedly be very beneficial and can enhance patient compliance, especially for those who have difficulty swallowing, such as geriatrics and pediatrics. FDSF can also be used for patients who need fast drug action (Zubaydah & Sahumena, 2021).

Pectin is a hydrophilic polymer that is often used in the manufacture of film preparations. Pectin has high hydration properties, enabling it to support faster dissolution of the preparation (Elshafeey & El-Dahmy., 2021). Films formed from pectin also exhibit optimal characteristics, with the highest diffusion value compared to other polymers (Patil & Daswadkar, 2020). However, the use of pectin alone results in slower disintegration time, and the resulting film does not meet good quality criteria, as it tends to be too thin. Carboxymethylcellulose sodium (CMC Na) is a hydrophilic polymer that can bind and absorb water, thereby accelerating disintegration time when combined with pectin (Hidayati *et al*., 2019). CMC Na also possesses high swelling properties, which allows it to form films with appropriate thickness (Rani *et al*., 2021). The combination of pectin and CMC Na complements each other, creating films with good integrity, strength, flexibility, rapid dissolution, and enhanced release of active ingredients (Hidayati *et al*., 2019).

In this study, polymer optimization was conducted in the Fast Dissolving Sublingual Film (FDSF) formulation of salbutamol sulfate to obtain the optimum formula for the combination of pectin and CMC Na. The method employed in this research is factorial design, which aims to determine interactions and achieve the optimal formula regarding the ratio between the amounts of orange peel pectin and CMC Na. This research is expected to yield a salbutamol sulfate FDSF preparation that accelerates the release of active ingredients and provides a rapid onset of action.

METHODS

1. Instrumentations and Materials

Instrumentations: Mortar, stamper, porcelain cup, measuring cup, beaker, stirring rod, petri dish, spatula, hotplate, analytical balance (AdventurerTM Ohaus USA), oven (Memmert, Germany), pH meter (Elemetron CP-302. Poland), micrometer screw, UV spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, USA), paddle type dissolution tester (Logan), Design expert software version 13.0.5.0, and validation method of analysis software. Materials: Salbutamol sulfate (Supriya Lifescience LTD, India), pectin (pectin from citrus peel Galacturonic acid ≥ 74.0%, Sigma Aldrich), CMC Na (PT. BrataChem), Na saccharin (PT. BrataChem), triethanolamine/TEA (PT. BrataChem), KH_2PO_4 (PT. Bintang Gemilang), NaOH (PT. Inalab Utama), and distilled water (PT. BrataChem).

2. Preparation of FDSF Salbutamol Sulfate

Preparation begins with dissolving pectin in distilled water, which is then mixed with CMC Na that has been prepared in hot water at 70ºC. TEA (triethanolamine) is added to the polymer mixture, followed by salbutamol sulfate, which has been dissolved in distilled water. The mixture is stirred until homogeneous. This mixture is then oven-dried at 50ºC for 20 hours. The dried film is released from the mold and cut into sizes of 2×1 cm^2, containing approximately 3 mg of salbutamol sulfate in each film.

FDSF formula with a film size of 2×1 cm² uses salbutamol sulfate at a dose of 3 mg. The mold used is a petri dish with a diameter of 9 cm, which has an area of 63.585 cm², allowing for the production of 32 films in one mold (**Table 1**).

Table 1: FDSF salbutamol sulfate dosage formula (1 mold)

Materials	Function	Formula			
		F(1)	F(A)	F(B)	F(AB)
Salbutamol sulfate	Active ingredients	0,096 g	0,096 g	0,096 g	0,096 g
Pectin	**Hydrophilic polymer**	**0,200 g**	**0,300 g**	**0,200 g**	**0,300 g**
CMC Na	**Hydrophilic polymer**	**0,010 g**	**0,010 g**	**0,015 g**	**0,015 g**
TEA	Alkalizing agent	0,030 g	0,030 g	0,030 g	0,030 g
Aquadest	Solvent	20 mL	20 mL	20 mL	20 mL

2.1 Organoleptic

The organoleptic test is a visual assessment based on the characteristics of the preparation, which includes testing for color, taste, texture, aroma, and surface conditions (Zubaydah & Sahumena, 2021).

2.2 Thickness

The thickness uniformity test is carried out because it is directly proportional to the accuracy of the film dose. This test was conducted by measuring three films using a screw micrometer at five points and then determining the average (Vardai, 2021). The thickness requirement for a 2×2 cm^2 film is a maximum of 100 µm, while for a 2×3 cm^2 film is a maximum of 350 µm. These conditions indicate that the 2×1 cm^2 film is expected to have a maximum thickness of 100 µm (Darusman *et al*., 2020; Wasilewska & Winnicka, 2019).

2.3 Surface pH

The pH test is very important to ensure that the preparation does not irritate the patient's oral cavity. The test was conducted by soaking the film in 5 mL of distilled water and measuring it with a pH meter. The pH requirement for a good sublingual film is between 5.5 and 7 (Susanti *et al*., 2024; Liu *et al*., 2017).

2.4 Disintegration Time

The disintegration time is a test to determine the time it takes for the film to disintegrate when administered sublingually. The test was conducted by placing the film on a petri dish, then adding 20 mL of phosphate buffer solution at pH 6.8, and recording the time it takes for the film to disintegrate (Vardai, 2021). The film is expected to disintegrate in ≤ 1 minute (Zubaydah & Sahumena, 2021).

2.5 Folding Endurance

The folding resistance test was conducted to determine the level of flexibility of the film. Films with excellent flexibility should have more than 300 folds at the same point with an angle of 180° (Wasilewska & Winnicka, 2019).

2.6 %Drug Content

The drug content was determined to assess the amount of salbutamol sulfate contained in each film. The stages involved dissolving the film in 10 mL of phosphate buffer solution at pH 6.8 using a magnetic stirrer. This solution was then pipetted to obtain 1 mL and subsequently diluted to a final volume of 10 mL. The concentration of salbutamol sulfate present in the film was then measured using

a UV-Vis spectrophotometer at its maximum wavelength of 276 nm (Zubaydah & Sahumena, 2021). The requirements for the content determination test state that the salbutamol sulfate must be not less than 90.0% and not more than 110.0% of the amount stated on the label (Depkes RI, 2020).

2.7 Formula Optimization

The data analysis used in this study to determine the optimum formula was based on a factorial design. The responses measured were disintegration time and folding resistance. The values obtained from each response were processed using Design Expert, and a general equation will be derived that states the relationship between factors and responses, namely $Y = b_0 + b_aX_A + b_bX_B + b_{ab}X_AX_B$. From this equation, the values of b_0, b_a, b_b, and b_{ab} can be calculated and then used to obtain a contour plot between disintegration time and folding resistance using Design Expert software. The contour plot results will show the optimum combination of pectin and CMC Na.

2.8 Optimum Formula Verification

The optimum formula obtained from the results using Design Expert software was formulated with three replications. The preparation was tested for disintegration time and folding resistance, and the response results were compared with the predicted responses from the factorial design method using a one-sample t-test on SPSS 22.0 software at a 95% confidence level. Data results that show significance > 0.05 indicate no significant difference.

2.9 Salbutamol Sulfate Release Study

The in vitro release test of salbutamol sulfate was conducted using a type 2 dissolution test device (paddle) at a speed of 100 ± 2 rpm. The media used was phosphate-buffered saline at pH 6.8, with a volume of 100 mL, maintained at a temperature of 37 ± 0.5°C. A sample solution of 5 mL was taken at a one-time point (60 seconds) (Kanna *et al*., 2023). This sample was analyzed with a UV-Vis spectrophotometer at the maximum wavelength of salbutamol sulfate, which is 276 nm, and the amount of active ingredient dissolved was calculated (Teaima *et al*., 2022). The requirement for the salbutamol sulfate release test is to dissolve no less than 85% (Q + 5%) (Depkes RI, 2020).

RESULTS AND DISCUSSION

1. The Physicochemical Characteristics

The prepared films with FDSF salbutamol sulfate composition (**Table 1**) were subjected to various physicochemical tests such as organoleptic, thickness, surface pH, disintegration time, folding resistance, and % drug content. The four film formulas were transparent, bitter, elastic, odorless, dry, and had a smooth surface (**Figure 1**).

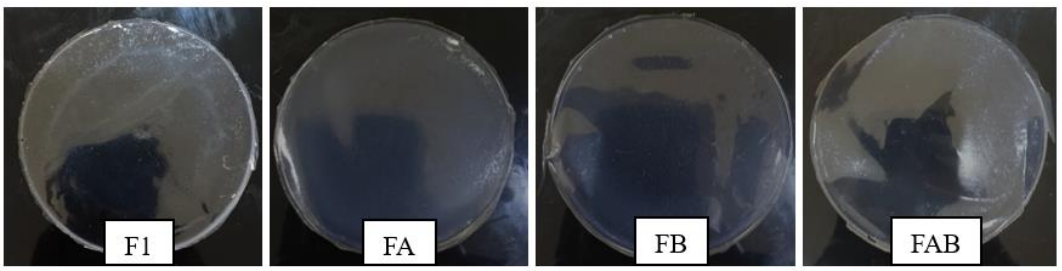

Figure 1. FDSF salbutamol sulfate (1 mold)

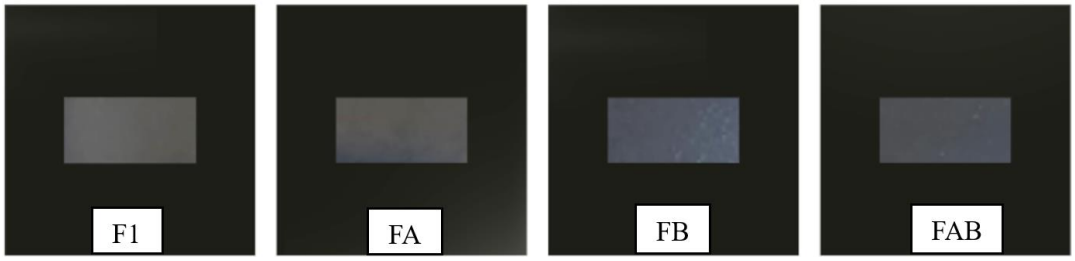

Figure 2. FDSF salbutamol sulfate (2×1 cm² film)

The requirement for the thickness of a 2×1 cm² film is a maximum of 100 µm (**Figure 2**), and the coefficient of variation should be less than 5% (Darusman *et al*., 2020; Wasilewska & Winnicka, 2019). The test results showed that all formulas met the requirements for film thickness uniformity (**Table 2**). Placing the film on a flat surface and drying it evenly can produce a film with uniform thickness.

Table 2: Salbutamol sulfate FDSF thickness test results

Formula	Thickness Uniformity* (mm)	Surface pH*	%Drug Content*
1	0.04	6.33	97.65
A	0.04	5.84	93.73
B	0.05	6.29	94.21
AB	0.05	5.85	97.57

* Data obtained with replication of 3 times

A good film preparation has a pH ranging from 5.5 to 7.9 (Susanti *et al*., 2024). Salbutamol sulfate is also stable at pH levels between 3.5 and 7 (Liu *et al*., 2017). This indicates that the pH requirement for FDSF salbutamol sulfate is between 5.5 and 7. The four film formulas tested for pH have a range between 5.84 and 6.33.

The % drug content requirement is to contain salbutamol sulfate in an amount not less than 90.0% and not more than 110.0% of the amount stated on the label (Depkes RI, 2020). The regression equation obtained from the standard curve is y = 0.0064x + 0.0221. The results show that all formulas meet the requirements for % drug content of salbutamol sulfate in FDSF preparations, with a range of 93.73% to 97.65%.

The disintegration time of film preparations is considered good if it is ≤ 60 seconds (Zubaydah & Sahumena, 2021). The folding resistance of the film preparation in the excellent category is more than 300 folds (Wasilewska & Winnicka, 2019). The results showed that all formulas had disintegration times and folding resistances that can be classified as good films (**Table 3**).

Table 3: The response testing results for optimization

Formula	Disintegration times (seconds) ± SD*	Folding endurance (folds) ± SD*
1	6.97 ± 0.67	362 ± 18.58
A	7.08 ± 0.68	726 ± 10.00
B	11.94 ± 1.82	674 ± 21.63
AB	4.27 ± 1.23	321 ± 24.43

* Data obtained with replication of 3 times

2. Disintegration Time

The disintegration time test results showed that FAB < F1 < FA < FB, with values of 4.27 ± 1.23; 6.97 ± 0.67; 7.08 ± 0.68; 11.94 ± 1.82 seconds, respectively. This indicates that the combination of pectin and CMC Na at high levels can disintegrate faster. The results demonstrated that the formula with the lowest disintegration time was obtained in formula AB, which suggests that when pectin and CMC Na are combined at high levels, their ability to bind water increases, allowing them to disintegrate more easily (Hidayati *et al*., 2019).

The results of this disintegration time test were analyzed using Design Expert version 13.0.5.0 software and the coded factors equation was obtained:

$$Y = 7.56 - 1.89A + 0.5417B - 1.95AB$$

This equation shows that pectin can reduce disintegration time, and the combination of both can further reduce disintegration time, while CMC Na can increase disintegration time.

The contour plot results showed that formulas with the same amount of pectin and CMC Na along the line would result in the same disintegration time (**Figure 3**). An increase in the amount of pectin, primarily from 200 to 250 mg, and a decrease in CMC Na from 15 mg to 10 mg led to a reduction in disintegration time. This contour plot also shows that an increase in the amount of pectin, mainly from 270 to 300 mg, and CMC Na from 11 mg to 15 mg leads to a decrease in disintegration time. The combination with the fastest disintegration time was pectin at approximately 300 mg and CMC Na at approximately 15 mg. Increasing the amount of both hydrophilic polymers, namely pectin and CMC Na, can enhance hydration, resulting in a faster disintegration time for the preparation.

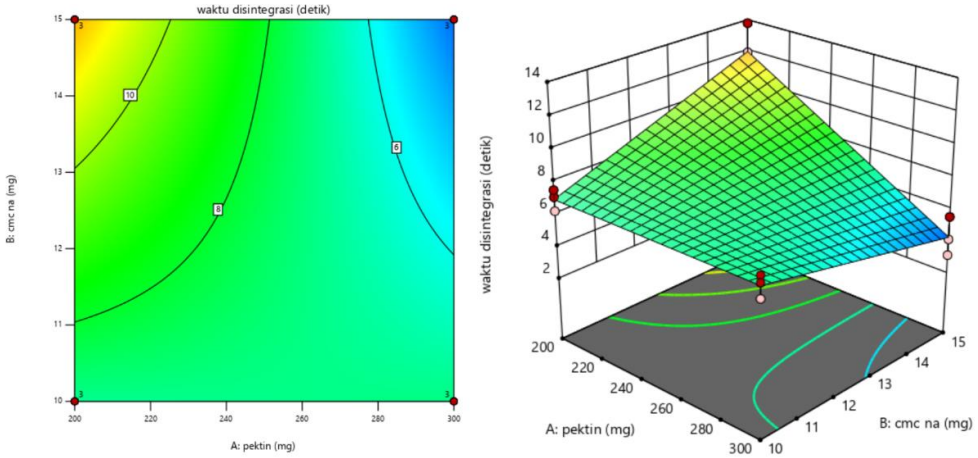

Figure 3. The results of contour plot disintegration time

The calculation of the effect of the disintegration time factor (**Table 4**) shows that the interaction of pectin and CMC Na is the dominant factor, with an effect of -3.89. This indicates that to produce a shorter disintegration time, a combination of pectin and CMC Na can be used.

Table 4: Effect of factors on disintegration time

Factor	Effect on disintegration time
Pectin (A)	-3,78
CMC Na (B)	+1,08
Interaction (AB)	-3,89

3. Folding Endurance

The folding endurance test results showed that FA > FB > F1 > FAB with values of 361.33 ± 18.58; 726.00 ± 10.00; 674.00 ± 21.63; and 321.00 ± 24.43, respectively. This indicates that the combination of high-level pectin and low-level CMC Na has the highest folding endurance or the best flexibility among the other formulas. The results demonstrate that the formula with the highest folding endurance is obtained in formula A, which suggests that when high levels of pectin are combined with low levels of CMC Na, it produces a film that easily binds water or moisture in an environment with a low level of swelling, thereby increasing its flexibility (Nurdiani *et al*., 2019; Rani *et al*., 2021).

The folding resistance test results were analyzed using Design Expert version 13.0.5.0 software, and the coded factors equation obtained was:

$$Y = 520.58 + 2.92A - 23.08B - 179.42AB.$$

This equation shows that pectin can increase folding endurance, while CMC Na and the combination of both can decrease folding endurance.

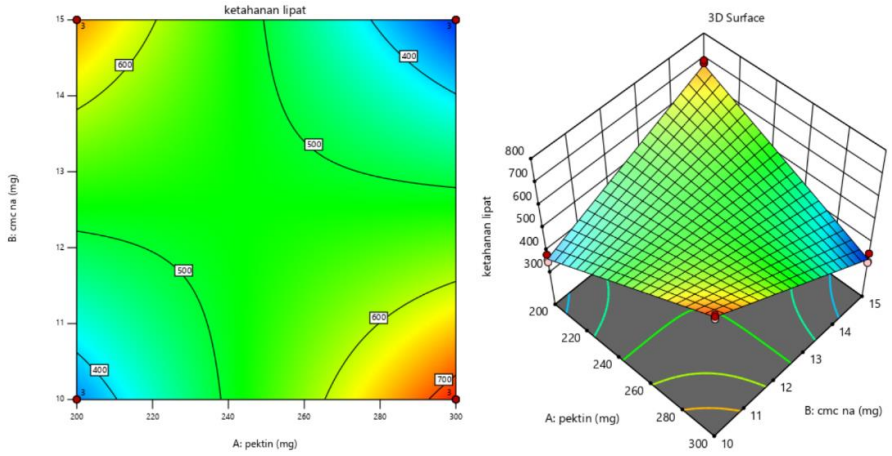

Figure 4. The results of contour plot folding endurance

The contour plot results show that the formula with the amounts of pectin and CMC Na along the line will produce the same folding endurance (**Figure 4**). An increase in the amount of pectin from about 260 to 300 mg, along with a decrease in CMC Na from about 12 to 10 mg, and an increase in pectin from about 200 to 240 mg with an increase in CMC Na from about 10 to 13 mg can improve the folding endurance of the film preparation. However, an increase in the amount of pectin from about 250 to 200 mg, along with an increase in CMC Na from about 13 to 15 mg, and an increase in pectin from about 200 to 240 mg with an increase in CMC Na from about 13 to 15 mg can reduce the folding endurance of the film preparation.

Table 5: Effect of factors on folding endurance

Factor	Effect on folding endurance
Pectin (A)	+5,84
CMC Na (B)	-46,17
Interaction (AB)	-358,84

The calculation of the effect of folding endurance factors (**Table 5**) shows that the interaction of pectin and CMC Na is the dominant factor, with an effect of -358.84. This indicates that the combination of the two is more dominant in causing a decrease in folding endurance, as indicated by the negative value.

4. Formula Optimization

Table 6: Optimum formula response criteria

Response	Kriteria	Goal	Importance
Disintegration time (seconds)	≤ 60 seconds	Minimize	+++++
Folding endurance (folds)	> 300 folds	Maximize	++

The response data for disintegration time and folding endurance were analyzed using Design Expert version 13.0.5.0 software to obtain the optimum formula for FDSF salbutamol sulfate preparation. The desired optimum formula criteria are to have a shorter disintegration time and higher folding endurance (**Table 6**).

Table 7: Solution to determine the optimum formula

Number	Pectin	CMC Na	Time disintegration	Folding endurance	Desira-bility	Desirability (w/o Intervals)	
1	**300.000**	**10.000**	**7.077**	**726.000**	**0.916**	**0.944**	**Selected**
2	300.000	10.043	7.052	722.505	0.915	0.942	
3	200.000	15.000	11.940	674.000	0.824	0.849	

This research resulted in three solutions shown in the Design Expert version 13.0.5.0 software (**Table 7**). The optimum formula obtained was a combination of pectin (300 mg) and CMC Na (10 mg), with a predicted disintegration time of 7.077 seconds and a folding endurance of 726,000 folds. The desirability value of this formula is 0.916, indicating that this formula has a high potential to provide the desired results, as it approaches 1. When viewed from the overlay plot, this formula is located in the yellow area, which indicates that the optimum formula can produce preparations according to the predicted desired characteristics (**Figure 5**).

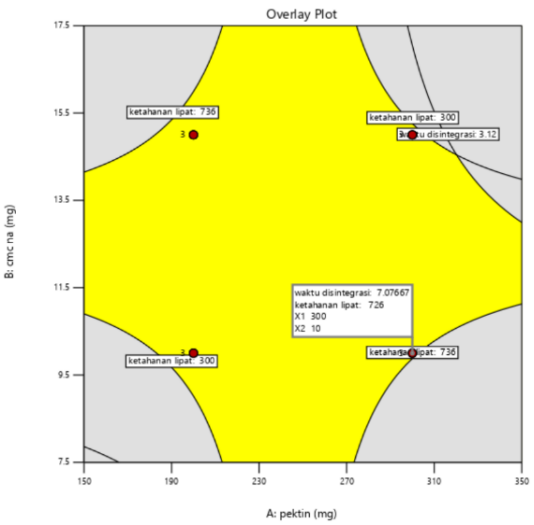

Figure 5. Overlay plot of optimum area determination

5. Optimum Formula Verification

The optimum formula, a combination of 300 mg of pectin and 10 mg of CMC Na, was formulated in three replicates. The preparation was tested for disintegration time and folding endurance. Data results using a one-sample t-test (**Table 8**) showed that the significance value for disintegration time was 0.058, and for folding resistance, it was 0.830. These results met the requirement of significance > 0.05. A significance value > 0.05 means not significantly different, whereas < 0.05 means significantly different.

Table 8: Optimum formula verification

Response	Prediction	Average ± SD*	Significance value (p)
Disintegration time (seconds)	7.077	4.67 ± 1.02	0.058
Folding endurance (folds)	726.000	725.33 ± 4.73	0.830

* Data obtained with replication of 3 times

6. Salbutamol Sulfate Release Study

The results of the salbutamol sulfate release study (**Table 9**) showed that 95.19 ± 1.30% of the drug was released within 60 seconds. The requirement for the salbutamol sulfate release test is to dissolve no less than 85% (Q + 5%) (Depkes RI, 2020). This indicates that the results have met the requirements.

Table 9: Optimum formula content determination results

Replication	Absorbance*	Drug concentration* (ppm)	Drug content* (%)
1	0.207	28.89	96.11
2	0.206	28.73	95.77
3	0.202	28.11	93.70
Average ± SD			95.19 ± 1.30 %

* Data obtained with replication of 3 times

CONCLUSIONS

The sole use of pectin in the formulation of Fast Dissolving Sublingual Films (FDSF) containing salbutamol sulfate can decrease disintegration time and increase folding endurance. CMC Na, when used alone, can increase disintegration time and decrease folding endurance. The interaction between the two can reduce both disintegration time and folding endurance. The optimal formula was achieved with 300 mg of pectin and 10 mg of CMC Na. The predicted response for the optimal formula was a disintegration time of 7.077 seconds and a folding endurance of 726.000 folds. The actual characteristics of the optimal formula showed a disintegration time of 4.67 ± 1.02 seconds, a folding endurance of 725.33 ± 4.73 folds, and a salbutamol sulfate release of 95.19 ± 1.30% within 60 seconds.

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CONFLICT OF INTEREST

There is no conflict of interest.

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Morpho-Anatomical and Secondary Metabolite Distribution Analysis of *Acriopsis Liliifolia* and *Dendrobium Mutabile* for Pharmacological Potential

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ABSTRACT

Orchids (*Orchidaceae*) are highly diverse plants known for their secondary metabolites. However, information on the distribution of secondary metabolites in plant tissues is still limited, especially in morpho-anatomical analysis using the SEM and TEM methods. This study analyzed the morpho-anatomical structure of *Acriopsis liliifolia* and *Dendrobium mutabile*, also examined the distribution and potential of their secondary metabolites. The methods used are macroscopic and microscopic morpho-anatomical observations, Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) analysis. Additionally, microchemical assay was conducted to analyzed the distribution of secondary metabolites and potential in the pharmacological field. The results show that the morphology and anatomy of both species have special adaptations to the epiphytic environment, with certain tissue structures that support the accumulation of secondary metabolites. The distribution of secondary metabolites is concentrated in certain tissues such as epidermis, trichomes, and secretory tissues. The secondary metabolite content produced was identified as having potential as a pharmacological agent that can be further developed in its application as nature-based medicine. This study provides insight into the relationship between tissue structure and accumulation of bioactive compounds that contribute to the development of orchid utilization in the fields of pharmacy and biotechnology.

Keywords: Orchid; potential nature-based medicine; Scanning Electron Microscope (SEM); Transmission Electron Microscope (TEM).

INTRODUCTION

Orchidaceae is a family of angiosperms consisting around 700 genera and more than 30,000 species in the world (Wang et al., 2024). In Indonesia, there are about 6,000 species distributed in 200 genera, with more than 5,000 of them being endemic species, especially in Papua, Kalimantan, and Java (Wati et al., 2023). On Java Island, various epiphytic and terrestrial orchid species are distributed in various ecosystems, ranging from tropical rainforests to rocky and mountainous areas. Orchids are known to have great ecological and economic potential, but the utilization of orchids in Indonesia is still limited, especially in the field of pharmacology. It was around 130 orchid species have

been studied as medicinal plants, while in Indonesia there are only 39 species known to have medicinal properties (Wati et al., 2023). The content that can be utilized as medicine mostly comes from secondary metabolite compounds.

There are interesting orchids need to further study including *Acriopsis liliifolia* and *Dendrobium mutabile*. These two species have in common in the term of natural habitat, which are epiphytic orchids. They were distributed in various tropical forest area throughout Indonesia, including Java Island. *Acriopsis liliifolia* is generally found in areas with high humidity and often grows attached to large tree trunks, while *Dendrobium mutabile* is found in medium to open canopied areas. *A. liliifolia* leaves contain secondary metabolite compounds such as flavonoids, phenolics, terpenoids, and alkaloids (Khoeriyah et al., 2024). Several previous studies have identified the presence of secondary metabolites in *Dendrobium* species, such as alkaloids, flavonoids, and phenolics, which have pharmacological activities, such as antioxidant and anti-inflammatory (Meng et al., 2023). Different environmental adaptations likely contribute to variations in secondary metabolite production, as epiphytic plants often produce bioactive compounds to survive environmental stresses. Therefore, it is important to understand how the morphological and anatomical structure of orchids relates to the distribution of secondary metabolites in plant tissues.

As epiphytic plants, orchids of the genera *Acriopsis* and *Dendrobium* possess distinctive morphological and anatomical characteristics compared to terrestrial species. In addition to environmental factors, the physical structure of plant tissues also plays a significant role in the production, distribution, and storage of secondary metabolites. The biosynthesis of secondary metabolites is influenced by the physiological state of the plant and environmental conditions through specific metabolic pathways (Paramanya et al., 2021). Therefore, understanding the morphology and anatomy of plant tissues is not only essential for taxonomic classification but also holds considerable importance in the context of bioactivity, pharmacology, and biotechnology. Many previous studies have focused on identifying secondary metabolites through chemical profiling methods (Khoeriyah et al., 2024) or in silico modeling approaches (Kinasih et al., 2022), without linking these findings to their biological localization or microscopic structural context. This has resulted in a lack of information regarding how biological structures facilitate the accumulation and function of secondary metabolites. Microscopic approaches such as Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) can reveal the structural details of tissues where these metabolites are stored. The visual data obtained from SEM and TEM can serve as a crucial foundation for further research using three-dimensional imaging technologies such as MicroCT and Cryo-Electron Microscopy (Cryo-EM) at the BRIN research facility.

The physiological adaptations are also employed by epiphytic orchids to ensure survival in their aerial environment. Each plant organ possesses distinct anatomical characteristics that support growth and functionality. Scanning Electron Microscopy (SEM) was performed on the external surfaces of the roots, leaves, and pseudobulbs of *Acriopsis liliifolia* and *Dendrobium mutabile* (**Figure 2**) to examine surface morphology. The SEM analysis of root samples revealed that the most prominent structure observed was the velamen. In *A. liliifolia*, the surface texture appeared more clearly defined and structured, whereas in *D. mutabile*, the texture was smoother and less porous. The outermost layer of aerial roots, which overlies the exodermis, is referred to as the velamen radicum (Hauber et al., 2020), and it is consistent with the root type found in both species. Velamen consists of multiple layers of dead cells with lignified secondary cell walls, which provide structural integrity and facilitate water absorption and retention (Idris et al., 2021). This porous tissue network primarily functions in absorbing moisture from the atmosphere through the process of imbibition. The absorbed water is subsequently

transferred to the living inner cells known as passage cells (Hauber et al., 2020). In the SEM images of *A. liliifolia* roots (**Figure 2a**), a polygonal pattern of densely arranged hollows was evident, forming a distinctive geometric structure. Further anatomical features may be observable through transverse section imaging. In contrast, SEM images of *D. mutabile* roots exhibited a denser and more layered velamen surface, with fewer visible pores compared to *A. liliifolia*. A characteristic feature of the velamen in *Dendrobium* species is the helical thickening of the cell walls within the velamen layers (Idris et al., 2021), which contributes to structural robustness and may influence water retention efficiency.

Following, Microchemical testing is a classic qualitative technique used to identify the presence of specific chemical compounds within plant tissues by applying targeted reagents. These reagents induce distinct reactions, such as color changes or crystal formation, which serve as indicators of particular compounds. Plants are composed of three major tissue systems: the ground tissue system, the dermal tissue system, and the vascular tissue system. Among these, the dermal tissue plays a crucial role in protecting the plant from environmental factors, with the epidermis acting as the main outer barrier. Within the cells and tissues of the epidermis, a diverse array of compounds can be found, including cutin, waxes, salts, lignin, resins, and various other specialized substances (M. F. Huda and Meishanti, 2023). These compounds vary not only in their composition but also in their specific locations, depending on the plant species. Microchemical testing involves the microscopic observation of plant tissue samples after reagent application, allowing researchers to localize chemical reactions and determine which tissues contain particular substances. Despite the significant advancements in modern analytical techniques, microchemical methods continue to hold value, particularly as rapid screening tools. They provide swift, preliminary insights into the presence of compounds within complex biological mixtures, supporting further, more detailed analyses (M. F. Huda et al., 2023).

The selection of *Acriopsis liliifolia* and *Dendrobium mutabile* as research samples was based on their unique habitat characteristics and known bioactive potential in their genus. Understanding the morphological structure, anatomy, and distribution of secondary metabolites in these two species can provide new insights related to adaptation mechanisms and the potential utilization of bioactive compounds. The purpose of this study is to provide information that is a comprehensive description of the morphology, anatomy, content of secondary metabolite compounds, their distribution patterns, and the potential of secondary metabolites in *Acriopsis liliifolia* and *Dendrobium mutabile* in the pharmacological field to further understand how plant structure relates to the storage and distribution of secondary metabolites.

METHODS

1. Plant Material

Acriopsis liliifolia and *Dendrobium mutabile*, both recognized as epiphytic orchids, were selected for this study. Epiphytic orchids are plants that grow on other plants for structural support, obtaining moisture and nutrients from the surrounding air and rainfall. To thrive in such environments, they exhibit a range of specialized morphological and physiological adaptations, including pseudobulbs for water storage, thickened cuticles to minimize water loss, and roots equipped with velamen tissue for enhanced water absorption and protection.

The plant materials used in this study were collected from a previous research project (BRIN-ACIAR) conducted in the karst mountain region of Purwosari, Kulon Progo, Yogyakarta Special Region. The collected samples were carefully selected and prepared for further analysis, ensuring they were

representative of natural conditions and suitable for the planned morphological and anatomical assessments.

2. Microchemical Testing

Microchemical tests were performed to detect specific phytochemical compounds within plant tissues, following histochemical and microchemical methods (Kumar et al, 2021; Sigh and Verma, 202). This technique requires only minimal sample amount, minimizing sample destruction during analysis. The procedure involves applying selected reagents to fresh or fixed plant tissue section, followed by examination under light microscope. The presence of particular secondary metabolites is indicated physical or chemical changes such as microscopic crystal formation, principate appearance, or color shift.

In this study part of the microchemical analyses were conducted at National Research and Innovation Agency facility. The equipment and materials used included a light microscope (Olympus CX21), object glass (Sail Brand, CAT. No. 7101), and cover glass (HERMA, 22 × 22 mm). The reagents employed were citroborate reagent for flavonoid testing, Dragendorff's reagent for alkaloid testing, 10% $FeCl_3$ solution for phenolic testing, and anisaldehyde reagent for terpenoid testing. All reagents were obtained from CV Kimia Jaya Labora. This study analyses targeted four major group of secondary metabolites, which are flavonoid, phenolic, alkaloids, and terpenoids. Specific reagents were used for each compound class, and the reaction were then carefully observed to determine the presence and distribution of these metabolite within various orchid tissues. The small amount reagent volume required, along with the flexibility to performed testing in the different location, made microchemical methods especially suitable for this research (Pete et al, 2022).

3. Scanning Electron Microscope (SEM) Analysis

Scanning Electron Microscopy (SEM) analysis was conducted at the BRIN laboratory located in Playen, Gunungkidul, Special Region of Yogyakarta, Indonesia. All equipment and materials used were provided by the laboratory facilities. The observed organs of orchids included the roots, leaves, and pseudobulbs. A series of procedures was conducted to preserve the integrity of the surface microstructure, based on a modified method adapted from several established protocols. For SEM observation, the best-preserved parts of each organ were selected and sectioned into thin slices with a thickness of 1–5 mm to facilitate efficient penetration during the drying and coating processes. The samples were then placed in sealed vial bottles covered with plastic wrap to prevent direct exposure to air and minimize contamination. The sealed vials containing the samples were placed in a low-pressure machine for 24 hours. This step was intended to reduce moisture content and minimize structural damage caused by atmospheric pressure during subsequent drying.

After the low-pressure treatment, the samples were further dried using a benchtop-pro system for three days. This drying technique represents a simpler and more cost-effective alternative to the critical point drying method employed by Lumaga et al. (2012). Once completely dried, the samples were coated with a thin layer of gold (Au) using a sputter coater set at 20 mA for 60 seconds. This coating procedure produced a conductive layer approximately 20–30 nm thick, which is essential to prevent surface charging during SEM scanning.

SEM observation was conducted using the following configuration: accelerating voltage (Vacc) of 3 kV, spot intensity (SI) at 30%, aperture size 4, high vacuum mode, and a secondary electron detector. Magnifications used were 25×, 500×, and 10,000×. This configuration allowed for the acquisition of high-resolution surface topography images. To identify the elemental composition of the tissue surface,

energy-dispersive X-ray spectroscopy (EDS) analysis was also performed using an accelerating voltage (Vacc) of 15 kV, spot intensity (SI) at 70%, aperture size 2, high vacuum, and a secondary electron detector at 200× magnification. These parameters supported the characterization of cell wall elements and residual secondary metabolites present on the tissue surface.

RESULTS AND DISCUSSION

1. Morphological of Orchids

Based on the findings of our study (**Table 1**), we observed that although *Acriopsis liliifolia* and *Dendrobium mutabile* showed the same epiphytic type in the natural habitat, they reveal morphological differences that reflect distinct survival strategies designed by their environment. Both species exhibit sympodial growth, characterized by the production of lateral shoots that enable them to expand across their host surfaces. This branching pattern enhances their ability to secure space, light, and moisture as a key resource for epiphytic orchids type.

Table 1: Morphological differences between *A. liliifolia* and *D. mutabile*

Morphological aspects	***Acriopsis liliifolia***	***Dendrobium mutabile***
Growth Patterns	Sympodial	Sympodial
Type of roots	Sticky roots	Sticky roots
Pseudobulb	Round, grow close in a clump	The shapes are bilateral compressed, with vertical growth and has internodes
Stem	None	Long, has a lot of branching, blackish brown,
Leaves	Growing from pseudobulb of 2-3 strands, ribbons average 21 cm long, green	Lancet, pointed tip, 4-9 cm long, adaxial green, abaxial green with purple outline
Flowers	Inflorescence panicle-bunches up to 60 cm long. at the tips and branches can grow flowers. Flowers up to 200, tepal white yellow with violet lines	grows at the end of the stem, in clusters of 10-12 flowers, white to purplish with a yellow lip

The data showed that while both species possess sticky roots for anchorage and nutrient absorption, the roots of *D. mutabile* were consistently longer. This extended root system likely enhances not only nutrient uptake but also the plant capacity for vegetative propagation through the formation of young plantlets. This suggests a reproductive strategy in *D. mutabile* that emphasizes clonal expansion alongside survival.

Acriopsis liliifolia does not possess a stem like *Dendrobium mutabile*, which develops a stem that can exceed 100 cm in height (**Figure 1**). Instead, *A. liliifolia* has branching structures from which the leaves emerge, characterized by a blackish-brown coloration. Stem elongation is associated with increased utilization of water and nutrients, which are essential for maintaining floral longevity and overall plant health (Li et al., 2022). Additionally, elongated stems enable the plant to access more effective light exposure, thereby enhancing photosynthesis. Leaves of *A. liliifolia* emerge directly from pseudobulbs, with each pseudobulb capable of producing 2–3 ribbon-shaped leaves, averaging 21 cm in length and bright green in color. In contrast, *D. mutabile* bears leaves along the extended stem, which are lanceolate with pointed apices, measuring approximately 4–9 cm in length. The adaxial (upper) surface is green, while the abaxial (lower) surface is green with distinctive purple stripes. These purple stripes are likely due to the accumulation of anthocyanins, a class of flavonoid pigments. In related

species such as *D. officinale*, pigmentation is regulated by multiple genes, including those encoding enzymes involved in the anthocyanin biosynthesis pathway (Zhan et al., 2020). The presence of flavonoids, as documented in related taxa, suggests that a similar metabolic pathway may be active in *D. mutabile*. Inflorescences of *A. liliifolia* grow in panicles reaching up to 60 cm in length, with up to 200 flowers per panicle. The flowers display yellowish-white tepals with violet striations. In *D. mutabile,* flowers are borne in clusters of 10–12 at the stem apex and range in color from white to pale purple, featuring a distinct yellow labellum (flower lip). The visually attractive flowers likely serve to attract insect pollinators (Teoh, 2021).

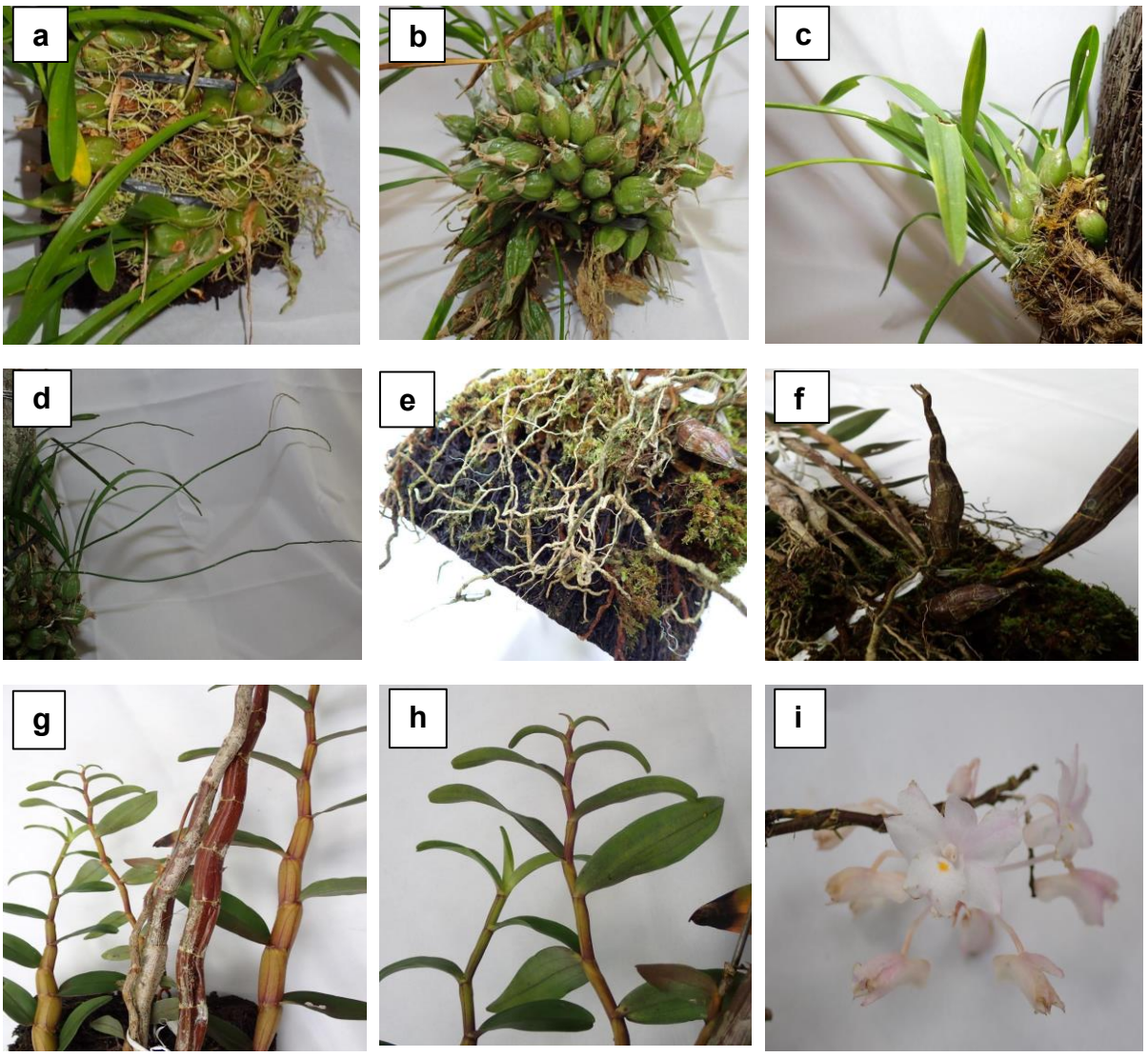


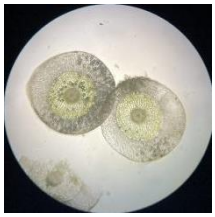
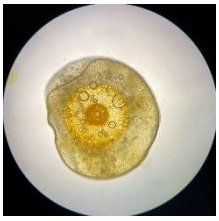
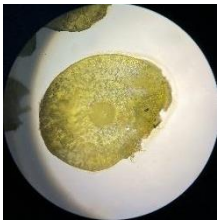
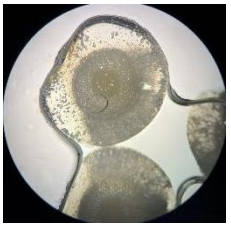
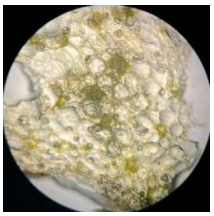
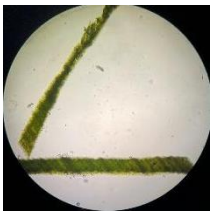
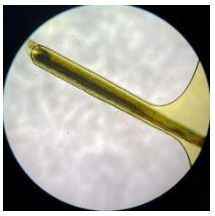
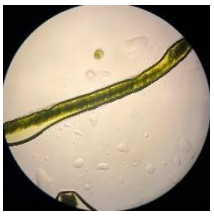
Figure 1. Morphological of *A. liliifolia* and *D. mutabile.* a) roots of *A. liliifolia,* b) pseudobulb of *A. liliifolia,* c) leaves of *A. liliifolia,* d) Inflorescence of *A. liliifolia,* e) roots of *D. mutabile,* f) pseudobulb of *D. mutabile,* g) stem of *D. mutabile,* h) leaves of *D. mutabile,* i) flowers of *D. mutabile.*

2. Phytochemical Compound in Organs of *Acriopsis Liliifolia* and *Dendrobium Mutabile*

Microchemical analysis of *A. liliifolia* (**Table 2**) revealed the presence of some secondary metabolites in various tissues including roots, pseudobulbs, and leaves. The tissues testes positive for flavonoids, alkaloid, phenolics, and terpenoid under 40x magnification using reagent sitroborat,

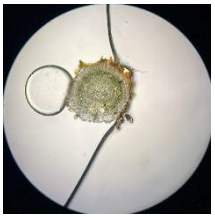
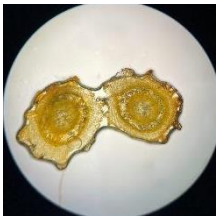
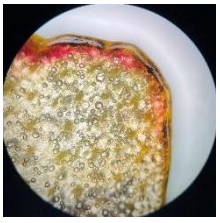
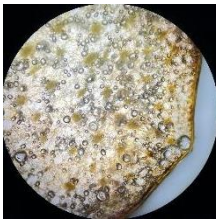
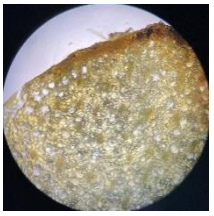
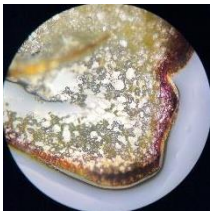
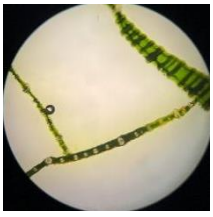
Dragendorff, FeCl3, and anysaldehide. This finding was aligned closely with the previous research project from Khoeriyah et al (2024), conducted detailed microchemical and LC-HRMS analysis of *A. liliifolia* using leaves and identifying similar classes of bioactive compounds. *A. liliifolia* orchid is part of Indonesia biodiversity, shows promising bioactive compounds with potential pharmacological uses. Hence, conserving orchids species is needed for both ecological and future natural product discovery.

Table 2: Microchemical testing of *Acriopsis liliifolia*

Control	Flavonoid	Alkaloid	Phenolic	Terpenoid
 Root of *A. liliifolia*	 Root of *A. liliifolia* Reagent: sitroborat	 Root of *A. liliifolia* Reagent: dragendorff	 Root of *A. liliifolia* Reagent: FeCl3 10%	 Root of *A. liliifolia* Reagent: anisaldehyde
 Pseudobulb of *A. liliifolia*	 Pseudobulb of *A. liliifolia* Reagent: sitroborat	 Pseudobulb of *A. liliifolia* Reagent: dragendorff	 Pseudobulb of *A. liliifolia* Reagent: FeCl3 10%	 Pseudobulb of *A. liliifolia* Reagent: anisaldehyde
 Leaf of *A. liliifolia*	 Leaf of *A. liliifolia* Reagent: sitroborat	 Leaf of *A. liliifolia* Reagent: dragendorff	 Leaf of *A. liliifolia* Reagent: FeCl3 10%	 Leaf of *A. liliifolia* Reagent: anisaldehyde

Similar to *A. liliifolia*, the result from *D. mutabile* (**Table 3**) showed the presence of all four tested secondary metabolite major group, which are flavonoids, alkaloids, phenolics, and terpenoids located in the roots, stems, and leaves under the 40x magnification microscope. These results showed consistency with the previous study from the *Dendrobium* genus, which is recognized for its rich phytochemical competition. Research from Xie et al (2021) identified terpenoid alkaloids like dendrobine in various *Dendrobium* species, compound known for their anti-inflammatory and analgesic effects. Study from Liu et al (2023) emphasized the therapeutic and antioxidant properties of phenolic compounds commonly found in *Dendrobium*. The detection of these metabolites in *D. mutabile* reinforces its potential as a valuable source of bioactive compounds and highlights the need for further pharmacological and clinical research to fully explore future safety medical application.

Table 3. Microchemical testing of *Dendrobium mutabile*

Control	Flavonoid	Alkaloid	Phenolic	Terpenoid
 Root of *D. mutabile*	 Root of *D. mutabile* Reagent: sitroborat	 Root of *D. mutabile* Reagent: dragendorff	 Root of *D. mutabile* Reagent: FeCl3 10%	 Root of *D. mutabile* Reagent: anisaldehyde
 Stem of *D. mutabile*	 Stem of *D. mutabile* Reagent: sitroborat	 Stem of *D. mutabile* Reagent: dragendorff	 Stem of *D. mutabile* Reagent: FeCl3 10%	 Stem of *D. mutabile* Reagent: anisaldehyde
 Leaf of *D. mutabile*	 Leaf of *D. mutabile* Reagent: sitroborat	 Leaf of *D. mutabile* Reagent: dragendorff	 Leaf of *D. mutabile* Reagent: FeCl3 10%	 Leaf of *D. mutabile* Reagent: anisaldehyde

3. Anatomical of orchids

Physiological adaptations are also employed by epiphytic orchids to ensure survival in their aerial environment. Each plant organ possesses distinct anatomical characteristics that support growth and functionality. Scanning Electron Microscopy (SEM) was performed on the external surfaces of the roots, leaves, and pseudobulbs of *Acriopsis liliifolia* and *Dendrobium mutabile* (**Figure 2**) to examine surface morphology.The SEM analysis of root samples revealed that the most prominent structure observed was the velamen. In *A. liliifolia*, the surface texture appeared more clearly defined and structured, whereas in *D. mutabile*, the texture was smoother and less porous. The outermost layer of aerial roots, which overlies the exodermis, is referred to as the velamen radicum (Hauber et al., 2020), and it is consistent with the root type found in both species. Velamen consists of multiple layers of dead cells with lignified secondary cell walls, which provide structural integrity and facilitate water absorption and retention (Idris et al., 2021). This porous tissue network primarily functions in absorbing moisture from the atmosphere through the process of imbibition. The absorbed water is subsequently transferred to the living inner cells known as passage cells (Hauber et al., 2020). In the SEM images of *A. liliifolia* roots (**Figure 2a**), a polygonal pattern of densely arranged hollows was evident, forming a distinctive geometric structure. Further anatomical features may be observable through transverse section imaging. In contrast, SEM images of *D. mutabile* roots exhibited a denser and more layered velamen surface, with fewer visible pores compared to *A. liliifolia* (**Figure 3**). A characteristic feature of the velamen in

Dendrobium species is the helical thickening of the cell walls within the velamen layers (Idris et al., 2021), which contributes to structural robustness and may influence water retention efficiency.

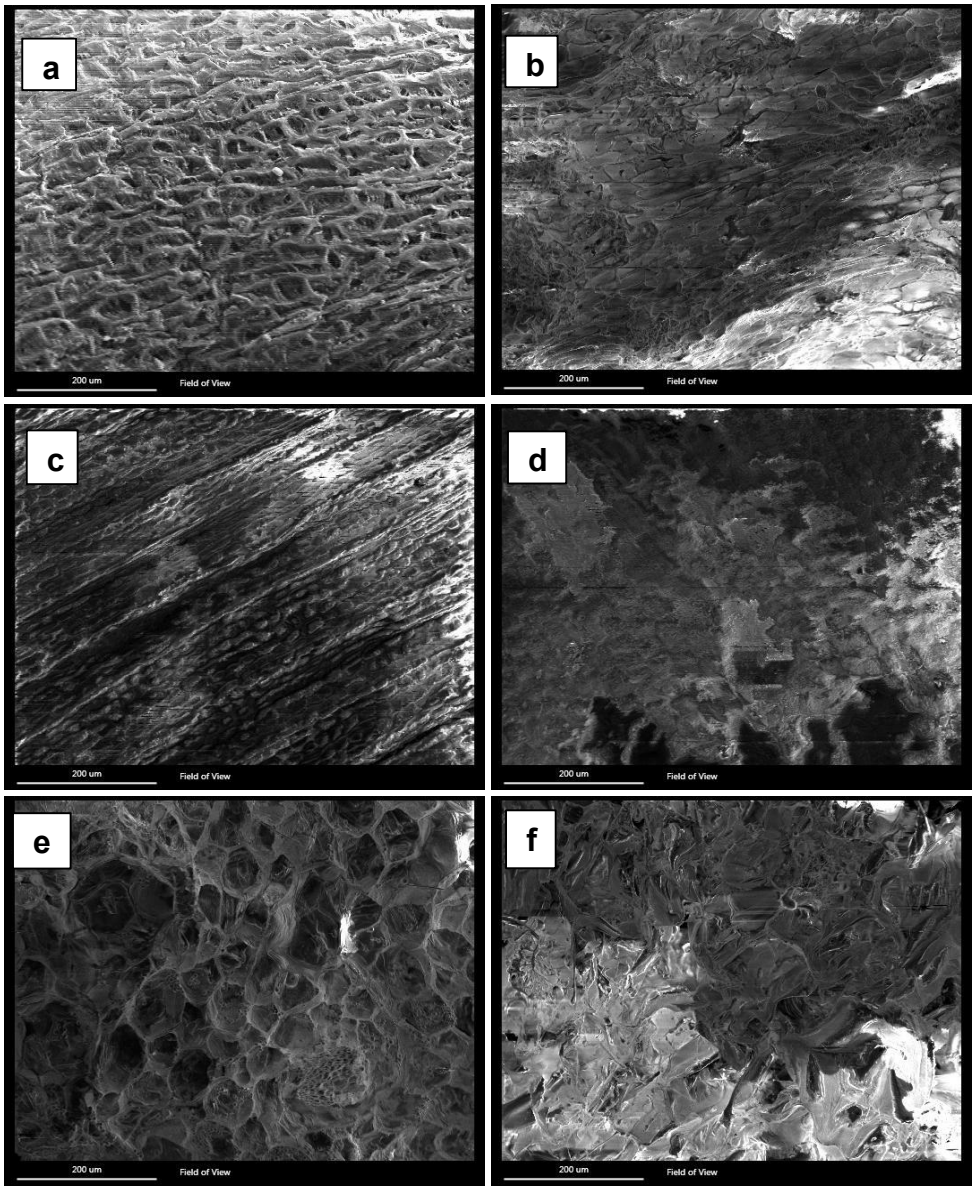


Figure 2. Field of View SEM of *A. liliifolia* and *D. mutabile.*
a) roots of *A. liliifolia,* b) roots of *D. mutabile,* c) leaves of *A. liliifolia,* d) leaves of *D. mutabile,*
e) pseudobulb of *A. liliifolia, f*) pseudobulb of *D. mutabile.*

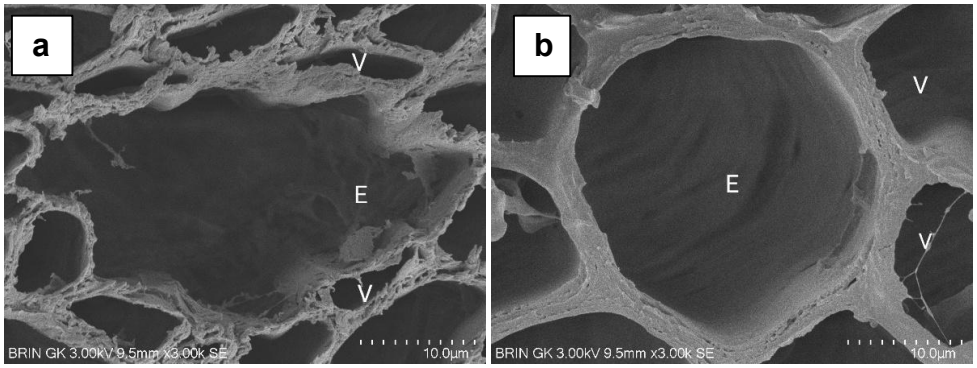


Figure 3. SEM images in cross section of *A. liliifolia* and *D. mutabile.*
a) roots of *A. liliifolia,* b) roots of *D. mutabile.* exodermis cell (E), velamen radicum (V).

In SEM imaging of *A. liliifolia* and *D. mutabile* leaves, different structures are seen (**Figure 2, c-d**). The surface of *A. liliifolia* leaves shows cells with elongated surfaces and have regular patterns. The shape of the cells that are parallel to the wavy contour allows that the cells are the leaf epidermis. No stomata, trichomes, or other parts are visible because the appearance is seen using a magnification of 200 μm. Higher magnification is needed to be able to see the entire leaf. On the surface of D. mutabile leaves, an uneven structure is seen indicating the structure of the epidermis covered by the cuticle. The cuticle is a layer of wax that is on the outside of the leaf surface as a protector (Muthukumar & Shenbagam, 2018). Its main function is to prevent water loss in the leaves during surface transpiration so that water use becomes more efficient, especially when water availability is reduced (Metusala et al., 2017). The cuticle looks thicker on the surface of *D. mutabile* leaves than *A. liliifolia* leaves, indicating a better defense to prevent water transpiration.

Anatomical observations of the pseudobulbs of *A. liliifolia* and *D. mutabile* (**Figure 2, e-f**) showed different results. The surface of the pseudobulb of *A. liliifolia* showed a structure with a pattern resembling sponge cells with cell walls that appeared quite thick and the boundaries between cells were very clear. It seems that this is a parenchyma tissue rich in starch granules. The function of this parenchyma tissue is to facilitate gas exchange and water storage in dry conditions (Muthukumar & Shenbagam, 2018). Cells that look dense and regular indicate their efficiency in maintaining turgor and allowing a stable diffusion process of water and gas. Similar to leaves, the outer surface of orchid pseudobulbs is generally coated with a cuticle to reduce excessive water loss and as a barrier against pathogens and physical damage to plants (Muthukumar & Shenbagam, 2018). The pseudobulb of *D. mutabile* shows a different surface from *A. liliifolia.* A more irregular and complex structure is seen, such as folds or dense and stacked fibers. It shows parenchymal tissue that has undergone morphological modification to support functions other than water storage. There are differences in contrasting colors between dark and light, indicating different density variations. In general, pseudobulbs consist of cuticles, parenchymal tissue, vascular bundles, and sclerenchymal cells (Muthukumar & Shenbagam, 2018). The thick cuticle structure helps protect the pseudobulb from drought, UV radiation, and pathogens (Matschi et al., 2020). The parenchymal tissue itself is responsible for storing water, minerals, and carbohydrates in the pseudobulb (Crang et al., 2018). It also facilitates photosynthesis and contributes to the carbon balance of plants (He, 2018). The transport of water and nutrients to all parts of the pseudobulb and connecting it to other organs is carried out by the vascular bundle (Crang et al., 2018). Sclerenchymal cells themselves are important for maintaining the integrity of the pseudobulb with structural support due to their lignified secondary walls (Crang et al., 2018). In order to see all of these parts, data with greater magnification and appropriate sample sections are required.

In addition to the SEM observations, Energy Dispersive X-ray Analysis (EDAX) was conducted to further examine the elemental composition of the samples. EDAX mapping provides information on the spatial distribution of elements within a given tissue, while the EDAX spectrum reveals the energy levels of X-rays emitted from the sample, allowing for identification of its elemental composition (Thakur et al., 2016). These analyses are typically performed in conjunction with Scanning Electron Microscopy. EDAX mapping enables visualization of specific element locations and their distribution patterns within the tissue. In contrast, the EDAX spectrum allows for more precise elemental identification and quantification, providing data on both the weight and atomic percentages of detected elements. The elemental analysis results can subsequently be used to infer potential relationships with the distribution of secondary metabolites within the plant tissues.

Figure 4 is the result of EDAX mapping analysis of roots, leaves, and pseudobulbs of *A. liliifolia* and *D. mutabile*. The test was conducted to show the type and relative amount of elements based on the X-ray signal energy emitted from one sample point. The chemical elements found from EDAX analysis in each part of the roots, leaves, pseudobulbs of *A. liliifolia* and *D. mutabile* are the same, only the amount is different. In the roots and pseudobulbs, the elements C, O, Ne, Na, Mg, Al, Si, S, Cl, and K were found. While in the leaves there were only elements C, O, Ne, Na, Mg, Al, Si, and S.

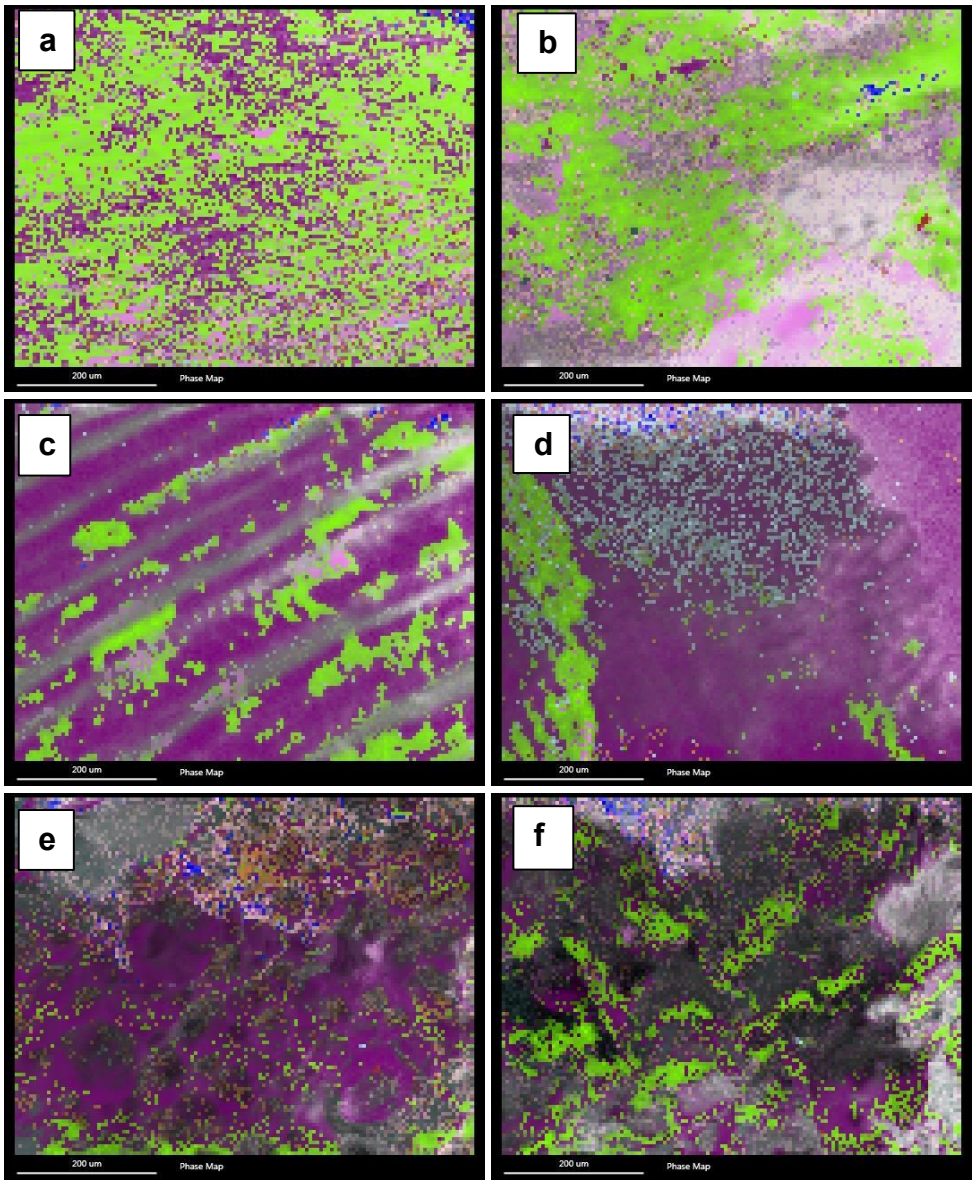
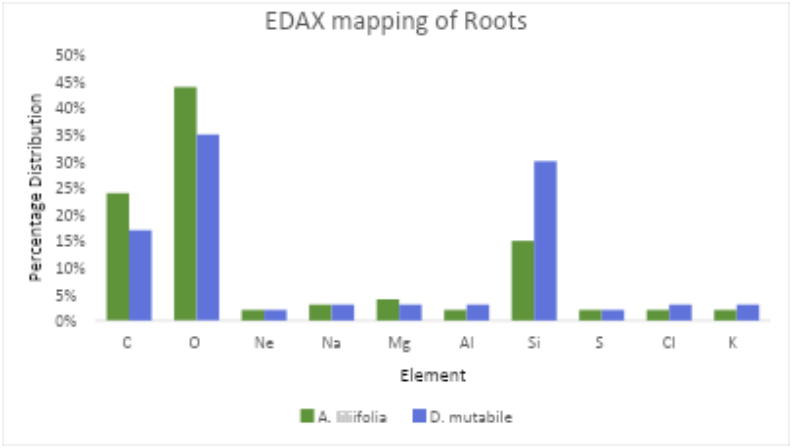


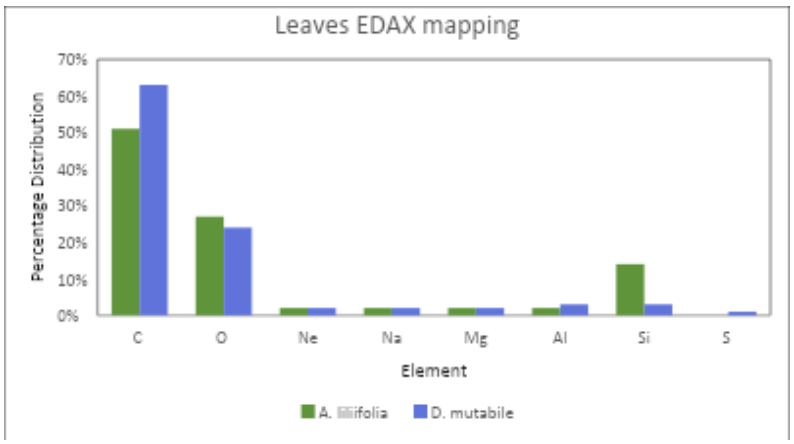
Figure 4. EDAX mapping SEM of *A. liliifolia* and *D. mutabile.* a) roots of *A. liliifolia,* b) roots of *D. mutabile,* c) leaves of *A. liliifolia,* d) leaves of *D. mutabile,* e) pseudobulb of *A. liliifolia, f*) pseudobulb of *D. mutabile.*

In the roots and pseudobulbs of both *A. liliifolia* and *D. mutabile*, oxygen was identified as the dominant element. Based on the results of EDAX mapping, the oxygen content in the roots of *A. liliifolia* was 44%, while in *D. mutabile* it was 35% (**Figure 5a**). In the pseudobulbs, oxygen content reached 36% in *A. liliifolia* and 40% in *D. mutabile* (**Figure 5c**). Oxygen plays a crucial role in the physiology of epiphytic orchids, particularly because their aerial root systems are capable of photosynthesis. The oxygen produced during this process can mitigate hypoxic conditions, especially in large root structures that tend to restrict oxygen diffusion (Brunello et al., 2024). This oxygen production is essential for supporting carbon metabolism, which in turn influences plant growth and overall health. Interestingly,

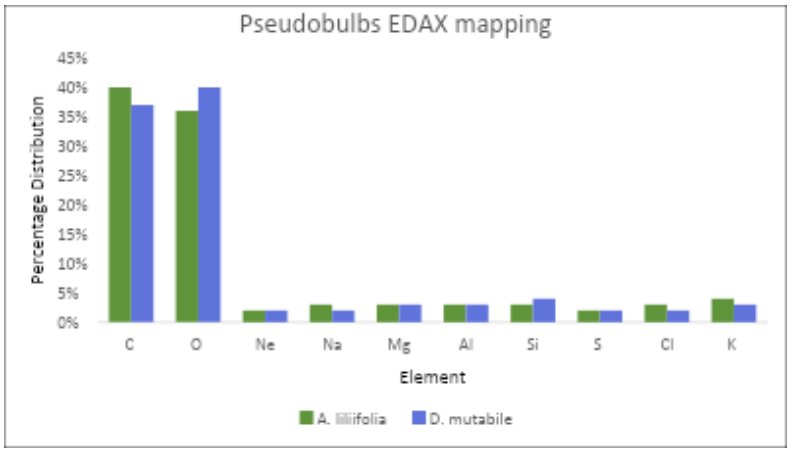
the oxygen content in the leaves was lower than that of carbon, with 27% detected in *A. liliifolia* and 24% in *D. mutabile* (**Figure 5b**). This discrepancy reflects a key physiological adaptation in epiphytic orchids, which have evolved specialized roots and pseudobulbs to thrive in nutrient-limited and exposed environments. In addition to roots capable of photosynthesis, the green pseudobulbs also contribute to photosynthesis and serve as storage organs for water, minerals, and carbohydrates (He, 2018). While leaves remain the primary organs for photosynthesis, the multifunctionality of orchid roots and pseudobulbs highlights their significant role in oxygen production and overall plant vitality.



a) EDAX mapping of roots



b) EDAX mapping of leaves



c) EDAX mapping of pseudobulb

Figure 5. Diagram EDAX mapping SEM of *A. liliifolia* and *D. mutabile.*
a) EDAX mapping of roots, b) EDAX mapping of leaves, c) EDAX mapping of pseudobulb.

Carbon (C) is also a dominant element across various plant parts of *A. liliifolia* and *D. mutabile*. The carbon content measured in the roots was 24% for *A. liliifolia* and 17% for *D. mutabile*, while in the leaves it reached 51% and 63% respectively. In the pseudobulbs, the carbon content was 40% in *A. liliifolia* and 37% in *D. mutabile*. Overall, the highest carbon concentration was found in the leaves. This finding is consistent with Ma et al. (2018), who reported that the average carbon content in leaves (46.85%) is generally higher than that in roots (45.64%) and other reproductive organs (45.01%). Carbon in plants originates from atmospheric carbon dioxide (CO_2), which is fixed during photosynthesis and subsequently converted into organic compounds such as sugars, lignin, cellulose, and other structural and functional molecules (Ma et al., 2028). Functionally, carbon is essential for building fundamental plant structures like cell walls, supporting metabolic functions, and serving as an energy reserve through carbohydrates and non-structural compounds such as starch and sugars (Ma et al., 2018).

In addition to the elements C and O, the element Si or Silicon has a high value, especially in the roots of *A. liliifolia* with a value of 15% and in *D. mutabile* 30%. The element Silicon accumulates in plant tissues, forming a protective layer that acts as a barrier against pathogens and environmental sensors (Ali et al., 2023). Silicon also helps relieve biotic and abiotic stress by increasing the structural integrity of plants and activating defense mechanisms (Mundada et al., 2021). The elements Ne, Na, Mg, Al, S, Cl, and K with small contents can help growth and adaptation to environmental stress with their respective functions.

Each chemical element detected is closely related to the biosynthesis of secondary metabolites in plants. Oxygen (O), in particular, plays a crucial role, as nearly all classes of secondary metabolites contain oxygen as part of their functional groups. Glycosides, for example, consist of sugar and non-sugar (aglycone) components, which may be phenolic or terpenoid in nature. These compounds contribute to plant defense and exhibit pharmacological properties, such as cardiac glycosides used in treating heart conditions. Terpenoids—oxygen-containing derivatives of terpenes—serve various ecological functions, including attracting pollinators and deterring herbivores, and they hold promise in pharmacology due to their antimicrobial activity (Alamgir, 2018). Phenolic compounds, another group of oxygen-rich secondary metabolites, contain benzene rings with one or more hydroxyl groups. Common phenolics such as flavonoids and tannins are known for their antioxidant properties and pharmacological potential (Alamgir, 2018). Oxygen is also essential in plant biosynthetic pathways, particularly in enzymatic reactions like hydroxylation, oxidation, and epoxidation, which modify primary metabolites into bioactive secondary compounds. A recent study demonstrated that increasing oxygen availability can enhance polyphenol biosynthesis, resulting in higher polyphenol content and improved antioxidant capacity (Dong et al., 2025). Therefore, maximizing the pharmacological utilization of polyphenols may involve targeting plant parts with high oxygen content, such as the roots, pseudobulbs, and leaves of *Acriopsis liliifolia* and *Dendrobium mutabile*.

Carbon forms the fundamental backbone of all organic compounds, including secondary metabolites. Composed of isoprene units (C_5H_8), terpenes and terpenoids are synthesized by plants as essential oils, pigments, flavor compounds, and natural defenses against herbivores and pathogens (Alamgir, 2018). The high carbon content observed in the roots, leaves, and pseudobulbs of *Acriopsis liliifolia* and *Dendrobium mutabile* suggests active secondary metabolite production, particularly due to the strong association between carbon and oxygen elements. In addition to C and O, silicon (Si) was the third most abundant element, especially in the roots of *A. liliifolia* and *D. mutabile*, with concentrations of 15% and 30%, respectively. Silicon plays a significant role in enhancing secondary metabolite metabolism, synthesis, and structural modification, contributing to plant stress tolerance (Farouk, 2022).

These metabolites—including phenolics, terpenoids, and nitrogen-containing compounds—are essential for plant defense against both biotic and abiotic stressors. Furthermore, silicon has been shown to influence the biosynthesis and regulation of these compounds, thereby improving the plant's resilience under various environmental pressures (Ahanger et al., 2020).

Magnesium (Mg) is an important macronutrient that plays a role in photosynthesis, enzyme activity, and tissue structural stabilization (Guo et al., 2016). Magnesium (Mg) significantly affects the biosynthesis of secondary metabolites, because Mg is a cofactor for some enzymes involved in metabolic pathways, including enzymes responsible for the biosynthesis of secondary metabolites. Research conducted by Rezaei et al., (2019), proves that Magnesium can be used in the form of magnesium oxide (MgO) which acts as an elicitor or trigger for the production of secondary metabolites. The secondary metabolites produced are flavonoids, phenolics, and other alkaloids. Aluminum (Al) plays a role in the production of secondary metabolites in plants, affecting the growth and biochemical pathways of various species. In the study of Webber & Mason (2016), in certain species aluminum affects the production of variations in phenolic compounds. Potassium (K) plays an important role in various physiological processes, including enzyme activation and osmotic balance which are very important for the synthesis of secondary metabolites. Optimal K levels can increase the yield and quality of secondary metabolites. In Saloner & Bernstein's (2022) study on cannabis plants, K significantly affected terpenoid concentrations, optimal levels encouraged high yields, but excessive K could reduce metabolite concentrations.

Sulfur (S) is an important element in plants in small amounts in the production of secondary metabolites and their utilization. Examples of secondary metabolite groups are glucosinolates, which act as antioxidants, anti-inflammatories, and antiapoptotic (Venditti & Bianco, 2020). These compounds can be applied as pharmacological products for Alzheimer's, Parkinson's, traumatic brain injury, and so on (Venditti & Bianco, 2020). Other elements in small amounts are not directly related to the production of secondary metabolites in plants. The elements Ne, Na, and Cl play a role in maintaining the survival of the plant.

Orchids species such as *A.liliifolia* and *D.mutabile* are known to produce a wide array of secondary metabolites that contribute to their ecological adaptation and pharmacological relevance. These compound are synthesized in various plant organs and have demonstrated diverse bioactives. Their distribution across leave, roots, stems, and pseudobulb indicate the complexity and specificity of metabolite biosynthesis in different tissues. Understanding the localization and fuction of these metabolie is essential for targeted extraction and utilization in pharmaceutical, netraceutical, and cosmetic industries. The following table (**Table 4**) summarizes the types of secondary metabolite identified in *A.liliifolia* and *D. mutabile* over their tissue distribution and the pharmacological potential.

Table 4: Secondary metabolite compounds, their distribution and potential

Secondary metabolite compounds	Spesies	Distribution	Potential	References
Alkaloid	*Acriopsis liliifolia, Dendrobium mutabile*	leaves, roots, pseudobulb, stem	anti-inflammatory, anti-tumor, immunomodulator, antibacterial, antioxidant, tyrosinase inhibitor its use in cosmetic applications	(Khoeriyah et al., 2024), (Li et al., 2024), (Song et al., 2022) (Arora et al., 2017),
Flavonoid	*Acriopsis liliifolia, Dendrobium mutabile*	leaves, roots, pseudobulb, stem	anti-carcinogenic, anti-inflammatory, neuroprotective effects	(Sut et al., 2017)
Phenolic	*Acriopsis liliifolia, Dendrobium mutabile*	leaves, roots, pseudobulb, stem	anticancer, neurodegeneration	(Sut et al., 2017), (Arora et al., 2017),
Terpenoid	*Acriopsis liliifolia, Dendrobium mutabile*	leaves, roots, pseudobulb, stem	antimicrobial, anti-rheumatic	(Sut et al., 2017)

The chemical elements contained in each part of *A. liliifolia* and *D. mutabile* have an important role in the biosynthesis and modification of secondary metabolites for plants. Many studies have shown that each secondary metabolite has pharmacological potential. In order to make a health product, the part of the plant used can be adjusted to the needs. Further studies are needed regarding the production process of pharmacological products that are in accordance with needs.

CONCLUSIONS

Acriopsis liliifolia and *Dendrobium mutabile* have specific morphological and anatomical adaptations that can support their epiphytic life. They have vascular aerial roots, pseudobulbs that function as water storage, and a thick cuticle layer on their leaves. SEM analysis shows the anatomical characteristics of these adaptation features. The results of EDAX mapping analysis showed that contained elements such as oxygen, carbon, and silicon with high concentrations play an important role in supporting the biosynthesis of secondary metabolites that have potential in pharmacological applications. Many secondary metabolites found are alkaloids, flavonoids, phenolics, and terpenoids that have potential as anti-inflammatory, anticancer, antimicrobial, and neuroprotective. For the development of pharmacological product applications from *A. liliifolia* and *D. mutabile*, further studies need to be carried out, especially regarding the isolation, characterization, and bioactivation tests of these secondary metabolites. Development of orchid culture methods to increase the production of bioactive compounds is also important. Collaboration between scientific fields can be maximized in research on the development of sustainable products in healthcare

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primarily responsible for the conceptualization, methodology, data analysis, experimental work, and the manuscript writing. SV contributed equally as a main contributor in the data interpretation and drafting of the manuscript. RH and CNH provided supporting contributions.

CONFLICT OF INTEREST

We declare that there is no conflict of interest to this research project.

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Feasibility Analysis of Production Facility Investment for Sex Hormone and Contraceptive Tablets

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ABSTRACT

According to data from the Indonesia Total Market Audit, the demand for sex hormone and contraceptive drug products has shown a consistent upward trend each year. Establishing a production facility for sex hormone tablets requires a large invesment; therefore, a feasibility study is necessary. This study aims to evaluate the business and investment feasibility of a production facility for sex hormone and contraceptive tablets using market analysis methods. The study includes an assessment of market, technical, managerial, legal, and financial-economic aspects. The analysis is based on projected investment costs and multi-year sales targets, conducted comprehensively by examining Indonesia's 2023 product market share and developing a corresponding product sales portfolio. The results indicate that the market potential for sex hormone tablets in Indonesia, based on IQVIA data from 2023, reached IDR 774,870,335,382, with a MAT GR of 18% and a three-year CAGR (2021–2023) of 9%. Financial analysis of the proposed 12-product portfolio yielded a Net Present Value (NPV) of IDR 191,886,357,660, an Internal Rate of Return (IRR) of 53%, and a Payback Period (PBP) of 2.13 years. A subsequent sensitivity analysis was conducted to assess changes in NPV due to fluctuations in Cost of Goods Sold (COGS) and revenue. The results showed that the investment remained feasible under a 30% increase in COGS and a 15% decrease in revenue. Based on these findings, the proposed investment plan is considered feasible for implementation.

Keywords: Contraception; Investment Feasibility Analysis; IRR; NPV; PBP; Sex Hormones

INTRODUCTION

The need for pregnancy programs and birth control continues growing in line with public awareness of the importance of family planning. Sex hormone drugs and contraceptives, such as combination tablets and progestins, have a strategic role in regulating fertility, treating hormonal disorders, and managing syndromes such as PCOS and endometriosis (Caselgrandi, 2022). Based on data from the Indonesia Total Market Audit, demand for these products tends to increase every year (IQVIA, 2023).

However, those products are dependence on imported and the limited of domestic production facilities are still major obstacles in terms of national market needs. This is reinforced by IQVIA data (2023) which shows the dominance of global brands in the sex hormone and contraceptive segments, while the contribution of local producers is relatively low. Therefore, strategic investment is needed to

develop domestic production facilities that meet CPOB standards (BPOM RI, 2024) and support the national pharmaceutical resilience (Ministry of Industry, 2021).

The production of sex hormone and contraceptive tablets must execute in separated facility. To establish a production facility requires a large investment, so a feasibility study is needed to avoid investments that turn out to be unprofitable in the future. Several previous studies have examined the feasibility of investment in the pharmaceutical sector, both for herbal products, cosmetics, and medical devices (Amitanael, 2015; Alfajriyanti, 2022; Purnatiyo, n.d.). However, no comprehensive study has been found that specifically analyzes the feasibility of investment in production facilities for sex hormones and contraceptives tablets in Indonesia. The limitations of previous studies open up new research opportunities to fill this gap.

This study aims to evaluate the business and investment feasibility of production facilities for sex hormones and contraceptives tablets using market, technical, legal, and financial aspects. Unlike previous research on investment in the pharmaceutical sector, this study focuses on a specific and strategic therapeutic segment, namely sex hormones and contraceptives, and uses financial sensitivity analysis that considers the dynamics of the local pharmaceutical market based on data from the Indonesia Total Market Audit.

METHODS

This research adopts an action research approach, in which the researcher is directly involved in identifying industrial challenges, developing potential solutions, and evaluating the feasibility of investment within pharmaceutical industry context. The study incorporates both primary and secondary data sources to ensure a comprehensive and credible feasibility analysis.

Primary data were derived from the researcher's direct engagement in the pharmaceutical industry, including internal observations, professional experience, and technical estimations concerning production capacity, equipment specifications, and workforce requirements. These data points also include projected unit demand growth (10% annually), targeted market share per product (10%), and estimated cost allocations—reflecting realistic conditions within the local pharmaceutical manufacturing environment.

Secondary data were obtained from established and reputable sources. Market data—such as total market value, Moving Annual Total Growth Rate (MAT GR), and the three-year Compound Annual Growth Rate (CAGR)—were extracted from the Indonesia Total Market Audit (ITMA) 2023 report published by IQVIA. Macroeconomic indicators such as inflation rates were sourced from Badan Pusat Statistik (Statistics Indonesia) for the years 2021 to 2023. Regulatory and compliance frameworks, including Good Manufacturing Practices (CPOB), were obtained from official government publications issued by the National Agency for Drug and Food Control (BPOM) and the Ministry of Health.

These datasets formed the foundation for technical, market, and financial analyses to determine the viability of the proposed investment. Financial modeling included projections for Capital Expenditure (CAPEX)—encompassing construction, machinery, licensing, and product development—as well as Operational Expenditure (OPEX), which covered raw materials, labor, marketing, and distribution costs. The financial evaluation utilized the following metrics over a 10-year projection period:

1. Net Present Value (NPV)

Net Present Value (NPV) is one of the most widely applied financial tools for assessing investment viability. It represents the sum of the present values of all projected future cash flows, discounted at a rate that reflects the cost of capital or the required return (Shou, 2022). NPV enables decision-makers to incorporate the time value of money into investment appraisals and provides a robust basis for comparing projects of varying scales and durations (Chen, 2022). The NPV formula is defined as follows:

$$NPV = \sum_{t=1}^{n} \frac{Bt - Ct}{(1+i)^t}$$

Where:

Bt = benefit in year t

Ct = cost in year t

n = economic life of the project (years)

i = discount rate

A positive NPV suggests that the investment is financially viable and should be accepted, while a negative NPV indicates the investment should be rejected.

2. Payback Period (PBP)

The Payback Period (PBP) measures the time required for the initial investment to be recovered through net cash inflows. It is a simple yet effective indicator of liquidity and risk. The shorter the payback period, the more favorable the investment (Juwitanigtyas, 2015). The formula is:

$$PBP = \frac{Initial\ Invesment}{Estimated\ Annual\ Cash\ Flow}$$

According to Titman et al. (2020), if the PBP is less than or equal to the economic life of the project, the investment is considered acceptable. Otherwise, it should be rejected.

3. Internal Rate of Return (IRR)

The Internal Rate of Return (IRR) is the discount rate at which the NPV of all cash flows equals zero. It represents the expected annualized rate of return for the investment (Titman & Martin, 2020). IRR is calculated using the following interpolation formula:

$$IRR = i' + \frac{NPV"}{NPV' - NPV"} \times (i" - i')$$

Where:

i' = lower discount rate

i" = higher discount rate

NPV' = NPV at the lower discount rate

NPV" = NPV at the higher discount rate

The investment is deemed feasible if the IRR exceeds the required rate of return, unfeasible if it is lower, and at breakeven if it equals the required rate.

4. Sensitivity Analysis

Additionally, a sensitivity analysis was performed to evaluate how changes in Cost of Goods Sold of the portfolio products (±15% and ±30%) and revenue (±15% and ±30%) would impact the investment viability, particularly the NPV values.

RESULTS AND DISCUSSION

1. Results

1.1 Market Aspect Analysis

Based on data from the Indonesia Total Market Audit (ITMA) 2023, the total market value for sex hormone and contraceptive tablets was IDR 774,841,295,382, contributed by a diverse range of products from various pharmaceutical companies. These products contain active ingredients indicated for oral contraception, menstrual disorders, endometriosis treatment, ovulation dysfunction, and estrogen replacement therapy.

An in-depth analysis of the existing products in the market was conducted to identify those with strong potential for further development from a market perspective. According to Michaeli et al. (2022), market value and competition are key factors that significantly influence the evaluation of pharmaceutical products, from early-stage development through to commercialization.

Table 1: Product Portfolio of Hormonal and Contraceptive Tablets Included in the Feasibility Study

No	Composition	Indication	Comparator Brand
1	Levonorgestrel 0.15 mg + Ethinylestradiol 0.03 mg	Oral contraception	Microgynon®
2	Drospirenone 3 mg + Ethinylestradiol 0.03 mg	Oral contraceptive, with amineralcorticoid and antiandrogenic effects also beneficial for women who experience hormone related fluid retention and resulting symptoms and for women with acne, and seborrhea	Yasmin®
3	Lynestrenol 0.05 mg	Contraception	Exluton®
4	Levonorgestrel 0.75 mg	Emergency contraception	Postinor®
5	Drospirenone 3 mg + Ethinylestradiol Betadex 0.02 mg	Oral contraception	Yaz®
6	Cyproterone Acetate 2 mg + Ethinylestradiol 0.035 mg	Treatment of moderate to severe acne related to androgen sensitivity (with or without seborrhoea) and/or hirsutism	Diane-35®
7	Norethisterone 5 mg	Dysfunctional bleeding, primary and secondary amenorrhea, premenstrual syndrome, cyclical mastopathy, timing of menstruation, endometrosis	Primolut N®
8	Clomifene Citrate 50 mg	Treatment of ovulary dysfunctional in women desiring pregnancy	Clomid®
9	Dydrogesterone 10 mg	Estrogen replacement therapy, progesterone deficiencies	Duphaston®
10	Dienogest 2 mg	Treatment of endometriosis	Visanne®
11	Estradiol Valerate 2 mg + Norgestrel 0.5 mg	Treatment of climacteric symptoms, as well as the prevention and management of postmenopausal conditions resulting from estrogen deficiency, such as osteoporosis and gynecological inflammation	Cyclo-Progynova®
12	Estradiol Valerate 2 mg	Hormone replacement therapy for the treatment of signs and symptoms of estrogen deficiency due to natural menopause or castration	Progynova®

The evaluation was based on several key indicators, including the Compound Annual Growth Rate (CAGR), Market Value, and Competition Level. The CAGR reflects long-term market growth and serves as a quantitative measure of market attractiveness. Market Value estimates the overall revenue opportunity within a segment, while Competition Level assesses the number of existing players.

Through this analysis, 12 products were identified as having strong potential for development and commercialization. These products were selected and compiled into a proposed product portfolio (**Table 1**). Each product in the portfolio was targeted to capture 10% of its respective market segment. Future demand was projected over a 10-year period, assuming an annual growth rate of 10% in unit sales. The projected first-year revenue for the 12-product portfolio was estimated at IDR 73,748,950,000.

1.2 Technical Aspect Analysis

In general, tablet production can be conducted by direct compression, dry granulation, or wet granulation. The manufacturing facility for sex hormone and contraceptive tablets would require equipment such as weigher, super mixer granulators, fluid bed dryers, bin mixers, tablet compressing machines, blistering, and stripping machines.

1.3 Managerial Aspect Analysis

The required production workforce was calculated based on the production target of the 12-product portfolio. A total of 36,580 man-hours were needed. With one employee working 1,464 hours per year, 25 operators were required. In addition, one process supervisor, one packaging supervisor, and one quality inspector were needed, resulting in a total of 28 personnel.

1.4 Legal Aspect Analysis

Before construction, permits such as the analysis of Upaya Pengelolaan Lingkungan dan Upaya Pemantauan Lingkungan (UKL UPL) and Persetujuan Bangunan Gedung (PBG) must be obtained. After the facility is completed, Sertifikat Laik Fungsi (SLF) and Cara Pembuatan Obat yang Baik (CPOB) certification must be secured. A facility certified for CPOB can be used for pilot production, with the resulting data used for product registration along with halal certification.

1.5 Financial Aspect Analysis

Table 2: Capital Expenditures (CAPEX)

No	Item Description	Amount (IDR)	Notes
1	Production machinery investment	19,672,096,000	Incurred in Year 0; covers major manufacturing equipment for hormonal tablet production.
2	Production facility construction	20,000,000,000	Incurred in Year 0; includes construction, design consulting, and project management.
3	Initial licensing and permits	1,000,000,000	Incurred in Year 0; includes building permit (PBG), environmental permit (UKL-UPL), and certificate of occupancy (SLF).
4	Office equipment	47,800,000	Incurred in Year 0; includes administrative tools and furnishings.
5	Product development	4,950,000,000	Incurred in Year 0; development of 12 hormonal and contraceptive tablet formulations.
	Total	46,669,896,000	

Project costs were divided into Capital Expenditures (CAPEX) and Operational Expenditures (OPEX). CAPEX included building investment, machinery, equipment, initial permits, and product development. OPEX included labor, raw and packaging materials, equipment maintenance, utilities (water, electricity, telephone), marketing, and distribution costs (Verburugge et al., 2006).

The required CAPEX was IDR 46,669,896,000 as shown in **Table 2**. OPEX was calculated as a percentage of the selling price as shown in **Table 3**. The first-year revenue from the 12-product portfolio was IDR 73,748,950,000. The resulting financial indicators were: NPV of IDR 191,886,357,660, IRR of 53%, and a Payback Period (PBP) of 2.13 years.

Table 3: Operational Expenditures (OPEX)

No	Item Description	Percentage	Notes
1	Manufacturing expenses	30% of COGS	Incurred annually in Years 1–10; includes raw and packaging materials, labor, and utilities.
2	Marketing expenses	30% of COGS	Incurred annually in Years 1–10; includes promotional programs and marketing activities.
3	Distribution expenses	10% of COGS	Incurred annually in Years 1–10; includes transportation, storage, and logistics.

1.6 Sensitivity Analysis

A sensitivity analysis was conducted to evaluate the robustness of the project under varying future conditions. Scenarios included a 15% and 30% increase or decrease in Cost of Goods Sold (COGS), and a 15% and 30% increase or decrease in revenue. Investment feasibility was reassessed using NPV, IRR, and PBP as indicators, shown at **Table 4**. Results showed that even with a 15% to 30% increase in COGS, the project remained feasible (NPV > 0), with a PBP ranging from 1.29 to 5.88 years. However, a 30% decline in revenue rendered the project unfeasible (NPV < 0). In contrast, scenarios with a 15% decrease, 15% increase, and 30% increase in revenue still yielded positive NPVs, with PBP ranging from 1.11 to 3.90 years.

Table 4: Sensitivity Analysis of Investment Feasibility Based on Changes in COGS and Revenue

Scenario	NPV (IDR)	IRR (%)	Payback Period (Years)
↓ 30% Cost of Goods Sold	361,176,568,021	85	1.29
↓ 15% Cost of Goods Sold	276,531,462,840	69	1.61
↑ 15% Cost of Goods Sold	107,241,252,479	35	3.12
↑ 30% Cost of Goods Sold	22,596,147,298	14	5.88
↓ 30% Revenue	–49,956,800,000	-	–
↓ 15% Revenue	70,964,778,830	27	3.90
↑ 15% Revenue	312,807,936,489	76	1.45
↑ 30% Revenue	433,729,515,319	98	1.11

A negative NPV indicates that the investment is financially unfeasible under the given scenario; therefore, IRR and Payback Period are not applicable.

2. Discussions

The findings of this study indicate that investment in a dedicated production facility for sex hormone and contraceptive tablets in Indonesia is feasible from both financial and strategic perspectives. The project demonstrates economic viability and presents an attractive opportunity within the hormonal

pharmaceutical sector, which aligns with the national policy direction stipulated in Presidential Instruction No. 1 of 2022, promoting the substitution of imported pharmaceutical products with domestically manufactured alternatives (Republic of Indonesia, 2022).

The favorable Net Present Value (NPV) achieved in this study is attributed to the projected revenue stream and a profit margin consistently maintained at a minimum of 30%. When compared to a similar feasibility study of an herbal production facility conducted by Dwika (2022), this investment demonstrates superior financial performance. The referenced study reported a lower NPV of IDR 71.8 billion, an Internal Rate of Return (IRR) of 22%, and a payback period of 2.81 years, in contrast to this project's IRR of 53% and payback period of 2.13 years.

From the market perspective, the consistent annual growth observed in this therapeutic segment—as evidenced by data from the 2023 Indonesian Total Market Audit (ITMA)—reflects rising demand, largely driven by increased public awareness of reproductive health and family planning (ITMA, 2023). The projected first-year revenue of IDR 73.7 billion across a portfolio of 12 products highlights significant commercial potential, assuming a 10% annual growth rate in demand. Achieving this target will depend on well-planned distribution strategies and customer retention programs, as suggested by Michaeli et al. (2022), who emphasized the critical role of market value and competition in early-phase pharmaceutical development decisions.

From the technical standpoint, the production of sex hormone and contraceptive tablets requires specialized equipment and segregated facilities to ensure compliance with Good Manufacturing Practices (GMP), known in Indonesia as Cara Pembuatan Obat yang Baik (CPOB). This is particularly essential given the potent and sensitive nature of hormonal active pharmaceutical ingredients (APIs). Although the investment in such technology and facility layout is considerable, it remains within standard industrial and is achievable with adequate planning and capital allocation.

The managerial aspect of the analysis highlights the importance of establishing an efficient organizational structure to support sustainable operations. Workforce calculations—including 25 production operators and 3 supervisory staff—ensure operational readiness while maintaining product quality and regulatory compliance. An efficient human resource plan is essential to control labor costs and optimize productivity.

From a regulatory perspective, the Indonesian legal framework requires several licenses both prior to and following facility construction. These include Analisis Upaya Pengelolaan Lingkungan dan Upaya Pemantauan Lingkungan (UKL UPL), Persetujuan Bangunan Gedung (PBG), Sertifikat Laik Fungsi (SLF), and GMP certification (CPOB). These regulatory milestones collectively ensure that the facility operates in accordance with national health, safety, and environmental standards.

The financial analysis yields strong indicators of viability: an NPV of IDR 191.8 billion, an IRR of 53%, and a payback period of 2.13 years. These figures signal an attractive return on investment. Moreover, the sensitivity analysis confirms the project's resilience under varying cost and revenue scenarios. While a 30% decline in revenue would result in a negative NPV, rendering the project unfeasible, moderate fluctuations in cost of goods sold (COGS) and revenue (±15%) still result in positive NPVs, indicating the presence of an adequate risk buffer.

Compared with prior feasibility studies within the pharmaceutical sector—most of which have focused on herbal products, cosmetics, or medical devices—this research offers a more targeted evaluation of a high-impact therapeutic segment with currently underutilized domestic production capacity. The findings address a significant gap in the literature and provide a comprehensive feasibility

assessment that supports Indonesia's strategic goals of achieving pharmaceutical self-sufficiency and reducing reliance on imported hormonal therapies.

In conclusion, investment in a specialized production facility for sex hormone and contraceptive tablets is not only financially viable but also strategically aligned with public health priorities and national industrial policy. This study can serve as a foundational reference for the formulation of both business strategies and public policies that aim to foster growth and resilience in Indonesia's pharmaceutical sector.

CONCLUSIONS

This study contributes to the limited academic literature on investment feasibility in Indonesia's pharmaceutical sector, particularly within the niche of hormone-based therapies. The analysis integrates technical, managerial, legal, market, and financial aspects, offering a comprehensive evaluation of a strategic manufacturing initiative. The findings align with Indonesia's national agenda of achieving pharmaceutical independence and reducing reliance on imported hormonal products (Ministry of Health, 2022; BPOM, 2023).

The investment in a dedicated production facility for sex hormone and contraceptive tablets was found to be financially viable, as indicated by a Net Present Value (NPV) of IDR 191.8 billion, exceeding the minimum threshold of financial viability (NPV > 0), an Internal Rate of Return (IRR) of 53%, and a Payback Period (PBP) of 2.13 years. Sensitivity analysis further demonstrated that the investment remained feasible under moderate changes in cost and revenue assumptions, supporting the project's financial robustness. In conclusion, investment in a dedicated production facility for sex hormone and contraceptive tablets is not only financially feasible—as evidenced by strong financial indicators such as a high NPV and IRR—but also aligned with public health priorities and national industrial policies (Presidential Instruction No. 1/2022).

Future studies may consider portfolio diversification into alternative dosage forms such as hard and or soft capsules, while also examining regulatory, operational, and policy-related challenges in scaling hormone manufacturing in Indonesia. Future research may explore the practical challenges of implementation and identify necessary policy support to strengthen the local hormone preparation production.

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CONFLICT OF INTEREST

The author is affiliated with a pharmaceutical company that granted access to the Indonesia Total Market Audit (ITMA) data utilized in this study. While the findings may potentially inform the company's future strategic or investment planning, all analyses and conclusions were conducted independently and solely for academic purposes. The author affirms that no direct conflict of interest exists that could compromise the objectivity or integrity of this research.

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Curcuma xanthorrhiza-Loaded Self-Emulsifying Drug Delivery System (SNEDDS)'s Interleukin-β Expression

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ABSTRACT

The immune system plays a crucial role in defending the body against pathogens. Herbal-based immunomodulators have gained increasing interest due to their natural origin and potential to provide broad immunological benefits. Turmeric (*Curcuma xanthorrhiza*) has been extensively studied for its immunostimulant properties, with a focus on produced IL-β therapies in macrophage cells. Turmeric has low solubility, hence the latest breakthrough in turmeric preparations is the SNEDDS. This research aims to assess the impact of *C. Xanthorrhiza*-Loaded SNEDDS on the immune response by measuring IL- β cytokine production in RAW 264.7 cells. *C. Xanthorrhiza*-Loaded SNEDDS was formulated using a high-energy method. The IL- β cytokine production evaluated using ELISA assay kits. The characterization test of SNEDDS briefly shows a transmittance value (99.75% ± 0.07), a particle size (84.7 nm ± 0.071), a polydispersity index (0.485 ± 0.173), and a zeta potential (-33.47 mV ± 0.060), indicating that the sample is nano-sized and stable. The IL- β production in SNEDDS at 100 µg/mL concentration is 0.754 ± 0.007, surpassing the cell control value of 0.250 ± 0.059. The result indicates that the sample enhance IL- β secretion in RAW 264.7 cells. The *C. Xanthorrhiza*-Loaded SNEDDS was stable and increased production of IL- β.

Keywords: Turmeric; SNEDDS; immunostimulant; interleukin-β

INTRODUCTION

Natural immunostimulants derived from medicinal plants have attracted considerable attention due to their bioavailability, lower side effects, and multifaceted mechanisms of action (Ulriksen et al., 2022; Zebeaman et al., 2023). One such plant is *Curcuma xanthorrhiza* Roxb. (*C. xanthorrhiza*), commonly known as temulawak or Javanese turmeric, is a traditional medicinal plant widely utilized in Indonesia and other Southeast Asian countries. It contains several bioactive compounds, including xanthorrhizol**,** curcumin, and secondary metabolic, which exhibit various pharmacological activities such as anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory effects (Hikmah and Triastuti, 2022; Yuandani et al., 2021).

The immune system plays a pivotal role in protecting the body from infections and diseases by recognizing and responding to pathogens (Kaur and Ghorai, 2022). One of the key mediators in this defense mechanism is IL-1β, a pro-inflammatory cytokine involved in the regulation of immune and inflammatory responses (Maghfirah et al., 2023). Dysregulation of interleukin-1 beta (IL-1β) is often

associated with various pathological conditions, including chronic inflammation, autoimmune disorders, and infectious diseases (Kaneko et al., 2019).

The fermentation of Javanese turmeric into ciders with the incorporation of *Acetobacter xylinum* effectively downregulated the gene expression of IL-1β, TNF-α, and chemokines, demonstrating superior anti-inflammatory effects compared to curcuminoid fractions (Mauren et al., 2016). Curcumin using phosphatidylserine-containing nanoparticles showed that particularly in directing them to macrophages and modulating their anti-inflammatory activity (Wang et al., 2016). Xanthorrhizol can suppress excessive IL-1β production in inflammatory models, thereby exerting anti-inflammatory effects (Simamora et al., 2024). Conversely, in immunocompromised or infected conditions, *C. xanthorrhiza* extracts have been shown to stimulate macrophage activity and enhance IL-1β production as part of an immunostimulant response. This dual role highlights the adaptogenic nature of the plant in modulating immune responses based on the physiological context (Zubaidah et al., 2023).

Despite its well-documented pharmacological benefits, the clinical application of *C. xanthorrhiza* remains limited due to several pharmaceutical and biopharmaceutical challenges. One of the primary drawbacks is its poor water solubility and low bioavailability, particularly of its major active constituents such as xanthorrhizol and curcuminoids (Rahmat et al., 2021). These lipophilic compounds tend to have limited absorption in the gastrointestinal tract, leading to suboptimal systemic exposure and reduced therapeutic efficacy. Moreover, the extensive first-pass metabolism further reduces the concentration of the active compounds reaching systemic circulation (Lee et al., 2024).

To overcome these limitations, advanced drug delivery strategies are necessary. One promising approach is the development of a SNEDDS, which is designed to improve the solubility and oral bioavailability of poorly water-soluble compounds. SNEDDS is an isotropic mixture of oils, surfactants, and co-surfactants that spontaneously forms nano-sized emulsions in aqueous environments such as the gastrointestinal tract (Cunha et al., 2020; Syukri et al., 2019). This system enhances drug dissolution, improves mucosal permeability, and protects active compounds from degradation in the harsh GI environment (Odriozola-Serrano et al., 2014).

C. xanthorrhiza has been widely recognized for its anti-inflammatory and immunomodulatory properties, recent advancements have focused on enhancing its bioavailability through lipid-based delivery systems, such as the SNEDDS with Labrasol (20%) as oil, Tween 20 (60%) as surfactant, and PG (20%) as co-surfactant (Fitriani et al., 2021a). There is limited to no data specifically evaluating the immunological response, particularly IL-1β expression following administration of *C. Xanthorrhiza*-Loaded SNEDDS form. This study presents a novel approach by not only optimizing the *C. Xanthorrhiza*-Loaded SNEDDS but also investigating its effect on IL-1β expression, a key pro-inflammatory cytokine involved in immune regulation. This dual focus introduces new insights into the immunomodulatory mechanisms of *C. xanthorrhiza* when delivered via advanced nanocarrier systems and contributes to the development of more effective plant-based immunotherapeutics.

METHODS

1. Materials and Tools

C. xanthorrhiza (Konimex, Indonesia), labrasol (Gattefose, France), polysorbate 20 (Tween 20) (Brataco, Indonesia), propylene glycol (Brataco, Indonesia), RAW 264.7 cell culture (American Type Culture Collection, USA) using Dulbecco's Modified Eagle Medium (DMEM) high glucose (Gibco®),

Fetal Bovine Serum (FBS) (Gibco®), Phosphate Buffered Saline (PBS) sterilized, 1% penicillin-streptomycin antibiotic (Gibco®), sodium bicarbonate (NaHCO3), water for injection, 70% alcohol, ELISA kit IL-1β (ABclonal Technology®). Positive control Lipopolysaccharide (Sigma®). Other necessary materials include cell culture flasks, conical tubes, blue tips, yellow tips, white tips, microtubes, 96-well plates, 24-well plates, and 70% alcohol.

Vortex (Heidolph Reax Top, Germany), centrifugator (Nuve-NF 400), glassware set (Pyrex), analytical balance (Metler Toledo), micropipette (Thermoscientific Finnpipette), UV-Vis spectrophotometer (Shimadzu UV Specthrophotometer, UV-1800), ultrasonicator (model 300 V/T, USA), Particle Size Analyzer (HORIBA Scientific Nano Partica SZ 100), hemocytometer (BD Facs Calibur), microplate well (Nunc, SaintNeots, England), hand tally counter, 5% CO_2 incubator (Memmert®), and ELISA reader (BIO-RAD Model 595).

2. Preparation of *C. Xanthorrhiza*-Loaded SNEDDS

C. Xanthorrhiza-Loaded SNEDDS formula was obtained from the previous study, comprising the carrier Labrasol (20%), Tween 20 (60%), and PG (20%). A quantity of 1500 mg of *C. Xanthorrhiza* extract was dissolved in the pre-prepared mixture using an ultrasonicator (model 300 131 V/T, USA) (Fitriani et al., 2021a).

3. Characterization of *C. Xanthorrhiza*-Loaded SNEDDS

The evaluation of *C. Xanthorrhiza*-Loaded SNEDDS is conducted through transmittance testing, particle size measurement, polydispersity index (PI), and zeta potential. Transmittance values are measured by diluting the *C. Xanthorrhiza*-Loaded SNEDDS 100 times with distilled water, and the transmittance is read using a UV-Vis spectrophotometer at a wavelength of 650 nm. Particle size, PI, and zeta potential determination are performed by diluting the *C. Xanthorrhiza*-Loaded SNEDDS 100 times with distilled water, followed by measurement using a particle size analyzer (Horiba SZ 100, Japan).

4. Cell line and Culture

RAW 264.7 cells were acquired from The American Type Culture Collection (ATTC), USA. These cells were precultured in a DMEM medium that included 10% (v/v) fetal bovine serum and a penicillin–streptomycin solution (100 µg/ml). The cultivation of RAW 264.7 cells took place in a humidified incubator with 5% CO_2 at 37°C. The cells were cultured and harvested during the logarithmic growth phase.

5. IL-β Production

The IL-β production test using the ABclonal® ELISA kit. RAW 264.7 cells ($1x10^5$ cells/well) were seeded in a 24-well plate and incubated for 3 hours. 100 µg/mL concentrations of the sample, LPS (1 µg/mL) as a positive control and DMSO control were then added and treated with an ELISA kit. The absorbance was evaluated at a wavelength of 450 nm.

6. Statistical Analysis

Data from all experiments were conducted with two replications (n=2), and the values are expressed as the mean and standard error (SE).

RESULTS AND DISCUSSION

1. Determination of *C. Xanthorrhiza*-Loaded SNEDDS

The formulation of the SNEDDS consists of oil, surfactant, cosurfactant, and turmeric extract, aiming to create a clear and monophasic preparation when mixed with an aqueous medium (Buya et al., 2020). The *C. Xanthorrhiza*-Loaded SNEDDS tested in this study was derived from previous research, with a drug loading of 23%, using Labrasol (20%) as the oil carrier, Tween 20 (60%) as the surfactant, and PG (20%) as the cosurfactant. This formula demonstrated stability, indicated by the absence of phase separation under extreme storage conditions. Accelerated resistance and stability tests also revealed that the *C. Xanthorrhiza*-Loaded SNEDDS remained resistant to dilution and stable over a 3-month storage period (Fitriani et al., 2021b).

The evaluation of *C. Xanthorrhiza*-Loaded SNEDDS was conducted through transmittance testing, particle size measurement, PI, and zeta potential (**Table 1**). The % transmittance value of *C. Xanthorrhiza*-Loaded SNEDDS formulation is 99.75% ± 0.07, falling within the range of 80%-100%, indicating a perfectly dispersed, clear, and transparent formulation. This suggests an increased particle surface area, potentially leading to faster drug absorption in the digestive tract (Cunha et al., 2020). Percent transmittance is a measurement of the optical clarity of diluted SNEDDS with water. Typically expressed as a percentage, transmittance measures how much light can pass through a sample, measured using UV spectrophotometry with water as the blank. Increased transmittance can be used to monitor the rate of self-emulsification, with the final percentage correlating with nanoparticle droplet size. Higher transmittance values indicate smaller particle sizes (Bali et al., 2010).

Table 1: Characterization of *C. Xanthorrhiza-Loaded SNEDDS*

C. Xanthorrhiza-Loaded SNEDDS	% transmittance	Droplet Size (nm)	Polydisperse Index	Zeta Potential (mV)
Mean ± SD	99.75 ± 0.070	84.7 ± 0.071	0.485 ± 0.173	-33.47 ± 0.060

The particle size of *C. Xanthorrhiza*-Loaded SNEDDS is small (<100 nm), specifically 84.7 nm±0.071, indicating that the formulation falls into the nanoparticle category. The polydispersity index, or homogeneity, is below 0.7 (0.485 ± 0.173), indicating that the SNEDDS formulation is homogeneous and stable (Chamieh et al., 2018; Syukri et al., 2019).

A high zeta potential value contributes to stability by resisting aggregation in a formulation. Generally, a zeta potential of ±45 mV characterizes a stable formulation. Negative values indicate the presence of free fatty acids, surfactants, and/or cosurfactants in a formulation. Additionally, negative values suggest a significant repulsion among droplets, preventing aggregation (Salimi et al., 2018). The thin mucus layer protects the gastrointestinal epithelial cells from xenobiotics and pathogens but also acts as a robust barrier to nanoparticles. Gastric mucus exhibits a negatively charged substructure made of sulfonate and sialic acid, hindering the diffusion of positively charged nanoparticles into deeper mucosal areas due to electrostatic interactions. Consequently, negatively charged nanoparticles penetrate the mucus more easily than positively charged nanoparticles (Corbo et al., 1990; Netsomboon and Bernkop-Schnürch, 2016). This contributes to the stability of the emulsion system. The zeta potential value for the turmeric SNEDDS formulation is -33.47 mV ± 0.060, indicating its stability.

2. IL-β Production

The present study aimed to evaluate the immunostimulatory activity of *Curcuma xanthorrhiza*, particularly in its SNEDDS formulation, by measuring the expression of IL-1β in vitro. IL-1β is a key pro-inflammatory cytokine and a marker of immune activation, often upregulated in response to pathogen exposure or immunostimulant agents such as lipopolysaccharides (LPS) (Galozzi et al., 2021).

Based on the results (**Figure 1**), the *C. Xanthorrhiza*-Loaded SNEDDS group demonstrated the highest IL-1β concentration (0.754 ± 0.022 pg/mL), significantly surpassing both the extract group (0.557 ± 0.096 pg/mL) and the basis of SNEDDS group (0.573 ± 0.183 pg/mL). This finding suggests that incorporating *C. xanthorrhiza* into a SNEDDS formulation substantially enhances its immunostimulatory activity. The improved performance may be attributed to increased bioavailability and cellular uptake of the active compounds. The cell control group and DMSO vehicle group exhibited low IL-1β expression levels (0.250 ± 0.176 pg/mL and 0.185 ± 0.062 pg/mL, respectively), indicating minimal baseline immune activation and confirming the suitability of the model. The medium-only group served as a true negative control (0.000 ± 0.048 pg/mL), while the LPS group (0.395 ± 0.044 pg/mL) functioned as a positive control, validating the assay's sensitivity to immune stimulation.

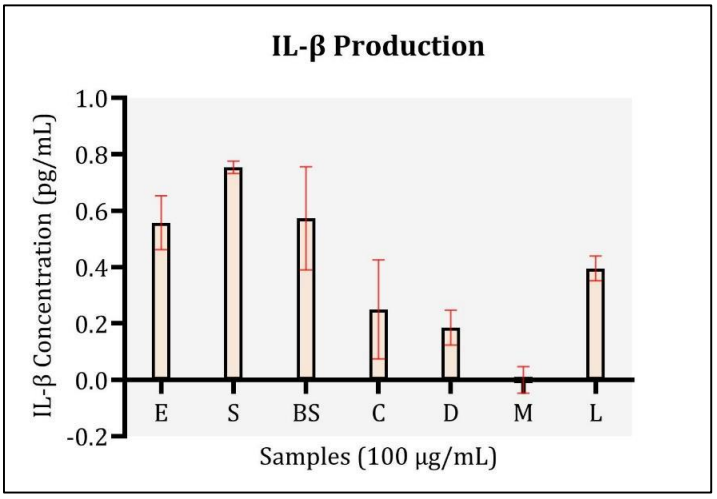

Figure 1. IL-β Production, E: *C. Xanthorrhiza* Extract; S: *C. Xanthorrhiza*-Loaded SNEDDS; BS: Basis of *C.* Xanthorrhiza-Loaded SNEDDS; C: Cell Control; D: DMSO; M: Medium; L: LPS

The extract and SNEDDS base groups also showed increased IL-1β expression compared to controls, the *C. Xanthorrhiza*-Loaded SNEDDS induced a markedly stronger response. This supports the hypothesis that SNEDDS enhances the delivery and immunological impact of lipophilic herbal compounds. Moreover, the relatively low standard error (SE) in the turmeric SNEDDS group (0.007) suggests consistent and reproducible results.

Curcumin is widely recognized for its anti-inflammatory properties. Several studies have demonstrated that its anti-inflammatory effects are mediated through the inhibition of key signaling pathways, including phosphatidylinositol 3-kinase (PI3K), toll-like receptor 4 (TLR-4), and nuclear factor-kappa B (NF-κB). This inhibition leads to a reduction in the expression of proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and IL-1β, as evidenced in both in vitro and in vivo models (Catanzaro et al., 2018; Shimizu et al., 2019).

Conversely, other studies have reported opposing outcomes, suggesting that curcumin may exert immunostimulatory effects by enhancing the production of TNF-α and Il-6 (Catanzaro et al., 2018;

Srivastava et al., 2011). Additional findings propose that curcumin functions as an immunomodulator, capable of eliciting both immunosuppressive and immunostimulatory responses depending on the physiological context and dose responses (Srivastava et al., 2011). Collectively, these observations indicate that curcumin's immunological effects are variable and context-dependent, supporting its classification as a bidirectional immunomodulatory agent.

Following immune system activation, immune cells begin to secrete various pro-inflammatory cytokines, such as TNF-α, interleukins (including IL-1β and IL-6), and interferon-gamma (IFN-γ) (Taniguchi & Karin, 2018). These cytokines contribute to an increase in vascular permeability, facilitating the recruitment of leukocytes to sites of tissue injury. This recruitment occurs through a series of coordinated steps, including margination, rolling, adhesion, transmigration, and chemotaxis. Once activated, leukocytes release prostaglandins and other inflammatory mediators, while also triggering the complement cascade leading to the generation of C3a and C5a fragments that play a critical role in pathogen elimination (Zhao et al., 2021).

These properties make *C. Xanthorrhiza*-Loaded SNEDDS a compelling candidate for further investigation as a natural therapeutic agent for managing immune related disorders (**Figure 2**). Specifically, its ability to modulate IL-1β expression presents a potential strategy for balancing immune responses in conditions characterized by either hyperinflammation or immune suppression.

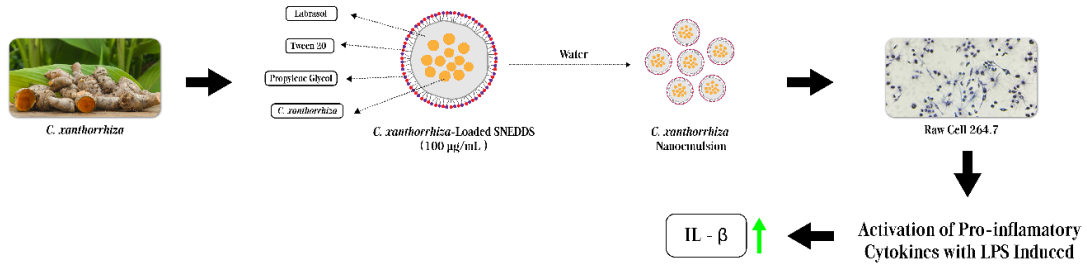

Figure 2. Schematic picture proposing that 100 µg/mL *C. Xanthorrhiza-Loaded* SNEDDS increased production of IL- β

CONCLUSIONS

These findings indicate that *C. xanthorrhiza* exhibits promising immunostimulatory effects, particularly when delivered via SNEDDS. The enhancement of IL-1β production supports its potential application as a natural immunotherapeutic agent. However, further studies are warranted to explore the underlying mechanisms, dose response relationships, and in vivo efficacy of this formulation.

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CONFLICT OF INTEREST

The author states there is no conflict of interest.

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Qualitative Identification of Compound in Red Betel Stem Using Histochemical Test and Phytochemical Screening

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ABSTRACT

The red betel plant is a medicinal plant that can be used for treatment purposes. The part of the red betel plant (*Piper crocatum* Ruiz & Pav.) that can be utilized is the stem. The aim of this study was to identify the compound content in the stem of *red betel* and whether it contains the same compounds as found in the red betel leaves. Qualitative identification was conducted using histochemical tests and phytochemical screening, which included tube tests and Thin Layer Chromatography (TLC). The histochemical test was performed to detect the presence of alkaloids, flavonoids, and tannins, followed by tube testing with the addition of specific reagents. The extraction of red betel stems was carried out using the maceration method with 96% ethanol as the solvent. The identification of the quercetin compound in the red betel stem extract was conducted using the TLC method with two different mobile phases: toluene:ethyl acetate:formic acid (7:2.5:0.5) and chloroform:ethyl acetate (9:1). The results from the histochemical and tube-based phytochemical screening showed that the red betel stem tested positive for alkaloids, flavonoids, and tannins. In the TLC phytochemical screening with the toluene:ethyl acetate:formic acid (7:2.5:0.5) mobile phase, the Rf value of quercetin was 0.3, while the Rf values obtained from the 96% ethanol extract of red betel stem were 0.28, 0.28, and 0.29, with spot colors differing from those of the quercetin reference compound. Meanwhile, in TLC with the chloroform:ethyl acetate (9:1) mobile phase, the quercetin reference compound was not eluted.

Keywords: Histochemical Test; phytochemical screening; red betel stem; TLC .

INTRODUCTION

The Indonesian people have long used medicinal plants for healing purposes, passed down through generations. These plants are utilized either as whole specimens or in parts (Tunny, 2022). The *Piper* plant possesses medicinal properties due to its content of secondary metabolites. These secondary metabolites are found in various parts of the plant, such as flowers, leaves, stems, bark, and roots. The secondary metabolites present in *Piper* contain unique compounds such as terpenes, phenylpropanoids, lignans, phenolics, and alkaloids. These compounds exhibit various biological activities, including antifeedant, antibacterial, antifungal, antiplatelet, antioxidant, anti-inflammatory, anti-amoebic, and insecticidal effects (Ozygit et al., 2023). The red betel (*Piper crocatum* Ruiz & Pav.) plant is a medicinal plant that can be used for treatment purposes. The leaf of red betel is reported to

have some pharmacological activities; containing compounds such as alkaloids, flavonoids, saponins, polyphenols, quinones, and essential oils (Nugroho et al.,2020).

The parts of the plant that can be used for medicinal purposes include the flowers, leaves, stem bark, seeds, or the outer skin. The stem is an essential part of the plant's structure and is often referred to as the plant's axis; it can be utilized as traditional medicine by the community (Helmina & Hidayah, 2021). In addition to detecting the presence of primary metabolites, histochemical tests are used to identify the presence of secondary metabolites in plant organs (Mulyaningsih et al., 2021). The presence of flavonoids, phenols, essential oils, saponins, tannins, and terpenoids is detected through histochemical testing. Phytochemical screening is the initial step in researching the chemical content of natural substances, involving qualitative testing of compounds in plants using test tubes and thin-layer chromatography methods (Saragih & Arsita, 2019).

The parts of the red betel that can be used medicinally are the leaves and stems, generally the leaves are used as medicinal ingredients, while the stems are less utilized. Understanding the phytochemical composition of different plant parts is essential for optimizing their medicinal use. This study focuses on identifying the bioactive compounds present in the stem of the red betel plant and compares them to those commonly found in the leaves, using histochemical analysis and phytochemical screening methods, including Thin Layer Chromatography (TLC).

METHODS

The plant determination was conducted at the Laboratorium Farmakognosi Fitokimia, Faculty of Pharmacy, Sanata Dharma University, where the herbarium specimen of *Piper crocatum* Ruiz & Pav is stored with specimen number: 01/LKTO/Far-USD/I/2024. The red betel stems used were semi-woody and originated from the Sleman area of Yogyakarta.

The histochemical test began with the preparation of the red betel stem by making cross-sections of fresh stems using a sliding microtome. The stem sections were then soaked in distilled water. The samples were treated with distilled water as a control, 5% NaOH for flavonoid detection, 10% $FeCl_3$ for tannin detection, and Dragendorff reagent for alkaloid detection. Color changes were observed under a light microscope equipped with OptiLab, and images were taken at magnifications of 4x10 and 10x10 (Ridwan et al., 2022; Nurhasanah & Iriani, 2021; Damayanti & Susanti, 2023).

Phytochemical screening was conducted using both test tube assays and Thin Layer Chromatography (TLC). For alkaloid testing, 2 grams of the test material were weighed and mixed with 10 ml of 1% HCl in a test tube. After cooling, the solution was filtered and divided into three tubes, each tested with Dragendorff's, Mayer's, and Wagner's reagents. For flavonoid testing, 10 ml of distilled water was added to 2 grams of the sample, heated for 5 minutes, then 10% NaOH was added. For tannin testing, 10 ml of distilled water was added to the sample, heated for 5 minutes, and 1% $FeCl_3$ was added (Fajri & Kristanty, 2022; Oktavia & Sutoyo, 2021; Hartini et al., 2013; Nofita & Dewangga, 2022).

Thin Layer Chromatography was carried out using silica gel $60F_{254}$ as the stationary phase, with the TLC plate activated in an oven at 110°C for 30 minutes. Two types of mobile phases were used: toluene:ethyl acetate:formic acid (7:2.5:0.5) and chloroform:ethyl acetate (9:1). A total of 2 microliters of the test sample was applied to the TLC plate, eluted to a distance of 7 cm, then removed and dried. Detection was performed using a UV spectrophotometer at wavelengths of 254 nm and 365 nm. Compound identification was done by observing the color and Retardation factor (Rf) values, where Rf

represents the ratio of the distance traveled by a compound from the origin to the distance traveled by the mobile phase from the same point (Patnaik, 2004).

RESULTS AND DISCUSSION

The cross-section of the red betel stem consists of the epidermis, chlorenchyma, parenchyma, collenchyma, peripheral vascular bundles, sclerenchyma, medullary vascular bundles comprising phloem and xylem, pith parenchyma, and mucilage canals (**Figure 1**). **Figure 2** shows the result of the histochemical test for alkaloids in the red betel stem at 100x magnification using a light microscope, along with a comparison between the control and the positive alkaloid reaction.

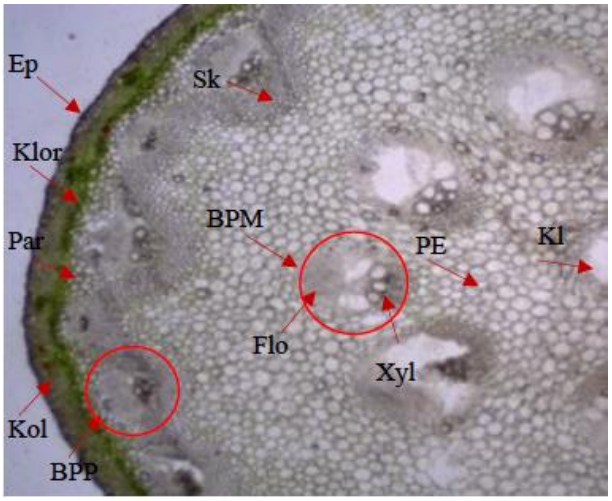
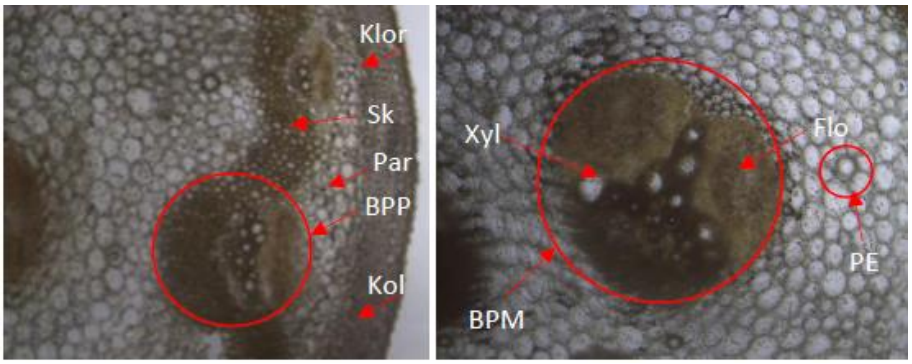


Figure 1. Cross section anatomy of red betel stem. Caption: Ep: epidermis; Klor: chlorenchyma; Par: parenchyma; Kol: collenchyma; BPP: peripheral vascular bundles; Sk: scherenchyma; BPM: medullary vascular bundles; Flo: phloem; Xyl: Xylem; PE: pith parenchyma; Kl: mucilage canals.

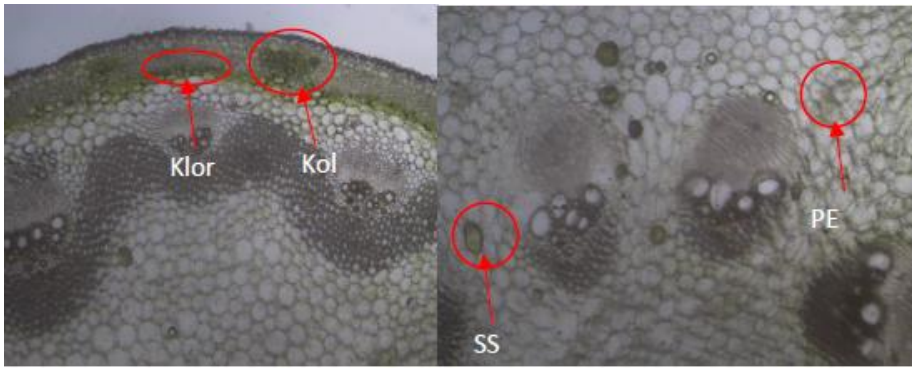


(a) (b)

Figure 2. The histochemical test for alkaloids in the red betel stem at 100x magnification using a light microscope Caption: Klor: chlorenchyma; Par: parenchyma; Kol: collenchyma; BPP: peripheral vascular bundles; Sk: scherenchyma (a); BPM: medullary vascular bundles; Flo: phloem; Xyl: Xylem; PE: pith parenchyma (b).

The red betel stem turned yellow when reacted with 10% NaOH, indicating a positive reaction for flavonoids. The formation of a yellow color indicates the presence of flavonoid compounds. The change in yellow color in the red betel stem occurs due to the formation of salts and quinoid structures which have longer conjugated double bonds (Muna, 2017). A positive reaction was also shown with the addition of 1% $FeCl_3$, which suggests the presence of tannins in the test material. A comparison between the control sample with no treatment and the positive flavonoid reaction under 40x magnification using

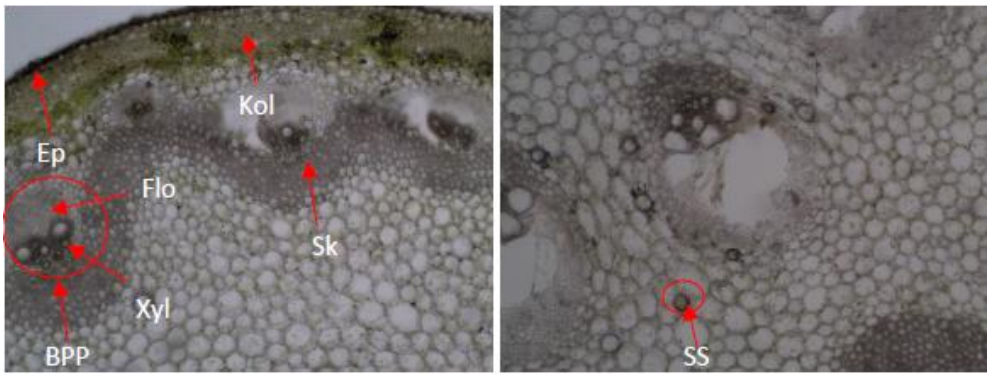
a light microscope is shown in **Figure 3**. The reaction between tannins in the red betel stem and $FeCl_3$ forms a complex compound with Fe^{3+} ions (Damayanti & Susanti, 2023).



(a) (b)

Figure 3. The histochemical test for flavonoid in the red betel stem at 100x magnification using a light microscope. Caption: Klor: chlorenchyma; Kol: collenchyma (a); SS: secretory cell; PE: pith parenchyma (b).

The test result using $FeCl_3$ reagent showed a color change to dark green, indicating the presence of tannins. **Figure 4** shows the result of the histochemical test for alkaloids in the red betel stem at 100x magnification, along with a comparison between the control and the positive reaction for tannins.



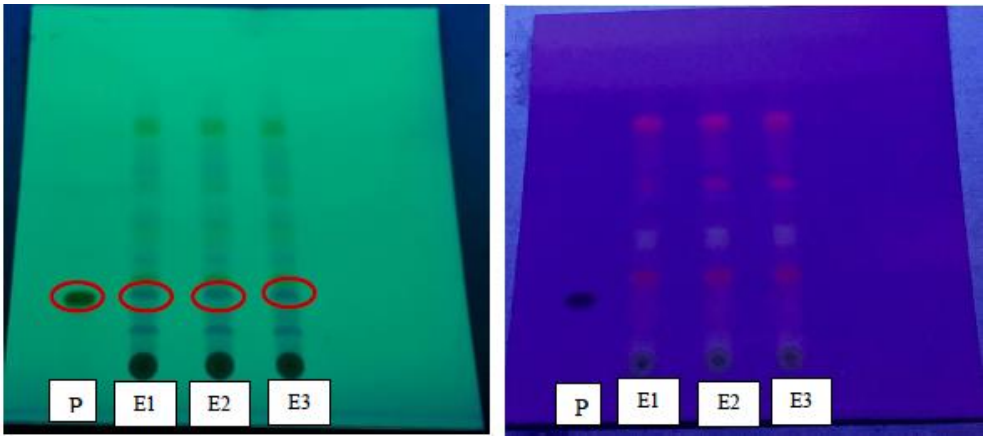
(a) (b)

Figure 4. The histochemical test for tannin in the red betel stem at 100x magnification using a light microscope. Caption: Kol: collenchyma; BPP: peripheral vascular bundles; Sk: scherenchyma; EP: epidermis; Flo: phloem; Xyl: Xylem (a); SS: secretory cell (b).

The histochemical tests conducted on red betel stem tissues confirmed the presence of alkaloids, flavonoids, and tannins, as evidenced by color changes when specific reagents were applied. These findings were consistent with the results from the tube-based phytochemical screening, which also indicated a positive presence of these three compound classes. This suggests that, similar to red betel leaves, the stem also contains bioactive secondary metabolites commonly associated with medicinal properties.

The extraction of red betel stems that has been done 3 times produced the amount of extract respectively as much as 1,567 grams with a yield of 10.447%; 1.9163 grams with a yield of 12.775%, and 2.1008 grams with a yield of 14.005%. The yield shows that the extraction of compounds from red betel stems produces a good amount of extract, namely more than 10%. The test tube results showed that the test material reacted positively with Dragendorff's reagent, indicated by a red color change and

the formation of a reddish-brown precipitate. The reaction with Wagner's reagent also yielded a positive result, evidenced by a brick-red color change and a reddish-brown precipitate. Alkaloids are basic compounds, and the addition of HCl can lead to the formation of alkaloid salts, which precipitate when Dragendorff's, Mayer's, or Wagner's reagents are added (Hanifa e al., 2021). The presence of alkaloids in red betel leaves has been reported.[12,17] This suggests that alkaloids are found in both the leaves and stems of red betel. The flavonoid test also showed a positive reaction: the addition of 10% NaOH caused a color change to yellow-orange. This color change occurs due to the formation of salts and the development of a quinonoid structure, which has a more extended conjugated double bond system (Theodora et al., 2019). Similar to alkaloids, flavonoids have also been reported in red betel leaves (Hartini et al., 2013; Alifah et al., 2023); indicating their presence in both the leaves and stems of red betel. The color change to greenish-black in the red betel stem upon treatment with $FeCl_3$ indicates the presence of tannins. Tannin compounds in simplicia powder bind with Fe^{3+} to form a complex, causing a greenish-black or bluish-black color change depending on the type of tannin present (Hanifa et al., 2021). A bluish-black color indicates the presence of gallotannins, whereas a greenish-black color indicates the presence of catechol-type tannins (Hartini et al., 2013). Tannins are found in red betel leaves (Alifah et al., 2023), as well as in the stems.

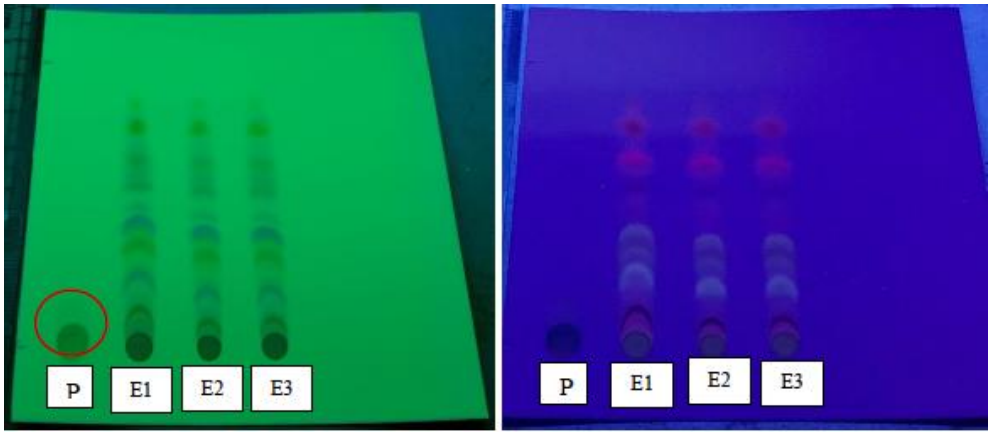


(a) (b)

Figure 5. The TLC using the toluene:ethyl acetate:formic acid (7:2.5:0.5) as mobile phase; UV_{254nm} (a) and UV_{365nm} (b) detection. Caption: P: quercetin; E1: 1st extract replication; E2: 2nd extract replication; E3: 3rd extract replication

In the TLC analysis using the toluene:ethyl acetate:formic acid (7:2.5:0.5) mobile phase (**Figure 5)**, the quercetin standard produced an Retardation factor (Rf) value of 0.3. The ethanol extract of the red betel stem yielded three spots with Rf values of 0.28, 0.28, and 0.29. Although these values were close to that of quercetin, the spot colors differed, suggesting that the compounds in the stem may be structurally similar to quercetin but are not identical. This implies the presence of flavonoid-like compounds in the red betel stem that may share some chromatographic characteristics with quercetin.

In contrast, the TLC analysis using chloroform:ethyl acetate (9:1) as the mobile phase (**Figure 6**) did not result in the elution of the quercetin standard, and therefore was not useful for identifying quercetin or related compounds in this solvent system. The reference compound quercetin was not eluted using the mobile phase of chloroform:ethyl acetate (9:1) because quercetin is a flavonol compound that contains five hydroxyl groups, which makes it polar (Hohakay et al., 2019)



(a) (b)

Figure 6. The TLC using the chloroform:ethyl acetate (9:1) as mobile phase; UV_{254nm} (a) and UV_{365nm} (b) detection. Caption: P: quercetin; E1: 1st extract replication; E2: 2nd extract replication; E3: 3rd extract replication

The mobile phase used consisted of 90% chloroform, which has a polarity index of 4.1, and 10% ethyl acetate, which has a polarity index of 4.4. Ethyl acetate is more polar compared to chloroform. Therefore, the use of a higher proportion of chloroform in the mobile phase composition results in a non-polar mobile phase. In thin-layer chromatography (TLC), the separation method is based on polarity differences—"like dissolves like." Polar analytes are more soluble in polar solvents and less soluble in non-polar solvents. Since quercetin is a polar compound, the eluent used should also be polar. In this study, the quercetin reference compound was not eluted with the chloroform:ethyl acetate (9:1) mobile phase due to differences in polarity, an unsuitable mobile phase composition, and possibly an inappropriate concentration of the quercetin reference compound. As a result, the Rf value and the similarity of the spot between the quercetin and the extract replications could not be compared.

Tabel 1: Secondary metabolites in leaves and stem of red betel

Secondary metabolite group	Presence in leaves	Presence in stem
Alkaloid	Reported	Confirmed via histochemical and tube tests (Dragendorff's, Wagner's)
Flavonoid	Reported	Confirmed via NaOH reaction and TLC analysis
Tanin	Reported	Confirmed via $FeCl_3$ reaction (greenish-black)

Both leaves and stems of red betel contain alkaloids, flavonoids, and tannins, indicating that the stem shares similar phytochemical constituents with the leaf (Tabel 1). However, TLC analysis suggests that flavonoids in the stem may differ structurally from those in the leaf, requiring further characterization. The comparable metabolite profile highlights the stem's potential as an alternative medicinal source.

CONCLUSIONS

The stem of the red betel plant (*Piper crocatum* Ruiz & Pav.) contains important secondary metabolites, including alkaloids, flavonoids, and tannins, as confirmed by histochemical and phytochemical screening methods. These findings demonstrate that the red betel stem holds phytochemical potential similar to the leaves and may be a valuable source of medicinal compounds, warranting further analysis for compound isolation and characterization.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Faloak and the Trilogy of Human Relations: Disaster Preparedness among the Atoin Meto Indigenous Community through the Practice of Faloak Consumption

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ABSTRACT

This study aims to explore such local knowledge by focusing on *Faloak* (*Sterculia quadrifida*, R.Br.), a medicinal plant endemic to Timor Island. The objective is to understand how the Atoin Meto people consume and conserve medicinal plants—particularly *Faloak*—through practices rooted in traditional knowledge that reflect an inherent readiness to protect these plants from disaster-related threats. Using an ethnographic approach and interpretative qualitative analysis, the study reveals two key findings:

(1) the consumption of medicinal plants is carried out with close attention to sustainability, employing specific methods embedded in spiritual beliefs and ritual practices; and (2) from these consumption and conservation practices emerges a deep understanding of the complexity of the human relational trilogy— between fellow humans, nature, and the Divine. These relationships are characterized by mutual respect and equality, interdependence and contradiction, care and potential harm. Such relational dynamics constitute a form of disaster preparedness within the Atoin Meto community, addressing intentional and unintentional disasters. This study contributes to the advancement of interdisciplinary approaches in disaster studies, promotes the recognition of local knowledge as a vital component of disaster risk management systems, and opens pathways for the integration of Indigenous knowledge into more inclusive and sustainable formal disaster risk governance frameworks.

Keywords: Atoin Meto; Faloak; healing practices; traditional healer; ancestors; relationality.

INTRODUCTION

Every Indigenous community possesses local knowledge systems that govern their relationship with the surrounding environment, and the Atoin Meto—or Atoin Pah Meto—of Timor Island, Indonesia, are no exception. In Indonesian, *Atoin* or *Atoni* means "person," *Pah* means "land," and *Meto* means "dry" or "without water" (Sawu 2004). The term *Atoin Pah Meto* thus refers to a large group of people who inhabit a dry region with barren soil and limited access to water resources. They are speakers of the Dawan language, or *uab meto*, which includes several closely related dialects (Grimes et al. 1997). This ethnic group is also commonly referred to as the Dawan people (Faimau 2009; Sawu 2004).

Geographically, the Atoin Meto predominantly occupy the western part of Timor Island, within the province of East Nusa Tenggara (Nusa Tenggara Timur or NTT). According Neonbasu (2016), referencing scholars such as Ormeling (1955), Nordholt (1971), and Hagèrdal(2012), during the period of indigenous princedoms, the Atoin Meto resided in ten traditional polities (swapraja) in western Timor. These included Biboki, Insana, Miomafo, Amanuban, Amanatun, Mollo, Amarasi, Amfoang, Fatuleu, Kupang, and Ambenu (now part of Timor-Leste). The majority of Atoin Meto are dryland farmers who cultivate crops such as maize and rice, and raise livestock, particularly cattle (Manafe 2016; Mullik and Jelantik 2009). For consistency, this paper uses the term *Atoin Meto* throughout.

As their name suggests, the Atoin Meto people inhabit arid regions. The dry conditions and low annual rainfall are characteristic features of Timor Island. The island's climate is strongly influenced by wind patterns originating from the Australian continent to the south, and from the Asian continent and the Pacific Ocean to the north. From June to September, the prevailing winds blowing toward Timor Island come from Australia. These air currents are generally hot and carry little moisture, resulting in a prolonged dry season. Conversely, from December to March, the winds that blow from Asia and the Pacific Ocean carry a high level of moisture, bringing about the rainy season during this period.

Climatological studies have shown that the rainfall patterns in West Timor are significantly influenced by prevailing wind systems and topography. For instance, Syarifudin et al., (2015) analyzed the temporal and spatial characteristics of rainfall distribution in West Timor, highlighting the impact of these physical factors on rainfall variability. Additionally, research by Sabuna et al. (2022) indicates that the region experiences a dominant dry period from April to November, with drought events often influenced by El Niño phenomena.

For communities who rely heavily on annual rainfall, the threat of drought remains one of the greatest dangers to their survival. To mitigate this risk, maintaining a harmonious relationship with nature becomes essential (Manafe 2016). This ecological harmony serves as a guarantee against the recurring threat of drought. As such, the Atoin Meto people are compelled to protect water springs (*oe*) and the surrounding natural environment by constructing and transmitting local knowledge rooted in the origin histories of each clan.

Water springs are regarded as being intrinsically connected with particular stones, referred to as *fatu kanaf* and *oe kanaf* (Manafe 2016). Manafe (2016) notes that each Atoni clan associates these two elements—stone and water—with the origin of their lineage. In ancestral narratives, when the clan founders first established their settlements after long migrations in search of new land, they brought with them a vessel of water and a sacred stone from their place of origin. The water would become the flowing spring near the settlement, typically located among the rocks in the surrounding landscape. This spring then served as the primary source of water for daily life and agricultural activity within the village.

The stone and water together are not only physical resources but also serve as markers of origin and identity—sacred elements that signify both ancestry and the continued vitality of the clan. These elements must be protected, as their preservation ensures the survival of the community (Manafe 2016; Neonbasu 2016). To safeguard the springs and their surrounding environment, the Atoin Meto apply local spiritual and normative concepts such as *leu* (sacred), *nuni* (taboo), and *pemali* (forbidden), which are reinforced through *bunuk* rituals—symbolic acts that involve hanging signs on trees as warnings to prevent damage or desecration (McWilliam 2005; Usbobo 2019)

Large trees in the forest and around water springs are protected under these concepts and rituals, as they play a crucial role in preserving water sources and maintaining soil integrity in the face of

drought. During the opening of new gardens (*lele tolas*), certain tree species are deliberately left uncut for the same ecological purpose (Foni 2008; Manafe 2016). In addition, some trees are protected from being felled because they serve specific functions: as nesting sites for bees, as materials for traditional house construction (Bria and Binsasi 2020), or because they are considered sacred. Among these is the sandalwood tree (*Santalum album*), which holds cultural and spiritual significance (Manafe 2016; McWilliam 2005).

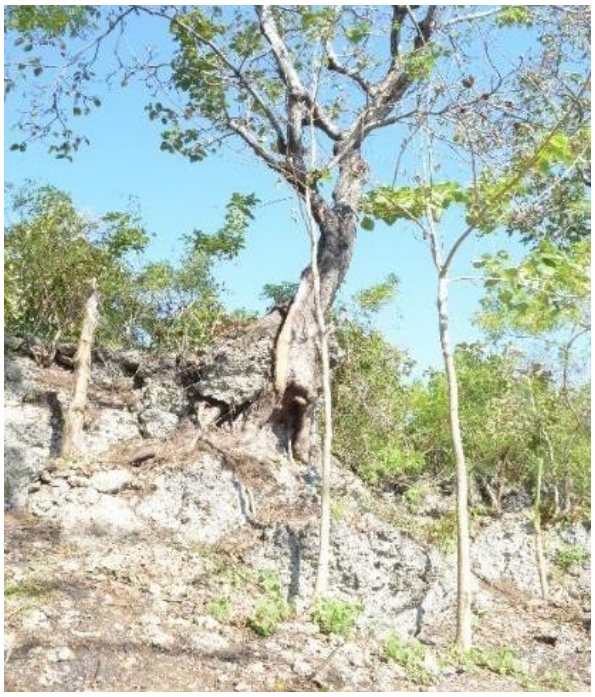

Figure 1. Faloak Tree.

One of the plants whose sustainability is actively safeguarded is *Faloak* (*Sterculia quadrifida* R.Br.), an indigenous species native to Timor Island, traditionally used for treating various ailments (Siswadi et al. 2016). It is well documented as a medicinal plant that has been consumed by local communities for generations. Scientific studies have demonstrated its hepatoprotective properties, offering protection for liver function (Rambung et al. 2017). *Faloak* is also believed to improve blood circulation, restore physical stamina, relieve back pain and gastric disorders, and is commonly used in postpartum blood-cleansing remedies (Siswadi et al. 2016; Siswadi and Saragih 2021). More recently, its leaves have been shown to exhibit significant antiglycemic activity (Dias et al. 2024). The most commonly used part of the plant is the bark. Each Atoin Meto community across Timor Island has developed its own distinct techniques for harvesting, processing, and consuming the bark of this valuable plant.

The diverse consumption patterns and harvesting techniques employed by different communities may pose a serious threat to the long-term survival of the *Faloak* plant (Rambung et al. 2016; Siswadi et al. 2016; Siswadi and Saragih 2021). As the plant becomes increasingly well-known for its medicinal value, its use has become more widespread, often leading to unsustainable harvesting practices. *Faloak* trees are frequently found in a critical condition, with their trunks severely damaged due to indiscriminate cutting. This situation endangers the survival of this endemic medicinal species of Timor Island. The threat is further exacerbated by the island's increasingly arid climate, marked by prolonged droughts, declining rainfall levels, and the lengthening of the dry season (Bendi 2024; Hima, Made, and Nasjono 2022; Maneno, Lestari, and Fallo 2023).

Mitigating environmental threats—particularly those endangering the survival of the *Faloak* plant—caused by natural disasters and destructive human consumption patterns is crucial for preserving ecological sustainability. Generally, mitigation strategies can be developed through two main approaches. The first is a modern, technology-based approach, which includes advanced tools such as Geographic Information Systems (GIS) (Mandal and Mani 2020; Wati et al. 2024), drones, remote sensing, machine learning algorithms, and environmental DNA (eDNA) analysis (Haryeni et al. 2024). The second is a conventional approach grounded in local knowledge systems. Examples include community-based forest conservation practices demonstrated by Candraningsih et al. (2018) in Tigawasa Village, Banjar District, Buleleng Regency, and by Syarif (2017) among the Karampuang Indigenous Community in Sinjai Regency, South Sulawesi, among others.

In light of these conditions, the survival of the *Faloak* plant, as well as other species, can be safeguarded through local knowledge encompassing harvesting techniques, care practices, and specific ritual protocols tied to their consumption, all of which are bound by certain mythical beliefs. For example, the sandalwood tree, as narrated by Manafe (2016) and Sa'u (2004) in Atoin Meto mythology, is said to have originated from the bones of Liurai Sonbai's younger sister, Bi Sobe Sonbai. The younger sister sacrificed herself to become various food plants and other vegetation to sustain Liurai Sonbai and his descendants. Consequently, whenever sandalwood is to be harvested, specific rituals must be performed with the knowledge and consent of the *usif* (traditional elders) and their descendants, who are regarded as the successors of Liurai Sonbai (Armini 2012; McWilliam 2005).

However, studies explicitly investigating local knowledge related to the use of the *Faloak* plant and its contribution to community-based disaster preparedness in the Timor Island region remain limited. Existing research tends to discuss the broader role of local knowledge in environmental conservation. For example, Lake et al. (2017) examined this role among the Dawan (Atoin Meto) people in North Central Timor Regency (Timor Tengah Utara, TTU), while Nope (2019) focused on the role of indigenous social structures in environmental conservation in Boti village, South Central Timor Regency (Timor Tengah Selatan, TTS). Similarly, Atanus et al. (2018) explored the concept of *Banul* as a form of local wisdom for environmental preservation in Tun'noe village, East Miomafo subdistrict, TTU.

This study aims to explore local knowledge with a specific focus on the *Faloak* plant, one of the endemic species of Timor Island. The research is conducted to understand the indigenous community's knowledge regarding the consumption and conservation of medicinal plants, including *Faloak*, as practiced by the Atoin Meto Indigenous Community (KMA) on Timor Island. This knowledge reflects a state of preparedness to safeguard the plant from natural disaster threats. Local knowledge is manifested in: (1) the consumption practices of *Faloak*, which always consider the sustainability of the medicinal plant through specific methods embedded within beliefs and rituals; and (2) an understanding of the complex trilogy of human relationships—relationships with fellow humans, with nature, and with the Divine. This local knowledge constitutes a form of community-based preparedness or mitigation against environmental degradation caused by natural disasters, thereby ensuring the preservation of the environment and particularly the survival of the *Faloak* plant on Timor Island.

METHODOLOGY

This study was conducted among the Atoin Meto community in the sub-districts of Eban, Naen Village, and Tunbakun in Timor Tengah Utara Regency (TTU). Data were collected through interviews with four traditional healers: two healers from Tunbakun, and one healer each from Eban and Naen. Additionally, interviews were conducted with six individuals currently consuming the *Faloak* plant, with

two respondents from each location—Eban, Naen, and Tunbakun. The study was carried out throughout 2019. An ethnographic approach combined with qualitative interpretative analysis was employed. This approach was considered appropriate for capturing traditional practices related to the use and consumption of *Faloak*, including harvesting techniques, medicinal use, and post-treatment ritual habits. The interpretative technique was used to understand the community's perspectives behind these practices. Interpretation in this study is regarded as a creative process of meaning-making from the data. As a creative process, interpretation is strongly influenced by the researcher's insight, imagination, and creativity (Brewer 2000). To maintain objectivity, interpretations were kept closely aligned with the reality of the research subjects by employing several recommended techniques (Brewer 2000), including: (a) verifying interpretations with the community to ensure accuracy according to local understanding; (b) maintaining a critical stance towards informant information; (c) seeking and examining alternative information from other informants; and (d) ensuring interpretations do not stray beyond the methodological, data, locational, and contextual boundaries where the data were collected.

Analysis was conducted using Tim Ingold's (2000) relational approach as elaborated in *The Perception of the Environment: Essays on Livelihood, Dwelling and Skill*. This relational approach was applied to understand the ongoing process of human-environment relations, which involve continuous and direct interactions with all elements of the environment, both human and non-human. According to Ingold (2000), cultural knowledge and bodily substance are understood as continuously produced in an ongoing process, reciprocally connected through active and sustained engagement with the land and its inhabitants—both human and non-human. Each participant plays a role in shaping relational forms and relational understanding throughout this continuous process.

RESULTS AND DISCUSSION

1. Treatment Patterns Using the *Faloak* Plant

As mentioned earlier in this paper, *Faloak* is a medicinal plant traditionally consumed by the community since ancient times. *Faloak* has been proven effective in protecting liver function (Rambung et al. 2017) and is believed to help restore stamina, relieve back pain, treat gastric disorders, and cleanse blood postpartum (Siswadi et al. 2016; Siswadi and Saragih 2021). The use of *Faloak* bark combined with *Maunlui* leaves (*Scurrula purpurrea* Dans.) is also reported to treat all stages of cancer (Rambung et al. 2017). The plant part most frequently used is the bark (Rambung et al. 2017; Siswadi et al. 2016; Siswadi and Saragih 2021). Each Atoin Meto community group in their respective villages or territories across Timor Island has developed distinct techniques in administering treatments using this plant. The treatment pattern involves the process of harvesting the medicinal plant, consumption methods, and several post- treatment practices.

1.1 Harvesting and Collection Techniques

The process of searching for and collecting *Faloak* bark can be performed either directly by the patient intending to consume it or by individuals who have been traditionally entrusted by the community as herbal healers. However, this process is more commonly conducted by traditional healers, although in recent developments, patients in need of the medicine have begun to harvest it themselves, following some of the harvesting techniques typically employed by the healers. Traditional healers are believed to possess certain spiritual powers that influence the efficacy of the medicine collected or prepared. Medicine collected without such spiritual involvement is believed not to result in healing (Adiyasa and Meiyanti 2021; Humaedi 2015; Setiawan and Kurniawan 2017; Suryaningsi 2015).

Typically, the harvesting process by traditional healers begins with a prayer inside the house, beside a stone that usually exists indoors as a symbol of ancestral presence. In traditional houses, such as the indigenous houses of the tribe, there is always a flat, round stone located beneath the main house pillar (ni ainaf). This stone serves as a place to offer sacrifices to the ancestors, including meat, food, and other offerings. The flat stone acts as a medium of communication with the ancestors (Dima, Antariksa, and Nugroho 2013; Fox 2006; Lang 2015; Sawu 2004). This custom is also practiced in modern dwellings, where a flat stone inside the house serves this communicative function (Fox 2006).

Two customary practices were observed. The first involves a prayer ritual performed during the slaughtering of a rooster. This ritual, called *Tekas*, is a ceremonial sacrifice of a chicken dedicated to the ancestors for specific purposes. The chicken is slaughtered, and its blood is dripped onto a stone while reciting a prayer addressed to the ancestors. The prayer expresses the intention to harvest a certain part of the plant for medicinal use, requesting the ancestors' presence and blessing so that the process of searching and collecting may proceed smoothly, and the medicine will cure the illness. The chicken meat is then cooked. Once cooked, a portion of the meat along with some rice is placed on a plate and set on the flat stone inside the house. Only after this offering is made may the meat and food be consumed. Through this ritual, the ancestors are called upon to witness and approve the family healing tradition carried out by the herbalist. The wording of the prayer can vary among healers. For example, a healer in Naen prays with the intention of summoning the ancestors as former healers who passed down the knowledge and practice of medicine to be present and personally conduct the process of searching and collecting the medicinal plants. The healer who prays acts only as an intermediary. During an interview, a traditional healer in Naen said:"

> "... ini saya sudah mohon memang di leluhur, di bapa, di nenek moyang memang to, jadi saya keluar itu
> (to collect plants as medicinal materials), bukan saya sendiri, saya dengan mereka, saya hanya pegang sa. Saat sampai itu, bapa harus bantu saya. Saya datang hanya pegang sa, baptua yang sendiri kerja (to collect plants)....' (interview, Naen 2019).

After the prayer, the search process begins. The healer must walk carefully to ensure that their shadow stays behind them and is not in front. Upon arriving at the intended Faloak tree, another prayer must be recited in front of the tree. Something similar is also expressed in the prayer said before taking parts of the plant. There is a custom by one of the healers in Naen to place a piece of money while praying. The money placed is usually just as a symbol, and the amount could be a one thousand or two thousand rupiah bill. In the Dawan language (uab meto), the prayer is as follows:

> "... Am hone ihomoba tokom ma nanoba paen jadi ho mepu ho kait a te usa nabala moe nabala. Au uplike au usi mnakam sa, ek ho ek ho kait, tef ho atam meup ho atem kait, au alaha uteb a huk be on le i, ho es mek namanon, ho es mu leko, ho es muloitan..." (interview, Naen 2019).

This prayer can be translated as: Father, I give you money, placing it at your seat, in your shelter. So you have the work and the ability that remain, which I remember. I hear what you say, I do what you say, that you use it according to your ability. I am only your extension, you are the one who works, you are the one who acts (to heal/cure)."

In the formulation of this prayer, there is an explicit belief in the role, ability, and presence of the healer's ancestors, known as *bapa* or the healer's elders, who assist or, more precisely, perform the

healing. In this context, the position and skills of the healer are merely a 'shadow' or an extension of their *bapa*. The healer is the visible shadow of their *bapa*, who exists as a spirit in the healing process. Another healer in Tunbakun recites a prayer while placing a banknote under the tree. The prayer is as follows:

> "... E Usi, fekau alekot, ho pal amleut. Kalo hom maneo tebes lo au ait ko le i au fena he naham he niun nait in naleok hele au anam neo anam nono ak lo ho muneo tebes luli tebes keso tebes (interview, Tunbakun 2019).

This prayer can be translated as, "Bapa, give me the good part, you take the bad/unwanted part. If it is truly true (having the ability to heal), I take this medicine to give to him so that after he drinks or eats it, he can recover, so I can say that you are truly sacred (having magical power) and strong (having strength/courage to heal)."

Figure 2. Common Practice of Peeling Faloak Bark on Timor Island

Furthermore, in the second customary practice, there are specific techniques and rules observed during the harvesting or peeling of the *Faloak* plant. The peeling process must be carried out vertically, from the bottom upward. This procedure must be conducted with great care, as the peeled bark must not fall to the ground. If the bark falls, it is believed to be a bad omen for the patient who is to be treated with it. In such cases, the medicine may prove ineffective, and the patient may ultimately succumb to their illness. In addition, there other important things to note in this process. The shadow of the person collecting the medicine or the healer must not cover the tree. They must stand facing the sun so that their shadow does not fall on the plant whose bark is to be taken. The bark of the Faloak plant must not be taken carelessly or in excessive amounts. One healer has a special rule regarding this. The amount of bark to be taken must be according to the need or purpose—meaning, only for the sick person in

question. The medicine must not be stored for others. If taken excessively, the healer believes there will be consequences to bear. Many people will come to ask for help to cure their illnesses. It is believed that the medicine will cause sick people to call and come for treatment from him. In an interview, he explained it as follows:

> "...Itu obat juga kalo kita ambil simpan, itu banyak orang yang sakit, datang minta tolong, kita tidak bisa duduk ko ini, nanti sebentar orang yang datang panggil, sebentar orang su datang ko minta tolong. Jadi ambil pas-pas untuk orang yang mau minum saja..." (interview, Tunbakun 2019).

Another healer only takes parts of the medicinal plant when a sick person comes to him. When asked by the research team to take parts of the plant to be shown and documented, he refused. He believes that if the medicinal plant is taken excessively, he himself will bear the illness. It is believed that the medicine will "ask" to be used, and he will be the one who falls ill and uses the medicine. In the prayer he recites in front of the tree before peeling it, he already states the purpose and intention of using the medicine, including who will use it. Therefore, the prayer binds him to only take the medicine for and according to the needs of the sick person who has been prayed for. In an interview, the healer explained this matter as follows:

> ".... seharusnya kita ambil (a part of the plant) itu harus pasang (use to heal) untuk orang sakit. Maksudnya memang harus begitu, ia, kalo tidak saya beban. Jadi memang ada adat (customary traditions), harus ada omong (the prayer), kita harus kasi tahu di (in front of) pohon, siapa yang sakit, baru kita cabut (cut the tree). Tidak ada, nanti saya yang kena. Ya itu, kecuali ada yang sakit, supaya kita pasang di situ, penyakit di mana kita pasang di situ..." (interview, Eban 2019).

The performance of prayers before and during the collection, as well as the rules for peeling, are related to the belief in the healing of the person to be treated and the safety of the healer themselves. These healers believe that if these rules are violated, they will suffer illness, bad luck, and so forth. The same treats also threaten the person being treated. Another healer in Tunbakun does not have traditions in line with the rites of traditional beliefs. He begins the process of searching for medicine and healing with prayer. The prayer is conducted according to the rites of the modern religion he adheres to, Catholicism. The prayer is intended to show his sincerity in helping with the healing, his belief in the role and power of God as the source of all things, including the plants to be taken as medicine, and to demonstrate God's providential power that will accompany the healing process toward recovery. After all these customs are observed, the process of taking parts of the plant is carried out. According to this healer, the process of collecting medicine can be done by anyone, including the person who wants to be treated. Sometimes, one of his children is assigned to collect and prepare the herbal medicine. However, in some cases he described, he openly informs the patient on how to gather the plants to be made into medicine by themselves.

2 Treatment Stage

After the part of the Faloak plant is collected, the medicinal material will be given to the patient to prepare the remedy themselves. However, some healers prepare the remedy by themselves, drying, cooking it, and then giving it to the patient to consume regularly over a certain period. Some healers use only the bark itself without mixing it with other plants for boiling and consumption. Meanwhile, other healers mix it with several other types of plants but do not disclose which plants are used. The preparation

process varies depending on the technique of each healer. There are healers, as noted by Rambung et al. (2017), who use a combination of Faloak bark with Maunlui leaves (Scurrula purpurrea Dans.), a type of mistletoe that lives symbiotically on Eucalyptus trees, to treat all stages of cancer.

The healers encountered generally have quite similar treatment techniques. The collected Faloak bark is then dried by sun-drying. Once completely dry, the Faloak bark is boiled in water for a certain period until the amount of water decreases and turns a cloudy red color. Typically, the ratio used is one handful of bark to 1–1.5 liters of water. The boiled water is then cooled and consumed regularly over a certain period. The same bark can only be re-boiled 1–2 times. Regular and routine consumption, using the same boiling pattern, is believed to effectively cure several diseases.

The healers mentioned earlier do not have specific prayers or rituals during the process of preparing and consuming the boiled Faloak bark water. However, another healer recommends that during the consumption of this medicine, there should also be prayers according to the patient's religion. These prayers are believed to help accelerate the healing process. This healer also openly advises that after consuming the medicine for a certain period, the patient should undergo a medical check-up at a health facility to assess their condition and verify the effectiveness of the medicine. According to him, this is important so that if the patient has not yet recovered, they can try other treatment methods, such as using different medicines or herbal mixtures, or by increasing the amount of medicinal plants consumed. He also advises the patient to follow general prohibitions that must be observed, such as avoiding meat, alcohol, and other medical restrictions depending on the patient's condition. Meanwhile, other healers do not impose particular prohibitions during the treatment process.

1.3 Post-Treatment Stage

Some healers do not have special rituals after the treatment, but others do have specific rituals performed after finishing the treatment or after the patient has consumed the Faloak bark decoction and no longer shows any physical symptoms caused by the illness. A healer in Naen has a special ritual, which he calls the ritual of "releasing or letting out the heat." This ritual is carried out inside the house, on top of a flat stone symbol (ceper) that serves as a medium of communication with the ancestors. The purpose of this ritual is to release all pain and illness that the patient has suffered so that the patient can achieve full recovery. The healer prays that the patient will no longer suffer from the same illness in the future. The main material used in this ritual is one rooster. The healer, as the ritual leader, slaughters the rooster while reciting prayers to the ancestors beside the flat stone.

Next, he takes all the leftover materials that were used during the process of boiling the medicine. These materials include the remaining firewood used to heat the water, a fan (usually made from woven lontar leaves) to ignite the fire, a pinch of ash from the burning, and the remnants of the boiled decoction. All of these are stored in a special place. Then, after finishing the prayer, these materials are taken to a river and discarded into the flowing water. Once again, he recites a prayer containing a request to the ancestors to leave the house after previously being invited to help in the healing process. The prayer formulation is:

> "...Taton hit ama, ije onle tak mensa oken, jadi hit mebpa oken, apoijen he tek maputul malala, poi tasaija mi noel hen sai neke, nao neke neo taes apunu nok niut apunu. Alaha telen manikin nok oetene. He naikan maput, naikan malal, naikanakini naika mabone (interview, Naen 2019).

This prayer can be translated as: "Inform the old father, this is because I have healed, so our work is done. I am taking these hot (illness) things down to the river to carry these hot things to the 'bad/dirty

sea' and to the place of the evil spirits that are bad, so that only the cold, the cool remains. So that the heat (illness) will no longer come, will not follow him again, and will not depend on him anymore."

In this ritual, the patient who has completed the treatment prepares a chicken to be slaughtered and an amount of money as a token of appreciation for the healer's service. Regarding the amount of money, the healer explains that every material used must be valued for its participation in the healing process, starting from the medicinal plants, firewood, and the healer's own effort. These three are symbolized by three stones of the hearth supporting the hot water pot, which is heated until it boils. The healer explains this symbolism as follows:

> "...Dia punya tungku itu 3 to yang pikul beban periuk untuk rebus air, jadi di masing-masing batu itu di taro uang, seribu, 5 ratus, karena tunggu itu, dia yang pikul beban, karena kita kasi naik periuk untuk tungku itu to, jadi itu tungku itu harus simpan uang, pas rebus obat itu..." (interview, Naen 2019).

A healer in Tunbakun and Eban has a custom/ritual called 'tendes' (pressing money into a tree). The healer performs this ritual on a special tree that has been used for this purpose for generations. According to him, the money given during this ritual is a form of appreciation for his services. The money cannot be used directly but a single bill is taken, a small piece is cut from one edge, then placed into a hole dug a few centimeters deep right beneath the tree. The placement of the cut piece of money is done while invoking the name of the ancestor who passed down the healing technique and saying that:

> "...ini engkau dapat engkau punya, saya dapat saya punya, engkau pakai di situ, saya pakai di sini..."

After this ritual is performed, the healer is allowed to use the money given to him as a token of appreciation for his services. He believes that he and/or his family will suffer adverse consequences if this custom is not followed. These consequences may include the illness of the patient being transferred to him or experienced by his family members. Alternatively, it could manifest as accidents during work, traveling, or other sudden illnesses believed to appear unexpectedly. Although simple, this practice is mandatory. After all these rituals and customs are completed, the healing process comes to an end.

2. The Trilogy of Human Relations, Conservation Practices, and Mitigation of Drought Hazards

A series of medicinal practices—from the process of collecting medicinal plants, consuming Faloak (Sterculia quadrifida R.Br.), to the post-consumption rituals—encompasses a dimension of mitigation against the threat of drought, whether occurring naturally or as a result of human-induced forest fires. This dimension is reflected in the ways various elements within the traditional medicinal system—humans, the environment, and ancestral entities—situate themselves within a complex and unique network of relations. These relationships are embedded within a dynamic that is at times conflicting, yet remain fundamentally reciprocal and mutually respectful. They are characterized by interdependence, complementarity, and mutual care. Such relational types unfold through sustained, direct interactions (Ingold 2000). In the relational approach, Ingold (2000), describes these as mutually constitutive relations. Identity, knowledge, and corporeal substance are not static, but are continually formed and reshaped through ongoing interaction with the land (environment) and with other beings—both human and non-human. Within the medicinal context, the healer, the natural environment where

Faloak grows and becomes a healing agent, and the invisible ancestral presence are all engaged in a process of mutual formation, strengthening, and affirmation of their respective identities, knowledge systems, and ontological substance. Neonbasu (2016) refers to this structure of human-environment-spirit interconnection as a relational trilogy— encompassing the relationship between humans and fellow humans, humans and nature, and humans and the invisible realm.

1.1 Human-to-Human Relations

The healer and the patient are engaged in this relational domain. Within Ingold's (2000) framework, both parties participate in a continual process of identity formation, knowledge reinforcement, and mutual recognition. This dynamic relationship is governed by a set of prohibitions and taboos that must be strictly observed throughout the healing process. Patients are generally forbidden from consuming oily or fatty foods. They are also prohibited from engaging in strenuous physical labour; they are advised not to work in the fields, herd livestock, or carry out routine activities. Instead, they are expected to observe complete rest. Additionally, patients are instructed to refrain from using modern medical treatments or pharmaceutical drugs during the course of traditional healing. Consultation with a medical doctor is only permitted once the traditional treatment process has concluded.

After the healing process concludes, it is customary for patients to offer a form of compensation to the healer. This compensation typically takes the form of basic household necessities such as rice, cooking oil, sugar, coffee, and other daily goods. For post-treatment rituals, it is also common for patients to bring a rooster. In practice, traditional healers never explicitly request money or set a fee for their services. However, they welcome and appreciate any offerings made in gratitude by those who have recovered.

The observance of prohibitions during the treatment process and the practice of giving compensation afterward are closely linked to the affirmation of the healer's identity. Through adherence to taboos and restrictions, patients recognize, affirm, and participate in the ongoing construction of the healer's identity. Such compliance serves as a tangible expression of acknowledgment and respect. Likewise, the receipt of offerings or compensation by the healer constitutes a material validation of their role, knowledge, and the healing process itself—an identity that is continuously reaffirmed by each person they treat. Meanwhile, the person receiving treatment also undergoes a process of identity, knowledge, and substantive formation throughout the healing process. This formation occurs through the healer's instructions regarding taboos, prohibitions, and the acceptance of any offerings provided. These interactions reaffirm the patient's identity as someone in need of healing, and emphasize the necessity of adhering to these prescriptions to ensure the effectiveness and success of the treatment.

1.2 The Relationship Between Humans and Nature

The relationship between the healer and Faloak as a medicinal plant is placed within the framework of the relationship between humans and nature. In Ingold's (2000) framework of thought, the interaction between the healer and the medicinal plant shapes identity, knowledge, and substance. The healer forms their identity as a healer, while Faloak forms itself as a medicinal plant. The healer finds their identity continuously shaped by ancestral heritage passed down to them, by the knowledge they possess to prepare various plants into healing medicines, and by their interaction with the patient. Similarly, Faloak continuously finds itself shaped as a medicinal plant in its relation to the healer and the sick person.

Ingold (2000) calls this the process of 'embodiment of identity' and the process of 'realization of potential,' experienced by both the healer and the medicinal plant (Faloak). Within this process of

embodying identity and realizing potential, an equal relationship is established between the healer and the medicinal plant in the act of healing. The healer and Faloak as a medicinal plant are two equal elements. Both are shaped and recognized for their roles, knowledge, and skills through interactions with the world beyond themselves. This equality gives rise to attitudes of mutual respect and appreciation.

There are three actions in the healing practice pattern as manifestations of this relationship, which have been carried out as part of traditional healing traditions. The first action is evident in the custom of performing rituals. In these rituals, a prayer is recited, and a piece of money is placed under the Faloak tree trunk before the plant part is taken or peeled. This action shows appreciation for the medicinal plant that will be harvested/peeled. A greeting is necessary to address the plant and the ancestors who guard it and have used it before. Permission must also be requested. The greeting and request for permission are contained in the formulation of the prayer. This permission and approval are needed because the harvesting and healing process using the medicinal plant demonstrates the magical ability (*Luli* or sacred/holy) and power (*Keso*—rooster/might) of the ancestral healer to cure. There is a value that must be paid in return for taking the plant, namely the manifestation/demonstration of the ancestral healer's magical ability to heal. The prayer formulation is recorded as follows:

> "... E Usi, fekau alekot, ho pal amleut. Kalo hom maneo tebes lo au ait ko le i au fena he naham he niun nait in naleok hele au anam neo anam nono ak lo ho muneo tebes **luli tebes keso tebes...**" (interview, Tunbakun 2019).

This formulation can be translated as: "Father, I give you what is good, you receive what is bad/not good. If it is truly true (that you have the ability to heal), I take this medicine to give to him/her so that after he/she drinks or eats it, he/she can be healed, so that I can say that you are truly sacred (have magical power) and strong (have strength/potency to heal)."

A form of gratitude is necessary for taking a certain part of the medicinal plant. This is symbolically manifested by placing a banknote. The amount of money is not the measure, as the usual amount placed is a one thousand rupiah bill. The money serves as a symbol of appreciation for the services of the medicinal tree and the ancestors or previous healers. Through this customary act, a reciprocal relationship of respect is established between the healer and the medicinal plant. The medicinal plant's existence and role are acknowledged—its identity and substance.

The second action is evident in the peeling of the Faloak plant. Every healer takes the bark of the Faloak plant by cutting from bottom to top, as shown in Figure 2. This technique is used by all the healers in this study. As seen in Figure 2, this technique reflects a posture of humility or bowing before the tree. The person harvesting the medicine bows while peeling the bark from bottom to top. Symbolically, this technique expresses the healer's respect toward the medicinal tree. They peel while humbling themselves to honor the plant.

The third action is seen in the amount of bark taken from the plant. There are rules about the quantity to be taken, as well as rules about its intended use. This regulation is tied to the belief that if the plant is taken excessively and without a clear or intended purpose, the healer will suffer illness, disaster, or even face death. The amount taken is estimated according to the need for a single treatment and taken only when there is a sick person who requires the medicine. This belief has been accepted and practiced as a hereditary tradition. In the ritual prayer formula at the beginning before the harvest, the purpose and intention of taking the bark are already stated or communicated to the tree and to the ancestors.

What can be seen from these three actions is how the healer positions themselves in relation to or in interaction with the medicinal plant during the process of harvesting the plant. The healer is not a subject who has power over the plant and can act arbitrarily because of that power. They must perform rituals, must humble themselves to respect the medicinal plant, and must obey the rules regarding the amount taken and its intended use. By doing so, the healer acknowledges that without the plant, they cannot cure the sick person. Without the medicinal plant, they are nobody. The medicinal plant, in its interaction or in the healing process, determines the healer's identity, existence, and knowledge as a healer (Ingold, 2000). The same applies to the Faloak plant. As a medicinal plant, the technique of harvesting its bark shapes its identity as a plant with specific healing properties. If it is not taken and used, it has no meaning.

The forms of actions that reflect the relationship between the healer and the medicinal plant carry a conservative value for the survival of the Faloak plant. If the Faloak plant is harvested or used outside of these actions, beyond the established relational patterns, the survival of the plant is threatened. The peeling of the Faloak bark is controlled through these forms of actions. Rituals, prayers, and peeling techniques serve as traditional conservation methods to protect the Faloak bark from indiscriminate harvesting. There must be rituals performed, the quantity and purpose must be appropriate, and care must be taken during the harvesting process. If the bark of the Faloak plant is continuously taken in uncontrolled amounts, combined with the hot and dry climate of Timor Island, the survival of the Faloak plant becomes endangered. This also serves as a mitigation effort against natural disasters caused by drought as well as disasters resulting from human actions, such as the threat of fire that endangers the survival of the Faloak plant.

1.3 The Relationship between Humans and Ancestors; the Unseen Figures

The relationship between humans and ancestors as unseen figures is evident in the bond established between the healer and their ancestors. This relationship ensures the success of the healing process. It is manifested through respect and obedience to ancestral traditions, especially those of the healer's ancestors, and all customary provisions during the healing process that the healer receives as ancestral heritage. These inherited provisions can be found, for example, in the preparation stage of the healing process, such as the ritual of harvesting medicinal plants, harvesting techniques, and preparation of the medicine. Additionally, they can also be found in the healing stage and in various habits and rituals carried out after the healing process.

As one example, there is the custom of **'tendes uang'** (placing money) practiced by healers from Tunbakun. Respect for ancestral traditions is symbolized by the act of placing a small cut portion of the money received as appreciation for their services beneath a special tree. This act is performed while uttering the name of the ancestor or previous healer who passed down the knowledge about medicinal plants. Afterward, the purpose of placing the money is expressed in a sentence, *"ini ambil lu punya, pake lu punya di situ, saya ju pake di sini*".

This act shows respect to the spirits of the previous healers. This respect is symbolically expressed through a small cut portion of the money. The ancestral spirit of the healer must be the first to receive and use this compensation; only afterward may the healer use it. If this custom is not observed, the healer believes that the ancestors will become angry with him, and he will suffer misfortune, illness, or other calamities. This practice must be carried out after the treatment process is complete, before the healer uses the compensation money received from the patient he helped. Such a tradition remains a necessary and well-preserved part of the series of healing actions.

Ancestors have a distinct role in this healing tradition. Their role and contribution are also framed within Ingold's (2000) relational concept. In this concept, the element of ancestors helps shape the identity, knowledge, and substance of the healer in a reciprocal relationship. The healer depends on the ancestors to form his identity. He always communicates with the ancestors through rituals, believing that all his actions are conducted with their consent and permission. This belief shapes his being, affirms his profession and actions, and guarantees the success of the healing. Without this, he would not dare to perform the treatment. All the knowledge and skills they possess are family inheritances maintained through closeness and obedience to ancestral traditions. These two aspects become key determinants of the healing's success, alongside the healer's own factors.

The relationship between humans and the Unseen is also reflected in the understanding of light and darkness as conditions for searching and harvesting Faloak. According to the tradition inherited from the ancestors, the gathering and collection of medicinal plants must be done at specific times, paying attention to the position of the healer's shadow. The healer's shadow must not precede their steps nor cover the medicinal plant to be taken. This rule can be understood based on the *Atoin Meto* community's understanding of light and darkness. In *Atoin Meto* culture, the concept of light and darkness is associated with their understanding of the bright world, called *Pah Meusine*, and the unseen dark world, called *Pah Maisokan*, or the dark/mystical world. The bright world symbolizes the presence of the inaccessible power of *Uis Neno* (*Afinit ma Aneset*), with the ever-burning firelight (*Apinat ma Aklahat*) (Neonbasu 2016).

The power of light is believed by the community to bring success in various activities. The power of the daytime is the blessing and permission from the ancestors. Meanwhile, the unseen dark world, called Pah *Maisokan* or the dark/spiritual world, is believed to be associated with evil spirits or Nitu that can disturb human life (Manafe 2016). With this understanding, the search must not be preceded by the shadow, and the technique of taking the plant must not be covered by the shadow because the shadow brings darkness, and darkness will cover the light. If covered by the power of darkness, the healing process will not run smoothly and will not bring recovery. Thus, the power of light, symbolizing the presence of the unreachable power of Uis Neno (Afinit ma Aneset), with the ever-burning firelight (Apinat ma Aklahat), will repel the power of darkness, which symbolizes the presence of the spiritual world, evil spirits, or Nitu that disturb human life (Neonbasu 2016). Light also represents the permission and approval of the ancestors that will bring healing. The healing system, which begins with the search and collection of medicinal plants, must not be controlled by the dark world, where evil spirits or Nitu dwell and disrupt the healing process. It must be carried out in the light, thus being under the power of *Uis Neno, Apinat ma Aklahat*, and the ancestors' permission, which will bring the light of life and healing in the treatment.

In addition to the concepts of light and darkness, the relationship between humans and the Unseen is also reflected in the understanding of heat and coolness. The ritual practice of releasing heat, commonly performed by healers after the completion of treatment, illustrates this belief. Some healers uphold this practice as an ancestral tradition that must be carried out. They perform a ritual to release the heat from the patient's body, symbolized by the disposal of leftover medicinal concoctions as well as the ashes or remnants of firewood used to boil the decoction. Heat is also released from the healer's own body, symbolized through the words of the ritual prayer for releasing heat.

This practice can be understood through the Atoin Meto community's conception of *menas* (heat) and *manikin* (coolness) (Manafe 2016; Neonbasu 2016; Sawu 2004). For the Atoin Meto people, balance occurs in the in-between state—between *manikin* (cool) and *menas* (hot). This intermediary condition

is referred to as *manikin nok oetene*, which can be interpreted as a state of freshness and warmth. It is understood as the ideal condition for the continuity of life.

In contrast, *menas* or heat is seen as a consequence of all kinds of activity, including the process of healing. *Menas* is also associated with all that is negative, bad, or unsuitable for life, as it may bring illness or even death. This heat must always be cooled and calmed into a state of coolness or *manikin ma oetene*, so that life can proceed. Therefore, the suffering of the patient, which is considered *menas* or heat and harmful to life, must be cooled (healed). The healing process itself—including the healer's activities that generate heat—must also be cooled. All of this must be cooled in order to bring about *manikin ma oetene*— a condition of balance desired by the ancestors and necessary for successful healing.

Adherence to ancestral traditions is regarded as a key factor in ensuring the success of healing practices. The ancestors are believed to desire conditions of light, coolness, and balance in order to bring about healing and sustain life. These states—light, coolness, and balance—represent the ideal environment in which all aspects of life can flourish: the healer may live safely without threats to their well-being, medicinal plants can grow under careful stewardship, and the ancestors continue to be honored. Such conditions are also essential for the sustainability of the Faloak plant. It remains protected and well- maintained while continuing to fulfill its role as a medicinal plant.

CONCLUSION

Relational approach—which emphasizes reciprocal relationships between individuals and the surrounding world, including the land (natural environment), unseen beings, and sociocultural settings—provides a framework in which the continuity of all forms of life can be sustained. The Faloak plant, which grows within the traditional healing system of the Atoin Meto people, exists in a reciprocal relationship with healers, patients, and ancestral spirits. These entities engage in mutual respect, co-formation, interdependence, and collective care. This network of relations cultivates a sociocultural climate that supports the sustainability of the Faloak plant in the context of Timor Island's dry and low-rainfall environment. Such a sociocultural climate functions as a form of mitigation against threats like drought and forest fires. It reflects the Atoin Meto indigenous community's disaster preparedness—both for intentional and unintentional hazards. This study contributes to the advancement of interdisciplinary approaches in disaster studies, promotes recognition of local knowledge as a crucial element in disaster risk management systems, and opens pathways for integrating indigenous wisdom into more inclusive and sustainable formal risk management frameworks.

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CONFLICT OF INTEREST

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Context Matters: Geographical and Gender Dimensions of Child Nutrition in Poor Households

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ABSTRACT

This study aims to understand why some children from poor households do not experience stunting, with a particular focus on the geographical and gender dimensions of child nutrition. The research was conducted in two villages on Timor Island—Lakamola (inland) and Nusakdale (coastal)—representing different geographic contexts within persistent poverty. A qualitative approach was employed through in-depth interviews, participatory observation, and focus group discussions with mothers, health workers, and community leaders. Findings reveal that the determinants of stunting and non-stunting among poor households go beyond food practices—such as the quality, diversity, and quantity of food provided to infants and young children—to include the caregiver's emotional responsiveness and affection during feeding episodes. Several challenges were identified, including difficult topographical conditions, heavy maternal workloads, limited financial support from partners, and seasonal fluctuations in food availability. Despite these, families demonstrated adaptive strategies by leveraging family and social support, including health facility staff, informal labor income, women's savings groups, and home gardens. This study highlights the importance of incorporating both geographic and gendered contexts into child nutrition interventions. Community-based approaches and the empowerment of women are essential to enhancing the resilience of poor households in mitigating the risk of stunting in a sustainable and culturally grounded manner.

Keywords: Rote Island, Stunting, Poverty, Gender Equity.

INTRODUCTION

Stunting can occur across all socioeconomic strata; however, the highest prevalence is found among economically disadvantaged groups (Beal et al., 2018)((Ayuningtyas et al., 2022; Che Omar et al., 2023; Eryando et al., 2022). Poverty is considered a significant contributing factor to stunting among children under five, although it does not function as an isolated determinant. Rather, it interacts with a range of other factors such as nutrition, health, sanitation, environmental conditions, parental behavior, and caregiving practices (Jokhu et al., 2024; Widyaningsih et al., 2022). According to the World Health Organization (WHO), the causes of stunting are multifaceted and complex, yet they can generally be categorized into four primary (basic) causes: household-related factors, infectious

diseases, breastfeeding practices, and complementary feeding practices. These four primary causes are shaped by broader community and societal contexts, which encompass economic and political structures, healthcare systems, education, socio-cultural norms, agricultural and food systems, as well as water and sanitation infrastructure (Che Omar et al., 2023; Flynn et al., 2020; Widyaningsih et al., 2022).

Understanding the relationship between poverty and child stunting within the framework of these contextual causes underscores the multidimensional nature of stunting as a public health issue in a given region (Che Omar et al., 2023; Flynn et al., 2020; Widyaningsih et al., 2022). Poor households are often unable to provide adequate nutrition for their children, which can lead to stunted growth. As a result, the child's development is impeded, ultimately affecting the quality of human resources. Stunting is also influenced by food accessibility and, to a significant extent—approximately 30 percent—by parental behaviors. Various practices during the first 1,000 days of life can increase a child's vulnerability to stunting. For example, many pregnant women lack awareness of stunting and do not believe that it can result from inadequate dietary practices, which leads to the absence of preventive measures from the early stages.

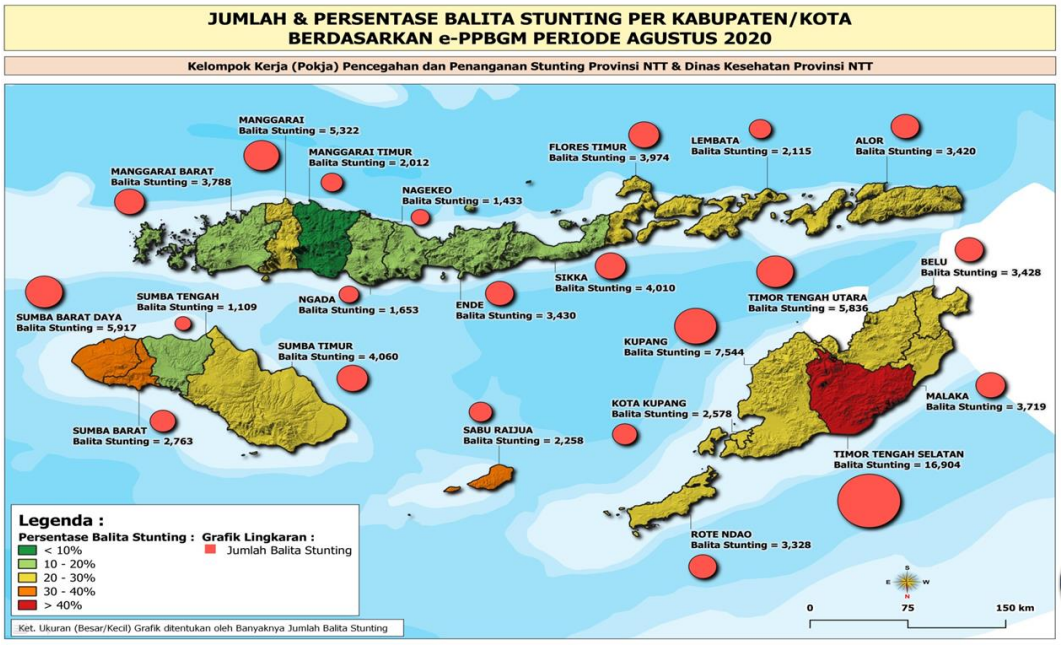

Figure 1. The Distribution of Stunting Prevalence in East Nusa Tenggara Province Based on e-PPGBM Data for the Year 2020

The discourse on stunting should not be confined solely to the domains of health, nutrition, and food security, which have traditionally formed the core focus of stunting interventions in Indonesia—namely, specific and non-specific nutritional interventions (Sanchez-Pimienta et al., 2021; Semba et al., 2008; Simeoni et al., 2017). When poverty is taken as the starting point of analysis, and when situated within the local context of Rote Ndao Regency, stunting must also be recognized as a social phenomenon, shaped by a wide range of complex, interrelated factors (**Figure 1** and **Figure 2**). In fact, there are instances where children suffer from stunting despite not coming from impoverished households, and conversely, many children from poor families do not experience stunting. In Rote Ndao, for example, several villages with high poverty rates exhibit surprisingly low stunting prevalence. In 2020, Lakamola Village in East Rote District reported a poverty rate of 99.16% but a stunting rate of only 11.01%. Similarly, Mbali Lendeiki Village in Ndao Nuse District, with the highest poverty rate in the district at 94.22%, reported a stunting rate of just 7.27%. This pattern is also evident in other villages, such as

Sotimori in Landu Leko District, Nusakdale, and Batulilok in Pantai Baru District, where poverty levels are high but stunting prevalence remains relatively low.

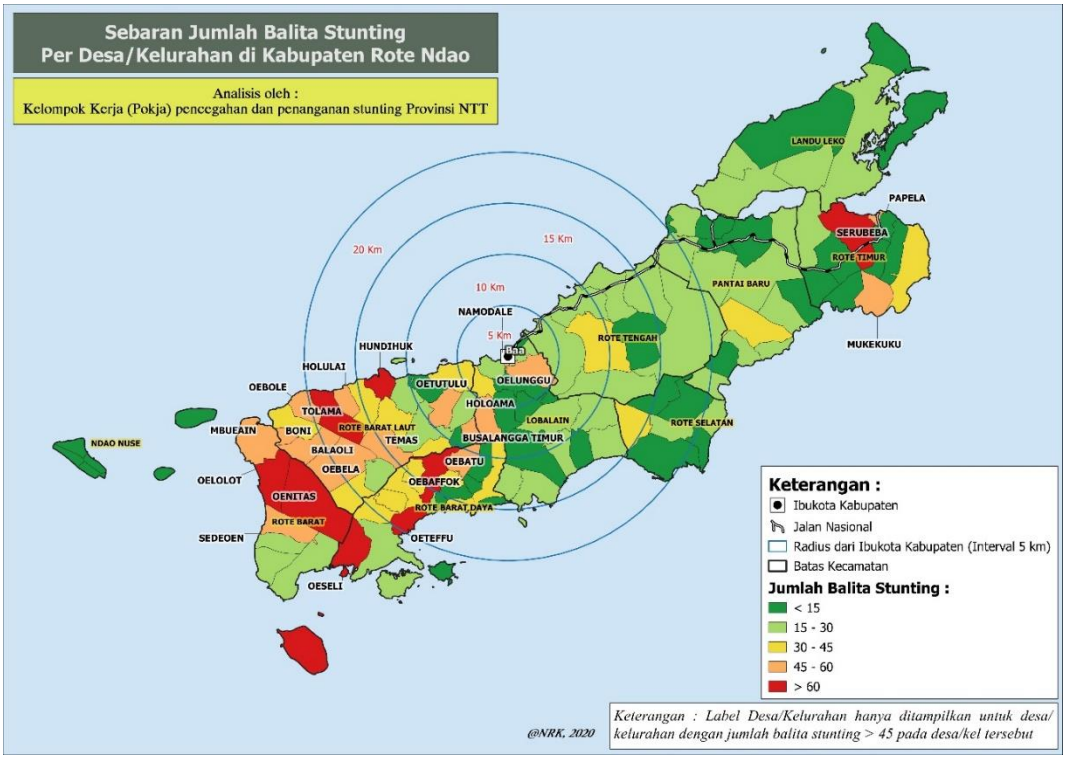

Figure 2. The Distribution of Stunted Children Under Five
by Village/Subdistrict in Rote Ndao Regency, 2021

Interestingly, these poverty and stunting patterns also reflect a geographical dimension, particularly in the distinction between coastal and non-coastal areas. Coastal villages often have higher poverty rates but lower stunting prevalence among children under five, while the reverse is observed in some non-coastal areas.

Another crucial dimension that is often overlooked in the stunting discourse is that of gender equity—particularly issues of women's roles, access, control, and empowerment. To date, most government efforts to address stunting have centered on health, nutrition, and dietary intake. However, women play a central role in early childhood care and development. A growing body of research has shown that stunting is closely linked to women's roles, both within families and in the broader community. Their strategic involvement significantly influences stunting outcomes (Metwally et al., 2021; Otekunrin et al., 2024; Ramlan et al., 2025; Sanchez-Pimienta et al., 2021; Semba et al., 2008; Yuhan et al., 2025).

Based on this situation, it is necessary to investigate and understand the underlying factors that explain why children from impoverished households do not experience stunting. This phenomenon should be analyzed within two interrelated contexts: (1) the context of poor households segmented by topographical location—namely, coastal and non-coastal areas—and (2) the context of women's empowerment. It is essential to explore how these two contextual dimensions interact and contribute to stunting outcomes in Rote Ndao Regency.

Understanding the role of geographic segmentation allows for an examination of how local ecological and livelihood systems—such as access to marine resources, food availability, and community resilience—might influence child nutrition and health despite economic hardship. Simultaneously,

examining women's empowerment—particularly their access to knowledge, healthcare, decision-making power, and community participation—sheds light on how maternal agency and caregiving practices can mitigate the risks of stunting. Together, these two contexts provide a more nuanced and multidimensional explanation of stunting patterns in Rote Ndao, moving beyond income-based determinants to incorporate sociocultural, gendered, and spatial dynamics.

METHODS

1. Methodological design

The study design was exploratory, descriptive and qualitative with a phenomenological approach.

2. Study setting and population

This study was conducted in communities characterized by high poverty rates but low stunting prevalence, specifically Lakamola Village in East Rote District and Nusakdale Village in Pantai Baru District, both of which exhibit high average poverty levels yet low rates of stunting. The study was carried out over a period of four months, from August 2021 to November 2021. The Informan for the study was purposively recruited from the same districts.

2. Data collection procedure

2.1 In-depth interviews

Data collection was conducted through in-depth interviews with three groups of key informants: mothers and fathers of children aged 0–23 months, healthcare workers (at Posyandu and Puskesmas), and local village government officials. Four administrative areas were purposively selected for this study. It was assumed that infant and young child feeding (IYCF) practices might vary across regions due to the district's morphology (variability in landscape). To capture the full range of IYCF practices and variations in stunting prevalence and poverty levels, two villages from East Rote District and two villages from Pantai Baru District were purposively selected.

In each village, participants were selected with the assistance of field coordinators at the district level (Rote District Development Planning Agency—Bappeda), based on official lists of poor households with stunted children provided by relevant government agencies and subsequently recommended by the local village authorities from the lower end of those lists.

The criteria for selecting key informants were as follows: (1) parents with children aged between 0 and 23 months; (2) willingness to participate in the study; (3) knowledge and personal experience related to infant and young child feeding practices; and (4) personal experience related to stunting management or maternal and child health. Recruitment of additional participants was discontinued once data saturation was reached and no new information emerged during interviews in each village.

2.2 Focus group discussion

One focus group discussion (FGD) was conducted in each of the two selected districts—East Rote and Pantai Baru. Each group consisted of seven to eight participants and was held in informal communal settings, such as a public square or under a tree, to ensure participant comfort and familiarity. The primary objective of the FGDs was to explore the socio-cultural practices influencing child feeding and dietary behaviors.

At the beginning of each session, the researcher welcomed the participants, invited them to introduce themselves, explained the purpose of the discussion, and emphasized the voluntary nature of participation. Participants were informed that the sessions would be audio-recorded and assured of the confidentiality of their responses.

The discussions were guided by a structured discussion guide developed to explore specific thematic areas related to infant and young child feeding. These themes included: definitions of a healthy diet; breastfeeding and exclusive breastfeeding practices; typical daily foods given to children; foods considered essential for child growth; feeding practices during the first year of life; caregiving and feeding practices during illness; household food distribution norms (e.g., who is served first); food taboos and restrictions during pregnancy and breastfeeding; foods recommended during pregnancy and lactation for maternal and child health; foods deemed inappropriate for young children; and foods avoided during illness.

Two research assistants— a male and a female local residents of Rote Island appointed by Bappeda—were recruited to assist with data collection. Their role was to observe the sessions, take detailed notes, and support the documentation process. The researcher moderated the discussions and encouraged open and candid participation, fostering an environment where all participants could share their experiences and perspectives freely.

2.3 Limited focus of participatory observation and documentation

In this study, a limited focus of participatory observation was employed as a complementary method to gain a deeper, contextual understanding of daily practices, behaviors, and interactions related to child care, feeding, and maternal roles within poor households in both coastal and non-coastal areas. This method allowed the researcher to be immersed in the natural settings of the participants, observing routines and practices as they occurred in everyday life—such as food preparation, breastfeeding, complementary feeding, and household decision-making.

The researcher actively engaged with the community by attending Posyandu sessions, informal household gatherings, and local events. Through this approach, the researcher was able to observe unspoken norms, cultural practices, and gender dynamics that may not be fully captured through interviews or focus group discussions alone. Field notes were systematically recorded, focusing on non-verbal cues, environmental contexts, intra-household interactions, and community responses to child nutrition and maternal empowerment. This approach helped triangulate findings from other data sources and provided a nuanced understanding of how socio-cultural, economic, and gender-related factors intersect in shaping stunting outcomes in poor communities.

3. Data analysis

This study employed the interactive model of data analysis as developed by Miles and Huberman (1994)(Alase, 2017; Ali et al., 2024), which is well-suited for qualitative research involving complex social phenomena in our research topic. Raw data derived from interviews, observations, FGD, and observations were reduced into meaningful categories or themes. Narrative summaries then adopted to identify relationships, contradictions, and gaps across locations (coastal vs non-coastal). An interpretative phase was introduced as the final step to draw conclusions. These conclusions were continuously tested against the raw data through the triangulation technique.

RESULTS AND DISCUSSION

1. Poor households with non-stunting children

In East Rote and Pantai Baru sub-districts, cases of stunted children are still found. In East Rote Sub-district, there are 258 stunted children across 11 villages, while the number of non-stunted (normal) children totals 1,057. In the two study villages, Lakamola and Panggodua, the number of stunted children is 9 in Lakamola and 14 in Panggodua. For non-stunted children, Lakamola recorded 86, while Panggodua recorded 99.

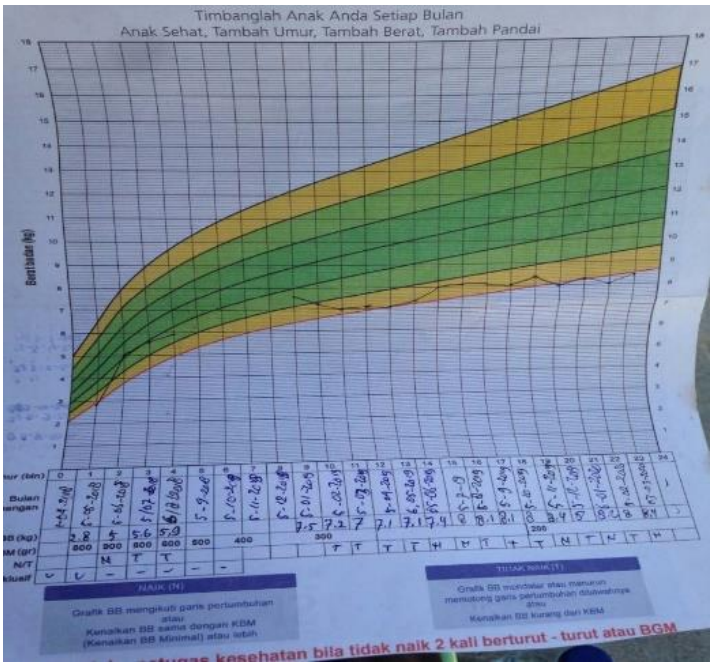

Figure 3. Data on children's height and weight measurements during visits to the integrated health post (posyandu) as recorded in the maternal and child health (MCH) pink book.

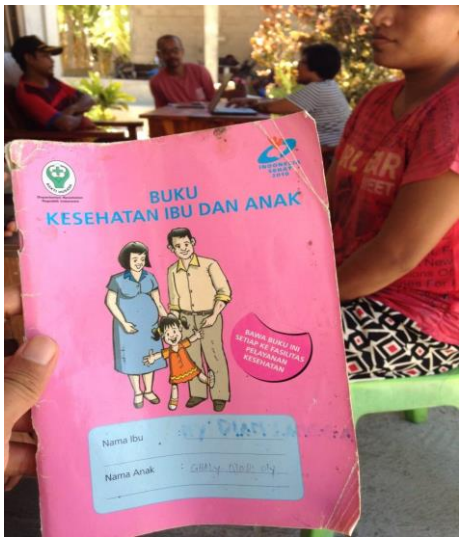

Figure 4. The Maternal and Child Health (MCH) Pink Book used to monitor women's and children's visits to the integrated health post (Posyandu).

Interestingly, data show that a significant proportion of the non-stunted children in these two villages come from economically disadvantaged or poor households. Previous studies on stunting have

often associated it with low parental education, particularly suggesting that parents with higher education levels are better able to access and comprehend information on child nutrition, including stunting prevention. Women with higher educational attainment are generally expected to more readily accept external information compared to those with lower education levels. However, this association is not consistently observed in the two villages in East Rote. In fact, women with lower levels of formal education do not always have stunted children, and conversely, women with higher education do not necessarily have non-stunted children.

This finding aligns with earlier research (Ni'mah & Muniroh, 2016) indicating that education level is not the sole determinant of child stunting. In both study villages, there are instances where children from economically stable families fall into the stunted category, while children from poor households are found to be non-stunted. This suggests the need to explore other contributing factors beyond economic and educational variables in understanding the persistence and variation of child stunting.

Some mothers with higher levels of education may work outside the home, limiting the time they can spend caring for their young children. In both study villages, it was observed that in some poor households, mothers who worked as farmers or village staff entrusted their children to their own parents during working hours. In such cases, the grandparents provided consistent supervision over the child's feeding and drinking practices.

Findings from interviews and field observations revealed that most children were breastfed exclusively for the first six months. After that period, complementary feeding typically included porridge mixed with vegetables grown in household gardens and fish sourced either from local markets or mobile vendors.

Lakamola and Panggodua villages, it was also found that some poor families lived with or near extended family, particularly grandparents, who played a vital caregiving role. The presence of these extended caregivers contributed to more consistent child supervision and was associated with protection against stunting. Another notable observation in both villages was the absence of food kiosks or shops selling processed snacks near residential areas. This might help explain the limited consumption of packaged or unhealthy snacks among toddlers in the area.

For daily water use—such as drinking, bathing, and washing—most households relied on wells or shared community water tanks. These environmental and caregiving contexts offer important insights into how non-stunting outcomes can emerge even in economically disadvantaged settings, suggesting that family support structures and local food practices can play a critical role in child nutrition and health.

Stunting cases in Pantai Baru Subdistrict amounted to 270 children, based on the collected data. In the study sites of Nusakdale and Batulilok villages, the number of stunted children was relatively low, with 5 stunted children in Nusakdale and 21 in Batulilok. Meanwhile, the number of non-stunted children reached 93 in Nusakdale and 41 in Batulilok. The study particularly focused on non-stunted children from economically disadvantaged households.

Observations and information gathered from the field indicated that most of these children were cared for directly by their parents. Even when the parents worked, they typically returned home to feed their children on time, and in some cases, they brought their children with them to the fields or gardens. As both villages are located in coastal areas, marine resources were utilized effectively, especially during pregnancy and postnatal periods. Importantly, both villages have access to freshwater sources, enabling the cultivation of vegetables, which supports adequate nutritional intake.

Furthermore, the study revealed discrepancies between non-stunting and variables such as father's education, father's occupation, maternal employment, number of children, birth spacing, and household food expenditure. Most fathers had relatively low levels of formal education, typically below high school. This finding aligns with prior research suggesting no significant correlation between paternal education and stunting incidence in children aged 1–2 years and 6–36 months. This may be attributed to fathers not playing a dominant role in household nutritional decisions. In contrast, cultural norms in Indonesia typically place the responsibility of childcare on mothers, thereby amplifying their influence on child health outcomes.

Cultural observations from both study districts suggest that, implicitly, household management in Rote is largely assigned to women, while men are expected to fulfill the role of economic provider. Despite the heavy workload observed among women, particularly in poor households, mothers demonstrated strong commitment and capacity to care for their children from pregnancy through childbirth, which appears to contribute significantly to the absence of stunting among their children.

Likewise, the occupation of the father or husband cannot be considered a definitive indicator of whether a child from a poor household will experience stunting. It is commonly assumed that fathers without stable employment contribute to low household purchasing power, which in turn may limit access to adequate food in both quantity and quality—potentially leading to nutritional deficiencies and suboptimal child development outcomes. However, field observations from this study reveal a different reality.

In the research locations, despite unstable income sources, many households successfully meet their nutritional needs by utilizing available natural resources. Home gardens are commonly used for growing vegetables, and access to marine resources is relatively easy due to the proximity to coastal areas. These practices help ensure a steady supply of nutritious food regardless of household income. Therefore, local food availability—particularly from home-based and natural sources—appears to mitigate the potential impact of economic constraints on child nutrition, highlighting the importance of contextual livelihood strategies in shaping child health outcomes.

In relation to breastfeeding practices, findings from the research sites indicate a consistent pattern among poor households whose children were not stunted. Most mothers reported providing exclusive breastfeeding for a maximum of up to six months, after which it was supplemented with complementary foods such as rice porridge. In cases where exclusive breastfeeding was not sustained, the primary reasons included limited maternal knowledge about its importance—particularly its role in enhancing infants' immunity against infections—and insufficient family support for the practice. As noted by Gunawan et al. (2016), exclusive breastfeeding is not the sole determining factor in the occurrence of stunting. Stunting among young children is influenced not only by the adequacy of breast milk or complementary feeding, but also by the quality of those foods.

In this study, both mothers of stunted and non-stunted children generally had low educational attainment. However, a lower education level does not necessarily correlate with having a stunted child, nor does higher education and financial stability guarantee a non-stunted child. Women with basic or secondary education may have received nutrition-related knowledge through health education at local Posyandu (integrated health posts), enabling them to apply this knowledge in caregiving, particularly in feeding practices.

Dietary diversity in non-stunted children from low-income families was generally low. Most children consumed homemade rice porridge with added salt. Instant porridge products (e.g., Sun, Milna)

were reportedly not well accepted by these children. Across the two subdistricts with differing characteristics, a common dietary pattern emerged: children consumed vegetables and fish as primary nutrient sources. In Batulilok and Nusakdale, which are coastal villages, fish was easily accessible, and the presence of freshwater sources supported vegetable gardening. In Lakamola and Pengodua, households mostly relied on leafy greens grown in home gardens or bought from local markets. Food diversity in these areas was also shaped by preference and habitual dietary practices.

Improved health promotion and awareness campaigns are necessary to shift community behavior and encourage attention to children's growth. Dietary diversity is influenced by internal factors—such as income, preferences, culture, religion, and nutrition knowledge—and external factors including agroecological conditions, food production and availability, distribution systems, and food marketing.

2. Shifts in Household Practices and Their Contribution to Stunting Prevention and Reduction

Findings indicate that women are sometimes positively influenced by significant female figures, such as grandmothers and mothers-in-law, in their breastfeeding practices. According to information obtained during interviews, most women reportedly breastfed their children beyond six months. Factors such as the surrounding environment, time availability, misconceptions, and prevailing beliefs were identified as key determinants of exclusive breastfeeding practices. These social "habits" or customary practices have a substantial impact on the quality of care that women provide to their children.

There is a collective awareness within Rote communities that the entire family bears responsibility for supporting a child's growth and development. The first year of a child's life is widely recognized by women as a particularly challenging period. Mothers typically introduce solid foods to their infants at around six months of age or slightly later, such as at seven or nine months. Regarding complementary feeding in the first year, most women reported that rice porridge was the initial food introduced, followed by other family foods. Their responses corroborated the types of foods regularly consumed in both sub-districts under study.

However, interviews also revealed that many of these diets were significantly lacking in animal protein and fruit sources. Limited financial resources were cited as a major barrier to the regular consumption of animal-based proteins. Some women mentioned the use of a locally available brown seaweed, referred to as *latu* in the local language, which they collected from the sea and prepared as a vegetable or mixed with rice porridge to feed their young children. This practice, passed down across generations, demonstrates that women possess inherited knowledge about appropriate infant feeding practices. Meat consumption was typically reserved for special occasions, such as communal celebrations or ceremonial events, as described by several interviewees.

The interviews also revealed that, in addition to receiving advice from grandmothers on infant and child care, many women regularly entrusted the care of their infants and young children to grandmothers. Two primary reasons emerged for this prolonged caregiving arrangement (lasting more than three months). First, some women were single mothers. Second, economic pressures and work obligations required parents—particularly mothers—to spend extended periods outside the home.

Furthermore, several women reported avoiding certain foods during pregnancy for a range of reasons, including safeguarding the pregnancy outcome, ensuring a smoother delivery process, preventing undesirable physical traits in the newborn, and respecting cultural taboos or elders' directives. For example, pregnant women in the villages of Nusakdale and Batulilok reported abstaining from a

particular type of seaweed (locally called *latu*) during the third trimester. In contrast, pregnant women in Lakamola and Pengodua were advised to limit their intake of moringa leaves (*marungga*) to avoid the risk of umbilical cord shrinkage, based on local beliefs.

These practices highlight the intersection between traditional knowledge, intergenerational caregiving roles, and culturally embedded dietary restrictions during pregnancy, which may influence maternal and child health outcomes.

3. Gender equity: issues of women's roles, access, control, and empowerment

3.1 Women's Roles: The Double Burden of Reproductive and Productive Labor

Field findings in Rote District reveal that women, particularly breastfeeding mothers, shoulder a double burden of labor. In addition to their reproductive roles—caring for children and breastfeeding—they are actively involved in the household economy, especially in the production of *gula air* (palm sugar syrup) (**Figure 5**). This productive work is non-negotiable, even for postpartum or nursing women, as it constitutes a primary source of household income.

Figure 5. A woman participating in the process of cooking *gula air* (palm sugar syrup) in her home kitchen.

As a result, these women often delegate childcare responsibilities to their mothers-in-law or grandmothers for extended periods. This arrangement, while normalized within the community, raises concerns about the quality of maternal bonding and caregiving, particularly during the critical first 1,000 days of a child's life.

3.2 Access: Limited Access to Time, Information, and Services

Women engaged in palm sugar production face significant barriers in accessing maternal and child health information, including knowledge on exclusive breastfeeding, complementary feeding, and age-appropriate childcare practices. The demanding nature of their daily work limits their participation in public health education sessions such as *Posyandu* (integrated health services post).

Nevertheless, women's involvement in informal savings and loans cooperatives among palm sugar workers indicates efforts to broaden economic access. Through these community-based financial mechanisms, women can obtain funds to purchase food and meet other household needs. However, these cooperatives have not yet evolved to support access to nutrition education or parenting skills training in a systematic way.

3.3 Control: Limited Authority in Decision-Making

Despite their central role in both caregiving and economic production, women in Rote often lack decision-making power over critical matters concerning their health and their children's well-being. Decisions such as the place of childbirth, dietary choices during pregnancy, or appropriate complementary feeding practices are frequently dictated by husbands or extended family members, especially in-laws.

The situation is further compounded when men leave the village to seek work as laborers in Kupang City. In their absence, women are expected to manage the household and generate income, yet they continue to have limited control over the resources they help produce. This dynamic reflects the entrenched structural vulnerability of women within the household economy.

3.4 Empowerment: Collective Agency and Adaptive Strategies

Despite these challenges, women in Rote exhibit forms of resilience and adaptive agency that represent key entry points for empowerment. Participation in community-based savings cooperatives illustrates a form of collective solidarity, enabling women to manage household needs despite financial hardship.

The intergenerational caregiving system—where grandmothers assume childcare duties—can be viewed as a cultural coping mechanism, though it warrants scrutiny in terms of caregiving quality and gendered labor distribution. Additionally, women's knowledge of local foods such as *latu* (brown seaweed), which is commonly prepared for children, reflects the presence of indigenous nutritional knowledge passed down through generations.

To support and sustain women's empowerment, it is necessary to implement capacity-building programs that address both productive and reproductive spheres. These may include diversifying women's income-generating activities, increasing access to nutrition and parenting education, and promoting gender-transformative interventions that encourage male involvement in childcare. Ultimately, creating space for shared responsibility in parenting and domestic labor is critical to achieving gender equity and improving child nutrition outcomes.

CONCLUSIONS

This study finds that addressing stunting and non-stunting among children in low-income households requires attention beyond food practices—namely, the quality, diversity, and quantity of foods offered to infants and young children. Equally critical is the caregiver's responsiveness and emotional engagement during feeding episodes. The study also reveals that multiple interrelated challenges—such as difficult natural topography, the heavy workload borne by women, limited financial support from partners, and seasonal fluctuations in food availability—are perceived to contribute to the persistence of stunting among children in poor households.

Family and social support, including assistance from health facility staff, informal income through casual labor, women's savings and loan groups, and home gardening practices, have been employed to mitigate these challenges.

The factors influencing child nutrition are multifaceted. Therefore, intervention strategies aimed at improving child nutrition must recognize the socially embedded nature of infant and young child feeding and should address broader economic constraints and social-environmental opportunities. These strategies must complement, rather than rely solely on, efforts to improve caregiver knowledge.

Moreover, men need to adopt a new perspective in building relational dynamics with women in child-rearing—one grounded in egalitarian values, mutual respect, and gender equity.

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CONFLICT OF INTEREST

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Faculty of Pharmacy Sanata Dharma University ICSNPH
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Interdisciplinary Learning for Pharmacy Students as a Means of Strengthening Interprofessional Collaboration in the Future

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ABSTRACT

In the era of globalization and rapid development of health sciences, interprofessional collaboration has become key in providing quality, comprehensive, and patient-centered healthcare services. However, pharmacy students in Indonesia still face limitations in understanding the roles of other health professions due to minimal interprofessional learning opportunities during their education. Therefore, interdisciplinary learning becomes a strategic solution to enhance pharmacy students' competence in working with other healthcare professionals. This paper highlights the importance of interdisciplinary learning in pharmacy education to strengthen interprofessional collaboration. Interdisciplinary learning enables pharmacy students to think critically and creatively, as well as develop communication and collaboration skills. Several strategies proposed in this paper include guest lectures, interdisciplinary seminars, interprofessional panel discussions, case simulations, field visits, and interprofessional collaborative projects. Analysis shows that despite challenges in implementing interdisciplinary learning, such as differences in professional cultures and resource limitations, the resulting opportunities are significant. Students involved in interdisciplinary learning have a better understanding of other professional roles, improved critical thinking abilities, and are better prepared to meet future healthcare service demands. Furthermore, with structured interdisciplinary learning designs, pharmacy students can develop broader academic and professional competencies, thereby contributing to the improvement of overall healthcare service quality.

Keywords: Interdisciplinary learning; pharmacy students; interprofessional collaboration; health education; learning strategies.

INTRODUCTION

In the era of globalization and advancements in science and technology in the health sector, the demands placed on healthcare professionals have become increasingly complex. The public expects healthcare services that are comprehensive, integrated, and patient-centered. To address these challenges, interprofessional collaboration among healthcare professionals has become a key strategy. Effective collaboration requires a solid understanding of each profession's roles and competencies, as well as the ability to work effectively within a team (World Health Organization [WHO], 2010).

Pharmacy, as one of the health professions, plays a significant role in delivering high-quality pharmaceutical care. Pharmacists are not only responsible for the provision and management of medications, but also for delivering drug information, providing counseling, and monitoring the effectiveness and safety of medication use. In order to fulfill these roles effectively, pharmacists must collaborate with other healthcare professionals such as physicians, nurses, nutritionists, and physiotherapists. Pharmacists are expected to support the implementation of Law No. 17 of 2023 by:

1. Ensuring the availability of medicines and vaccines in healthcare services;
2. Optimizing the management of pharmaceutical supplies, medical devices, and consumable medical materials according to standards;
3. Utilizing digital technology and information systems;
4. Enhancing their role in health services, particularly in promotive and preventive efforts; and
5. Supporting the use of domestic medicines and medical devices. (Ministry of Health of the Republic of Indonesia, 2022; Indonesian Pharmacists Association, 2023)

However, interprofessional collaboration in Indonesia still faces several challenges. One of the major issues is the lack of understanding of the roles and responsibilities of each profession. Pharmacy students, during their education, are often taught pharmaceutical sciences in isolation, with limited or no opportunities to interact and learn alongside students from other healthcare disciplines. This lack of interprofessional exposure can result in poor understanding of the roles and competencies of other professions, ultimately hindering effective collaboration.

According to the Standards for the Pharmacist Profession as stated in the Decree of the Minister of Health No. HK 01.07/MENKES/13/2023, a pharmacist's competencies are built on a foundation of professionalism, self-awareness, and continuous development, supported by effective communication skills, and grounded in scientific knowledge of pharmaceutical sciences, biomedical sciences, humanities, and public health. These competencies also include the skills required to manage pharmaceutical practice optimally. (Indonesian Pharmacists Association, 2023; Noor et al., 2020; Ministry of Health, 2023). This decree explicitly highlights that pharmacist competencies are developed through collaboration with other health professions.

Therefore, efforts must be made to strengthen interprofessional collaboration from an early stage, particularly through interprofessional education (IPE). IPE is a learning process that involves students from various health professions learning with, from, and about each other. Through IPE, students can develop a clearer understanding of each profession's roles and competencies and build the skills necessary to collaborate effectively as members of healthcare teams. (Interprofessional Education Collaborative Expert Panel, 2016)

1. Interdisciplinary learning

Interdisciplinary learning is an educational approach that integrates knowledge and methods from multiple disciplines to understand complex phenomena or problems. In the context of health education, interdisciplinary learning refers to a learning process that involves students from various health professions learning together and interacting with one another (Hall & Weaver, 2001).

This approach differs from multidisciplinary learning, which merely involves collecting information from different disciplines without deep integration. In interdisciplinary learning, students are encouraged to think critically and creatively, connecting knowledge across disciplines to solve complex

problems. It also emphasizes the development of collaboration and communication skills, as students must work together with peers from other fields to achieve common learning objectives (Klein, 2010).

Interdisciplinary learning is characterized by several key features:

1 **Integrative**: It combines knowledge from different disciplines to understand complex issues or phenomena.
2. **Collaborative**: It engages students from various health professions in shared learning experiences and interactions.
3. **Contextual**: It links learning materials to real-world contexts, enabling students to see the relevance of their learning to actual practice.
4. **Reflective**: It encourages students to reflect on their learning experiences, thereby fostering a deeper understanding of the subject matter. (Spelt et al., 2009)

Interdisciplinary learning is particularly important for pharmacy students for several reasons:

1. **The Complexity of Health Problems**: Health issues are becoming increasingly complex and multidimensional. Patients may face not only physical ailments but also psychological, social, and economic challenges. To provide comprehensive pharmaceutical care, pharmacists need to understand all these aspects.
2. **The Expanding Role of Pharmacists**: The pharmacist's role is no longer limited to compounding and dispensing medications. Pharmacists now provide drug information, conduct patient counseling, and monitor the effectiveness and safety of medication use. To perform these roles effectively, they require knowledge and skills from various disciplines.
3. **The Need for Collaboration**: Healthcare is delivered by multidisciplinary teams. Pharmacists must collaborate with other healthcare professionals such as physicians, nurses, nutritionists, and psychologists to provide the best possible care. Effective collaboration depends on a clear understanding of each profession's roles and competencies.
4. **Public Expectations**: Society is becoming more critical and well-informed. People now expect high-quality, patient-centered healthcare services. To meet these expectations, pharmacists must achieve high levels of competence—competence that can be fostered through interdisciplinary learning. (Remington et al., 2020; Hepler & Strand, 1990)

LEARNING STRATEGIES

1. Guest Lectures and Interdisciplinary Seminars

This can be implemented by inviting experts from various disciplines—such as physicians, nurses, nutritionists, psychologists, and health law professionals—to deliver lectures or seminars for pharmacy students. The topics may cover complex health issues, such as managing patients with chronic diseases, medication use in geriatric patients, or ethical issues in pharmaceutical services. One example is the topic of *Medication Use in Patients with Comorbid Conditions*. Students from different health disciplines can collaborate to understand how various coexisting medical conditions (e.g., diabetes, hypertension, depression) affect medication selection and management. This topic aims to enhance understanding of drug interactions, side effects, and the importance of a holistic approach to treating patients with comorbidities. (Harden, 1998)

This aligns with **Social Learning Theory**, which emphasizes the importance of learning from others, including subject matter experts. Guest lectures and interdisciplinary seminars thus offer

pharmacy students the opportunity to learn directly from professionals across different fields, expanding their perspectives and understanding of complex health issues.

Research has identified several benefits:

1. Enhancing students' understanding of the roles and competencies of other professions,
2. Broadening students' perspectives on health problems from multiple viewpoints,
3. Strengthening students' critical and analytical thinking skills,
4. Facilitating professional networking with experts from various disciplines.

2. Interprofessional Panel Discussions

This activity involves panel discussions with students from various disciplines such as pharmacy, medicine, nursing, nutrition, and psychology. The discussions may focus on complex patient cases or current issues in healthcare. An example topic is *Ethics and Professionalism in Healthcare*. Students can engage in discussions and debates on complex ethical issues such as informed consent, patient confidentiality, conflicts of interest, and end-of-life decision-making. This fosters awareness of the importance of ethics and professionalism and helps students develop the ability to deal with ethical dilemmas. (Buring et al., 2009)

This approach is consistent with **Constructivist Theory**, which posits that knowledge is constructed through social interaction. These activities allow students to interact and exchange ideas with peers from other disciplines, deepening their understanding of complex healthcare issues. Reported benefits include:

1. Improving students' communication and collaboration skills with other health professionals,
2. Enhancing students' critical thinking and collaborative problem-solving abilities,
3. Increasing students' understanding of the values and perspectives of other professions.

3. Interprofessional Case Simulations

In this activity, pharmacy students collaborate with students from other disciplines to solve complex patient cases in a simulated setting. Simulations may involve standardized patients or detailed case studies. An example is the topic of *Pain Management*. Students learn together about various aspects of pain management, including pain assessment, pharmacological and non-pharmacological approaches, and the roles of each profession in delivering comprehensive care. This activity helps students improve their skills in providing patient-centered and multidisciplinary pain management. (Shrader et al., 2016)

This aligns with **Experiential Learning Theory**, which asserts that the most effective learning occurs through direct experience. Interprofessional simulations give students firsthand experience in collaborating with other health professionals in managing complex patients.

Research highlights the following benefits:

1. Enhancing students' ability to apply knowledge and skills in real-life contexts,
2. Strengthening students' ability to work in interprofessional teams,
3. Improving students' clinical decision-making skills.

4. Interprofessional Field Visits

This activity involves pharmacy students visiting hospitals, primary care clinics, or other healthcare facilities together with students from other disciplines. The purpose is to give students the

opportunity to observe how professionals from different health fields collaborate to provide patient care. (Kolb, 1984; Frenk et al., 2010). This is in line with **Contextual Learning Theory**, which suggests that the most effective learning is anchored in real-world contexts. Interprofessional field visits allow students to witness how classroom theories are implemented in clinical practice.

Benefits include:

1. Enhancing students' understanding of the roles and responsibilities of each profession within the healthcare team,
2. Improving students' understanding of healthcare service delivery workflows,
3. Strengthening students' ability to interact with patients and healthcare providers. Studies have shown that these visits can significantly increase students' comprehension of team-based healthcare delivery and enhance their interprofessional communication skills.

5. Interprofessional Collaborative Projects

This involves pharmacy students working with students from other disciplines on public health-related projects, such as health campaigns, drug development, or research on medication safety and efficacy. An example project is *Patient Counseling and Education*. Students from different disciplines collaborate to learn about effective communication, empathy, and counseling techniques, enabling them to deliver clear and accurate information to patients about medications, medical conditions, and necessary lifestyle changes. The goal is to improve students' ability to provide effective patient counseling, which contributes to better treatment adherence and health outcomes. (Barr et al., 2005; Oandasan & Reeves, 2005)

This strategy aligns with **Project-Based Learning Theory**, which holds that the most effective learning occurs through real-world projects relevant to students' lives.

Benefits of interprofessional collaborative projects include:

1. Improving students' ability to work within interprofessional teams,
2. Enhancing students' skills in planning, executing, and evaluating projects,
3. Increasing students' understanding of public health challenges.

6. Opportunities in Interdisciplinary Learning:

6.1 Challenges

a. Differences in professional culture and language

Each discipline has its own professional culture and language, which can pose barriers to communication and collaboration among students and healthcare professionals from diverse backgrounds. These differences may affect the interpretation of information, decision-making, and overall team interaction (Hall, 2005).

b. Scheduling and facilities

Coordinating schedules that accommodate participants from various study programs and institutions can be a significant challenge. Likewise, the availability of adequate facilities, space, and resources must also be considered (Gilbert, 2005).

c. Assessment and evaluation

Developing comprehensive assessment methods to evaluate students' interprofessional competencies—including communication, collaboration, and understanding of each profession's role—is a considerable challenge. Assessments should address both individual and team aspects and take into account the contributions from various disciplines (Freeth et al., 2005).

d. Resistance to change

Some educators and students may feel comfortable with traditional learning methods and resist the changes introduced by interdisciplinary learning. A lack of understanding of the benefits of interdisciplinary education can also be a barrier (Hammick et al., 2007).

e. Limited resource

Interdisciplinary learning often requires greater resources than traditional education, including competent instructors, relevant learning materials, and supporting technologies (Hammick et al., 2007).

6.2 Opportunities

a. Enhanced graduate competencies

Interdisciplinary learning offers students the opportunity to develop competencies aligned with the demands of the professional world, such as communication, collaboration, problem-solving, and decision-making skills in interprofessional team contexts (Reeves et al., 2013).

b. Improved healthcare quality

Through interdisciplinary learning, students learn to work collaboratively in interprofessional teams, which can improve overall healthcare quality, including more accurate diagnoses, more comprehensive care plans, and better patient outcomes (Institute of Medicine, 2015).

c. Continuous professional development

Interdisciplinary learning can serve as a foundation for continuous professional development among healthcare workers, enabling them to enhance their interprofessional competencies throughout their careers (Thistlethwaite, 2012).

d. Innovation in health education

Interdisciplinary learning promotes innovation in health education, including the development of integrated curricula, active learning methods, and interprofessional competency assessment (Frenk et al., 2010).

e. Networking and collaboration

Interdisciplinary learning provides opportunities for students and healthcare professionals to build networks and collaborate with peers from various disciplines, which can be valuable for career development and research (Barr, 2005).

6.3 Interdisciplinary Learning Design for Pharmacy Students

To enable interdisciplinary learning for pharmacy students—who will eventually collaborate with students from other health disciplines—it is essential to design learning activities that facilitate such collaboration. The proposed design can be illustrated in Table 1. and Table 2.

Table 1: Learning Activities Design

	Semester							
	1	2	3	4	5	6	7	8
Guest Lectures								
Interdisciplinary Seminars								
Interprofessional Panel Discussions								
Interprofessional Case Simulations								
Interprofessional Field Visits								
Interprofessional Collaborative Projects								

Table 2: Example of a Proposed Design for Interdisciplinary Learning Materials
For Pharmacy, Medical, and Nursing Students
Main Theme: Chronic Disease Management – An Integrated Approach to Diabetes Mellitus

GUEST LECTURER (SEMESTER 3 DAN 4)	
Topic 1 **Comprehensive Approach to Diabetes Mellitus** **Speakers:** Endocrinologist, Clinical Pharmacist, and Diabetes Specialist Nurse	**Topic 2** **Effective Communication in Interprofessional Teams** **Speakers:** Health communication expert and care management specialist
Content: • Pathophysiology and diagnosis of diabetes from a medical perspective • Current pharmacological therapies for diabetes • Long-term management of diabetic patients • Challenges in cross-professional care coordination	**Content:** • SBAR communication model (Situation-Background-Assessment-Recommendation) • Care transitions and continuity of information among healthcare professionals • Strategies to overcome interprofessional communication barriers • Integrated documentation and health information systems
INTERDISCIPLINARY SEMINAR (SEMESTER 3 DAN 4)	
Topic 1 **Evidence-Based Clinical Decision Making** **Sessions:** • Analysis and evaluation of current medical literature on diabetes • Implementation of clinical guidelines in collaborative practice • Complex case presentations by students from the three disciplines	**Sessions:** • The influence of socioeconomic factors on treatment adherence • Cultural perspectives in diabetes care • Community intervention strategies for diabetes prevention and management
INTERPROFESSIONAL CASE DISCUSSIONS (SEMESTER 5 DAN 6)	
Topic 1 **Diabetes with Macrovascular Complications**	**Topic 2** **Diabetes with Microvascular Complications**
Case Study: A patient with diabetes and coronary heart disease **Discussion Focus:** • Identification of medical, pharmacological, and nursing issues • Prioritization of interventions by each discipline • Potential drug interactions and prevention strategies • Integrated discharge planning and patient education	**Case Study:** A patient with diabetic nephropathy and retinopathy **Discussion Focus:** • Collaborative approaches to preventing organ damage • Adjustment of therapy regimens based on organ function • Continuity of care from hospital to community • Role of each profession in long-term monitoring

INTERPROFESSIONAL CASE SIMULATIONS (SEMESTER 5 DAN 6)	
Topic 1 **Management of Patients with Diabetic Ketoacidosis (DKA)**	**Topic 2** **Integrated Patient Counseling and Education**
Simulation: Emergency scenario with a DKA patient **Student Roles:** • **Medical:** Medical assessment and stabilization • **Pharmacy:** Dosage calculation, insulin preparation, and rapid interventions • **Nursing:** Patient monitoring, protocol implementation, and care coordination	**Simulation:** Counseling a newly diagnosed diabetes patient **Student Roles:** • **Medical:** Explaining diagnosis and clinical implications • **Pharmacy:** Medication counseling, insulin use, and glucose monitoring • **Nursing:** Daily self-management teaching and disease adaptation support
INTERPROFESSIONAL FIELD VISITS (SEMESTER 7)	
Topic 1 **Visit to an Integrated Diabetes Center**	**Topic 2** **Visit to a Community Health Center**
Observasi model perawatan terintegrasi **Activities:** • Attend multidisciplinary team meetings • Review diabetes patient management systems • Analyze communication flows between professionals	Observasi program manajemen diabetes berbasis komunitas **Activities:** • Participate in diabetes screening programs • Observe patient education and support programs • Analyze challenges in primary-level diabetes care
INTERPROFESSIONAL COLLABORATIVE PROJECTS (SEMESTER 7)	
Topic 1 **Development of an Integrated Diabetes Management Protocol**	**Topic 2** **Public Health Campaign on Diabetes Prevention**
Project: Multidisciplinary teams of students create a management protocol **Protocol Components:** • Pharmacological therapy algorithm • Monitoring and follow-up plan • Integrated referral system • Interprofessional documentation standards	**Project:** Collaborative student teams design a public health campaign **Campaign Components:** • Educational materials from medical, pharmacological, and nursing perspectives • Community outreach programs • Evaluation of campaign impact • Health policy recommendations

Table 2 provides an explanation of how the learning design facilitates integration across various activities, as follows.

(1) Continuity of theme: The theme of diabetes is consistently explored from multiple perspectives across all activities.

(2) Progressive development:

a) *Guest lectures* provide a theoretical foundation
b) *Seminars* deepen conceptual understanding
c) *Case discussions* apply knowledge in clinical contexts
d) *Simulations* develop practical skills
e) *Field visits* offer exposure to real-world applications
f) *Collaborative projects* synthesize all prior learning experiences

(3) Ongoing reflection: Each activity includes a reflection component that connects previous learning experiences with new ones.

(4) Learning portfolio: Students create a developmental portfolio documenting their learning journey across all activities.

(5) Integrated assessment: Assessment encompasses both individual and team contributions throughout the entire sequence of activities.

Upon completing this series of activities, students will be able to enhance their knowledge, skills, and professional attitudes, as presented in **Table 3**.

Table 3: Student Competencies in Interdisciplinary Learning: Knowledge, Skills, and Professional Attitudes

Knowledge	Skills	Professional Attitude
• Identify the unique roles and contributions of each profession within the healthcare team • Explain the scientific basis for managing conditions from a multidisciplinary perspective	• Communicate effectively with members of an interprofessional team • Integrate profession-specific clinical skills in the context of interprofessional care • Participate effectively in team-based decision making	• Respect the perspectives and contributions of other professions • Develop a professional identity within a collaborative context • Demonstrate commitment to evidence-based, patient-centered practice

CONCLUSION

Interdisciplinary learning serves as a strategic solution to enhance the competencies of pharmacy students in collaborating with other healthcare professionals. Through this educational approach, pharmacy students gain a deeper understanding of the roles of other professions, improve their critical thinking skills, and become better prepared to meet the demands of future healthcare services

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INTERNATIONAL CONFERENCE ON SUSTAINABLE NATURAL PRODUCTS IN HEALTHCARE

"Interdisciplinary Approaches from Lab. to Clinical Breakthroughs"

The proceeding is centered on the theme Interdisciplinary Approaches from Lab to Clinical Breakthroughs, highlighting research on sustainable natural products and their applications in advancing healthcare services. The theme aims to foster innovation and collaboration across disciplines—bridging the gap between laboratory research and clinical implementation. The proceedings highlight several critical research domains including phytochemistry and natural product isolation, structure-activity relationship studies, nanotechnology applications in natural product delivery, personalized medicine approaches using natural compounds, sustainability and green chemistry in natural product development, regulatory science and quality assurance, and economic analysis of natural product-based therapeutics. These diverse areas of investigation reflect the multidisciplinary nature of contemporary natural product research and its expanding role in modern healthcare systems.

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