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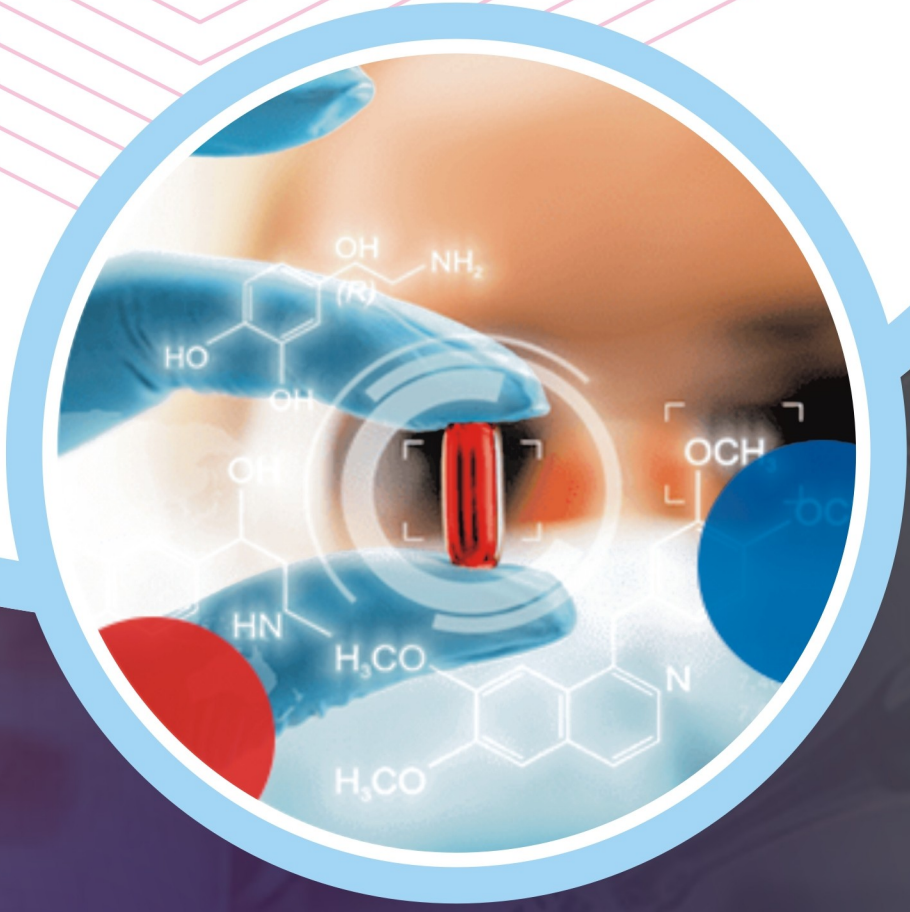

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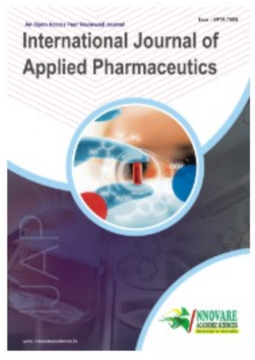

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Original Article

A NOVEL TOPICAL GEL OF THE FRESH YOUNG COCONUT HUSK ETHANOLIC EXTRACT DEDICATED FOR DIABETIC WOUND HEALING

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ABSTRACT

Objective: This study aimed to develop and to optimize the formulation of novel Fresh-Young Coconut-Husk-Ethanolic-Extract (FYCHEE) gels by Response Surface Methodology (RSM) of a Box Behnken Design (BBD), and to investigate the activity of novel FYCHEE topical gels on accelerating the Diabetic Wound Healing (DWH).

Methods: The FYCHEE was prepared by maceration using 96% ethanol and was standardized. Gels were formed by incorporating the FYCHEE into the gel-forming system containing Diethylene-Glycol Monoethyl-Ether (DGME), Carbopol 940, and Triethanolamine (TEA). Sixteen experimental runs were set using BBD. Physical properties (viscosity, pH and spread ability) were further characterized. The data were analyzed and optimized using "rsm" package of the R software. The predicted model of DGME, Carbopol 940 and TEA composition was further validated. The *in vivo* study of the activity of FYCHEE gels on accelerating DWH was conducted on groups of male Wistar rats in 12 d of topical application of FYCHEE gels containing 5%, 10%, 20% extracts.

Results: The FYCHEE gels performed light-brown semisolid texture with varied physical characteristics. The RSM generated visualization of response surface plots based on three model equations. The novel FYCHEE gels with optimum responses have been well developed (DGME: Carbopol 940: TEA = 8:0.8:0.6). The formulation based on predicted composition showed 1.194±0.136 Pa. s (viscosity), 5.715±0.213 (pH) and 4.997±0.265 cm (spread-ability). The FYCHEE gels showed significant results on accelerating the wound closure in 12 d, with percentage of (88.798±4.697) %, (91.685±3.124) %, and (93.060±1.687) % for FYCHEE of 5%, 10%, and 20%, respectively. These results were similar to the positive control ($p>0.05$).

Conclusion: The novel FYCHEE gels were successfully fabricated with standardized FYCHEE. The RSM of the BBD was applied satisfactorily to optimize the formula. Further study of FYCHEE gels on accelerating DWH revealed promising results of the FYCHEE gels to be potentially developed as diabetic wound healing topical preparation.

Keywords: Formula, Optimization, Ethanolic extract, Coconut husk, Topical gel

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INTRODUCTION

Diabetic condition due to the uncontrolled glucose levels may get unmanageably complicated by impaired wound healing, which may cause fatal condition of tissue death and gangrene infection leading to amputation [1, 2]. Many efforts have been done to develop a wide series of wound healing therapies to overcome this condition, including exploring the pharmacological benefits of natural products [3]. A number of plants and phytoconstituents which were potential for Diabetic Wound Healing (DWH) has been comprehensively reviewed and studied [4–6]. However, the potential of coconut on DWH has not been thoroughly investigated. Coconut (*Cocos nucifera* L) is known as a multi-benefit tropical fruit in Indonesia [7]. Empirically, young coconut water is used as an antidote of some toxicity cases [8]. In terms of healing the diabetic wound, the virgin coconut oil and the liquid smoke of coconut shells appear to be the only materials from coconut which has been widely reported [9–11]. The husk of coconut has a high content of tannin. It was suggested that total tannin in the extract of husk was up to 141.5 ±5.08 mg Tannic Acid Equivalents (TAE)/g [12]. Tannin shows astringency, antioxidant and anti-inflammatory effects; therefore, the extract of the young coconut husk is potential to develop as a wound-healing agent [13, 14].

To be practically applicable, a topical formulation needs to be carefully designed for the extract of the young coconut husk. A topical hydrogel system is favorable as it provides many valuable features for wound healing, such as: high hydration and cooling effect, firm adhesion, and ease of application [15]. A number of gel-forming systems have been explored in order to provide suitable delivery systems for diabetic wound healing [16]. However, to the best of our knowledge, topical gel formulation which incorporates the plant extracts has not been extensively studied. As the extract of the young coconut husk is acidic due to the content of tannic acid, the quality of the gel product may be significantly affected by this property. Therefore, material selection of the gel-forming system must focus on the capability of the product to withstand the acidic environment and to create a supporting system for the stability of the extract. In addition to that, viscosity and the spread-ability of the gel are two essential physical properties as these properties may affect the physical stability and ease of product application as well as the filling process in manufacturing area [17–19]. These properties are significantly governed by the gel-forming system in the formulation.

Response Surface Methodology (RSM) in the Box Behnken Design (BBD) is a promising tool for the formulator in designing the formula to predict the composition with the optimum responses. Multiple responses optimization is meticulously facilitated in BBD to investigate more than two independent variables at three different levels [20]. BBD uses fewer number of experiments than any other optimization, such as central composite design, resulting in efficiency in carrying out the experiments [21].

To ensure the effect of accelerating the wound recovery, it is imperative to evaluate the activity of the topical hydrogel containing Fresh Young Coconut Husk Ethanolic Extract (FYCHEE) on the wound contraction.

Punch biopsy is one of wound models which can be applied to assess the effectivity of wound healing topical preparation [22].

This study aimed to provide good quality of FYCHEE and to optimize the formula by applying the response surface methodology in BBD. The optimization focused on the composition of the gel-forming system containing Diethylene Glycol Monoethyl Ether (DGME), Carbopol 940 and Triethanolamine (TEA), which provides the optimum responses of viscosity, pH and spread-ability. The computational optimization was assisted by an open-source R software package called "rsm" [23] which was evident in implementing BBD [18, 24]. Furthermore, an *in viv*o study was conducted with the objectives to confirm the potential effects of the FYCHEE gel in accelerating DWH.

MATERIALS AND METHODS

Materials

Fresh young coconut husk; Diethylene Glycol Monoethyl Ether (DGME; Sigma Aldrich, Singapore; pharmaceutical grade); ethanol 96% (grade A for extraction; General Labora, Indonesia); Carbopol 940, Triethanolamine (TEA), methylparaben (nipagin), Butyl Hydroxy Toluene (BHT), these materials are technical grade, purchased from General Labora, Indonesia), demineralized water.

Methods

Young coconut husk extraction and characterization

Fresh young coconuts were thoroughly washed and longitudinally cut into 2 (two) pieces. The shell and green outer skin were removed. Each piece was then horizontally divided into 4 (four) parts. Every part was sliced at 1 cm long and then grated with an electric grater (Bison, Indonesia). The grated husk was then mechanically macerated in 96% ethanol (in ratio 1:10) by using an orbital shaker (Innova 2100, Germany), with the speed of 150 rpm at room temperature for 4 h. The liquid extract was then vacuum-filtered and undergone solvent evaporation by a vacuum rotary evaporator (Buchi, Switzerland) at 35 °C and 45 mmHg pressure, resulting in the semi-concentrated extract. The semi-concentrated extract was further evaporated to obtain the dry extract.

To characterize the extract, the organoleptic observation was carried out, followed by the yield calculation of the semi-concentrated and dry extracts. Water content was determined on the semi-concentrated extract, while for the dry extract, Loss on Drying (LOD) was examined. In order to facilitate the ease of mixing, the semi-concentrated extract was selected for topical gel formulation (at concentration of 10% w/w gel).

FYCHEE gel formulation

The fabrication of the FYCHEE gels was initiated by a gelation process of Carbopol 940 in demineralized water containing preservative, which was then kept aside at room temperature for 24 h. The half-made gel was then added with TEA, BHT and DGME and homogenously mixed by an overhead stirrer (IKA Works, Malaysia) at 650 rpm for 3 min to obtain a viscous gel system. The FYCHEE was further incorporated into the gel system and continuously mixed at 650 rpm for 2 min. The gel was then contained in a light-resistant glass jar and kept at 2-8 °C for 24 h prior to physical characterization.

Table 1: The basic formula of the FYCHEE gel

Ingredients	Weight (g)
Carbopol 940	1
Water	78
Nipagin	0.1
TEA	0.4
BHT	0.1
Ethanol	0.4
DGME	10
FYCHEE	10

The composition of the excipients was organized based on the BBD stated below.

Experimental design

The BBD model allowed 16 runs of experiments with three different independent variables selected, which were DGME, Carbopol 940, and TEA, in three levels. The dependent variables optimized were viscosity (Pa. s), pH and the spread-ability (cm). The experimental variables for the response surface methodology are shown at table 2.

Table 2: Experimental variables in three levels

Variables	Levels		
	Low	Medium	High
x_1: DGME (g)	8	10	12
x_2: Carbopol 940 (g)	0.8	1	1.2
x_3: TEA (g)	0.2	0.4	0.6

Physical characterization of FYCHEE gels

Organoleptic characterization and pH confirmation

Visual organoleptic characterization was carried out for the extracts and the FYCHEE gels. This characterization involved color, odor, and consistency. To confirm the pH of the semi-concentrated extract and FYCHEE gels, a calibrated-pH meter (WTW pH 3110 SET2, Germany) was used. In brief, the probe was directly immersed in the samples for 30 seconds and the pH could be read on the display. The gels should have delicate, light-brown semisolid consistency, with pH of 4.5-5.9.

Determination of viscosity

A Merlin VR viscometer (Rheosys, USA) was used to determine the viscosity of the FYCHEE gels, with cone and plate mode 2°/30 mm, operating at 50 rpm, at room temperature. The measurement was replicated, with a delay time of 20 seconds, zero-shear time of 20 seconds and the integration time of 10 seconds [17]. The viscosity of 0.5-1.5 Pa. s was preferable.

Spread-ability measurement

The spreading diameter of 1 (one) g of the FYCHEE gel, which was sandwiched in between two glass plates horizontally for 1 minute, with the upper plate loaded with metals thereby weighing 125g [25], was measured by a ruler. The spreading diameter was the average diameter measured in 4 directions passing the center point. The diameter should be 4-7 cm.

Statistical analysis of the BBD

The BBD model of 16 runs and the response surface methodology were designed with the aid of R open-source software version 4.4.1 and Rstudio version 2024.04.2 Build 764 equipped with "rsm" package [23]. The optimization of multiple responses and the desirability analysis were executed to obtain a composition of Carbopol 940, triethanolamine, and DGME which resulted in optimum responses of physical characteristics (pH, viscosity, and spreadability). Statistical analysis was carried out with 95% confidence interval.

Stability evaluation of the FYCHEE gels

In-use stability of FYCHEE gels (E5%, E10%, E20%) was carried out parallel with the *in vivo* activity study. FYCHEE gels were stored at two different storage conditions: cold storage (2-8 °C) and at room temperature. (28-33 °C). The observation was based on the alteration of viscosity, spread-ability and pH in 14 d. The data was

statistically analyzed using the paired t test for the condition on day 1 and the condition on day 14.

The *in vivo* study of the activity of FYCHEE gels on accelerating DWH

Experimental design

The study was conducted following the approval of animal ethical clearance by the Ethic Committee of Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Indonesia on October 1st, 2024, number KE/FK/1523/EC/2024.

Healthy male Wistar rats (2.5-3 mo; 200g-250g BW) were acclimatized under 12h light/dark cycles at room temperature with 70% RH and caged individually, fed by standard nutritious pellets. The rats were allowed to drink clean fresh water *ad libitum*.

The induction of diabetic condition was carried out by injecting Streptozotocin (45 mg/kg BW) and Nicotinamide (110 mg/kg BW) intraperitoneally. After 72 h, the diabetic condition of the rats (blood glucose level at ≥ 250 mg/dL) was confirmed by measuring the blood glucose level using Glucose-Oxidase Peroxidase (GOD-PAP).

The groups of rats were then divided into 6 (six) groups @ 3 rats.

The first group was the Untreated Non-Diabetic Group (UTNDG), which was set as a reference of wound healing normal response. The second group involved the diabetic rats with untreated wounds (Untreated Diabetic Group; UTDG). The group of Positive Control (PC) was treated by the commercial product (Lukajel®). The other three groups were the group treated with FYCHEE gels containing 5% extract (E5%), 10% extract (E10%), and 20% extract (E20%).

The hairs on the dorsal surface were removed to provide hairless space for observation. All rats were then anaesthetized by intramuscular injection of ketamine HCl (50 mg/kg BW) and xylazine (10 mg/kg BW) prior to punch biopsy (diameter of 5 mm). Punch biopsy was carried out two times on each side of the rats and the wound was kept open for 24 h to let the exudates dry. Approximately 200 mg of the topical preparation was then applied on the wound with the aid of a metal mini spatula, twice a day for 11 consecutive days.

The observation of the wound contraction was carried out every three days, on day 3, day 6, day 9, and day 12. The wounds were captured perpendicularly using a digital camera (Samsung Galaxy S23, South Korea) at around 20 cm height, with the scaling aid of millimeter-scale paper. The area of the wounds was further analyzed using ImageJ software (National Health Institute, U. S). The percentage of wound contraction was calculated by: [(wound area on the day 0 – wound area on the day X): wound area on the day 0] [22, 26].

On day 9, after being visually observed, one rat of each group was sacrificed, and the wounded skin tissues were then removed and fixed in 10% formalin for histological observation.

Fixed tissues were further embedded in paraffin, sectioned at the thickness of 5µm using a microtome, then stained with hematoxylin and eosin. The sections were examined under a light microscope for the evaluation of histopathological parameters. Three parameters were selected those are the number of inflammatory cells (represented by neutrophils), the epithelium generation, and the number of vessels generated (angiogenesis).

Data presentation and statistics analysis

The data are presented as the mean±SD for physical characteristics with n=3 replications/formula and the mean±SD for wound healing activity study with n=5 wounds/group. Sample size was justified according to Arifin *et al.* [27] with the formula of 10/k+1 as the minimum number of animals per group. Normally distributed data were analyzed using either ANOVA or unpaired t test. For the non-parametric data, a Wilcoxon test and Kruskal Wallis applied. Significant differences were considered if $p<0.05$. All data were statistically analyzed using Analysis-ToolPak on the MS Excel 2019 (Microsoft, USA). Statistical analysis was carried out with 95% confidence interval. In terms of the activity of accelerating wound contraction, comparisons were made among gels and between the gels and control groups of the diabetic-induced rats.

RESULTS

Coconut husk extraction and standardization

The organoleptic observation revealed that the semi-concentrated extract was brown and transparent liquid, while the dry extract showed a sticky-brown semisolid texture (fig. 1).

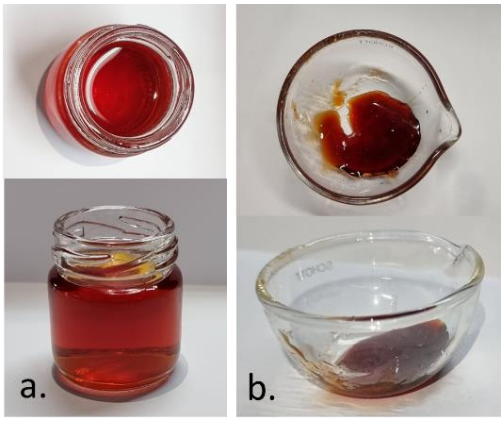

Fig. 1: Visual appearance of the FYCHEE: a. Semi-concentrated extract; b. Dry extract

The yield of the semi-concentrated extract was 5.03±0.21 ml per 500 ml solvent with water content of (86.88±0.006) %. The pH of the semi-concentrated extract was 4.59. In terms of the dry extract, the yield was 5.07±0.590 % per 50g husk and the LOD was 13.81±0.866 %.

Table 3: Design of experimental results for DGME, carbopol, and TEA composition in the FYCHEE gel formulation

Run order	Standard order	Independent variables			Dependent variables		
		DGME (x_1)	Carbopol (x_2)	TEA (x_3)	Viscosity (y1)	pH (y2)	Spread ability (y3)
1	6	12	1.0	0.2	0.316	4.403	4.750
2	3	8	1.2	0.4	0.674	4.314	4.750
3	1	8	0.8	0.4	0.326	4.686	5.400
4	16	10	1.0	0.4	0.364	4.436	5.225
5	8	12	1.0	0.6	0.810	5.091	4.537
6	2	12	0.8	0.4	0.412	4.950	5.850
7	9	10	0.8	0.2	0.138	4.302	6.000
8	11	10	0.8	0.6	0.689	5.123	5.400
9	4	12	1.2	0.4	0.526	4.629	5.400
10	15	10	1.0	0.4	0.445	4.471	5.650
11	7	8	1.0	0.6	1.295	5.180	4.500
12	12	10	1.2	0.6	1.197	5.269	3.887
13	5	8	1.0	0.2	0.357	4.488	5.500
14	14	10	1.0	0.4	0.610	4.529	5.300
15	10	10	1.2	0.2	0.096	4.656	5.425
16	13	10	1.0	0.4	0.869	4.352	5.475

Topical gel fabrication, design of experiment, and physical characterization

Sixteen runs of the Box Behnken model were executed with DGME, Carbopol 940, and TEA as the independent variables. The FYCHEE gels were then physically characterized in terms of viscosity, pH, and spread-ability as the dependent variables. The visual appearance of the topical gels was light brown semisolid with varied values of the observed physical properties (table 3).

Response surface methodology

Responses in terms of viscosity, pH and spreadability in uncoded units were evaluated using RSM (table 4). Contour plots and response surfaces were generated and depicted in fig. 2.

The acceptance criteria for optimization were in the range of 0.5-1.5 Pa. s for viscosity, 4.5-5.9 for pH and 4-7 cm for the spread ability. Based on the desirability analysis, with the composite desirability value of 0.673, it was predicted that a composition of DGME: Carbopol 940: TEA = 8: 0.8: 0.6 might result in optimum responses.

The predicted responses were 0.827 Pa. s for the viscosity, 5.304 for the pH and 4.988 cm for the spread-ability. Validation of the prediction was further carried out and the observed values were closed to the prediction and met the acceptance criteria (table 5). The visual appearance of topical gels of the ethanolic extract of fresh young coconut husk was transparent, light-brown semisolid (fig. 3).

Table 4: Results of RSM modeling for viscosity, pH, and spread-ability

Responses	R^2	Equation
Viscosity (Pa. s)	0.896	$y1 = -3.464+0.028\ x_1+6.972\ x_2-0.412\ x_3-0.146\ x_1x_2-0.278\ x_1x_3+3.438\ x_2x_3+0.009\ x_1^2-3.152\ x_2^2+2.096\ x_3^2$
pH	0.840	$y2 = 9.416-0.382\ x_1-6.038\ x_2-2.279\ x_3+0.032\ x_1x_2-0.003\ x_1x_3-1.300\ x_2x_3+0.019\ x_1^2+3.059\ x_2^2+6.703\ x_3^2$
Spread-ability (cm)	0.840	$y3 = 5.785+0.749\ x_1-8.241\ x_2+6.477\ x_3+0.125\ x_1x_2+0.492\ x_1x_3-5.863\ x_2x_3-0.052\ x_1^2+3.672\ x_2^2-9.534\ x3^2$

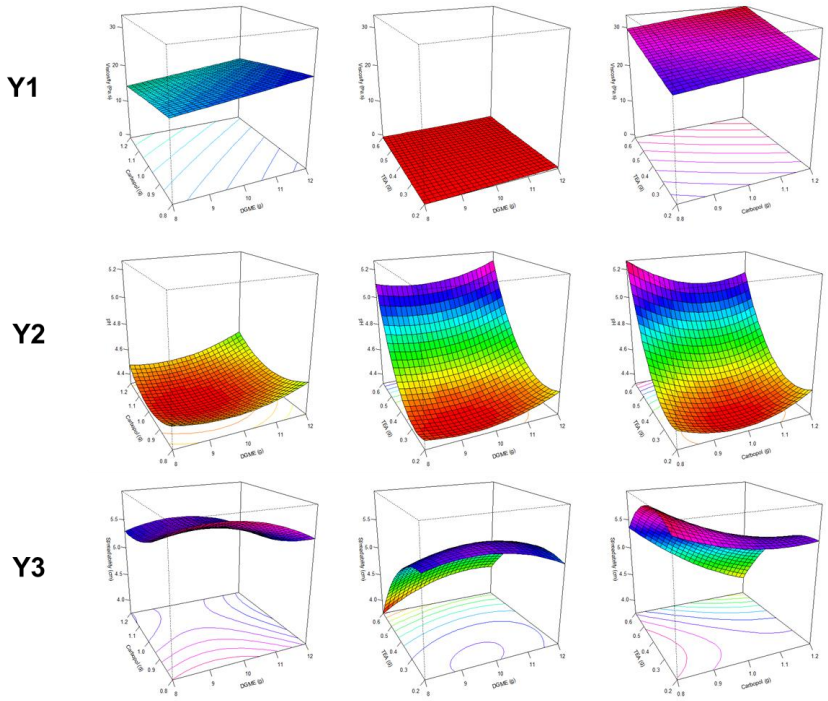

Fig. 2: Visualization of response surface plot for viscosity (y1), pH (y2), and spread-ability (y3)

Fig. 3: Visual appearance of optimized topical gels of ethanolic extract of fresh young coconut husk

Table 5: Desirability analysis for topical gel formulation containing young coconut husk ethanolic extract

Responses	Optimization setting			Composite desirability	Prediction	Validation results
	Lower	Target	Upper			
Viscosity (Pa. s)	0.5	1.0	1.5	0.673	0.827	1.194±0.136
pH	4.5	5.2	5.9		5.304	5.715±0.213
Spread-ability (cm)	4.0	5.5	7.0		4.988	4.997±0.265

Note: validation data are presented as x̄±SD with 3 replicates

Stability evaluation of the FYCHEE gels

FYCHEE gels were successfully developed with noticeable physical properties. The viscosity of FYCHEE containing 5% extract was the highest among three formulations, with the consequence of being the least spreadable (table 6), whereas the characteristics of viscosity and spread-ability of FYCHEE with 10 and 20% extract were comparable. Focusing on the pH, since the pH of the extract was acidic, the higher the concentration tends to give more acidic gels, although FYCHEE 5% and 10% were similar.

Table 6: Properties of FYCHEE gel 24h after fabrication

FYCHEE concentration (%)	Viscosity (Pa. s)	Spread-ability (cm)	pH
5	1.442±0.0199	4.050±0.0577	4.790±0.0290
10	0.951±0.0147	4.925±0.0957	4.949±0.0156
20	0.850±0.005	5.025±0.0957	4.493±0.007

Note: Data are presented as x̄±SD with 3 replicates

In 14 d of storage, the FYCHEE gel spread-ability increased on both cold and at room temperature condition as a result of decreasing viscosity (table 7). In terms of pH, the values were slightly decreased. The stability of the FYCHEE was moderate with the viscosity shift at around 18% for cold storage and approximately 25% at room temperature.

Table 7: Stability of FYCHEE gel after 14 d of storage

FYCHEE (%)	Cold storage condition		Room temperature storage condition		
	Viscosity shift (%)	Spread-ability shift (%)	Viscosity shift (%)	Spread-ability shift (%)	pH shift
5	16.596±1.3723	30.384±5.1061	34.98±9.3683	37.55±2.2036	1.846±0.8329
10	27.548±4.1368	31.267±7.7530	26.908±5.8970	15.88±8.4454	2.765±0.4536
20	22.302±4.3265	24.015±6.4347	21.34±5.1532	20.21±6.3966	2.951±0.1734

Note: Data are presented as x̄±SD with 3 replicates

The *in vivo* study of the activity of FYCHEE gels on accelerating diabetic wound healing

Fig. 4A depicts visual appearance of wound contraction over time. The skin with normal condition (Untreated Non-Diabetic Group; UTNDG) showed natural recovery of wound healing without any treatments over 12 d, whereas the diabetic wounds, as shown on the Untreated Diabetic Group (UTDG) were getting exaggerated due to massive inflammation on the first stage of wound healing. Rapid increase of contraction evidenced on day 9 on the FYCHEE gels and PC (table 9). FYCHEE gels provided significant recovery profile from day 3 to day 12, like the positive control ($p>0.05$). Different concentrations of FYCHEE in gels unaffected the wound contraction on the day 12 ($p>0.05$).

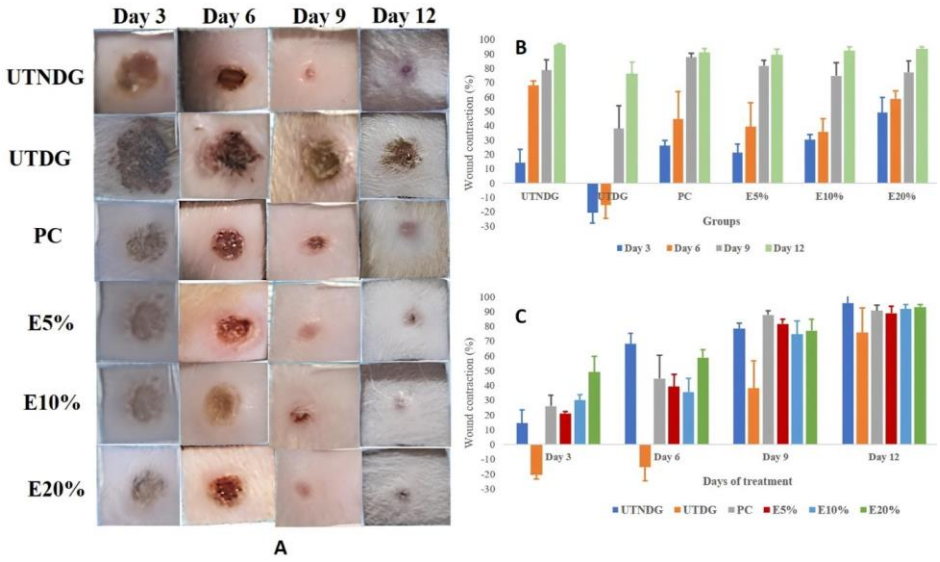

Fig. 4: *In vivo* study of DWH acceleration by FYCHEE: A. Macroscopic appearance of the wound contraction on day 3, 6, 9, 12. B. Percentage of wound contraction on each day of observation. C. Wound closure tendency over 12 d. UTNDG: Untreated Non-Diabetic Group; UTDG: Untreated Diabetic Group; PC: Positive Control group; E5%: wound applied with FYCHEE 5% gel; E10%: wound applied with FYCHEE 10% gels; E20%: wound applied with FYCHEE 20% gels

Table 9: Diabetic wound healing process

Days	Wound contraction (%)					
	UTNDG	UTDG	PC	E5%	E10%	E20%
Day 3	14.34±8.975	-20.68±.057	25.95±3.552	21.16±5.912	30.19±3.566	49.08±10.720
Day 6	68.24±2.706	-15.16±9.417	44.76±18.776	39.23±16.555	35.64±9.012	58.76±5.492
Day 9	78.59±7.391	38.00±15.696	87.65±2.724	81.68±3.756	74.61±9.100	77.09±7.799
Day 12	95.79±1.214	75.76±8.370	90.58±3.223	88.80±4.697	91.69±3.124	93.06±1.687

Note: UTNDG: Untreated Non-Diabetic Group; UTDG: Untreated Diabetic Group; PC: Positive Control group; E5%: wound applied with FYCHEE 5% gel; E10%: wound applied with FYCHEE 10% gels; E20%: wound applied with FYCHEE 20% gels. Data are presented as $\bar{x}$±SD with 5 replicates

The groups of treated diabetic wounds showed significant recovery from day 9 (fig. 4B and fig. 4C). Moreover, there is strong evidence that the FYCHEE gels accelerated the diabetic wound contraction showing by the significant higher percentage over time compared to the untreated diabetic wound control group. FYCHEE, with 20% extract, managed to contract the wound at almost 100% on the day 12, showing a comparable result to the positive control.

Reepithelization of the treated wounds on the day 9 was confirmed by the H and E images of the wound-fixed tissue (fig 5). Epithelia layer appeared on top of skin of normal wound (UTNDG; 5A). However, fig 5B depicted that the epithelial layer was still unable to grow on the untreated diabetic group (UTDG) on day 9. On the positive control (PC; 5C) the epithelial layer appeared to grow well, and similar appearance was also noticed at the FYCHEE gels in all concentrations (fig 5D-5F).

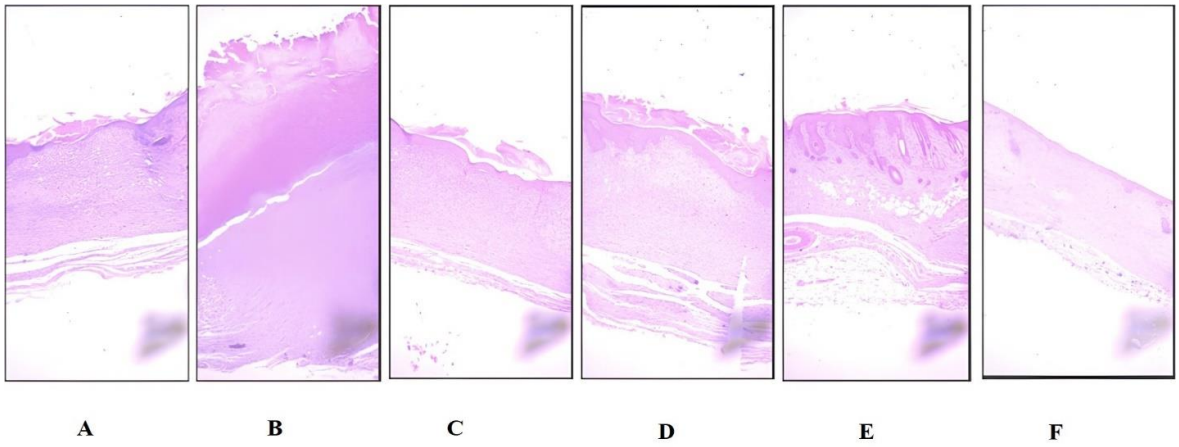

Fig. 5: Re-epithelization progress on day 9 of diabetic wound healing. A. Untreated Non-Diabetic Group (UTNDG); B. Untreated Diabetic Group (UTDG); C. Positive Control group (PC); D. Wound applied with FYCHEE 5% gel (E5%); E. Wound applied with FYCHEE 10% gels (E10%); F. wound applied with FYCHEE 20% gels (E20%)

Fig. 6 illustrates the condition of wound tissue on day 9. The untreated non-diabetic (UTNDG) wound showed only few neutrophils without angiogenesis (fig. 6A), however, massive inflammation was still evidenced on the untreated diabetic group (UTDG; fig. 6B), shown by the huge amounts of neutrophils as the inflammation cell marker. Interestingly, the angiogenesis was also enormously increased, indicating the natural effort of homeostatic response to the wound (see inserted fig. on fig 6B). The wounds treated with FYCHEE gels revealed less neutrophils compared to the UTDG, but similar to the control group (fig.6C), although the vascularization was still ongoing (fig 6D-F). The appearance of neutrophils was decreasing on the wound applied with FYCHEE gels with the least was on the FYCHEE 20% gels.

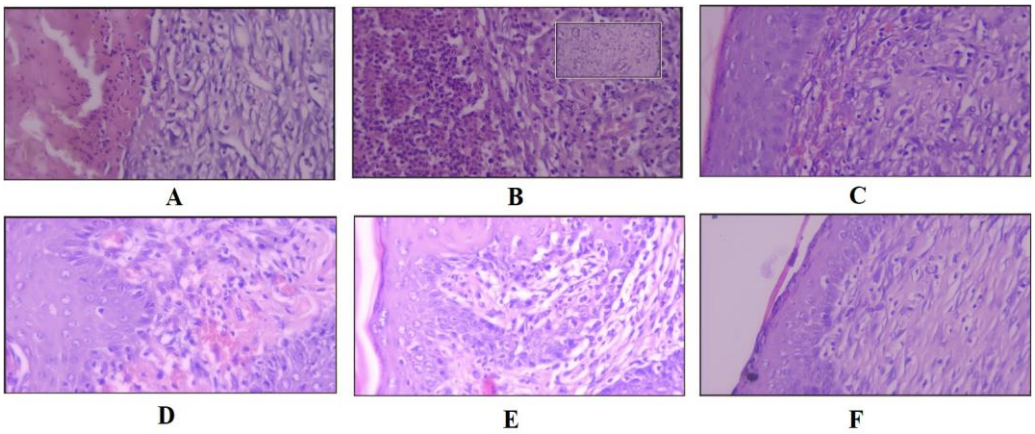

Fig. 6: Histological images focused on the neutrophils and the angiogenesis on the day 9 of diabetic wound healing. A. Untreated Non-Diabetic Group (UTNDG); B. Untreated Diabetic Group (UTDG); C. Positive Control group (PC); D. Wound applied with FYCHEE 5% gel (E5%); E. Wound applied with FYCHEE 10% gels (E10%); F. wound applied with FYCHEE 20% gels (E20%)

DISCUSSION

Plant-based diabetic wound healing formulation has recently gained much interest due to the biocompatibility and abundant renewable resource of the active ingredients. This current study highlights the significance of novel topical gel formula optimization for delivering FYCHEE, which is potential as diabetic wound healing accelerator. DGME, Carbopol 940, TEA were selected as the experimental independent variables on providing the optimum responses of pH, viscosity and spreadability. This study also aimed to evaluate the capacity of FYCHEE gels to accelerate diabetic wound healing.

The ethanolic extract of fresh young coconut husk was successfully collected using mechanical maceration for 4 h. The solvent underwent massive evaporation to generate semi-concentrated and dry extracts. The standardization had been carried out in terms of yield, solvent reduction and loss on drying. The pH of the semi-concentrated extract was also confirmed, which was approximately 4.5. The semi-concentrated extract was further selected to be applied in the formulation as it was easier to mix with other components.

Carbopol 940 is a common gel-forming agent, which the viscosity can be enhanced by neutralizing the pH using an alkalizing agent such as TEA. The addition of TEA will ionize the carboxylic groups therefore it creates repulsion in between branches, hence forming the 3D structure of gel [28]. The combination of Carbopol 940-TEA provides a rigid transparent form of gel [29] which is expected to be able to deliver the ethanolic extract of fresh young coconut husk dedicated for diabetic wound healing. The addition of DGME, a stabilizer, is found to increase the viscosity of the gel system and stabilizes it. The application of those materials may influence the physical characteristics of the gel formulation especially on pH, viscosity, and spread-ability. Therefore, it is imperative to optimize the formula to provide targeted responses based on acceptance criteria.

Acidic pH of the products must be scrutinized as it will provide a supporting atmosphere for the stability of extract and encourage the wound repairing acceleration. An acidic wound microenvironment with pH 4 has been suggested for a better scheme of wound healing therapy [30]. In this study, targeted pH was on the range of 4.5-5.9. Viscosity of product also plays an important role on the formulation, as the optimum viscosity will assist the manufacturing handling and ease the application on the wound [17, 19]. Spread ability, which is represented by the diameter of the gel applied under 125g weight [25], as a function of viscosity, will also contribute to the optimum way of application the semisolid preparation. Viscosity of 0.5-1.5 Pa. s and the spread-ability of 4-7 cm were targeted based on our previous study [22] and some preliminary experiments.

Response surface methodology of BBD has been satisfactorily applied to optimize the formula with 3 (three) factors of the gel-forming system, which were DGME, Carbopol 940 and TEA (x1, x2, x3) in three different levels of low, medium, and high. The observed responses which were the physical characteristics of the formula involved viscosity, pH and spread ability, served as y1, y2, and y3, respectively. Those three factors in three levels were modelled using the "rsm" package of the R open-source software program to create 16 experimental runs with various compositions, hence various values of each type of response. The model equations of viscosity, pH, and spread-ability were $y1 = -3.464 + 0.028\ x_1 + 6.972\ x_2 - 0.412\ x_3 - 0.146\ x_1x_2 - 0.278\ x_1x_3 + 3.438\ x_2x_3 + 0.009\ x_1^2 - 3.152\ x_2^2 + 2.096\ x_3^2$ ($R^2 = 0.896$), $y2 = 9.416 - 0.382\ x_1 - 6.038\ x_2 - 2.279\ x_3 + 0.032\ x_1x_2 - 0.003\ x_1x_3 - 1.300\ x_2x_3 + 0.019\ x_1^2 + 3.059\ x_2^2 + 6.703\ x_3^2$ ($R^2 = 0.840$), and $y3 = 5.785 + 0.749\ x_1 - 8.241\ x_2 + 6.477\ x_3 + 0.125\ x_1x_2 + 0.492\ x_1x_3 - 5.863\ x_2x_3 - 0.052\ x_1^2 + 3.672\ x_2^2 - 9.534\ x3^2$ ($R^2 = 0.840$), respectively. Those equations then were considered to provide response surface plots. Response surface plots of combination of two factors resulted in various surface profiles (fig. 2). Three perspective plots of each response showed the different properties of each surface.

In this current study, it was found that the composite desirability of 0.673. This value was generated from the consideration of three responses namely viscosity, pH, and spread ability. Response of viscosity was set as a target response with the lower, target, and upper of 0.5, 1.0, and 1.5 Pa. s, respectively. Response of pH was set as a target response with the lower, target, and upper of 4.5, 5.2, and 5.9, respectively. Response of spread-ability was set as a target response with the lower, target, and upper of 4.0, 5.5, and 7.0 cm, respectively. Desirability analysis with a value between 0-1 is a statistical tool to optimize the formula based on the target setting of each response [31]. The most expected responses will generate the desirability values close to 1 [32].

It is important to verify the RSM prediction by performing an empirical study of validation of the experimental design. In this current study, the optimized composition of DGME, Carbopol 940, and TEA were applied in the formulation of the topical gel. Three predicted responses were then evaluated. The results of the desirability analysis as well as the validation study were presented in table 5. The visual appearance of topical gels obtained from the optimized condition was also presented in fig. 3. It was found that the validation of the prediction and the observed values were closed and met the acceptance criteria.

The in-use stability results showed that the percentage of alteration of physical properties such as viscosity, which would impact the spread-ability was quite high. The viscosity of the gels reduced over time by around 25% both at cold storage and at room temperature. FYCHEE gels were formulated with quite low viscosity, as it considered to be able to facilitate the release of the active ingredients faster [33, 34]. However, this consideration might cause the risk of the instability of gels on the storage. The high viscosity of Carbopol 940 as gel gel-forming agents would be well performed at neutral pH [35]. The lower the pH of the system would reduce the viscosity [36]. On the other side, the stability of tannin as polyphenol molecules would be endangered in alkaline atmosphere [37]. Furthermore, the acidic pH of wound healing topical preparation was convincingly emphasized to manage faster recovery of wound healing [30]. Incorporating such acidic FYCHEE appeared to be challenging as it undeniably decreased the pH of the system overtime, resulting in the decrease of viscosity of gels. Moderate results of stability evaluation indicated that the formulation study was in the right direction, but the development must be progressing on how to enhance the stability of FYCHEE gels in storage. Exploring the potential of other gel forming agents and suggesting an encapsulation technique for the extract may be of interest for the future research to enhance the stability of the gels.

Although the in-use stability showed moderate results, an *in vivo* study of diabetic wound healing acceleration revealed that the FYCHEE gels successfully accelerated the wound contraction (table 9 and fig. 4). Wounds contracted at almost 100% on day 12. E5% results were comparable to PC, whereas E10% and E20% showed higher percentage. The astringency effect of tannin as the main component aided with the anti-inflammatory effect of other flavonoids was predicted to be the main roles on diabetic wound contraction acceleration, supported with gel formulation as the prominent vehicle [14, 22, 38, 39].

The novel FYCHEE gels were successfully formulated. Further development of the formulations, especially on the enhancement of gel stability, needs to be done to ensure the quality of FYCHEE gels. Moreover, future studies of long-term stability, clinical trials, and scaling up of commercialization are encouraged to emphasize the FYCHEE contribution to the alternative solution of diabetic wound treatment, with lateral objective of increasing the economic value of the young coconut husk.

CONCLUSION

The "rsm" package of the R open-source program could satisfactorily assisted in conducting the response surface methodology of BBD to optimize the FYCHEE gel formula. A predicted composition of DGME: Carbopol 940: TEA of 8: 0.8: 0.6 could yield the topical gel of ethanolic extract of fresh young coconut with optimized responses which met the target of formula (1.194±0.136 Pa. s for the viscosity; 5.715±0.213 for the pH; 4.997±0.265 for the spread ability) with the composite desirability of 0.673. The FYCHEE gels evidenced to accelerate the diabetic wound on diabetes-induced Wistar rats with similar results to the positive control.

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AUTHORS CONTRIBUTIONS

C. M. R. R. N. and F. D. O. contributed to conceptualization, data acquisition, data analysis and interpretation, supervision as well as article writing and critical reviewing. Y. D., C. P., M. R. G. contributed to data acquisition, data analysis, and critical reviewing.

CONFLICT OF INTERESTS

Declared none

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