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by Yustina Sri Hartini

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Anticancer drug screening of natural products: *In vitro* cytotoxicity assays, techniques, and challenges

Agustina Setiawati[✉], Damiana Sapt Candrasari, F.D. Erika Setyajati, Vincentia Krisnina Prasetyo, Dewi Setyaningsih, Yustina Sri Hartini

Faculty of Pharmacy, Sanata Dharma University, Paingan, Maguwoharjo, Depok, Sleman, Yogyakarta 55282, Indonesia

ABSTRACT

Natural products include several diverse compounds that have been found to be effective against cancer. Discovering anticancer compounds in nature is a multistep and complex process that requires pre-clinical and clinical studies. Only a few of the available natural products are used to treat cancer since most of them have very high complexity and low bioavailability. Therefore, the process of anticancer drug discovery requires a straightforward and effective method to assess anticancer activity using *in vitro* assays. This review summarizes various cell-based assays and techniques used to measure cell viability, migration, and apoptosis, focusing in particular on the principles, mechanisms, advantages, and disadvantages of each assay to provide a preliminary platform for cancer drug discovery.

KEYWORDS: Drug discovery; Cancer; Natural products; Screening; *In vitro* assay; Cytotoxicity; Migration; Apoptosis

1. Introduction

Cancer chemotherapy has been used since the mid-20th century^[1]. Although over 200 anticancer biopharmaceuticals have been approved, with many more in preclinical or clinical trials, tumor therapies are still emerging^[2]. Natural products have been widely studied for their anticancer activities for more than 50 years. For example, vinblastine and vincristine were isolated from active compounds of the Madagascar periwinkle *Catharanthus roseus* G. Don. (Apocynaceae). Other drugs isolated from plants include etoposide, paclitaxel, topotecan, and irinotecan^[3–5]. Chemical compounds found in natural products induce canonical processes such as apoptosis and non-canonical cell death mechanisms such as ferroptosis, pyroptosis, and necroptosis^[6]. Approximately 600

natural products and dietary substances exhibit *in vitro* anticancer activity against various forms of cancer, such as breast, leukemia, lymphoma, prostate, liver, lung, and myeloma^[7]. Such findings have driven attempts to screen natural products as chemopreventive agents.

Anticancer drug discovery is a long complex process involving the screening of new entities, assessment of anticancer activity, production and formulation, *in vivo* toxicity testing in animals, and pre-clinical trials and has unpredictable results^[8]. The discovery of chemical compounds from natural products that are suitable for use as anticancer drugs has several limitations, including low yield and regulations on plant extraction, implemented in various countries^[9]. Moreover, only a small fraction of cytotoxic natural products are effective in clinical applications. Therefore, anticancer drug discovery from natural products requires a straightforward and effective method to assess anticancer activity. Additionally, the biodiversity of natural products suggests great potential for the discovery of novel natural anticancer agents.

Despite enhancements in molecular targeting in pre-clinical drug development, testing new molecules requires cytotoxic assays to quickly assess a particular chemical compound^[10]. Thus, the need for improved *in vitro* assays to evaluate and predict cytotoxic effects is increasing in anticancer drug discovery. Furthermore, investigating

¹ to whom correspondence may be addressed. E-mail: nina@usd.ac.id
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apoptosis in cancer cells is critical to evaluating cytotoxic activity. The efficacy of anticancer agents is assessed by their ability to differentiate between cancerous and non-cancerous cells and selectively promote apoptosis[11].

Selecting the most suitable drug screening assay is crucial to minimizing the failure rate in drug discovery. This review highlights *in vitro* anticancer assays used for natural products during the new drug discovery. Diverse methods are reviewed in detail and their advantages and disadvantages are considered.

2. Progress in anticancer drug screening

Anticancer drug screening aims to identify natural metabolites that exhibit antitumor activity. Selected compounds are usually toxic, and their anticancer activity is assessed in humans during the clinical phase[24]. In pre-clinical trials, various platforms can be designed using *in vitro* and *in vivo* testing. However, the ideal platform should be fast, simple, inexpensive, and with predictable pharmacodynamic activity. Between 1955 and 1975, *in vivo* tests approved by the National Cancer Institute Developmental Therapeutics Program were the method of choice for anticancer drug screening.

Before the 1950s, transplantable mouse tumors were utilized to prescreen anticancer drugs, followed by human xenografts to better predict anticancer activity[12]. In the early 1990s, this prescreening assay was replaced by the human *in vitro* cell model[24] using 60 different types of human cell lines that screened up to 20 000 compounds per year for anticancer activity. Furthermore, anticancer drug testing is an arduous and expensive process, requiring up to 15-20 years and costing approximately US\$ 900 million to test 5 000 compounds during the initial screening steps, with only one or two compounds eventually being clinically approved[13,14].

Several natural products from plants and antimicrobial fermentation have resulted in the discovery of effective anticancer agents. Vincristine, vinblastine, and vindesine are vinca alkaloids with potent anticancer activity[5]. Other compounds derived from microbial fermentation include actinomycin D, doxorubicin, mitomycin C, and bleomycin[15]. Some of the recent advances in anticancer drug discovery include the use of chemical compound library synthesis and high-throughput screening (HTS), which can simultaneously screen many compounds[16]. However, discovering chemicals from natural sources has several drawbacks during the isolation and separation procedures, such as low product yield and regulations regarding plant extractions implemented by different countries[9]. Drug repurposing is another promising strategy for administering marketed therapeutic drugs used for other ailments to treat cancers. For example, in our previous study, the anti-inflammatory drug celecoxib showed cytotoxicity against HeLa cervical cancer cells by inhibiting p53, a tumor suppressor gene[17]. However, most synthetic

compounds have unpredictable results[16]. Therefore, finding new anticancer entities from natural products is receiving great attention in countries such as Indonesia, which has high plant biodiversity.

The success of anticancer drug development is dependent on the close integration of three major components: targeting, treatment, and technology. Thus, anticancer drug discovery and development have shifted from cytotoxic chemotherapy to personalized medicine, in which molecular-targeted therapy is exploited[18]. Researchers are now focusing on platforms for cancer treatment that involve specific targets of cancer cells. Targeted treatment deploys multiple molecular mechanisms, including proliferation inhibition, immune regulation, apoptosis induction, metastasis suppression, and multidrug resistance reversal[19]. One example of modern molecular-targeted therapy is trastuzumab which treats human epithelial receptor (HER2)-overexpressing breast cancer[20]. Estrogen receptors and HER2 are the most effective breast cancer therapies to date[21].

Despite improvements in molecular targeting in pre-clinical drug development, the testing of new molecules requires cytotoxic assays to quickly assess the chemical compounds under investigation[10]. Thus, improved *in vitro* assays are required to evaluate and predict cytotoxic effects during anticancer drug discovery. Furthermore, the investigation of apoptosis in cancer cells is an effective way to evaluate the cytotoxic activity of chemical and natural compounds. The efficacy of anticancer agents is determined by their ability to differentiate between cancer and non-cancer cells and selectively promote apoptosis[11].

3. *In vitro* cytotoxic assays

Several methods have been established to measure cytotoxic activity and calculate the number of viable cells during anticancer drug screening[22]. Cell viability is defined as the proportion of healthy cells within a population. In general, cell viability assays are employed to measure the response of cells to toxic agents and are widely used for drug screening. Various markers have been used to measure metabolically active cells, such as enzyme activity, cell membrane permeability, cell adherence, adenosine triphosphate production, co-enzyme production, and nucleotide uptake activity[22]. Here, we discuss seven *in vitro* cytotoxic assays: cellular enzyme- and protein-based assays, electric cell-substrate impedance sensing, cell-based assays dependent on DNA synthesis, dye exclusion assays, colony formation assays, real-time cell proliferation monitoring, and live-cell imaging.

3.1. Dye exclusion assay

Dye exclusion assays exploit the permeability of the cytoplasmic membrane to measure the number of viable cells[23] by applying

various dyes, such as trypan blue, eosin, Congo red, and erythrosine B, to cell culture. Viable cells can prevent the passage of these molecules through the membrane owing to their high selectivity, whereas dead cells cannot[24]. Trypan blue has become one of the most feasible methods for measuring cell viability. With concentrations ranging from 0.04%-0.4%, this inexpensive and efficient dye is especially useful for monolayer cell culture models[25]. Trypan blue is a negatively charged molecule that enters dead cells through their damaged membrane, thereby turning them blue. Meanwhile, living cells exclude the dye, thus appearing clear under a light microscope[22,26]. Eosin Y and Congo red are also effective dyes for visualizing cells under a light microscope; Eosin Y selectively stains the cytoplasm, collagen, and muscle fibers, whereas Congo red stains the cytoplasm[27,28].

3.2. Colorimetric assays

One of the hallmarks of cancer is uncontrolled cell proliferation, which augments the number of viable cells in a system. Cell-based proliferative assays are based on cellular enzyme activity, DNA synthesis, membrane integrity, and adenosine triphosphate levels, which are indicators of both viable and dead cells[29]. Several colorimetric and fluorometric assays have been developed for cancer drug screening. These methods are based on the measurement of biochemical markers to determine cellular metabolic activity. A

substrate undergoes a measurable color change by an intracellular enzyme in living cells, such that the number of colored cells is proportional to that of viable cells. Applying colorimetric assays to natural products is challenging since some extracts or fractions are inherently colorful, thereby limiting assessment of their efficacy under visible light.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is a widely used colorimetric assay for initial drug discovery. MTT can pass through the cell membrane owing to its lipophilic side groups and net positive charge. The tetrazolium salt is then reduced to an insoluble formazan complex by the nicotinamide adenine dinucleotide (NADH) dehydrogenase enzyme, which is present in the mitochondria of viable cells at 37 °C (Figure 1A). The solubilizing agents sodium dodecyl sulfate, acidified isopropanol, dimethyl sulfoxide, and dimethylformamide dissolve the formazan complex and are then quantitatively measured at 570 nm using a spectrophotometer[24]. Solubilization of the formazan complex is considered a time-consuming step and requires modification. Dead cells lose their ability to convert MTT to formazan products; therefore, the intensity of color change is directly proportional to the viable cell number in the culture[30]. In some cases, cell lysis is crucial for dissolving formazan because crystals are formed intracellularly. However, the mechanism underlying the solid formazan crystal dissolution remains unclear.

To overcome the time-consuming process of dissolving the

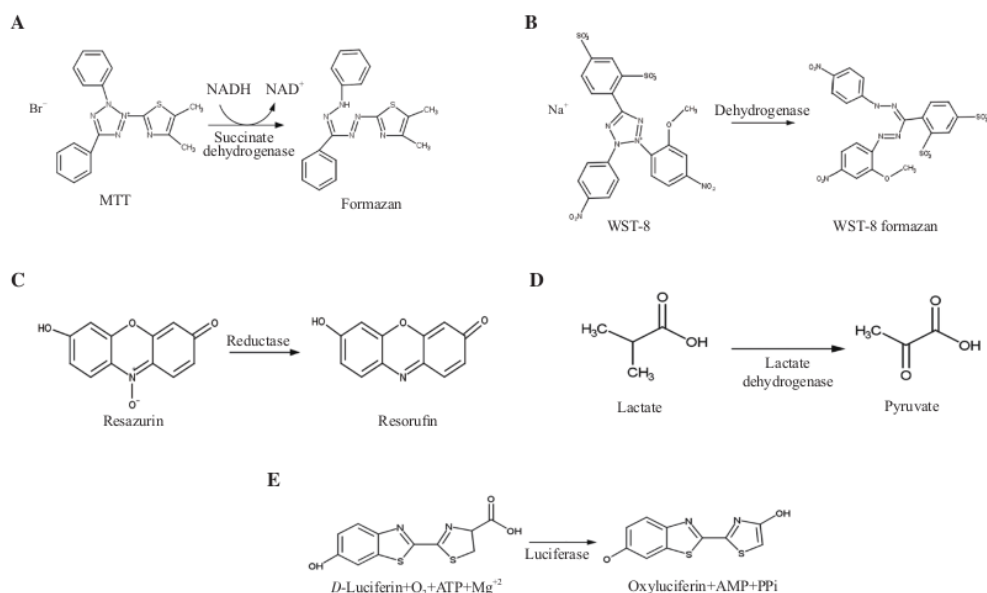


Figure 1. (A) Conversion reaction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. (B) Conversion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) to the formazan salt. (C) Conversion of resazurin to resorufin. (D) Reduction of lactate to pyruvate. (E) Luciferin oxidation to oxyluciferin. ATP: adenosine 5'-triphosphate; AMP: adenosine monophosphate. The structures are drawn using MarvinSketch.

formazan complex, water-soluble tetrazolium (WST) assays have been developed, the most common one being the WST-8 assay [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]. WST-8 was first used by Tominaga in 1999 as a second-generation tetrazolium salt. This salt has high solubility due to the negatively charged sulfone groups on the phenyl rings, which compensate for the positive charge of the tetrazolium ring (Figure 1B)[31]. This negatively charged dye can permeate cells and is extracellularly reduced to tetrazolium[30]. The number of viable cells is proportional to the amount of reduced WST-tetrazolium, quantified by measuring absorption at 450 nm[30], thus allowing real-time assays to be performed[32].

Another type of dye, resazurin, which was discovered by Weselsky, is non-fluorescent and converted to resorufin by NADH, nicotinamide adenine dinucleotide phosphate (NADPH), or flavin adenine dinucleotide (FADH)-dependent oxidoreductase enzymes in viable cells[30]. In the late 1920s, cell metabolic activity was used to measure bacterial contamination. Since then, redox dyes have been used to determine the active metabolism of cells for various applications. Resazurin is a physiological buffer-soluble compound with deep blue color in a solution that allows its direct application in cell culture[33]. Once resazurin diffuses through the cell membrane, it is converted to resorufin, which emits a pink color (Figure 1C). When excited at 579 nm, resorufin has an emission wavelength of 584 nm[24].

Lastly, the lactate dehydrogenase (LDH) assay quantifies viable cells based on LDH activity interconverting pyruvate to lactate, accompanied by the oxidation of NADH to NAD⁺ in the medium (Figure 1D)[29,34]. In various human tissues, five isoforms of LDH are present in different quantities and with different specificities and kinetics[34]. Cell numbers can be quantified by releasing LDH through cell lysis and measuring the absorbance values at 490 nm, which represents all LDH activity[34]. Thus, modification of the LDH assay can determine the percentage of cell death and inhibition in a single experiment[35].

3.3. Adenosine 5'-triphosphate (ATP) assay

ATP is a principal molecule of energy storage in living cells and is synthesized in the mitochondria. ATP is transported intracellularly and supplies energy for biochemical processes in cells. Since it is an indicator of living cells, measuring the total cellular ATP enables determination of cell viability and cytotoxicity[22]. During cell death, ATP synthesis is halted, and the remaining ATP is degraded by ATPase as the cells lose their membrane integrity[24]. The ATP assay, also called the luciferase assay, is based on the oxidation reaction of luciferin with cellular ATP, catalyzed by added natural firefly luciferase (Figure 1E). The reaction produces light proportional to

the number of viable cells and can be measured using a luminometer.

The luminescence signal is relatively stable and can be measured within a few hours. Overall, the assay is sensitive and can measure a signal when there are more than 50 cells that exist. Moreover, ATP assay can predict the sensitivity of platinum-resistant epithelial ovarian cancer to anticancer agents[36]. However, it cannot distinguish ATP depletion due to the cytotoxic or cytostatic effects of the drugs, or changes in the cell number or metabolic activities[37].

3.4. Colony formation assay (clonogenic assay)

The colony-forming assay, also known as the clonogenic assay, is an *in vitro* assay based on the ability of a single cell to form a colony of at least 50 cells[38,39]. This assay is recognized as the gold standard for ionizing radiation therapy in cellular systems since it was first introduced by Puck and Marcus in 1956[39]. Despite this reputation, colony-forming assay is a time-consuming method that measures the number of colonies through manual counting under a light microscope[38]. This assay can detect the cytotoxic effect of any agent, regardless of its mechanism, if it affects the cell's ability to produce a lineage or kills reproductive cells as a result of chromosome damage and apoptosis. Although many different assays can detect various processes that affect cell numbers, such as proliferation[40], apoptosis[57], and senescence[42], colony-forming assay can only measure the ability of cancer cells to form colonies after treatment.

3.5. Electric cell-substrate impedance sensing

Electric cell-substrate impedance sensing is used to study real-time cell attachment, growth, morphology, function, and motility on a solid substrate[43,44]. This technique investigates the invasiveness of cancer cells, barrier function of endothelial cells, cell-cell and cell-extracellular matrix (ECM) interactions, signaling pathways for drug recovery, and wound-healing processes[44,45]. Impedance increases during the adherence and spread of cells over the electrodes (Figure 2). However, it decreases during wound healing and cell death. In electric cell-substrate impedance sensing experiments, cells are directly seeded in a confluent state on integrated gold-film electrodes. The proximity of the cell layer to the thin gold electrodes results in extremely sensitive measurements. However, there is no basolateral fluid compartment owing to cell adherence to the electrode[45]. In a previous study, the electrode was designed as a microelectrode array consisting of many uniform single microelectrodes on a shared platform. Every microelectrode could target heterogeneous cell properties instead of providing collective cell responses with fluctuating signals[47]. Therefore, repetition of the set measurements and proper fitting methods for the obtained average data are required.

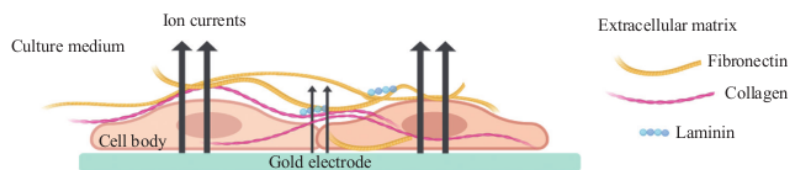


Figure 2. The current pathway in adherence cells at high frequency in electric cell-substrate impedance sensing (>40 kHz), modified from Benson *et al*[46].

4. Cell migration and invasion assay

In biology, migration is a process in which a cell moves within tissues or between different organs. Thus, invasion is defined as the cellular penetration of tissue barriers by passing through the basement membrane and infiltrating the interstitial tissue[44]. In experimental cell biology, however, migration is defined as the movement of cells on two-dimensional (2D) surfaces without any obstruction. On the contrary, invasion is defined as cell movement in a three-dimensional (3D) matrix because the cell reciprocally modifies its shape and interacts with the ECM[44,48]. Cell migration can regulate normal physiological processes, particularly embryonic and branching morphogenesis, nervous system development[49], gastrulation[50], vascular development[51], wound healing, and immunocyte movement[52]. However, unregulated cell migration might contribute to many pathological phenomena, such as inflammation and cancer metastasis[53]. Cancer cells spread from the primary tumor mass through the blood and lymphatic systems by crossing the basement membranes and endothelial walls to colonize distant organs[53,54]. Hence, understanding cellular migration and invasion is important for fighting cancer. Numerous studies on cancer drug discovery have investigated the role of migration and invasion when studying the effects of novel therapeutic compounds on metastasis progression[54]. In this section, we review *in vitro* assays that are suitable for HTS and less expensive than *in vivo* assays.

4.1. Scratch assay

The *in vitro* scratch assay is an easy, simple, and economical method for measuring cell migration[56]. The first step is to create a scratch or wound on a 2D monolayer cell culture with a tip or insert; then images are captured, and the gap is measured at the beginning and end of specific interval times[55,56]. Cell movement can be calculated from the residual gap between scratched cells, shown as the percentage of wound closure at different time points[57]. The timescale is usually no longer than 24 h to avoid taking into account the effects of cell proliferation and changes in cell survival. Limitations of this assay include the possibility of uneven gaps in the scratch, scratched cells reattaching to the gap area, and the surface

of the polystyrene plate being accidentally scraped off[58]. To solve these issues, cells are seeded directly onto cell culture inserts without wounding[53] scratching the monolayer culture[55,59]. This assay is employed to assess the effects of both soluble compounds and solid materials on cell migration[57].

4.2. Live imaging

Live imaging has been used to track cancer cells to understand the mechanism by which their highly dynamic migratory cell motility is affected by physical, chemical, and molecular aspects[60]. Individual cells are tracked to analyze their collective dynamics and the mechanism by which they contribute to cancer metastasis. Cells are labeled either *ex vivo* or *in vivo* and then individually tracked using optical imaging. The *ex vivo* labeling includes radioactive, paramagnetic, or fluorescent tracers, whereas *in vivo* labeling uses radioactive, fluorescent, or luminescent imaging[61]. The direction and distribution of cell migration are plotted in an angular histogram, later referred to as the rose diagram. A previous study showed that untreated cancer cells moved more randomly than treated cells (Figure 3)[62].

4.3. Transwell assay

One way of determining the effectiveness of anticancer drugs is by investigating their influence on cell migration and invasion. The Transwell assay employs a chamber known as the Boyden Chamber, which is separated into two compartments using a filter pore membrane 3–12 μm in diameter, through which cells migrate[44,63]. The inserted membrane is coated with a collagen I-containing ECM to mimic biological conditions. One limitation of this assay is the high cost of ECM-coated membranes, which was considered in a previous study[9] that developed a paper scaffold to replace the culture platform[44]. Cells are cultured in the upper chamber and can migrate through the membrane pores into the lower chamber, which contains higher serum concentrations or attractants. The cells that have migrated are fixed and stained with a cytological dye (hematoxylin, toluidine blue, or crystal violet) and then quantified. Another method counts migrated cells by dissociating them from the membrane and staining them with a fluorescent dye[64].

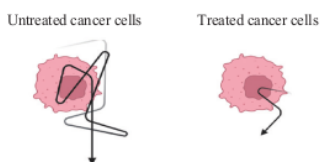


Figure 3. The direction and movement of untreated and treated cancer cells.

5. Apoptosis assay

The word “apoptosis” is derived from Greek and means “falling off,” which references the falling leaves in autumn. It describes the condition under which cells pursue regulated cell death after receiving certain stimuli[65]. Cells undergoing apoptosis exhibit cytoplasmic shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing, and the formation of apparently intact apoptotic bodies that are efficiently phagocytosed by surrounding cells and degraded within lysosomes[65]. Apoptosis is orchestrated and regulated by cellular processes under physiological and pathological conditions[66]. Cancer involves the transformation of a normal cell into a malignant cell while avoiding any type of cell death, particularly apoptosis[67]. Impairment of apoptotic pathways is critical in carcinogenesis, and many novel therapies targeting apoptosis are feasible for application in cancer treatment[66]. Measurement of apoptosis is a critical evaluation of the cytotoxic activity of chemical or natural compounds. Since cancer therapy aims to induce cell death without extensive damage to normal cells, studies that focus on the genes involved in apoptotic signaling have played an important role in oncology research[11].

Thus, the development of apoptosis assays in cancer studies has enhanced the discovery of cancer drugs from natural products. The efficacy of anticancer agents is determined by their ability to differentiate between cancerous and non-cancerous cells and selectively promote apoptosis[11]. Various methods that measure morphological and biomolecular processes, such as DNA content and fragmentation, loss of membrane integrity, other morphological cell features are used to detect apoptosis[68]. Apoptosis assays are the most commonly used method to investigate the anticancer activity of compounds from natural products.

5.1. Double-staining method

In the double-staining method, two fluorescent dyes are applied to cells (acridine orange and ethidium bromide) to identify apoptosis-associated membrane changes, which are then observed under a fluorescence microscope[11]. This protocol can also accurately distinguish between cells at different stages of apoptosis. Acridine orange enters the cells, intercalates into the DNA of normal and early

apoptotic cells, and produces a green fluorescent color. In contrast, ethidium bromide can only penetrate late apoptotic and dead cells through the damaged membrane and bind to DNA fragments or apoptotic bodies to emit an orange-red fluorescence[69].

5.2. Comet assay

The comet assay is a simple, rapid, and sensitive tool that measures DNA damage in eukaryotic cells. This assay, also known as single-cell gel electrophoresis and microgel electrophoresis, uses a microelectrophoresis technique for direct visualization of DNA damage in individual cells[70]. Östling and Johanson introduced this assay in 1984 to observe the effect of irradiation on cells. The cells are lysed, and an electric current is applied to pull the DNA from the nucleus. The DNA is then stained with a fluorescent dye, and the damaged DNA is revealed. The name of this assay is derived from the comet-like appearance of the fragmented DNA[70,71].

5.3. TUNEL assay

Apoptosis is often detected by identifying DNA breakage. DNA fragments of 180-200 bp in size can be separated into a ladder-like line by agarose gel electrophoresis. However, this assay cannot identify the source of fragmented DNA at the single-cell level in a mixed-cell population[72]. TUNEL stands for terminal deoxynucleotidyl transferase dUTP nick end labeling and is a method to detect the blunt 3' hydroxyl ends of double-stranded DNA breaks using terminal deoxynucleotidyl transferase (TdT)[73,74]. This enzyme catalyzes the labeled attachment of deoxyuridine triphosphates (dUTPs) to DNA termini breaks, which are further detected using immunoperoxidase techniques.

This assay is relatively simple and easy to perform, and TUNEL assay kits for single-cell analysis in cell cultures and tissue samples are widely available. The TUNEL assay is a reliable method for detecting apoptosis in a histochemical model for tissues and adherent suspension cell lines. The sensitivity of this assay is due to the use of specific markers, such as bromodeoxyuridine labeling (BrdU), fluorescein labeling, thymidine analogs, 5'-ethynyl-2-deoxyuridine labeling (EdU)[73]. DNA breakage is not a unique feature of apoptosis and also exists in necrosis[75,76]. Therefore, the accuracy of the TUNEL assay in detecting apoptosis, in particular, has been examined in several studies.

5.4. Flow cytometry

Flow cytometry is the most common method for identifying dead cells, revealing the mode (apoptosis or necrosis) as well as the mechanism of cell death[77]. Hallmarks of apoptosis include membrane blebbing, nucleus fragmentation, decreased cellular

time, and the formation of apoptotic bodies. In contrast, the morphological features of necrosis are early plasma membrane rupture, rapid cytoplasmic, nuclear swelling, and organelle breakdown, which induce immune responses[78,79]. Although apoptosis and necrosis have distinct morphological features, distinguishing between these processes is difficult[80].

Flow cytometry measures changes in cell morphology and chromatin condensation using laser light beam scattering[77]. Annexin V propidium iodide (PI) staining enables distinction between apoptosis and necroptosis at the single-cell level. Annexin V is a polypeptide that binds to phospholipids and phosphatidylserine but cannot enter the cell. At an early apoptotic stage, cells stain annexin V positive and PI negative due to their intact membrane preventing PI staining. In contrast, membrane damage occurs at a late apoptotic or early necroptotic stage, which enables the PI membrane-impermeable dye to bind directly to DNA[80]. To distinguish cellular phenotypes precisely, such as proliferation, division, necrosis, and apoptosis (Figure 4A & 4B), imaging flow cytometry has been developed to combine the strengths of microscopy and flow cytometry, which enables the high-throughput characterization of cells in populations on a more precise microscopic scale[41].

6. Challenges and insights of anticancer drug screening of natural products

Current methods are insufficient for the effective *in vitro* HTS of small molecules and characterization of the molecular metastatic cascade complex. Most studies on cell migration are limited to endpoint assays. New *in vitro* time-lapse microscopy approaches, complex metrics analysis, and the downstream interpretation of motility findings are daunting but essential challenges for researchers in this field.

6.1. Escaping the apoptosis mechanism

Numerous plant-derived compounds such as quercetin, resveratrol,

lycopene, kaempferol, curcumin, epigallocatechin gallate, and capsaicin can kill cancer cells by triggering apoptosis *via* inhibiting several important pathways, including the death receptor apoptotic pathway (extrinsic apoptosis pathway), mitochondrial pathway (intrinsic apoptotic pathway), NF- κ B signaling pathway, MAPK signaling pathway, and PI3K-Akt pathway[81,82]. However, solid tumor-derived cell lines under treatment can recover from apoptosis during a process called “anastasis,” from the Greek word meaning “resurrection”[73]. Tumor-derived cell lines are used to identify outliers that appear as “dead cells” under conventional pre-clinical assays. These outliers remain viable and active, secrete growth factors, resist chemotherapy, and proliferate actively. Although some natural products might induce non-canonical cell death, such as ferroptosis, cancer cells can be killed by radicals or oxidants induced by drugs[6]. Therefore, using natural products as chemopreventive agents remains a promising strategy.

6.2. Compound complexity of natural products

Natural product-based chemical compounds are a cocktail of substances capable of targeting multiple cancer signaling pathways and serve as a vigorous form of cancer therapy by increasing effectiveness, preventing drug resistance, and decreasing the necessary dose to achieve responses[83]. Additionally, natural products are typically low in cost and have few side effects, making them attractive as cancer treatments. Many natural products have multiple cancerous cell targets. For instance, curcumin[84,85], astragaloside IV[86], and emodin[87,88] can induce apoptosis in cancer cells and modulate the immune response to tumor formation. Additionally, paclitaxel, one of the most common naturally derived chemotherapeutic-targeting microtubules, also successfully inhibits angiogenesis by suppressing vascular endothelial growth factors at low concentrations[89].

However, having multiple targets comes at a price, and natural products might have low efficacy at low concentrations, necessitating anticancer drug discovery based on HTS. Complex mixtures of compounds, including phenolic compounds and plant

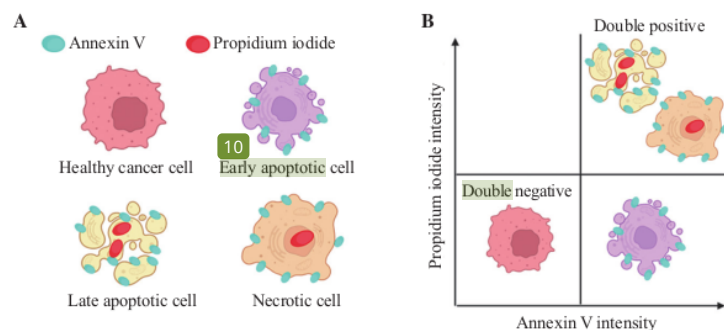


Figure 4. Schematic illustration of the staining strategy in flow cytometry. (A) Identification of healthy cancer cells (double-negative), early and late apoptosis (annexin V-positive and double-positive, respectively), and necrotic (double-positive) cell populations. (B) Gating illustration of the cells in flow cytometry. Adapted from Pietkiewicz *et al*[80].

tannins, function as protein binders, which might give false-positive results in HTS. Therefore, crude plant extracts cannot be used⁹. However, detanninization might reverse this problem by removing the interfering tannins. Despite the potential of natural products in anticancer therapy, most are considered immature candidates for drug therapy, and interest in natural products in anticancer drug discovery is declining⁹⁰. Hence, a novel strategy is to utilize natural products in medicinal chemistry. In identifying lead compounds, bioactivity-directed fractionation can be applied in anticancer drug discovery. A refined approach involves pre-fractionating a crude extract into an almost pure compound for HTS¹³. As discovery methods for treatments develop, the interest in natural product research has been guided towards molecular-targeted compounds. To date, small-molecule targeted compounds have shown distinct selectivity in every type of cancer cell. Thus, their small size (molecular weight less than 1000) allows them to bind to multiple intracellular and extracellular targets by oral administration^{91,92}.

6.3. Bioavailability issues of compounds derived from natural products

While the use of natural products lowers the cost of cancer care, their clinical use is limited due to their low solubility, rapid metabolism, and poor bioavailability⁹³. Although natural products have significant effects *in vitro* using cell cultures, as well as *in vivo* in animal models, their clinical efficacy is low⁹⁴. For instance, genistein inhibits breast and prostate cancer cells by regulating vital genes involved in cell proliferation, apoptosis, the cell cycle, oncogenesis, and signaling pathways⁹⁵. However, the clinical trial did not reveal breast cancer inhibition in healthy high-risk Western women, suggesting its low efficacy in breast cancer therapy⁹⁶. The limiting factor for genistein efficacy is poor bioavailability due to extensive phase II metabolism via glucuronidation and sulfonation in the intestine and liver⁹⁷.

Curcumin is a well-known chemopreventive compound found in turmeric species and exhibits numerous *in vitro* and *in vivo* anticancer activities in various types of cancer⁹⁸. However, studies in humans have revealed poor bioavailability, even at high doses (12 g/day). The low curcumin plasma level is a consequence of poor absorption, low aqueous solubility, and extensive metabolism, resulting in its rapid elimination from the human body⁹⁸. Although curcumin is considered safe to consume and pharmacologically active against cancer, its pharmacokinetic properties revealed poor bioavailability after oral administration in human studies. One study suggested that 1.0 g/kg of curcumin provided 0.13 g/mL in the plasma after 15 min, reaching a maximum of 0.22 µg/mL after 1 h, followed by a rapid decline to below the limit of detection within 6 h⁹⁸. Therefore, designing a curcumin delivery system with good bioavailability is a promising strategy for cancer chemoprevention.

Another strategy to improve natural product bioavailability is to use nanotechnology. Unfortunately, drug nano-formulations can raise toxicity levels and are often rapidly eliminated by the mononuclear phagocytic system⁹⁹. Currently, exosome delivery is considered a novel strategy for improving the pharmacokinetics of natural products. Exosomes are extracellular vesicles with 30-150 nm in diameter secreted by various types of cells, including cancer cells. They have a high drug-carrying capacity that is non-immunogenic and have a low-toxicity profile. Exosomes extend drug circulation in the blood, reducing the elimination rate and protecting drugs from degradation. Thus, application of this exosome and the functional and safety properties of exosome delivery should be elucidated⁹⁹.

7. Conclusion and prospects

Natural products are promising sources of biologically active compounds that could be used against cancer cells. The discovery of new natural product-based anticancer entities with a high degree of biodiversity is still receiving enormous attention in Indonesia. To enhance new anticancer drug discoveries, cell-based *in vitro* assays and techniques have been well developed. Cytotoxic assays based on cellular metabolic activity and cell migration assays are still popular for screening anticancer agents from natural products. Furthermore, apoptosis has been extensively studied to investigate the response to chemotherapy and to improve therapeutic outcomes as a goal of translational and personalized medicine. However, screening anticancer compounds from natural products has limitations, such as their ability to escape from the apoptosis mechanism, their natural compound complexity, and poor bioavailability. Therefore, only a few pure compounds from cytotoxic natural products demonstrate effects in clinical cancer therapy. Thus, the goal of anticancer drug screening of natural products is to overcome these limitations by designing effective drug delivery methods and focusing on bioassay-guided molecular-targeted compounds. The bioassay-guided isolation addressing cancer cells could be a solution for specifically targeting active compounds and avoiding random screening. Another strategy could be to use a bioinformatics approach and perform *in silico* studies before *in vitro* anticancer screening to predict compounds that will interact with the target protein in the cancer cells. From the many well-developed assays, choosing the correct one is key to success in anticancer drug screening. This review summarizes the different *in vitro* assays used in anticancer drug discovery to aid researchers in selecting those most appropriate.

Conflict of interest statement

We declare no conflict of interest in this review.

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Authors' contributions

AS was responsible for the main concept, literature search, manuscript outline, and writing the introduction and future perspective. DSC wrote the *in vitro* cytotoxic assay section. FDES and VKP produced the chemical reactions and cartoons in the figures. DSC, DS, and YSH were responsible for literature search, manuscript preparation, and manuscript editing.

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