Trop J Nat Prod Res, June 2024; 8(6):7459-7465 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

The Cytotoxic Effects, Stimulation of p53, Caspase 3, and Bax by Potent Fractions Derived from the *Leptastrea purpurea* Sponge

Haryanto Susanto[1,2]*, Nashi Widodo[3], Masruri Masruri[4], Siti Mariah Ulfa[4], Nur Fitriana[5], Rollando Rollando[2]

[1]*Doctoral Student, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia.*
[2]*Pharmacy Department, Faculty of Health Sciences, Universitas Ma Chung, Malang, Indonesia.*
[3] *Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia.*
[4]*Department of Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia.*
[5]*Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research and Innovation Agency, Bogor, Indonesia.*

ARTICLE INFO

Article history:
Received 11 April 2024
Revised 23 May 2024
Accepted 31 May 2024
Published online 01 July 2024

ABSTRACT

Breast cancer remains a significant challenge in oncology, necessitating the exploration of novel therapeutic options. This study investigates the anticancer effects of ethanol and n-hexane fractions derived from the methanol extract of the *Leptastrea purpurea* sponge on T47D breast cancer cells. Employing cytotoxicity assays (MTT and WST-1), mitochondrial transmembrane potential assays, and protein expression analysis of p53, Caspase-3, and Bax, we aimed to elucidate the mechanisms underlying their potential anticancer activity. LC-MS/MS analysis was utilized to identify active compounds within the fractions. The results of the experiment demonstrate that the ethanol fraction exhibits significant efficacy against T47D breast cancer cells, with an IC_{50} value of 25.36 µg/mL indicating potent cytotoxicity. At a concentration of 1508 µg/mL, it induces cell death in 95% of T47D cells, highlighting its specificity in targeting breast cancer cells. Comparative analysis with cisplatin reveals a 9.92% higher rate of cell death induced by the ethanol fraction, suggesting superior apoptosis induction capability. Flow cytometry results show increased p53 expression at concentrations of 754 and 1508 µg/mL, with a concentration-dependent pattern. Furthermore, notable increases in Bax protein expression at concentrations of 377 and 754 µg/mL suggest the involvement of the ethanol fraction in regulating apoptosis. In conclusion, the ethanol fraction demonstrates promising potential as a cytotoxic and apoptosis-inducing agent for breast cancer therapy. These findings underscore the importance of exploring natural products from marine sources as potential anticancer agents.

***Keywords*:** Cytotoxic, P53, Caspase 5, Bax, *Leptastrea purpurea*

Introduction

Breast cancer, a leading cause of cancer-related mortality, demands innovative therapeutic strategies due to its widespread impact, affecting both women and men.[1] In 2023, an estimated 1,958,310 new cancer cases and 609,820 cancer-related deaths in the United States underline the urgent need for advancements in cancer treatment.[2] Among the various subtypes, triple-negative breast cancer (TNBC) is particularly challenging to treat because it does not respond to standard breast cancer therapies, making it a focal point for research.[3]

Cisplatin has shown promise in treating TNBC, but its application is severely restricted by cardiotoxic side effects, limiting its long-term usability.[4] This underscores a significant gap in treatment options for TNBC, necessitating the development of alternative therapies that are both effective and have fewer adverse effects.[5]

Marine organisms, with their unique biochemical properties, have emerged as a promising source of novel anti-cancer compounds.

*Corresponding author. E mail: haryanto63@student.ub.ac.id
Tel: +628885558822

Citation: Susanto, H, Widodo, N, Masruri, M, Ulfa SM, Fitriana, N, Rollando, R. The Cytotoxic Effects, Stimulation of p53, Caspase 3, and Bax by Potent Fractions Derived from the *Leptastrea purpurea* Sponge Trop J Nat Prod Res. 2024; 8(6): 7459-7465. https://doi.org/10.26538/tjnpr/v8i6.19

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The sponge *Leptasrea purpurea*, located on Kondang Merak beach in East Java, Indonesia, has garnered attention for its potential in yielding new cancer-fighting drugs.

The rich chemical diversity of marine species provides a vast reservoir of natural substances that could lead to more effective and less toxic cancer therapies.[6]

Research in cancer prevention has increasingly focused on antioxidants and apoptosis mechanisms.[7] Antioxidants neutralize free radicals-highly reactive molecules with unpaired electrons that can damage DNA, proteins, and lipids-thus protecting cells from damage and reducing cancer risk and progression.[8] Apoptosis, the programmed cell death process, maintains cellular balance and tissue integrity by eliminating dysfunctional cells, making it a crucial target for cancer therapies.[9,10]

This research aims to bridge the gap by identifying new compounds that can effectively induce apoptosis and enhance antioxidant defense mechanisms in cancer cells, particularly TNBC. By exploring compounds derived from *Leptasrea purpurea*, the study seeks to understand their potential efficacy and mechanisms of action as alternative treatments for breast cancer. Advancing this research is vital for oncology, offering hope for less toxic and more effective therapeutic options for patients battling this challenging disease.

Materials and Methods

Materials

The 4T1 (ATCC® HTB-133) breast cancer cells were sourced from Brawijaya University, Indonesia, and cultured in a 100 ml medium of RPMI supplemented with HEPES, sodium bicarbonate, 10% FBS, 1% Penicillin Streptomycin, and 0.5% Fungizone. Tripsin-EDTA 0.25%

aided cell preparation. MTT reagent for cytotoxicity was diluted in PBS, while washing utilized PBS (pH 7.4) with HPLC grade KCl, NaCl, Na_2HPO_4, and KH_2PO_4. The stopper reagent comprised 10% b/v SDS in 0.01 N HCl. WST-1 reagent solution included WST-1, 1-methoxy PMS, and hydroxyethyl)-1-piperazineethanesulfonic acid buffer. Annexin-FITC was diluted in a binding buffer containing HEPES, NaCl, KCl, $MgCl_2$, $CaCl_2$, and propidium iodide. All components were meticulously sourced and prepared for the experiments.

Collection and identification of sponge

The sponge *Leptastera purpurea* was collected in July 2023 from Kondang Merak Beach, Malang Regency, East Java, Indonesia (112° 30' 19.80" E - 8° 24' 14.14" S). Dr. Rollando, a botanist, verified the authenticity of the sponge samples and stored voucher specimens (FA:089-MACHUNG-2023) in the Pharmacognosy Laboratory of the Department of Pharmacy at Ma Chung University.

MTT cytotoxicity assay

The cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) test. At the beginning, $8x10^3$ T47D cells were placed in every well of a 96-well plate. On the subsequent day, the cells were subjected to different doses of ethanol, n-Hexane, and cisplatin over a period of 24 hours. Afterwards, 100 microliters of MTT reagent with a concentration of 0.5 milligrams per milliliter were introduced to the cells and allowed to incubate for a period of 2 to 4 hours. After the incubation period, a solution containing SDS and 0.01N HCl was applied to each well. The absorbance at a wavelength of 595 nm was later determined using an ELISA reader, and the percentage of cell viability was computed based on the absorbance data. The IC_{50} value was determined by employing linear regression analysis to demonstrate the correlation between the concentration of the sample and the percentage of cell viability.[11]

WST-1 cytotoxicity assay

In the WST-1 assay, the quantity of formazan dye produced is directly proportional to the metabolic activity of cells. The test was conducted according to the instructions provided by the manufacturer. Concisely, cells were placed in clear 96-well plates and promptly or the following day, exposed to various quantities of particles or similar extracts for either 0 or 24 hours. Afterwards, the cells were rinsed two times with DPBS 1X and placed in a new solution containing 10% WST-1 reagent for a duration of 2 hours. The absorbance was quantified at a wavelength of 450 nm using a multiplate reader, with a reference wavelength of 690 nm being utilized and subtracted. The complete visible spectrum was recorded for certain tests. The results are expressed as relative WST-1 activity, with a value of 1.0 corresponding to the absorbance obtained in the control cultures.[12]

Mitochondrial transmembrane potential assay

The mitochondrial transmembrane potential (ΔΨm) was analyzed using Rhodamine 123 dye (Rh123). It identifies the reduction of ΔΨm, which is linked to mitochondrial death. Loss of mitochondrial membrane potential (ΔΨm) leads to a decrease in the fluorescence of Rh123. The cells treated with AECIR were stained using 10 µL of Rh123 dye (10 µg/mL Rh123 in PBS). The plate was thereafter placed in an incubator at a temperature of 37 °C for a duration of 30 minutes. Afterward, the dye was eliminated by rinsing with PBS before being examined under a fluorescence microscope at a magnification of 200.[13]

Apoptosis assay

A total of $5x10^5$ cells were placed into each well of a six-well tissue culture plate and cultured for 24 hours. The cells were exposed to fractions and cisplatin incubated for 24 hours. Following incubation, both adherent and detached cells were harvested and subjected to centrifugation at a speed of 2000 revolutions per minute for a duration of 3 minutes. Subsequently, the cells were rinsed twice with cold phosphate-buffered saline (PBS). The cells were suspended in 500 µl of Annexin V buffer and thereafter exposed to Annexin V and propidium iodide for a duration of 10 minutes at a temperature of 37 °C. The cells that had been treated were subsequently analyzed using FACS flow cytometry. The bivariate analysis of FITC-fluorescence (FL-1) and PI-fluorescence (FL-3) revealed distinct cell populations. Cells that were negative for both FITC and PI were classified as viable cells. Cells that were positive for FITC but negative for PI were categorized as apoptotic cells. Cells that were negative for FITC but positive for PI were identified as necrotic cells. Lastly, cells that were positive for both FITC and PI were classified as late apoptotic cells.[5]

Expression of p53 and caspase 3 proteins with flow cytometry

T47D cells (1×10^6) were collected and rinsed once in cold PBS. The cell pellets were immobilized in 75% ethanol at a temperature of 4 °C for the duration of one night and subsequently rinsed in cold PBS. Next, the pellets were mixed with 1 mL of a 50 mg/L PI solution, along with 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton X-100. The cell samples were placed in a dark environment and kept at a temperature of 4 °C for a minimum of 15 minutes. Subsequently, they were examined using a FACScan flow cytometer.

Expression of Bax protein with western blotting

T47D cells (2×10^6) were pre-incubated with or without 2 millimoles per liter of 3-methyladenine (3-MA) for 1 hour, and then treated with 64 micromoles per liter of oridonin for 12, 24, 36, or 48 hours. Both adherent and floating cells were gathered and subjected to Western blot analysis, following the previously reported protocol. In summary, the T47D cells were rinsed with cold PBS and dissolved using a lysis solution containing 1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 2 mmol/L leupeptin, and 1 mmol/L aprotinin. The protein concentration was quantified using the Bio-Rad DC protein assay. The protein lysates underwent separation using a 12% SDS-PAGE technique and were subsequently transferred to a nitrocellulose membrane. The membranes were immersed in a blocking buffer solution containing 5% skimmed milk in PBS. Subsequently, they were left to incubate overnight with primary antibodies, and then treated with secondary antibodies conjugated with horseradish peroxidase. The color was generated using diaminobenzidine (DAB).[14]

Compounds analysis

The tandem MS system and LC-MS/MS methodology were employed in conjunction with the UHPLC to analyze our samples. A series of specialized instruments were utilized to conduct the LC-MS analysis: LC-30AD binary pumps, CTO-10ASvp column furnace, DGU-20A3R degasser, and SIL-30AC autosampler. To accomplish the separation of compounds, a reversed-phase C18 Inertsil ODS-4 analytical column measuring 150 mm x 4.6 mm x 3 µm was utilized. The column operated at a temperature of 40 °C. Mobile phase A, consisting of H_2O, ammonium formate (5 mM), and formic acid (0.1%), and mobile phase B, comprising methanol, ammonium formate (5 mM), and formic acid (0.1%), were utilized in the elution gradient. Compounds were eluted through the utilization of a gradient composed of these mobile phases. To maintain the flow rate, the solvent flow was adjusted to 0.5 mL/min, and sample introduction utilized a fixed injection volume of 4 µL. The electrospray ionization (ESI) source was utilized to induce ionization in the air pressure. In order to optimize the conditions for ESI, the following parameters were established: a desolvation line (DL) temperature of 250 °C, an interface temperature of 350 °C, a heat block temperature of 400 °C, and nebulizing gas and drying gas flow rates of 15 mL/min and 3 mL/min, respectively.[15]

Statistical analysis

The cytotoxic activity of both ethanol and n-hexane fractions on T47D breast cancer cells was evaluated across five independent replicates. The effects were quantified using MTT and WST-1 assays, which are standard methods for assessing cell viability and cytotoxic response in vitro. The results are expressed as the mean ± standard deviation (SD) to provide a clear indication of the central tendency and variability of the data. This statistical presentation allows for a comprehensive understanding of the cytotoxic potential and consistency of the response induced by each fraction.

Results and Discussion

Cytotoxic impact of fractions on T47D

The ethanol fraction has a lower IC_{50} value than the n-hexane fraction when tested against T47D cells, as shown in Table 1. Based on the lower IC_{50} value, it looks like the ethanol fraction is more effective at killing T47D cells than the n-hexane fraction. Furthermore, an IC_{50} value below 100 µg/mL indicates that the ethanol fraction has potential as a significant cytotoxic agent.[16] This suggests that the fraction is effective at inhibiting T47D cell growth at relatively low concentrations.

The MTT assay method is based on the enzymatic reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into formazan salt by the enzyme succinate dehydrogenase found in the mitochondria of viable cells. Afterwards, a stopper reagent is introduced, which causes the cell membrane to rupture and the formazan salt to dissolve. The resulting formazan salt is subsequently quantified based on its absorbance. Greater absorbance values indicate a bigger number of viable cells, resulting in increased cell viability. The principle underlying the WST-1 assay method lies in its utilization of a water-soluble tetrazolium salt, WST-1, to assess cellular viability or metabolic activity. Upon introduction into cell cultures, WST-1 is enzymatically reduced by the activity of cellular enzymes, particularly those found within the mitochondria of metabolically active cells. This reduction process converts WST-1 into a formazan dye, which is soluble in aqueous solutions. The intensity of the resulting color formation is directly proportional to the metabolic activity of the cells and can serve as an indicator of cell viability.[17]

Several factors may contribute to the differences in MTT and WST-1 assay results between the ethanol and n-hexane fractions. Firstly, certain components within the ethanol fraction might interact differently with the MTT and WST-1 substrates. These interactions might change how tetrazolium is reduced and how formazan products are made, which could change the results of both tests in the end. Additionally, MTT and WST-1 may exhibit different reaction rates under various conditions. The process of tetrazolium reduction to formazan may occur at varying speeds, depending on factors such as pH, temperature, and enzyme concentration within the cells. These differences in reaction kinetics could lead to discrepancies in the final results between the two assays. Furthermore, the cytotoxic effects of components within the ethanol fraction or their interactions with other compounds in the culture medium may vary in their impact on both types of tests. For instance, a particular compound might be more effective in inhibiting the reduction process of MTT compared to WST-1. In summary, variations in substrate interactions, reaction kinetics, and the cytotoxic influence of components within the ethanol fraction can account for the observed differences in MTT and WST-1 assay results. These factors highlight the importance of considering multiple assay methodologies and conducting thorough validations to ensure robust and reliable conclusions.[18]

The cellular morphology was examined after treatment with the ethanol fraction. The administration of the ethanol fraction and cisplatin resulted in alterations in the physical structure of T47D cells. Cell nuclei were seen to undergo shrinkage, with some cells displaying symptoms of cell death, resulting in an overall decrease in cell count (Figures 1b and 1e). In contrast, cells that were not treated exhibited a typical shape (Figure 1a). This suggests that the ethanol fraction has the capacity to cause structural alterations in T47D cells, which could be a sign of its potential as a substance that is poisonous to cells. Therefore, the morphological response observed in T47D cells following treatment with cisplatin and the ethanol fraction may be influenced by various mechanisms, including DNA damage, activation of the apoptosis pathway, mitochondrial dysfunction, oxidative stress, and interaction with cellular signaling pathways.[19]

Mitochondrial transmembrane potential result

The results indicate that more than 95% of cells experienced changes in mitochondrial membrane potential after being treated twice with the IC_{50} concentration of the test compound (Figure 2). This high percentage suggests that the treatment significantly affects mitochondrial function in the majority of cells. Rhodamine 123 is a fluorescent dye commonly used to assess mitochondrial membrane potential. It accumulates in the mitochondria in a membrane potential-dependent manner. A decrease in fluorescence indicates a decrease in mitochondrial membrane potential, while an increase indicates an increase in potential.[20] Cisplatin is a chemotherapy drug known to induce cellular stress and apoptosis, often by disrupting mitochondrial function. The comparison reveals that the test compound, at its IC_{50} concentration, induces changes in mitochondrial membrane potential in a much larger proportion of cells compared to cisplatin. Specifically, while cisplatin administration causes changes in mitochondrial membrane potential in more than 21% of cells, the test compound affects over 95% of cells (Figure 3). This suggests that the test compound may have a more potent impact on mitochondrial function than cisplatin. The observed difference between the effects of the test compound and cisplatin on mitochondrial membrane potential is described as very significant. This suggests that the test compound may represent a novel and potentially more effective approach for targeting mitochondrial function in the context of cellular treatment or therapy. The results of this analysis could have significant implications for further research and development of treatments targeting mitochondrial function. Understanding how the test compound affects mitochondrial membrane potential may provide insights into its mechanism of action and potential therapeutic applications.[21]

Table 1: The results of cytotoxicity assay against T47D cell line

Fraction	IC_{50} MTT Assay (µg/ml)	IC_{50} WST-1 Assay (µg/ml)
Ethanol	25.36 ± 0.19	753.98 ± 3.82
n-Hexane	630.63 ± 2.31	806.16 ± 4.14

Values area means ± SD, n = 5 replicates

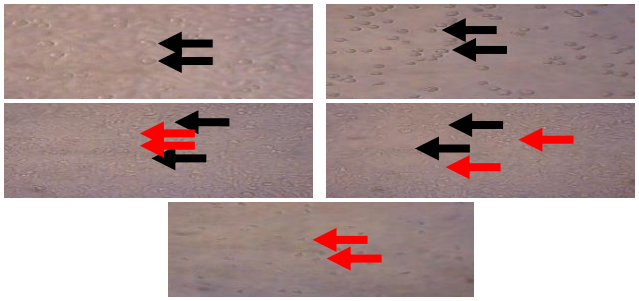


Figure 1: a. Cell control; b. Cisplatin; c. Ethanol fraction (377 µg/mL); d. Ethanol fraction (754 µg/mL); e. Ethanol fraction (1508 µg/mL); The black arrows indicate the normal living cell, whereas the red arrows indicate the cell morphology changing

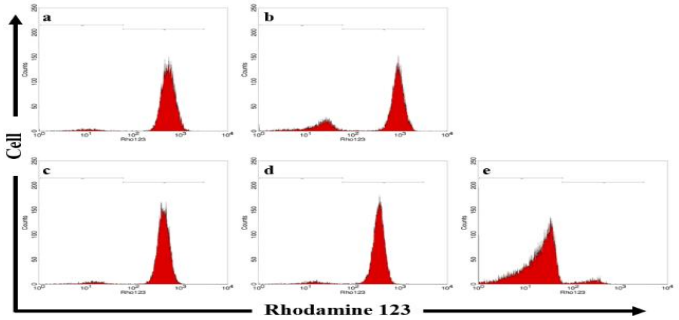


Figure 2: Mitochondrial transmembrane potential result. a. Cell control; b. Cisplatin; c. Ethanol fraction (377 µg/mL); d. Ethanol fraction (754 µg/mL); e. Ethanol fraction (1508 µg/mL)

Apoptosis analysis

The study aimed to assess the influence of the ethanol fraction and cisplatin on the growth of T47D breast cancer cells during a 24-hour timeframe. The inquiry employed the Annexin V assay in combination with flow cytometry to detect the initiation of apoptosis in the treated cells.[22] Annexin V, a protein, has a high attraction for negatively charged phospholipids found on cell membranes. In addition, propidium iodide (PI) staining, which specifically binds to DNA, was used to differentiate between cell death caused by apoptosis and necrosis.[23] Figure 4 displays the results of the apoptosis induction assay performed using flow cytometry. It shows the percentage of cell death caused by apoptosis or necrosis after treatment with the ethanol fraction and cisplatin.

After being exposed to the ethanol fraction and cisplatin, the examination of cell death percentages shows that untreated cells have a viability rate of 95.06% and a matching cell death rate of 4.94%. After being treated with the ethanol fraction, the recorded rate of cell death is 4.81%, but treatment with cisplatin results in a cell death rate of 7.82%. The results indicate that the ethanol fraction treatment leads to a 9.92% increase in cell death compared to cisplatin, indicating that the ethanol fraction triggers apoptosis.

The T47D breast cancer cell line is a subtype that is defined by having wild-type caspase-3 and caspase-7, as well as positive expression of estrogen receptor (ER) and progesterone receptor (PR). Additionally, it has a mutant form of the p53 protein.[24] The induction of apoptosis in these cells is hypothesized to occur through p53-independent pathways.[25] Cisplatin is known for its ability to reduce the levels of Bcl-2 protein in T47D breast cancer cells, leading to the formation of crosslinks in DNA and consequent apoptosis caused by DNA damage. Decreased expression of Bcl-2, a protein that prevents cell death, reduces cell survival and increases sensitivity to chemotherapeutic drugs.[26] Future study should focus on identifying the precise proteins implicated and elucidating the molecular mechanisms that explain the synergistic interactions among the extract components, p53 protein expression, Bcl-2, and NF-κB in T47D breast cancer cells in a laboratory setting.

Expression of p53 and caspase-3 proteins

Flow cytometry analysis was conducted to assess the expression levels of p53 and caspase-3 proteins. The findings revealed a notable upregulation in the expression of all three proteins in T47D cells treated with cisplatin compared to the control group (Figure 5). Specifically, the flow cytometry results indicated a significant increase in p53 expression in T47D cells following treatment with extract concentrations of 754 and 1508 µg/mL. However, at lower concentrations (377 µg/mL), the observed p53 expression did not exhibit a significant difference compared to untreated cells. Consistent with the effects of cisplatin, all three concentrations of the extract led to a significant enhancement in caspase-3 protein expression.[27]

Expression of Bax protein with western blotting

Western blot analysis was employed to assess the expression levels of the Bax protein. The findings revealed a significant increase in Bax protein expression following treatment with concentrations of 377 and 754 µg/mL. However, it was observed that conducting Bax protein expression analysis via western blotting was not feasible in cells treated with higher concentrations. This limitation arises due to the cell destruction that occurs at high treatment concentrations, potentially leading to the mixing of cell proteins in the medium. Consequently, during protein extraction, the protein may be lost along with the medium (Figure 6). These observations were further supported by alterations in cell morphology data, indicating that administration of the highest treatment concentration resulted in cell destruction, with only a few cells exhibiting altered morphology able to survive and adhere to the petri dish.[28]

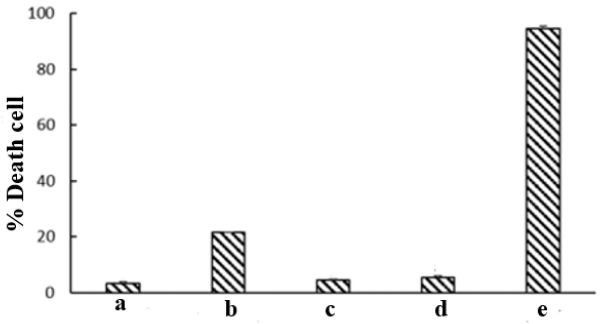


Figure 3: Effects of compounds on T47D cells. a. Cell control; b. Cisplatin; c. Ethanol fraction (377 µg/mL); d. Ethanol fraction (754 µg/mL); e. Ethanol fraction (1508 µg/mL)

Table 2: Compounds have been identified from the fractions

Fraction	Component	Chemical Formula	Neutral Mass (Da)	Retention Time (min)
Ethanol	Valine	$C_5H_{11}O_2$	117.0790	0.51
	14-Acetoxy-7*β*-angeloyloxynotonipetranone	$C_{22}H_{32}O_5$	376.22500	5.68
	14-Deoxy-11-oxoandrographolide	$C_{20}H_{28}O_5$	348.1937	6.90
	(E)-Labda-8(17),12-diene-15,16-dial	$C_{20}H_{30}O_2$	302.2246	8.09
	Arachidonic acid	$C_{20}H_{32}O_2$	304.2402	9.87
	Linolenic acid	$C_{18}H_{30}O_2$	278.2246	9.49
	Candidate Mass (BLP-E1)	$C_{32}H_{51}O_7$	561.3666	7.58
	Candidate Mass (BLP-E2)	$C_{31}H_{47}O_3$	481.3556	8.24
		$C_{33}H_{51}O_3$	509.38690	9.32
n-Hexane	Linolenic acid	$C_{18}H_{30}O_2$	278.2246	9.49
	Arachidonic acid	$C_{20}H_{32}O_2$	304.2402	9.87
	Bis(2-ethylhexyl)phthalate	$C_{24}H_{38}O_4$	390.2770	11.32
	Candidate Mass (BLP-H1)	$C_6H_{15}NO_3$	149.1052	0.48
	Candidate Mass (BLP-H1)	$C_{20}H_{41}NO_3$	343.3086	7.89
	Candidate Mass (BLP-H2)	$C_{22}H_{45}NO_3$	371.3399	8.66

Compounds analysis result

Analyses on the ethanol fraction revealed the presence of nine identified compounds (Table 2). There are six compounds identified by name, namely valine, 14-acetoxy-7β-angeloyloxynotonipetranone, 14-deoxy-11-oxoandrographolide, (E)-labda-8(17),12-diene-15,16-dial, arachidonic acid, and linolenic acid (Figure 7). Then, the analysis results on the hexane fraction showed the presence of six identified compounds. There are three compounds identified by name, namely linolenic acid, arachidonic acid, and bis(2-ethylhexyl)phthalate (Figure 8). Directly, valine itself does not possess an anticancer mechanism. However, as part of a protein, valine plays a role in various biological processes within the human body. Several studies have indicated that a diet rich in specific amino acids, including valine, may influence metabolism and the growth of cancer cells. However, this relationship is complex and requires further research to understand its precise role.[29] The compound 14-acetoxy-7β-angeloyloxynotonipetranone, found in *Plectranthus hadiensis*, has been reported to exhibit cytotoxic activity against MDA-MB-231S cancer cell line cells, with an IC_{50} value of 5.5 µM (2.15 µg/mL).[30] The compound 14-deoxy-11-oxoandrographolide, a derivative of andrographolide found in *Andrographis paniculata*, has been shown to possess cytotoxic activity against HepG2 liver cancer cells with an IC_{50} value of 2.82 µg/mL. Additionally, 14-Deoxy-11-oxoandrographolide has demonstrated the ability to inhibit the formation of new blood vessels required for tumor growth, a process known as angiogenesis.[31] (E)-Labda-8(17),12-diene-15,16-dial is a compound belonging to the group of labdanoids, commonly found in various plant species. This compound has been shown to induce apoptosis mechanisms through the p53 pathway.[32] Linolenic acid has demonstrated inhibition of the proliferation of OS-RC-2 cells, a type of human RCC cell line. PPAR-γ activation and COX-2 inhibition are two signaling pathways involved in the action of ALA on OS-RC-2 cells.[33]

Conclusion

The ethanol fraction is highly effective against T47D breast cancer cells, with an IC_{50} value of 25.36 µg/mL indicating strong cytotoxicity. It induces cell death in 95% of T47D cells at 1508 µg/mL, confirming its ability to target breast cancer cells. Compared to cisplatin, it causes 9.92% more cell death, suggesting superior apoptosis induction. Flow cytometry shows increased p53 expression at 754 and 1508 µg/mL, with a concentration-dependent effect. Bax protein expression also increases significantly at 377 and 754 µg/mL, indicating the ethanol fraction's role in regulating apoptosis. Overall, the ethanol fraction holds promise as a cytotoxic and apoptosis-inducing agent in breast cancer therapy.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors would like to thank the Ma Chung University for support through chemicals and laboratory.

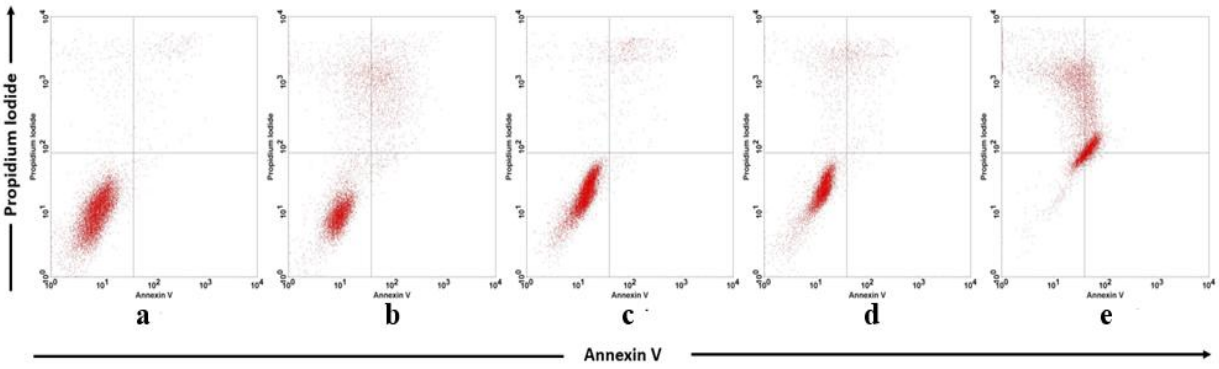


Figure 4: Effects of compounds on T47D cells. a. Cell control; b. Cisplatin; c. Ethanol fraction (377 µg/mL); d. Ethanol fraction (754 µg/mL); e. Ethanol fraction (1508 µg/mL)

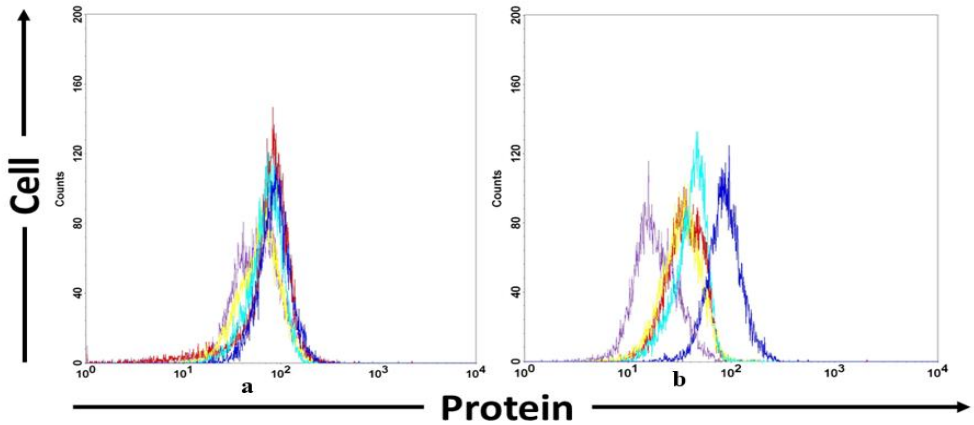


Figure 5: Expression of p53 and caspase 3 proteins with flow cytometry. a. expression p53; b. expression caspase 3. Peak description: Purple = control, Red= cisplatin, yellow= half IC_{50}, tosca= IC_{50}, blue= 2xIC_{50}

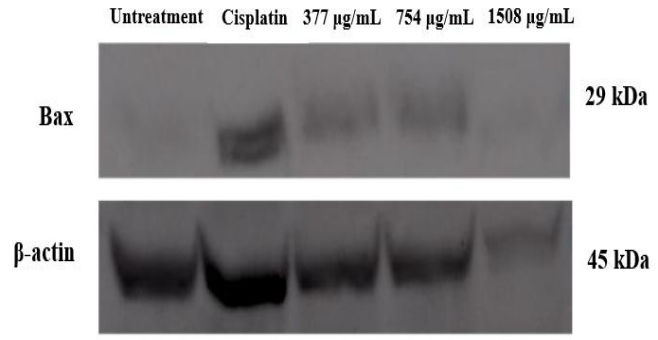
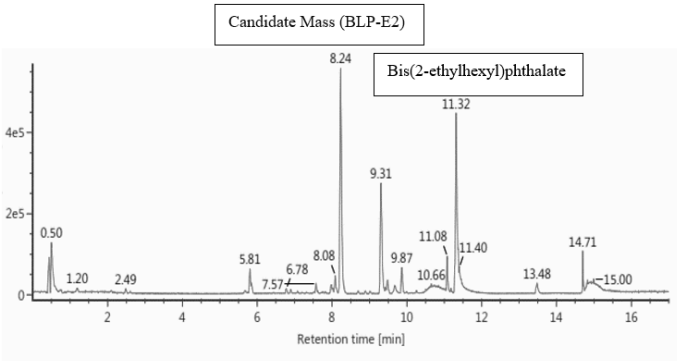


Figure 6: Analysis results of 5.23 µg/mL cisplatin; 377 µg/mL; 754 µg/mL; 1508 µg/mL fraction with western blotting on apoptosis marker protein (Bax)



***F*igure 7:** LC MS/MS chromatogram of ethanol fraction

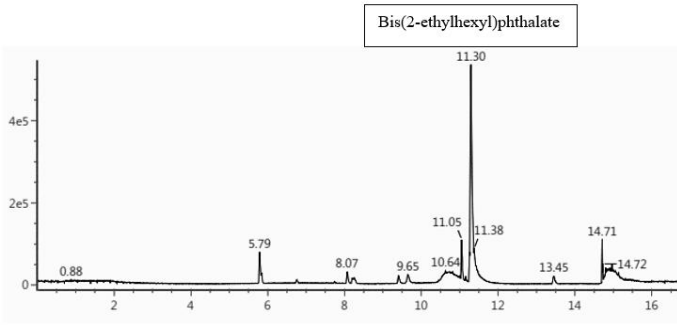


Figure 8: LC MS/MS chromatogram of n-hexane fraction

References

1. Rollando R, Monica E, Aftoni MH. *In vitro* Cytotoxic Potential of *Sterculia quadrifida* Leaf Extract Against Human Breast Cancer Cell Lines. Trop J Nat Prod Res. 2022 1;6(8):1228–32.
2. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023;73(1):17–48.
3. Fitriana N, Rifa'i M, Masruri, Wicaksono ST, Nashi Widodo. Anticancer effects of *Curcuma zedoaria* (Berg.) Roscoe ethanol extract on a human breast cancer cell line. Chem Pap. 2023;77(1):399–411.
4. Zhang Z, Zhang H, Li D, Zhou X, Qin Q, Zhang Q. Caspase-3-mediated GSDME induced Pyroptosis in breast cancer cells through the ROS/JNK signalling pathway. J Cell Mol Med. 2021;25(17):8159–68.
5. Rollando R, Monica E, Afthoni MH, Warsito W, Masruri M, Widodo N. A Phenylpropanoid Compound from the Seeds of *Sterculia quadrifida* and its Cytotoxic Activity. Trop J Nat Prod Res. 2023;7(6):3203–8.
6. Rollando R, Warsito W, Masruri M, Widodo W. *Sterculia foetida* Leaf Fraction Against Matrix Metalloproteinase-9 Protein and 4T1 Breast Cancer Cells: *In-Vitro* and *In-Silico* Studies. Trop J Nat Prod Res. 2021;5(1):113–21.
7. Rollando R, Monica E, Afthoni MH, Warsito W, Masruri M, Widodo N, Zainul R. In-vitro and In-silico Studies of a Phenylpropanoid Compound Isolated from *Sterculia quadrifida* Seeds and Its Inhibitory Effect on Matrix Metalloproteinase-9: http://www.doi.org/10.26538/tjnpr/v7i7.30. Trop J Nat Prod Res. 2023;7(7):3490–5.
8. Hayes JD, Dinkova-Kostova AT, Tew KD. Oxidative Stress in Cancer. Cancer Cell. 2020;38(2):167–97.
9. Gulcin İ. Antioxidants and antioxidant methods: an updated overview. Arch Toxicol. 2020;94(3):651–715.
10. Hariono M, Rollando R, Karamoy J, Hariyono P, Atmono M, Djohan M, Wiwy W, Nuwarda R, Kurniawan C, Salin N, Wahab H. Bioguided Fractionation of Local Plants against Matrix Metalloproteinase9 and Its Cytotoxicity against Breast Cancer Cell Models: In Silico and In Vitro Study. Mol Basel Switz. 2020;25(20).
11. Hariono M, Rollando R, Yoga I, Harjono A, Suryodanindro A, Yanuar M, Gonzaga T, Parabang Z, Hariyono P, Febriansah R, Hermawansyah A, Setyani W, Wahab H. Bioguided Fractionation of Local Plants against Matrix Metalloproteinase9 and Its Cytotoxicity against Breast Cancer Cell Models: In Silico and In Vitro Study (Part II). Mol Basel Switz. 2021;26(5):1464.
12. Guney Eskiler G, Ozman Z, Haciefendi A, Cansaran-Duman D. Novel combination treatment of CDK 4/6 inhibitors with PARP inhibitors in triple negative breast cancer cells. Naunyn Schmiedebergs Arch Pharmacol. 2023;396(5):1031–41.
13. Özerkan D. The Determination of Cisplatin and Luteolin Synergistic Effect on Colorectal Cancer Cell Apoptosis and Mitochondrial Dysfunction by Fluorescence Labelling. J Fluoresc. 2023;33(3):1217–25.
14. Rollando R, Yuniati Y, Monica E. Bioactive Potential of *Cephalosporium* sp. a Fungal Endophyte Isolated from *Phyllanthus niruri* L. Trop J Nat Prod Res. 2023;7(4):2749–55.
15. Liu Y, VanAernum Z, Zhang Y, Gao X, Vlad M, Feng B, Cross R, Kilgore B, Newman A, Wang D, Schuessler HA, Richardson DD, Chadwick JS. LC-MS Approach to Decipher a Light Chain Chromatographic Peak Splitting of a Monoclonal Antibody. Pharm Res. 2023;40(12):3087–98.
16. Hariono M, Nuwarda RF, Yusuf M, Rollando R, Jenie RI, Al-Najjar B, Julianus J, Putra KC, Nugroho ES, Wisnumurti YK, Dewa SP, Jati BW, Tiara R, Ramadani RD, Qodria L, Wahab HA. Arylamide as Potential Selective Inhibitor for Matrix Metalloproteinase 9 (MMP9): Design, Synthesis, Biological Evaluation, and Molecular Modeling. J Chem Inf Model. 2020;60(1):349–59.
17. Dunavári EK, Kőházy A, Vecsernyés M, Szalma J, Lovász BV, Berta G, Lempel E. Does Preheating Influence the Cytotoxic Potential of Dental Resin Composites? Polymers. 2024;16(2):174.
18. Xu W, Shi D, Chen K, Palmer J, Popovich DG. An improved MTT colorimetric method for rapid viable bacteria counting. J Microbiol Methods. 2023;214:106830.

19. Li Y, Zhang T, Song Q, Gao D, Li Y, Jie H, Huang P, Zheng G, Yang J, He J. Cisplatin ototoxicity mechanism and antagonistic intervention strategy: a scope review. Front Cell Neurosci. 2023;17:1197051.
20. Spurlock B, Hanumanthu VS, Mitra K. Strategy of Isolating 'Primed' Tumor Initiating Cells Based on Mitochondrial Transmembrane Potential. Bio-Protoc. 2021;11(5):e3945.
21. Margineantu DH, Gregory Cox W, Sundell L, Sherwood SW, Beechem JM, Capaldi RA. Cell cycle dependent morphology changes and associated mitochondrial DNA redistribution in mitochondria of human cell lines. Mitochondrion. 2002;1(5):425–35.
22. Guefack MGF, Talukdar D, Mukherjee R, Guha S, Mitra D, Saha D, Das G, Damen F, Kuete V, Murmu N. *Hypericum roeperianum* bark extract suppresses breast cancer proliferation via induction of apoptosis, downregulation of PI3K/Akt/mTOR signaling cascade and reversal of EMT. J Ethnopharmacol. 2024;319(Pt 1):117093.
23. Li ZF, Feng JK, Zhao XC, Liu W, Gu SA, Li R, Lu YL, Mao RJ, Xia LL, Dong LL, Zhang LW, Ruan JY, Liu J, Li GF, Li T, Sun R, Qiu SL, Zheng ZZ, Dong T. The Extract of Pinellia Ternata-Induced Apoptosis of Leukemia Cells by Regulating the Expression of Bax, Bcl-2 and Caspase-3 Protein Expression in Mice. Transplant Proc. 2023;55(9):2232–40.
24. Wu Y, Li Z, Wedn AM, Casey AN, Brown D, Rao SV, Omarjee S, Hooda J, Carroll JS, Gertz J, Atkinson JM, Lee AV, Oesterreich S. FOXA1 Reprogramming Dictates Retinoid X Receptor Response in ESR1-Mutant Breast Cancer. Mol Cancer Res. 2023;21(6):591–604.
25. Yusuf H, Novia H, Fahriani M. Cytotoxic activity of ethyl acetate extract *of Chromolaena odorata* on MCF7 and T47D breast cancer cells. Narra J. 2023;3(3):e326.
26. Rollando R. Combination of *Hedyotis corymbosa* l. and *Tinospora crispa* ethanol extract increase cisplatin cytotoxicity on t47d breast cancer cells. Asian J Pharm Clin Res. 2018;171–7.
27. Rollando R, Warsito W, Masruri M, Widodo W. *Pterygota alata* (Roxb.) R.Br. Bark Fraction Induced Intrinsic Apoptotic Pathway in 4T1 Cells by Decreasing Bcl-2 and Inducing Bax Expression. Pak J Biol Sci. 2021;24(2):172–81.
28. Quarato G, Mari L, Barrows NJ, Yang M, Ruehl S, Chen MJ, Guy CS, Low J, Chen T, Green DR. Mitophagy restricts BAX/BAK-independent, Parkin-mediated apoptosis. Sci Adv. 2023;9(21):eadg8156.
29. An S, Yan S, Zhang Y, Xu H, Gan T, Liu Q. Is It Possible to Establish a Tumor-Suppressive Microenvironment With Glycine and Valine Supplement? Cancer Control J Moffitt Cancer Cent. 2020;27(1):1073274820954453.
30. Ntungwe E, Domínguez-Martín EM, Teodósio C, Teixidó-Trujillo S, Armas Capote N, Saraiva L, Díaz-Lanza AM, Duarte N, Rijo P. Preliminary Biological Activity Screening of *Plectranthus* spp. Extracts for the Search of Anticancer Lead Molecules. Pharmaceuticals. 2021;14(5):402.
31. Gao S, Tan H, Gang J. Inhibition of hepatocellular carcinoma cell proliferation through regulation of the cell cycle, AGE RAGE, and leptin signaling pathways by a compound formulation comprised of andrographolide, wogonin, and oroxylin a derived from *Andrographis Paniculata*(Burm.f.) Nees. J Ethnopharmacol. 2024;118001.
32. Shun MC, Yu W, Gapor A, Parsons R, Atkinson J, Sanders BG, Kline K. Pro-apoptotic mechanisms of action of a novel vitamin E analog (alpha-TEA) and a naturally occurring form of vitamin E (delta-tocotrienol) in MDA-MB-435 human breast cancer cells. Nutr Cancer. 2004;48(1):95–105.
33. Yang L, Yuan J, Liu L, Shi C, Wang L, Tian F, Liu F, Wang H, Shao C, Zhang Q, Chen Z, Qin W, Wen W. α-linolenic acid inhibits human renal cell carcinoma cell proliferation through PPAR-γ activation and COX-2 inhibition. Oncol Lett. 2013;6(1):197–202.