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Potential Cytotoxic Activity of Methanol Extract, Ethyl Acetate, and n-Hexane Fraction from *Clitoria ternatea* L. on MCF-7 Breast Cancer Cell Line and Molecular Docking Study to P53

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ABSTRACT

Breast cancer is a condition where the cells in breast tissue lose control and multiply uncontrollably. In this study, MCF-7 breast cancer cells were tested for cytotoxic activity using the MTT assay and the active compound's interaction with the p53 protein was tested in silico. The most active fraction was found to be the ethyl acetate fraction, with an IC50 value of 1.730 µg/mL and a selectivity index of 2.485. However, the selectivity index was less than 3, and Vero cells showed changes in morphology with the addition of the ethyl acetate fraction. GC-MS was used to identify 19 compounds in the ethyl acetate fraction, and in-silico tests were performed on 5 potential anticancer compounds. Lipinski's Rule of Five test showed that only 3 of these compounds could undergo molecular docking. The results indicated that Anethole compound can interact with p53 protein, while Cinnamaldehyde, (E)- can interact with p21 protein.

Key word: Breast Cancer, *Clitoria ternatea* L., GC-MS, MCF-7 Cells, Molecular docking

INTRODUCTION

Cancer is one of the leading causes of death worldwide. According to Globocan data, in 2018, there were 18.1 million new cases with a death toll of 9.6 million, where 1 in 5 men and 1 in 6 women in the world experience cancer. The data also states that 1 in 8 men and 1 in 11 women die from cancer. The incidence rate of cancer in Indonesia (136.2/100,000 population) ranks 8th in Southeast Asia, while in Asia, it ranks 23rd [1]. Breast cancer is a disease characterized by uncontrolled growth and cell division in the breast, leading to the formation of a tumor that can damage surrounding tissue [2). Current treatments for cancer include surgery, chemotherapy, and radiotherapy, with commonly used drugs being antimetabolites, DNA interactive agents, and anti-tubulin [3]. Chemotherapy involves the use of cytotoxic drugs to destroy cancer cells, but multidrug resistance (MDR) can reduce their efficacy. To increase the sensitivity of cancer cells and reduce chemotherapy side effects, some researchers are testing natural ingredients as potential chemopreventive agents to be used in combination with chemotherapy.

Clitoria ternatea L., also known as Butterfly Pea Flower, is a member of the Fabaceae family, characterized by pod-type fruits and commonly found in tropical regions such as Southeast Asia. Due to their widespread distribution, Fabaceae plants have various uses, including as a source of food, feed, reforestation, and traditional medicines (known as Tanaman Obat Keluarga or TOGA.

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This study aims to investigate the potential of *Clitoria ternate* L. as an anticancer agent in the MCF-7 breast cancer cell line. Chemopreventive agents can prevent and inhibit cancer cell development and help in the recovery of cancer patients. They work by inducing cell cycle arrest, triggering apoptotic processes, or inhibiting protein expression in the multidrug resistance mechanism [4]. The findings of this study may pave the way for Butterfly Pea Flower to be used as an alternative treatment for breast cancer.

EXPERIMENT

Materials and instrumentation

In this research, the materials used were the MCF-7 breast cancer cell line (Gadjah Mada University, Yogyakarta), vero cells (Gadjah Mada University, Yogyakarta), *C. ternatea* L. which were determined at Balai Materia Medica Batu, methanol (Jaya Makmur Kimia, Surabaya), ethyl acetate (Ma Chung University), n-hexane (Ma Chung University), DMEM sachet Hepes, $NaHCO_3$, 1N HCl, 1N NaOH, sterile aquabidest, PBS (Phospate Buffered Saline), DMSO (Gadjah Mada University, Yogyakarta), MTT (3,4,5 dimethylthiazole-2-yl)-2,5 diphenyl tetrazolium bromide) (Gadjah Mada University, Yogyakarta), trypsin-EDTA 0.025% (Gadjah Mada University, Yogyakarta).

In this research, the instruments used were analytical balance, rotary evaporator, incubator, inverted microscope, microplate reader, vortex, cell counter, small dropper pipette, beaker, erlenmeyer, centrifugator, petri dish, water bath, tissue culture dish, cryo tube, conical tube, hemocytometer, glass jar, stirring rod, orbital shaker, whatman filter paper, filter cloth, desiccator, vial, 6 well plate, 96 well plate, falcon tubes 15 ml, eppendorf tubes, eppendorf micropipette, Gas Chromatography and Mass Spectroscopy (GC-MS) and laminar air flow (LAF) cabinets.

Extraction

The methanol extract of *C. ternatea* L. was obtained through a process of maceration with methanol at a ratio of 1:3 (w/v) for 48 hours, followed by filtration and another maceration process for 24 hours. The resulting methanol extract was then collected, and the residual solvent was removed using a rotary evaporator at 60 ℃. The extract from the rotary evaporator was then placed in a porcelain dish and in a water bath to obtain an extract with a sticky texture [5].

Fractionation

Methanol extract was fractionated using the trituration concept. The methanol extract was then removed using a rotary evaporator and evaporated using a water bath, then ethyl acetate was added and stirred until two phases were produced. The dissolved phase is called the ethyl acetate fraction and the insoluble phase is then added with n-hexane and stirred until the n-hexane fraction is obtained.

Cytotoxic activity test using MTT assay

To ensure cleanliness, all equipment and materials are cleaned with 70% alcohol before being placed in the Laminar Air Flow. The DMEM media is divided into aliquots in a 15 mL tube to prevent contamination. 2 mL of cells are combined with 12 mL of DMEM media and observed under an inverted microscope to confirm cell presence. After incubating for 24 hours at 37°C and 5% CO_2, 100 µL of the extract and fractions (400, 200, 100, 50, 25, 12.5, 6.25, and 3.125 g/mL) were added to different wells of a 96-well plate containing Vero cells and MCF-7 cells. The plate is then incubated for 48 hours under the same conditions. MTT

and 10% SDS stopper reagent are added to each well, and the absorbance is measured using a microplate reader at a 550 - 600 nm [6].

Identification of compounds using GC-MS

The structure of an active sample was determined using GC-MS. The sample was dissolved in methanol and injected into an Agilent 7890A GC connected to an MS detector. A glass column measuring 35×950 mm was used with helium as a carrier gas. The GC-MS was operated at an initial temperature of 50°C for 2 minutes, then increased to 300°C at a rate of 5°C/min, and held at a final temperature of 300°C. The running time was 30 minutes. The NIST and WELLY databases compared the identified compounds to the TOX Library [7].

Molecular docking test

The first step in this study involved the preparation and validation of the protein. A suitable protein was selected based on criteria such as resolution and Ramachandran values, using PyMOL software. Two proteins, PDB 6HQU and PDB 2ZVW, were identified based on the mechanism of action of MCF-7 breast cancer cells. The compounds produced from the GC-MS test were searched for SMILES and downloaded from the PubChem website. Their literature activity was screened using various online tests such as PASS, ADMET, and Lipinski rule of 5 to determine their potential as medicinal compounds. The protein was then prepared using BIOVIA Discovery Studio to remove water molecules, followed by docking with the PyRx application. The results with the smallest binding affinity and RMSD values, preferably 0, were selected. Finally, the interaction between the compounds and native ligands was visualized using the BIOVIA Discovery Studio application [8].

RESULT AND DISCUSSION

Plant determination

In this study, the *C. ternatea* L flower used as the sample was identified at the UPT Laboratory of Herbal Materia Medica Batu, as part of the determination process which aims to minimize errors in collecting plants used in research by matching and equating them with previously identified plants.

Cytotoxic test

The MTT assay method is used to determine the cytotoxicity of samples by observing the color change of the MTT reagent, which is reduced in response to active cells that have metabolized the reagent through succinate dehydrogenase in the mitochondria. The formazan product formed is an indicator of living cells, and the addition of 10% DMSO is required to stop the reaction and dissolve the formazan crystals. The result is tabulated on Table 1.

Table 1. Results of cytotoxicity test of extract and fractions on MCF-7 and Vero cells

Sample	IC_{50} MCF-7 (µg/mL)	IC_{50} Vero (µg/mL)	Selectivity Index
Methanol Extract	2.57±0.16	2.58±0.12	1.01
Ethyl Acetate Fraction	1.73±0.75	4.30±0.80	2.49
n-Hexane Fraction	1.88±0.67	2.00±0.25	1.06

The methanol extract, ethyl acetate, and n-hexane fraction of *C. ternatea* L. flower were found to have cytotoxic effects on MCF-7 breast cancer cells, as evidenced by decreased cell viability and morphological changes. The ethyl acetate fraction from *C. ternatea* L. flower

exhibited the most potent cytotoxic activity against MCF-7 breast cancer cells. This effect was likely attributed to bioactive compounds in the fraction that targeted the cancer cells through specific molecular groups. However, the low cytotoxic effect could be due to the potential limited activity of the active compounds present in the sample, suggesting that further fractionation and isolation of compounds are necessary [9].

The selectivity index values were calculated for the methanol extract, ethyl acetate fraction, and n-hexane fraction from *C. ternatea* L. flower. These values indicate that these samples are not highly selective in killing cancer cells, as their values are less than 3. However, of the three compounds, the ethyl acetate fraction has the highest selectivity index value, which is closer to the ideal range. A high selectivity index value indicates a more effective and safer drug for in vivo treatment. When the selectivity index value is relatively low (<1), it suggests that the sample may be toxic and unsuitable for use as medicine. If the value is between 1 and 10, it requires re-evaluation in a different environment to confirm its potential as a medicine [10].

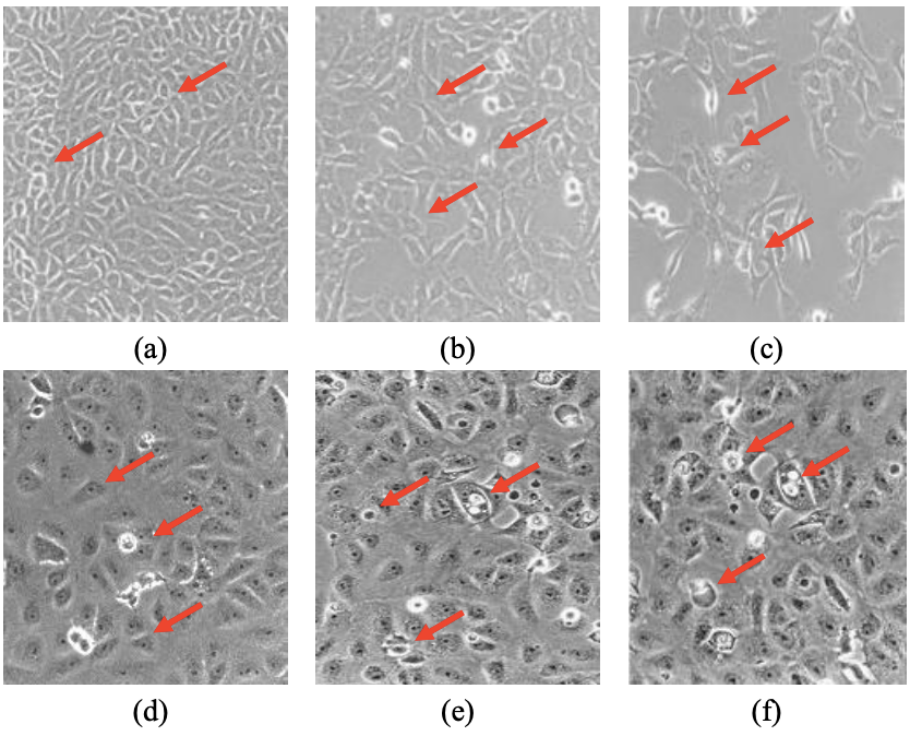

Figure 1. The effect of ethyl acetate fraction in variation times of treatment on MCF-7 cell (a-c) dan Vero cell (d-f). The observations were made using an inverted microscope with a magnification of 100x. (a) Control cell MCF-7; (b) 24 Hours treatment; (c) 48 Hours treatment; (d) Control cell Vero; (e) 24 Hours treatment; (f) 48 Hours treatment. The red arrow indicated the morphological alteration.

Changes in cell morphology can be used to determine the effectiveness of a compound in treating cells. To compare the effects of a compound with normal cell morphology, the morphological changes of MCF-7 cancer cells were examined using an inverted microscope (Figure 1). The use of the ethyl acetate fraction of *C. ternatea* L. resulted in a change in cell density and cell membrane damage, which was more evident in the 48-hour treatment than in the 24-hour treatment. However, the treatment did not visibly affect the cell nucleus. To

verify that the cells without treatment remained unaffected, the morphology of treated and untreated cells was compared. The IC_{50} value and morphology showed that the cytotoxic effect of the ethyl acetate fraction on Vero cells was less significant, as indicated by the absence of significant cell membrane damage, damage to the cell nucleus, or difference in cell density in the 24-hour and 48-hour treatments.

GC-MS analysis

GC-MS stands for Gas Chromatography-Mass Spectrometry, which is an analytical technique used to separate and identify compounds in a sample. The chromatogram is displayed in Figure 2.

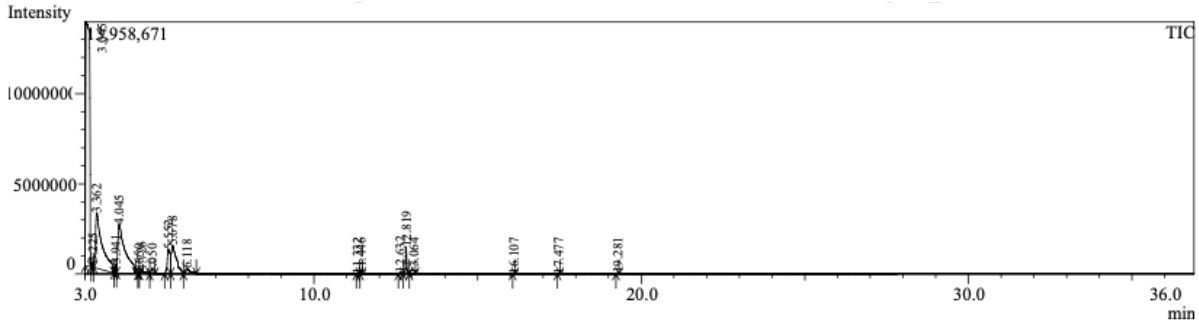

Figure 2. GC chromatogram of ethyl acetate fraction

Table 2. Compounds identified in the ethyl acetate fraction.

Peak number	Compounds name	Molecular Formula	Retention time (min.)	Percentage (%)
1	Pentanoic acid	$C_6H_{10}O_3$	3.045	46.70
2	Propanoic acid	$C_5H_{10}O_2$	3.225	19.19
3	Toluene	C_7H_8	3.941	18.24
4	Butyl ester	$C_6H_{12}O_2$	4.650	0.71
5	Ethylbenzene	C_8H_{10}	5.552	3.98
6	p-Xylene	C_8H_{10}	5.678	7.24
7	o-Xylene	C_8H_{10}	6.118	1.16
8	Naphthalene	$C_{10}H_8$	11.332	0.03
9	Estragole	$C_{10}H_{12}O$	11.446	0.02
10	Cinnamaldehyde	C_9H_8O	12.632	0.13
11	Anethole	$C_{10}H_{12}O$	12.819	1.58
12	Naphthalene, 1-methyl-2,4,6-Tris(1,1-dimethylethyl)-4-methylcyclohexa-2,5-dien-1-one	$C_{19}H_{32}O$	16.107	0.02
13	Phthalic acid, 2-chloropropyl ethyl ester	$C_{13}H_{15}ClO_4$	17.477	0.01
14	Sulfurous acid cyclohexylmethyl hexyl ester	$C_{13}H_{26}O_3S$	19.281	0.06

The analysis of the ethyl acetate fraction revealed the presence of 14 detected compounds (Table 2), with the major compounds being pentanoic acid (46.70%), propanoic acid (19.19%), toluene (18.24%), and p-xylene (7.24%) (Table 2). Literature review indicates

that almost all the chemical compounds found in the ethyl acetate fraction have cytotoxic activity. Additionally, our study found that there are cinnamaldehyde compounds in the fraction that exhibit both antioxidant and cytotoxic activity on MCF-7 and T47D cell lines.

In Silico Prediction of Ethyl Acetate Fraction from *Clitoria ternatea* L. using Molecular Docking

The testing process starts by searching for SMILES and downloading the PDB files of the compounds identified from the GC-MS test. Based on existing literature, these compounds are then screened through the PASS test to identify their activity. The ADMET test is conducted to predict their pharmacokinetic properties and potential toxicity. Overall, these tests aim to evaluate the potential of the twenty compounds for further development as therapeutic agents.

According to the PASS test results, only five out of nineteen compounds from the ethyl acetate fraction of *C. ternatea* L. have the potential to act as anti-cancer agents, specifically as an apoptosis agonist, caspase 3 stimulant, and TP53 expression enhancer that contribute to the apoptotic mechanism. Caspase-3, an executor caspase associated with apoptosis, is activated by caspase inhibitors, namely caspase-8 and caspase-9. TP53 plays a critical role in determining whether cells with DNA damage or abnormalities will repair themselves or undergo apoptosis. When DNA damage occurs, the p53 protein delays the cell from progressing to the next phase to provide time for DNA repair. If the damage is severe, the p53 protein initiates the cell death program or apoptosis.

Table 3. Results of molecular docking test of compounds within ethyl acetate fraction

Compound	Protein	Binding Affinity (kcal/mol)
Estragole	6HQU	-4.9
Cinnamaldehyde	6HQU	-4.9
Anethole	6HQU	-5.1
Control	6HQU	-7.8
Estragole	2ZVW	-3.4
Cinnamaldehyde	2ZVW	-3.6
Anethole	2ZVW	-3.5
Control	2ZVW	-2.4

Based on the Lipinski test, only three of the five compounds were suitable for molecular docking. Two proteins, DNA repair and recombination protein RadA (PDB 6HQU) and the crystal form of proliferating cell nuclear antigen 2 (PDB 2ZVW), were chosen as targets for molecular docking from several proteins. After determining the binding affinity value, the interaction between the compound and the native ligand was checked using the Biovia Discovery Studio application. The best compounds were selected and can be found in Table 3.

The 6HQU protein was chosen as a target because it functions in DNA repair and recombination and interacts with the p53 protein. It is essential for maintaining genome stability and has a role in the cell cycle, DNA repair, and apoptosis. The protein is also related to the anticancer drug anthracycline, which inhibits DNA replication and transcription. Only Anethole had good binding affinity among the three compounds tested,

with a score of -5.1 compared to the control ligand's score of -7.8. However, this still indicates that the native ligand is more active than Anethole since less energy is required for it to attach to the protein's active site.

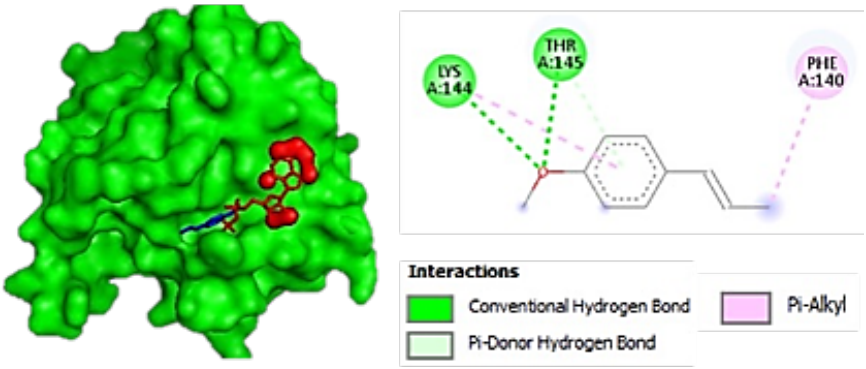

Figure 3. The anethole interaction with receptor

The interaction of anethole compounds (Figure 3) with the same amino acid residues (LYS144, THR145) as the original ligand compounds in the binding pocket on 6HQU suggests that Anethole has the potential for DNA repair and RadA recombination protein that interacts with p53 protein. This interaction can potentially affect the cell cycle, DNA repair, and induce apoptosis of the MCF-7 breast cancer cell line. The amino acid residues in the Anethole compound, including LYS144 and THR145, formed hydrogen bonds, while the PHE140 amino acid residue had hydrophobic bonds.

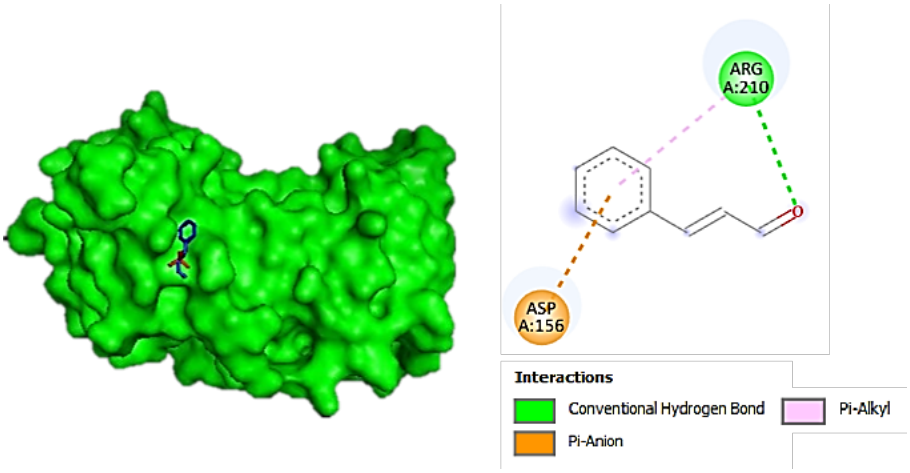

Figure 4. The cinnamaldehyde with receptor

The 2ZVW protein, also known as proliferating cell nuclear antigen 2 (PCNA 2), interacts with a peptide of p21 protein and plays a role in DNA synthesis and repair. The p21 protein is involved in the molecular mechanism that enables the p53 protein to halt the cell cycle, allowing for DNA repair and apoptosis. The Cinnamaldehyde compound, was found to have good binding energy with a binding affinity of -3.6 and was able to bind more easily to the receptor or active site of the 2ZVW protein than the control ligand due to its higher activity (Figure 4).

The results of molecular docking analysis using BIOVIA Discovery studio showed that Cinnamaldehyde compound interacts with the identical amino acid residues (ARG210, ASP156) as the original ligand compounds that bind to the 2ZVW receptor binding pocket. However, its binding affinity is lower than that of the control ligand, indicating that Cinnamaldehyde has the potential to inhibit cell proliferation and play a role in DNA synthesis and repair. The compound shares the same amino acid residue, ARG210, with the control ligand, which forms hydrogen bonds.

CONCLUSION

The most effective cytotoxic activity against the MCF-7 breast cancer cell line was observed with the ethyl acetate fraction of *C. ternatea* L. using the MTT Assay method, with an IC_{50} value of 1.73±0.75 and a selectivity index of 2.49. The addition of the ethyl acetate fraction to MCF-7 cells caused changes in cell morphology but did not affect the morphology of Vero cells. In silico testing was carried out on 5 compounds with potential as anticancer agents, but only 3 compounds passed Lipinski's Rule of Five test for molecular docking. The results showed that anethole compounds can interact with p53 protein and cinnamaldehyde, (E)- can interact with p21 protein.

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