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Cytotoxic Activity of Cambodian Leaves Extract *(Plumeria acuminate)* on Breast Cancer Cells and COX-2 Targeted Prediction of Its Chemical Contents

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Abstract

Cambodian leaves are suspected to contain stigma sterol which may target Cyclo-Oxygenase-2 (COX-2) or Estrogen Receptor (ER) to contribute to its cytotoxic activity on breast cancer cells. This study aimed to determine the potential of Cambodian leaf compounds and extracts as chemopreventive agents for luminal breast cancer with a molecular target of COX-2. Ethanol was used to extract the active compound of Cambodian leaves. The study on chemical activity against COX-2 employed molecular docking with Molecular Operating Environment (MOE) and the cytotoxic property of Cambodian leaf extract (CLE) on T47D was determined using the trypan blue exclusion method. The extraction yielded as 4.87% w/w CLE. Thin layer chromatography showed that Cambodian leaves contain sterol. Molecular docking confirmed that several sterol compounds have greater affinity to COX-2 than native ligands indicating that they are potent as COX-2 inhibitors. They are Stigmast-7-en-3-ol, Lupeol Acetate, and Lupeol carboxylic acid with docking scores of -14.3874, -13.8098, and -14.1045 kcal/mol respectively. The CLE exhibited cytotoxic activity on T47D cells with an IC_{50} value of 18 µg/mL. Therefore, CLE has a potential effect as a chemopreventive agent for breast cancer and potentially as a COX-2 inhibitor.

Keywords: *Cambodian leaf extract, breast cancer, COX-2 inhibitor, chemopreventive.*

INTRODUCTION

Breast cancer therapy, including luminal breast cancer, often causes numerous side effects, as its mechanism of action lacks selectivity, consequently posing a risk of harming normal cells (Patel, 2018). Consequently, there is a requirement for a chemopreventive compound that can complement chemotherapy medications, leading to a more effective treatment outcome and a reduction in undesirable side effects. Chemopreventive agents not only obstruct the initiation of cancer but also aid in the recovery of cancer patients, promoting

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their return to a healthy state. Chemopreventive substances can be prepared from medicinal plants and their secondary metabolite derivatives, including compounds like flavonoids, flavonols, saponins, terpenoids, alkaloids, steroids, and various others with the potential to inhibit cancer growth. (Yu, *et al* ., 2018).

Breast cancer has various potential targets in prevention, one of which is COX-2 (Harris, *et al*., 2014). The relationship between COX-2 and chemopreventive agents is an area of interest in cancer research and prevention. COX-2 is an enzyme that plays a role in the development and progression of certain types of cancer. Specifically, overexpression of COX-2 has been observed in various cancer types including breast cancer (Harris, 2009). In breast cancer, COX-2 is overexpressed at every stage of its development (Hugo, *et al*., 2015).

Plumeria sp., a plant native to Cambodia, had many activities including anti-inflammatory, cytotoxic, pro-apoptotic, and antioxidant properties. *P. accuminata*, *P. alba*, *P. rubra*, *P. lancifolia*, *P. drastic*, and *P. phagidenica*, are known for their medicinal activities. Cambodian leaf extract contains various compounds, including steroids, flavonoids, tannins, alkaloids, and glycosides triterpenoids, which may function as anti-inflammatory agents (Gupta, *et al*., 2018). There are also indications of additional potentials within Cambodian leaf extract that warrant further exploration.

P. obtuse exhibits pharmacological activities, including the ability to induce cell apoptosis (Gai, *et al*., 2016), and the presence of metabolites with anti-tumor properties has been documented (Riaz, *et al.*, 2020). Moreover, the methanolic extract of *Plumeria acuminata* is reported to have significant anti-inflammatory effects in animal models with carrageenan-induced edema, both in acute and chronic settings. Additionally, *P. rubra* has exhibited cytotoxic activities against breast cancer cells. Consequently, experimental research is needed to assess the potential of compounds found in Cambodian leaf extract as inhibitors of COX-2 and to investigate the potential molecular mechanisms through which the active compound in Cambodian leaves (*P. acuminate*) may interact with COX-2 enzyme.

MATERIALS AND METHODS

Material Collection, Determination, and Extraction

The Cambodian leaves utilized in this study were sourced from the Gunungkidul regency and determined at the Laboratory of the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The collected leaves were sliced into small pieces and dehydrated for 10 h at a temperature range of 28-47°C using a dehydrator. Subsequently, the leaves were subjected to pollination with a pollinator and filtered through a 40 mesh sieve.

The extraction procedure in this study used maceration technique. The extraction process utilized digital scales, a dryer, a rotary evaporator, a grinding machine, a measuring cup, an erlenmeyer, a dehydrator, a grinder, and a maceration flask. A total of 200 grams of Cambodian leaf powder were weighed and placed into an erlenmeyer. The sample and compound had a ratio of 1:4, and were then dissolved in PA ethanol. The Cambodian leaves were macerated for 20 h and then filtered using a vacuum press separation technique (Shofi, *et al*., 2020).

Identification of Phytochemical Profile

Identification of phytochemical compounds in Cambodian leaves was carried out by thin-layer chromatography. 5 mg Cambodian leaf extract was dissolved in 500 µl so the solution contained 10.000 µg/mL CLE. The standard solution for TLC was ursolic acid (Sigma, Missouri St. Louis, USA) which was spotted 1 cm from the extract's spot. The stationary phase that was used for TLC is the F254 silica gel plate (Merck, Darmstadt, Germany). The

mobile phase was toluene and ethyl acetate with a ratio of 8:2. Then the spot result was visualized in visible light which was documented with a camera.

Active Compound Activity Test

The active compound test evaluates the inhibitory effect of the active compound through the KNIME (Konstanz Information Miner) software using the TeachOpenCADD workflow template. The active compounds tested were stigmast-7-enol, lupeol acetate, lupeol carboxylic acid, and ursolic acid. Data on the structure of the compound to be tested for its inhibitory effect can be obtained by entering the SMILES code for the Cambodian leaf extract from PubChem (https://pubchem.ncbi.nlm.nih.gov).

Molecular Docking

Molecular docking was conducted to assess the binding affinity of the four identification compounds present in the Cambodian leaf extract. We obtained data from the molecular docking simulation and visualized the interactions using the MOE 2010 software, licensed by the Faculty of Pharmacy at UGM. We acquired the COX-2 model with the code 3LN1 from PDB. The MOE provides a direct description of the compound structure to be docked by entering the SMILES code for the CLE compound from PubChem (https://pubchem.ncbi.nlm.nih.gov).

In the molecular docking procedure, the validation of the molecular docking technique takes place. This validation phase involves reattaching the original ligand to the receptor, which was initially detached. The docking method is deemed successful if it produces Root Mean Square Deviation (RMSD) value of 2 (Puratchikody, *et al*., 2016).

Cytotoxic Assay with Direct Counting

The cytotoxic assay utilized a 24-well plate, micropipette, microtube, and hemocytometer. Each well of the 24-well plate was seeded with $2x10^4$ T47D cells and incubated for 24 h in Dulbecco's Modified Eagle Medium (DMEM) at 37°C, with CO_2 levels of 0.5%. A cambodian leaf extract test solution was added to each well. After 24 h of incubation, the cells were washed 2-3 times with PBS in each well. Then, the Trypsin-EDTA solution was evenly added and incubated in the incubator for 3 minutes. Next, media was added to inactivate trypsin. The cells were resuspended using a micropipette until they were released. Finally, the cells were transferred into a new sterile microtube, and trypan blue was added to the cell suspension solution. Cell suspension treated with trypan blue was pipetted into a hemocytometer and counted under a light or inverted microscope using a counter. Viable cells (unstained) were then counted under a light microscope. The number of viable cells in each sample treatment was normalized to the number of untreated (control) cells, and the data was plotted on a concentration vs. % cell viability graph. The IC_{50} value was calculated using this data.

Analysis Method

1. Yield Calculation

The Cambodian leaf extract that has been obtained is weighed to determine the total yield using the following formula:

%Extract yield=(Cambodian Leaf Extract gained weight (g)/sample weight (g))x100%

2. Compound Interaction Strength

Four compounds were collected as possible binding interactions on COX-2. The compound that had a lower docking score than the native ligand COX-2 was concluded as the compound with the greatest interaction strength with the COX-2.

3. Cytotoxic assay

The data was obtained in the form of variations in the concentration of Cambodian leaf extract and the viability cells. The percentage of cell viability was calculated using the data of cell viability results for each concentration compared

with the results of cell viability in the control group. Then calculated the concentration of IC_{50} using the linear regression method between the concentration of the extract and the percentage of living cells.

%cell viability=[(Absorbance by treatment-Absorbance control media)/(Absorbance control cells-Absorbance control media)]x100%

RESULTS

Determination, Extraction, and Chemical Detection

The cambodian leaves were obtained from the Gunungkidul area for the extraction process and based on the determination test showed that the sample was indeed *Plumeria acuminate*.

The extraction method was maceration and obtained weight of 9.75 grams and an extract yield of 4.87% w/w. The Cambodian leaf extract (CLE) is a blackish green thick extract (Figure 1A). This extract was analyzed by thin layer chromatography (TLC). Reagan standard for TLC used ursolic acid that sterol's derivatives. The result showed that cambodian leaves extract had the same Rf value with ursolic acid and also gave a positive reaction with Liebermann burchard reagent (Figure 1B-C). The Rf value is 4.5. It means that Cambodian leaves contain sterol. This result confirmed the previous research that the Cambodian leaves contain four phytochemical compounds, including Stigmast-7-en-3-ol, Lupeol acetate, ursolic acid, and lupeol carboxylic acid (Farooque, *et al* ., 2012).

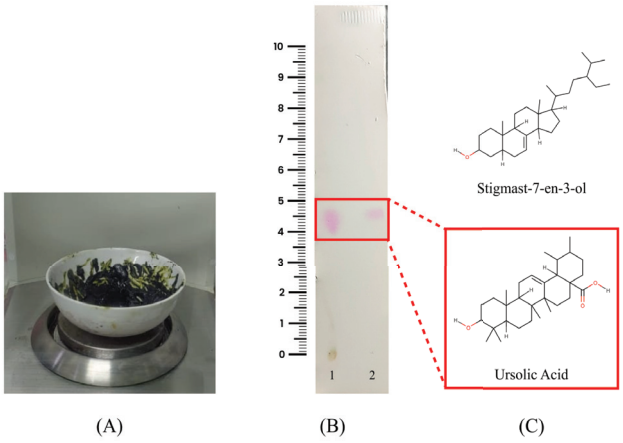

Figure 1. Cambodian leaf extract and phytochemical profile. The cambodian leaf extract (A) was obtained by maceration using ethanol PA (1:4) with 4.87% yield percentage. The extract was identified by thin layer chromatography (B1) and showed the same stain (Rf value : 4.5) as Ursolic Acid Standard (B2) and also gave the positive reaction with Liebermann burchard reagent. Both stigmast-7-en-3-ol and ursolic acid showed the similar chemical structure (C).

Active Compound Activity Test

We then analyzed the main compounds, stigmast-7-en-3-ol, lupeol acetate, ursolic acid, and lupeol carboxylic acid (Farooque, *et al*., 2012), in Cambodian leaves using machine learning KNIME to obtain compounds that were predicted to have inhibitory activity against COX-2.

The results of screening potential compounds of cambodian leaf extract which have activity as COX-2 inhibitors with KNIME resulted in an overall accuracy >70% which is 0.978 and the ROC curve with p-value >0.7 which is 0.907 which means that the method used is valid. The results of the analysis showed that all compounds have inhibitory activity against COX-2 (Table 1).

Table 1. Potential compounds of cambodian leaf extract which have activity as COX-2 inhibitors.

Compound	Inhibition Prediction
Lupeol acetate	0.59
Stigmast-7-en-3-ol	0.57
Lupeol carboxylic acid	0.57
Ursolic acid	0.54

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Molecular Docking

Prediction of compound activity is also done using molecular docking where compounds with low bond energies will be easier to bind to the proteins. The test of the interaction strength of compounds in Cambodian leaf extract with COX-2 was carried out by molecular docking through the MOE application. Based on the results of the molecular docking test, the compounds that have the lowest docking scores are stigmast-7-en-3-ol, lupeol acetate, lupeol carboxylic acid, and ursolic acid (Table 2).

Celecoxib (CEL) was used as a native ligand of COX-2 in performing molecular docking and showed a docking score of -13.3398 kcal/mol (Table 2). CEL, celecoxib ligand is a COX-2 inhibitor that is used as a reference for comparison with the test compound. If the docking score of the compound being tested has a more negative value than the ligand, it can be said that the compound being tested has a strong potential to become a COX-2 inhibitor. In addition to the docking value, the estimated interaction between the test compound and native ligand with COX-2 was also obtained.

Table 2. Docking scores of compounds against COX-2.

Ligand	Score (kcal/mol)
CEL (native ligand)	-13.3398
Stigmast-7-en-3-ol	-14.3874
Lupeol acetate	-14.1045
Lupeol carboxylic acid	-13.8098
Ursolic acid	-13.2388

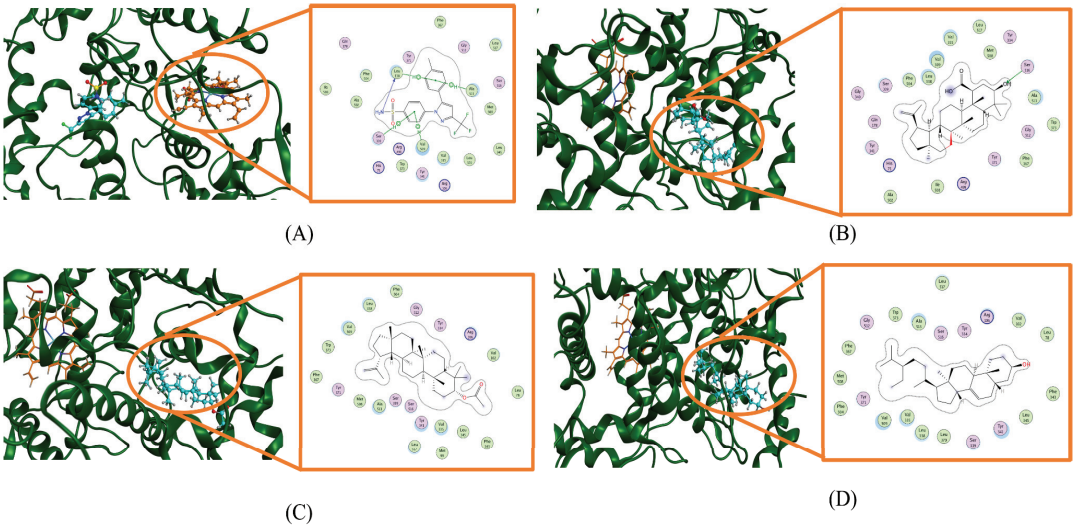

Figure 2. The 2D and 3D visualization of the interaction for several compounds in Cambodian leaf extract with COX-2. Molecular docking was performed using MOE as described in the methods. The interaction visualization of native ligand CEL (A) of COX2 compared to the interaction of Lupeol carboxylic acid (B), Lupeol Acetate (C), Stigmast-7-en-3-ol (D) to COX-2.

The results showed that three compounds were potent as COX-2 inhibitors, namely Stigmast-7-en-3-ol, Lupeol Acetate, and Lupeol Carboxylic Acid. The three compounds illustrated the interactions between COX-2 and related compounds showing the tight interaction at the same site with the native ligand with the lower docking score (Figure 2).

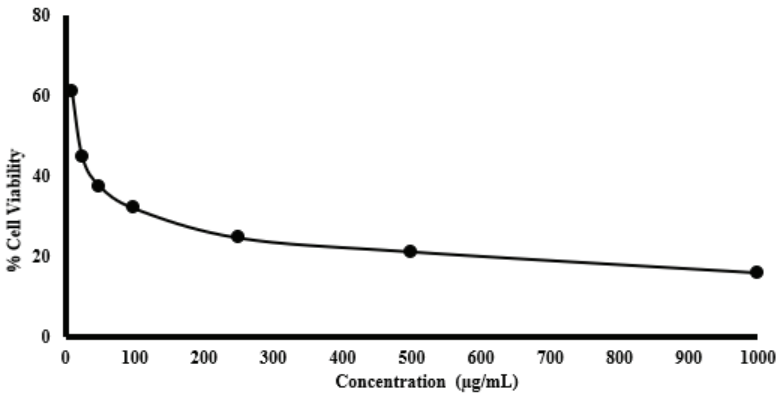

Figure 3. The cytotoxic effect of Cambodian leaf extract on T47D. The cytotoxic activity of Cambodian leaf extract on T47D cells was assessed using the direct counting for 24 h. Cambodian leaf extract at the respective concentrations was tested for its cytotoxicity on T47D cells using the direct counting method.

Cytotoxic Assay with Direct Counting

A cytotoxic assay was conducted to determine the cytotoxic ability of Cambodian leaf extract against T47D breast cancer cells which were assessed with the IC_{50} parameter. The IC_{50} value is the concentration of the test material that gives 50% growth inhibition to living cells. The result showed that CLE suppressed the growth of T47D cells progressively with the IC_{50} value of 18 µg/mL (Figure 3). This means that Cambodian leaf extract has a strong inhibitory activity on the growth of luminal breast cancer cells.

DISCUSSION

The cambodian leaf extract was obtained from maceration with ethanol pa in a ratio of 1:10. Ethanol is used because it is universal, safe to use, and can dissolve the compounds containing Cambodian leaves well, especially to target sterol compounds. This system looked effective to obtain the sterols compound, especially ursolic acid. This result would be interesting for further study concerning the phytochemical exploration of this plant as a source of ursolic acid or other steroid compounds (Farooque, *et al* ., 2012). The cytotoxic test also confirmed that this extract showed a strong cytotoxic activity against T47D cells, the luminal breast cancer cell line. However, which the compounds of the extract contribute significantly to the cytotoxic activity should be more detailed investigation further. The KNIME analysis may help to guide the possible target of action of the potential compounds. Our research also confirmed that the four compounds of sterols could target COX-2. This information is also supported by the result of molecular docking studies that showed high interaction between the compounds and COX-2 over the native ligand. Even Though this evidence is a virtual study that needs further experimental study, this finding could give insight into the basic molecular mechanism of the CLE as an anti cancer agent.

This research open the possibility that Cambodian compounds strongly contribute to the cytotoxic effect of the CLE for breast cancer or may the other type of cancers that highly express COX-2. However, we know that this experiment used T47D cells that also express Estrogen Receptor. The steroid compounds perhaps also target the ER as the estrogen-like compounds. This additional target could amplify the cytotoxic effect if they have antagonistic activity. This possibility will be interesting for further investigation.

The Cambodian tree is commonly found in tropical countries, including Indonesia. This

plant is also easily cultivated as a garden plant or public plant but still limited to be utilized as a source of pharmacological materials. This report gives important information to additional usage of this plant in health care, especially in cancer treatment. This report should be extended to gain more comprehensive information regarding its potential as an anti-cancer agent with expanding in using more cancer types and the study of molecular targets mechanism.

CONCLUSION

Cambodian leaf extract is cytotoxic to T47D breast cancer cells. This extract may contain ursolic acid that interacts tightly to COX-2 better than celecoxib. Cambodian leaf extract has a potential effect as a chemopreventive agent for breast cancer in correlation with COX-2.

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