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Original Research Article

Ficus deltoidea Ethanol Extract Cytotoxic Activity on MCF-7 Treated Doxorubicin: An *In Silico* and *In vitro* Approach

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ABSTRACT

Female breast cancer is the most frequently diagnosed cancer worldwide, and the number of cases will increase in the next 20 years. Doxorubicin is an effective first-line chemotherapy for breast cancer. Unfortunately, continuous administration of doxorubicin can decrease the sensitivity of cancer cells to the drug. The use of herbal medicines as a valuable and efficient approach to cancer treatment is increasing. Several studies have confirmed the anti-breast cancer activity of extract *Ficus deltoidea* (FD), but no study has revealed the cytotoxicity of extract FD in MCF-7 breast cancer cell lines that have been exposed to doxorubicin repeatedly. This study aims to evaluate the cytotoxic potential of ethanolic extracts from the *F.deltoidea* leaves against MCF-7/Dox. In addition to assessing the total flavonoid contents in the extract. We performed a molecular docking analysis. The 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) assay is conducted using doxorubicin treated-MCF-7 cell lines (MCF-7/Dox). The total flavonoid content is 130.77 mg QE/g extract of FD. Based on molecular docking, kaempferol-3-*O*-*β*-D-glucuronopyranosyl methyl ester interacted best with Bax and Bcl-2 proteins compared to 11 compounds. The IC_{50} value of the FD extract on MCF-7/Dox was 2.85 ppm ± 0.47. Our results provide valuable insight into FD as an herb to be developed as an alternative or additional therapy to reduce sensitivity in patients with breast cancer.

Keywords: B-cell lymphoma-2-associated X, B-cell lymphoma 2, *Ficus deltoidea*, Antibreast cancer.

Introduction

Female breast cancer is the most frequently diagnosed cancer worldwide, and the number of cases is predicted to continue to increase in the next 20 years [1]. Breast cancer is influenced by a balance between cell proliferation (growth and division) and apoptosis (programmed cell death) [2]. Apoptosis is vital for eliminating damaged or unnecessary cells, preventing them from becoming cancerous and balancing cell growth [3, 4]. B-cell lymphoma-2-associated X (Bax) and B-cell lymphoma 2 (Bcl-2) protein families are key regulators in apoptosis. In patients with breast cancer, Bcl-2 protein expression is significantly increased [5]. Bcl-2 is an anti-apoptotic protein that binds to pro-apoptotic proteins such as Bax under normal conditions. When the cell is damaged or has no viable signal, anti-apoptotic and pro-apoptotic interactions will be inhibited [6].
Common treatments for cancer often have severe side effects that can decrease a patient's overall well-being, result in non-adherence to chemotherapy protocols, and contribute to the development of drug resistance [7–9]. Doxorubicin is a highly effective chemotherapy treatment that is often used as the initial line of treatment for breast cancer. However, prolonged administration of doxorubicin can reduce the responsiveness of metastatic cancer cells to the medication. The sensitivity of breast cancer to chemotherapy can decrease after 6-10 months of doxorubicin treatment. [10, 11] Chemotherapy resistance is a significant challenge in breast cancer treatment. One of the key mechanisms by which cancer cells become resistant to chemotherapy is by evading apoptosis. [10, 12].
Some patients with cancer use natural-derived anti-cancer drugs because they believe they have fewer side effects and are more affordable [13]. Several studies showed plant extract had anti-cancer effects even after doxorubicin exposure [8,9,14]. *Ficus deltoidea* (FD), or Tabat barito, is abundantly distributed throughout Southeast Asia, including Malaysia, Indonesia, and Thailand. [15] The leaves of the plant have been used in traditional medicine to treat cancer, diabetes, inflammation, diarrhea, infections, wound treatment, rheumatism, and sores. [16, 17] Unfortunately, there is a lack of data on the anti-breast cancer activity of this plant extract in addressing the chemoresistance issue. To investigate the anti-cancer effect of extract FD, we performed molecular docking and MTT assays using doxorubicin-treated MCF-7 cells (MCF-7/Dox). [18, 19] In addition, phytochemical Screening, Total Flavonoid Content (TFC) Assay, and Ultra-Performance Liquid Chromatography Tandem Quadrupole Time-of-Flight Mass Spectrometry (UPLC-QTOF MS/MS) analysis were conducted.

Materials and Methods

Materials
Ethanol 70%, MTT, dimethyl sulfoxide (DMSO), Dulbecco Modified Eagle Medium (DMEM) and doxorubicin were purchased from Sigma-Aldrich, US. MCF-7 cell lines (iCell h-129) No. Lot 20240115 was purchased from PT iCell Bioscience Inc. Trypan blue, trypsin EDTA

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0,5%, Phosphate buffer saline (PBS), Fetal Bovine Serum 10% (v/v), penicillin-streptomycin (10 000 U/mL, and 25 mg/mL Fungizone antimycotic were purchased from Gibco, USA.

Collection and Identification of Plant Material:
Leaves of *F.deltoidea* were collected from Purworejo Regency, Indonesia, in December 2023. The plant was taxonomically verified by Head of Department Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia from the Universitas Indonesia Biota Collection Herbarium in Depok City, Indonesia, with voucher No. JI24-P-46. The leaves were washed in running water, dried, powdered, and stored in an airtight container for further analysis [20].

Extraction process
The *Ficus deltoidea* (FD) leaves were dried and ground into a fine powder. The dried leaf powder (700 g) was soaked in 70% ethanol for 24 hours. The extracted filtrate was collected and then evaporated in a rotary evaporator at a temperature of 50ºC and pressure of 200 mbar (Buchi, Switzerland) until it became a semisolid extract [21].

Phytochemical Screening
The phytochemical screening test was conducted according to Harborne [22] for alkaloids, anthraquinones, flavonoids, tannins, saponins, and terpenoids. Alkaloid test with Mayer, Dragendorff, and Wagner reagents; anthraquinone with Borntrager test; flavonoid test with Shinoda test; tannin test with gelatin-salt test; and test with ferrous (III) chloride; saponin test with froth test; terpenoid test with Liebermann-Burchard reagent [20].

Total Flavonoid Content (TFC) Assay
A total of 10 mg of the FD extract was used in the study. A standard curve was created using quercetin concentrations ranging from 50 to 300 ppm. The absorbance of each solution was measured using a microplate reader at a wavelength of 415 nm. The total flavonoid values were interpreted as milligrams of Quercetin Equivalents (QE) per gram of plant extract [20].

UPLC-QTOF MS/MS Analysis

The extract was dissolved in methanol and filtered with millipore 0.2 microns, then 5 µL of the filtrate was injected into UPLC-QTOF MS/MS Samples were run through HSS T3 columns, 2.1 x 100 mm, 1.8 µm, and elution using formic acid 0.1% water-acetonitrile as the mobile phase at a rate of 0.6 ml/min. The autosampler temperature used was 15 ºC, while the chromatography column temperature used was 40 ºC [23].

Prediction of Pharmacokinetic and Physicochemical Compounds

Pharmacokinetic characteristics and physicochemical compounds were analyzed using absorption, distribution, metabolism, excretion, and toxicity (ADMET Lab) (https://admet.scbdd.com/), small-molecule pharmacokinetics prediction (pkCSM) (https://biosig.lab.uq.edu.au/pkcsm/prediction), and SwissADME (http://www.swissadme.ch). [24, 25]

Molecular Docking
Autodock Vina 1.5.7 was used as the docking software, and Discovery Studio was used for visualization. The proteins used were Bax (PDB IDs: 4SO0) and Bcl-2 (PDB ID: 2w3l) obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). The grid box dimensions were validated, with the dimensions for Bcl-2 being x = 60, y = 60, and z =60, centered at x = 38.905 Å, y=26.949 Å, and z=-12.545 Å. The grid box dimensions for Bax were x = 40, y = 40, and z =40, centered at x = 19 Å, y=-9 Å, and z=-12 Å. [26, 27]

Cell culture
MCF-7 cell lines were grown in DMEM supplemented with 10% Fetal Bovine Serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and passaged when the cells were approximately 80% confluent. After the cells reached log-phase, MCF-7 cells were repeatedly exposed to doxorubicin 0.05 µM for 16 days and called MCF-7/Dox. [28, 29] MCF-7/Dox would be used for further study.

Cytotoxicity Assay
MTT testing was performed to determine the IC_{50} of *F. deltoidea* extracts in MCF-7/Dox cells. The extract was added to cells and incubated for 24 hours. After treatment, 100 µl of MTT reagent (2 mg/ml) was added to each well, incubated at 37°C for 3 hours and added 100 µl DMSO to dissolve the formazan crystals. Then, a microplate reader was used at a wavelength of 595 nm. [30]

Data analysis
All treatments were performed in duplicate. The MTT cell proliferation assay results were calculated using the GraphPad PRISM ®9.0 software (GraphPad Software, Inc.).

Result and Discussion

Extract Characterization
One of Indonesia's therapeutically beneficial plants for cancer is *Ficus deltoidea* (FD). [21, 31, 32]. FD leaves extraction using ethanol 70% solvent obtained a yield value of 11.74%. Several studies have shown that the yield value of FD extract with ethanol or methanol solvents is 5-20%[33]. Based on phytochemical screening, the FD extract contained flavonoid, phenol, saponin, and triterpenoid compounds. The phytochemical screening results are presented in Table 1.

Table 1: Phytochemical screening of the *Ficus deltoidea* leaves extract

Chemical Compounds	Result
Alkaloid	Negative (-)
Anthraquinone	Negative (-)
Flavonoid	Positive (+)
Phenolic	Positive (+)
Saponin	Positive (+)
Tannin	Positive (+)
Triterpenoid	Positive (+)

Total Flavonoid Content Analysis
Flavonoids are among the most notable phenolic compounds found in FD extracts. [17, 34–37] Research has demonstrated that these compounds influence the advancement of cancer cells, such as those found in breast cancer, via modifications to proteins and changes to signaling pathways. [38] This study measured the total amount of flavonoids in FD extract, which was expressed as milligrams of Quercetin equivalents per gram of extract. The extract has a total flavonoid content equivalent to 130.77 mg of quercetin per gram. The flavonoid content was determined by a colorimetric method that utilized $AlCl_3$ and quercetin as a reference standard. The method was defined by the equation y = 0.005 - 0.2982 and a correlation coefficient of R = 0.9624.

Metabolite Profiling of Flavonoid Compounds from Ficus deltoidea Extract and its Drug similarity analysis
Metabolite profiling of extract FD using UPLC-QTOF MS/MS obtained 83 compounds. In this study, only 11 selected compounds were used for further study.[39] The eleven compounds identified through UPLC-QTOF MS/MS were examined in order to assess their likelihood of being a pharmaceutical compound according to Lipinski's Rule of Five. Lipinski's five rules comprise a set of criteria, including a molecular weight not exceeding 500 Daltons, a limit on hydrogen bond acceptors to 10, a limit on hydrogen bond donors to 5, a molar refractivity between 40 and 130, and a lipophilicity (log P) of less than 5. Compliance with Lipinski's rule of five (RO5) is essential for drug probability testing on potential compounds in the drug discovery process. The results of drug similarity analyses are presented in Table 2. The results of the predictions for the physicochemical data reveal that Lipinski's rule is violated by 5 out of 11 compounds. Among the 11 compounds investigated, apigenin, episappanol, izalpinin, and pachypodol were the only ones that conformed to Lipinski's rule of five

Table 2: Physicochemical properties of selected flavonoid compounds

Compounds	MW	LogP	Rotatable Bond	HBA	HBD	TPSA
5-Methyl kaempferol	300.26	1.67	2	3	6	100.13
Nicotiflorin	594.52	0.02	6	9	15	249.20
Apigenin	270.24	3.02	1	3	5	90.90
Baicalin	446.36	1.11	4	6	11	187.12
Episappanol	304.29	0.91	2	5	6	110.38
Izalpinin	284.26	2.58	2	2	5	79.90
Kaempferol 3-O-β-D-glucuronopyranosyl methyl ester	448.38	0.72	4	7	11	190.28
Pachypodol	344.32	3.61	4	2	7	98.36
Rhamnazin	330.29	2.19	3	3	7	109.36
Skullcapflavone I	314.29	2.96	3	2	6	89.13
Isovitexin-3''-O-glucopyranoside	432.38	0.21	3	7	10	181.05

Notes: MW: Molecular Weight; TPSA: The Polar Surface Area; HBD: Hydrogen Bond Donor; HBA: Hydrogen Bond Acceptor

Table 3: Molecular Docking Results of Selected Compounds from Extract *F.deltoide*

Molecules	ΔG (kcal/mol)	Ki (μM)	Hydrogen Bonds
BAX			
Kaempferol 3-O-β-D-glucuronopyranosyl methyl ester	-10.77	0.013	ARG134, GLU 131, GLN 28, LEU47
Isovitexin-3''-O-glucopyranoside	-9.51	0.107	ALA42, ARG 134, LEU47
Pachypodol	-9.26	0.163	ALA42, ARG 134; GLN 32; LEU 47
Apigenin	-9.12	0.198	ALA, 42; GLN, 32; PRO, 130
Rhamnazin	-9.01	0.249	ARG 134, ALA 42, GLN 32, LEU 47
5-Methyl kaempferol	-8.73	0.400	ALA 42, ARG 134, GLN 32, MET 137
Skullcapflavon I	-8.5	0.589	ALA42, GLN 28, LEU47
Episappanol	-8.04	1.28	ALA42, ALA35, LEU47, MET 137
Izalpinin	-7.99	1.38	ALA42, ARG 134
Nicotiflorin	-7.98	1.4	ALA42, ALA35, GLU41, GLN32, and PRO130
Baicalin	-6.59	14.65	ARG 134, GLU41, PRO130
BCL-2			
Kaempferol 3-O-β-D-glucuronopyranosyl methyl ester	-7.78	1.97	ALA108, ASP99, and ARG105
Isovitexin-3''-O-glucopyranoside	-7.08	6.42	ASN 122, GLU29, GLU 124, VAL 121, SER126
Rhamnazin	-6.13	3.22	GLU95, MET74
Episappanol	-6.04	37.63	ASN 122, VAL 121
5-Methyl kaempferol	-5.87	49.99	ALA108, GLU95, and MET74
Skullcapflavon I	-5.85	51.56	ALA108, GLU95
Baicalin	-5.71	65.2	ASP99, LEU96, and GLU95
Izalpinin	-5.45	101.83	ARG123, GLU119, HIS79
Nicotiflorin	-5.24	143.18	ALA108, ARG105, ASN102, GLU95, LEU96
Apigenin	-5.24	143.18	ALA 108, GLU95
Pachypodol	-5.20	153.69	ASN102, ARG105

The molecular weights of the compounds in Table 2 range from 270.24 to 594.52. Molecular weight is an important parameter that affects the distribution, metabolism, and excretion of compounds in the body.[40] Large molecules tend to lack membrane permeability, which is essential for gastrointestinal absorption after oral dosing.[41] Nicotiflorin has the highest molecular weight of 594.52, whereas apigenin has the lowest molecular weight of 270.24.

The number of hydrogen bond acceptors (HBA) in these compounds ranged from 3 to 9, and the number of hydrogen bond donors (HBD) ranged from 5 to 15. Hydrogen bond acceptors and donors influence the solubility of compounds in water and their interactions with biological molecules.[42] Nicotiflorin has the highest number of HBDs and HBA at 15 and 9, indicating a high capacity for hydrogen bond interactions. In this study, Pachypodol had the highest LogP of 3.61, indicating high lipid solubility, whereas nicotiflorin had the lowest LogP of 0.02, indicating low lipid solubility. LogP values indicate the lipophilicity or solubility of the compounds in lipids. Lipophilicity may affect compound bioavailability, toxicity, and penetration. [43]The surface areas of the compounds ranged from 79.90 to 249.20. Surface area influences the interaction of compounds with biological membranes and their pharmacokinetic properties. [44] Nicotiflorin has the highest surface area of 249.20, indicating that this compound has the potential for more extensive interaction with biological environments than the other compounds.

Molecular modelling of selected flavonoid compounds with Bax and Bcl-2 Receptor

The energy Gibbs (ΔG) and Inhibition constant (ki) values are used as criteria to determine the ability of a compound to bind to a target protein during molecular docking. The results of the molecular docking analysis are presented in Table 3. In this study, the target proteins were Bax and Bcl-2. The 2D and 3D visualizations of Kaempferol-3-*O*-*β*-D-glucuronopyranosyl methyl ester interaction with the protein target are presented in Figure 1. Kaempferol-3-O-β-D-glucuronopyranosyl methyl ester exhibited strong interactions with protein targets. Compared with all the selected compounds, kaempferol-3-*O*-*β*-D-glucuronopyranosyl methyl ester had the most negative ΔG and lowest ki in its interaction with Bax and Bcl-2. The ΔG values of kaempferol-3-*O*-*β*-D-

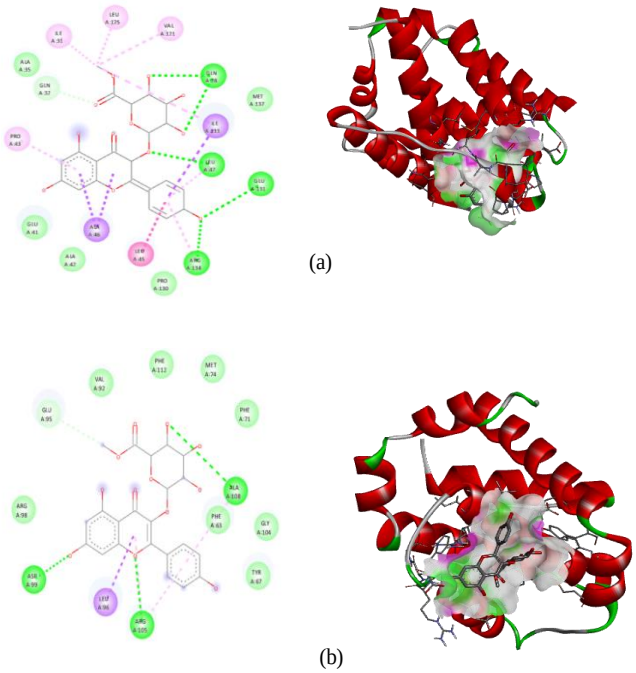

Figure 1: Three-Dimensional (3D) and Two-Dimensional visualizations of kaempferol-3-O-β-D-glucuronopyranosyl methyl ester Interaction with protein targets Bax and Bcl-2 (a) Bax (b) bcl-2

Glucuronopyranosyl methyl ester binding to the Bax and Bcl-2 proteins were 10.77 and 7.78 kcal/mol, respectively. The compound interacts with the Bax protein to form hydrogen bonds with the amino acid residues ARG134, GLU131, GLN28, and LEU47. In the Bcl-2 protein, kaempferol-3-*O*-*β*-D-glucuronopyranosyl methyl ester forms hydrogen bonds with the protein at amino acid residues ALA108, ASP99, and ARG105. Covalent and non-covalent interactions through hydrogen, van der Wall, and electrostatic and hydrophobic bonding can mediate interactions between phenolic compounds such as flavonoids and proteins. The primary interaction between a flavonoid and protein is hydrophobic interaction and hydrogen binding. [45]

Cytotoxicity of FD Extract on MCF-7 Treated Cells

We conducted a cytotoxicity assay using MTT assay to confirm the anti-breast cancer activity of extract FD on MCF-7 cells treated with doxorubicin (MCF-7/Dox). In the MTT assay, purple formazan crystals were formed between the MTT reagent and the mitochondrial enzymes of living cells. The succinate dehydrogenase enzyme is transformed into purple formazan crystals. The intensity of the purple color is directly proportional to the number of living cells. [46]

Based on the cytotoxicity assay, the IC_{50} value of the FD extract on MCF-7/Dox was 2.85 ± 0.47 ppm. A previous study showed that FD extract could inhibit colon cancer cell line HCT-116 with IC_{50} 5.41 ppm. Meanwhile, ethyl acetate extract of *Ficus deltoidea* could inhibit HCT-116 with IC_{50} value 54.89 ppm. [32, 47] Additionally, the nonpolar extract of *F.deltoidea* also showed an anti-cancer effect against prostate cancer cell line, DU145 and LNCaP with IC_{50} values individually 14 ppm and 23 ppm.[48]

The cytotoxic effect of FD extract is probably due to its flavonoid compounds. Based on UPLC-QTOF MS/MS, the FD extract contains flavonoids with anti-cancer effects, such as kaempferol and isovitexin. Flavonoids can inhibit cancer cell proliferation and invasiveness by several mechanisms, such as modulating the activity of reactive oxygen species (ROS)-scavenging enzymes, inducing cell cycle arrest, and promoting apoptosis and autophagy.[38]

Conclusion

Ficus deltoidea has the potential to be developed as an anti-cancer herbal remedy, as evidenced by its strong IC_{50} values in inhibiting MCF-7 cancer cells that have been exposed to doxorubicin. The mechanism of F. *deltoidea* is suspected to be mediated by flavonoid compounds such as kaempferol and isovitexin. It has been observed that *F. deltoidea* can reduce the viability of MCF-7/Dox cells. However, these findings require further studies to confirm the mechanisms and responsible compounds. This research can be a reference for future research *on F. deltoidea* as an anti-cancer agent.

Conflicts of Interest

The authors have declared that there is 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.

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