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Chemopreventive Potential of *Calotropis gigantea* Root Fraction on MCF-7 Cells: *In Vitro* and *In Silico* Study

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Abstract

Cancer remains a major global health issue due to its high mortality rate. Chemotherapy, though commonly used, often causes severe side effects by damaging both cancerous and healthy cells. Therefore, safer and more selective chemopreventive agents from natural sources are needed. This study aims to evaluate the chemopreventive potential of the ethanol fraction of biduri root (EFBR, *Calotropis gigantea* L.) through *in vitro* and *in silico* approaches. Phytochemical screening was used to identify bioactive compounds. Molecular docking using AutoDock Vina assessed calotropin's interaction with Bcl-2 and HER-2 proteins. Antioxidant activity was measured by the DPPH method, and cytotoxic activity was tested on MCF-7 breast cancer cells and normal Vero cells using the MTT assay. EFBR was found to contain cardiac glycosides, flavonoids, terpenoids, saponins, and tannins. Docking results showed strong binding of calotropin to Bcl-2 (-9.3 kcal/mol) and HER-2 (-8.1 kcal/mol). EFBR showed weak antioxidant activity (IC_{50}: 518.93 µg/mL) but moderate cytotoxicity against MCF-7 (IC_{50}: 159.80 µg/mL), with low toxicity on Vero cells (IC_{50}: 2703.61 µg/mL) and a high selectivity index (SI: 16.91). These results indicate that EFBR has potential as a selective chemopreventive agent against breast cancer.

Keywords: Calotropis gigantea *L., molecular docking, MTT assay, DPPH, MCF-7.*

INTRODUCTION

Cancer is one of the main problems in health sector and a disease with a relatively high mortality rate (Nurhayati & Lusiyanti, 2014). In 2022, 23.8% of new cancer cases and 15.4% of cancer-related deaths in women were recorded. Breast cancer was the leading cause of cancer-related death among women. In Indonesia, the prevalence of breast cancer reached 30.1%, with a mortality rate of 19.8% (Globocan, 2022).

Cancer treatment methods that widely used are chemotherapy, radiotherapy and surgery. The treatment that will be given to the patient is based on the type and stage of the cancer. The most commonly used treatment for advanced cancer is chemotherapy (Nussbaumer, *et al*., 2011). Chemotherapy is a chemical agent that can inhibit and kill cancer cells (Remesh, 2012). However, cancer treatment with chemotherapy has several disadvantages. Chemotherapy not only kills cancer cells, but it also can affects the normal cells,

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resulting in quite severe side effects. In addition, the cost of chemotherapy is expensive (Wijaya & Muchtaridi, 2017). Therefore, it is necessary to investigate and develop the potential candidate for new cancer drugs that are safe and effective with minimal side effects derived from natural ingredients, especially from plants.

One of the plants that potential to be developed as a chemopreventive agent for breast cancer is biduri root (*Calotropis gigantea* L.). Biduri (*Calotropis gigantea* L.) contains several active compounds including tannins, flavonoids, saponins, terpenoids, steroids, alkaloids, cardiac glycosides, calotroxin, calotropin D1, calotropin D2, procerosterol, and taraxsterol (Chandrawat & Sharma, 2016). Empirically, people have used biduri to treat wounds, canker sores, itching, fever, cough and increase appetite (Faradilla & Maysarah, 2019). Based on the research that conducted by (Tian, Xie and Zhu, 2018; Zhou, *et al*., 2019) biduri root contains a compound named calotropin which can act as anticancer agent.

In this research, the ethanol fraction of biduri (*Calotropis gigantea* L.) root was developed as an anticancer agent by testing its cytotoxic and antioxidant activity. This research was started by making ethanol extract of biduri root using 70% ethanol and then fractioned. The reason for using ethanol fraction in this research is because based on previous research that was conducted by (Rafiqah, Mastura and Hasibuan, 2019), ethanol fraction has the potential to be active in extracting compounds that have anticancer properties. Furthermore, identification of the compound in biduri roots was carried out using phytochemical screening to determine the active compounds contained in biduri roots. Then, the ethanol fraction of biduri root was tested for its cytotoxicity activity against MCF-7 cancer cells using MTT Assay.

MCF-7 cells are one of the cells used to test anticancer agents in breast cancer by *in vitro* method. The *in silico* test was conducted using molecular docking method towards Bcl-2 and HER-2 proteins. Bcl-2 was chosen because it is a protein that plays an important role in the antiapoptotic process of cells, while HER-2 plays an important role in cell regulation in breast. Then the antioxidant activity test of the biduri roots ethanol fraction tested with 1-1-difenil-2-pikrilhidrazil (DPPH) method. This study is expected to support the previous research and add a new reference regarding the benefits of herbal plants as chemopreventive agent against breast cancer.

The aim of this study is to evaluate the cytotoxic and antioxidant activities of the ethanol fraction of biduri root (EFBR), and to explore its preliminary molecular interactions through *in silico* docking, as a potential chemopreventive agent against breast cancer.

This study is expected to support previous findings and contribute new insight into the potential of herbal plants as alternative therapies for breast cancer.

METHODS

Extraction and Fractionation

Biduri roots (*Calotropis gigantea* L.) were collected from Jenu, Tuban, East Java. The roots were washed, sliced, and dried under indirect sunlight for 4–7 days. Once dried, they were ground into powder, yielding 670 g of root powder.

The powder was macerated in 6.7 L of 70% ethanol (1:10 ratio) at room temperature for 5 days with daily stirring, followed by re-maceration for 2 additional days. The combined extract volume obtained from maceration and re-maceration was 3.975 L.

Liquid-liquid partitioning was performed using ethyl acetate in a 1:1 ratio to obtain the EFBR. This process yielded 1.4 L of liquid EFBR. The fraction was concentrated using a rotary evaporator at 60°C and thickened in a water bath to remove residual solvent, producing 28.772 g of viscous EFBR. The final yield was calculated at 10.9% (Febriansah & Dewayanti, 2021).

Phytochemical Screening

Phytochemical tests were conducted on the ethanol fraction of Calotropis gigantea root to detect the presence of secondary metabolites:

1. Cardiac Glycosides: Keller-Killiani test using glacial acetic acid with $FeCl_3$ and concentrated H_2SO_4.
2. Flavonoids: 200 mg sample treated with magnesium powder and concentrated HCl, then heated in a water bath for 15 minutes.
3. Alkaloids: 200 mg sample added to 5 mL 2N HCl, heated and cooled, then divided into two test tubes and tested with Mayer's and Dragendorff's reagents.
4. Terpenoids/Steroids: Sample mixed with ethyl acetate, evaporated, then treated with acetic anhydride and concentrated H_2SO_4.
5. Saponins: 200 mg sample shaken in 10 mL hot water, left for 10 minutes, then treated with 2N HCl.
6. Tannins: 200 mg sample boiled in 10 mL water for 5 minutes, then 3–4 drops of $FeCl_3$ were added.

In Silico Molecular Docking

Docking was carried out using AutoDock Vina with supporting tools: Discovery Studio Visualizer (DSV), MGLTools, Open Babel, Python, and YASARA. The 3D structures of Bcl-2 (PDB ID: 1G5M) and HER-2 (PDB ID: 3PP0) were downloaded from the Protein Data Bank (www.rcsb.org).

Proteins were cleaned in DSV by removing ligands, water, and other non-receptor molecules. Ligands were either extracted from protein residues or downloaded as .sdf files and converted to .pdb using Open Babel. All files were converted to .pdbqt format in MGLTools, hydrogens were added, and grid box parameters were defined.

Docking was executed using a configuration file (conf.txt) specifying ligand and receptor coordinates. Conformations with the lowest binding affinities and RMSD < 2 Å were selected. Binding interactions were visualized and analyzed in Discovery Studio (Febriansah & Dewayanti, 2021).

Antioxidant Activity Test (DPPH Method)

A 0.4 mM DPPH solution was prepared by dissolving 15.8 mg of DPPH in 25 mL of methanol and diluting to 100 mL. The EFBR stock solution (1000 µg/mL) was prepared by dissolving 100 mg of the ethanol fraction in 100 mL of methanol. Quercetin (100 µg/mL) was used as a positive control.

Serial dilutions were prepared for EFBR (100–500 µg/mL) and quercetin (1–5 µg/mL). Each sample (2 mL) was mixed with 2 mL of DPPH solution and 6 mL of methanol, vortexed, and incubated in the dark at room temperature for a predetermined operating time. Absorbance was measured at 513 nm using a UV-Vis spectrophotometer. All treatments were performed in triplicate. Antioxidant activity was calculated as % inhibition, and IC_{50} values were determined by linear regression (Febriansah & Dewayanti, 2021).

Cytotoxicity Test (MTT Assay)

All instruments were sterilized via autoclaving (121°C, 15 psi, 20 min) and handled under a UV-sterilized laminar airflow hood.

Cell Culture:

MCF-7 (human breast cancer) and Vero (normal) cells were thawed, transferred into complete DMEM (10% FBS, 2% penicillin-streptomycin, 0.5% fungizone), and incubated at 37°C with 5% CO_2. Cells were harvested at confluency using 0.25% trypsin, centrifuged, and resuspended in culture media to reach 5×10^3 cells/100 µL.

Sample Preparation:

EFBR stock solution (105 µg/mL in 0.2% DMSO) was serially diluted. MCF-7 breast cancer cells were treated with EFBR at concentrations ranging from 7.81 to 250 µg/mL, while Vero normal cells were treated at 15.63 to 500 µg/mL. As a positive control, doxorubicin was

tested on MCF-7 cells at concentrations ranging from 0.625 to 10 µg/mL.

Cells (5×10^3/well) were seeded into 96-well plates and incubated for 24 h. After exposure to EFBR or doxorubicin for another 24 h, MTT solution (0.5 mg/mL) was added and incubated for 4 h. Formazan crystals were dissolved in 0.1N HCl in SDS, and absorbance was read at 595 nm. IC_{50} values were calculated to assess cytotoxicity and selectivity (Febriansah & Dewayanti, 2021).

RESULT

Extraction and Fractionation

The EFBR was successfully obtained through a combination of maceration and ethyl acetate liquid-liquid partitioning. From 670 g of powdered root material, an ethanol extract volume of 3.975 L was produced, followed by fractionation to yield 1.4 L of EFBR. After concentration, the final yield of the ethanol fraction was 10.9%. This yield is considered moderate and aligns with reported extraction yields from other plant roots using similar hydroalcoholic solvents. However, it is important to note that the use of alternative solvents such as methanol, dichloromethane, or sequential extraction strategies may potentially enhance both the extraction efficiency and biological activity of the resulting fractions, as observed in related phytochemical studies (Pandey & Tripathi, 2014; Tiwari, *et al.*, 2011).

The choice of 70% ethanol followed by ethyl acetate partitioning was based on its efficiency in selectively extracting semi-polar to polar secondary metabolites, including flavonoids, terpenoids, cardiac glycosides, and phenolic compounds, which were identified in the phytochemical screening of EFBR. Ethyl acetate, in particular, is widely used in natural product isolation due to its intermediate polarity, which enriches compounds with known bioactivities, especially those relevant to anticancer properties (Eloff, 1998). This fractionation strategy aimed to concentrate the active constituents while minimizing non-polar and inactive compounds, thereby increasing the likelihood of detecting biological activity in subsequent assays.

Table 1. Phytochemical screening result.

Phytochemical test	Reagent	Result
Cardiac glycosides	$CH_3COOH + FeCl_3 + H_2SO_4$	+
Flavonoid	Mg + strong HCl	+
Alkaloid	Mayer	-
	Dragendorff	-
Terpenoid and Steroid	Anhydrous acetic acid + strong H_2SO_4	+
Saponin	H_2O + HCl	+
Tannin	$FeCl_3$	+

Phytochemical Screening

Phytochemical tests confirmed the presence of several secondary metabolites in EFBR, including cardiac glycosides, flavonoids, terpenoids, tannins, and saponins (Table 1). Alkaloids were not detected using Mayer or

Table 2. Molecular docking results between ligands and receptors.

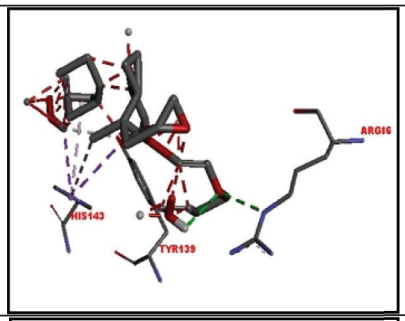
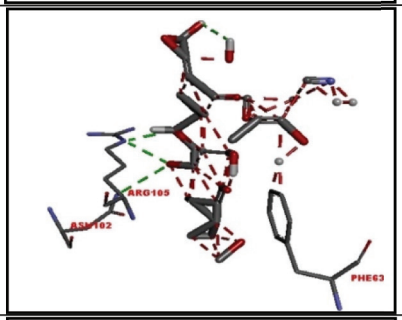
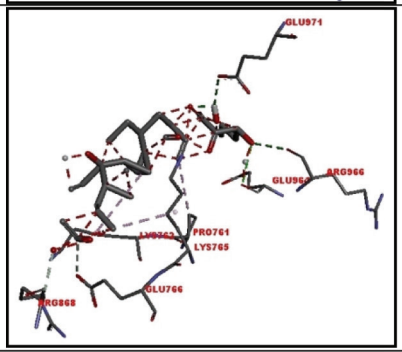
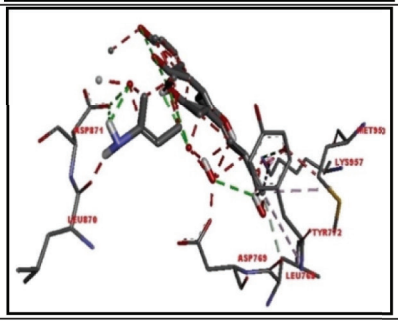
Receptor	Compound	Conformation	RMSD Value	Docking Score (kcal/mol)
Bcl-2	Calotropin	2	1.613	- 8.1
	Doxorubicin	8	1.373	- 6.5
HER-2	Calotropin	5	1.836	- 7.5
	Doxorubicin	2	1.246	- 7.2

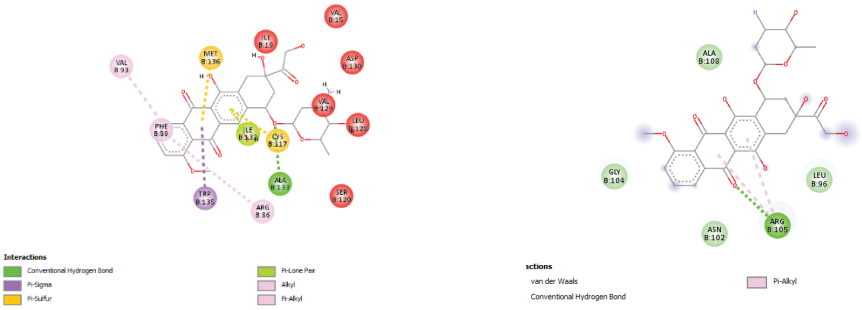
Dragendorff reagents. These findings suggest the extract contains compounds known to exhibit cytotoxic and antioxidant activities.

In Silico Test by Molecular Docking

Molecular docking revealed that calotropin had a stronger binding affinity to Bcl-2 (−8.1 kcal/mol) compared to doxorubicin (−6.5 kcal/mol), suggesting potential for pro-apoptotic activity. With HER-2, calotropin also showed a slightly better docking score (−7.5 kcal/mol) than doxorubicin (−7.2 kcal/mol), although this result is exploratory due to the HER-2–negative status of MCF-7 cells. All docking poses had RMSD values <2 Å, indicating good structural reliability (Table 2).

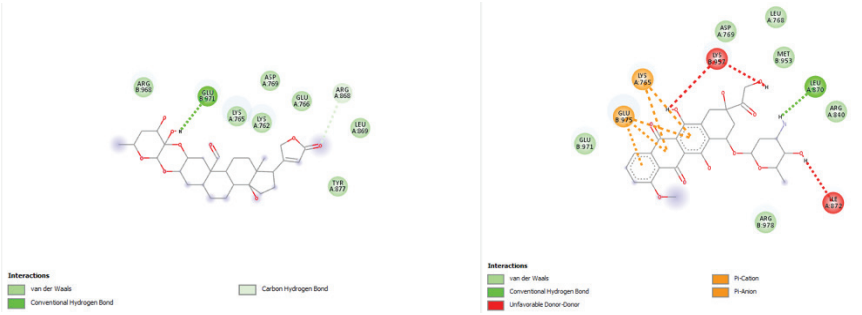
Table 3. Interpretation of interaction between test compounds and protein.

Protein	Compound	Amino Acid Binding Potential	3D Visualization
Bcl-2	Calotropin	Arg86, His143, Tyr139	
	Doxorubicin	Arg105, Asn102, Phe63	
HER-2	Calotropin	Arg868, Arg966, Glu766, Glu966, Glu971, Lys762, Pro761	
	Doxorubicin	Asp769, Asp871, Leu768, Leu870, Lys957, Met953, Tyr772	



A B

Figure 1. 2D visualization of amino acid interaction between test compounds (A) calotropin; (B) doxorubicin and Bcl-2 protein.



A B

Figure 2. 2D visualization of amino acid Interaction between test compounds (A) calotropin; (B) doxorubicin and HER-2 protein.

Antioxidant Activity Test with DPPH Method

The DPPH assay showed EFBR had weak antioxidant activity with an IC_{50} value of 518.93 µg/mL (R^2=0.9954), while quercetin, the positive control, exhibited very strong antioxidant activity (IC_{50}=2.24 µg/mL, R^2=0.8411) (Table 4; Figures 3 and 4). The weak antioxidant result suggests other mechanisms, such as apoptosis induction, may contribute more significantly to EFBR's anticancer effect.

Table 4. Antioxidant assay IC_{50} value.

Sample	Linear Regression Equation	IC_{50} Value	Description
EFBR	Y=0.0875x+4.594 R^2=0.9954	518.925	Weak
Quercetin	Y=9.8897x+27.833 R^2=0.8411	2.241	Very Strong

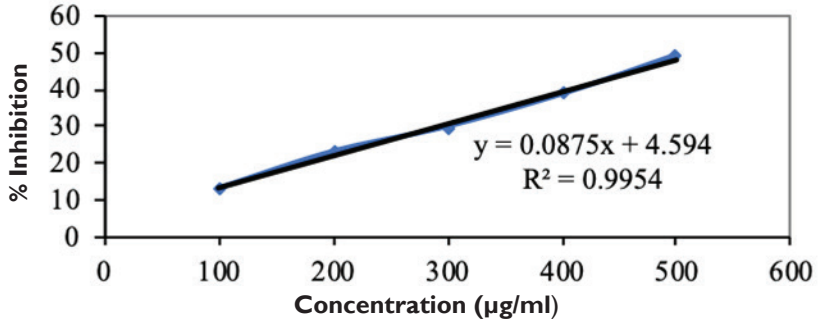


Figure 3. EFBR inhibition chart.

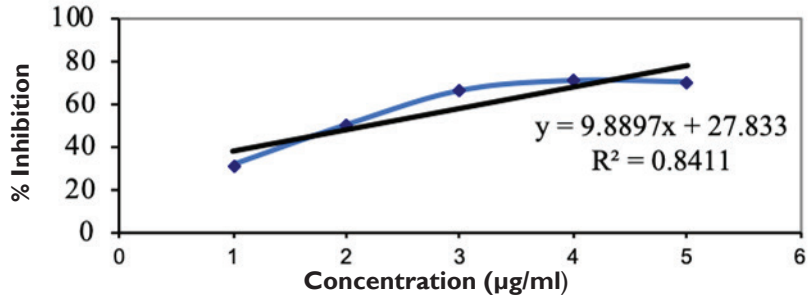


Figure 4. Quercetin inhibition chart.

Cytotoxic Test with MTT Assay

EFBR demonstrated moderate cytotoxicity against MCF-7 cells with an IC_{50} of 159.80 µg/mL (R^2=0.848) and induced a dose-dependent reduction in cell viability (Table 5; Figure 5). In contrast, doxorubicin was more potent, with an IC_{50} of 22.19 µg/mL (R^2=0.6921) (Figure 7). Morphological changes consistent with cytotoxic effects were observed under an inverted microscope (Figures 8 and 10). On Vero normal cells, EFBR had a high IC_{50} of 2703.61 µg/mL (R^2=0.7068), indicating low toxicity (Figures 6 and 9).

Table 5. IC_{50} value in cytotoxic test.

Target	Linier Regression Equation	IC_{50} Value	Description
MCF-7 Cancer Cells (EFBR Treatment)	Y=-0.094x+65.021 R^2=0.848	159.797	Quite Toxic
MCF-7 Cancer Cells (Doxorubicin Treatment)	Y=-1.0281x+72.818 R^2=0.6921	22.194	Toxic
Vero Normal Cells	Y=0.0083x+72.44 R^2=0.7068	2703.614	Non-Toxic

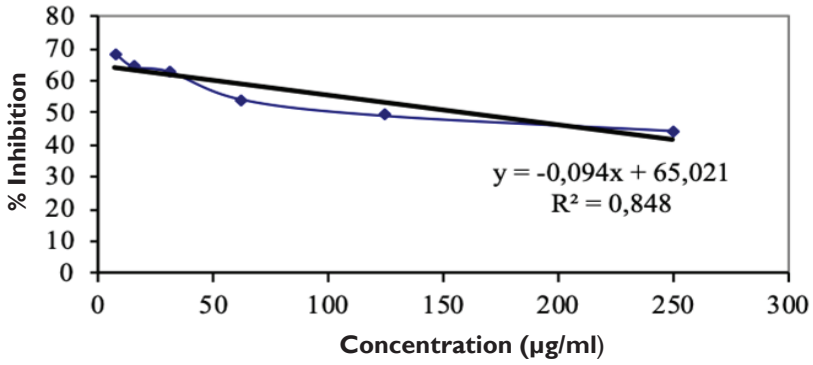


Figure 5. Graph of living cell percentage after EFBR treatment on MCF-7 cancer cells.

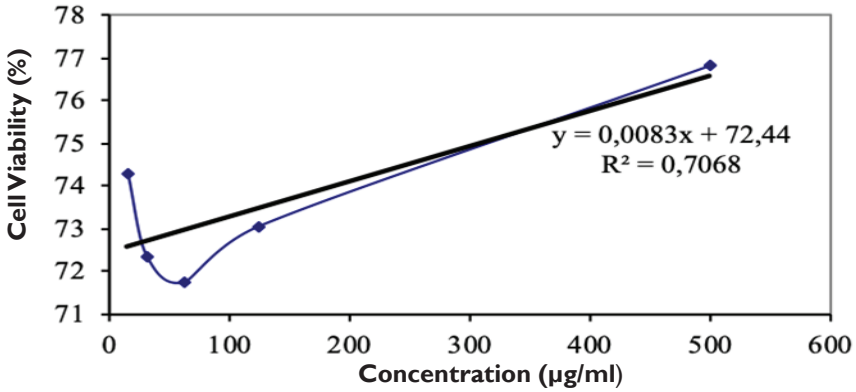


Figure 6. Graph of living cell percentage after EFBR treatment on Vero normal cells.

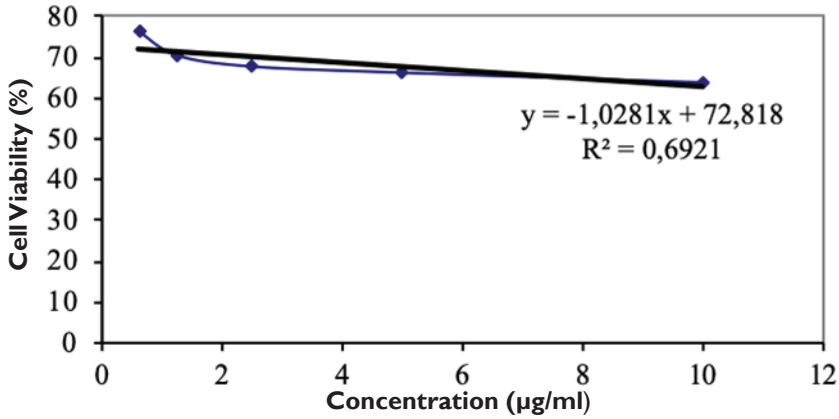


Figure 7. Graph of living cell percentage after doxorubicin treatment on MCF-7 cancer cells.

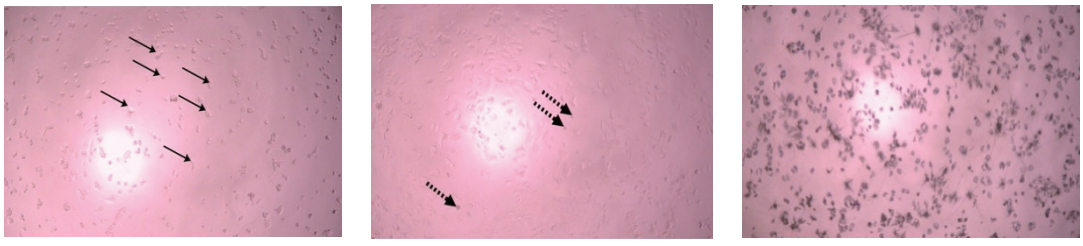
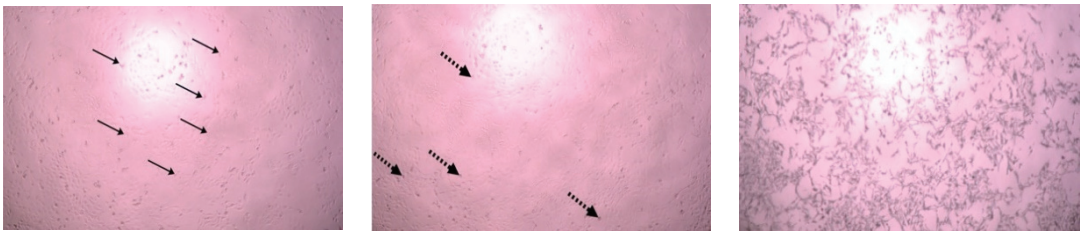
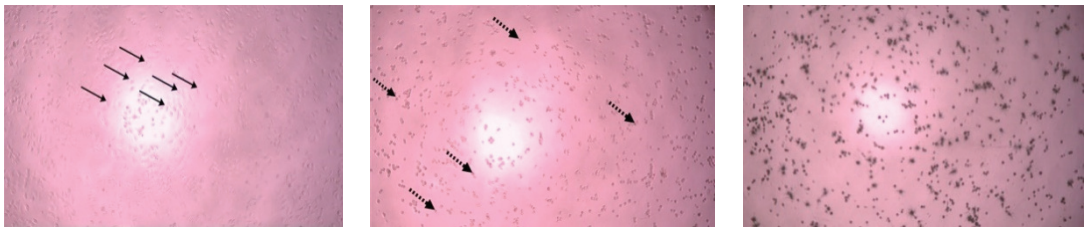


Figure 8. Morphological changes of MCF-7 cells on treatment with EFBR (a) before being treated; (b) after being treated; (c) after being treated and incubated as well as the addition of MTT reagent. (⟶) living cells; (⇢) dead cells.



a b c

Figure 9. Morphological changes of Vero normal cells on treatment with EFBR (a) before being treated; (b) after being treated; (c) after being treated and incubated as well as the addition of MTT reagent. (⟶) living cells; (⇢) dead cells.



a b c

Figure 10. Morphological changes of MCF-7 cells on treatment with doxorubicin (a) before being treated; (b) after being treated; (c) after being treated and incubated as well as the addition of MTT reagent. (⟶) Living Cells; (⇢) Dead Cells.

Selectivity Test

Table 6. Selectivity index value on selectivity cells.

Target	IC_{50} Value	SI	Description
MCF-7 Cancer Cells	159.79	16.91	Selective
Vero Normal Cells	2703.61		

The calculated Selectivity Index (SI) was 16.91, well above the SI ≥ 3 threshold typically used to define selective cytotoxicity (Table 6). EFBR has strong potential as a selective anticancer agent.

DISCUSSION

This study investigated the anticancer potential of the EFBR through a combination of phytochemical screening, *in silico* molecular docking, antioxidant (DPPH) assay, and cytotoxicity testing (MTT assay) on MCF-7 and Vero cells. The presence of bioactive secondary metabolites such as flavonoids, terpenoids, cardiac glycosides, and tannins indicated possible pharmacological activity. Calotropin, a cardenolide compound previously identified in Calotropis gigantea, was selected as a representative ligand for molecular docking. The docking study targeted two cancer- related proteins, Bcl-2 (PDB ID: 1G5M) and HER-2 (PDB ID: 3PP0), with doxorubicin included as a reference ligand due to its well documented use

as an anticancer drug. Bcl-2 was selected because of its role as an antiapoptotic protein regulating mitochondrial apoptosis pathways. Overexpression of Bcl-2 in cancer cells suppresses programmed cell death and contributes to cancer progression, making it a crucial therapeutic target (Arora, *et al*., 2024). Calotropin demonstrated a stronger binding affinity to Bcl-2 (−8.1 kcal/mol) than doxorubicin (−6.5 kcal/mol), suggesting greater potential to induce apoptosis. Although HER-2 was included due to its overexpression in 20–30% of breast cancers and its association with metastasis and treatment resistance (Zhou, *et al*., 2023), it is important to clarify that MCF-7 cells used in this study are HER-2 negative, falling under the luminal A subtype. Therefore, the HER-2 docking results (calotropin −7.5 kcal/mol vs. doxorubicin −7.2 kcal/mol) are exploratory and do not directly correlate with the *in vitro* model. All docking results showed RMSD values <2 Å, validating the pose accuracy, though RMSD is a measure of prediction reliability rather than ligand affinity (Forli & Olson, 2012). To improve target specificity in future studies, network pharmacology is recommended as a strategy to systematically identify biological targets relevant to calotropin's mechanism of action (Hopkins, 2008).

Following docking analysis, EFBR was tested for antioxidant activity using the DPPH free radical scavenging method. According to (Pai, Shukla and Kikkeri, 2014) free radicals are atoms or molecules with one or more unpaired electrons. This causes these compounds to be very reactive in finding electron pair by binding and attacking electrons in surrounding molecules such as proteins, lipids and DNA When free radicals do not bind to any antioxidants, the oxidation reaction will continue and can form a cascade that can cause cancer (Andarina & Djauhari, 2017).

To prevent the accumulation of free radicals in the body, antioxidant compounds are needed so it can neutralize, reduce and inhibit the formation of new free radicals in the body. Antioxidants will become electron donors, so that free radicals become paired and stop damaging the body. This study aims to determine the ability of EFBR to inhibit or capture free radicals from DPPH. The principle of DPPH free radical scavenging method is based on the reduction of methanol and purple solution of DPPH free radical by inhibition of free radical. When the purple DPPH solution meets the electron donor material, the DPPH will be reduced and can cause the purple color to fade and replaced with a yellow color that comes from the picryl group (Tristantini, *et al*., 2016). The intensity of purple color lost then measured by a UV-Vis spectrophotometer with a maximum wavelength (Tristantini, *et al*., 2016). The comparison used in this study was quercetin as a positive control. Quercetin is a flavonol belonging to one of the subclasses of flavonoids. Various biological effects of quercetin have been idely studied, such as anticancer, antiviral and anti-inflammatory (Li, *et al*., 2016). In addition, quercetin has been known as a source of antioxidants (Siswarni, Putri and Rinda, 2017).

After optimization of DPPH, the absorbance were measured with wavelength of 513 nm. The absorbance value obtained then made a graph of the relationship between the percentage of inhibition and the concentration. From this graph, a linear regression equation were obtained. The EFBR equation is y=0,0875x+4,594 (R2=0.9954) and the quercetin equation is y=9.8897x+27.833 (R2=0.8411) which can later be used to calculate the IC_{50} which is a parameter used to determine the amount of antioxidant activity. The test result shows that EFBR has a weak antioxidant activity with an IC_{50} value of 518.925 µg/mL, while the IC_{50} value of quercetin is 2.241 µg/mL as written in Table 4. This shows that quercetin has a very strong antioxidant activity. This aligns with the complex nature of EFBR, which contains a mixture of compounds whose combined antioxidant capacity may be diluted compared to single, purified flavonoids.

The cytotoxicity of EFBR was assessed

using the MTT assay on MCF-7 breast cancer cells and Vero normal cells. This method is used to measure the level of cytotoxicity of a substance with the principle of reducing the yellow tetrazolium salt. The MTT assay method will form an enzymatic reaction in the form of a yellow tetrazolium salt which then reacts with living cells and forms purplish blue formazan crystals. Formazan crystal formation is helped by the presence of the succinate dehydrogenase enzyme (Doyle & Griffiths, 2000). The color change method is intended to detect the presence of cell proliferation. In proliferating cells, mitochondria will absorb MTT so that the cells will turn purple due to the formation of tetrazolium or formazan crystals (Depamede & Rosyidi, 2009).

Living cells are indicated by the formation of purple formazan. The darker purple color that is formed, the more cells are alive (Siregarr & Hadijono, 2000; Vajrabhaya & Korsuwannawong, 2018). The purpose of this test was to determine the potential of EFBR as a cytotoxic agent and to determine the level of its selectivity in normal cells against MCF-7 breast cancer cells. The test parameter used is IC_{50} value. This value indicates the concentration value that results in a 50% cells proliferation inhibition and indicates the cell potential which is a benchmark for conducting cell kinetics observation tests.

MCF-7 breast cancer cells were treated with EFBR and doxorubicin as a positive control while Vero normal cells were only treated with EFBR with several concentrations. Then observations were made on the morphological changes that occurred visually using an inverted microscope and compared with cells without treatment. The results obtained in MCF-7 cells before being given EFBR and doxorubicin treatment were oval in shape and after EFBR treatment were given, the cells shape changed to an irregular oval, while after being given doxorubicin treatment, they were round with a smaller size. The Vero normal cells before being given EFBR treatment were round and after being given EFBR treatment, the results were slightly oval.

Furthermore, quantitative analysis was carried out with absorbance readings using an ELISA reader and the absorbance value was obtained. The absorbance value is then made a linear regression equation to find the IC_{50} value. Based on the IC_{50} value data in Table 5, EFBR exhibited moderate cytotoxic activity on MCF-7 cells, with an IC_{50} value of 159.79 µg/mL. A dose-dependent decrease in cell viability was observed, where the lowest tested concentration (7.81 µg/mL) resulted in 70.18% cell viability, while the highest (250 µg/mL) reduced viability to 44.47%. In contrast, the reference compound doxorubicin showed a stronger cytotoxic effect, with an IC_{50} of 22.19 µg/mL. At concentrations of 0.625 µg/mL and 20 µg/mL, cell viability was reduced to 74.53% and 63.82%, respectively, indicating less than 40% inhibition at the upper tested dose.

These findings align with previous studies, where doxorubicin demonstrated IC_{50} values ranging from 0.3–5 µg/mL against MCF-7 cells depending on exposure time and formulation. For instance, Mishra, *et al*. (2021) reported an IC_{50} of 3.3 µg/mL after 24 h treatment with free doxorubicin, while Yang, *et al.* (2015) found values as low as 0.5 µg/mL for nanoparticle-encapsulated doxorubicin. The higher IC_{50} observed in this study may be due to differences in experimental conditions such as incubation time or compound solubility.

Despite having a higher IC_{50} than doxorubicin, EFBR demonstrated low toxicity toward Vero normal cells, with an IC_{50} of 2703.61 µg/mL. Across the tested concentration range (15.63–500 µg/mL), cell viability remained above 70%, indicating that EFBR kills less than 30% of normal cells. This contrasts with the selective cytotoxicity observed in MCF-7 cells and suggests EFBR has a favorable safety profile.

The SI, calculated as the ratio of IC_{50} in

normal cells to cancer cells, was 16.91. An SI≥3 is considered indicative of selective cytotoxicity toward cancer cells Sirait *et al*., 2019. Therefore, EFBR demonstrates promising selective toxicity, supporting its potential as a chemopreventive or therapeutic agent against breast cancer. While its activity is lower than that of doxorubicin, EFBR's natural origin, complex phytochemical composition, and low toxicity to normal cells make it a valuable candidate for further investigation (Sirait, *et al.*, 2019). Compared to other plant-derived extracts tested on MCF-7 cells, such as Annona muricata SI=10.7 (Restuati, *et al*., 2021) and Tinospora crispa SI=4.8 (Kurniawan, *et al*., 2020), EFBR demonstrates a notably higher selectivity.

Bcl-2 and HER-2 proteins are important molecular targets involved in the pathogenesis and progression of breast cancer, particularly in MCF-7 cells, which express hormone-responsive signaling pathways associated with tumor proliferation and survival. Bcl-2 functions as an anti-apoptotic protein by inhibiting mitochondrial outer membrane permeabilization and preventing cytochrome-c release, thereby suppressing caspase activation and apoptosis. Overexpression of Bcl-2 has been associated with prolonged cancer cell survival and resistance to chemotherapeutic agents. In contrast, HER-2 is a receptor tyrosine kinase that promotes cellular proliferation, migration, and survival through activation of downstream signaling pathways, including PI3K/Akt and MAPK. HER-2 overexpression contributes to aggressive tumor progression, increased metastatic potential, and poor prognosis in breast cancer patients. Therefore, inhibition of Bcl-2 and HER-2 may represent a promising therapeutic strategy for suppressing breast cancer cell growth and inducing apoptosis in MCF-7 cells (Adams & Cory, 2007; Loibl & Gianni, 2017; Moasser, 2007; Yip & Reed, 2008)

Despite promising cytotoxicity and selectivity, the study has limitations. The extract was not tested on other cancer cell lines such as HER-2 positive SK-BR-3 or triple-negative MDA-MB-231, limiting generalizability. Additionally, apoptosis-specific assays (*e.g.*, Annexin V/PI, caspase activity) and mechanistic studies were not conducted. The complex composition of EFBR also complicates identification of the active constituents responsible for cytotoxicity. Future studies should employ bioassay-guided fractionation, network pharmacology analysis, and detailed apoptosis/cell-cycle assays to clarify mechanisms of action. Moreover, statistical comparisons across treatment groups (*e.g*., ANOVA with post hoc testing) should be more explicitly presented in figures and results tables to strengthen interpretation.

Overall, EFBR shows selective cytotoxicity toward MCF-7 cells, likely through non antioxidant mechanisms such as apoptosis induction, with calotropin as a promising candidate compound. Further *in vitro* and *in vivo* validation is necessary to fully elucidate its therapeutic potential and safety profile.

CONCLUSION

This study concludes that the ethanol fraction of biduri root (EFBR) contains cardiac glycosides, flavonoids, terpenoids, saponins, and tannins, as revealed by phytochemical screening. Molecular docking results showed that calotropin, a compound in EFBR, exhibited strong and stable binding to Bcl-2 and HER-2 proteins, with docking scores of –8.1 and –7.5 kcal/mol, respectively. EFBR demonstrated weak antioxidant activity, with an IC_{50} value of 518.925 µg/mL. In cytotoxicity tests, EFBR showed moderate toxicity against MCF-7 breast cancer cells (IC_{50}=159.797 µg/mL) and low toxicity toward Vero normal cells (IC_{50}=2703.614 µg/mL). The selectivity index (SI) of 16.91 indicates that EFBR selectively targets cancer cells over normal cells, suggesting its potential as a chemopreventive agent.

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