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Antioxidant and Anticancer Activity of *Dillenia serrata* Thunb Ethanol Extract Against MCF-7 Breast Cancer Cell Line

Rahmawati[1]*, Zulkifli[4], Tri Rini Nuringtyas[2], Riris Istighfari Jenie[3], Laurentius Hartanto Nugroho[2]

[1]Doctoral Program of Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2]Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
[3]Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia
[4]Faculty of Pharmacy, Universitas Muhammadiyah Makassar, Makassar, Indonesia

Abstract

Women's breast cancer incidence rate in Indonesia ranks number one with 12 per 100,000 cases, with luminal A as the dominant subtype. Currently, chemotherapeutic agents have limitations that lead to inefficiencies in therapy, therefore it is necessary to develop more effective and efficient chemopreventive agents. Plant secondary metabolites can provide pharmacological effects that can be used as chemoprevention agents. Secondary metabolites of *D. serrata* may have pharmacological effects as antioxidants and cytotoxic. This study aims to determine the antioxidant properties and cytotoxic activity of *D. serrata* ethanolic extract on the MCF-7 breast cancer cell line. The leaves of *D. serrata* were macerated, while the bark and root samples were refluxed with 96% ethanol as solvent. All extracts were evaporated with a rotary evaporator. Qualitative evaluation of the phytochemical content of leaf ethanolic extract, bark ethanolic extract, and root ethanolic extract was done using the standard tube test method. The antioxidant assay was carried out using the DPPH. The cytotoxic activity was determined *in vitro* using an MTT assay against the MCF-7 cell line with a series of concentrations from 12.5–400 µg/mL. Doxorubicin was the positive control treated at a 3.125–100 µg/mL concentration. The antioxidant activity showed that leaf extract had the highest antioxidant activity, followed by root and bark extract, with IC_{50} values of 95.66, 270.5, and 335.96 ppm, respectively. Leaf ethanolic extract and root ethanolic extract's cytotoxic ability is considered moderate cytotoxic with IC_{50} values of 493.17 and 229.82 µg/mL, respectively. Amongst the ethanolic extract from the leaf, bark, and root of *D. serrata*, the leaf ethanolic extract has the best anti-oxidant activity and the bark ethanolic extract was the most cytotoxic one against MCF-7 cells.

Keywords: *Antioxidant, Cytotoxic, Dillenia serrata, MCF-7.*

INTRODUCTION

Cancer is one of the leading causes of death with the number of sufferers increasing every year. Cancer is a degenerative disease characterized

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*Corresponding author: rahmawati.sbi@mail.ugm.ac.id

by the condition of cells that grow continuously without control and have the ability to metastasize pathologically. Globocan data in 2020 states that breast cancer ranks first with an incidence rate of cases (16.6%) of the total 396,914 new cases of cancer in Indonesia, and 9.6% of female breast cancer cases end in death (International Agency for Research on Cancer, 2021). Malignancy in breast cancer occurs due to disruption of the cell growth system in breast tissue originating from the lobules or ducts (Subarkah, 2018).

Chemotherapy is one of the treatments for breast cancer patients. However, currently available chemotherapeutic agents have several limitations, such as resistance events, adverse effects, and inadequate efficacy (Pearce, *et al*., 2017). As a result, therapeutic inefficiency occurs, so it is necessary to develop more effective and efficient chemopreventive agents. Plants generally have active substances in the form of secondary metabolites and can provide pharmacological effects that can be used as chemoprevention agents. One approach to finding chemopreventive compounds is exploring natural materials, for example, *D. serrata*. Plant *D. serrata* belonging to the Dilleniaceae family, is an endemic plant to Sulawesi. Almost all parts of *D. serrata* are used traditionally for therapeutic purposes. The bark is used as an ingredient in traditional medicine, such as for treating fever and wounds (Irnawati, *et al*., 2017), and has bioactivity as an anti-inflammatory (Jalil, *et al*., 2015). The vitamin C content in the fruit extract of *D. serrata* is 68.3% (Illing, *et al*., 2019). Therefore, it has the potential as an antioxidant. The main function of antioxidants is to neutralize free radicals, this becomes one of the mechanisms of cancer chemoprevention, to halt the progression of cancer (George, *et al*., 2021). *Dillenia indica* f. elongata has been known to have anticancer potential due to the chemical content of betulinic acid (Boparai, *et al*., 2016). Therefore, we hypothesized that *D. serrata*, which is in the same genus as *Dillenia indica* f. elongata, has the same chemical content and cancer chemopreventive activity toward breast cancer. This research is a preliminary study that aimed to examine the antioxidant and cytotoxic activity of the ethanolic extract of *D. serrata* against the MCF-7 cell line as a Luminal A subtype breast cancer cell line model.

METHODS

Sample Preparation

Plant *D. serrata* was obtained from Porodoa Hamlets, Mappedeceng District, North Luwu Regency, South Sulawesi, Indonesia. Geographically located at 2˚ 23’ 55”-2˚ 41’ 54” South Latitude and 120˚ 21’ 9”-120˚32’ 40” East Longitude. The determination of the plant was performed in the Laboratory of Pharmaceutical Biology, Universitas Indonesia Timur. The plant leaves were extracted by using the maceration method, while the bark and root were extracted by using the reflux method. All samples were extracted using 96% ethanol as solvent. The extract was evaporated with a rotary evaporator at 40ºC. We then calculated the yield percentage of each extract.

Phytochemical screening

Phytochemical content in leaf ethanolic extract, bark ethanolic extract, and root ethanolic extract were tested qualitatively using the appropriate method reported by (Sabandar, *et al*., 2020). The screening was carried out to test the content of alkaloids, flavonoids, saponins, steroids, tannins, and terpenoids using Lieberman Burchard reagent, Magnesium (Mg), concentrated HCl (Merck, Darmstadt, Germany), Dragendroff’s reagent (Merck), HCl 1% (Merck), and 0.1% FeCl3.

Antioxidant Activity Assay

The antioxidant activity test was carried out using the DPPH method (1,1-diphenyl-1-picrylhydrazyl) with a concentration of 5, 10, 20, 40, and 80 ppm. L-Ascorbic acid (Sigma-Aldrich, St. Louis, Missouri, USA) was used as the standard solution. The absorbance was measured at a wavelength of 516.30 nm using a UV-Vis

Table 1. Yield percentage of each extract of *D. serrata* Thunb.

Sample	Fresh Sample mass (g)	Simplicia mass (g)	Extract mass (g)	Yield (%)
Leaf	1000	180	34.60	19.22
Bark	1980	724	47.59	6.57
Root	2640	907.2	77.11	8.45

spectrophotometer. We determined the antioxidant potential of the samples using the following equation (Adesanwo, *et al.*, 2013):

$$DPPH\ (\%)=((A0-A1)x100\%)/A0$$

Where: A0=Absorbance of standard
A1=Absorbance of sample

We then calculated the inhibitory concentration 50 (IC_{50}) of the antioxidant activity based on linear regression analysis of the DPPH attenuation percentage vs. the extract concentration. The IC_{50} value represents the amount of sample concentration required to reduce 50% of the DPPH radicals.

Cell Culture

The MCF-7 cell line was obtained from the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada. Cells were grown in RPMI-1640 (Roswell Park Memorial Institute) (Sigma-Aldrich) medium, 10% FBS (Himedia Ref RM 10432), sodium bicarbonate (Sigma-Aldrich), 1% penicillin-streptomycin and 0.5% fungizone (Gibco).

Cytotoxic Assay

In vitro cytotoxic activity of extracts was observed using the MTT assay method based on the description of Jenie, *et al*. (2018) with modification. Cytotoxicity was carried out to determine the IC_{50} value with a sample concentration of 400, 200, 100, 50, 25, and 12.5 µg/mL and doxorubicin concentration 100, 50, 25, 12.5, 6.25, and 3.125 µg/mL. In the cytotoxic test, the test solution was prepared by dissolving 10 mg of extract in 100 µL of DMSO and 900 µL of culture medium. Then the test solution and doxorubicin were made in various concentrations. The treatments consisted of media control, cell control, test solution (extract), and doxorubicin. 100 µL of cell suspension was added to control cells, and 100 µL of complete culture medium was added to control media, then cell cultures were incubated at 37°C, with 5% CO_2 content for 24 h. After 24 h, the culture medium was discarded and then washed with PBS (Sigma-Aldrich) and added 100 µL of MTT solution (Sigma), then incubated for 3-4 h. Then 100 mL of 10% SDS was added to stop the MTT reaction in each well, then shaken for 5 minutes, wrapped in aluminium foil, and incubated for 24 h at room

Table 2. Qualitative analysis of phytochemical content in *D. serrata* extract.

Phytochemical	LEE	BEE	REE
Alkaloid	+	+	-
Flavonoid	+	+	+
Saponin	+	+	+
Tanin	+	+	+
Terpenoid	-	+	+
Steroid	+	-	-

Note:
(+) positive reaction
(-) negative reaction
Leaf Ethanolic Extract (LEE)
Bark Ethanolic Extract (BEE)
Root Ethanolic Extract (REE)

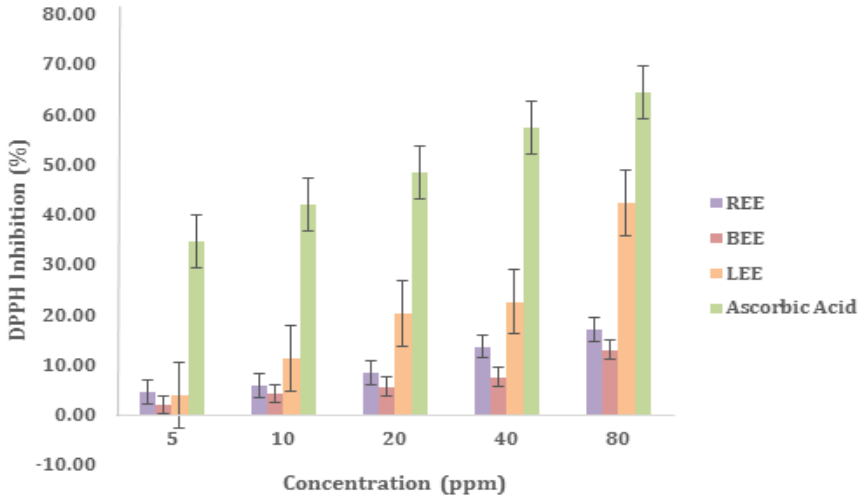

Figure 1. Antioxidant activity of *D. serrata* ethanolic extract. Root Ethanolic Extract (REE); Bark Ethanolic Extract (BEE); Leaf Ethanolic Extract (LEE) of D. serrata, and ascorbic acid as standard were tested for their antioxidant activity using DPPH Method. Values are mean ±SE, n=3, *p<0.05.

temperature. The absorbance of the solution in each well was read with an Elisa reader at a wavelength of 595 nm (Bio-Rad). The percentage of MCF-7 living cells was calculated from the absorbance data.

Percentage of Viable Cells=((a-b))/((c-b))x100%

Where: a=absorbance of sample
b=absorbance of medium
c=absorbance of control

RESULT

Extraction and Phytochemical Screening

We extract the leaf, bark, and root of the *D. serrata* using 96% ethanol and then calculated the yield. The highest yield percentage was from the leaf ethanol extract of 19.22% (Table 1). The high yield of the ethanolic extract of the leaves shows that there are many compounds (polar and non-polar) found in the ethanolic extract of the leaves. The number of active compounds that are attracted to the extraction process depends on the solvent used, the appropriate extraction method, and the surface area of the simplicia.

The phytochemical screening of root ethanolic extracts was proven to contain flavonoids, saponins, tannins, and terpenoids (Table 2) The bioactive compounds that can be attracted by solvents depend on the polarity of the solvent. Ethanol solvent has a high polarity so that it can attract polar compounds such as phenolics, flavonoids, alkaloids, glycosides, terpenoids, and steroids (Hanin & Pratiwi, 2017). Although terpenoids can be attracted by ethanol, not all compounds including terpenoids will be attracted by ethanol. This can be seen from the phytochemical screening results that leaf ethanolic extract is not positive for terpenoids but the bark ethanolic extract and root ethanolic extract was positive for terpenoids which was indicated by a change in color after being given the reagent compound. Not all plant organs have the same bioactive compounds, this is because the biosynthesis of secondary metabolites

Table 3. Antioxidant activity of ethanolic extract of *D. serrata* based on DPPH method.

Sample	Regression equation	R Value	IC_{50} (ppm)±SE
Root Extract Y	=0.2865x+1.7897	0.9469	270.50±2.36
Bark Extract Y	=0.1957x+0.9125	0.9702	335.96±1.86
Leaf Extract Y	=0.608x+2.2003 0	.9757	95.66±6.48
Ascorbic Acid	Y=1.32x+14.316	0.8665 3	2.34±5.30

often occurs in specific tissues or cells and is highly dependent on the level of differentiation and development (Kuntorini & Nugroho, 2009).

Free Radical Scavenging Assay

The results of the antioxidant activity test based on the percentage of DPPH inhibition of LEE, BEE, and REE and the IC_{50} value is shown in Figure 1.

Figure 1 shows antioxidant testing among the three extracts, it turns out that leaf ethanolic extract has the highest antioxidant activity with an IC_{50} value that is closer to ascorbic acid. This result indicated that leaf ethanolic extract has a strong antioxidant activity. On the other hand, bark ethanolic extract and root ethanolic extract showed a weak level of antioxidant activity. Leaf ethanolic extract's antioxidant activity is correlated with its high flavonoid content as shown in Table 1 Phenolic compounds and flavonoids are known as secondary metabolites of plants with great antioxidant properties (Tungmunnithum, *et al*., 2018). Moreover, the high antioxidant activity correlated with phenol, flavonoid, and tannin contents (Agustina, *et al*., 2016). The major compound of *D. serrata*, namely, betulinic acid showed weak free radical scavenging ability (Adesanwo, *et al*., 2013). Betulinic acid is classified as a triterpene which is widely found in the bark and roots of *D. serrata* (Jalil, *et al*., 2015; Sabandar, *et al*., 2020). The antioxidant activity of an extract is strongly influenced by the major compounds contained in it, both synergistic effects and side effects antagonists (Crespo, *et al*., 2019). Therefore,

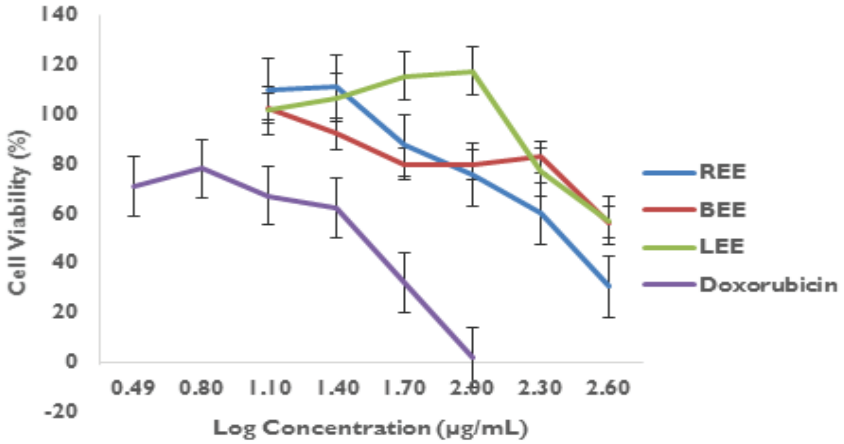

Figure 2. Cytotoxic activity of *D. serrata* ethanolic extract. Root Ethanolic Extract (REE); Bark Ethanolic Extract (BEE); and Leaf Ethanolic Extract (LEE) of *D. serrata* cytotoxic activity on MCF-7 cells using MTT method with doxorubicin as a positive control. Cells were treated with various concentrations of samples for 24 h before assessment with MTT assay as described in the Methods. Values are mean ±SE, n=3, *p<0.05.

Table 4. Result of cytotoxic test of extract of *D. serrata* and doxorubicin.

Sample	IC_{50} (µg/mL)±SE
Root Extract Ethanol	229.82±12.61
Bark Extract Ethanol	727.78±6.30
Leaf Extract Ethanol	493.17±9.73
Doxorubicin	32.34±11.89

the weak antioxidant activity of bark ethanolic extract and root ethanolic extract may be due to the presence of betulinic acid which exerts the opposite effect on DPPH radical scavenging activity.

In Vitro Cytotoxicity Assay

We performed a cytotoxicity assay using the MTT method. Based on the assay, the IC_{50} value of leaf ethanolic extract, bark ethanolic extract, and root ethanolic extract, respectively, were 493.17, 731.14, and 229.82 µg/mL. The cytotoxic ability of leaf ethanolic extract and root ethanolic extract is classified as moderate cytotoxic with an IC_{50} range of 100-500 µg/mL (Weerapreeyakul, *et al*., 2012).

Bioactive compounds from the alkaloid and flavonoid groups in leaf ethanolic extract and terpenoids in root ethanolic extract are known as a group of compounds that have cytotoxic activity, inhibit cell growth, and induce apoptosis in various cancer cell lines (Widiyastuti, *et al*., 2018).

DISCUSSION

In this study, we explored the antioxidant and cytotoxicity activity of various parts of *Dillenia serrata* toward MCF-7 breast cancer cells. The root, bark, and leaf of the plant were extracted using 96% ethanol and analyzed. Qualitative phytochemical analysis of *D. serrata* showed that the plant contains many compounds such as alkaloid, flavonoid, saponin, terpenoid, and steroid, however, the compounds were not present in all parts of the plant. Dilleniaceae plants contain various flavonoids and phenolic compounds. Phenol, flavonoids, and tannin are secondary metabolite compounds that have antioxidant activity (Sen & Chakraborty, 2011). Phenolic compounds have antioxidant activity and act as a reductor and hydrogen donor, neutralize singlet oxygen, and bind metal. Flavonoids act as an antioxidant because it is a good reductor so they can prevent oxidative stress. On the other hand, tannin act as an antioxidant by binding free radical, metal, and protein with enzyme activity (Tiwari, *et al*., 2011). The position and number of free hydroxyl radical groups determine their potency.

The antioxidant activity of the extract was demonstrated through the DPPH test. The result showed that the ethanol extract of *D. serrata* leaves had better antioxidant activity than other extracts with a strong level of antioxidant activity. This is in accordance with what was stated by Rahayu, *et al*. (2019), most of the flavonoids and phenols were found in the leaves and have the potential as a source of antioxidants, and are also supported by a study from Sabandar, *et al*. (2017), quercetin compounds are found in the leaves of all types of Dillenia sp. Quercetin is a type of flavonoid antioxidant compound contained in plant foods. The high antioxidant activity of leaf ethanolic extract may be due to this compound. Therefore, further research is needed to determine the types of antioxidant compounds contained in *D. serrata* leaves. Several studies reported that low antioxidant intake or low blood levels of antioxidants increases the risk of different diseases such as the risk of cancer doubled (Percival, 1998). Therefore, antioxidants

can protect health from oxidative stress. Oxidative stress causes different diseases through four critical steps; membrane lipid peroxidation, protein oxidation, DNA damage and disruption in reducing cell equivalents; which leads to cell destruction, altering signaling pathways. Oxidative stress has implications for various diseases such as cancer, cardiovascular disease, diabetes, neurological disorders, and neurological disorders (Sen & Chakraborty, 2011). Several antioxidant compounds were found to increase the cytotoxic effect of drugs on malignant cells through the apoptotic pathway by damaging the DNA of cancer cells, so there may be potential complementary effects with chemotherapy and antioxidants (Singh, *et al*., 2018).

The cytotoxic activity of the extract was carried out *in vitro* by MTT assay. The results showed that treatment of root ethanol extract for 24 h of incubation showed better cytotoxic activity than other extracts, although the level of cytotoxic activity was still lower than doxorubicin IC_{50} 32.34 µg/mL. Root ethanolic extract's cytotoxic ability is caused by the content of existing metabolites such as flavonoids, tannins, saponins, and terpenoids. Several studies have shown that flavonoids can be developed as chemotherapy agents against breast cancer because they can treat cancer cells by targeting their anticancer effects, such as inhibiting cell growth, protein kinase activity, and inducing apoptosis (Park, *et al*., 2022; Magne Nde, *et al*., 2015; Şöhretoğlu, *et al*., 2021). Many natural terpenoid compounds have been shown to significantly inhibit proliferation, migration, apoptotic resistance, tumor angiogenesis or metastasis in different breast cancer cells/tumors *in vitro* and *in vivo* (Ateba, *et al*., 2018). In fact, currently, paclitaxel is one of the most successful natural terpenoid clinical drugs against metastatic breast cancer (Negi, 2021).

Three triterpenoids koetjapic acid, 3-oxoolean-12-en-30-oic acid, and betulinic acid were found in the roots and bark of *D. serrata* (Jalil, *et al*., 2015). Several studies have reported that betulinic acid is able to induce apoptotic cell death in cancer cells by triggering the mitochondrial pathway of apoptosis and has high selectivity so that non-malignant cells and normal tissues are not affected by betulinic acid, therefore, in the future, it is potential to be developed as a chemotherapeutic agent (Fulda, 2008; Ali-Seyed, *et al*., 2016). Betulinic acid of *D. suffruticosa* induced cell cycle arrest in MCF-7 cells via the p53/p21 pathway and induced apoptosis by increasing the ratio of Bax/Bcl-2 protein (Foo, *et al*., 2015). Thus, we predicted that betulinic acid of *D. serrata* involves in the cytotoxicity activity against MCF-7 cells.

CONCLUSION

Based on these results, we concluded that amongst the leaves, bark, and root of *D. serrata*, the ethanolic extract of the leaves showed the strongest antioxidant activity and the ethanolic extract of the roots showed the highest cytotoxicity against MCF-7 cells.

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