Research Article

Exploring the Antioxidant and Anti-proliferative Effects of *Murraya koenigii* (L.) Leaves Methanol Extract on T47D Breast Cancer Cell Lines

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ABSTRACT

Background: *Murraya koenigii* (Curry) leaves are herbal plants that have bioactive compounds such as phenolics, flavonoids, and alkaloids that function as antioxidants and anti-cancers.

Aims: This study aimed to quantitatively determine the bioactive compounds in curry leaves by calculating phenolic, flavonoid, and alkaloid content. Its potential as an antioxidant and anti-proliferative compound in T47D breast cancer cell lines was also analyzed.

Methods: *Murraya koenigii* leaves extract was obtained by maceration using 80% methanol (1:5 w/v), then the antioxidant and anti-proliferative test was carried out using the 1,1-Diphenyl-2-Pycrylhidracyl (DPPH) and MTT (-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, respectively, followed by flow cytometry to determine the apoptotic activity of the extract on T47D breast cancer cell lines.

Results: The results for the bioactive compounds in the methanol extract of *Murraya koenigii* leaves were 156.62±1.49 mg/g phenolics, 99.19±0.25 mg/g flavonoids, and 2.90±0.01 mg/g alkaloids. The IC50 value for antioxidant activity was 25.058±2.2 µg/ml and showed an anti-proliferative effect on T47D cells in a dose-dependent manner with IC50 74.71±5.45 µg/ml for cytotoxicity. Furthermore, flow cytometry showed that a concentration of 1/16 IC50 has the best results for apoptosis.

Conclusion: The methanol extract of *Murraya koenigii* leaves has the potential as an antioxidant and anti-proliferative agent and can induce apoptosis of T47D cells.

Keywords: *Antioxidant; Anti-proliferative; Cytotoxicity; Murraya koenigii; T47D Breast Cancer*.

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1. Introduction

Oxidative stress is the accumulation of free radicals in the body. Free radicals are compounds with unpaired electrons produced during the ATP formation process in mitochondria (Martemucci et al., 2022). They include reactive oxygen species (ROS), superoxide anion ($O2^{-\cdot}$), and hydroxyl radicals (OH). Furthermore, they can damage cellular protein, lipid, and DNA, which significantly affects cellular changes and leads to carcinogenesis. This condition is caused by free radicals that react freely and continuously with DNA, causing modification and cross-linking of proteins. Oxidative damage to proteins can cause changes in enzyme activity, receptors, and affect the permeability of the transport membrane. Additionally, the damaged membrane can cause these molecules to become reactive due to the loss of electrons, thus affecting cell function (Zaric et al., 2023). The human body has antioxidant mechanisms to counteract the formation of free radicals. However, under certain conditions, such as the effects of an unhealthy lifestyle, it can lead to the accumulation of free radicals in the body and cause oxidative stress (Gupta et al., 2020). Uncontrolled oxidative stress can lead to diseases in the body, one of which is the development of cancer cells (Pizzino et al., 2017). In such conditions, the body needs exogenous antioxidants to suppress free radicals and prevent oxidative stress.

Murraya koenigii (L.) or curry leaves are herbal plants commonly used as cooking spices. In addition to enhancing appetite, the leaves also possess high antioxidant capabilities (Fakriah et al., 2019; Suthar et al., 2022). Antioxidants play an important role in reducing free radicals and minimizing cellular damage. Their main function is to donate electrons or hydrogen atoms to convert free-radical compounds into stable forms, and they also inhibit the rate of autoxidation. Organic compounds such as Vitamin C and E, carotenoids, flavonoids, as well as phenolics are a group of non-enzymatic antioxidants that are obtained from consumed food sources, including vegetables, fruits, or spices. Foods that are high in these compounds have stronger antioxidant properties (Muscolo et al., 2024).

Several studies on *Murraya koenigii* (L.) have been carried out to determine its medical property and develop its potential in the health sector. The phenolic and flavonoid compounds from its methanol extract have an aromatic ring structure containing a hydroxyl group (Luna-Guevara et al., 2018), which plays an important role in scavenging free radicals (Jomova et al., 2024). Furthermore, they affect cell division, indicating their potential as an anti-proliferative compound (Guo et al., 2017; Sun et al., 2016). Similarly, the alkaloid content in curry leaves extract has the potential as an anti-proliferative compound (Liu et al., 2018). This is related to their benefits as an anticancer compound and is influenced by cell treatment (Ismail et al., 2016). Previous research has shown that curry leaves extract can inhibit the growth of several cancer cells such as DLD-1 (colon cancer cells), PC3 (prostate cancer cells), HepG2 (human carcinoma cells), and U937 (human leukemic monocyte lymphoma cell line) (Arun et al., 2017). Additionally, ethanol extract of curry leaves can inhibit the proliferation of ER receptor negative breast cancer MDA-MB-231 cells at a concentration of 14.4 µg/mL (Ismail et al., 2016). Meanwhile, in ER receptor positive breast cancer cells MCF7, curry leaves can inhibit cell proliferation at high concentrations. The cytotoxicity of curry leaves on MCF7 cells is not significant (Arun et al., 2017).

Aside from MCF7, there are other breast cancer cells that have ER receptor expression, namely T47D cells (Orrantia-Borunda et al., 2022). The anti-cancer activity of curry leaves on T47D cells is still unknown. Although they have the same hormone receptor expression, T47D and MCF7 have many different characteristics, one of which is in p53 gene expression (Soleimani & Sajedi, 2020).

This study determined the content of phenolic, flavonoid, and alkaloid compounds in curry (*Murraya koenigii* (L.)) leaves methanol extract and investigated the antioxidant and anti-proliferative abilities in the T47D breast cancer cell line. T47D cells were selected because they have positive estrogen receptors (ER), in which approximately 80% of breast cancer cases are ER-positive (Sun et al., 2016).

2. Methods

Sample Preparation

Curry leaves were obtained from a single plant originating from Banda Mesen, Banda Sakti, Lhokseumawe, Aceh, and was identified in the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, Indonesia. They were dried at room temperature to determine the constant weight and ground to a powder, which was further extracted with 80% methanol (1:5 g/l). The solution was macerated using a shaker incubator (200 rpm; 28-30°C) for 48 hours then filtered with Whatman No.1 and concentrated in a rotary evaporator (50ºC; 20Pa). The extract was dried in an oven at 50ºC until all the solvent had evaporated and stored at 4-8°C.

Phytochemical Compound

The total phenolic content (TPC), total flavonoid content (TFC), and total alkaloid content (TAC) in the methanol extract of curry leaves were determined using the Alara et al., (2018) method with modifications. Folin-Ciocalteu reagent was adopted for the total phenol content and gallic acid was used as a standard solution. For sample preparation, the extract (0.5 – 2 mg) was dissolved in methanol (25 ml) and allowed to stand for 10 minutes, and then the solution was filtered. Subsequently, the total phenolic content was determined by adding 1 ml of sample with 0.2 ml of Folin-Ciocalteu reagent 0.6 ml of 0.2 mM Na_2CO_3, vortexed for 5 minutes, and allowed to stand for 120 minutes. Solution absorbance was measured by a spectrophotometer with λ 765 nm and the data were analyzed using linear regression.

Total flavonoid content was tested using Quercetin as a standard. Then extract (1 g) was dissolved in 10 ml methanol then homogenized and allowed to stand for 30 minutes, and then filtered with a vacuum filter. Afterward, the 0.1 ml filtered solution was mixed with 0.1 ml of 2% Al_2Cl_3, homogenized with a vortex, and allowed to stand for 60 minutes, then 1 ml of distilled water was added. Red color is formed given that the sample contains flavonoids. Solution absorbance was measured by a spectrophotometer with λ 420 nm and the data were analyzed using linear regression.

Total alkaloid content was tested using atropine as a standard solution. The extract (0.1 g) was dissolved in 10 ml DMSO and mixed with 1 ml HCl 2 N, 2 ml bromocresol green, and 5 ml phosphate buffer, then the solution is homogenized and allowed to stand for 1 hour. Afterward, it was transferred to a separating funnel and mixed with chloroform (10 ml) and shaken until a layer is formed. Subsequently, 1 ml upper phase solution was diluted with chloroform to a volume of 5 ml and the sample absorbance was measured by a spectrophotometer with λ 470 nm. To obtain the pytochemical compound results at least three independent experiments was performed.

The phytochemical content data of curry leaves methanol extracts were analyzed using a simple linear regression with the equation y= a+bx where y is the absorbance value, and x is the sample concentration.

Antioxidant Assay

An antioxidant test was carried out in a dark room using the DPPH method. The antioxidant used as a comparison in the test is vitamin C, which was mixed with curry leaves extract dissolved in methanol. The variation of extract concentrations to be tested are 10, 20, 40, 60, 80, and 100 g/ml. Furthermore, vitamin C was used as a comparison at various concentrations such as 1, 2, 3, 4, and 5 g/ml. For each variation concentration in the sampel and vitamin C were repeated three times. The test was carried out by mixing the 0.1 mM (Mr 394.32 g/mol) DPPH with the extract solution (1:1), homogenized using a vortex, and incubated for 30 minutes in a dark room. The absorbance value was measured using a spectrophotometer with λ 515 nm (Sujana & Wijayanti, 2022) and the inhibition value was calculated by the following formula:

$$\% \, Inhibition = \frac{Ab \ control - Ab \ Sample}{Ab \ control} x \ 100\%$$

Cell Culture Preparation

The T47D breast cancer cells were used and obtained from the Gaharu Research Team, Faculty of Biology, Universitas Gadjah Mada, Special Region of Yogyakarta, Indonesia. The DMEM was used as a medium with high glucose, 10% FBS, and 1% Penicillin-Streptomycin. Cells were cultured in a 25T flask and incubated in a CO_2 incubator at 37ºC, 85% humidity, and 5% CO_2 (Pratitis et al., 2024).

Anti-proliferation Assay

Cells were grown in 96 well plates containing 5 x 10^4 cells/well and incubated for 24 hours. They were treated with extracts dissolved in DMSO at concentrations of 400, 200, 100, 50, 25, and 10 µg/ml with a volume of 100 µl/well in triplo. There are two controls such as the normal control and solvent, which were used to see the effect of DMSO (0.1%) on cells. The treated cells were incubated for 24 hours in a CO_2 incubator, then given 100 µl/well of MTT (0.5 mg/ml) solution and incubated for 4 hours until formazan crystals were formed. Then, 10% SDS in 0.01 M HCl was added at 100 µl/well to stop the MTT reaction and the cells were incubated for 24 hours at room temperature in the dark. Furthermore, the absorbance was measured using an ELISA reader with λ 595 nm (Sujana and Wijayanti, 2022). The absorbance results were used to calculate the cytotoxicity data using probit analysis in Ms. Excel and the IC50 value was obtained using linear regression from the percentage of viable cells on the probit analysis curve. The percent of cell viability was calculated with the following formula:

$$\%\ Cell\ Viability = \left(\frac{Ab\ sample - Ab\ blank}{Ab\ control - Ab\ blank}\right) \times\ 100\%$$

Meanwhile, the percentage of cell inhibition was calculated with the following formula:

$$\%\ Cell\ Inhibition = \left(100 - \frac{Ab\ sample - Ab\ blank}{Ab\ control - Ab\ blank}\right) \times\ 100\%$$

Apoptosis Assay

The apoptotic method was adopted using flow cytometry based on Alshehade et al., (2024) with modifications. Cells were grown in 6 well-plates containing 5 x 10^5 cells/well and incubated for 24 hours. Afterward, they were treated with extract at concentrations 1/16 IC50 with exposure durations of 4 and 18 hours. The cells were harvested, fixed using cold PBS, and centrifuged; then, annexin reagent and propidium iodine (PI) were given and incubated for 15 minutes at 4ºC. They were then transferred into a flowcyto-tube and read with a FACS Calibur flow cytometer (Gunathilaka et al., 2021).

3. Results

Bioactive Content of Murraya koenigii (L.) Leaves Methanol Extract

The calculation of the bioactive contents in methanol extract of *Murraya koenigii* (L.) with a calibration curve showed a total phenolic content (R2 = 0.99), total flavonoid content (R2 = 0.95), and total alkaloid content (R2 = 0.99) (Table 1). These indicate that the highest content of bioactive compounds was phenolic content, followed by flavonoids and alkaloids in smaller amounts (Table 1).

Table 1. Total phenolic, flavonoid, and alkaloid content of methanol extract of *Murraya koenigii* (L.) leaves.

Parameters	**Result**
Total Phenolic Content (TPC)	156.62±1.49mg GAE/g
Total Flavonoid Content (TFC)	99.19±0.25mg QE/g
Total Alkaloid Content (TAC)	2.90±0.01mg AE/g

This result showed that the phenolic and flavonoid content of methanol extract of *Murraya koenigii* (L.) has a higher value compared to the extraction with ethanol solvent that had been reported, in which the ethanol extract contains 58.48±5.46mg GAE/g and 27.97±7.2mg QE/g of phenolic and flavonoid, respectively (Abeysinghe et al., 2021). The result is also higher than *Murraya koenigii* (L.) leaves extracted with MEA (Microwave Assisted Extract), which produced 120.60±14.8mg GAE/gr and 85.23±2.5mg QE/g of phenolic and flavonoid content, respectively (Parithy et al., 2021). Furthermore, the results from Salomi & Manimekalai (2016) showed that *Murraya koenigii* (L.) leaves extract with ethanol solvent using a soxhlet method had a higher alkaloid content (99.09mg AE/g) but lower phenolic (142.32 mg GAE/g) and flavonoid (45.76mg QE/g).

Antioxidant Activity of Murraya koenigii (L.) Leaves Methanol Extract

The IC50 value of methanol extract (R^2 = 0.995) and vitamin C (R^2 0.94) as scavenger free radicals are shown in Table 2. The Antioxidant Activity Index (AAI) value of methanol extract was 1 < AAI < 2 and vitamin C is > 2. Furthermore, its antioxidant ability is directly proportional to the concentration of the given extract, indicating that a higher concentration results in a higher ability (Figure 1).

The principle of the DPPH method is mixing antioxidant compounds that can donate hydrogen with stable free radicals (DPPH) to the extent that they are quenched (Gulcin and Alwasel, 2023). Vitamin C was used as a comparison due to its high antioxidant activity. The extracts with antioxidant activity will result in a decreased color of the DPPH solution and the absorbance value also decreases (Gangwar et al., 2014). When the electron number of DPPH is high, the solution is dark purple. The given extract functions as an electron donor binding to free DPPH to become neutral, indicated by the fading of the DPPH solution's purple color (Sukweenadhi et al., 2020).

Table 2. Scavenging activity and antioxidant Activity Index (AAI) value of methanol extract of *Murraya koenigii* & Vitamin C.

Samples	**IC50**	**AAI**
Methanol extract of curry leaves (*Murraya koenigii*)	25.059±2.2	1.596
Vitamin C	4.46±0.16	8.96

The methanol extract of *Murraya koenigii* (L.) had strong antioxidant activity as shown in Table 2 and AAI is used to standardize antioxidant test results. The determination of the antioxidant activity can be grouped into four based on the AAI value: low AAI < 0.5; moderate 0.5 < AAI < 1; strong 1 < AAI < 2, and very strong AAI > 2. AAI is obtained by dividing the DPPH concentration used and the IC50 value (Scherer & Godoy, 2009). This study result is not significantly different from previous reports that the ethanol extract of *Murraya koenigii* (L.) has an IC50 value of 21.4-49.5 g/ml (Ma et al., 2016). Furthermore, the ability of the methanol extract to scavenge free radicals was better than aqueous extract which obtained an IC50 value of 36.46 g/ml (Rehana et al., 2017). The results of the extract with several solvents indicate that *Murraya koenigii* (L.) leaves have antioxidant properties. Furthermore, a higher content of phenolic and flavonoid compounds in an extract result in a stronger antioxidant activity (Augusto et al., 2014; Moalin et al., 2011)

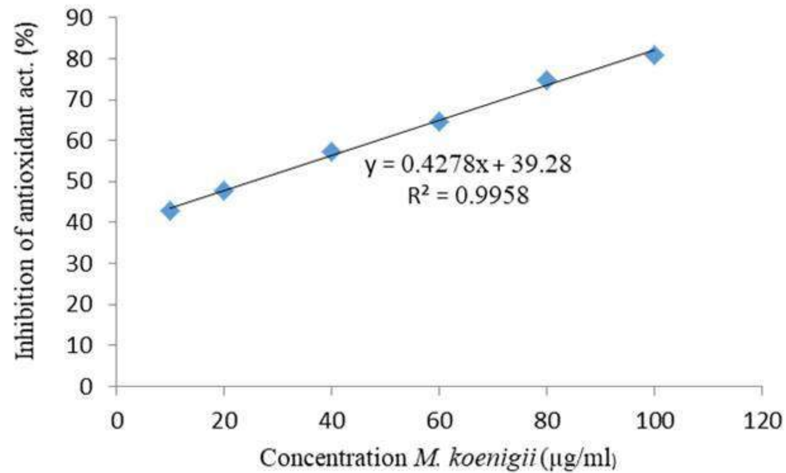

Figure 1. Antioxidant activity of methanol extract of Murraya *koenigii* leaves.

Anti-proliferative effect of Murraya koenigii (L.) Leaves Methanol Extract

The anti-proliferative test using MTT assay showed that administering extract affected the growth of T47D cells. The dose-dependent manner in cell inhibition is shown in Figure 2, and the IC50 value calculation of cytotoxicity was moderate 74.71±5.45 μg/ml. According to Yeap et al. (2015), *Murraya koenigii* (L.) extract can reduce the viability of breast cancer cells in humans (MDA-MB321) and mice (4T1) with an IC50 of 2.4 mg/ml and 1.5 mg/ml, respectively.

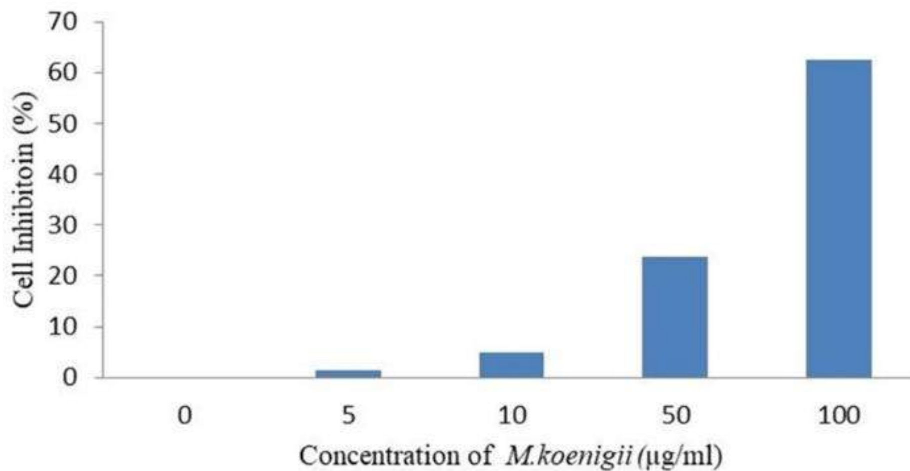

Figure 2. Proliferation inhibition of T47D breast cancer cell line in different concentrations of Murraya *koenigii* leaves extract (5, 10, 50, and 100 μg/ml).

Furthermore, the results showed that administering concentration affected cell viability. Noolu & Ismail, (2015) showed that anti-proliferation ability of *Murraya koenigii* (L.) extract is associated with the presence of flavonoid compounds, and there is a possibility of proteasome inhibitory activity in cancer cells that triggers cell death by flavonoid compounds.

Apoptosis Induction of Murraya koenigii (L.) Leaves Methanol Extract

The flow cytometry results showed that the methanol extract induced apoptosis and necrosis in T47D cells at a concentration of 1/16 IC50 with incubation times of 4 and 18 hours. The apoptotic analysis using flowcytometry (Figure 3) showed that the administration of the extract induces apoptosis of T47D cells and alkaloid content plays an important role in this activity. Several studies mention some alkaloid compounds in the leaves of *Murraya koenigii (L.)* have anticancer properties because they can induce cell apoptosis, these include mahanine and koenimbine, which are a group of carbazole. According to Bhattacharya et al., (2010), the carbazole alkaloid content in the extract of *Murraya koenigii (L.)* has the potential as a proteasome inhibitor and can induce apoptosis. They trigger changes in mitochondrial membrane pores, allowing apoptotic proteins such as cytochrome C to be transfused into the cytosol, thus activating caspase 3 and 7, which cause apoptosis (Kamalidehghan et al., 2015; Noolu et al., 2013).

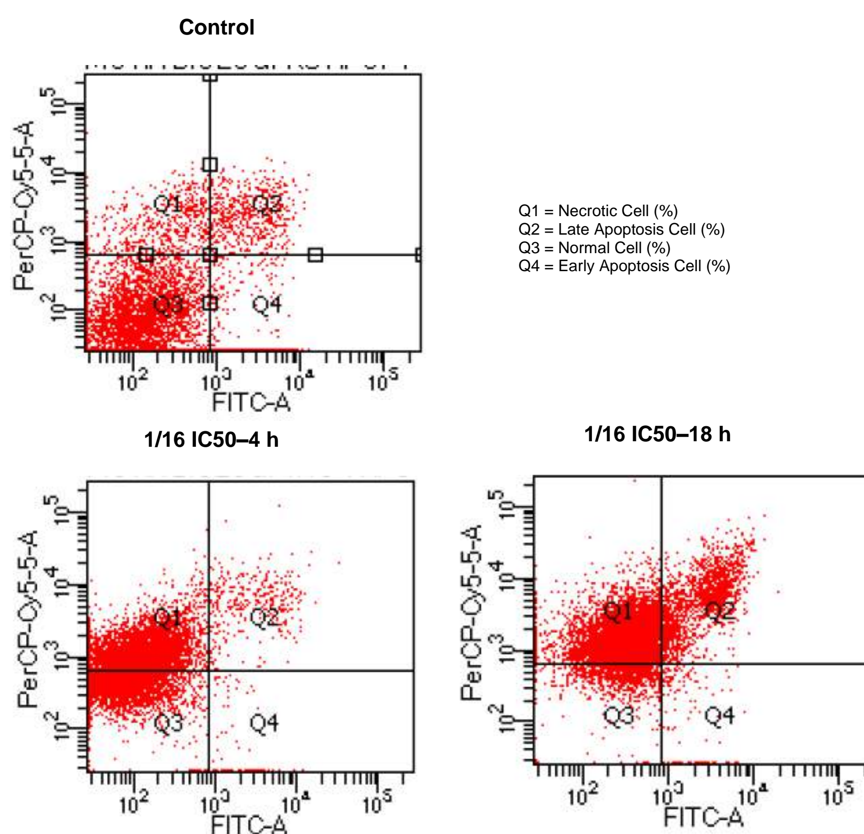

Figure 3. Apoptosis in flow cytometry analysis with different incubation times.

4. Discussion

Different solvents and extraction methods will produce different bioactive components. In this study, the highest bioactive content in methanol extract of *Murraya koenigii (L.)* leaves was phenolic content, followed by flavonoids and alkaloids in smaller amounts (Table 1). The types of bioactive components are the same as the results of ethanol extract using the soxhlet method by Salomi & Manimekalai (2016) but with different percentages of content in which flavonoids were the smallest amount than phenolic and alkaloid. The methanol extract of *Murraya koenigii (L.)* had strong antioxidant activity. The strong antioxidant activity of the methanol extract is attributed to its phenolic and flavonoid content, which can inhibit the oxidation of molecules and stop the chain reactions that can cause cell damage (Arjun et al., 2017). The role of phenolic compounds is to counteract free radicals by donating hydrogen atoms bind to electrons (Minatel et al., 2017). In flavonoid compounds, the hydroxyl group function as a hydrogen atom donor to free radical molecules, resulting in the formation of a more stable venoxyl radical, which then reacts again with alkoxyl (RO*) and forms a stable quinone structure (Banjarnahor & Artanti, 2014).

The methanol extract of *Murraya koenigii (L.)* leaves have anti-proliferation and anti-apoptotic effect (Fig.2 and 3). According to Bhattacharya et al., (2010), one of the alkaloid contents in curry leaves extract, namely, mahanine can inhibit the proliferation of prostate cancer cells. It can also stop the cycle in the G0/G1 phase in glioblastoma cancer cells. So, the anti-proliferation ability of *Murraya koenigii (L.)* methanol extract in T47D breast cancer cell line may be related to the alkaloid content. Furthermore, the carbazole alkaloid content in the extract of *Murraya koenigii (L.)* has the potential as a proteasome inhibitor and can induce apoptosis. They trigger changes in mitochondrial membrane pores, allowing apoptotic proteins such as cytochrome C to be transfused into the cytosol, thus activating caspase 3 and 7, which cause apoptosis (Kamalidehghan et al., 2015; Noolu et al., 2013).

5. Conclusion

Conclusively, methanol extract of *Murraya koenigii (L.)* leaves contains phenol, flavonoid, and alkaloid, which can induce apoptosis of T47D breast cancer cells. It also has the potency of strong antioxidant and anti-proliferative agents.

Conflict of Interest

There is no conflict of interest.

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