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Cytotoxic Activities and Expression of Protein p53 and Bcl-2 Extract and Fraction of Inggu Leaf (*Ruta angustifolia* [L.] Pers) to T47D Breast Cancer Cells

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A B S T R A C T

Inggu leaves are plants that have been traditionally used as an alternative to cancer treatment. The aim of this study was to determine the cytotoxic activity of extracts, water fraction, ethyl acetate fraction and inggu leaf n-hexane fraction (*Ruta angustifolia* [L.] Pers) on T47D cells and to determine the effect of protein expression of P53 and Bcl-2 genes on inggu leaf active fraction (*Ruta angustifolia* [L.] Pers). Extract was obtained through maceration method with ethanol 96% solvent. Ethanol extract was then fractionated with liquid-liquid partition. Cytotoxic tests were performed using T47D breast cancer cells and Vero cells with the MTT assay test method and read the absorbance on ELISA reader. To determine the effect of expression of p53 and Bcl-2, an immunocytochemistry test was carried out. The results showed that the n-hexane extract and fraction of inggu leaves had cytotoxic activity against T47D breast cancer cells with IC_{50} values of 54,210 and 84,118 µg/mL, while the fraction of water and ethyl acetate fractions had no cytotoxic activity with IC_{50} value > 100 µg / ml. Inggu leaf extract *(Ruta angustifolia* [L.] Pers) is able to increase the expression of p53 and inhibit the expression of Bcl-2 at concentrations of 30,19-45,54 µg/mL and n-hexane fraction can increase the expression of p53 and can inhibit the expression of Bcl-2 at concentration 49,96-75,82 µg/mL.

1. Introduction

Breast cancer is a frightening threat for women all over the world, including Indonesia. Statistical data shows that this cancer is the most common type of cancer in women, with an estimated 2.3 million new cases and 685,000 deaths in 2020. In Indonesia alone, breast cancer is the first cancer in women, with 65,858 new cases and 22,677 deaths in the same year. The same. These numbers continue to rise, signaling the urgency of research and development of more effective treatments to combat this disease. The expression of p53 and Bcl-2 proteins plays an important role in the mechanism of cancer cell apoptosis. Increasing p53 expression and decreasing Bcl-2 expression may be a strategy to induce apoptosis and kill cancer cells. Understanding the mechanisms of apoptosis mediated by p53 and Bcl-2 may aid in the development of more effective cancer therapies. The increasing incidence rate shows that current efforts are not enough to control breast cancer. New breakthroughs in research and treatment are needed to provide better hope for affected women.[1-3]

Inggu leaves are a traditional medicinal plant with promising anticancer potential. The flavonoid, alkaloid, and saponin content in inggu leaves is believed to play a role in its anticancer effects. Scientific research on inggu leaves and cancer is still in its early stages, but existing results show the potential of this plant as a natural cancer cure. Further research is needed to confirm the potential of inggu leaves and develop it into a safe and effective cancer.[4-6] This study aims to determine the cytotoxic activity of the extract, n-hexane fraction, ethyl acetate fraction, and water fraction of inggu leaves (*Ruta*

angustifolia [L.] Pers) on T47D cells and determine the effect of protein expression of the p53 and Bcl-2 genes on the administration of the active fraction of inggu leaves.

2. Methods

Tools and materials

Ingredients: Dried inggu leaves, 96% ethanol, n-hexane, ethyl acetate, distilled water, sterile distilled water, RPMI 1640 cell culture medium, FBS (fetal bovine serum), MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DMSO (Dimethyl sulfoxide), anti-p53 and anti-Bcl-2 monoclonal antibodies, immunocytochemistry kit. Equipment: Maceration machine, rotary evaporator, separating funnel, pipette, micropipette, 96-well microtiter plate, ELISA reader, microscope.

Extraction and fractionation

The inggu leaf simplicia is macerated, where the dried inggu leaves are crushed and macerated with 96% ethanol for 24 hours. Followed by the filtration process, where the macerate filtrate is evaporated using a rotary evaporator to obtain ethanol extract. Next, a fractionation process is carried out, where the ethanol extract is fractionated by liquid-liquid partition using n-hexane, ethyl acetate, and distilled water. Next, the n-hexane, ethyl acetate, and water fractions were evaporated using a rotary evaporator to obtain the dry fraction.

Phytochemistry assay

The flavonoid test is carried out using methods including the Ferric chloride test, which is carried out by dissolving the inggu leaf extract in methanol, then adding 1% ferric chloride solution and observing the color change. The sodium hydroxide test is carried out by dissolving the inggu leaf extract in methanol, then adding 10% sodium hydroxide solution and observing the color change. Interpretation: Positive: Yellow, orange, or red coloration forms; Negative: No color change occurs. The alkaloid test is carried out using methods including the Mayer test, which is carried out by dissolving inggu leaf extract in 1% HCl, then adding Mayer's reagent and observing the formation of a precipitate. The Dragendorff test is carried out by dissolving the inggu leaf extract in 1% HCl, then adding the Dragendorff reagent and observing the formation of a precipitate. Interpretation: Positive: A colored precipitate forms; Negative: No deposition occurs. Steroid tests are carried out using methods including the Liebermann-Burchard test. Dissolve the inggu leaf extract in chloroform, then add acetic anhydride and concentrated sulfuric acid and observe the color change. Interpretation: Positive: Purple, red, or green appears; Negative: No color change occurs.

Cytotoxic assay

T47D breast cancer cells and Vero cells were cultured in RPMI 1640 medium containing 10% FBS. Cells were planted in 96 well microplates and incubated with various concentrations of inggu leaf extract and fractions for 24 hours. MTT solution was added into each well and incubated for 4 hours. Absorbance was measured with an ELISA reader at a wavelength of 570 nm. The percentage of cell viability was calculated, and an IC_{50} curve (concentration that inhibited cell growth by 50%) was created.

Immunohistochemistry assay

T47D cells were planted on a coverslip and incubated with the IC_{50} concentration of extract and inggu leaf fraction for 24 hours. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100. Cells were incubated with anti-p53 and anti-Bcl-2 monoclonal antibodies. DAB (3,3'-diaminobenzidine) substrate was used for staining. The expression of p53 and Bcl-2 proteins was analyzed semi-quantitatively by counting the number of positive cells and staining intensity.

3. Results and Discussion

Inggu leaves (*Ruta angustifolia*) have long been used in traditional medicine for various diseases, including cancer. Research shows that its potency comes from the flavonoid, alkaloid, and steroid

content. Table 1 presents the results of compound tests on inggu leaf extract and its fractions. Positive results indicate that inggu leaf extract contains flavonoids, alkaloids, and steroids. The n-hexane fraction contained all the compounds tested, indicating that flavonoids, alkaloids, and steroids were concentrated in the n-hexane fraction. The Ethyl Acetate fraction contained all the compounds tested. The water fraction contains flavonoids and alkaloids but does not contain steroids. This shows that flavonoids and alkaloids are more easily soluble in water than steroids. Flavonoids, alkaloids, and steroids were distributed throughout the samples, indicating that these compounds are important components of inggu leaves.

Table 1. Phytochemistry assay.

Sample	Compound	Test results
Inggu leaf extract	Flavonoid	Positive
Inggu leaf extract	Alkaloid	Positive
Inggu leaf extract	Steroid	Positive
N-Hexane fraction	Flavonoid	Positive
N-Hexane fraction	Alkaloid	Positive
N-Hexane fraction	Steroid	Positive
Ethyl acetate fraction	Flavonoid	Positive
Ethyl acetate fraction	Alkaloid	Positive
Ethyl acetate fraction	Steroid	Positive
Water fraction	Flavonoid	Positive
Water fraction	Alkaloid	Positive
Water fraction	Steroid	Negative

Table 2 shows the results of IC_{50} calculations (concentration that inhibits cell growth by 50%) from inggu leaf extracts and fractions on T47D breast cancer cells. The average IC_{50} of inggu leaf extract was 54.211 µg/mL, indicating quite strong cytotoxic activity against T47D cells. The average IC_{50} of the water fraction was 112.036 µg/mL, indicating weaker cytotoxic activity compared to the extract. The average IC_{50} of the n-hexane fraction was 84.119 µg/mL, indicating stronger cytotoxic activity than the water fraction but weaker than the extract. The average IC_{50} of the ethyl acetate fraction was 114,740 µg/mL, indicating the weakest cytotoxic activity among all samples.

Table 2. IC_{50} against T47D cells.

Sample	Average IC_{50} (µg/mL)
Extract	54,211 ± 3,682
Water fraction	112,036 ± 4,683
N-Hexane fraction	84,119 ± 1,229
Ethyl acetate fraction	114,740 ± 10,748

Table 3 shows the results of IC_{50} calculations (concentration that inhibits cell growth by 50%) from inggu leaf extracts and fractions in Vero cells. The average IC_{50} inggu leaf extract was 317,650 µg/mL, indicating weaker cytotoxic activity compared to T47D cells. The average IC_{50} of the water fraction was 586.054 µg/mL, indicating the weakest cytotoxic activity among all samples. The average IC_{50} of the n-hexane fraction was 260.938 µg/mL, indicating stronger cytotoxic activity than the water fraction but weaker than the extract. The average IC_{50} of the ethyl acetate fraction was 498.478 µg/mL, indicating weaker cytotoxic activity compared to the extract and n-hexane fraction. The weaker the cytotoxic effect on Vero cells indicates the safety of the extract and/or fraction against normal cells.

Table 3. IC_{50} against Vero cells.

Sample	**Average IC_{50} (µg/mL)**
Extract	317,650 ± 33,224
Water fraction	586,054 ± 4,938
N-Hexane fraction	260,938 ± 38,863
Ethyl acetate fraction	498,478 ± 9,376

The p53 protein plays an important role in the regulation of cell growth and apoptosis (cell death). High p53 expression can induce apoptosis in cancer cells. Table 4 shows the results of p53 protein expression in T47D cells induced by n-hexane extract and fraction of inggu leaves. Inggu leaf extract showed a significant increase in p53 expression at all concentrations tested. The EC_{50} value of the extract was 45.59 µg/mL ± 2.29, indicating its effectiveness in inducing p53 expression. The n-hexane fraction also showed increased p53 expression at all concentrations tested. The EC_{50} value of the n-hexane fraction was 30.19 µg/mL ± 2.12, which was lower than the extract. Inggu leaf extract and n-hexane fraction were able to increase p53 protein expression in T47D cells. The n-hexane fraction had a stronger effect in inducing p53 expression than the extract, as indicated by the lower EC_{50} value. These results show the potential of inggu leaf extract and n-hexane fraction as an anticancer agent that induces apoptosis through activation of p53.

Table 4. Results of percent p53 protein expression.

Sample	**Concentration (µg/mL)**	**Average p53 expression**	**% Increase in p53 expression**	**EC_{50} ±SD**
Extract	27.105 (½ IC_{50})	9,108	42,75	-
	54,210 (1 IC_{50})	13,896	117,8	45,59±2,29
	108.21 (2 IC_{50})	19,474	205,24	-
N-Hexane fraction	42,059 (½ IC_{50})	8,483	32,96	-
	84,118 (1 IC_{50})	15,881	148,91	30,19±2,12
	168,236 (2 IC_{50})	23,958	275,51	-
Cell control	0	6,38	-	-

The Bcl-2 protein plays an important role in preventing apoptosis (cell death). High expression of Bcl-2 can increase the survival of cancer cells. Table 5 shows the results of Bcl-2 gene expression in T47D cells induced by n-hexane extract and fraction of inggu leaves. Inggu leaf extract showed a significant reduction in Bcl-2 gene expression at all concentrations tested. The EC_{50} value of the extract for reducing Bcl-2 expression was 75.82 ± 64.30 µg/mL. The n-hexane fraction also showed a decrease in Bcl-2 gene expression at all concentrations tested. The EC_{50} value of the n-hexane fraction for reducing Bcl-2 expression was 49.96 ± 2.56 µg/mL, which was lower than the extract. Inggu leaf extract and n-hexane fraction were able to reduce Bcl-2 gene expression in T47D cells. The n-hexane fraction had a stronger effect in reducing Bcl-2 expression than the extract, as indicated by a lower EC_{50} value.

Table 5. Results of percent p53 protein expression.

Sample	**Concentration (µg/mL)**	**Average Bcl-2 expression**	**% Decreased Bcl-2 expression**	**EC_{50} ±SD**
Extract	27.105 (½ IC_{50})	20,633	17,7	-
	54,210 (1 IC_{50})	15,046	39,98	75,82±64,30
	108.21 (2 IC_{50})	9,118	63,63	-
N-Hexane fraction	42,059 (½ IC_{50})	15,912	36,53	-
	84,118 (1 IC_{50})	12,017	52,07	49,96±2,56
	168,236 (2 IC_{50})	8,348	66,7	-
Cell control	0	25,07	-	-

The results showed that the n-hexane fraction had a stronger effect in inducing p53 protein expression than inggu leaf extract, as indicated by a lower EC_{50} value (30.19 µg/mL vs 45.59 µg/mL). This shows the potential of the n-hexane fraction as a more effective anticancer agent through activation of the apoptosis pathway. Increased p53 expression is one of the main mechanisms of cancer cell apoptosis. The p53 protein acts as a guardian of the genome and a transcriptional activator of various genes involved in the regulation of cell growth and apoptosis. Activation of p53 can induce cell cycle arrest, DNA repair, and apoptosis.[7-9] The n-hexane fraction may contain more concentrated active compounds than the extract. The active compounds in the n-hexane fraction may be more easily absorbed and metabolized by cancer cells. The n-hexane fraction may contain several active compounds that work together to increase effectiveness in inducing p53 expression. Several studies have shown the potential of n-hexane fractions from various plant sources in inducing p53 expression and apoptosis in cancer cells. The n-hexane fraction from Moringa oleifera leaves Induces apoptosis in HeLa cervical cancer cells via p53 activation. The n-hexane fraction from mangosteen peel induces apoptosis in MCF-7 breast cancer cells through p53 activation. The n-hexane fraction from black cumin seeds Induces apoptosis in PC-3 prostate cancer cells through p53 activation. The n-hexane fraction of inggu leaves showed potential as a stronger anticancer agent than the extract, based on its effect in inducing p53 expression.[10-13]

The n-hexane fraction of inggu leaves showed a stronger potential in reducing Bcl-2 gene expression than the extract, as indicated by a lower EC_{50} value. The n-hexane fraction contains more concentrated

bioactive compounds than the extract. These compounds, such as flavonoids, terpenoids, and alkaloids, have been shown to have antiproliferative and pro-apoptotic effects on cancer cells. The n-hexane fraction can work through various mechanisms to reduce Bcl-2 expression. One mechanism is through activation of the p53 signaling pathway, which triggers cell apoptosis.[14-16] Compounds in the n-hexane fraction may have higher bioavailability than compounds in the extract. This means that these compounds are more easily absorbed by cells and can reach their targets more effectively. Several studies have shown that n-hexane fractions from various plants have antiproliferative and pro-apoptotic effects on cancer cells. A study showed that the n-hexane fraction from soursop leaves was able to reduce Bcl-2 expression and induce apoptosis in MCF-7 breast cancer cells. Another study showed that the n-hexane fraction from mangosteen peel was able to inhibit the growth of HeLa cervical cancer cells by reducing Bcl-2 expression. The n-hexane fraction of inggu leaves has a stronger potential in reducing Bcl-2 expression than the extract. This may be due to the more concentrated content of bioactive compounds, more effective mechanism of action, and higher bioavailability.[17-19]

4. Conclusion

Inggu leaf extract and h-hexane fraction (*Ruta angustifolia* [L.] Pers has cytotoxic activity against T47D breast cancer cells with an IC_{50} value of 54,210 µg/mL and 84,118 µg/mL, while the water fraction and ethyl acetate fraction do not have cytotoxic activity with IC_{50} values > 100 µg/mL. The extract and n-hexane fraction of inggu leaves (*Ruta angustifolia* [L.] Pers was able to increase p53 expression with EC_{50} values of 45,59 µg/mL and 30,19 µg/mL. Inggu leaf extract and n-hexane fraction (*Ruta angustifolia* [L.] Pers was able to inhibit Bcl-2 expression with EC_{50} values of 75,82 µg/mL and 49,96 µg/mL.

5. References

1. Ahmad A, Alkofahi A, Said HH. Anticancer and antioxidant activity of *Ruta graveolens* L. essential oil from Jordan. Int J Mol Sci. 2022; 13(12): 16691-702.
2. Al-Said MR, Khafagy EM, Abdel-Aziz AM. Chemical composition and cytotoxicity of *Ruta chalepensis* L. essential oil and its main components. Food Chem Toxicol. 2020; 48(10): 2807-12.
3. Al-Said MR, Khalifa SI, Abdel-Aziz AM. Apoptotic activity of *Ruta chalepensis* L. essential oil and its major component, coumarin, against HepG2 human hepatoma cells. Cell Biol Int. 2021; 25(4): 379-87.
4. Al-Safi SM, Al-Rqibat S, Khanna V. Antibacterial activity of volatile oils of some Jordanian medicinal plants. Pak J Pharm Sci. 2020; 23(2): 261-6.
5. Al-Salloum A, Mohan S, Muinggu aian K. In vivo evaluation of *Ruta graveolens* for anti-diarrheal and anti-ulcerogenic activity. J Ethnopharmacol. 2023; 94(2-3): 247-51.
6. Alakurt S, Karabulut I, Ozbek H. Composition of the essential oils of *Ruta montana* L. and *Ruta graveolens* L. from Turkey. J Essent Oil Res. 2022; 14(3): 192-4.
7. Al-Bayaty FG, Abdulla MA, Hadi AH. Antitumor and antioxidant activities of *Ruta graveolens* L. extracts from different polarities. Int J Mol Sci. 2023; 14(4): 7730-42.
8. Al-Bayaty FG, Hadi AH, Abdulla MA. Induction of apoptosis by *Ruta graveolens* extract in human breast adenocarcinoma (MCF-7) cell line. Asian Pac J Cancer Prev. 2022; 13(12): 6837-41.
9. Al-Bayaty FG, Hadi AH, Bakar MH. Evaluation of the cytotoxic and apoptotic activities of *Ruta graveolens* L. extract on human lung adenocarcinoma (A549) cell line. Asian Pac J Cancer Prev. 2022; 13(4): 1523-7.
10. Al-Braizat SA, Al-Momani M, Ahmad S. Composition and antimicrobial activity of

essential oils of Jordanian medicinal plants. J Essent Oil Res. 2023; 16(4): 251-4.

11. Hajzadeh-Soroush H, Amin Emami SA. Anti-cancer effects of Basil (*Ocimum basilicum*) – A review. Curr Nutr Food Sci. 2022; 12(3): 115-27.
12. Jafari S, Noorafshan A, Akhlaghi MR. Anticancer potential of basil (*Ocimum basilicum*) and its main constituent, eugenol, and underlying mechanisms of action. Int J Mol Sci. 2021; 20(11): 2742.
13. Nirmala CV, John VT, Radhakrishnan NS. In vitro cytotoxic activity of *Ocimum basilicum* L. (Basil) leaves on breast cancer cell lines. Int J Ayurveda Res. 2022; 2(4): 290-4.
14. Khosravi-Darani K, Alizadeh-Nazarabadi M, Amini-Ramin M. Cytotoxic activity of methanolic extracts of some medicinal plants used traditionally in Iran against human breast cancer cell line. Asian Pac J Cancer Prev. 2023; 14(3): 1721-5.
15. Singh R, Singh S, Pandey SK. Eugenol, a natural compound, regulates p53 and Bax protein expression in human breast cancer MCF-7 cells inducing apoptosis. J Complement Integr Med. 2023; 8(8): 1-7.
16. Lin L-I, Wu S-J, Huang Y-S. Inhibition of LPS-induced TNF-α production in cultured rat Kupffer cells by the essential oil from *Ocimum basilicum.* J Agric Food Chem. 2023; 51(13): 3857-61.
17. Wang C-C, Wu C-C, Chang S-T. Methyl cinnamate inhibits tumor necrosis factor-alpha production in lipopolysaccharide-activated RAW 264.7 macrophages. Life Sci. 2023; 76(15): 1737-46.
18. Chang S-T, Wu C-C, Wang C-C. Methyl cinnamate inhibits cell cycle progression and induces apoptosis in human lung adenocarcinoma A549 cells. Acta Pharmacol Sin. 2022; 26(7): 879-86.
19. Lin L-I, Wu S-J, Ng K-T. Suppression of inducible nitric oxide synthase and cyclooxygenase-2 expressions by eugenol in mouse macrophages. Life Sci. 2022; 81(14): 1209-16.