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Cellular Mechanism of the Cytotoxic Effects of *Medinilla speciosa* (Parijoto) Methanol Extract on MCF-7/HER-2 Cells is Through Increased Caspase-9 Protein and Decreased Vascular Endothelial Growth Factor Expression

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ABSTRACT

Breast cancer is the leading cause of death in women around the world. *Medinilla speciosa* (Parijoto) contains quercetin, which has been linked to cancer prevention. However, there have been no previous reports on the cytotoxic effect of *Medinilla speciosa* on resistant breast cancer cells, such as MCF-7/HER-2. The study was therefore conducted to investigate the cytotoxic effects and mechanism of action of *Medinilla speciosa* methanol fruit extract (*MSMFE*) on MCF-7/HER-2 cancer cells, both alone and in combination with cisplatin. MCF-7/HER-2 and Vero cell lines were obtained for the *in vitro* study. Methanol extract was prepared from *Medinilla speciosa* fruits. Different concentrations of the extract and cisplatin were tested against the MCF-7/HER-2 cells. For the combination treatment, 88 µg/mL of the *MSMFE* was added to cisplatin in two different concentrations (2.4 and 8 µM). The MTT assay was employed to evaluate the cytotoxic effects of the extract. Cell viability and expression of caspase-9 and VEGF proteins were analyzed. The results showed that the *MSMFE* had an IC_{50} value of 175±0.962 µg/mL on MCF-7/HER-2 cells, indicating a cytotoxic effect. After *MSMFE* administration, there was an increase in caspase-9 protein expression and a decrease in VEGF expression, and the combination of *MSMFE* and cisplatin lowered cell viability by 53%. The findings of the study revealed that when combined with cisplatin, *Medinilla speciosa* methanol fruit extract may have synergistic cytotoxic effects on MCF-7/HER-2 cancer cells. It may also induce apoptosis through increased expression of the caspase-9 protein and inhibit angiogenesis by lowering VEGF expression.

Keywords: Caspase-9, Cisplatin, MCF-7/HER2, *Medinilla speciose*, Parijoto, Vascular endothelial growth factors (VEGF).

Introduction

Breast cancer is the leading cause of death in women worldwide.[1] According to GLOBOCAN data from 2018, the incidence of breast cancer in Indonesia is estimated to be 42.1 per 100,000 people, with a mortality rate of 17 per 100,000 people on the average.[2] In Indonesia, the median age of breast cancer diagnosis is lower (48 years old), and a disproportionately higher percentage of patients are under 40 years old. The prevalence of breast cancer in young women is relatively low. In developed countries, the median age of diagnosis is 68 years old, while it is 48 years old in Indonesia, where more than 5,000 women in their 40s are diagnosed with breast cancer each year. However, there are a few studies in Indonesia that look at biological traits, the administration of care, and clinical results.[3] To lower mortality due to the high incidence of breast cancer, better treatments must be administered with fewer side effects. Surgery, radiation therapy, chemotherapy, and radiochemotherapy are all options for treating breast cancer. The use of chemotherapeutic drugs such as trastuzumab, doxorubicin, cisplatin, cyclophosphamide, and paclitaxel is one of the most popular treatments for breast cancer.

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Trastuzumab monoclonal antibodies have caused resistance in MCF-7 breast cancer cells. In some breast cancer cases, HER-2 takes on a deformed shape, further indicating the existence of P95. However, P95 does not have the extracellular domain required by trastuzumab for binding, thereby making it resistant to HER-2. Therefore, it is necessary to develop drugs that are molecularly targeted to prevent the activation of HER-2 expression at ATP-binding sites in breast cancer cells.[4] A platinum derivative called cisplatin is frequently used to treat breast cancer and is extremely effective, but it also has several negative side effects, including nausea and vomiting, chronic and acute nephrotoxicity, anemia,[5] acute cochlear toxicity (ototoxicity), and resistance.[6] The combination of chemotherapy and lowering the dose of cisplatin given to patients is a method that can be utilized to reduce the side effects and cellular resistance. Combination therapy can be used to boost therapeutic effectiveness while lowering toxicity brought on by higher doses of chemotherapeutic drugs (co-chemotherapy). Co-chemotherapy is a cancer therapy strategy that involves the combination of a compound with a chemotherapy agent.[7] In cancer therapy, it is possible and often useful to combine numerous medications simultaneously.[8] Co-chemoprevention is a non-toxic method of treating cancer that lessens toxicity and negative effects on healthy cells. The mechanism of anticancer medications, in particular anti-angiogenesis agents, has recently been the focus of the majority of anticancer research.

Within the human body, angiogenesis, the formation of a new blood vessel, is regulated by two types of molecules: pro-angiogenic molecules such as vascular endothelial growth factors (VEGFs) and anti-angiogenic molecules. VEGFs are required in a number of health and disease conditions. The critical behaviors of VGEFs in wound

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healing, embryonic development, and lymphangiogenesis have been widely investigated and studied. The majority of solid tumors, particularly breast cancer, are now the primary focus of anti-angiogenic medication.[9] Hypoxia-inducible factor α (HIF-1α) is regarded as a crucial regulator of angiogenesis in breast cancer. It activates the expression of VEGF in breast cancer.[10] Furthermore, increased angiogenesis, which could be measured by VEGF expression or the density of blood vessels, is an independent negative prognostic factor in early breast cancer.[11] A highly conserved family of proteases known as caspases plays a crucial role in apoptosis by cleaving proteins to cause cell death.[12] The executioner caspases, caspase-3, caspase-6, and caspase-7, which accelerate many of the hallmark processes of apoptosis, are activated by caspase-9 and other initiator caspases.[13] Although earlier studies indicated that HER-2 is cleaved by caspase,[14] the precise mechanisms and functional effects of HER-2 proteolysis are yet unknown.

Medinilla speciosa (Parijoto) is a type of shrub with red fruit, which has been empirically used as an enhancer of fertility in women, an anti-inflammatory,[15] and anticancer medication.[16] Secondary metabolites found in its fruits, including quercetindic flavonoids, tannin phenolic compounds, glycosides, and saponins, have been suggested to have antitumor properties.[17] Flavonoid and phenolic compounds are antioxidants that have an antitumor function. Tannins have the potential as anticancer compounds. Also, glycosides, and saponins can inhibit abnormal cell proliferation. *Medinilla speciosa* cytotoxic tests have been performed in the past using T47D breast cancer cells, which have an IC_{50} value of 614.50 µg/mL or are moderately cytotoxic.[18] There have not been previous reports on the cytotoxic effect of *Medinilla speciosa* fruit extract against breast cancer cells that have proven resistant, including MCF-7/HER-2 cells.

The study was aimed at determining the cytotoxic effects of *Medinilla speciosa* methanol fruit extract alone and in combination with cisplatin (a chemotherapeutic drug) on MCF-7/HER-2 cells. The cytotoxic effect's mechanism of action was also investigated.

Materials and Methods

Source of cell lines

In this *in vitro* study, MCF-7/HER-2 and Vero cells were used. The cells were obtained from the Cancer Chemoprevention Research Center, Gadjah Mada University, Indonesia. The cell density was calculated using the hemocytometer on the confluent growing cells (± 80%) in TCD, which were planted in a 96-well microplate at 1×10^4 cells/well.

Ethical clearance

Ethical clearance for the study was obtained from the Ethics Committee of the Department of Medicine, Universitas Muhammadiyah Surakarta (No. 1815/A.1/KEPK-FKUMS/I/ 2019).

Source of plant material

The seeds of *Medinilla speciosa* were collected on June 15, 2020, from Mount Muria, Colo, Kudus, Central Java. The seeds were authenticated and deposited in the herbarium unit of the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, with voucher 17. After drying, the seeds were processed into powder form.

Preparation of Medinilla speciosa methanol fruit extract

Five hundred grams (500 g) of the powder *Medinilla speciosa* were extracted with 500 mL of methanol using the maceration method. The maceration process was carried out for 5 days, followed by 3 days of re-maceration. The filtrate was evaporated with a vacuum rotary evaporator at 50 ºC. To obtain a thick methanol fruit extract of *Medinilla speciosa*, additional evaporation was carried out using a water bath at 40–90ºC.

Preparation of culture media

The MCF-7/HER-2 cell culture medium was prepared using 1 L of Dulbecco's Modified Eagle Medium (DMEM), by combining complete medium consisting of 10 mL of 10% FBS (Gibco), 3.7 g NaH_2CO_3, 2 g Hepes, 1.5 mL of 1.5% penicillin-streptomycin (Gibco), 0.5 mL of 0.5% fungizone (Gibco), 100 µg/mL hygromycin, and DMEM Hi Glucose 1640 (Gibco). Thereafter, 1 L of distilled water was added. The solution was then aseptically stirred and filtered using a 0.22 µm sterile microfilter in laminar airflow chamber, and its acidity was determined to be between 7.2–7.4.[16,18] To make 1 L of M199 medium for the Vero cell culture, the complete medium consisting of 10 mL of 10% FBS (Gibco), 3.7 g NaH_2CO_3, 2 g Hepes, 1.5 mL of 1.5% penicillin-streptomycin (Gibco), 0.5 mL of 0.5% fungizone (Gibco), 100 µg/mL hygromycin, and M199 (Gibco) was combined. Then, 1 L of distilled water was added. The solution was then stirred and aseptically filtered using a 0.22 µm sterile microfilter in a laminar airflow chamber and its acidity was determined to be between pH 7.2 and 7.4.

Preparation of test solutions and treatments

Stocks of *Medinilla speciosa* methanol fruit extract were made using extracts that had been dissolved in DMSO, with 1000 µg/ml in 1 mL made for various solvent polarity phases of the extract; 200 µg/ml in 1 mL made for cisplatin, and the remaining stocks were freshly prepared. *Medinilla speciosa* methanol fruit extract test solutions for three different polarity solvent phases were used to treat the cells in concentrations of 50, 100, 150, 200, 250, 300, 350, and 400 µg/ mL and cisplatin in concentrations of 1, 5, 10, 25, 50, and 100 µg/mL. All the solutions were made in Control Medium (CM) and DMEM. Laminar airflow cabinet was used to aseptically prepare the test solutions.

Determination of the cytotoxic effect of Medinilla speciosa methanol extract by MTT assay

An MTT colorimetric assay was applied to evaluate the cytotoxic effect of the *Medinilla speciosa* methanol fruit extract, alone and in combination with cisplatin (a chemotherapy agent), on MCF-7/HER-2 cells. The DMEM control media was prepared and MCF-7/HER-2 cells at a density of approximately $1x10^6$ cells per well were seeded into 96-well plates. The DMEM was added, and the cell condition was observed under a microscope. It was then incubated at 37°C with 5% CO_2. The hygromycin antibiotic agent was then added to the media at a final concentration of 100 µg/mL following the attachment of the cells to the bottom of the tissue culture dish, which was indicated by the enlargement of the cytoplasm of the cells. Treatment for Vero cells was the same as for MCF-7/HER-2 cells.

In the single cytotoxicity assay, the number of cells was calculated and diluted with CM and DMEM as needed. A total of 100 µL of cells were then transferred into the well and incubated overnight. After discarding the media and washing it with phosphate buffer saline (PBS), 100 µL of new media containing a series of test solutions were added to the well and incubated for 24 hours. After incubation, the media were removed and washed with PBS. A 100 µL/well CM containing MTT reagent (5 mg/10 mL) was added and incubated for 3 h. Then, 10% of sodium dodecyl sulphate (SDS) stopper in 0.01 N HCl was added and read with an ELISA reader at 595 nm wavelength. It was possible to obtain absorption that represented the absorbance of MCF-7/HER-2 living cells. The percentage of living cells was calculated by making a curve between the percentage of living cells and the log levels to obtain the $IC5_0$ value of the test solutions on MCF-7/HER-2 cells.[17,19] The significance percentage test of living cells was calculated with the following formula:

$$\%\ \text{live cells} = \frac{\text{Absorbance of cells with control media} - \text{absorbance of treatment}}{\text{Control cell absorbance} - \text{control media absorbance}} \times 100\%$$

To obtain linearity between the concentration log and the percentage of living cells, IC_{50} concentrations were then calculated by using Microsoft Excel. IC_{50} is the concentration that causes the death of 50% of the cell population so that its cytotoxicity potential can be determined. The selectivity index (SI) was calculated by dividing the IC_{50} values of *Medinilla speciosa* methanol fruit extracts into MCF-7/HER-2 cells by the IC_{50} values of Vero cells. The SI demonstrates the increasingly selective index of *Medinilla speciosa* methanol fruit

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extract. A selective index of ≥3 indicates safety, as opposed to a selective index of ≤3 denoting non-selective.[20]

In the combined cytotoxicity assay, confluent cells (± 0%) from TCD were harvested and seeded in the 96-well microplates at a density of 1×10^4 cells/well. The cells were then incubated for 24 h in an incubator at 5% CO_2 until the cells were ready to be treated by using a test compound. Before treatment, the media contained in the plate were removed by using a micropipette and the cells were then washed with 100 µL/well of 1x PBS. Cells received a test solution with a predetermined concentration series. The administration of the test solution was carried out in a triple amount of 100 µL/well. Cells were incubated again for 24 h and washed with 1x PBS. Then, 100 µL of CM containing 0.5 mg/mL MTT was added to each well. The cells were then re-incubated for 3–4 h in an incubator. MTT reagent was degraded into the deep purple formazan crystals in living cells. Following this, formazan crystals were dissolved by using 100 µL of SDS solution per well and incubated overnight at room temperature in the dark. Thereafter, the plate was shaken with a horizontal shaker for 10 min and read with an ELISA reader at a wavelength of 595 nm. The combined cytotoxic test was done by using the *Medinilla speciosa* methanol extract and cisplatin. As evidenced by the combination matrix simulation in Table 1, the concentration used was below the IC_{50}.

Table 1: Cytotoxic effect of *Medinilla speciosa* methanol extract and cisplatin on MCF7/HER2 cells by combined assay.

IC_{50} of Cisplatin (µM)	**IC_{50} of *Medinilla speciosa* methanol extract (µM)**		
	1/8	**1/4**	**½**
1/8	1/8 : 1/8	1/8 : ¼	1/8 : ½
¼	¼ : 1/8	¼ : ¼	¼ : ½
½	½ : 1/8	½ : ¼	½ : ½

Immunocytochemical analysis

Cells with a density of 5×10^4 cells/well were plated on a coverslip in a 24-well plate and were then incubated until they were 70% confluent. The cells were treated and re-incubated for 15 h. Thereafter, the cells were washed with PBS. Cold methanol was then added, and the cells were incubated in the freezer for 10 min. The remaining methanol was then discarded; PBS and distilled water were used to wash the fixed cells twice. After 10 minutes at room temperature with three drops of hydrogen peroxidase solution added, it was rinsed three times with PBS. Three drops of pre-diluted blocking serum were applied to the cells and incubated for 10 minutes at room temperature. Primary monoclonal antibodies, specifically anti-caspase-9 and VEGF (Proteintech®; diluted 1:100), were added and incubated at room temperature before being incubated overnight at 4°C. After three washes with PBS, cells were incubated for 20 minutes with three drops of biotin-conjugated secondary antibodies (biotin-conjugated biotinylated universal secondary antibody). The cells were washed again with PBS three times. Thereafter, 60 µL reagents containing the streptavidin-horseradish peroxidase (ThermoFisher®) enzyme complex were added at room temperature and incubated for 10 min. The cells were washed again with PBS three times before being added to 60 µL of DAB solution and incubated for 10 min at room temperature. Then, after being washed with distilled water, the cells were dipped in Mayer's hematoxylin solution and incubated for 1 min. Thereafter, the cells were washed again with distilled water. The coverslip was dipped in alcohol and then xylol. After drying, the coverslip was placed on the slide and dropped with glue (mounting media). The prepared slides were observed with a light microscope. Positive cells appeared to have brown cytoplasm (not the nucleus). The blue color in the cytoplasm indicated the absence or low level of expression, which was not detected in the cells.

Statistical analysis

Cell viability in the single and combined cytotoxicity assays was analyzed by using one-way analysis of variance (ANOVA) with the Statistical Package for Social Sciences (SPSS; version 7) software. The data were further analyzed with Tukey's HSD analysis ($p < 0.005$).

Results and Dıscussıon

The MTT assay was used to conduct a cytotoxic analysis on MCF-7/HER-2 cells. It was noted that the MTT assay is a cytotoxic test method that is relatively fast and sensitive and can be used to measure large samples with a high level of accuracy.[2,10] The basic principle of the MTT assay is presented in Figure 1. Based on the principle of MTT reduction reaction, the *Medinilla speciosa* methanol extract cytotoxic potential was determined by using the IC_{50} parameter, which is a concentration that can cause the inhibition of cell growth by 50% of the population. The difference in color produced after adding MTT reagents was observed in the test concentration in the well. Based on the observations, the development of formazan crystals, which build up in living cells, caused the purple color. With more living cells, there would be more metabolically active cells, which would increase formazan crystal formation and purple color intensities.[21,22] Mitochondria of dead cells in the MTT cytotoxicity test were unable to carry out respiration, so they did not produce the tetrazolium succinate reductase enzyme, which could reduce MTT reagents into formazan crystals. Dead cells consequently formed a slightly purple formazan, which eventually turned pale pink.[23,24]

The morphology of the MCF-7/HER-2 cancerous cells that received the treatment of *Medinilla speciosa* methanol fruit extract changed with an increase in extract concentrations (Figure 2). There was an imbalance between the MCF-7/HER-2 cell groups after receiving cisplatin treatment, which resulted in the production of dead cells that were spherical or globular. When compared to the cell control, the dead cells were more noticeable after receiving cisplatin treatment than the untreated cells (cell control). The morphology of cells administered cisplatin showed significant alterations in cell death compared to cell control. Changes in the cell morphology by the rounding indicated that the cells were undergoing death.

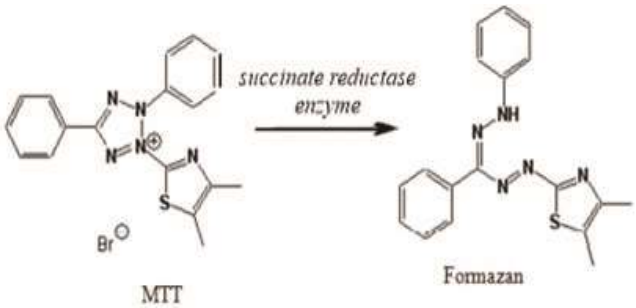

Figure 1: The reduction reaction of MTT into formazan by the reductase enzyme.

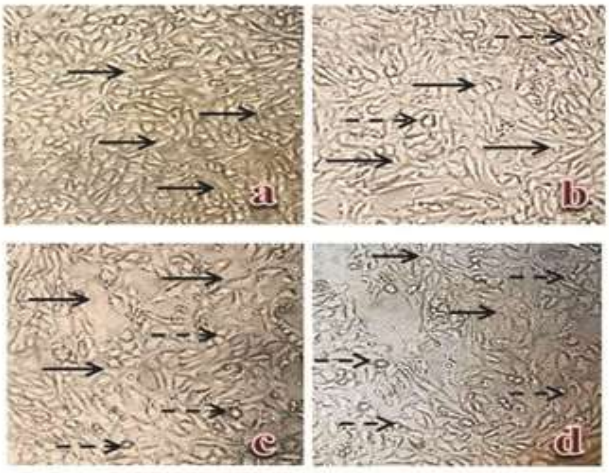

Figure 2: Cytotoxic effect of *Medinilla speciosa methanol extract* on MCF-7/HER-2 cells.
(a) Cells without treatment. (b) EMP 100 µg/mL. (c) EMP 150 µg/mL. (d) EMP 200 µg/mL

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At a concentration of 8 µg/ml, about 40% of cells have died, whereas, at a concentration of 10 ppm, they were still alive. More and more cells were still dying, and at a concentration of 25 µg/ml, the majority of cells began to die. Therefore, cisplatin is specifically used as a positive control due to its effectiveness in killing MCF-7/HER-2 cancer cells. Cisplatin is also an anticancer drug due to its potential in possible cytotoxic categories.

Medinilla speciosa methanol fruit extract had a linear regression of y = -0.0872x + 65.28, R2 = 0.9627, according to the equation for linear regression (Figure 2). In the cytotoxic activity test, the cisplatin positive control had a linear regression of y = 2.0486x + 82.676, R2 = 0.9243 as shown in Figure 3. The IC_{50} value obtained after the administration of the *Medinilla speciosa* methanol fruit extract was 175 ± 0.962 µg/mL, whereas the IC_{50} values obtained after the administration of cisplatin were 16±1.07 µM. Based on IC_{50} values, cytotoxic activity is divided into three categories: potential cytotoxic (IC_{50} < 100 µg/mL), moderate (100 µg/mL < IC_{50} < 1000 µg/mL), and non toxic (IC_{50} > 1000 µg/mL).[25] The IC_{50} values obtained from the cytotoxic test results of *Medinilla speciosa* methanol fruit extract fell under the category of moderate cytotoxicity. Meanwhile, cisplatin used as a positive control is classified in the category of potential cytotoxic or has good anticancer content, with an IC_{50} value of 16 µg/mL. According to the National Cancer Institute, a substance has anticancer activity if its IC_{50} value is less than 20 µg/ml. Potentially cytotoxic compounds can be employed as anticancer agents, while moderately cytotoxic substances can be utilized as co-chemoprevention agents or to inhibit or reduce the growth of cancer cells.[5,26] The *Medinilla speciosa* methanol fruit extract's IC_{50} value is therefore categorized as moderate (Table 2), making it a co-chemoprevention agent. Due to the presence of secondary metabolites, particularly flavonoids, tannins, and saponins, which have the potential to be anticancer agents, *Medinilla speciosa* methanol fruit extract is thought to have cytotoxic properties. By inducing apoptosis or non-apoptosis, saponins can have cytotoxic effects on cells.[27] Hydroxyl radicals, superoxide, and anti-lipoperoxidants can all be reduced by flavonoid molecules.[6,28]

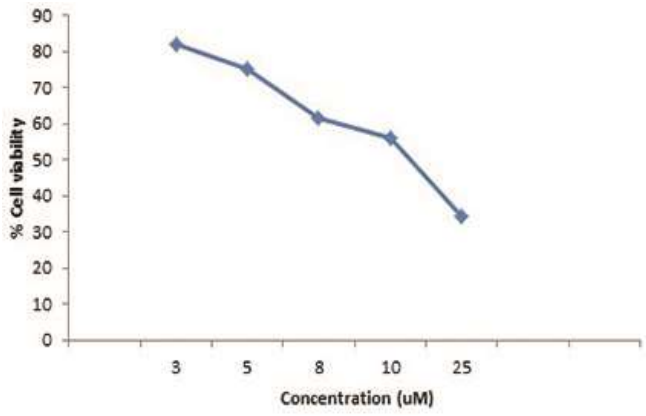

Figure 1: Cytotoxic activity of cisplatin on MCF-7/HER-2 cells.
The panel showed a decrease in cell viability after cisplatin treatment for 24 hours. The experiments were independently carried out for three times.

Table 2: Single cytotoxic test results of *Medinilla speciosa* methanol extract and cisplatin

Sample	IC_{50}	Category
MSME	175±0.962 µg/mL	Moderate cytotoxic
Cisplatin	16±1.07 µM	Strong cytotoxic

MSME: *Medinilla speciosa* methanol extract

Criteria in selecting compounds that have the potential to be anticancer agents are not only tested on cancer cells to observe their cytotoxic effects, but their selectivity against normal cells (Vero cells) is also needed. This is conducted to determine the safety of test extracts that have anticancer effects on normal cells. An extract with a SI value of ≥3 selectively inhibits the proliferation of cancer cells while being non-toxic to healthy cells. If a compound has a value of ≤3, it can be classified as having no selectivity. The selective extract allows for further usage as a possible anticancer agent because it only affects cancer cells while not affecting healthy cells.[7,29] As shown in Table 3, *Medinilla speciosa* methanol fruit extract was selective (SI = 30), so it is safe in normal cells, whereas cisplatin as a positive control has a non-selective value of SI = 1.3 because cisplatin is an anticancer agent that is toxic to both cancer and normal cells.[28,30] The ability of every phytochemical compound to act as a co-chemoprotection in inhibiting cancer growth must be understood as the result of a combination of various intracellular processes and not a single biological response. The phytochemical isolation procedure can be used to identify the effects of each active component of *Medinilla speciosa* methanol fruit extract, and each component's cytotoxicity is tested against cancer cells.[29,31]

Cisplatin has a cytotoxic effect on MCF-7/HER-2 cells with an IC_{50} value of 16 µM. By employing concentrations lower than the IC_{50} value, the concentration of the combined treatment was determined. Cisplatin concentrations (2, 4, and 8 µM) were combined with 88 µg/mL *Medinilla speciosa* methanol fruit extract. Following the combined treatment, the morphological alterations and reduced cell density are depicted in Figures 3A–C. Compared to single cisplatin treatment, the combined treatment significantly ($p < 0.05$) decreased cell viability. Statistical analysis revealed a significant ($p < 0.05$) difference between cisplatin, *Medinilla speciosa* methanol fruit extract, and the combined treatments (Figure 4). The combination of 8 µM cisplatin with 88 µg/mL *Medinilla speciosa* methanol fruit extract significantly ($p < 0.05$) reduced the viability of MCF-7/HER-2 cells compared to the combinations with 2 and 4 µM cisplatin concentrations. Cell viability was reduced by 53% when 8 µM cisplatin and 88 g/mL *Medinilla speciosa* methanol fruit extract were combined for 24 h. Cell viability from a single *Medinilla speciosa* methanol fruit extract and cisplatin treatment above is considered to be 100%. This indicated that the plant extract and cisplatin do not show cytotoxic effects on MCF-7/HER-2 cells at low concentrations. However, at low concentrations, the combination of *Medinilla speciosa* methanol fruit extract and cisplatin significantly ($p < 0.05$) reduced cell viability. These observations suggest that the fruit extract can be utilized in combination with cisplatin to treat MCF-7/HER-2 cells.

Table 3: The index selectivity values of *Medinilla speciosa* methanol extract and cisplatin

Sample	SI	Selectivity
MSME	30.008	Selective
Cisplatin	1.235	Not selective

MSME: *Medinilla speciosa* methanol extract

The mechanism of cytotoxic effects resulting from the administration of *Medinilla speciosa* methanol fruit extract was investigated by determining the protein target of VEGF and caspase-9. VEGF plays a role in angiogenesis through increased endothelial cell migration, methane monooxygenase activity, and endothelial cell mitosis.[4,25] By binding to tyrosine kinase receptors in most cancers and embryonic tissue, high VEGF levels can be used to diagnose angiogenesis.[3,20] Caspase-9 induces apoptosis through the activation of the intrinsic pathway caused by several stimulants, including chemotherapy and radiation. An apoptosome complex containing zymogen monomers activates caspase-9.[19]

The induction of *Medinilla speciosa* methanol fruit extract at a concentration of 175 µg/mL can reduce the VEGF expression compared to the control, as shown in Figure 5. This demonstrates how *Medinilla speciosa* methanol fruit extract can decrease VEGF expression by inhibiting angiogenesis. As shown in Figure 5, some of the stages of angiogenesis inhibition include the inhibition of bFGFG,

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VEGF, circulating endothelial progenitor cells, inhibiting the enzymes of matrix metalloproteinases, which can degrade the basement membrane of blood vessels, inhibiting the proliferation, activation, and differentiation of endothelial cells.[4,16] Neoplastic cells release several pro-angiogenic factors, such as collagenase and plasminogen activators, that cause disruption of the basement membrane of the surrounding vasculature and trigger angiogenesis. In addition, these molecules act as chemotactic factors for endothelial cells and enhance the differentiation of the circulating bone marrow progenitor cells into endothelial cells. As the series progresses, a new basement membrane formation is observed, and pericytes are recruited to support the new blood vessel. Interestingly, angiogenesis facilitates the spread of the tumor cells and, hence, mediates metastasis.[5,17]

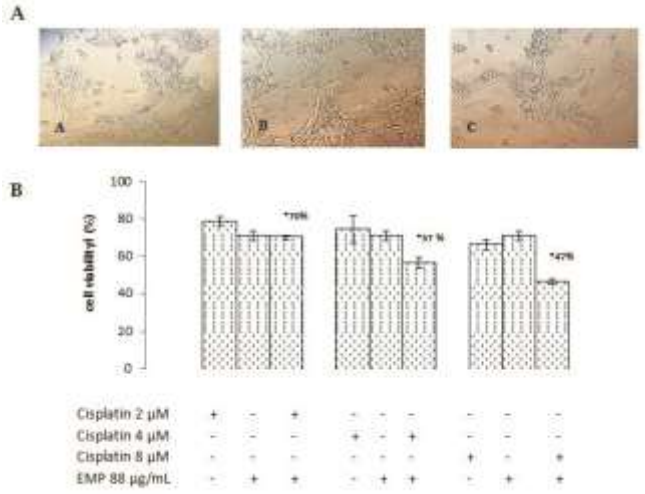

Figure 2: Cytotoxic effects of *Medinilla speciosa* methanol extract and cisplatin in combination treatment on MCF-7/HER-2 cells.

A. Cytotoxic Effects of EMP and Cisplatin Combination Treatment on MCF-7/HER-2 Cells and observed under an inverted microscope at a 100× magnification. (a) cell treatment with 8 µg/ mL cisplatin (b) cell treatment with 88 µg/ mL EMP (c) cell treatment with combination of 8 µM cisplatin with 88 µg/ mL EMP

B. Citotoxic effect of EMP and Cisplatin combination treatment on MCF-7/HER-2 cells using MTT Assay by viability cell observed.

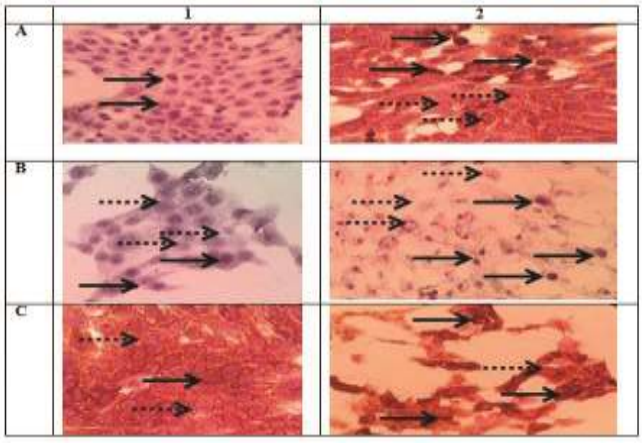

Figure 5: Immunocytochemistry of VEGF (A.1) and Caspase-9 on MCF-7/ HER-2 Cells (A.2).

(A1) Cell control with vehicle treatment. (B1) treatment with cisplatin 8 µg/mL. (C1) cell treatment with combination of 8 µM cisplatin with 88 µg/ mL EMP.

(A2) Cell control with vehicle treatment. (B2) treatment with cisplatin 8 µg/mL. (C2) cell treatment with combination of 8 µM cisplatin with 88 µg/ mL EMP.

The majority of solid tumors, such as breast cancer, overexpress VEGF-A, making it the dominant target for anti-angiogenic drugs. The results of the present study indicated that *Medinilla speciosa* methanol fruit extract can lower VEGF expression compared to controls, suggesting that it may be a viable anti-angiogenic agent. The expression profile of the protein cysteinyl aspartic-acid-protease-9 (caspase-9) can also be used to determine the exact mechanism by which *Medinilla speciosa* methanol fruit extract causes cytotoxicity in MCF-7/HER-2 cells. Caspase-9 is the upstream direction of the intrinsic apoptotic pathway. The intrinsic route contains a variety of non-receptor-mediated stimuli that result in intracellular signals that directly attack the intracellular target of the mitochondria. The expression of caspase-9 after the *Medinilla speciosa* methanol fruit extract administration showed apoptotic induction activity through the mitochondrial pathway. The results of the immunocytochemical test demonstrated that the induction of apoptosis by the administration of 175 g/mL *Medinilla speciosa* methanol fruit extract increased the expression of the caspase-9 protein. In the cytoplasm (not the nucleus), positive cells appeared brown. The blue color in the cytoplasm indicated the absence or low expression that was not detected.

Conclusion

The findings of the present study revealed that *Medinilla speciosa* methanol fruit extract exhibits cytotoxic effects on MCF-7/HER-2 cells. Cell viability was lowered by 53% when the fruit extract and cisplatin were combined. VEGF expression decreased and caspase-9 protein expression increased following administration of *Medinilla speciosa* methanol fruit extract.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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