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Cytotoxic effects of parijoto (*Medinilla speciosa* Reinw. Ex. Bl.) methanol extract combined with cisplatin on WiDr colon cancer cells through apoptosis induction

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ABSTRACT Parijoto (*Medinilla speciosa* Reinw. Ex. Bl.) is a medicinal plant with cytotoxic effects on cancer cells in vitro. As only a limited number of studies have reported the effect of parijoto on colon cancer cells, this study initially aimed to measure the total flavonoid levels and potential cytotoxic effects of parijoto methanol extract (PME) through cell viability assays and expression of the apoptotic protein on WiDr colon cancer cells as a model. PME cytotoxic activity was determined by conducting a cytotoxicity test on WiDr colon cancer cells using the MTT assay. The synergistic cytotoxic effects of the PME and cisplatin were tested to obtain the combination index (CI) value. Apoptosis was analyzed by flow cytometry, and the apoptotic protein expression was observed by immunocytochemical tests. Furthermore, quercetin as a major flavonoid in PME was measured using a UV–Vis spectrophotometer. The results showed that PME had a moderate cytotoxic activity with an IC_{50} of 198.64±1.6 µg/mL, whereas the IC_{50} of cisplatin was 2.34±0.7 µg/mL. The PME with cisplatin combination test showed a strong synergistic effect with a CI value of <1 (0.1-0.4). The combination showed increased apoptosis properties compared to PME treatment alone. In addition, immunocytochemistry showed that PME alone or in combination with cisplatin increased the pro-apoptosis proteins (p53 and caspase-9) and suppressed Bcl-2 expression. Moreover, the cell viability value increased as the PME concentration decreased. The administration of PME led to changes in cell morphology, lower cell density, and a decreasing number of living cells. Therefore, the combination of PME and cisplatin had a strong synergistic effect in inducing apoptosis.

KEYWORDS apoptosis; cisplatin; cytotoxic; parijoto methanol extract; WiDr colon cancer cells

1. Introduction

Parijoto (*Medinilla speciosa* Reinw. Ex. Bl. Fam. Melastomaceae) is one of about 375 species of Medinilla genus widespread in Africa and Asia-pacific (Mabberley 2017). Parijoto fruit contains alkaloids, flavonoids, saponins, tannins, glycosides, terpenoids, and anthocyanins (Wijayanti and Ardigurnita 2019; Niswah 2014; Sa'adah et al. 2017). According to Tusanti et al. (2014), parijoto ethanol extract showed moderate cytotoxicity in vitro against T47D breast cancer cells with an IC_{50} value of 614.50 µg/mL. Besides possessing a potential cytotoxic effect against cancer cells, parijoto has also benefited as an antihyperlipidemic agent, antibacterial, and antioxidant (Sa'adah et al. 2017; Wahyuni et al. 2019; Wachidah 2013).

Cancer is a disease with a high prevalence characterized by the uncontrolled growth of abnormal cells (American Cancer Society 2017). Based on data from the World Health Organization (2018), there are 1.8 million cases of colon cancer in the world. A total of 30,017 new cases of colon cancer occurred in Indonesia. Colon cancer is a malignant tumor growth that can damage DNA and healthy tissue around the colon and rectum (Tedja and Abdullah 2013). Colon cancer cells undergo irregular molecular mechanisms of cell division and apoptosis, such as inefficient control of cell proliferation, unstable genetic and chromosome structures, changes in differentiation programs, and impaired apoptosis control (Pritchard and Grady 2011). Apoptotic function abnormalities are associated with colon cancer and its resistance to chemotherapy and radiotherapy. Disruption of apoptosis regulation can increase tumorigenesis and lead to colon cancer resistance (Abraha and Ketema 2016).

One of the pro-apoptotic proteins that act as a marker of apoptosis involving the mitochondria is caspase. Mitochondrial pathways are activated by various cytotoxic drugs, DNA damage, deficiency of growth factors, oxidative stress, excess of Ca^{2+}, and oncogenes activation (Mendelsohn et al. 2015). They are regulated by

forming the mitochondrial permeability transition pore, which is composed of Bcl-2 family members and a voltage-dependent anion channel. Cytochrome-c (Cyt-c) then associates apoptosis protease-activating factor 1 and caspase-9 to form an apoptosome complex. Activation of caspase-9 and/or caspase-8 leads to caspase-3 cleavage, activation of endonucleases, and finally, nuclear DNA fragmentation, which characteristic of apoptosis (Redza-Dutordoir and Averill-Bates 2016).

In colon cancer cells, Bcl-2 family proteins are central regulators of the intrinsic pathway, which suppresses or promotes changes in mitochondrial membrane permeability required to release Cyt-c and other apoptogenic proteins (Bostan et al. 2016). Increased expression of the anti-apoptotic protein Bcl-2 family proteins may lead to poor prognosis in patients with colonic adenocarcinoma, providing a multidrug resistance phenotype (Huang and Linda 2015). One of the apoptosis inducers in colon cancer cells is the p53 protein. The molecular mechanisms of cell death induction by p53 to suppress cancer development include transcription regulation of pro-apoptosis PUMA, the formation of oxidative free radicals in mitochondrial components, reduction of COX-2/PGE2 synthesis, and induction of death receptors (Edagawa et al. 2014). On the other hand, p53 mutation occurs at position 273 in WiDr cells, resulting in a change in the arginine residue to histidine (Noguchi et al. 1979). However, p21 in normal WiDr cells allows cell cycle termination. Apoptosis in WiDr cells can occur through p53-independent pathways (Liu et al. 2006).

Some of the therapeutic agents used in colon cancer treatment, e.g., fluoropyrimidines, cisplatin, oxaliplatin, and irinotecan, have been shown to cause resistance in killing cancer cells. The number of cancer cells may increase by modulation of survival cell components, such as proliferative proteins or anti-apoptosis factors (Haider et al. 2020). In addition, side effects, such as nausea, vomiting, hair loss, swelling, sores in the mouth and throat, drastic weight loss, and memory problems, also occur following chemotherapy with cisplatin (He et al. 2016). Thus, it is essential to find new target-based molecules and new therapeutic approaches in colon cancer by determining the cellular mechanisms responsible for inducing apoptosis in cancer cells (Thornthwaite et al. 2013). It can be done through a combination of chemotherapy, which has the cytotoxicity potential for additive or synergistic tumors. The combination of natural compounds with anticancer drugs (co-chemotherapy) can increase the effectiveness of cancer treatment, especially in highly invasive colon cancer cells. In contrast, the use of natural compounds can reduce cytotoxic side effects in non-tumor cells (Fox et al. 2012).

One of the plants that potentially be developed as a co-chemotherapy candidate is parijoto (*M. speciosa*). Parijoto possesses antioxidant activity. The ethyl acetate fraction, methanol fraction, and methanol extract of the fruits display IC_{50} values at 20.34, 46.65, and 48.24 µg/mL, respectively (Wachidah 2013). Forni et al. (2019) reported a positive correlation between the antioxidant activity of plants and their anti-proliferative effects, such as flavonoids that exhibit various biological activities, including anti-inflammatory, cytoprotective activities, and some are known to act as anticancer agents. There are limited studies regarding the cytotoxic mechanism of parijoto fruit against colon cancer cells. This study aimed to observe the cytotoxic effect of parijoto methanol (PME) extract on WiDr cancer cells, which was confirmed by the induction pattern of apoptosis through the expression of the p53, Bcl-2, and caspase-9 proteins, then proceed with quantification of quercetin levels in PME.

2. Materials and Methods

2.1. Sample collection and extract preparation

Parijoto fruits obtained from Kudus, Central Java, Indonesia, were stored in a cooler bag to transport to the laboratory. The fruits were dried at 25 °C and then blended for optimum extraction. The extraction method was conducted based on Vifta and Advistasari (2018). The fruit powder (500 g) was extracted with 500 mL of methanol (Merck, Germany) by the maceration for 24 hours (h) at 37 °C. Upon filtration, the filtrate was evaporated using a rotary evaporator at 45 °C to form a thick extract. The remaining solvent in the viscous extract was evaporated using a water bath. The extract was stored at −20 °C until use. This study was conducted at the Parasitology Laboratory, Faculty of Medicine, Nursing and Public Health, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia.

2.2. Drug preparation and treatment

Cisplatin (1 mg) (Kalbe, Indonesia) was dissolved in dimethyl sulfoxide (DMSO) (0.1 mL) (Merck, Germany) and used as a comparison compound in colonic cancer cytotoxicity tests. The solvent concentration in the culture medium was not more than 0.5% in each experiment.

2.3. Cell culture

Human colon adenocarcinoma (WiDr) cancer and normal epithelial cell lines (Vero cells) were obtained from Parasitology Laboratory, Faculty of Medicine, Nursing and Public Health UGM. Both cell lines were cultured in the RPMI medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) with 5% CO_2 supply at 37 °C.

2.4. Cytotoxicity test with the MTT assay

The MTT assay (Sigma Aldrich, USA) was carried out for 24 h to measure the cytotoxic effect of parijoto fruit methanol extract (PME) and cisplatin on WiDr cancer cells and normal cells. Up to 1×10^4 cells/well were grown in 96-well plates. The cell suspension (100 µL) was transferred into the wells and observed in a microscope to see the distribution of cells. Cells were then incubated in an incubator for 24 h so that the cells recovered after harvesting. The concentration series of PME (50–400 µg/mL) and

cisplatin (0–10 µg/mL) was added (100 µL) to the well in the triplicate, then re-incubated in a CO_2 incubator for 24 h. At the end of the incubation time, cell conditions were documented for each treatment under an inverted microscope. The cell media was discarded, and the cells were washed with 1× phosphate buffered saline (PBS). Then, 10 µL of MTT (0.25 mg/mL) was added to each well. Cells were re-incubated for 4 h in a CO_2 incubator until formazan crystals were formed. When the formazan crystals had formed, 10% SDS in 0.1 N HCl was added as a stopper. The absorbance was then measured using an ELISA microplate reader (Bio-Rad microplate reader Japan) with a spectrophotometer UV-Vis (Genesys, Thermo Scientific, USA) at 595 nm. The log concentration and cell viability were plotted on a logarithmic graph, then used to determine the IC_{50} value (Wulandari et al. 2021).

Based on the IC_{50} value from a single cytotoxicity test, the concentration series was made of ½, ¼, and ⅛ of IC_{50} for PME and cisplatin. Equation (1) is used to evaluate the combination synergism based on research conducted by Reynolds and Maurer (2005).

$$\text{Combination Index (CI)} = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} \quad (1)$$

Dx1 and Dx2 are concentrations of one compound needed to exert an effect (IC_{50} on the growth of WiDr cells), D1 and D2 are the concentrations of the two compounds to give the same effect.

2.5. Apoptosis measurement by flow cytometry

WiDr cells in the exponential growth phase were treated with PME at ⅛ or ½ IC_{50} for 72 h, then harvested by trypsinization and washed twice with ice-cold PBS. The cells were then resuspended with 100 µL of the binding buffer. In the next stage, 5 µL of Annexin-V FITC and 5 µL of propidium iodide (PI) were added to each tube and incubated for 10 min at room temperature in the dark. Finally, 400 µL of the binding buffer was added to each tube, and the data from 10,000 cells per sample were collected and analyzed on a flow cytometer (BD Accuri C6) within 1 h. The results were compared with those for untreated control cells (Haryanti et al. 2017).

2.6. Immunocytochemical test against p53, Bcl-2, and Caspase-9 proteins

Cells at a 5×10^4 cells/well density were plated on a coverslip in a 24-well plate and incubated until 70% confluent. The cells were treated and re-incubated for 15 h. At the end of the incubation time, the cells were washed with PBS, cold methanol was added, and the cells were then incubated in the freezer for 10 min. After discarding the methanol, the cells were washed with PBS twice, washed with distilled water twice, incubated with hydrogen peroxidase solution for 10 min, and then washed three times. Solution or cell suspension then dripped with prediluted blocking serum and incubated for 10 mins. In the treatment group, the primary anti-p53 monoclonal (ThermoFisher Scientific, Cat #MA5-12557), anti-Bcl-2 (ThermoFisher Scientific, Cat #TA806591), and anti-caspase-9 antibodies (ThermoFisher Scientific, Cat #MA1-16842) were added and then incubated overnight. After being washed with PBS three times, the cells were incubated with a secondary antibody conjugated with biotin (biotinylated universal secondary antibody) (ThermoFisher Scientific, Cat #31806) for 20 min. After being washed, a reagent containing the streptavidin-horse radish peroxidase enzyme complex was added to the cells and incubated for 10 min. The cells were rewashed with PBS three times, dripped with DAB solution, and incubated for 10 min. After being washed with distilled water, the cells were incubated with the Mayer–Hematoxylin solution for 1 min. The coverslip was then dipped in alcohol and xylol after washing with distilled water. After drying, the coverslip was placed on a slide and dripped with mounting media. The coverslip was closed with a slide for further observation with a light microscope (Noviantari et al. 2020).

2.7. Quantification of quercetin levels in PME

The PME and a quercetin standard were plated using a capillary tube on thin-layer chromatography (TLC, Silica gel GF 254) plate, then eluted with the appropriate mobile phase. The mobile phase used was chloroform: acetone: formic acid (10:2:1 v/v), which was detected with a 10% $FeCl_3$ spray reagent. Furthermore, the determination of quercetin levels begins with determining the maximum wavelength of quercetin in the wavelength range of 400–600 nm, and the maximum wavelength was obtained at 439 nm. A calibration curve was created by dissolving 10 mg of quercetin in 10 mL of methanol and diluting it into serial concentrations of 12.5, 25, 50, 75, and 100 µg/mL. The blank solution was 0.5 mL methanol and the test solution used 0.5 mL PME. Each solution (0.5 mL) was added with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of each solution was measured at 439 nm.

2.8. Analysis of cytotoxicity test data

The data obtained were in the absorbance of each well, which was then converted into cell viability. The cell viability was calculated using equation (2) based on research conducted by Doyle and Bryan (1998).

$$\text{Cell viability (\%)} = \frac{(Treated\,Cell\,Absorbance - Media\,Absorbance)}{(Control\,Cell\,Absorbance - Media\,Absorbance)} \times 100\% \quad (2)$$

2.9. Selectivity measurement

Selectivity was determined using the Selectivity Index (SI) parameter with the following equation (3) according to Prayong et al. (2008):

$$\text{SI} = \frac{Vero\ cells\ IC_{50}\ value}{Cancer\ (WiDr)\ cells\ IC_{50}\ value} \quad (3)$$

2.10. Apoptosis analysis

Data from flow cytometry were analyzed using Microsoft Excel 2010. The percentage of cell death, including early apoptosis, late apoptosis, and necrosis, was displayed in a bar graph. In addition, the induction of cell death by the test solution was known by comparing single and combination treatments with control cells.

2.11. Observation of p53, caspase-9, and Bcl-2

Protein expression was descriptively qualitatively observed, where cells expressing the proteins would give a brown color in the nucleus, while those that did not express the proteins or had low expression levels would give a purple color in the nucleus.

$$\text{Percentage of cells with protein expression} = \frac{Cells\ with\ protein}{Total\ cells} \times 100\% \quad (4)$$

As mentioned in equation (4), the calculation of protein expression based on research conducted by Zakinah et al. (2017) was carried out on a minimum of 100 cells from each point of view. Furthermore, the observation of the samples was carried out from three different points of view for each sample and documented with a camera.

2.12. Analysis of total flavonoid levels

Total flavonoid levels were calculated using equation (5) according to Chang et al. (2002) as follows:

$$\%F = \frac{c \times V \times f \times 10^{-3}}{m} \times 100\% \quad (5)$$

F is the total flavonoid with AlCl3 methods, c is the quercetin equivalent (µg/mL); V is the total extract volume (mL), f is the dilution factor, and m is the sample mass (mg).

2.13. Statistical analysis

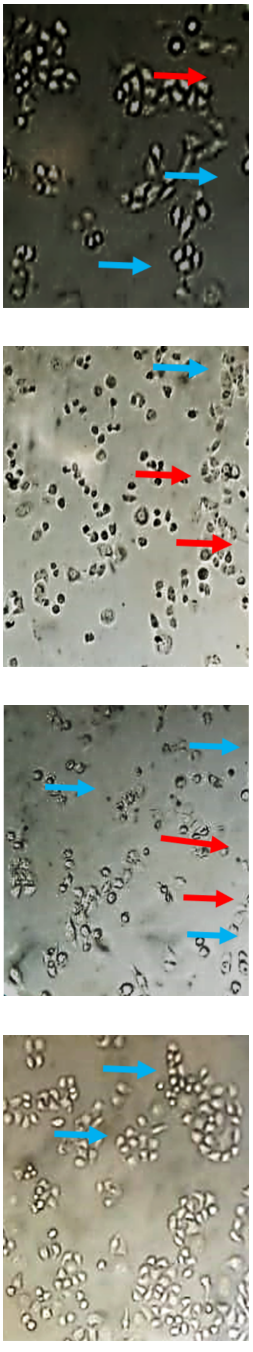
The quantitative data resulting from the combination treatment of cell viability were analyzed statistically with the Analysis of Variance (ANOVA) test followed by the Tukey HSD test using SPSS 16.

3. Results and Discussion

3.1. Assessment of WiDr cell viability following treatment with PME, cisplatin, or their combination

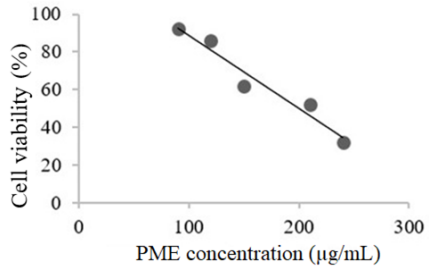
The relationship between cisplatin concentration and cell viability is presented in Figure 1 and Figure 2. The determination of cisplatin activity through linear regression obtained a linear equation of y = −2.3562× + 55.518 with an R-value of 0.9752. Based on the linear regression, the IC_{50} values of cisplatin and PME were 2.34 ± 0.7 µg/mL and 198.64 ± 1.6 µg/mL, respectively. Therefore, the linear equation obtained for PME cytotoxicity activity was y = −0.3868x + 127.47 with an R-value of 0.9505. The IC_{50} value is a value that indicates the death of 50% of the cell population so that its potential cytotoxicity can be known (Mazumder et al. 2020).

Viable WiDr cells have a polygonal shape and attach to the bottom of the well, while dead WiDr cells are round and smaller, scattered, and do not attach to the bottom of the well (Anandani et al. 2018). PME selectively inhibited colon cancer cells compared that of to normal cells (Vero cells). The results showed that there were differences between control cells and PME-treated cells. The living WiDr cells were polygonal in the control group, and

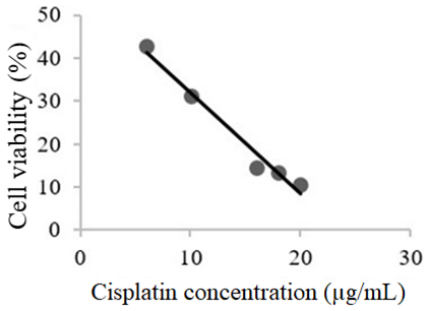


(a) (b) (c) (d)

FIGURE 1 Morphology of WiDr cells treated with PME and observed under an inverted microscope at a 100× magnification. (a) PME 90 µg/mL. (b) PME 150 µg/mL. (c) PME 210 µg/mL. (d) Control cells. Blue arrows indicate living cells, red arrows indicate dead cells.



(a)



(b)

FIGURE 2 Relationship between cell viability and PME concentrations in the range of 70–225 µg/mL (a), and cisplatin concentrations in 5–20 µg/mL (b).

the cell density was higher. In addition, cell viability value was increased with the high concentration of PME administration. The administration of PME affects the cell morphology, decreases cell density, and decreases the number of living cells with increasing concentrations (Figure 1), but had less effects on Vero cells (Figure 3). Based on the test results, the IC_{50} value of PME was 198.64±1.6 µg/mL. Moreover, our results showed that the IC_{50} value in the Vero cell was 5,258.42 µg/mL. According to Weerapreeyakul et al. (2012), natural material is categorized as having very strong cytotoxicity if it has an IC_{50} value of <10 µg/mL; strong cytotoxicity if it has an IC_{50} value between 10–100 µg/mL; and moderate cytotoxicity when it has an IC_{50} value of 100–500 µg/mL.

The safety level of an anticancer compound against normal cells is confirmed by determining the value of its selectivity compared to normal cells. Extracts are classified as selective if they have an SI value of ≥3 and as less selective if the SI value is <3 (Sutejo et al. 2016). The required SI value is ≥3, which indicates that the extract has cytotoxic activity against cancer cells with minimal effect on normal cells and can be further developed as a chemoprevention agent (Sutejo et al. 2016).

TABLE 1 Cytotoxic effects of test compounds and their IC_{50} values

Test compound	IC_{50} in WiDr cells (µg/mL)	IC_{50} in Vero cells (µg/mL)	Selectivity Index
PME	198.64±1.6	5,258.42±0.7	26.46
Cisplatin	2.34±0.7	21.07±0.9	9.00

As shown in Table 1, the selectivity analysis showed that PME selectively toxic to WiDr colon cancer cells and had minimal effect on Vero cells with an SI value of 26.47 (SI ≥ 3). The IC_{50} PME in WiDr cells was higher than that of normal Vero cells. This indicates that PME cytotoxicity in WiDr cancer cells is stronger than Vero cells. The SI value after PME treatment and after treatment with cisplatin was 26.46 and 9, respectively. A compound with a cytotoxic effect and high selectivity can be developed as a chemoprevention agent because the compound is able to distinguish between cancer cells and normal cells (Sholikhah et al. 2018).

TABLE 2 The combination index value of PME and cisplatin combination on WiDr cells

PME (µg/mL)	Cisplatin (nM)			
	29	58	117	234
24.8	0.14	0.14	0.13	0.10
49.6	0.37	0.47	0.43	0.66
99.3	0.74	0.97	0.88	0.94

We next tested the cytotoxicity of PME and cisplatin combination through the cytotoxicity test PME and cisplatin combination. Combination treatment of PME and cisplatin has a synergistic effect (Reynolds and Maurer 2005) at cisplatin concentration of 200 nM and all PME concentrations (24.8, 49.6, and 99.3 µg/mL), with CI values of 0.14–0.97 (Table 2). These results revealed that PME could increase the sensitivity of WiDr cells toward cisplatin.

3.2. Effect of PME, cisplatin, and its combination on apoptosis

The confirmation of the cytotoxicity test results was carried out through the apoptosis induction test using flow cytometry. Cell detection using Annexin-V and PI showed the living cells and dead cells in early apoptosis, late apoptosis, and necrosis (Figure 4). PME induces less cell death than cisplatin. The higher cisplatin cytotoxic effect by PME through apoptosis induction mechanisms. The flow cytometry results of PME in WiDr cells are present in Figure 4 (n=3).

The PME apoptosis induction test on WiDr cells showed that untreated cells (control) showed more living cells (95.61%) than dead cells (4.39%). Meanwhile, cells treated with PME experienced death up to 25.8%, and after the combination treatment of ⅛ IC_{50} PME and 7/8 IC_{50} cisplatin, the cell death raised to 60.6%. The percentage of cell mortality due to cisplatin treatment was 96.7%. The reduction in cell death after combination was 36.1% compared to cisplatin alone. Cell necrosis in the combination treatment increased by 18.3% compared to the cisplatin treatment alone. PME induces less cell death than cisplatin. Therefore, the high cytotoxicity of cisplatin by PME probably occurs through apoptosis induction mechanisms. The apoptosis induction was confirmed through immunocytochemical tests by looking at the expression profiles of Bcl-2, p53, and caspase-9.

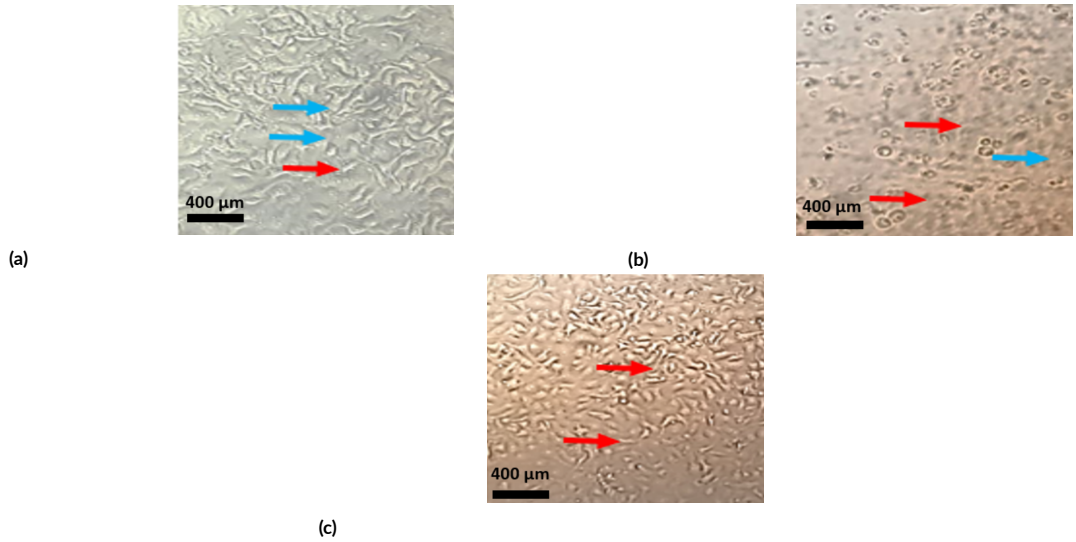


FIGURE 3 The morphology of Vero normal cells was observed under an inverted microscope at a magnification of 100×. (a) PME 1,000 µg/mL. (b) Cisplatin 20 µg/mL. (c) Control cells. Blue arrows indicate living cells, red arrows indicate dead cells.

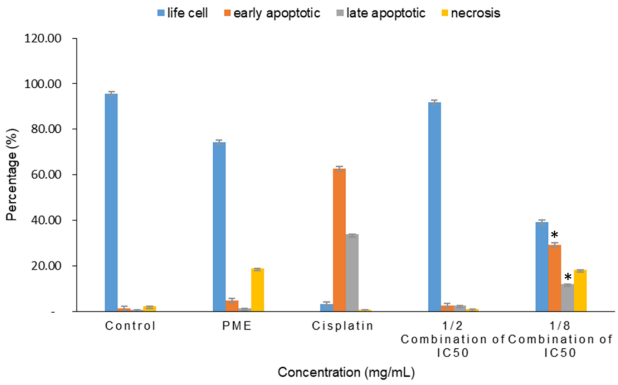


FIGURE 4 Analysis of the percentage of cell death after treatment with PME, cisplatin, and the combination of both. WiDr cells were treated with PME, cisplatin, and the combination for 72 h using Annexin-V FITC and propidium iodide staining. Significant difference (p <0.05) compared with untreated WiDr cells is indicated by the increase of apoptotic The observation was conducted for three times (n=3).

3.3. *Effect of PME, cisplatin, and their combination on Bcl-2, p53, and caspase-9 expressions in WiDr cell*

The anti-apoptotic protein Bcl-2 was highly expressed in WiDr cells, and it was correlated with the low effect of the chemotherapy agent (Figure 5- 7). Therefore, we select PME in this study because of the quercetin compound.

TABLE 3 Standard Rf value of quercetin and PME

Standard	Rf		hRf	
	Standard	PME	Standard	PME
Quercetin	0.7	Rf 1 = 0.11 Rf 2 = 0.23 Rf 3 = 0.53 Rf 4 = 0.77	70	Rf 1 = 11 Rf 2 = 23 Rf 3 = 53 Rf 4 = 77

Quercetin increased the expression of p53 and caspase 9 proteins while suppressing the expression of Bcl-2.

3.4. *Quantification of the quercetin content in PME*

The quantification results show that PME contains the secondary metabolite quercetin (Figure 8, Table 3). PME has a total flavonoid content of 21.86 ppm or 2.73% g/g of flavonoids which is equivalent to quercetin. The data used were in the form of five serial concentrations, which then the linear regression calculation was carried out with absorbance. The linear regression obtained from quercetin standards was y = 0.0074x − 0.0324 with an R-value of 0.9999 (Figure 9). Quercetin standard calibration curve equation was used to determine the total flavonoid levels contained in PME. The absorbance value obtained from the PME was then calculated with linear regression.

Quercetin levels measurement was carried out using UV–Vis spectrophotometry at 400–600 nm (Fawwaz et al. 2017). Determination of the maximum wavelength ensures the wavelength required to produce maximum absorption. The maximum absorption will also produce maximum sensitivity and minimize errors. The results showed that the maximum wavelength of the quercetin standard was 439 nm and quercetin was used as a standard as it is a flavonoid (Azizah et al. 2014). The results also showed that the total flavonoid concentration was 21.81 ppm or 2.73% g/g of flavonoids calculated as quercetin. Stevens et al. (1994) stated that quercetin was able to inhibit the growth of WiDr cells with an IC_{50} value of 56 µg/mL.

3.5. *Discussion*

Cisplatin is a chemotherapy agent in the treatment of colon cancer. In this study, cisplatin was used as a positive control. To overcome resistance, cisplatin is commonly used with some other drugs in treating colon cancer, ovarian cancer, colorectal cancer, biliary tract cancer, lung cancer,

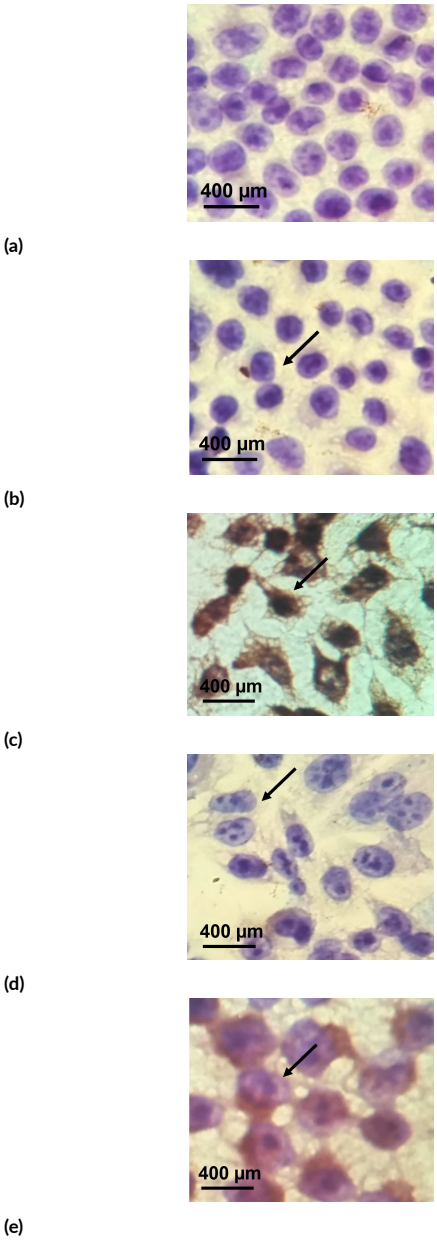


FIGURE 5 Effect of PME, cisplatin, and their combination on Bcl-2 expression in WiDr cells. A total of 5×10^4 cells were seeded on coverslips in 24-well plates and were treated for 18 h, followed by staining with an anti-Bcl2 primer antibody. (a) Cell control without primary antibody Bcl-2. (b) Cell control with vehicle treatment. (c) Cisplatin 2 µg/mL. (d) PME 198 µg/mL. (e) Combination of cisplatin 0.3 nM (1/8 IC_{50} cisplatin) and PME 173 µg/mL (7/8 IC_{50} PME). The analysis was done under a light microscope at a magnification of 400× (a, b, d) or 1,000× (c, e).

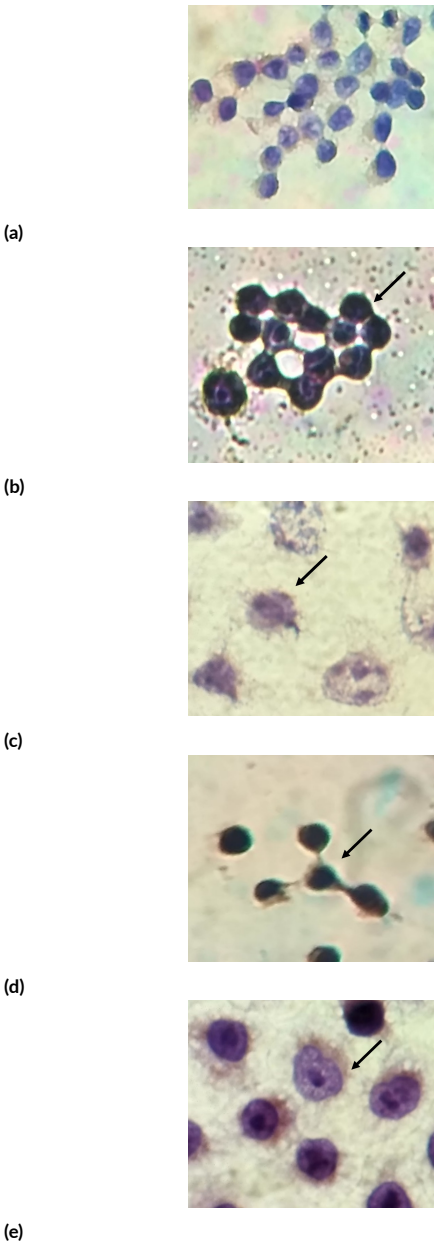


FIGURE 6 Effect of PME, cisplatin, and their combination on p53 expression on WiDr cells. A total of 5×10^4 cells were seeded on coverslips in 24-well plates and were treated for 18 h, followed by immunocytochemistry with an anti-p53 primer antibody. (a) Cell control without primary antibody. (b) Cell control with vehicle treatment. (c) treCisplatin 2 µg/mL. (d) PME 198 µg/mL. (E) Combination of cisplatin 0.3 nM (1/8 IC_{50} cisplatin) and PME 173 µg/mL (7/8 IC_{50} PME). The observation was carried out under a light microscope at a magnification of 400× (a, b, d) or 1,000× (c, e).

gastric cancer, pancreatic cancer cell lines, and urothelial bladder and cervical cancer. Cisplatin enters the cells by passive diffusion through the plasma membrane and by active transport mediated by several membrane transporters (Spreckelmeyer et al. 2014; Hall et al. 2008). Combinations of cisplatin with compounds that interfere with specific cisplatin resistance factors have been tested in various preclinical cancer models. Notably, p53 and p53-mediated DNA damage responses might be used to target biochemical modulators in colorectal cancer cells. Effects of chemotherapeutic drugs are also influenced by the efficiency of DNA repair. Exploiting DNA repair might be another strategy in cancer therapy (Herůdková et al. 2017; Rawlinson and Massey 2014; Shen et al. 2013).

Based on that, the exploration of PME and cisplatin apoptotic mechanism, either alone or in combination, were directed to inhibit Bcl-2 expression. The qualitative ob-

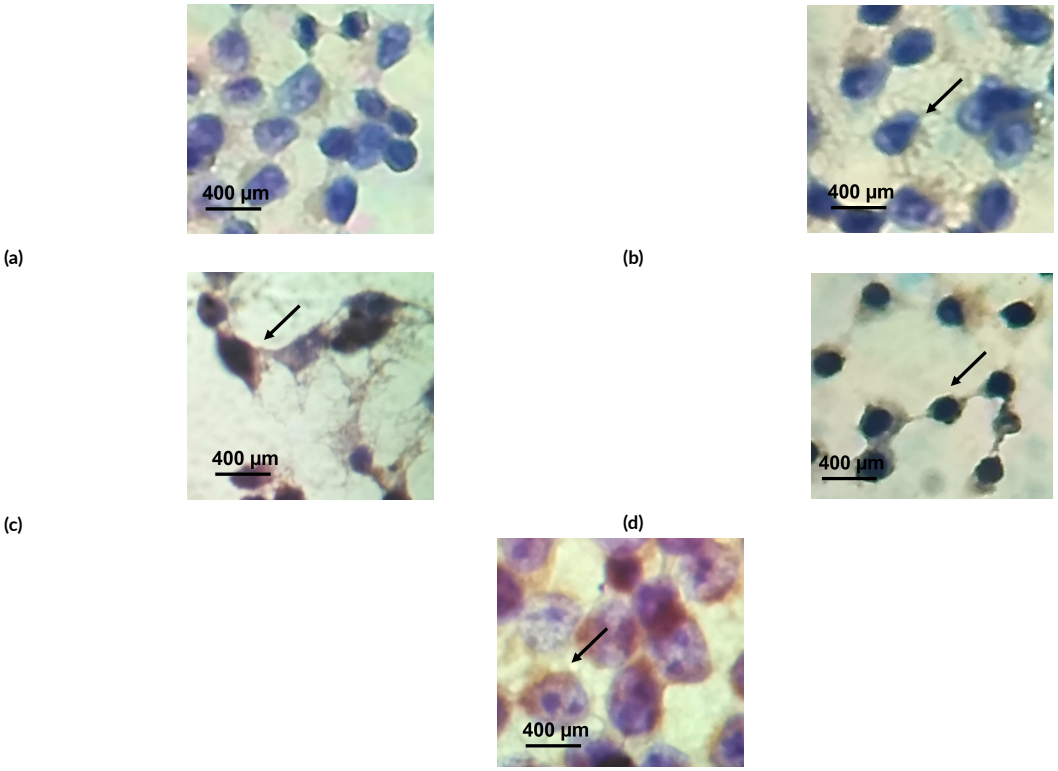


FIGURE 7 Effect of PME, cisplatin, and their combination on the caspase-9 expression on WiDr cells. A total of 5×10^4 cells were seeded on coverslips in 24-well plates, treated for 18 h, and stained by immunocytochemistry with an anti-caspase-9 primer antibody. (a) Cell control without primary antibody. (b) Cell control with vehicle treatment. (c) Cisplatin 2 µg/mL. (d) PME 198 µg/mL. (E) Combination of cisplatin 0.3 nM (1/8 IC_{50} cisplatin) and PME 173 µg/mL (7/8 IC_{50} PME). The observation was done under a light microscope at a magnification of 400× (a, b, d) or 1,000× (c, e).

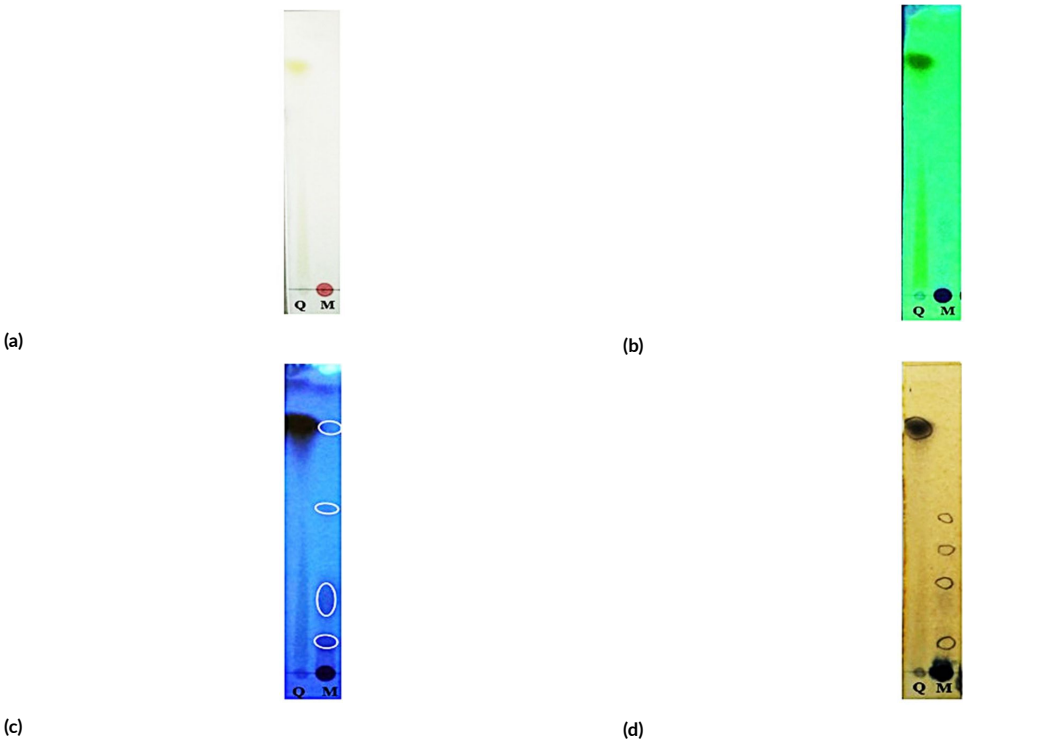


FIGURE 8 Standard TLC profile of quercetin (Q) and PME (M). Stationary phase: silica gel GF254; mobile phase: chloroform-acetone-formic acid (10:2:1). (a) Visible light. (b) UV254. (c) UV366. (d) 10% $FeCl_3$ spray reagent.

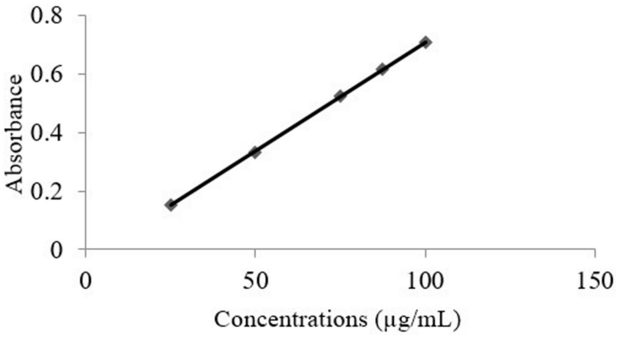


FIGURE 9 Relationship between concentration and absorbance of quercetin standards.

servation showed the decrease of Bcl-2 expression after a single treatment of 198.64 µg/mL PME, 2.34 µg/mL cisplatin, and the combination compared with control cells (Figure 3). The decrease in Bcl-2 expression following combination treatment was more than a single treatment of PME and cisplatin, which was shown by the low intensity of brown color in the cytoplasm compared with treatment with cisplatin alone. Therefore, both PME and cisplatin contributed to Bcl-2 expression in combination treatment. Furthermore, we also observed the control treatment of PME, combination of PME + cisplatin, and cisplatin against Vero cells (Figure 2). Each treatment showed changes in cell morphology. Vero cells were polygonal in shape and had higher cell density in the control cells. The morphology of Vero cells was round when compared to the treatment group.

We selected WiDr cells based on a previous study that demonstrated expression of the p53 in vitro by fermented black rice bran extract (Ashcroft and Vousden 1999). Immunocytochemistry was used to detect the p53 expression level in treated WiDr cells and control cells. The p53 protein detection was performed to confirm the apoptosis induction mechanisms by PME, cisplatin, and their combination. The p53 protein is a known potent tumor suppressor and is maintained at low levels in unstressed cells (Ashcroft and Vousden 1999). In stimulated cells, p53 is increased and acts as a transcription factor to upregulate the expression of many genes, including cell-growth-arrest and apoptosis-associated genes (Roos and Kaina 2013). The present study found that PME significantly increased p53 expression in WiDr cells. This may be due to the differences in p53 expression levels, the qualitative status of p53, and other cellular contexts, which have been suggested to influence p53 to stimulate cell cycle arrest or apoptosis (Haupt et al. 2003; Zuckerman et al. 2009).

We analyzed anticancer against proteins that oppose the proliferation of colon cancer cells, namely, p53, from the chemical composition of PME and to determine the apoptotic of colon cancer cells. p53 protein plays a crucial role in response to cellular stress, such as exposure to carcinogens. This protein inhibited the proliferation of abnormal cells to prevent the development of neoplasms. On the other hand, protein inactivity can cause malignancy until the cancer is malignant (Hu et al. 2003). Besides, p53 also regulates apoptosis, inhibits angiogenesis, and regulates DNA repairment. p53 is a mutation in cancer and can be in the form of degradation of p53, loss of the ability of p53 induces cell cycle arrest or apoptosis, and loss affinity of p53 to bind damaged DNA. These conditions increase p53 and decrease COX-2 on the cancer cell cycle. Therefore, the growth and development of colon cancer cells (WiDr) can be inhibited (Zhu et al. 2015; Zambetti et al. 1992).

The intensity of the brown color indicated the presence of p53 expression both in the cytoplasm and in the nucleus. An increased level of ROS characterizes the mechanism of cancer cell death treated with quercetin and p53 expression, decreased Bcl-2 expression, mitochondrial membrane depolarization, caspase-3 cleavage, and DNA fragmentation (Edagawa et al. 2014; Abraha and Ketema 2016; Sanchez-Gonzalez et al. 2011). New drug compounds may induce apoptosis by activating caspases, Fas, Bax, Bid, APC, or molecules that promote colon cancer cell survival (mutant p53, Bcl-2, or COX-2). Caspase proteins are used as markers of apoptosis through mitochondrial regulation (Mendelsohn et al. 2015). Activation of caspase-9 and/or caspase-8 causes caspase-3 cleavage, endonuclease activation, and nuclear DNA fragmentation, which are characteristic of apoptosis (Redza-Dutordoir and Averill-Bates 2016). The immunocytochemical tests showed that PME treatment alone or a combination with cisplatin could increase the expression of the pro-apoptotic p53 and caspase-9 proteins and suppress Bcl-2 expression. The decrease in Bcl-2 expression in the PME single treatment was probably due to the presence of quercetin in PME. Quercetin binds directly to the BH3 domain of Bcl-2 and Bcl-xL proteins, thereby inhibiting their activity and promoting cancer cell apoptosis (Primikyri et al. 2014).

The induction of apoptosis by PME is thought to involve secondary metabolites contained in PME; one of them is quercetin. Quercetin is a secondary metabolite involved in suppressing cancer cell activity, oxidative stress, proliferation, and metastasis. Another bioactive compound in PME is tannin. At the present study, we focus on quercetin, which is a pro-apoptotic compound specifically inhibits the growth of colon cancer cells compared to that of to normal cells (Sanchez-Gonzalez et al. 2011). The anti-tumor effect found on SW480 colon cancer cells is associated with inhibition of cyclin D and surviving expression, as well as the Wnt/beta-catenin signaling pathway (Mabberley 2017; Tai et al. 2014). Quercetin has anti-proliferative effects against breast, ovarian, and colon cancer cells in vitro (Neuhouser 2004). Quercetin also induced inhibition of Akt phosphorylation was coupled with a significant decrease of Bcl-2 and Bcl-XL because active or unphosphorylated BAD is known to induce apoptosis by inhibiting anti-apoptotic Bcl-2 family members allowing pro-apoptotic proteins to be aggregate and initiate cytochrome c release and subsequent caspase-9 activation (Cheng et al. 2001). Moreover, Akt is known to inhibit caspase-9 activity and inhibit the expression of

death ligands on the cells. Quercetin may exert its anticancer effect through several mechanisms, including acting as an antioxidant, inducing apoptosis, acting as an anti-inflammatory agent, and modulating signaling pathways (Inoue et al. 2004).

4. Conclusions

PME has moderate cytotoxic activity with an IC_{50} value of 198.64 ± 1.6 µg/ml. The combination of PME with cisplatin showed a strong synergistic effect with a CI value of <1. Flow cytometry test results showed that the combination of PME and cisplatin increased apoptosis induction compared to PME treatment alone. The immunocytochemical tests showed that cisplatin increased the expression of the pro-apoptosis p53 and caspase-9 proteins and suppressed Bcl-2 expression compared to PME. The quantification of total flavonoids in PME was 21.86 ppm or 2.73% g/g of flavonoids, calculated as quercetin.

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Authors' contributions

ANA, FP designed the study. ANA, FP, RKS carried out the laboratory work. ANA, FP, RKS analyzed the data. FP, RKS wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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