Synergistic Cytotoxicity Effect by Combination of Methanol Extract of Parijoto Fruit (*Medinilla speciosa* Reinw. ex. Bl) and Cisplatin against HeLa Cell Line

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Abstract

As one of the leading causes of death in worldwide, cervical cancer requires the effective therapies to reduce its mortality rate. One of the chemotherapy agents that frequently used in the treatment is cisplatin. However, due to drug resistance and its side effects, an agent that can be combined with cisplatin is needed. Parijoto fruit (*Medinilla speciosa* Reinw.ex.Bl) contains secondary metabolites compounds that have potential as anticancer. The study aims to determine the cytotoxic effect of methanol extract of Parijoto fruit calculated from the IC_{50} value and the synergicity of the combinational treatment with cisplatin evaluated from the Combination Index (CI) value and its cell viability by using MTT assay. Results showed that methanol extract of Parijoto fruit (MEP) performed cytotoxic effect on HeLa cell line with IC_{50} of 209.6 μg/mL while the value of IC_{50} of cisplatin against HeLa cells amounted to 12.8 μg/mL. The combination of 26.205 ppm (1/8 IC_{50}) of MEP and 1.601 ppm (1/8 IC_{50}) of Cisplatin performed synergistic effect on HeLa cell line with the CI value of 0.69. From the above results, it can be concluded that MEP is potential as co-chemotherapy agent based on the synergistic cytotoxicity effect with cisplatin.

Keyword: *cytotoxic, Medinilla speciosa, cisplatin, co-chemotherapy, MTT*

INTRODUCTION

Cancer is one of the leading causes of death in the world. In 2015, around 8.2 million deaths were caused by cancer, one of which was cervical cancer. According to data center and information (INFODATIN, 2015), cervical cancer has the second highest percentage of new cases experienced by women in the world by 14% (INFODATIN, 2015). Several treatments have been made to reduce mortality due to this disease, one of them is by the use of cisplatin, a chemotherapy drug. However, the use of cisplatin is limited because of drug resistance and many side effects (Sihotang, 2008). Combination therapy is the right choice to overcome the limitations of cisplatin use and natural ingredients can be used as its co-chemotherapy agents.

One of the natural ingredient has potential to be explored is Parijoto fruit (*Medinilla speciosa* Reinw.ex.Bl). It contains secondary metabolites in the form of flavonoids, alkaloids, tannins, saponins, glycosides and terpenoids (Vifta, *et al.*, 2018).

Some *in vitro* studies show that these metabolites have anticancer activity. In a previous study it was reported that the ethanolic extract of Parijoto fruit has a cytotoxic effect on T47D breast cancer cells with an IC$_{50}$ value of 614.50 μg/mL (Tussanti, *et al.*, 2014). This study will examine the cytotoxic effect of the methanol extract of Parijoto fruit (MEP) and its combination treatment with Cisplatin against cervical cancer using the HeLa cell line. The treatment will be compared with vero cell line, asa normal cell model. This combination is expected to provide a synergistic effect in increasing the sensitivity of the chemotherapy agent and reducing the toxicity arising from cisplatin. The cytotoxic effect of MEP was calculated from the IC$_{50}$ value and the synergy of its combination with cisplatin can be seen from its Combination Index (CI) value parameters.

MATERIAL AND METHODS

Sample

Parijoto fruits (*Medinilla speciosa*, Reinw. ex Bl.) were taken from the Muria Mountain Area, Colo Village, Dawe District, Kudus Regency, Central Java, Indonesia. Plants were identified at the Biology Laboratory of Faculty of Math and Science, UNS. Cisplatin (Kalbe, Jakarta, Indonesia) was obtained from the Dr. Moewardi District Hospital, Surakarta. HeLa cell and Vero cell are obtained from the collection of Cancer Chemoprevention Research Center (CCRC) Faculty of Pharmacy, Universitas Gadjah Mada.

Cell Culture

Roswell Park Memorial Institute (RPMI) 1640 (Gibco, New York, USA) media containing Foetal Bovine Serum (FBS) 10% (v/v) (Gibco), penicillin streptomycin 1% (v/v) (Gibco) was used. Trypsin-EDTA in concentration of 0.25% was used to harvest the adherent cells. Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Missouri, USA) was used to dissolve the extract. The 3-(4,5-dimethyltiazol-

2-il)-2,5 diphenyltetrazolium bromide (MTT) (Sigma) was used for MTT assay. The stopper used was sodium dodecyl sulfate (SDS) 10% (Merck, Frankfurt, Germany) in 0.01 N HCl (Merck).

Sampel Preparation

Parijoto fruit was macerated with 500 mL methanol (Brataco, Cikarang, Indonesia) for 2x24 h. It continued by re-macerated with three equal solvents of 200 mL per each for 24 h. The macerate is collected and evaporated with a rotary evaporator at a temperature of 500^{0}C andre-concentrated by heating it over a water bath until a viscous extract is obtained.

Cytotoxic MTT Assay

MEP were dissolved in DMSO and was made a series of concentrations between 30-240 μg/mL for cytotoxic tests of HeLa cells. Cytotoxic tests on vero cells were made in series of concentrations of 7.81; 15.63; 31.25; 62.5; 125; 250; 500; 1000 μg/mL. The concentration of the cisplatin test solution was made in series of concentrations between 1-20 μg/mL. HeLa cells ($10x10^{3}$/well) were cultured to 96 well-plates in RPMI media, then incubated at 37^{0}C for 24 h. Then, MEP and cisplatin were added in DMSO, incubated in a 5% CO_2 incubator for 24 h. MTT solution (0.5 mg/mL in 100 μL of culture media) was added to each well, incubated for 4 h at 37^{0}C. A 10% SDS stopper in 0.01 N HCl was added and incubated at room temperature for 24 h and protected by light. Thus absorbance measurements is conducted with enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 595 nm. The data obtained is in the form of absorbance which is converted into percent of living cells. Formula for living cells:

(sample absorbantion-medium absorbantion)/ (control absorbantion-medium absorbantion)x100%

The results of cell viability versus sample concentration are graphed linear regression equations to obtain the function equation y=bx+a

then the IC_{50} value is obtained. Based on the IC_{50} value of the single cytotoxic test, the series concentration was made as much as 1/2 IC_{50}, 1/4 IC_{50}, 1/8 IC_{50} both the MEP and cisplatin, To evaluate the synergism of a combination using equation:

$$\text{Combination Index} = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

Dx_1 and Dx_2 are concentrations of one single compound needed to exert an effect (IC_{50} on the growth of HeLa cells), D_1 and D_2 are the concentrations of the two compounds to give the same effect. The results of the CI obtained are then interpreted according to Table 1.

Table 1. Interpretations of CI

Combination Index	Interpretation
<0.1	Very strong sinergistic
0.1-0.3	Strong sinergistic
0.3-0.7	Sinergistic
0.7-0.9	Moderate sinergistic
0.9-1.1	Aditif
1.1-1.45	Moderate Antagonist
1.45-3.3	Antagonist
>3.3	Strong Antagonist

Index Selectivity Test

To examine whether the extract is safe against normal cells, the selectivity index analysis was performed using the formula:

$$\text{Selectivity index} = (IC_{50} \text{ vero cell line})/(IC_{50} \text{ cancer cell line})$$

Table 2 shows the interpretations of index selectivity.

Table 2. Interpretations of Index Selectivity

Index Selectivity	Interpretation
Selectivity index >=3	Selective
Selectivity index <3	Non Selective

Ethics Approval

This study was approved by the Research Ethics Commission of the Faculty of Medicine, Universitas Muhammadiyah Surakarta (FK UMS) proven through Ethical clearance approval number: 1814/A.1/KEPK-FKUMS/I/2019.

RESULT

Cytotoxicity Effect of MEP on HeLa Cells

MEP is obtained with a weight of 20.2 grams with red extract color and a yield of 4.05%. The cytotoxic effect test on HeLa cells is a preliminary test using the MTT assay method whose absorption results will give a purple color and the absorbance measured with the ELISA reader. The absorbance results are used to calculate the IC_{50} value, which is the ability of a compound that can cause growth inhibition in 50% of the cell population. IC_{50} values of MEP obtained in the treatment of 209.6±3.55 μg/mL. While the IC_{50} value of cisplatin in HeLa cells was 12.08±1.06 μg/mL. The percentage of viability results are presented in Figure 1.

Cytotoxicity Effect of Combination of MEP and Cisplatin on HeLa Cells

Synergistic cytotoxic effects are seen by combining MEP with cisplatin. IC_{50} values of MEP and cisplatin were used as guidelines for determining the concentrations. The concentration variations used were 1/2 IC_{50}, 1/4 IC_{50}, 1/8 IC_{50}. The results of the calculation of the CI value can be seen in Table 3. The results of the CI show the combination of MEP and cisplatin is synergistic with a combination of 26.205 ppm (1/8 IC_{50}) of MEP+1.601 ppm (1/8 IC_{50}) of cisplatin with a percentage of cell viability of 80.58 %.
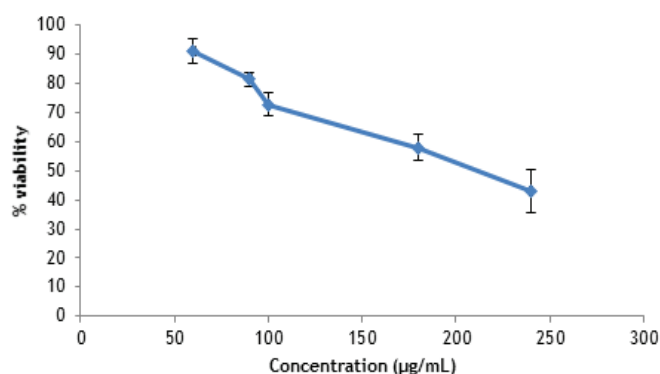
Figure 1. Cytotoxicity effect of MEP against HeLa cells. Cells were seeded in 96 well plates with a density of 10×10^3/ well and incubated for 24 h. Cells were treated with MEP in concentration series of 30 µg/mL; 60 µg/mL; 120 µg/mL; 210 µg/mL; 240 µg/mL. Cells viability were obtained from the conversion of absorbance values by MTT treatment as described in the method. Data are representative of values from three independent experiment. The value represent mean plus minus SE.

DISCUSSION

This study aims to determine the cytotoxic effect of MEP that can be seen from the IC_{50} parameters and the synergy of its combination with cisplatin against HeLa cervical cancer cells in terms of its CI value parameters. The yield analysis shows that the methanol solvent has a high percentage of yield (20.2 grams). These results indicate that the methanol solvent has a higher ability to extract the active component of the Parijoto fruit. The high percentage of yield in methanol solvents is thought to be due to the large content of polar phytochemical compounds in Parijoto fruit. Parijoto fruit has been shown to contain flavonoid compounds with groups similar to flavonol compounds (Vifta, *et al.*, 2018). These results are strengthened by qualitative TLC test data previously carried out, namely methanol extract of positive parijoto fruits containing secondary metabolite compounds of quercetin (Unpublish data, 2019). The total presence of this compound is thought to be one of the influences in cytotoxic effects of Parijoto fruit extracts.

The results of a single cytotoxic test of the MEP showed that the extracts had a moderate cytotoxic effect on HeLa cells. A compound that has an IC_{50} value of less than 100 µg/mL is a compound with a potential cytotoxic effect, a compound that has an IC_{50} value of 100-1000 µg/mL can be said to be a compound with a moderate cytotoxic effect. The compound has no cytotoxic effect if the IC_{50} value more than 1000 µg/mL (Prayong, *et al.*, 2008). The MEP has the highest cytotoxic effect on HeLa cells seen from the lowest IC_{50} parameter. The high cytotoxic effect on MEP is probably due to the fact that the methanol solvent dissolves more polar secondary metabolites such as quercetin. In a cytotoxic test the combination of synergistic activity from the combination of MEP (26.205 ppm or 1/8 IC_{50}) and cisplatin (1.601 ppm or 1/8 IC_{50}) is likely to result from the same action target or different action target in inducing cell apoptosis. The mechanism of cell apoptosis is thought to involve components of secondary metabolites contained in Parijoto fruit.

Tabel 3. Synergistic cytotoxic effects are seen by combining MEP with cisplatin

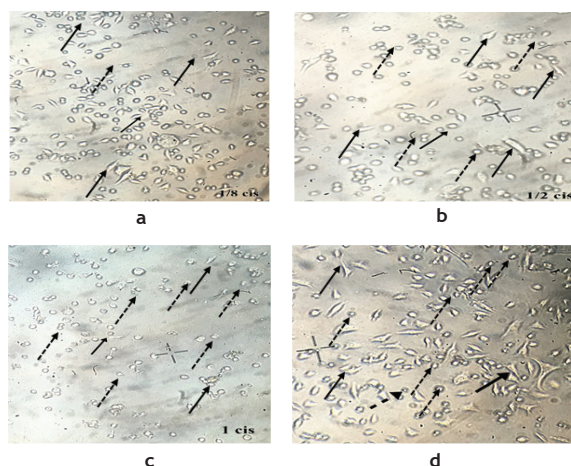| MEP (µg/mL)	Cisplatin (µM)		
	1.601	3.202	6.405
26.205	0.69	1.83	1.79
52.41	1.00	2.75	1.80
104.82	0.93	1.19	1.09

Figure 2. Effect of MEP in combination with cisplatin on HeLa cel viability. Cells with density of $10x10^3$/well were seeded on 96 well plates. Morphology and cytotoxic effects of HeLa cells after 24 h treatment. a: combination of MEP 26.205 ppm (1/8 IC_{50}) and cisplatin 1.601 ppm (1/8 IC_{50}), b: combination of MEP 104.82 µg/mL (1/2 IC_{50}) and cisplatin 6.405 µg/mL (1/2 IC_{50}), c: single cisplatin 12.81 µg/mL (1 IC_{50}), d: control or untreated cells. The black arrow (→) indicates living cells, meanwhile the doted arrow (--→) indicates dead cells.

This is reinforced by the presence of quercetin in the methanol extract of Parijoto fruit. Quercetin triggers apoptosis in cancer cells by inducing p53 protein expression and involves modulation of cell cycle regulating proteins, activating the B cell lymphoma-2 (Bcl-2) (Siegelin, *et al.*, 2008). This activation will cause inhibition of signal transduction from growth factors which will then induce cell apoptosis (Wickremasinghe, *et al.*, 1999). Quercetin can also inhibit HeLa cell proliferation through cell cycle arrest in the G2/M phase. While the mechanism of action of cisplatin via the intrinsic pathway causes damage to the DNA of cancer cells regulated by the Bcl-2 protein. This DNA damage results in the release of cytochrome C from the mitochondria, thereby activating procaspase 9 through the interaction of apoptosis promoting activating factor-1 (APAF-1) and active apoptosome formation (Dasari, *et al.*, 2014).

The value of CI in the treatment of high cisplatin concentrations tends to approach the additive effect to the antagonist this is due to the cisplatin mechanism that experiences resistance in cells. Cell resistance causes cisplatin to not damage cell DNA so that p53 protein cannot be activated. Activation of p53 protein will trigger programmed cell death (cell apoptosis). Failure to activate the p53 protein will cause an uncontrolled proliferation of cancer cells. Therefore the existence of a combination of a natural compound with chemotherapy drugs is expected to reduce resistance in cancer cells and increase the occurrence of apoptosis in the development of cancer therapy (Mutiah, 2014). Based on Table 4, MEP is categorized as having high selectivity while cisplatin as a positive control is categorized as not selective. A compound can be categorized selectively if it has a Selectivity index value ≥3 and categorized as not selective if the

Table 4. Results of Index Selectivity values.

Treatment	IC_{50} value treatment in HeLa cells (µg/mL)	IC_{50} value treatment in Vero cells (µg/mL)	Index Selectivity Values	Interpretation
MEP	209.64	52.58	25.08	Selective
Cisplatin	12.08	21.06	1.74	Non Selective

Selectivity index value less than 3. A compound that has cytotoxic activity and has a high selectivity can be developed into a chemopreventive agent because it can distinguish between cancer cells and normal cells from the needs of Adenosine Triphospat (ATP) of these cells. Cancer cells will require large ATP energy to replicate quickly and actively. Chemopreventive agents work to block energy production by attaching to the mitochondria of cancer cells.

CONCLUSION

In a single treatment, MEP has cytotoxic effect againts HeLa cell with the IC_{50} values of 209.6 μg/mL and in the combinational treatment with cisplatin, it showed theis synergistic effect with CI value of 0.69. Based on this study, MEP is potential co-chemotherapy agent based on the synergistic cytotoxicity effect with cisplatin, particularly in the cervical cancer treatment. To strengthen this conclusion, the further study should be conducted.

ACKNOWLEDMENT

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