Antioxidant Activity and Cytotoxic Potential of Parijoto
(*Medinilla speciosa* (Reinw ex BL)) Fruit Fractions on HeLa Cell Line

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Abstract

Parijoto (*Medinilla speciosa* (Reinw ex BL)) is one of the Indonesian indigenous plants widely used as traditional medicine. A previous study showed that ethanol and methanol extracts of Parijoto fruit could inhibit T47D breast cancer cells. This study explores the antioxidant and cytotoxic activities of Parijoto fruit extract and fractions on HeLa cell line. The fruits were extracted using ethanol 70% and fractionated by hexane and ethyl acetate. Furthermore, the fraction was analyzed for secondary phytochemical metabolite content using thin-layer chromatography and staining reagents. The antioxidant and cytotoxic activities were determined using the DPPH scavenging assay and the MTT assay, respectively. The ethanol extract and fraction contained flavonoid and tannin compounds. Ethanol extract, ethanol fraction, and ethyl acetate fraction of Parijoto fruit had an antioxidant activity with IC\textsubscript{50} values of 77.3, 88.64, and 46.61 μg/mL, respectively. Ethyl acetate fraction showed the highest activity on HeLa cells with an IC\textsubscript{50} value of 45.57 μg/mL compared to ethanol extract, ethanol fraction and n-hexane fraction with an IC\textsubscript{50} value of 233.43, 700.75, and 534.30 μg/mL, respectively. Based on these results, the ethyl acetate fraction of Parijoto fruit had the potency to be explored further to elucidate their cytotoxicity mechanism in HeLa cells.

Keywords: antioxidant activity, cytotoxicity, *Medinilla speciosa*, Parijoto fruit fractions.

INTRODUCTION

Cancer is the second leading cause of mortality in the world after heart disease and is responsible for 9.6 million deaths in 2018. Cervical cancer, cancer that causes the highest death rate, represents 6.6% of all cancers that attack women with 570,000 new cases and 311.00 deaths in 2018 (World Health Organization, 2018). Recently, various cancer therapies, such as chemotherapy, radiotherapy, and surgery, have been developed (Manuaba, 2010). The chemotherapy has a mechanism in inhibiting the cell as a whole, both cancer cells and normal cells. However, this treatment causes some side effects such as hepatotoxicity, cardiotoxicity, nausea and vomiting, diarrhea and anorexia (Huang, *et al*., 2017; Oun, *et al*., 2018). Therefore, the discovery of an effective and safe chemopreventive agent should be explored.

The discovery of natural and safe chemopreventive agents is still ongoing these days. For...
thousands of years, raw materials from plants have been used as medicine (Krueger, 2005). One widely used plant as medicine by some people in Indonesia is the ‘Parijoto’ fruit (*Medinilla speciosa* Reinw. ex. Bl). Parijoto fruit is one of the most widely cultivated plants in the Muria Mountains, Kudus Regency. People in there generally consume this fruit as mouth ulcer medication.

Phytochemical components in parijoto fruit include flavonoids, saponins, kardenolin, terpenoid and tannins (Ministry of Health RI, 2006; Vifta and Advistasari, 2018). These compounds could inhibit the cancer growth. Flavonoids have anticancer properties because they prevent cancer cell growth and tumour expansion (Ren, *et al*., 2003). Anti-tumor effects of saponins have been reported in several cancer cells (Man, *et al.*, 2010; Yang, *et al.*, 2018). A previous study conducted by Tussanti, *et al.* (2014) found that the parijoto methanol extract’s has cytotoxic effect on breast cancer cells (T47D). In addition, parijoto fruit has a vigorous antioxidant activity (Wachidah, 2013). However, the cytotoxic activity of parijoto fruits on cervical cancer remains unclear. Therefore in this study we explored the cytotoxic activity of Parijoto fruits on HeLa cells.

In this study, a phytochemical test was carried out to ensure the presence of flavonoids and phenolic in Parijoto fruit fractions. Furthermore, the potential of antioxidant and cytotoxic activity against HeLa cervical cancer cells will also be examined in vitro. This study can be used as a scientific basis for empirical evidence of Parijoto to be developed as chemopreventive agent.

**MATERIALS AND METHODS**

**Materials**

Parijoto fruits were collected from Kudus, Central Java, Indonesia. The fruits were identified in the Faculty of Pharmacy UAD, Indonesia, under Nr.13/Lab.Bio/B/III/2020. Extraction and fractionation solvents used in this study were ethanol, hexane, and ethyl acetate (technical grade). Materials used for phytochemical assay were acetyl acid (Merck, Darmstadt, Germany), n-butanol (Merck); NH4OH, FeCl3 (Merck); HCl (Mallinckrodt, Blanchardstown Dublin, Ireland), Dregendorf reagent, NaOH (Merck), TLC silica gel GF254 (Merck), and rutin (Sigma-Aldrich, Germany). Materials used for antioxidant activity and cytotoxic assays were 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (Merck), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Bio basic, Canada) and sodium dodecyl sulfate (SDS) (Merck) as stopper reagent. The cervical cancer cell line (HeLa) was propagated using the following growth media: RPMI 1640 (Gibco, New York, USA).

**Extraction and Fractionation Procedures**

Approximately 375 mg of dried parijoto fruit were firstly macerated with 1:6 of ethanol 70% for 5 days. Afterword, the residue was then re-macerated for 3 days. The filtrate was evaporated using a rotary evaporator at 60°C under vacuum to get a crude extract. The filtrate was then partitioned with ethyl acetate to obtain the crude extract. Furthermore, the filtrate was fractioned using hexane and ethyl acetate in a ratio of 1:1 three times. The hexane containing extract was further partitioned with ethyl acetate to produce ethyl acetate fraction and ethanolic fraction and the fractions filtrate was evaporated undevreduced pressure to obtain the crude hexane, ethyl acetate and ethanol fraction (Phang, *et al.*, 2013; Tusanti, *et al.*, 2014).

**Phytochemistry Evaluation**

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolite of parijoto fruit fraction such as flavonoid, alkaloids and tannin. Phytochemical analysis was analyzed using the TLC silica gel 60F254 plate with mobile phase, butanol: acetic acid: water (3:1:1 v/v/v). The samples were determined under UV254 and UV366 lights before and after spraying with ammonia vapor to identify fla-
vonoids (Jork, 1990). The reagent dragendrof and FeCl3 were used to identify alkaloids and tannin (Ayoola, et al., 2008; Tiwari, et al., 2011).

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging activity was analyzed according to Kaulika and Febriansah (2019). Sample of ethanol extract, hexane fraction, ethyl acetate fraction, and the ethanolic fraction was diluted to final concentrations of 60, 50, 40, 30, and 20 µg/mL on methanol. One mL of a 0.4 mM DPPH methanol solution was added to give 5 mL of sample solutions of different concentrations and homogenized using a vortex, then incubated at room temperature for 30 minutes. Absorbance was analysed at 517 nm using a spectrophotometer. DPPH solution was used as a control, methanol was used as a blank, and vitamin C was used as a positive standard (Kaulika and Febriansah, 2019). The percentage of inhibition activity was calculated as [(A0 − A1)/A0] × 100, where A0 was the absorbance of the control, and A1 was the absorbance of the extract/standard. The absorbance value was processed into percentage antioxidant to perform IC50 calculations (Phang, et al., 2013).

Cytotoxic Assay In Vitro

The samples were diluted on DMSO and were added by serial concentrations (12.5-200 µg/mL). Doxorubicin (15.25-250 ppm) was used as a positive control, untreated cells were used as negative control, and medium control. The HeLa Cell lines were transferred onto micro titer-plate in 7.5x10^3 cells/well and incubated for 24 h in a CO2 incubator. Samples were added by serial dilution (12.5-200 ppm) then incubated for 24 h in a CO2 incubator. The medium was removed, then the MTT 5 mg/mL was put in PBS solution 100 µl/well and incubated for 3-4 h in a CO2 incubator until formazan was formed. Furthermore, the 0.1% SDS was put in 0.01N HCl as a stopper and incubated for 24 h. The viability cells were analysed by ELISA reader at 595 nm. The absorption value was converted to % cell viability, and IC50 was calculated by the equation y=bx+a obtained from the linear regression between the concentration and % cell viability (Riss, et al., 2013).

Figure 1. TLC profile after ammonia vapourized. (A) Rutin standart (B) Ethyl acetate fraction (C) Ethanolic extract (D) Ethanolic fraction and (e) n-hexane fraction. TLC system: stationary phase silica gel F254, mobile phase butanol: astic acid: water (3:1:1 v/v/v).
Table 1. TLC test’s results of extract and fraction of Parijoto fruit.

| Sample          | Spot Number | Rf value | Visible Light | UV 254 nm | UV 366 nm |
|-----------------|-------------|----------|---------------|-----------|-----------|
| Routine         | 1           | 0.83     | Brownish-yellow | Brown     | Brown     |
| Ethyl Acetate Fraction | 2           | 0.92     | Brown         | Brown     | Fluorescent bluish |
| Ethyl Acetate Fraction | 3           | 0.81     | Brown         | Brown     | Blush     |
| Ethanol Extract | 4           | 0.31     | Brown         | Brown     | -         |
| Ethanol Extract | 5           | 0.91     | -             | Brown     | -         |
| Ethanol Fraction| 7           | 0.30     | Brown         | Brown     | -         |
| n-hexane fraction | 6           | 0.85     | Light brown   | -         | -         |

RESULTS

Phytochemical Analysis

The yield of Parijoto’s thick ethanol extract (*Medinilla speciosa* Reinw. ex. Bl) was 8.89% w/w, and the yield of the ethanol, ethyl acetate, n-hexane fractions was 66% w/w, 28% w/w, and 6% w/w, respectively.

Based on the identification of compounds using TLC, ethanol extract, ethyl acetate, ethanol fraction & n-hexane fraction were considered to contain flavonoids as there were brown spots after being steamed with ammonia in visible light, 254 nm UV light, and a bluish glow on 366 nm UV light (Harborne, 1987; Jork, 1990).

The results of phytochemical screening showed that the ethanol extract, ethyl acetate fraction, and ethanol fraction positively contained flavonoids and tannins. Meanwhile, Parijoto fruit extract and fraction negatively contained alkaloids.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The Parijoto fruit extract and fraction have antioxidant activity based on DPPH scavenging assay. These compounds have ability to reduce free radicals and have in IC$_{50}$ value of 46.61 µg/mL–88.64 µg/mL (Table 3). The ethyl acetate fraction has the strongest antioxidant activity compare to ethanol extract and ethanol fraction.

Cytotoxic Activity

In terms of the value, the ethanol extract, ethanol fraction, ethyl acetate fraction and n-hexane fraction obtained 233.43 µg/mL, 700.75 µg/mL, 45.57 µg/mL, and 534.30 µg/mL, respectively. According to Weerapreeyatkul, et al. (2012), the ethyl acetate fraction of parijoto fruit had activity as an anticancer agent in the strong cytotoxic category with an average in the range of 10-100 µg/mL, ethanol extract had moderate cytotoxicity with an average in the range 100-500 µg/mL, and ethanol fraction and n-hexane fraction were categorized as non-toxic compared to the positive control. Meanwhile, doxorubicin was categorized as very strong cytotoxicity with a value <10 µg/mL. Compare with the Parijoto extract and other fractions, the ethyl acetate fraction had the highest cytotoxic activity against HeLa cancer cells with a value of 45.57 µg/mL.

Table 2. Phytochemical screening.

| Secondary metabolites | Ethanol Extract | Ethyl Acetate fraction | Ethanolic Fraction | n-hexane fraction |
|-----------------------|-----------------|------------------------|--------------------|-------------------|
| Flavonoid             | +               | +                      | +                  | +                 |
| Tanin                 | +               | +                      | +                  | +                 |
| Alkaloid              | -               | -                      | -                  | -                 |
### Tested Compounds Linear Regression Equations IC$_{50}$ (µg/mL) Statement

| Tested Compounds               | Linear Regression Equations                      | IC$_{50}$ (µg/mL) | Statement  |
|-------------------------------|-------------------------------------------------|------------------|------------|
| Vitamin C                     | $y = 0.8795x + 40.932$                            | 10.31            | Very Strong|
| Ethanol Extract               | $y = 1.5761x - 71.974$                           | 77.39            | Strong     |
| Ethanol Fraction              | $y = 1.3688x - 71.333$                           | 88.64            | Strong     |
| Ethyl Acetate Fraction        | $y = 2.1739x - 51.068$                           | 46.61            | Very Strong|

Changes in cell morphology were characterized by changes in the shape of cells, which were initially identical round, oval, and taper that later became irregular after the treatment.

**DISCUSSION**

Parijoto fruits have a considerable potential to be developed into chemo-preventive agents. The results of this study showed that Parijoto fruit contained flavonoids and tannins (Table 2). Previous study also revealed that Parijoto fruit contained flavonoids, saponins, kardenolin and tannins (Octaviani, 2016; Tusanti, et al., 2014; Vifta and Advistasari, 2018). The antioxidant activity and cytotoxic effect was alike due to flavonoids and tannins in Parijoto fruit. There were large amounts of flavonoid compounds that could bind free radicals; thus, many flavonoids from extracts and fractions of Parijoto fruit could contribute hydrogen to DPPH as free radicals (Amić, et al., 2003; Es-Safi, et al., 2007).

The ethyl acetate fraction in parijoto fruit was proven to have a toxic cytotoxic activity with an IC$_{50}$ value of 45.57 µg/mL (Table 4). Compounds with an IC$_{50}$ value of 10-100 µg/mL were strong.

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![Figure 2. Percentage of HeLa living cell after sample treatment.](image-url)
cytotoxic activity (Weerapreyakul, et al., 2012). The characteristic of HeLa cells is the degradation of p53 due to the binding of the HPV E6 protein complex to cellular factor E6AP which causes proteasome-mediated degradation (Buitrago-Pérez, et al., 2009). Several study showed that ethyl acetate fraction from plants was able induced p53 wild type protein. This may cause the ethyl acetate fraction has strong cytotoxic activity against HeLa cell (Husnaa, et al., 2017). It also corresponded to the ethyl acetate fraction’s antioxidant activity with an IC₅₀ value of 46.61 µg/mL (Table 3). These IC₅₀ values were included in the group of compounds with robust antioxidant activity as they had an IC₅₀ value of <50 µg/mL (Mardawati, et al., 2008).

Based on the results of this study, the relationship between antioxidant effects and cytotoxic effects was directly proportional, where the more substantial the antioxidant activity is, the greater the cytotoxic effect will be. The ethanol extract of Parijoto fruit was proved to have a cytotoxic effect on T47D cells (Tusanti, et al., 2014), and hydrolyzable tannin had the strongest cytotoxic effect on human leukemia cells (Yang, et al., 2000).

### Table 4. Results of cytotoxic activity (IC₅₀).

| Tested Compounds          | Linear Regression Equations | IC₅₀ (µg/mL) | Statement          |
|---------------------------|----------------------------|-------------|--------------------|
| Doxorubicin               | $y = -24.304x + 91.108$    | 1.69        | Very strong cytotoxicity |
| Ethyl Acetate Faction    | $y = -61.613x + 152.2$     | 45.57       | Strong cytotoxicity |
| Ethanol Extract           | $y = -0.2126x + 99.629$    | 233.43      | Moderate cytotoxicity |
| Ethanol Fraction          | $y = -0.0798x + 105.92$    | 700.75      | Less cytotoxicity   |
| N-Hexane fraction         | $y = -0.0899x + 98.034$    | 534.30      | Less cytotoxicity   |

Figure 3. HeLa cells’ morphology with ethyl acetate fraction treatment. (A) Before being treated with a sample (B) After being treated with a sample (C) After treatment as well as incubation and addition of MTT reagent. Living cells (→) Dead cells (→→).
The antioxidant activity and the resulting cytotoxic effect may be due to the flavonoid and tannin content in Parijoto fruit. Many flavonoids and tannins compounds were dissolved in a semipolar solvent such as ethyl acetate. Components of phenolic compounds such as flavonoids, tannins, and phenols are examples of several phenolic compounds that have antioxidant activity, and it can ward off free radicals that become one of the causes of cancer (Meenakshi, et al., 2011). The cytotoxic activity indicates that flavonoid compounds can increase the inhibitory effect on cancer cells so that the ethanol extract and ethyl acetate fraction of Parijoto fruit has the potential to be developed as a chemopreventive agent in the treatment of cervical cancer. The growth of cancer cells can be inhibited in the presence of antioxidants as they have similar inhibition mechanisms at the cellular level. The flavonoid action, which has anti-cancer properties, includes inhibition of carcinogenesis’ inactivation, cell cycle inhibition, angiogenesis inhibition, cell proliferation, and apoptotic mechanisms (Abotaleb, et al., 2018; Chahar, et al., 2011). The cytotoxic activity mechanism of tannin induces apoptosis in HL-60 cells and HS-683 cells by increasing ROS production, activation of pro-capcase 3, and capcase 9 (Yang, et al., 2000; Zhang, et al., 2018).

CONCLUSION

The ethanol extract and parijoto fruit fraction contained secondary phytochemical metabolites of flavonoids and tannins. The ethyl acetate fraction of parijoto was a robust antioxidant activity with an IC\(_{50}\) value of 46.61 µg/mL, and cytotoxic activity was toxic to HeLa cervical cancer cells with an IC\(_{50}\) value of 45.57 µg/mL. Based on these results, the ethyl acetate fraction of Parijoto fruit had the potency to be explored further to elucidate their cytotoxicity mechanism in HeLa cells.

ACKNOWLEDGMENT

The author would like to thank Culture Cell Laboratory UMY for providing the cell collection and support; thus, this research was well conducted. This research was funded by Hibah Kemitraan LP3M UMY grant 2019 from Universitas Muhammadiyah Yogyakarta.

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