Evaluation of total phenolic, flavonoid contents, antioxidant and cytotoxicity activities of various parts of *Phaleria macrocarpa* (Scheff.) Boerl fruit

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**Abstract.** The objective of this study was to evaluate the total phenolic and flavonoid contents and to investigate antioxidant and cytotoxic activities of ethanol extract of pericarp, mesocarp and seeds of *Phaleria macrocarpa* (Scheff.) Boerl. The total phenolic content was determined as Gallic acid (GAE) equivalent, and flavonoid contents were determined as Quercetin (QE) equivalent. 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was used to measure the antioxidant activity. The cytotoxicity activities were tested against breast cancer cell lines T47D using WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene sulfonate) assay. The ethanol extract of pericarp, rich in flavonoid (43.88 ± 2.33), exhibited the most potent antioxidant activities to DPPH (IC₅₀: 16.01 μg/mL). However, a high amount of phenolic content (66.57 ± 2.33 mg GAE/g) was found in the ethanol extract of *P. macrocarpa* mesocarp. The ethanol extract of *P. macrocarpa* seeds exhibited high cytotoxic activities against T47D cell lines in a dose-dependent manner compared to mesocarp and pericarp extracts. These results suggested that the ethanol extract of *P. macrocarpa* seeds could be used as an anticancer against T47D cell lines.

1. Introduction

Traditional herbal remedy has been commonly used to treat of several illnesses and health promotion in the worldwide [1]. The ethnopharmacological effect has proven that traditional medicine can be used as an alternative treatment to treat cancer and other diseases. Traditional herbal medicine is easily found in nature, give less side effect, relatively safe and low cost [2,3]. Thus, traditional herbal medicine has a great interest among scientist in the developing country [3]. Herbal medicine is the natural sources of several bioactive compound including phenols, flavonoids, tannins, alkaloids, lignins and glycosides. Interestingly, flavonoids and phenolic compound contained in the plant are responsible for the antioxidants activities [4]. In cancer disease, the use of the natural product could inhibit the abnormal production of free radicals and thus reducing the progression of cancer.

*Phaleria macrocarpa* (family Thymelaceae), usually recognized as Mahkota Dewa in Indonesia, is a tropical plant which easily found in several areas of Indonesia [5]. All parts of *P. macrocarpa* including fruit, seeds, stems and leaves have been used traditionally for treat flu, heart disease, allergy, cancer, impotence, diabetes, liver and lung disease [5,6]. Several studies evaluated biological activities
of P. macrocarpa extract to prove traditional claims including anti-inflammation, anti-tumor, anti-diarrhoeal, anti-hyperglycemia, vasodilator, antioxidant, anti-viral, antibacterial and anti-fungal effect [6,7].

Several studies have been conducted regarding P. macrocarpa antioxidant activities. However, the comparative study from various parts of the plant's fruit is limited. Therefore, the current work aimed to analyze the total phenolic and flavonoid contents, and investigate antioxidant and inhibitory activities of ethanol extract of the seeds, mesocarp and pericarp of P. macrocarpa against breast cancer cell line. This study could promote the establishment of the basis for further research focusing on the development of the natural product to treat several diseases.

2. Materials and methods

2.1. Plant collection
Phaleria macrocarpa fruits were obtained from UPT. Materia Medica Batu, Malang, Indonesia during October 2019. The samples were cleaned and separated into some part (mesocarp, seeds and pericarp). Then, the samples were dried at 50°C for 2 days before the extraction.

2.2. Plant extraction
The dried seeds, mesocarps and pericarps of P. macrocarpa were grinded separately. The powdered samples were extracted three times, with 95% ethanol by the maceration method. Extraction was conducted for 3 days at room temperature in the dark place. Then, the extract was evaporated using rotary evaporator at 50°C. The obtained extract was kept at -20°C until further used.

2.3. Total Phenolic Contents (TPC)
Total phenolic content of ethanol extract of seed, mesocarps and pericarps of P. macrocarpa were measured according to the Folin-Ciocalteu methods [8]. Twenty μL of each extract was added with 100 μL of 10% (w/v) Folin–Ciocalteu reagent in a 96 well plate. After 5 min, 75 μL of sodium carbonate (75 g/L) was added and incubated at 25°C in darkness for 2 h. Then, the absorbance was measured at 765 nm compared to a blank. Gallic acid was used as a standard for the calibration curve. The phenolic contents were expressed as mg Gallic acid equivalent (mg GAE/g) of extract.

2.4. Total Flavonoid Contents (TFC)
Total flavonoid contents of ethanol extract of seed, mesocarps and pericarps of P. macrocarpa were measured according to Sasipriya and Siddhuraju [9] and some modification by Ablat et al. [2]. Fifty μL extract was added with 70 μL of distilled water and 15 μL of 5% sodium nitrite solution in a 96-well plate. Then, the samples were incubated for 5 min at 25°C followed with the addition of 15 μL of 10% (w/v) AlCl3 solution and 6 min of incubation. Then, 100 μL of 1 M sodium hydroxide solution was added and followed by the measurement of absorbance at 510 nm against the blank using microplate reader (BioTek Instruments, Inc., USA). Quercetin has used a standard for the calibration curve, and the results were expressed as mg Quercetin equivalents (mg QE/g) of extract.

2.5. DPPH radical-scavenging activity
The free radical scavenging activity of each extract was measured by DPPH radical as adopted by Marghitas et al. [10] with slight modification by Ablat et al. [2]. Forty μL of each sample with concentrations ranged from 0.05 to 2 mg/mL were added and mixed with 200 μL of DPPH in ethanol (50 μM). The mixture was incubated for 15 min in the darkness at 25°C. The absorbance level was measured at 517 nm against the blank using a microplate reader (BioTek Instruments, Inc., USA). Ascorbic acid was used as a standard, and the control was ethanol. All experiments were performed in triplicate. The inhibition activity of each extract was calculated by the following equation:

\[ Inhibition \text{ activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample or standard}}}{A_{\text{control}}} \times 100\% \]  

(1)
The calculation of the percentage of inhibition activity was used to obtain equitation \( y = mx + c \). Then, the IC50 value was calculated. Ascorbic acid was used as standard (mg/mL).

2.6. Cell culture
T47D human breast cancer cell lines were providing by Dr. Satuman, Faculty of Medicine, Brawijaya University, Indonesia. Cells were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 \( \mu \)g/mL streptomycin. T47D cells were maintained at 37\(^\circ\)C under a humidified 5% CO\(_2\).

2.7. Cell viability assay
The effect of the different part of \( P. \) macrocarpa fruit on cell viability was determined using 4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate (WST-1) assay. In brief, T47D were seeded (5 x 10\(^3\) cells/well) in 96-well culture plates (NEST Scientific USA Inc.) for 24 h. Cells were treated with ethanol extract of the different part of \( P. \) macrocarpa fruit in complete serum medium at 37\(^\circ\)C. After 24 h treatment, the cells were washed with phosphate buffer saline, and WST-1 was added to each well (5 \( \mu \)l/well). The absorbance was determined at 450 nm against the blank using a microplate reader (BioTek Instrumens, Inc., USA). The percentages of cell viability of T47D cells were calculated:

\[
\text{% cell viability} = \frac{\text{Absorbance sample} - \text{Absorbance blanko}}{\text{Absorbance control} - \text{Absorbance blanko}} \times 100
\]

2.8. Data analysis
Data are expressed as the mean ± standard error (SE). Data were analyzed using SPSS 16.0 version, Chicago, USA). Differences between extracts were analyzed by one-way analysis of variance (ANOVA) with Tukey post-test. \( P < 0.05 \) was considered statistically significant. All experiments were performed in triplicate.

3. Results

3.1. Total phenolic and flavonoid contents
The highest quantity of phenolic content was detected in the ethanol extract of \( P. \) macrocarpa mesocarp (66.57 ± 2.33 mg GAE/g). While the lowest amount of phenolic content was detected in \( P. \) macrocarpa seeds (51.18 ± 2.54 mg GAE/g) (Table 1). Largely, the phenolic content of all extracts was significantly high, which strongly correlated with the antioxidant activity. This present work also exposed that all extracts were rich in phenols but low in flavonoids. When compared TFC value between each extract, \( P. \) macrocarpa pericarp has a high flavonoid content (43.88 ± 2.33 mg QE/g).

**Table 1.** Total phenolic and flavonoid content in various part of \( P. \) macrocarpa fruit.

| Samples      | TPC (mg GAE/g) | TFC (mg QE/g) |
|--------------|----------------|---------------|
| PM. Pericarp | 52.01 ± 2.11\( ^a \) | 43.88 ± 2.33\( ^c \) |
| PM. Mesocarp | 66.57 ± 2.33\( ^b \) | 24.17 ± 0.68\( ^b \) |
| PM. Seed     | 51.18 ± 2.54\( ^a \) | 18.96 ± 0.37\( ^a \) |

Data are mean ± SE \((n = 3)\). Value with different letters in the same column indicated significant different \((P < 0.05)\). TPC: total phenolic content expressed as mg gallic acid equivalent (mg GA/g); TFC: total flavonoid content expressed as mg quercetin equivalent (mg QE/g). PM: *Phaleria macrocarpa*.

3.2. Antioxidant activity
Antioxidant activity of the seed, mesocarp and pericarps of \( P. \) macrocarpa was evaluated by DPPH free radical scavenging method. The scavenging activity was determined by inhibitory concentration
50% (IC$_{50}$) values which indicated that the concentration required to scavenge 50% DPPH radicals. The lower IC$_{50}$ indicated a high radical scavenging activity. The ethanol extract of pericarp possessed the strongest antioxidant activity to DPPH (IC$_{50}$ = 16.01 µg/mL) followed by mesocarp extract (IC$_{50}$ = 19.50 µg/mL) and seed extract (IC$_{50}$ = 80.24 µg/mL).

Table 2. Antioxidant activities of the various part of P. macrocarpa fruit

| Samples          | DPPH (IC$_{50}$ µg/mL) |
|------------------|------------------------|
| Ascorbic acid    | 11.22                  |
| PM. Pericarp     | 16.01                  |
| PM. Mesocarp     | 19.50                  |
| PM. Seed         | 80.24                  |

Data are mean ± SE (n = 3). Value with different letters in the same column indicated significant different (P < 0.05).

As noted in Table 1 and 2, P. macrocarpa pericarp contained a high level of flavonoid content and exhibited potent activity to scavenge DPPH. It was suggested that flavonoid compounds might be the main contributor against DPPH free radicals in P. macrocarpa pericarp. However, the phenolic content may be responsible as a contributor against DPPH in the mesocarp extract. Although the antioxidant activity of the extracts was lower than ascorbic acid as standard, it was evident that the pericarp and mesocarp of P. macrocarpa could serve as scavengers of free radicals.

3.3. T47D cells viability

The results showed the ethanol extract of P. macrocarpa seeds extract exhibited good inhibitory activity in a dose-dependent manner (Figure 1, Table 3). At a low concentration (50 µg/ml), the pericarp extract could decrease the T47D cell viability. However, the increasing concentration of pericarp extract from 100-500 µg/ml leads to an increase in cell viability. It was indicated that pericarp extract has a specific concentration to decrease T47D cell viability.

![Figure 1. The viability cells of T47D after treatment with various part of P. macrocarpa fruit.](image-url)
As indicated before that *P. macrocarpa* pericarp has a high antioxidant activity and might be expected to have good inhibitory activity against T47D. However, this study revealed that the ethanol extract of *P. macrocarpa* pericarp at a high concentration leads to an increase in the viability cell of T47D cell lines. Therefore, it was indicated that the high antioxidant level in pericarp extract was not able to inhibit the growth of T47D cells.

| Table 3. The viability of the T47D cell line after treatment with various part of *P. macrocarpa* fruit |
|-------------------------------------------------|
| Various part of *P. macrocarpa* | Concentration (µg/ml) | % Viability cell (mean ± SD) |
|---------------------------------|------------------------|------------------------------|
| Pericarp                        | 0                      | 100.0 ± 0.03                 |
|                                 | 50                     | 38.66 ± 0.02                 |
|                                 | 100                    | 60.77 ± 0.05                 |
|                                 | 125                    | 58.00 ± 0.05                 |
|                                 | 250                    | 48.08 ± 0.02                 |
|                                 | 500                    | 55.29 ± 0.11                 |
| Mesocarp                        | 0                      | 100.0 ± 0.03                 |
|                                 | 50                     | 52.65 ± 0.01                 |
|                                 | 100                    | 52.58 ± 0.01                 |
|                                 | 125                    | 52.02 ± 0.00                 |
|                                 | 250                    | 52.37 ± 0.02                 |
|                                 | 500                    | 44.36 ± 0.02                 |
| Seeds                           | 0                      | 100.0 ± 0.03                 |
|                                 | 50                     | 51.23 ± 0.04                 |
|                                 | 100                    | 49.12 ± 0.05                 |
|                                 | 125                    | 49.26 ± 0.04                 |
|                                 | 250                    | 47.68 ± 0.03                 |
|                                 | 500                    | 44.85 ± 0.07                 |

4. Discussion

The secondary metabolites such as flavonoid and the other phenolic compounds are naturally occurring compounds from plants, which both compounds have been stated on their optimal antioxidant, antibacterial, anti-inflammation, anticancer and other pharmacological application [11]. Phenolics and flavonoids also are the most abundant phytochemical compounds with antioxidant properties from plants [12]. Several studies have been conducted to quantify the phenolic and flavonoid content of *P. macrocarpa* fruit. However, the correlation between total phenolic and flavonoid contents with antioxidant and inhibitory activities of this plant is still limited.

This study found that the total phenolic contents were greater in the ethanol extract of mesocarp than the seed and pericarp extracts of *P. macrocarpa*. However, the total flavonoid content was highest in the *P. macrocarpa* pericarp. The higher content of flavonoids in *P. macrocarpa* pericarp was strongly correlated with their antioxidant activity, which possessed the most potent scavenging activity against DPPH. The previous study revealed that the methanol extract of *P. macrocarpa* dried fruit was rich in total flavonoids [13]. The major flavonoids compound including naringin, myricetin, kaempferol, and rutin were found in the pericarp while naringin and quercetin were present in mesocarp and seed. The antioxidant activity of *P. macrocarpa* fruit mainly caused by the existence of phenolic and flavonoid compounds [14]. Another extract of *P. macrocarpa* fruit such as butanol extract and ethyl acetate fraction also possessed a best antioxidant activity [15,16].

It has been demonstrated that *P. macrocarpa* fruits exhibited cytotoxic activity on several human cancer cell line. In our study, T47D cells was treated with various part of *P. macrocarpa* fruit extract to determine which the potent inhibitory agent against T47D. The ethanol extract of *P. macrocarpa*
seeds was effective to decrease T47D cell viability in a dose-dependent manner than pericarp and mesocarp. This study also showed that pericarp extract at a specific concentration (50 µg/ml) reduced the cell viability of 38.66 ± 0.02 (Table 3). However, a high dose of pericarp extract could not reduce T47D cell viability. It was indicated that the highest antioxidant levels of *P. macrocarpa* pericarp could not inhibit T47D proliferation.

Furthermore, this research is in line with Hendra et al. [14], that the seed of *P. macrocarpa* exhibited a potential anticancer against MCF-7. The cytotoxicity effect of *P. macrocarpa* seed was higher than mesocarp and pericarp with IC₅₀ of 25.5±1.74, 26.2±2.01, and 25.5±1.37 µg/ml, respectively. Another research by Lay et al. [15] reported that the methanol extract *P. macrocarpa* fruits have a good cytotoxicity activity against breast cancer cell line such as MDA cell line (IC₅₀: 20.3±3.71 µg/ml at 24 h). Other fraction of the *P. macrocarpa* seed also reported that the methanol, chloroform and ethyl acetate extract of *P. macrocarpa* seed exhibited good cytotoxic effect against MCF-7 cells and Ca Ski cell at 24, 48 and 72 h. However, the hexane fraction of *P. macrocarpa* seed has a moderate cytotoxic effect [17]. Additional studies are needed to identify the active antioxidant and anticancer compound in *P. macrocarpa* seeds extract.

5. Conclusion
In summary, these results suggested that the ethanol extract of *P. macrocarpa* pericarp could be considered as an anticancer agent against T47D cell lines. More methodological work is required to evaluate the anticancer effect of *P. macrocarpa* seeds in the vivo experiment.

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