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The Extraction and Activity Test of Bioactive Compounds in *Phaleria macrocarpa* as Antioxidants

David Andrean, Susiana Prasetyo, Anastasia Prima Kristijarti, Tedi Hudaya*

Graduate Programs in Chemical Engineering, Parahyangan Catholic University, Merdeka 30, Bandung 40117, Indonesia

Abstract

Phaleria macrocarpa, one of native Indonesian plants, contains various bioactive compounds and thus possesses antioxidant, antimicrobial, or even anti cancer attributes. This study is the first stage of ongoing 3 years project to isolate and quantify the antioxidant content in the fruit of *Phaleria macrocarpa*, which possessed higher antioxidant activity than berry fruits, especially for natural food preservative application. In particular, the optimum condition for the extraction process using methanol was the main purpose of this study. The raw material pretreatment (drying) method prior to extraction was also investigated. The bioactivity of the extract obtained from each method was then compared to commercial dried *P. macrocarpa* by phytochemical screening methods. Tray and air drying methods resulted in the most complete phytochemical content followed by sun drying, in which the alkaloid were lost. Commercial dried *P.macrocarpa* showed similar phytochemical contents with sun drying specimen, but with additional lost of saponin compounds. The antioxidant contents were measured as DPPH equivalencies which showed tray drying method had the best antioxidant activity of 0.200 μmol DPPH/mg (dry basis), followed by air drying and sun drying of 0.117 and 0.037 μmol DPPH/mg (dry basis), respectively. Two important extraction process parameters were optimized using central composite experimental design by varying operation temperatures from 35 to 50 ^{o}C and F:S (g/mL) ratio from 1:20 to 1:50. The response surface analysis showed a maximum response point (F:S=0.02 and T= 50 ^{o}C), at which the highest antioxidant content (yield) of 0.3234 μmol DPPH/mg dry basis could be achieved.

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Keywords: *Phaleria macrocarpa, extraction, phytochemical, DPPH, antioxidant*

* Corresponding author. Tel.: +62-22-2032700
E-mail address: t_hudaya@yahoo.com.au

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1. Introduction

Phaleria macrocarpa, one of many native Indonesian plants, possess various bioactive compounds related to antioxidant, antimicrobial, or even anti cancer properties. *Phaleria macrocarpa* plants are very suitable to be cultivated in the tropics at an altitude of 10-1000 meters above sea level. The plant is relatively small with about 1.5 to 3 meters and can be grown throughout the year[1]. The *P.macrocarpa* fruit availability in Indonesia was abundant enough, with an annual production reached 11.236 tons in 2012[2]. The fruit of *Phaleria macrocarpa* is empirically believed as a potent medicine to treat some diseases such as high blood pressure, diabetes, gout and so forth. Moreover, various investigations[3-5] reported that secondary metabolites of this plant such as tannin, saponin, phenolic compounds, flavonoid, terpenoid and alkaloid play a major role as antioxidants, anti-inflammatory, antimicrobial agents and also have cytotoxic activity. In addition, *Phaleria macrocarpa* fruit extract possessed higher antioxidant activity than berry fruits. Hendra et al.[6] reported that EC_{50} concentration of pericarp extract of *Phaleria macrocarpa* fruit was 0.142 mg/mL compared to 0.42, 0.44, and 0.81 mg/mL for blueberry, blackberry, and strawberry fruit extract[7], respectively. Therefore, the extract of the fruit offers a great potential application in pharmaceutical areas. However, based on clinical evidence, the fruit extract so far showed only antihistamine and antialergic attributes[8]. In particular, the optimum conditions (F:S and temperature) for the extraction process of *P.macrocarpa* fruit using methanol is the main purpose of this study. The drying methods as raw material pretreatment is also established and chosen based on phytochemical screening and the antioxidant yield of the extract.

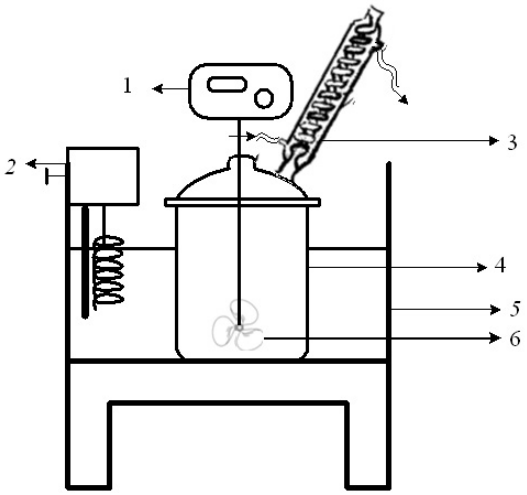
2. Materials and Methods

2.1. Materials

Fresh *P.macrocarpa* fruit was bought from traditional plantations in Subang, Indonesia. The original moisture content of fresh fruits ranged between 78-80%. After slicing and seeds separation, the fruits were dried with three different methods namely air drying at room temperature, tray drying at 40 ºC, and sun drying until its moisture content below 10% (wet basis). The dried *P.macrocarpa* were then grinded prior to extraction. Commercial dried *P.macrocarpa* was bought in a traditional drug store in Bandung, Indonesia for phytochemical activity comparison. Methanol (99% in purity), as the extraction solvent, was provided by Brataco Chemicals (Bandung, Indonesia).

2.2. Extractor

Experiments were conducted in a 1-L laboratory scale extractor (Figure 1). The extractor was equipped with an agitator to provide intense mixing during extraction process, a condenser for recondensing solvent vapours due to elevated temperature, and a water bath equipped with a thermostat to ensure constant temperature. Extraction was carried out for 360 min, and during the process samples (5 mL each) were withdrawn then evaporated at 45ºC for yield analyses purpose. After the extraction process, the mixture was separated sequentially from residual solid by filtration using Whatman 41 filter paper, followed by centrifugation at 6000 RPM for 15 min and filtration using Whatman 42. The solid free mixture was then evaporated using Rotary Vacuum Evaporator (Buchii, Switzerland) at 50 ºC with gradual depressurization from 30 kPa to 7 kPa.



1	Agitator
2	Thermsostat
3	Condensor
4	Extractor
5	Water bath
6	Impeller

Fig. 1. Schematic diagram of laboratory scale extractor

2.3. Antioxidant analysis

DPPH (*1,1-Diphenyl-2-picryl hydrazyl*) assay method was used to determine antioxidant activity. DPPH (Sigma Aldrich) stable free radical reacted with antioxidant specimens to give colour changes from purple to pale yellow due to the formation of more stable radical specimen[9, 10]. DPPH analysis was conducted to 100 ppm extract concentration using 0.1 mM DPPH which were prepared using HPLC grade methanol (Merck). The extract to DPPH volume ratio was = 1:1[9] with total volume of 5 mL[11]. After 4 hours incubation period, the absorbance of each sample was measured by LW scientific UV-Vis Spectrophotometer (model UV-200 RS) at 515 nm[12-14]. DPPH assay results were presented in DPPH equivalencies (µmol DPPH/mg) calculated by determining moles of DPPH that reacted with plant extracts, then divided by mass of reacted extracts or mass of dry matter which represented antioxidant activities and antioxidant yield, respectively

2.4. Qualitative phytochemical screening

The following phytochemical screenings were performed with triplicates for each extract to determine the presence of particular bioactive compounds.

a) Detection of Saponin (foam test)[15]

50 mg extracts were diluted with distilled water up to 20 mL. The suspension was then shaken in a test tube for 15 min. Formation a two cm layer of foam indicated the presence of saponin.

b) *Detection of Flavonoid (alkaline reagent test)*[16]

Extracts were treated with few drops of sodium hydroxide (2M). The formation of intense yellow-brownish colour, which became colourless on the addition of hydrochloric acid (2M) indicates the presence of flavonoid.

c) *Detection of Phenolic Compounds and Tannin (ferric chloride test)*[17]

50 mg extracts was dissolved in 5 mL distilled water. Into this solution, a few drops of 5% ferric chloride solution was added. The formation of dark green colour indicated the presence of phenolic compounds.

d) *Detection of Phytosterol (Libermann-Burchard's test)*[15, 17]

50 mg extracts was dissolved in 2 mL of acetic anhydride. Afterwards, a few drops of concentrated sulphuric acid were added slowly along the test tubes. An array of colour changes indicated the presence of phytosterol.

e) *Detection of Alkaloid (Mayer's test)*[15-17]

The solvent-free extract (50 mg) was dissolved in hydrochloric acid (2M). To a few mL of this solution, a few drops of Mayer's reagent were added by the side of test tube. A white creamy precipitates indicated the presence of alkaloid compounds. Mayer reagent's was made by dissolving mercuric chloride (1.358 g) in 60 mL distilled water and 5.0 grams of potassium iodide was dissolved in 10 mL of distilled water. These two solutions were mixed and made up to 100 mL with distilled water.

2.5. Experimental design

Two important extraction process parameters were optimized using Central Composite experimental design with 5 center points by varying operation temperatures from 35 to 50 ^{o}C and F:S ratio from 0.02 to 0.05 g/mL. Prior to optimization process, the comparison of extracts quality was conducted for various drying methods (sun drying, air drying and tray drying at 40 ºC) in order to choose the best drying method which gave the most of active extract.

3. Results and discussion

Various drying methods were investigated to find out the most suitable method based on the quality of the extract produced. Experimental results showed that tray drying and sun drying method could result in final moisture contents below 10 wt % (wet basis) within 8 h and 6 days drying time, respectively. On the other hand, air drying method could not provide final moisture contents below 10% (wet basis) after 2 weeks drying due to humid local weather conditions. The moisture content was neccessarily reduced below 10 wt%, so that the microorganisms and enzymatic activity could not function due to low water content, and hence the dried fruits could be stored for extended periods of time[18].

The extractions of dried *P.macrocarpa* from various drying methods were carried out with ratio F:S=1:40 (g/mL) at room temperature until it reached the equilibrium. The mixture with 500 mL solvent was stirred using a 72 Watt agitator at 400 rpm. There was an obvious colour change in the solution from transparent to light yellow. The increasing yellow colour intensity showed the increasing concentration of bioactive compounds extracted in solution. Subsequently, the mixture was filtered and dried using a vacuumed Rotary Vacuum Evaporator at 50 ºC with gradual depressurization from 30 kPa to 7 kPa until red gummy and sticky extracts formed. The phytochemical activity tests of each extract were conducted, and then compared to commercial dried *P. macrocarpa*.

Table 1. Phytochemical screening results

Phytochemical	Tray Drying	Air drying	Sun drying	Commercial (dried)
Phenolic	+	+	+	+
Flavonoid	+	+	+	+
Saponin	+	+	+	-
Alkaloid	+	+	-	-
Phytosterol	+	+	+	+
Tannin	+	+	+	+

(+) = presence of phytochemical constituents

(-) = absence of phytochemical constituents

Phytochemical components in the extracts such as phenolic, flavonoid, saponin, alkaloid, phytosterol and tannin were determined (Table 1). The phytochemical screening experiments showed that tray drying and air drying

methods provide the most complete phytochemical content followed by sun drying, in which the alkaloid were lost. Commercial dried *P.macrocarpa* showed similar phytochemical contents with sun drying specimen, but with additional lost of saponin compounds.

Saponin, a hydrophilic species[19], was not found in the commercial dried samples probably because sliced fruits were usually soaked in water prior to the separation of seeds. In addition, Lydon et al.[20] showed that UV irradiation could cause alkaloid compounds to be degraded.

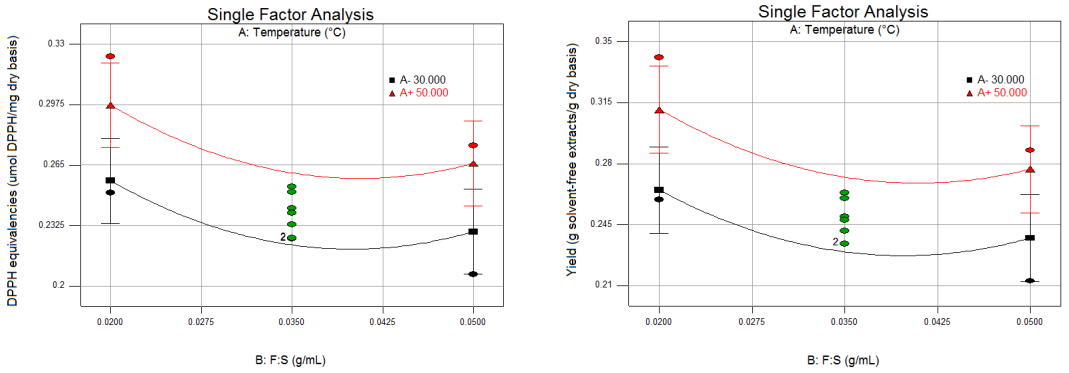
These phytochemical screening results were also reflected by DPPH equivalencies measurement which showed tray drying method resulted the best antioxidant yield of 0.200 µmol DPPH/mg (dry basis), followed by air drying and sun drying of 0.117 and 0.037 µmol DPPH/mg (dry basis), respectively. Atlabachew et al.[21] reported that prolonged drying time could deteriorate alkaloid contents and thus lowered the antioxidant content. Consequently, the air dried fruit contained significantly lower antioxidant content. Meanwhile, the combination of UV and prolonged drying time in the sun drying was detrimental.

Table 2. Experimental Results and Central Composite Design (5 center points) with Results

Run	F:S (g/mL)	T (°C)	Oleoresin Yield (g solvent-free extract/g dry basis)	DPPH equivalencies (µmol DPPH /mg dry basis)
1	0.035	40	0.2415	0.2331
2	0.035	40	0.2477	0.2392
3	0.05	30	0.2127	0.2062
4	0.02	30	0.2594	0.2502
5	0.014	40	0.2929	0.2843
6	0.035	25.9	0.2495	0.2417
7	0.035	40	0.2341	0.2254
8	0.035	54.1	0.2601	0.2504
9	0.035	40	0.2341	0.2257
10	0.05	50	0.2876	0.2754
11	0.035	40	0.2633	0.2535
12	0.056	40	0.2764	0.2663
13	0.02	50	0.3409	0.3234

In order to compare the extract's activity between different drying treatments, DPPH equivalencies values could be converted to another basis such µmol DPPH/mg solvent free extract. Even though tray drying method could give the best antioxidant yield, this method resulted in antioxidant activity which was not satistically (using LSD method) different compared to sun drying. However, tray drying method was significantly different compared with air drying. The antioxidant activity values of 0.969 µmol DPPH/mg solvent-free extract was measured for tray drying, while air drying and sun drying resulted in 0.958 and 0.964 µmol DPPH/mg solvent-free extracts respectively. For comparison, Huang et al.[7] reported EC_{50} value (the effective concentration of sample to scavenge 50% DPPH) for blueberry, blackberry and strawberry of 0.42, 0.44, and 0.81 mg/mL (equivalent to 0.362, 0.346 and 0.188 µmol DPPH/mg solvent-free extract), respectively. Therefore, the berries showed lower antioxidant activity compared with *P.macrocarpa* fruit extract.

Although the extract's antioxidant activity were comparable, the sun drying specimen lost around 80% of its antioxidant content. Since the end results of this study were meant to be applied in small and medium scale industries, the air drying would be the better option because both capital and energy cost could be minimized. This method also produced relatively high yield with the comparable antioxidant activity of the extract in comparison with tray drying. However, if sufficient capital was available the combination between air and tray drying method would overcome the drawbacks of the former method.

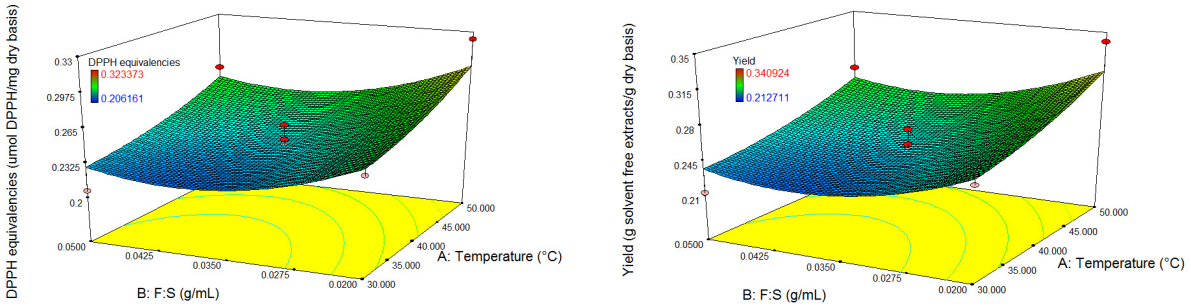


(a) (b)

Fig 2. Single factor analysis for DPPH equivalencies and (a) and yield (b)

P. macrocarpa fruits for the following experiments were dried using tray drying method due to much shorter drying time and the best antioxidant yield. Extraction experiments were conducted based on central composite experimental design (with 5 center points) for temperature and F:S ratio factors. Table 2 showed the design and results of those experiments.

Response surface methods and single factor analysis were conducted based on data from Table 2. Single factor analysis (Fig 2) was utilized to identify the significance of each process variable, F:S ratio and temperature, on oleoresin yield and DPPH equivalencies. Both F:S ratio and temperature gave significant effect (p-value <0.1) on DPPH equivalencies, but yield was only affected by extraction temperature (ANOVA not shown). At small F:S ratio, the increasing temperature have significant effect on both responses, while at higher F:S ratio the effect of temperature was not significant. Figure 2 indicated there were no interaction between F:S ratio and temperature on both responses. Both of process parameters provided the same trend on the responses. Along with F:S ratio reduction and temperature increment, better yield and DPPH equivalencies were acquired.



(a) (b)

Fig 3. 3D Surface plot for DPPH equivalencies (a) and yield (b)

Both 3D surface plot (Fig 3) showed nonlinear response surface with maximum response point at F:S=0.02 (1:50) and T=50 ^{o}C. In addition, there might be a minimum stationary point slightly beyond the T axis below 30 ^{o}C. Multilinear regression using a standard quadratic model followed by differentiation predicted the minimum stationary point for DPPH equivalencies of 0.2181 µmol DPPH/mg dry basis at T=24.3 ºC and F:S=0.04 (g/mL).

Similarly, the minimum yield of 0.2251 g solvent-free extracts/g dry basis at T=24.4 ºC and F:S=0.04 was also found. Additional run was conducted at the maximum response point and consistent results were obtained. Therefore, the best condition within the experimental boundaries were F:S=1:50 (g/mL) and T=50 ^{o}C, at which oleoresin yield of 0.3409 g solvent-free extracts/g dry basis and antioxidant yield of 0.3234 μmol DPPH/mg dry basis could be obtained.

4. Conclusion

Tray and air drying methods resulted in the most complete phytochemical content followed by sun drying, in which the alkaloid were lost. Meanwhile, commercial dried *P.macrocarpa* showed similar phytochemical contents with sun drying specimen, but with additional lost of saponin compounds. These results were also reflected by DPPH equivalencies which showed tray drying method had the best antioxidant yield of 0.200 μmol DPPH/mg (dry basis), followed by air drying and sun drying of 0.117 and 0.037 μmol DPPH/mg (dry basis), respectively. Although tray drying method could give the best antioxidant yield, tray drying method provided DPPH equivalencies (based on antioxidant activity) which is not significantly different statistically compared to sun drying, but significantly different with air drying method. The response surface analysis showed a maximum response point (F:S=0.02 and T= 50 ^{o}C), at which the highest antioxidant content (yield) of 0.3234 μmol DPPH/mg dry basis could be achieved.

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