ALKIMIA : Jurnal Ilmu Kimia dan Terapan

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Effect of Temperature on Antioxidant Activity of *Phaleria macrocarpa* Fruits Via Green Hydrothermal Extraction

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Abstract. *Phaleria macrocarpa* (God's Crown) is a medicinal plant rich in bioactive compounds, including minerals, vitamins, alkaloids (such as vincristine), and flavonoids. These compounds have shown potential in treating various ailments. This study aimed to optimize the extraction of bioactive compounds, particularly flavonoids and phenols, from *P. macrocarpa* fruit. Various extraction methods were employed, including hydrothermal extraction at different temperatures and decoction. The resulting extracts were analysed for total flavonoid and phenol content and antioxidant activity. Colorimetric methods, using $AlCl_3$ and Folin-Ciocalteu reagents, were used to determine the respective contents. Antioxidant activity was assessed using the DPPH assay. Hydrothermal extraction at 175°C provided the highest levels of flavonoids (4.04 mg QE/1% extract) total phenols (30.12 mg GAE/1% extract) and antioxidant activity (77.73%). However, further increases in temperature led to a decline in these parameters, indicating thermal degradation of heat-sensitive bioactive compounds.

Keywords : Antioxidant activity, phenols, flavonoids, *phaleria macrocarpa*, extraction

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Introduction

Phaleria macrocarpa is a plant with promising health benefits. It exhibits antioxidant properties, protecting cells from damage, and may also have anti-inflammatory and immune-boosting effects [1], [2]. The potent bioactivity shown by *P. macrocarpa* is due to abundant secondary metabolites, notably flavonoids and phenols. These compounds have been well-documented for their potent antioxidant properties, enabling them to neutralize free radicals and protect cells from oxidative damage [3]. Recent analyses have confirmed exceptionally high levels of these compounds in *P. macrocarpa* fruit. For example, one study found that oven-dried *P. macrocarpa* fruits contained approximately 55.4 mg gallic acid equivalent (GAE)/g total phenolics and 15.5 mg quercetin equivalent (QE)/g flavonoids, yielding about 84.5% inhibition in a DPPH antioxidant assay [4]. Based on Pratiwi's research (2023), the flavonoid extract from *P. macrocarpa* fruit has shown significant potential in reducing IL-17A levels, comparable to the commonly used endometriosis drug, dienogest [5]. Likewise, aqueous soxhlet extracts of the fruit were reported to have very high flavonoid content (89.9 mg QE per 100 mL extract) [6]. In the context of phenolic compounds, the extraction method significantly influences their yield. These findings indicate that *P. macrocarpa* fruit is a potent source of natural antioxidants (phenolic and flavonoid compounds) that can scavenge free radicals and support cellular health.

The use of organic solvents in extraction, while effective in isolating various compounds, is frequently associated with toxicity and environmental hazards. Organic solvents like methanol, ethanol, and hexane can be detrimental to human health and pollute the environment if not handled appropriately. On the other hand, water provides a safer and more sustainable option for extraction. Water-based extraction methods significantly reduce contamination risks and are simpler to manage [7], [8], [9]. In particular, hydrothermal (subcritical water) extraction has several significant advantages over other extraction methods. One of its primary advantages is its ability to extract bioactive compounds at high temperatures and pressures, which can increase extraction efficiency, accelerate the process and solubilize a wide range of phytochemicals [10], [11], [12]. Additionally, this method is often used due to water's ability to have selective polarity, allowing water to act as a polar or non-polar solvent depending on the applied conditions. Under certain conditions, water can behave similarly to ethanol or methanol and can extract various phytochemicals from plant biomass, including medicinal plants [13], [14], [15]. Indeed, multiple studies report that subcritical water extraction yields higher total phenolic and flavonoid contents than conventional methods [12]. Such green extraction approaches improve efficiency and selectivity while avoiding toxic solvent use.

To date, P. macrocarpa fruit extracts have largely been prepared with organic solvents or fixed drying conditions, and no studies have systematically optimized hydrothermal extraction conditions for this material [4], [6]. In particular, the effect of extraction temperature on the yield of antioxidant phenolics and flavonoids from *P. macrocarpa* fruit has not been examined. Optimizing this parameter is scientifically important, because identifying the ideal hydrothermal temperature will maximize recovery of valuable antioxidants while minimizing degradation [12]. This study aims to optimize the extraction of flavonoids and phenols from *P. macrocarpa* fruit.

Experimental

Materials and Equipment. The materials used in this study included powdered P. mcarocarpa fruits obtained from Balai Materia Medica, Batu. The plant material was authenticated at the UPT Lab. Herbal BMM, Batu. The chemical used included ethanol (Merck), distilled water, concentrated hydrochloride acid (HCl, 37%, Merck), Magnesium powder (Merck), NaOH (Merck), Folin-Ciocalteu reagent, AlCl3 (Merck), CH3COONa (Merck), 1,1-diphenyl-2-picrylhydrazyl (DPPH, Smart Lab), gallic acid standard (Sigma), and quercetin standard (Sigma).

The equipment used included an analytical balance (US Solid Precision Balance), hydrothermal reactor (stainless-steel autoclave using inner teflon 100 mL capacity), whatman No. 1 filter paper, a set of laboratory glassware. Quantitative analysis was performed using a UV-Vis Spectrophotometer (Shimadzu UV-T70).

Hydrothermal Extraction. Hydrothermal extraction was carried out following an adaptation of previous studies on subcritical water extraction of

medical plants [10], [15], with modifications to accommodate P.macrocarpa samples. This method has been widely applied for plant-based antioxidant extraction, although this is the first systematic optimization for P. macrocarpa using this technique.

Four grams of powdered plant were mixed with 60 mL of distilled water in a hydrothermal reactor. The mixture was heated at temperatures of 150°C, 175°C, and 200°C for 30 minutes. After cooling to room temperature, the resulting extracts were filtered through whatman No. 1 filter paper and stored at 4 °C for subsequent analysis.

Decoction Method. A decoction method was employed for extraction, using distilled water. Four grams of powdered P. macrocarpa were boiled in 60 mL of distilled water at 100 °C in a glass beaker on hotplate for 30 minutes. The mixture was cooled and filtered using Whatman No. 1 filter paper.

Phytochemical Assay

Flavonoid. A one-millilitre aliquot of P. macrocarpa extract was introduced into a test tube. Thereafter, three to four drops of concentrated hydrochloric acid and one gram of magnesium powder were added to the aliquot. The formation of a reddish-black, yellow, or orange coloration indicates a positive outcome for the presence of flavonoid compounds [16].

Phenols. One millilitre of P. macrocarpa extract was transferred to a test tube. Subsequently, three to four drops of ferric chloride were added to the extract. The development of a dark blue or black hue signifies a positive result for the presence of phenolic compounds [17].

Determination of Total Flavonoid Content. This method was adapted from Sembiring et al. (2017). A stock solution of quercetin with a concentration of 100 ppm was prepared and subsequently diluted to generate a series of standard solutions. Each standard solution was treated with aluminium chloride and sodium acetate, followed by a 30-minute incubation period. The absorbance of each solution was measured spectrophotometrically at a wavelength of 425 nm. A calibration curve was constructed to correlate the concentration of quercetin with the corresponding absorbance values.

A 1% solution of P. macrocarpa extract was prepared and subjected to the same experimental protocol. The total flavonoid content of the extract was quantified by comparing its absorbance to the established calibration curve [18].

Determination of Total Phenol Content. A series of standard solutions containing gallic acid were prepared. Each standard solution was reacted with Folin-Ciocalteu reagent, followed by the addition of sodium hydroxide. After a 30-minute incubation period, the absorbance of each solution was measured at 735 nm using spectrophotometer. A calibration curve was constructed to correlate the concentration of gallic acid with the corresponding absorbance values.

A portion of each P. macrocarpa extract was subjected to the same experimental protocol. The total phenol content of the extracts was quantified by comparing their absorbance to the established calibration curve [18].

Evaluating Antioxidant Activity. Antioxidant evaluation was performed using the DPPH method following a procedure adapted from Hidayat and Fatmawati (2019). A 6 x 10-5 M solution of DPPH radical was prepared, and its maximum absorbance wavelength was determined at 520 nm. Each extract (0.5 mL) was mixed with 2 mL of DPPH solution and incubated in the dark for 30 minutes at room temperature. As a positive control, ascorbic acid (100 ppm) was prepared and treated in the same way as the sample extracts. The blank was prepared by mixing 2 mL of DPPH solution with 0.5 mL of ethanol. The antioxidant activity of each sample was determined by calculating the percentage inhibition of DPPH radical absorption using the following formula [19]:

$$\% \text{ Absorbance} = \frac{A_0 - A_s}{A_0} x100\%$$

Where: A0 = Absorbance of control (DPPH solution without sample); As = Absorbance of sample or positive control.

Result and Discussion

Extraction of *P. Macrocarpa.* Extraction was performed using a variety of methods, including hydrothermal extraction at 150°C, 175°C, and 200°C, as well as traditional methods such as boiling. The results of the hydrothermal extraction demonstrated a correlation between increasing temperature

and increasing color intensity of the extract. This phenomenon can be attributed to the accelerated extraction process at higher temperatures, which results in an increased concentration of extracted components.

Phytochemical Screening. A qualitative phytochemical screening was performed to identify secondary metabolites, specifically flavonoids and phenols, present in P. macrocarpa extracts. The results, summarized in Table 1, demonstrate that all extracts, obtained through various hydrothermal extraction methods (150°C, 175°C, 200° C) as well as conventional methods (boiling), contain both classes of compounds. The presence of flavonoids was confirmed by a positive colorimetric test, characterized by the formation of a red or orange flavylium salt upon the addition of HCl and Mg. The qualitative test results for flavonoids are visually represented in Figure 1.

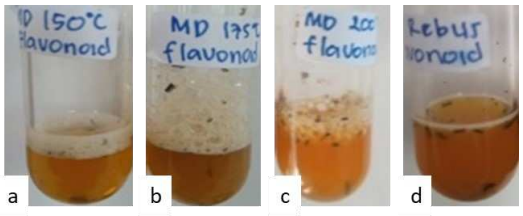

Figure 1. Flavonoid qualitative test results of *P. Macrocarpa*: (a) 150°C extract, (b) 175°C extract, (c) 200°C extract, (d) extract boiling

Similarly, the presence of phenols was confirmed by a positive colorimetric test, evidenced by a dark colour change upon the addition of FeCl3. The qualitative test results for phenols are visually represented in Figure 2. This suggests the presence of catechol tannins, which are derivatives of phenolic compounds. The reaction involves the complexation between Fe3+ ions from FeCl3 and ortho-dihydroxy (catechol) groups in phenol compounds during phytochemical screening with FeCl3. The ferric ion coordinates with one or more catechol groups via their hydroxyl functional group, forming mono-, bis-, or tris-catecholate complexes. These complexes exhibit intense color change ranging from green to black, associated with ligand-to-metal charge transfer transmitions (Figure 3) [20]. These results agree with the findings of Kurang and Malaipada (2021), who also observed a black colour change in the presence of phenols on P. macrocarpa extract [21].

Total Flavonoid. The total flavonoid content was quantified using a colorimetric method

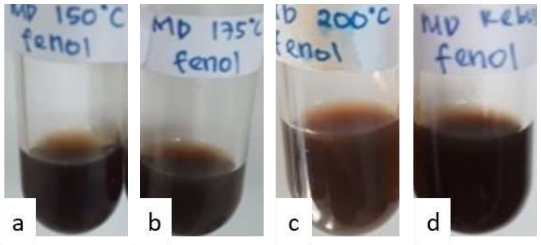

Figure 2. Phenol qualitative test results of *P. Macrocarpa* fruit: (a) 150°C extract, (b) 175°C extract, (c) 200°C extract, (d) extract boiling

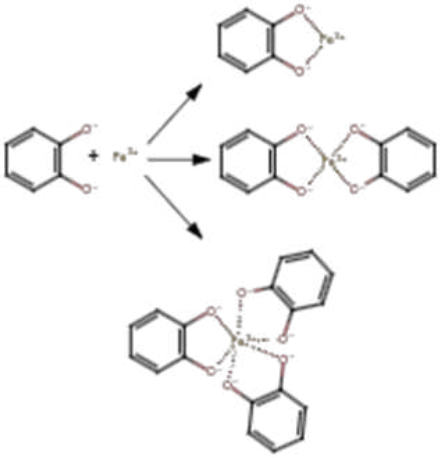

Figure 3. Mechanism of ferric ion (Fe^{3+}) complexation with catechol groups in phenolic compounds

that relies on the formation of coloured complexes between flavonoids and aluminium chloride ($AlCl_3$). Hydrothermal extraction at 175°C proved to be the optimal method for extracting flavonoids from P. macrocarpa, yielding a total flavonoid content of 4.04 mg quercetin equivalents per 1% extract. At higher temperatures, such as 200°C, a decline in flavonoid content was observed. This reduction can be attributed to the thermal degradation of flavonoids, which are susceptible to high temperatures and oxidation. This finding is in agreement with the observations reported by Prihandini and Purnama (2021), which identified optimal total flavonoids content at temperatures between 140-170 °C in hydrothermal extraction. Further increase to 200 °C led to a decrease in flavonoid content (3.24 mg QE/1%), likely due to thermal degradation, as supported by general stability studies of plant extracts [22]. The test results of total flavonoid content of P. macrocarpa fruit extract can be seen in Table 2.

Total Phenol Content. The total phenol content was quantified using the Folin-Ciocalteu method, which relies on an oxidation-reduction reaction between polyphenols and phosphomolybdic acid/ phosphotungstic acid in the reagent. This reaction

Table 1. Phytochemical screening test results

Secondary metabolites	Hydrothermal			Boiling
	150°C	175°C	200°C	
Flavonoids	+	+	+	+
Phenols	+	+	+	+

Description:

Sign (+): The sample contains the tested secondary metabolite compound
Sign (-): Samples do not contain the tested secondary metabolite compounds

results in a colorimetric change from yellow to blue. Among the various extraction methods tested, hydrothermal extraction at 175°C yielded the highest total phenolic content (30.12 mg GAE/1% extract). Phenolic content increased with temperature up to 175 °C, followed by a decrease at 200 °C due to thermal degradation. In hydrothermal extraction, phenolic compounds can undergo thermal degradation, leading to a decrease in their concentration and antioxidant activity. This degradation is often due to the breakdown of phenolic structures and the formation of new, less beneficial compounds [23]. The test results of total phenol content of crown fruit extract can be seen in Table 3.

Antioxidant Activity Test. The P. macrocarpa extract was added to a DPPH solution and subsequently analyzed using UV-Vis spectrophotometry at a wavelength of 520 nm. The percentage inhibition values obtained from this analysis quantify the extract's capacity to inhibit free radical activity. The antioxidant potential of the fruit extracts, as measured by the DPPH assay, was found to be significant, with inhibition rates ranging from 66.974% to 77.727% (Table 4). The extract processed at 175° C demonstrated the highest antioxidant activity. The improved extraction at 175 °C could be attributed to enhances cell wall disruption and increase water diffusivity, which facilitates the release of intracellular bio-actives like phenolic and flavonoids. At this temperature, subcritical water reduces its dielectric constant, enhancing the solubility of moderately polar bio-actives, including flavonoid and phenolic compounds, thereby contributing to maximal antioxidant activity as observed in the DPPH assay. These compounds can act as potent antioxidants by donating hydrogen atoms and reducing free radicals [24]. Similar trends were also reported by Prihandini and Purnama (2021) in their hydrothermal extraction study Moringa oleifera leaves, where antioxidant activity peaked between 140-170 °C, followed by a decline at higher temperatures [22]. A comparable pattern was described by Antony and Farid (2022), Who demonstrated that phenolic and flavonoid-rich extracts showed maximal DPPH radical scavenging activity at moderate subcritical temperatures, while activity decreased beyond 180 °C due to thermal degradation and the formation of degradation products. This antioxidant activity decreased with increasing temperature, as observed in the 200°C extract. This decline can be attributed to the thermal degradation of phenolic and flavonoids compounds, which are known to be sensitive to prolonged high-temperature exposure, as previously reported. This temperature-dependent pattern is consistent with prior subcritical water extraction studies, where antioxidant capacities increased up to moderate temperatures (100–200 °C), but declined beyond due to thermal degradation and Maillard reaction product formation [23]. This study is the first to systematically optimize hydrothermal extraction conditions for P. macrocarpa fruit and confirm 175 °C as the optimal temperature for maximizing antioxidant yield.

Table 2. Test results of total flavonoid content of *P. Macrocarpa* fruit extracts

Sample	Flavonoid Content (mg QE/1% extract)
P. Macrocarpa extract 150 °C	2,66
P. Macrocarpa extract 175 °C	4,04
P. Macrocarpa extract 200 °C	3,24
Boiling	2,10

Table 3. Test results of total phenol content of *P. Macrocarpa* extracts

Sample	Phenol Content (mg GAE/1% extract)
P. Macrocarpa extract 150°C	24,66
P. Macrocarpa extract 175°C	30,12
P. Macrocarpa extract 200°C	27,49
Boiling	26,84

Conclusion

This study demonstrated that hydrothermal extraction is an effective green extraction method for obtaining bioactive compounds from P. macro-

Table 4. %Inhibition values of *P. macrocarpa* fruit extracts

Sample	Absorbance 1	Absorbance 2	Absorbance 3	Mean ± SD	% Inhibition
Blank (DPPH Only)	0.728	0.724	0.726	0.726 ± 0.003	-
Ascorbic acid	0.115	0.118	0.118	0.117 ± 0.002	83.885
P. macrocarpa extract 150 °C	0.240	0.237	0.237	0.238 ± 0.004	67.281
P. macrocarpa extract 175 °C	0.160	0.163	0.163	0.162 ± 0.003	77.727
P. macrocarpa extract 200 °C	0.243	0.238	0.239	0.240 ± 0.005	66.974
Boiling	0.207	0.203	0.205	0.205 ± 0.003	71.736

carpa fruit. The quantitative analysis showed that increasing extraction temperature up to175 °C enhanced the yield of total flavonoids (4.04 mg QE/1% extract) and total phenols (30.12 mg GAE/1% extract), along with the highest antioxidant activity (77.73% DPPH inhibition). However, higher temperatures (200 °C) led to a decrease in these parameters, suggesting that excessive heat can degrade bioactive compounds (heat-sensitive compounds). Compared to boiling, hydrothermal extraction at 175 °C provided higher yields of antioxidant compounds and activity. These findings highlight the importance of optimizing extraction conditions to maximize antioxidant yield and the recovery of valuable bioactive compounds from P. macrocarpa fruit using a water-based, organic solvent-free method.

Acknowledgements

The authors are grateful for the laboratory support from Chemistry Laboratory, Universitas Islam Kadiri-Kediri.

Author Contributions

All the authors have contributed sufficiently to this research, from coming up with the idea, collecting the data, supervising, and producing the paper.

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