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In-Vitro Evaluation of Antioxidant Activity of *Phaleria macrocarpa* using Different Extraction Process and Determination of Functional Groups in the Extract by FTIR (Fourier Transform Infrared Spectroscopy)

Shifa Nur Anisya[1], Fithri Choirun Nisa[1], Liew Kai Bin[3] and A. B. M. Helal Uddin[2]*
[1]Department of Food Science and Biotechnology, Faculty of Agricultural Technology, University of Brawijaya, Malang, Indonesia
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, International Islamic University Malaysia (IIUM), Kuantan Campus, Kuantan 25200, Pahang, Malaysia
[3]Department of Pharmaceutical Technology & Industry, University of Cyberjaya, Selangor, Malaysia
*Corresponding author (e-mail: abmhelal@iium.edu.my)

P. macrocarpa is often known as "God's crown" due to its great nutritional importance for human health. *P. macrocarpa* fruit can delay and avoid oxidative cell damage in the body by its antioxidant properties. In the meantime, there is still lacking yield, antioxidant activity, and functional properties of *P. macrocarpa* fruit in the sonication method using water and ethanol. So, this study aims to compare *P. macrocarpa* fruit yield, and antioxidant properties and to identify its functional group. Crude ethanol extract and water extract of *P. macrocarpa* fruit have been studied for Ferric Reducing Antioxidant Power (FRAP) and 2,2- Diphenyl-1-Picrylhydrazyl (DPPH) antioxidant activities against Ascorbic acid as a standard. The study also determined the functional group using FTIR (Fourier-Transform Infrared Spectroscopy) assay.The most effective *P. macrocarpa* fruit yield was obtained in the water extract compared to the ethanol extract. In addition, the ethanol extract showed a higher FRAP (54.285±1.139 mgAAE/g) effect than the water extract. Besides, the DPPH activity of ethanol extract (46.113±1.535 µg/mL) was higher than water extract (59.406±2.089 µg/mL) and lower than standard Ascorbic acid (8.287±0.125 µg/mL). Moreover, some functional groups such as -OH, aliphatic CH, aromatic CH, aromatic C=C and C=N were also identified in both *P. macrocarpa* fruit extracts. The results confirmed that the sonication method using ethanol and water could influence the yield of *P. macrocarpa* fruit extracts as well as antioxidant activities with different functional groups.

Keywords: *P. macrocarpa* fruit; extraction process; antioxidant activity; functional groups; FTIR

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Traditional medicinal plants are often regarded as the most reliable natural sources of drugs. The medicinal components derived from plants have long been regarded as a therapeutic option for the treatment of different ailments and in basic health care [1]. In many countries of the world, herbal medicines have been used for a variety of ailments since antiquity by traditional medicine practitioners due to cultural beliefs and good biomedical properties [2]. Indonesia is one of the countries with the largest agricultural sector in the world. However, the condition of land is already a problem because it decreases gradually regarding the public construction. The public construction creates more employment opportunities in the industry at the time. Along with it, the impact of construction produces bad environmental conditions such as pollution and free radicals.

Plants contain many phytochemicals that could be used as traditional medicine to treat both long-term and short-term illnesses. These phytochemicals, which include alkaloids, tannins, flavonoids and terpenoids, have a variety of biologically beneficial properties, including antihyperglycemic, anticancer, antibacterial, antioxidant, antidiarrheal, analgesic, and wound healing [3]. The *Phaleria macrocarpa* (Scheff.) Boerl plant belongs to the Thymelaeaceae family sometimes referred to as "Mahkota Dewa," "God Crown," or "Pau." In several South Asian nations, including Indonesia, Malaysia, and the Philippines, it is well-known and commonly utilized as a medicinal herb. *P. macrocarpa* fruit is used to treat allergies, cancer, impotence, diabetes mellitus, haemorrhoids, stroke, heart and liver diseases, acne, blood-related diseases, kidney disorders, migraine, and several skin ailments [4].

Some advanced extraction techniques have been widely investigated as water extraction and solvent extraction. The most prevalent molecule on earth, water has positive effects on both environmental impact and safety. Since the quality is affordable, non-flammable, and non-toxic, it opens prospects for

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clean processing and pollution control while adhering to standards that get constantly stricter [5]. The choice of extraction method is an essential factor to achieve quality and higher yields. Ultrasound/sonication proved effective for cleaning, degassing solvents, and extracting adsorbed metal and organic pollutants from environmental samples. By reducing intermolecular interactions and hastening the dissolution of target compounds, such as phenolics and antioxidants in extracts, sonication in the extraction process aims to increase the extraction efficiency of the target chemical [6].

Antioxidants play a crucial role in neutralising free radicals to protect cells from oxidative stress and help reduce the risk of chronic conditions [7]. The analysis of antioxidants is carried out to determine which compounds that linked to curing various diseases. Plant extracts contain antioxidants that are essential because they have been associated with a variety of disorders, including antihyperglycemic effects through the suppression of protein glycation, the deactivation of several enzymes, and modifications in the collagen basement membrane of pancreatic β *-cells* [8].

The current study was designed to evaluate *in-vitro* DPPH and FRAP antioxidant activities. Our goal is to explore a suitable extract with a higher yield and enriched with components to exhibit antioxidant activity in *P. macrocarpa* through *in-vitro* antioxidant evaluation and determine the functional groups that are related to antioxidant compounds.

MATERIALS AND METHODS

Chemical and Reagents

Ethanol, Methanol (LCMS grade), Folin-Ciocalteu's reagent, Hydrochloric acid (37%), Ascorbic acid, Sodium acetate, Acetic acid, Dimethyl sulfoxide, 2,2-diphenyl-1 picrylhydrazyl, 2,4,6-tris (2-pyridyl)-s-triazine, Iron (III) chloride.

Collection and Preparation of *P. macrocarpa* Fruit Extract

P. macrocarpa dry sliced fruits were collected from Mohd Shukri, Jalan Guar Perahu 2, Taman Guar Perahu, Penang, Malaysia. The flesh parts of dried *P. macrocarpa* fruits were separated from their seed and shell parts. The flesh was dried at 50°C using a laboratory dryer machine to remove moisture. Dried flesh was crushed into powder form by universal cutting mill machine and stored in a plastic container at room temperature for further analysis.

Water Extraction of *P. macrocarpa* Fruit

25 g of dried powder was poured into a 500 ml glass bottle and added up to 250 ml water, mixed well. The mixture was kept overnight and sonicated at 60°C for 2 hours using an ultrasonic cleaner. It was filtrated using cotton a ball in a funnel and afterwards, another porcelain Buchner funnel. The filtered part was collected in falcon tubes and kept in a freezer at 80°C before being concentrated by freeze drying for 7 days to drive its extract. Crude extracts were kept at room temperature for further analysis. The percentage yield of the extract was calculated in **equation (1)**:

$$Yield\ (\%) = \frac{W_1-(W_1-W_\alpha)}{W_1} x\ 100 \qquad (1)$$

W_1 = Weight of dry *P. macrocarpa* fruit powder

Wα=Weight of extract after rotary evaporation

Ethanol Extraction of *P. macrocarpa* Fruit

25 g of dried powder was poured into a 500 ml glass bottle and added up to 250 ml water, mixed well. The mixture was kept overnight and sonicated at 60°C for 2 hours using an ultrasonic cleaner. It was filtrated using filtrate paper (Whatman No.42) in a Buchner funnel with the help of gas from a water pipe. The filtered part was collected in a round bottom flask and concentrated by rotary evaporator at 50°C with 50-90 rpm. Crude extracts were transferred into a falcon tube and kept at room temperature for further analysis. The percentage yield of the extract was calculated in **equation (1).**

Determination of Antioxidant Activity using FRAP Assay

The method was followed by [9] with minor modifications. The FRAP solution was prepared freshly by mixing 0.1 M acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM $FeCl_3$ in a ratio of 10:1:1 respectively and incubated for 10 min at 37°C in an oven. Different concentrations (500 to 3.906 µg/mL in methanol) of ascorbic acid were used to prepare the calibration curve. Ethanol extract (500 µg/mL) and water extract (500 µg/mL) of *P. macrocarpa* fruit were dissolved in DMSO. Then, 20 µl of each *P. macrocarpa* fruit extract and different concentrations of ascorbic acid (500 to 3.906 µg/mL) were placed in a 96 microwell plate and added 40 µl of fresh FRAP solution. Subsequently, 140 µl of distilled water was added and mixed gently. Reagent blank was prepared with 40 µL of FRAP reagents made up to 200 µL with distilled water. It was incubated in the dark at room temperature for 20 min. The absorbance of all samples and standards was taken reading at 593nm using a multi-detection microplate reader.

Determination of Antioxidant Activity using DPPH Assay

The DPPH assay was described by [10] with slight changes. 3.94 mg of DPPH was dissolved in 50 mL methanol. Different concentrations of water extract

and ethanol extract were dissolved in dimethyl sulfoxide (DMSO). 50 µl of each extract was placed in a 96 microwell plate and 150 µl DPPH (0.1 mM) was added to each well. An equal amount of methanol was added to the control and kept in the dark at room temperature for 30 minutes. Ascorbic acid was used as a standard to measure the antioxidant activity of water and ethanol extract. A control reaction was carried out without the test sample. The absorption was measured at 517 nm using a multi-detection microplate reader.

FTIR Spectroscopy Analysis

The Fourier-Transform Infrared Spectroscopy (FTIR) analysis of *P. macrocarpa* fruit extracts was performed according to [9] with slight modification. The equipment used was Perkin Elmer Inc and scanned in the mid region of 4000 cm-1 to 400 cm-1. Each sample was scanned using an Attenuated Total Reflectance (ATR) system. The freeze-dried extracts were evaluated in their natural state, without any preparation, by placing them directly on the diamond ATR crystal.

Data Analysis

Unpaired T-test analysed the percentage of yield extract and FRAP assay data. Meanwhile, data from the DPPH assay was analysed by one-way analysis of variance and Dunnett's multiple comparisons test. All data was expressed as means ± SD. Statistical significance and graphs were determined by GraphPad Prism (version 7.00). Linear regression and IC_{50} were also determined using Microsoft Excel.

RESULTS AND DISCUSSION

Extraction Result

An unpaired T-test determined the percentage yield of extracts. The T-test result for the extraction yield of *P. macrocarpa* is shown in **Figure 1**. The results showed that the water extract and ethanol extract of *P. macrocarpa* were significantly (****p<0.0001) different from each other. The percentage yield of the ethanol extract was found to be 12.130±0.243 gm and 22.860± 0.845 gm for water extract. The yield of water extract was higher than ethanol extract in the sonication method due to solvent polarity [11]. The results demonstrated that water was a suitable extraction solvent for *P. macrocarpa* fruits according to the extract yield.

The extraction methods were followed by sonication to obtain the maximum extraction yield. It was pointed out that raising the temperature (up to 60°C) leads to a decrease in solvent viscosity, resulting in increased cavitation and vapour pressure of the solvent within the tissue. Consequently, this maximises the contact area between the particles and solvent 12]. Each extraction was carried out in three batches to produce a high amount of yield. The yield collection has shown the characteristic of dry *P. macrocarpa* fruit flesh was brownish coloured with a sticky-viscous texture.

At elevated temperatures, the plant's cellular structure facilitates a faster absorption of the solvent, causing the cell walls to rupture and release their contents into the solvent. This results in an enhanced extraction yield. However, higher temperatures can also lead to the degradation of thermolabile materials and the evaporation of volatile substances, ultimately leading to a reduction in yield [13].

FRAP Assay Result

The FRAP assay was determined by unpaired T-test. The result for the FRAP assay of *P. macrocarpa* is shown in **Figure 2.** The result showed that the water extract and ethanol extract of *P. macrocarpa* were significantly (***p<0.001) different from each other.

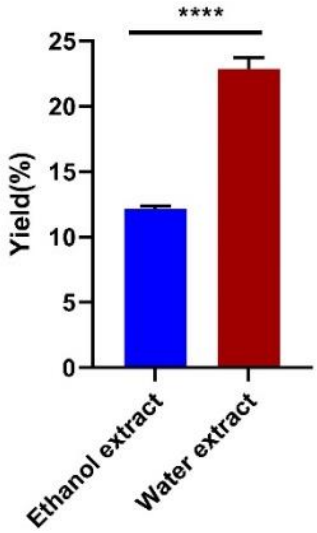

Figure 1. T-test analysis for percentage yield of *P. macrocarpa* extracts.

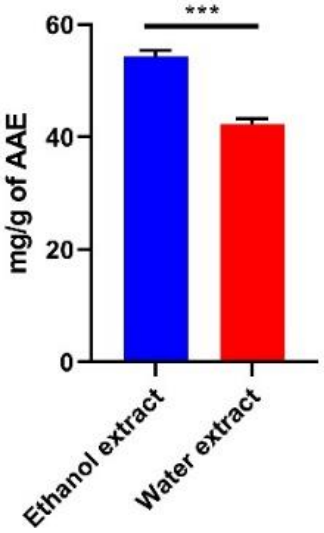

Figure 2. T-test analysis for FRAP assay of *P. macrocarpa* extracts.

The FRAP value of ethanol extract was found to be 54.285±1.139 mg/g of AAE and 42.213±1.103 mg/g of AAE for water extract. It reflected that the ethanol extract was suitable for the antioxidant activity of *P. macrocarpa*.

It can generally be concluded that the extracts obtained with ethanol contain higher levels of polyphenols and flavonoids. Secondary metabolites found in ethanol extract are polyphenols alkaloids, triterpenoids, flavonoids, saponins, tannins and quinones [14]. These secondary metabolites possess many biological remedial benefits such as antioxidant, anticancer, antiviral, analgesic, antimicrobial and antidiarrheal [15]. Some secondary metabolites namely myricetin, rutin, quercetin, naringin and kaemferol were isolated and identified from methanolic extract of *P. macrocarpa* fruits (Mia et al., 2021; Mia et al., 2022). Scientists assert that highly polar solvent extracts, particularly ethanol, enable the extraction of phenolic chemicals by generating more polar media. The ability of antioxidant chemicals to reduce Fe^{3+} to Fe^{2+} served as the basis for determining their antioxidant activity, which corresponded to their ferric reducing identity. Since the chemicals that act as antioxidants are more soluble in solvents that are less polar than water, using pure water as a solvent to extract them is less effective [16].

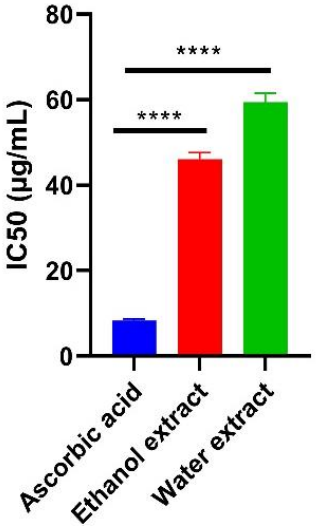

Figure 3. T-test analysis for DPPH assay of *P. macrocarpa* extracts.

Table-1. IC_{50} value for DPPH assay of water and ethanolic extracts.

Name of extracts	IC_{50} (µg/mL)
Water extract	59.406±2.089
Ethanolic extract	46.113±1.535
Standard (Ascorbic acid)	8.287±0.125

2,2- Diphenyl-1-Picrylhydrazyl (DPPH) Activity

The DPPH assay was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. The result for the DPPH assay of *P. macrocarpa* is shown in **Figure 3.** The result showed that the IC_{50} occurred at 59.406±2.089 µg/ml for water extract and 46.113±1.535 µg/ml for ethanol extract. The extract's inhibitory capacity was evaluated in comparison to well-known antioxidants (ascorbic acid), and the result was comparable as shown in **Figure 3 and Table-1.** The findings reflects that the ethanol extract is suitable for the antioxidant activity of *P. macrocarpa*.

The ethanol extract can identify more metabolite compounds than water extracts because it has the same level of polarity. [17] reported that ethanolic extract from the *Populus balsamifera* bud contains more phenolic and flavonoid compounds and also confirmed the higher amounts through HPLC than water extract. Due to the presence of hydroxyl groups, flavonoids are also polar, making them highly soluble in polar solvents like ethanol [18]. The ethanolic extract might show a better antioxidant effect than a water extract as ethanol can more effectively extract certain compounds, such as polyphenols and flavonoids, which are known for their antioxidant properties. Additionally, ethanol can penetrate cell walls more efficiently, extracting a wider range of antioxidants from plant material compared to water extraction.

Analysis of Antioxidant by FTIR

The FTIR spectrum was used to identify the functional group of the bioactive compounds based on the peak value in the region of infrared radiation as seen in **Table 2**. The FTIR spectrum of water *P. macrocarpa* extract is shown in **Figure 4** and ethanol *P. macrocarpa* extract is shown in **Figure 5.** The absorption at 3310.25 cm-1 is due to the stretching O-H symmetric that is present in the ethanol extract. The bands at 3267.22 cm-1 and 2973.13 cm-1 are due to the symmetric stretching O-H and stretching C-H that are present in the water extract. The band at 2936.2 cm-1 is due to the stretching of saturated (sp3) hydrocarbon that is present in both extracts. The band at 2884.3 cm-1 and 1615.24 cm-1 are due to the stretching C-H and the bending mode of C=C, that present in the water extract. The bands at 1592.71 cm-1, 1575.34 cm-1, 1558.91 cm-1, and 1515.73 cm-1 are assigned to amino acids present in the ethanol extract. A band at 1513.88 cm-1 represents the aromatic C=C in the water extract.

Table 2. List of different functional groups present in water and ethanol extracts of *P. macrocarpa* fruit.

Wavenumber (cm-1)	Chain	Presence	
		Ethanol Extract	Water Extract
3310.25	O-H	v	
3267.22	O-H		v
2973.13	sp3 C-H		v
2936.2	sp3 C-H	v	v
2884.3	sp3 C-H		v
1615.24	C=C		v
1592.71	C=N	v	
1575.34	C=N	v	
1558.91	C=N	v	
1515.73	C=C	v	
1513.88	C=C		v

Note: **"v"** denoted presence of groups

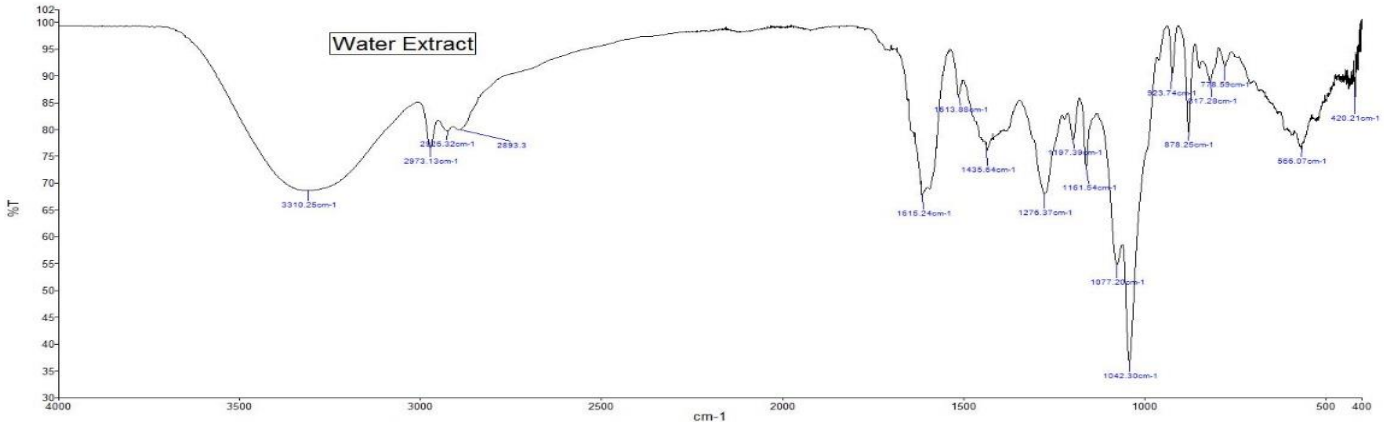

Figure 4. FTIR spectra of *P. macrocarpa* water extract.

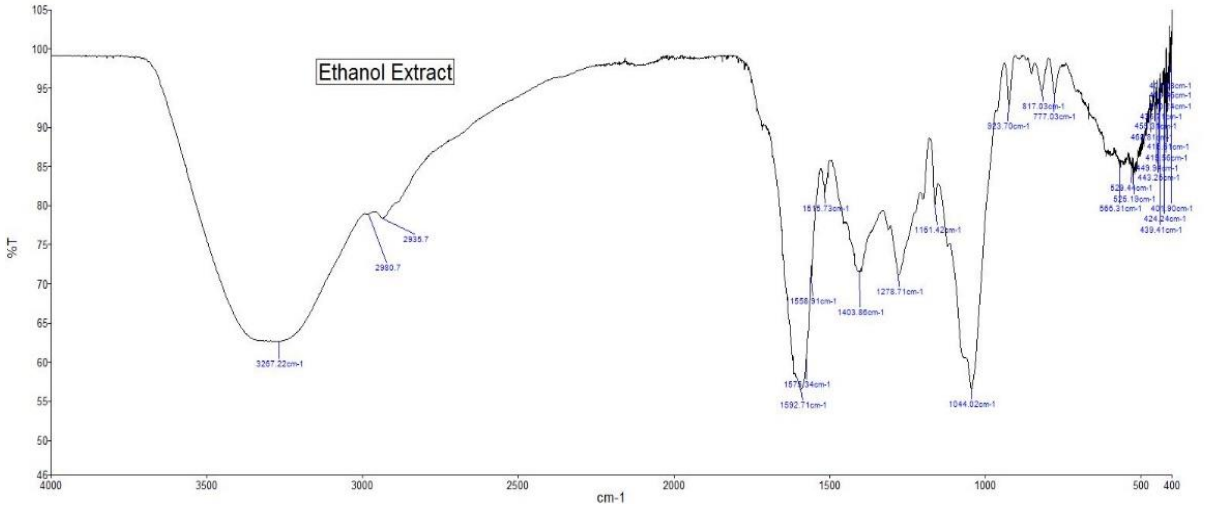

Figure 5. FTIR spectra of *P. macrocarpa* ethanol extract.

CONCLUSION

This research on the antioxidant activity of *P. macrocarpa* helps to develop effective herbal remedies as an antioxidant activity to delay oxidation in the body systems. The result shows that *P. macrocarpa* fruit extract possesses a higher antioxidant activity compared to the water extract. There are several functional groups in *P. macrocarpa*, such as O-H, C-H, C=C, and C=N, that act as an antioxidant. Therefore, this fruit extract has the potential to be further developed for functional food-making to cure some diseases.

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