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Phytochemical Screening, Total Phenolic Content and Antioxidant Activity of Leaf Extract of *Muntingia calabura*

Noor Hidayah Pungot[1*] and Nur Syafiqah Atikah Nazaharuddin[2]
[1]Faculty of Applied Sciences, School of Chemistry and Environment,
Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia
[2]Faculty of Applied Sciences, School of Chemistry and Environment,
Universiti Teknologi MARA(UiTM) Cawangan Negeri Sembilan, Kampus Kuala Pilah, Pekan Parit Tinggi,
72000 Kuala Pilah, Negeri Sembilan, Malaysia
[*]Corresponding author (e-mail: noorhidayah977@uitm.edu.my)

Muntingia calabura or locally known as *kerukup siam* in Malaysia belongs to the *Muntingiaceae* family. This study aimed to investigate the potential application of *M. calabura* leaves from Melaka and extraction using different non-polar solvents from a previously study by conducting the preliminary analysis on phytochemical screening, total phenolic content, and antioxidant activity. The phytochemicals were extracted by sequential maceration by using *n*-hexane, ethyl acetate, and methanol. The phytochemical screening was performed using various chemical tests and established standard procedures. Meanwhile, the total phenolic content and antioxidant activity of the extracts were assessed by using Folin-Ciocalteau method and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, respectively. Flavonoids, phenols, triterpenes, and steroids were present in the extract using *n*-hexane. The methanol extract contained flavonoids, phenols, steroids, triterpenes, tannins, reducing sugars, and saponins. The methanol extract exhibited the highest phenolic content at 8.20 mg GAE/g. Among the tested extracts, the methanol extract demonstrated strong DPPH radical scavenging activity with an IC_{50} value of 167.70 µg/mL. This study confirmed the presence of various phytochemicals in *M. calabura* leaves from Melaka that possessed good potentials as antioxidants. This finding would benefit future research and exploration on biochemical profiles in different usages/applications especially in pharmaceutical and cosmeceutical commercialisation.

Key words: *M. calabura*; phytochemical screening; total phenolic content; antioxidant activity; DPPH

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Free radicals are molecules produced by cells when the body processes food and reacts to the environment. Excessive production of free radicals can damage cells and body functions [1]. Free radicals are linked to many health issues such as diabetes, cancer, and heart diseases[2]. In order to prevent this damage, the body needs antioxidants. Antioxidants are stable molecules that remove free radicals and repair damaged cells. Natural and synthetic antioxidants are being used to restrain free radicals. However, the use of synthetic antioxidants needs to be taken seriously, as some studies have shown toxic and carcinogenic effects on the human body [3]. Besides, natural antioxidants can act as nutraceuticals to terminate free radical chain reactions in biological systems, which in turn provide health benefits [4]. Naturally occurring antioxidants isolated from plants consist of secondary metabolites such as flavonoids, phenolics, tannins, quinones, glycosides, and terpenes. Recently, it was reported that these species have high potentials in the cosmeceutical industry such as collagen [5] due to good antioxidant activities. Not only in skin care consumable but the leaves of plants are also in high demand for healthy drinks such as tea [6], due to the antioxidant activities contained in plants.

Muntingia calabura was introduced from the tropical America to Southeast Asia as this tree is well-adapted to the local environment, where it is often grown as roadside trees [7]. *M. calabura* is called Jamaican cherry, but in Malaysia it is known as *kerukup siam* [8]. *M. calabura* is from the *Muntingiaceae* family that consists of three genera, with each genus only contains one species. The genera are *Dicraspidia*, *Muntingia*, and *Neotessmannia.* The species *M. calabura* is a quickly developing tree of thin extents, achieving a stature of around of 3-12 m in height, with fan-like branches and is sagging, hence bringing about its layered tree shape. The leaves are evergreen of around 4-14 cm long and 1-4 cm wide. The leaves are basic, ovate-lanceolate, dim green, and minutely shaggy on the upper side, asymmetry of leaf-cutting edge base, and leaf edge serrate. The flowers have white petals and are 1.25-2 cm wide with five green sepals, five white petals, and numerous conspicuous

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Figure 1. *M. calabura* tree, leaves, fruits and flower
Source: Zakaria *et al*. (2015)

yellow stamens. The fruits are 1-1.25 cm in diameter, with red or at times orangey-yellow smooth skin. A few thousand small seeds are found in the delicate mash, yet are too fine to be visible when eaten [9], as shown in Figure 1.

M. calabura has numerous utilisations in customary medication. For example, in Peruvian old medicine, the bark and flowers are used as antiseptics to diminish gastric ulcer, decrease swelling of the prostate, and reduce cerebral pain and flu. In addition, the plant is used as a sedative agent, tonic, nascent chilly or sedatives, antidyspeptics, and antispasmodics in Southeast Asia. In Mexico, the plant is utilised to treat mouth pimples, stomachache, and measles. This plant is reported to have antibacterial, antidiabetic, anti-inflammatory, hypotensive, antiviral, inhibition of platelet-activating factor, antioxidant, cytotoxicity, antiproliferative, anticoagulant, wound healing, gastroprotective, antidiarrheal, antinociceptive, and antipyretic activities at different concentrations [10].

Phytochemical screening, total phenolic conte nt, and antioxidant activity in *n*-hexane extract of *M. calabura* leaves from Melaka have not been studied until today. Previous research only reported the phyto chemical screening and antioxidant activity of *M. calabura* leaf extract using chloroform for non-polar extract, and the samples were collected only from Selangor, Malaysia [11-16]. It is generally recognized that the antioxidant activity of phenolic compounds is affected by the chemical compositions in the plants, which depend on many factors, such as geographic variation, environmental conditions, and extraction method. Besides, extraction parameters such as solvents, temperature, the material to solvent ratio, and extraction time significantly impact the yield and biological activities of the plant extract [17]. Therefore, in this study, the leaves of *M. calabura* were collected from a different location. The location was in Melaka, Malaysia.

This research was carried out to determine the phytochemicals in *M. calabura* leaves from Melaka, Malaysia and their correlation with phenolic content and antioxidant activity. The extraction was performed using various solvents such as *n*-hexane, ethyl acetate, and methanol. The results of this study offered opportunities to identify *M. calabura* populations with targeted health-promoting compoun ds and also to establish future plantations with better pharmaceutical and food uses.

EXPERIMENTS

1. Materials and Instruments

The chemicals used were: acetone, ethanol, methanol, *n*-hexane, ethyl acetate, chloroform, and acetic anhydride (purchased from HmBG chemicals); gallic acid, ascorbic acid, ammonia, sulfuric acid, Fehling solutions A and B, hydrochloric acid, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), iodine, potassium iodide, and iron(III) chloride (purchased from R&M Chemical Supplier); 2,2-Diphenyl-1-picrylhydrazyl (purchased from Sigma-aldrich); and sodium hydroxide pellets (purchased from QReC). The instrument used was T80/T80+ UV-Visible

spectrometer (500–700 nm).

2. Plant Material Collection

Fresh *M. calabura* leaves were collected in August 2018 in Alor Gajah, Melaka. The surface of the leaves was cleaned, washed with distilled water, and allowed to air-dry for two weeks at room temperature. The air-dried samples were then ground into powder using an electrical grinder.

3. Extraction of Plant Extract

The extracts were prepared by macerating of 100 grams of the powdered sample in 800 mL of methanol, ethyl acetate, and n-hexane, using an orbital shaker for 72 h at room temperature. Subsequently, the extracts were filtered and concentrated using a rotary evaporation (IKA HB 10) at 40°C. Crude extracts were stored in sealed containers until further analyses. Concentrated extracts were weighed to find the extraction efficiency on dry weight basis. The extraction efficiency was calculated as follows:

$$\text{Extraction efficiency (\%)} = \frac{\text{Final dry weight of extract}}{\text{Initial weight of dried plant material}} \times 100$$

4. Phytochemical Screening

Phytochemical screening tests were performed to evaluate the presence of phytoconstituents in *M. calabura* leaf extracts using standard methods.

4.1 Tests for alkaloids (Wagner's Test)

For alkaloid tests, 0.5 g of extracts was dissolved in 15% dilute hydrochloric acid and filtered. Wagner's reagent (iodine in potassium iodide) was used to test the mixtures. The formation of brown/reddish precipitate indicated the presence of alkaloids.

4.2 Tests for steroids and triterpenes (Liebermann Burchard Test)

For steroid and triterpene tests, 0.5 g of samples was mixed with a few drops of acetic anhydride. The mixtures were boiled and cooled for further analyses. Concentrated sulfuric acid was added from the side of the test tubes, and the formation of a brown ring at the junction of the two layers was observed. The appearance of deep red color in the lower layer indicated a positive test for triterpenes. The formation of green color at the upper layer was a positive test for steroids.

4.3 Tests for tannins (Ferric Chloride Test)

For tannin tests, 0.5 g of samples was boiled in 20 mL of water in a test tube, filtered, and a few drops of 0.1% ferric chloride were added. A blue-black or a brownish-green coloration indicated the presence of tannins.

4.4 Tests for reducing sugars (Fehling's Test)

For reducing sugar tests, 0.5 g of samples was dissolved in 5 mL of distilled water and filtered. The mixtures were hydrolyzed with 1 mL of dilute hydrochloric acid, neutralized with 1 mL of ethanol, and heated with a mixture of 1 mL of Fehling's A & B solutions. A red precipitate indicated the presence of reducing sugars.

4.5 Tests for phenols (Ferric Chloride Test)

For phenol tests, 0.5 g of samples was treated with 3-4 drops of 5% ferric chloride solution. A dark green coloration indicated the presence of phenols.

4.6 Tests for saponins (Froth Test)

For saponin tests, 0.5 mg of extracts was diluted with 20 mL of distilled water and the mixtures were shaken in measuring cylinders for 15 min. The formation of an emulsion indicated the presence of saponins.

4.7 Tests for flavonoids (Alkaline Reagent Test)

For flavonoid tests, 0.5 mg of extracts was treated with a few drops of sodium hydroxide solution. Yellow coloration, which became colorless on the addition of a few drops of sulfuric acid, indicated the presence of flavonoids.

5. Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) analyses of methanol, ethyl acetate, and *n*-hexane extracts of *M. calabura* leaves were carried out using Folin-Ciocalteau method. The stock solution of gallic acid was prepared by dissolving 0.01 g of gallic acid in 100 mL of distilled water. The stock solution was pipetted into a 100 mL volumetric flask. After that, distilled water was added up to the mark of 100 mL. Then, the mixture was left in the dark for 1 h. The absorbance was measured at 760 nm against a blank solution. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

6. Antioxidant Assay

Antioxidant activity was determined by using DPPH free radical scavenging assay. The assay was accomplished as previously described with minor modifications [18]. The methanolic DPPH solution was prepared to achieve a final concentration of 50 µM (1 mg/50 mL). The samples in methanol (0.2 mL) with the concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.63, and 7.81 µg/mL were mixed with the DPPH solution (3.8 mL). The mixtures were allowed to react for 30 min. The absorbance of the reaction mixtures was recorded at 517 nm. Ascorbic acid was

used as the standard antioxidant, while methanol and the DPPH solution were used as controls. The inhibition was calculated using the following formula:

$$\% \text{ Inhibition DPPH} = \left[\frac{A_{\text{DPPH blank}} - (A_{\text{sample}} - A_{\text{blank sample}})}{A_{\text{DPPH blank}}}\right] \times 100$$

Where, A_{DPPH} is the absorbance of DPPH reagent and MeOH, A_{sample} is the absorbance of the sample with DPPH solution reagent, and $A_{blank\ sample}$ is the absorbance of the sample without DPPH solution reagent. The experiments were carried out in triplicate and were expressed as mean ± standard deviation. The IC_{50} value was obtained from the graph of scavenging activity (%) versus concentration of samples.

RESULTS AND DISCUSSION

1. Percentage Yields of Plant Extracts

The extraction efficiencies using different solvents are shown in Table 1.

The methanol extract had the highest yield at 8.96%, followed by the ethyl acetate (6.42%) and *n*-hexane (5.31%) extracts. The highest yield of the methanol extract could be due to the nature of the secondary metabolites that dissolved better in polar solvents [19]. A previous study reported the following yields: aqueous (9%), chloroform (9%), methanol (8%), and petroleum ether (7%) [20]. Non-polar petroleum ether (7%) is more efficient compared to non-polar *n*-hexane (5.31%), due to the high relative polarity value of petroleum ether (0.117) compared to that of *n*-hexane (0.009). In this study, methanol was efficient in extracting phytochemicals from *M. calabura* leaves.

2. Phytochemical Screening

The phytochemical screening of the *M. calabura* leaf extracts revealed the existence of different constituents in all extracts, as shown in Table 2.

The methanol extract of *M. calabura* leaves contained saponins, flavonoids, tannins, reducing sugars, phenols, triterpenes, and steroids. For the ethyl acetate extract, only flavonoids, phenols, and steroids were detected. While for the *n*-hexane extract, flavonoids, phenols, triterpenes, and steroids were detected. Meanwhile, no alkaloids were detected in all three extracts of *M. calabura* leaves in Melaka, Malaysia. A previous study reported that no alkaloids were detected in most of the solvent extracts of *M. calabura* leaves in Malaysia, but present in *M. calabura* leaves originated from Indonesia [21]. It might be due to the external factors (light, temperature, soil water, soil fertility, and salinity) that affect plants to produce bioactive substances [22].

Table 1. *M. calabura* extraction efficiencies using various solvents

Solvents	Initial weight (g)	Final weight (g)	Yield (%)
n- Hexane	89.50	4.75	5.31
Ethyl acetate	97.34	6.25	6.42
Methanol	100.04	8.96	8.96

Table 2. Phytochemical analyses of *M. calabura* leaf extracts using various solvents

Phytochemical Tests	*M. calabura* leaf extract		
	n-Hexane	Ethyl acetate	Methanol
Flavonoids	+	+	+
Phenols	+	+	+
Alkaloids	-	-	-
Steroids	+	+	+
Triterpenes	+	-	+
Tannins	-	-	+
Reducing sugars	-	-	+
Saponins	-	-	+

(+): indicates presence of the phytochemical constituent

(-) : indicates absence of the phytochemical constituent

Table 3. Total phenolic content of *M. calabura* leaf extracts

Crude extracts	mg GAE /g
n - Hexane	2.80±0.0
Ethyl acetate	4.42±0.0
Methanol	8.20±0.0

Data represent mean ± standard deviation of three replicate experiments

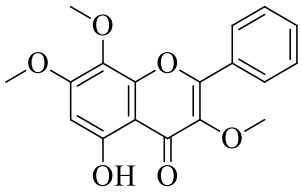

Figure 2. 5-hydroxy-3,7,8-trimethoxyflavone

3. Total Phenolic Content

The phenolic compounds in *M. calabura* leaves in alkaline medium reacted with Folin-Ciocalteu reagent to develop a blue complex that could be measured using a visible light spectrophotometry at 760 nm [23]. The total phenolic content (TPC) of three crude extracts of *M. calabura* leaves is tabulated in Table 3.

The highest TPC was found in the methanol extract (8.20 mg GAE/g) followed by the ethyl acetate (4.42 mg GAE/g) and *n*-hexane (2.80 mg GAE/g) extracts. The TPC of the extracts was in the order of: methanol > ethyl eacetate > *n*-hexane. The major compound contributing to the TPC was 5-hydroxy-3,7,8-trimethoxyflavone [24], as shown in Figure 2. This compound was extracted from the leaves of *M. calabura* that have polar sites interaction (hydrogen bonds) with the solvent, which increased the solvation of compounds in the solvent [25, 26].

4. DPPH Radical Scavenging Activity

The radical scavenging potential of the crude extracts of *M. calabura* along with ascorbic acid (AA) as the positive control are shown in Table 4. All extracts were active against DPPH radical as their inhibition were more than 50% at the concentration of 1000 µg/mL.

All extracts were found to scavenge DPPH radical in a concentration-dependent manner. The inhibition of all extracts at 1000 µg/mL of 88%-95% were almost equal to that of the positive control, AA (95%). From the 50% inhibitory concentration (IC_{50}) of the extracts, the methanol extract possessed the highest activity with the IC_{50} value of 100 µg/mL, followed by the ethyl acetate and *n*-hexane extracts with the IC_{50} values of 404.03 and 408.80 µg/mL, respectively. All the IC_{50} values were higher than that of the positive control of 10.68 µg/mL. The radical scavenging activity of the extracts was in the order of methanol > ethyl acetate > *n*-hexane.

It was reported that the IC_{50} values obtained from *M. calabura* leaf extracts were 496.18±4.56 µg/mL for petroleum ether extract, 107.99±6.24 µg/mL for chloroform extract, 79.96±0.91 µg/mL for ethanol extract, and 97.638±2.06 µg/mL for aqueous extract in comparison to ascorbic acid of 40.43±3.95 µg/mL [4]. The leaf extract of *M. calabura* from Melaka, Malaysia with high total phenolic content exhibited high antioxidant activity when assessed using DPPH radical scavenging assay. The linear correlation between high TPC and antioxidant activity observed was in line with those reported in previous studies [12,15].

Table 4. DPPH radical scavenging activity of crude extracts of *M. calabura* leaves

Crude extracts	**Inhibition at 1000 µg/mL (%)**	**IC_{50} (µg/mL)**
n - Hexane	90.23±0.5a	408.80±0.5a
Ethyl acetate	88.35±0.6a	404.03±0.7a
Methanol	95.12±0.07a	167.70±0.6a
AA	95.83±0.3a	10.68±0.9a

[a]Data represent mean ± standard deviation of three replicate experiments
Positive controls: AA – Ascorbic acid

CONCLUSION

The phytochemical analyses of leaf extracts of *M. calabura* from Melaka revealed the presence of seven phytochemicals, namely saponins, flavonoids, tannins, reducing sugars, phenols, triterpenes and steroids. The methanol extract recorded the highest extraction yield and good DPPH radical scavenging activity. Therefore, *M. calabura* could be regarded as a promising plant species for natural sources of antioxidants with potential value for future development and commercialisation of products in cosmeceutical and healthy supplement industries.

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