Effect of Extraction Conditions on The Phytochemical Properties of *Clinacanthus nutans* Using Pressurized Hot Water Extraction (PHWE) Technique

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Abstract. *Clinacanthus nutans* is an important herb species that is widely cultivated in the region of Southeast Asia. It is also known as snake grass, or ‘belalai gajah’ in malay [1-3]. The leaves of *C. nutan* can be used to make a refreshing juice or tea, or even consumed raw as ‘ulam’, a traditional malay salad. In this work, the leaves, stems, and a mixture of both leaves and stems of *C. nutan* are first dried in an oven at 50°C for 24 hours and then grounded into a powder. Then, pressurized hot water extraction (PHWE) is used to extract phytochemicals from the powdered samples at 120°C for 20 minutes. This work investigates the effects of three important parameters for extraction; the sample particle size (<63 to 500μm), solvent-to-sample ratio (10:2 to 50:2 v/w), and sample weight (0.5 to 3.0g) on the extracted phytochemicals’ total phenolic compounds (TPC), total flavonoid compounds (TFC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. The experiments are carried out in triplicate and the results are analyzed using Minitab. Phytochemicals extracted from 2g leaf powder samples of particle size <63μm using a solvent-to-sample ratio of 50:2 (v/w) resulted in the most favorable results for TPC, TFC, and DPPH scavenging activity. The results are found to be significant with the

1. Introduction

*Clinacanthus nutans* is an important herb species that is widely cultivated in the region of Southeast Asia. *C. nutan* belongs to the Acanthaceae family and is also known as snake grass or ‘belalai gajah’ in malay [1-3]. The leaves of *C. nutan* are traditionally consumed raw as ‘ulam’, a traditional malay salad, or made into a refreshing juice or tea. In addition, this herb is known to have excellent, fast-acting anti-inflammatory activity, antibiotic, antiallergic, antimutagenic and therefore is used as a topical cream for the relief of minor skin inflammation and insect bites [4-6].

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C. nutans leaves extract is rich in natural antioxidant due to the presence of the phytochemical compounds [6-8]. Phenolic and flavonoid compounds have been known for their ability to scavenge free radical reaction redox reaction due to their properties that can act as reducing agents, hydrogen donors, singlet oxygen quenchers and also other biological effect that provide health benefits for human beings [7].

Extraction is defined as a separation process to separate a solute from one phase into another. Existing conventional techniques such as soxhlet, soaking, and maceration require the use of an organic solvent which is expensive, hazardous to health, and bad for the environment [8]. In addition several extraction methods require high temperatures and long extraction times which can cause thermal degradation of bioactive compounds [9].

Recently, green technology applications that preclude the use of toxic organic solvents are gaining interest. PHWE is one such green technology as it uses water in place of organic solvents. The type of compound extracted through this process is highly dependent on the water temperature where the highly polar compounds are obtained at low temperatures and lowly polar compounds are obtained at high temperatures [9;12]

Our previous study shows that potential of water as the extraction solvent can be further exploited with the used of pressurised hot water extractor. The results reveal that all water extracts either cold (room temperature) or hot maceration at 60°C of stem, leaves and mixture of C. nutans contain low TPC, TFC and DPPH scavenging activity compared to PHWE [11]. Therefore, the objective of this study is to evaluate the effects of extraction condition on the phytochemical properties of C. nutans using PHWE by manipulating its particle size, solvent-to-sample ratio, and sample weight.

2. Methodology

2.1. Chemicals
Gallic acid and quercetin standards were purchased from Sigma (Malaysia). DPPH, Foln -Ciocalteu reagent, aluminium chloride -6-hydrate, sodium nitrate, and sodium carbonate were purchased from HmbG (Germany).

2.2. Sample preparation
Fresh C. nutans were generously provided by farmers in Kampung Wang Tepus, Jitra, Kedah. The plants were first washed thoroughly under running tap water to clean dirt and other contaminants. Then they were cut and separated into three groups; stems, leaves and a mixture of stems and leaves. Next, the samples were dried in an oven at 60 °C for 24 hours, then grounded using a mechanical grinder (Mill Powder Tech, Taiwan), and finally sieved to four different particle sizes (< 63, 125, 250, and 500μm).

2.3. Pressurized hot water extraction
PHWE were carried out on the three sample groups (stems, leaves, and stem-leaf mixture). Experiment were carried out using one-factor-at-one-time method to investigate the effect of different particle size (<63, 125, 250, and 500μm), different solvent-to-sample ratios (10:2, 20:2, 30:2, 40:2, and 50:2 v:w) and sample weight (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g). All the extraction processes were carried out at temperature of 120 °C and pressure of 4 bars for 20 min. The extracts were then transferred into plastic containers wrapped in aluminum foil and stored at -20 °C freezer until further use.

2.4. Determination of total phenolic content (TPC)
The total phenolic content was measured using the Foln -Ciocalteu colorimetric method [14-15]. 0.1 mL of plant extract was mixed with 0.2 mL of Foln -Ciocalteu reagent and 8 mL of water followed by incubation at room temperature for 3 min. Then 1 mL of 20% sodium carbonate was added into the
mixture and further incubated for 30 min at room temperature in the dark for the color to develop and measured the absorbance at 765 nm using UV-Vis spectrophotometer. The total phenol concentration was expressed in terms of gallic acid equivalents (GAE), and calculated based on a gallic acid standard curve.

2.5. Determination of total flavonoid content (TFC)
The total flavonoid content of the *C. nutans* was determined using aluminium chloride (AlCl₃) [16]. 0.5 mL of the extract was mixed with 2 mL of distilled water and 0.15 mL of 5% sodium nitrate (NaNO₂). After 5 min, 0.15 mL of 10% aluminium chloride-6-hydrate (AlCl₃·6H₂O) was added. Then after another 5 min, 1 mL of 1 M sodium hydroxide (NaOH) was added. The solution was well mixed and then kept for 15 min. The absorbance at 415 nm was measured using UV-Vis spectrophotometer. The total flavonoid content was expressed in terms of mg QUE/g DM, and calculated based on a quercetin standard curve.

2.6. Determination of DPPH scavenging activity
Accurately, 200 μL of the extract was mixed with 2.5 mL of 60 μM ethanolic DPPH. A control was also prepared containing 200 μL of ethanol and 2.5 mL of 60μM ethanolic DPPH. The mixture was mixed thoroughly and incubated for 30 min in the dark. Then, the absorbance at 517 nm was measured using a UV-Vis spectrophotometer. The DPPH scavenging activity (%) was calculated using Equation 1 [11].

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\text{DPPH scavenging activity} = \frac{A_{0} - A_{t}}{A_{0}} 
\]

(1)

2.7. Statistical analyses
Minitab® Ver.18 was used to calculate the mean and standard deviation for all measurements. The data was subjected to one way analysis of variance (ANOVA) with the significance level of 0.05 [15]. The data were presented as mean values ± standard deviation (n=3).

3. Results and discussion

3.1. Effect of particle size on TPC, TFC, and DPPH of *C. nutans* extract
PHWE was carried out on 2 g of *C. nutans* leaf, stem, or mixture samples at a temperature of 120 °C for 20 min with 50 ml of distilled water as the solvent. Figure 1 A-B shows the effect of particle size on the total phenolic compounds (TPC) and total flavonoid compound (TFC) of the leaf, stem and mixture extracts.

The results show that TPC and TFC are inversely proportional to particle size. Smaller particle size results in better yields of phytochemicals because the higher surface-area-to-volume ratio allow better contact between the solvent and plant material during extraction. The TPC and TFC in *C. nutans* extracts is the highest when it is from samples with the smallest particle size (<63 μm). The TPC and TFC yield from the leaf sample is also significantly higher compared to that from the stem and mixture samples. This is because the leaves are also the site of synthesis of phenolic and flavonoid compounds and therefore they are expected to contain a higher concentration of these compounds.

As reference, Bakar et al. [16] found that extracts from different parts of *Mangifera pajang* and *Artocarpus odoratissimus* have different levels of the bioactivities and chemical compounds present and Ismail et al. [17] also obtained different TPC and TFC values for extracts from different parts of *Strobilanthes crispus*.

Figure 1C shows the effect of particle size on the DPPH scavenging activity of the leaves, stems, and mixture from *C. nutans* extract. As particle size increases, the DPPH scavenging activity decreases. Also, the DPPH scavenging activity of *C. nutans* leaf extract is significantly higher than that for the stem and mixture extracts. The high DPPH scavenging activity of the leaves may be
attributed to the presence of high amounts of phenolics and flavonoids which contribute antioxidant activity.

To conclude, the smallest particle size is preferable to get the highest yield of TPC, TFC, and DPPH scavenging activity. The results were analyzed using analysis of variance (ANOVA) with $p < 0.05$ and were found to be statistically significant. Samples of particle size <63 μm is used in all subsequent experiments.

**Figure 1.** Effect of particle size on A) total phenolic compounds, B) total flavonoid compound, C) DPPH scavenging activity of different part of *C. nutans* extract; leaf ( ), stem ( ), mixture ( ). Error bars represent standard deviation from measurements ($n = 3$) and different lower case letters mean they are statistically different ($p < 0.05$).

### 3.2. Effect of solvent to sample ratio on TPC, TFC, and DPPH of *C. nutans* extract

Figure 2A-B shows the effect of solvent-to-sample ratio on the extraction yield of TPC and TFC from the leaves, stems and mixture of *C. nutans*. The yield of phenolic and flavonoids increases with solvent-to-sample ratio. Using a higher solvent to solid ratio would increase extraction efficiency. This is because the concentration gradient between the solvent and sample only decreases slowly as the extraction process continue to run due to the higher volume of solvent used and as a result, the mass transfer rate from the sample to the solvent also only decreases slowly and the overall extraction rate is
higher [18]. The best solvent-to-sample ratio is found to be 50:2 (v/w) which is also the maximum ratio used in this study. If the solvent-to-sample ratio is low, the solvent will quickly become saturated, resulting in decreased TPC and TFC yields. The DPPH scavenging activity is also found to increase with solvent-to-sample ratio (Figure 2C) with the ratio of 50:2 (v/w) resulting in the best % activity in all extracts. Moreover, it is observed that extracts from leaves have the highest rate of DPPH scavenging activity as well. Extracts obtained from leaf samples with a solvent-to-sample ratio of 50:2 have the highest TPC and TFC content which are responsible for its high antioxidant activity. The results were analyzed using analysis of variance (ANOVA) with p < 0.05 and were found to be statistically significant. The solvent-to-sample ratio of 50:2 (v/w) is used in all subsequent experiments.

Figure 2. Effect of solvent-to-sample ratio on A) total phenolic compounds, B) total flavonoid compound, and C) DPPH scavenging activity of different part of *C. nutans* leaf ( ), stem ( ), and mixture ( ) extracts. Error bars represent standard deviation from measurements (n = 3) and different lower case letters mean that they are statistically different (p < 0.05).

3.3. Effect of sample weight on TPC, TFC, and DPPH scavenging activity of *C. nutans* extract
Figure 3A-C shows the effect of sample weight on the TPC, TFC and DPPH activity of *C. nutans* leaf, stem, and mixture extracts using PHWE. The results were analyzed using analysis of variance.
(ANOVA) with $p < 0.05$ and were found to be statistically significant. The best sample size to use is 2 g of *C. nutans* leaves.

The results show that the phytochemical yield increases with weight of the sample and reach maximum with 2 g and slowly decline afterward. A higher sample weight means a higher sample volume and therefore higher surface area that is in contact with the solvent; and that there is simply more material to extract the phytochemicals from. Phytochemicals would then be extracted at a higher rate to a higher yield. Again because of the TPC and TFC are highest for the extract from the leaf sample of 2 g and because they contribute to antioxidant activity, the DPPH scavenging activity would be the highest which is exactly what was observed. The decline in TPC, TFC, and DPPH scavenging activity when the sample weight is more than 2 g is not fully understood and warrants further investigation in future works. It is expected that TPC, TFC and DPPH scavenging activity reach its maximum and saturated when sample weight is more than 2 g due to the constant amount of solvent used.

![Figure 3. Effect of sample weight on A) total phenolic compounds, B) total flavonoid compound, C) DPPH scavenging activity of different part of *C. nutans* extract; leaves ( ), stem ( ), mixture ( ). Error bars represent standard deviation from measurements in triplicate (n = 3).](image-url)
values followed by different letters were statistically different (p < 0.05).

Our results exhibit that *C. nutans* is promising sources of natural antioxidants. A significant correlation between antioxidant properties and total phenolic and flavonoid compounds were found, indicating that compounds were the major contributor to the antioxidant properties of this plant extract. Antioxidants are widely used in the food industry to prevent or delay the oxidation of fats and oils. The global trend to avoid or reduce the use of synthetic additives, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), has resulted in a need to identify natural alternative sources of food antioxidants [19]. Therefore, there is a growing interest in the use of natural antioxidants in the food industry, not only for application as preservatives but also for the potential benefits to human health.

4. Conclusion

The present study outlines the effects of particle size, solvent-to-sample ratio, and sample weight to determine the optimum extraction parameters of *C. nutans* using PHWE. Among the samples tested, *C. nutans* leaf extracts contain significantly higher amount of TPC and TFC and therefore better DPPH scavenging activity compared to the stem and mixture extracts. The suggested optimum extraction parameters for all the samples are particle size of <63μm, solvent-to-sample ratio of 50:2 (v/w) and weight of 2 g. Further confirmation of the optimum conditions for the extraction of *C. nutans* using PHWE are suggested to be carried out either using Design of Experiment or the kinetic extraction approach. Utilization of plant extract as an alternative to conventional drugs and synthetic products contribute to increase interest in research and industrial application of medicinal plants.

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