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Comparative Study on the Extraction Methods of Clinachanthus nutans for Total Phenolic Content, Total Flavonoid Content and DPPH Scavenging Activity

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ABSTRACT

Clinacanthus nutans, which belongs to the family of Acanthaceae is often used as herbal remedies in complementary and alternative medicine. This study was conducted to determine the total phenolic content, total flavonoid content and the DPPH scavenging activity of the different parts of C. nutans (stem, leaves, and mixture of both stem and leaves) using different type of solvents, which is absolute methanol, 80% of methanol and distilled water on different extraction methods. The total phenolic content, total flavonoid content and DPPH scavenging activity were evaluated using the Follin-Ciocalteu, aluminium chloride and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay respectively, and correlation of the three responses were analyzed using Principal Component Analysis. From the results, it can be concluded that regardless the extraction method used, 80% methanol is the most potential solvent to be used in the extraction of C. nutans compared to absolute methanol and distilled water. However, the potential of water as the extraction solvent can be exploited with the used of pressurised hot water extractor. Leaves and mixture of C. nutans have a similar potential; both contain high TPC and TFC but lower DPPH activity. On the other hand, stem of C. nutans has high DPPH activity but lower TPC and TFC.

Keywords: Antioxidant, Belalai Gajah, *Clinachanthus Nutans*, DPPH Scavenging Activity, Total Chlorophyll, Total Phenolic Content.

1. INTRODUCTION

Clinicanthus nutans locally known as Snake Grass (English), Belalai Gajah (Malay), Phaya Plong Tong (Thai) is a species of plant in Acanthaceae family. It has been used in traditional medicine and reported to be used for cancer prevention and treatment (Yong *et al*., 2013). In addition, this herb also exhibits an excellent and rapid-acting anti-inflammatory activity which makes *C .nutans* become a topical product for the relief of minor skin inflammation and insect bites. Generally, it was utilized as a herb to cure snake chomp (Sakdarat *et al*., 2009). In traditional ways, *C. nutans* can either brew as a tea or blend the leaves to extract its juice. By using modern technology, more *C. nutans* extract can be obtained by various extraction methods.

Extraction as the term used pharmaceutically involves the separation of medicinally active portions of plant or animal tissue from the inactive or inert components by using selective solvents in standard extraction procedures. The products obtained from the plants are relatively impure liquids, semisolids or powders intended only for oral or external use (Ncube *et al*., 2008). Recently, many new techniques of extraction have come up. The extractions that have been used are maceration with cold and hot solvent, ultrasonic-assisted extraction, Soxhlet extraction,

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microwave-assisted extraction and pressurised liquid extraction (Anand *et al*., 2005). All these extraction techniques are influenced by varies parameters such as temperature, time and pressure and solvent used. Temperature is the essential parameter that needed to be considered. The utilization of high temperature in plants extraction of the plant sample is generally expected to prevent the compounds from deteriorating due to moisture content or existing enzyme, bringing about extended time span or higher movement of the active compound (Nantitanon *et al*., 2010).

Furthermore, besides temperature, the solvent is another important parameter that needed to be considered in extraction. Among the pure solvents, methanol was the most efficient solvent (Boeing *et al*., 2014) for extraction of antioxidant compounds, followed by water, ethanol and acetone. Methanol is the most efficient solvents for the extraction due to the better solvation of antioxidant compounds present in the medicinal plant as a result of interactions (hydrogen bonds) between the polar sites of the antioxidant molecules and the solvent. Ethanol was less efficient in the extraction of antioxidant compounds than methanol, regardless of the fact that their polarities were comparative. This may be due to the low solvation gave by ethanol, probably because of the presence of the ethyl radical that is longer than the methyl radical present in methanol, resulting in a lower solvation of antioxidant molecules (Boeing *et al*., 2014).

Antioxidant compounds in food play an important role as a health protecting element. The benefits of antioxidants are very important to good health and have been suggested that antioxidant diminish the danger for chronic diseases including cancer and heart disease. Essential sources of normally happening antioxidant are entire grains, fruits and vegetables. Plant-sourced nutrition antioxidant like vitamin C, vitamin E, carotenes, phenolic acids and phytoestrogens have been perceived as having the possibility to diminish disease risk. A large portion of the antioxidant compound in a normal eating routine obtains from plant sources and have a place with different classes of the compound with a wide mixture of physical and chemical properties. A few compounds, for example gallic acid have strong antioxidant activity, while others, for example, the mono-phenols are weak antioxidant (Miller, *et al*., 2000).

The extraction methods play an important role to ensure high quantity and quality of an extract. Lots of studies have been carried out to extract *C. nutans* using a different method of extraction such as infusion, maceration, digestion, decoction, percolation, soxhlet extraction, counter-current extraction, ultrasound-assisted and supercritical fluid extraction. All these methods are varying in extraction parameters such as solvent, temperature and pressure. Most of the studies conducted so far focus on the ability of the *C. nutans* extract on bioactivity potential such as anti-cancer, antioxidant, anti-viral but not on the extraction method itself. There is a very limited study conducted on the efficiency of extraction method to extract different part of *C. nutans* for antioxidant activity. Therefore, this study was conducted to compare the ability of various extraction methods to extract different part of *C. nutans* for its antioxidant activity.

2. MATERIAL AND METHODS

2.1 Standards and Solvents

Standards of the gallic acid and quercetin were purchased from Sigma (Malaysia). DPPH, Folin-Ciocalteu reagent and all other solvents of analytical grade were purchased from Merck (Malaysia). Aluminium chloride-6-hydrate, sodium nitrate and sodium carbonate were purchased from HmbG (Germany).

2.2 Plant Sampling and Preparation

C. nutans plant was collected from Kampung Wang Tepus, Jitra, Kedah. The plant was washed under running tap water and divided into three parts; leaves (L), stem (S) and a mixture of both leaves

and stem (M). Then, it was dried and ground using a mechanical grinder (Mill Powder Tech, Taiwan) into a fine powder before use for the extraction process.

2.3 Plant Extraction

The extraction of the plants was carried out using different type of solvents, which is absolute methanol, 80% of methanol and distilled water on different extraction methods.

2.3.1 Cold Maceration

About 2 g of ground *C. nutans* was soaked in 20 mL of solvents in a universal bottle. The mixture was left at room temperature for 20 min. The extract solution was filtered by using Whatman No. 1 filter paper and kept at 4˚C until further used.

2.3.2 Hot Maceration

About 2 g of ground *C. nutans* was soaked in 20 mL of solvents in a universal bottle. The mixture was incubated in water bath at 60˚C for 20 min. The extract solution was filtered by using Whatman No. 1 filter paper and kept at 4˚C until further used.

2.3.3 Ultrasonic-Assisted Extraction

Approximately 2 g of ground *C. nutans* was added with 20 mL of solvents in a universal bottle and sonicated using Elmasonic X-tra 70H, 3kHz ultra-sonicator. The sample was left for 20 min. The extract solution was filtered using Whatman No. 1 filter paper and kept at 4˚C until further used.

2.3.4 Microwave-Assisted Extraction

About 2 g of ground *C. nutans* was filled with 20 mL of solvents in a round bottle flask. The flask was introduced into the microwave cavity and fitted with a condenser. A focused-type, open-vessel microwave system (Star System 2, CEM Matthews, USA) operating at 800 W maximum power with a frequency 2.45 GHz was used. The microwave power applied was intermittent with power on for 20 min. The extract solution was filtered by using Whatman No. 1 filter paper and kept at 4˚C until further used.

2.3.5 Pressurized Hot Water Extraction

About 2 g of ground *C. nutans* was filled with 20 mL of water in a vessel and attached to the Pressurized Hot Water Extractor. The extraction condition was set up at a temperature of 121 °C, the pressure of 4 bars and extracted for 20 min. The extract solution was filtered by using Whatman no. 1 filter paper and kept at 4˚C until further used.

2.3.6 Soxhlet Extraction

Solvent extraction was carried out in a Soxhlet apparatus for 6 hr using 150 mL of solvents over about 2 g of ground *C. nutans*. Afterward, extracts were subjected to rotary vacuum evaporation at 40 °C to remove the solvent. The extract solution was kept at 4 °C until further used.

2.4 Total Phenolic Content (TPC)

The total phenolic content was measured using Folin-Ciocalteu colorimetric method (Mustapa *et al*., 2015). Plant extract 0.1 mL was mixed with 0.2 mL of Folin-Ciocalteu reagent and 8 mL of water and incubated at room temperature for 3 min, following the addition of 1 ml of 20% sodium carbonate to the mixture. The mixture was incubated at room temperature in the dark for the development of color for 30 min. The absorbance was measured at 765 nm using a

spectrophotometer (Helios Zeta, Thermo Scientific, USA). The total phenols concentration was expressed in terms of gallic acid equivalents (GAE), based on the standard curves.

2.5 Total Flavonoid Content

The total flavonoid content of the *C. nutans* was determined by using aluminium chloride ($AlCl_3$) (Vijayalaxmi *et al*., 2015). About 0.5 mL of the extracts was added with 2 mL distilled water and mixed well with 5% of 0.15 mL sodium nitrate ($NaNO_2$). After reacting for 5 min, 0.15 mL of 10% aluminium chloride-6-hydrate ($AlCl_3.6H_2O$) solution was added. After another 5 min, 1 mL of 1 M sodium hydroxide (NaOH) was added. The reaction solution was well mixed, then, kept for 15 min and the absorbance was determined at 415 nm using UV-VIS spectrophotometer. Quantification was done using the quercetin as the standard and the results were expressed in mg QUE/g DM.

2.6 DPPH Scavenging Activity

Approximately, 200 µL of the extract was mixed with 2.5 mL of 60 µM ethanolic DPPH solution. Control was prepared to contain 200 µL of ethanol and 2.5 mL of 60 µM ethanolic DPPH solution. The mixture was mixed thoroughly and incubated for 30 min in dark condition. Then, the absorbance was measured at 517 nm by using spectrophotometer (Helios-Zeta Thermo Scientific, United State). The DPPH scavenging activity (%) was calculated as Eq. 1.

$$DPPH\ scavenging\ activity\ (\%) = \left(1 - \frac{Absorbance\ of\ sampe\ at\ 517\ nm}{Absorbance\ of\ control\ at\ 517\ nm}\right) x\ 100 \quad (1)$$

2.7 Principal Component Analysis

Correlation between TPC, TFC and DPPH activity were analysed using Principal Component Analysis from Minitab® 17 software.

3. RESULTS AND DISCUSSION

3.1 Total Phenolic Content

Fig. 1 shows the TPC in the various part of *C. nutans* extracted with different solvents and extraction methods were varies from 4-19 mg GAE/g DM. TPC in the *C. nutans* extracts reported to be in the range of 7-13 mg GAE/g DM (Mustapa *et al*., 2015). Therefore, in this study, extracts containing 15 mg GAE/g DM and above were considered to have a high content of TPC. Among all the extracts, most water and 80% methanol extracts of the leaves and mixture contain high TPC content except for hot maceration and microwave-assisted extraction compared to the stem. This phenomenon can be explained by the nature of the solvent used in the extraction, most phenolic compounds are highly attracted to a highly polar solvent such as water and 80% methanol (Oszoy., 2008)

Interestingly, all water extracts of the stem contain low TPC compared to leaves and mixture extracts except for PHWE. A study conducted on TPC for *Momordica chantia* (Kubola *et al*., 2008), nettle (Otles *et al*., 2012) and *Zingiber officinale* (Ghasemzadeh *et al*., 2010) also in agreement with this study which found that TPC in the stem was significantly low compared to the leaves. However, all the samples have a significantly high TPC when extracted with PHWE. This suggested that high temperature with pressure preserved the phenolic compounds from being degraded (Jamial, 2010).

Comparing the extraction methods employed, regardless the solvent used, hot maceration, microwave-assisted extraction and soxhlet extraction were not favourable method to extract total phenolic from *C. nutans*.

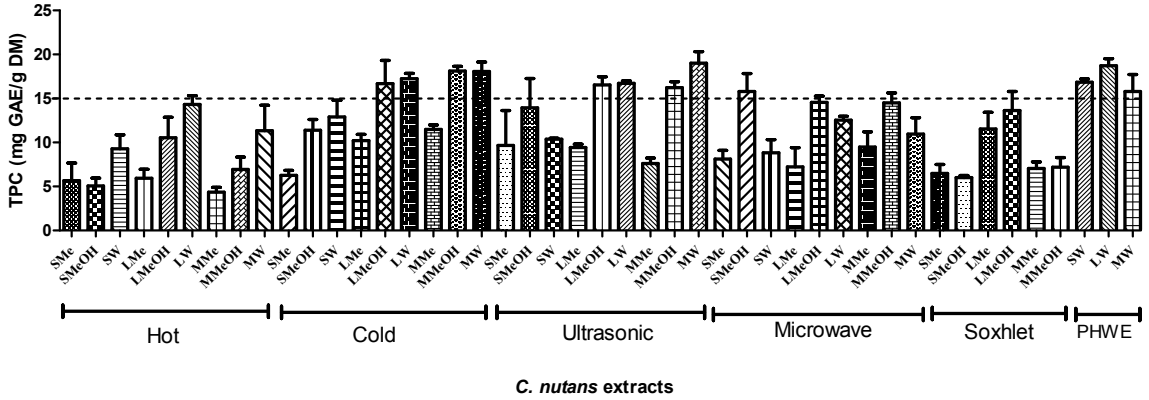

Figure 1. Total phenolic content (mg GAE/g DM) of various part of *C. nutans* extracts with the different type of solvents. Values were expressed as Mean ± SEM of three determinations where S=stem, L= leaves, M=mixture, Me= absolute methanol, MeOH=80% methanol and W= water.

3.2 Total Flavonoid Content

TFC in the *C. nutans* extracts were reported to be in the range of 6-17 mg Que/g DM and methanol was the best solvent to extract TPC compared to hot water extraction (Kuen, 2013). In this study, TPC was recorded were in the range of 3.65-18.07 mg Que/g DM. The lowest TFC was in the mixture of *C. nutans* extracted by hot maceration using water while the highest TFC was in the mixture of cold maceration extracted with 80% methanol and water (18.07±0.90 and 18.07±1.40 mg Que/g DM respectively).

Extracts, which contain TPC with a concentration of 15 mg GAE/g DM and above were considered to have high TPC. From the results, it can be concluded that cold extraction was the best method to extract TFC in *C. nutans.* Leaves and mixture of *C. nutans* contain higher TPC compared to the stem. This result was in agreement with a study conducted by Fong (2015) which found that cold aqueous extraction of *C. nutans* leaves had the highest TPC and suggested that cold extraction with water is the best method to prepare a crude extract of *C. nutans* leaves with maximum phenolic and flavonoids.

As for TPC, the TFC content in PHWE extracts also contain high TFC content except for stem extract. This suggested that increasing temperature with pressure not only increases phenolic compounds, it also increases the flavonoid compounds to be extracted.

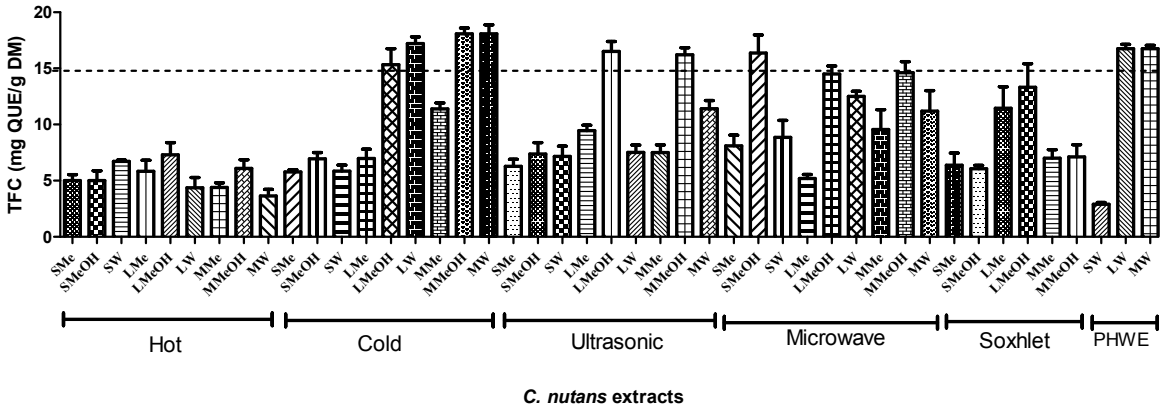

Figure 2. Total flavonoid content (mg GAE/g DM) of various part of *C. nutans* extracts with the different type of solvents. Values were expressed as Mean ± SEM of three determinations where S=stem, L= leaves, M=mixture, Me=absolute methanol, MeOH=80% methanol and W=water.

3.3 DPPH Scavenging Activity

DPPH scavenging activity of the *C. nutans* extracts was shown in Fig. 3. It can be observed that regardless the extraction method used, water extracts have the lowest DPPH activity compared to methanol and 80% methanol except for PHWE. Methanol and 80% methanol extracts were almost similar in the extraction capability for DPPH activity. Among all the extracts, 16 extracts have a strong scavenging activity with the scavenging activity ≥ 80% as suggested by Vimala *et al*.(2003).

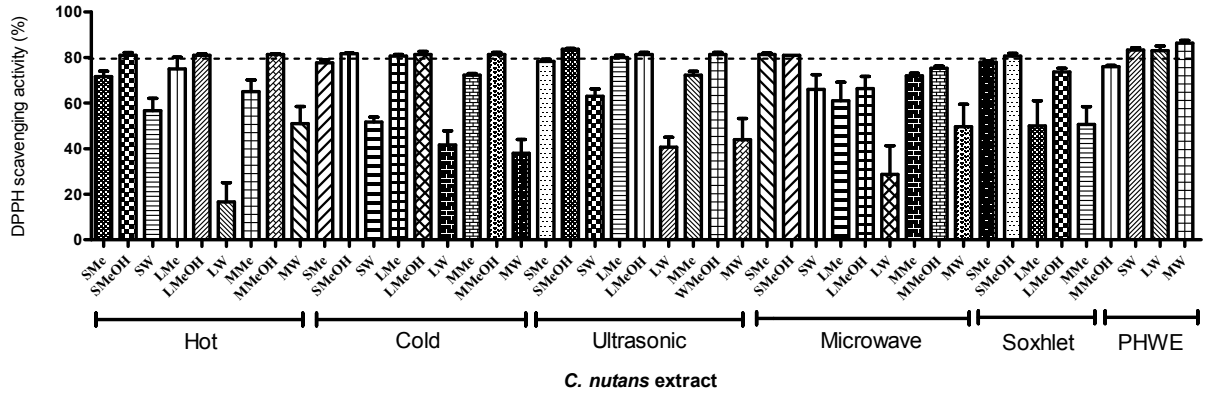

Figure 3. DPPH scavenging activity of various part of *C. nutans* extracts with a different type of solvent. Values were expressed as Mean ± SEM of three determinations where S=stem, L= leaves, M=mixture, Me=absolute methanol, MeOH=80% methanol and W=water.

3.4 Correlation between TPC, TFC and DPPH of the *C. nutans* extracts

The principal component analysis was carried out to describe the correlation between TPC, TFC and DPPH activity of the extracts. Only selected data were used in the analysis. The data must at least have one of the criteria; TPC ≥ 15 mg GAE/g DM, TFC ≥ 15 mg Que/g DM and/or DPPH activity ≥ 80%. These values were selected from the literature review as recommended as average content of TPC and TFC in *C. nutans* extract (Mustapa *et al*., 2015; Kuen, 2017) and for DPPH activity, it was referred as strongly scavenged when scavenging activity ≥ 80% (Vimala *et al*., 2003). Means of all data were plotted against the first two components (Fig. 4). Correlation of the attributes generated from the data of the first component, accounting for 63.5% of total variation, separated stem extracts from leaves and mixture extracts. The second component, accounting for 26.9% of total variation, separated the leaves and mixture extracts. Regardless of the extraction method used, the samples were discriminated into three groups based on the solved and part of the plant used. Most of the water extracts were in the same group except for pressurized hot water extracts. All the samples in this group have the lowest TPC, TFC and DDPH activity. The second group consisted of all the 80% methanol extracts of the stem except for few exceptions. Interestingly stem extracts of PHWE also belong to this group. Extracts in this group have higher DPPH activity but lower TPC and TPC compared to the other groups. The third group consisted of most of 80% methanol extracts of the leaves and mixture of *C. nutans* and also the pressurized hot water extract of leaves and mixture. This group contains high TPC and TFC but lower DPPH activity.

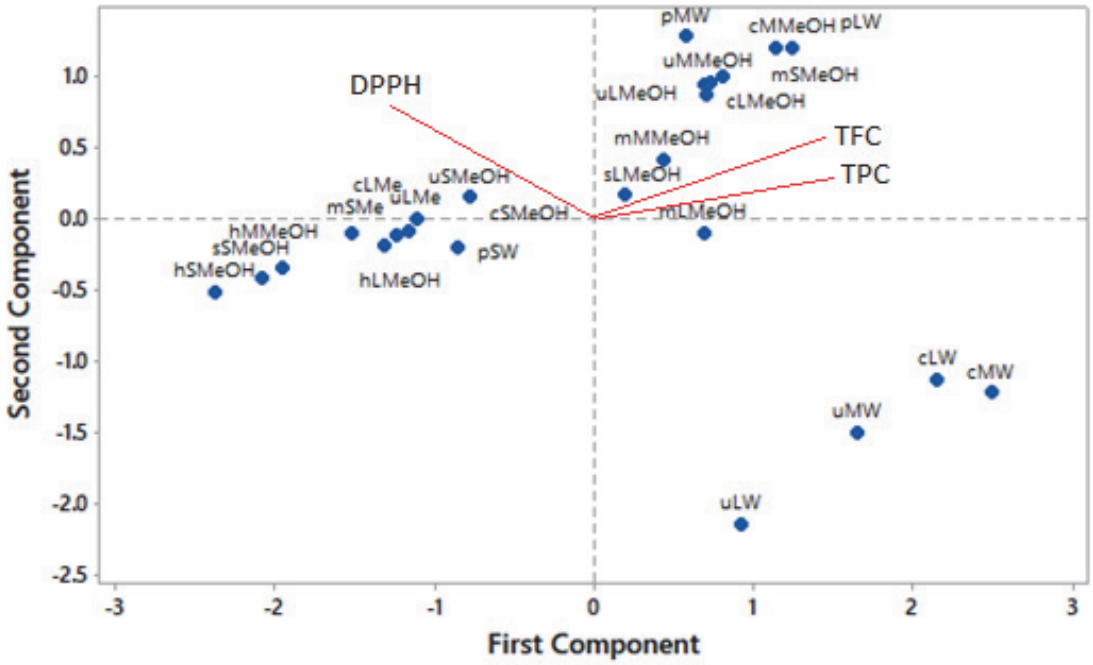

Figure 4. Principal component analysis of the *C. nutans* extracts for TPC, TFC and DPPH scavenging activity.

4. CONCLUSION

It can be concluded that regardless of the extraction method used, 80% methanol is the most potential solvent to be used in the extraction of *C. nutans* compared to absolute methanol and water. However, the potential of water as the extraction solvent can be further exploited with the used of pressurised hot water extractor. Leaves and mixture of *C. nutans* have the similar potential; both contain high TPC and TFC but lower DPPH activity. On the other hand, stem of *C. nutans* have high DPPH activity but lower TPC and TFC.

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