Evaluation of Antioxidant Activity of Clinacanthus nutans (Acanthaceae)

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ABSTRACT

Clinacanthus nutans is listed as one of the five medicinal plants in the National List of Essential Drugs A.D. in 1999. It is nowadays widely used for consumption in daily diet and in folk medicine. The central objective of the current research is to evaluate the antioxidant activities of ethyl acetate and methanol extract from Clinacanthus nutans' leaves, stems and roots. Antioxidants are reported to prevent oxidative damage caused by free radical and can be used in the prevention of cardiovascular and anti-inflammatory diseases. The total flavonoid contents are detected using 10% aluminium chloride and expressed as catechins equivalents (CE). Meanwhile, the total phenolics were determined using Folin-Ciocalteu assay and expressed as gallic acid equivalent (GAE). As a result, the ethyl acetate root extract was found to possess the highest total phenolic content (29.333±1.701 mg GAE/g) while the ethyl acetate leaf extract had the highest total flavonoid content (33.600±1.442 mg CatE/g). The methanol leaf extract possessed the highest free radical scavenging activity, with the lowest IC50 of 1.203 ± 0.0898 mg/ml. This study suggests that Clinacanthus nutans could be considered as a significant source of natural antioxidant.

Keywords: Clinacanthus nutans, Antioxidant activity, Acanthaceae

INTRODUCTION

Malaysia possesses great biodiversity in terms of its flora species. The country is rich with numerous medicinal plants with high medicinal values and is well-known as one of the 12 mega-biodiverse countries in the world. Undoubtedly, by consuming fruits, vegetables and other derivative from plants gives a lot of benefits to human health due to the contribution of the bioactive molecules. Plants are considered as the main source to get bioactive compounds and they are able to synthesize a variety of low molecular weight compounds called
secondary metabolite. The phenolic and flavonoid compounds are a major group of phytochemicals which show oxidative effect (Pietta, 2000). Therefore, it is worthwhile to study the local traditional medicinal plant in the tropical rainforest in Malaysia.

The presence of reactive oxygen species (ROS) can increase the oxidative stress in human body that cause the chronic illness such as asthma, diabetes, cardiovascular disease and cancers (Paravicini and Touyz, 2008; Klaunig and Kamendulis, 2004; Zhang et al., 2009). Free radicals are molecular species that have a single unpaired electron in the outermost shell. The free radicals that have gained most attention in the biological system are superoxide anion radical, hydrogen peroxide, oxygen singlet, hydroxyl radical, hypochlorite, peroxynitrite anions, alkylperoxyl, and nitric oxide radical that can cause oxidative damage in living body (Wang et al., 2002). The formation of ROS in the human body is due to the daily inhalation of oxygen from atmosphere (Tai et al., 2011). There are two effects of ROS on the human body in which it is involved in the immune system to kill pathogen, as well as it can trigger chronic diseases mentioned previously.

We can protect our body from excessive production of oxidants by consuming antioxidant supplements (V'agi et al., 2005), because antioxidant can prevent oxidative damage by free radicals that induce oxidative stress (Ozsoy et al., 2008). During the extensive production of oxidants, it causes the imbalance between oxidant and antioxidant; thus, the body is no longer secured from tissue injury through oxidative damage to the macromolecules like proteins, lipids and nucleic acids (Naqvi et al., 2013). According to the Ozsoy et al. (2008), polyphenol is an effective antioxidant compound that has been tested in vitro for free radical scavenging activity and is comparable to tocopherol and ascorbate. The roles played by polyphenol as antioxidant are they possess high hydrogen reactivity as electron donor, function as chain-breaking where polyphenol derived radical is able to stabilize and delocalize the lonely electron and it is also able to terminate the Fenton reaction by chelating transition metal ions (Rice-Evans et al., 1997).

The increasing popularity and demand to obtain natural antioxidants from herbal products has spurred researchers to look into the value of medicinal plants. Clinacanthus nutans has been chosen in this study since it has gained public interest recently with a claim that can be used to cure cancer disease. It is a medicinal herb belonging to the family of Acanthaceae, which appeared as a small shrub, native to Asia and often cultivated (Sakdarat et al., 2009). Yet, it
has been listed as one of five medicinal plants in the National List of Essential Drugs A.D. in 1999 (Shuyprom, 2004). To the best of our knowledge, there is no such previous study on the exploration of antioxidant activity of *C. nutans* whole plant.

**MATERIALS AND METHODS**

**Materials**

1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany), Folin-Ciocalteu (Merck, Germany) sodium hydroxide (R&M Chemicals, United Kingdom), sodium nitrite (System, Malaysia), aluminium chloride hydrated (Techno Pharchem, India), dimethyl sulfoxide (DMSO) (J. T. Baker, USA), sodium carbonate (System, Malaysia), gallic acid (Merck, Germany), butylated hydroxytoluene, BHT (Sigma-Aldrich, Germany), (+)-catechin (Sigma-Aldrich, Germany), ascorbic acid and α-tocopherol were the chemical reagents used for the assay tests. Solvents for extraction were methanol and ethyl acetate (Analytical grade) obtained from Fisher Chemical, UK. Measurements of absorbance were done using a Thermo Scientific Genesys 20 Visible spectrophotometer.

**Collection and preparation of plant material**

Fresh *Clinacanthus nutans* plants were purchased from Pasar Tamu Kolombong, Sabah, Malaysia. The samples were washed thoroughly in running tap water to remove soil particles and washed with sterile distilled water for a final cleansing. Identification of the plants material was carried out by a botanist, Dr. Berhaman Ahmad from the School of International Tropical Forestry, Universiti Malaysia Sabah. The plant specimen (Voucher no: ACCN001/13) of the collected plant materials was deposited at the Herbarium of School of International Tropical Forestry, of the same department. The whole plants were divided into leaf, stem and root parts and dried overnight in room temperature. The plants were then further freeze-dried for three days to make sure the plants have dried completely and ground into fine powder. The powdered samples were stored in blue capped bottle and keep in -80°C freezer until use for analysis.

**Plant sample extraction**

Ten grams of powdered samples were extracted with 100 ml methanol and ethyl acetate, respectively, using maceration technique in incubator shaker for three days at 180 rpm, room temperature. The extracts were then filtered through a Whatman filter paper with 0.45 µm pore size and evaporated under rotary evaporator at low pressure to get dried samples. The dried samples were then stored at -4°C until further use.
Determination of Total Phenolic Compounds (TPC)
Determination of total phenolic compounds is conducted according to Slinkard and Singleton (1977) and Ozsoy et al. (2008). Total soluble phenolic in methanol and ethyl acetate extracts of C. nutans were determined by using Folin-Ciocalteu reagent. Aliquots (0.1 ml) of the plants extracts with concentration range (5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml) were transferred into the test tubes and made up with 4.6 ml of the distilled water. After that, 0.1 ml of the Folin-Ciocalteu reagent, where it is previously diluted 3-fold with distilled water, were added with 0.3 ml of the 2% Na₂CO₃ solution, and the tubes were vortexed and allowed to stand for 2 hours before reading absorbance at 760 nm by using UV-Vis Spectrophotometer against a blank containing 0.1 ml of the extraction solvent. In order to get the amount of total phenolic compounds, the gallic acid standard solution’s calibration curve were first plotted covering the concentration range of between 0.05 mg/ml and 0.4 mg/ml. Thus, the total phenolic compounds was calculated as mg gallic acid equivalents (GAE) from the gallic acid calibration curve and expressed as mg gallic acid/g dry weight (DW) of the plant samples. Each sample was carried out in triplicate analyses and the date was presented as average ± SD of the three absorbance reading.

Determination of Total Flavonoid Content (TFC)
Total flavonoid content was measured by using the method described by Sakanaka et al. (2005) and Ozsoy et al. (2008). Aliquots (0.25 ml) of the plant extracts with concentration range from 0.625, 1.25, 2.5 and 5 mg/ml or (+)-catechin standard solution (15, 31, 62.5, 125, 250 µg/ml) was mixed with 1.25 ml of distilled water in a test tube. After that, 75 µl of 5% (w/v) sodium nitrite solution was also added into the tube. After 6 min, it was followed by the addition of 150 µl of a 10% (w/v) aluminium chloride and the mixture was allowed to stand for another 5 min before 0.5 ml of 1M NaOH was added. Before taking the absorbance reading at 510 nm, the mixture was made up to 2.5 ml with distilled water and vortexed. The reading was taken immediately after mixing. The mean (±SD) result of triplicate analyses was expressed as mg of (+)-catechin equivalents of total extractable compounds.

DPPH Radical Scavenging Activity
The DPPH radical scavenging activity was determined by using the method described by Brand-Williams et al. (1995) and Ozsoy et al. (2008). Aliquots (0.1 ml) of each extract (0.0625, 1.25, 2.5, 5 mg/ml), gallic acid (0.016, 0.032, 0.0625, 0.125 mg/ml) and BHA (0.016, 0.032, 0.0625, 0.125, 0.25, 0.5 mg/ml), α-tocopherol (0.016, 0.032, 0.0625, 0.125 mg/ml) and ascorbic acid (0.016, 0.032, 0.0625, 0.125 mg/ml) in DMSO was added with 3.9 ml of 6x10⁻³ M
methanolic solution of DPPH. The mixture was vortexed and allowed to stand in dark condition at room temperature for 30 min. The absorbance of the solution was then measured spectrophotometrically at 517 nm against methanol. The extracts were measured in triplicates and the average absorbance reading was taken. Besides that, two controls were used throughout the analyses i.e. negative control (containing all reagents except the test sample) and one positive control (using the reference antioxidants). The ability to scavenge DPPH radical was calculated by using the following equation:

\[
DPPH \text{ radical scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}}\right) \times 100\%
\]

**RESULTS AND DISCUSSION**

**Extraction method**

Maceration technique was applied for the extraction of *C. nutans* with methanol and ethyl acetate in this study since it is cost-effective, fast and simple and can enhance the selectivity during extraction (Tiew et al., 2014). However, the ratio between dry sample and solvent must be enough in order for successful extraction of the samples.

**Total Phenolic Content and Total Flavonoid Content**

The content of extractable phenolic compounds in extracts were determined from regression equation of calibration curve \( y=0.9329+0.0011, R^2=0.9966 \). The phenolic content was determined from the regression equation and expressed as gallic acid equivalent (GAE mg/g DW) which varied between 14.600 to 29.333 mg/g DW. These results were compared with the previous study carried out by other researchers. The result given in Table 1 showed that ethyl acetate root extract possessed the highest total phenolic content (29.333±1.701 mg/g DW). The difference in TPC between different parts of *C. nutans* had been reported by Lee et al. (2014). Hence, it is not surprising that different organs of the plant have different levels of the bioactivities or chemical constituents present which was reported by the study conducted by Abu Bakar et al. (2009) for *Mangifera pajang* and *Artocarpus odoratissimus*. Overall, the amount of total phenolic shown in table was higher than total flavonoid content. This is because flavonoid is sub-class of phenolic (Tiew et al., 2014).
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The content of extractable flavonoid content in extracts were also determined from regression equation of calibration curve $y=6.6883x-0.0038$, $R^2=0.999$ and expressed as catechin equivalent (CatE mg/g DW) which varied between 2.133 to 33.600 mg/g DW. Ethyl acetate leaf extracts give surprisingly higher of total flavonoid content compared to the other extract with 33.600±1.442 mg CatE/g DW. This suggested that ethyl acetate extracts of *C. nutans* contained less polar flavonoids. As stated by Alothman *et al.* (2009), the least polar solvents are considered to be suitable solvent for the extraction of lipophilic phenols and flavonoids compounds. A comparison of the findings reported from previous studies shows differences in the antioxidative activity, total phenolic content and total flavonoid content of the species (Table 2). These contradictory findings may be due to the differences in solvent used, method of extraction, parts of plant being analyzed, even climatic condition and cultural practices, maturity at harvest and storage condition of the plants (Lee *et al.*, 2014). Besides that, Lee *et al.* (2014) also conducted study to evaluate the antioxidant activity of *Strobilanthes crispus* from two different locations, Pusat Pembangunan Komoditi Sendayan, Seremban, Negeri Sembilan and University Agriculture Park (TPU), UPM. It was surprisingly to note that same species under same method extraction and preparation having different TPC value. This is because different environmental or geographical variations (sunlight, water stress intensity of rain, air humidity, temperature) (Ahmed *et al.*, 2012) influenced the plant metabolism which directly affect synthesis and accumulation of primary and secondary metabolism thus give different bioactivity in the plants (Hong *et al.*, 2008).

<table>
<thead>
<tr>
<th>Extract</th>
<th>PC (mg/g DW)</th>
<th>FC (mg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf (MET)</td>
<td>23.133 ± 0.115</td>
<td>6.733 ± 0.115</td>
</tr>
<tr>
<td>Stem (MET)</td>
<td>20.933 ± 2.318</td>
<td>5.267 ± 0.115</td>
</tr>
<tr>
<td>Root (MET)</td>
<td>14.600 ± 0.400</td>
<td>9.200 ± 0.346</td>
</tr>
<tr>
<td>Leaf (EA)</td>
<td>27.800 ± 1.510</td>
<td>33.600 ± 1.442</td>
</tr>
<tr>
<td>Stem (EA)</td>
<td>16.867 ± 0.306</td>
<td>2.133 ± 0.115</td>
</tr>
<tr>
<td>Root (EA)</td>
<td>29.333 ± 1.701</td>
<td>4.200 ± 0.200</td>
</tr>
</tbody>
</table>

### Table 1

Total phenolic content (PC) (as gallic acid equivalents) and total flavonoids content (FC) (as catechin equivalents) of methanol and ethyl acetate extracts from *C. nutans* leaves, stems and roots (5 mg/ml)
The concept of free radical scavenging of a compound is the donation of hydrogen atom of the compound to stabilize the free radical. DPPH method is one of the common methods to evaluate antioxidant activity of extracts because it is a quick, reliable and reproducible method (Koleva et al., 2002). The DPPH free radical scavenging activity was expressed as \( IC_{50} \) value (Table 3). \( IC_{50} \) is defined as the concentration of the antioxidant extract required to scavenge 50% of the DPPH radical. Result of free radical scavenging is presented in Figure 1. The free radical scavenging activity of the extract was lower compared to the positive control using antioxidant reference compound such as ascorbic acid, gallic acid, BHT and \( \alpha \)-tocopherol. The result showed that all parts of the \( C. \) nutans plant exhibited potential antioxidant activity varied from 1.203 to 4.005 mg/ml. The lower the \( IC_{50} \) value, the stronger is the extract to scavenge free radical. The \( C. \) nutans extract showed a moderate scavenging activity with the maximum effect of 1.203±0.090 mg/ml for methanol leaf extract and the potency was approximately 0.06 times of ascorbic acid, 6.5\times10^{-19} \) times gallic acid, 0.07 times \( \alpha \)-tocopherol and 0.12 times BHT. This could be explained that the leaf organ synthesized antioxidant compounds and moved them from leaves to the storage site via xylem and phloem in the stem through long distance translocation (Hartmann, 1996).

### Table 2
Comparison of total phenolic content (PC), total flavonoid content (FC) and \( IC_{50} \) done by previous researchers for \( C. \) nutans

<table>
<thead>
<tr>
<th>Extract</th>
<th>Conc.</th>
<th>PC (mg/g DW)</th>
<th>FC (mg/g DW)</th>
<th>( IC_{50} )</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf (MET)</td>
<td>5 mg/ml</td>
<td>2.68</td>
<td>-</td>
<td>1126.63 ( \mu g/ml )</td>
<td>Lee et al., 2014</td>
</tr>
<tr>
<td>Stem (MET)</td>
<td>5 mg/ml</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
<td>Lee et al., 2014</td>
</tr>
<tr>
<td>Leaf (MET)</td>
<td>10 mg/ml</td>
<td>1.77 ± 0.008</td>
<td>0.04 ± 0.001</td>
<td>-</td>
<td>Ho et al., 2013</td>
</tr>
<tr>
<td>Leaf (MET)</td>
<td>10 mg/ml</td>
<td>0.78 ± 0.005</td>
<td>0.21± 0.005</td>
<td>1.33 ± 0.001 ( mg/ml )</td>
<td>Tiew et al., 2014</td>
</tr>
<tr>
<td>Leaf (ETOH)</td>
<td>-</td>
<td>23.5</td>
<td>-</td>
<td>ND</td>
<td>Yuan et al., 2012</td>
</tr>
<tr>
<td>Leaf (CHLF)</td>
<td>100( \mu g/ml )</td>
<td>-</td>
<td>-</td>
<td>7852.63 ± 449.90</td>
<td>Yong et al., 2013</td>
</tr>
<tr>
<td>Leaf (ETOH)</td>
<td>300( \mu g/ml )</td>
<td>-</td>
<td>-</td>
<td>110.4 ± 6.59 ( \mu g/ml )</td>
<td>Pannangpetch et al., 2007</td>
</tr>
</tbody>
</table>

Note: EA = ethyl acetate, MET = methanol, ETOH = ethanol, CHLF = chloroform, PC = phenolic content, FC = flavonoid content, Conc. = concentration, ND = not detected and DW = dry weight.

### DPPH Free Radical Scavenging
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CONCLUSION

As a conclusion, *Clinacanthus nutans* was found to possess antioxidant activity. The identification of corresponding bioactive compounds that contribute to this bioactivity will be further investigated. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use.

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