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Research article

CHEMICAL COMPOSITION, TOTAL PHENOLICS AND ANTIOXIDANT ACTIVITIES OF CRUDE LEAF EXTRACT FROM *Clinacanthus nutans* (BURM. F.)

Samiaa J. Abdulwahid-Kurdi[1], Nurfazila Binti Saulol[2], Zailina Binti Hashim[3], Annas Bin Salleh[4], Goh YongMeng[5], Mahdi Ebrahimi[5], Falah Hasan Baiee[6], Nur Hafizah Mohammed[7]

[1]Department of General Sciences, Faculty of Education, Soran University, Delyzian, Soran, Erbil, Iraq.
[2]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia.
[3]Department of Environmental and Occupational Health, Faculty of Medicine and Health Sciences, UniversitiPutra Malaysia, 43400 Serdang, Selangor, Malaysia.
[4]Department of Veterinary Laboratory Diagnostics, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[5]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, University Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia.
[6]Department of Clinical Science, Faculty of Veterinary Medicine, University of Kufa, Kufa 54003, Najaf, Iraq.
[7]Livestock Science Research Center, MARDI Kluang, 86000Kluang, Johor, Malaysia
Corresponding author: Samiaa J. Abdulwahid-Kurdi: E-mail: samiaa.abdulwahid@gmail.com

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ABSTRACT

Clinacanthus nutans (Burm. f.), one of the important species in the Acanthaceae family, is utilized as an essential herbal remedy and is located in tropical Asia. This plant contains a wide variety of beneficial chemicals, according to a phytochemical analysis. The objective of this research is to examine the phytochemical phenols, flavonoids, and antioxidant activity of Clinacanthus nutans leaf extract. Total carbohydrates (71.05±0.09%), crude protein (12.32±0.09%), moisture (6.91±0.01%), and fat content (1.01±0.01%) were the main nutrients in the leaf that were measured. At 500, 1000, and 2000 mg/mL, the 2,2′-diphenyl-1-picrylhydrazyl (DPPH)% inhibition of crude methanolic leaf extract was significantly lower than that of ascorbic acid; at the same dosages, the ABST% inhibition of methanolic leaf extract was lower than that of butylated hydroxytoluene (BHT). At a 2000 mg/ml dosage, leaf extract significantly increased total phenol (17.47±0.61 mg GAE/g) and flavonoid (8.23±0.11 mg RU/g) compared to 1000 and 500 mg/ml. It has been reported that 12 phenolic compounds, including 4 phenolic acid compounds, 5 flavonoids, and 3 unknown chemicals, were found using liquid chromatography with mass spectrometry (LC-MS/MS). The results demonstrate that increasing concentrations of methanolic leaf extract improves antioxidant capacity against various reactive oxygen species. Understanding the traditional use of herbal medicines, preserving them for future use, and safeguarding them for good purposes all depend on an understanding of their photochemistry.

1.Introduction

Herbal treatments, which have been used for millennia to treat illnesses and preserve health, depend on valuable plants that may be farmed for food, medicine, and other uses (Hou et al., 2024). Currently, herbal medicines provide primary care for 80% of people in developing countries (Abdulwahid-Kurdi et al., 2023). Diseases that involve the transfer of electrons or hydrogen and produce reactive free radicals are linked to oxidative stress. One of the important qualities of antioxidants is their ability to shield the body from the harmful effects of free radicals. Accordingly, antioxidants have a critical role in maintaining cell integrity and safeguarding metabolic pathways related to lipid, protein, and glucose metabolism (Rajendra et al., 2014). Plants that include high levels of antioxidants—known as phytochemicals have been demonstrated to help prevent and treat several ailments (Nwozo et al., 2023). Phytochemicals typically exhibit biological action within their host plants, supporting the growth of the plant or strengthening its defenses against pathogens, animals, and intruders. Phytochemicals are produced through the primary or secondary metabolism of plants. According to Dula and Zelalem (2018), terpenoids, alkaloids, and phenolic compounds are secondary constituents, while proteins, common sugars, and chlorophyll are the primary ingredients. Synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene are restricted due to potential harm to essential organs (Abdulwahid, 2020). Synthetic medications can have negative effects and are expensive; there is a growing need for alternative treatments, such as herbal cures (Sajjad, 2018). This screening is going to make it easier to identify and assess the various bioactive chemicals present in the plants, potentially contributing to the identification of particular medicinal attributes (Zgórka et al., 2021). According to Qing-hua et al. (2012), knowing the compositions of medicinal plants and related products is crucial to comprehending their nutritional value. Malaysia is endowed with tropical rainforests that are home to a vast array of medicinal plant species, in addition to its high biodiversity. Among them is the well-known Thai medicinal herb, *Clinacanthus nutans*. Mostly found in Sabah, in eastern Malaysia, this plant is also known by its English name, Sabah Snake Grass. Researchers in Malaysia have studied *Clinacanthus nutans* in several ways since the plant's popularity began in 2011 (Roeslan et al., 2012). Phytochemical substances found in *Clinacanthus nutans* have a variety of biological actions, including anti-hyperglycemic and anti-hyperlipidemic properties, and improve sperm quality (Abdulwahid-Kurdi, 2024). However, numerous variables, such as the plant's age, leaf development, and environmental circumstances, can alter the phytochemical concentration in herbs and medicinal plants (Abdulwahid, 2019). For instance, the leaves of *Clinacanthus nutan*s grown at higher elevations with lower air temperatures have a higher total phenolic and flavonoid content compared to those grown at higher air temperatures (Fong, 2015). Recently, many techniques were used to calculate the total flavonoid and phenolic content. It has been established that phenolic compound detection by liquid chromatography with mass spectrometry (LC-MS/MS) is a sensitive and precise method compared to classical techniques (Costa et al., 2015). It is believed that regular evaluations of the polyphenol profile and antioxidant capacity are beneficial for preserving product quality and public health. Thus, the objective of this study is to ascertain the methanolic leaf extract of *Clinacanthus nutans* (MECN) and its proximate analysis, antioxidant activities and polyphenol profiles.

2. Materials and methods

2.1. Plant materials

Fresh leaves of *Clinacanthus nutans* (Burm. f.) Lindau was acquired from a botanical garden in Ladang 10, Universiti Putra Malaysia, Selangor, Malaysia with latitude 2.991299 and longitude 101.708182, as shown in Figure 1. The botanical identity of *Clinacanthus nutans* was characterized by the Phytomedicine

Herbarium, Institute of bioscience, University Putra Malaysia, Selangor (Voucher no. SK2942/15).

Figure 1. Leaves of *Clinacanthus nutans* (Burm. f.).

2.1.1. Proximate analysis

Proximate analysis was conducted on ash, moisture, protein, fat, and carbohydrate contents in the leaf of *Clinacanthus nutans* samples (AOAC, 2005). All tests were performed in triplicates.

2.1.2. Determination of moisture content

Approximately 5 g of dried leaves of *Clinacanthus nutans* samples were weighed using an aluminum dish and placed in an oven at 105˚C overnight according to the AOAC method (2005). The moisture content was calculated as W1 = weight of the wet sample (g), W2 = weight of dry sample (g).

$$Moistuer\ \% = \ 1 - \frac{W2}{W1} \times 100 \quad (1)$$

2.1.3. Determination of ash content

Five grams of dried *Clinacanthus nutans* leaves were burned at 550°C for the entire night in a furnace (Furnace 62700, Barnstead/ Thermolyne, USA). The remaining inorganic material was then cooled down in a desiccator and weighed. Where W2 = weight of ash, W1= weight of sample.

$$Ash\ \% = \frac{W1}{W2} \times 100 \quad (2)$$

2.1.4. Determination of crude protein

The total nitrogen content of *Clinacanthus nutans* leaf was determined using the official method of the Association of Official Analytical Chemists (AOAC, 2005). About 1 g of raw material was hydrolyzed with 15 mL concentrated sulfuric acid (H_2SO_4) containing two copper catalyst tablets (mixture of potassium sulphate and copper sulphate in 10:1 ratio) in a heat block (Kjeltec™ 2200, Auto Distillation Unit, FOSS Tecator, Sweden) for 2 hours at 420˚C. Before neutralization and titration, the solution was cooled down, and H_2O was added to the hydrolysates. The green colored receiver solution was titrated with 0.096 N of hydrochloric acid until the color changed to red. The percentage of nitrogen present in the sample was calculated using the formula below: Where S = volume of used hydrochloric acid for sample, B = volume of used hydrochloric acid for blank, W = weight of sample.

$$\text{Nitrogen}\ \% = \frac{0.1\times(\text{S}-\text{B})\times 14}{\text{W}\times 1000} \times 100 \quad (3)$$

$$\text{Crude protein } \% = \text{ Nitrogen } \% \times 4.4 \quad (4)$$

2.1.5. Determination of crude fats

Approximately 1-2 g of the dried leaves of *Clinacanthus nutans* samples was weighed, wrapped in Whatman filter paper, and placed neatly in a thimble. The thimble was placed on a thimble holder. Then, the sample was covered with a thin layer of cotton. The thimble was moved to thimble support. The weight of the pre-dried extraction cup was measured. About 50 mL of hexane was poured into an extraction cup using a measuring cylinder. The extraction cup was then placed on a cup holder attached to Soxtec™ 2050 automated Analyzer (FOSS Analytical, Denmark) according to the official method of the Association of Official Analytical Chemists (AOAC, 2005). After that, the extraction cup was transferred to an oven and dried at 105 °C for 1 hour. The extraction cup was cooled down in a desiccator to room temperature (25 ± 0.5 °C) and weighed (W3) (AOAC, 1997). The percentage of crude fat in the sample was calculated using the formula below: Where W3 = weight of extraction cup and residue (g), W2 = weight of the extraction cup (g), W1= weight of sample (g).

$$\text{Crude fat } \% = \frac{\text{W3} - \text{W2}}{\text{W 1}} \times 100 \quad (5)$$

2.1.6. Determination of crude carbohydrates

The percentage of crude carbohydrates in the sample was calculated using the formula below:

$$Total\ carbohydrate = 100 - (moisture + protein + fat + ash) \quad (6)$$

2.2. Preparation of methanolic leaf extract of Clinacanthus nutans

With a few adjustments, the extracts were made using Lau et al. (2014)'s methodology. After being thoroughly cleaned under running water, the leaves of *Clinacanthus* nutans were allowed to air dry for a week in the sun. These leaves were ground into a fine powder using an electric grinder (RT-08, Rong Tsong Precision Technology Co., Taiwan) and oven-dried for 24 hours at 40ºC. They were then sealed in an airtight container. One gram of the sample was used for every 20 milliliters of methanol, or a ratio of 1:20 (w/v), to extract the powdered leaves using 80% methanol (by adding 20% of distilled water). After being macerated in methanol for 72 hours, the powdered *Clinacanthus nutans* leaves were shaken with a rotary shaker (Liquid Brushless DC motor clock). Next, cotton wool, Whatman No. 1 filter paper (Whatman No.1, Fitchburg, WI, USA), and a cloth filter were used to separate the methanol solution from the powdered leaves. Subsequently, the methanol extract was concentrated at 40ºC using a rotating evaporator (R-215, Buchi, Flawil, Switzerland) at compact pressure. The concentrated methanol extract was held at -80ºC for storage before being lyophilized and dried into a powder using a Labconco Free zone 6 Plus Freeze Dryer. It was then stored at -20ºC. 15.92% (w/w) was the yield that was obtained from the MECN.

2.2.1. Total phenolic contents

Total phenol content was determined using 2 g/L of the dried methanolic leaf extract of *Clinacanthus nutans* (MECN) was diluted to prepare solutions at 500, 1000, and 2000 mg/mL. The Folin-Ciocalteu assay, described by Iqbal and Bhanger (2006), was slightly modified to measure the total phenol content. The amount of phenolic in the extracts was then measured using gallic acid equivalents (mg GAE/g extract).

2.2.2. Total flavonoid contents

After diluting 2 g/L MECN, the final concentrations were 500, 1000, and 2000 mg/mL. The aluminum chloride colorimetric method described by Yusri et al. (2012) was slightly modified and utilized to measure the total flavonoid concentration (TFC) in extracts. TFC in extracts was expressed as rutin equivalents in mg per gram extract (mg RU/g extra

2.2.3 Chromatography analysis

The AB Sciex 5500Q Trap LC/MS-MS system, which includes a degasser, binary pump, autosampler, and column heater, was used to perform the LC-ESI-MS/MS analysis. According to Ramirez et al. (2014), the ESI Turbo interface operated in a negative mode, which is more selective and effective for characterizing phenolic compounds. The mass fragmentation was based on ACD/Labs' sophisticated chemometric mass fragmentation predictive software. Using a combination of liquid chromatography and mass spectrometry (ESI-LC-MS/MS) and analysis of their ultraviolet (UV), retention time, mass spectra, and comparison with our standard library data, which comprises 500 established compounds, the phenolic acids and flavonoids compounds in *Clinacanthus nutan*s leaf extract were identified.

2.2.4. 2, 2-Diphenyl-2 picrylhydrazyl assay

Under ambient circumstances, 0.4 mL of diluted 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) was allowed to react with 0.1 mL of each extract of MECN and ascorbic acid at concentrations of 500, 1000, and 2000 mg/mL. The DPPH manufacturing method was adapted with minor changes from Thaipong et al. (2006). 50 milliliters of methanol were used to dissolve 4.2 milligrams of DPPH powder to create the DPPH solution. Before interacting with the extract, this solution was vigorously shaken to allow the DPPH to dissolve completely, and it was then allowed to sit at room temperature for two hours in a dark environment. The final solutions were then allowed to sit at room temperature in the dark for 60 minutes. For every test, triplicates were subsequently completed. Next, a wavelength was used to read the absorbance.

$$\textbf{DPPH radical scavenging activity } \% = \frac{(\textbf{Abs control} - \textbf{Abs sample})}{\textbf{Abs control}} \times \mathbf{100} \quad (7)$$

2.2.5.2-2'-azinobis (3-ethyl-benzothiazoline -6-sulfonic acid) assay

0.9 mL of diluted 2-2'-azinobis (3-ethyl-benzothiazoline -6-sulfonic acid) (ABTS) was mixed with approximately 0.1 mL of each MECN and BHT solution at concentrations of 500, 1000, and 2000 mg/mL, respectively. Potassium sulfate (2.6 mM) and 7.4 mM ABTS were combined to create the ABST test. To calculate the total antioxidant, the ABST was utilized. With few modifications, the technique was taken from Fidrianny et al. (2013). The final solutions were allowed to sit at room temperature for five minutes to initiate the process. A spectrophotometer was utilized to measure the absorbance at a wavelength of 734 nm.

The standard was ABTS solution, while the blank was 95% methanol. For each extract and the standard, the test was run three times. Every procedure measurement was carried out. Where: A_{734} sample = absorbance of ABTS radical and leaf extract or BHT, A_{734} blank = absorbance of ABTS radical and methanol.

$$\text{ABST radical scavening activity } \% = \frac{1-\text{A734 (sample)}}{A734\,(blank)} \times 100 \quad (8)$$

2.3. Statistical analysis

Before statistical analysis, the current study employed the PROCUNIVARIATE procedure of SAS software to check for conformance with normality in the entire data. The means ± standard error of the means (SEM) are displayed for each result in the tables. The data was analyzed using Graph Pad Prism version 7 (Prism 7.0, Graph Pad Software Inc., CA, USA) using either a one-way or two-way analysis of variance (ANOVA). Tukey's post hoc comparison test was used to clarify statistically significant differences between the group means. We used a 95% confidence threshold while conducting statistical procedures. In addition, the graph was made using the Graph Pad Prism program.

3. Results and discussion

3.1. Proximate analysis

It has been reported by numerous studies that increased consumption of high-antioxidant vegetables results in risk reduction of some long-term diseases (Costacou and Mayer-Davis, 2003). This study assessed the proximate

analysis of fresh leaves of *Clinacanthus nutans*. The MECN after extraction with 80% of methanol is freeze-dried and then 3 concentrations of 500, 1000, and 2000 mg/mL were prepared. Next, antioxidant-potent molecules in MECN are compared with synthetic antioxidants. The result of this study suggested that MECN possesses antioxidant properties and 12 phenolic compounds, including good sources of mineral value. The estimations of carbohydrate, protein, fat, moisture, and ash in the proximate analysis of *Clinacanthus nutans* leaf are shown in Table 1.

Table 1. Proximate analysis of leaves *Clinacanthus nutans*.

Nutritional value (% dry weight)	Leaf
Moisture	6.91 ± 0.01
Ash	8.71±0.08
Protein	12.32±0.09
Fats	1.01 ±0.01
Carbohydrate	71.05±0.09

All the values are means of three replicates and are reported as expressed as mean ± SEM (n=3).

The primary nutrient present in the leaf was total carbohydrates with the highest value (71.05±0.09%). The crude protein was 12.32±0.09%, the moisture content was 6.91±0.01%, and the fat content was the lowest (1.01±0.01%). Proximate analysis is necessary to determine the nutrient content which is crucial for promoting metabolic health. *Clinacanthus nutans* leaves have low moisture content and this indicates low vulnerability to infections from microbes and a probable long shelf-life. The high ash content also indicates that it could be a good source of minerals (Sarega et al., 2016b). The minimum percentages of crude fat in *Clinacanthus nutans* may be profitable for people who are suffering from chronic diseases like obesity and diabetes (Sarega et al., 2016b). In comparison with our findings, Moses et al. (2015) reported that *Clinacanthus nutans* leaves were collected from the Kundasang area in Ranau, which is located in the East of Peninsular Malaysia. The crude protein, ash, and carbohydrate contents of the freeze-dried, unfermented herbal tea were high at 12.39±0.39%, 22.43±0.53%, and 63.40±0.53%, respectively, with lower fat content (2.23±0.10%). Whereas, *Clinacanthus nutans* was collected from Yik Poh Ling Herb Farm (YPL) in Negeri Sembilan, showed lower ash (6.33±0.25%), protein (8.79±0.02%), fat (2.08±0.09%) but higher carbohydrate (76.08±0.02%) content (Kong and Abdullah, 2017). This indicates that *Clinacanthus nutans* from different places revealed different nutrition compositions. The authors concluded that relatively higher carbohydrate and lower crude fats content in *Clinacanthus nutans* concerning a different location. Consequently, the green vegetables examined in this study are excellent providers of protein. According to Yu et al. (2015), our findings demonstrate that fresh leaves of *Clinacanthus nutans* represent a novel type of wild vegetable with low-fat content and high protein and carbohydrate content.

3.2. Total phenolic

Using a linear gallic acid standard curve (y = 7.7538 x 0.21; r2 = 0.99), the amount of extractable phenolic compounds in the MECN was ascertained. Using Folin-Ciocalteu's reagent, determine the total phenolic contents in the MECN. The total phenolic content of the methanolic extracts is also shown in Table 2, with levels ranging from 3.47, 8.44, and 17.64 mg GAE/g extract at 500, 1000, and 2000 mg/mL. After doing a one-way ANOVA and Tukey's post hoc tests, there was a significant difference in the total phenolic contents [F =

39.9, p = < 0.0001]. The total phenolic content was found to increase along with the MECN concentrations. The total phenolic content and antioxidant activity were measured using the solvent extraction technique. According to Mustapa et al. (2015), when the equivalent proportion of ethanol-water concentration is used as the extracting solvent, the balance of high and moderate polar structures in the phenol compounds leads to an outstanding impact on the extraction. This could explain why fruits and vegetables' phenolic components dissolve more readily in methanol. Although the polarity of methanol and ethanol were similar, methanol was more effective in extracting antioxidant compounds than ethanol. This may be because methyl radical in methanol is shorter than ethyl radical in ethanol, leading to a higher solvation of antioxidant molecules. Boeing et al. (2014) demonstrated that methanol and water were the most efficient solvents for the extraction. This occurs as a result of the interactions of the hydrogen bond between the polar sites of the solvent and antioxidant molecules. An 80% methanol has been reported by Ismail et al. (2017) as the ideal solvent for extraction due to higher polar properties obtained when mixed with water and preferable in the determination of antioxidant, phenolic, and flavonoid activities. The results of Sulaiman et al. (2015) and Ismail et al. (2017), who demonstrated increased antioxidant activity in *Clinacanthus nutans* followed by total phenolic and flavonoid content, are consistent with the current study. This could be the outcome of more than only phenolic compounds' antioxidative properties, such as those of sterols, terpenes, and saponin. (Odchimar et al., 2016). Differences in values of the total phenolic content of MECN have been reported by several researchers, for example, Tiew et al. (2014) reported a lower value 0.78±0.01 mg GAE/g dry weight extract at stock: 10 mg/mL, using methanol maceration, while Ghasemzadeh et al. (2014) reported that 6.840±0.470 to 15.460±1.231 mg/g dry weight using methanol and freeze dryer. However, Sarega et al. (2016a) reported higher value of 73.33±12.18 to 48.84±1.33 mg GAE/g using aqueous and methanolic leaf extract. Environmental conditions, saline soil, extraction method, genetic variation of the traits, the growth, yield and secondary metabolites of plants affect phenolic compounds (Ismail et al., 2017). Hence, studies by Sarega et al. (2016a) have shown that bioactive compounds of *Clinacanthus nutans* leaf extract are potentially effective for health benefits against several chronic diseases. This finding indicated that most antioxidant activities from plant sources are derived from phenolic compounds.

3.3. Total flavonoids

Using an aluminum colorimetric test method, the total flavonoid content of MECN at various doses was assessed in this study. The standard utilized was rutin (y = 0.313x, r2 = 0.94). A significant difference was found in the total flavonoid contents in Table 2, as indicated by a one-way ANOVA and Tukey's post hoc test (F = 14.8, p = < 0.007).

Table 2. Total phenolic and flavonoid contents in different concentration of MECN.

Concentration of MECN	Total phenol mg GAE/g	Total flavonoid mg RU/g
500 mg/mL	3.47±0.08^{c}	1.60±0.04^{c}
1000 mg/mL	8.44±0.11^{b}	3.99±0.08^{b}
2000 mg/mL	17.64±0.61^{a}	8.23±0.11^{a}
p-value	< 0.0001	< 0.007

Values are expressed as mean ± SEM (n=3). ^{a,b,c} Means within a column with different superscript letters are significantly different (p < 0.05, ANOVA) between groups by Tukey's test. MECN: methanolic extract of *Clinacanthus nutans*, GAE: gallic acid equivalent, RUT: Ruten

Consequently, the highest flavonoid content was found in the methanol extract at 2000 mg/mL (8.23 mg RE/g), while methanolic extract showed the lowest flavonoid content (1.62 mg RU/g) at 500 mg/mL. Flavonoids have hydroxyl groups which are functional in

mediating the antioxidant activities via free radical scavenging activity and chelation of metal ions. Also, differences in values of the total flavonoids content of *Clinacanthus nutans* have been reported by various researchers, Tiew et al. (2014) found that 0.21±0.005 mg QE/g dry extract by using methanol maceration for the extraction procedure. However, Ghasemzadeh et al. (2014) showed 3.27±0.33 to 6.32±0.74 mg QE/g dry weight while Fong (2015) showed higher total flavonoid 14.02±1.68 to 27.72±0.14 mg QE/g of dry extract using methanol for extraction. A direct comparison between the findings of previous studies and the current results tends to be less logical, because the difference in value might be due to several reasons, variations strongly influence first bioavailability of flavonoid in plant type and growth, season, light intensity, high temperature and food preparation were responsible for total flavonoid content in the *Clinacanthus nutans* (Aherne and O'Brien, 2002). Second, variations in the extraction technique, solvent used, and extract concentration could be the cause of the value discrepancy. In addition, because flavonoids are labile substances that readily undergo degradation reactions in aqueous conditions, their degradation during the extraction process may also be a significant factor (Mustapa et al., 2015). Moreover, the extraction competition between flavonoids and chlorophyll from plant leaves as a function of ethanol concentration may also be contributing factors. According to Fernandes et al. (2013), the harvesting period of Calendula officinalis affected the synthesis of five flavonoid components. Flavonoids are beneficial in a range of human illnesses because, once absorbed, they regulate several biological functions such as protein synthesis, cell proliferation, differentiation, and angiogenesis (Di Carlo et al., 1999).

3.4. Phenolic compounds in MECN by LC-ESI-MS/MS

The LC-ESI-MS/MS profiles of MECN at 280 nm are displayed in Figure 2. By matching the molecular ions (M-H+) or (M-H) of the polyphenol compounds using LC-ESI-MS, the identity of the compounds was verified. This was accomplished by contrasting the mass spectrometric data with information from the literature and the LC-MS library. A total of 12 phenolic compounds were examined in the range of 3–12 retention times (RT) in the total ion chromatogram (See Table 3, Figure 2). These compounds were composed of approximately 4 phenolic acid compounds and 5 flavonoids.

Table 3. Phenolic compounds identified in MECN samples by LC-MS/MS with ESI. RT; retention time, [M-H]- molecular mass of MECN extract on the loss of one proton measured by SIM.

Peak No	RT (min)	[M-H]- (Frag. MS2 m/z)	Molecular formula	Compounds	Polyphenol class
1	3.19	147.11(147.01)	C9H8O2	Cinnamic acid	Phenolic acid
2	4.08	315.15 (315.08)	C7H6O4	Protocatechuic acid	Phenolic acid
3	4.79	563.61 (564.06, 383.14, 297.23)	C26H28O14	6-C-glucosyl-8-C-arabinosyl apigenin or arabinosyl-glucosyl apigenin isomere	Flavonoid
4	5.02	562.98 (563.12, 383.43, 353.48, 297.53)	C26H28O14	6-C-glucosyl-8-arabinosyle apigenin or arabinosyl-glucosyl apigenin	Flavonoid
5	5.20	533.53 (383.13, 353.19, 297.27)		Gendarucin A	Flavonoid

6	6.01	328.62 (329.23, 229.12, 211.12)	C16H10O8	3,3-di-O-methyle ellagic acid	Phenolic
7	6.73	197.23 (197.04, 133.02)	C9H10O5	Ethyl gallate	Phenolic
8	8.66	269.26 (269.22)	C15H10O5	Apigenin	Flavonoid
9	9.37	831.82 (831.49)		Unknown	
10	9.78	981.79 (982.30, 920.37, 906.42, 555.28, 540.33)		Unknown	
11	10.69	715.49 (715.49 699.50, 461.48)		Unknown	
12	11.3	301.27 (228.99,172.01, 116.95)	C15H10O7	Quercetin	Flavonoid

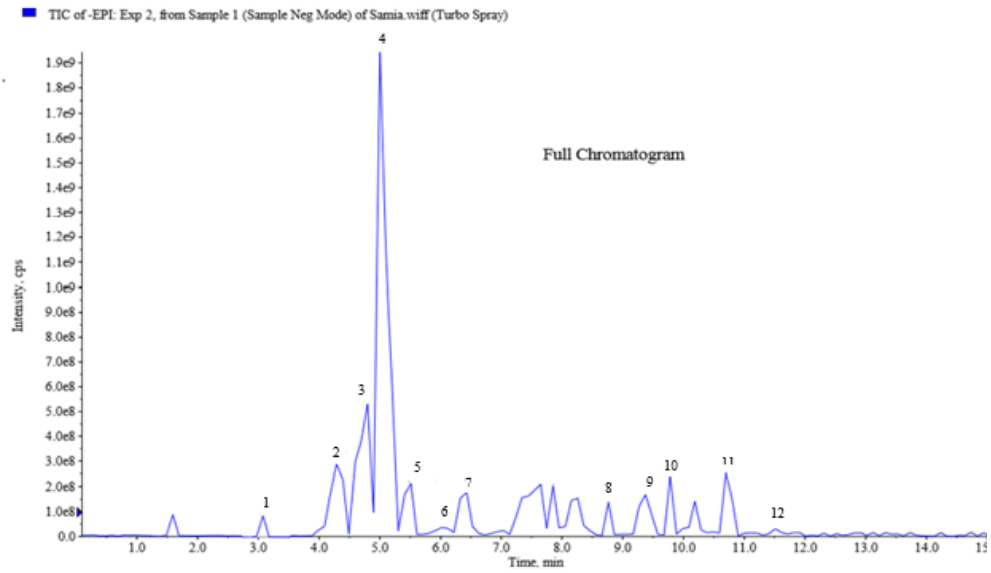

Figure 2. LC-MS chromatogram at 280 nm of MECN. The x-axis shows retention time in minutes, the y-axis shows the intensity. The peak number are consistent with those indicating in Table 3. 1, Cinnamic acid; 2, Protocatechuic acid; 3, 4, 6-C-glucosyl-8-C-arabinosyl apigenin or arabinosyl-glucosyl apigenin isomere; 5, Gendarucin A; 6, 3,3-di-O-methyle ellagic acid; 7, Ethyl gallate; 8, Apeginin; 9,10,11, Unknown; 12, Quercetin.

Phenolic acid is represented by peaks 1, 2, 6, and 7, and flavonoid is represented by peaks 3, 4, 5, 8, and 12. Because they needed different instruments to identify, the other three unknown substances were not detected under the same circumstances.

Examining the polyphenol profiles in MECN samples, the current work has demonstrated that, when LC-MS/MS data analysis is performed under the previously mentioned conditions, it is possible to distinguish each and every type of polyphenol according to its molecular weight (Table 3). Some of the flavonoid compounds, including quercetin-3-O-rutinosid, were found to be attached to sugar moieties, which makes them more soluble in water. On the other hand, the bulk of the detected polyphenols were classified

as phenolic acid, which explains the high phenolic content (Ferreres et al., 2013). Moreover, 9 polyphenolic compounds were identified in MECN; we hypothesized that these compounds would have potent anti-oxidative effects. Cinnamic acid which is known as phenolic acid derivatives is important and promising compounds to possess potent antioxidant properties and potential for development into drugs (Sova, 2012). Accordingly, protocatechuic acid beside antioxidant properties was found potent antibacterial, anticancer, antihyperlipidemic, antidiabetic and anti-inflammatory (Lende et al., 2011). It has been found that certain flavonoid molecules, such as quercetin-3-O-rutinosid, are connected to sugar moieties, which increases their solubility in water. However, most of the polyphenols found were identified as phenolic acid, accounting for the high phenolic content (Ferreres et al., 2013). Many studies have also proven the efficiency of quercetin in inhibiting cancer cell growth (Lee et al., 2015b). While 5 flavonoids were identified, in which two 6-C-glucosyl-8-arabinosyle apigenin or arabinose-glucosyl apigenin isomeres were detected in MECN. These findings highlight the need for more research on flavonoid C-glycosides, as these compounds have a variety of biological actions that may be crucial for the use of *Clinacanthus nutans* in both nutritional and medicinal applications. The quantity of hydroxyl groups in their structures is the cause of their biological activity. *Clinacanthus nutans* has also been included by the Malaysian government in the National Key Economic Areas proprietary list (Narayanaswamy and Ismail, 2015), demonstrating its potential in the supplementary and pharmaceutical industries.

3.5. Antioxidants activity

DPPH radical scavenging activity was detected at different concentrations of MECN and compared with ascorbic acid. Two-way ANOVA showed a significant interaction between the effect of treatment concentrations and scavenger activity of DPPH [$F = 32.91$, $p = < 0.0001$]. Tukey's post hoc comparison test showed a significantly higher scavenger activity percentage of DPPH in ascorbic acid compared to the MECN. 36.14%±0.45, 53.62%±0.67, 75.33%±0.88 percentage scavenger activity was observed in *Clinacanthus nutans*, while ascorbic acid showed percentage inhibition of 47.99%±0.67, 75.32%±0.44, 93.71%±0.44 at concentrations of 500, 1000 and 2000 mg/mL, respectively (Figure 3). These findings showed that the rise in concentrations is still correlated with the increase in scavenging activity in methanolic extract. The radical scavenging activity of MECN (500, 1000 and 2000 mg/mL) was compared with those of ascorbic acid and BHT expressed as % against DPPH and ABTS, respectively. DPPH assay was performed to determine the radical scavenging activity of the MECN extracts against stable DPPH.

Antioxidants can be assessed easily and effectively by the use of DPPH (Viuda-Martos et al., 2010). The MECN was evaluated based on comparing the percentage scavenging activity of DPPH radical formation by extracts to that ascorbic acid. Ascorbic acid has the highest percentage scavenging activity than MECN because ascorbic acid is a pure antioxidant compound. A study conducted by Khanam et al. (2012) showed that ranging from scavenging activity from 33% to 72% for 8 leafy vegetables, for instance komatsuna, mizuna, bok choi, mitsuba, salad spinach, lettuce, red amaranth and green amaranth, while methanolic leaf extract at different concentration had shown a higher scavenging activity from 36.1% to 75.33% compare to eight leafy vegetables.

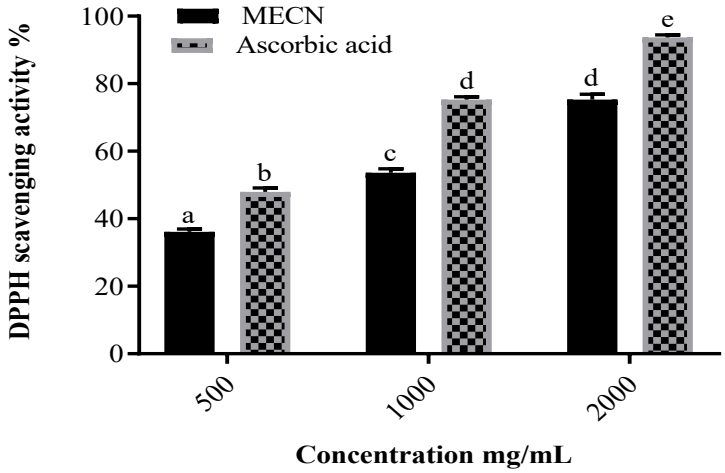

Figure 3. Anti-oxidant activity (%) of MECN compared with ascorbic acid at 500, 1000 and 2000 mg/mL by using DPPH. Values are express as mean ± SEM (n = 3). [a,b,c,d,e] Means with different letters are significantly different ($p < 0.05$, ANOVA) between groups by Tukey's test. MECN: methanolic leaf extract of *Clinacanthus nutans*.

Results from the current study suggest that high levels of DPPH depended on the age of leaves, total phytochemical and flavonoid contents were noticed for younger leaves of *Clinacanthus. nutans* compared to older leaves (Raya et al., 2015).The scavenging activity of DPPH increased concerning the increasing extract concentration of 500, 1000, and 2000 mg/mL, as observed in control ascorbic acid (Kim and Son, 2011). In addition, for hydrophobic assessments, the DPPH method is more sensitive than alternative approaches (Kaur and Kapoor, 2001). This is in agreement with the present study, which showed that 80% of MECN exhibits higher antioxidant activity (Sarega et al., 2016a). There was a positive correlation between the total phenolic content and DPPH assay ($r = 0.98$; p (two-tailed) < 0.001). In the same way, Sulaiman et al. (2017) demonstrated a robust association between the *Clinacanthus nutans* leaves' total phenolic content and the DPPH test. This observation leads to the conclusion that MECN showed greater DPPH at 2000 mg/mL, indicating excellent hydrogen and electron-donating activity in DPPH. During the ABTS assay, the cationic chromophore ($ABTS^{\bullet+}$) showed some level of stability but it reacted vigorously with molecules yielding electrons and atoms like phenolic compounds. This led to the disappearance of the blue-green color of this radical. The decolonization of the ABTS+ determined the antioxidant activity in MECN and the reduction of the radical cation as the percentage inhibition was measured at 734 nm.

Two-way ANOVA revealed a significant interaction between the effect of treatment concentrations and the percentage of ABST [$F = 14.19$, $p = 0.0007$]. Tukey's post hoc comparison test showed a significantly higher ABTS radical scavenging activity in BHT compared to MECN. The free radical scavenging activity of MECN revealed percentage inhibition of 46.03%±0.34, 64.65%±0.48, 81.87% ±0.31, while BHT showed percentage inhibition of 63.83% ±0.81, 82.23%±1.02, 92.12%±1.32 at concentrations of 500, 1000 and 2000 mg/mL, respectively (Figure 4).

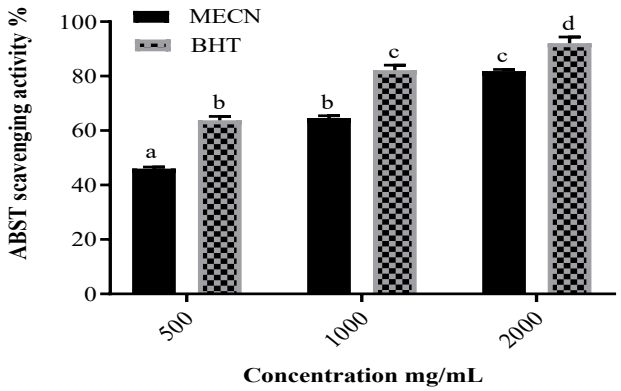

Figure 4. Anti-oxidant activity (%) of MECN compared with ascorbic acid at 500, 1000 and 2000 mg/mL by using ABTS. Values are expressed as mean ± SEM (n = 3). [a,b,c,d]Means with different letters are significantly different ($p < 0.05$, ANOVA) between groups by Tukey's test. MECN: methanolic leaf extract of *Clinacanthus nutans*, BHT: butylated hydroxytoluene.

Table 4. Pearson correlation coefficient between antioxidant activity and total phenol and flavonoid content.

	Total phenol	**Total flavonoid**	**DPPH**	**ABST**
Total phenol	1			
Total flavonoid	0.99*	1		
DPPH	0.98*	0.99*	1	
ABST	0.97*	0.98*	0.99*	1

Correlation is significant at the 0.01 level, p (2-tailed) = < 0.001

Additionally, the inhibition percentage was significantly higher in BHT compared to MECN at concentrations of 500, 1000 and 2000 mg/mL These results showed that the increase of scavenging activity in MECN remains coordinated with the increase of concentrations. The Pearson correlation coefficient was used to determine the relationship between antioxidant activity and total phenolic content in MECN. The results showed that phenolic and flavonoid contents in MECN correlated significantly with antioxidant assays, namely DPPH and ABST (Table 4). The presence of phenolic compound could be a result of this occurrence. The anti-oxidative status of numerous biological specimens has been ascertained by using ABST systems (Kim and Son, 2011). According to the current ABST method result, BHT and MECN prevent ABST from oxidizing. According to Re et al. (1999), the antioxidant content and reaction time affect the suppression of radical cation absorption, which is a measure of antioxidant activity. The ABST assay and total phenolic content showed a favorable connection ($r = 0.97$; p (two-tailed) < 0.001). Highly pigmented foods including cherry, spinach, plum, and red cabbage have significantly higher antioxidant capacity as determined by the ABST assay than by the DPPH assay, according to Floegel et al. (2010). ABST applicable to antioxidant systems that are hydrophilic and lipophilic. However, the DPPH test can be applied to hydrophobic systems since it employs a radical that has been dissolved in organic media (Kim et al., 2002). Comparing the ABST to the DPPH assay, the current study also revealed a better antioxidant capacity, which

may be related to the different pigment types found in the MECN.

4. Conclusions

This study looked investigated MECN's antioxidant capabilities and phenolic composition. Twelve phenolic compounds were found when the MECN was screened using the LC-MS/MS technique. The present results indicate that when the concentrations of several reactive oxygen species (ROS) were increased, MECN's antioxidant capacity increased. MECN showed less antioxidant power against a wider range of ROS than either ascorbic acid or BHT. The levels of phenolics and flavonoids in MECN were positively correlated with its antioxidant potential. Nevertheless, these findings warrant further investigations of this natural product specifically the bioactive components as sources of nutraceutical ingredients**.**

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