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Antioxidant and LC-QToF-MS/MS analysis of polyphenols in polar and non-polar extracts from *Strobilanthes crispus* and *Clinacanthus nutans*

[1]Tan, H. M., [1]Leong, K. H., [2]Song, J., [1]Mohd Sufian, N. S. F., [3]Mohd Hazli, U. H. A., [4]Chew, L. Y. and [3]*Kong, K. W.

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Malaya, 50603, Kuala Lumpur, Malaysia*
[2]*Key Lab of Chinese Medicine Resources Conservation, State Administration of Traditional Chinese Medicine of the People's Republic of China, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, China*
[3]*Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia*
[4]*School of Biosciences, Taylor's University, No. 1 Jalan Taylor's, 47500 Subang Jaya, Selangor Darul Ehsan, Malaysia*

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Abstract

Strobilanthes crispus and *Clinacanthus nutans* are popular herbal plants in the Southeast Asian region. The present work was aimed at determining the antioxidant activities and the associated components in the leaf extracts of both species using polar and non-polar solvents namely water, methanol, ethyl acetate, and hexane. The total phenolic content (TPC) and total flavonoid content (TFC) were higher in the leaf extracts of *S. crispus* as compared to *C. nutans*. Among the solvents, methanol was the best solvent in extracting the antioxidant components for *S. crispus* (TPC: 159.85 ± 0.89 mg GAE/g extract and TFC: 955.47 ± 2.66 mg RE/g extract). However, for *C. nutans*, its methanolic extract yielded the highest TPC (36.39 ± 0.17 mg GAE/g extract), whereas ethyl acetate yielded the highest TFC (229.61 ± 7.81 mg RE/g extract). The high levels of both TPC and TFC contributed to the antioxidant activities of *S. crispus* extract as reflected in the methanolic extract attaining the highest level of antioxidant activities, measured by ferric reducing antioxidant power (FRAP) (6.84 ± 1.12 mmol Fe^{2+}/g extract), DPPH radical scavenging (IC_{50}: 203.60 ± 7.28 µg/mL), and Trolox equivalent antioxidant capacity (TEAC) (1.01 ± 0.01 mmol TE/g extract) assays. This contrasted with *C. nutans* which showed lower antioxidant activities owing to its lower TPC and TFC. Correlation analysis revealed significant correlations ($p < 0.05$, $r = 0.915$ - 0.985) between both TPC and TFC in *S. crispus* and antioxidant activities. However, only TPC of *C. nutans* showed a significant correlation with FRAP values ($r = 0.934$). Further tentative identification of the constituents in the extracts using HPLC-ESI-QToF-MS/MS revealed the existence of 20 polyphenolic compounds in both *S. crispus* and *C. nutans*, which were likely responsible for their antioxidant activities. In addition, 15 polyphenolic compounds classified as chalcones, isoflavanoids, flavones, and flavonols have not been previously reported in both species. The methanolic extracts of both species yielded a higher content of antioxidants, with *S. crispus* offering a richer source of dietary antioxidants as compared to *C. nutans*. However, further study is needed to identify their bioactivities in relation to their bioactive components.

Introduction

Plants are rich sources of natural bioactive compounds that possess antioxidant potential (Ghasemzadeh *et al.,* 2011) which can significantly delay or inhibit oxidative damage from exposure to oxidative radicals. Biologically, antioxidant compounds such as phenolic acids and flavonoids are essential to protect against the damaging effects of free radicals that lead to premature aging, cardiovascular diseases, and central nervous system disorders. Hence, research on antioxidants is still of growing interest because of their ability to scavenge free radicals like peroxide, hydroperoxide, or lipid peroxyl, thus playing an important role in the prevention of various diseases (Adebiyi *et al.,* 2017).

Herbal plants which possess potent antioxidant compounds that can be beneficial in maintaining health have been traditionally consumed since ancient times. *Strobilanthes crispus* ("*pecah beling*") and *Clinacan*

*Corresponding author.
Email: kongkm@um.edu.my

thus nutans ("*belalai gajah*") are both popular herbal plants in the Southeast Asian region, particularly in Malaysia. They are traditionally consumed as a herbal infusion by the local population for general maintenance of health. Previous studies have identified that these plants have anti-inflammatory, anti-carcinogenesis, anti-viral, anti-diabetes, and antioxidant properties (Ghasemzadeh *et al.,* 2015; Sarega *et al.,* 2016; Alam *et al.,* 2016). *S. crispus* and *C. nutans* have been reported to contain high amounts of polyphenols, saponins, triterpenoids, phytosterols, sulphur-containing glucosides, and lipids which are believed to be responsible for the plants' bioactivities (Khoo *et al.,* 2015; Ghasemzadeh *et al.,* 2015; Sarega *et al.,* 2016; Alam *et al.,* 2016). However, the specific phytochemicals responsible for their antioxidant properties are not yet well studied. Hence, the present work attempted to further determine the phytochemicals of *S. crispus* and *C. nutans* that may be responsible for their antioxidant activities, particularly polyphenols which are well-known as major antioxidants in plants.

Although the antioxidant activities of the plants have been previously reported, they are limited to polar extracts mainly prepared using water, methanol, and ethanol, while the identification of the bioactive compounds in both species using HPLC-QToF-MS/MS has yet to be done (Lusia Barek *et al.,* 2015; Ghasemzadeh *et al.,* 2015; Khoo *et al.,* 2015; Sarega *et al.,* 2016; Alam *et al.,* 2017). Previous studies on the bioactive compounds in both plants utilising spectrophotometric and HPLC analysis identified only limited bioactive compounds. Hence, in the present work, the leaves of *S. crispus* and *C. nutans* were subjected to extractions using solvents of different polarities namely water, hexane, ethyl acetate, and methanol. Their antioxidant activities, total phenolic content, and total flavonoid content were assessed (Kong *et al.,* 2012). Further HPLC-ESI-QToF-MS/MS analysis was adopted to tentatively identify the polyphenolic compounds in the plant extracts.

Materials and methods

Analytical reagents and chemicals

Methanol, ethyl acetate, hexane, dimethyl sulfoxide (DMSO), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), aluminium chloride, sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium acetate, rutin, gallic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2'-azino-bis(3-thylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), iron chloride ($FeCl_3$), iron sulphate ($FeSO_4$), hydrochloric acid (HCl), potassium peroxodisulphate, and Trolox were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA) and other general suppliers. Milli-Q water was used throughout the study.

Sample preparation and extraction

Both *S. crispus* and *C. nutans* were collected from Bertam Ulu, Melaka, Malaysia in February 2018 (Figure 1). The matured leaves (leaves with a dark green colour) of the plants were separated from the stems, while the young shoots with a light green colour were excluded. The selected leaves were thoroughly washed with tap water to remove any dust and impurities. Then, the leaves were air dried at room temperature (23 - 26°C) using a fan for 2 - 3 h prior to being freeze-dried. After that, the dried samples were ground into powder using an electronic blender, and sieved using a 1 mm mesh sieve. The powdered samples were stored at -20°C until further analysis.

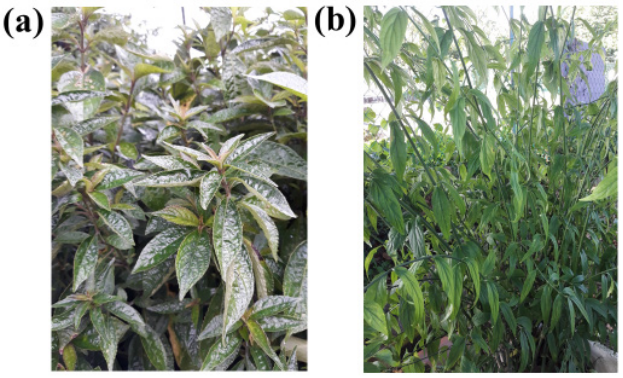

Figure 1. The leaves of (a) *Strobilanthes crispus* and (b) *Clinacanthus nutans*.

Plant extractions were achieved by weighing 2 g of the powdered samples, and soaking them individually in 40 mL of solvents with varying polarity such as water, methanol, ethyl acetate, and hexane. The samples were subjected to a constant shaking of 200 rpm at 25°C for 24 h in an incubator shaker (News Brunswick Scientific Innova 4300, New Jersey, USA) to ensure complete extraction (Mohd Hazli *et al.,* 2019). The crude extracts were then individually filtered through a Whatman filter paper (No. 4), and the organic solvents were evaporated using a rotary evaporator (Buchi Labortecnik R-215, Flawil, Switzerland) at 40°C. For water extracts, the filtrates were dried using a freeze-dryer. Finally, the dried extracts were weighed to calculate the extraction yield, which was later expressed in a percentage (%). Prior to further analysis, the dried extracts were initially dissolved in 100% dimethyl sulfoxide (DMSO) as stock solutions, and further diluted to 20% DMSO

with Milli-Q water as working solutions.

Determination of total phenolic content (TPC)

The TPC of the extracts was determined using the Folin-Ciocalteu method in accordance with the Singleton and Rossi method (1965), using a 96-well plate. Briefly, 25 µL of 1 N Folin-Ciocalteu reagent was mixed with 50 µL of the diluted sample (1 mg/mL). Then, the mixture was left to stand at room temperature for 5 min. Next, 100 µL of Na_2CO_3 solution (0.57 M) was added, and the solution was made up to a final volume of 250 µL by adding Milli-Q water. Finally, the absorbance was measured at 760 nm (Tecan Infinite 200 PRO microplate reader, Männedorf, Switzerland) after 2 h reaction time. A standard calibration curve of gallic acid (0 - 0.4 mg/mL) was plotted using the same procedure, and the results of the extracts were expressed as mg gallic acid equivalent (GAE)/g dried extract.

Determination of total flavonoid content (TFC)

The TFC of the extracts was measured using an aluminium chloride colorimetric assay as described by Kong *et al.* (2012). A total of 100 µL of the diluted sample was mixed with 10 µL of 5% $NaNO_2$. The mixture was incubated for 5 min before the addition of 10 µL of 10% $AlCl_3$. After 6 min, 100 µL of 1 M NaOH was added and diluted to a volume of 250 µL by adding Milli-Q water for the measurement of absorbance at 510 nm. A standard calibration curve of rutin (0 - 0.4 mg/mL) was plotted using the same procedure, and the results of the extracts were expressed as mg rutin equivalent (RE)/g dried extract.

Determination of DPPH radical scavenging activity

A DPPH assay was conducted using the method described by Brand-Williams *et al.* (1995) with some modifications, using a 96-well plate. A mixture of 195 µL of 100 µM methanolic DPPH solution with the diluted sample at different concentrations (0 - 1000 µg/mL) was individually prepared, and kept in the dark for 30 min. After that, absorbance of the reaction mixture was read at 515 nm. Standards (gallic acid and rutin) were included at different concentrations (0 - 1000 µg/mL) for comparison. All results were expressed as a percentage (%) of the DPPH radical scavenging activity, which was calculated using Eq. 1:

$$\text{Scavenging activity (\%)} = (A_{control} - A_{sample} \text{ or } A_{standard}) / A_{control} \times 100 \quad \text{(Eq. 1)}$$

where, $A_{control}$ = absorbance of DPPH radicals without sample or standard, and A_{sample} or $A_{standard}$ = absorbance of DPPH radicals with sample or standard.

The value of a 50% inhibitory concentration (IC_{50}) was subsequently obtained and expressed as µg/mL, corresponding to the concentration of a sample or standard that scavenged 50% of the DPPH radicals.

Determination of ferric reducing antioxidant power (FRAP)

The reducing power of the extracts was determined following the method of Benzie and Strain (1996) with slight modifications. Initially, three reagents, 300 mM acetate buffer at pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ were prepared. These reagents were mixed at a ratio of 10:1:1 (v/v/v) to produce a complete FRAP reagent. Next, 5 µL of the plant extract was added to 300 µL of the FRAP reagent, incubated for 30 min at 37°C, and absorbance was measured at 593 nm. The results were calculated based on a calibration curve using $FeSO_4$ (0 - 2 mM) and expressed as mmol Fe2+/g dried extract.

Determination of Trolox equivalent antioxidant capacity (TEAC)

In a procedure described by Re *et al.* (1999), a stock solution of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations was prepared by mixing 10 mL of Milli-Q water with 7 mM $ABTS^{\bullet}$ and 2.45 mM potassium peroxodisulphate. Then, the mixture was incubated in the dark at room temperature for 12 - 16 h. To prepare a fresh $ABTS^{\bullet}$ working solution, the stock solution was diluted with Milli-Q water to obtain an absorbance of 0.70 ± 0.05 at 734 nm. Subsequently, 3 µL of the extract was added to 300 µL of the working solution, and thoroughly mixed. After 6 min, the absorbance was measured at 734 nm. Standards (gallic acid and rutin) were used as positive controls, and the percentage of antioxidant capacity was calculated using Eq. 2:

$$\text{Antioxidant capacity (\%)} = (A_{+ABTS} - A_{sample} \text{ or } A_{standart}) / A+_{ABTS} \times 100 \quad \text{(Eq. 2)}$$

where, A_{+ABTS} = absorbance of ABTS radical cations without sample or standard, and A_{sample} or $As_{tandard}$ = absorbance of ABTS radical cations with sample or standard.

Calculation of the TEAC value was based on a calibration curve using different concentrations of Trolox (0 - 1.6 mM), and the results were expressed as mmol Trolox equivalent (TE)/g dried extract.

HPLC-ESI-QToF-MS/MS analysis of polyphenolic compounds

An HPLC-ESI-QToF-MS/MS system consisting of an HPLC 1260 Infinity coupled to an ESI-QToF-MS/MS 6530 model (Agilent Technologies, USA) was used to separate and tentatively identify polyphenols in the extracts through existing database matching (METLIN, Agilent Technologies, USA). An Agilent InfinityLab Poroshell 120 EC-C18 column (3.0 × 150 mm, 2.7 µm), set at a temperature of 40°C was used for the separation of compounds in the extracts. A total of 2 µL of extract was injected into the system and allowed to separate at a flow rate of 0.7 mL/min. The mobile phase was a mixture of water with 0.1% v/v formic acid (solvent A) and acetonitrile (solvent B) (Mohd Hazli *et al.,* 2019). Both positive and negative modes were conducted using a gradient elution as follows: 0 - 2 min, 0 - 10% B; 2 - 5 min, 10 - 15% B; 5 - 10 min, 15 - 25% B; 10 - 25 min, 25 - 60% B; 25 - 40 min, 60 - 80% B; and 40 - 60 min, 80 - 100% B. The MS parameters were set as: nebuliser gas, 40 psi; dry gas, 10 L/min; dry temperature, 300°C; acquisition range, m/z 50 to 1700, spray voltage of +/- 3000 V, cone voltage at 100 V with an MS scan rate of 2 s/scan, whereas the auto-MS/MS data acquisition was performed at collision energies of 10, 20, and 40 eV. Standard calibrants consisting of purine ($C_5H_4N_4$, m/z 121.0509), hexakis-(1H, 1H, 3H-tetrafluropropoxy), and phosphazene ($C_{18}H_{18}0_6N_3P_3F_{24}$, m/z 922.0098) were applied during data acquisition for real-time mass correction and ion suppression monitoring.

Statistical analysis

All experiments were independently conducted in triplicate. Data were expressed as mean ± standard deviation (SD), and statistically analysed using SPSS statistical software, version 23 (SPSS Inc, Chicago, Illinois, USA). One-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference test were used to compare means among groups. Values of $p < 0.05$ were considered as statistically significant. The Pearson correlation test was used to analyse the relationship between TPC and TFC and antioxidant activities measured using different assays.

Results and discussion

Extraction yield

To extract phytochemicals of different polarities from the leaves of *S. crispus* and *C. nutans*, four different solvents were used, namely water, methanol, ethyl acetate, and hexane. Different types of solvents were employed owing to the varying solubility of the phytochemicals in the solvents (Ngo *et al.,* 2017). Hence, this would have affected the extraction yield as different types of phytochemicals were extracted from the plants (Zlotek *et al.,* 2016). For example, less polar compounds in a plant are better extracted with non-polar solvents such as hexane and ethyl acetate, whereas polar solvents such as methanol and water are mostly used to extract alkaloids, polyphenols, and saponins (Jadid *et al.,* 2017).

The yields for the four different solvent extracts are shown in Table 1. The extraction yields of *S. crispus* were in a range of 7.5% (hexane extract) to 16.55% (water extract). Among the four extracts, the water extract yielded the highest percentage, followed by methanol and ethyl acetate, with the lowest being hexane extract. For *C. nutans*, the water extract (18.4%) was also the highest in yield while ethyl acetate extract gave the lowest yield (6.5%). This indicated that the content of the polar compounds was higher than that of non-polar compounds in both plant species. However, other compounds, such as protein and carbohydrates that possess higher solubility in methanol and water may have been extracted, which may have contributed to a higher extraction yield with these solvents (Do *et al.*, 2014). A lower extraction yield in non-polar solvent extracts indicated a low lipid soluble component in both plants. Even though solvent polarity can affect the extraction yield, the extraction yield itself had no influence on the antioxidant activities of the sample. This was shown by the methanolic extracts of both plants which had lower extraction yields but yielded higher antioxidant activities than the water extracts. Nonetheless, the information regarding the extraction yield will provide the basis for product quality and quantity monitoring for the nutraceutical and pharmaceutical industries.

Total phenolic content

Tomsone *et al.* (2012) reported that the recovery of polyphenols from plants is influenced by the solubility of phenolic compounds in the extraction solvent used. The TPC of both plants was highest in the methanolic extracts, followed by the water, ethyl acetate, and hexane extracts (Table 1). The high phenolic content in the methanolic extract may be due to the ability of methanol to form hydrogen bonds with the phenolics (Felhi *et al.,* 2017), as opposed to hexane. Generally, polar alcohol-based solvents like methanol and ethanol would yield a higher amount of TPC than the aqueous extract (Tomsone *et al.,* 2012; Zlotek *et al.,* 2016). However, this is not always true as Ghasemzadeh *et al.* (2015) reported that the aqueous

Table 1. Extraction yields, polyphenolic contents, and antioxidant activities of *S. crispus* and *C. nutans* extracted with solvents of different polarities.

Sample	Extraction solvent	Yield (%)	TPC (mg GAE/g extract)	TFC (mg RE/g extract)	DPPH radical scavenging activity IC_{50} (µg extract/mL)	FRAP (mmol Fe^{2+}/g extract)	TEAC (mmol TE/g extract)
S. cripsus	Water	16.55 ± 2.61^{a}	45.63 ± 0.32^{b}	50.43 ± 1.28^{d}	> 1000	1.22 ± 0.06^{d}	0.02 ± 0.03^{d}
	Methanol	13.00 ± 0.50^{a}	159.85 ± 0.89^{a}	955.47 ± 2.66^{a}	203.6 ± 7.28^{a}	6.84 ± 1.12^{c}	1.01 ± 0.25^{c}
	Ethyl acetate	7.67 ± 0.76^{b}	41.46 ± 0.88^{c}	242.20 ± 3.67^{b}	> 1000	1.38 ± 0.08^{d}	0.04 ± 0.01^{d}
	Hexane	7.50 ± 1.80^{b}	14.19 ± 0.23^{d}	99.55 ± 2.74^{c}	> 1000	1.28 ± 0.07^{d}	0.09 ± 0.05^{d}
C. nutans	Water	18.42 ± 0.95^{z}	35.01 ± 0.66^{y}	11.90 ± 3.56^{x}	922.99 ± 190.44^{z}	1.12 ± 0.03^{w}	0.06 ± 0.01^{v}
	Methanol	14.64 ± 1.52^{y}	36.39 ± 0.17^{z}	91.43 ± 2.62^{y}	> 1000	1.49 ± 0.12^{x}	0.10 ± 0.03^{v}
	Ethyl acetate	6.50 ± 1.80^{x}	32.01 ± 0.68^{x}	229.61 ± 7.81^{z}	> 1000	1.24 ± 0.02^{w}	0.19 ± 0.02^{w}
	Hexane	6.83 ± 0.76^{x}	5.65 ± 0.20^{w}	10.49 ± 1.24^{x}	> 1000	0.51 ± 0.01^{v}	0.14 ± 0.05^{wv}
Positive control	Gallic acid	NA	NA	NA	7.07 ± 0.03^{cx}	29.17 ± 0.25^{az}	4.29 ± 0.01^{az}
	Rutin	NA	NA	NA	61.18 ± 0.44^{by}	19.92 ± 0.38^{by}	1.67 ± 0.09^{by}

TPC: total phenolic content, TFC: total flavonoid content, GAE: gallic acid equivalent, RE: rutin equivalent, NA: not applicable, DPPH: DPPH radical scavenging activity, FRAP: ferric reducing antioxidant power, and TEAC: Trolox equivalent antioxidant capacity. Data are mean ± standard deviation. Means with different superscript lowercase letters ($^{a\text{-}d}$ and $^{x\text{-}z}$) within the same column of the sample are significantly different ($p < 0.05$).

extract of S. crispus displayed a higher level of TPC than alcohol-based solvent extracts. The TPC of the water extract in the leaves of *S. crispus* in the present work was higher (45.63 mg GAE/g extract) than the TPC (8.33 mg GAE/g extract) reported by Qader *et al.* (2011). Contrary to the study of Latiff et al. (2017), the phenolic content of the *C. nutans* leaf methanolic extract in the present work (36.39 mg GAE/g extract) was lower than the previous report using 70% methanol as solvent (125.83 mg GAE/g extract).

Total flavonoid content (TFC)

Similar to the observations of TPC, the extraction of flavonoids was significantly dependent on the type of solvent used. For *S. crispus*, the highest flavonoid content was found in the methanolic extract (955.47 mg RE/g extract), whereas the lowest was in the water extract (50.43 mg RE/g extract). Our findings also revealed that the hexane extract yielded a much lower TFC than the methanolic and ethyl acetate extracts. This may be due to the low solubility of semi-polar flavonoids in polar aqueous media as well as apolar media (Antolovich *et al.*, 2000). Despite the high content of flavonoids in the methanolic extract, Ghasemzadeh *et al.* (2015) demonstrated that a water extract had a higher TFC than an alcohol-based solvent, which gave better antioxidant activity.

In contrast, the flavonoid content was highest in the ethyl acetate extract followed by the methanolic, water, and hexane extracts of the *C. nutans*, with flavonoid yields ranging from 10.49 to 229.61 mg RE/g extract (Table 1). The relatively higher yield of flavonoids in the ethyl acetate and methanolic extracts of *C. nutans* was likely due to the higher solubility of the flavonoids in these solvents (Alam *et al.,* 2017). The varied content may be related to many factors such as the age of the plant, maturity of the leaves, environmental factors, and the parts of the plant being studied. According to Ghasemzadeh *et al.* (2014), both the phenolic and flavonoid content of *C. nutans* were higher in young leaves than in mature ones.

Antioxidant activities

The antioxidant activities of the extracts of *S. crispus* and *C. nutans* were assessed using three different antioxidant assays, namely ferric reducing antioxidant power (FRAP), Trolox-equivalent antioxidant capacity (TEAC), and the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging method. Since a single antioxidant assay may not be sufficient to reveal the overall antioxidant potential of the plant extracts, the three different assays were adopted (Prasad *et al.,* 2013). In addition, a single antioxidant assay may not encompass the antioxidant ability of the plant extracts, as different phytochemicals may act as antioxidants through

various mechanisms of action such as oxidative-reductive capacity or free radical scavenging ability (Gan *et al.,* 2010).

DPPH radical scavenging activity

The DPPH radical scavenging activity of the plant extracts and reference standards (gallic acid and rutin) was determined based on the concentration required to inhibit 50% of the DPPH radicals (Table 1). The ability of the samples to donate hydrogen ions in neutralising DPPH radicals was monitored through colorimetric measurements (changes in colour from purple to yellow) (Zhang *et al.,* 2017). The IC_{50} values of gallic acid and rutin were 7.07 and 61.18 µg/mL, respectively, exhibiting stronger scavenging activities than the different extracts of *S. crispus* and *C. nutans*. Among the various sample extracts, only the methanolic extract of *S. crispus* showed DPPH radical scavenging potential (IC_{50} = 203.6 µg/mL), whereas the water, ethyl acetate, and hexane extracts did not show any potential (IC_{50} > 1000 µg/mL) at the tested concentrations. This may be attributed to the low content of antioxidant compounds in the water, ethyl acetate, and hexane extracts (Kong *et al.,* 2012).

Similarly, only the water extract of *C. nutans* showed DPPH radical scavenging activity (IC_{50} = 922.99 µg/mL), whereas the other solvent extracts did not show such potential (IC_{50} > 1000 µg/mL). The results are in close agreement with another study by Latiff *et al.* (2017) which reported an IC_{50} of 953.22 µg/mL in their leaf extract of *C. nutans*. However, the IC_{50} of *C. nutans* water extract in the present work was more than four-fold higher than the methanolic extract of *S. crispus*, indicating a weaker DPPH radical scavenging ability.

Besides the IC_{50} values, the DPPH results were also presented as a percentage of DPPH radical scavenging activity across different concentrations (Figure 2a). The scavenging activity (%) of the *S. crispus* extracts showed a more gradual dose-dependent increase as compared to the standards (gallic acid and rutin), which rapidly increased to a plateau at concentrations below 200 µg/mL, indicating their high potency as antioxidants. The DPPH radical scavenging activities in the *S. crispus* extracts were more active in the polar extracts (water and methanol) as compared to the non-polar ones (ethyl acetate and hexane).

The DPPH radical scavenging activities of the extracts of *C. nutans* also showed a concentration-dependent relationship except for the ethyl acetate extract (Figure 2b), where a declining trend was observed when the concentration exceeded 500 µg/mL. This phenomenon was previously reported by Kong *et al.* (2012) where some antioxidants may act as

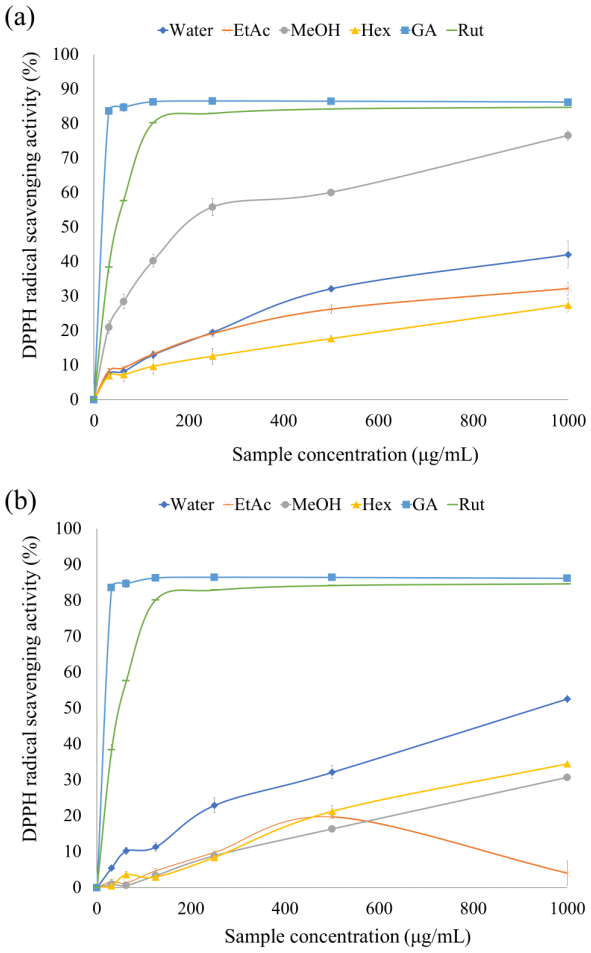

Figure 2. DPPH radical scavenging activities of (a) *S. crispus* and (b) *C. nutans* extracted with solvents of different polarities. Water: water extract, EtAc: ethyl acetate extract, MeoH: methanolic extract, Hex: hexane extract, GA: gallic acid, and Rut: rutin.

pro-oxidants. The ethyl acetate extract of *C. nutans* was found to be high in flavonoids (Table 1), which may cause pro-oxidant effects depending on the number of hydroxyl groups in the flavonoids, the existence of transition metal ions, and their concentrations in the extract (Procházková *et al.,* 2011).

Ferric reducing antioxidant power (FRAP)

The FRAP value was expressed as mmol/Fe^{2+} g extract since the assay assesses the ability of an antioxidant to reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) (Gliszczyńska-Świgło, 2006). The FRAP value of *S. crispus* extracts ranged from 1.22 to 6.84 mmol Fe^{2+}/g extract, with the highest reducing power in the methanolic extract, followed by the ethyl acetate, hexane, and finally water extracts. This is likely due to the higher amount of total phenolics and flavonoids in the methanolic extract that can donate electrons to reduce the Fe^{3+} to Fe^{2+} ions. Despite the methanolic

Table 2. Tentative identification of polyphenols in *S. crispus* methanolic extract using LC-QToF-MS/MS.

C	Tentative compound	RT	Mode	Precursor ion (m/z)	Fragment ion (m/z)	MW (g/mol)	Formula	Chemical classification
1.	Bidenoside B	13.687	$[M-H]^-$	477.1773	191.8997, 273.0868, 384.6101	478.1846	$C_{24}H_{30}O_{10}$	Chalcone glycoside
2.	8-p-Hydroxybenzylquercetin	13.968	$[M+HCOO]^-$	453.0815	187.0508, 343.1575	408.0833	$C_{22}H_{16}O_8$	Flavones and Flavonols
3.	Torosaflavone C	14.198	$[M+HCOO]^-$	453.0815	343.1556, 445.184	408.0833	$C_{22}H_{16}O_8$	Flavones and Flavonols
4.	5-Hydroxy-7,8-dimethoxyflavanone 5-rhamnoside	14.248	$[M-H]^-$	445.1504	266.3647, 383.1368	446.1576	$C_{23}H_{26}O_9$	Flavones and Flavonols
5.	3,6-Dimethoxy-6”,6”-dimethyl-3’,4’-methylenedioxypyranol [2,3:7,8] flavone	20.745	$[M-H]^-$	407.1141	197.0764, 309.0855	408.1214	$C_{23}H_{20}O_7$	Flavones and Flavonols
6.	Euchrenone b3	25.063	$[M-H]^-$	461.1584	197.027, 325.1859	462.1663	$C_{27}H_{26}O_7$	Isoflavonoid
7.	Scutellarein 7-glucuronide-6-ferulate	27.157	$[M+HCOO]^-$	683.1262	325.1789, 404.2747, 533.8258, 673.0743	638.128	$C_{31}H_{26}O_{15}$	Flavones and Flavonols
8.	Quercetin 3-(2'''-galloylglucosyl)-(1->2)-alpha-L-arabinofuranoside	28.054	$[M-H]^-$	747.1393	280.4614, 475.7874, 496.7559, 737.0983	748.1462	$C_{33}H_{32}O_{20}$	Flavones and Flavonols
9.	Lupinisol C	30.200	$[M+Cl]^-$	473.136	148.9531, 257.1505, 325.1837	438.1672	$C_{25}H_{26}O_7$	Isoflavonoid
10.	Patuletin 3-(6''-(E)-feruloylglucoside)	31.322	$[M-H]^-$	669.1463	248.9649, 325.1879, 455.131	670.1534	$C_{32}H_{30}O_{16}$	Flavones and Flavonols
11.	Quercetin 3-methyl ether 7- glucuronide	31.392	$[M+HCOO]^-$	537.0871	311.0585, 480.9549	492.0889	$C_{22}H_{20}O_{13}$	Flavones and Flavonols
12.	Quercetin 3-(6”-methylglucoronide)	31.745	$[M+HCOO]^-$	537.0871	267.0687, 284.9924	492.0889	$C_{22}H_{20}O_{13}$	Flavones and Flavonols
13.	Vitexin 2''-O-rhamnoside 6''- acetate	33.671	$[M+Cl]^-$	655.1431	248.9574, 304.9046, 347.08	620.1737	$C_{29}H_{32}O_{15}$	Flavones and Flavonols
14.	Veronicafolin 3-glucosyl-(1- >3)-galactoside	34.285	$[M+Cl]^-$	719.1566	443.1067, 563.1459, 583.1428	684.1872	$C_{30}H_{36}O_{18}$	Flavones and Flavonols
15.	Quercetin 3-sophoroside-7-glucuronide	38.947	$[M+HCOO]^-$	847.1818	325.184, 611.1484, 691.1607	802.181	$C_{33}H_{38}O_{23}$	Flavones and Flavonols

extract having the highest FRAP activity among the different solvents used, it was still lower than the standards (gallic acid and rutin), indicating that the methanolic extract had a moderate antioxidant potential as compared to the standards. Notably, the moderate levels of phenolics and flavonoids in the aqueous extract did not show a comparable ferric reducing power, which was also reported by Do *et al.* (2014). Nevertheless, Qader *et al.* (2011) reported otherwise, and such variable observations may be due to different procedures in the extractions or agronomic conditions of the collected samples.

On the other hand, *C. nutans* methanolic extract showed the highest ferric reducing activity and the hexane extract the least, which is in agreement with a previous study where the methanolic extract gave the highest FRAP value followed by the ethyl acetate and hexane extracts of a similar plant (Alam

Table 3. Tentative identification of polyphenols in *C. nutans* methanolic extract using LC-QToF-MS/MS.

No.	Tentative compound	RT	Mode	Precursor ion (m/z)	Fragment ion (m/z)	MW (g/mol)	Formula	Chemical group
1.	8-Chloro-5,7,4'-trihydroxy-3-C-methylflavanone	12.740	$[M-H]^-$	319.0393	169.08, 187.0916	320.0465	$C_{16}H_{13}ClO_5$	Flavones and Flavonols
2.	5-Hydroxy-7,4'-dimethoxy-6,8-di-C-prenylflavanone 5-O-galactoside	14.825	$[M-H]^-$	489.1778	237.1255, 321.1120, 393.2744	490.185	$C_{25}H_{30}O_{10}$	Flavones and Flavonols
3.	3,4,7-Trihydroxy-5-methoxy-8-prenylflavan 7-O-beta-D-glucopyranoside	15.194	$[M-H]^-$	533.2035	237.1284, 475.2331	534.2108	$C_{27}H_{34}O_{11}$	Flavones and Flavonols
4.	Xanthoangelol C	21.958	$[M-H]^-$	365.14	174.9559, 197.0242, 297.1526	366.1466	$C_{22}H_{22}O_5$	Chalcone and dihydrochlacone
5.	Euchrenone b3	24.520	$[M-H]^-$	461.1616	198.0391, 325.1838, 393.1645, 455.1427	462.1674	$C_{27}H_{26}O_7$	Isoflavonoid
6.	Scutellarein 7-glucuronide-6-ferulate	27.154	$[M+HCOO]^-$	683.126	325.1924, 430.8549, 673.0561	638.1286	$C_{31}H_{26}O_{15}$	Flavones and Flavonols
7.	Quercetin 3-(2'''-galloylglucosyl)-(1->2)-alpha-L-arabinofuranoside	28.013	$[M-H]^-$	747.1399	278.9129, 492.7362, 737.0868	748.1468	$C_{33}H_{32}O_{20}$	Flavonoid glycoside
8.	Patuletin 3-(6"-(E)-feruloylglucoside)	31.331	$[M-H]^-$	669.1467	248.9566, 325.1785, 483.999, 673.0679	670.1537	$C_{32}H_{30}O_{16}$	Flavones and Flavonols
9.	Vitexin 2"-O-rhamnoside 6"- acetate	33.360	$[M+Cl]^-$	655.1437	248.9606, 419.1, 519.1267, 655.1439	620.1744	$C_{29}H_{32}O_{15}$	Flavones and Flavonols
10.	Veronicafolin 3-glucosyl-(1- >3)-galactoside	34.282	$[M+Cl]^-$	719.1569	443.1022, 563.1345, 583.1521	684.1864	$C_{30}H_{36}O_{18}$	Flavones and Flavonols
11.	Quercetin 3-sophoroside-7- glucuronide	37.099	$[M+HCOO]^-$	847.1803	651.1552, 691.1601	802.1805	$C_{33}H_{38}O_{23}$	Flavones and Flavonols
12.	Chalconaringenin 2'-O-glucoside 4'-O-gentobioside	50.996	$[M+Cl]^-$	793.1991	543.9599, 765.9689, 785.9776	758.2296	$C_{33}H_{42}O_{20}$	Flavones and Flavonols

et al., 2017). Similarly observed in *S. crispus*, the FRAP values of *C. nutans* methanolic leaves extract corresponded with its high phenolic and flavonoid contents. The FRAP value in the present work (1.49 mmol Fe^{2+}/g extract) was higher than that observed in the buds of the plant (0.45 mmol Fe^{2+}/g extract) as reported by Ghasemzadeh *et al.* (2014). The differences could be attributed to the different biosynthesis of bioactive compounds in the various parts of the plant.

Trolox equivalent antioxidant capacity (TEAC)

The ABTS radical cation scavenging capacities of the plant extracts were expressed as Trolox equivalent antioxidant capacity (TEAC) (Table 1). The antioxidant capacity was measured by the ability of the extract to scavenge the oxidised free radical, ABTS•, through the donation of hydrogen atoms from hydroxyl groups which results in the loss of the blue/green colour of the ABTS• (Arts *et al.,* 2004; Saeed *et al.,* 2012). This assay has the advantage of determining the antioxidant potential of both hydrophilic and lipophilic antioxidant compounds over different types of sample, including plant extracts (Alam *et al.,* 2013).

Similar to the DPPH and FRAP results, the highest TEAC value was observed in the *S. crispus* methanolic extract (1.01 mmol TE/g extract), which was also observed in previous studies in which methanol was an efficient solvent in extracting

antioxidants from the species (Rajendrakumar *et al.,* 2014; Agnel Ruba and Mohan, 2016). Although the methanolic *S. crispus* extract had a lower TEAC value as compared to the standards (gallic acid and rutin), it was still able to serve as a potent radical scavenger.

As for *C. nutans*, the ethyl acetate extract showed the highest TEAC value with the water extract showing the lowest, which is in agreement with another study conducted by Sarega *et al.* (2016). The higher TPC in the ethyl acetate extract might have been responsible for the slightly higher ABTS radical scavenging capacity. Nevertheless, the TEAC value in *C. nutans* was higher than the methanolic extracts of some Chinese medicinal plants such as *Chrysanthemum indicum* (0.05 mmol TE/g extract), *Ginkgo biloba* (0.08 mmol TE/g extract), and *Centipeda minima* (0.02 mmol TE/g extract) (Song *et al.,* 2010).

Correlation analysis

The linear correlations (*r*) between the polyphenols and antioxidant activities in *S. crispus* and *C. nutans* were determined using Pearson correlation analysis. Correlation with DPPH was done using DPPH radical scavenging activity at 1000 µg/mL. Statistically significant ($p < 0.05$) and high positive correlations were observed between the TPC and TFC of *S. crispus* and the FRAP values (r = 0.976 and 0.985, respectively), TEAC values (r = 0.937 and 0.956, respectively), and DPPH values (r = 0.982 and 0.915, respectively). This supports our hypothesis that phenolics and flavonoids significantly contribute to the antioxidant activities of *S. crispus*. Our previous findings also reported the ability of the hydroxyl group present in phenolic compounds to act as reducing agents to neutralise free radicals and exert their antioxidant activities (Kong *et al.,* 2012).

For *C. nutans*, the TPC showed a statistically significant high positive correlation with the FRAP values (r = 0.934), but no significant correlations with the TEAC and DPPH values. This demonstrated that the phenolic compounds were the major contributor of ferric reducing antioxidant activities in the *C. nutans* extracts, which agrees with a study by Lusia Barek *et al.* (2015). However, the antioxidant activities of the plant extracts may not be solely attributed to the phenolic and flavonoid contents, as other antioxidant secondary metabolites such as vitamins and carotenoids may contribute to the activities (Javanmardi *et al.,* 2003). On the other hand, the antioxidant activity of TEAC was mainly contributed by the flavonoids in the extract, as a statistically significant positive correlation between the TFC and the TEAC value was observed (r = 0.663). These findings are in agreement with Chew *et al.* (2011) who showed a similar correlation between the TFC and FRAP value, although Lusia Barek *et al.* (2015) did not observe such a correlation. These findings revealed that the flavonoids in *C. nutans* act as electron donors in contributing to its antioxidant activities. On the other hand, a statistically significant negative correlation was observed between the TFC and DPPH of *C. nutans* (r = -0.925). This may be due to the pro-oxidant activities of flavonoids that was mentioned earlier (Procházková *et al.,* 2011).

Identification of polyphenolic compounds

Advanced tandem HPLC techniques such as HPLC-ESI-QToF-MS/MS serve as powerful tools to rapidly identify phytochemicals in a complex mixture. Combining the separation power of HPLC and the ability of MS to detect multiple components with high sensitivity is a valuable technique in the simultaneous determination of phytochemicals in plant extracts (Glauser *et al.,* 2013). Many studies have reported the bioactive compounds in *S. crispus* and *C. nutans* (Khoo *et al.,* 2015; Ghasemzadeh *et al.,* 2015; Sarega *et al.,* 2016; Alam *et al.,* 2016). However, the studies were mostly carried out using the spectrophotometry method or HPLC analysis. Hence, limited compounds were identified in both plants due to the limited standards available, particularly the polyphenolic compounds. The advantage of HPLC-ESI-QToF-MS/MS in the present work offers another dimension of separation which spectrophotometry or HPLC alone would not be able to achieve, leveraging on the fact that the different fragmentation patterns of different compounds can assist in discriminating them. In addition, the high sensitivity of HPLC-ESI-QToF-MS/MS allows for the detection of low abundance compounds which normally would be masked by high abundance compounds when analysed with the HPLC and spectrophotometry methods. Therefore, the application of a more advanced HPLC-ESI-QToF-MS/MS method coupled with identification using the available database will hopefully shed more light on the plants' bioactive compounds, which is important for future research on their bioactivities. HPLC-ESI-QToF-MS/MS analysis through the matching of MS/MS data with the existing database (METLIN) was able to tentatively identify 20 polyphenolic compounds in the methanolic extracts of *S. crispus* (Table 2) and *C. nutans* (Table 3). The

identified compounds were bidenoside B, 8-p-hydroxybenzylquercetin, torosaflavone C, 5-hydroxy-7,8-dimethoxyflavanone 5-rhamnoside, 3,6-dimethoxy-6”,6”-dimethyl-3’,4’-methylenedioxypyranol [2,3:7,8] flavone, euchrenone b3, scutellarein 7-glucuronide-6-ferulate, quercetin 3-(2'''-galloylglucosyl)-(1->2)-alpha-L-arabinofuranoside, lupinisol C, patuletin 3-(6''-(E)-feruloylglucoside, quercetin 3-methyl ether 7-glucuronide, quercetin 3-(6”-methylglucoronide), vitexin 2''-O-rhamnoside 6''-acetate, veronicafolin 3-glucosyl-(1->3)-galactoside, quercetin 3-sophoroside-7-glucuronide, chalconaringenin 2'-O-glucoside 4'-O-gentobioside, 8-chloro-5,7,4’-trihydroxy-3-C-methylflavanone, 5-hydroxy-7,4’-dimethoxy-6,8-di-C-prenylflavanone 5-O-galactoside, 3,4,7-Trihydroxy-5-methoxy-8-prenylflavan 7-O-beta-D-glucopyranoside, and xanthoangelol C. To the best of our knowledge and based on literature mining, 15 tentatively identified polyphenolic compounds classified as chalcones, isoflavanoids, flavones, and flavonols have not been previously reported for both species, with the exception of quercetin and vitexin (Ghasemzadeh *et al.,* 2015; Khoo *et al.,* 2015; Alam *et al.,* 2017). The polyphenolic compounds reported in both species were mainly flavonoids, which fall under the subgroup of flavones and flavonols. Many of these flavones and flavonols, either aglycones or glycosides, present in plants are well known for their antioxidant activities, for example quercetin (Kong *et al.,* 2012), vitexin (Nasma *et al.,* 2018), patuletin (Corrêa *et al.,* 2018), and scutellarein (Meng *et al.,* 2008).

Conclusions

The antioxidant activities and tentative polyphenolic constituents of *S. crispus* and *C. nutans* leaves extracted with different solvents were determined. *S. crispus* gave overall better polyphenolic content and antioxidant activities as compared to *C. nutans*. The methanolic extracts of both species were ideal in yielding higher amounts of phenolics and flavonoids. Further HPLC-ESI-QToF-MS/MS analysis was able to tentatively identify 20 polyphenolic compounds in the extracts of both species. However, further confirmative analysis using standards is needed to ascertain the presence of these polyphenolic compounds as well as to quantify their amounts. Although the antioxidant activities of the methanolic leaf extracts in both species were not as high as those of gallic acid and rutin, these plants still serve as potential sources of antioxidants that can benefit the nutraceutical and pharmaceutical industries.

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