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Analysis of the phytochemical contents and anti-oxidative properties of *Stenochlaena palustris*

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Abstract

Stenochlaena palustris (Burm. f.) Bedd, a popular edible fern in Southeast Asia, is considered nutritious and beneficial for human health. It is believed that the presence of several micronutrients and antioxidants might be the reason for its speculated beneficial effects and its usage in traditional medicine. In order to assess the nutritional properties of this particular herb, we have investigated the presence of several phytochemicals in *S. palustris* along with their total phenolics (TP) and total flavonoids (TF) contents. The total phytochemicals were extracted using hydrophilic and hydrophobic solvents [polar (ethanol), partially polar (ethyl acetate), and non-polar (hexane)], and their antioxidative properties were determined. The results obtained showed that the leaf of *S. palustris* extracted using ethanol and ethyl-acetate contained higher phenolics and flavonoids when compared with other parts of the plant extracts. The ethanolic leaf extracts exhibited the highest antioxidant activities in all of the methods used to determine antioxidative property as compared to other parts of the plants, with FRAP, ABTS, and DPPH assays value of 17.95 mM Fe2+/g, 0.062 mM TE/g, and IC_{50} of 24.24 µg/ml, respectively. The IC_{50} value of the sample was comparable with that of ascorbic acid which was used as standard antioxidant. In the present work, we have used special types of *S. palustris* which was collected from a site free from environmental toxins and human intrusion. Though this is a common herb in this part, but information on phytochemicals of *S. palustris* is lacking, especially from this part of Borneo. Our findings address the comparison between different solvent extracts followed by their bioactive properties.

Introduction

Oxidative stress is a known causative factor for several human diseases. Free radicals, or reactive oxygen species (ROS), are often generated in the human body as metabolic by-products, which are subsequently neutralised or removed from the body by endogenous enzymatic reactions or dietary micronutrients. However, intrinsic defence systems are often unable to protect vital biomolecules following the activation of excess oxidative stress inducers. The increased incidence of oxidative stress-related diseases among the human population has prompted the consumption of increasing amounts of natural antioxidants in human diets. Existing information has suggested that the elevation of free radicals and ROS may contribute to several complicated diseases including cancers, metabolic disorders, and cardiovascular diseases (Lobo *et al.,* 2010; Chen and Keaney, 2012; He and Zuo, 2015; Panieri and Santoro, 2016). Furthermore, several studies have demonstrated that the consumption of foods that are rich in antioxidants can prevent human diseases (Rathore *et al.,* 2011; Jurikova *et al.,* 2017; Panya *et al.,* 2018). Ferns have been utilised as a food source and in folk medicines since ancient times in many parts of India (Yumkham *et al.,* 2017), Malaysia (Yusuf, 2010), and some African countries (Maroyi, 2014). Ferns are rich sources of phytochemicals, containing bioactive components that belong to the flavonoid, phenolic, terpenoid, and alkaloid families (Li *et al.,* 2008; Lee *et al.,* 2014; Abdul Wahab *et al.,* 2015). Ferns have received increased attention in the field of herbal medicine due to their wide range of pharmacological applications, including the treatment of eczema and wound healing (Yadav *et al.,* 2012), as anti-inflammatory (Yonathan *et al.,* 2006), antimicrobial and antioxidant agents (Proestos *et al.,* 2005; Goswami and Ram, 2017), and in the treatment of diabetes (Ajikumaran *et al.,* 2006; Sathiyaraj *et al.,* 2015). *Stenochlaena palustris*, which is locally known as 'Kalakai' in the Borneo region, and as 'Paku' in Malaysia (Holttum and Ridley, 1968), is an edible creeping fern that can be found widely across Malaysia and Australia. The sterile fronds are available

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throughout the year, and are edible, with broad pinnae and sharply toothed margins. However, fertile fronds have thin, long pinnae that bear spores which are not edible and are seasonal. Young fronds are light green in colour, with a red tonality, and are softer than mature fronds. The young sterile fronds of the fern are used in traditional medicines to treat ailments such as skin disease, diarrhoea, fever, and gastric ulcer (Piggott, 1988). A nutritional analysis of *S. palustris* has suggested that this plant represents a good source of minerals, including potassium and phosphorus with additional nutritional values (Hoe and Siong, 1999). The present work was designed to investigate the phytochemical contents of *S. palustris*, especially the total phenolic contents (TPC) and the total flavonoid contents (TFC) using three different solvents, and to assess the antioxidant properties of the extracts.

Materials and methods

Plant material

Stenochlaena palustris was collected in March 2018, in the Kuala Belait district of Brunei Darussalam. This site (latitute 4.5800, longitude 114.3963) was once covered with a peat swamp forest but has now become a degraded land due to logging and several severe fires. This site is regularly flooded; therefore, the soil is rich with organic substances and abundant water is available. The closest city is Kuala Belait, located approximately 5 km south of the site, and the surrounding area is covered with a pristine, tropical peat swamp forest, with no human disturbances. The sampling site was least accessible to humans with minimal or no exposure to pollutants.

Soxhlet extraction

The stems and leaves of *S. palustris* (20 g samples) were used for Soxhlet extraction, and separately dissolved in 250 mL of each solvents (*n*-hexane, ethyl acetate, and ethanol). The extraction process was allowed to run for several rounds over 24 h. The extract was filtered and concentrated using a rotatory evaporator (Buchi Rotary Evaporator, R-205, Switzerland). The final concentrated dried extract was stored at -20°C until further use.

Preliminary (qualitative) phytochemical analyses

A preliminary phytochemical analysis of the *S. palustris* extract was performed to qualitatively investigate the presence of secondary metabolites such as flavonoids, terpenoids, saponins, tannins, and phenolics. The standard procedures described by Trease and Evans (1989) were used for these analyses.

Test for terpenoids (Salkowski's test)

Chloroform (2 mL) was mixed with 1 mL of plant extract, and 3 mL of concentrated sulphuric acid (H_2SO_4) was carefully added to this mixture, without jerking. The development of a reddish-brown colour at the interface indicated the presence of terpenoids.

Test for flavonoids

To 1 mL of the extract, 5 mL of diluted ammonia was added. Upon the addition of 1 mL of concentrated H_2SO_4, a transient yellow colour solution was produced in the samples that contained flavonoid. This was further confirmed by adding few drops of 1% aluminium solution that produced stable and permanent yellow colour, thus indicating the presence of flavonoids.

Test for saponins (frothing test)

Sample (1 mL) containing 1 mg/mL of extract was mixed with 1 mL of distilled water in a test tube, and mixed vigorously. The formation of a foamy froth indicated the presence of saponins.

Test for tannins

Sample (1 mL) containing 1 mg/mL of extract was mixed with 9 mL of distilled water, and boiled for 5 min in a water bath. This reaction mixture was allowed to cool to room temperature, and then filtered with 3 mm Whatman filter paper to remove debris. Next, 1 mL of 0.1% ferric chloride was added to the filtered mixture, and the appearance of a brownish-green colour indicated the presence of tannins.

Test for phenols

The extract was diluted with distilled water in a ratio of 1:4, and few drops of 10% ferric chloride solution were added. The appearance of dark green colour indicated the presence of phenols.

Total flavonoid contents

The total flavonoid contents were determined using the method described by Zhishen *et al.* (1999). To 1 mL extract, 3 mL of methanol, 200 µL of 10% aluminium chloride, 200 µL of 1 M sodium acetate, and 5.6 mL of distilled water were added. The reaction mixture was incubated at 30°C for 30 min, and the absorbance was measured at 420 nm using a spectrophotometer, and compared against a blank. Synthetic quercetin was used as the standard. The total contents of flavonoid compounds were calculated using the standard curve derived from quercetin standard, and expressed as quercetin equivalents (QE) (Zhishen *et al.,* 1999).

Total phenolic contents

The total phenolic contents were determined using the Singleton method with slight modifications. To 1 mL extract with a concentration of 1 mg/mL, 5 mL of 10% Folin-reagent dissolved in water, and 5 mL of 7.5% $NaHCO_3$ were added (Singleton *et al.,* 1999). The samples were incubated at 25°C for 30 min to complete the reaction. The absorbance was recorded at 70 nm, and the mean absorbance value was used for TPC quantifications. Gallic acid was used as the standard, and a standard curve was used to calculate the TPC. All the experiments were performed in triplicate, and the TPC expressed as gallic acid equivalents (mg of GAE/g of extract).

Scavenging activity (DPPH) assay

The scavenging activity of the extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Shen *et al.,* 2010). DPPH in methanol produces a deep violet colour. An antioxidant compound donates an electron to DPPH causing its reduction and changing the colour from deep violet to yellow. A fresh solution of 1 M DPPH was prepared in methanol. Next, 3 mL extract was mixed with 1 mL of DPPH solution, and allowed to stand in the dark for 30 min. The absorbance was recorded at 517 nm, and the percent inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = (A - B / A) \times 100$$

where, A = absorbance of pure DPPH in oxidised form, and B = absorbance of the sample mixed with DPPH.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay described by Benzie and Strain (1996) was used to determine the total antioxidant activity of the *S. palustris* extract. Stock solutions were prepared including 10 mM of 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) in 40 mM of hydrochloric acid, a 300 mM of acetate buffer, pH 3.6, and 20 mM of ferric chloride solution. A fresh working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ, and 2.5 mL of ferric chloride. The solution was heated to 37°C before use. *S. palustris* extract (100 µL) was allowed to react with 2 mL of FRAP reagent for 30 min in the dark. Then, the absorbance of the reaction mixture was measured at 593 nm. The results were expressed in mM Fe (II)/g dry mass (Benzie and Strain, 1996).

ABTS (2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) antioxidant assay

An antioxidant assay kit (catalogue number CS0790, Sigma Aldrich) was used to determine the antioxidant activity of the *S. palustris* extract. Using synthetic Trolox, a standard curve was generated and used to quantify the antioxidant properties. A working solution of 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was prepared by adding 25 µL of 3% hydrogen peroxide solution to 10 mL of ABTS substrate solution. In a 96-well plate, 10 µL of Trolox standards and samples were added to each well, combined with 20 µL of myoglobin solution (provided with the kit). To each well, 150 µL of ABTS substrate was added and incubated at room temperature for 5 min. The reaction was stopped by adding 100 µL of stop solution, and the absorbance was measured at 405 nm using a microplate reader.

Statistical analysis

SPSS Statistics version 16 was used to analyse the obtained data. All experiments were performed in triplicate, and the values were expressed as mean ± standard deviation. An analysis of variance (ANOVA), followed by Tukey's *post hoc* test was used to determine significant differences, with *p*-values less than 0.05 was considered significant.

Results and discussion

Flavonoids and phenolic compounds are secondary metabolites with a variety of biological activities, and widely found in plants. The consumption of fruits and vegetables that contain high levels of phenolic compounds is often recommended because phenolic compounds represent the most efficient natural antioxidants, and play important roles in the prevention of cardiovascular disease, ageing, and the effective scavenging of oxygen free radicals. Scientists have investigated the phytochemical contents of a variety of natural products, including edible fruits and vegetables based on existing information. A preliminary phytochemical analysis of *S. palustris* leaf and stem extracts was performed by Trease and Evans (1989). Recent advances in this field have suggested that the phytochemical compounds in plants may vary depending on the landscape, the presence of environmental toxins, and the availability of soil nutrients. In the present work, we have investigated the phytochemical properties of *S. palustris* collected from a location believed to be free from environmental pollutants and human intrusion. As depicted in Table 1, the preliminary (qualitative) phytochemical analyses of *S. palustris* revealed the presence of flavonoids, saponins, terpenoids, and

phenols, which are known antioxidants with free radical scavenging capacities. Following this, we measured the TPC and TFC. Finally, we assessed their biochemical properties using several techniques.

Table 1. Preliminary (qualitative) results of the phytochemical constituents of *Stenochlaena palustris* extracts.

Chemical constituent	Ethanol leaf	Ethanol stem	Ethyl acetate leaf	Ethyl acetate stem	Hexane leaf	Hexane stem
Flavonoid	+	+	+	+	+	+
Saponin	+	+	+	+	+	+
Tannin	+	+	+	+	+	+
Terpenoid	+	+	+	+	+	+
Phenol	+	+	+	+	+	+

+ = present, – = absent.

Total phenolic and flavonoid contents

Our observations suggested that ethanolic *S. palustris* leaf extracts showed the highest TPC contents at 3.80 mg GAE/g, when compared with those for ethyl acetate or hexane extracts. This result may be due to the nature of ethanol, which can dissolve hydrophilic and some hydrophobic compounds. Ethanolic extracts of young *S. palustris* leaves have been reported to contain higher TPCs than root extracts (Kusmardiyani *et al.,* 2016), whereas the methanolic extracts of both young and mature fronds were also found to contain high TPCs (Chear *et al.,* 2016). In the present work, we observed that TPCs significantly differed among leaf and stem of *S. palustris* extracts made with different solvents ($p < 0.05$). The *S. palustris* extract obtained using hexane, which is a partially polar solvent, demonstrated the lowest TPCs at 1.59 and 1.89 mg GAE/g for leaves and stems, respectively, which could be due to the fact that most phenolic compounds are soluble in polar solvents, whereas the reduced polar properties of hexane cannot dissolve most phenolics. When we examined the other two solvents (ethanol and ethyl acetate), which are polar, the TPCs were higher for those extracts as expected.

The TFC was expressed as QE, as shown in Table 2. Our statistical analysis suggested no significant difference between the ethyl acetate leaf and stem extracts, which were found to have 3.96 and 3.23 mg QCE/g, respectively. The ethyl acetate extracts had higher TFC values when compared with TPC values. Similar findings were previously reported for *S. palustris* extracts derived from plants collected in peninsular Malaysia (Chai *et al.,* 2012; Chear *et al.,* 2016); however, the sampling sites were not well-described or illustrated, and the levels of environmental pollutants were not assessed. The present results implied that flavonoids in *S. palustris* extracts are less-polar or semi-polar in nature. Therefore, the observed differences in TFCs may be due to differences in the solvent polarity. This view is also supported by reports that ethyl acetate was the most efficient solvent for the extraction of polyphenols from grape seeds and modified *boyang-hawan-o-tang* herbs (Spigno *et al.,* 2007). Polyphenols refer to a large group of phytochemicals that contain more than one phenolic hydroxyl group. Phenolic compounds present in plants have a wide range of medical uses. Phenolics can scavenge free radicals due to the presence of hydroxyl groups. Because polyphenols are known for their antioxidant properties, we employed several standard experimental

Table 2. Total phenolic, flavonoid, and antioxidant contents of *Stenochlaena palustris* extracts.

***Stenochlaena palustris* extracts**	**TPC (mg GAE/g)**	**TFC (mg QCE/g)**	**DPPH IC_{50} (µg/ml)**	**FRAP (mM Fe2+/g)**	**ABTS (mM)**
Ethanol leaf	3.80 ± 0.22^{a}	2.15 ± 0.005^{c}	24.24 ± 0.174^{e}	17.95 ± 0.026^{a}	0.61 ± 0.0023^{a}
Ethanol stem	2.30 ± 0.09^{b}	1.85 ± 0.030^{c}	89.96 ± 0.527^{d}	3.82 ± 0.184^{d}	0.62 ± 0.0017^{a}
Ethyl-acetate leaf	2.65 ± 0.11^{b}	3.96 ± 0.072^{a}	159.1 ± 0.116^{c}	7.54 ± 0.056^{b}	0.46 ± 0.0105^{d}
Ethyl-acetate stem	2.19 ± 0.06^{bc}	3.23 ± 0.034^{b}	178.27 ± 0.390^{b}	6.79 ± 0.140^{c}	0.56 ± 0.0122^{c}
Hexane leaf	1.59 ± 0.04^{d}	3.20 ± 0.332^{b}	323.43 ± 0.450^{a}	2.94 ± 0.356^{e}	0.57 ± 0.0025^{bc}
Hexane stem	1.89 ± 0.3^{bcd}	2.75 ± 0.061^{b}	323.50 ± 0.095^{a}	2.93 ± 0.114^{e}	0.56 ± 0.0080^{bc}
Ascorbic acid	-	-	28.8 ± 0.011^{f}	-	-

Values are mean ± standard deviation of triplicate ($n = 3$). Different superscript letters in a column indicate significance difference ($p < 0.05$) using one-way ANOVA followed by Tukey's *post-hoc* test.

methods to confirm the presence of polyphenolic compounds by measuring their antioxidant properties.

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Free radical scavenging property of the S. palustris extracts

Studies have shown that free radicals play important roles in the development of heart diseases, cancers, ageing, and immune system damages (Asimi *et al.,* 2013). Antioxidants that inhibit the oxidation rate and protect against cellular damages can eliminate these unstable free radicals. Orthodox antioxidant drugs have been used to treat oxidative stress-related diseases such as stroke, cancers, and Alzheimer's disease (Devasagayam *et al.,* 2004). In the present work, the antioxidant properties of *S. palustris* extracts were evaluated using the FRAP assay, followed by assessing their abilities to scavenge free radicals using DPPH and ABTS assays.

DPPH is a dark-purple, crystalline powder, composed of stable free radical molecules that is commercially available and has been widely used to assess the free radical scavenging properties of plant extracts. DPPH scavenging techniques were used to determine the IC_{50} values of the extracts, in which extracts containing increased levels of antioxidants generated increased levels of reduced DPPH, which was visible as the transition from a purple to a yellow colour. Because the IC_{50} value is inversely related to the antioxidative property, a lower IC_{50} value indicated increased free radical scavenging power. The lowest IC_{50} value was obtained for the ethanolic leaf extract at 24.8 µg/mL, whereas the highest IC_{50} values were obtained for the hexane leaf and stem extracts at 323.40 µg/mL for both (Table 2). As expected, we observed negative correlations between the IC_{50} values and the TPCs of the extracts.

Antioxidant assay using radical cationic ABTS+ scavenging capacity

Following the previous experiment, we employed a commercially available antioxidant assay kit (Cat: CS0790, Sigma-Aldrich) to improve the acceptability of our findings. This kit detects the conversion of oxidised ABTS+ to reduced ABTS. In the presence of antioxidants, ABTS+ is reduced with a consequent decrease in the blue-green colour, and the decolourisation is proportionate to the antioxidant free radical scavenging property. Thus, all experimental extracts were tested for their abilities to scavenge ABTS+ radical cations (Table 2). The ethanolic stem and leaf extracts displayed the highest scavenging activities with values of 0.62 and 0.61 mM TE/g, respectively, whereas the ethyl acetate leaf extracts showed the lowest activity at 0.46 mM TE/g.

Further confirmation of radical scavenging property

We utilised the FRAP assay during which the antioxidants in the extracts reduced Fe^{3+} to Fe^{2+} in the presence of TPTZ. Upon reduction, Fe^{2+} interacts with TPTZ to produce a blue colour, which was used to assess the antioxidative properties of the extracts. The reducing powers of the *S. palustris* extracts are shown in Table 2. Increased reducing power indicates stronger antioxidant activity. We utilised this technique to confirm our previous findings regarding the antioxidative properties of our experimental extracts, and this technique has been used by numerous studies to address the antioxidative property of the plant extracts (Gülçın *et al.,* 2003; Odabasoglu *et al.,* 2005; Duan *et al.,* 2007; Stojanović *et al.,* 2010; Irshad *et al.,* 2012; Samaradivakara *et al.,* 2016). In the present work, we determined that the reducing power of the ethanolic leaf extract of *S. palustris* was significantly higher ($p < 0.05$; 17.95 mM/g) than those for the *S. palustris* extracts using other solvents, as depicted in Table 2. We have also observed no significant difference between hexane leaf and stem extracts ($p > 0.05$) at 2.94 and 2.93 mM/g, respectively, and the low value observed for hexane extracts could be due to the low polarity of the solvent.

Conclusion

Stenochlaena palustris are grown naturally and free from synthetic fertiliser and pesticides, and these readily available natural herbs are a good source of dietary antioxidant. Though such findings have been reported earlier, inconsistency and discrepancy in their phytochemical properties prompted us to re-evaluate these characteristics. It was observed that TPC and TFC for the extracts showed a correlation with FRAP values, and an inverse association with IC_{50} values. However, ABTS assay suggested the presence of substantial antioxidative properties of all the experimental extracts. Though FRAP assay suggested highest antioxidative property of ethanolic leaf extract, and this was lowest in hexane leaf and stem extracts, the DPPH assay-derived IC_{50} values were significantly different among these samples. In order to optimise the experimental doses and selection of the most effective extract for further *in vivo* experiments, these factors should be taken into consideration.

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