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DETERMINATION OF ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC CONTENT IN DIFFERENT PARTS OF MEDICINAL FERN, *BLENCHUM ORIENTALE*

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Abstract

Medicinal value of pteridophytes has been known to man for more than 2000 years. *B. orientale* is an edible fern that is used as a food and in traditional medicine. This study assessed the total phenol content (TPC) and antioxidant activity by three different assays (DPPH, FRAP and ORAC) of the 50% aqueous acetone extracts of *B.oriental* parts sampled from two selected location (UKM Fern Garden and Chini Forest). The extracts analyses were prepared from the young fronds, mature fronds, rhizome, and fiddlehead of the sampling fern. ANOVA test at $P < 0.05$ determines significant differences between various parts extract. It was found the TPC of young frond extract of *B. oriental* had the highest contents (1360 and 1643 mg GAE/100g DW) in UKM Fern Garden and Chini Forest respectively. While the antioxidant activity of plant extracts by DPPH, FRAP assay and ORAC value for all parts of *B. oriantle* was very high at both locations. It illustrated that there is strong antioxidant activity for all parts of plant, especially for Chini Forest and that correlate to diversity of climatic and geomorphological features. A good linear significant correlation coefficient was detected between the TPC and FRAP ($p < 0.01$), and among the assays were compared with each other (DPPH, FRAP and ORAC), ($p < 0.05$) from both location. These findings provide scientific evidence to support its traditional medicinal importance which is potentially rich sources of natural antioxidants.

Key words: Medicinal Ferns, Total Phenolic Content*, Blenchum orientale*

Introduction

Over the recent years, the effects of antioxidants on illnesses have been investigated. Antioxidants inhibit and scavenge radicals, thereby protecting humans against infections and degenerative diseases. Antioxidants can be defined as any substrate that significantly delays or prevents oxidation when present at low concentration compared with an oxidizable substrate (lipid, protein, carbohydrate, or DNA) (Halliwell 1999). Numerous studies have also established the relationship between consumption of antioxidant-rich plants and prevention of human diseases (Rathore *et al.* 2011). A natural antioxidant from medicinal plant is safer for the human body because it is a less harmful alternative to a synthetic antioxidant.Malaysia is known for its green tropical vegetation and forest, and its diverse nature is believed to possess medicinal values. Ferns have been used by humans as food and medicine since ancient times (Ghosh 2004; Lee & Shin 2010). Ferns are a group of non-flowering plants known as Pteridophytes. The fern species in Malaysia are estimated to be 1136 (Bidin & Jaman 1999).As the nutritional contents of the fern are comparable or even superior to those of some common leafy vegetables and medicines, we explore the potential of ferns as a low-cost functional medicine particularly for developing countries (Chang *et al*., 2010). *Blechnum orientale* is an important medicinal fern belonging to the family Blechnaceae, is a widespread fern in Southeast Asian with a rapid growth rate and large aboveground biomass. This fern has been used in traditional Chinese, Indian, and Malay medicine, as well as a food source plant since ancient times (Benjamin & Manickam 2007). The fern is used in the treatment of diarrhea and stomach problems (Vasudeva 1999).It is considered a cure for intestinal worms and bladder complaints in India and Polynesia, and as a diaphoretic and aromatic in the Philippines (Dixit & Vohra 1984). The rhizome is used as an anthelmintic in China. The diuretic properties of this fern have been put to good use in treating swellings. The information and knowledge gained from this study are expected to increase the awareness of using natural antioxidants besides synthetic antioxidants. Despite the varied uses of ferns in traditional medicine, no published report exists on their antioxidant activity. Therefore, the dire need of the hour is to discover or identify medicinal plants, rich in antioxidants. Medicinal ferns can be economic, natural and easily affordable by all the people. Thus, The present study was taken up for explore scientifically the antioxidant potential and total phenolic content of different parts of *B. orientale* fern that were collected from two different growth location(UKM Fern Garden and Chini Forest. All the assays were carried out in triplicate and the average value was considered.

Fig. 1 : *B. orientale* fern: (a) fiddlehead, (b) whole plant

Materials and Methods

Samples Collection

The fern samples in this study were randomly collectedin October 2013 from two study sites, the first site was from, UKM Fern Garden (Taman Paku Pakis) and the second site was Chini Forest reserve. Healthy and green plants were selected for the collection of plant parts. The plant was identified based on taxonomical parameters and their vernacular names.

In the present study, the plants used are similar to those generally used in medical preparations in traditional Malay medicine. Fern samples from two different plants (three replicate) were collected and stored in polyethylene plastic bags. The samples of the plant were directly washed gently with deionized distilled water for approximately 3 minutes to remove soil particles adhered to the plants. Plant sample divided into rhizome, mature fronds, young fronds and fiddlehead.

It was oven dried at temperature 40°C for 48 h. The dried sample was then pulverized using a mechanical grinder (sharp EM-II, Malaysia) and passed through a standard 20 mesh size sieve (particle size 0.5 mm) The homogenized oven-dried ground material was kept in air-tight container labeled and stored at 4°C until required for analyses.

Extraction of Antioxidant

The fine powder of fern parts was extracted using50% of aqueous acetone. Acetone-water solvent was a good solvent to extraction of less polar phenolics. About one gram of each sample (mature frond, young fronds, rhizome and fiddlehead) were weighed in universal bottles and 10 ml solvent was added at room temperature and then swirled with a magnetic stirrer at a speed of 1,000 rpm for 30 min. All extracted samples were centrifuged using a table top centrifuge (MLX 210, Thermo-line, China) at 4750 g for 15 min. All extractions were carried out in three replicates. The supernatant was collected and kept in –20°C until the experiment commenced.

Total Phenol Content (TPC) Assay

Total phenolic concentration in the different plant parts extract were quantified by Folin-Ciocalteu procedure which is considered as one of the best methods for the determination of TPC. According to (Musa *et al*., 2011), about 10 µL parts extract of *B. orientale* apart extracts were added to 0.5 ml diluted Folin-Ciocalteu reagent. The samples (Ferns extracts with Folin-Ciocalteu reagent) were left for 5 min before 1 ml 7.5% sodium carbonate (w/v) was added. The absorbance were taken at 765 nm wavelength with a spectrophotometer (Epoch, Biotek, USA), after 2 hours in the dark place. These data were used to estimate the total phenolic content using a standard calibration curve prepared by plotting absorbance against five points concentration: 20, 40, 60, 80, 100 ppm of Gallic acid in methanol to estimate the activity capacity of samples. The result was means of three reading expressed as mg of Gallic acid equivalents per 100 g of dry sample (mg GA/100 g of DW).

DPPH Radical Scavenging Activity (DPPH) Assay

The free radical scavenging activities by antioxidant in the plant extracts were evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals following the procedure described by (Brand-Williams *et al*., 1995), with slight modification. This method widely used to predict the ability of compounds to act as free radical scavengers or hydrogen donors to radicals is based on the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (Cos et al. 2002). As the electron of a radical pairs off with hydrogen donation from the free radical scavenging antioxidant, the absorption strength will be decreased. This resulted in decolorization that is stoichiometric with the number of electrons captured.

A stock solution was prepared by dissolving 40 mg DPPH in 100 ml methanol and kept at-20°C until used. About 350 ml stock solution was mixed with 350 ml methanol to obtain the absorbance of 0.70±0.01 unit at 517 nm wavelength by using spectrophotometer (Epoch, Biotek, USA). About 10 µL of *B. orintale* extracts with 1 ml methanolic DPPH solution prepared were kept 30 min for scavenging reaction in the dark. The difference in absorbance between the test sample and control (DPPH) expressed as percentage inhibition is taken as antioxidant activity AOA was determined as follows:

Inhibition (%) = A blank – A sample / A blank × 100

where, A is the absorbance. All determinations are performed in triplicate

Ferric Reducing Antioxidant Power (FRAP) Assay

The determination of antioxidant activity through FRAP was carried out according to the method of (Musa et al. 2011) was followed with slight modification. FRAP reagent was prepared fresh as using 300 m Macetate buffer, pH 3.6 (3.1 g sodium acetate trihydrate, plus16 ml glacial acid made up to 1:1 with distilled water); 10 mM TPTZ (2,4,6-tris (2-pyridyl)-*s*-triazine), in 40 mM HCL; and 20 mM FeCl3•6H2O mixed in a ratio of 10:1:1 and then incubated at 37°C for 10 minutes prior to the analysis to give the working reagent. About 1 mL FRAP reagent was added to 10µL parts extract of *B. orientale*. After adding FRAP reagent, plates were incubated for 30 minutes at room temperature. The absorbance of blue complex (ferrous tripyridyltriazine), formed from the reaction was measured at 595 nm wavelength with spectrophotometer. The antioxidant activity in this study was expressed as milligram Trolox equivalents per hundred grams of plant material on dry basis (mgTE/100g). Linear standard calibration curve of Trolox ranging from 0-100 mM Trolox was set up to estimate the activity capacity of samples. The higher absorbance of the reaction mixture indicates higher reductive potential.

ORAC Antioxidant Activity (ORAC) Assay

The ORAC assay was measured according to the method of (Arya *et al*., 2012), with slight adjustments. The method measures the antioxidant scavenging activity against peroxyl radical. All analyses were conducted in phosphate buffer pH 7.4 and at 37°C. The ORAC assay was carried out on a fluorescence microplate reader (FLUO star Omega, BMG LABTECH, Multi-Detection Microplate Reader, Germany). Peroxyl radical was generated using 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) which was prepared fresh for each run. Fluorescein (FL) was used as the substrate. Fluorescence microplate reader was used at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

B riefly, Proper dilutions 50µL of the sample extraction were dissolved with 950 µL PBS, Serial dilutions of the standard Trolox were prepared as (50, 25, 12.5, 6.25, 3.12 mM). Subsequently, 150 µL of fluorescent sodium salt solution was added, and the plate was incubated for 20 minutes at 37°C. For the blank, 25µl of phosphate buffer was used instead of a sample. AAPH solution (25 µL) was added to make up a total volume of 200 µL/well. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) as compared to the blank area in which no antioxidant is present. Data were collected every 2 minutes for 2 hours and were analysed by computing the differences of areas in the fluorescent falloff curve (AUC) between the blank and the sample. Values were expressed as Trolox equivalents per 100 grams of sample (µmol TE/100g).

Statistical Analysis

All data were expressed as mean ± standard deviation and were done in triplicate independent analyses. Data were analyzed using one-way ANOVA using SPSS version 20 (SPSS Inc., Chicago, Illinois, USA). analysis of variance (ANOVA) followed by Duncan's test for comparison, as a post hoc test to analyze the different parts of the plant while independent samples.

Results and Discussion

Total Phenolic Content (TPC)

The plants were initially characterized by the amount of phenolic compound they contained. Results are calculated as Gallic acid equivalent (GAE) of a sample using a standard curve. A linear calibration curve was constructed within the range of 20–100 mg/ml with an R^2 value of 0.9963. The contents of phenolic compounds in 50% aqueous acetone extracts of mature fronds, young fronds, rhizomes and fiddleheads of the wild fern, *B. orientale* are shown in figure 2.The young fronds of *B. orientale* contained higher values of TPC than the plant's mature fronds, rhizomes, and fiddleheads. The young fronds of *B. orientale* from Chini Forest and UKM Fern Garden exhibit maximum TPC values (1643 and 1360 mg GAE/100g DW), followed by the mature frond (1545 and 1244mg GAE/100g DW), fiddlehead (1170 and 1152 mg GAE/100g DW) and rhizome had the lowest content (1098 and 825.53 mg GAE/100g DW) respectively, which is comparable with the TPC obtained in ethanolic extracts of other wild edible plants, such as *Conyza sumatrensis* (Stem and leaves) possess 1566, *Artemisia dubia* (Stem and leaves) 1424, *Dolichandrone serrulata* (Flower) 1325 and *Vaccinium sprengelii* (Leaves) 954.20 mg of GAE/100g DW which were reported by (Phomkaivon & Areekul 2009).

Interestingly, comparison among different parts of *B. orientale* showed their different phenolic contents. This finding could be attributed to the variation in the nature and distribution of phenol contents within different parts of the same plant. Previous studies showed that the developmental stage of a plant may affect the biosynthesis pathways of phenolic compounds, thereby affecting the total phenolic and flavonoid contents (Križman *et al*., 2007). The plants sampled from the remote area of Chini Forest

demonstrated higher TPC than those from the urban area of UKM Fern Garden. Differences in growing conditions, such as climate and soil state, increase in organic matter, rainfall rate, and topography of the land in the two different locations significantly affected bioactive compounds and could be cause of the significant differences between the TPC values of the investigated fern.

The results strongly suggest that phenolics are very important components of the plant and some of its pharmacological effects could beat tributed o the presence of its valuable constituents. The results from this study showed that the constituents of this plant are having antioxidant and pharmacological effects.

Antioxidant Substances

Many antioxidant assays with varied chemical backgrounds and mechanisms have been established and used to evaluate the antioxidant potential of pure compounds and plant extracts. The use of multiple assays for evaluating the antioxidant potential of extracts provides useful and informative data.

DPPH Radical Scavenging Activity (DPPH) : DPPH is a stable nitrogen-centered free radical compound that is widely used to assess the free radical scavenging activity ability of various chemicals, including single compounds, food, and plant extracts (Yamaguchi *et al*., 1998). Figure 3 shows the scavenging effect of *B. oriental* extracts of different parts have high values for all samples at both locations. These values of radical scavenging activities in *B. orientale* extracts collected from Chini Forest were ranked as; young fronds > rhizome > mature fronds > fiddlehead with 90.24%, 89.56%, 87.88% and 85.52%, respectively. The young fronds of *B. orientale* in UKM Fern Garden obtained the highest scavenging activity (89.22%) followed closely by rhizome (88.21%) with no significant difference between them, and fiddlehead with no significant difference with rhizome (87.54%) and mature frond had the lowest (86.87%). This is consistent with the result by (Naik *et al*., 2013) reported that the methanol extract of *B. orientale* showed good antioxidant properties against DPPH with (80%) and this provides an empirical scientific evidence to support its traditional medicinal importance. The present study found that the DPPH value of the plant sampled from Chini Forest was significantly higher ($p<0.05$) than that sampled from UKM Fern Garden. The high radical-scavenging activity of *B. orientale* extracts may be related to the high amounts of phenolic compounds as the plant sample from Chini Forest contained higher phenolic content than the plant from UKM Fern Garden.

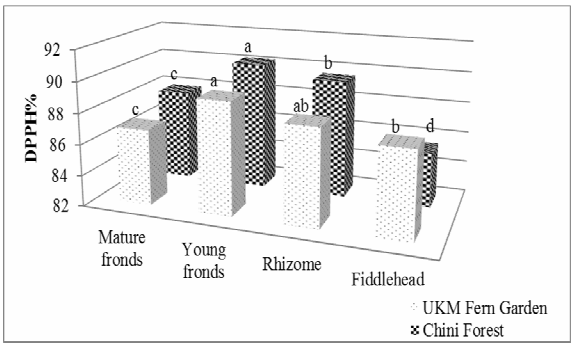

Fig. 3 : DPPH of different parts of *B. orientale* from Chini Forest and UKM Fern Garden in 50% aqueous acetone (Mean value, n=3)

Note: Means within samples with different alphabet letters are significantly different, means with same alphabet letters within each location are not significantly different ($p>0.05$)

Ferric Reducing Antioxidant Power (FRAP) : The FRAP assay was originally developed by (Benzie & Strain 1996), to measure reducing power in plasma, but the assay has also been adapted and used to assess the "antioxidant power" of food and biological samples and plant extracts by its ability to reduce ferric ions (Pellegrini *et al*., 2003). In the present study, we evaluated the ferric reducing potential of *B. orientale* plant parts. expressed as Trolox equivalent capacity (TE). Analysis of the different parts extracts showed that the plant extracts exhibited a significantly different ($p<0.05$) reducing ability to ferric compounds.

As depicted in Figure 4, the ferric compound was significantly reduced by *B. orientale* sampled from Chini Forest, as the Ferric reducing abilities recorded were 1,077 mg TE/100g DW (young fronds), 978.13 mg TE/100g DW (mature frond), 836.82 mg TE/100g DW (rhizome) and 763.56 mg TE/100g DW (fiddlehead). Similarly, *B. orientale* sampled from UKM Fern Garden showed 838.91 mg TE/100g DW (young fronds), 758.17 mg TE/100g DW (fiddlehead)**,** 707.28 mg TE/100g DW (mature fronds) and 537.71 mg TE/100g DW (rhizome). These values are in agreement with the results obtained in a previous study (Lai *et al*., 2009), in which the *B. orientale* contained (1,098 mg GAE/100g) in methanol extract. furthermore, (Naik *et al*., 2013) showed that the frond of *B. orientale* presented 534.05 mg equivalent of ascorbic acid at dry weight in methanol extract. The origin of the plants collected was significantly affected ($p < 0.01$) FRAP value in both plants.

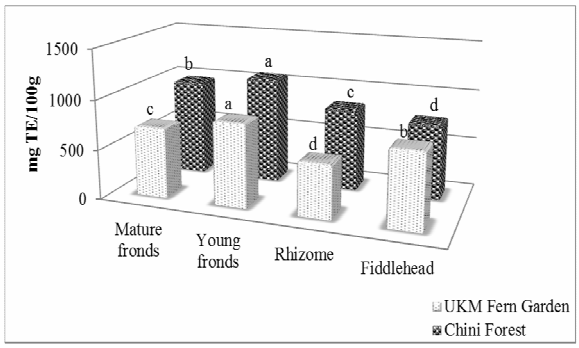

Fig. 4 : FRAP value of different parts of *B. orientale* from Chini Forest and UKM Fern Garden in 50% aqueous acetone (Mean value, n=3)

Note: Means within samples with different alphabet letters are significantly different, means with same alphabet letters within each location are not significantly different (p>0.05)

Oxygen Radical Absorbance Capacity (ORAC) : Oxygen radical absorbance capacity (ORAC) method, which was developed by (Cao *et al*., 1993), can measures the ability of antioxidant compounds to confer protection against oxidation by the peroxyl radical generator AAPH. The ORAC assay is important because it utilizes a biologically relevant radical source (Prior *et al*., 2003). These techniques present different results across different crop species and laboratories. As depicted in Figure 5, the ORAC result obtained from this study showed a very strong antioxidant activity in the extracts of *B. orientale* parts sampled from Chini Forest. The highest ORAC value among all the parts was obtained in the rhizome extract (80729 µmol TE/100g DW) and mature fronds (80368 µmol TE/100g DW), with no significant difference between them, this was followed by young fronds (66830 µmolTE/100g DW) and fiddlehead (57842 µmol TE/100g DW). These values are similar to those obtained in (Tavares *et al*., 2010), which evaluated five endemic Macaronesian species, from the Azores and Madeira/Canary Islands. Result of *B. orientale* sampled from UKM Fern Garden showed that the mature fronds extracts had the highest ORAC value (82441 µmolTE/100g DW) than the other extracts of the three parts and this was followed by rhizome (58456 µmol TE/100g DW), young fronds (51225 µmol TE/100g DW) and then by fiddlehead (40836 µmol TE/100g DW). There is no data available in the literature related with the ORAC value of *B. orientale*, The result of this plant is more comparable to the result obtained in (Ghosh Das 2011), which reported that spices like turmeric powder showed the highest ORAC value of (79,400 µmol TE/100g) followed by ginger (65,100 µmol TE/100g), cumin (65,000 µmol TE/100g), cinnamon (64,900 µmol TE/100g), Malabathrum leaf (59,300 µmol TE/100g), and caraway (46,900µmolTE/100g).

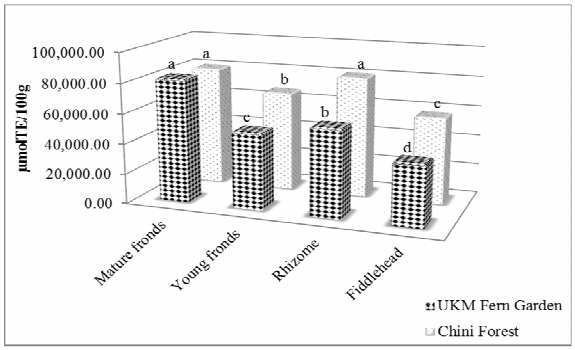

Fig. 5 : ORAC capacity of different parts of *B. orientale* from Chini Forest and UKM Fern Garden in 50% aqueous acetone, Mean (n=3)

Note: Means within samples with different alphabet letters are significantly different, means with same alphabet letters within each location are not significantly different (p>0.05)

The Correlation between Total Phenolic Content and Antioxidant Activity

The relationship between the antioxidant activity of phenolic compounds and free radical scavenging depends on the arrangement of the functional groups in the nuclear structure (Cao *et al*., 1997; Pannala *et al*., 2001). The total phenolic compounds (GAE values) of the plant extracts were correlated with the antioxidant activity as shows in Table 1, the Pearson's correlation coefficient of the total various extracts of the *B. orientale* parts sampled from Chini Forest shows significant positive correlation coefficient between the TPC and FRAP assay of plant extracts (r=0.937, $p < 0.01$), these strong correlation can be attributed to the fact that both assays rely on the same reaction mechanism. A positive significant correlation (r = 0.627) which is significant at ($P < 0.05$) was observed between the DPPH scavenging activity and the FRAP assay.

The different correlations obtained from total various parts of *B. orientale* sampled from UKM Fern Garden revealed a highly significant positive correlation between the TPC and the FRAP assays (r = 0.944, $p<0.01$), a positive significant correlation between TPC and ORAC capacity (r =0.819, $p<0.01$). In addition, a high positive correlation between ORAC capacity and FRAP (r =0. 610, $p < 0.05$). Thus, we conclude that the antioxidant capacity of the extract could be attributed to the bioactive compounds of the samples, not only to phenolic contents. The antioxidant activity could also be attributed to the presence of other antioxidantsecondary metabolites, such as volatile oils, carotenoids, and vitamins, which contribute to the residual ratio of the

antioxidant capacity. Previous studies showed a high correlation between TPC and antioxidant activities (using DPPH and FRAP assays),thereby confirming that phenols contribute to the antioxidant power of plants extracts. Many reports revealed the direct correlation between antioxidant activities and reducing powers of the components of some plants. (Javanmardi et al. 2003) found a linear positive correlation between antioxidant activity and total phenolic content of basil extract (r = 0.71) and (Katalinic et al. 2006) found a significant linear correlation (r = 0.9825) between the TPC and related FRAP of 70 medicinal plant extracts.

Table 1 : Correlation coefficients between phenolic content and antioxidant activity of total parts of *B. orientale*

		DPPH	FRAP	TPC	ORAC
UKM Fern Garden	DPPH	1			
	FRAP	0.222	1		
	TPC	0.093	0.944**	1	
	ORAC	-0.334	0.610*	0.819**	1
Chini Forest	DPPH	1			
	FRAP	0.627*	1		
	TPC	0.355	0.937**	1	
	ORAC	0.065	-0.218	-0.311	1

*Significant p<0.05, ** strong significant p<0.01

Conclusion

This study has provided an empirical evidence of the role of DPPH, FRAP, ORAC and TPC assays in providing essentially identical information in regard to the antioxidant capability of fern extracts.The results obtained demonstrated that young fronds of *B. orintale* had the highest total phenolic content and antioxidant activity compared to other parts but all the different parts of ferns contained high antioxidants that are beneficial to the human health. The TPC and total antioxidant content of different parts of *B. orientale* were significantly affected at the locations where the plant was growing. The plant collected from Chini Forest had higher TPC and antioxidant activity compared with the plant collected UKM Fern Garden, and this could be related to the different growing conditions such as soil, geographical and environmental conditions.

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