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Bioactives in three Philippine edible ferns

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Ferns in the Philippines have been used by native people as food and tea since the early times but over time their values have been lost in the Filipino diet. The present study was aimed at bringing back the lost value of ferns by demonstrating their important components needed for health and wellness in food. To do this, we determined the antioxidants, proteins and phytochemicals in three edible species of ferns, namely: *Diplazium esculentum* (Retz.) Sw., *Marsilea crenata* Presl and *Stenochlaena palustris* (Burm. *f.*) Bedd. These edible ferns were collected from the wild in several places in Mindanao and were propagated in the pteridogarden in Central Mindanao University (CMU), Musuan, Bukidnon. The young fern fronds which are the part used for food were tested in the CMU Natural Sciences Laboratories. Test results showed quantifiable level of antioxidant activity by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay with *D. esculentum* exhibiting the highest antioxidant activity relative to ascorbic acid (83%). Laboratory test results also showed protein content as high as 4.4 mg/g for *M. crenata*. All the three fern species showed positive to alkaloids, saponins, phenolics and flavonoids and only *D. esculentum* showed definite positive to terpenes. *M. crenata* and *S. palustris* showed faint bands for terpenes.

Keywords: ferns, antioxidants, proteins, phytochemicals, Mindanao, Philippines

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INTRODUCTION

Starch-based foods have been the main staple food in the developing countries including the Philippines for supply of protein and energy. This is recognized by the Food and Agricultural Organization (FAO) as the cause of protein deficiency in the populace. Many Filipinos eat vegetables but they seem to choose only those that are palatable to them. This practice led to under-exploitation of a wide range of vegetables most especially local and indigenous vegetable materials.

Some ferns in the Philippines had been used by native people as foods, teas, and various forms of medicines for a long time (de Winter & Amoroso 2003). In Canada and Northeastern America, the species fiddle heads (*Pteridium aquilinum* and *Matteuccia struthiopteris*) have antioxidant activity twice that of blueberries, which is claimed to have the highest antioxidant activity among fruits. In fact, Amoroso (1990) reported 10 indigenous edible ferns in Mindanao, Philippines and some of these species are prepared as green vegetable or salad as part of the diet. Furthermore, the qualitative data on these ferns showed the presence of active principles such as alkaloids, tannins, glycosides, saponins and organic acids (Amoroso 2013) but no quantitative data had been reported on their antioxidant activity, protein content and their relative component proteins. Since the active principles otherwise known as phytochemicals are more likely responsible for the antioxidant activity in plants, the present study was conducted to determine and compare the phytochemicals, the antioxidant and protein content of the three indigenous edible species of ferns, viz. *Diplazium esculentum* (Retz.) Sw., *Marsilea crenata* Presl and *Stenochlaena palustris* (Burm. *f.*) Bedd.

MATERIALS AND METHODS

Plants of *D. esculentum, M. crenata and S. palustris* collected from the wild in different places in Mindanao were cultivated and propagated in the Central Mindanao University (CMU) Fernery and in the Mt. Musuan Botanical and Zoological Garden, CMU. Subsequently, the tender fronds of these species were obtained from these cultivated plants (Figure 1) and were used in this study.

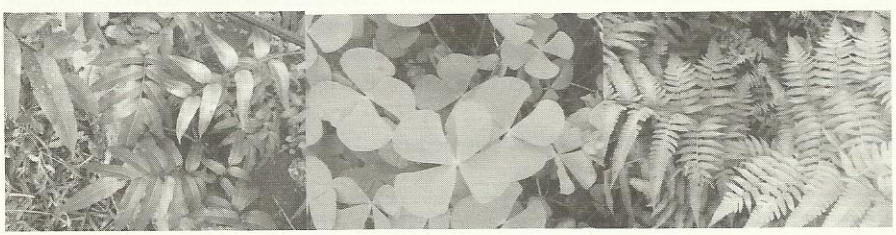

Figure 1. Three species of Philippine edible ferns used in this study (left to right): *Stenochlaena palustris* (Burm. *f.*) Bedd. (*Hagnaya/Diliman*), *Marsilea crenata* Presl (*Apat-apat*/clover fern) and *Diplazium esculentum* (Retz.) Sw. (*Pako*).

Determination of bioactive components (antioxidants and protein)

Preliminary test for antioxidant by the ferric chloride test. In this study, a preliminary test was made for the antioxidant activity in each fern species by the ferric chloride ($FeCl_3$) test. Briefly, fern fronds were added with boiled distilled water and centrifuged immediately to separate the fronds from the supernatants. 250 µL supernatant was added with a drop of 10% $FeCl_3$ solution. Results were evaluated by visual inspection.

Test for antioxidant activity, phytochemical screening and protein content. The functional activity of the three edible fern species were determined. The fern fronds of each species were harvested, washed to remove dirt and debris, weighed and air-dried in the Natural Sciences Laboratories, CMU. Once dried, the samples were cut into homogeneous fine pieces. The ground samples were soaked in methanol for 48 hr and filtered to obtain the methanol extract and subsequently concentrated at 40°C. The concentrated methanol extracts were tested for antioxidant activity by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, phytochemical screening to determine the organic components in the active solvent fractions by TLC (thin layer chromatography), and protein content by the Bradford assay.

a. Measurement of DPPH free radical scavenging activity of the methanol/ aqueous extracts. The free radical scavenging activity of the three fern species were determined and their activities were compared against ascorbic acid. A new micro-assay method was developed using 96-microtiter well configuration with DPPH. The assay is based on the reaction of DPPH in the presence of a free radical scavenger (antioxidant). The sample was added to the DPPH solution and the reaction was made to proceed in the dark for 30 min. The decoloration from purple solution after addition of a free radical scavenger from the sample or from the standard was monitored at 517 nm using Molecular Devices® Spectramax 250 spectrophotometer. In this micro-assay configuration, ascorbic acid was run in parallel with the samples and with reaction being monitored on the same 96-well plate and read at 517 nm at once. Shortly, the concentrated extract was diluted in 15:5:2 solvent mixture of dimethyl sulfoxide (DMSO), ethanol and water. Ascorbic acid and DPPH solutions were prepared in parallel to serve as positive and negative controls, respectively. This test was conducted using the method used by Mosquera (2007) with some modifications. The 96-well plate was automatically shaken to mix in the microplate reader compartment prior to reading the Absorbance (A) of samples and controls at 517 nm. Scavenging activities were measured using this formula:

$$\% \text{ Activity} = [(A_{control} - A_{extract\ or\ difference}) / A_{control}] \times 100\%$$

Results from this test were further calculated to obtain the ORAC (Oxygen Radical Absorbance Capacity) in order to compare the results from this study with published data of some agricultural food materials.

b. Phytochemical screening by TLC. The organic components of the aqueous extracts of the young tender fern fronds were determined by TLC. The tests for the presence of alkaloids, phenolic compounds, saponins, terpenes and flavonoids were

performed using different spray reagents. Commercially available TLC plates were used to separate organic compounds present in the solvent extract.

Using fine capillary tubes, a spot on the TLC plate at least 2 cm from the edge were made with the extract. The chromatogram was developed in a chamber with a tightly closed lid containing the 99.8% methanol as solvent system. When the solvent almost reached the top of the plate, the plate was removed from the chamber and allowed to dry. The dried TLC plates were developed using Potassium ferricyanide-ferric chloride, Dragendorff and Acetic anhydride-sulfuric acid chloride as spray reagents to determine the presence of each of the phytochemicals. Subsequently, images of the TLC plates were documented.

c. Protein content by the Bradford assay. The 0.1N NaOH crude extracts from the three species were tested for total protein content by the Bradford assay. The method was a microassay in a 96-microtiter well configuration which was also developed for this study. The assay was based on the binding of the Coomassie dye reagent to side chains of basic and hydrophobic amino acid residues in proteins. All natural proteins contained these amino acid residues hence considered to detect all proteins in a sample, with a difference in color intensity at various concentration of proteins. Binding of the dye was monitored as development of a deep blue coloration which was monitored at 595 nm and determined using the Molecular Devices® Spectramax 250 spectrophotometer.

The assay used Bovine Serum Albumin (BSA) as standard, which was run at different concentrations in parallel with the samples. The protein concentration in the indigenous fern extracts was determined by interpolating their absorbance values against the BSA standard curve run at that particular assay.

RESULTS AND DISCUSSION

Determination of bioactive components (antioxidants, phytochemicals and proteins).

a. Screening for antioxidants by the Ferric Chloride ($FeCl_3$) test. Results from this qualitative screening test indicated that all three edible fern species contained antioxidants as evidenced by brownish green to intense dark purple/blue coloration with precipitate (Table 1). This result is not unexpected considering that ferns in general are a group of plants that survived the Paleozoic times and since had kept adapting to many more changes in the environment than other vascular plants. As such, they had been reported to produce many useful secondary metabolites, considered antioxidants, than other vascular plants (Wallace et al. 1991). In this screening test, *S. palustris* had the most intense coloration or relatively high antioxidant content followed by *D. esculentum* and *M. crenata.*

Test for antioxidant activity, phytochemicals and protein content

a. Test for antioxidant activity. Results plotted for comparison and ranking for percent activity are shown in Figure 1. The highest percent activity at 83% was observed in *D. esculentum,* followed by *M. crenata.* The data obtained in this assay also quantitatively confirmed the positive results for antioxidants in the $FeCl_3$ screening test for these three species of indigenous edible ferns.

Table 1. Appearance of edible fern extracts upon addition of 10% $FeCl_3$.

Scientific Name	Color change	Remark
Marsilea crenata Presl	Orange to greenish brown with precipitate	+
Stenochlaena palustris (Burm. *f.*) Bedd.	Red to purple with precipitate	+
Diplazium esculetum (Retz.) Sw.	Light orange to greenish brown with precipitate	+

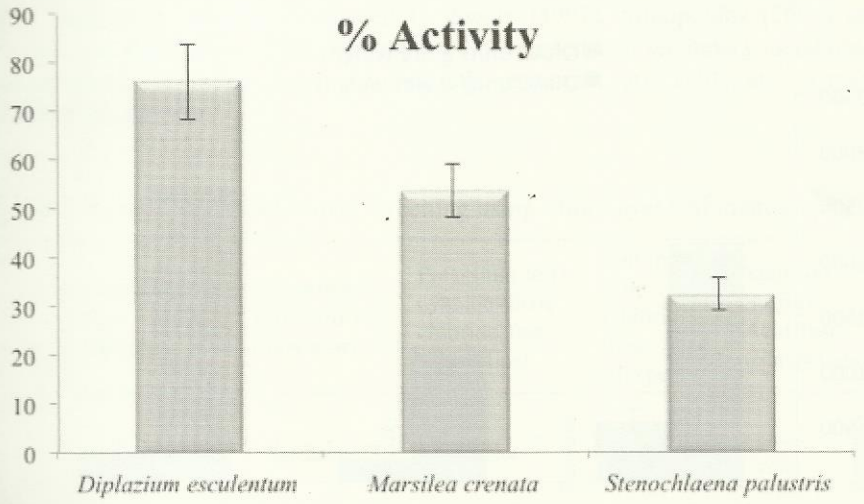

Figure 1. Ranking of percent antioxidant activity of three edible ferns by the DPPH assay.

It is also important to express the antioxidant activity of the indigenous ferns when used as fresh vegetables. In this case, the activity obtained from the DPPH assay was calculated relative to wet weight (tender fresh fronds) and dry weight. Data per wet weight showed *M. crenata*, a favorite vegetable in the Visayan region but not yet part of the diet in Mindanao, gave the highest value (Table 2, Figure 2). Values for *D. esculentum* and *S. palustris* per wet weight are relatively close. Data obtained in the present study are promising when compared to published values for commercial vegetables and fruits in the US [US Department of Agriculture (USDA) Bulletin - How to Count Antioxidants].

Naturally the antioxidant unit values became higher upon drying of the tender young fronds with the highest obtained in *D. esculentum* followed, by *M. crenata*. As expected, there was variability in the level of drying to a final weight depending on the moisture content of the tender young fronds as well as the nature of the sample. Hence, extra care was done to maintain consistency in this step of the study, and at the same time, to avoid any microbial growth contamination in the process.

Table 2. Antioxidant activity of three edible fern species by the DPPH assay.

Sample	ORAC unit[2]/g dry weight	ORAC unit[2]/g wet weight
Marsilea crenata Presl	2365 ± 111	588 ± 25
Stenochlaena palustris (Burm. *f.*) Bedd.	1440 ± 70	248 ± 12
Diplazium esculentum (Retz.) Sw.	3359 ± 309	336 ± 31

[2] Oxygen Radical Absorbance Capacity (ORAC) based on Trolox as standard – 442000 micromole equivalents per 100-gram Ascorbic Acid by the DPPH assay (A. Prakash et al. 2011; A. Miller et al. 2000). Data are mean ± SD of samples (n = 2)

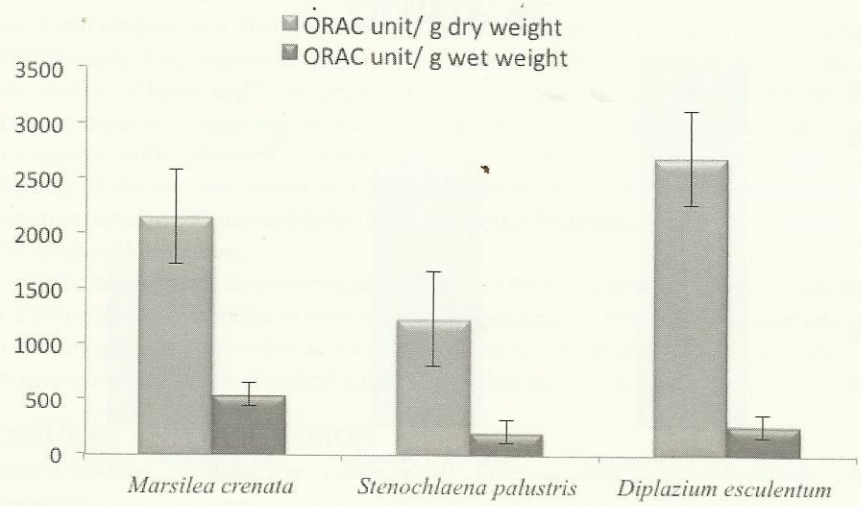

Figure 2. Comparison of antioxidant activity of the three edible ferns per sample weight.

DPPH results indicating levels of antioxidant activity in the three fern species are supported by previous studies on other species of ferns (Garcia et al. 2006, Chen et al. 2007, Ding et al. 2008, Hafidh et al. 2009, Shin & Lee 2010). In addition, functional activities of ferns for human health are being studied in the US, Canada and in Southeast Asia. Results from intervention clinical trials with single compounds, however, have not supported any protective effect implying that the functional activities function synergistically (Carr & Frei 1999, Halliwell 2000). In fact in Canada, the fiddle heads of *P. aquilinum and M. struthiopteris* have been reported to have antioxidant activity twice that of blueberries.

The strong antioxidant activities of the three species of indigenous edible ferns are in accordance with data reported by researchers from Korea (Shin & Lee 2010) who screened 37 ferns and fern allies. They reported that frond and rhizome extracts of *Polystichum lepidocoulon* and *P. polyblepharum* contained more than 13% of total polyphenols. Several ferns, *i.e.* Dryopteridaceae, Osmundaceae and Woodsiaceae, also showed vigorous antioxidant activities in scavenging DPPH and

2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radicals, indicating that most ferns have huge potential abilities as antioxidants.

All these data from different studies are supported by the fact that since ferns have survived from Paleozoic times, they have adapted many more changes of environment than other vascular plants (Wallace et al. 1991). As such, they are expected to have many useful secondary metabolites which have not been discovered in other plants (Zhao et al. 2007, Shinozaki at al. 2008).

b. Phytochemical screening by Thin Layer Chromatography. Results on phytochemical screening showed the presence of alkaloids, saponins, phenolic compounds, flavonoids and terpenes in *D. esculentum* by TLC (Table 3). These data support the studies conducted by Swain (1997), Simopoulos (2004) and Bennet and Wallsgrove (2006) who reported the presence of secondary metabolites polyphenols, flavonoids, terpinoids, steroids, saponins, fatty acids, etc. in many edible plant species.

Table 3. Results of phytochemical screening using Thin Layer Chromatography.

Species	Dragendorff's Test (Alkaloids)		Liebermann-Burchard Test (Saponins)		Potassium ferric cyanide-ferric chloride Test (Phenolics)		Antimony (III) Chloride Test (Terpenes)		Vanillin/ Sulfuric Acid Test (Flavonoids)	
	Spot color	Remarks	Spot color	Remarks	Spot color	Remarks	Spot color	Remarks	Spot color	Remarks
Marsilea crenata	brown orange	+	pink	+	blue	+	faint band	-	light red	+
Stenochlaena palustris	brown orange	+	pink	+	blue	+	faint band	-	light red	+
Diplazium esculentum	brown orange	+	pink	+	blue	+	reddish brown	+	light red	+

c. Protein content by the Bradford assay. Data from the Bradford test showed an efficient extraction method resulting in protein concentrations ranging from 0.578 to 0.671mg/mL (Table 4, Figure 3).

Table 4. Total protein content of the three edible ferns by the Bradford assay[1].

Sample	Protein concentration, mg/mL	mg protein/g dry weight	mg protein/g wet weight
Marsilea crenata	0.628 ± 0.005	17.58 ± 0.14	4.39 ± 0.04
Stenochlaena palustris	0.671 ± 0.010	13.42 ± 0.21	2.30 ± 0.04
Diplazium esculentum	0.646 ± 0.011	18.09 ± 0.30	1.81 ± 0.03

[1]Protein concentration was determined by the Bradford assay using Bovine Serum Albumin (BSA) as standard. Data are mean ±SD of samples (n = 2).

The variability obtained for total protein per gram of dried fern fronds is contributed mainly by the degree of the resulting amount of water lost in the drying process. This is highly dependent on the nature of the fern fronds. The loss of water content ranged from 75 to 90% with *D. esculentum*, being the highest (90%) after air-drying (Table 5).

Table 5. Relevant information during air-drying of the three edible ferns.

Sample	Wet weight (g)	Dry weight (g)	% weight loss
Marsilea crenata	153.83	38.44	75.02
Stenochlaena palustris	697.71	119.78	82.83
Diplazium esculentum	489.47	49.04	90.00

Conducting a quantitative protein test for ferns is not common considering the appropriate method for such a test as well as the use of expensive protein standard usually used for plants (purified plant enzyme). It has been reported however, that several health effects of fern are currently well known, *e.g.* glycoprotein from bracken fiddle head has immune function (Park et al. 1998) and acidic polysaccharide extract of dried bracken fern fiddle head has anti-complementary activity (Oh et al. 1994). In the present study, the 96-well micro Bradford method was specifically optimized to provide consistent result and with the use of Bovine Serum Albumin (BSA) as standard. The positive data for proteins obtained are further supported by results from the SDS-PAGE (related study, data not shown), which determined the relative component proteins present in each species of edible ferns.

CONCLUSION AND RECOMMENDATIONS

Three species of indigenous edible ferns from the wild and propagated in the CMU Fernery and in Mt. Musuan Botanical and Zoological Garden, CMU were used in this study. All these indigenous species of edible ferns showed quantifiable level of antioxidant activity by the DPPH assay. *Diplazium esculentum* exhibited

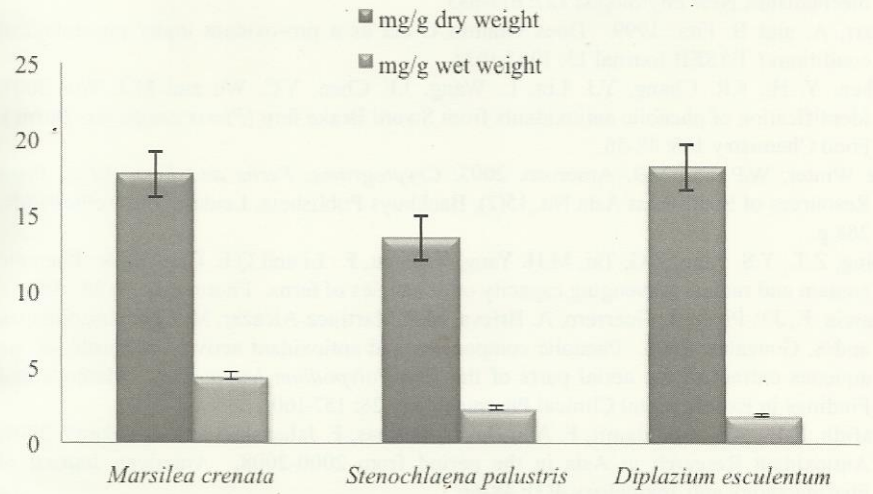

Figure 3. Comparison of total protein content in three edible ferns relative to sample weight.

the highest antioxidant activity of 83% relative to ascorbic acid and *M. crenata* had the highest antioxidant activity per gram of tender fresh fronds. TLC confirmed the presence of alkaloids, phenolic compounds, flavonoids and saponins in all three species with terpenes observed only in *D. esculentum*. The three edible ferns are good sources not only of antioxidants and phytochemicals but also of proteins as high as 4 mg/g fresh tender fronds.

To further maximize the values of these three indigenous edible species of ferns, the following recommendations are made here: (1) Identify farmer-cooperators for mass cultivation of the three ferns; (2) Conduct a cost-and-benefit analysis for each of the species of ferns; (3) Prepare a comprehensive Fern Cookbook, and (4) the expansion of the study capturing Good Collection and Agricultural Practices (GACP) and the medicinal properties of these species of ferns.

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