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Evaluation of the antioxidant properties of the leaf extracts of Philippine medicinal plants *Casuarina equisetifolia* Linn, *Cyperus brevifolius* (Rottb) Hassk, *Drymoglossum piloselloides* Linn, *Ixora chinensis* Lam, and *Piper abbreviatum* Opiz

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Abstract. In our continuing search for natural antioxidants, the decoction, ethanol, and 50:50 ethanol:water extracts of the medicinal plants *Casuarina equisetifolia* Linn, *Cyperus brevifolius* (Rottb) Hassk, *Drymoglossum piloselloides* Linn, *Ixora chinensis* Lam, and *Piper abbreviatum* Opiz found in different areas in Mindanao are evaluated for their antioxidant potentials. The decoction and 50:50 ethanol:water extracts of *C. equisetifolia* and *I. chinensis* showed considerable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity ranging from 80.06 to 91.79% when compared to the commercial antioxidant butylated hydroxytoluene (BHT). The total phenolics content of the leaf extracts ranges from 34.67 to 285.62 gallic acid equivalence with the ethanol extract of *D. piloselloides* exhibiting the highest phenolics content at 285.62 mg g^{-1} sample. The ethanol extract of *D. piloselloides* also demonstrated the highest total antioxidant capacity, 87.54 ascorbic acid equivalents and 149.43 butylated hydroxytoluene equivalents. The results indicate that *C. equisetifolia*, *I. chinensis*, and *D. piloselloides* can be important sources of antioxidants that may offer protection from the harmful effects of oxidative stress.

Key Words: Plant-based systems, radical scavenging activity, total phenolics content, total antioxidant capacity, oxidative stress.

Introduction. Throughout the ages, nature has been the source of medicinal products for the treatment of a wide range of diseases. Plants, in particular, have formed the foundation of intricate traditional medicine systems, with many useful drugs developed from plant sources. These plant-based systems continue to play an essential role in healthcare and their use by different cultures has been extensively documented (Moerman 1986). Natural products possessing medicinal properties present in traditional herbs and medicinal plants have been established to be the reason of the extensive consumption of herbal remedies and healthcare preparations (Hoareau & DaSilva 1999).

It is now well established that a series of oxygen-centered free radicals and other reactive oxygen species (ROS) contribute to the pathology of many disorders including atherogenesis, neurodegeneration, chronic inflammation, cancer and physiological senescence (Ani et al 2006). It is believed that foods rich in antioxidants play an essential role in the prevention of diseases because the damaging actions of free radicals can be blocked by antioxidant substances which scavenge the free radicals and detoxify the organism (Alam et al 2012). Because of this, there is a considerable increase in the research of naturally occurring antioxidants for use in food or medicinal materials as an alternative to synthetic antioxidants which are being restricted due to their possible toxicity (Namiki 1990). In our continuing search for naturally-occurring bioactive compounds, the extracts of five selected medicinal plants *Casuarina equisetifolia* Linn, *Cyperus brevifolius* (Rottb) Hassk, *Drymoglossum piloselloides* Linn, *Ixora chinensis* Lam, and *Piper abbreviatum* Opiz found in the different areas in Mindanao are evaluated for their antioxidant potential. The establishment of a scientific basis for the claimed

medicinal properties of these plant species is important and may provide opportunities for the discovery and development of new plant-based drugs for the pharmaceutical industry and medicine.

C. equisetifolia Linn belongs to the Casuarinaceae family and is a large, tall and straight evergreen tree that can grow up to 20 meters high with a crown that is narrowly pyramidal, resembling some of the conifers in appearance. In the Philippines, it is commonly known as “agoho”. The following phytoconstituents have been isolated from the plant so far; kaempferol, quercetin (El-Ansari et al 1977) alicyclic acids (shikimic and quinic acid), amino acids (Madhusudanamma et al 1978), taraxerol, lupenone, lupeol, gallic acid, sitosterol (Rastogi & Mehrotra 1998), catechin and gallocatechin (Roux 1957). The plant is used as an astringent (Mhaskar et al 2000), as a treatment for diarrhea (Chopra et al 1956), dysentery, cough, ulcers, toothache, and diabetes (Prajapati et al 2003). In a study performed by Ahsan et al (2009), the leaf and bark methanol extract of *C. equisetifolia* exhibited inhibition of bacterial growth against *Bacillus proteus*, *Shigella sonnei,* and *Klebsillea* sp*.* Studies on the bark and wood of *C. equisetifolia* revealed their substantial anticancer and anthelmintic properties (Aher et al 2006).

C. brevifolius is an annual herb belonging to the Cyperaceae family with slender stems, 10 to 50 cm high, usually scattered, rising from slender creeping rootstocks. It is commonly known as “kyllinga weed” in English and “pugo-pugo” in the Visayan dialect of the Philippines. Folkloric applications of this plant include treatment for colds with fever, cough, bronchitis, swelling pain in the throat, malaria, snake bites, furuncles, and sprains. The study on the crude hydro-ethanolic extract and fractions of the rhizome of *C. brevifolius* findings indicate that it exerts a weak sedative and an interesting anxiolytic-like effect in mice and suggest its potential usefulness for the treatment of anxiety in humans (Hellión-Ibarrola et al 2012).

D. piloselloides of the Polypodiaceae family is a climbing epiphyte with slender rhizomes covered with peltate scales, creeping on trunks and branches. It is known as “dragon scales” in English and “pagong-pagongan” or “lagolo” in the Philippines where this plant can be found throughout the country. Ground leaves of *D. piloselloides* are used for coagulating blood and arresting capillary hemorrhages. It is also used for eczema and leaf decoction is used for making lotion for small pox or as poultice for headaches. Decoction of leaves is also taken internally for treatment of body pains. Antibacterial activity study of the water, ethanol and chloroform extracts of *D. piloselloides* showed that only the chloroform and ethanol extracts had mild activity against the *Trichophyton* spp. and the water extract was devoid of any activity. The antifungal activity was statistically less potent than griseofulvin and fluconazole or itraconazole. The antibacterial activity against gram positive bacteria was detected only in water extract against *Staphylococcus aureus* (Somchit et al 2011).

I. chinensis is a flowering plant of the Rubiaceae family with about 500 species in the genus Ixora and is commonly known as “santan-tsina” in the Philippines. There are numerous cultivars differing in flower color (yellow, pink, orange) and plant size. In the Philippines, infusion of fresh flowers, drunk *ad libitum*, is said to be good for incipient tuberculosis and for hemorrhage (Stuart 2014). The Malays use decoction of the root after childbirth while the Indonesians use decoction of the roots for bronchial disorders and flower decoction for amenorrhea and hypertension. In Vietnam, roots, stems, leaves, and flowers are used for irregular menses, high blood pressure, tuberculosis, hemoptysis, rheumatism, and acne (Stuart 2014).

P. abbreviatum, commonly known in the Philippines as “buyo-buyo”, is a dioecious vine with smooth branches. The paste of leaves is used externally to treat splenomegaly (enlargement of the spleen) in the Philippines. Fruits are used for coughs and colds and it is also used for flatulence. Benzene, chloroform, ethyl acetate, acetone, ethanol and water extracts of the fruits of *P. cubeba*, *P. retrofractum*, *P. chaba*, *P. longrum*, and *P. nigrum* exhibited good antibacterial activity against *Staphylococcus albus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus megaterium* (Khan & Siddiqui 2007).

Material and Method

Plant materials. Fresh leaf samples of the medicinal plants were collected from different localities in the Lanao Provinces of the Philippines. Identification and authentication of the collected test plants was done by Dr. Alicia E. Tabaranza from the Department of Biology, MSU - Iligan Institute of Technology, Iligan City, Philippines. Fresh plant materials were washed under running tap water, air-dried for three to four weeks, homogenized to fine powder, and stored in airtight containers.

Plant extraction. Fresh plant part was thoroughly cleaned, washed under tap water, rinsed with distilled water, cut into small pieces, and boiled in sufficient amount of distilled water (1:2) for 5 minutes. The resulting mixture was then filtered, cooled, freeze-dried, and stored in air-tight sample containers. Pulverized plant materials were soaked in an adequate amount of 95% ethanol and another set was soaked in 50:50 ethanol:water for 72 hours. The mixture was then filtered, concentrated *in vacuo* using rotatory evaporator, and then weighed to give the ethanol extract and the 50:50 ethanol:water (EtOH:H_2O) extract.

Spectrophotometric measurements. The ultraviolet absorbance measurements involved in the assays were done using a Lasany Double Beam LI-2800 Microprocessor UV-Vis Spectrophotometer.

DDPH radical scavenging test. DPPH radical-scavenging activity assay was performed as described by Lee & Shibamoto (2001) where the test samples were compared with that of a known antioxidant, butylated hydroxytoluene (BHT). Different concentrations (500, 100, 50, and 25 ppm) were mixed with 3 mL of methanolic solution of DPPH (0.1 mM). The mixture was shaken vigorously and then allowed to stand at room temperature for one hour. The reaction of the DPPH radical was estimated by measuring the absorption at 517 nm against methanol as blank in the spectrophotometer. Three trials were done for each sample. The percent of DPPH discoloration of the samples was calculated according to the formula:

$$Antiradical\ activity = \frac{A_{control} - A_{sample}}{A_{control}}\ x\ 100$$

where $A_{control}$ and A_{sample} are the absorbance values of the control and test sample, respectively. The effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) was obtained by linear regression analysis of dose-response curve plotting between percent inhibition and concentration.

Total antioxidant activity test. The total antioxidant activity of all the extracts was evaluated by the phosphomolybdenum method described by Prieto et al (1999). A 0.3 mL extract solution (200 µg/mL), dispensed into test tubes was added separately with 3.0 mL reagent solution (6M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate). The test tubes were incubated at 95°C for 90 minutes, cooled to room temperature, and the absorbance was measured at 695 nm using a spectrophotometer. Ascorbic acid and butylated hydroxytolune were used as reference standards and the antioxidant activity was expressed as ascorbic acid equivalents (AAE) and Butylated Hydroxytoluene Equivalents (BHTE) determined from established linear equations. Three trials were done for each test sample.

Total phenolics content test. The total phenolics content of the extracts was determined using the method of Makkar et al (1993). 0.1 mL (0.5 mg mL^{-1}) of sample was combined with 2.8 mL of 10% Na_2CO_3 and 0.1 mL of 2N Folin-Ciocalteu reagent. After 40 min absorbance at 725 nm was measured. Total phenolics content was expressed as gallic acid equivalence (GAE) in milligrams per gram of sample by

computing with standard calibration curve constructed for different concentrations of gallic acid (25-500 mg g^{-1}) and results were reported in GAE.

Results and Discussion

DPPH radical scavenging assay. The free radical scavenging activity of all the leaf extracts was evaluated by its ability to reduce DPPH which is a stable free radical. Results of the assay expressed as percent of antiradical activity are summarized in Table 1.

Table 1

DPPH radical-scavenging activities of the plant leaf extracts at various concentrations expressed as percent antiradical activity

Plant/Standard	*Extracts*	*Antiradical activity, %**				
		Code	*25 ppm*	*50 ppm*	*100 ppm*	*500 ppm*
C. equisetifolia	Decoction	CeD	6.55	14.17	23.05	91.79
	Ethanol	CeE	1.81	1.93	4.42	22.89
	EtOH:H_2O	CeA	2.82	5.76	7.59	52.36
C. brevifolius	Decoction	CbD	3.26	2.65	5.01	21.30
	Ethanol	CbE	0.00	0.34	0.42	4.48
	EtOH:H_2O	CbA	0.87	0.73	1.21	7.36
D. piloselloides	Decoction	DpD	4.63	4.79	9.37	42.56
	Ethanol	DpE	0.00	0.11	0.19	1.43
	EtOH:H_2O	DpA	0.70	1.57	2.42	8.99
I. chinensis	Decoction	IcD	7.13	13.24	22.44	80.06
	Ethanol	IcE	0.92	0.84	1.65	17.78
	EtOH:H_2O	IcA	7.88	18.82	36.16	90.10
P. abbreviatum	Decoction	PaD	2.33	2.91	4.60	21.62
	Ethanol	PaE	1.43	1.54	1.90	14.25
	EtOH:H_2O	PaA	1.14	1.14	1.69	6.26
BHT	-	-	27.92	54.03	81.13	94.16

* - mean of triplicate analysis.

It is observed that the free radical scavenging activity of the leaf extracts exhibited an increasing trend as the concentration of the leaf extract increases. Among the tested leaf extracts, the highest radical-scavenging activity at the 500 ppm concentration was seen in the decoction of *C. equisetifolia* (CeD, 91.79%) followed by the 50:50 EtOH:H_20 extract of *I. chinensis* (IcA, 90.10%). The antioxidant activities of the leaf extracts at 25, 50, and 100 ppm concentrations are well below the values of the standard at the same concentration and therefore, are not comparable to that of the standard BHT. The lowest radical-scavenging activity was found in the ethanol extract of *D. piloselloides* (DpE, 1.43%). The decoction and 50:50 EtOH:H_20 extracts of two plant species *C. equisefolia* (CeD, 91.79% and CeA, 52.36%) and *I. chinensis* (IcD, 80.06% and IcA, 90.10%) showed considerable DPPH radical-scavenging activity and ranked as the top four extracts in the order CeD > IcA > IcD > CeA.

Total antioxidant capacity test: Phosphomolybdenum method. As it is illustrated in Figure 1 & 2, the crude ethanol extract of *D. piloselloides* (DpE, 149.43 BHTE, 87.54 AAE) has the highest total antioxidant activity while the 50:50 EtOH:H_2O extract of *C. brevifolius* (CbA, 3.19 BHTE, 0.00 AAE) has the lowest antioxidant activity. The order of the total antioxidant capacity of the top five extracts compared to both standards, in decreasing order is: DpE (87.54 AAE, 149.43 BHTE) > CeE (37.69 AAE, 77.06 BHTE) > IcE (34.58 AAE, 72.55 BHTE) > PaE (20.73AAE, 52.44 BHTE) > CeD (18.95 AAE, 49.866 BHTE).

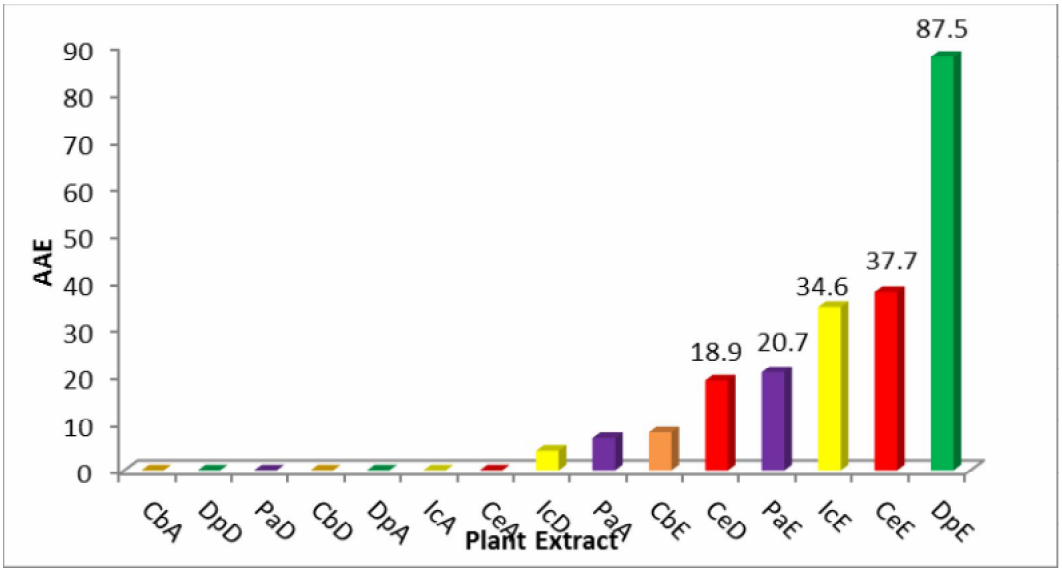

Figure 1. Total antioxidant capacities of the leaf extracts at 200 ppm concentration expressed as ascorbic acid equivalents (AAE).

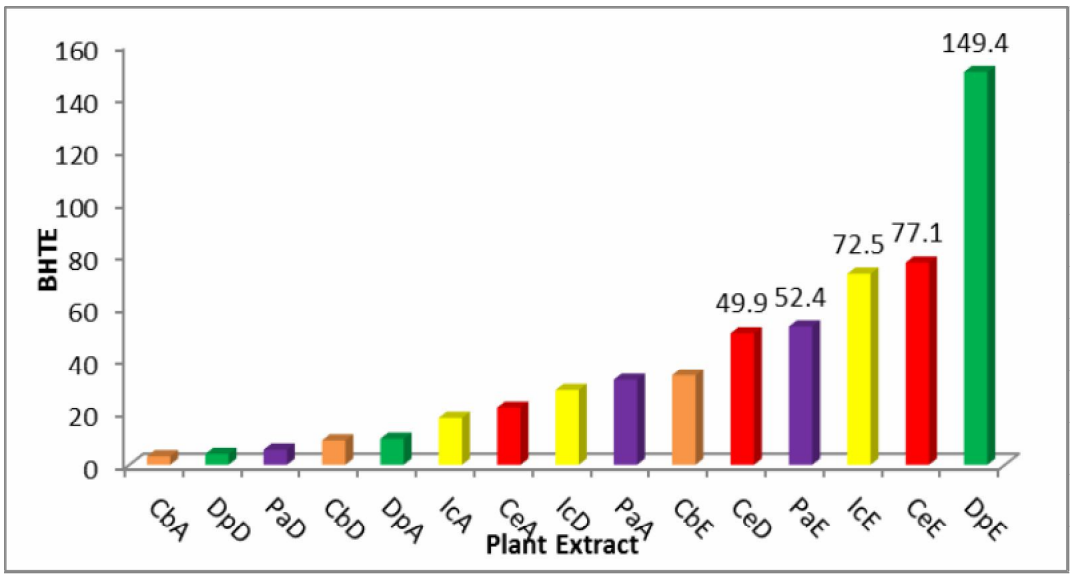

Figure 2. Total antioxidant capacities of the leaf extracts at 200 ppm concentration expressed as butylated hydroxytoluene equivalents (BHTE).

Total phenolics content. Figure 3 illustrates the total phenolics content of the various leaf extracts. The amount of total phenolics varied considerably and ranged from 88.00 to 285.62 GAE. The ethanol extract of *D. piloselloides* (DpE, 285.62) was found to contain the highest phenolic content and the lowest was found in the ethanol extract of *C. brevifolius* (CbE, 34.67). The five extracts with the highest phenolic contents in decreasing order are: DpE (285.62) > CbA (195.14) > PaA (155.14) > IcA (154.67) > CeE (144.67).

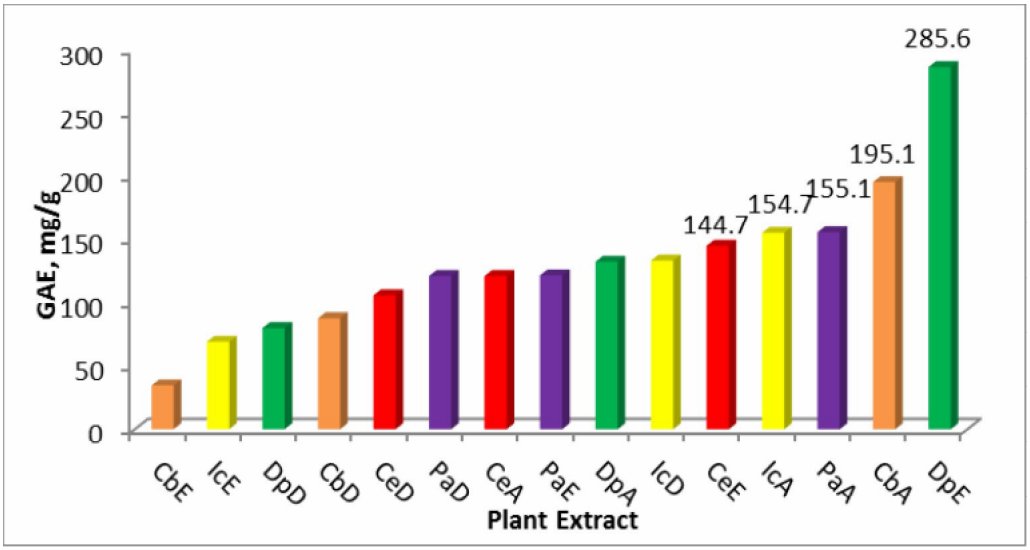

Figure 3. Total phenolics content of the leaf extracts at 500-ppm expressed as gallic acid equivalence (GAE).

An antioxidant is a molecule (or an ion, or a relatively stable radical) that is capable of slowing or even preventing the oxidation of other molecules. In living systems, various metabolic processes and environmental stresses generate numerous reactive species such as free radicals and reactive oxygen species. Free radicals are responsible for causing a large number of diseases but substantial evidence indicates that food containing antioxidants may be of major importance in disease prevention (Alam et al 2012). The continuing growth of the market of antioxidants reflects the hope to cure the wide range of diseases that are believed to be caused or promoted by 'oxidative stress' (Halliwell & Gutteridge 2007; Niki 2011; Dotan et al 2009). A great number of research studies have been carried out relating to the antioxidant activity of numerous plant extracts. Secondary compounds of higher plants have been demonstrated in *in vitro* experiments to protect against oxidative damage by inhibiting or reducing free radicals and reactive oxygen species (Larson 1988).

A number of methods and variations have been developed and applied for the measurement of antioxidant capacity and efficacy. One such method is the DPPH radical scavenging activity assay which is frequently used for the assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds (Huang et al 2005). Compared with other methods, the DPPH assay has many advantages, such as good stability, credible sensitivity, simplicity and feasibility (Jin et al 2006). In its radical form, DPPH absorbs at 517 nm, but upon reduction with an antioxidant, its absorption decreases due to the formation of its non-radical form (Blois 1958). Consequently, the radical-scavenging activity can be monitored in the presence of a hydrogen donating antioxidant as a decrease in absorbance of DPPH solution.

The total antioxidant capacity of the leaf extracts of five medicinal plants was evaluated spectrophotometrically by the phosphomolybdenum method, which is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at 695 nm. The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoids (Prieto et al 1999).

Phenolic compounds are secondary plant metabolites. Studies have shown polyphenols to be more active antioxidants *in vitro* than tocopherols and ascorbate. This can be attributed to their structures which are perfect for free-radical scavenging activity (Rao et al 2010). The antioxidant properties of polyphenols are due to their capacity to act as strong hydrogen or electron donors. Moreover, polyphenol radicals are capable of stabilizing unpaired electrons and chelating transition metal ions (Rice-Evans et al 1997).

The total phenolics assay measures a sample's reducing capacity and numerous publications applied the total phenolic assay by Folin-Ciocalteu reagent and other antioxidant capacity assays and often found excellent linear correlations between the total phenols and the antioxidant activity of plant extracts (Ryan & Ray 2004). Literature suggests that the antioxidant activity of plant extracts is mainly due to the presence of phenolic compounds, which may exert antioxidant effects as free radical scavengers, hydrogen donating sources, singlet oxygen quenchers, and metal ion chelators (Shahriar et al 2013).

Conclusions. Results (Table 1) show that the activities of the decoction and 50:50 EtOH:H_2O extracts of two plant species *C. equisetifolia* and *I. chinensis* have considerable DPPH radical-scavenging activity at 500 ppm concentration. It can be said that there was a reduction in the concentration of DPPH due to the scavenging ability of the extracts of *C. equisetifolia* and *I. chinensis* and thus, increasing the percentage of the free radical inhibition. This assay can also be used as a guide for fractionation and isolation of potential antioxidant compounds from the mentioned plant species.

From results, it has been observed that the crude ethanol extract of *D. piloselloides* has the highest total antioxidant activity followed by the crude ethanol extracts of *C. equisetifolia* and *I. chinensis* suggesting promising sources of useful bioactive compounds from these three plant species.

Results of this study show that the crude ethanol extract of *D. piloselloides* contains the highest phenolic concentration. It is proposed that phenolic compounds could possibly be significant components of the tested plant extracts as all of the plant extracts exhibited antioxidant activities in varying amounts.

This study has shown that some plants used in traditional medicine in the Philippines possess antioxidant potential in varying degrees suggesting the presence of secondary metabolites having antioxidant properties. These plants have the potential to be developed further as plant-based herbal supplements. However, no clear trend was observed among the plant extracts evaluated using different antioxidant potential assays. The inconsistency in the obtained antioxidant activity values depending on the method used shows that different assays determine different aspects of the antioxidant capacity. This is in agreement with studies stating that a number of methods and variations have been developed for the measurement of antioxidant capacity and efficacy, but very often there is a lack of correlation between activities determined on the same material by different assays and between activities determined by the same assay in different laboratories (Niki 2011).

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