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Cytotoxicity and Antioxidant Activity of *Equisetum debile* Roxb. (Equisetaceae) from Bukidnon, Philippines

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ABSTRACT
*The potential of Equisetum debileRoxb. as source of lead compounds for drug development was investigated. The bioactivity of the decoction (**EdD**), ethanolic - (**EdE**), chloroform- (**EdC**), hexane - (**EdH**), and aqueous (**EdA**) extracts of E. debile were determined through Brine Shrimp Lethality Test (BSLT) and in vitro antioxidant assays: Total Antioxidant Capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity, and Total Phenolics Content. The chloroform extract (**EdC**) of E. debile exhibited moderate toxicity against Artemiasalinanauplii with an LC_{50} value of 190.55 mg/L and gave the highest total antioxidant capacity (354.05 mg BHT Equivalents/g sample and 330.20 mg Ascorbic Acid Equivalents/g sample). **EdC** also gave a remarkably higher phenolics content (713.72 mg Gallic Acid Equivalents/g sample) among the extracts, showed DPPH radical scavenging activity of 78.13% and consequently the lowest EC_{50} value (295.02 mg/L). The results indicated that the chloroform-soluble extract of E. debile is the most bioactive and can be further purified to identify compounds responsible for its bioactivity.*
***Keywords*:** *Equisetum debile*, brine shrimp lethality, antioxidant property, cytotoxicity

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INTRODUCTION

The importance of traditional medicinal plants and traditional health systems has been continuously recognized and is gaining increasing attention even up to present. Today, plant-based system has continued to play an essential role in solving the healthcare problems of the world. According to World Health Organization (WHO), about 80% of people worldwide rely on herbal medicines for some parts of their primary health care[1]. However, only about five percent of the total plant species have been thoroughly investigated to ascertain safety and efficacy of traditional medicine[2]. Hence, investigation on plants of medicinal importance is growing phenomenally.

In the Philippines, there are over 1000 plant species considered to be of medicinal use[3]. Among these plants is *Equisetum debile* Roxb., a fern ally under family Equisetaceae. Ethnobotanical reports have shown polypus, jaundice, hepatitis, urinary troubles, and cancers of breast, liver, intestine, stomach, kidneys and tongue[4].However, limited scientific studies were conducted to support therapeutic claims and medicinal value of *E. debile*. Thus, cytotoxic and antioxidant properties of *E. debile* is worth investigating.

During the process of oxygen utilization in normal physiological and metabolic processes, approximately 5% of oxygen gets univalently reduced to oxygen-derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these radicals are known as reactive oxygen species (ROS)[5].

ROS exert oxidative stress towards the cells of a body inducing tissue damage leading to various physiological disorders[6]. It has been reported that ROS has the capability to initiate the peroxidation of membrane lipids and other crucial biomolecules like nucleic acids, proteins and carbohydrates. In fact, there is an increased evidence for the participation of ROS in the pathology of various diseases like cancer, diabetes, autoimmune diseases, neurodegenerative diseases, aging, among others[7]. In nature,

however, plants have been considered a reservoir of naturally occurring antioxidants. Antioxidants neutralize the effect of free radicals through scavenging ROS, inhibiting ROS formation, binding transition metal ions and preventing formation of OH^- and/or decomposition of lipid hydroperoxides, repairing damage tissues, or by any combination of the above[6].At present, attention to harness the natural and non-toxic compounds with antioxidative activity from plant materials has been intensified[8]. Various methods are developed to investigate antioxidant properties of different samples. Yet, no single method is adequate for determining the antioxidant capacity of food[9] as different methods may give diverse results. Thus, different methods must be used to take into account the various mechanisms of antioxidant action[10].

The1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay is the most rapid, simple, inexpensive, and widely reported method for screening of antioxidant activity of many plant drugs. This assay involves the reduction of free radical DPPH by a free radical scavenger and has been widely used to investigate scavenging activity of several natural compounds such as phenolics or anthocyanins[11]. Several studies have used DPPH radical scavenging activity for assaying antioxidant activity of plant extracts [12,13,14]. Phosphomolybdenum Method is a spectroscopic method for the quantitative determination of antioxidant capacity, through the formation of phosphomolybdenum complex. In this assay, Mo(VI) is reduced to Mo(V) by the sample analyte leading to a subsequent formation of a green phosphate/ Mo(V) complex at acidic pH which has a maximum absorbance at 695 nm[15]. The higher absorbance value indicates higher antioxidant activity.The Folin-Ciocalteu (FC) method is used to measure the total phenolics content. FC method is based on a chemical reduction of the reagent, which isa mixture of tungsten and molybdenum oxides. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 765 nm. The intensity of the light absorption at that wavelength is proportional to the concentration of phenols[16].

In this study, Brine Shrimp Lethality Test (BSLT) has been used to evaluate the cytotoxic properties of *E. debile*. BSLT, a general bioassay capable of detecting a broad spectrum of bioactivity present in crude extracts, has been demonstrated to correlate well with cytotoxicity and other biological properties[17]. In the Cell Culture Laboratory of Purdue Cancer Center, BSLT has been used as a pre-screen test for a panel of six human solid tumor lines and for the record, over 300 antitumor and pesticidal natural products were now isolated[18]. Moreover, BSLT has been used as a simple tool to guide screening and fractionation of physiologically active plant extracts and has appeared to be predictive of cytotoxicity and pesticidal activity[19]. This study evaluates the cytotoxic and antioxidant property of the whole plant sample of *E. debile*.

MATERIALS AND METHODS

Sample Collection and Preparation

Whole plant **s**amples of *Equisetum debile*Roxb. were collected from Kalasungay, Malaybalay, Bukidnon (8° 10' 27" North, 125° 6' 14" East) in July 2012 and were submitted to Professor Edgardo C.Aranico of the Biological Sciences Department, MSU-IIT for authentication and identification. Fresh whole plant samples were washed with tap water, rinsed with distilled water, and air-dried.

Preparation of Crude Extracts

About 2 kg of air-dried plant samples was pulverized using sterile electric blender, weighed, and soaked with about 15 L of 95% ethanol for 3 days. To obtain the crude ethanol extract (**EdE**), the resulting solution was then filtered, concentrated *in vacuo* at temperature not exceeding 40°C, and weighed. A portion of the **EdE**(48.1686 g) was then sequentially partitioned in hexane:water (1:1) and chloroform:water (1:1) solutions. The hexane-soluble, chloroform-soluble and water-soluble portions were individually concentrated *in vacuo* and weighed to give hexane - (**EdH**), chloroform -(**EdC**) and aqueous-soluble (**EdA**) extracts, respectively. For the preparation of plant decoction (**EdD**), 630.0 g of fresh and clean whole plant sample of *E. debile*Roxb. were cut into pieces and boiled in sufficient amount of water (1:2) for 5 minutes. The mixture was then filtered, cooled, freeze-dried, and stored in glass containers.

Brine Shrimp Lethality Assay

The assay was carried out employing the principle and protocol previously described by McLaughlin and Krishnaraju with slight modifications [4,18]. Four tests solutions (1000-, 500-, 100- and 10 mg/L) were prepared and tested for each plant extract. Ten previously hatched brine shrimps were transferred to each test tube and sterilized sea water was added up to the mark corresponding to 5.00 mL. Control experiments using podophyllotoxin (positive) and seawater (negative) were simultaneously performed for the four concentrations. The number of dead and alive nauplii was counted 24 hours for chronic toxicity data. The results were evaluated and the LC_{50} (median lethal concentration) values were

determined using the Reed-Muench Method[20]. Tests for each sample concentration were done in triplicates.

DPPH Free Radical Scavenging Assay

The DPPH free radical scavenging activity of the *E. debile* extracts were determined by comparison with known antioxidant butylated hydroxytoluene (BHT) and ascorbic acid (AA)[21].A 3.00 mL of DPPH (0.1 mM) methanolic solution was added in to each of the various concentrations (500-, 100-, 50-, 25 mg/L) of the extracts. The mixtures were then shaken vigorously and allowed to stand under dark condition at room temperature for one hour. Using a UV-Vis spectrophotometer (Lasany-LI-2800), absorbance of the solutions was measured at 517 nm against methanol as a blank. Each determination was carried out in triplicate. The percent (%) DPPH inhibition was calculated according to the formula:

$$\%\ Inhibition = \frac{Absorbance_{control} - Absorbance_{sample}}{Absorbance_{control}}\ x\ 100$$

A calibration curve was drawn for the BHT, AA, and sample extracts using Least Squares Method. The equation of the line was then used to calculate the EC_{50} (concentration required to obtain a 50% antioxidant effect) of the extracts.

Total Antioxidant Capacity Assay

The total antioxidant activity of the *E. debile* extracts was evaluated by the Phosphomolybdenum Method[15]. A 0.30 mL of 200 mg/L ethanolic solution of *E. debile* was added with 3.00 mL of the reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate). The mixtures were then incubated at 95^{o}C for 90 minutes, allowed to cool to room temperature. Absorbance was then measured at 695 nm using a UV spectrophotometer (Lasany-LI-2800). The total antioxidant activity of the various extracts was expressed as milligram Ascorbic Acid Equivalents per g sample (mg AAE/g sample) and milligram Butylated Hydroxytoluene Equivalents per gram sample (mg BHTE/g sample), which were derived from the calibration curve established using ascorbic acid and butylated hydroxytoluene as reference standards. All analyses were conducted in triplicates and results were reported as means of the triplicate analysis.

Total Phenolics Content Assay

The total phenolics content of the various *E. debile* extracts were determined by Folin-ciocalteu Method as described by Makkar et al.[22].In separate test tubes, 0.10 mL of 500 mg/L (in ethanol) extract was combined with 2.80 mL of 10% Na_2CO_3 and 0.10 mL of 2N Folin-Ciocalteu reagent. The mixture was then set aside for 40 minutes before the absorbance was measured at 725 nm. The total phenolics content was measured as milligram Gallic Acid Equivalents per gram sample (mg GAE/g sample) which was derived from the linear equation of the calibration curveusing various concentrations (25-, 50-, 100-, 200 mg/L) of gallic acid as working standards. Analysis was done in triplicates.

RESULTS AND DISCUSSION

Toxicity Against Brine Shrimp Nauplii

Analysis of the brine shrimp nauplii mortality data was carried out using the Reed-Muench Method[20] in order to estimate the toxicity of the extracts. Results of the analysis are shown in Table 1.

Table 1. Percent mortality of brine shrimp nauplii and toxicity (LC_{50}, mg/L) of various *E. debile* extracts

Extract	Percent Mortality of Brine Shrimp after 24-h Exposure*				LC_{50} (mg/L)
	10 mg/L	100 mg/L	500 mg/L	1000 mg/L	
EdE	0.00	18.03	42.59	75.47	588.84
EdH	0.00	6.67	100.00	100.00	208.93
EdC	0.00	16.13	97.14	100.00	190.55
EdA	0.00	0.00	26.19	70.73	758.88
EdD	0.00	0.00	4.08	40.63	>1000

* mean of triplicate analysis

The LC_{50} values after 24-hour exposure of the nauplii are in the order of 190.55 mg/L (**EdC**) < 208.9 3 mg/L (**EdH**)<588.84 mg/L (**EdE**)<758.88 mg/L (**EdA**)<greater than 1000 mg/L (**EdD**). Brine Shrimp Lethality Test is predictive of cytotoxicity and pesticidal activity[19]. Crude extracts resulting in LC_{50} of less than 250 mg/L are considered significantly active and has the potential for further investigation[23]. The data obtained indicate relatively higher toxicity of EdC and EdH compared to the other extracts. Although the obtained results suggest moderate toxicity among the crude extracts, presence of potential antitumor or pesticidal components in both chloroform- and hexane-soluble extracts may still be

considered. These findings are supported by some studies which have reported that methanolic extracts of *E. debile*aerial plant part exhibited moderate activity against brine shrimp[24]and shown antifungal and allelopathic properties[25]. Furthermore, the results suggest that the toxicity of *E. debile* extracts, particularly the **EdC** and **EdH**, against brine shrimp nauplii is extract type and concentration-dependent.

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity, expressed as percent DPPH inhibition, various concentrations of *E. debile* extracts at is presented in Table 2.

Table 2. Percent DPPH inhibition of various concentrations of *E. debile* extracts

Extract	% Inhibition*				EC_{50}, mg/L
	25 mg/L	50 mg/L	100 mg/L	500 mg/L	
EdE	1.77	1.97	1.97	5.22	>500
EdH	1.08	1.38	1.58	4.33	>500
EdC	7.98	14.29	28.18	78.13	295
EdA	5.80	7.52	9.69	15.13	>500
EdD	5.62	7.61	7.79	13.59	>500
Ascorbic acid	18.98	48.47	99.21	100.00	53
Butylated hydroxytoluene	15.07	39.51	51.10	87.55	123

*mean of triplicate analysis

The DPPH radical scavenging activity of the *E. debile* extracts increases along with the increase in their concentrations (Table 2). Among the extracts, **EdC** gave the highest DPPH radical scavenging activity. The chloroform extract showed 78.13% DPPH radical inhibition compared to 100% and 87.59% for ascorbic acid and BHT, respectively. Consequently, the required extract concentration to obtain 50% radical inhibition (EC_{50}) is lowest for **EdC** (295 mg/L). The results obtained are consistent with the findings of Sarkar where *E. debile* extract has shown significant free radical scavenging activity[12]. The said activity of *E. debile* extract may be attributed to the known antioxidants, i.e. sterols, ascorbic acid, silicic acid, phenolic acid and flavonoids, present in the plant [12, 26].

Total Antioxidant Capacity

The total antioxidant capacity of the *E. debile* extracts is presented in Figure 1.

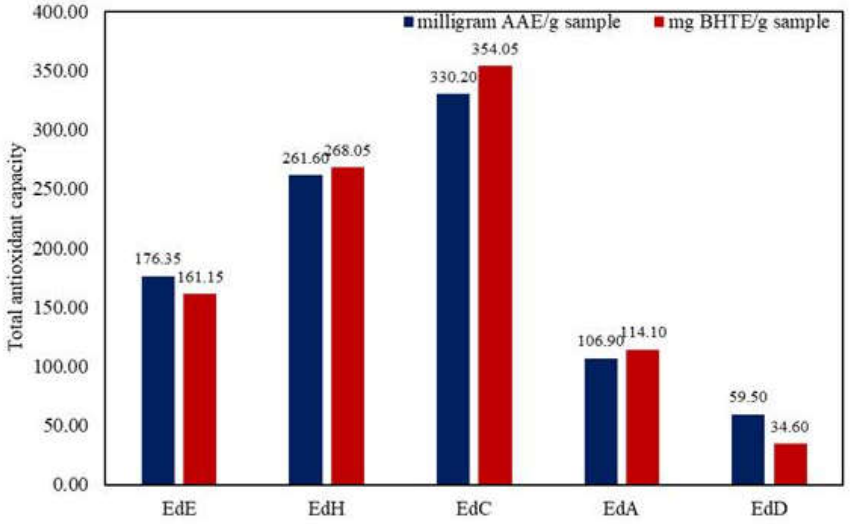

Figure 2. Total antioxidant capacity of various *E. debile* extracts.

The total antioxidant capacity of the *E. debile* extract was found to decrease in the order **EdC>EdH>EdE>EdA>EdD**. The good antioxidant capacity of **EdC** compared to the other crude extracts can be attributed to the antioxidant compounds in the plant sample that has high extractability in chloroform. Moreover, the values for BHTE were found relatively higher than their corresponding AAE values (Figure 2). This can be explained by taking into consideration that major contributor to the total antioxidant capacity of terrestrial plants are lipid-soluble. Other compounds that might contribute to total lipid-soluble antioxidant capacity are carotenoids, flavonoids, and cinnamic acid derivatives[16].

Total Phenolics Content

The total phenolics content (in mg GAE/g sample) of the various extracts of *E. debile* are shown in Figure 3.

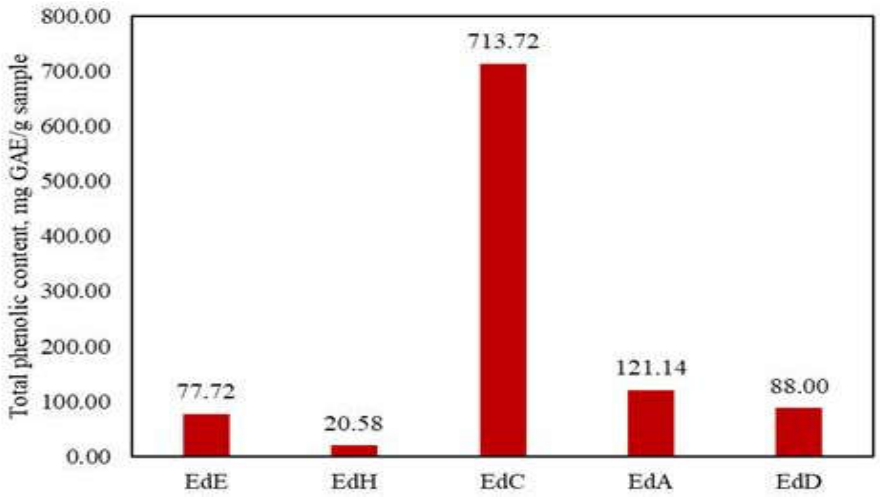

Figure 3. Total phenolic content of various *E. debile* extracts.

The chloroform-soluble fraction (**EdC**) showed the highest level of phenolic content (713.72 mg GAE/g sample) among the five samples (Figure 3). This finding is supported with the known fact that most phenolic compounds have intermediate polarity and are expected to be soluble in moderately polar solvent like chloroform. Phytochemical studies found that *E. debile* contains phenolic acids and flavonoids [27,28]. It is noteworthy that the trend of the results for DPPH radical scavenging activity and total phenolics are similar. The results imply a strong correlation between DPPH radical scavenging activity and the levels of phenolic compounds in the extracts[29]. Accordingly, phenolic compounds are active quenchers of DPPH radical in a concentration-dependent manner [30,31].

CONCLUSION AND RECOMMENDATION

LC_{50} values for BSLT showed relatively higher toxicity of **EdC** and **EdH** against brine shrimp nauplii. The **EdC**gave the highest total antioxidant capacity value. On the other hand, a strong correlation between DPPH radical scavenging activity and total phenolics content was observed. **EdC** which has remarkably higher phenolics content than the other extracts exhibited the highest radical scavenging activity of and consequently the lowest EC_{50} value. The results of the BSLT and *in vitro* antioxidant assays indicate that the chloroform-soluble extract (**EdC**) is the most bioactive and can be a possible source of lead compounds for drug development. Further purification of the extract is recommended to identify the compounds responsible for its bioactivity.

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