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ORIGINAL ARTICLE **OPEN ACCESS**

Antioxidant Activity, Total Phenolic Content, and GC-MS Analysis of the root of Kawilan (*Embeliaphilippinensis*A. DC.)

Nyzar Mabeth O. Odchimar[1], Olga M. Nuñeza[1*], Mylene M. Uy[2], W.T.P.S.K. Senarath[3]
[1]Department of Biological Sciences, Mindanao State University – Iligan Institute of Technology, Philippines
[2]Department of Chemistry, Mindanao State University – Iligan Institute of Technology, Philippines
[3]Department of Botany, University of Sri Jayewardenepura, Sri Lanka
*Corresponding author's **Email:** olgamnuneza@yahoo.com

ABSTRACT
The root of "kawilan" (Embeliaphilippinensis) is known by the Talaandig tribe of Bukidnon, Philippines to cure tumors, cysts, and few types of cancer yet it lacks scientific authentication of its medicinal properties. In the present study, the antioxidant activity of aqueous and methanol extracts of E. philippinensis roots was evaluated by determining the free radical scavenging activity using 2,2 diphenyl-2-picrylhydrazyl hydrate (DPPH)assay method and substantiated by total phenolics content (TPC) by Folin-Ciocalteu's assay method. GC-MS analysis of methanol extract was also performed to identify the specific semi-volatile chemicals present. Both aqueous and methanol root extracts showed high DPPH inhibition activity at high concentrations. However, the aqueous root extract of E. philippinensis showed higher DPPH inhibition activity than methanol root extract. The TPC assay showed higher phenolics content on the methanol extract. Furthermore, GC-MS analysis revealed the presence of bioactive compounds with known antioxidant properties: (a) 9,12-octadecadienoic acid, methyl ester, (b) eicosane, (c) hexadecane (d) hexadecanoic acid, methyl ester, (e) 9, 12, octadecatrienoic acid, (Z,Z), (f) 9, 12, 15- octadecatrienoic acid, (Z,Z,Z), (g) n-hexadecanoic acid, (h) squalene, (i) phenol, 2,4-bis (1,1-dimethyl), and (j) 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester. Results indicate that E. philippinensis possesses antioxidant properties, which may account for its folkloric medicinal uses.
Keywords: *bioactive compounds, DPPH assay, Folin-Ciocalteu's assay, free radical scavenging, medicinal.*

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INTRODUCTION

Free radicals are generated from the by-products of normal metabolic endogenous processes [1] and from the reaction with exogenous sources such as oxygen which generates reactive oxygen species (ROS) [2]. ROS such as superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide have unpaired electrons which make them highly unstable and highly reactive [3]. Being unpaired and aggressive, they attempt to steal electrons from other neighboring molecules which can lead to a destructive chain reaction and eventually causes oxidative stress in the body [4]. Oxidative stress is the overproduction of free radicals in the body system, which is capable of reversibly or irreversibly damaging compounds of all biochemical classes, including nucleic acids, proteins and free amino acids, lipids and lipoproteins, carbohydrates and other macromolecules [5]. Thus, oxidative stress is implicated in the pathogenesis of ageing, immunodepression, atherosclerosis, inflammation, diabetes, cardiovascular, and neurological disorders [6, 7]. Importantly, in carcinogenesis and tumor-bearing cancer, oxidative stress has been implicated [8]. It has a particular role in DNA damage leading to various mutations [9, 10]. Specifically, reactive oxygen species acts directly or indirectly by altering gene expression particularly the DNA binding of transcription and signaling factors [11] which at some rate causes the onset or contribute to the metastatic potential of cancer and/or tumor formation [2]. However, studies have shown that free radicals, *in vitro*, can be stabilized by the application of an anti-oxidant. Antioxidants are chemical substances that inhibit free radical activities by donation of the missing electrons without joining the chain of reactions [7]. There are synthetic and naturally occurring anti-oxidants. The synthetic antioxidants, however, impose potential health risks and toxicity [12]. They have been reported to induce liver and kidney dysfunction, and carcinogenic effect in experimental animals, thus, naturally occurring antioxidants are preferred [13]. They occur naturally in plants mainly in the

form of phenolic compounds [14]. Plant phenolics are reported as the most abundant secondary metabolites found in plants, with approximately 8,000 known to date [15]. Phenolics (phenols or polyphenols) are chemical component responsible for a plant's color, flavor, odor and oxidative stability [16]. They are compounds possessing one or more aromatic rings with one or more hydroxyl groups [17]. Flavonoids, tannins, and phenolic acids are the major groups of compound belonging to plant phenolics [18]. These compounds are known to have anti-inflammatory and anti-carcinogenic property [19] as well as free-radical scavenging property [20]. Thus, damaging effects of free radicals can be prevented by treatment using naturally occurring medicinal plant which is already scientifically accepted [21]. Hence, plant diet rich in phenolics is gaining medical interest. *In vitro,* antioxidant activity of a medicinal plant can be investigated through 2, 2 diphenyl-2-picrylhydrazyl hydrate (DPPH) assay method which is the most used method in determining the free radical scavenging property of the plant extract [22]. DPPH is a stable free radical which readily accepts an electron or hydrogen radical donated from the antioxidants, which converts and neutralizes the free radical 2-2 diphenyl-2-picryl hydrazyl hydrate to 1-1 diphenyl-2-picrylhydrazine [23]. Neutralization of DPPH is indicated in the discoloration of the reaction mixture from violet to yellow [24]. *Embeliaphilippinensis*, from the family Primulaceae, is a woody climbing vine which can be found in the deep forest of Mt. Kitanglad, Philippines. It's roots, by decoction, is one of the most used herbal medicines of the Talaandig tribe of the Philippines being traditionally used as sedative, and anti-hypertensive, but prominently used for eliminating tumors or cysts, and treating few types of cancers [25]. There were no recent scientific investigations on *E. philippinensis* roots and its antioxidant activity. Thus, the present study aimed to determine the antioxidant properties of the root of *E. philippinensis* using DPPH assay method with determination of its total phenolics content.

METHODOLOGY

Fresh roots of *Embeliaphilippinensis*were collected at the deep forest of Lilingayon, Valencia City, Bukidnon, Philippines. Prior to collection, Gratuitous Permit from Department of Environment and Natural Resources of the Philippines and an official permission from the local government were obtained. The collected roots were washed thoroughly and air-dried until crispy. The dried plant materials were pulverized using an electric blender and were subjected to two types of extraction: decoction and maceration. In decoction, 10 grams of powdered root of *E. philippinensis* were allowed to boil with 400 ml distilled water on a medium heat until the volume was reduced to approximately 50 ml. In maceration, same amount of powdered root was soaked with 100 ml of 70% methanol in a shaker (100 rpm) continuously for seven days at 26°C. Both filtrates were collected by passing through Whatman no.1 filter paper and were subjected to rotary evaporator, 60°C for decoction filtrates and 50°C for maceration filtrates, to obtain the aqueous and methanol crude extracts. The crude extracts were stored in a tight glass container at 4°C until used. The samples were then subjected to DPPH assay method to determine the free radical scavenging activity of the plant extracts.DPPH (2, 2 diphenyl-2-picrylhydrazyl hydrate) (Sigma) scavenging activity was determined using a spectrophotometric method [26]. Freshly prepared DPPH solution was used. Reaction mixture was prepared using 2.5 ml of $6.5x10^{-5}$ M DPPH solution and 0.5 ml of sample extracts dissolved in methanol. Methanol was used as the control. Samples were tested in four concentrations (0.25 mg/ml, 0.50 mg/ml, 1.0 mg/ml, and 2.0 mg/ml) with three replicates. Both set-ups were placed untouched for 30 minutes in the dark at room temperature. Absorbance was measured at 517 nm using UV-Vis spectrophotometer (SHIMADZU UV mini 1240). The percentage of DPPH radical scavenging activity was determined using the equation mentioned below. IC_{50} value was also calculated.

$$\% \text{ DPPH scavenging activity} = (A_{control} - A_{sample} / A_{control}) \times 100$$

To further lend support on the antioxidant activity of *E. philippinensis* root extracts, total phenolics content and GC-MS analysis were done to determine and evaluate possible compound responsible for the activity. The total phenolics content was determined using Folin-Ciocalteu's assay [26]. A 100 µL of the extract in methanol (1mg/mL) was mixed with 1.0 mL of distilled water and 0.5 mL of Folin-Ciocalteu's reagent (1:10 v/v). After mixing, 1.5 mL of 2% aqueous sodium bicarbonate was added and the mixture was allowed to stand for 30 min with intermittent shaking. The absorbance was measured at 765nm using a spectrophotometer (SHIMADZU UV mini 1240). A standard calibration plot of absorbance values of Gallic acid was measured at different concentrations (64 µg/ml, 32 µg/ml, 16 µg/ml, and 8 µg/ml, 4 µg/ml). Methanol was used as a blank and the assay was carried out in triplicates. GC-MS analysis was performed according to Chipiti*et al.* [13] with modifications. The methanol root extract of *E. philippinensis* was analyzed and wasdiluted with chloroform. The sample was subjected to Agilent Technologies 7890A GC system coupled with (an Agilent) 5975C Mass Selective detector. A HP-5MS capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) was used. The carrier gas was helium. The injector

temperature was set at 320°C. The initial oven temperature was at 70°C which was programmed to increase to 280°C at the rate of 10°C/min with a hold time of 4 min at each increment. Injections of 1 µL were made in split mode with a split ratio of 100:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230°C, quadrupole temperature 150°C, solvent delay 3 min and scan range 33-550 amu. The compounds were identified by direct comparison of the mass spectrum of the analyte at a particular retention time to that of a reference standard found in the National Institute of Standards and Technology (NIST) library. At least 80% similarity index was considered significant [27]. Total GC-MS running time was 45 minutes.

RESULTS

Table 1 shows the scavenging activity of the extracts which are evident in both the aqueous and the methanol extracts indicated by the concentration dependent increase in the % inhibition. It also showed that the aqueous root extract of *E. philippinensis* (IC_{50}= 0.3286 mg/ml)has lower IC_{50} value than the methanol extract (IC_{50}= 0.6203 mg/ml).

Table 1.DPPH – Radical Scavenging Activity of *E. philippinensis* root extracts.

% Activity					
Extract	0.25mg/ml	0.5mg/ml	1mg/ml	2mg/ml	IC_{50} (mg/ml)
Aqueous	42.60±0.98*	58.59±1.30	72.79±0.79	74.14±0.98	0.3286
Methanol	43.79±2.74	44.92±2.09	53.36±0.93	55.90±1.44	0.6203
Ascorbic Acid	65.17±0.93	72.20±0.39	73.39±0.30	83.86±1.32	<0.2500

*mean±SD; n=3

To further ascertain the antioxidant activity of *E. philippinensis* root extracts, total phenolics content was assessed since phenolics are compounds with known free radical scavenging activity [28]. The total phenolics content, calculated using a standard curve, of *E. philippinensis*aqueous and methanol root extracts is presented in Table 2. The results showed that the aqueous extract have lower phenolics content at 42.16 mg GA/g extract than methanol extract with 131.10 mg GA/g extract.

Table 2. Total Phenolics content of *E. philippinensis* root extracts.

Total Phenolics Content, GAE	
Extracts	mg GA /g of extract
Aqueous	42.16±0.03*
Methanol	131.10±0.001

*mean±SD; n=3

Meanwhile, the GC-MS analysis of the methanol root extract of *E. philippinensis* revealed the presence of (a) 9,12-octadecadienoic acid methyl ester, (b) eicosane, (c) hexadecane (d) hexadecanoic acid methyl ester, (e) 9, 12-octadecatrienoic acid, (f) 9, 12, 15- octadecatrienoic acid, (g) n-hexadecanoic acid, (h) squalene, (i) 2,4-bis(1,1-dimethyl) phenol, and (j) 1,2-benzenedicarboxylic acid, bis (2-methylpropyl) ester (Table 3) which are previously known to possess antioxidant activity.

Table 3. Possible Compounds Found in the Methanolic Extract of *E. philippinensis* roots.

Name of Compound	Similarity index (%)	Retention time (minutes)
9,12-octadecadienoic acid methyl ester	98	20.810
1,2-benzenedicarboxylic acid bis(2-methylpropyl) ester	97	18.896
Eicosane	97	19.874
Hexadecane	97	24.957
Hexadecanoic acid methyl ester	95	19.169
9, 12-octadecadienoic acid	95	21.187
9, 12, 15-octadecadienoic acid	94	20.869
n-hexadecanoic acid	92	19.525
Squalene	92	27.035
2,4-bis(1,1-dimethyl) phenol	90	14.646

DISCUSSION

The concentration-dependent increase in the DPPH percent inhibition of the aqueous and methanol root extracts of *E. philippinensis* indicates that it has a free radical scavenging property, thereby, antioxidant activity. Moreover, IC_{50} which is the concentration of sample at which the inhibition percentage reaches 50%, has an inverse relationship to the activity of a sample. The lower the IC_{50} value, the higher is the activity. Results indicated that the aqueous extract has higher free radical scavenging activity than the alcohol extract. This is in agreement with the study conducted by Chanda *et al.* [29] in which decoction extract of *Syzygium cumini* leaves showed higher free radical scavenging activity than the 80% methanol extract. The results of total phenolics content of *E. philippinensis* aqueous and methanol root extract are opposite to that in the free radical scavenging assay. The methanol extract exhibited higher total phenolics content than the aqueous extract. Other studies have shown the same inverse relationship results on free radical scavenging activity and total phenolics content for aqueous and methanol extracts [30, 31]. This suggests that phenolic compounds are not the predominant antioxidant components in *E. philippinensis* aqueous root extract. Other compounds with antioxidant properties like alkaloids [32], terpenes [33, 34], sterols [35, 36], saponin [18], and many more may have contributed to these activities exhibited by the methanol extract against DPPH radical. In addition, Folin-Ciocalteau reagent is sensitive in recognizing oxidizable phenolics only [37]. Moreover, phenolics content of the sample can be negatively affected by other competing pre-existing oxidants present during the reaction period [15]. The aqueous extract of *E. philippinensis* may contain several pre-existing oxidants which can affect the results of Folin-Ciocalteau assay. This may explain the negative correlation between aqueous and methanol extract's DPPH scavenging activity and total phenolics content.

The GC-MS analysis of the methanol root extract of *E. philippinensis* revealed the presence of Squalene, which is a known anti-carcinogenic and antioxidant [38], thus, the free radical scavenging activity of the plant is expected. Moreover, the GC-MS also revealed the presence of 2, 4-bis (1, 1-dimethyl) phenol, which is the only known phenolics identified. The other compounds belong to hydrocarbons, alkanes, esters, fatty acids, and terpenes. This further indicates that phenolic compounds arenot the only group of compound contributing to the antioxidant activity of the *E. philippinensis* roots.

CONCLUSION

The results from the study indicated that the aqueous and methanol extracts of *E. philippinensis*roots have antioxidant activities as shown in the DPPH scavenging activities of the extracts. This study also revealed that aside from phenolics there are other compounds which may be responsible for the antioxidant activity of the methanol extract. The free radical scavenging effects of the aqueous extract were higher than the methanol extract suggesting that the use of the ethnobotanical preparation (decoction) of the root of *E. philippinensis*in the treatmentof the diseasecan be supported.

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