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GC-MS analysis and evaluation of *in vitro* antioxidant potential and total phenolics content of wild hops (*Flemingia strobilifera* (L.) W. T. Aiton)

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Abstract

Wild hops (*Flemingia strobilifera* Linn.) is a shrub belonging to Fabaceae family. The leaves of *F. strobilifera are* commonly used by the Subanen, the indigenous group in Lapuyan, Zamboanga del Sur, Philippines to treat inflammation. In this study, the hydromethanolic (80%) and aqueous leaf extracts of *F. strobilifera* were evaluated for their antioxidant activity and total phenolics content. The active semi-volatile components of 80% methanol leaf extract were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). DPPH radical scavenging activity was used to determine the potential of this plant as anti-oxidant. Total phenolics content was determined using Folin-Ciocalteu reagent and calculated as gallic acid equivalence. GC-MS analysis revealed the presence of eight compounds. Both the 80% methanol and aqueous extracts of *F. strobilifera* showed significant scavenging activity with low IC_{50} values of 0.299 mg/mL and <0.25 mg/mL, respectively. There was positive correlation between the scavenging activity percentage and the total phenolics content only in the aqueous extract of *F. strobilifera* while 80% methanol extract showed negative correlation between inhibition percentage and total phenolics content which can be attributed to the solvent used and method used in quantification of phenolics. Nevertheless, the results suggest that these leaf extracts are potent source of antioxidant compounds and may serve as natural anti-inflammatory agents.

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Introduction

Free radicals cause oxidative stress and induce DNA damage, protein carbonylation, and lipid peroxidation which result into several health-related problems such as aging, cancer, Parkinson's disease (Garg *et al.*, 2013), and inflammation (Halliwell, 1999). Free radical-induced diseases can be alleviated by the application of antioxidants. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Furthermore, there is a widespread agreement that the synthetic antioxidants such as butylatedhydroxytoluene, butylatedhydroxyanisole, gallic acid esters, and tertiary butylatedhydroquinone must be replaced with natural antioxidants since synthetic antioxidants impose health risk and toxicity and have low solubility along with moderate antioxidant activity (Peteros and Uy, 2010; Bhaskar *et al.*, 2009; Garg *et al.*, 2013). Thus, there is a need to develop potential sources of natural antioxidants.

Medicinal plants indeed have great importance to human health and the society in the treatment of a wide range of diseases. According to Chanda *et al.* (2011), the use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Aside from being useful and economically essential, plants are also safe from harmful side effects caused by synthetic drugs (Azlim *et al.*, 2010). Moreover, plants that exhibit free radical scavenging activity can also inhibit inflammatory agents (Miguel, 2010).

Flemingia strobilifera (L.) W.T. Aiton (wild hops) which belongs to family Fabaceae is traditionally used by the Subanen tribe in the treatment or suppression of inflammation. Biochemical assessments reveal the presence of chalcone, flavonoid glycosides, aurone glycosides, epoxy chromenes, lipids, phenolic compounds, tannins and phytosterols in F. *strobilifera* (Madan *et al.*, 2008; Ghalot *et al.*, 2011; Ramcharan *et al.*, 2010). There have been several studies conducted on the roots, however, leaves are less studied. Hence, the present study aims to assess the antioxidant activity, total phenolics content, and the compounds present in the leaf extracts of F. *strobilifera* which may validate the traditional use of this plant as anti-inflammatory agent.

Materials and methods

Collection of Plant Material

The leaves of *F. strobilifera* were collected from the Municipality of Lapuyan, Zamboanga del Sur, Philippines (Fig. 1.).

Fig. 1. Map of the Municipality of Lapuyan, Zamboanga del Sur, Philippines (https://maps.google.com.ph/, 2015).

The leaves were washed properly under running tap water and distilled water, air dried, powdered, and stored in an airtight container.

Preparation of Extracts

Maceration

Fifteen grams of the dried and powdered leaves were soaked in 150 mL 80% methanol in a mechanical shaker at 100 rpm for seven days. The solution was filtered and the filtrates were collected and evaporated using a rotary evaporator at 50 °C. The crude extract obtained was then stored in a freezer (Amarowicz *et al.,* 2003).

Decoction

A 400 mL volume of distilled water was added to 15.0 g of powdered leaves of *F. strobilifera*. The mixture was boiled to obtain 100 mL. The decoction was filtered and the obtained filtrates were subjected to evaporation in a rotary evaporator at 50°C. The resultant extract was lyophilized to remove excess water then placed in air tight containers and stored in the freezer for further use.

DPPH radical scavenging activity of leaf extracts

Antioxidant activity of the *F. strobilifera* leaf extracts was quantified by measuring the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The DPPH (Sigma) scavenging activity of the extracts was determined using spectrophotometry which was adopted from the method of Perera *et al.* (2013).

Reaction mixture was prepared using 2.5mL of 6.5x10-5 M freshly prepared DPPH solution and 0.5 mL of extract dissolved in methanol. Extracts were tested in four concentrations (0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, and 2.0 mg/mL) with three replicates at room temperature in a dark condition. Absorbance was measured at 540 nm using UV-Vis spectrophotometer (SHIMADZU UV mini 1240). Methanol was used as control. Ascorbic acid was used as the reference standard. The percentage of DPPH radical scavenging activity was determined using the equation:

% scavenging activity= A_0-A_s/ A_0 x 100

Determination of Total Phenolics Content

Total phenolics content of the extracts was determined using the protocol of Formagio *et al.* (2014). An aliquot of 100µL of extract in methanol (1.0 mg/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 minutes with intermittent manual shaking. Total phenolics content is expressed as gallic acid equivalence (GAE) in mg gallic acid per gram of extract. The methanol solution served as blank. All assays were carried out in triplicate. The total phenolics content was determined from a standard calibration plot of absorbances of gallic acid measured at different concentrations (4.0 ug/mL, 8.0 ug/mL, 16.0 ug/mL, 32.0 ug/mL, 63.0 ug/mL).

GC-MS Analysis of leaf extract

For the preparation of extracts for the analysis, 1.0 mg of the crude extract was diluted with a mixture containing 0.5 mL absolute methanol and 0.5 mL dichloromethane to separate chlorophyll. The upper layer with no chlorophyll was transferred to another tube. A 10.0 µL was then taken and was further diluted with 1.0 mL chloroform. The GC-MS analysis was carried out using Agilent Technologies 7890A GC system coupled to 5975C Mass Selective detector, and driven by Agilent Chemstation software and with HP-5MS 30mx 0.25mm x 0.25 µm df capillary column. The carrier gas was ultra-pure helium at a flow rate of 1.0mL/ min. and a liner velocity of 37 cm/s. The temperature of the injector was set at 320°C. The instrument was set to an initial temperature of 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. An aliquot of 1 µL sample was injected in a split mode 100:1. The mass spectrometer was operated in the electron ionization mode at 70eV and electron multiplier voltage at 1859V. Other MS operating parameters were: ion source temperature 230°C, quadruple temperature °C, solvent delay 3 min and scan range 22-550 amu (automatic mass unit). 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. Total GC running time was 45 min (Chipiti *et al.,* 2015).

Statistical analysis

All experiments were carried out in triplicates. Data were expressed in Mean ± standard deviation. Statistical analysis was done with SPSS software (version 20.0). Difference at $p < 0.05$ was considered statistically significant.

Results and discussion

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of leaf extracts

The leaf extracts of *F. strobilifera* exhibited significant radical scavenging activity (Table 1).

Table 1. Average DPPH scavenging activity of hydroalcohol and aqueous leaf extracts of *F. strobilifera* at different concentrations.

Extracts	% Activity				
	0.25 mg/mL	0.5 mg/mL	1 mg/mL	2 mg/mL	IC_{50} mg/mL
80% methanol	42.00± 2.36	64.57± 0.53	73.09± 0.50	77.73± 1.25	0.299
aqueous	61.73± 1.54	67.16± 1.14	71.30± 1.67	74.28± 1.41	<0.25
Ascorbic acid*	65.17± 3.95	72.20± 0.39	73.39± 0.30	83.86± 1.32	<0.25

*standard.

The scavenging activity of the leaf extracts was dose-dependent wherein the % activity increased as the concentration of the leaf extract was increased. Both 80% methanol and aqueous leaf extracts exhibited high activity which is more or less comparable to the standard (ascorbic acid) wherein the aqueous extract has values ranging from 61.73± 1.54 to 74.28± 1.41 mg/mL and the methanol extracts with values ranging from 42.00± 2.36 to 77.73± 1.25 mg/mL. Moreover, the low IC50 value of the aqueous extract (<0.25 mg/mL) is the same with the standard (ascorbic acid) with IC50 value of 0.25 mg/mL. The IC50 of the methanol extract (0.299 mg/mL), however ,is slightly higher than the standard but is still considered low. According to Figueroa *et al.* (2014), low IC50 value denotes high ability of the extracts to act as DPPH scavenger. Thus, the results indicate that the 80% methanol and the aqueous leaf extracts of *F. strobilifera* are potent antioxidants.

Table 2. Total phenolic content of extracts expressed in mean gallic acid equivalence (GAE).

Sample	Extracts	GAE (mg/g extract)
F. strobilifera	80% methanol	12.49± 0.020
	aqueous	102.98± 0.003

Total Phenolics Content

In order to further confirm the antioxidant activity of the extracts of *F. strobilifera,* their total phenolics content was evaluated. Table 2 shows the total amount of phenolics of the two extracts expressed as Gallic Acid Equivalence (GAE). Apparently, the total phenolics content of the aqueous extract is higher than the 80% methanol extract with GAE values 102.98± 0.003 and 12.49± 0.020, respectively.

Phenols comprise the largest group of secondary metabolites that have been reported to have antioxidant properties which play an important role in lipid peroxidation and other biological activities (Sanadhya *et al.,* 2013). They are very important in plants because of their scavenging ability due to their hydroxyl groups. In addition, the antioxidant ability of phenols is due to their redox properties which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or

decomposing peroxides (Osawa, 1994).

As observed, there is a positive correlation between DPPH radical scavenging activity and the total phenolics content of aqueous leaf extracts of *F. strobilifera*. The aqueous extract exhibited higher radical scavenging activity and showed considerably higher amount of total phenolics than 80% methanolic extract. The result obtained is in contrast with the findings of Othman *et al.* (2014) that alcohol extracts exhibited higher total phenolics content compared to the water extracts.

Table 3. Compounds found in the 80% methanolic leaf extract of *F. strobilifera*.

Compound	Similarity Index (%)	Abundance (%)
Thiocyanic acid, ethyl ester	72	0.74
Limonene	60	0.41
Phenol, 3,5-bis (1,1-dimethylethyl)	74	0.92
Eicosane	78	0.73
Heptacosane	68	0.90
Hexanedioic acid, bis (2-ethylhexyl) ester	62	2.71
Hexacosane	94	3.50
Nonadecane	98	6.31

However, the finding in this study supports the work of Vuong *et al.* (2013) where water was shown to be an effective solvent for extraction of polyphenols from papaya leaves contributing to the high total phenollics content of the extract. On the other hand, there is a weak correlation between DPPH radical scavenging and total phenolics content of 80% methanol where the extract exhibits DPPH radical scavenging activity but was found to contain relatively low amount of phenolics. This might be due to the method used in the quantification of phenols. The Folin-Ciocalteu assay gives a crude estimate on the total phenolics content of an extract, whereas the DPPH free radical scavenging assay is not only specific to polyphenols (Prior *et al.,* 2005). In addition, Folin-Ciocalteu reagent measures not only the total phenolics content but also other oxidation substrates. The other oxidation substrates present in a given extract sample may interfere with the total phenolics measurement in an inhibitory, additive, or enhancing manner. The inhibitory effects could be due to the oxidants competing with Folin-Ciocalteu reagent. Moreover, various phenolic compounds respond differently in the DPPH assay, depending on the number of phenolic groups they have (Singleton and Rossi, 1965). Furthermore, Tahawa *et al.* (2007) suggested that negative correlation between antioxidant activity and total phenolics content might be due to the fact that total phenolics content does not necessarily incorporate all the antioxidants that may be present in an extract. These findings may help explain the negative correlation obtained between the DPPH scavenging activity and the total phenolics content of the 80% methanol leaf extract observed in the present study.

GC-MS Analysis of Leaf Extract

There were eight active compounds detected that could contribute to the medicinal property of the plant (Table 3). Among the eight compounds identified in this study, the major compounds are hydrocarbons nonadecane (6.31%), hexacosane (3.50%), and hexanedioic acid, bis (2-ethylhexyl) ester (2.71%) whereas thiocyanic acid, ethyl ester, limonene, phenol, 3, 5-bis (1, 1-dimethylethyl), eicosane, and heptacosane are present in lesser amount.

Although present in lesser quantity, the compounds limonene, eicosane, and hexanedioic acid, bis (2-ethylhexyl) ester might also contribute to the antioxidant capacity of the 80% methanol extract (Sermakanni and Thangapandian, 2012; Kazemi, 2015). Nonadecane is reported to have high

antioxidant activity (Dandekar *et al.,* 2015) and antidiabetic activity (Hamidi *et al.,* 2012). Hexacosane is found to have antimicrobial properties (Rukaiyat *et al.,* 2015). Eicosane is found to have antioxidant and antidiabetic action (Hamidi *et al.,* 2012; Chang *et al.,* 2004). Hexadecanoic acid, bis (2-ethylhexyl) ester is reported to have antioxidant activity (Sermakanni and Thangapandian, 2012).

Conclusion

The aqueous and hydroalcoholic leaf extracts of *F. strobilifera* showed significant radical scavenging activity which is comparable to the standard used. These extracts may serve as potential sources of natural antioxidants. Relatively, aqueous extract had high antioxidant activity and total phenolics content. The 80% methanol extract, however, exhibited high antioxidant activity but had low total phenolics content which might be due to the method of quantification used. Moreover, the active components detected might contribute to antioxidant activity of the leaf extracts, thus can be a potent anti-inflammatory agent.

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