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In-vitro antioxidant activities of the stem bark extract fractions of *Bridelia ferruginea*

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ABSTRACT
Bridelia ferruginea stem bark generally used in Indigenous folk medicine for diverse uses was evaluated scientifically to elucidate the antioxidant activity of various fractions *in-vitro* to validate its folkloric usage. *In vitro* antioxidant properties of the extract fractions were evaluated using the free radical scavenging activities by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) with ascorbic acid as control. The total antioxidant activity results indicated that, the inhibition percent of aqueous extract was significantly higher than the inhibition percent of ethanol and ethyl acetate in the DPPH methods. A higher IC_{50} (0.85 mg/ml compared to 1.34 mg/ml ethanolic extract and 1.41mg/ml of Ethyl acetate) value was observed. The aqueous extract was better in Fe^{2+}chelating activity and higher in the content of total phenol as compared to ethanolic and ethyl acetate extracts. However, the ethyl acetate extract had significantly higher ($P < 0.05$) hydroxyl radical scavenging activity with the concentration for 50% inhibition (IC) value of 0.86 mg/mL, 1.24 mg/mL for ethanolic extract and 2.16 mg/mL for aqueous extract. The results indicated that all the extracts showed excellent Nitric oxide scavenging activities with a close range of IC_{50} values of 0.83, 0.99 and 1.83 mg/mL for aqueous, ethanolic and ethyl acetate extract. The results suggest that *Bridelia ferruginea* stem bark possesses varied degrees of antioxidant activity *in vitro* and has the potential to be developed into dietary supplements and synergically modified with synthetic antioxidants.
Keywords: Ethanolic extract, Aqueous extract, Ethyl acetate extract, *Bridelia ferruginea,* and Antioxidant activity.

INTRODUCTION

Antioxidants have been found to play a major role in protecting the human body against damage induced by reactive free radicals (Halliwell and Gutteridge, 1990; Mates *et al*., 1999) by reacting with free radicals, chelating and also by acting as oxygen scavenger (Shahidi and Wanasundara, 1992; Buyukokuroglu *et al*., 2001). Antioxidant compounds play an important role in our body due to favorable effects on human health. Consumption of food containing phytochemicals with potential antioxidant properties can reduce the risk of human diseases (Temple, 2000) since Overproduction of free radicals in certain conditions can cause an imbalance, leading to oxidative damage to large biomolecules such as lipids, DNA, and proteins (Liu,2002). Natural antioxidants that are present in plants and herbs are responsible for inhibiting or preventing the deleterious consequences of oxidative stress since synthetic antioxidants have been limited because of their toxicity (Valentao *et al*., 2002).

Bridelia ferruginea belongs to the family Euphorbiaceae which is commonly found in Savannah regions (Ekanem *et al*., 2008). It is usually a gnarled shrub which sometimes reaches the size of a tree in suitable condition. Its common names are Kizni (Hausa), Marehi (Fulani), Iralodan (Yoruba), Ola (Igbo); and Kensange Abia (Boki). Its habitat is the Savannah, especially in the moister regions extending from Guinea to Zaire and Angola. The tree is 6 - 15 m high, up to 1.5 m in girth and bole crooked branching low down. The bark is dark grey, rough and often marked scaly (Rashid *et al*., 2000). A decoction of the leaves has been used to treat diabetes. It is also used as purgative and a vermifuge (Cimanga *et al*., 1999). The bark extract has been used for the coagulation of milk and also lime juice for the formulation of a traditional gargle "egun efu" (Orafidiya *et al*., 1990). It is also reported of having potential for water treatment (Kolawole and Olayemi, 2003). In Togo, the roots of the plant are used as chewing sticks and the root bark is used for intestinal and bladder disorder remedies as well as skin diseases (De Bruyne *et al*., 1997). Other reported activities of the bark extract include typanocidal (Iwu, 1984), molluscidal (Adeoye *et al*., 1988), antimicrobial (Olajide *et al*., 1999) and anti-inflammatory (Ndukwe *et al*., 2005). Antimicrobial properties of stem bark of B. ferruginea against facultative Gram negative rods have been reported by (Ndukwe *et al*., 2005). The plant was found to contain Alkaloids, Tannins, Terpenoids, Glycosides, Flavonoids, Saponins, Anthraquinones and Steroids. The activities of the methanol, petroleum ether and chloroform bark extracts of the B. ferruginea against some potential pathogenic organisms have been extensively investigated (Iwu, 1984); (Adeoye *et al*., 1988); (Olajide *et al*., 1999). *Bridelia ferruginea* has a great antioxidant potential which can be used to protect the body against damage caused by free radicals which is regularly produced in vivo and oxidative stress induce these free radicals (Oloyede and Babalola, 2012).

Due to the widespread consumption of *Bridelia ferruginea*, it's of great significance and necessity that research focuses on discovering potent natural and effective antioxidants to replace the synthetic ones.

MATERIALS AND METHODS

Plant

Fresh stem bark peelings of *Bridelia ferruginea* were collected at a farm in the suburbs of Ado Ekiti, Nigeria. The plant was identified and authenticated by a plant scientist in the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria and a voucher specimen was deposited accordingly at the herbarium of the Department of Plant Science, Ekiti State University, Ado-Ekiti, Nigeria.

Chemicals

Chemicals and reagents used such as 1,10-phenantroline, gallic acid, Folin-coicalteau's reagent were produced from Sigma-Aldrich, Inc(St Loius, MO), trichloroacetic acid (TCA), thiobarbituric acid (TBA) and ethylenediaminetetraacetic acid (EDTA) was sourced from Sigma-Aldrich, Cheme GmbH(Stein-heim, Germany), ditroniphenyl hydrazine (DNPH) from ACROS Organics (New Jersey,USA), hydrogen peroxide, methanol, acetic acid and $FeCl_3$ were sourced from BDH Chemicals Ltd.,(Poole, England), thiourea, $CuSO_4.5H_2O$, H_2SO_4, Sodium carbonate, $AlCl_3$, potassium acetate, Sodium Nitroprusside, Tris-Hcl buffer, $FeSO_4$, potassium ferricyanide and ferric chloride.

Extraction

Preparation of Ethanolic extract

The stem bark were air-dried in the laboratory at ambient temperature (30 ± 2°C) for 10 days, pulverized using a laboratory mechanical grinder (Christy and Norris limited, machine type 8) and the fine powders obtained stored until further use. 120g of the powdered sample was extracted with solvent combination (via maceration) of 70% ethanol for 48hrs. One litre of 70% ethanol was used. The mixture was decanted and filtered using sterile whatman paper No 1. The filterate measured up to 600mls and evaporated to dryness using a freeze dryer to obtain 40% yield ethanolic residue. The crude extract was later subjected to bioassay analyses.

From the stock solution, concentrations of 10mg/ml, 20, 40, 80, 100, and were obtained by serial dilution. These were stored until further use.

Preparation of Ethyl Acetate extract

The stem bark were air-dried in the laboratory at ambient temperature (30 ± 2°C) for 10 days, pulverized using a laboratory mechanical grinder (Christy and Norris limited, machine type 8) and the fine powders obtained stored until further use. 120g of the powdered sample was extracted with solvent combination (via maceration) of 70% ethanol for 48hrs. One litre of 70% ethanol was used. The mixture was decanted and filtered using sterile whatman paper No 1. The filterate measured up to 600mls and evaporated to dryness using a freeze dryer to obtain 40% yield ethanolic residue. 30g ethanolic extract was weighed and reconstituted in distilled water of 300mls. The mixture was divided into equal parts of 150mls each and to each portion, 75mls of Petroleum Ether is added. This is mixed thoroughly, turned into separating funnels (250mls size) and left to stand. The aqueous top layer is decanted and to each fraction, 100mls of Ethyl acetate is added to wash. After the first wash, the decanted aqueous fraction is washed again with 100mls of Ethyl acetate. This procedure is repeated for the second time and the total mixture measured up to 310mls and evaporated to dryness using a freeze dryer to obtain 4% yield. The crude extract was later subjected to bioassay analyses. From the stock solution, concentrations of 10mg/ml, 20, 40, 80, 100, were obtained by serial dilution. These were stored until further use.

Preparation of Aqueous extract

The stem bark were air-dried in the laboratory at ambient temperature (30 ± 2°C) for 10 days, pulverized using a laboratory mechanical grinder (Christy and Norris limited, machine type 8) and the fine powders obtained stored until further use. 50g of the powdered sample was extracted with distilled water of 500mls (via maceration) for 48hrs. The mixture was decanted and filtered using sterile whatman paper No 1. The filterate measured up to 425mls and evaporated to dryness using a freeze dryer to obtain 10% yield aqueous residue. The crude extract was later subjected to bioassay analyses. From the stock solution, concentrations of 10mg/ml, 20, 40, 80, 100, were obtained by serial dilution. These were stored until further use.

Experimental protocol

Determination of Total Phenolic Contents

Total phenolics were determined using Folin-Ciocalteu reagent (Single-ton and Slinkard 1977) Samples (200 m L) were introduced into test tubes, and then 1.0 mL Folin-Ciocalteu's reagent and 0.8 mL sodium carbonate (7.5%) were added. The absorbance of all samples was measured at 760 nm after incubating at 30C for 1.5 h. Results were expressed as milligram of gallic acid equivalent (GAE) per gram of fresh weight.

Fe+ 2 Chelating Assay

The Fe^{2+} chelating ability of the seed extracts was measured by the ferrous iron-ferrozine complex at 562 nm (Decker and Welch 1990). The reaction mixture containing $FeCl_2$(2 mmol/L) and ferrozine (5 mmol/L) along

with extracts (50–500 m g/mL) was adjusted to a total volume of 0.8 mL with d, mixed and incubated for 10 min at room temperature. The absorbance of the mixture was read at 562 nm against a blank. EDTA was used as positive control. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was given below with the formula:

$$\% \text{ Chelation activity} = ([A_o\text{-}A_1]/ A_o) \text{ X } 100$$

where $A_0$was the absorbance of the control and A_1 was the absorbance in the presence of the sample and standards.

DPPH Radical Scavenging Activity

The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable DPPH free radical, was determined by the method described by Hatano *et al.* (1988). An aliquot (0.5 mL) of the DPPH solution (50 mg/mL) was diluted in 4.5 mL of methanol, and 0.1 mL of the crude extracts at various concentrations (10–100mg/mL) was added. The mixture was shaken vigorously and allowed to stand for 45 min in the dark. The decrease in absorbance was measured at 517 nm against a blank (without extract) in a spectrophotometer. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and calculated using the following equation:

$$\% \text{ Radical scavenging activity} = ([A_o - A_1] / A_o) \times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample and standards.

Determination of NO radical scavenging ability

Sodium Nitropruside in aqueous solution at physiological pH 7.0 spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent [1.0mL sulfanilic acid reagent(0.33% prepared in 20% glacial acetic acid at room temperature for 5mins with 1mL of naphethylenediamine dichloride (0.1% w/v)]. Scavengers of NO compete with oxygen, leading to reduce production of NO. 5mM sodium nitroprusside in phosphate- saline was mixed with the extract, before incubation at 25^{o}C for 150min. Thereafter the reaction mixture was added to Greiss reagent. Before measuring the absorbance at 546nm, relative to the absorbance of standard solution of potassium nitrate treated in the same way with Greiss reagent. (Jagetia *et al*., 2004).

OH radical scavenging ability

The ability of the extract to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the method of Halliwell and Gutteridge (1992). Briefly, freshly prepared extract (0-100µl) was added to a reaction mixture containing 120µl, 20mM deoxyribose, 400µl, 0.1M phosphate buffer pH 7.4, 40µl, 20mM hydrogen peroxide and 40µl, 500µM FeSO4**,** and the volume was made to 800µl with distilled water. The reaction mixture was incubated at 37^{O}C for 30min and the reaction was stopped by the addition of 0.5ml of 2.8% TCA, this was followed by the addition of 0.4ml of 0.6% TBA solution. The tubes were subsequently incubated in boiling water for 20min. The absorbance was measured at 532nm in spectrophotometer.

Statistical analysis: Data were expressed as Mean ± SE of duplicate measurements with one-way Analysis of Variance (ANOVA) Analysis of Variance (ANOVA) with the help of software SPSS 16.0 for windows. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

DPPH ASSAY

FIG 1 below shows the radical scavenging activity of the ethanolic, aqueous and ethyl acetate extracts of *B.ferruginea* tested against DPPH. The extract exhibited a strong antioxidant activity in the order of decreasing magnitude; Aqueous> Ethanolic > Ethyl Acetate. (0.85, 1.34, and 1.41) mg/ml were obtained as the IC_{50} values of the extracts, hence conferring greatest potency on the Aqueous extract and also showed its ability to quench the stable DPPH radical.

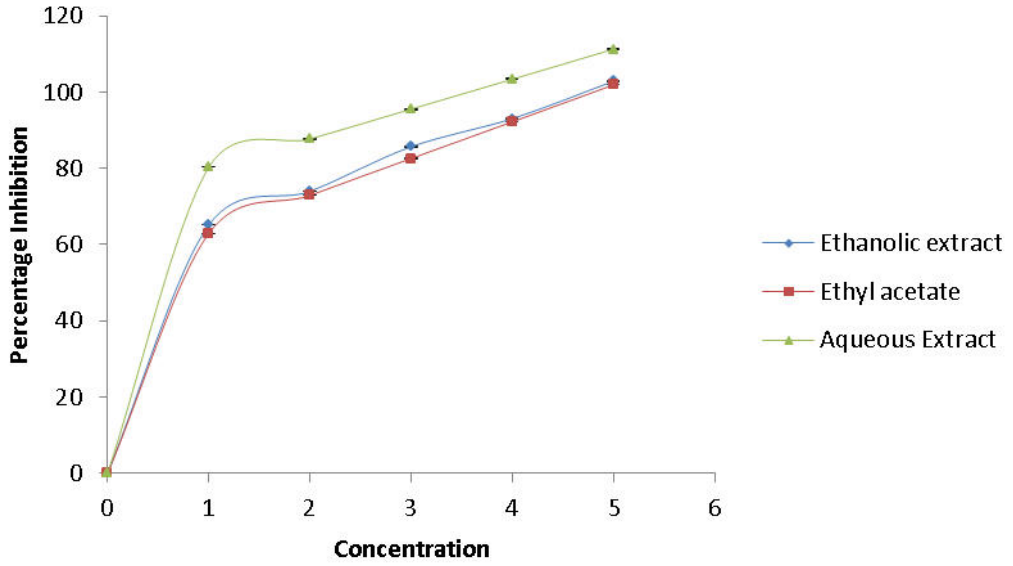

FIG 1: DPPH radical scavenging activities of various *B. ferruginea* extracts

IRON CHELATION

Metal ions play an important role in the acceleration of oxidation of important biological molecules, for instance they may catalyze the formation of first few radicals that can lead to propagation of the radical chain reaction in lipid peroxidation (Gordon 1990). **FIG 2** below shows the Iron chelating ability of the ethanolic, aqueous and ethyl acetate extracts of *B.ferruginea*. The extract exhibited a strong activity in the order of Increasing magnitude; Ethyl Acetate < Ethanolic < Aqueous. 2.92mg/ml, 1.93mg/ml, and 1.72mg/ml were obtained as the IC_{50} values of the extracts, hence conferring greatest potency on the Aqueous extract and also showed its ability to Chelate metals by forming a complex with Iron. Chelating agents inhibit the radical mediated oxidative chain reactions in biological or food systems, and consequently improve human health, and food quality, stability and safety. In addition, plant phenolic compounds have also been found to be good metal ion chelators (Van acker *et al.* 1996).

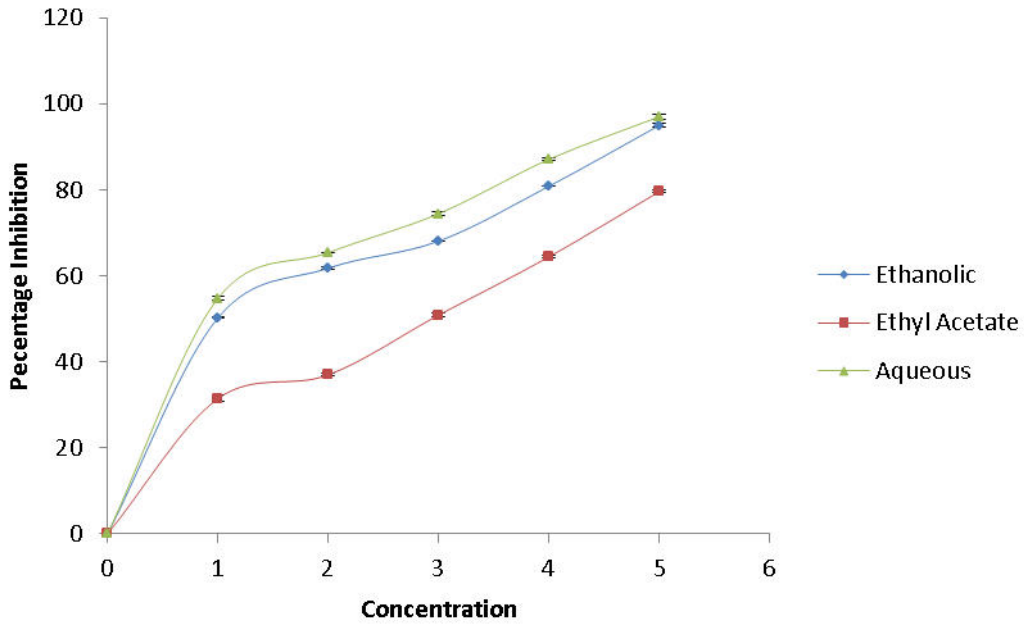

FIG 2: Iron chelating abilities of various *B. ferruginea* extracts

OH RADICAL

FIG 3 below shows the radical scavenging activity ethanolic, aqueous and ethyl acetate extracts of *B.ferruginea* against OH radical. The extract exhibited a strong activity in the order of Increasing magnitude; Aqueous < Ethanolic <. Ethyl Acetate. Oxidative damage to lipids and DNA is of particular significance in carcinogenesis. *B.ferruginea* with its antioxidant activity was expected to protect against OH mediated damage of deoxyribose. The extracts afforded significant protection (p<0.05) against both iron and hydrogen peroxide induced damage of deoxyribose with Ethyl acetate being the most potent.

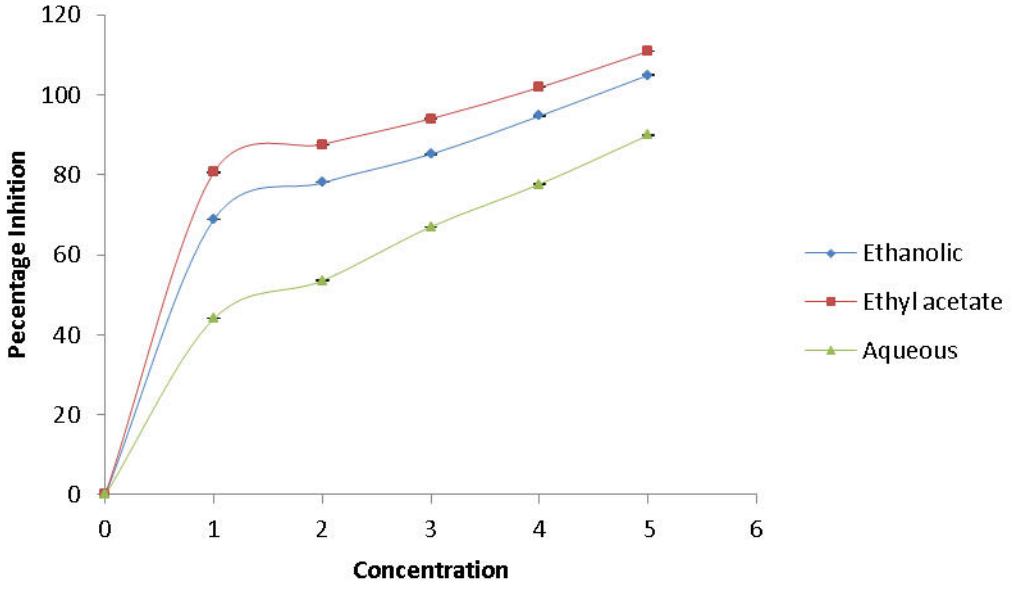

FIG 3: OH radical scavenging activities of *B. ferruginea* extracts

NO ASSAY

Nitric oxide is a very unstable species under the aerobic condition. It reacts with O_2 to produce the stable product nitrates and nitrite through intermediates through NO_2, N_2O_4 and N_3O_4. It is estimated by using the Griess reagent. In the presence of test compound, which is a scavenger, the amount of nitrous acid will decrease. The percentage inhibition of aqueous extract showed an IC_{50} value of 0.83mg/ml which is the most potent while the ethanolic extract (0.99mg/ml) and ethyl acetate (1.03mg/ml) followed the ranks in that order as the least potent. It was observed that the differences were within a particular range (**FIG 4**)

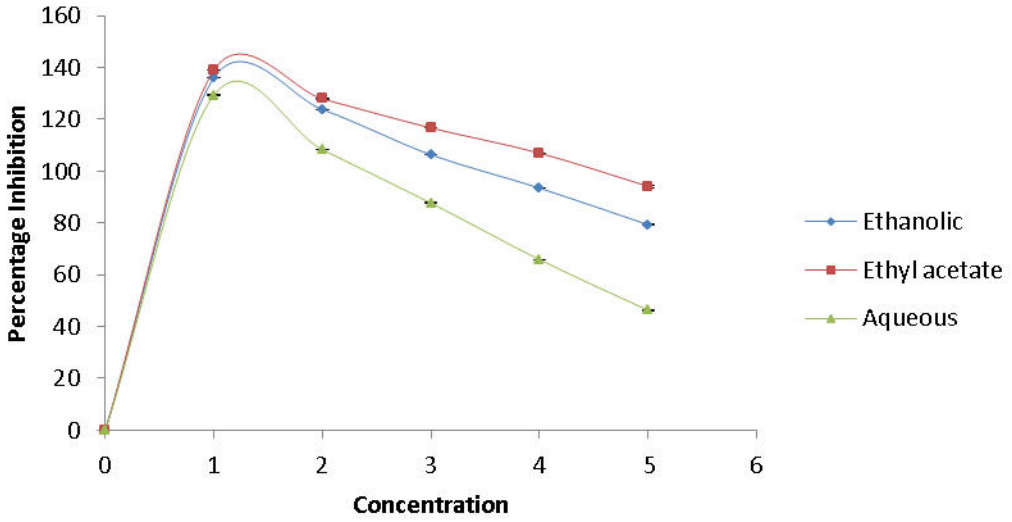

FIG 4: NO scavenging activities of various *B. ferruginea* extracts

Table 1. Phenolic Contents of the Ethanolic, Ethyl Acetate and Aqueous extracts of *Bridelia ferruginea* (BF) stem bark.

	Ethanol	Ethyl acetate	Aqueous
	Total phenolic of extracts (mg GAE/100g)	Total phenolic of extracts (mg GAE/100g)	Total phenolic of extracts (mg GAE/100g)
1(10mg/ml)	0.13 ± 0.02	3.87 ± 0.41	5.19 ± 0.10
2(20mg/ml)	0.16 ± 0.00	3.66 ± 0.05	6.10 ± 0.13
3(40mg/ml)	0.17 ± 0.00	3.29 ± 0.14	6.30 ± 0.00
4(80mg/ml)	0.20 ± 0.01	2.89 ± 0.16	6.58 ± 0.18
5(100mg/ml)	0.21 ± 0.01	2.28 ± 0.01	7.67 ± 0.04

Results are expressed as means of duplicates ± standard deviation

The phenolic contents in different extracts varied significantly based on increasing concentrations. Increasing phenolic content was obtained with increasing concentration of the ethanol, ethyl acetate and water extracts. The aqueous extract had the highest ($P < 0.05$) phenolic content, followed by the ethyl acetate and ethanolic extracts (Table 7). The results indicate that the amounts of polyphenolics (mg GAE/100g) increased with an increased concentration from 10-100mg/ml in all extracts. The high phenolic content of the ethylacetate fraction is in accord with Bhandari *et al.,* 2008 and Shobana *et al.,* 2009. The correlation between phenol content and antioxidant activity were also reported by Batomayena *et al.,* 2012.

CONCLUSION

In this study, the highest antioxidant activity, DPPH scavenging activity, metal chelating activity, Hydroxyl radical scavenging activity, Nitric oxide assay and the content of total phenol were obtained in the aqueous extract. On the other hand, the antioxidant activities in the ethanolic extract was quite competing in the overall result, although all the extracts of this plant contain varying degrees of antioxidants. *Bridelia ferruginea* can thus be suggested to have the potential to be developed into dietary supplements and synergically modified with synthetic antioxidants.

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