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Full Length Research Paper

Phytochemicals and *in vitro* antioxidant potentials of defatted methanolic extract of *Holarrhena floribunda* leaves

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Plant-based dietary components and additives are known to protect cells from deleterious effect of reactive oxygen species (ROS). Proximate, phytochemical and antioxidant potentials of methanolic extract of defatted *Holarrhena floribunda* (G.Don) leaves were assessed using *in vitro* systems such as, 1,1 diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, nitric oxide radical scavenging and inhibition of lipid peroxidation. Total antioxidant activity was measured using phosphomolybdenum method. Total phenol content and the reductive potential of the extract were also evaluated. The results of the proximate analysis revealed that the leaf contained 0.23% moisture, 12.8% ash, 9.62% crude fat, 23.3% crude fibre, 21.17% protein and 32.68% carbohydrate while the phytochemical constituents included alkaloid, saponin, tannin and cardiac glycosides. The leaf extract of *H. floribunda* (G. Don) exhibited scavenging activity with IC_{50} of 12.63, 1,377.00 and 244.00 µg/ml for DPPH, hydroxyl radical and nitric oxide radical respectively. The extract conferred 50% protection at the concentration of 73.80 µg/ml on lipid peroxidation induced by $FeSO_4$ in egg yolk. Total antioxidant capacity equivalent of gallic acid and vitamin C were 195.57 and 519.28 µg/mg of extract respectively and total phenol content equivalent of gallic acid was 1427.87 µg/mg. The reductive potential increased with increasing concentration of extract. The results obtained from this study reveal that the extract is rich in antioxidant components with several mechanisms of eliciting antioxidant actions which provide scientific basis for its use in folk medicine.

Key words: Reactive oxygen species, *Holarrhena floribunda*, antioxidant, phytochemical.

INTRODUCTION

Generally, human beings live in a highly oxidative environment and many processes involved in metabolism may result in the production of more oxidants (Rui and Boyer, 2004). It has been estimated that there are more than ten thousand oxidative hits to DNA per cell per day in humans (Ames et al, 1985). For protection, against free radicals, organisms are endowed with endogenous (catalase, superoxide dismutase, glutathione peroxidase/reductase) and exogenous (vitamins C and E, β – carotene, uric acid) defense systems. However, these systems are not sufficient in situations (oxidative stress, contamination, uv-exposure etc) where the production in free radicals significantly increases (Mondon et al, 1999).

Most of the protective effects of plants on living cells have been attributed to their non-nutrient constituents such as carotenoid, flavonoids, isoflavonoid and phenolic acids. These findings have led to an increased emphasis on cancer prevention strategies in which these dietary factors are utilized (Keith, 2000). Different phytochemicals have been shown to possess a range of activities, which may help in protecting against chronic diseases like cancer, regulate inflammatory and immune response and protect against lipid oxidation (Hollman and Katan 1997; Liu, 2003).

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Holarrhena floribunda is a tree of 17 m high by 1 m girth of deciduous forest and savanna woodland. It is commonly found in Senegal, Nigeria and some parts of West Africa. The tree yields a large number of alkaloid. Total alkaloids are greatest in the roots (2%), decreasing in the stem (1 – 1.5%) and least in the leaves (< 1.0%) (Kerharo and Adams, 1974). The leaves of this plant have been employed as an anti-malarial (Banquet and Debray, 1974). In Ivory Coast, the leaves and the bark are used for treating diarrhea and the leaves alone for amenorrhea and management of diabetes (Kerharo and Adams, 1974).

Even though the leaves of this plant have been employed as concoctions in treating various ailments in folk medicine, there is paucity of information as to the phytochemicals contained in the plant and therefore significance to the antioxidant activities. This study, however, sought to evaluate the phytochemical constituents of *H. floribunda* leaves extract and its antioxidant potential using different *in vitro* antioxidant assays.

EXPERIMENTALS

Materials

1,1-Diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid, sodium nitroprusside, sodium dodecyl sulphate and sulphanilic acid were purchased from Sigma Co. (St. Louis, USA), Folin-Ciocalteu, 2-deoxy-D-ribose, ascorbic acid, gallic acid, ammonium molybdate, trichloroacetic acid, potassium ferricyanide, 95% methanol and N-hexane from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals used were of analytical grade.

Collection of plant material

H. floribunda leaves were collected at University of Ibadan, Ibadan and authenticated by Dr. Ayodele of Department of Botany, University of Ibadan, Ibadan.

Preparation of extract

The leaves were air- dried in ambient temperature. 500 g of the dry leaves were soaked in 1.5 L N-Hexane to remove fat (defat) for 3 h. The defatted plant sample were re-soaked in 1.5 L 70% methanol for a week and then filtered using whatman (No 1) filter paper. The filtrate was lyophilized at the Obafemi Awolowo University Central laboratory.

Proximate analysis

Crude protein, fat, crude fiber and total ash contents in the dried leaves were determined using the methods described by Association of Official Analytical Chemists (AOAC, 1990). Carbohydrate (nitrogen free extract) was determined by difference; that is, the sum of the crude protein, fat, crude fibre and total ash deducted from 100.

Phytochemical analysis

Mayer, Dragendoff, Wagner and picric reagents were used to test for Alkaloid. Frothing test for saponin, ferric chloride test for tannin while Salkowski test for cardiac glycosides according to Adeoye and Oyedapo (2004).

DPPH radical assay

The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method of Braca et al. (2001). Defatted methanolic extract (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percent inhibition activity was calculated as [(Ao – Ae)/Ao] x 100, where Ao = absorbance without extract; and Ae = absorbance with extract.

Lipid peroxidation and thiobarbituric acid reactions

A modified thiobarbituric acid reactive species (TBARS) assay (Ohkowa et al., 1979) was used to measure the lipid peroxide formed using egg yolk homogenate as lipid rich media (Ruberto et al., 2000). Egg homogenate (0.5 ml of 10%, v/v) and 0.1 ml of extract were added to a test tube and made up to 1 ml with distilled water; 0.05 ml of FeSO4 (0.07 M) was added to induce lipid peroxidation and the mixture incubated for 30 min. Then, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate were added and the resulting mixture was vortexed and then heated at 95°C for 60 min. After cooling, 5.0 ml of butan-1-ol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation percent by the extract was calculated as [(1-E)/C] x 100 Where C is the absorbance value of the fully oxidized control and E is the absorbance in presence of extract.

Hydroxyl radical scavenging assay

The assay was performed as described by Halliwell et al. (1987) with some modifications. All solutions were prepared fresh essentially. 1.0 ml of the reaction mixture contained 28 mM 2-deoxy-2-ribose (dissolved in phosphate buffer, pH 7.4), 500 µl of various concentrations of *H. floribunda* (1,400 - 2,600 µg/ml), 200 µm $Fecl_3$ and 1.04 mM EDTA (1:1 v/v), 1.0 mM H_2O_2 and 1.0 mM ascorbic acid. After incubation period of 1 h at 37°C the extent of deoxyribose degradation was measured by the TBA reaction (Ohkowa et al., 1979).

Nitric oxide radical inhibition activity

Nitric oxide, generated from sodium nitroprusside in aqeous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Marcocci et al., 1994; Green et al., 1982). The reaction mixture (3 ml) containing sodium nitroppruside (10 mM) in phosphate buffer saline (PBS) and the extract from (100 - 1000) µg/ml was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H_3PO_4 and 0.1% (w/v) naphthylethylene diamine hydrochloride) was added. The absorbance of the chromophore formed was measured at 546 nm.

Determination of total antioxidant capacity

The assay is based on the reduction of molybnedum (VI) to molybnedum (V) by the extract and the subsequent formation of a green phosphate Mo (V) complex at acid pH (Priesto et al., 1999).

Extract 0.3 ml was combined with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 min and cooled down to room temperature. The absorbance was measured

at 695 nm against reagent blank. The antioxidant activity was expressed as the number of equivalent of ascorbic acid and gallic acid.

Determination of total phenol

The total phenol content was determined by mixing 0.5 ml of the extract with 2.5 ml 10% Folin-ciocateus reagent (v/v) and 2.0 ml of 7.5% sodium carbonate was added after 3 min. The reaction mixture was incubated at 45°C for 40 min and the absorbance was measured at 765 nm with gallic acid as standard (Singleton et al., 1999).

Reducing power

The reducing power of *H. floribunda* extract was determined according to the method of Oyaizu (1986). Different concentrations of the extract (100 - 1000 µg) in 1 ml of distilled water was mixed with phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 min. 10% (w/v) trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.1% $FeCl_3$ (0.5 ml) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated reducing power. Gallic acid was used as a standard.

Statistical analysis

All the experimental results were mean ± S.D of three parallel measurements. Regression curve analysis was used to evaluate IC_{50} values using Graphpad prism version 5.02 statistical software.

RESULTS AND DISCUSSION

Evaluation of the antioxidant property of extracts of plants used in folk medicine is necessary and important. This is partly due to renewed interests in the use of concoctions and/or decocotions made from these plants in alternative medicine and to scientific evidences implicating positive roles of the antioxidant components of these plants being protective against reactive oxygen species related to pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity. Many synthetic antioxidant compounds have been demonstrated to exhibit toxic and /or mutagenic effects, hence attention is now being shifted toward the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidants activity (Aruoma and Cuppett, 1997). Among these are flavonoids and other phenolic compounds of plant origin have been reported as scavengers and inhibitors of lipid peroxidation (Formica and Regelson, 1995).

The results of the proximate analysis showed that the leaf extract contained 0.23% moisture, 12.8% ash, 9.62% crude fat, 23.3% crude fibre, 21.17% protein and 32.68% carbohydrate (32.68 %) (Figure 1). These values differ from those of *Telfaria occidentalils, Manihot esculentum* reported by Taiga et al. (2008) and *Ceratotheca sesamoides* (Fasakin, 2004) in that the leaves of *H. floribunda* contain appreciable levels of crude fibre, protein and carbohydrate (Figure 1).

The phytochemical constituents of the leaf of *H. floribunda* include alkaloid, saponin, tannin and cardiac glycosides as revealed by the positive reactions of these molecules (Table 1). The presence of these compounds explains the ability of the leaf extract to inhibit the activities of various radicals.

DPPH free radical scavenging method has been widely applied for evaluating antioxidant activity in a number of studies (Brad-Williams et al., 1995). The method relies on the reaction of DPPH that is characterized as a stable free radical with deep violent colour and any substance that can donate hydrogen atom to DPPH thus reduces it to become stable diamagnetic molecule. The colour changes from purple to yellow and its absorbance at wavelength 517 nm decreases. The methanolic extract of defatted *H. floribunda* leaves inhibited DPPH free radical in a dose dependent manner with IC_{50} values of 12.63 µg/ml compared with vitamin C 6.53 µg/ml (Table 3a). Kamlesh et al. (2007) reported IC_{50} values of *Sida rhombifolia* for root, leaf, whole plant and stem extracts to be 546.1, 852.8, 983.8 and 1,222.5 µg/ml respectively. Likewise, *Calendula officinalis* flower extract was reported to have IC_{50} value of 100 µg/ml (Preethi et al., 2006), while De and Dasgupta (2006) reported different IC_{50} values for eleven leafy vegetables of India origin. The previously reported DPPH scavenging abilities were lower than that obtained for the methanolic leaf extract of *H. floribunda*.

Egg yolk lipids undergo rapid non enzymatic peroxidation when incubated in the presence of ferrous sulphate with subsequent formation of malonodialdehyde (MDA) and other aldehydes that form pink chromogen with TBA absorbing at 532 nm (Kosugi et al., 1987). Peroxidation of lipids has been shown to be cumulative effect of reactive oxygen species, which disturb the assembly of the membrane causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes (Nigam and Schewe, 2000). The extract exhibited strong lipid peroxidation inhibition in a concentration dependent manner (Table 3b). The IC_{50} value for the inhibition of lipid peroxidation is 73.80µg/ml. This activity is higher than that of red pepper [*Capsicum annuum var. aviculare* (Tepin)] reported by Oboh and Rocha (2007) using brain and liver as media of peroxidation. There is no significant difference in the IC_{50} values of three varieties of aqueous leaf extract of *Piper betle* leaf except Kauri with 14.8 µg/ml using egg yolk medium as reported by Dasgupta and De (2003). This result suggests that *H. floribunda* leaf extract could play a role in protecting the physicochemical properties of mem-

brane bilayers from free radical induced severe cellular dysfunction.

Hydroxyl radical is the most reactive among reactive oxygen species (ROS); it has the shortest half life

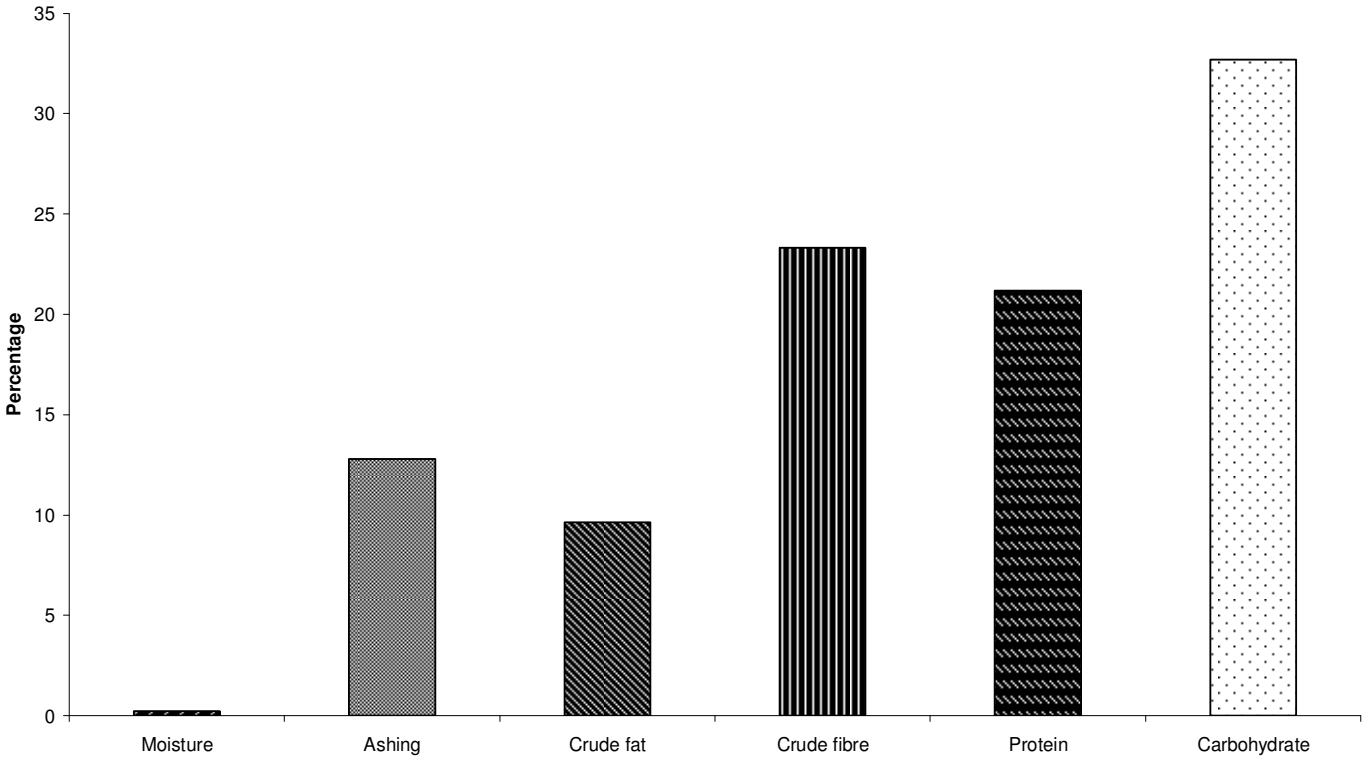

Figure 1. Proxoimate analysis of *H. floribunda* leaves.

Table 1. Phytochemical screening of *H. floribunda* leaves.

Test	Extract	Inference
Alkaloid	Positive to Mayer, Dragendoff Wagner and Picric reagents	Alkaloid
Froth	Positive to Froth	Saponin
Salkowski	Reddish-brown colour at the interference	Cardiac Glycoside
Tannins	Turned blue-black precipitate on addition of $FeCl_3$	Tannin

Table 2. Total antioxidant capacity and total phenol in *H. floribunda* leaves.

parameter	Equivalent to ascorbic acid µg/mg plant material	Equivalent to gallic acid µg/mg plant material
Total antioxidant capacity	519.28	195.57
Total phenol	ND	1427.87

ND: not determined.

Table 3a. DPPH radical scavenging activity of defatted methanolic extract of *H. floribunda* leaves.

Sample	Concentration (µg/ml)	% Inhibition ± SD (n = 3)	Regression equation	IC_{50} (µg/ml)
Extract	15.63	48.08 ± 0.41	Y = 0.913x + 38.47	12.63
	31.25	73.99 ± 0.73	(r^2 =0.92)	
	62.25	92.94 ± 0.31		
Vitamin C	03.50	27.08 ± 0.96	Y =7.79x – 0.845	6.53

	04.50 06.50	33.24 ± 0.04 50.13 ± 0.41	(r^2 =0.99)	

Table 3b. Prevention of lipid peroxidation by defatted methanolic extract of *Holarrhena floribunda* leaves.

Sample	Concentration (µg/ml)	% Inhibition ± SD (n = 3)	Regression equation	IC_{50} (µg/ml)
Extract	24.00 48.00 95.00	57.47 ± 0.55 73.34 ± 0.42 99.97 ± 1.08	Y = 0.9x + 20.73 (r^2 = 0.8256)	73.80

Table 3c. Inhibition of nitric oxide radical by defatted methanolic extract of *H. floribunda* leaves.

Sample	Concentration (µg/ml)	% Inhibition ± SD (n = 3)	Regression equation	IC_{50} (µg/ml)
Extract	500.00 600.00 1000.00	23.48 ± 0.06 52.01 ± 0.04 65.17 ± 0.03	Y = 0.28x – 18.21 (r^2 = 0.9996)	244

Table 3d. Hydroxyl radical scavenging activity of defatted methanolic extract of *H. floribunda* leaves.

Sample	Concentration (µg/ml)	% Inhibition ± SD (n = 3)	Regression equation	IC_{50} (µg/ml)
Extract	1,200.00 1,300.00 1,500.00	38.97 ± 0.05 41.68 ± 0.70 59.57 ± 0.85	Y = 0.07x – 48.77 (r^2 = 0.9557)	1377
Vitamin C	10.00 20.00 30.00	40.16 ± 0.21 48.32 ± 0.32 56.38 ± 0.43	Y = 0.81x + 32.40 (r^2 = 0.9944)	22.10

compared with others and is considered to be responsible for much of the biological damage in free radical pathology (Mahinda et al., 2006). The radical has the capacity to cause strand breakage in DNA, which contributes to carcinogenesis, mutagenesis and cytotoxicity (Hochestein and Atallah, 1988). The deoxyribose method is a com-mon method for determining the rate constant of hydroxyl radical reactions (Gutteridge and Halliwell, 1988). Ferric-EDTA incubated with H_2O_2 and ascorbic acid at pH 7.4 produces hydroxyl radicals in free solution and can be detected by their ability to degrade 2-deoxy-2-ribose into fragments on heating with TBA at low pH to form a pink chromogen (Halliwell et al., 1987). Nevertheless, if a hydroxyl radical scavenger is added to the reaction mixture, it will compete with deoxyribose for the avail-ability of hydroxyl radicals, thus reducing the amount of MDA formation (Roberto et al., 2007). The *H. floribunda* leaf extract prevented the degradation of 2-deoxy-2-ribose with IC_{50} of 1,377.00 µg/ml compared with 22.10 µg/ml for vitamin C as standard (Table 3d). There is no significant difference in the activity exhibited by the extract compared to the activity of C*yperus rotundus* reported by Yazdanparast and Ardestani (2007) and is similar to the value reported for water extract of *Propolis* (Nagai et al., 2003). Therefore, methanolic leaf extract of *H. floribunda* has the potential to scavenge hydroxyl radicals at the stage of initiation and protect the biomembrane from H^+ abstraction that might lead to lipid peroxidation.

Nitric oxide (NO) is an essential molecule required for several physiological processes like neural signal transmission, immune response, vasodilation and control of blood pressure (Wink et al., 1991). However, high concentration of NO may result in several pathological conditions including cancer (Ganesh and Manjeshwar, 2004). NO molecule in an aerobic condition reacts with oxygen to produce intermediates such as NO_2, N_20_4, N_3O_4, the stable products nitrate and nitrite (Lala, 1998) and peroxynitrite when reacted with superoxide (Marcocci et al., 1994). These products are highly genotoxic; causing deamination of guanine, cytosine, adenine and several other enzyme impairments like DNA ligase and DNA alkyltransferase.

Sodium nitroprusside in aqueous solution at physiological pH generates nitric oxide, which interacts with oxygen to produce nitrate ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitric oxide. The leaf extract in this study showed significant inhibition of NO with IC_{50} value of 244.00 µg/ml Table 3c. The activity exhibited by the extract is significantly higher than those previously reported for some extracts

(Kamlesh et al., 2007; Preethi et al., 2006; Roberto et al., 2007).

Total antioxidant capacity of *H. floribunda* leaf extract is expressed as the number of equivalents of ascorbic acid and gallic acid. The phosphomolybdenum method is quantitative since the antioxidant activity is expressed as

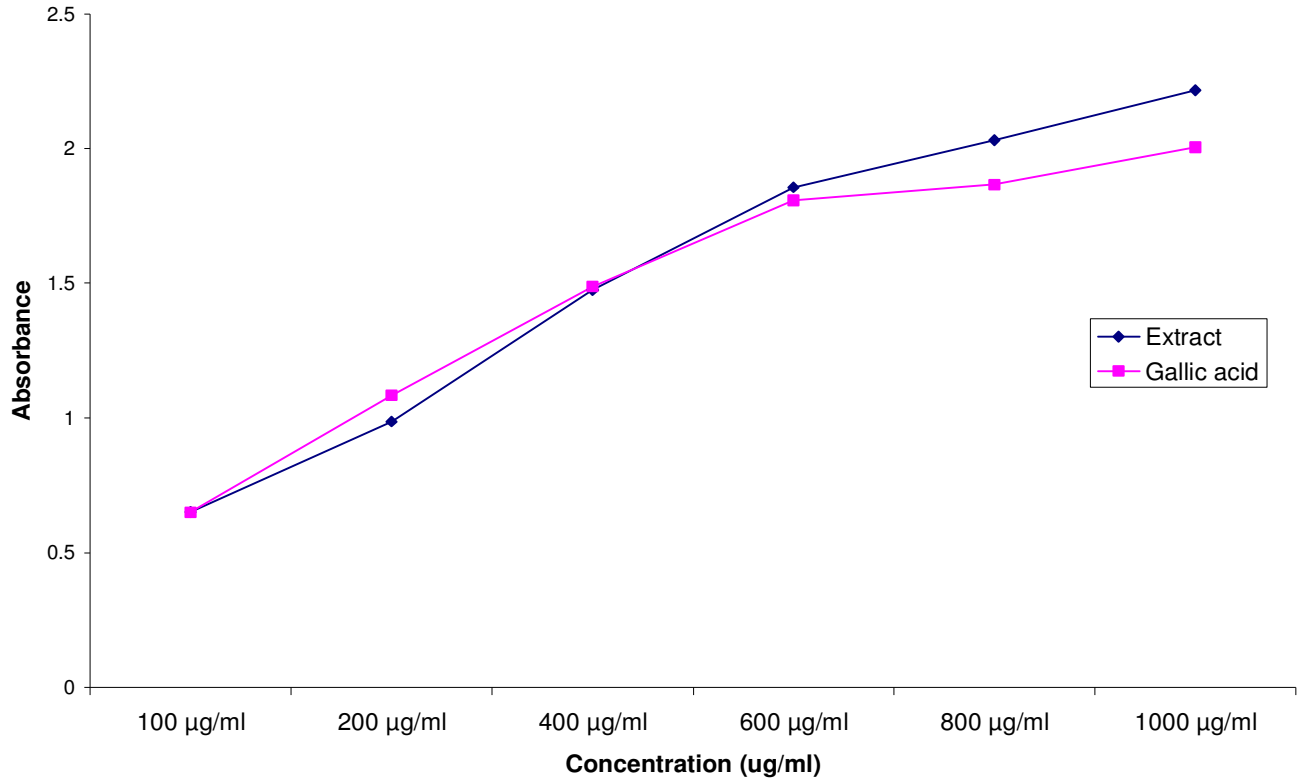

Figure 2. Reducing power of the *H. floribunda* leaves extract and gallic acid.

the number of equivalents of ascorbic acid (Singleton et al., 1999). The method is based on the reduction of Mo (VI) to Mo (V) by the extract at the acid pH. Total antioxidant equivalent to gallic acid is 195.57 µg/ mg while vitamin C is 519.28 µg/ mg (Table 2).

Plant phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activities (Lopez-velez et al., 2003) and are believed to prevent many degenerative diseases including cancer and atherosclerosis (Roginsky, 2003). Total phenol content of the leaf extract expressed as gallic acid equivalent is 1427.87 µg/ mg. The higher antioxidant activities, however, exhibited by the extract of *H. floribunda* however, might not be unconnected with the presence of phenolic compound.

The reducing ability of a compound generally depends on the presence of reductones (Pin-Der-Duh, 1998), which exhibit antioxidative potential by donating a hydrogen atom for breaking the free radical chain (Gordon, 1990). The methanolic leaf extract of *H. floribunda* leaves confirmed the presence of reductones with increase in the absorbance as the amount of the extract increases (Figure 2). There is no significant difference in the level of reducing ability between the extract and gallic acid as standard within the concentrations used. The extract, however, showed significantly higher reducing potential than reported activities of different extracts of *Sargassum siliquastrum* (Sun-hee et al., 2007).

In conclusion, the results of this study suggest that the defatted methanolic leaf extract of *H. floribunda* has potential as an antioxidant agent. Further investigation on the isolation and characterization of the antioxidant components is however required.

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