Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(12); 1951-1958

ISSN: 0975-4873

Research Article

In Vitro Antioxidant Activity and High Performance Liquid Chromatography fingerprint of *Adansonia digitata* Stem Bark Extracts

Adeoye Akinwunmi O[1,3*], Boligon Aline A[2], Athayde Margareth L[2], Bewaji Clement O[3]

[1]*Department of Biochemistry, Federal University Oye-Ekiti, Ekiti State, Nigeria.*
[2]*Department of Industrial Pharmacy, Federal University of Santa Maria, Build 26, room 1115, Santa Maria, CEP 97105-900, Brazil.*
[3]*Department of Biochemistry University of Ilorin, Kwara State, Nigeria.*

Available Online: 15th December, 2016

ABSTRACT

Background: Medicinal plants have been utilized to treat acute and chronic disorders for many years. The basis for such utilization however is based on the potential use of phytochemicals to manage a plethora of chronic diseases including cancer, inflammatory diseases and cardiovascular abnormalities. This study investigated the flavonoid, total phenolic content, and antioxidant potentials of aqueous and methanolic extracts of *Adansonia digitata* stem bark. Methods: High Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) was used to quantify the phenolic acids and flavonoids present in *Adansonia digitata* stem bark extracts. Phytochemical composition of the extract was evaluated and standard protocols were used to estimate the total phenols and total flavonoid content of the extract. DPPH (2, 2-diphenyl -1-picrylhydrazyl), hydroxyl radical (OH^-) radicals scavenging activity, and the ability of the extracts to chelate ferrous ions as well its reductive potential was also evaluated. Results: The presence of saponins, tannins, alkaloids, flavonoids, terpenoids and cardiac glycosides were confirmed in the extracts. The methanolic extract showed highest phenolics and flavonoids content with 2.15 ± 0.01µg/ml of tannic acid equivalent and 4.68 ± 0.00 µg/ml of quercetin equivalent respectively. Aqueous extract had the lowest IC_{50} value 20.45, for hydroxyl (OH) radical scavenging activity and the highest FRAP value. The IC_{50} values of DPPH radical scavenging activities are 18.22 and 14.62 and ferrous-ion chelating activities are 38.75 and 28.95 for aqueous and methanolic extract respectively. The difference in DPPH, ferrous-ion and hydroxyl radical scavenging activities of aqueous and methanolic extract were significant. Methanolic extract had the highest antioxidant activities. The HPLC-DAD analysis showed significant levels of flavonoids and phenolic acids. Conclusion: The free radical scavenging potentials of this plant probably contribute to the effectiveness of the plant as a medicinal plant. The secondary metabolites in the plant have been found to be of medicinal importance both in preventive and curative medicine.

Keywords: High performance liquid chromatography, *Adansonia digitata*, antioxidant, flavonoids, phenolics.

INTRODUCTION

The oxygen consumption inherent in cells growth leads to the generation of series of oxygen free radicals. Highly active free radicals and their uncontrolled production are responsible for numerous pathological processes such as cell tumour and coronary heart diseases[1,2,3]. Oxidative stress plays a major role in the development of chronic and degenerative diseases such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases. Generation of highly reactive oxygen species (ROS) is an integral feature of normal cellular function[4]. Damage due to free radicals caused by ROS leads to several damaging effects as they can attack lipids, protein/enzymes, carbohydrates, and DNA in cells and tissues. They induce undesirable oxidation, causing membrane damage, protein modification, DNA damage, and cell death induced by DNA fragmentation and lipid peroxidation[4]. Antioxidants have been known to play protective role in human body against deleterious effects of reactive free radicals. They can significantly delay or prevent the oxidation of easily oxidizable substances[5,6]. Natural antioxidants are classified according to their mechanism of action as chain-breaking antioxidants which scavenge free radicals or inhibit the initiation step or interrupt the propagation step of oxidation of lipid and as preventive antioxidants which slow the rate of oxidation by several actions but do not convert free radicals[7,8, 9,10,11]. Phenolic compounds are secondary metabolites of plants that also act as antioxidants by many potential pathways such as free radical-scavenging, oxygen radical absorbance, and chelating of metal ions[12]. They are naturally present in fruits and vegetables and also used as supplements or medicines. Research has shown that fruits and vegetables contain other antioxidant nutrients, in addition to vitamins C and E, and carotenoids, which significantly contribute to their total antioxidant capacity[13,14]. The major part of those antioxidant nutrients is polyphenolic compounds, which are components of

**Author for Correspondence: akinwunmi.adeoye@fuoye.edu.ng*

fruits and vegetables having strong antioxidant capacity[15,16].

Flavonoids have a wide range of biological activities, such as cell-proliferation-inhibiting, apoptosis-inducing, enzyme-inhibiting, antibacterial, and antioxidant effects. Some findings indicate that flavonoids possess various clinical properties, such as antiatherosclerotic, antiinflammatory, antitumour, antithrombogenic, antiosteoporotic, and antiviral effects[17,18]. Interest in traditional medicines has grown in recent years as they are typically low in toxicity, rarely produce complications and have beneficial pharmacological activities[19]. Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly have supported the idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems[13,20].

Adansonia digitata, commonly called baobab is most widespread of the *Adansonia* species on the African continent, and is majorly found in the hot, dry savannahs of sub-Saharan Africa. Different parts of *Adansonia digitata* solvent extracts have been reported to posses medicinal properties and are currently been used in treatment of various ailments such as diarrhea, fever induced malaria, inflammation, scurvy, cough, dysentery, small pox and measles, kidney and bladder diseases, blood clearing and asthma[21]. This study examined the flavonoid, total phenolic content, and antioxidant potentials of aqueous and methanolic extracts of *Adansonia digitata* stem bark.

MATERIALS AND METHODS

Preparation of Adansonia digitata extract

The stem of *Adansonia digitata* (Bombacaceae) was collected from Ido-Ekiti, Ekiti State Nigeria. The plant was identified and authenticated by in the herbarium unit of Forest Research Institute of Nigeria (FRIN) with identification number FHI 109806. The stem bark peels were air-dried at room temperature to avoid possible degradation or denaturation of their putative compounds. The air-dried stem bark of *Adansonia digitata* was blended to powder using an electric blender. This was stored in a glass container. Blended air-dried stem bark was soaked in sufficient volume of methanol for 72 hours at room temperature. It was continually stirred after each 24 hours. After 72 hours, the mixture was then filtered and the filtrate was concentrated using rotary evaporator at 40^0C. The concentrate was heated over a water bath to obtain a solvent free extract, which was stored in a refrigerator at 4^0C.

Phytochemical screening

The extract was screened for the presence of secondary metabolites and constituents using conventional protocols for detecting the presence of alkaloids, tannins, saponins terpenoids, flavonoids, cardiac glycosides Phylobatannins, Anthraquinones and steroids[22].

Determination of total phenolic content

The Folin–Ciocalteu reagent assay was used to determine the total phenol content by Hong et al.,[23]. 0.1ml of extracts of the plant of different concentrations was rapidly mixed with 0.1ml of Folin-Ciocalteu reagent, followed by the addition of 0.3ml sodium carbonate (15%w/v) solution. The mixture was incubated in the dark for 30mins. The absorbance of the blue colour was read at 760nm after 30mins on a spectrophotometer. Tannic acid was used as standard for the calibration curve. The total phenol content was expressed as mg/g Tannic acid equivalents (TAE) after calculation using the following equation: $C = (cV)/m$, where, C = total phenol contents, mg/g plant extract in TAE, c = concentration of tannic acid obtained from calibration curve (mg/ml), V = the volume of the sample solution (ml), m = weight of the sample (g). All tests were conducted in triplicate.

Determination of total flavonoid

Total flavonoid of the stem bark extracts was determined using the method described by Kumaran and Karunakaran[24] with slight modifications. Briefly, 0.5ml of extract solution (1mg/ml) and standard (quercetin) at different concentrations were taken in test tubes. 3.0ml of methanol followed by 0.1ml of 10% aluminum chloride solution was added into the test tubes. 200ml of 1M potassium acetate solution was added to the mixtures in the test tubes. Furthermore, each reaction test tube was then immediately diluted with 2.8ml of distilled water and mixed to incubate for 30 min at room temperature to complete reaction. The absorbance of pink colored solution was measured at 415nm using a spectrophotometer against blank methanol. Total flavonoid content of the extract was expressed as quercetin equivalents (QE) after calculation using the following equation: $C = (cV)/m$

where C = total flavonoid content, mg/g plant extract in QE, c = concentration of quercetin obtained from calibration curve (mg/ml), V=the volume of the sample solution (ml), m=weight of the sample (g). All tests were conducted in triplicate.

2.4 Quantification of compounds by HPLC-DAD

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column. *Adansonia digitata* extracts (aqueous and methanolic extracts) at a concentration of 15 mg/ml was injected by means of a model SIL-20A Shimadzu Auto sampler. Separations were carried out using Phenomenex C_{18} column (4.6 mm x 250 mm x 5 μm particle size). The mobile phase was water with 2% phosphoric acid (v/v) (solvent A) and HPLC grade acetonitrile (solvent B) at a flow rate of 0.6 mL/min and injection volume 40 μl. The composition gradient was: 2% solvent B reaching 15% at 10 min; 30% solvent B at 25 min, 65% solvent B at 40 min and 98% solvent B at 45 min, followed by 50 min at isocratic elution until 55 min. At 60 min the gradient reached the initial conditions again, following the method described by Colpo et al.,[25] with slight modifications. The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.025 – 0.300 mg/ml. Quantifications were carried out by integration of the peaks using the external standard method, at 270 nm for gallic acid; 325 nm for caffeic acid

and chlorogenic acid; and 366nm for quercitrin, rutin, kaempferol, luteolin and apigenin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 500 nm). All chromatography operations were carried out at ambient temperature and in triplicate. Limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al.,[26]. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

DPPH (1,1, Diphenyl 2-Picryl Hydrazyl) assay

The antioxidant activity by DPPH assay was assessed according to the method of Molyneux [27]. To 1ml of various concentration of the extract 1ml of DPPH 0.1mM was added in a test tube. Tannic acid was used as the standard for comparison. After incubation for 30 mins in dark at room temperature, absorbance was recorded at 517nm. The percent DPPH radical scavenging was calculated with the equation: % DPPH radical scavenging = [(Absorbance control – Absorbance sample)/Absorbance control] × 100.

Hydroxyl radical scavenging activity

The hydroxyl radical (OH) scavenging activity was measured by the method of Jin et al., [28]. The hydroxyl radical was generated in a mixture of 1.0ml of 0.75mM 1, 10-phenanthroline, 2.0 ml of 0.2 M sodium phosphate buffer (pH7.4), 1.0ml of 0.75mM FeSO4 and 1.0ml of H_2O_2 (0.01%,v/v). After addition of 1.0ml sample solution, the mixture was incubated at 37ºC for 30 min. Then, the absorbance of the mixture at 536nm was measured. Deionized water and mannitol were used as the blank and positive control respectively. The scavenging activity on OH^- was calculated by the following equation: Scavenging activity (%) = (Abs. sample – Abs. blank)/(Abs_0 Abs. blank)*****100 where Abs_0 is the absorbance of the deionized water instead of H_2O_2 and sample in the assay system.

Iron chelating ability

The in vitro Fe2+ chelating ability of plant extract was assayed according to the method of Minnoti and Aust,[29] with slight modification. 900µl of FeSO4 (500µM) and 150µl of extract were incubated for 5 min at room temperature, 78µl of 1,10-phenanthroline (0.25 % w/v, aqueous) was added. The absorbance of the orange colour solution was read at 510nm with a spectrophotometer. The principle of the assay is based on distruption of o-phenanthroline-Fe^{2+} complex in the presence of chelating agent. Ethylene diamine tetra aceticacid (EDTA) was used as the standard. The in vitro Fe^{2+} chelating ability of the sample was calculated by using the following formula: Chelating ability (%) = (Acontrol - Asample)/(Acontrol) x 100.

Where; Acontrol = the absorbance of the control (reaction mixture in the absence of sample) i.e. FeSO4 alone.

Asample = the absorbance of the reaction mixture (sample, FeSO4 and 1,10-phenanthroline)

Reductive potential

Reducing power of the extracts were measured by the direct reduction of $Fe^{3+}(CN^-)^6$ to $Fe^{2+}(CN^-)^6$ and was determined by absorbance measurement of the formation of the Perl's Prussian Blue complex following the addition of excess Fe^{3+}. Different concentrations of extracts in 0.5ml of distilled water were mixed with 1.25mL of 0.2M, pH 6.6 sodium phosphate buffer and 1.25ml of potassium ferricyanide [$K_3Fe(CN)^6$] (1%). The mixture was incubated at 50°C for 20min. After 20 min incubation, the reaction mixture was acidified with 1.25ml of trichloroaceticacid (10%). Finally, 0.5ml of $FeCl_3$ (0.1%) was added to this solution and the absorbance was measured at 700nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates greater reductive capability.

Table 1: Phytochemical screening of *Adansonia digitata* stem bark extract.

Phytochemicals	Aqueous Extract	Methanol Extract
Alkaloids	+	+
Saponin	+	+
Tannin	+	+
Steroids	-	-
Phlobatannin	-	-
Anthraquinone	-	-
Terpenoids	+	+
Cardiac glycosides	+	+
Flavonoids	+	+

Key: += Presence of constituent; - = Absence of constituent

Statistical analysis

Results calculated from triplicate data were expressed as means ± standard error of means (SEM). Data were analyzed using one-way analysis of variance followed by Newman-Keuls comparison of means. The significance level was set at $p < 0.05$. Statistical Analysis, Graphing, curve fitting, and IC_{50} were performed using Graph Pad Prism (ver.5.0a).

RESULTS AND DISCUSSION

Photochemical screening

The preliminary routine means of evaluating the medicinal relevance of plants is through the assessment of phytochemical analysis. It is believed generally that there is a direct relationship between total phenol content and antioxidant activity[30]. The flavonoids which are widespread in plants, naturally contributes to the free radical scavenging activity together with other phenolics[31]. In table 1, both aqueous extract of *Adansonia digitata* (AEAD) and methanolic extract of *Adansonia digitata* (MEAD) showed the presence of alkaloids, saponin, tannin, terpenoids, cardiac glycosides and flavonids. This is a confirmation of an earlier work[32].

Total phenol and total flavonoid contents

The total phenol contents of AEAD and MEAD are 1.00 ± 0.00 and 2.15 ± 0.01, and the total flavonoid contents are 4.37 ± 0.00 and 4.68 ± 0.00 respectively (Table 2). The differences in total phenolic content of samples were significant ($p < 0.05$). The different antioxidant activities of medicinal plants can be ascribed to their total phenolic concentrations. A number of research works have been

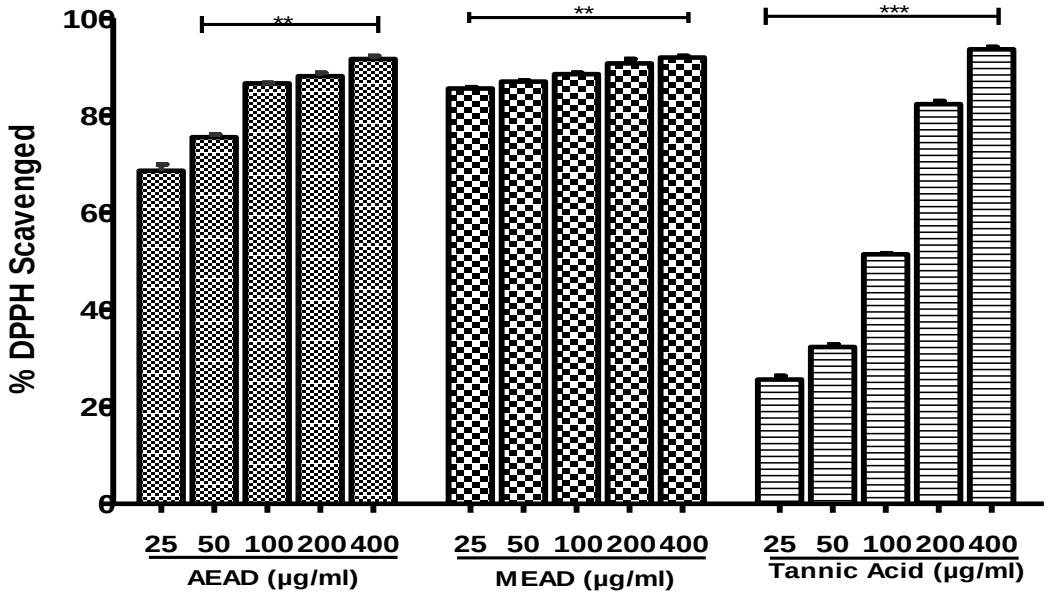

Figure 1: DPPH free radical-scavenging activities of aqueous and methanolic extracts of *Adansonia digitata* stem.
†The results are mean ±SEM of three parallel measurements.
**P<0.05 vs 0 µg/ml; ***P<0.01 vs 0 µg/ml

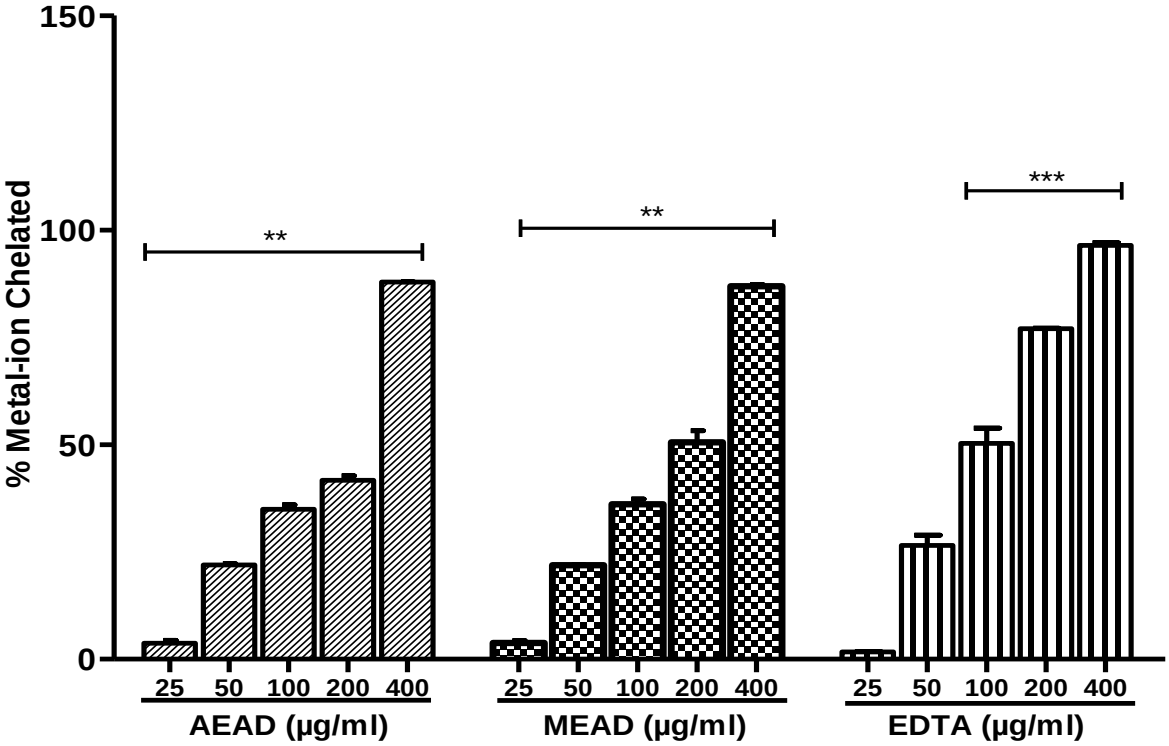

Figure 2: Ferrous ion-chelating activities of aqueous and methanolic extracts of Adansonia digitata stem.
†The results are mean ±SEM of three parallel measurements.
**P<0.05 vs 0 µg/ml; ***P<0.01 vs 0 µg/ml

done on the effects of phenolic compounds on total antioxidants and correlations between phenolic compounds and total antioxidants[2,5,3, 6,11]. The results of this study agree with the observation from the previous work. There was a good correlation between the total phenolic and flavonoid content and the scavenging of DPPH radical in AEAD and MEAD extract (Figure 1). These results indicated that the radical scavenging capacity of each extract might be mostly related to their concentration of phenolic hydroxyl group. The antiradical activity of phenolic compounds depends on their molecular structure, on the availability of phenolic hydrogens and on the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation[33,34]. Thus, free radical-scavenging activity is greatly influenced by the phenolic composition of the sample.

Phenolic acids and flavonoid composition

The results of the components of *Adansonia digitata* stem bark extracts as revealed by the high performance liquid chromatography (HPLC) is shown in table 3 below.

Due to the relationship between free radical scavenging capacity of medicanal plants, many analytical methodologies have been published for the determination of antioxidant ability. Phenolics in fruits have been monitored by HPLC[35] or colorimetrically using Folio-ciocalteu reagent[36]. Several assays have been used to evaluate total antioxidant capacity of foods and food products including spectrophotometric methods.

HPLC finger printing has become an important tool for assessing the phytohemicals present in herbal samples. It is a chromatographic technique which is used to separate, identify, quantify and purify individual components of a mixture and it provides quantitative and qualitative information to researchers as well as enables the screening of samples for the presence of new compounds. In tables 3, the various phenolic acids and flavonoids present in the extracts were identified and their relative quantities estimated. The amount of phenolic acids and flavonoids found in the MEAD were higher than the AEAD.

Scavenging of DPPH radical

DPPH is a free radical compound that has been widely used to determine the free radical-scavenging ability of various samples. DPPH decreases significantly upon exposure to proton radical scavengers[37]. The DPPH free radical- scavenging activities of AEAD and MEAD are presented in fig. 1. MEAD showed a high radical scavenging ability with the lowest IC_{50} value of 14.62 (Table 2.). This showed that the MEAD was a better scavenger of DPPH radical. The radical scavenging ability of the extracts is concentration dependent and is comparable to the standard, tannic acid at 400 µg/ml.

Metal ion-chelating assay

The main mechanism of ion-chelating activity is the ability to deactivate and/or chelate transition metals which can promote the Fenton reaction and hydroperoxide decomposition. EDTA exhibited an excellent ferrous ion-chelating capacity at all concentration when compared to the extracts. Among the extract, MEAD had the highest ferrous ion-chelating ability with the lowest IC_{50} value of 28.95 when compared to AEAD IC_{50} value of 38.75. fig 2. The difference in IC_{50} values of both the AEAD and MEAD are statistically significant ($p < 0.05$). The two extracts exhibited ferrous ion-chelating ability in a concentration dependent manner.

Hydroxyl radical-scavenging assay

Hydroxyl radicals are reactive oxygen species that initiate peroxidation of lipid membranes. $^{\bullet}OH$ is one of the most damaging free radicals in the body and can be important mediator of damage to cell structures, nucleic acids, lipids and proteins[38]. AEAD showed higher hydroxyl radical scavenging ability than MEAD. The difference in OH radical scavenging in the two extracts is significant statistically ($p < 0.05$). The hydroxyl radical scavenging capability of AEAD was comparable to mannitol at all concentrations (fig.3).

Ferric-reducing antioxidant power (FRAP) assay

The results obtained from the screening of the reductive potential of AEAD and MEAD is shown in fig.4. The reduction of Fe^{3+} to Fe^{2+} in the presence of AEAD and MEAD was employed to determine the reductive capability of the extract. AEAD showed concentration dependent reducing property with the highest absorbance of 0.564 at 400 µg/ml when compared to the absorbance of MEAD 0.407 at 400 µg/ml. AEAD exhibited higher reducing effect than the MEAD. The difference in the reducing power of AEAD and MEAD is significant ($P < 0.05$).

The reducing power of a compound is mainly due to the availability of hydrogen atoms that could be donated to a free radical and convert it to a more stable product. Through this, the chain reaction initiated by the free radical could be effectively terminated. The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity[39]. The transformation of Fe^{3+} to Fe^{2+} in the presence of either the extract or the standard (ascorbic acid) is a measure of reducing capability[40]. Increased absorbance of the reaction mixture correlates with greater reducing power.

Table 2: Total phenol and total flavonoid content and IC_{50} values of aqueous and methanolic extract of *Adansonia digitata* stem bark in antioxidant properties

Activity/ Sample	DPPH scavenging (mg/ml)	Metal ion-chelating (mg/ml)	Hydroxyl radical (mg/ml)	Total Phenol (mg/g Tannic acid equivalent)	Total Flavonoid (mg/g Quercetin equivalent)
Aqueous extract	18.22^{b}	38.75^{b}	20.45^{a}	1.00 ± 0.00^{b}	4.37 ± 0.00^{a}
Methanol extract	14.62^{a}	28.95^{a}	30.43^{b}	2.15 ± 0.01^{a}	4.68 ± 0.00^{a}

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$

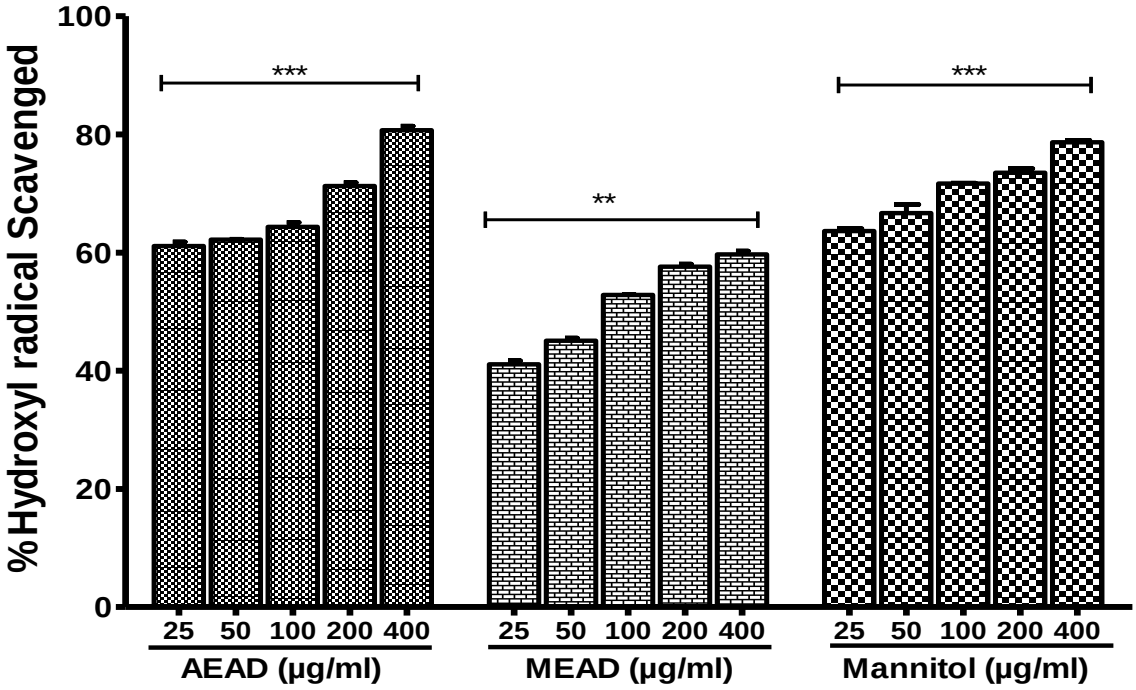

Figure 3: Hydroxyl radical-scavenging activities of aqueous and methanolic extracts of Adansonia digitata stem.
[†]The results are mean ±SEM of three parallel measurements.
[**]P<0.05 vs 0 µg/ml; [***]P<0.01 vs 0 µg/ml

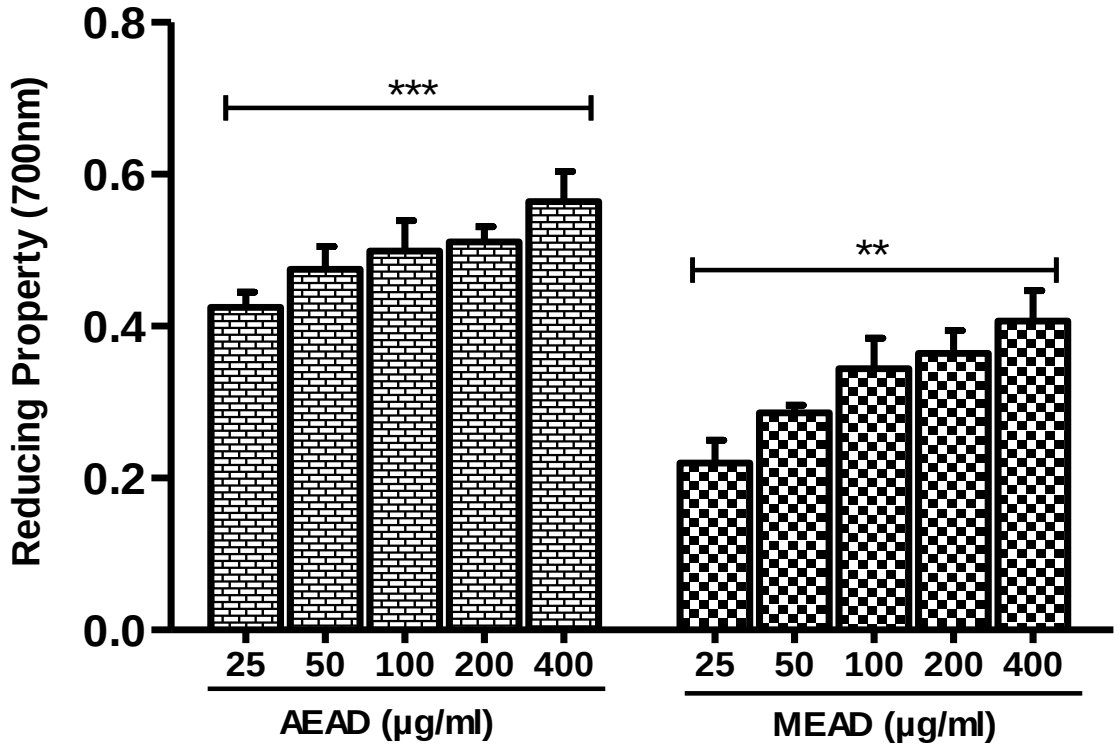

Figure 4: Ferric-reducing antioxidant power of aqueous and methanolic extracts of Adansonia digitata stem.
[†]The results are mean ±SEM of three parallel measurements.
[**]P<0.05 vs 0 µg/ml; [***]P<0.01 vs 0 µg/ml

Table 3: Phenolic acids and flavonoid composition of *Adansonia digitata* stem bark extract.

Compounds	Aqueous (mg/g)	Methanolic (mg/g)
Gallic acid	0.63 ± 0.01^{a}	2.29 ± 0.03^{a}
Chlorogenic acid	0.67 ± 0.01^{a}	0.73 ± 0.01^{b}
Caffeic acid	1.84 ± 0.03^{b}	0.25 ± 0.02^{c}
Rutin	0.59 ± 0.02^{a}	1.87 ± 0.01^{d}
Quercitrin	2.38 ± 0.01^{c}	1.85 ± 0.03^{d}
Kaempferol	2.41 ± 0.03^{c}	3.69 ± 0.01^{e}
Luteolin	1.87 ± 0.01^{b}	4.05 ± 0.01^{f}
Apigenin	0.60 ± 0.01^{a}	1.81 ± 0.02^{d}

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$

CONCLUSION

From the findings of this study, methanolic extract had the highest radical scavenging activity and ferrous ion-chelating activity with appreciable amount of phenolic acids and flavonoids. A significant relationship was obtained between antioxidant activity and phenolic content indicating that phenolic compounds contribute significantly to antioxidant activity of the plant. The high antioxidant activity exhibited by *Adansonia digitata* stem bark extracts provided justification for the therapeutic use of this plant in folk medicine due to the phytochemical constituents. The results therefore suggest that the extract could be a potential source of natural antioxidant that could be of great importance for the treatment of radical related diseases.

REFERENCES

1. Karadenz, F., Burdurlu, HS., Koca, N (2005). Antioxidant activity of selected fruits and vegetables grown in turkey. Turk. J. Agric. 29: 297-303.
2. Barros, L., Joao Ferreira, M., Queiros, B., Ferreira, IC., Baptista, P. (2007). Total phenol, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushroom and their antioxidant acitivities. Food Chem. 413-419.
3. Jagadish, LK., Krishnan, VV., Shenbhagaraman, R., Kaviyarasan, V (2009). Comparative study on the antioxidant, anticancer and antimicrobial property of *Agaricus bisporus* imbach before and after boiling. Afr. J. Biotechnol. 8: 654-661.
4. Ravindra, P.S., Shashwat, S., Suman, K. (2004). Free Radicals and Oxidative Stress in Neurodegenerative Diseases: Relevance of Dietary Antioxidants. JIACM. 5(3): 218-25.
5. Atrooz, OM., (2009). The antioxidant activity and polyphenolic contents of different plant seeds extracts. Pakistan J. Biol. Sci. 12: 1063-1068.
6. Kim, JH., Kim, SJ., Park, HR., In Choi, J., Cheoul, Ju Y., Chang, Nam K., Kim, SJ., Lee, SC. (2009). The different antioxidant and anticancer activities depending on the colour of oyster mushroom. J. Med. Plants Res. 3:1016-1020.
7. Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, JA., Deemer, EK. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and Ferric reducing antioxidant power (FRAP) assays: A comparative study. J. Agric. Food Chem. 50: 3122-3128.
8. Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Hawkins Byrne, D. (2006). Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Comp. Anal. 19: 669-675.
9. Othman, A., Ismail, A., Abdul Ghani, N., Adenan I. (2007). Antioxidant capacity and phenolic content of cocoa beans. Food Chem. 100: 1523-1530.
10. Hodzic Z; Pasalic H; Memiseric A; Srabovic M; Saletovic M (2008). The influence of total phenolic content on antioxidant capacity in the whole grain extracts. Euro. J. Sci. Res. 28: 471-477.
11. Semalty, M., Semalty, A., Joshi, GP., Rawat, MSM. (2009). Comparison of in vitro antioxidant activity of *Trigonella foenum-graecum and T. corniculata* seed. Res. J. Phytochem. 1-5.
12. Halliwell, B., Aeschbach, R., Loliger, J., Aruoma, OI. (1995). The characterization of antioxidants. Food Chemical Toxicity. 33: 601–617.
13. Cao, G., Sofic, E., Prior, R. L. (1996). Antioxidant capacity of tea and common vegetables. Journal of Agricultural and Food Chemistry. 44: 3426–3431.
14. Wang, H., Cao, G., Prior, RL. (1996). Total antioxidant capacity of fruits. Journal of Agricultural and Food Chemistry. 44: 701–705.
15. Cao, G., Sofic, E., Prior, R. L. (1997). Antioxidant and pro-oxidant behavior of flavonoids: Structure-activity relationships. Free Radical Biology and Medicine. 22: 749–760.
16. Wang, H., Cao, G., Prior, RL. (1997). Oxygen radical absorbing capacity of anthocyanins. Journal of Agricultural and Food Chemistry. 45: 304–309.
17. Cook, N. C., Samman, S. (1996). Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary resources. The Journal of Nutritional Biochemistry. 7: 66–76.
18. Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. Pharmacology and Therapeutics. 96: 67–202.
19. Endo, J., Nakamura, Y. (1993). Reconsideration of the concepts of the large intestine and the small intestine in Chinese traditional medicine. Journal of Japanese History of Medicine. 39: 157–168.
20. Ness, AR., Powles, JW. (1997). Fruit and vegetables and cardiovascular disease: a review. Int. J. Epidemiol. 26: 1-13.
21. Van Wick B. E. and Gericke N. (2000). People's Plants - A Guide to useful Plants of southern Africa. Briza Publications, Pretoria.
22. Trease, A., Evans, WC. (1989). Trease and Evans Pharmacognosy. 13th ed. London, Bailiere Tindall. pp. 342- 383.
23. Hong, Y., Sava, V., Yang, S.M., Yang, P.C., Huang, G.W.S. (2001). Isolation and characterization of

melanin pigments derived from tea and tea polyphenols. Food Chem. 73: 177-184.
24. Kumaran, A., Karunakaran, R.J. (2007). In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. LWT-Food Sci Technol. 40: 344–352.
25. Colpo, E., Vilanova, C.D.D.A., Reetz, L.G.B., Duarte, M.M.M.F., Farias, I.L.G., Meinerz, D.F., Mariano, D.O.C., Vendrusculo, R.G., Boligon, A.A., Corte, C.L.D., Wagner, R., Athayde, M.L., Rocha, J.B.T. (2014). Brazilian nut consumption by healthy volunteers improves inflammatory parameters. Nutrition. 30: 459-465.
26. Boligon, A.A., Piana, M., Schawnz, T.G., Pereira, R.P., Rocha, R.B.T., Athayde, M.L. (2014). Chromatographic Analysis and Antioxidant Capacity of *Tabernaemontana catharinensis*. Natural Product Communications. 9 (1): 61-64.
27. Molyneux, P., (2004). The use of stable free radical diphenyl picryl-hydrazyl (DPPH) for estimating antioxidant activity. J Sci Tech. 26: 212-219.
28. Jin, M., Cai, Y.X., Li, JR., (1996). 1,10-Phenanthroline Fe2+ oxidative assay of hydroxyl radical produced by H2O2/Fe2+. Prog Bio Biophys. 23: 553-555.
29. Minnoti, G., Aust, S.D., (1997). An investigation into the mechanism of citrate–Fe2+ dependent lipid peroxidation. Free Rad Biol Med. 3: 379–387.
30. Chanda, S., Dave, R., (2009). In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. Afr J Microbiol Res. 3(13):981-996.
31. Halliwell, B., Gutteridge, JMC. (1984). Oxygen toxicology, oxygen radicals, transition metals and disease. Biochem J. l:219.
32. Fasola, TR., Iyamah, PC. (2014). Comparing the Phytochemical Composition of Some Plant Parts Commonly Used in the Treatment of Malaria. *Int. J. Pure Appl. Sci. Technol.*, 21(1) pp. 1-11.
33. Catherine, A., Nicholas, JM., Rice-Evans., George, P. (1996). Structureantioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol. Med. 20: 933 – 956.
34. Ramarathnam, N., Ochi, H., Takeuchi, M. (1997). Antioxidant defense system in vegetable extracts. *In:* F. Shahidi, Edition, Natural Antioxidants; chemistry, health effects and applications, AOCS Press, Champaign, IL, pp. 76 – 87.
35. Tung, TY., Wu, JH., Kuo, YH., Chang, ST. (2007). Antioxidant activities of natural phenolic compounds from Acacia confuse bark. Bioresour. Technol. 98:1120- 1123.
36. Faujam, H., Noriham, A., Norrakiah, AS., Babji, AS. (2009). Antioxidant acivity of plants methanolic extracts containing phenolic compound. Afr. J. Biotechnol. 8:484-489.
37. Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. Bioscience, Biotechnology, and Biochemistry. 62: 1201–1204.
38. Valko, M., Leibfritz, D., Moncola, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease Review. Int. J.Biochem. Cell Biol. 39:44-84.
39. Reichenbach, G., Momi, S., Gresele, P. (2005). Nitricoxide and its antithrombotic action in the cardiovascular system. Curr. Drug Targets Cardiovasc. Haematol. Disord. 5:65-74.
40. Halliwell, B. (1991). Reactive oxygen species in living systems: Source, biochemistry and role in human disease. Am.J. Med. 91:14–22.