Journal of Medicinal Plants Research Vol. 5(31), pp. 6839-6848, 23 December, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals
DOI: 10.5897/JMPR11.1238

Full Length Research Paper

In vitro antioxidant properties of aqueous and ethanolic extracts of walnut (*Juglans regia)*

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Accepted 1 November, 2011

Walnut has been reported to exhibit several pharmacological properties and widely used in folkloric medicine for the traditional treatment of ailments. Hence, this study sought to investigate the antioxidant properties of the aqueous and ethanolic extract of walnut seed using several antioxidant parameters. Both extracts were tested for free radical scavenging ability against 2, 2 diphenyl-1-picryl hydrazyl (DPPH) radical, ferric reducing and iron (II) chelating ability. Furthermore, the ability of both extracts to offer protective benefit against lipid peroxidation was assessed. Moreover, the phytochemical constituents (total phenolics, flavonoids and vitamin C) of both extracts were measured. Generally, ethanolic extract was found to exhibit a significantly higher antioxidant activity than do aqueous in all parameters determined. Result showed that both extracts demonstrated potent free radical scavenging and ferric reducing ability and iron chelating activity *in vitro.* Moreover, both extracts demonstrated potent inhibitory effect on proxidant induced oxidative assault against cerebral and hepatic lipid. The phenolic content of *Juglans regia* was estimated to be 35.22 ± 0.75 mg/g (GAE) for ethanolic and 20.26 ± 0.55 mg/g (GAE) for aqueous extract whereas the flavonoid content was estimated to be 20.02 ± 0.12 mg/g (QE) for ethanolic and 14.82 ± 0.15 mg/g (QE). In addition, the vitamin C contents were 18.22 mg/g and 10.18 mg/g respectively for ethanolic and aqueous extract of dried *J. regia*. From the foregoing, the wide usage of walnut seed as remedy for ailment in folk medicine may be due to its high phytochemical constituents and potent antioxidant properties *in vitro*. Hence, information from this study would provide the underlying mechanisms through which walnut seed exhibit its pharmacological properties which when exploited could be helpful in the management of degenerative diseases.

Key words: Phytochemical, oxidative assault, proxidant.

INTRODUCTION

Walnut (*Juglans regia* L) is a member of Juglandaceae family and one of the finest nuts of temperate regions (Ozcan, 2009) with highly promising economic value for the food industry (Martinez et al., 2008). Its nuts have been reported to exhibit unique organoleptic characteristics (Lopez et al., 1995), hypocholesterolemic (Sabate and Fraser, 1994; Abbey et al., 1994; Savage, 2005; Dogan and Akgulb, 2005; Pereira et al., 2008) and antihypertensive effect (Sabate and Fraser, 1994; Mexis et al., 2008; Arranz et al., 2008). The bark of *J. regia* is regularly used as miswaks for teeth and mouth cleaning (Ibrar et al., 2007). Moreover, walnut has been reported to exhibit both antibacterial (Emira et al., 2011) and antifungal properties (Emira et al., 2010). The increasing interest in walnuts may be due to its significant economical value and medicinal importance for human health because of their biochemical composition of polyunsaturated fatty acids, especially (linoleic, oleic acid) and high protein value (Savage et al., 2001). They also contain other beneficial components like plant protein (for example, arginine, leucine), carbohydrates (for example, dietary fibre), vitamins (for example, vitamin A and E), pectic substances, minerals (magnesium, potassium, phosphorus, sulphur, copper and iron), plant

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sterols and phytochemicals (Kris-Etherton et al., 1999; Savage et al., 2001; Prasad, 2003; Colaric et al., 2006). An ever-growing body of research has shown that walnuts play a significant role in reducing the risk of heart disease. The heart benefits of walnuts include lowering cholesterol, reducing inflammation and improving arterial function. Walnuts have been reported to reduce cell adhesion molecules associated with atherosclerosis. This effect enhances the circulatory system, aiding in the prevention of heart disease. According to the researchers, walnuts are the first whole food to show such cardiovascular benefits (Sabate et al., 1993; Abbey et al., 1994).

Clinical trials have consistently shown that including walnuts as part of a cholesterol-lowering diet improves lipid and lipoprotein profiles (Sabate et al., 1993; Abbey et al., 1994; Chisholm et al., 1998; Zambon et al., 2000; Spiller et al., 1992; O'Byrne et al., 1997; Kris-Etherton et al., 1999; Curb et al., 2000; Morgan and Clayshulte, 2000; Edwards et al., 1999). In fact, a systematic review of well-designed nut intervention studies estimated that consuming a moderate fat diet (approximately 35 percent of calories) including (50 to 100 g) of nuts per day, especially almonds, peanuts, pecans, or walnuts, significantly lowered the total cholesterol content (Mukuddem-Petersen et al., 2005).

Besides, walnuts have been recently discovered to exhibit antidiabetic potential helping the body address one of the problems associated with early stage type 2 diabetes- insulin resistance- which hinders the absorption of glucose from the bloodstream into human cells (Fukuda et al., 2004). Considering the potential health benefits of walnut, the present study sought to investigate the effect of solvent of extraction on the antioxidant properties of walnut and unravel its *in vitro* antioxidant mechanisms of action with a view to providing useful explanation on the widely reported pharmacopotency of nuts.

MATERIALS AND METHODS

Chemical reagents

Thiobarbituric acid (TBA) was obtained from Sigma (St. Louis, MO). 2, 2-diphenyl -1-picrylhydrazyl (DPPH) and 1, 10 phenanthroline were obtained from Fluka Chemie and Merck (Germany). All other chemicals were obtained from standard chemical suppliers and were of analytical grade.

Plant material

Walnut fruits *J. regia* L were collected from a farm close to The Federal University of Technology Akure (Nigeria) campus, and were identified at the Crop Soil and Pest Management Department of the Federal University of Technology, Akure, Nigeria. The fruits were than deshelled and the nuts collected, chopped and air- dried. The dried nuts were pulverized using a blender and the powdered nuts were stored in polythene bags and placed at room temperature until they were used.

Preparation of plant extracts

Five grams each of powdered nuts were weighed in two separate extraction bottle and one hundred milliliters of solvents (ethanol and water) was added to the bottles containing the powdered nuts respectively and left for 24 h to allow for extraction. Thereafter, the solutions were filtered separately using a Whatman filter paper. The extracts were stored air tight in a refrigerator until required for use. This serves as the stock solution for all determinations.

Animals

Male adult Wistar rats (200 to 250 g) were used. The animals were used according to the standard guidelines of the Committee on Care and Use of Experimental Animal Resources.

Determination of total phenol contents

The total phenol contents of the nuts of *J. regia were* determined according to the method of Singleton et al. (1999) by mixing (0 to 1.0 ml) of the extracts with equal volume of water; 2.5 ml Folin-Ciocalteau's reagent and 2 ml of 7.5% sodium carbonate were subsequently added, and the absorbance was measured at 765 nm after incubating at 45°C for 40 min. The amount of phenols in the nut extract was expressed as gallic acid equivalent (GAE).

Determination of total flavonoid content

The total flavonoid content of *J. regia* was determined using quercetin as a reference compound according to Meda et al. (2005). Briefly, 0 to 500 µl of stock solution of both ethanolic and aqueous extract was mixed with 50 µl of aluminium trichloride and potassium acetate. The absorption at 415 nm was read after 30 min at room temperature. Standard quercetin solutions were prepared from 0.01 g quercetin dissolved in 20 ml of ethanol. All determinations were carried out in triplicate. The amount of flavonoids in both extracts was expressed as quercetin equivalent (QE).

Vitamin C content

The level of vitamin C in *J. regia* was determined colorimetrically as described by Jacques-Silva et al. (2001). Briefly, an aliquot of both extracts (200 µl) was incubated for 3 h at 38°C then 1 ml H_2SO_4 65% (v/v) was added. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/ml), and the absorbance of the colored product was measured at 520 nm.

Standard ascorbic acid solutions were prepared from 20 mg of standard ascorbic acid dissolved in 10 ml of water and ethanol for aqueous and ethanolic extracts respectively. The content of ascorbic acid in both extracts per gram of dried sample was estimated from the standard ascorbic acid.

Free radical scavenging ability

The free radical scavenging ability of *J. regia* against 2, 2-diphenyl -1- picrylhydrazyl (DPPH) free radicals were evaluated according to Gyamfi et al. (1999). Briefly, 600 µl of extract (0 to 100 µM) was mixed with 600 µl, 0.3 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was measured at 516 nm.

Table 1. Antioxidant constituents of *J. regia* extracts.

Parameter	Ethanolic	Aqueous
Total phenol	35.22 ± 0.75 mg/g (GAE)	20.26 ± 0.55 mg/g (GAE)
Total flavonoid	20.02 ± 0.12 mg/g (QE)	14.82 ± 0.15 mg/g (QE)
Ascorbic acid	18.22 ± 0.45 mg/g (AscE)	10.18 ± 0.21 mg/g (AscE)

GAE- Gallic acid equivalent; QE - Quercetin equivalent; Asc - Ascorbic acid equivalent.

Reducing property

The reducing property was determined by assessing the ability of both aqueous and ethanolic extract of *J. regia* to reduce $FeCl_3$ solution as described by Pulido et al. (2000). Briefly walnut extract (0 to 250 µl of stock) was mixed with 250 µl, 200 mM Sodium phosphate buffer (pH 6.6) and 250 µl of 1% Potassium ferrocyanide, the mixture was incubated at 50°C for 20 min, thereafter 250 µl, 10% trichloroacetic acid was added, and subsequently centrifuged at 650 rpm for 10 min, 1000 µl of the supernatant was mixed with equal volume of water and 100 µl of 0.1 g/100 ml ferric chloride, the absorbance was later measured at 700 nm, a higher absorbance indicates a higher reducing power.

Fe^{2+} Chelating assay

The Fe^{2+} chelating ability of both aqueous and ethanolic extract of *J. regia* was determined using a modified method described by Puntel et al. (2005). Freshly prepared 500 µmol/L $FeSO_4$ (150 µl) was added to a reaction mixture containing 168 µl of 0.1 mol/L Tris-HCl (pH 7.4), 218 µl saline and extract (0 to 100 µM). The reaction mixture was incubated for 5 min, before the addition of 13 µl of 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe (II) chelating ability was subsequently calculated with respect to the reference (which contains all the reagents without seed extracts).

Lipid peroxidation

Rats were decapitated under mild ether anesthesia and the cerebral (whole brain) and hepatic (liver) tissues were rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris-HCl, pH 7.4 (1/10, w/v). The homogenates were centrifuged for 10 min at 4000 g to yield a pellet that was discarded and a low-speed supernatant (S1). An aliquot of 100 µl of S1 was incubated for 1 h at 37°C in the presence of both walnut extracts, with and without the prooxidants, iron [final concentration (10 µM)] and sodium nitroprusside (SNP) (final concentration 30 µM). This was then used for lipid peroxidation determination. Production of thiobarbituric acid reactive species (TBARS) was determined as described by Ohkawa et al. (1979), excepting that the buffer of the color reaction has a pH of 3.4. The color reaction was developed by adding 300 ml 8.1% sodium dodecyl sulfate (SDS) to S1, followed by sequential addition of 500 ml acetic acid/HCl (pH 3.4) and 500 ml 0.8% thiobarbituric acid (TBA). This mixture was incubated at 95°C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared to that of the controls.

Statistical analysis

The results were expressed as mean ± SD of three-four independent experiments performed in triplicate and were analyzed by one-way analysis of variance, followed by Duncan's multiple range test. Differences between groups were considered significant when $p < 0.05$.

RESULTS

Antioxidant constituents of *Juglans regia*

The antioxidant constituents of *J. regia* that was determined in the present study as shown in Table 1 include total phenols, flavonoids and vitamin C. The phenolic content of *J. regia* was estimated to be 35.22 ± 0.75 mg/g (GAE) for ethanolic and 20.26 ± 0.55 mg/g (GAE) for aqueous extract whereas the flavonoid content was estimated to be 20.02 ± 0.12 mg/g (QE) for ethanolic and 14.82 ± 0.15 mg/g (QE). In addition, the vitamin C contents were 18.22 ± 0.45 mg/g and 10.18 ± 0.21 mg/g respectively for ethanolic and aqueous extract of dried *J. regia*. GAE denotes gallic acid equivalent while QE denotes quercetin equivalent.

Antioxidant mechanisms of plants

In order to better ascertain the antioxidant potentials of *J. regia,* several antioxidant mechanisms such as reducing property, metal chelating ability, free radical scavenging properties and inhibition of lipid peroxidation were employed. Generally, *J. regia* exhibited potent antioxidant action in a concentration dependent manner. However, ethanolic extract demonstrated a better antioxidant properties than aqueous extracts in all parameters determined.

Free radical scavenging ability

Figure 1 shows the free radical scavenging property of the seeds of *J. regia.* Apparently, the ethanolic extract exhibited potent free radical scavenging activities which was significant ($P < 0.05$) at all dilutions of the stock solution used.

Reducing property

The reducing property of *J. regia* is presented in Figure 2

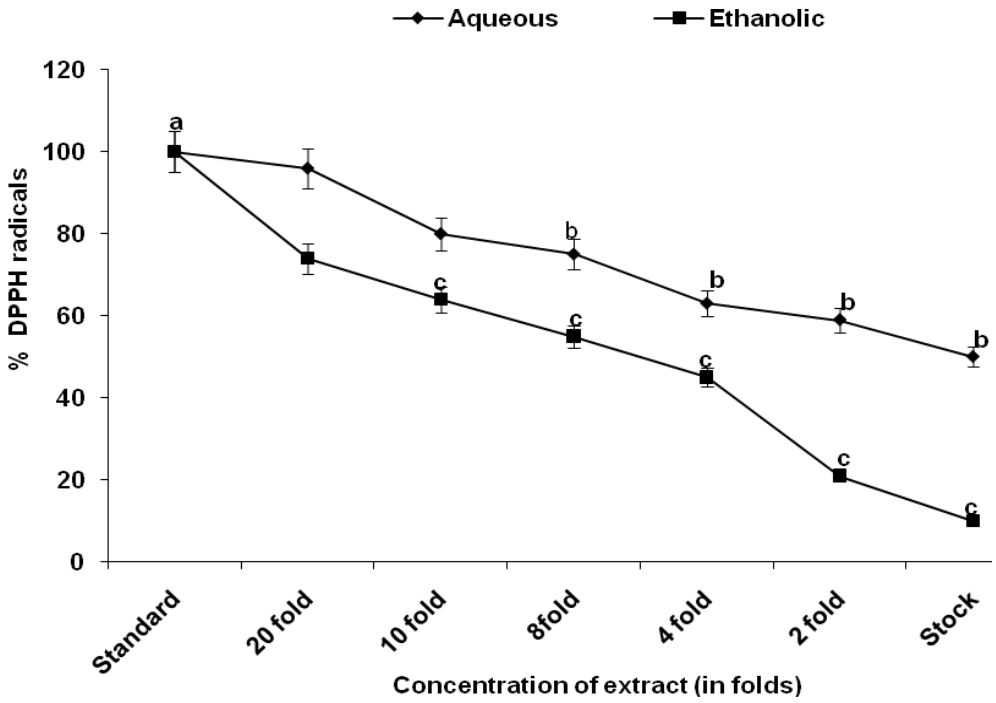

Figure 1. Free radical scavenging properties of walnut extracts. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

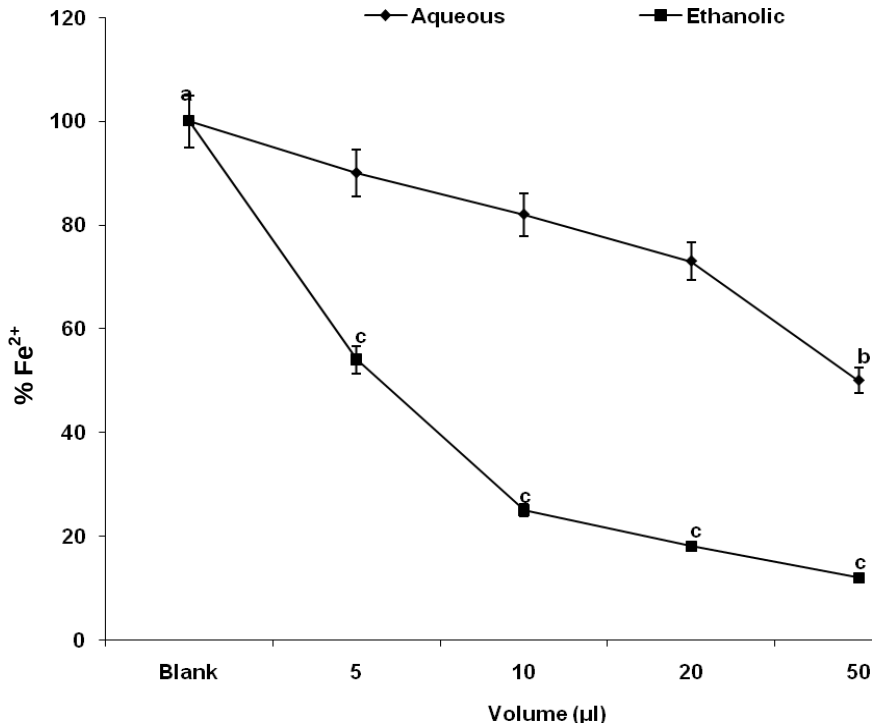

Figure 2. Iron (II)- chelating properties of walnut seed extracts. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

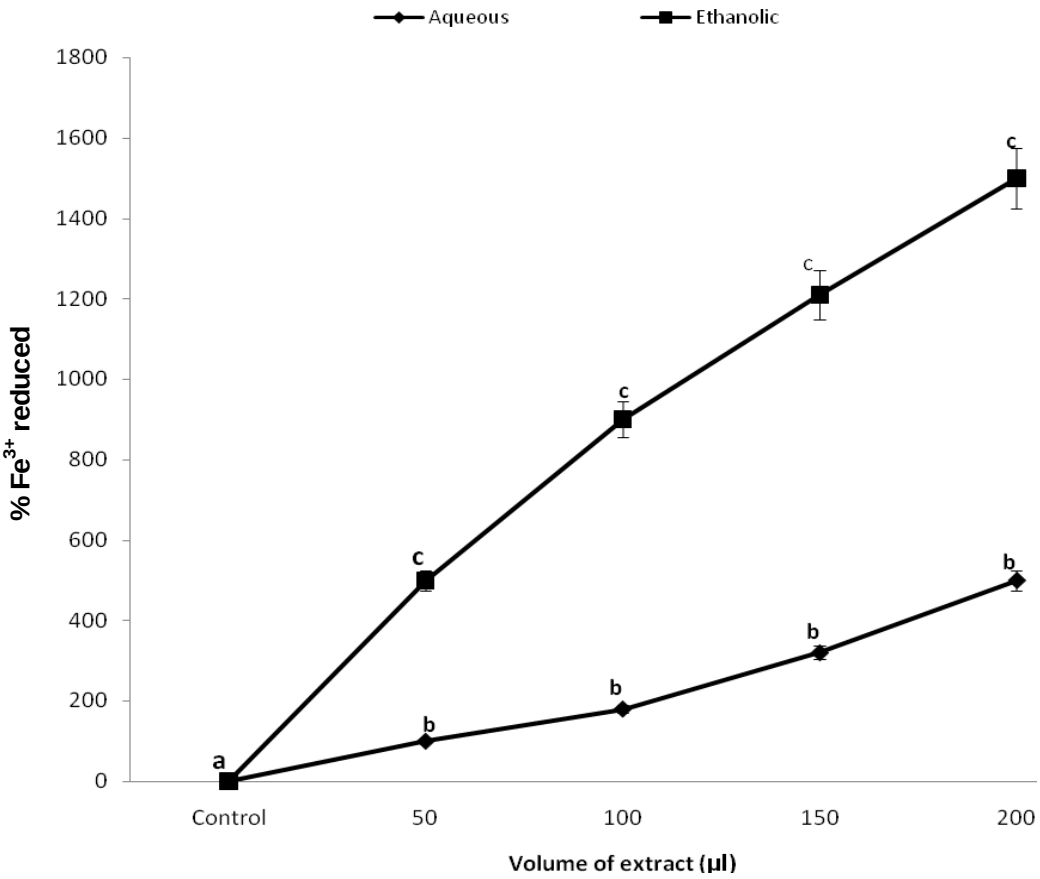

Figure 3. Ferric reducing properties of walnut seed extract. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

One-way ANOVA revealed that *J. regia* is rich in free electrons and readily supplies such electrons to Fe^{3+}, thereby reducing Fe^{3+} to Fe^{2+}. This reductive ability of *J. regia* was significant ($P < 0.05$) at the least volume of extract tested.

Fe^{2+} chelating ability

Figure 3 shows the Fe^{2+} chelating properties of *J. regia.* A one-way ANOVA followed by Duncan's test shows that the ethanolic extracts of seeds of *J. regia* greatly chelated Fe^{2+} when compared to control ($P < 0.05$).

Lipid peroxidation

Figures 4 and 5 and show the effect of *J. regia* on lipid peroxidation subjected to oxidative assaults induced by iron and SNP respectively. Figures 4a and 5a show that when brain lipids were subjected to stress-induced peroxidation either caused by Fe^{2+} or sodium nitroprusside in the presence of *J. regia* the extract exerted a significant inhibitory effect on the peroxidation processes. Similarly, Figures 4b and 5b shows that when hepatic lipids were subjected to oxidative stress, *J. regia* was able to significantly inhibit the peroxidation of hepatic lipids in a fashion similar to that observed when cerebral lipids were used. One-way ANOVA revealed that irrespective of the prooxidant or lipid types, the inhibitory effect of *J. regia* was significant at the lowest volume of extract tested ($P < 0.05$). However, Figures 4 and 5 generally revealed that ethanolic extract was more potent than aqueous in the inhibition of proxidant induced lipid peroxidation regardless of the tissue or proxidant employed for oxidative assault.

DISCUSSION

Plants have been shown to be efficacious in the management of diseases. In fact, the use of plant parts

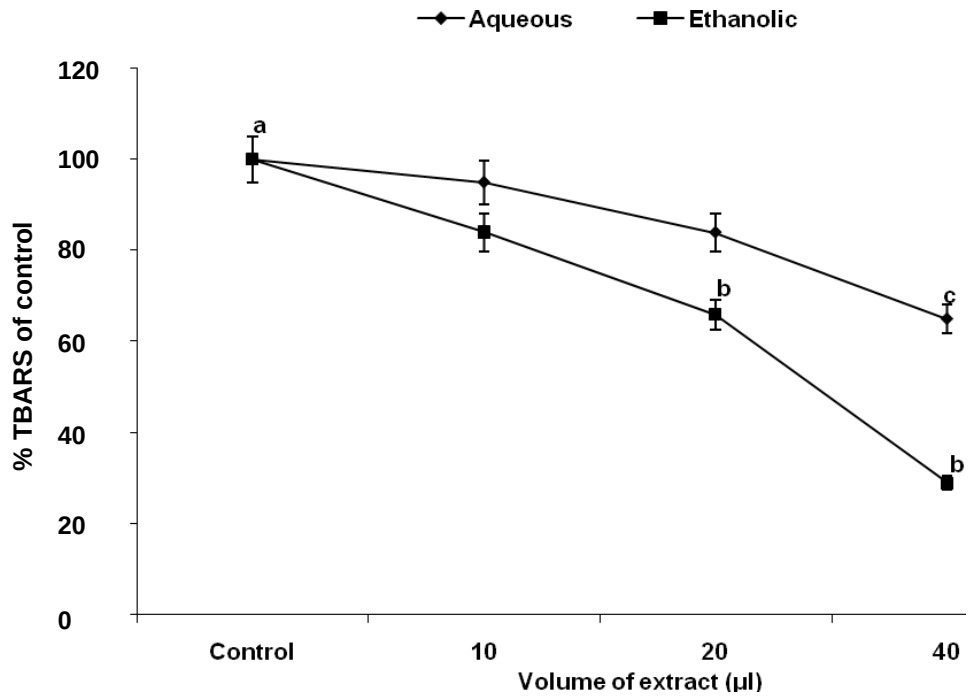

Figure 4a. Inhibitory effect of walnut extracts on Fe^{2+}-induced hepatic lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

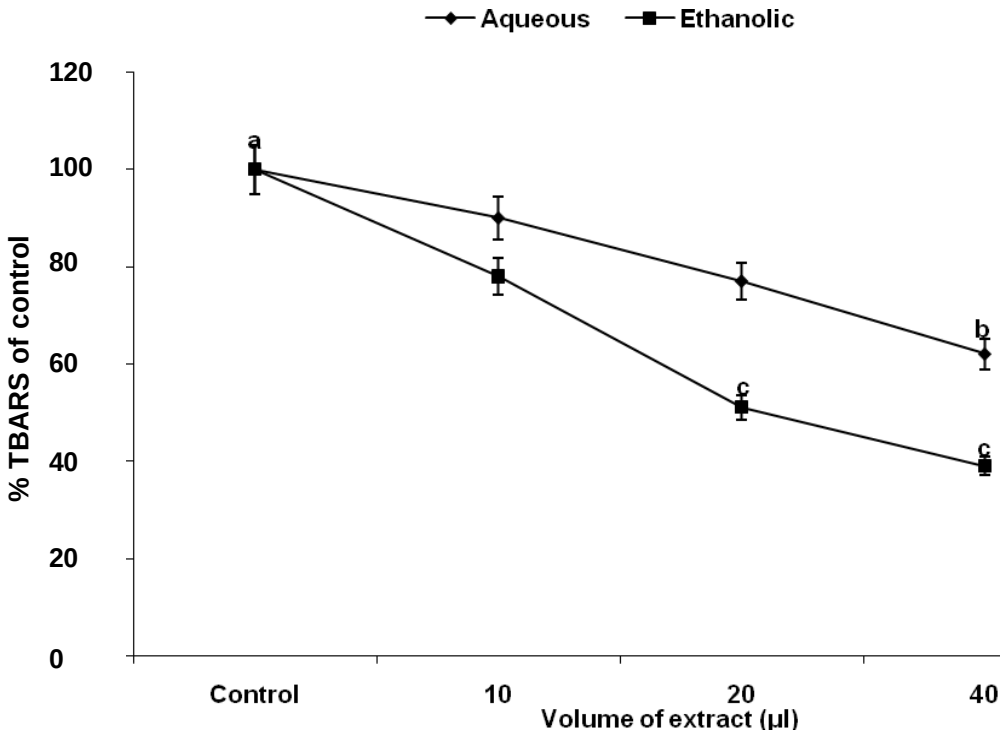

Figure 4b. Inhibitory effect of walnut extract on Fe^{2+}-induced cerebral lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

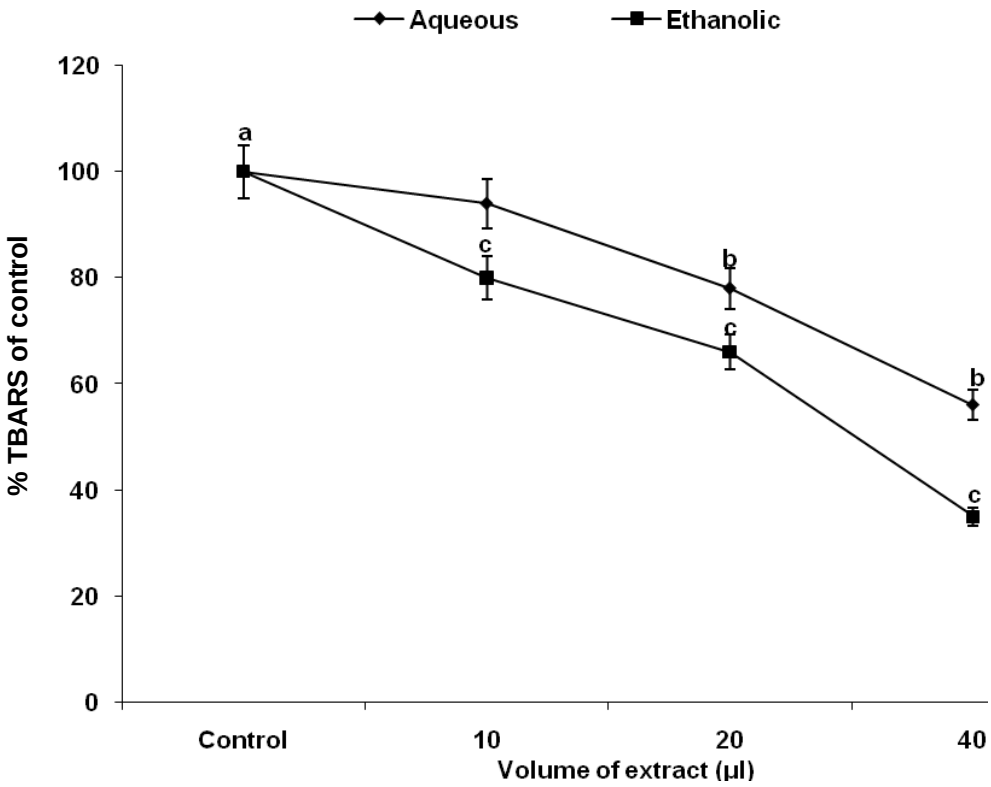

Figure 5a. Inhibitory effect of walnut extract on SNP - induced hepatic lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

as traditional remedy for ailments has been from antiquity. Hence, efforts at identifying and exploiting potentially beneficial plants are on the increase. Although several authors have reported the potential benefits of walnut (Curb et al., 2000; Morgan and Clayshulte, 2000; Edwards et al., 1999) however, little is known about the mechanisms involved in its *in vitro* antioxidant effects. In view of this, we intend to unravel the antioxidant mechanisms of walnut. The phytochemical constituents of the extracts of the seed of *J. regia* show that it is rich in phenolic acids, flavonoids and Vitamin C (Table 1). However, a more striking observation is that the ethanolic extract contained significantly higher phytochemical constituents (Phenolics, flavonoids and ascorbic acid) than aqueous extracts. Although the reason for this observation is largely obscure, it could be due to solvent effect. In this respect, it could be speculated that most of the phytoconstituents that confer antioxidant properties on walnut are more soluble in ethanol than water, hence they are extracted more in the presence of ethanol, leading to a higher phenolics, flavonoid and ascorbic acid content than the aqueous. The presence of these phytochemicals confer some antioxidant properties on plants which could be assessed by several *in vitro* antioxidant parameters.

One of the *in vitro* antioxidant parameters used for testing the potency of plants is their ability to scavenge free radicals. The mechanism involves the protonation of the unstable 2, 2-diphenyl -1-picryl hydrazyl (DPPH) radicals turning it to stable diamagnetic molecule which is visually noticeable as a discoloration from purple to golden yellow. Interestingly, walnut seed extracts demonstrated marked radical scavenging activity (Figure 1). However, ethanolic extract showed a significantly higher radical scavenging effect than the aqueous extract. While the reason behind this observation is still not completely understood, it could be attributed to the difference in the chemistry of the two solvents. Meanwhile, the antioxidant properties of plants are largely dependent on the phytochemical constituents. For instance, phenolics and flavonoids are commonly known for their antioxidant activity. They modify the body's reactions to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory, antimicrobical and anticancer activity (Balch and Balchi, 2000), and may be useful in therapeutic roles (Jisika et al., 1992). From the foregoing, it would be rational to expect that the extract with a higher content of these phytochemicals would exhibit higher antioxidant properties. Hence, the ethanolic extract was a Better radical scavenger than aqueous

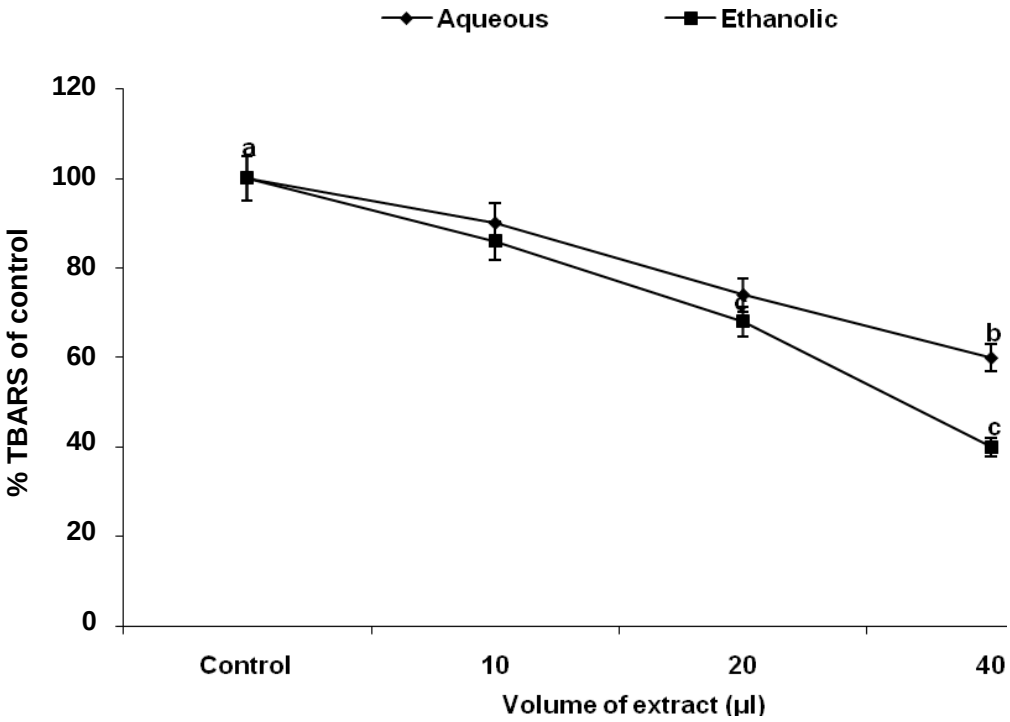

Figure 5b. Inhibitory effect of walnut extract on SNP - induced cerebral lipid peroxidation. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$.

probably due to its higher phytochemical content. Moreover, the ability of agents to chelate transition metals specifically iron (II) has been considered a defence mechanism of antioxidant agents. Interestingly, Figure 2 showed that only ethanolic extract demonstrated a marked iron (II) chelating effect. This may be related to its higher phenolic and flavonoid content since phenols and flavonoids have been reported to be good chelators of iron (Omololu et al., 2011). Hence, the presence of these secondary metabolites must have conferred potent iron chelating property on the ethanolic extract.

Another antioxidant mechanism of agents is their ability to deactivate and reduce transition metals. Similar to other observations, ethanolic extract demonstrated potent ferric reducing power than the aqueous (Figure 3). The reason may be hypothesized to be related to its higher phytochemical constituents. Earlier reports have shown that phenols and flavonoids exhibit potent ferric reducing power (Omololu et al., 2011). Hence, the presence of these phytochemicals must have been responsible for the higher reducing power.

Furthermore, lipid peroxidation, a type of oxidative degeneration of polyunsaturated lipids, has been implicated in a variety of pathogenic processes (Slater, 1972; Dianzani and Ugazio, 1978). The reaction involves the formation of thiobarbituric acid reactive substances (TBARS). Hence, antioxidants are assessed based on their ability to prevent the formation of TBARS via lipid peroxidation. However, since proxidants differ in their mechanism of causing oxidative havoc to macromolecules specifically lipids, a good antioxidant should be able to inhibit proxidant-induced lipid peroxidation regardless of the proxidant employed. Hence, two proxidants were employed in this study to investigate the antioxidant potentials of *J. regia* to offer protective benefits to lipids subjected to several oxidative assaults.

Meanwhile, iron has been reported to cause deleterious effect on biological macromolecules by reacting with superoxide anion ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) to produce the hydroxyl radical ($OH^{\bullet}$) via the Fenton chemistry (Graf et al., 1984). These radicals can also lead to the formation of other reactive oxygen species (ROS) (Klebanoff et al., 1992). Interestingly, Figure 4a and b respectively showed that *J. regia* extract exhibited marked inhibitory effect against Fe^{2+} -induced cerebral and hepatic lipid peroxidation. Although, both extracts demonstrated marked inhibitory effect against TBARS formation, ethanolic extract showed a higher inhibitory effect probably due to the its higher phenolics and flavonoid content. Better still, it could be speculated that *J. regia*, being a good iron chelator must have prevented the oxidation of iron (II), thereby preventing the generation of hydroxyl radical and inhibiting oxidative

assault in the process. Moreover, reports have shown that sodium nitroprusside (SNP) elicit cytotoxic effect through the release of cyanide and/or nitric oxide (NO) (Rauhala et al., 1998). NO has been implicated in the pathophysiology of strokes, traumas, seizures and Alzheimer's, and Parkinson's diseases (Castill et al., 2000; Prast and Philippou, 2001). Besides, light exposure promotes the release of NO from SNP through a photodegradation process (Arnold et al., 1984; Singh et al., 1995), and data from the literature have shown that after the release of NO and SNP, $[NO–Fe–(CN)^{5-}]^{2-}$ is converted to iron containing $[(CN)5–Fe]^{3-}$ and $[(CN)^{4-}Fe]^{2-}$ species (Loiacono and Beart, 1992). After the release of NO, the iron moiety may react with SNP, which could lead to the formation of highly reactive oxygen species, such as hydroxyl radicals via the Fenton reaction (Graf et al., 1984). The fact that *J. regia* extract inhibited SNP- induced lipid peroxidation (Figure 5a and b) may indicate that extracts possibly prevented the breakdown of SNP to its constituents thereby offering protective shield to both cerebral and hepatic tissues since the toxic constituents are presumably prevented from being released.

From the foregoing, the antioxidant properties of walnut could be wholly attributed to its phytochemical constituents. Since it has been earlier reported that walnut is rich in alkaloids, flavonoids, tannins, saponnins and glycosides; and also possess antibacterial properties as well as physiological activity (Sofowora, 1993). All these phytochemical constituents among others could be speculated to confer such potent antioxidant effect on its extracts and could be said to be responsible for its widely reported therapeutic relevance. Hence, walnut remains a potential candidate with potent antioxidant properties that could help in the management of degenerative diseases in which oxidative stress has been implicated in their etiology.

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