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Full Length Research Paper

Antioxidant properties of free and bound phenolic extract of the leaves of *Jatropha tanjorensis in vitro*

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The study is aimed at investigating the antioxidant properties of the free and bound phenols of the leaves of *Jatropha tanjorensis*. Antioxidant properties of the leaves were determined by various assays. Results showed that the free (FP) and bound phenols (BP) content were 294 and 432 mg/g (GAE), respectively while the flavonoid content of (FP) and (BP) were 32.72 ± 1.37 and 77.24 ± 4.45 mg/g (QE), respectively. Further results show that both FP and BP demonstrated potent but dose-dependent free radical scavenging activity against both hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Moreover, both phenolic extracts displayed significant ($P < 0.05$) Fe(II)-chelating and ferric reducing properties even at the least dose used. Finally, both extracts demonstrated a significant ($P < 0.05$) inhibitory effect against lipid peroxidation intentionally induced by Fe^{2+} in rat's brain and liver. From the foregoing, it is rational to attribute the wide usage of the leaves of *J. tanjorensis* in folkloric medicine to its high phenolic content. Hence, information from this study could be exploited in the global fight against degenerative diseases, whose etiology has been linked to oxidative stress.

Key words: Free phenols, bound phenol, antioxidant, free radical, oxidative stress.

INTRODUCTION

Polyphenols are a wide and complex group of secondary plant metabolites which are essential for the physiology of plants, having functions in growth, structure, pigmentation, pollination, allelopathy, and resistance for pathogens and predators (Harborne, 1986; Bravo, 1998; Manach et al., 2004). Polyphenols have attracted the interest of the researchers because of their antioxidant capacity. They have long been recognised to possess anti-allergic, anti-inflammatory, antiviral and anti-proliferative, anticarcinogenic and antioxidant (Harborne, 1994; Frankel et al., 1993; Rice-Evans et al., 1996; Robards et al., 1999; Sharma et al., 1994; Stavric, 1994). Reports have shown that there is an inverse relationship between the intake of flavonoids and the risk of coronary heart disease (Hertog et al., 1993a, 1995; Knekt et al., 1996), stroke (Keli et al., 1996), lung cancer (Knekt et al., 1997; Le Marchand et al., 2000), and stomach cancer (Garcia-Closas et al., 1999).

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In view of the recognition of the potent antioxidant properties of polyphenols, researchers have been tailoring their efforts towards identifying plants with potent antioxidant properties that could be exploited for the management of degenerative diseases. Meanwhile, *Jatropha tanjorensis* has received a lot of attention due to its potential health benefits, availability and affordability (Omoregie and Osagie, 2007; Omobuwajo et al., 2011). *J. tanjorensis* have also been shown to exhibit antibacterial activity (Iwalewa et al., 2005). In fact, earlier reports have shown that *J. tanjorensis* is rich in antioxidant nutrients like phosphorus, selenium, zinc and vitamins C and E (Omobuwajo et al., 2011). Meanwhile, there is dearth of information on the contribution of the phenolic component of the plant to its antioxidant properties. From the foregoing, it is pertinent to unravel the contribution of the phenolic component of the plant to its widely reported pharmacopotency, with a view to gaining insight into the underlying mechanism behind the vast therapeutic relevance of *J. tanjorensis*. Interestingly, there is a link between phytochemical constituents of plants and their antioxidant effect. Hence, information from this study

could act as a panacea in the global combat against degenerative diseases, especially if they are exploited for therapeutic purposes.

MATERIALS AND METHODS

Chemical reagents

Thiobarbituric acid (TBA) was obtained from Sigma (St. Louis, MO). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 1,10-phenanthroline and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Fluka Chemie and Merck (Germany). All other chemicals were obtained from standard chemical suppliers and were of analytical grade.

Plant

J. tanjorensis leaves were collected from Ikare-Akoko, Ondo State, Nigeria, in November 2010 and identified at the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria.

Preparation of samples

The leaves of *J. tanjorensis* were air-dried and powdered with the aid of a blender. Extraction of the soluble free phenols (FP) was conducted as reported previously by Chu et al. (2002) with minor modifications. Dry powder (2 g) was solubilized in methanol-water (80:20, v/v), sonicated and homogenized at room temperature for 1 h 30 min. The solution was filtered through Whatman filter paper, using a Buchner funnel under vacuum. The filtrate was then evaporated using a rotary evaporator (Bibby RE-200B) under vacuum at 40°C to obtain the FP extract. On the other hand, bound phenols (BP) was extracted according to the method of Krygier et al. (1982) with slight modifications. Briefly, residues recovered from the extraction of FP were dried and hydrolyzed with 4 M NaOH at room temperature under shaking. The mixture was acidified to pH 2 with concentrated HCl, extracted four times with ethyl acetate, pooled and evaporated at 40°C to dryness under vacuum to yield BP extract. The yield of extraction was calculated as follows:

(DWe / DWs) × 100

Where, DWe is the dry weight of extract after evaporation and DWs, the weight of the dry powdered leaf.

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 content

The total phenol content of the extracts was determined according to Singleton et al. (1999), using gallic acid as standard. Appropriate dilutions of FP and BP were oxidized with 2.5 ml of 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45°C and the absorbance was measured at 765 nm. The amount of phenol present in the extracts was expressed as gallic acid equivalents (GAE).

Determination of total flavonoid content

The total flavonoid content of both extracts was determined as reported by Meda et al. (2005) with slight modification. Briefly, 0.5 ml of FP and BP was mixed with 0.5 ml methanol, 50 µl of 10% $AlCl_3$, 50 µl of 1 M potassium acetate and 1.4 ml water and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm and the total flavonoid was calculated using quercetin as standard, and expressed as quercetin equivalent (QE).

Free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH radical was evaluated as described by Gyamfi et al. (1999). Briefly, an appropriate dilution of FP and BP (1 ml) was mixed with 1 ml of 0.4 mM methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm. The DPPH free radical scavenging ability was subsequently calculated with respect to the reference (which contains all the reagents without the test sample).

Reducing property

The reducing property was determined by assessing the ability of FP and BP extract of *J. tanjorensis* to reduce $FeCl_3$ solution as described by Pulido et al. (2000). Briefly, FP and BP 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 2 0 min, thereafter 250 µl 10% trichloroacetic acid (TCA) 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% (w/v) $FeCl_3$, the absorbance was later measured at 700 nm, a higher absorbance indicates a higher reducing power.

Fe^{2+} chelating property

The Fe^{2+} chelating ability of FP and BP extract of *J. tanjorensis* was determined using a modified method described by Puntel et al. (2005). Freshly prepared 500 µmol/L FeSO4 (150 µl) was added to reaction mixture containing 168 µl of 0.1 mol/L Tris-HCl (pH 7.4), 218 µl saline and extract (0 to 4.5 mg/ml). 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 extract).

Hydroxyl radical-scavenging activity

The hydroxyl (OH) radical-scavenging activity of *J. tanjorensis* extracts against deoxyribose degradation was measured according to Halliwell et al. (1987). Briefly, (0.3 to 4.5 mg/ml) were added to a reaction mixture containing 80 µl potassium phosphate buffer (20 mM, pH 7.4), 120 µl 30 mM deoxyribose. Thereafter, 80 µl of 1 mM $FeSO_4$ and H_2O_2 were added to generate OH radicals needed to induce deoxyribose degradation. The reaction mixture was then incubated at 37°C for 1 h after which, 0.8 ml 2.8% (w/v) TCA and 0.4 ml 0.8% (w/v) TBA were added. The mixture was then heated for 20 min, cooled and absorbance measured at 532 nm.

Table 1. Total phenolic and flavonoid content of *J. tanjorensis.*

Sample	%	Yield phenol [mg/g (GAE)]	Flavonoid [mg/g (QE)]
FP	85	294.41±10.2	32.72±1.37
BP	15	432.31±14.7	77.24±4.45

Lipid peroxidation

Lipid peroxidation was measured according to Ohkawa et al. (1979), with slight modification. Rats were decapitated under mild ether anesthesia and the whole brain and liver were rapidly removed, placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged for 10 min at 4000 × g to yield a pellet that was discarded, and a low-speed supernatant (S1). 100 µl of S1 was mixed with a reaction mixture containing 30 µl of 0.1 M Tris-HCl buffer (pH 7.4) and FP and BP extracts (0.3 to 4.5 mg/ml). Lipid peroxidation was initiated by the addition of $FeSO_4$ (final concentration 10 µM) and sodium nitroprusside (SNP) (final concentration 30 µM), and the reaction mixture incubated for 1 h at 37°C. The colour reaction was developed by adding 200 µl 8.1% sodium dodecyl sulphate (SDS), and sequential addition of 500 µl of acetic acid/HCl (pH 3.4) and 500 µl 0.8% TBA. The mixture was then incubated at 95°C for 1 h to produce thiobarbituric acid reactive species (TBARS), a coloured product that was read spectrophotometrically at 532 nm.

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 *J. tanjorensis*

Table 1 showed the yield extract obtained for FP and BP isolated from *J. tanjorensis* leaves. The percentage yield of FP and BP were 85 and 15%, respectively. Meanwhile, the total phenolic content of FP and BP was estimated to be 294.41 ± 10.20 mg/g (GAE) and 432.31 ± 14.71 mg/g (GAE), respectively. Whereas, the flavonoid content of the FP and BP were 32.72 ± 1.37 mg/g (QE) and 77.24 ± 4.45 mg/g (QE), respectively.

DPPH scavenging activity

The DPPH free radical scavenging ability of FP and BP is as shown in Figure 1. One-way ANOVA revealed that both FP and BP demonstrated potent free radical scavenging ability with IC_{50} of 1.1 and 0.92 mg/ml, respectively. Apparently, there was no significant ($P < 0.05$) difference between FP and BP in their free radical scavenging activities.

Fe^{2+} - Chelating ability

The Fe^{2+} - chelating properties of FP and BP extracts of *J. tanjorensis* is as shown in Figure 2. Both extracts showed a marked ($P < 0.05$) but dose dependent Fe^{2+} - chelating effect. Obviously, both FP and BP had the same inhibitory concentration (IC_{50}) value of 1.12 mg/ml, showing that there was no significant difference in their chelating property.

Ferric reducing property

Figure 3 showed the ferric reducing property of FP and BP extracts of *J. tanjorensis*. Apparently, it revealed that there was no significant difference between the reducing power of FP and BP. However, both extracts demonstrated potent ferric reducing power.

Hydroxyl radical scavenging ability

The ability of FP and BP extracts of *J. tanjorensis* to protect deoxyribose against degradation by hydroxyl radical is as shown in Figure 4. One way ANOVA showed that both extracts displayed potent hydroxyl radical scavenging effect.

Lipid peroxidation

The inhibitory effect of FP and BP extracts of *J. tanjorensis* against Fe^{2+}- induced hepatic and cerebral lipid peroxidation is as shown in Figure 5a and b. It shows that FP and BP were able to significantly stem-down the proxidative effects of Fe^{2+}, regardless of the tissue in question. Apparently, Figure 5a and b indicated that there is no significant difference between the inhibitory ability of FP and BP.

DISCUSSION

Globally, researchers are on the lookout for agents that could ameliorate the menace of oxidative stress which has been found as a culprit in almost all human pathologies. This is rather necessary, as the conventional

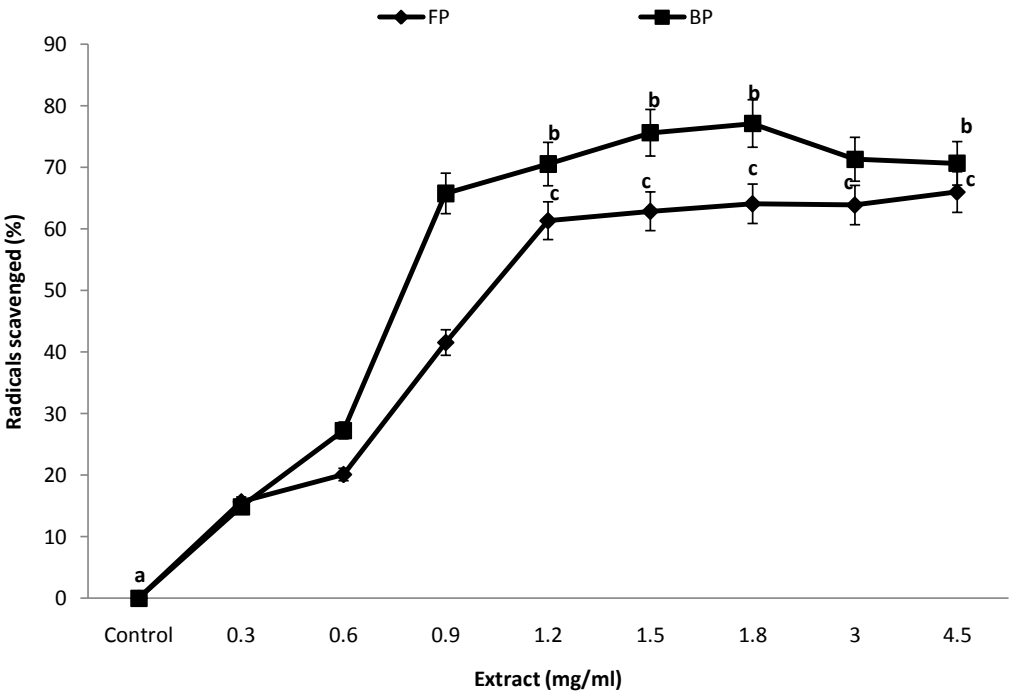

Figure 1. Free radical scavenging ability of FP and BP extract of *J. tanjorensis*. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$. FP indicates free phenol while BP indicates bound phenols.

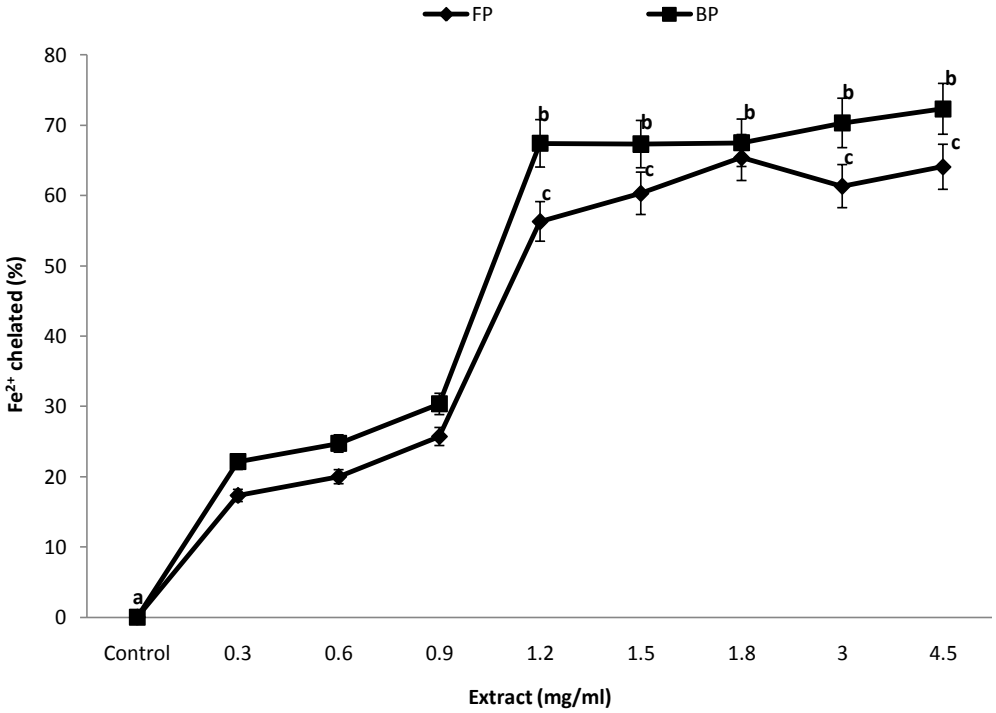

Figure 2. Fe^{2+}-chelating properties of FP and BP extract of *J. tanjorensis*. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$. FP indicates free phenol while BP indicates bound phenols.

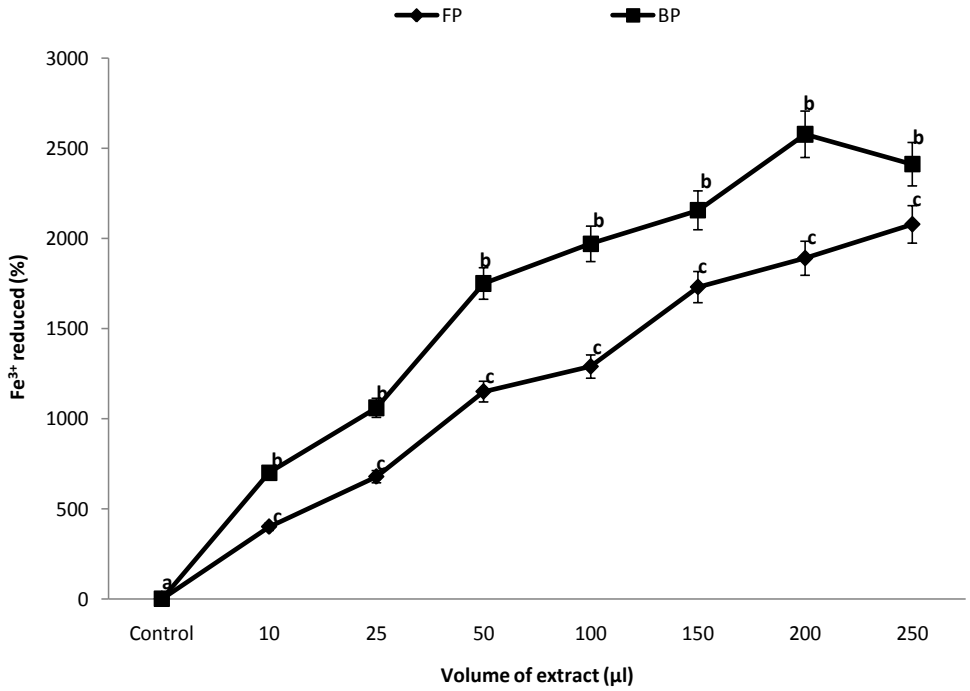

Figure 3. Ferric reducing properties of FP and BP extract of *J. tanjorensis.* Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$. FP indicates free phenol while BP indicates bound phenols.

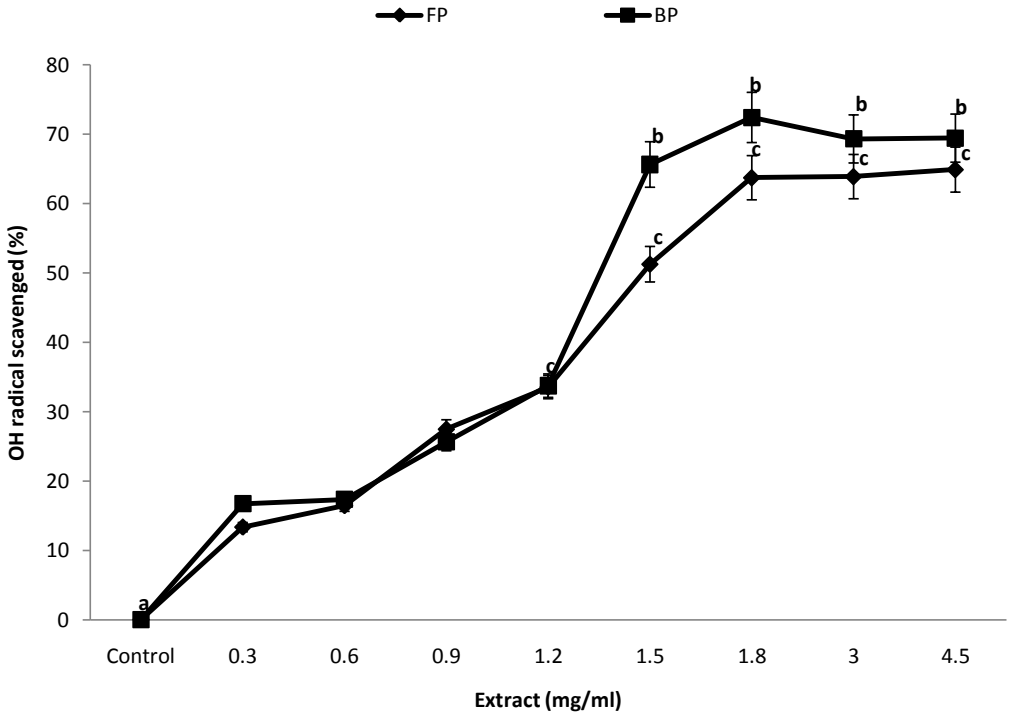

Figure 4. Hydroxyl radical scavenging ability of FP and BP extract of *J. tanjorensis.* Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$. FP indicates free phenol while BP indicates bound phenols.

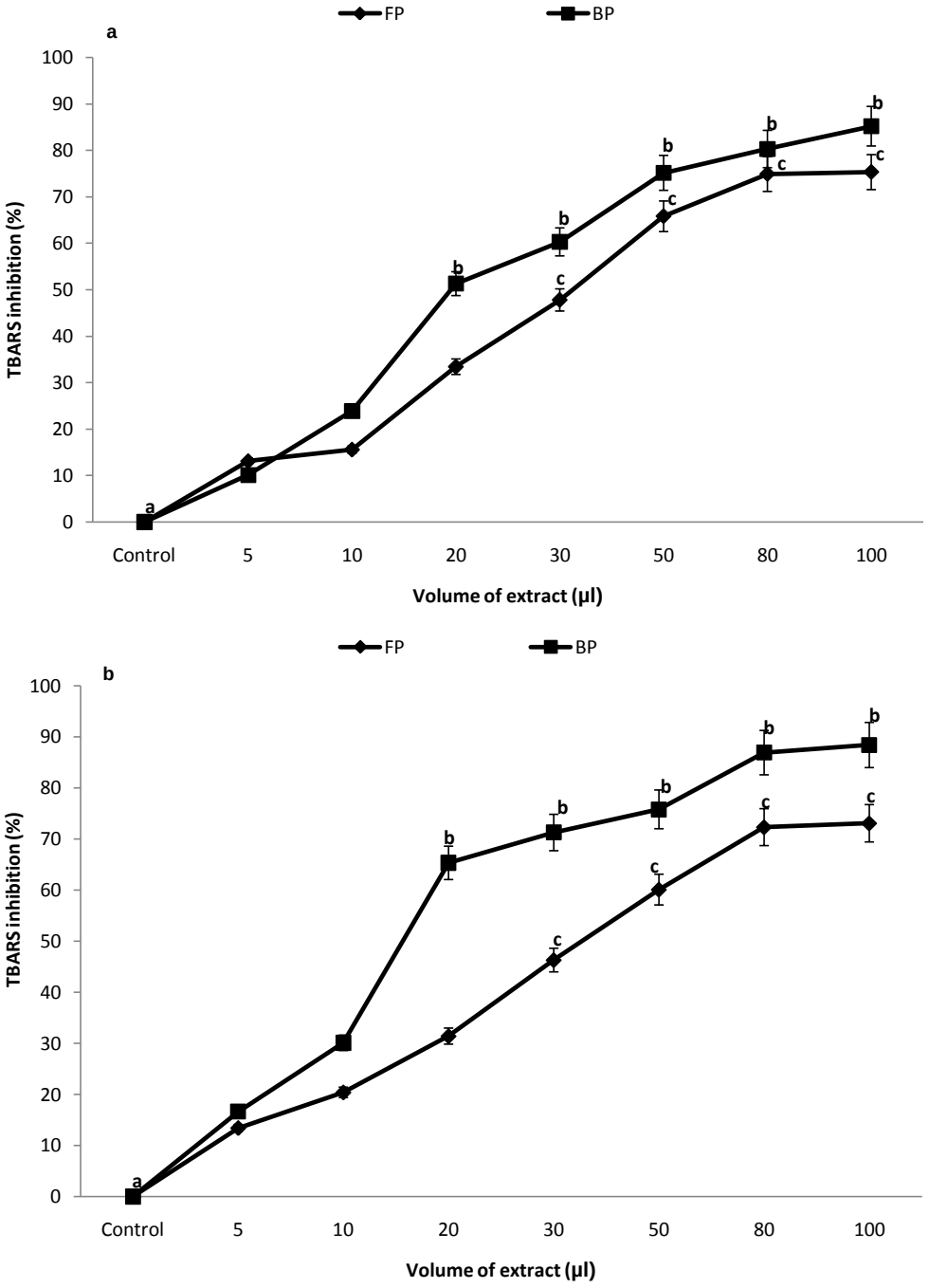

Figure 5. Inhibitory effect of FP and BP extract of *J. tanjorensis* on Fe^{2+}- induced lipid peroxidation in rat liver, (b) inhibitory effect of FP and BP extract of *J. tanjorensis* on Fe^{2+}- induced lipid peroxidation in rat brain. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate. 'b' and 'c' indicate a significant difference from the control 'a' at $p < 0.05$. FP indicates free phenol while BP indicates bound phenols.

synthetic drugs are not easy to come by. Hence, efforts are now being tailored at discovering plants with potent antioxidant properties which could be harnessed and exploited for therapeutic purposes. Of course, the antioxidant properties of plants are intricately related to their phytochemicals. Recently, these bioactive substances, especially the polyphenols, have been found to be responsible for the antioxidant properties of plants (Bravo, 1998; Omobuwajo et al., 2011). Hence, there is an increased interest in the isolation of the polyphenolic

components of plants that could be used in the management of degenerative diseases. Interestingly, Table 1 showed that the leaves of *J. tanjorensis* is rich in both free and bound phenols and flavonoids. This may partly explain the rationale behind its widespread usage in folkloric medicines for the treatment of ailments. However, it is not enough to know what is responsible for the pharmacopotency of *J. tanjorensis* leaves, without unraveling the mechanism involved in its therapeutic effects earlier reported. Hence, we tested the *in vitro* antioxidant properties of FP and BP extracts of *J. tanjorensis,* with a view to gaining an insight into the mechanism(s) involved in its antioxidant action.

Meanwhile, the DPPH radical scavenging activity has been extensively used for screening antioxidants ranging from fruits, cereals and vegetable juices or extracts (Ayoola et al., 2006). Therefore, the ability of the FP and BP extract to scavenge DPPH radicals were investigated and presented in Figure 1. DPPH is an unstable diamagnetic molecule that attains stability through protonation. This stability is visually noticeable by an abrupt discoloration from purple to golden yellow. Figure 1 clearly showed that both FP and BP extracts are potent radical scavengers. Although the reason behind this observation is not completely understood, it is logical to speculate that both free and bound phenols are potent antioxidants, since there was no significant difference between their radical scavenging ability. Furthermore, the observation further corroborates the widely speculated report that one of the mechanisms of antioxidant activity of polyphenols is through radical scavenging (Ayoola et al., 2006). Apart from scavenging radicals, antioxidants could chelate transition metals specifically Fe(II) as a measure of its antioxidant potency. They do this by forming a complex with Fe, thereby preventing the initiation of lipid peroxidation (Oboh and Rocha, 2008). This general metal chelating ability of phenolic compounds is also probably related to the high nucleophilic character of the aromatic rings, rather than to specific chelating groups within the molecule. Interestingly, Figure 2 showed that both extract demonstrated a marked iron chelating effect. This may be related to their high phenolic and flavonoid content since phenols and flavonoids have been reported to be good chelators of iron (Omololu et al., 2011). Besides, the capacity of agents to reduce Fe^{3+} to Fe^{2+} has been used as a measure of the antioxidant efficacy of agents. The mechanism involves donation of proton to Fe^{3+} and its consequent reduction to Fe^{2+} after protonation. Meanwhile, it has been suggested that, the higher the total polyphenolic content, the greater is the antioxidant activity of plant extract (Abu-Amsha et al., 1996).

In line with this assertion, Figure 3 showed that both FPand BP extracts exhibited markedly high reducing power, probably because of their high concentration in the leaves of *J. tanjorensis*. Although the precise reason for this observation is still unknown, it is rational to speculate that reduction is a component of the antioxidant mechanism of *J. tanjorensis*. Moreover, tran-sition metals such as Cu^{+} and Fe^{2+} can interact with H_2O_2 to generate hydroxyl radical through the Fenton reaction (Michalak, 2006). Hence, antioxidants are also assessed based on their ability to prevent the formation and/or scavenge hydroxyl radical. Figure 4 revealed that both extracts significantly scavenged the $^{\bullet}OH$ radical produced. This observation could be attributed to the high phenolic content of the leaves of *J. tanjorensis* which must have shielded Fe^{2+} from interacting with H_2O_2, consequently inhibiting Fenton reaction. Hence, it is pertinent to mention that hydroxyl radical scavenging is one of the components of the antioxidant mechanisms of *J. tanjorensis*. This could probably explain its use as traditional remedies to several pathological conditions.

Furthermore, phytochemicals can act as antioxidants by preventing damages to cell membrane and cellular oxidative processes that may give rise to diseases (van Acker et al., 1998; Oboh and Akindahunsi, 2004). Hence, antioxidants are usually assessed by their ability to offer protective shields to lipids intentionally assaulted with proxidants. One routinely used proxidant is Fe^{2+} since it can catalyze one-electron transfer reactions that generate reactive oxygen species (ROS), such as the reactive OH radical. Despite the proxidative power of Fe(II), Figure 5a and b showed that both FP and BP markedly inhibited Fe^{2+}-induced hepatic (Figure 5a) and cerebral (Figure 5b) lipid peroxidation. This observation could be attributed to the potent Fe(II) chelating effect of both FP and BP, through which they must have prevented the interaction Fe^{2+} with the lipid, thereby halting lipid peroxidation process. From the foregoing, we could trace the wide usage of the leaves of *J. tanjorensis* for the treatment of diseases in folkoric medicine to its high polyphenolic content. Hence, we could speculate that *J. tanjorensis* utilizes several antioxidant mechanisms to elicit its pharmacological effect.

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