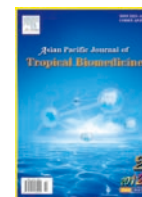




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Phytochemical analysis and antioxidants activities of aqueous stem bark extract of *Schotia latifolia* Jacq

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ABSTRACT

Objective: To evaluate the phytochemical constituents and antioxidant activities of aqueous extract of *Schotia latifolia* (*S. latifolia*) bark locally used for the treatment of oxidative stress-induced ailments in South Africa. **Methods:** The antioxidant and free radical scavenging activity of aqueous extract of the plant was assessed against 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and the ferric reducing agent. Total phenolics, flavonoids, flavonols and proanthocyanidins were also determined to assess their corresponding effect on the antioxidant activity of this plant. **Results:** The activities of plant extract against DPPH, ABTS and NO radicals were concentration dependent with IC₅₀ value of 0.06, 0.05 and 0.05 mg/mL, respectively. The reducing power of the extract was greater than that of butylated hydroxyl toluene (BHT) and ascorbic acid which were used as standard drugs in a concentration dependent manner. The total phenolics content of the aqueous bark extract was (193.33±0.03 TE/g), followed by flavonoids (72.70±0.01 QE/g), proanthocyanidins (48.76±0.00 CE/g) and flavonols (47.76±0.21 QE/g). Phytochemical analysis revealed the presence of percentage tannin (11.40±0.02), alkaloid (9.80±0.01), steroids (18.20±0.01), glycosides (29.80±0.01) and saponins (6.80±0.00). The results exhibited a positive linear correlation between these polyphenols and the free radical scavenging activities. **Conclusions:** Our findings provide evidence that the crude aqueous extract of *S. latifolia* is a potential source of natural antioxidants and this justifies its uses in folkloric medicines.

1. Introduction

Free radicals are chemically unstable atoms that cause damage to lipid cells, proteins and DNA as a result of imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzymes[1]. They are known to be the underlying cause of oxidative stress which is grossly implicated in the pathogenesis of various diseases such as cancer, diabetes, cardiovascular diseases, aging and metabolic syndrome[2,3]. Examples of these radicals include superoxide anions, hydroxyl, nitric oxide and hydrogen peroxide radicals. These radicals can be scavenged by the protective role of natural and synthetic antioxidant agents. Meanwhile, the ingestion of several

synthetic antioxidants such as BHT and BHA has been reported toxic to man[4]. The use of natural antioxidant has gained much attention from consumers because they are considered safer than synthetic antioxidants. Recently, there has been a worldwide trend towards the use and ingestion of natural antioxidants present in different parts of plants due to their phytochemical constituents[5,6]. Oyedemi *et al*[7] attributed the antioxidant activity observed in the aqueous stem bark extract of *S. henningii* to the presence of flavonoids, flavonols, phenols and proanthocyanidins. These compounds have been reported in several studies of medicinal plants to quench free radicals or decompose formation of peroxides owing to the presence of conjugated rings or carboxylic acid[8]. Furthermore, some plant constituents such as saponins, alkaloids, glycosides and tannins have also been documented to exhibit various biological activities including anti-inflammatory, anti-atherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial and antiviral activities[9]. However, majority of

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these plants have not been investigated for their antioxidant potency.

Schotia latifolia (*S. latifolia*) Jacq (Fabaceae), commonly known as bush boer-bean belongs to the genus *Schotia*. It is a tree which grows up to 3 m high in dry and scrubby habitat but may reach 15 m when growing in moist areas. The plant is found in bush-veld, scrub, forests and forest margins, in the Western and Eastern Cape of South Africa^[10]. The bark of *S. latifolia* is locally used for the management of heartburn, hangover and diarrhoea^[11]. It is also used in livestock for the management of red-water disease^[12,13]. The infusion of the extract was recently reported by the traditional healers for the management of obesity, diabetes, hypertension, chest pain and arthritis^[14].

To the best of our knowledge, there was no scientific report to give credence to the ethnomedicinal usage of this plant for the management of various ailments. Therefore, the present study was aimed to provide information on the quantitative compositions of the phytochemicals and antioxidant activities of the aqueous extract of *S. latifolia* bark in order to provide scientific basis to justify its therapeutic usage.

2. Materials and methods

2.1. Plant collection

The stem bark of *S. latifolia* was collected in April, 2010 from Amathole Mountain Eastern Cape Province of South Africa. The plant was identified by using scientific literature^[15] and authenticated by Professor Grierson DS of Botany Department, University of Fort Hare, Alice. The specimen voucher (BLESS1/2010) was deposited at the Giffen Herbarium of the University. The bark was oven dried at 40 °C for 14 days and pulverized in a plant mill and stored in an airtight container for further use.

2.2. Preparation of extract

The powdered plant material (20 g) was extracted in 200 mL of distilled water on a mechanical shaker (Labotec Scientific Orbital Shaker, SA) for 48 h. The extract was filtered using a Buchner funnel and Whatman No. 1 filter paper and sterile cotton wool. The filtrate of the extract was quickly frozen at -40 °C and dried for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give the yield of 2.3 g and later reconstituted in distilled water to give the required concentrations needed in this study.

2.3. Chemicals

1, 1-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS), butylated hydroxyl toluene (BHT), rutin, potassium persulphate, sodium nitroprusside, hydrogen peroxide, sulfanilic acid,

glacial acetic acid, naphthylethylenediamine dichloride, gallic acid, tannic acid and quercetin were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Other chemicals used including ferric chloride, HCl, Dragendorff's reagent, methanol, chloroform, H₂SO₄, Folin-Ciocalteu reagent, Na₂CO₃, vanillin, aluminium chloride, potassium acetate, phosphate buffer, K₃Fe(CN)₆, trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA) were purchased from Merck, USA. All the chemicals used in this study were of analytical grade.

2.4. Determination of total phenolics

The method described by Wolfe *et al*^[16] with little modification by using Folin-Ciocalteu reagent was used to determine total phenolics content in aqueous extract of *S. latifolia* bark. A volume of 0.5 mL of the extract (1 mg/mL), was mixed with 2.5 mL of 10% Folin-Ciocalteu and 2 mL of Na₂CO₃ (75% w/v). The resulting mixture was vortexed for 15 sec and incubated at 40 °C for 30 min for colour development. The absorbance of total phenolics was measured at 765 nm using Hewlett Packard, UV/visible light. Total phenolics content was expressed as mg/g tannic acid equivalent (TE) using the expression from the calibration curve $Y=0.121 6x$, $R^2=0.936 512$, where x is the absorbance and Y is the tannic acid equivalent in mg/g. The experiment was conducted in triplicate and the results were expressed as mean±SD values.

2.5. Determination of total flavonoids

Total flavonoid was determined using the method of Ordonez *et al*^[17] on the formation of a complex flavonoid-aluminium. A volume of 0.5 mL of 2% AlCl₃-ethanol solution was mixed with 0.5 mL of the extract (1 mg/mL). The resultant mixture was incubated for 1 h for yellow colour development which indicated the presence of flavonoid. The absorbance was measured at 420 nm using UV-VIS spectrophotometer. Total flavonoid content was calculated as quercetin equivalent (mg/g) using the equation obtained from the curve $Y=0.255x$, $R^2=0.981 2$, where x is the absorbance and Y is the quercetin equivalent.

2.6. Determination of total flavonols

Total flavonols content was determined using the method of Kumaran and Karunakaran^[18]. A volume of 2 mL of the plant extract (1 mg/mL) was mixed with 2 mL of AlCl₃ prepared in ethanol and 3 mL of 50 g/L sodium acetate solution. The mixture was incubated at 20 °C for 2.5 h after which the absorption was measured at 440 nm. Total flavonoids content was calculated as quercetin (mg/g) using the following equation based on the calibration curve $Y=0.025 5x$, $R^2=0.981 2$, where x is the absorbance and Y is the quercetin equivalent.

2.7. Determination of proanthocyanidin

Total proanthocyanidin was determined using the procedure of Sun *et al.*[19]. The mixture of 3 mL of vanillin–methanol (4% v/v) and 1.5 mL of hydrochloric acid was added to 0.5 mL from 1 mg/mL of aqueous extract and then vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidin content was expressed as catechin (mg/g) using the following equation of the curve $Y=0.582 5x$, $R^2= 0.9277$, where x is the absorbance and Y is the catechin equivalent.

2.8. Determination of tannins

Tannin determination was done according to the method of AOAC[20] with some modifications. One–fifth gram (0.20 g) of the sample was added to 20 mL of 50% methanol. This was shaken thoroughly and placed in a water bath at 80 °C for 1 h to ensure a uniform mixing. The extract was filtered into a 100 mL volumetric flask, followed by adding 20 mL of distilled water, 2.5 mL of Folin–Denis reagent and 10 mL of 17% aq. Na_2CO_3 was also added and thoroughly mixed together. The mixture was made up to 100 mL with distilled water, then mixed and allowed to stand for 20 min. The bluish–green color developed at the end of the reaction mixture of different concentrations ranging from 0–10 ppm. The absorbance of the tannic acid standard solutions as well as sample was measured after color development at 760 nm using the AJI–C03 UV–VIS spectrophotometer. Results were expressed as mg/g of tannic acid equivalent using the calibration curve: $Y = 0.059 3x - 0.048 5$, $R^2 = 0.982 6$, where x was the absorbance and Y was tannic acid equivalent.

2.9. Determination of saponins contents

The determination of saponins was done following the method of Obadoni and Ochuko[21]. Five grams of fine powder (plant sample) was dispersed in 50 mL of 20% v/v ethanol prepared in distilled water and the mixture was heated over hot water bath at 55 °C for 4 h with continuous stirring. The residue collected after filtration was re–extracted with another 50 mL of 20% ethanol and reduced to 20 mL over hot water bath at boiling temperature. The concentrated solution obtained was shaken vigorously with 10 mL of diethyl ether in a separating funnel; the aqueous layer was collected for purification process and repeated. Twenty millilitre of but–1–ol was added to the filtrate and then washed with 10 mL of 5% w/v aqueous sodium chloride. The whole mixture was heated to evaporation on hot water bath and later oven dried at 40 °C to a constant weight. The percentage saponins content of the sample was calculated using the formula.

$$\% \text{ Saponins} = \frac{\text{Weight of final filtrate}}{\text{Weight of sample}} \times 100$$

2.10. Determination of alkaloids

Alkaloids were quantitatively determined according to the method of Harborne[22]. Two hundred mL of 10% acetic acid in ethanol was added to 5 g powdered plant sample, covered and allowed to stand for 4 h. The filtrate was then concentrated on a water bath to 1/4 of its original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was completed and the whole solution was allowed to settle. The collected precipitates were washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed. The alkaloid content was determined using this formula:

$$\% \text{ Alkaloid} = \frac{\text{Final weight of sample}}{\text{Initial weight of extract}} \times 100$$

2.11. Determination of steroids contents

Steroid content of the plant sample was determined using the method described by Trease and Evans[23]. A portion of 2 mL was taken from a solution of 2.5 g of powdered plant material prepared in 50 mL of distilled water after vigorous shaking for 1 h. The extract solution was washed with 3 mL of 0.1 M NaOH (pH 9) and later mixed with 2 mL of chloroform and 3 mL of ice cold acetic anhydride followed by adding two drops of concentrated H_2SO_4 cautiously. The absorbance of both sample and blank were measured spectrophotometrically at 420 nm.

2.12. Determination of reducing power

The reducing power of the extract was evaluated according to the method of Kumar and Hemalatha[24]. A volume of 1 mL of the extract was prepared in distilled water or BHT or vitamin C (0.2–1.0 mg/mL) and mixed thoroughly with the mixture of 2.5 mL of 0.2 mM phosphate buffer (pH 7.4) and 2.5 mL of $\text{K}_3\text{Fe}(\text{CN})_6$ (1% w/v). The resulting mixture was incubated at 50 °C for 20 min, followed by the addition of 2.5 mL of TCA (10% w/v) and the mixture was centrifuged at 3 000 rpm for 10 min. The upper layer of the solution was collected and mixed with 2.5 mL of distilled water and later with 0.5 mL of ferrous chloride (0.1% w/v). The absorbance was measured at 700 nm against a blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

2.13. Nitric oxide scavenging activity

The method of Ebrahimzadeh *et al.*[25] was used to determine the antiradical activity of aqueous bark extract of *S. latifolia* against nitric oxide radical. A volume of 2 mL of sodium nitroprusside prepared in 0.5 mM phosphate buffer

saline (pH 7.4) was mixed with 0.5 mL of plant extract or BHT or rutin at various concentrations (0.2–1.0 mg/mL). The mixture was incubated at 25 °C for 150 min. An aliquot of 0.5 mL of the solution was added to 0.5 mL of Griess reagents [(1.0 mL of sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthyethylenediamine chloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was then measured at 540 nm. The amount of nitric oxide radical was calculated using the equation:

NO radical scavenging activity = [(Abs control– Abs sample)/ (Abs control)] × 100, where Abs control is the absorbance of NO radical + methanol; Abs sample is the absorbance of NO radical + sample extract or standard.

2.14. DPPH scavenging assay

The method of Shen *et al*[26] was used for the determination of scavenging activity of DPPH radical in the extract solution. A portion of 0.135 mM DPPH prepared in methanol containing 0.002–0.5 mg of the plant extracts and standard drug (BHT and rutin). The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the plant on DPPH was calculated using the equation:

DPPH scavenging activity (%) = [(Abs control – Abs sample)/(Abs control)] × 100, where Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample extract or standard.

2.15. ABTS scavenging activity

The scavenging activity of plant extract against ABTS radical was determined by following the method described by Re *et al*[27]. The stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in equal volumes were allowed to stand in the dark for 12 h at room temperature. The resultant ABTS⁺ solution was diluted by mixing 1 mL of freshly prepared ABTS⁺ solution to obtain an absorbance of (0.076±0.001) units at 734 nm after 7 min. The percentage inhibition of ABTS⁺ by the plant extract was calculated and compared with BHT and rutin. The percentage inhibition of ABTS⁺ by the plant extract was calculated using the equation:

ABTS scavenging activity = [(Abs control– Abs sample)/ (Abs control)] × 100, where Abs control is the absorbance of ABTS⁺ radical + methanol; Abs sample is the absorbance of ABTS⁺ radical + sample extract or standard.

2.16. Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates and were subjected to analysis of variance using the student Minitab release version 12, Windows 95. Significant levels were

tested at $P < 0.05$.

3. Results

The stem bark extract of *S. latifolia* was evaluated for the phytochemical constituents which revealed the percentage composition of tannins, alkaloids, saponins, steroids and glycoside to be (11.40±0.02), (9.80±0.01), (6.80±0.00), (18.20±0.01) and (19.33±0.03), respectively as shown in Table 1. Total phenolics (193.94 TE/g), flavonoids (72.69 QE/g), flavonols (47.67 QE/g) and proanthocyanidins (48.76 CE/g) contents were also presented in Table 1. Among the phytochemicals evaluated glycoside and steroids showed prominent contents while alkaloids and saponins contained the least contents. The plant extract showed high concentration of phenolics content followed by flavonoids whereas both flavonols and proanthocyanidins exhibited similar concentrations. The high content of phenolics compounds in this plant may be responsible for the strong antioxidant activity observed in this study.

The antioxidant activity of *S. latifolia* extract was determined by measuring its ability to transform Fe³⁺ to Fe²⁺. The reducing power was confirmed by the changes of yellow colour of the test solution to various shades of green and blue depending on the concentration of the plant extract. The reducing power of the extract and the standard drugs increased with an increase in concentration though the plant extract had higher antioxidant activity than the BHT and ascorbic acid used as reference drugs (Figure 1).

Table 1

The phytochemical constituents of aqueous stem bark extract of *S. latifolia* (mean±SD).

Phytochemical constituents	Amount of compound (%)	Extract equivalents
Tannin	11.40±0.02	ND
Alkaloid	9.80±0.01	ND
Saponin	6.80±0.00	ND
Glucoside	29.80±0.01	ND
Steroid	18.20±0.01	ND
Total phenolics (TE/g)	193.33±0.03	Tannic acid
Total flavonoids (QE/g)	72.70±0.01	Quercetin
Total flavonols (QE/g)	47.76±0.21	Quercetin
Total proanthocyanidins (CE/g)	48.76±0.00	Catechin

ND: Not detected; TE: Tannic acid equivalent; QE: quercetin equivalent; CE: Catechin equivalent.

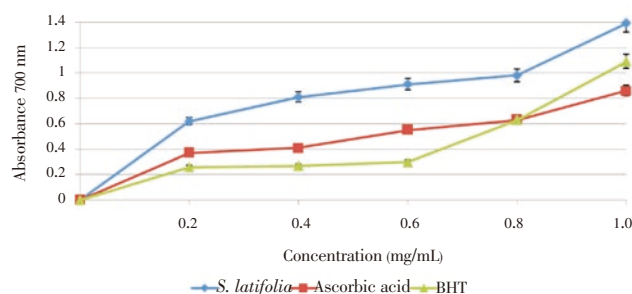


Figure 1. Reducing power activities of the aqueous extract of *S. latifolia* bark in comparison with BHT and ascorbic acid.

The results of DPPH radical scavenging activity of the extract and the standard drugs (BHT, gallic acid and ascorbic acid) were presented in Figure 2. The percentage inhibitory activity of free radicals by 50% has been used widely as a parameter to measure antioxidant activity. In this study, both plant extract and standard drugs significantly reduced the DPPH radical with increasing concentrations. The percentage inhibition of the DPPH radical by the extract, BHT, gallic acid and ascorbic acid at 0.2 mg/mL was 79.14%, 67.91%, 90.83% and 55.20% while the IC_{50} values were 0.126, 0.147, 0.110 and 0.181 mg/mL, respectively. The scavenging activity of the plant extract was comparable to that of gallic acid, BHT and ascorbic acid as shown in this study.

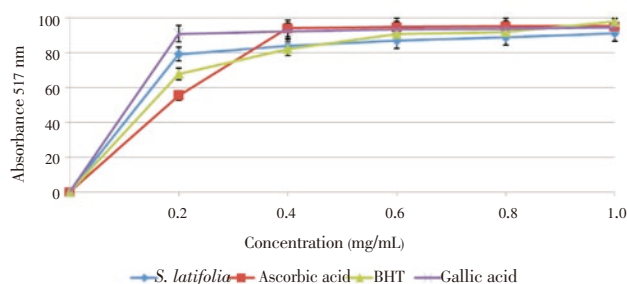


Figure 2. DPPH radical scavenging activities of the aqueous extract of *S. latifolia* bark in comparison with BHT, ascorbic acid and gallic acid.

Figure 3 showed the scavenging activity of *S. latifolia* stem bark extract against ABTS radical in a concentration dependent manner. A comparable scavenging activity was observed between the extract and the standard drugs (BHT and rutin). At 0.2 mg/mL, the percentage inhibition of the extract, BHT and rutin was 94.94%, 84.38% and 85.36%, respectively. The IC_{50} values of the standard BHT and rutin at 0.2 mg/mL were 0.124 and 0.117 mg/mL, respectively while that of the extract was 0.105 mg/mL. Both plant extract and standards recorded high inhibitory activities at all the concentrations tested in an increasing order.

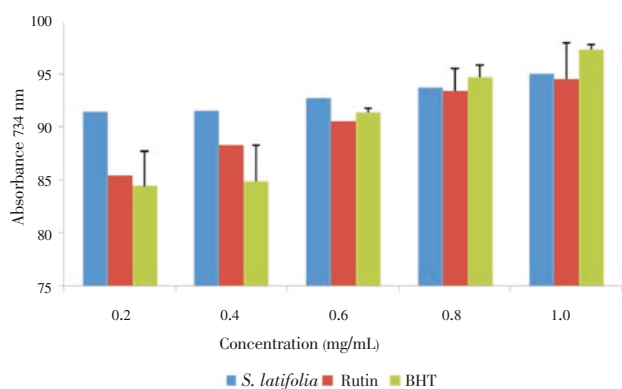


Figure 3. ABTS radical scavenging activity of the aqueous extract of *S. latifolia* bark in comparison with standards (BHT) and rutin.

The scavenging activity of the extract against nitric oxide released by sodium nitroprusside was investigated and the result was shown in Figure 4. The percentage inhibitory activity of the extract, BHT and rutin against nitric oxide

radical was 81.83%, 89.53% and 89.73% at 0.2 mg/mL while the IC_{50} values were 0.150, 0.105 and 0.121 mg/mL, respectively. The inhibitory effect of the extract was comparable to the standard drugs used in this study.

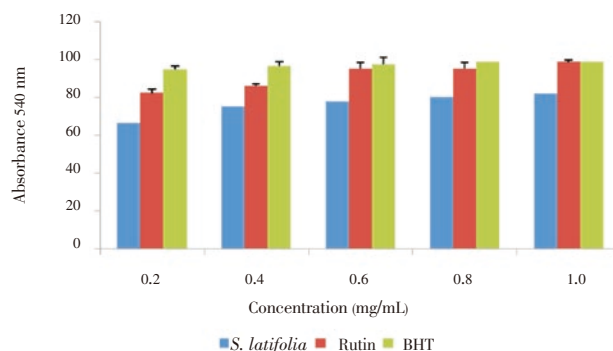


Figure 4. Nitric oxide scavenging activities of the aqueous extract of *S. latifolia* bark in comparison with BHT and Rutin.

4. Discussion

Phenolics compounds are well known as antioxidant and scavenging agents against free radicals associated with oxidative damage[28]. The presence of these compounds such as tannins, flavonoids, proanthocyanidins and phenols in *S. latifolia* extract may give credence to its local usage for the management of oxidative stress induced ailments. Tannins have been used traditionally for the treatment of diarrhoea, hemorrhage and detoxification[14,29]. The composition of tannins as observed in this study may justify its traditional usage for the management of diarrhoea. Flavonoids are important secondary metabolite of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that pharmacological effect of flavonoids is correlating with their antioxidant activities[30]. Furthermore, the ethnomedicinal usage of *S. latifolia* extract might be attributed to the high concentration of flavonoids and therefore it could support its usage for the management of hypertension, obesity and diabetes. The antioxidant activity of proanthocyanidins has been demonstrated to be 50 times greater than vitamin C and 20 times greater than vitamin E[31]. It has also been shown that proanthocyanidins help to protect body from tissue damage, cancer, and to improve blood circulation by strengthening the capillaries, arteries and veins[31–33]. Therefore, the concentration of this compound as shown in this study could contribute synergistically to the significant antioxidant potency of this plant and thus may support the local usage for the treatment of radical related diseases. Alkaloids and saponins have a history of pharmacological effects for their analgesic and antispasmodic effects thus it explains why traditional healers of South Africa used *S. latifolia* extract for the management of chest pain and arthritis among other diseases[34,35].

The reducing power of the extract was evaluated by the transformation of Fe^{3+} to Fe^{2+} through electron transfer ability

which serves as a significant indicator of its antioxidant activity. The reductive activity of the extract and the standard drugs was increased with increasing concentration which is confirmed with increasing absorbance at 700 nm. The antioxidant activity of plant extract was significantly higher than that of the standard drugs used in this study. Findings from this study showed that the antioxidant activity is well correlated with the amount of phenolics constituent found in the extract. Therefore, phenolics compounds as depicted in *S. latifolia* are good electron donors and could terminate the radical chain reaction by converting free radicals to more stable products.

The reaction of plant extract with purple coloured DPPH radical converted the radical to α, α diphenyl- β -picryl hydrazine due to the extract antioxidant property. The degree of discolouration indicates the potential of the plant extract to scavenge free radical due to its ability to donate hydrogen proton. The concentration-dependent curve of DPPH radical scavenging activity of *S. latifolia* compared well with ascorbic acid, gallic acid and BHT used as standard drugs. The result obtained from this study concurred with the findings of Igbiosa *et al*[36–44] and Oyedemi *et al*[7] on *Jatropha carcus* and *Strychnos henningsii*, respectively who attributed the antioxidant potential of these plants to high concentration of phenolics compounds. Consequently, the strong antioxidant activity of *S. latifolia* as shown in the present study might be related to the high contents of phenolics compounds.

ABTS radical is a blue chromophore produced by the reaction of ABTS and potassium persulphate after incubation in the dark environment. The reactions of extract with this pre-formed radical cation discolored the blue chromophore with increasing concentrations. The scavenging activity of ABTS and DPPH radicals by the extract was found to be similar at the highest concentration. This is contrary to the several opinions that plant with DPPH scavenging ability may not inhibit ABTS radical which is due to their different system of preparation and solubility[36–44].

Nitric oxide is a key signalling molecule that played a crucial role in the pathogenesis of various diseases associated with inflammation[45]. It is a free radical generated from sodium nitroprusside in aqueous solution at physiological pH and reacts with oxygen to form oxides of nitrogen. Nitrite is one of the oxides of nitrogen which was significantly inhibited by plant extract through direct competition with oxygen and other oxides of nitrogen in the reaction medium[46]. The scavenging activity of plant extract against nitric oxide formation was comparable to the standard drugs used in this study. This observation gives an indication of strong antioxidant potential of the extract which is confirmed with reducing power, DPPH and ABTS radicals. It can be inferred that the presence and the quantity of antioxidant compounds in *S. latifolia* could justify the observed results. Thus it may give support to the traditional usage of this plant for the treatment of diseases caused by inflammation and cellular damage.

In conclusion, the high antioxidant activity exhibited by *S. latifolia* extract provided justification for the therapeutic use

of this plant in folkloric medicine due to the phytochemical constituents. The present data suggest that this extract could be a potential source of natural antioxidant that could be of great importance for the treatment of radical related diseases and age associated diseases. Further studies are needed to identify the unknown phenolics components to establish their pharmacological properties using appropriate assay models.

Conflict of interest statement

We declare that we have no conflict of interest.

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