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EVALUATION OF ANTIOXIDANT ACTIVITY, TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENTS OF *BREONADIA SALICINA* HEPPER AND J.R.I. WOOD (RUBIACEAE) LEAF EXTRACTS

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ABSTRACT

Medicinal plants are rich in phenolic compounds making them important natural antioxidants capable of combating numerous chronic and degenerative diseases. This research aimed at determining the total phenolic content, total flavonoid contents as well as antioxidant activity of *Breonardia salicina* leaves extracts. Folin-Ciocalteu method was used for the total phenolic and total flavonoid contents determination while the antioxidant activity was determined using 2,2-Diphenyl-1-picrylhydrazyl(DPPH) and2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) that is ABTS free radical scavenging methods. The total phenolic contents of the leaf extracts and fractions were 212.5, 181.7, 201.3 and 123.4 mg/g Gallic acid Equivalence at y = 0.0068x - 0.0228 (R^2 = 0.9992) for the ethanol leaf extract, ethyl acetate fraction, n-butanol fraction and aqueous fraction respectively. The total flavonoid contents at y = 0.0098x + 0.0341 (R^2 = 0.9931) were 58.7, 68.1, 57.7 and 17.1 mg/g Rutin respectively for the ethanol leaf extract, ethyl acetate fraction, n-butanol fraction and aqueous fraction. The antioxidant activity for the ethanol leaf extract, ethyl acetate fraction, n-butanol fraction and aqueous fraction were 205.4, 281.7, 176.3 and 118.7 mg/g Trolox Equivalence respectively using DPPH method at y = 0.9891x - 1.996 (R^2 = 0.9938) and 195.4, 275.8, 196.5 and 143.1 mg/g Trolox equivalence using ABTS at y =0.8039x + 0.2045 (R^2 = 0.9996).The leaf extract and fractions had high phenolic and flavonoid contents which are consistent with the antioxidant activity and therefore,justified the use of the plant in the treatment of many diseases that are known to respond to antioxidantion.

KEYWORDS: ***Breonadia salicina*; Total Phenolic Content; Total Flavonoid Contents; Antioxidant**

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INTRODUCTION

Phenolic acids have been reported to be one of the major contributors to the sensory quality, colour, nutritional and antioxidant properties of the plant kingdom in general and medicinal plant in particular[1]. Phenolic compounds especially phenolic acids and flavonoids are health enriching and found mostly in plants [2]. They provide plants with their antioxidant properties [3] and prevent conditions triggered by free radicals [4,5], due to their antioxidant activities on the free radicals [6]. Antioxidants are natural or synthetic that intercept damage to cells caused by the free radicals or unstable molecules that the body manufactures in response to environment or pressures [7]. These free radicals, which are referred to as reactive oxygen species (ROS) perhaps, are the major cause of degenerative diseases such as cancer and neurodegenerative diseases [8]. Natural antioxidants are metabolites that are mostly from plant origin [9], and are often phenolic and organic acids [10, 11]. *Breonardia salicina* is an evergreen plant that grows up to 20 m in height [12]. It is commonly called *Matumi* and locally *Kadanyar rafi* by Hausa communities of Northern Nigeria [13]. It is widely distributed in tropical and subtropical parts of the world including but not limited to Saudi Arabia, Yemen, Madagascar, Ethiopia and Nigeria [14]. The plant is ethno medically used to treat cancer, gastrointestinal diseases, fevers, headaches, arthritis, diabetes, inflamed wounds and ulcers [15]. This study was therefore carried out to determine the Total phenolic contents, total flavonoid contents and antioxidant capacity of the leaf extract and fraction of the plant.

METHODS

Collection, Identification and Preparation of the Leaf of *Breonadia salicina*

Breonadia salicina was identified first on the field using its morphological features around *Kudingi* Village, Giwa Local Government Area, Kaduna state, Nigeria. Sample of the plant was then collected and taken to Herbarium Unit of the Department of Botany, Ahmadu Bello University, Zaria for proper identification and authentication. A voucher specimen number of ABU900383 was given. Thereafter, sufficient quantities of the leaf were obtained for further studies. The leaves were washed and all foreign matters removed, air-dried under shade, communited to powder form using pestle and mortar, and then stored in an airtight container.

Extraction of the Plant Material

The dried powdered leaf sample of the *B. salicina* (300 g) was macerated with 1L of 95% ethanol using mechanical shaker (Stuart Scientific Flask Shaker, Great Britain) at 25^0C, 200 rpm for 6 hours. The extract obtained was filtered with a Whatman filter paper No 1, and then evaporated to dryness using rotary evaporator (Buchi Labortechnik) at 50^0C and reduced pressure. The dried extract was weighed and transferred into an airtight container and kept properly in a desiccator for further use.

Fractionation of the Aqueous Ethanolic Extract

The extract (2.5 g) was suspended in 500 mL of water and sonicated at 20 ^{0}C for 10 minutes. Thereafter, n-hexane (300 mL) was extracted with ethyl acetate (300 mL) as described above. The same procedure was repeated using n-butanol. The ethyl acetate, n-butanol and aqueous fractions were concentrated over a water bath, transferred into sample bottles and kept for further use.

Determination of Total Phenolic Content (TPC) of the Extract

The total phenolic content of the ethanol extract (LEE), ethyl acetate fraction (EAL), n-butanol fraction (NBL) and the aqueous fraction (AQL) were determined using modified method [16].The solutions of the extracts standard (gallic acid) and negative control (methanol), each 25µL were added to 125 µL Folin-Ciocalteu reagent (10 x diluted in distilled water) and incubated at room temperature for 5 min. A saturated solution of $NaHCO_3$ (100 µL of 7.5%) was mixed to the reaction mixture in a 96-well plate, incubated for 2 hours. The entire tests were carried in triplicate at different concentrations. Absorbance was measured at 725nm spectrophotometrically using microplate reader (synergy H1, Biotec.). Gallic acid solution (1mg/mL) was used to prepare the standard curve and the result was express as gallic acid equivalent (GAE).

Determination of Total Flavonoid Content (TFC) of Extract

Total flavonoid content (TFC) of the ethanolic extract (LEE), ethyl acetate fraction (EAL), n-butanol fraction (NBL) and the aqueous fraction (AQL) were all determined using method described with rutin as standard. Absorbance was measured at 435nm spectrophotometrically using (microplate reader synergy H1, Biotec.). The total flavonoid content of the samples was expressed as µg rutin equivalent (RE).

Determination of the Antioxidant Activities of Extract

DPPH Radical Scavenging Assay

DPPH radical scavenging activity of the ethanolic extract (LEE), ethyl acetate fraction (EAL), n-butanol fraction (NBL) and the aqueous fraction (AQL) were all determined using a method modified [17]. Absorbance of each solution measured at 517 nm using a microplate reader. Gallic acid was used as the standard (positive control). The percentage of radical scavenging activity was calculated as follows:

$$\text{Inhibition (\%)} = \frac{A\ Control - A\ Sample}{A\ Control} \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the test extracts.

ABTS Radical Cation Scavenging Assay

The ABTS radical cation scavenging of the leaf ethanolic extract (LEE), ethyl acetate leaf fraction (EAL), n-butanol leaf fraction (NBL) and the aqueous leaf fraction (AQL) were all determined using the modified method [18]. The ABTS (7 mM) and potassium persulfate solutions (2.45 mM) were prepared and mixed together, incubated for 8-hours in the dark. The stock solution was then diluted with methanol and its absorbance adjusted to 0.900 (± 0.02) at 745 nm at 30°C. 300 uL (125- 2000ug/mL in methanol) for each of the sample was mixed with the ABTS working solution and measured the absorbance. The percentage scavenging property of the samples and the standard was calculated thus:

$$\text{Scavenging effect (\%)} = \frac{Control\ Absorbance - Sampl\ \ Absorbance}{Control\ Absorbamce} \times 100$$

Statistical analysis

Data were expressed as Mean ± Standard deviation (SD). Mean, Standard Deviation and linear regression R^2values were determined using Microsoft Excel software 2013. One-way analyses of variance (ANOVA) was carried out followed by post hoc test (Dunnett's) using SPSS 17.0 (SPSS. Inc, Chicago IL, USA).

RESULTS

Extraction yield

The 95% ethanol cold maceration of the dried powdered leaf yielded 14.14%.

Fractionation of Ethanol Extracts

Four (4) fractions were obtained, after successive fractionation with three solvents, these are; hexane (HX), ethyl acetate (EA), and n-butanol (NB). The fractions were HXL, EAL, NBL and AQL respectively.

Total Phenolic Contents (TPC)

Total Phenolic contents of the extracts/fractions (LEE, EAL, NBL and AQL) of the *B. salicina* were determined using Gallic acid as the standard phenolic acid. Gallic acid equivalence was calculated for each of the extract/fraction using a standard regression curve of the gallic acid with R^2 value of 0.9992 and (y = 0.0068x - 0.0228), the results are shown in Table 1 below:

Total Flavonoid Content (TFC)

Total Flavonoid contents (TFC) of the extracts/fractions (LEE, EAL, NBL and AQL) of the *B. salicina* were determined using Rutin as the standard flavonoid. Rutin equivalence was calculated for each of the extract/fraction using a standard regression curve of the rutin with R^2 value of 0.9931 and (y = 0.0098x + 0.0341), the results are shown in Table 2 below:

Free Radical Scavenging Activities by DPPH

Free radical scavenging power (Antioxidant property) of the extracts/fractions (LEE, EAL, NBL and AQL) of the *B. salicina* were determined using DPPH method with Trolox as the standard antioxidant. Trolox antioxidant equivalence were calculated for each of the extract/fraction using a standard regression curve of Trolox with R^2 value of 0.9938 and standard equation (y = 0.9891x - 1.996), the results are shown in Table 3 below:

Free Radical Scavenging Activities by ABTS

Free radical scavenging power for the extracts/fractions (LEE, EAL, NBL and AQL) of the *B. salicina* were also determined using ABTS method with Trolox as the standard antioxidant. Trolox antioxidant equivalence were calculated for each of the extract/fraction using a standard regression curve of Trolox with R^2 value of 0.9996 and standard equation (y = 0.8039x + 0.2045), the results are shown in Table 4 below:

DISCUSSION

Breonardia salicina is a popular medicinal plant that has been use for the treatment of many diseases [13]. The medicinal values of the medicinal plants

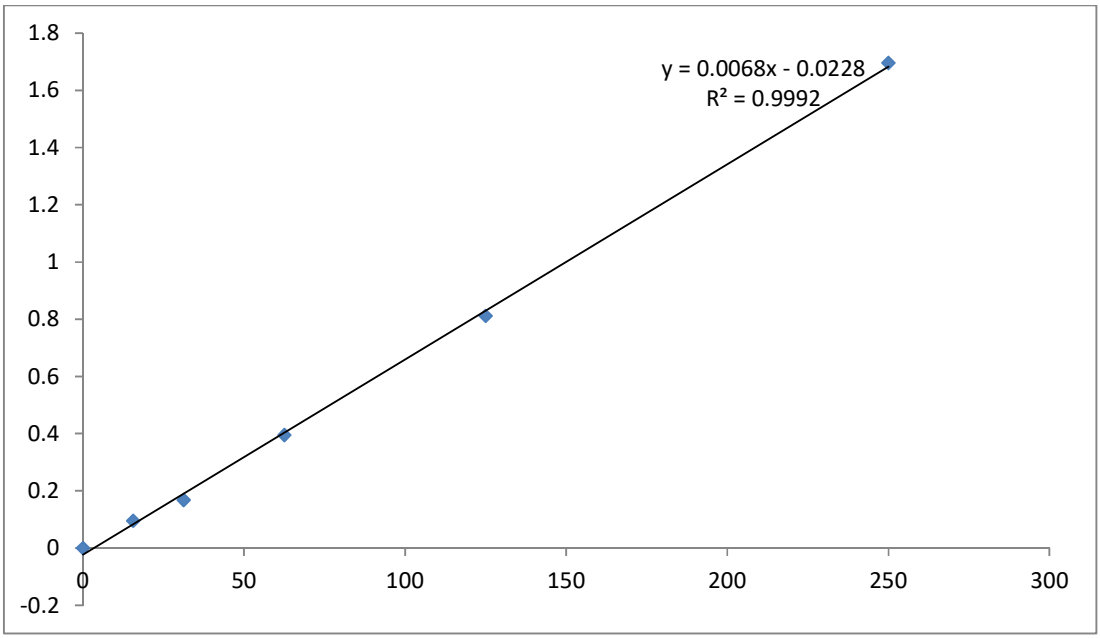

Figure 1: Standard Curve of the Gallic acid for the TPC determination of the extracts

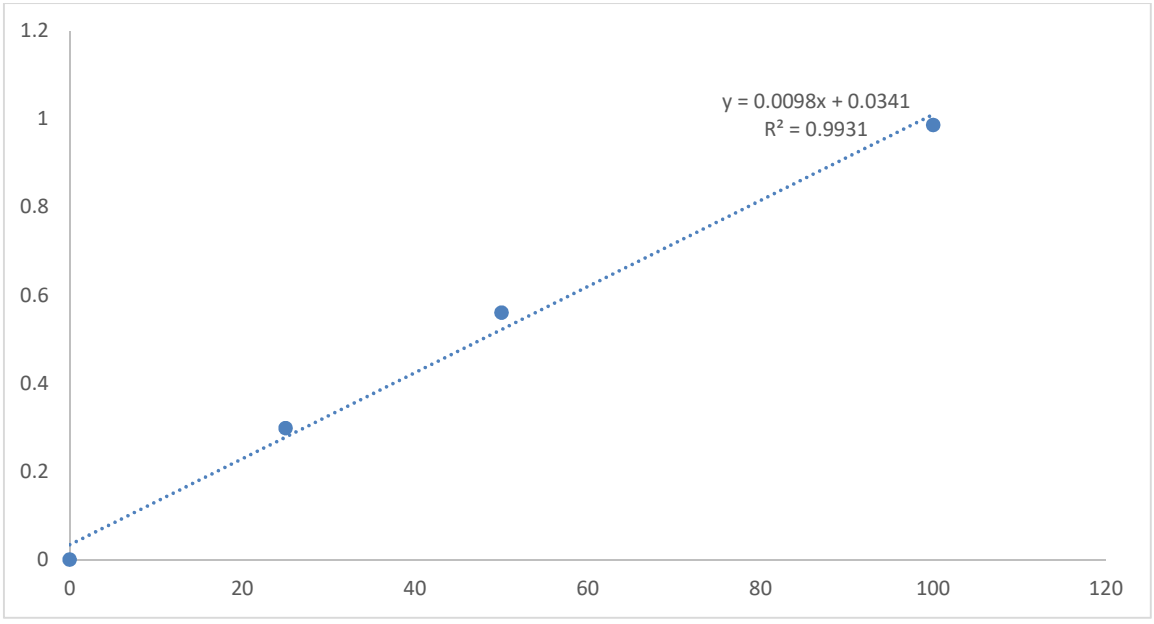

Figure 2: Standard curve of rutin for the TFC determination of the extracts

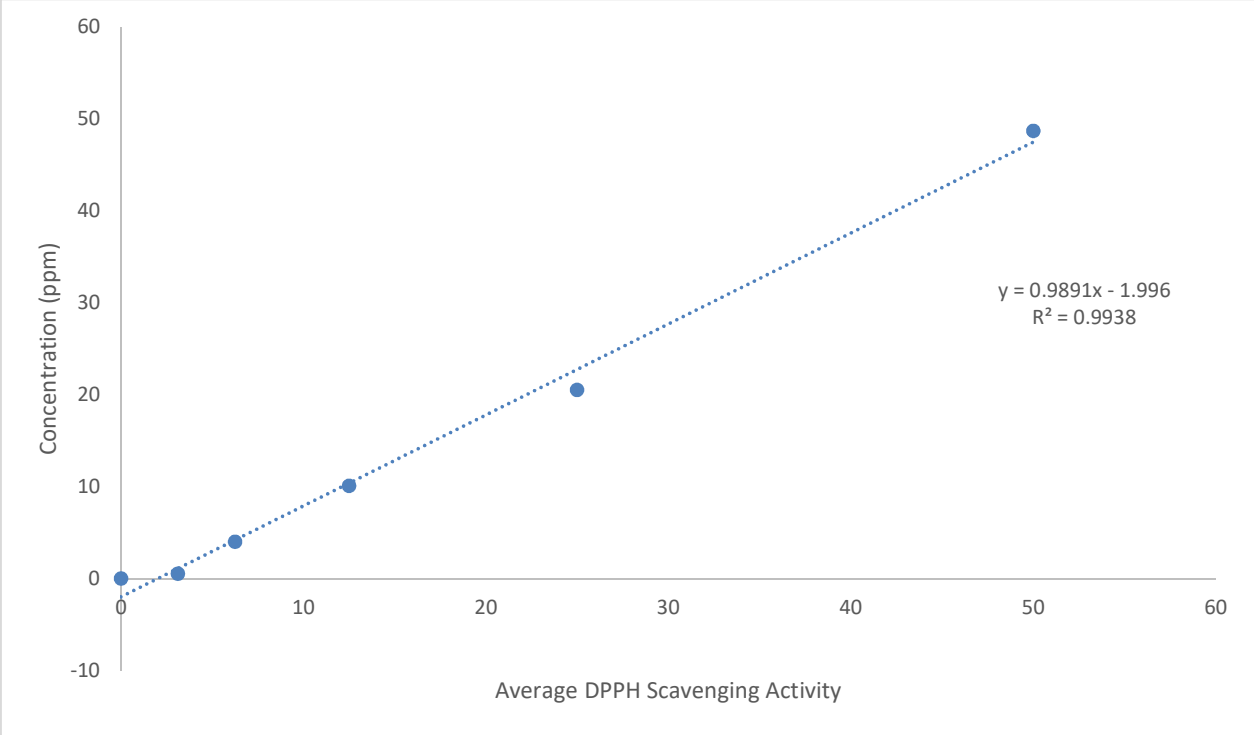

Figure 3: Standard curve of Trolox for the determination of mg equivalence of scavenging activities of the extracts

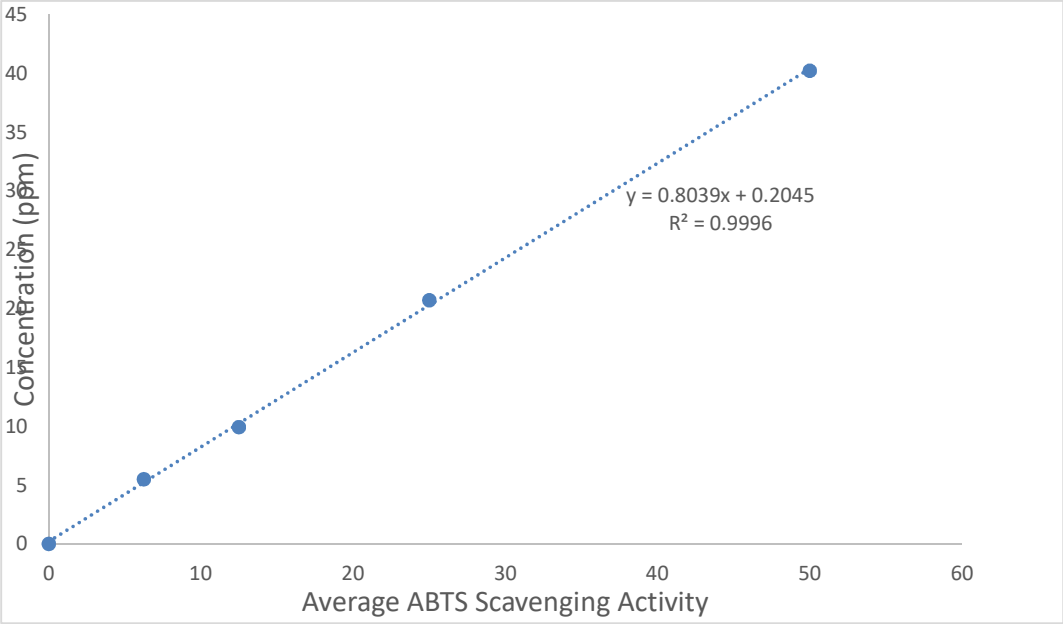

Figure 4: Standard curve of Trolox for the determination of scavenging activities of the extracts and fractions of the leaf of the *B. salicina*

Table 1: **Total Phenolic Contents (TPC) of the extracts**

		mg GAE/g Extract ± SD			
Con µg/ml	0	400	800	1600	P value*
LEE	0.0	51.4±3.5	101.4±2.1	212.5 ± 3.1	<0.005
EAL	0.0	40.9±2.5	93±0.6	181.7 ± 3.9	<0.005
NBL	0.0	49.7±3.5	101.2±3.5	201.3 ± 3.5	<0.005
AQL	0.0	36.9±2.1	72.8±2.9	123.4 ± 6.5	<0.005

Key: Values are means **±** SD of 3 replicates. LEE = 95% Leaf Ethanol Extract, EAL = Leaf Ethyl acetate Fraction, NBL = n-Butanol Leaf Fraction, AQL = Aqueous Leaf Fraction

* All concentrations of the extracts tested had significantly high mg GAE/g Equivalence at $p < 0.05$ using one way ANOVA post hoc (Dunnett's test)

Table 2: Total Flavonoid Contents (TFC) of the extracts

		mg Rutin TFC/g Extract ± SD			
Con µg/ml	0	400	800	1600	P value*
LEE	0.0	13.9±3.1	31.4±3.2	58.7 ± 4.1	<0.005
EAL	0.0	19.5±2.3	37.05±0.6	68.1 ± 2.9	<0.005
NBL	0.0	15.4±2.9	29.4±2.5	57.7 ± 2.5	<0.005
AQL	0.0	3.9±4.1	8.55±1.9	17.1 ± 3.5	<0.005

Key: Values are means **±** SD of 3 replicates. LEE = 95% Leaf Ethanol Extract, EAL = Leaf Ethyl acetate Fraction, NBL = n-Butanol Leaf Fraction, AQL = Aqueous Leaf Fraction

* All concentrations of different extracts had significantly high mg Rutin/g Equivalence at $p < 0.05$ using one way ANOVA post hoc (Dunnett's test)

Table 3: Antioxidant activities of the extracts by DPPH

		mg Trolox Equivalent ± SD			
Con µg/ml	0	200	400	800	P value*
LEE	0.0	55.7±2.5	101.7±2.1	205.4 ± 0.6	<0.005
EAL	0.0	77.2±1.5	141.3±0.6	281.7 ± 0.8	<0.005
NBL	0.0	40.5±2.5	85.4±1.5	176.3 ± 2.8	<0.005
AQL	0.0	28.2±2.1	56.3±1.9	118.7 ± 2.7	<0.005

Key: Values are means **±** SD of 3 replicates. LEE = 95% Leaf Ethanol Extract, EAL = Leaf Ethyl acetate Fraction, NBL = n-Butanol Leaf Fraction, AQL = Aqueous Leaf Fraction.

* All concentrations of different extracts had significantly high mg Trolox/g Equivalence at $p < 0.05$ using one way ANOVA post hoc (Dunnett's test)

Table 4: Antioxidant of the extracts by ABTS

		mg Trolox Equivalent ± SD			
Con µg/ml	0	200	400	800	P value*
LEE	0.0	47.1±1.5	102.4±2.4	195.4 ± 0.8	<0.005
EAL	0.0	69.7±3.5	132.0±0.9	275.8 ± 0.6	<0.005
NBL	0.0	51.7±2.5	91.6±3.1	196.5 ± 1.1	<0.005
AQL	0.0	32.3±1.6	73.5±1.9	143.1 ± 1.8	<0.005

Key: Values are means **±** SD of 3 replicates. LEE = 95% Leaf Ethanol Extract, EAL = Leaf Ethyl acetate Fraction, NBL = n-Butanol Leaf Fraction and AQL = Aqueous Leaf Fraction

* All concentrations of different extracts had significantly high mg Trolox/g Equivalence at $p < 0.05$ using one way ANOVA post hoc (Dunnett's test)

are primarily due to the abundant phenolic compounds present. These phenolic compounds are reported to be major contributors to the sensory quality, colour, nutritional and antioxidant properties of edible food from the plant kingdom [1]. Phenolics and flavonoids are health enriching compounds found mostly in plants, particularly in fruits and vegetables [2]. They provide plants with their antioxidant properties [3] and prevent conditions triggered by free radicals[4,5]. Valued obtained in the studies were in conformity with the reported studies [19]. Antioxidants are natural or synthetic central basics that intercept or mitigates damage to cells caused by free radicals or unstable molecules that the body manufactures in response to environment or pressures [7]. These free radicals, which are refer to as reactive oxygen species (ROS) perhaps, are the major cause of degenerative diseases such as cancer and neurodegenerative diseases [8]. Natural antioxidants are metabolites that are mostly from plant origin and are often phenolic and organic acids [10, 11]. DPPH assay was based on the measurement of the scavenging capacity of antioxidants towards a stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH). The odd electron of the nitrogen atom from antioxidants to the corresponding hydrazine [20]. The DPPH and ABTS results presented as percent scavenging activity. Their scavenging activities increased with increased concentration (Table 3 and 4),same trend was observed and reported [21] where increased in scavenging activity increases with concentration. A better scavenging activity in the DPPH and ABTS antioxidant assays observed with the leaf extracts of the plant. The DPPH radical scavenging activity ranged from; 43.4 mgTrolox Equivalence to 281.7 mgTrolox Equivalence (Table 3). The ABTS antioxidant assay for the extracts and fractions of the leaf of the plant gave values ranging from 97.4 mgTrolox Equivalence to 275.80mgTrolox Equivalence (Table 4). The performance of the extracts and fractions of the plant using the two antioxidants assays shows strong positive correlation. This result is in agreement with the previous studies on a plant of the family [22].

CONCLUSION

The present study suggests that *Breonardia salicina* leaves possesses good antioxidant activity. Thus, the leaf could be a potential source of natural antioxidant that could have great importance as therapeutic agent in preventing or slowing down the progress of oxidative stress related degenerative diseases.

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