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CHEMICAL PROFILING AND ANTIOXIDANT STUDIES ON THE LEAF OF *BREONADIA SALICINA* HEPPER AND J.R.I. WOOD (RUBIACEAE)

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Key words: *Breonadia salicina*, Rubiaceae, antioxidant, flavonoids, phenolic acids.

Abstract

This study evaluated the antioxidant properties of the leaves of *Breonardia salicina* and the profiling of its chemicals. The leaves of the plant are widely used ethnobotanically for the treatment of cancer, gastrointestinal diseases, fevers, headaches, arthritis, diabetes, inflamed wounds, and ulcers. The plant is an evergreen growing along riverbanks, streams, and river tributaries, belonging to the family Rubiaceae. The plant was collected and identified, samples and fractions of the samples were then evaluated for antioxidant activity using DPPH and ABTS and chemical profiling of the sample was performed using LC–ESI-MS/MS. Antioxidant equivalence of the leaf extracts/fractions of the plant at R^2 value of 0.9938 and standard equation (y = 0.9891x - 1.996) was found to be highest at 281.7 ±0.8 mg Trolox Equivalent and lowest at 118.7 ±2.7 mg Trolox Equivalent. The LC–ESI-MS/MS of the sample identified 22 compounds with their structures, belonging to different classes including flavonoids, glycosides, phenolic acids, triterpenoids, amides, and sulphonamides. The identified compounds are of medicinal importance, which is undoubtedly responsible for the antioxidant and anticancer properties of the plant. The good antioxidant values obtained revealed the possible use of the plant in the treatment of many diseases that are known to respond to antioxidation.

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Introduction

Phenolic compounds especially Phenolic acids and flavonoids are health enriching and found mostly in plants (CERVENKA et al. 2018). They provide plants with their antioxidant properties (TUNGMUNNITHUM et al. 2018) and prevent conditions triggered by free radicals (AKINOLA et al. 2014, ZAHID et al. 2018), due to their antioxidant ion on the free radicals (CARVALHO-SIVA et al. 2013).

Antioxidants are natural or synthetic central basics that intercept or mitigates damage to cells caused by the free radicals or unstable molecules that the body manufactures in response to environment or pressures (KASOTE et al. 2015). These free radicals, which are also referred to as reactive oxygen species (ROS) perhaps, are the major cause of degenerative diseases such as cancer and neurodegenerative diseases (LIU et al. 2018). They can be managed using a synthetic or natural approach. The synthetic antioxidants such as vianol and embanox have been useful in managing degenerative free radical complication. However, due to their associated side effect and attendant problems such as accumulation in tissues, people are forced to look for an alternative approach. This issue makes natural antioxidants more popular.

Natural antioxidants are metabolites that are mostly from plant origin (LOURENÇO et al. 2019), and are often phenolic and organic acids (CROZIER et al. 2007, EL-KASHAK et al. 2017). This study therefore was 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 leaves of *Breonadia salicina*

Breonadia salicina was first identified 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 transported to Herbarium Unit of the Department of Botany, Ahmadu Bello University, Zaria for proper identification and authentication. The plant collected identified as *Breonadia salicina* and a voucher specimen number of ABU900383 given and deposited in the Herbarium of the Department. Sufficient quantities of the leaf obtained for further studies. The leaves were garbled and all foreign matters were removed. The material was air-dried in the shade, comminuted into a powder form using a pestle and mortar, and then stored in an airtight container.

Extraction of the leaves of *Breonadia salicina*

The dried powdered leaf sample of the *B. salicina* (300 g) each macerated with 1 L of 95% ethanol using mechanical shaker (Stuart Scientific Flask Shaker, Great Britain) at 25°C, 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 (Büchi Labortechnik) at 50°C and reduced pressure. The dried extract then weighed, and the percentage yield calculated. The extract transferred into an airtight container and kept properly in a dessicator for further use.

Fractionation of the aqueous ethanolic extract of the leaf of *B. salicina*

The extract of the leaf (2.5 g) suspended in 500 mL of water and sonicated at 20°C for 10 minutes. Thereafter, n-Hexane (300 mL) added and then shaken using the mechanical shaker (Stuart Scientific Flask Shaker, Great Britain) at 20°C, 200 rpm for 30 minutes. The mixture was transferred to a separating funnel, allowed to stand and the hexane fraction was collected, and then evaporated to dryness using rotary evaporator (Büchi Labortechnik) at 50°C. The aqueous portion then 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 the antioxidant activities of *Breonadia salicina* leaf extract DPPH radical scavenging assay

DPPH radical scavenging activity 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 method described by (BLOIS 1958), modified by CHAN et al. (2018). Absorbance of each solution measured at 517 nm using a microplate reader. Gallic acid used as the standard (positive control). The percentage of radical scavenging activity was calculated as follows:

$$\% \text{ inhibition} = [(A_{control} - A_{sample})/(A_{control})] \cdot 100,$$

where:
$A_{control}$ – the absorbance of the control
A_{sample} – 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 method described by (Re et al. 1999). 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 μL (125–2000 μg/mL in methanol) for each of the sample mixed with the ABTS working solution and measured the absorbance. The percentage scavenging property of the samples and the standard calculated thus:

$$\text{Scavenging effect [\%]} = [(\text{control absorbance (ABTS)} - \text{sample absorbance} / (\text{control absorbance})] \cdot 100.$$

Liquid Chromatography–Mass Spectroscopy of the ethyl acetate leaf fraction (EAL) of *Breonadia salicina* (LC–MS/MS) analysis sample preparation

The ethyl acetate leaf fraction (EAL) of *B. salicina* leaf (1 mg) dissolved in 1 mL of LCMS grade methanol as the master stock (MS) and 10 μg/mL as the working stock (WS) in methanol before analysis. All samples were filtered with a 0.22 μm PTFE membrane filter and transferred to 2 ml vials for analysis.

Liquid Chromatography–Mass Spectroscopy procedure

The sample analysed using ultra-performance liquid chromatography with high-resolution mass spectrometry (LC–MS/LC–HRM) for the identification of compounds. The method used employed reversed-phase chromatography with a gradient range of solvent strengths. The online high-resolution accurate mass (HRAM) fragmentation library contains highly curated MS/MS and MSn spectra from different collision types and collision energies. Cloud Search was integrated into the compound discoverer along with other tools, such as predicted compositions based on high-resolution full MS and ChemSpider search, that helped partially identify the compounds. Built-in FISH scoring was used to verify hits from ChemSpider against the MS2 data.

Operating conditions

The results of the (LC–MS/LC-HRMS) subjected to the Thermo Scientific Compound Discoverer software version 3.1 for online compound database matching using cloud and ChemSpider.

Results

Extraction of the powdered leaf of *Breonadia salicina*

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

Fractionation of ethanol extracts of the leaf of *B. salicina*

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.

Free radical scavenging activities of the leaves of *Breonadia salicina* 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 ($y = 0.9891x - 1.996$).

Table 1

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 (donnet test).

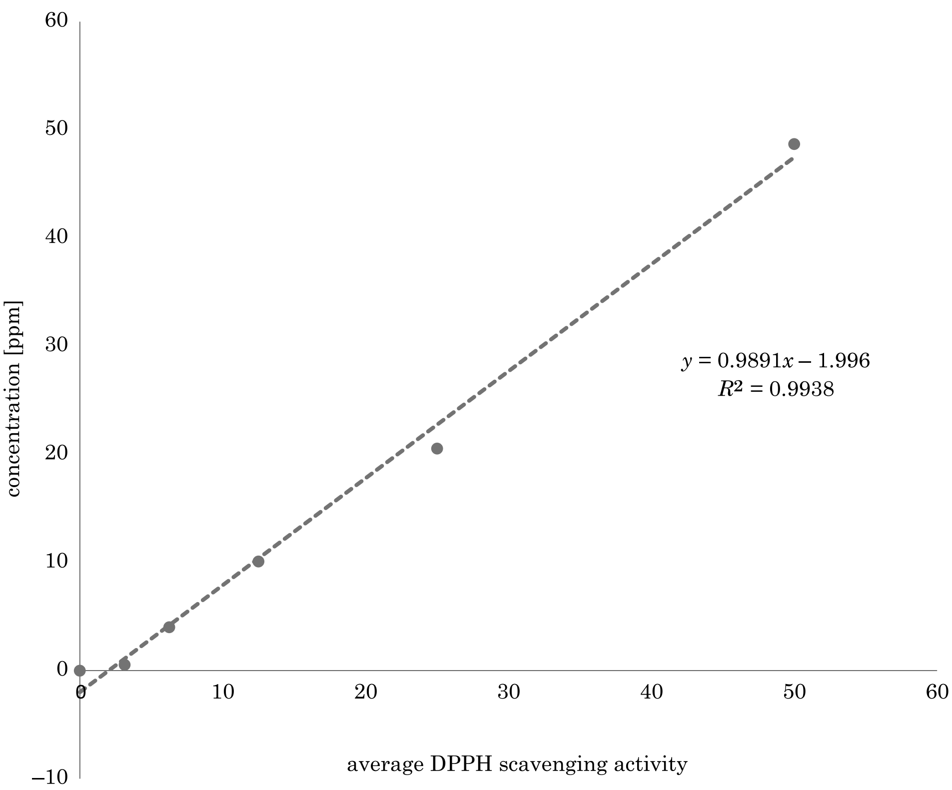

Fig. 1. Standard curve of Trolox for the determination of mg equivalence of scavenging activities of the extracts and fractions for the leaf of *Breonadia salicina*

Free radical scavenging activities for the leaves of *Breonadia salicina* 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 ($y = 0.8039x + 0.2045$) – Figure 2.

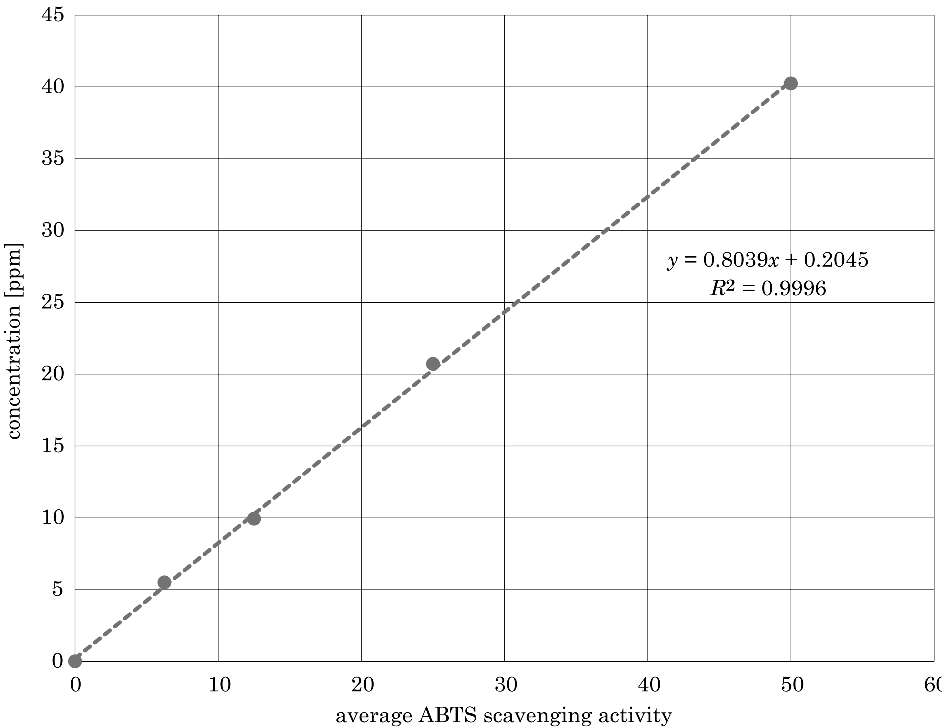

Fig. 2. Standard curve of Trolox for the determination of scavenging activities of the extracts and fractions of the leaf of the *B. salicina*

Table 2

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 (Donnet test).

Isolation of chemical compounds from the ethyl acetate fraction of the *B. salicina* Leaf Using LC–ESI-MS/MS Analysis

Twenty-two (22) chemical compounds were isolated using LC–MS/MS identified with the assistance of Thermo Scientific Compound Discoverer software version 3.1 for online compound database matching using cloud and ChemSpider. The twenty-two (22) compounds with their chemical structures, belonging to different classes of chemical compounds; includes flavonoids, glycosides, phenolic acids, triterpenoids, amides and sulphonamides as shown in Table 3.

Table 3

Chemical compounds of the Ethyl Acetate Leaf fraction (EAL) of *Breonadia salicina* using LC–ESI-MS/MS

S/N	Structure of the compound	Molecular Formula	Name of the compound	Molecular weight	RT [min.]
1	2	3	4	5	6
1.	O, OH, HO, O, OH, HO, OH, O, OH	$C_{16}H_{18}O_9$	chlorogenic acid	354.0944	4.09
2.	HO, OH, H, HO, O, O, H, HO, OH, O, O, OH, O	$C_{17}H_{24}O_{11}$	methyl1 (hexopyranosyloxy) 5-hydroxy-7-(hydroxymethyl) 1,4a,5,7 atetrahydrocyclopenta [c]pyran-4-carboxylate	404.1310	6.04
3.	OH, H, HO, O, O, H, HO, OH, O, OH, OH, O	C_{16} $H_{22}O_{10}$	geniposidic acid	374.1207	3.80
4.	OH, OH, HO, OH, HO, O	C_7 $H_{12}O_6$	D-(-)-Quinic acid	192.0621	4.08
5.	OH, HO, OH, OH, O, HO, O, HO, O, OH, O, O, OH, HO, OH	C_{27} $H_{30}O_{16}$	rutin	610.1532	9.50

cont. Table 3

1	2	3	4	5	6
6.		$C_{17}H_{23}ClN_2O_4$	2-[(2S,3R,4S,5R)-5-{[(3-Chlorobenzyl)amino] methyl}-3,4-dihydroxytetrahydro-2-furanyl]-N-cyclopropylacetamide	390.1159	3.64
7.		$C_{19}H_{30}O_8$	3-Hydroxy-3,5,5-trimethyl-4-(3-oxo-1-buten-1-ylidene) cyclohexyl β-D-glucopyranoside	386.1938	4.47
8.		$C_{25}H_{24}O_{12}$	4,5-Dicaffeoylquinic acid	516.1263	12.29
9.		$C_{36}H_{36}O_{18}$	2-(3,4-Dihydroxyphenyl)-5,-7-dihydroxy-4-oxo-4H-chromen-3-yl 6-deoxy-2-O-{6-O--[(2E)-3-(4-hydroxyphenyl)--2-propenoyl]-β-D-glucopyranosyl}-α-L-glucopyranoside	756.1897	16.62
10.		$C_{21}H_{20}O_{12}$	quercetin-3β-D-glucoside	464.0955	9.63

cont. Table 3

1	2	3	4	5	6
11.	HO, HO, H, HO, H, O, HO, HO	$C_{30}H_{48}O_6$	arjungenin	504.3448	18.64
12.	HO, OH, OH, HO, O, HO, HO, O, O, HO, OH, HO, OH, HO, O, OH	$C_{27}H_{30}O_{15}$	5,7-Dihydroxy-2-(4-hydroxyphenyl)-6,8-bis[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]-4H--chromen-4-one	594.1584	10.35
13.	OH, O, H, O, H, HO	$C_{30}H_{46}O_4$	18-ß-Glycyrrhetinic acid	470.3392	22.44
14.	O, N, N, O, N, N, O, Cl	$C_{21}H_{19}Cl$-N_4O_3	1-Benzyl-8-(4-chloro-3-methylphenoxy)-3,7-dimethyl-3,7-dihydro-1H-purine-2,6-dione	410.1178	6.04
15.	HO, H, H, O, HO	$C_{30}H_{48}O_3$	ursolic acid	456.3595	25.49
16.	HO, H, H, O, HO	$C_{30}H_{48}O_3$	oleanolic acid	438.3490	25.43
17.	S, N, N, N, H, H, O, S, N, H, O	$C_{23}H$-$_{28}N_4O_2S_2$	N-({(2R,4S,5R)-5-[1-Methyl-3-(2-thienyl)-1H-pyrazol--5-yl]-1-azabicyclo[2.2.2]oct-2-yl}methyl)-1-phenylmethanesulfonamide	456.1608	8.90

cont. Table 3

1	2	3	4	5	6
18.		$C_{25}H_{20}N_4O_2$	SSR161421	386.1727	19.07
19.		$C_{19}H_{30}O_8$	3-Hydroxy-3,5,5-trimethyl--4-(3-oxo-1-buten-1-ylidene) cyclohexyl β-D-glucopyranoside	386.1938	4.47
20.		$C_{26}H_{50}O_4$	Bis(2-ethylhexyl) sebacate	426.3703	29.38
21.		$C_{18}H_{15}ClN_8O_2S$	N1-(4-Chlorophenyl)-2-({-4-methyl-5-[(3-pyrazin-2-yl--1,2,4-oxadiazol-5-yl) methyl]-4H-1,2,4-triazol--3-yl}thio)acetamide	884.1550	6.06
22.		$C_{30}H_{50}O_2$	betulin	442.3808	27.15

Discussion

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 (Kasote et al. 2015). These free radicals, which are also referred to as reactive oxygen species (ROS) perhaps, are the major cause of degenerative diseases such as cancer and neurodegenerative diseases (Liu et al. 2018). They can be managed using a synthetic or natural approach. The synthetic antioxidants such as vianol and embanox have been useful in managing degenerative free radical complication. However, due to their associated side effect and attendant problems such as accumulation in tissues, people forced to look for an alternative approach. This issue makes natural antioxidants more popular.

Natural antioxidants are metabolites that are mostly from plant origin (Lourenço et al. 2019), and are often phenolics and organic acids (Crozier et al. 2007, El-Kashak et al. 2017). 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 (Kedare and Sigh 2011). The DPPH and ABTS results presented as percent scavenging activity. Their scavenging activities increased with increased concentration (Table 1 and Table 2). Sawale et al. (2017) observed the same trend of increased scavenging activity with an increase in concentration on the family. A better scavenging activity in the DPPH and ABTS antioxidant assays observed with the ethyl acetate leaf fraction. The DPPH radical scavenging activity ranged from; 43.4 mg Trolox Equivalence to 281.7 mg Trolox Equivalence for the leaf, stem-bark and the root extarcts and fractions. The ABTS antioxidant assay for the extracts and fractions of the leaf, stem-bark and the root of the plant gave radical scavenging effects ranging from 97.4 mg Trolox Equivalence to 275.80 mg Trolox Equivalence. 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 that of Matuszewska et al. (2018). The LC–ESI-MS/MS of the EAL fraction of the *B. salicina* identified 22 compounds with their structures, belonging to different classes including flavonoids, glycosides, phenolic acids, triterpenoids, amides and sulphonamides as shown Table 3. Most of the compounds have been reported to have good antioxidant activities. The identified compounds are of medicinal importance, which are responsible for the antioxidant and anticancer properties of the plant.

Conclusion

The present study suggests that *Breonardia salicina* leaf possesses potent antioxidant activity, which could be due to the various phenolic compounds profiled. Thus, it shows that the leaf may 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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