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Original Research Article

Phytochemical Investigation and *In vitro* Antioxidant Potency of Root Bark of *Brenania brieyi* Fractions

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ABSTRACT

Excessive generation of oxidant species is a leading factor in the pathogenesis of several diseases and disorders. We investigated the phytochemicals and antioxidant activity of methanol, and chloroform fraction of the root bark of *Brenania brieyi* using standard *in vitro* analytical methods. Our results show the percentage proximate composition as moisture (15.33 ± 3.03), ash (4.00 ± 0.02), crude fiber (7.60 ± 0.80), fats (2.00 ± 0.10), proteins (6.65 ± 0.35), and carbohydrate (64.00 ± 1.06). Total phenols were 547.67 ± 5.51; 522 ± 13.32 µg/100 g GAE and flavonoids were 62.60 ± 13.50; 8.29 ± 2.61 mg/100g for methanol, and chloroform fractions respectively. 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity in percentage (%) at 500 µg/mL was: 96.44 ± 0.79; 95.19 ± 1.98, and 54.56 ± 2.06 for methanol fraction, chloroform fraction, and ascorbic acid respectively. Percentage total antioxidant capacity was 85.33 ± 0.89; 54.79 ± 8.21; 65.17 ± 8.27 at 31.3 µg/mL but increased at 1000 µg/mL to 89.32 ± 4.26; 64.47 ± 6.60; 94.51 ± 4.08 for methanol fraction, chloroform fraction, and ascorbic acid, respectively. The reduction of ferric ions by methanol fraction was comparable to gallic acid. Methanol fraction portrayed better antioxidant potential in all the antioxidant models used as revealed from its IC_{50} values of 19.10 and 1.12 µg/mL compared with 26.00 and 11.74 5 µg/mL obtained in chloroform fraction in DPPH, and TAC assays respectively. Our results suggest that *Brenania brieyi* root bark possesses antioxidant activity, and maybe employed as drug target in averting oxidative stress complications.

Keywords: *Brenania brieyi*, Antioxidants activity, Phytochemicals, Proximate components, Oxidative stress.

Introduction

Reactive nitrogen species including nitric oxide, peroxynitrite, and alkylperoxinitrite, and singlet oxygen, superoxide anion, hydroxyl ion, and hydrogen peroxide[1,2] which are reactive oxygen species play remarkable roles in gene expression, receptor activation[3] as well as in cell signaling in both intracellular and extracellular pathways.[1,4] The body is equipped with a wide array of endogenous antioxidant enzymes[1,4,5] which protect the body against tissue injury by regulating the level of oxidant species.[6] Besides, an optimal level of glutathione is a pre-requisite for catalytic activities of other antioxidants.[7] However, under some physiological conditions, the rate of production of the oxidants far overpowers the scavenging capacity of this antioxidant leading to oxidative stress.[2] Oxidative stress has been established as a predisposing factor for the onset and complications of several diseases.[8] Accumulating evidence from several studies attributed the onset of these diseases to oxidation of lipids, proteins, DNA, and RNA.[3]

Despite the approval of usage of many synthetic antioxidants by the US Food and Drug Administration,[2] there is still a remarkable increase in the use of natural antioxidants from plants due to their biocompatibility, less toxicity, and low cost[6,9] coupled with the World Health Organization promotion on the use of herbal drugs.[10]

Plants primary metabolites (including proximate composition) as well as secondary metabolites (phytochemicals) are recently employed as pharmaceutical agents in disease prevention and management.[11-13] Among plant phytochemicals, phenolics and flavonoids have been identified as important antioxidants involved in neutralization of free radicals, quenching of singlet oxygen, chelating of metal ion,[6] and stimulation of expression of endogenous antioxidants[5]. The mechanisms behind the antioxidant potential of phenolics and flavonoids are through hydrogen donation from their hydroxyl group, and subsequent delocalization of the unpaired electrons to maintain stability.[14]

Though the antioxidant activity of several medicinal plants has been studied extensively, many species of herbal plants are still left unexplored. *Brenania brieyi* is a member of the Rubiaceae family found mostly in Cameroon, Nigeria, and Gabon. Traditionally, it is used as a remedy for fever, pain, impotency, swelling, and endocrine disorders.[15] Preliminary spectroscopic studies carried out on root bark of *Brenania brieyi* with FTIR and GC-MS *by Odo et al*.[15] identified numerous compounds such as pentadecanoic acid, 9, 12-hexadecanoic acid, 9-Octadecanoic acid, squalene, and octadecanoic acid which are known to exert antioxidative, anti-inflammatory and immunostimulatory activities. Thus, *Brenania brieyi* is chosen in this study due to the paucity of information on its antioxidant activity notwithstanding the numerous antioxidative compounds found in the plant. In this study, we investigated phytochemicals present in the root bark fractions of *Brenania brieyi* which is the basis of antioxidant potency and also its potential in scavenging free radicals and oxidant species using *in vitro* models.

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Materials and Methods

Chemicals

Analytical grade chemicals were used in this research, and they were sourced from British Drug Houses, England, and Sigma-Aldrich Inc., UK while the standard drugs ascorbic acid and gallic acid were obtained from Qualitex, India.

Plant identification and extraction

The root bark of *B. brieyi* used for this study was collected from Anambra State, Nigeria from June to October 2015. It was identified by a taxonomist Mr. Felix Nwafor of the Department of Pharmacognosy, and Environmental Medicine, University of Nigeria, Nsukka, Enugu State of Nigeria. A voucher specimen of the root bark with identification number PCG/UNN/0327 was deposited in the taxonomist herbarium.

The root bark was shade dried, pulverized, 1793 g was extracted with 6000 mL of methanol, and 3000 mL of chloroform (ratio of 2:1) for 48 h with intermittent shaking. The extract was filtered and shaken with 0.2 volume of distilled water. The two fractions obtained were separated with a separating funnel and evaporated with a rotary evaporator at a temperature of 45ºC before storing in a refrigerator.

Proximate composition

Proximate composition of the plant which measures the amount of moisture, ash, crude fibers, crude fats, proteins, and carbohydrates contents were determined by AOAC.[16]

Total phenols

This was determined with the method of Harbone[17] by measuring the absorbance of a mixture of 1.5 mL of folin ciocalteau reagent and 1.5 mL of Na_2CO_3 (2%) and 0.5 g of each fraction incubated for 15 at 765 nm wavelength. The experiment was done in triplicate.

Total flavonoids

Flavonoid–aluminum complex formation was used to quantify the flavonoid contents of the fractions as described by Harborne[17].

In vitro antioxidant activity

1, 1- diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

The DPPH radical scavenging activity was investigated as described in Koleva *et al*.[18] This was done by adding 1 mL of DPPH (0.078 mM, dissolved with methanol) to 1 mL of each fraction and ascorbic acid (standard) (7.8 – 500 µg/mL also dissolved in methanol) as well as 1 mL of control (methanol). The absorbance was measured at a wavelength of 517 nm with a spectrophotometer after I h incubation period. The experiment was done in triplicate.

The formula below was used to determine the percentage inhibition of DPPH radical activity.

$$\text{Inhibition of DPPH activity (\%)} = \frac{Absorbance of control - absorbance of test}{Absorbance of control} \; x\; 100$$

Total antioxidant capacity

This was determined with Saeed *et al.*[19]method. A volume, 1 mL of reagent solution (prepared by adding 1: 1: 1 of 28 mM sodium phosphate, 600 mM sulphuric acid, and 4 mM ammonium molybdate) was pipetted into 0.1 mL of each fraction (313 - 1000µg/ml), ascorbic acid (standard drug), and distilled water (control). The test tubes were covered with aluminum foil, incubated for 1½ h in a water bath at 95ºC, and the absorbance measured spectrophotometer at 765 nm wavelength. Triplicate determination was done. The control was used to quantify the fraction's total antioxidant capacity (TAC) as follows:

$$\text{Total antioxidant capacity (\%)} = \frac{Absorbance of control - absorbance of test}{Absorbance of control} \; x\; 100$$

Ferric reducing power activity

The efficacy of the fractions in reducing ferric ion to ferrous ion was evaluated by the Oyaizu [20]method. Phosphate buffer (0.5 mL) with pH 6.6 and potassium hexacyanoferrate were pipetted into various test tubes containing the fractions and gallic acids (31.5-1000 µg/mL) while the control test tube contains distilled water in place of fraction/gallic acid. The solution was mixed and incubated at 50ºC for 20 min in a water bath. After incubation, the reaction was stopped with 10 % TCA (0.5 mL) and centrifuged. 1 mL of the supernatant was mixed with 0.1 mL $FeCl_3$ (0.01%). The absorbance of the mixture was taken at 700 nm after 10 min incubation at room temperature.

Statistical analysis

Data obtained from the research work was analyzed by one-way analysis of variance (ANOVA), and post hoc multiple comparisons with the aid of statistical package for social sciences (SPSS) version 18 while IC_{50} values were generated with graph pad prism version 6. Results were presented as mean ± SD with $p \leq 0.05$ taken as the significance threshold.

Results and Discussion

Proximate composition (%) found in the root bark of *B. brieyi* are moisture (15.33 ± 3.03), ash (4.00 ± 0.02), crude fiber (7.60 ± 0.80), crude fat (2.00 ± 0.10), crude protein (6.65 ± 0.35), and carbohydrate (64.00 ± 1.06) as shown in Table 1. The proximate composition of plant materials is important as they show their respective percentages of moisture, ash, crude fiber, crude fats, proteins, and carbohydrates. Studies have reported that these nutrients are of immense benefit to the body: Moisture is known to aid biochemical reactions. Accumulating evidence from previous studies has identified the direct relationship between ash content, and the composition of minerals in food samples.[24] Ash acts as secondary messengers[21]. Crude fiber facilitates the digestion of food, enhances the immune system, [23]and excretion of cholesterols,[22] This could be the basis for the efficacy of crude fiber as a remedy for obesity, diabetes, and gastrointestinal tract diseases.[21] An appreciable low amount of crude fats was observed in the root bark of *B. brieyi*. This was found to be relatively smaller when compared with 3.75 ± 0.30% and 2.88 ± 0.20% seen in raw and fermented roots of *Lagenaria breviflora* respectively by Gbenge *et al*.[24] and 2.27% in roots of *Ageratum conyzoides.*[23] Although fats play an essential role as a precursor of steroid hormones, components of biomembrane, the storage form of fat-soluble vitamins (ADEK), and also regulate body temperature, excess body fats have been reported to have adverse medical, psychological, and social implications[25]. The amount of fats present in food samples is an index of its quality and extent of rancidity.[24] Invariably, the low-fat content seen in the root bark of *B. brieyi* is of immense benefit to minimize the extent of rancidity in oil and also circumvent fat-related complications when consumed.[26] The observed value of crude proteins in our study was lower than what was obtained in raw and fermented roots of *Lagenaria breviflora* respectively by Gbenge *et al.*[24] Proteins help in the growth, and differentiation of cells,[21] while the high carbohydrate content (64.00 ± 1.06%) compared with 36.84% obtained in roots of *Ageratum conyzoides*,[23] makes the root bark good sources of energy.

Results obtained in the phytochemical analysis showed that the fractions contain 547.67 ± 5.51; 542 ± 13.32 µg/100 g GAE of total phenols and 62.60 ± 13.50; 8.29 ± 2.61 mg/100 g flavonoids for methanol, and chloroform fractions respectively (Table 2**)**. Phenols and flavonoids have been reported to have antioxidant activity.[5,6,27,28] Antioxidant rich-foods are required as preventive and management agents for oxidative stress-related disorders.[29] Among the natural antioxidants, polyphenols especially phenols and flavonoids are used as a marker of the antioxidant capacity of foods.[30] Remarkably, polyphenols inhibit pro-inflammatory gene expression which offers protection against inflammatory-related diseases.[31] Specifically, reports from research studies have unveiled the efficacy of phenols in inhibiting the development of cancer, brain disorders, and atherosclerosis while flavonoids are known modifiers of the body's response to viral infection, allergies, inflammation, and cancer[5,32] The

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mechanism behind the action of polyphenol is through stimulation of the transcription factor Nrf2 required for the protection of cells against oxidative damage,[3] regulation of mediators and cell signaling pathways[33] as well as scavenging of radicals.[5] The aromatic rings and hydroxyl groups found in these compounds make them a good hydrogen/electron donor(s).[34] Hence, they might prevent biomembrane oxidation thereby ameliorating oxidative stress-induced diseases.[35] Imperatively, the high amount of these phytochemicals in the fractions might make them good candidate for combating all the aforementioned diseases. Total phenol obtained in our study was higher than 358 ± 1.20, 336 ± 2.52, and 381 ± 1.13 obtained in *Anchomonas diffomis* methanol, acetone, and n-butanol root extracts, respectively.[36] In the same vein, the flavonoids contents in our methanol fraction was higher than that of *Brachychiton populneu* extracts.[37]

Stabilization of DPPH is one of the commonly used models for evaluating *in vitro* antioxidant activity of compounds.[38] The fractions of the root bark of *B. brieyi* at varying concentrations (7.8 -500 µg/mL) had very high DPPH radicals scavenging activity which is comparable with a standard antioxidant drug, ascorbic acid, Optimal activities of 96.44 ± 0.79%, 95.19 ± 1.98% and 54.56 ± 2.06% were recorded for methanol fraction, chloroform fraction and ascorbic acid, respectively at 500 µg/mL (Table 3). The IC_{50} value obtained showed their scavenging activity in this order: Methanol > chloroform > ascorbic acid (Figure 1). This observed inhibition of DPPH activity might be a result of the transfer of electron/hydrogen ions from phytoconstituents found in the plant. Earlier studies have shown that increase in DPPH activity has a positive correlation with polyphenol content of the test substance[37] and could be extrapolated to extent of the radical reduction *in vivo*. Hence, the observed antioxidant potency of the fractions makes them good supplement to working in synergy with the endogenous antioxidant. Root bark fractions of *B. brieyi* scavenged DPPH radical more than methanol and chloroform extracts of *Carica papaya* flowers[30], stem bark extracts of *Irvingia gabonens*,[28] and *Cissus aralioides* leaf extract.[35] However, scavenging activity of *C. tora with* IC_{50} value of 9.898 µg/mL[5] is significantly ($p < 0.05$) higher than 19.10 µg/mL and 26.00 µg/mL observed in methanol and chloroform fractions respectively in the present study.

Activity of methanol fraction increased in concentration-dependent manner from 85.33 ± 0.90% inhibition at 31.3 µg/mL to 89.32 ± 4.26% at 1000 µg/mL in TAC model. However, in chloroform fraction, 31.3, and 61.5 µg/mL had 54.79 ± 8.20% and 58.58 ± 5.60% inhibitory activities, respectively. Notably, a decrease in TAC inhibition was seen at 125 µg/mL (53.09 ± 4.39%), but a subsequent increase in a concentration-dependent manner was recorded from 250-1000 µg/mL. From the R^2, TAC of ascorbic acid had a strong positive correlation (0.92) with an increase in concentration, followed by methanol fraction (0.84), and then chloroform fraction (0.49) (Table 4). Methanol fraction had the highest TAC activity followed by ascorbic acid, and then chloroform fraction as shown in the IC_{50} values (Figure 2). The observed inhibition in this assay portrays the potential of the fractions in reducing Mo (VI) to Mo (V) which is measured in TAC assay.[36]Invariably, this could be anchored on the availability of phenols and flavonoids in the fractions which has been established to have a significant correlation with phosphomolybdenum assay.[37]

In the FRAP assay, methanol fraction also had significantly ($p < 0.05$) higher reducing power compared with the standard (garlic acid), and chloroform fraction (Figure 3). This is an indication of the high potency of methanol fraction in donating electrons required in ferric ion reduction[28]. Interestingly, from the above premise, the high ferric reducing power of *B. brieyi* methanol fraction suggests that it could be effective under physiological conditions, in reducing ferric ion and other oxidant species. This might prevent the induction of oxidative stress by the labile iron pool in the body[39] thereby circumventing other complications of communicable diseases associated with oxidative stress.[40] Also, maintaining iron homeostasis prevents the etiology of neurological diseases associated with iron overload.[41]

Table 1: Proximate composition of the root bark of *B. brieyi*

Parameter	Amount (%)
Moisture	15.33 ± 2.03
Ash content	4.00 ± 0.02
Crude fiber	7.60 ± 0.80
Crude fat	2.00 ± 0.11
Protein	6.65 ± 0.35
Carbohydrates	64.42 ± 1.06

Results are presented as means ± SD (n = 3)

Table 2: Total phenols and flavonoids contents of the root bark of *B. brieyi* fractions

Extracts	Total phenols (µg/100 g GAE)	Total flavonoids (mg/100 g QE)
Methanol	547.67 ± 5.51	62.60 ± 13.50
Chloroform	522.00 ± 13.32	8.29 ± 2.61

GAE = gallic acid equivalent, QE = quercetin equivalent.

Table 3: DPPH radical scavenging activity of the root bark of *B. brieyi* fractions

Conc. (µg/mL)	Methanol fraction	Chloroform fraction	Ascorbic acid
7.8	52.49 ± 5.81^{a}	44.43 ± 7.61^{a}	45.10 ± 4.39^{a}
15.6	50.09 ± 2.71^{a}	44.51 ± 4.68^{a}	42.64 ± 3.64^{a}
31.3	41.60 ± 0.91^{a}	50.18 ± 3.17^{ab}	49.82 ± 4.01^{a}
62.5	57.44 ± 9.08^{b}	47.04 ± 5.74^{a}	51.31 ± 4.76^{a}
125	59.75 ± 3.22^{b}	57.56 ± 2.24^{b}	53.21 ± 2.93^{a}
250	82.70 ± 3.52^{c}	91.22 ± 1.09^{c}	52.50 ± 1.77^{a}
500	96.44 ± 0.79^{c}	95.19 ± 1.98^{c}	54.56 ± 2.06^{a}
R^2	0.67	0.72	0.81

Values are presented as mean ± SD (n = 3) Mean values having different letters down the column are significantly ($p < 0.05$) different while those with the same letters down the column are not significantly different ($p > 0.05$).

Table 4: Total antioxidant capacity of the root bark of *B. brieyi* fractions

Conc.(µg/ml)	Methanol fraction	Chloroform fraction	Ascorbic acid
31.3	85.33 ± 0.89^{a}	54.79 ± 8.21^{a}	65.17 ± 8.27^{a}
62.5	87.23 ± 0.91^{a}	58.58 ± 6.00^{a}	80.44 ± 3.29^{b}
125	86.33 ± 0.41^{a}	53.09 ± 4.39^{a}	89.62 ± 0.35^{b}
250	87.62 ± 1.70^{a}	55.98 ± 8.99^{a}	87.33 ± 1.75^{b}
500	89.30 ± 7.28^{a}	59.18 ± 8.35^{ab}	93.21 ± 5.53^{bc}
1000	89.32 ± 4.26^{a}	64.47 ± 6.60^{b}	94.51 ± 4.08^{c}
R^2	0.84	0.49	0.92

Values are presented as mean ± SD (n = 3) Mean values having different letters down the column are significantly ($p < 0.05$) different while those with the same letters down the column are not significantly different ($p > 0.05$).

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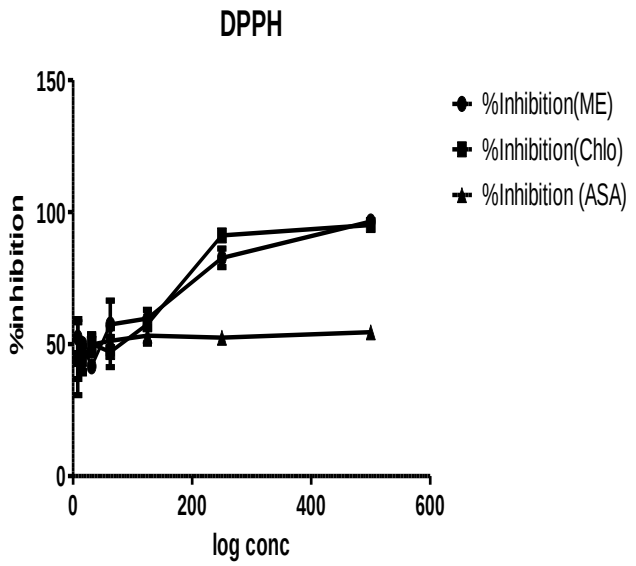

Figure 1: IC_{50} values of DPPH activity of the root bark of *B. brieyi* fractions

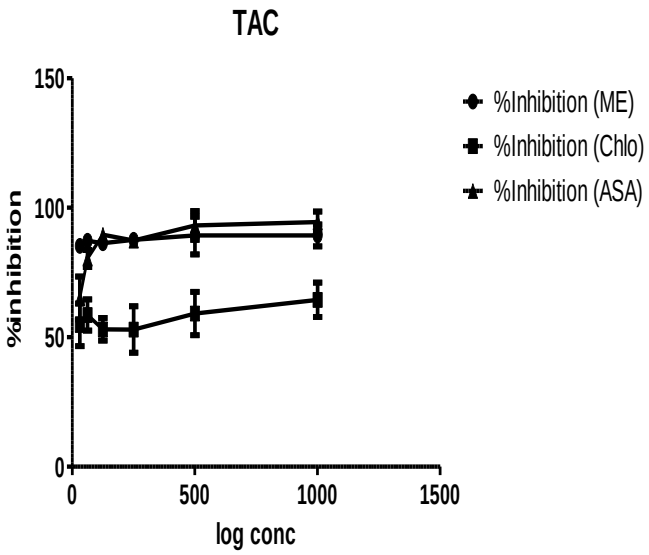

Figure 2: IC_{50} values of TAC activity of the root bark of *B. brieyi* fractions

Note: ME = methanol fraction , Chlo = chloroform fraction while and ASA = ascorbic acid.

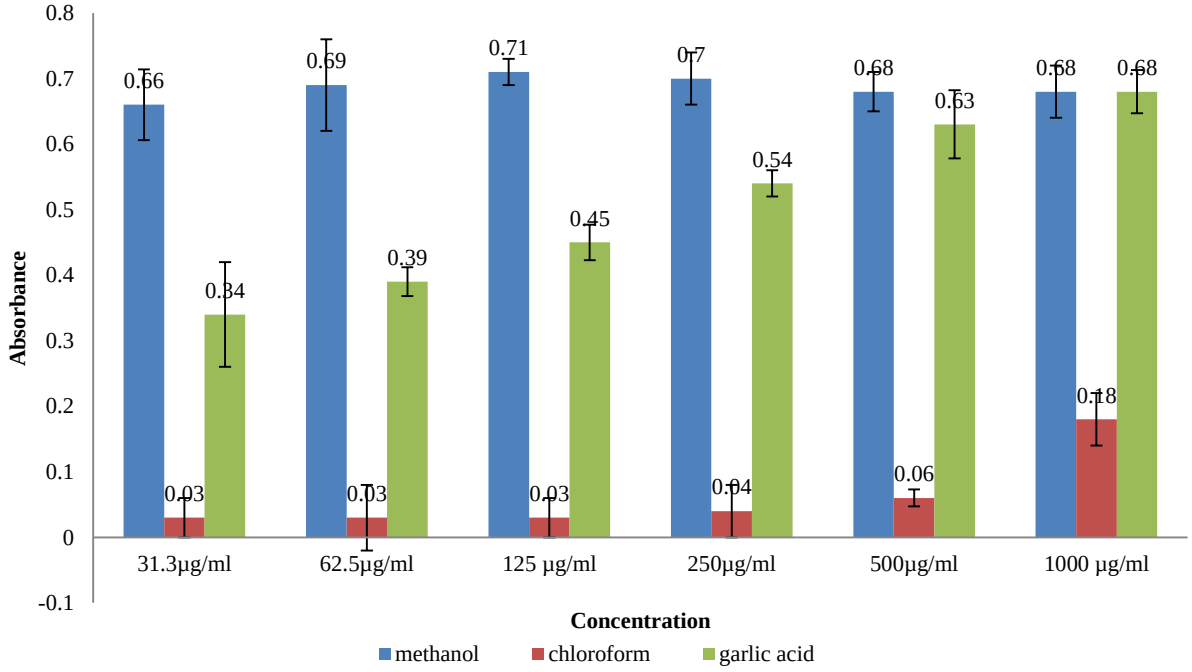

Figure 3: Ferric reducing capacity of the root bark of *B. brieyi* fraction

The antioxidant capacity of root bark of *B. brieyi* fractions could be as a result of its phenols, flavonoids, and other antioxidant compounds earlier identified from the plant through GC-MS.[15] Several studies have established a relationship between antioxidant activity with quantity of polyphenols[42]. FRAP reduction capacity recorded in methanol fraction was significantly lower than what was found in 50-200 µg/ml of *C. tora* leaf by Aryal *et al*.[5] but higher than what was reported in *Anchomonas diffomis* methanol extract.[36]

Conclusion

Results from our study unveiled the antioxidant potential of the root bark of *B. brieyi* fractions. This potency might be due to its phytoconstituents. However, further studies are required to determine its *in–vivo* antioxidant activity and also the exert mechanisms of action.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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