Phytochemical studies, *in vitro* antioxidant and antiproliferative of the stem bark of *Boswellia dalzielii* hutch

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Abstract:

This work aims to evaluate the total phenolic and flavonoid contents, and antioxidant and Antiproliferative activities of the stem bark of *Boswellia dalzielii*. Hundred gram (100 g) of methanolic extract was re-dissolved in 70% methanol and partitioned exhaustively with different solvent hexane and ethyl acetate in a separating funnel; and this method gave three fractions, hexane fraction, ethyl acetate fraction and aqueous extract. The ethyl acetate fraction was subjected to Accelerated Gradient Chromatographic due to its higher activity over the hexane fraction and four sub-fractions were obtained. Standard methods were used to determine flavonoid and phenolic contents of the methanolic, aqueous, ethyl acetate and hexane fractions and their sub-fractions. Standard methods were used to determine flavonoid and phenolic contents of the methanolic, aqueous, ethyl acetate and hexane extracts and their sub-fractions. The antioxidant property of the extracts was determined using DPPH radical scavenging and FRAP assay. Growth inhibitory activity was carried out on the crude extracts and sub-fractions using *Sorghum bicolor* seeds. The phenolic content was found to be highest in sub-fraction C (481.20 ± 10.13 mg GAE/g) and flavonoid contents were found to be highest in methanolic extract (142.17 ± 4.82 mg RE/g). *Boswellia dalzielii* stem bark exhibited antioxidant capacity; and the highest antioxidant activities were recorded from aqueous extract with the IC_{50} 1.58 and methanol extract IC_{50} 1.99 using DPPH. FRAP assay exhibited antioxidant capacity with EC_{50} 1.00 for aqueous extract and sub-fraction D EC_{50} 1.25. The antiproliferative, sub-fractions C and D at 125 µg/ml gave the highest percentage of inhibition (90%) followed by sub-fraction B (50%) at 250 µg/ml. These results further showed that the stem bark of *Boswellia dalzielii* has antioxidant activities and antiproliferative activity on the seeds of *Sorghum bicolor*; and therefore possess likely an anticancer component which needs further anticancer screening.

Keywords: **Antioxidant, Antiproliferative, *Boswellia dalzielii, Sorghum bicolor* seeds, Stem bark, total phenolic content, total flavonoid content**

Introduction

Many plants in Africa have been used as sources of remedies for many diseases. Africans are still keeping this old tradition of using plants extract as remedies for their health needs [1]. *Boswellia dalzielii* Hutch is a plant from the genius *Boswellia* and the family of *Burseraceae*; it is a tree plant that is commonly found in North-Western Nigeria and often used, among the locals population as a source of ethno-medicine [2, 3, 4, 5]. In Cameroon, people use the leaves of *B. dalzielii* to protect maize, millet and *sorghum* against weevils' attacks [6]. The extract of the leaves is used for the treatment of diarrhea and the gum resin of this plant is used locally for fumigation of clothes and houses [7]. The leaf extract is used in the treatment of bilharziasis and it is given to pregnant women in Niger as an oxytocic [8]. The root and the stem bark aqueous extract are used as antidote to snake bite and as arrow poison [9,10]. The root decoction, boiled along with *Hibiscus sabdariffa,* is used for the treatment of syphilis. The root decoction with *Daniellia oliveri* is used on wounds treatment. The gum resin is used along with other medicines as a stomachic and for the treatment of venereal diseases [9]. When burned the stem bark, serves as a fumigant and deodorant [10]. The use of the stem bark of *Boswellia dalzielii* to treat fever, rheumatism and gastrointestinal disorders has also been reported [11, 12]. The stem bark is boiled in large quantities to make a wash for septic sores. It also serves as part of a multi-component prescription for treating leprosy [10, 13]. In Nigeria (Adamawa state), the fresh bark is eaten to induce vomiting and to relieve symptoms of giddiness and palpitations. *Boswellia carteri* Birdw have shown to be a weak antioxidant [14] and it is reported that *boswellic* acids of *Boswellia serrata* Roxb exhibited anticancer activity in different types of cancer like, prostate cancer, skin cancer, brain tumor and blood cancer [15].

WHO recommends the use of plant-based medicines as an alternative medicine; especially in developing countries [14]; plants also contain a variety of phytochemicals such as phenolics and flavonoids, which provide important health benefits [16]. Antioxidants play a very important role to protect the body against oxidative stress and free radical damages which are the cause of various ailments such as diabetes, heart diseases, cancer, brain dysfunction, weakened immune system and many more. In a study done on the antioxidant activity of many plant extracts, it was found that phenolic and flavonoid compounds are mainly responsible for the antioxidant and free radical scavenging effect of the plants [12]. Intake of sufficient amounts of antioxidants is necessary to prevent free radical-induced oxidative stress [16].

Among various medicinal uses of the stem bark of *Boswellia dalzielii* reported, there is no record in the literature about the quantitative antioxidant capacity and antiproliferative activity. Therefore, this study was conducted to determine total phenolic, total flavonoid, antioxidant activities and Antiproliferative activity of the stem bark of *Boswellia dalzielii*.

2. Materials and Methods

2.1. Collection and Identification of Plant

The plant material was collected from Jos, Plateau State, Nigeria during the dry season (between December and March 2015) and was authenticated by comparing with voucher specimen (Number: UJ/PCG/HSP/89B13), deposited at the Herbaria of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Jos, Jos, Nigeria.

2.2. Preparation of Extracts

The bark of *Boswellia dalzielii* collected was chopped into small pieces and air-dried to obtain the dried plant material. A 1kg of the dried plant material was soaked in 3.7 litters of methanol-70%. This was filtered after 24 hours (maceration). The filtrate obtained was evaporated to dryness using Bibby vacuum rotary evaporator (RE 100) to leave 236.0452 g (23.60 %) of crude extract.

2.3. Organic Solvent Partitioning of the Methanol Extract

Hundred gram (100 g) of the extract was re-dissolved in 70% methanol and partitioned exhaustively with solvent hexane (5 X 200ml) in a separating funnel. The hexane layer was collected first, followed by the ethyl acetate. This was repeated until a clear lower layer was obtained. The hexane, ethyl acetate and aqueous fractions were concentrated to dryness on a rotary evaporator and their respective yields noted. After concentration, 2.2457 g (0.22%) of hexane, 17.4318g (1.17%) of the ethyl acetate and 803225 g (8.03%) of the aqueous fractions obtained were kept for further work.

2.4. Accelerated Gradient Chromatography (AGC) of Ethyl Acetate Fraction of the Stem Bark

Fifteen grams (15 g) of ethyl acetate fraction was subjected to Accelerated Gradient Chromatographic procedure using hexane (100%), hexane-ethyl acetate (1:1), ethyl acetate (100%), ethyl acetate-methanol (1:1), methanol (100%) solvent systems gave four (4) sub-fractions noted A, B, C and D which were concentrated to dryness and weights noted. The fractions obtained were concentrated to dryness and the respective weights noted. Analytical thin layer chromatographic analyses of the fractions were carried out on commercial TLC aluminium precoated plate of Silica gel GF254 using; ethyl acetate-hexane-chloroform-methanol (10:20:30:40). After development, the plates were sprayed with anysaldehyde and subsequently heated for 5mins.in an oven maintained at 100°C. The coloured spots were noted and their Rf values were recorded. Similar spots were bulked based on the Rf values, intensity, colour and size of the spots.

2.5. Total Phenolic Compounds Determination

Total phenolic content of the extracts were evaluated by a colorimetric method utilizing Folin-Ciocalteu reagent according to the method described by Adedapo et al. (2008). Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent thereby producing blue coloured complex. The phenolic concentration of extract, fractions and sub-fractions was evaluated from a gallic acid calibration curve. 500 µL aliquots of 10, 20, 30, 40, 50, and 60 µg/mL methanolic gallic acid solutions were mixed with 2.5 mL Folin–Ciocalteu reagent (diluted ten-fold) and 2.5 mL (75 g/L) sodium carbonate.

The tubes were vortexed for 10 sec and allowed to stand for 2 hr at 25 ^{o}C. After incubation at 25 °C for 2 hr, absorbance was measured at 765 nm against reagent blank using the Shimadzu Uv-Vis Spectrophotometer 1650. Japan. Total phenolic content was expressed as mg gallic acid equivalent/g. All determinations were performed in triplicate [17].

2.6. Total Flavonoid Content Determination

The total flavonoid content of the extract was measured by employing aluminium chloride colorimetric assay reported by Huma *et al*., (2014). Aluminium chloride colorimetric method was used for determination of total flavonoid contents. One mL of plant extract was diluted with 4 ml of distilled water in a volumetric flask. Initially 0.3 ml of 5% $NaNO_2$ was added in the mixture. Then at 5th minute 10% $AlCl_3$ (0.3 ml) and at 6th minute 2 ml of NaOH (1M) were added. Total volume made up to 10 ml by addition of distilled water then mixed well. Absorbance of the reaction mixture was measured at 510 nm using a Uv-Vis spectrophotometer (SHIMADZU UV-1650 spectrophotometer, Japan). The total flavonoid content in the plant extract was expressed as milligram of rutin equivalents (RE) per g of extract. All the determinations were carried out in triplicates [18].

2.7. DPPH Radical Scavenging Assay

The antioxidant activity (free radical scavenging activity) of the extract, fractions and sub-fractions (A, B, C, and D) on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were determined according to the method reported by Odumosu *et al*., (2015). The following concentrations of extract were prepared in methanol; 500, 250, 125, 62.50, 31.25, 15.62, 7.81, 3.91, 1.95 and 0.98 µg/mL. 2 mL of each concentration was mixed with 4 mL of 50 µM DPPH solution in methanol in triplicate. The mixture was vortexed for 10 sec to homogenize the mixture and test tubes were incubated for 30 min at room temperature in the dark and the absorbance was measured at 515 nm using Uv-vis spectrophotometer (SHIMADZU UV-1650 spectrophotometer, Japan). Lower absorbance readings of the reaction mixture indicate higher free radical scavenging activity. Gallic acid, ascorbic acid and rutin were used as standards at the following concentrations 100, 50, 25, 12.5, 6.2, 3.1, 1.5, 0.7, 0.3, & 0.1 µM. Blank solutions were prepared by mixing 2 mL of methanol with 4 mL of 50 µM DPPH solutions in methanol. The difference in absorbance between the test and the control (DPPH in methanol) was calculated and expressed as % scavenging of DPPH radical. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\% \text{ of antioxidant} = \frac{Ab - As}{Ab} x\ 100$$

Ab: absorbance of blank
As: absorbance of the sample

Finally, the IC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the separate linear regression plots of the mean percentage of the antioxidant activity against concentration of the test extract (µg/mL) [19].

2.8. Ferric Reducing Antioxidant Power Assay (FRAP).

The FRAP assay was used to estimate the reducing capacity of stem bark of Boswellia dalzielii extracts, according to the method reported by Shwetha (2012); The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl, 2.5 ml of 20 mM $FeCl_3$. $6H_2O$ and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37°C. 900 µL FRAP reagent was mixed with 90 µL water and 30 µL of the extract. The reaction mixture was incubated at 37°C for 30 minutes and the absorbance was measured at 593 nm using Uv-Vis (SHIMADZU UV-1650 spectrophotometer, Japan. The values are expressed as mmol $FeSO_4$ equivalents per gram of sample. The measurements were done in triplicate [20].

2.9. Determination of Antiproliferative Activity of Extract, Fractions and Sub-Fractions on Sorghum Bicolor Seeds.

The method of Ikpefan and Ayinde, (2013) was used for determination of antiproliferative activity on *Sorghum bicolor* seeds. *Sorghum bicolor* seeds obtained from Terminus market, in Jos Plateau State Nigeria was cleansed with absolute alcohol for 1 minute and finally with distilled water and air dried before use. The viability of the seeds was determined by their ability to sink in water. Those that remained submerged in water were removed and dried for use. A 5ml of respective *B. dalzielii* extracts in distilled water at concentration of 500 µg/ml, 250 µg/ml and 125 µg/ml were poured into 9 cm wide Petri dishes laid with cotton wool and filter paper (Whatman No 1). Twenty (20) seeds of sterilized *Sorghum bicolor* were spread in sterile Petri dishes containing cotton wool. The Petri dishes were incubated at room temperature in the dark for 96 hours. After incubating for 96 hours the number of seeds germinated in each Petri dish were counted and recorded. The control seeds were only treated with distilled water containing no extracts. The experiments were carried out in triplicates while the Statistical analyses were carried out using SPSS statistics T-test (version 20).The percentage of seeds germinated at 96 hours was calculated as:

$$\% \text{ seed germination} = \frac{NGS}{TNSU} x\ 100$$

NGS: number of germinated seeds

TNSU: total number of seeds used

The percentage of inhibition of seed germination at 4 days was calculated as:

% inhibition of seed germination= 100- % seed germination [21].

2.10. Statistical Analysis

All data were analysed by standard computer programs SPSS statistics T-test (version 20.) and are expressed as mean±S.E.M. significant differences were evaluated using t-test. P<0.05 was considered significant.

3. RESULTS

The amounts of Phenolics and flavonoids contents in the extract, fractions and sub-fractions of *Boswellia dalzielii* are reported as mg of gallic acid equivalent (GAE) per g and mg of rutin equivalents (RE) per g of extract, fractions and sub-fractions see, **Table 1**.

***Table 1**. Phenolic contents and flavonoids contents of extract, fractions, and sub-fractions of Boswellia dalzielii stem bark.*

Samples	TPC (mg GAE/g extract)	TFC (mg RE/g extract)
Methanolic extract	373.9 ± 27.2	142.2 ± 4.8
Aqueous fraction	318.8 ± 33.4	130.6 ± 9.8
Ethyl acetate fraction	356.4 ± 28.0	112.9 ± 8.2
Hexane fraction	157.6 ± 13.9	123.8 ± 14.4
Sub-Fraction A	125.0 ± 7.7	63.7 ± 1.1
Sub-Fraction B	339.2 ± 13.4	71.7 ± 2.9
Sub-Fraction C	481.2 ± 10.1	129.9 ± 10.7
Sub-Fraction D	331.8 ± 7.8	97.5 ± 5.9

Values are expressed as mean ± SD (n=3). GAE-Gallic acid equivalents; RE-Rutin equivalents, TPC-Total phenolic contents, TFC-Total flavonoid contents.

***Table 2.** DPPH (IC_{50}) and FRAP (EC_{50}) radical scavenging activity of Boswellia dalzielii stem bark.*

Samples	DPPH scavenging activity (IC_{50})	FRAP scavenging activity (EC_{50})
Vitamin C*	0.39	-
Rutin**	1.99	1.12
Gallic acid***	10.00	-
Methanolic extract	1.99**	4.78**
Hexane fraction	50.11*,**,***	120.22**
Ethyl acetate fraction	3.16**	5.62**
Aqueous fraction	1.58**,***	1.00**
Sub-fraction A	501.10*,**,***	251.18**
Sub-fraction B	6.31**,***	11.22**
Sub-fraction C	5.01**,***	3.98**
Sub-fraction D	7.94**,***	1.25**

Key: * have p < 0.05 and they are statistically significant.

Table 3. *Anti-proliferative activities of B. dalzielii stem bark extracts and Sub-fractions*

Treatment (µg/ml)		Mean ± SEM	% Seed germination	% Inhibition
Methanolic extract	500	14.66 ± 0.88	70	30
	250	17.66 ± 0.33	85	15
	125	15.66 ± 1.45	75	25
Aqueous fraction	500	14.00 ± 2.08	70	30
	250	16.33 ± 0.66	80	20
	125	16.00 ± 2.00	80	20
Ethyl acetate fraction	500	17.66 ± 0.66	85	15
	250	16.33 ± 1.76	80	20
	125	17.00 ± 0.57	85	15
Hexane fraction	500	19.00 ± 0.57	95	5
	250	18.66 ± 0.88	90	10
	125	19.33 ± 0.66	95	5
Sub-Fraction A	500	18.33 ± 0.88	90	10
	250	19.66 ± 0.33	95	5
	125	20.00 ± 0.00	100	0
Sub-Fraction B	500	15.00 ± 2.08	75	25
	250	10.66 ± 0.33	50	50
	125	11.33 ± 0.33	55	45
Sub-Fraction C	500	3.00 ± 1.52	15	85
	250	7.00 ± 1.52	35	65
	125	2.66 ± 0.33	10	90
Sub-Fraction D	500	4.33 ± 0.88	20	80
	250	8.66 ± 1.33	40	60
	125	2.00 ± 0.57	10	90
Control			100	0

4. DISCUSSION

Table 1 reports results of phenolic content and flavonoids content of extract, fractions and sub-fractions of *Boswellia dalzielii* stem bark, the methanolic extract has the greatest content of phenolics and flavonoids compounds, whereas the hexane extract has the lowest content of TPC and ethyl acetate extract, the lowest content of TFC. It was recorded that for all extracts and sub-fractions, sub-fraction C of *Boswellia dalzielii* stem bark has the highest total phenolics contents (481.20 ± 10.13 mg GAE/g) whereas sub-fraction A has the least total phenolics contents (125.04 ± 7.73 mg GAE/g). Methanolic extract of *Boswellia dalzielii* stem bark has the highest total flavonoid contents, 142.17 ± 4.82 mg RTE/g and the least total flavonoid contents were obtained from sub-fraction A (63.71 ± 1.06 mg RE/g).

One of the quick methods to evaluate antioxidant activity is the scavenging activity on DPPH, a stable free radical and widely used [22] and others methods are used to evaluate the antioxidant activity.

Table 2 showed the IC_{50} and EC_{50} of extract, fractions and sub-fractions of *Boswellia dalzielii* stem bark. The methanolic, ethyl acetate and aqueous fractions of this plant exhibited antioxidant potential when compare their IC_{50} with the IC_{50} of the standard Vitamin C, Gallic acid and Rutin. Sub-fractions B, C and D showed better antioxidant activities when compare to standard Gallic acid by DPPH scavenging assay method. The aqueous fraction exhibited antioxidant activity with the lowest IC_{50} than gallic acid and rutin, and possessed the most DPPH radical scavenging activity among all fractions and subs-fractions. Sub-fraction A with an IC_{50} above 500 µg/ml, exhibited the least DPPH radical scavenging activity.

The DPPH radical scavenging activity of *Boswellia dalzielii* stem bark may be attributed to the presence of phenolic and flavonoids contents. The phenolic constituents found in vegetables and spices have received considerable attention due to their antioxidant activity. The antioxidant activity of phenolic constituents has been attributed to their oxido-reduction properties, which play an important role in the adsorption and neutralization of free radicals [23]. The methanolic extract, fractions and sub-fractions showed antioxidant activities because the solvent used has a greater affinity in extracting phenolics and flavonoids compounds while the less polar hexane solvent, has no affinity for the extraction of these compounds. This quantitative antioxidant activity confirm the results found by Alemika (2004) [3] on qualitative antioxidant of stem bark of *Boswellia dalzielii* and the gum resin of *Boswellia dalzielii* found by Ojerinde (2010) [24].

The results of antioxidant activities of ferric reducing antioxidant power of methanolic extract, hexane, ethyl acetate and aqueous fractions and all sub-fractions A, B, C and D of stem bark of *Boswellia dalzielii* are showed in table 2. FRAP assay was used to determine the antioxidant activity of stem bark of *Boswellia dalzielii*. In this assay, ferric ions are reduced to ferrous ions in the presence of an antioxidant (or, a reducing agent) which form a blue-colored ferrous tripyridyltriazine complex (Fe^{2+}-TPTZ) at pH 3.6. The change is monitored spectrophotometrically at 593 nm [25].

The aqueous fraction exhibited the highest FRAP antioxidant capacity content with EC_{50} 1.00 µg/ml followed by methanolic 4.78 µg/ml, ethyl acetate 5.62 µg/ml and hexane fraction 120 µg/ml, and all sub-fractions the highest activity were observed in sub-fraction D with EC_{50} 1. 25 µg/ml, followed by sub-fraction C, B and A (3.98 µg/ml, 11.22 µg/ml and 251.18 µg/ml) respectively. Aqueous fraction and sub-fraction D activities observed were the same with standard antioxidant rutin 1.12 µg/ml. The FRAP essay activity observed for extracts and sub-fractions Showed antioxidant activity when compared with the ferric reducing potential of the standard antioxidant rutin.

Tables 3 give the results of percentage of antiproliferative activity of *Sorghum bicolor* seeds against the extract, fractions and sub-fractions of *Boswellia dalzielii* stem bark. These methods are of tremendous value because they are simple, rapid, reproducible, time and material saving. They are experimental procedures which can be carried out in laboratories where appropriate human cell lines are not readily available. The methods can be used to screen and eliminate many medicinal plants that may be claimed to treat tumour related ailments.

Research work into natural products with probable antitumour effects involves the use of simple bench-top assay models which are cheap, simple and rapid [21].

From the results of tests for antiproliferative activity it was observed that sub-fractions C and D at a dose of 125 µg/ml has highest activity with 90% inhibition of seed germination at 96 hours, hexane fraction and sub-fraction A at a different dose of 250 µg/ml and 500 µg/ml respectively give the same percentage of inhibition 10%. This result indicates that the biological activity of *Boswellia dalzielii* stem bark resides in the sub-fractions C and D. Therefore, this further confirms its application for the treatment of diseases such as liver problem, brain dysfunction and cancer. It also confirms the similarity of *B. dalzielii* stem bark in activity with its congeners such as *B. carterii* and *B. serrata* [26]. These results confirm again the work of Ojerinde (2010) [24] on antiproliferative activity of gum resin of *Boswellia dalzielii*, and it is probably that the plant *Boswellia dalzielii* has an antiproliferative activity.

5. CONCLUSION

Extracts and sub-fractions of *Boswellia dalzielii* Hutch stem bark showed high contents of TPC and TFC. The phenolic and flavonoid compounds in the extracts are responsible for the activity of DPPH scavenging and FRAP essay activity observed on the extracts and sub-fractions. These are translated to a good antioxidant activity of the plant stem bark *B. dalzielii* as methanol a polar solvent support the extraction of polar compounds such as poly-phenols, responsible for antioxidant activity. The extract, fractions and sub-fractions were found to possess antioxidants activities and radical scavenging effect on the DPPH and FRAP. Sub-fractions C and D showed a good antiproliferative activity with 90% growth inhibitory activity of seeds of *Sorghum bicolor*. Therefore, the results of the present study indicated that the stem bark of *Boswellia dalzielii* can be used as alternative therapeutic to suppress oxidative stress and cancer progression.

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