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Phytochemical Evaluation and *In-Vitro* Antibacterial Properties of the Methanolic Leaf Extract of *Boswellia dalzielii* Hutch. (Burseraceae)

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ABSTRACT

The upsurge of multi-drug resistance by microbes is at alarming rate and poses serious challenges in the field of medical and pharmaceutical industries. This study was aimed to determine the active components and assess the antibacterial potency of methanolic leaf extract of *Boswellia dalzielii.* The plant specimen was collected, identified, prepared and cold macerated using 95% of methanol as solvent. The resulted crude extract was further assayed for anti-bacterial potency; adopting agar well diffusion protocol against some selected human pathogenic isolates viz *S. aureus, S. pyogenes, B. Subtilis*, *Corynebact. Spp. K. pneumoniae, S. typhi,P. aeruginosa* and *E. coli*. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using the standard protocol of microdilution assay. The antimicrobial assay of the crude extract showcased that *B. dalzielii* leaf is susceptible against all the tested pathogens by exhibiting significant degrees of inhibition zone of diameter (0.25±0.26to 27.16±0.76 mm). The positive control (ciprofloxacin) showed superior activity against the tested isolates (27.06±0.35 to31.03±0.64 mm). The MIC/MBC was observed at 25.0 to 6.25 mg/mL and 25.0 to 12.5 mg/mL against bacterial studied. *S. aureus* is the most susceptible pathogen with MIC/MBC values at 6.25/12.5 mg/ml respectively while *S. Pyogene, B. subtilis, P. aeruginosa* were inhibitory at 12.5 mg/ml and bactericidal at 25mg/ml. *Corynebact. Spp.* and *K. pneumoniae* had the moderate MIC/MBC at 25mg/ml. The phytochemical analysis revealed the presence of alkaloids, flavonoids, cardiac glycosides, saponins, steroids, terpenoids, resins, tannins and carbohydrates. This study showed that *B. dalzielii* leaf has a strong antibacterial activity against Gram +ve and Gram -ve bacteria tested; this provides a scientific justification to the traditional healers for the utilization of the plant in the management of different ailments caused by the tested pathogens.

Keywords: Phytochemical Evaluation, Antibacterial property, *Boswellia dalzielii,* leaf extract, *in-vitro*

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1. INTRODUCTION

It is eminent that diseases caused by pathogenic microbes are prevalent globally, which account for high rate of morbidity and mortality, particularly in the developing nation like Nigeria. In recent times, there has been an increasing trend in the emergence of resistance to chemotherapeutic products and series of conventional antibiotics have lost their efficacy as a result of advancement in microbial strains resistant, mainly through the countenance of resistance genes [1,2,3,4] coupled with insufficiency, exorbitance and adverse side effects of some conventional drugs.

According to Abdulmumin et al. [1], most standard antibiotics at some points are associated with adverse effect on the host together with suppression of healthy immune response, depletion of advantageous gut and mucosal microbes, hypersensitivity, allergic reactions and other setbacks. These have prompted to the necessity for urgent search and development of new pharmaceuticals to combat infectious ailments and curtail antimicrobial resistance.

Medicinal plants are the nature's pharmacy for compound substances which have distinctive biological activities that are valuable in the management of human illness [1]. According to recent literature, many people are still depending on medicinal plants for therapeutic purposes due to accessibility, cheapness and less side effect[5].

Boswellia dalzielii (*B. dalzielii*) is a popular tree plant of the savannah forest belonging to the family Burseraceae. The plant is commonly called Frankincense tree. It is locally known as "arrarabi", in Hausa; "Kaushi" in Kanuri; "debro" in Babur/Bura; "mofu" in Marghi all in Nigerian languages [4]. The plant is popularly known as "piangwogu" in Upper Volta and Ghana; it is also called "etan" in Ethiopia, "libanos" in Greek and "luban" in Arabic [4]. *B. dalzielii* is widely distributed in dry climates of tropical and subtropical regions, such as Northern Nigeria, Northern Ivory Coast, Cameroon, Upper Volta, Togo, Burkinafaso, Benin, Ethiopia, Poland, Czech Republic, India and Ubangishari [4,6].

B. dalzielii has been reported to have wide array of medicinal values in folk medicine as antiseptic, anti-arthritic, wound healing, antimalaria, antidiarrhea, anti-inflammatory, antibacteria, anti-fungal, anti-trypanosomal, anti-hepatitis, anti-HIV/AIDS, antidotes to arrow poison and for the treatment of rheumatism, leprosy, gastrointestinal troubles [5,7,8]. The plant is also reported for the treatment of dental problems, swellings, bronchitis, coughs, gastric disorder, asthmatic attack, pulmonary diseases and skin ailments [4,9] and as immune booster [6].

The biopharmaceuticals are derivatives of plant and animal materials which are of paramount important to human, animal and public health as well. It is therefore of interest to scrutinizing this plant organ (leaf) for its natural biological active compounds and antimicrobial efficacy as well as validation of the claims from ethnomedicinal uses of the plant.

2. MATERIALS AND METHODS

2.1 Collection, Identification and Preparation of Plant

The fresh leaf of *B. dalzielii* was collected from Shongom Local Government Area, Gombe State, Nigeria. It was identified and authenticated by a plant Taxonomist from the Department of Biological Sciences, University of Maiduguri, Nigeria. The herbarium specimen was deposited at Research Laboratory, Department of Pure and Applied Chemistry with voucher number #534 provided. The leaf of the plant was cleaned, air-dried under shade for fourteen days and pulverized into fine powder and then coded "plant material".

2.2 Extraction of Plant Material

The air-dried powdered plant material (250 g) was extracted exhaustively with 95% methanol in distilled water using cold maceration method. The crude extract was concentrated to dryness at reduced pressure using rotary evaporator.

2.3 Phytochemical Analysis

The preliminary phytochemical screening of the crude extract to identify secondary

metabolites such as alkaloids, tannins, flavonoids, terpenes, saponins, cardiac glycosides, anthraquinones, resins, steroids, carbohydrates were carried out using standard procedures by [10,11,12,13,14].

2.3.1 Test for Alkaloids

The extract 0.5g was stirred with 5ml of 1% aqueous hydrochloric acid on water bath and filtered. The filtrate was divided into two different labelled test tubes (A and B).

(i) Dragendorff's Test

To the test tube labelled A, a few drops of Dragendorff's reagent was added, occurrence of orange-red precipitate indicate the presence of alkaloids [10].

(ii) Mayer's Test

To the test tube labelled B, a few drops of Mayer's reagent was added, formation of buff colored indicates the presence of alkaloids [10].

2.3.2 Test for Flavonoids

(i) Ferric Chloride Test

Two hundred milligram (200mg) of the extract was boiled with 10ml distilled water and filtered. Few drops of 10% ferric chloride solution were added to the 2ml of the extract, a green-blue or violet color indicates the presence of phenolic hydroxyl group [11].

(ii) Shinoda's Test

The extract (0.5g) was dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips was then added to the filtrate followed by few drops of conc. HCl. A pink, orange or red to purple coloration indicate the presence of flavonoids [14].

(iii) Lead Ethanoate Test

The extract (0.2g) was boiled with distilled water and filtered. 3ml of lead ethanoate was added to 5ml of the filtrate. The appearance of a buff colored precipitate indicated the presence of flavonoids [10].

(iv) Sodium Hydroxide Test

The extract (0.2g) was dissolved in distilled water and filtered. 2ml of 10% aqueous sodium hydroxide was added to the filtrate to produced yellow coloration. Changes in color from yellow to colorless on addition of dilute hydrochloric acid indicate the presence of flavonoids [11].

2.3.3 Test for Steroids

(i) Salkowski's Test

The extract (0.5g) was admixed in 2ml of chloroform. Sulphuric acid was added and the appearance of a reddish-brown color at the interface indicates the presence of steroidal nucleus [12].

(ii) Libermann's-Bruchard's Test

The extract (0.5g) was dissolved in 2ml of acetic anhydride and cooled. Sulphuric acid was then carefully added. A color change from violet to blue indicated the presence of a steroidal nucleus [12].

2.3.4 Test for Cardiac Glycosides

(i) Keller-Kelliani's Test

The extract (0.5g) was dissolved in 2ml of glacial acetic acid containing few drops of ferric chloride solution. This was then added with 1ml of concentrated sulphuric acid. A brown ring obtained at the interface indicates the presence of cardiac glycosides [11].

2.3.5 Test for Anthraquinone Glycosides

(i) Free Anthraquinone Glycosides

The extract (0.5g) was extracted with hot water for 5 minutes, filtered while hot, cooled and extracted with chloroform; 5ml of 10% ammonium solution was added to the separated chloroform. A pink coloration in ammoniacal phase was not shown which indicated the absence of free anthraquinone glycosides [11].

(ii) Combined Anthraquinone glycosides

The aqueous layer, 10ml from above was heated with 5ml of 10% sulphuric acid filtered while hot. The filtrate was shaken with chloroform. 5ml of 10% ammonia solution was added to the filtrate. A pink coloration in the ammoniacal layer was not shown and then indicated the absence of combined anthraquinone glycosides [11].

2.3.6 Test for Tannins

(i) Test for Hydrolysable Tannins

The extract (0.5g) was boiled in 10ml of distilled water for 5 minutes and filtered. The filtrate was made up of 10ml with distilled water. To 2ml of the filtrate was added10ml of distilled water and 2 drops of 10% ferric chloride solution. A blue black was taken as evidence for the presence of hydrolysable tannins [11].

(ii) Test for Condensed Tannins

To the filtrate from above, a drop of bromine water was added and an orange precipitate was taken as evidence for the presence of condensed tannins [11].

2.3.7 Test for Terpenoids

Two hundred milligram (200mg) of the extract was dissolved in ethanol. 1ml of acetic anhydride was added followed by addition of concentrated H_2SO_4. A color changes from pink to violet showed the presence of terpenoids [12].

2.3.8 Test for Saponins

(i) Frothing Test

The extract (5ml) aliquot was placed in a test tube and shaken vigorously for five minutes. The presence of frothing and time taken for the froth to disappear was taken as an indication for the presence of saponin [13].

2.3.9 Test for Carbohydrates

(i) Molisch Test

The extract (0.5g) was dissolved in 10 ml water, 2drops of 10% naphthol was added to about 2ml of the dissolved extract, 2ml of concentrated sulphuric acid was added gently down the side of the test tube. A deep violet coloration at the interface indicates the presence of sugars [11].

(ii) Fehling Test

The aqueous layer of each extract (5ml) was added to 5ml of Fehling solution and boiled for few minutes. Appearance of a brick red precipitate was taken as an indication for the presence of reducing sugars [11].

2.4 Antimicrobial Assay:

A total of eight microorganisms were used in this study: four Gram positive bacteria (*S. aureus, Corynae. spp., S. pyogene, and B. subtilis*) andfour Gram negative bacteria (*S. typhi, E. coli, K. pneumonia* and *P. aeruginosa*). Standard antibiotic used was Ciprofloxacin (50 mg/ml) produced by Oxiod Ltd., Hampshire, England. These organisms were clinical isolates obtained from the Department of Medical Microbiology University of Maiduguri Teaching Hospital (UMTH), University of Maiduguri- Nigeria.

2.4.1 Preparation of Standard Inoculums for Antimicrobial Assay

The stock cultures were maintained on nutrient agar (NA) slants, active cultures for the experiments were prepared by transferring a loopful of microbial cells from the stock cultures into bottles containing sterile Mueller-Hinton Broth (MHB) for bacteria in order to revive them by culturing overnight at 37°C. The overnight cultures were further sub cultured with freshly prepared MHB and the turbidity was adjusted to $BaSO_4$ turbidity standard which is equivalent to 0.5 McFarland standards in order to obtain the microbial density consistent with $1.5x10^8$ CFU/mL cells for bacteria [1].

2.4.2 Preparation of the Crude Extract

The crude extract was prepared in four different concentrations of 50, 100, 200 and 400 mg/ml by dissolving 0.5 g, 1.0 g, 2.0 g and 4.0g, respectively into 10 ml each of 95% methanol in distilled water (v/v) as working concentrations.

2.4.3 *In-vitro* Antimicrobial Susceptibility Assay of the Crude Extract

The susceptibility test of the extract was done adopting agar-well diffusion protocol as described by [15]. The microorganisms were maintained on agar slants. The inoculum was prepared by subjecting the test organisms in nutrient broth and inoculated for 24 h at 35°C. After inoculation, the broth cultures were diluted to 1:1000 for Gram +ve bacteria and 1:5000 for the Gram -ve bacteria. One milliliter of the diluted cultures was inoculated into 19 ml sterile molten nutrient agar (48°C) and poured into sterile petri-dishes. These were swirled gently and then allowed to solidify. Afterwards, holes of 9 mm diameter were bored into the solidified and inoculated nutrient agar plates using a sterile number VI cork borer. All the holes were filled with equal volumes of 0.1 ml (50, 100, 200, 400 mg/hole) of the crude extract. The reference antibiotic (ciprofloxacin) was used. This standard disc was placed on already bacteria-inoculated nutrient agar plates for an hour to allow the extract diffuse into the agar. Thereafter, the plates were then incubated overnight at 35°C bacterial strains. At the end of the incubation period, inhibition zones were recorded in millimeters as the diameter of growth-free zones around the bored holes using a transparent meter rule. The crude extract and the standard antibiotic were independently tested in triplicate. Diameters of zones of inhibition ≥10 mm exhibited by plant extract was considered active [15,16]. The MIC and MBC of the extract were equally carried out to buttress the scientific investigation of the antimicrobial sensitivity of the plant.

2.4.4 Determination of Minimum inhibitory concentration (MIC)

The MIC was determined using the nutrient broth dilution technique as described by[17]. The minimum inhibitory concentration value was determined for the microorganisms that were sensitive to the extract under study. The extract was first diluted to the highest concentration (100 mg/ml) in 95% methanol in distilled water (v/v) and then two-fold serial dilution of the crude extract was then made to a concentration ranging from 3.123 to 50 mg/ml using nutrient broth (by dissolving 13 g/L). The concentration was then varied depending on the extracts and level of activity. The extracts were inoculated with 1 ml suspension of the organisms and thereafter incubated at 35°C.

2.4.5 Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined using the broth dilution technique previously described by [17] as adopted by [3, 15] by assessing the test tubes resulting from MIC determinations. A loopful of the content of each test tube was inoculated by streaking on a solidified nutrient agar plate and then incubated at 35°C for 24 hours and observed for bacterial growth. The lowest concentration of the sub-culture that shows no bacterial growth was considered the minimum bactericidal concentration.

2.5 Statistical Analysis of Data

The results of antimicrobial data were presented as Mean ± standard error mean (SEM) using GraphPad InStat software, version 2003 [18].

3. RESULTS AND DISCUSSIONS

The results of qualitative phytochemical screening of the crude leaf extract revealed the presence of alkaloids, flavonoids, saponins, terpenoids, resins, tannins, cardiac glycosides, steroids, phenolicsand carbohydrates while anthraquinones were absent (Table 1). The presence of these chemicals revealed in this study were in agreement with the report by some researchers [4,9,19,20]. These metabolites were responsible for the physiological and chemotherapeutic effects exhibited by plant extractives both *in vitro* and *in vivo* [4]. The manifestation of phytochemical ingredients like glycosides, terpenes, alkaloids, saponins, steroids and tannins are of medically importance since they have antimicrobial potency [1]. Alkaloids have been shown to possess antibacterial, antidiabetic and anticancer activities [21]. Tannins have been reported to inhibit growth of microorganisms by precipitating microbial protein and making nutritional protein unavailable to them [22].

Table 1: **Results of phytochemical screening of methanolic leave extract *B. dalzielii***

S.N.	Phytochemical Content	Result
1.	Test for Alkaloids	
	Dragendroff's reagent	+
	Mayer's reagent	+
2.	Test for flavonoids	
	Shinoda's test	+
	Ferric chloride	+
	Lead acetate	+
3.	Test Tannins	+
4.	Test for Resins	+
5.	Test for Saponins Frothing	+
6.	Test for Carbohydrates	
	Molish's Test	+
	Test for combine reducing Sugar	+
	Test for Monosaccharide	+
7.	Test for Phlobatanins	+
8.	Test for Anthroquinones	
	Combine Anthroquinones	-
	Free Anthroquinones	-
9.	Test for Phenolic	+
10.	Test for Terpenoids	+
11	Test for Steroids	
	Libermann's-Bruchard's	+
	Salkwoski's Test	+
12	Test for Cardiac Glycosides	
	Keller-Killiani's Test	+

Key: (+) = Present, (-) = Absent

The antimicrobial activities of flavonoids have been attributed to their ability to complex with extra cellular, soluble protein and to complex with bacterial cell wall proteins [23]. Flavonoids have shown antifungal, antibacterial as well as anti-inflammatory activity [4,24]. Saponins are known to have antimicrobial properties. Saponins and terpenes have antimicrobial and curative properties against various pathogens. Terpenoids and glycosides play the role of protective against different pathogens like

insects, fungi and bacteria [25]. Steroids are known for their antibacterial activity especially associated with membrane lipids and causes leakages from liposomes [21].

The results of antimicrobial susceptibility test is presented in Table 2; the DIZ exhibited by the crude extract against Gram +ve bacteria were found in the range of 10.96±0.68 to 27.16±0.76 mm and Gram -ve bacteria (0.25±0.26 to 25.33±0.12 mm) compared with the standard drug ciprofloxacin within the range of (27.06±0.35 to 31.13±0.30 mm). This observation is in line with the fact that plant extractive with diameters of inhibition zones ≥ 10 mm is considered active as described by [15].

The antimicrobial activity of this plant against the tested organisms; Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa, Streptococcus pyogenes, Klebsiella pneumoniae*, *Bacillus subtilis* and *Corynebacterium species* proved effective against them. This may be the reason why it has been used by traditional medical practitioners in the management of different ailments in northeastern Nigeria, especially Gombe State.

From this study, the extract showed most potent activity against Gram positive bacteria (*S. aureus*) than Gram negative bacteria. The extract showed activity dose dependent across the all pathogens as seen from comparative dosage analysis on extract concentrations.

The results of antibacterial properties of this study were in agreement of [4,20,21,22,26] where aqueous and methanolic extracts of *B. dalzielii* leaf and stem-bark demonstrated antibacterial properties especially on these microbes studied.

Table 2: Results of Antimicrobial Susceptibility test of crude methanolic leaf extract of *Boswellia dalzielii*

S.N.	Microorganisms	Concentration (mg/ml)/Diameter of inhibition zone (Mean ± SEM) (mm)				Std Drug(Cipro) [50mg/ml]
		400	200	100	50	
1.	*Staphylococcus Aureus*	27.16±0.76	22.66±0.57	19.26±0.20	14.86±0.58	30.13±0.30
2.	*Streptococcus Pyogene*	24.86±0.58	19.33±0.57	15.36±0.25	12.69±0.66	31.03±0.64
3.	*Bacillus Subtilis*	24.66±0.57	20.33±0.57	16.00±0.69	12.06±0.64	30.16±0.35
4.	*Corynebacterium Species*	22.66±0.57	19.26±0.15	14.03±0.50	10.96±0.68	28.19±0.51
5.	*Escherichia Coli*	10.33±0.57	0.33±0.15	0.25±0.20	0.25±0.26	30.13±0.34
6.	*Salmonella Typhi*	10.26±0.15	0.33±0.20	0.46±0.25	0.46±0.26	28.13±0.30
7.	*Klebsiella pneumoniae*	24.66±0.57	20.30±0.19	15.03±0.56	11.06±0.68	27.06±0.35
8.	*Pseudomonas aeruginosa*	25.33±0.12	20.25±0.15	16.03±0.76	12.09±0.29	28.19±0.35

Key: Cipro = Ciprofloxacin n=3

4.1 MIC and MBC of the Susceptible Microorganisms

The MIC and MBC values against tested pathogens (Gram-positive and Gram-negative bacteria) ranged from 12.5 to 25 (Table 3 and Table 4). From this result, it has shown that crude extract was more potent to Gram positive bacteria. The extract has also shown a remarkable bacteriostatic and bactericidal effect on Gram +ve and Gram -ve bacteria with MIC / MBC at 6.25 / 12.5 mg / ml against *S. aureus*, *S. pyogene, K. pneumoniae, B. subtilis,*

Corynebacterium spp. has shown bactericidal effect at 12.5 concentration. According to Abdulmumin et al. [1], when the MIC/MBC values are low, it signifies that the drug material is more potent. Therefore, the efficacy and potency displayed by this extract have also confirmed that the plant possessed very important metabolites which can be used for natural antibiotics agents against the resistance pathogens studied.

Table 3: Minimum Inhibitory Concentration (MIC) of methanol leaf extract of *Boswellia dalzielii*

S.N.	Microorganisms	Concentration of Extract (mg/ml)				
		50	25	12.5	6.25	3.125
1.	*Staphylococcus aureus*	-	-	-	α	+
2.	*Streptococcus pyogene*	-	-	α	+	+
3.	*Bacillus subtilis*	-	-	α	+	+
4.	*Corynebacteria species*	-	α	+	+	+
5.	*Klebsiella pneumoniae*	-	α	+	+	+
6.	*Pseudomonas aeruginosae*	-	-	α	+	+

Key: α= MIC value, + = Growth, - = No growth

Table 4: Minimum Bacteria Concentration (MBC) of methanol leaf extract of *Boswellia dalzielii*

S.N.	Microorganisms	Concentration of Extract (mg/ml)				
		50	25	12.5	6.25	3.125
1.	*Staphylococcus aureus*	-	-	β	+	+
2.	*Streptococcus pyogene*	-	β	+	+	+
3.	*Bacillus subtilis*	-	β	+	+	+
4.	*Corynebacteria species*	-	β	+	+	+
5.	*Klebsiella pneumoniae*	-	β	+	+	+
6.	*Pseudomonas aeruginosae*	-	-	+	+	β

Key: β= MBC value +=turbid/bacterial growth -=No turbid/no bacterial growth

5. CONCLUSION

The phytochemical analysis has revealed the presence of alkaloids, flavonoids, tannins, saponins, terpenoids, resins and carbohydrates. The results of the *in vitro* antimicrobial studies proved that the extract possessed remarkable antibacterial properties against resistant microbes especially on *S. aureus, P. aeruginosa, K. pneumoniae, B. subtilis* and *Corynebacterium species.* The bacteriostatic and bactericidal effect of this plant is highly recommended for phytopharmaceutical importance for drug discovery and development. Isolation and characterization of the active compounds responsible for the antibacterial properties is also recommended.

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Conflict of Interest
There is no conflict of interest for any reason among the Authors at all.

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