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Phytochemical Analysis and *In vitro* Antimicrobial Assay of the Methanolic Stem Bark Extract of *Boswellia dalzielii* Hutch. (Burseraceae)

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Abstract The methanolic stem bark extract of *Boswellia dalzielii* (BDME) were phytochemically screened and investigated *in vitro* against six Gram negative bacteria (*S. typhi, E. coli, K. pneumoniae, S. dysentriae, P. aeruginosa and P. mirabilis*), four Gram positive bacteria (*S. aureus, Corynae. spp., S. pyogene and B. subtilis*) and four fungal strains (*C. albicans, A. flavus, A. niger* and *Rhizopus spp.*) using hole-in-plate disc diffusion technique. The qualitative phytochemical examinations of the crude extract revealed the presence of alkaloids, aloes, cardenolides, cardiac glycosides, terpenoids, cyanogenic glycosides, flavonoids, steroids, resins, saponins, tannins, higher fatty acids and carbohydrates. The *in vitro* antimicrobial assay revealed that, the DIZ against Gram -ve, Gram +ve and fungal strains ranged from 7.33±0.33 to 16.66±0.33 mm, 8.33±0.33 to 17.00±0.00 mm and 9.00±0.00 to 17.33±0.33 mm respectively. The overall susceptibility showed that BDME was more susceptible to fungal strain (*C. albicans*) than Gram -ve and Gram +ve, although the inhibitory effect in some particular cases insignificant (P>0.05) against *E. coli, S. typhi, S. dysentriae, P. mirabilis, S. aureus, Corynaebacterium spp.* at highest dosage (200 mg/hole) compared with standard antibiotic Erythromycin (5µg). The MIC, MBC and MFC results revealed that Gram +ve, Gram -ve and fungal strains ranged from 12.5 to 50 mg/ml, 12.5 to 25 mg/ml and 6.25 mg/ml respectively. The BDME was more inhibitory and fungicidal at 6.25 mg/ml against *C. albicans*. The extract has also shown a remarkable inhibitory and bactericidal effect on Gram +ve and Gram -ve bacteria at 12.5 mg/ml against *Corynae. spp., S. dysentriae* and *B. subtilis* respectively. The sensitivity pattern of BDME across the strains of the pathogens studied compared with standard antibiotics (Ciprofloxacin, Erythromycin and Gentamicin) and antifungal (ketoconazole) were susceptible to Gram +ve bacteria with the percentage activity of 100 %, Gram -ve bacteria (66.67 %) and fungal strains (100 %). The spectral intensity index (SII) expressed by the extract was found to be 7.27 mm against all the pathogens. These sensitivities observed were due to the presence of the secondary metabolites in the plant.

Keywords phytochemical, antimicrobial, *Boswellia dalzielii*, microorganisms, *in vitro*

Introduction

Boswellia dalzielii Hutch is a 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. 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 [1,2,3]. *B. dalzielii* is

widely distributed in dry climates of tropical and subtropical regions, such as Northern Nigeria, Northern Ivory Coast, Cameroon, Upper Volta, Togo, Burkina faso, Benin, Ethiopia, Poland, Czech Republic, India and Ubangishari [1-5]. The tree has a characteristic smooth, pale brown bark that peels off in ragged papery patches, which on rapping exudes a whitish fragrant resin. It grows up to 13 m high [1]. The small, white flowers, which may appear while the tree is leafless, are fragrant.

B. dalzielii has wide array of medicinal values in folk medicine as antiseptic, anti-arthritic, wound healing, antimalaria, antidiarhoea, 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 [3-9]. The plant is also reported for the treatment of dental problems, swellings, bronchitis, coughs, gastric disorder, asthmatic attack, pulmonary diseases and skin ailments, among others [6, 10]. According to [6,11], *B. dalzielii* has shown a potent immunological effect and immune modulatory activity both *in vitro* and *in vivo* in northern Cameroon. This study is designed to investigate the phytochemical constituents and antimicrobial (antibacterial and antifungal) activities of *B. dalzielii* in order to validate its medicinal use in ethno-medicine.

Materials and Methods

Collection and Identification of Plant

The stem bark of *B.dalzielii* was collected from Bagale village, Giere Local Government Area, Adamawa State, Nigeria (Long. 14^{o} 23.32' E; Lat. 11^{o} 01.22' N).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 the Post Graduate Research Laboratory, Department of Chemistry with voucher number #340 provided. The stem bark of the plant was cleaned, chopped into pieces, air-dried under shade for fourteen days and pulverized into fine powder and then coded "plant material".

Extraction of Plant Material

The air-dried powdered plant material (2000 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 and the extract coded 'BDME"- *Boswellia dalzielii* Methanolic Extract.

Phytochemical Screening

0.2 g each of the BDME was subjected to qualitative chromogenic phytochemical screening to test for the presence of the following secondary plant metabolites: alkaloids, flavonoids, saponins, tannins, glycosides (cardiac, steroidal), terpenes/terpenoids, fatty acids, resins, aloes, cyanogenic glycosides etc. as described by [12-15].

Antimicrobial Studies

A total of fourteen microorganisms were used in this study: four Gram positive bacteria (*S. aureus, Corynae. spp., S. pyogene,* and *B. subtilis*), six Gram negative bacteria (*S. typhi, E. coli, K. pneumoniae, S. dysentriae, P. aeruginosa and P. mirabilis*) and four fungal strains (*C. albicans, A. flavus, A. niger* and *Rhizopus spp.*). Standard antibiotics and antifungal discs used were Ciprofloxacin (5µg/disc), Erythromycin (5 µg/disc), Gentamicin (10 µg/disc) and ketoconazole (10 µg/disc), 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.

The BDME was subjected to preliminary antimicrobial evaluation against the pathogens stated above using the hole-in-plate disc diffusion technique as described by [16, 17]. The crude extract was prepared in four different concentrations of 25, 50, 100 and 200 mg/ml by dissolving 0.25g, 0.5 g, 1.0 g and 2.0 g, respectively into 10 ml each of 95 % methanol in distilled water (v/v) as working concentrations.

The microorganisms were maintained on agar slants. The inocula was prepared by subjecting the test organisms in nutrient broth and inoculated for 24 h at 35 ^{O}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 ^{O}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 (25, 50, 100, 200 mg/hole) of the crude extract. The reference antibiotics and antifungal that were used include Ciprofloxacin (5 µg/disc); Erythromycin (5 µg/disc); Gentamicin (10 µg/disc) and ketoconazole (10 µg/disc). These standard discs were 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 37 °C and 35 °C for the fungi and bacterial strains respectively. At the end of the incubation period, inhibition zones were recorded in millimitres as the diameter of growth-free zones around the bored holes using a transparent metre rule. The crude extract and the standard antibiotics/antifungal were independently tested in triplicate. Diametres of zones of inhibition ≥10 mm exhibited by plant extracts were considered active [17, 18]. The activity index, percentage activity, spectral intensity index, MIC, MBC and MFC of the BDME were equally carried out to buttress the scientific investigation of the antimicrobial sensitivity of the plant.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using the nutrient broth dilution technique as described by [17, 19]. The minimum inhibitory concentration value was determined for the microorganisms that were sensitive to the extracts under study. Each 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 ^{o}C and 37 ^{o}C for bacterial and fungal strains respectively.

Determination of Minimum Bactericidal Concentration (MBC)

The MBC was determined using the broth dilution technique previously described by [17, 19] as adopted by Usman *et al.* (2007); Sodipo *et al.* (2016) 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 ^{o}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.

Determination of Minimum Fungicidal Concentration (MFC)

The MFC was determined using the broth dilution technique [17, 19] 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 37 ^{o}C for seven days and observed for fungal growth. The lowest concentration of the sub-culture that shows no fungal growth was considered the MFC.

Statistical Analysis of Data

The generated antimicrobial data were presented as Mean ± standard error mean (SEM). Statistical analysis to determine the mean differences among the DIZ exhibited were expressed using one-way analysis of variance (ANOVA) with student - Newman - Keul's multiple comparison test using GraphPadInStat [20], where $P<0.05$ considered significant.

Results and Discussion

The qualitative phytochemical examinations of the crude revealed the presence of alkaloids, aloes, cardenolides, cardiac glycosides, terpenoids, cyanogenic glycosides, flavonoids, steroids, resins, saponins, tannins, higher fatty acids and carbohydrates (Table 1). These metabolites were responsible for the physiological and chemotherapeutic effects exhibited by plant extractives both *in vitro* and *in vivo* [21-23]. Alkaloids have been shown to possess antibacterial, antidiabetic and anticancer activities [24-25]. Tannins have been reported to inhibit growth of microorganisms by precipitating microbial protein and making nutritional protein unavailable to them [26]. The

antimicrobial effects of flavonoids have been attributed to their ability to complex with extra cellular, soluble protein and to complex with bacterial cell wall proteins [27]. Flavonoids have shown antifungal, antibacterial as well as anti-inflammatory activity [28, 29]. 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 andbacteria [29-31]. Steroids are known for their antibacterial activity especially associated with memebrane lipids and causes leakages from liposomes. The presence of these constituents in the stem bark of *B. dalzielii* suggests that the plant is pharmacologically active, thus supporting the claims by traditional healers.

Table 1: Phytochemical constituents of methanol stem bark extract of *Boswellia dalzielii*

S/No	Phytochemicals	Test	Results CM
1	Alkaloids	Dragendorff's	+
		Mayer's	+
		Wagner's	-
2	Aloes		+
3	Anthroquinones	Borntrager's	-
	Free Anthroq.	Borntrager's	-
	Combined Anth.	Borntrager's	-
4	Cardenolides	Legal's	+
		Keller-Kilianis	+
5	Cardiac glycosides	Salkwoski's	+
		Liebermann-Burchard	+
6	Terpenoids		+
7	Cyanogenic glycoside		+
8	Flavonoids	Shinoda's	+
		Ferric chloride	+
		Lead acetate	+
		NAOH	-
9	Higher fatty acids		+
10	Phlobatannins		-
11	Resins		+
12	Saponins	Frothing	+
		Fehling's	+
13	Tannins	Ferric chloride	+
		Lead acetate	+
		Gold beater's	+
14	Steroids	10% HCl	-
15	Carbohydrates		
	i. General test	Molisch's	+
	ii. Monosaccharides	Barfoed's	+
	iii. Free reducing sugar	Fehling's	+
	iv. Combined reducing sugar	Fehling's	+
	v. Soluble starch		+
	vi. Ketoses	Salivanoff's	+
	vii. Pentoses		+

Key: CM= Crude methanolic extract, - =absent, + =present

The results of the diameters zone of inhibition (DIZ) of BDME is presented in Table 2; the DIZ exhibited by BDME against Gram +ve bacteria were found in the range of 8.33±0.33 to 17.00±0.00 mm, Gram -ve 7.33±0.33 to 16.66±0.33 mm while 9.00±0.00 to 17.33±0.33 mm was recorded as range of values against fungal strains studied. This observation is in line with the fact that plant extractive with diameters of inhibition zones ≥10 mm is considered active as described by [17,18].

The susceptibility data revealed that BDME was more susceptible to fungal strain (*C. albicans*) than Gram –ve and Gram +ve bacteria, although the inhibition in some particular cases insignificant (P>0.05) against *E. coli, S. typhi, S. dysentriae, P. mirabilis, S. aureus, Corynaebacterium spp.* at highest dosage (200 mg/hole) compared with standard antibiotic Erythromycin (5µg). The extract showed activity dose dependent across the all pathogens as seen from comparative dosage analysis on extract concentrations which revealed that significant difference (P<0.05, 0.01,0.001) were observed at 100 and 200 mg/ml on most pathogens (bacteria and fungus). No significant difference (P>0.05) was noted between Gentamicin, Erythromycin and BDME at 200 mg/ml against *S. dysentriae* suggesting that the BDME at higher doses could contain phytocompounds that these bacteria and fungal strains are susceptible to; while variable differences were observed at lower doses, this trend is in line with the report of [32]. Also, no significance difference (P>0.05) effects was noted as shown at 100 mg/hole between ketoconazole and BDME against *C. albicans* but at highest dosage of the extract (200 mg/hole), the DIZ was higher (17.33±0.33 mm) compared to ketoconazole (15.40±0.33 mm). The comparative studies among the various doses of the BDME have shown that, there were no significant (P>0.05) effects noted as shown at 25 and 50 mg/hole against *P. aeruginosa, P. mirabilis, S. aureus, S. pyogene, B. subtilis* (Table 2). The remaining activity revealed significant (P<0.05, 0.01, 0.001) difference compared to all antibiotics and antifungal considered in this study. This pattern of comparison is an indicative of the presence of phytochemicals of similar or more than the antibiotics [32, 33]. The inhibitions of these pathogens (Gram +ve, Gram -ve and Fungal Strains) were dose dependent. The three fungal strains (*A. niger, A. flavus* and *Rhizopus spp.*) have showed resistance against extract concentrations under study.

Table 2: Susceptibility pattern of BDMEat various concentrations on some microorganisms compared to that of standard antibiotics

	Concentrations (mg/hole) /Diameters of Inhibition Zone Mean±SEM (mm)						
Microorganisms	**25 mg/hole**	**50 mg/hole**	**100 mg/hole**	**200 mg/hole**	**Ery. 5µg**	**Cip. 5µg**	**Gen. 10µg Ketaconazole**
E. coli	0.00±0.00^{a}	0.00±0.00^{a}	8.33±0.33^{b}	11.66±0.33^{c}	12.66±0.33^{c}	20.33±0.33^{d}	18.00±0.00^{d}NT
S. typhii	0.00±0.00^{a}	0.00±0.00^{a}	8.00±0.00^{b}	10.00±0.00^{c}	10.66±0.33^{c}	21.66±0.88^{d}	17.00±0.57^{e}NT
S.dysentriae	9.00±0.00^{a}	11.33±0.33^{b}	12.66±0.33^{b}	16.33±0.33^{c}	17.00±0.00^{c}	24.00±0.00^{d}	16.00±0.00^{c}NT
P. aeruginosa	9.00±0.00^{a}	10.33±0.33^{a}	12.66±0.33^{b}	15.00±0.00^{c}	12.00±0.00^{b}	25.66±0.33^{d}	19.33±0.33^{e}NT
K. pneumonia	8.66±0.33^{a}	10.66±0.33^{b}	14.00±0.00^{c}	17.00±0.00^{d}	13.33±0.33^{c}	25.00±0.00^{e}	15.00±0.00^{c}NT
P. mirabilis	8.33±0.33^{a}	9.33±0.33^{a}	12.33±0.33^{b}	15.33±0.33^{c}	14.00±0.00^{c}	20.00±0.57^{d}	17.66±0.57^{e}NT
S. aureus	8.33±0.33^{a}	8.66±0.33^{a}	10.33±0.33^{b}	12.66±0.33^{c}	13.00±0.00^{c}	25.00±0.00^{d}	17.00±0.00^{e}NT
S. pyogene	7.33±0.33^{a}	8.33±0.33^{a}	9.33±0.33^{a}	11.00±0.00^{b}	21.00±0.57^{c}	20.00±0.57^{c}	19.00±0.00^{c}NT
B. subtilis	7.66±0.33^{a}	8.66±0.33^{a}	10.66±0.33^{b}	13.66±0.33^{c}	17.33±0.57^{d}	25.00±0.00^{e}	21.66±0.57^{f}NT
Cory. Spp.	8.66±0.33^{a}	11.33±0.33^{b}	13.33±0.33^{c}	16.66±0.33^{d}	17.00±0.00^{d}	28.66±0.33^{e}	20.00±0.00^{f}NT
C. albicans	9.00±0.00^{a}	11.00±0.00^{b}	14.00±0.57^{c}	17.33±0.33^{d}	NT	NT	NT 15.40±0.33^{c}
A. niger	-	-	-	-	NT	NT	NTNT
A. flavus	-	-	-	-	NT	NT	NTNT
R. spp.	-	-	-	-	NT	NT	NTNT

Key: Data are mean±SEM, n=3, *E. Coli=Escherichia coli. S. typhii=Salmonella typhii, S. dysentriae = Shigella dysentriae, P. aeruginosa= Pseudomonas aeruginosa, K. pneumonia=Klebsiella pneumonia, P. microbilis= Proteus microbilis, S. aureus=Staphylococcus aureus, S. pyogene =Streptococcus pyogene, B. subtilis=Bacillus subtilis, C. spp.=Corynebacteria species, C. albicans= Candida albicans, A. niger= Aspergellus niger, A. flavus= Aspergellus flavus, R. spp.=Rhizopus species.* NT= not tested.- = no activity. Means with different superscript along same row are significantly (P<0.05) different.

MIC, MBC and MFC of the Susceptible Microorganisms

The MIC, MBC and MFC values against tested pathogens (Gram positive, Gram negative and fungal strains) ranged from 12.5 to 50, 12.5 to 25 and 6.25 mg/ml respectively (Table 2). From this result, it has shown that BDME was more susceptible to fungal strains since the value recorded was lowest at 6.25 mg/ml for both MIC and MFC against *C. albicans*. The extract has also shown a remarkable inhibitory and bactericidal effect on Gram +ve and Gram -ve bacteria with MIC/MBC at 12.5 mg/ml against *Corynae. spp*., *S. dysentriae* and *B. subtilis* respectively. The sensitivity pattern of the crude extract (Table 3) had showed a remarkable percentage activity of Gram –ve (66.67%), Gram +ve (100%) and fungal strains (25%) respectively compared with standard drugs used. *C. albicans* had showed the highest activity index (119.61%) compared to ketoconazole while *P. aeruginosa and K. pneumonia* had 97.90% and 94.37% against erythromycin standard. The overall spectral intensity index (SII) was 7.27 mm across all the pathogens which also indicated good sensitivity of the extract [31]. The activity of the extract is even superior to Gentamicin. The antimicrobial activities displayed by this extract have also confirmed that the plant possessed very important metabolites which can be used for natural antibiotics and antifungal agents against the resistance pathogens.

Table 2: MIC, MBC and MFC concentrations of methanol stem bark extract of *Boswellia dalzielii* against susceptible organisms

S/No	Organisms		Concentration (mg/ml)					
			100	**50.00**	**25.00**	**12.50**	**6.25**	**3.125**
1	*Staphylococcus aureus*	-	β	α	+	+		
2	*Sreptococcus pyogene*		-	β	α	+	+	+
3	*Bacillus subtilis*	-	-	-	αβ	+	+	
4	*Corynebacterium species*	-	β	-	αβ	+	+	
5	*Escherichia coli*	-	β	α	+	+	+	
6	*Salmonella typhii*	-	β	α	+	+	+	
7	*Shigella dysentriae*		-	-	-	αβ	+	+
8	*Pseudomonas aeruginosa*	-	β	α	+	+	+	
9	*Klebsiella pneumonia*		-	-	-	α	+	+
10	*Proteus microbilis*		-	β	α	+	+	+
11	*Candida albicans*	-	-	-	-	αμ	+	

Key: α = MIC, β = MBC,αβ= MIC and MBC,αμ =MIC and MFC, - = not turbid/no growth, + = turbid/ growth

Table 3: Sensitivity Pattern of the Crude Methanolic Stem Bark Extract of *B. dalzielii* against Some Microorganisms compared with standard antibiotics and antifungal

S/N	Microorganisms	Activity Index (%)	Spectral Activity Index	
1	*E. coli*	39.47^{a}	24.58^{b}	27.76^{c}
2	*S. typhii*	42.21^{a}	20.78^{b}	26.47^{c}
3	*S. dysentriae*	72.53^{a}	51.38^{b}	77.06^{c}
4	*P. aeruginosa*	97.90^{a}	45.78^{b}	60.77^{c}
5	*K. pneumoniae*	94.37^{a}	50.32^{b}	83.87^{c}
6	*P. mirabilis*	80.93^{a}	56.65^{b}	64.16^{c}
7	*S. aureus*	76.88^{a}	43.46^{b}	58.81^{c}
8	*S. pyogene*	42.85^{a}	35.99^{b}	47.36^{c}
9	*B. Subtilis*	58.63^{a}	37.63^{b}	46.91^{c}
10	*Coryn. Spp.*	73.50^{a}	43.60^{b}	62.48^{c}
11	*C. albicans*	-	63.75^{b}	119.61^{d}
***Percentage activity (%)**		**%G+ = 100**	**%G- =66.67**	**%FS=100 %T=63.89 7.27 mm**

Key: %G+ = percentage Gram positive; %G- = percentage Gram negative; %FS=percentage fungal strains; % T= percentage total computed with: a =Erythromycin, b = Ciprofloxacin, c =Gentamicin, d=Ketoconazole, * =susceptible value ≥7 mm, - = no activity.

Conclusion

The phytochemical analysis has revealed the presence of alkaloids, flavonoids, tannins, saponins, steroids, cardiac glycosides, resins, cardenolides, fatty acids and carbohydrates, some of which have associated with antibacterial and antifungal properties. The results of the *in vitro* antimicrobial studies showed that the extract possesses antibacterial and antifungal properties. This is evident from the results of susceptibility test, MIC, MBC, MFC, activity index, percentage activity and spectral intensity index when compared with the standard antibacterial and antifungal drugs.

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