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Antimicrobial Efficacy of Methanolic fraction of Shea nut on selected Skin Pathogens and Characterization of the Bioactive Compounds

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ABSTRACT

This study aimed to investigate the antimicrobial activities of methanolic extracts of *Vitellaria paradoxa* nut against dermatophytes and characterize its bioactive compounds. Isolates used in this study were *Candida albican* ATCC14503, *Trichophyton rubrum* ATCC28188, *Trichophyton mentagrophytes* ATCC9533 and *Staphylococcus aureus* ATCC25925. Antimicrobial assay and minimum inhibitory concentration (MIC) were carried out using agar plug, agar well diffusion and broth dilution techniques. Bioactive compounds were characterized using Gas Chromatography-Mass Spectrometry (GC-MS). Phytochemical screening revealed alkaloids, tanins and saponins as active phytoconstituents of *V. paradoxa*'s antimicrobial activity. Methanolic extract at 200 mg/ml showed mean clear zone of 25.0 mm and 29.0 mm against *S. aureus and C. albican* respectively. For *T. rubrum*, no growth observed after 14 days while *T. mentagrophyte* showed radial growth at day 12 of incubation. Highest activity of standard antibiotics against *S. aureus* was obtained with Meropenem (25.0 mm). Griseofulvin, itraconazole and fluconazole at 150, 25 and 100 mg/ml respectively inhibited *T. rubrum* while for *T. mentagrophyte*, at 350, 25 and 200 mg/ml respectively after 14 days incubation. MIC for *S. aureus* and *C. albican* were observed at 150 mg/ml and 100 mg/ml respectively. *T. rubrum* and *T. mentagrophyte* in comparison to conventional antifungals revealed Itraconazole among other antifungals exhibited lowest MIC value (25 mg/ml) against both fungi. However, MIC for extract was recorded at 150 mg/ml against *T. rubrum* while *T. mentagrophyte* did not indicate any MIC effect. Some bioactive compounds present in extract includes, E- 15 Heptadecenal, possess antifungal activity, Methyl palmitate possess anti-inflammatory, Palmitic acid possesses antifungal activities. The species of dermatophytes used in this present study has never been reported.

Keywords: Antimicrobial; Dermatophytes; Bioactive; Antibiotics; Antifungal

INTRODUCTION

The widespread of microbial resistance to antibacterial and antifungal drugs has become a major intimidation, and a challenge that leads to the reducing of antibiotics effectiveness[1]. Advocacy has increased to create awareness for the growing rate of antimicrobial resistance (AMR). According to Serwecinska[2], antibiotics, which have capability to hinder the progression and growth of microbes and/or kill infections of humans and animals caused by bacteria, they are also used in non-medical procedures.

The use of antimicrobials has over several years facilitated medical improvements; nonetheless, the incessant rise of microorganism resistance to antimicrobials limits the capacity of treating infections and edges the energies targeted at achieving worldwide health coverage as well as the health-associated sustainable development goal (SDG). The antimicrobial resistance (AMR) is becoming a neglected universal crisis which entails imperative responsiveness and actions[3].

The constituents of most antimicrobial therapeutic drugs administered today are derived from either bacteria, fungi, or chemical synthesis[4,5]. Globally, the use of locally constituted alternative and traditional therapeutic agents is a common discourse however, this has not been fully explored due to the Eurocentric dominance of the therapeutic interventions in Nigeria. In order to salvage the ugly situation created by the surging AMR among clinical bacterial and fungal isolates, many researchers have started

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to exploit the use of plants and plant extracts for their capacity to prevent the growth of these organisms[6,7].

Shea butter is usually gotten from *Vitellaria paradoxa* tree nuts, which belongs to the Sapotaceae family. These nuts are bean-shaped, tiny, rigid, and brown in color. The raw unrefined shea butter is derived from stepwise processes including crushing, roasting, cooking and filtering to eliminate impurities while refined shea butter is treated with chemicals like kojic acid, a known bleaching agent. Shea butter has been used as ointments, gels or creams, skin moisturizer, pastes and so on, containing active ingredients that are either uniformly dispersed and not[8]. Medically, shea butter is used in treatment of rashes, skin inflammation, dermatitis, rheumatism and irritation[9]. Some also use in cooking food, in soaps and candles making[10]. According to reports, the shea butter plant parts generally are effective antimicrobials against bacterial and fungal infections[11].

Skin is a natural forte of abode for microbes and also the first wall to counter antagonism from the environment and pathogens. There also is the dynamic structure organized by the immune system that is resident in the skin, which is vital to the control of an infection, resolution of impairment as well as maintaining homeostasis of the tissue[12]. Dermatophytes and other microorganisms responsible for many of these skin infections include the genera *Trichophyton, Epidermophyton, Microsporum, Staphylococcus, Candida, Trichoderma, Chrysosporium* and so on[13]. This study aimed to investigate the antimicrobial efficacy of methanolic shea nut extract against some selected pathogens including dermatophytes.

MATERIALS AND METHODS

Sample collection

Shea nut sample was obtained from various identified locations within the natural ecological regions of Shea tree within Ilorin metropolis, Kwara state Nigeria.

Collection of isolates

Fungal isolates, *C. albican* ATCC 14503, *T. rubrum* ATCC 28188 and *T. mentagrophytes* ATCC 9533 were obtained from American Typed Cultured Center (ATCC) United State of America. *S. aureus* ATCC 25925 was obtained from Microbiology Department, University of Ilorin, Nigeria.

Solvent Extraction

Extract from nut of Shea plant was obtained from solvent extraction procedure as described by Ajala et al.[14] using a Soxhlet apparatus with n-Hexane, Methanol and Petroleum ether as the solvents and rotary evaporator was used to concentrate the filtrate.

Proximate and phytochemical analysis

Proximate analysis was carried out using a previously reported method[15]. The moisture content was determined by drying five grams of ground samples of were weighed into a sterile aluminum dish, weight of the dish and weight of un-dried sample (in duplicate) were taken. This was transferred into an oven at 100°C for 3 hours respectively. Ash content determination was carried out by placing two grams of the sample placed in a muffle furnace which is maintained at 550 °C for 5 hours, while crude fibre content was estimated by consecutive acid and alkali digestion followed by washing, drying, ashing at 550 °C. Crude fat was obtained by exhaustively extracting about 30 grams of the sample in a Soxhlet apparatus using n-hexane solvent. Crude protein (% total Nitrogen 6.25) was determined by Kjeldahl method. The carbohydrate content was determined by difference.

For phytochemical analysis of shea nut, the different phytoconstituents present in the methanolic extracts of nut was screened qualitatively[14].

Antimicrobial activity of methanolic extract of shea butter against selected isolates

Agar well technique was employed in the assay of the extract against *S. aureus* and *C. albican*. Standardized isolates were spread on sterile Mueller Hinton and Sabouraud dextrose agar plates for growth of bacterial and fungal isolates respectively. Wells were made into the agar plates using sterile corkborer of 8.0 mm in diameter. The methanolic extract of 200 mg/ml concentration was introduced into the wells for bacterial isolates, plates were left for about one hour for diffusion to occur before incubating at 37°C for 24 hours. For *T. rubrum* and *T. mentagrophyte*, agar plugs of uniform size (diameter, 8 mm) were picked using a sterilized corkborer from the Petri dish having full growth isolates and inoculated at the middle of every Petri dish containing 200 mg/ml kept at room temperature for 14 days. After incubation, radial growth of fungal mycelium was observed was measured and recorded accordingly. Control used includes standard antibiotics disk and ketoconazole as positive control, while the negative control was 4% dimethyl sulfur oxide (DMSO) used as diluent. The diameter of zones of clearance around the wells and disks were measured to show the degree to which the test organisms are susceptible to the sample[15].

Minimum Inhibitory, bactericidal and fungicidal concentrations

The minimum inhibitory concentration (MIC) of the methanolic extract against test organisms was determined using the modified method of Ahmed et al.[15]. Extract (1ml) at concentrations of 350, 300, 250, 200, 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 mg/ml was added each to 1 ml of sterile

nutrient broth in different test tubes respectively. Eighteen hours culture (50 μl) was adjusted to 0.5 MacFarland standard with approximate cell number of 1.0 x 10^8cfu/ml and inoculated in each test tube. The tubes were incubated at 37°C for 24 hours in the case of S. aureus ATCC 25925. For dermatophytes, spore suspension of each of the isolate was collected into sterile saline water as described by Ahmed et al.[15] and 1 ml of extract at concentrations of 350, 300, 250, 200, 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 mg/ml was added separately to 1 ml of sterile Sabouraud dextrose broth in different test tubes respectively. Spore suspension (50 μl) was inoculated into each of the tubes and incubated at room temperature for 14 days. Control tubes included reference antibiotics, growth medium and test isolates while a blank was set containing only sterile broth. The tube with the lowest concentration of the extracts (highest dilution) which had no detectable bacterial and fungal growth when compared with the control tube was considered the MIC.

Characterization of bioactive compounds

Gas Chromatography-Mass Spectrometry was done as described by Khan and Bhadauria[16] using SHIMADZU QP2014 ULTRA apparatus operated in EI mode at 70ev. A Restek-5MS column ($30\,m \times 0.25\,mm \times 0.25\,\mu m$) was employed in the determination of bioactive compounds present in the extract. The temperature of oven was projected at 60 °C raised to 280 °C at 5 °C min-1 and held for 2 min, then 250 °C to 280 °C and held for 14 min. The injector temperature was 290 °C with normal injection mode. The flow rate of carrier gas, helium was 1.00 ml min-1. Total running time for GC was 30 min. The compounds were identified by comparing the mass spectra data with data stored in GC-MS library.

RESULTS AND DISCUSSION

Proximate and phytochemical analysis

The result of proximate analysis of shea nut subjected to varying treatment (dry and roasted, dried and freshly crushed) is presented in Table 1. Moisture content was seen to be highest in fresh nuts (19.56 ± 0.03) and lowest in the dried and roasted nuts (6.8 ± 0.01). This is in line with report by Ukpanukpong et al. (2016) that moisture content of shea nut was within the range of 6.33 ± 3.21 to 6.33 ± 3.21. It was obvious from the result that drying as a form of treatment reduced the moisture content of the nuts s compared to the fresh nuts. This also suggests that drying with roasting is an efficient treatment capable of preserving the nuts from microbial invasion for a longer time without spoilage. This is because higher moisture content results in increased microbial activity, since microorganism require moisture as a growth condition. Ash content was highest in dried and roasted nuts (4.93 ± 0.01) and lowest in freshly crushed nuts (3.72 ± 0.01). From literature, ash content connotes availability of minerals in a sample[17,18]. This information suggests that the dried and roasted nuts may be higher in mineral content than the other treatment method which exhibited lower ash contents. The ash content values in this study is in line with Abdul-Mumeen et al.[19] who reported ash content ranging from 4.16 to 4.44 in the analysis of shea nut cakes. The fact that higher mineral composition of sample is a function of ash content was further confirmed by Abdul-Mumeen et al.[19] where the shea nut sample with higher ash content contained more number of minerals than there were in the ones with lower ash content. From literature, shea butter comprises of a vast array of fatty acids and according to Maanikuu and Peker[20] the presence of relatively high amount of saturated fatty acids in shea butter gives it anti-inflammatory property which in turn confers on it great healing potential. The oil-soluble components of the fats in shea butter do not undergo saponification on contact with alkali. From this explanation, it is imperative to state that the exposure of extract from the shea nuts may be inhibitory against pathogens.

In this present study, five classes of phytochemicals were observed as follows; Saponins, tannins, alkaloids, terpenoids and reducing sugars (Table 2). From literatures, the medicinal effect of plants is as a result of certain chemical substances which possess bioactive uses. Saponins, known to possess detergent-like properties which may disrupt microbial cell membrane thus rendering them permeable to harmful substances. The antimicrobial activity of tannins is explained by their ability to penetrate bacterial cell wall thereby interfering with metabolism[21]. Terpenes has been reported to be able to inhibit crucial processes in microbial survivability[22]. Generally, the mechanism of action of most terpenes remains largely unknown. However, a study by Griffin et al.[23] reported that terpenes able to disrupt oxygen uptake as well as oxidative phosphorylation in microbial cells. The presence of reducing sugars can cause bacterial cells to lose water by osmosis which could result in cell lysis. From literature, alkaloids exert their inhibitory effects by various mechanisms, some of which are: disruption of virulent genes, inhibition of microbial destructive enzymes, inhibition of biofilm formation etc.[24].

Evaluation of activity of methanolic extract against selected pathogens

The antimicrobial assay carried out of different extracts obtained from the solvent extraction processed revealed that only methanolic fraction showed activities against the test pathogens and is presented in Table 3. Methanolic extract inhibited both *S. aureus* (25.0mm) and *C. albican* (29.0mm) with 24 hours of exposure with the highest susceptibility by *C. albican*. The closeness in susceptibility demonstrated by *S. aureus* and *C. albican* may be due to the fact that *C. albican* are yeast and hence act like vegetative form of bacteria such as *S. aureus*. From the antifungal assay, no radial growth was

Table 1: Proximate analysis of different sheanut samples

Property	Treatment/Sample		
	Dried and roasted	Dried	Fresh crushed
Moisture content (%)	6.8 ± 0.01	7.84 ± 0.01	19.56 ± 0.03
Ash content (%)	4.93 ± 0.01	4.62 ± 0.001	3.72 ± 0.01
Crude protein (%)	3.21 ± 0.01	3.28 ± 0.01	4.26 ± 0.01
Crude fibre (%)	4.18 ± 0.01	4.12 ± 0.01	3.14 ± 0.01
Fat & oil content (%)	3.86 ± 0.01	3.86 ± 0.01	2.96 ± 0.01
Carbohydrate (%)	58.28 ± 0.04	58.30 ± 0.03	57.39 ± 0.04
Calorific value (%)	1188.91 ± 0.02	1173.63 ± 0.02	1140.79 ± 0.31

Table 2: Qualitative phytochemical analysis ofdifferent shea nut samples

Property	Extraction solvent					
	Methanolic			Ethanol		
	Roasted &dried	Dried	Fresh crushed	Roasted &dried	Dried	Fresh crushed
Saponin	++	+	+	+	+	+
Tannins	++	+	+	+	+	+
Alkaloid	++	+	+	+	+	+
Terpenoid	++	+	+	+	+	+
Reducing Sugar	++	++	++	+	+	+

observed at all for the first 10 days, owing to the growth pattern of *T. rubrum* and *T. mentagrophyte*. However, only *T. rubrum* exhibited total susceptibility towards the extract with no radial growth observed after 14 days indicating 100% growth inhibition of the isolate. *T. mentagrophyte* resisted the extract by demonstrating increased radial growth as the time incubation time increased. The resistance exhibited by *T. mentagrophyte* in this present study is in accordance with findings by Taghipour et al.[25] indicating that *T. mentagrophyte* and *T. interdigitile* demonstrated emergent antifungal drug resistance and global spread of such resistance mechanisms.

Minimum inhibitory concentration of methanolic extract against *S. aureus* was observed at 150g/ml (Table 4) and compared favourably with standard antibiotics, as *S. aureus* resisted all conventional antibiotics used except meropenem (Table 5). The effectiveness of meropenem on this isolate further confirm that meropenem is one of the strongest antibiotics in the treatment of infections caused by *S. aureus*. Several researchers have also reported the sensitivity of multidrug resistant *S. aureus* to meropenem[26]. The MIC assay with *C. albican* revealed that the extract exhibited the lowest MIC value (100mg/ml) in comparison to all other test antifungals with lowest MIC of 150mg/ml (Table 6). The MIC assay against *T. rubrum* and *T. mentagrophyte* in comparison to conventional antifungals revealed that Itraconazole among other antifungals exhibited the lowest MIC (25mg/ml) value against both fungi (Table 7). However, MIC for the extract was recorded at 150mg/ml against *T. rubrum* only. *T. mentagrophyte* did not indicate any MIC effect, confirming its resistant nature as suggested by Taghipour et al.[25].

Characterization of chemical compounds present in Methanolic extract

The chemical compounds detected in the methanolic extract are presented in Table 8. Some of the compound reported to possess antimicrobial activities include E- 15 Heptadecenal possess antifungi activity[27], Methyl palmitate possess anti-inflammatory[28], Palmitic acid possesses both antifungal and anti-inflammatory activities[29], Oleic acid possess anti-inflammatory activity[30]. 10- Nonadecenoic acid[31], cis-13-Eicosenoic acid[32], 2-Monoolein[33].

Table 3: Antimicrobial activity of methanolic extract on selected isolates

Incubation time (day)	Zone of Inhibition/radial growth (mm)							
	S. aureus		*C. albican*		*T. rubrum*		*T. mentagrophyte*	
	ME	Ctr	ME	Ctr	ME	Ctr	ME	Ctr
1	25.0	0.0	29.0	0.0	0.0	0.0	0.0	0.0
2	NA	NA	NA	NA	0.0	0.0	0.0	0.0
3	NA	NA	NA	NA	0.0	0.0	0.0	0.0
4	NA	NA	NA	NA	0.0	0.0	0.0	0.0
5	NA	NA	NA	NA	0.0	0.0	0.0	0.0
6	NA	NA	NA	NA	0.0	0.0	0.0	0.0
7	NA	NA	NA	NA	0.0	0.0	0.0	0.0
8	NA	NA	NA	NA	0.0	0.0	0.0	0.0
9	NA	NA	NA	NA	0.0	0.0	0.0	0.0
10	NA	NA	NA	NA	0.0	0.0	0.0	19.5
11	NA	NA	NA	NA	0.0	20.5	10.5	22.0
12	NA	NA	NA	NA	0.0	23.0	16.0	27.0
13	NA	NA	NA	NA	0.0	28.0	26.5	31.5
14	NA	NA	NA	NA	0.0	36.0	30.5	34.0

Key: ME- Methanolic extract; Ctr- Control; NA- Not Applicable

Table 4: Minimum inhibitory concentration of various concentrations of methanolic extract of *V. paradoxa* against *S. aureus* after 24 h

Concentration (mg/ml)	*S. aureus* 25923
Control (0)	+
1.56	+
3.125	+
6.25	+
12.5	+
25	+
50	+
75	+
100	+
150	–
200	–

Key: + = Turbid growth; – = No growth

Table 5: Comparison between standard antibiotics and methanolic extract *V. paradoxa* nut against *S. aureus* 25923

Antibiotic / Extract	Conc	ZOI (mm)	CLSI breakpoints		
			R	I	S
Amoxicillin	10μg	0.00	≤25	28-36	≥37
Tetracyclin	30μg	0.00	≤24	24-30	≥30
Cotrimoxazole	30μg	10.50	≤12	13–14	≥15
Gentamacin	10μg	9.50	≤12	13-14	≥15
Cefuroxine	30μg	0.00	≤27	27-35	≥35
Cefazolin	30μg	0.00	≤29	29-35	≥35
Ceftriaxone	30μg	0.00	≤27	27-35	≥35
Cefotaxine	30μg	13.50	≤25	25-31	≥31
Ciprofloxacin	5μg	0.00	≤15	16–20	≥ 21
Meropenem	10μg	25.00	≤29	29-37	≥37
Amikacin	30μg	0.00	≤17	15-16	≤14
Levofloxacin	5μg	0.00	≤15	16-18	≥19
Vancomycin	30μg	15.00	-	-	-
Methanolic extract	75 mg/ml	12.0 ± 0.71			
	100 mg/ml	13.5 ± 0.35			
	150 mg/ml	18.0 ± 2.83			
	200 mg/ml	25.0 ± 0.71			

Conc- Concentration; ZOI- Zone of Inhibition

Table 6: Minimum inhibitory concentration of various concentrations of methanolic extract of *V. paradoxa* against *C. albican* after 24 h

Concentration (mg/ml)	Methanolic extract	Griseo fluvin	Itracon azole	Flucon zole
Control (0)	+	+	+	+
1.56	+	+	+	+
3.125	+	+	+	+
6.25	+	+	+	+
12.5	+	+	+	+
25	+	+	+	+
50	+	+	+	+
75	+	+	+	+
100	–	+	+	+
150	–	–	+	–
200	–	–	–	–
250	–	–	–	–
300	–	–	–	–
350	–	–	–	–

Key: + growth; - No growth

Table 7: Minimum inhibitory concentration of various concentrations of methanolic extract of *V. paradoxa* against *T. rubrum* 28188 and *T. mentagrophyte* 9533 after 14 days

Concentration (mg/ml)	*T. rubrum*				T. mentagrophyte			
	M	G	I	F	M	G	I	F
Control (0)	+	+	+	+	+	+	+	+
1.56	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+	+
12.5	+	+	+	+	+	+	+	+
25	+	+	–	+	+	+	–	+
50	+	+	–	+	+	+	–	+
75	+	+	–	+	+	+	–	+
100	+	+	–	–	+	+	–	+
150	–	–	–	–	+	+	–	+
200	–	–	–	–	+	+	–	–
250	–	–	–	–	+	+	–	–
300	–	–	–	–	+	+	–	–
350	–	–	–	–	+	–	–	–

Key: M = Methanolic extract; G = Griseofluvin; I = Itraconazole; F = Fluconazole; + = Aerial growth; – = No aerial growth

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Table 8: Chemical Compounds detected in *V. paradoxa* nut extracts

RT	Compound name	Methanolic extract
30.98	E- 15 Heptadecenal	1.08
38.39	Methyl palmitate	10.02
39.02	Palmitic acid	10.78
39.71	cis-Oleic Acid	19.96
41.23	10-Nonadecenoic acid, methyl ester	0.64
41.32	cis-13-Eicosenoic acid	9.42
41.44	Eicosanoic acid	16.29
41.79	Dasycarpidan-1-methanol, acetate 2 (Octadecyloxy) ethyldocosanoic	3.51
41.91	acid ester	0.86
42.39	Docosanoic acid	2.68
43.04	N-Butylcinnamamide	2.20
43.49	2-Monoolein	22.55

CONCLUSION

The report from the present study indicated that dried and roasted methanolic extract of *V. paradoxa* had the highest mineral composition and lowest water activity which may have contributed to enhancing phytochemical properties of extract and extending its shelf-life respectively. Furthermore, the dried and roasted extract used for antimicrobial assay was observed to contain bioactive chemical compounds which have been previously reported to possess antimicrobial property. These phytochemicals may have been responsible for the efficacy reported in this study against bacteria, yeast and dermatophytes.

However, it is pertinent to note that antifungal activity was not recorded for *T. mentagrophyte* and this may be owing to the presence of resistance genes as reported by Taghipour et al.[25] that *T. mentagrophyte* harbour mutation in the squalene epoxidase (SQLE) gene. From the study, dried and roasted methanolic extract of shea nut is recommended as an effective antifungal agent against skin infections caused by *S. aureus* and *T. rubrum* at 150mg/ml, and *C. albican* at 100mg/ml.

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