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Antimicrobial Activity of *Zanthoxylum holtizianum* (Engl.) Waterm and *Zanthoxylum lindense* (Engl.) Kokwaro Growing in Bagamoyo District, Coast Region, Tanzania

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Abstract

Global emergence of antimicrobial resistance in developing countries like Tanzania is aggravated by misuse of antimicrobials in prophylaxis and treatment of both, animal and human diseases and lack of effective antimicrobial resistance surveillance. However, some microbes are also intrinsically resistant to antimicrobials, hence, antimicrobial resistance is unrelenting war and search for new effective antimicrobials is an unending pursuit. Tanzania being a country rich in biodiversity could provide novel molecules as antimicrobials or leads for effective antimicrobials. The current study aimed at determining the antimicrobial potential of various extracts of two *Zanthoxylum* species growing in Tanzania.

Petroleum ether, dichloromethane and methanolic extracts of the leaves, stem bark and roots of *Z. holtizianum* and *Z. lindense* were screened for antimicrobial activity against several Gram positive bacteria, Gram negative bacteria and yeasts using agar disc diffusion method. The minimum inhibitory concentrations (MICs) for the active extracts were also determined using broth microdilution method.

The stem bark and root extracts showed good activity against all tested Gram positive bacteria including *Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus anthracis* and *Bacillus cereus,* with inhibition zones ranging from 7-15 mm but were inactive against all the tested Gram negative bacteria and the two yeasts. The most susceptible bacteria was *S. aureus* with MICs ranging from 2.5- 0.625mg/ml, the lowest MIC was displayed by the methanolic root extract of *Z. lindense.* The two *Zanthoxylum* species have shown varied degrees of antimicrobial activity which could be explored further.

Key words: Antimicrobials, *Staphylococus aureus,* Tanzania, *Zanthoxylum holtizianum*, *Zanthoxylum lindense.*

INTRODUCTION

Infectious diseases including those due to microorganisms are among the leading causes of death and disability worldwide [1]. The management of these diseases is undermined by global emergence of antimicrobial resistance [www.who.int/drugresistance/documents/surveillancereport /en/, 2, 3,]. Causes of antimicrobial resistance include both human and non human factors. Misuse of antimicrobial agents in prophylaxis and treatment of both, animal and human diseases, lack of effective antimicrobial resistance surveillance, lack of quality antimicrobials and poor infections control especially in developing countries are human factors which could be intervened [4, 5]. However, non human factors, including adaptability of microbes to various environments through mutation, some microbes being intrinsically resistant to antimicrobials and presence of resistance genes in the environment are beyond human control [5-8]. All this implies that antimicrobial resistance cannot become completely extinct. Hence, as long as infectious microbes co-exist with humans search for new effective antimicrobials is a continuous pursuit.

Apart from antimicrobial resistance new emerging infectious diseases mostly due to HIV and AIDS, is another major health concern [9, 10]. Furthermore, most of the commonly used drugs are associated with side/toxic effects and drug-drug interactions [11]. Affordability is another disturbing factor, especially with microbial resistance, the cost of new effective antimicrobial agents may not be affordable by the majority of developing countries including Tanzania [12]. Antimicrobial resistance has led to decreased development of new effective antimicrobials by the pharmaceutical industries which have, in turn, stimulated exploration of other alternative sources [13]. Tanzania has a rich biodiversity which can be used as a starting point for alternative source of effective antimicrobials.

The genus *Zanthoxylum* (Rutaceae) is reported to comprise 250 species consisting of shrubs and trees [14] which are widely used in different parts of the world including, Africa, Asia and America, to treat a number of human and animal diseases [14, 15, 16]. The herbarium, at the Department of Botany, University of Dar es Salaam has documented10 *Zanthoxylum* species found in Tanzania (Table 1). Roots and stem barks of *Zanthoxylum holtizianum* (Engl.) Waterm and *Zanthoxylum lindense* (Engl.) Kokwaro have been used by inhabitants of coastal regions of Tanzania, to treat infectious diseases. To our knowledge only scanty information is available on these plants.

However, in Tanzania, *Z. chalybeum*, *Z. zanthoxyloides* and *Z. deremense,* have been reported to be used to treat various health conditions including epilepsy, fungal infections, stomach ache, fever, hernia, headache, toothache, body and

limb swelling, malaria, asthma, chest pains, infertility, heart pains, diabetes, abscess and sickle cell [17-22]. Moreover, of the species found in Tanzania, *Z. chalybeum* is the most studied and it has been reported to have antimalarial [23], antifungal [19], brine shrimp lethality [20] and cytotoxic activities [24].

The review of the genus *Zanthoxylum* shows that it is associated with a number of biological activities including hepatoprotective, antiplasmodic, cytotoxic, anthelminthic, antiviral, antiproliferative, antibacterial, antifungal, leishmanicidal, hepatotoxicity, larvicidal, anti-inflammatory, analgesic, antinociceptive and antioxidant activities [14].

In the perspective of the current study a number of species including *Z. chalybeum*, *Z. usambarense, Z. fagara, Z. elephantiasis, Z. martinicense*, *Zanthoxylum chiloperone* var. *angustifolium* have been reported to exhibit antimicrobial activity [19, 25, 26, 27, 28, 29]. Furthermore, closely related compounds; dihydrochelerythrine and chelerythrine isolated from *Zanthoxylum rhetsa* and *Zanthoxylum clava-herculis,* respectively, exhibited a strong activity against various strains of methicillin resistant *Staphylococcus aureus* with MICs ranging from 8µg/ml to 16µg/ml. Dihydrochelerythrine was also active against *Escherichia. coli* [30, 31].

The current study, therefore, explored the antimicrobial activity of various extracts of different plant parts of *Zanthoxylum holtzianum* and *Zanthoxylum lindense* collected in Bagamoyo district, Coastal region, Tanzania.

MATERIALS AND METHODS

Plant Collection and authentication

Various plant parts including, leaves, stem barks and roots were collected from plants growing wild at Saadan National Park, Bagamoyo, Coast region, Tanzania, in December 2012. Identification of the plants was done by a botanist at the herbarium of the Department of Botany, University of Dar es Salaam, where voucher specimens were deposited.

Preparation of the extracts

Roots and stem barks were cut into small pieces and leaves were separated from stems and dried at room temperature for several weeks. The dried plant materials were ground using a blender, to appropriate size based on their botanical structures. The ground plant materials were consecutively, exhaustively macerated with petroleum ether, dichloromethane and methanol at room temperature. The resultant extracts were concentrated at ≤50ºC *in vacuo* using Buchi rotary evaporator. The extracts were kept in a refrigerator until needed for antimicrobial screening.

Antimicrobial screening

Test organisms

Test organisms included four Gram positive bacteria; *Streptococcus pyogenes* (clinical isolate), *Staphylococcus aureus* (ATCC 25923), *Bacillus anthracis* (NCTC 10073) and *Bacillus cereus* (clinical isolate), four Gram negative bacteria; *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603) and *Salmonella typhi*, (NCTC 8385) and two yeasts; *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (clinical isolate) which were obtained from the Departments of Pharmaceutical Microbiology, School of Pharmacy, and Biological and Preclinical Studies, Institute of Traditional Medicine (ITM), atMUHAS.

Inocula

Prior to the sensitivity testing each of the bacteria strains and fungi were separately cultured repeatedly on tryptone soya agar (Oxoid Ltd. UK) and Sabouraud's dextrose agar (Oxoid Ltd. UK), respectively in order to ensure viability. From these cultures few colonies were suspended in normal saline and their densities adjusted to 0.5 MacFarland; this was the inocula used for seeding the agar plates used for sensitivity testing.

Disc diffusion

Sterile filter paper discs (6 mm, Whatman No. 20) were impregnated with 10µl of the extract solution (500mg/ml in DMSO) and allowed to dry. Gentamicin (10µg) and fluconazole (100µg) susceptibility test discs were used as positive control for bacteria and fungi, respectively. The test disc and positive control discs were placed on the surface of the seeded plates. The plates were incubated over night at 37ºC for bacteria and 30^{o}C for fungi. The inhibition was zones were recorded. The tests were done in triplets to check for consistency.

Determination of Minimum Inhibitory Concentrations (MICs)

Determination of MICs was accomplished through microdilution susceptibility test utilizing a 96 well microtitre plate consisting of twelve columns and eight rows [32]. Test extracts were prepared by dissolving 100mg into 1ml DMSO and diluted with 4ml of distilled water to make a concentration of 20mg/ml. Double and normal strength tryptone soya broth (Oxoid Ltd. UK) were prepared according to the manufacturer's instruction. Fifty (50) µl of the prepared double strength media were placed in each well of the first row of the microtitre plate whereas the normal strength media was placed in the rest of the wells. Fifty (50) µl of various test samples were introduced into the first row of the microtitre plate and mixed well, followed by a two- fold dilution down the column. Test microorganisms (50µl) of appropriate density, were subsequently added to each well to attain a final density of 0.5 MacFarland. The adjusted final concentrations of the various extracts test samples ranged from 5mg/ml to 3.8 x10^{-3}mg/ml while for positive control (Gentamicin sulphate) ranged from 1.256 x 10 $^{-3}$ to 9.76x 10 $^{-6}$mg/ml and Dimethyl sulphoxide (5%) was used as the negative control. The plates were then incubated for 24 hours at 37ºC after which they were removed from the incubator and 40 µl of 0.2 mg/ml p- iodonitrotetrazolium (INT) chloride dye was added, followed by further incubation of the plates for 30 minutes. The lowest concentrations which showed no purple colour (indicating absence of growth) were taken as the minimum inhibitory concentrations (MICs).

Table 1: *Zanthoxylum* species found in Tanzania

S/N	Subspecies	Life Form	Distribution	Status
1	*Zanthoxylum chalybeum* Engl.	S	All over the Country	N
2	*Zanthoxylum deremense* (Engl.) Kokwaro	S	Tanga, Kilimanjaro, Coast, Morogoro, and Southern Regions	N
3	*Zanthoxylum gilletii* (De Wild.) P.G.Waterman	T	Tanga, Kilimanjaro and Southern Highlands of Tanzania	N
4	*Zanthoxylum holtzianum* (Engl.) P.G.Waterman subspcecies *holtzianum*	S	Tanga, Kilimanjaro, Coast, Morogoro, and Southern Regions	N
	Zanthoxylum holtzianum (Engl.) P.G.Waterman subspecies *tenuipedicellatum* Kokwaro	S	Southern Regions	E
5	*Zanthoxylum leprieurii* Guill. & Perr.	T	Southern Regions	N
6	*Zanthoxylum lindense* (Engl.) Kokwaro	S	Coast, Morogoro, and Southern Regions	E
7	*Zanthoxylum paracanthum* (Mildbr.) Kokwaro	C	Cetral zone (Dodoma & Singida)	E
8	*Zanthoxylum rubescens* Hook.f.	S	Rukwa, Mpanda, Tabora, Kigoma and Southern Highlands of Tanzania	N
9	*Zanthoxylum trijugum* (Dunkley) P.G.Waterman	S	Rukwa, Mpanda, Tabora, Kigoma and Cetral zone	N
10	*Zanthoxylum usambarense* (Engl.) Kokwaro	T	Lake zone, Arusha, Manyara, Kilimanjaro and Tanga	E

Table 2: Percentage extract yields for different plant parts using different solvents.

S/N	Plant Species	Plant part	Solvent	%yield
1.	*Z. holtzianum*	Leaves	Petroleum ether	0.60
2.	*Z. lindense*	Leaves	Petroleum ether	0.40
3.	*Z. holtzianum*	Leaves	Dichloromethane	1.50
4.	*Z. lindense*	Leaves	Dichloromethane	2.80
5.	*Z. holtzianum*	Leaves	Methanol	6.50
6.	*Z. lindense*	Leaves	Methanol	7.70
7.	*Z. holtzianum*	Stem bark	Petroleum ether	0.24
8.	*Z. lindense*	Stem bark	Petroleum ether	0.99
9.	*Z. holtzianum*	Stem bark	Dichloromethane	1.80
10.	*Z. lindense*	Stem bark	Dichloromethane	1.50
11.	*Z. holtzianum*	Stem bark	Methanol	7.00
12.	*Z. lindense*	Stem bark	Methanol	5.30
13.	*Z. holtzianum*	Roots	Petroleum ether	1.30
14.	*Z. lindense*	Roots	Petroleum ether	0.20
15.	*Z. holtzianum*	Roots	Dichloromethane	2.30
16.	*Z. lindense*	Roots	Dichloromethane	1.50
17.	*Z. holtzianum*	Roots	Methanol	4.50
18.	*Z. lindense*	Roots	Methanol	5.00

Table 3: Inhibition zones for the various extracts of *Zanthoxylum* species

Microoganism	Test sample/ inhibition zones in mm																			
	a	**b**	**c**	**d**	**e**	**f**	**g**	**h**	**i**	**j**	**k**	**k**	**l**	**m**	**n**	**o**	**p**	**q**	**G**	**F**
S. aureus	0	0	0	0	0	0	7	7	7	9	8	13	9	9	11	9	8	9	20	NA
S. pyogenes	0	0	0	0	0	0	11	10	7	8	10	8	9	7	8	9	7	9	30	NA
B. anthracis	0	0	0	0	0	0	8	8	7	9	9	9	12	11	15	12	11	15	23	NA
B. cereus	0	0	0	0	0	0	8	9	8	11	10	9	10	11	15	11	10	13	20	NA
E. coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	NA
K .pneumoniae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	NA
P. aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
S. typhi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	16	NA
C. albicanas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	0
C. neoformans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	0

1: *Z. lindense* LP (Leaf Pet. ether); 2: *Z. lindense* LD (Leaf dichloromethane); 3: *Z. lindense* LM (Leaf methanol); 4:*Z. holtizianum* LP; 5: *Z. holtizianum* LD;6: *Z. holtizianum* LM; 7: *Z. Lindense* BP (Bark Pet. Ether); 8: *Z. lindense* BD (Bark dichloromehane); 9: *Z. lindense* BM (Bark methanol); 10: *Z. holtizianum* BP; 11: *Z. holtizianum* BD; 12: *Z. holtizianum* BM; 13: *Z. Lindense* RP (Root Pet.ether); 14: *Z. lindense*RD (Root dichloromethane); 15: *Z. lindense* RM; 16: *Z. holtizianum* RP; 17: *Z. holtizianum* RD; 18: *Z. holtizianum* RM; G: Gentamicin; F: Fluconazole; NA: Not applicable

Table 4: Minimum inhibitory zones for various extracts *of Zanthoxylum* species

Plant species	Plant part	Extracting solvent	Minimum inhibitory concentrations (MICs) mg/ml			
			S. aureus	*S. pyogenes*	*B. anthracis*	*B. cereus*
Z. holtizianum	Stem Bark	Pet. ether	1.25	>5	>5	>5
Z. lindense	Stem Bark	Pet. ether	2.5	>5	>5	>5
Z. holtizianum	Stem Bark	Dichloromethane	2.5	>5	>5	>5
Z. lindense	Stem Bark	Dichloromethane	2.5	>5	>5	>5
Z. holtizianum	Stem Bark	Methanol	2.5	>5	>5	>5
Z. lindense	Stem Bark	Methanol	1.25	>5	>5	>5
Z. holtizianum	Roots	Pet. ether	2.5	>5	>5	>5
Z. lindense	Roots	Pet. ether	1.25	>5	>5	>5
Z. holtizianum	Roots	Dichloromethane	1.25	>5	>5	>5
Z. lindense	Roots	Dichloromethane	2.5	>5	>5	>5
Z. holtizianum	Roots	Methanol	1.25	>5	>5	>5
Z. lindense	Roots	Methanol	0.625	>5	>5	>5
Gentamicin			3.12×10^{-4}	1.56×10^{-4}	6.28×10^{-4}	6.28×10^{-4}

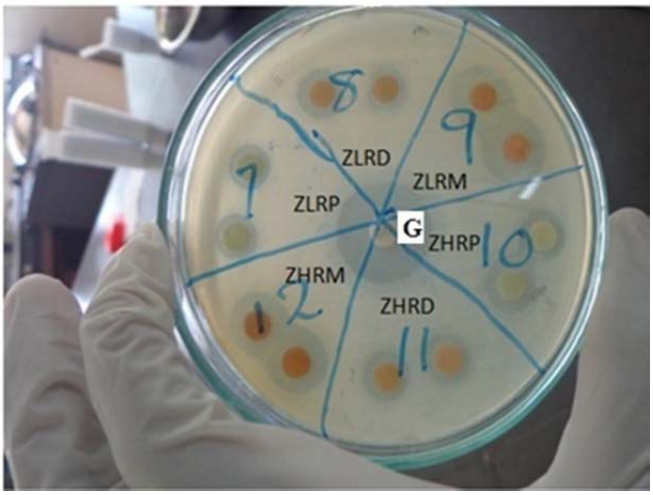

Figure1: Petri dish showing inhibition zones of various extracts against S. aureus*

*ZL: *Z.lindense*; ZH: *Z. holtizianum*; R: Root; D: Dichloromethane; P: Petroleum ether; M: Methanol; G: Gentamicin

RESULTS

The percentage yields of the extracts were as indicated in Table 2; they ranged from 0.2% w/w (petroleum ether extract of *Z. lindense* roots) to 7.7% w/w (methanol extract of *Z. lindense* leaves). All extracts were tested for antimicrobial activity by the disc diffusion method and the inhibition zones were as indicated in Table 3. Figure 1 also shows the inhibition zones displayed by the extracts against *S. aureus.* Stem bark and root extracts of both *Zanthoxylum* species were inactive on all tested Gram -ve bacteria but displayed good activity against all tested Gram +ve bacteria with inhibition zones ranging from 7mm to15mm. However, leaf extracts for both species had no activity against all tested microorganisms. Furthermore, all the tested extracts were inactive against the test yeasts, *Candida albicans* and *Cryptococcus neoformans.*

The minimum inhibitory concentrations (MICs) were determined for all active extracts using broth microdilution method and the results are as indicated in Table 3. *Staphylococcus aureus* was the most sensitive bacteria with MICs ranging from 2.5 to 0.625mg/ml.

DISCUSSION

The two studied *Zanthoxylum* species yielded substantial amounts of extracts using various solvents (Table 2). The maximum yield (7.7 %w/w) was displayed by *Z. lindense* leaves (methanol) while the minimum was 0.2 %w/w from the roots of the same species (petroleum ether). The bark and root extracts of both species showed good activity against all tested Gram positive bacteria including *S. pyogenes* (clinical isolate), *S. aureus* (ATCC 25923), *B. anthracis* (NCTC 10073) and *B. cereus* (clinical isolate), with inhibition zones ranging from 7 to 15mm (Table 3). However, most extracts were devoid of the activity against the tested Gram negative bacteria and the yeasts. In addition, all leaf extracts, exhibited no antimicrobial activity (Table 3) against all tested microorganisms. Reports from previous study [28] done on methanolic extracts and essential oils from fruits of *Z. zanthoxyloides* and *Z. leprieurii* from Cameroon had shown significant inhibition zones varying from 11 to 15.66 mm whereby Gram positive bacteria were more susceptible compared to Gram negative bacteria. It is, therefore, not surprising that in the current study Gram negative bacteria were less susceptible since the presence of extra membrane permeability barrier with multiple efflux pumps and antibiotic modifying enzymes make them difficult to be killed [33]. However, a previous study on non polar root bark extracts of *Z. chalybeum* from Kenya reported inhibition zones of 13.17mm and 9.33mm for *B. cereus* and *S. aureus,* respectively, values falling within the inhibition zone range determined in this study. Contrary to the current study the extract from the Kenyan plant also inhibited the growth of *P. aeruginosa* which was resistant in this study [29].

On the other hand all studied extracts were inactive against fungi but previous studies on different *Zanthoxylum* species reported activities against a number of fungi. For instance, the alkaloidal extract of the stem bark of *Z. chiroperone* var *angustifolium* exhibited good activity against several fungi including *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton mentagrophytes* var. *interdigitale* using a bioautographic method. Moreover, isolated alkaloids had a broader spectrum of activity, whereby *Aspergillus fumigatus*, *A. niger, A. terreus, Candida albicans*, *C. tropicalis*, *C. glabrata*, *Cryptococcus neoformans*, *Geotrichum candidum*, *Saccharomyces cerevisiae*, *Trichosporon beigelii*, *Trichosporon cutaneum* and *Trichophyton mentagrophytes* var. *interdigitale* were all inhibited [27]. Also, the non polar root bark extracts *Z. chalybeum* from Kenya was reported to be active against *C. albicans,* with an inhibition zone of 8.17 mm (29). Essential oil hydrodistilled from fruits of *Z. xanthoxyloides* from Cameroon was also reported to be active against a plant pathogenic fungus *Phytophthora megakarya* [34]. Furthermore, an aqueous methanolic extract of the root bark of *Z. chalybeum* from Tanzania displayed moderate activity against *Candida albicans* (ATCC90028) [19].

In previous studies roots and stem or root bark extracts of various *Zanthoxylum* species were also found active [19, 27, 31]. Despite the fact that leaf extracts in this study were inactive, in a previous study leaf, fruit, stem bark and root extracts of Northern prickly ash, *Zanthoxylum americanum* from Canada were reported to display similar activities against opportunistic and pathogenic fungi including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* [35].

All bark extracts of *Zanthoxylum holtizianum* were more active than respective extracts of *Zanthoxylum lindense* for *Staphylococcus aureus*, *Bacillus anthracis* and *Bacillus cereus*. In most cases *Zanthoxylum holtizianum* root extracts displayed either equal or stronger activity than respective extracts of *Zanthoxylum lindense.* The differences in activities between the studied species and those previously reported may be attributed to presence of different chemical constituents in these species.

The active extracts were used to determine the minimum inhibitory concentrations which for most of the tested microorganisms were above 5mg/ml and they were between 2.5 - 0.625 mg/ml for *S. aureus.* The lowest minimum inhibitory concentration being displayed by the methanolic root extracts of *Z. lindense*. More studies are required so as to isolate the active components.

CONCLUSION

The current study has shown that all stem bark and root extracts of both *Zanthoxylum holtizianum* and *Zanthoxylum lindense* displayed good activity against Gram positive bacteria including *S. pyogenes*, *S. aureus*, *B. anthracis* and *B. cereus.* They were however, devoid of activity against Gram negative bacteria and yeasts. Furthermore it was noted that leaves were devoid of activity; this is a negative attribute in view of conserving the biodiversity. The obtained results call for further studies on *Zanthoxylum* species found in Tanzania.

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