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ANTIMICROBIAL AND BRINE SHRIMP LETHALITY OF EXTRACTS OF *TERMINALIA MOLLIS* LAWS

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Abstract

Using the disc diffusion method it was demonstrated that extracts of the leaves, stem and roots of *Terminalia mollis* Laws (Combretaceae) have antibacterial activity against *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (NCTC 10662), *Klebsiella pneumoniae* (NCTC 9633), *Salmonella typhi* (NCTC 8385), and *Bacillus anthracis* (NCTC 10073) and antifungal activity against *Candida albicans* (Strain HG 392), and local strains of *Aspergillus flavus* and *Cryptococcus neoformans*. The root extracts were the most active followed by the stem, and leaf extracts. Extracts of the three parts also exhibited cytotoxicity to brine shrimp larvae with LC_{50} values ranging from 26.3 to 58.1, l0.4 to 69.5, and 42.9-101.3µg/ml, for the root, stem, and leaf extracts, respectively. The results support the traditional uses of extracts of this plant for the management of bacterial and fungal infections.

Key words: *Terminalia mollis*, Antimicrobial activity, Traditional medicine.

Introduction

The family Combretaceae consists of 18 genera, the largest of which is *Combretum* with about 370 species, and *Terminalia* with about 200 species (McGaw et al., 2001). Plant species from the two genera, especially *Combretum* are widely used in traditional medicine throughout Africa (Kokwaro, 1993; Baba-Mousa et al., 1999; McGaw et al., 2001; Fyhrquist et al., 2002; 2004; Moshi and Mbwambo, 2005).

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Recent studies on the genus *Terminalia* have generated reports of useful biological activities, including antimicrobial (Katerere et al., 2002; Carpano et al., 2003; Bonjar, 2004a,b; Fyhrquist et al., 2002; 2004; Masoko et al., 2005; Moshi and Mbwambo, 2005), antidiabetic (Kaur et al., 2003), anti-HIV (Martino et al., 2002), antioxidant (Cheng et al., 2003), anticancer (Saleem et al., 2002), promotion of wound healing (Mukherjee et al., 2003), antimalarial (Sanon et al., 2003), anti-Herpes simplex type 2 (Cheng et al., 2002), and antidiarrhoeal activity (Abdullah et al., 2001), to mention a few. A number of the already established biological activities indicate the potential this genus has in the management of conditions associated with the HIV infection. Having recognized this potential, efforts are now ongoing to study the antimicrobial activities of Combretaceae plants that grow in Tanzania and are used in traditional medicine (Fyhrquist et al., 2002; 2004; Moshi and Mbwambo, 2005).

In the present study we are reporting work that was done on *Terminalia mollis* Laws (Combretaceae) (Syn:*Terminalia tolurosa* F. Hoffm., or *Terminalia spekei* Rolfe). This is a savanna woodlands tree 11 – 26 m tall, with a black gray, deep fissured bark (Dale and Greenway, 1961). It is widely used in Tanzania for the treatment of malaria and as an adjunct therapy for HIV patients, to treat diarrhea and bacterial infections. In Bukoba (northwestern Tanzania) where it is known as "Muongora" it has similar traditional uses as the related species *Terminalia sericea* Burch. Ex. DC., whose antimicrobial activity was recently reported (Moshi and Mbwambo, 2005). There is up to now very scanty information about this plant in the literature. One study in west Africa reported the molluscicidal activity of a root bark extract (Sofowora, 1980), and another reported that a 50% aqueous ethanol extract of both the leaf and root bark have antifungal activity against *Trichophyton mentagrophytes* (MIC 0.25 mg/ml), and *Epidermophyton floccosum* (MIC 0.5 mg/ml), but did not show activity against *Microsporum gypseum* and *Candida albicans* (Baba-mousa et al., 1999). A recent study reported the presence of antifungal activity in different extracts of the leaves (Masoko et al., 2005). The current study explores further the antibacterial and antifungal activities of parts of the plant, and has included test for cytotoxicity, using brine shrimps (Meyer et al., 1982).

Materials and Methods

Materials

Petroleum ether, dichloromethane, ethyl acetate, butanol, and ethanol were purchased from Fisher Scientific, UK, Ltd (Bishop Meadow Road, Loughborough, Leicestershire, LE 11 5RG, UK). Saboraud's dextrose agar (SDA) and Mueller Hinton agar were purchased from Oxoid Ltd (Basingstoke, Hampshire, England), while dimethylsulfoxide (DMSO) was purchased from Sigma (Poole, Dorset, England). Brine shrimp eggs were bought from Dohse Acquaristic, Bonn (Aus Dem Hause Dohse Acquaristik), Germany. Sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast.

Collection of plant material

The plant was first collected in 1999 from Tabora and identified by Mr. Selemani of Botany Department, University of Dar es Salaam (voucher no. IMPP

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002-0067). The material for this study was collected in Kagera region and identified by the same person (Voucher no. ZHM 2). Both vouchers are kept in the Herbarium of the Institute of Traditional Medicine, Muhimbili University College of Health Sciences.

Extraction procedure

The stem and whole roots of *Terminalia mollis* were separately chopped into small pieces, and ground into fine particles. The fine particles of the stem (854.0 g) and roots (753.0 g) were sequentially extracted with petroleum ether (5.0 l), ethylacetate (5.0 l), 1:1 mixture of dichloromethane/methanol (2.5 l each), methanol (5.0 l), and distilled water (5.0 l). The aqueous eluent (3.8 l) was concentrated *in vacuo* at 30^0C to 1.0 l. The later was twice partitioned with 500 ml butanol. Solvents were completely dried *in vacuo*, and freeze dried to afford butanol and water extracts, respectively. The extract yields were (stem, roots) as follows: Petroleum ether (2.0; 2.4), Ethylacetate (8.6; 11.0), 1:1 dichloromethane: methanol (225.0; 22.0), methanol (21.0; 200.0), butanol (2.0; 23.6), aqueous (1.0; 9.8) g, respectively. The leaves were dried in the shade and 825 g extracted by different solvents to afford extracts of petroleum ether (42 g), 1:1methanol:dichloromethane (52 g), Methanol (49 g), butanol (42 g), and water (50 g).

Antimicrobial tests

Antibacterial and antifungal activities were tested by the disc-diffusion method (Singh et al, 2002). Six standard bacteria, *Staphylococcus aureus* (NCTC 6571), *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (NCTC 10662*), Klebsiella pneumoniae* (NCTC 9633), *Salmonella typhi* (NCTC 8385), and *Bacillus anthracis* (NCTC 10073) and the fungi, *Candida albicans* (Strain HG 392), and local strains of *Aspergillus niger*, *Aspergillus fumigatus, Aspergillus flavus, Cryptococcus neoformans* and *Penicillium* Spp.were used. Filter paper discs (Whatman No. 1; 5 mm diameter) were impregnated with crude extracts (5 mg/disc) or standard drugs (20 μg/disc ampicillin, 10 μg/disc gentamicin; for bacteria) and clotrimazole (20 μg/disc; for fungi). The discs were overlayed on Mueller Hinton agar plates (for bacteria) and Saborauld's dextrose agar plates (for fungi) and incubated at 37 ^{O} C, for 24 h in the case of bacteria and Candida and for 48 h in the case of the other fungi. The discs were tested in triplicate, including one with a solvent blank and 3 for the standard drugs. Inhibition zones were calculated as the difference between disc diameter (5 mm) and the diameters of inhibition (Hewitt and Vincent, 1989). The mean inhibition zones were used to calculate the activity index. Activity index (AI) was calculated as the mean inhibition zone for test sample divided by the mean inhibition zone for the standard drug (Singh et al, 2002).

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Brine shrimp lethality test

The brine shrimp lethality test (BST) was used to predict the presence, in the extracts, of cytotoxic activity (Meyer et al., 1982). Assay procedures and analysis of results was done as reported earlier (Moshi and Mbwambo, 2005).

Results

Antimicrobial activity

Tables 1-3 show that extracts of the root, stem and leaves of *Terminalia mollis* exhibited antibacterial and antifungal activity. With the exception of the stem petroleum ether extract which exhibited antifungal activity against *Cryptococcus neoformans,* all the petroleum ether extracts had no activity against bacteria and fungi. Butanol, dichloromethane: methanol (1:1), and aqueous extracts exhibited antifungal activity against *Candida albicans* and *Cryptococcus neoformans*. The highest activity against *Candida albicans* was found in the stem extracts, while root extracts showed the highest activity against *Cryptococcus neoformans*. The aqueous extract exhibited the highest activity against both *Candida albicans* and *Cryptococcus neoformans* for all the three plant parts. All the stem extracts were inactive against *Aspergillus* species and the *Penicillium* species (Table 1).

Root extracts (Table 2) exhibited the best antibacterial and antifungal activity. Most of the root extracts, except petroleum ether, were active against all the bacteria used and three fungi, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus flavus*. The leaf ethylacetate, methanol, butanol and dichloromethane: methanol (1:1) extracts were active against *Salmonella typhi, Staphylococcus aureus*, and *Bacillus anthracis* (Table 3). They had no activity against *Escherichia coli, Vibrio cholera* and *Pseudomonas aeruginosa*. The aqueous extract was active against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Brine shrimp lethality

Tables 4-6 showed results of the activity of the root, stem and leaf extracts against brine shrimp larvae, respectively. The butanol extract of the stem wood with an LC_{50} (95% confidence interval) of 10.4 (5.9–18.3) μg/ml was the most toxic. The LC_{50} of the butanol extract of the stem was lower than that of the standard drug cyclophosphamide which gave an LC_{50} of 16.3 (10.6-25.2)μg/ml. The stem extracts gave LC_{50} values between 35.0 (18.3-66.9) and 69.5 (51.9-93.1) μg/ml. The root extracts gave LC_{50} (95% confidence interval) values between 26.3 (18.8-37.0) and 58.1 (43.9-76.8) μg/ml. The leaf extracts were less toxic to brine shrimps. The aqueous, methanol, and petroleum ether extracts gave LC_{50} (95% confidence interval) values between 42.5 (29.1-62.0) and 101.3 (69.8-146.8)μg/ml.

Table 1: Antibacterial and antifungal activity of *Terminalia mollis* stem wood extracts

Organisms tested on		PE	ET	M	Bu	1:1 M:D	AQ	Clotr 20 µg/disc	Gent 10 µg/disc	Amp 20 µg/disc
Escherichia coli	IZ	-	-	-	-	-	-	-	-	21.0±1.0
	AI									1.0
Salmonella typhi	IZ	-	4.7±0.6	7.0±0	7.0±0	6.7±0.5	-	-	-	20.0±1.0
	AI		0.2	0.3	0.3	0.3				1.0
Vibrio cholera	IZ	-	-	-	-	-	-	-	-	17.0±1.0
	AI									1.0
Staphylococcus	IZ	-	4.7±1.0	8.3±1.0	12.7±0.6	5.7±0.6	11.7±1.1	-	-	20.0±1.0
aureus	AI		0.2	0.4	0.6	0.3	0.6			1.0
Bacillus anthracis	IZ	-	6.7±0.6	5.0±0.6	5.3±0.6	5.3±1.0	-	-	-	10.3±1.5
	AI		0.7	0.5	0.5	0.5				1.0
Pseudomonas	IZ	-	-	-	10.0±0	-	9.7±0.6	-	12.0±1.0	-
aeruginosa	AI				0.8		0.8		1.0	
Candida albicans	IZ	_	13.3±0.6	15.0±1.0	9.0±1.0	15.0±0	10.0±0	15.0±1.0		
	AI		0.9	1.0	0.6	1.0	0.7	1.0		
Aspergillus niger	IZ	-	-	-	-	-	-	15.0±1.0		
	AI							1.0		
Aspergillus fumigatus	IZ	-	-	-	-	-	-	25.0±1.0		
	AI							1.0		
Aspergillus flavus	IZ	-			-	-	-	20.0±0.6	1.00	
	AI									
Cryptococcus	IZ	1.3±0.6	7.0±1.0	5.3±0.6	5.0±1.0	5.7±0.6	0.3	6.7±1.1	15.0±1.0	
neoformans	AI	0.1	0.5	0.3	0.3		0.4	1.0		
Penicillium spp.	IZ	-	-	-	-	-	-	20.0±1.0	1.0	
	AI									

Results are reported as inhibition zones (IZ; mm) with the corresponding activity index (AI). Inhibition zones are presented as mean±SD (n = 3). Inhibition zones (IZ) exclude the disc diameter (5 mm); Activity index (AI) = Inhibition zone of test sample divided by inhibition zone of a standard drug. B = butanol extract; AQ = Aqueous extract; M = methanol extract; Amp=ampicillin; D = dichloromethane; ET = Ethyl acetate; P = petroleum ether;.clotr = clotrimazole

Table 2: Antibacterial and antifungal activities of Terminalia *mollis* root extracts

Organisms tested	IZ AI	**P**	**ET**	**M**	**Bu**	**1:1 M:D**	**AQ**	**Clotr 20 µg/disc**	**Gent 10 µg/disc**	**Amp 20 µg/disc**
Staphylococcus aureus	IZ	-	19.3±0.7	19.3±0.6 1.3	20.0±1.0 1.3	14.7±0.6 1.0	10.0±0	-	-	15.0±1.0
	AI		1.3				0.7			1.0
Escherichia coli	IZ	-	11.0±1.0	17.7±1.1	15.7±2.5	8.0±1.0 0.4	12.3±1.1	-	-	21.0±1.0
	AI		0.5	0.8	0.7		0.6			1.0
Pseudomonas aeruginosa	IZ	-	13.7±0.6	20.0±1.0 1.3	19.3±0.7 1.2	12.7±0.6	14.0±1.0 0.9	-	15.0±1.0 1.0	-
	AI		0.9			0.8				
Salmonella typhi	IZ	-	11.0±0.6 0.5	19.7±0.6	16.0±0	13.3±1.1 0.7	14.0±1.0 0.7	-	-	20.0±1.0
	AI			1.0	0.8					1.0
Vibrio cholera	IZ	-	12.0±1.0 1.0	14.3±0.6 1.1	14.3±0.6	11.0±1.0 0.9	11.3±1.1 0.9	-	-	12.0±0
	AI				1.2					1.0
Bacillus anthracis	IZ	-	12.5±1.1 1.1	15.7±0.6	15.7±1.0	15.0±0	17.3±2.5 1.7	-	-	10.0±1.0
	AI			1.6	1.6	1.5				1.0
Candida albicans	IZ	_	9.7±0.6	14.0±1.9 0.7	7.0±1.0	12.3±0.6	11.7±1.5	20.0±0	-	-
	AI		0.5		0.3	0.4	0.6	1.0		
Aspergillus niger	IZ	-	-	-	-	-	-	15.0±1.0	-	-
	AI							1.0		
Aspergillus fumigatus	IZ	-	-	-	-	-	-	25.0±1.0	-	-
	AI							1.0		
Aspergillus flavus	IZ	-	10.0±1.0	16.0±1.0	15.8±2.08	15.0±1.0	-	20.0±0	-	-
	AI		0.7	0.8	0.8	0.7		1.0		
Cryptococcus neoformans	IZ	-	5.3±0.6	9.7±0.6	2.0±1.0	10.0±0	13.0±0 0.6	20.0±1.0 1.0	-	-
	AI		0.5	0.5	0.1	0.5				
Penicillim spp	IZ	-	-	-	-	5.0±1.0	-	20.0±1.0	-	-
	AI					0.2		1.00		
Mucor ssp.	IZ	-				5.3±0.6	8.7±1.1	20.0±1.0		
	AI					0.3	0.4	1.0		

Results are reported as inhibition zones (IZ; mm) with the corresponding activity index (AI). Inhibition zones are presented as mean±SD (n = 3). Inhibition zones (IZ) exclude the disc diameter (5 mm); Activity index (AI) = Inhibition zone of test sample divided by inhibition zone of a standard drug. B = butanol extract; AQ = Aqueous extract; M = methanol extract; Amp=ampicillin; D = dichloromethane; ET = Ethyl acetate; P = petroleum ether;. clotr = clotrimazole

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Table 3: Antibacterial and antifungal activities of *Terminalia mollis* leaf extracts

Organisms tested	IZ AI	**PE**	**M**	**Bu**	**1:1 M:D**	**AQ**	**Clotr 20 µg/disc**	**Gent 10 µg/disc**	**Amp 20 µg/disc**
Staphylococcus aureus	IZ	-	-	-	13.3±2.9	16.3±1.5	-	-	20.0±1.1
	AI				0.7	0.9			1.0
Escherichia coli	IZ	-	-	13.7±1.1	-	-	-	-	20.0±1.1
	AI			0.7					1.0
Pseudomonas aeruginosa	IZ	-	-	-	-	-	-	15.0±1.15	-
	AI							1.0	
Salmonella typhi	IZ	-	-	-	5.3±0.6	-	-	10.0±0.6	-
	AI				0.5			1.0	
Shigella	IZ		-	-	-	-	-	10.0±0	-
	AI							1.0	
Klebsiella Pneumoniae			-	-	-	-	-	10.0±1.0	-
								1.0	
Vibrio cholera	IZ	-	-	-	ND	-	-	-	12.0±0.6
	AI								1.0
Bacillus anthracis	IZ	-	-	2.3±0.6	-	5.3±1.1	-	-	20.0±0.6
	AI			0.1		0.3			1.0
Candida albicans	IZ	_	-	7.7±0.6	6.7±2.9	15.0±0	30.0±1.0	-	-
	AI			0.2	0.2	0.5	1.0		
Aspergillus fumigatus	IZ	-	-	4.0±1.0	-	-	30.0±0.6	-	-
	AI			0.1			1.0		
Aspergillus niger	IZ		ND	ND	ND	-	15.0±1.0	-	-
	AI						1.0		
Aspergillus flavus	IZ		ND	ND	ND	-	20.0±1.0	-	-
	AI						1.0		
Cryptococcus neoformans	IZ	-	-	8.0±1.0	5.0±0	15.0±1.0	30.0±1.0	-	-
	AI			0.3	0.2	0.5	1.0		

Results are reported as inhibition zones (IZ; mm) with the corresponding activity index (AI). Inhibition zones are presented as mean±SD (n = 3). Inhibition zones (IZ) exclude the disc diameter (5 mm); Activity index (AI) = Inhibition zone of test sample divided by inhibition zone of a standard drug. B = butanol extract; AQ = Aqueous extract; M = methanol extract; Amp=ampicillin; D = dichloromethane; ET = Ethyl acetate; P = petroleum ether;. clotr = clotrimazole

Discussion

Recently the antimicrobial and cytotoxic activities of extracts of *Terminalia sericea* roots, which shares similar ethnomedical uses with *Terminalia mollis,* were reported (Moshi and Mbwambo, 2005). They are both used in the treatment of diarrhea in HIV patients, and in addition *Terminalia mollis* is used in the treatment of malaria.

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Table 4: Brine shrimp lethality of *Terminalia mollis* root extracts.

Extract type	**LC_{50} µg/ml**	**95% CIs**
Petroleum ether extract	26.3	18.8-37.0
Ethyl acetate extract	51.0	39.0-66.9
Dichloromethane:methanol extract (1:1)	58.1	43.9-76.8
Methanol extract	34.0	23.9-48.3
Butanol extract	29.2	20.9-40.9
Aqueous extract	35.2	26.3-47.2

The results are reported as LC_{50} values in µg/ml with the corresponding 95% confidence intervals (95% CI).

Table 5: Brine shrimp lethality of *Terminalia mollis* stem wood extracts.

Extract type	**LC_{50} µg/ml**	**95% CI**
Pet ether extract	35.0	18.3-66.9
Ethylacetate extract	69.5	51.9-93.1
Butanol extract	10.4	5.9-18.3
Dichloromethane:methanol extract (1:1)	35.6	26.0-48.8
Methanol extract	49.7	38.1-65.0
Aqueous extract	54.9	42.6-70.8

The results are reported as LC_{50} values in µg/ml with the corresponding 95% confidence intervals (95% CI)

Diarrhoea can be caused by different agents, including bacteria, fungi, and some protozoa. Bacteria that may cause diarrhea include *Escherichia coli, Salmonella typi,* and *Vibrio cholera,* while for fungi *Candida albicans* is responsible. Extracts of the roots were effective against bacteria and fungi that may cause diarrhea thus supporting the traditional uses for this purpose. The extracts were also effective against other important fungi like *Cryptococcus neoformans*, which is a fungus of importance in HIV patients as it is causes Cryptococcal meningitis. The results of this study are in agreement with those from another study which reported that leaf extracts of the plant have antifungal activity (Masoko et al., 2005).

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Table 6: Brine shrimp lethality of *Terminalia mollis* leaf extracts.

Extract type	**LC_{50} µg/ml**	**95% CI**
Petroleum ether extract	93.5	64.9-134.6
Dichloromethane:methanol extract (1:1)	42.9	31.7-57.9
Methanol extract	101.2	69.8-146.8
Butanol extract	42.5	29.1-62.0
Aqueous extract	101.3	71.4-143.9

The results are reported as LC_{50} values in µg/ml with the corresponding 95% confidence intervals (95% CI).

Using the microdilution method they detected antifungal activity in all the extracts, polar and non-polar, unlike in this in which the petroleum ether extracts were largely inactive against both bacteria and fungi.

Plants of the Combretaceae family are known to be sources for combretastins (Rogers and Verotta, 1996), which have potent anticancer activity. However, given the low predictive nature of the brine shrimps test that was used in this study it is difficult to directly relate the high toxicity with the presence, in the extracts, of activity with useful anticancer compounds. At best we can only speculate about this possibility as has been indicated before (Meyer et al., 1982). The petroleum ether extracts of the roots and stem exhibited high toxicity on brine shrimps, but hardly did they exhibit antibacterial and antifungal activity. This may indicate selectivity, although with the disc diffusion method there could be problems of diffusion through the agar by non-polar extracts hence failure to detect zone of inhibition. It is however, noteworthy mentioning that the petroleum ether extract was dissolved in DMSO, so the solution would not have difficulty to diffuse through the agar.

In conclusion, the detection of antibacterial and antifungal activity in extracts of *T. mollis* supports the traditional uses of this plant for the treatment of bacterial and fungal infections. The brine shrimp results suggest need for follow up with tests on cancer cell lines.

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