Antibacterial and cytotoxic activities of *Terminalia stenostachya* and *Terminalia spinosa*

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Abstract: Plants that belong to the *Combretaceae* family have long history of use in the traditional medicine systems of Africa and Asia for treatment of diseases and conditions associated with HIV/AIDS-opportunistic infections. The objective of this study was to investigate the biological activities of extracts of *Terminalia stenostachya* Engl. & Diels and *Terminalia spinosa* Engl. (*Combretaceae*), to verify the rationale for their use by traditional health practitioners in the treatment of HIV/AIDS patients in Tanzania. Extracts of the leaves, stem barks and roots of *T. stenostachya* and extracts of stem barks and roots of *T. spinosa* have all shown strong activity against a number of standard microbial strains including *Mycobacterium madagascariense* and *Mycobacterium indicus pranii*, *Streptococcus faecalis*, *Staphylococcus aureus*, *Vibrio cholera, Bacillus anthracis, Bacillus subtilis, Klebsiella pneumoniae, Salmonella typhi, Pseuodomonas aeruginosa* and *Escherichia coli*. All extracts from the two plant species showed strong antimycobacterial activity against test organisms. The stem and root bark extracts were more active than leaves against both gram positive and negative bacteria. With the exception of two extracts from stem barks of *T. spinosa*, all other extracts from *T. stenostachya* and *T. spinosa* that were tested exhibited less activity against brine shrimp larvae with LC_{50} values ≥100μg/mL compared to cyclophosphamide, a standard anticancer drug. These results provide an indication that these plants may possess therapeutically potent antimicrobial compounds worth further development.

Keywords: Antibacterial, cytotoxic, *Terminalia stenostachya*, *Terminalia spinosa*, Combretaceae, HIV/AIDS

Introduction

Traditional medicine health care system continues to play an important role in primary health care for majority of the people in the developing countries. Records indicate that at least 70% of the general population in sub-Saharan Africa consults traditional health practitioners not only for their primary health care needs but also for treatment of chronic diseases like cancer and HIV and AIDS (Mills *et al*., 2006). This high rate of depending on medicinal plants demands for thorough evaluation of herbal medicines to establish their safety and efficacy and hence justify their wide usage.

Plants of the *Combretum* and *Terminalia* genera constitute majority of the Combretaceae family that are widely represented in Tanzania. At least 55 and 17 species of *Combretum* and *Terminalia*, respectively, are reported to be growing in Tanzania ranging from climbers, shrubs and big trees (Wickens, 1973). Most of these species are also found in other parts of tropical and warm temperate regions of the world (McGaw *et al*., 2001). Traditional healers throughout Eastern to Southern and Western Africa use *Combretaceae* species for treatment of conditions like, abdominal disorders, backache, bilharzia, cancer,

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coughs, colds, conjunctivitis, diarrhoea, dysentery, dysmenorrhoea, fever, gastric ulcers, general weakness, venereal diseases, headaches, heart diseases, hypertension, jaundice, leprosy, nosebleeds, oedema, pneumonia, skin diseases, sore throats, dental caries, diabetes, dropsy, enteralgia, eye diseases, general fatigue, hiccups, jaundice, loss of appetite, malaria, menorrhagia, tuberculosis, tumours, wasting and yellow fever (McGaw *et al*., 2001; Masoko *et al*., 2005; Eloff, 2008). The wide range of diseases/conditions that are treated through use of *Combretaceae* plants potrays their potential for treatment of HIV and AIDS related opportunistic infections.

Terminalia is a genus comprising of 200-250 species that are widely distributed in tropical areas of the world (Pettit, *et al*., 1996; McGaw *et al*., 2001). Their wide application in traditional medicine systems of Africa and Asia have necessitated increased investigation of many plants of this genus for a wide range of biological activities. Several plant species of *Terminalia* have been evaluated and established as having antibacterial, antifungal and antidiabetic activities (Eloff, 1999; Fyhrquist *et al*., 2002; Masoko *et al*., 2005; Mbwambo *et al*., 2007; Eloff, 2008). Furthermore, some of these plants have been reported to exhibit cytotoxicity (Lee *et al*., 1995; Fyhrquist *et al*., 2006) and for the treatment of cancers (Hartwell, 1982; Petit *et al*., 1996). Plants of the genus *Terminali*a have likewise been reported as having *in vitro* inhibitory activity on HIV-1 reverse transcriptase (Kusumoto *et al*., 1995; Asres *et al*., 2005; Tshikalange *et al*., 2008; Desai *et al*., 2009). Phytochemical work on some of these plant species have been carried out and secondary metabolites including cyclic triterpenes and their derivatives, flavonoids, tannins and other aromatics were identified, characterized and some of these substances have been proven to exhibit antifungal, antibacterial, anti-cancer, hepatoprotective, antioxidant and anti-HIV-1 activities (Petit *et al*., 1996; Asres *et a*l., 2005).

T. stenostachya is a small tree or shrub 4-5m high, usually found in wooded grassland of Malawi, Mozambique, Tanzania, Zambia and Zimbabwe (Wickens, 1973). In Zimbabwe, *T. stenostachya* is reported for its use against epilepsy and as antidote for various poisons (Rogers & Verotta, 1996). In a study conducted on ethnobiomedical and antimicrobial screening of Tanzanian plants of the *Combretaceae* from Mbeya, Tanzania, it was established that *T. stenostachya* had no ethnobiomedical records but when screened, it was found to exhibit antimicrobial activity against *Staphylococcus aureus*, *S. epidermidis*, *Baccillus subtilis* and *Enterobacter aerogenes* (Fyhrquist *et al*., 2002). Recently, however, based on interview with traditional healers in Tanga, Tanzania, it was revealed that root decoction of this plant is used for managing HIV/AIDS patients).

T. spinosa commonly is a tree of up to 20m high widely distributed in dry bush wooded grassland in Tanzania, Uganda, Kenya, Somalia and Sudan (Wickens, 1973). Leaves of this plant are traditionally used by the Zaramo people of Coast region in Tanzania against malaria (Chhabra *et al*., 1989). Methanolic extracts of stem barks of *T. spinosa* have been shown to be active against *Helicobacter pylorrii* and against two fungi pathogens of the genera *Candida* and *Aspergilous* and other fungi (Fabry *et al*., 1996a,b, 1998). Furthermore, the same extracts were shown to exhibit antiprotozoal activity against *Acanthamoeba castellanii* which causes granulomatous brain lesions (Fabry *et al*., 1996a). Additionally, stem wood and stem bark of *T.spinosa* have been established to have significant activity against both chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* (Omulokoli *et al*., 1997).

The objective of this study therefore is to investigate antibacterial and cytotoxicity activities of *T. stenostachya* and *T. spinosa* as part of our continued efforts to evaluate plants of the *Combretaceae* for their potential for managing HIV and AIDS patients.

Materials and Methods

Collection, preparation and extraction of plant material

Roots and stem barks of *Terminalia spinosa*, (voucher specimen with collection No: 3602) were collected from Chalinze in the Coast Region of Tanzania. Roots, stem barks and leaves of *Terminalia stenostachya* (voucher specimen with collection No: 3614) were collected from Handeni, Tanga, Tanzania. Both plant samples were collected in May 2010 and their voucher specimens have been deposited at the herbarium, Department of Botany, University of Dar es Salaam. All plant parts were air-dried, pulverized and extracted by maceration sequentially using Dichloromethane:Methanol (1:1), Acetone (100%) and Ethanol (80%). The extracts were dried under *vacuo* using rotary evaporator and/or freeze-dried to complete dryness before testing.

Materials

Dichloromethane was from UNILAB (UNILAB®, Nairobi, Kenya), ethanol (absolute) from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands) Methanol and Acetone from Sigma-Aldrich GmbH, Germany) whereas Dimethyl sulfoxide (DMSO) was from Sigma® (Poole, Dorset, UK). Tryptone Soya broth and Saboraud's broth were from HIMEDIA® (Himedia Laboratories Pvt Ltd, Mumbai, INDIA). *M. madagascariense* (DSM 44641) and *M. indicus pranii* (DSM 45239) were obtained from the Germany Resource Centre for Biological materials (DSMZ, Braunschweig, Germany). *Staphylococcus aureus* (NCTC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 29953), *Salmonella typhi* (NCTC 8385), *Vibrio cholera* (clinical isolate), *Bacillus anthracis* (NCTC10073), *Bacillus subtilis* (clinical isolate), *Klebsiella pneumonia* (clinical isolate) and *Streptococcus faecalis* (clinical isolate) were obtained from the Department of Microbiology, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania. Iodonitrotetrazolium chloride was bought from SIGMA® (Sigma- Aldrich®, St Louis, USA). The Brine Shrimps eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam coast.

Testing for antimicrobial activity

A total of 15 plant extracts from *T. spinosa* and *T. stenostachya* were screened against eleven bacteria *viz* two *Mycobacteria*, four gram positive bacteria and five gram negative bacteria. Minimum inhibitory concentrations (MICs) were determined by microdilution method (Eloff, 1998) using 96-well microtitre plates. The plates were first preloaded with 50 µL of the broth media in each well followed by addition of 50 µL of the extract (100 mg/mL) into the first wells of each row tested to make a total volume of 100µl in the first wells. The mixtures in the first wells were thoroughly mixed and then serially diluted up to the final wells where 50 µL was discarded. Thereafter, 50µL of the bacterial suspension (0.5 MacFarhland standard turbidity) was then added in each well to make the final volume of 100 µL in each well. The rows containing Gentamicin sulphate (50 – 0.024 µg/mL) was used as a standard positive drug, DMSO as negative control while the rows with broth and bacteria only was used to monitor bacterial growth. The plates were then incubated at 37 °C for 24h. For each extract, MICs were determined by adding 40µL of 0.02% *p*-iodonitrotetrazolium (INT) chloride dye in each well followed by incubation for 1h at 37°C.

Bacterial growth was indicated by a change in pink colour. The lowest concentration which showed no bacterial growth was considered as MIC.

Brine shrimps lethality test

Brine shrimps lethality test (BST) was used to predict the presence of cytotoxic compounds in the extract (Meyer *et al*., 1982). Briefly, stock solutions (40 mg/mL) of all extracts were prepared by dissolving them in DMSO. Different levels of concentrations (240, 120, 80, 40, 24 and 8μg/mL) were prepared by drawing different volumes from the stock solutions and then added into vials, each containing ten brine shrimps larvae. The volume was then adjusted to 5mL with artificial sea water prepared by dissolving 3.8 g/L. Each level of concentration was tested in duplicate. The negative control contained brine shrimp, artificial sea water and DMSO (0.6%) only. Cyclophosphamide was used as a standard test drug. The vials were incubated under light for 24h. The dead larvae were counted and mean was subjected to probit analysis using Fig P computer program (Biosoft Inc, USA).

Data analysis

The mean results of the percentage mortality were plotted against the logarithms of concentrations using the Fig P computer program. Regression equations obtained from the graphs were used to obtain LC_{16}, LC_{50}, LC_{84} and the 95% CI values (Litchfield and Wilcoxon, 1949). An LC_{50} of >100 μg/mL was considered to represent an inactive compound or extract.

Results

Antimicrobial activity

All extracts exhibited an activity at least against one organism tested (Tables 1). Extracts from both, stembark and roots of *T. spinosa* were found to be highly active against all bacteria except *S. faecalis* and *E. coli* (MIC ≥ 5 mg/mL). The root and stem bark of *T. stenostachya* were more active (MIC 0.3-2.5 mg/mL) than the leaves (MIC ≥ 2.5 mg/mL) except for mycobacteria tested. High activity was observed with all extracts from the two plant species against mycobacteria with MIC range of 0.3-1.25 mg/mL. In general gram positive bacteria were equally susceptible as gram negative bacteria to all extracts suggesting their broad range of activity.

Table 1: Antibacterial activity of extracts from stem barks and roots of *T. spinosa*

Plant	**Minimum Inhibitory Concentrations, MIC (mg/mL)**										
Extracts	MA	MI	SF	SA	EC	PA	BA	BS	VC	KP	ST
SBA	0.625	0.313	6.25	0.781	6.25	1.563	1.563	1.25	0.391	0.781	0.625
SBE	1.25	0.313	6.25	0.781	6.25	3.125	1.563	1.25	0.391	1.5625	1.25
SBDM	0.625	0.625	5.0	5.0	5.0	2.5	2.5	2.5	2.5	0.625	2.5
RA	0.625	1.25	2.5	2.5	2.5	1.25	1.25	0.625	2.5	2.5	1.25
RE	1.25	0.625	1.563	0.781	3.125	3.125	1.563	0.626	0.391	0.781	0.625
RDM	0.313	0.625	1.25	1.25	5.0	1.25	0.625	1.25	2.5	-	2.5

Key: MA=*M. madagascariense*; MI= *M. indicus;* S.F= *S. faecalis*; S.A= *S. aureus;* EC= *E.coli*; P.A= *P. aeruginosa*; B.A= *B. anthracis*; BS= *B. subtilis*; VC=*V. cholera;* KP= *K. pneumonia*; ST= *S. typhi*

SBA= Stem bark acetone (100%) extract; SBE= Stem bark ethanol (80%) extract; SBDM= Stem bark dichloromethane:methanol (1:1) extract; RA= Root acetone (100%) extract; RE= Root ethanol (80%) extract; RDM= Root dichloromethane:methanol (1:1) extract.

Brine shrimp Activity

With the exception of two extracts from stem barks of *T. spinosa*, brine shrimps resultsshows that all extracts from *T. stenostachya* and *T. spinosa* exhibited less activity against brine shrimp larvae with LC_{50} values >100µg/mL (Table 2). Dichloromethane:Methanol extract (1:1) and 80% ethanol extract from *T. spinosa* stem barks exhibited LC_{50} values of 99.535µg/mL and 75.818µg/mL, respectively. Of the all extracts tested, dichloromethane: methanol (1:1) extract from *T. stenostachya* leaves was found to be the least active (LC_{50} >500µg/mL) whereas ethanol (80%) extract from *T. spinosa* stem barks was found to be the most active LC_{50}=75.818µg/mL.

Table 2: Antibacterial activity of extracts from roots, stem barks and leaves of *T. stenostachya*

Plant	Minimum Inhibitory Concentrations, MIC (mg/mL)										
Extracts	MA	MI	SF	SA	EC	PA	BA	BS	VC	KP	ST
LE	0.625	1.25	5.0	5.0	5.0	2.5	2.5	2.5	1.25	5.0	5.0
LA	0.625	0.625	5.0	2.5	5.0	2.5	2.5	2.5	1.25	5.0	5.0
LDM	0.625	0.625	5.0	5.0	5.0	2.5	2.5	2.5	1.25	5.0	5.0
RE	0.625	0.313	1.563	0.391	3.125	3.125	0.781	0.313	0.781	0.391	0.391
RA	0.156	0.313	3.125	0.781	1.563	1.563	0.391	0.313	0.781	0.781	0.781
RDM	0.625	0.625	2.5	1.25	2.5	1.25	0.625	1.25	0.625	2.5	2.5
SBE	0.625	0.625	2.5	1.25	2.5	2.5	0.625	0.625	0.625	2.5	2.5
SBA	0.625	1.25	2.5	1.25	2.5	2.5	0.625	1.25	0.625	2.5	1.25
SBDM	1.25	1.25	2.5	1.25	2.5	2.5	2.5	2.5	2.5	2.5	2.5

Key: MA=*M. madagascariense*; MI= *M. indicus;* S.F= *S. faecalis*; S.A= *S. aureus;* EC= *E.coli*; P.A= *P. aeruginosa*; B.A= *B. anthracis*; BS= *B. subtilis*; VC=*V. cholera;* KP= *K. pneumonia*; ST= *S. typhi*

LE= Leaves ethanol (80%) extract; LA= Leaves acetone (100%) extract; LDM = leaves Dichloromethane: Methanol(1:1); RE= Root ethanol (80%); RA= Root acetone (100%); RDM= Root dichloromethane:methanol (1:1) extract; SBE = Stem bark ethanol (80%); SBA= Stem bark acetone (100%); SBDM= Stem bark dichloromethane: Methanol (1:1) extract

All root extracts, acetone stem barks extract and dichloromethane: methanol (1:1) stem barks extracts from *T. stenostachya* were however, not tested against brine shrimp larvae because of the lack of enough extracts.

Table 3: **Brine Shrimp Activity of Extracts from *T. spinosa* and *T. stenostachya***

Plant extracts	LC_{50} (µg/ml)	95% Confidence interval	Regression coefficient (r)
TSRA	323.9746	233.579 – 449.353	0.991434
TSRE	380.0919	258.366 – 558.735	0.997705
TSRDM	126.367	100.932 – 158. 211	0.949285
TSSBA	101.498	80.682 – 127.684	0.976658
TSSBE	75.818	59.935 – 95.910	0.967860
TSSBDM	99.535	75.234 – 131.685	0.968541
TSLA	350.240	203.628 – 602.413	0.978276
TSLE	142.667	106.23 - 191.60	0.967297
TSLDM	545.523	325.491 – 914.296	0.966892
TSBE	133.315	100.996 – 175.976	0.964430
Cyclophosphamide	16.365	12.006 – 22.305	0.994929

Key: TSRA= *T. spinosa* root acetone (100%) extract; TSRE= *T. spinosa* root ethanol (80%) extract; TSRDM= *T. spinosa* root dichloromethane:methanol (1:1) extract; TSSBA = *T. spinosa* stem bark acetone (100%) extract; TSSBE= *T. spinosa* stem bark ethanol (80%) extract; TSSBDM= *T. spinosa* stem bark dichloromethane:methanol (1:1) extract; TSLA = *T. stenostachya* leaves acetone (100%) extract; TSLE = *T. stenostachya* leaves ethanol (80%) extract; TSLDM = *T. stenostachya* leaves dichloromethane:methanol (1:1); TSBE = *T. stenostachya* stem bark ethanol (80%) extract.

Discussion

Records on ethnobiomedical informations indicates that *T. stenostachya* is used against epilepsy and as antidote for various poisons (Rogers & Verotta, 1996), whereas leaves of *T. spinosa* are used by the Zaramo people of Coast region in Tanzania against malaria (Chhabra *et al*., 1989). Pharmacological screening of methanolic extracts of roots of *T. stenostachya* have revealed antimicrobial activity against *Staphylococcus aureus*, *S. epidermidis*, *Baccillus subtilis* and *Enterobacter aerogenes* (Fyhrquist *et al*., 2002). Screening for biological activity for *T. spinosa* have shown that extracts of stem wood and stem bark of *T. spinosa* exhibited significant activity against both chloroquine-resistant and chloroquine-sensitive strains (Omulokoli *et al*., 1997*).* Antifungal and antibacterial activities have been further reported in methanolic extracts of young branches of *T. spinosa* (Fabry *et al*., 1996a, 1998). Methanolic extracts of stem barks of *T. spinosa* have also been reported to exhibit activity against *Helicobacter pylorrii,* against two fungi pathogens of the genera *Candida* and *Aspergilous* and against a protozoa *Acanthamoeba castellanii* (Fabry *et al*., 1996b).

Results from the present study have supported previous studies and in addition have shown that root and stembark extracts of both *T. spinosa* and *T. stenostachya* and leaf extracts of the latter have considerable activity against a wide range of microorganisms. The antibacterial activity exhibited by these extracts specifically against *Mycobacterium* spp., namely, *M. madagascariense* and *M. indicus pranii*, indicate that extracts from the two plants could be effective against *M. tuberculosis* since the two *Mycobacterium* sub-species used in this study share several clinically important properties that characterize *M. tuberculosis* (Kadza *et al*., 1992; Chaturved *et al*., 2007). In the whole the antiprotozoal, antifungal, and antimicrobial activity that have so far been shown by extracts of various parts of both the plants corroborate well with the traditional uses for treatment of HIV/AIDS-associated secondary infections particularly diseases like tuberculosis, malaria, stomach ulcers and candidiasis among several others.

The brine shrimp test results have shown that the majority of extracts that were tested had mild and/or no toxicity. These results provide an indication that extracts from the two plants are generally safe and could be traditionally used with possibly no obvious toxic effects to the intended population. However, further studies on safety of these extracts may be necessary before they are used by a larger population.

Based on results from this study, it can be concluded that extracts from both *T. stenostachya* and *T. spinosa* have shown a wide range of antibacterial activity. The strong activities that these extracts have shown specifically against mycobacterium indicate that these plants may be a good source of antimicrobial compounds worth further development.

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