Antimycobacterial and cytotoxicity activities of *Moringa oleifera* Lam extracts

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ABSTRACT

This study has evaluated antimycobacterial activity of *Moringa oleifera* Lam extracts against *Mycobacterium indicus pranii* and *Mycobacterium madagascariense* using two folds broth microdilution method. It further evaluated a potential cytotoxicity against brine shrimp larvae. In the assays, petroleum ether, ethyl acetate and methanol extracts from roots, stem bark, leaves and seeds of *M. oleifera* were screened against test organisms. Of all tested samples, the leaf ethyl acetate extract of *M. oleifera* showed the highiest activity against *M. madagascariense* with minimum inhibition concentration (MIC) value of 0.37381 mg/ml while the seeds ethyl acetate extracts had MIC value of 0.37381 mg/ml against both *M. madagascariense* and *Mycobacterium indicus pranii*. The root extract exhibited weak activity against *M. madagascariense* strains with MIC value of 6.25 mg/ml, lower than other extracts. The remaining extracts were moderately active against the two mycobacteria strains. In the cytotoxicity assay, the root barks of *M. oleifera* exhibited significant toxicity against brine shrimp larvae with LC_{50} value of 26.639 µg/mL.

KEYWORDS: *Moringa oleifera*; anti-mycobacterial; Tuberculosis; cytotoxicity.

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INTRODUCTION

Tuberculosis (TB) caused by mycobacterium is the global health disaster which kills millions of people and affects mostly young adults in their most productive years (Cosivi *et al.*, 1998). It is believed that, one person is infected with tuberculosis in every second (Heimbeck, 1928). In Tanzania, Tuberculosis continues to be among the major public health problem. According to Chum, (1991) and Mfinanga *et al.*, (2008), the number of TB cases has steadily increased from 11,753 in 1983 to about 65, 665 in 2004. TB is the leading killer of people living with HIV, with about one in four deaths among people with HIV dies from TB (Mukadi *et al.*, 2001).

There are many challenges in fighting TB, one being the appropriate treatment of the disease (Ginsberg and Spigelman, 2007). Standard TB treatment involves up to 2 years of medication which is very damaging to patient's health due to high levels of drug toxicity (Hannan *et al.*, 2011). Due to this treatment longevity, TB patients frequently stop the treatment even before it is completed, allowing the bacteria to mutate (Petrini and Hiffner, 1999). As a result, new and potent strains of TB are emerging and are resistant to the available antibiotics, posing a major health risk. Multidrug-resistant tuberculosis (MDR-TB) requires a lengthier and more difficult treatment. The treatment of MDR-TB is 100 times more expensive making it to be a most deadly infectious disease in developing countries (Selgelid, 2008). Therefore, there is a fundamental need to explore alternative anti-TB agents.

The antimicrobial properties of plants have been investigated worldwide and the results are very promising (Cowan, 1999; Duffy and Power, 2001; Hammer *et al.,* 1999; Smith-Palmer *et al.,* 1998). Hence medicinal plants should be investigated to understand their antimicrobial properties and safety. *Moringa oleifera* (Moringaceae) is one of the medicinal plants which possess variety of biological properties like anti-inflammatory (Ezeamuzie *et al.,* 1996), hepatoprotective (Pari and Kumar, 2002) and anti-hypertensive (Faizi *et al.,* 1995). Owing to its therapeutic versatility, this study was designed to evaluate the antimycobacterial efficacy of extracts from various parts of this medicinal plant against *M. indicus pranii* and *M. madagascariense*. In this study the potential cytotoxicity of the extracts was also evaluated against brine shrimps larvae.

METHODOLOGY

Collection and Preparation of Plant Materials

Moringa oleifera parts viz leaves, seeds, flower, roots and stem bark were collected from Lushoto, Tanga Region, Tanzania. The plants were identified by Mr. Haji Selemani, a botanist from the Department of Botany, University of Dar es Salaam and the voucher specimen (MOLT 2143) was kept at Nelson Mandela African Institute of Science and Technology, Arusha. The leaves were air dried at room temperature (29 ^{o}C – 30 ^{o}C) till dryness. The root barks were washed with clean running tap water to remove soil, together with stem bark and seeds separately were exposed under the sun until complete dryness. Plant materials were pulverized to obtain powders for use during extraction.

Reagents, media and test organisms.

Ethanol (absolute), petroleum ether and ethyl acetate were bought from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Sigma® (Poole, Dorset, UK). Cyclophosphamide was purchase from Khandelwal laboratories pvt ltd, 79/87 D.LADPATH, Mumbai 400033 India. The Brine Shrimps eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating sea water collected from the Indian Ocean, along the Dar es Salaam Coast, Ciprofloxacin was purchased from astra lifecare (India) Pvt Ltd. Iodonitrotetrazolium chloride was purchased from SIGMA (Sigma Aldrich, St Louis, USA. Nutrient agar and broth were purchased from Hi Media Laboratories Pvt Ltd (Mumbai-India). *Mycobacterium indicus pranii* and *Mycobacterium madagascariense* were supplied by Department of Microbiology and Immunology-Muhimbili University of Health and allied Sciences (MUHAS).

Extraction

Sequential extraction was done using solvents in increasing order of polarity, starting with petroleum ether, ethyl acetate and finally methanol. 1kg of each powdered plant part was soaked in 2.5L of an appropriate solvent for 24h. After 24h the extracts were filtered off and concentrated *in vicuo* using rotary evaporator. The extracts were collected, poured into air tight bottles and stored in refrigerator at -4°C until further use.

Brine Shrimps Lethality Test

The brine shrimp lethality test (BST) was used to predict the potential cytoxicity property of extracts. The experiment was set according to Meyer *et al.* (1982). Briefly, stock solutions (40

mg/mL) of all extracts were prepared in DMSO. Different levels of concentrations (240, 120, 80, 40, 24 and 8 µg/mL) were prepared by drawing different volumes from the stock solutions and then added into vials. The volume was then adjusted to 5 mL with artificial sea water prepared by dissolving 3.8 g of sea salt in1 L of distilled water. Thereafter, shrimps larvae were introduced into each vial for assaying. Each level of concentration was tested in triplicate. The negative control contained shrimp larvae, artificial sea water and DMSO (0.6%) only. The vials were incubated under light for 24 h. The dead larvae were counted and mean values were subjected to 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 LC16, LC50, LC84 and the 95% CI values. An LC50 value >100 µg/mL is considered to represent an inactive compound or extract (Gupta *et al.*, 1996).

Anti-Mycobacterial Test

Sub-culturing of Mycobacterium species

The Mycobacterium strains were sub-cultured in Middlebrook 7H9 broth base supplemented with glycerol. This was followed by suspending about 1.2 g of Middlebrook 7H9 broth base in 240 mL of distilled water in a Scotch bottle (500 mL) followed by addition of 1 ml of glycerol. The mixture was heated and later autoclaved at 122°C for 15 minutes. The mixture was left to cool to 31 and 35°C under lamina flow hood, before separately being inoculated with *M. madagascariense* (MM) and *M. indicus pranii (*MIP) respectively. Hence MM was incubated at

31°C while MIP was incubated at 37°C. The optimal growth of the bacteria cultures was observed after 5 days.

Determination of minimum inhibition concentration (MIC) of extracts

The two fold broth microdilution method was used to determine the MIC values of extracts against *M. Indicus pranii* and *M. madagascariense* as described by Eloff (1998). Bacterial inoculums were prepared from five days grown cultures in middle brook 7H9 broth base containing 0.1% tween 80 and the turbidity was adjusted to the equivalent of 0.5 McFarland units to "approximately 1.2 x108 CFU/ml". The concentration of stock solution of all test extracts before serial dilutions was 100 mg/ml. The extracts were serially diluted two folds with a broth base containing 0.1% tween 80. The serial dilution was performed by addition of 40 µl of extracts into the first well which had 50 µl of broth base, and thereafter mixed well and transferred 50 µl of the first well sample-broth base mixture to next and subsequent wells of each row. The remaining 50 µl of the mixture was discarded from the last well of the row. This was followed by the separate inoculation of 50 µl of mycobacteria cultures in each well, to complete a twofold broth microdilution. Two additional wells were used as growth controls, while a row with inoculums and control drugs were used as positive control. The inoculated microtiter plates were incubated at 31°C for MM and 37°C or MIP for 24 hours. To determine the MIC values of extracts, 40µl (0.2 mg/ml) iodonitrotetrazolium (INT) chloride salt was added into each well and plates incubated at 31 and 37°C for 1 hour. The minimal inhibitory concentration (MIC) value of each extract was read at the concentration where a marked reduction in color formation due to bacterial growth inhibition was noted. Positive control used in this study was Ciprofloxacin.

RESULTS AND DISCUSSION

Brine Shrimp Lethality Test

The brine shrimp lethality test (BST) was used to predict cytotoxicity properties of plant extracts and also possible presence of compounds with potential anticancer activity (Moshi *at el*, 2010). This assay has revealed that out of fifteen extracts, twelve extracts were less toxic to brine shrimp larvae (Table 2). The root bark ethyl acetate and methanolic extract of *M. oleifera* was more toxic to shrimps larvae with LC_{50} value of 26.639 µg/ml and 36. 485 µg/ml respectively (Table 2). Stem bark ethyl acetate and methanolic extract were also significantly toxic against shrimps with LC_{50} 59.247 µg/ml and 77.758 µg/ml respectively. Other test samples were far less toxic to shrimps with LC_{50} > 100 µg/mL compared to a standard anticancer drug cyclophosphamide which had LC_{50} value of 16.40 µg/ml. There was no mortality in the negative control groups indicating that the assay yielded valid results. Brine shrimp results obtained from stem and roots justifies its traditional use as anti-tumor (Ganatra *et al.,* 2012).

Anti-mycobacterial Activity

Two non-pathogenic *Mycobacterium* species namely, *M. madagascariense* (MM) and *M.indicus pranii* (MIP) were used to determine the anti-mycobacterial potential of different extracts of *Moringa oleifera* parts. The *in vitro* antibacterial assay showed that *M. oleifera* extract is more active against MM. except for root extracts. Of the tested extracts, the seed ethyl acetate extract of *M. oleifera* exhibited higher anti-mycobacterial activity against *M. indicus pranii* and *M. madagascariense* with MIC value of 0.3125 mg/ml for both organisms, while the leaves ethyl acetate extracts of *M. oleifera* showed the same activity against *M. madagascariense*. The stem bark ethyl acetate extract had MIC value of 1.5125 mg/mL against *M. indicus pranii*. Root back extract of *M. oleifera* showed the lowest activity with MIC value of 6.25 mg/ml against *M.*

madagascariense. The rest of the tested extracts showed moderate activity with MIC values ranging from 1.5125 mg/ml - 3.125 mg/ml against test organisms (Table 1).

Table 1. Lethality assay of *Moringa oleifera* extracts against brine shrimp larvae

Plant	Extracts	%Mortality	LC_{50} µg/ml	CI 95	Regression equation	R^2
	PE	872.475	103.796	24.404-149.674	Y=54.079logx-59.033	0.870
Leaf	EtOAc	17,192.08	697.18	401.83-1209.607	Y=35.92logx-52.133	0.936
	MeOH	1,168.033	117.14	75.968-180.633	Y=50.063logx-53.566	0.937
	PE	451.38	117.02	90.748-150.897	Y=85.284logx-126.39	0.900
Stem back	EtOAc	375.893	59.247	41.841-83.894	Y=62.313logx-60.46	0.969
	MeOH	954.149	77.758	48.499-124.69	Y=45.919logx-36.821	0.91
	PE	1501.790	180.183	120.847-268.653	Y=54.295logx+72.474	0.9724
Root back	EtOAc	264.475	26.639	4.7629-39.532	Y=50.158logx-21.502	0.930
	MeOH	135.796	36.485	28.482-46.737	Y=87.601logx-86.843	0.958
	PE	665.223	129.128	94.808-175.872	Y=70.23logx-98.257	0.987
Seed	EtOAc	874.842	203.288	155.538-265.697	Y=78.887logx-132.08	0.943
	MeOH	1,614.45	201.029	135.739-297.724	Y=55.263logx-77.285	0.888
DMSO		NM	-	-	-	-
CPA		88.195	16.40	12.006 - 22.305	Y = 69.9logx -34.936	0.9949

Key: PE =petroleum ether; EtOAc =ethyl acetate and MeOH=methanol; NM=No mortality at all levels of concentration tested; LC_{50}= Lethal concentration (concentration to kill 50% of test organisms); UCL= Upper Confidence limit; LCL= Lower Confidence limit; R^2= regression coefficient; Y=Regression equation; CPA= Cyclophosphamide; DMSO= Dimethyl sulfoxide.

The present study has shown that extracts from the different part of *M. oleifera* have both cytotoxic and anti-mycobacterial activities. Out of the extracts tested in an anti-mycobacterial assay, thirteen extracts showed significant activities against *M. indicus pranii* and *M. madagascariense*. Phytochemical screening of *M,oleifera* indicates that it contain mainly

triterpenoids and alkaloids (Shahriar *et al.*, 2012), phenolic, tannin, phenol and saponins (Makkar and Becker, 1997), all of which have been shown to possess antimicrobial activity (Doughari *et al.*, 2007).

Table 2: Minimum Inhibition Concentration (MIC) of *M. oleifera* against the selected mycobacterium

Plant part	Extract	MIC (mg/mL)	
		MIP	**MM**
Leaf	PE	3.125	3.125
	EtOAc	1.5125	0.37381
	MeOH	1.5125	3.125
Stem bark	PE	3.125	3.125
	EtOAc	3.125	1.5125
	MeOH	3.125	3.125
Root bark	PE	3.125	6.25
	EtOAc	1.5125	6.25
	MeOH	3.125	6.25
Seed	PE	1.5125	3.125
	EtOAc	0.37381	0.37381
	MeOH	1.5125	1.5125
DMSO		12.5	12.5
CP		<0.01	<0.046

Key: MIP= *Mycobacteria Indica Prenii*; MM = *Mycobacteria madagascariensis*, PE = petroleum ether; EtOAc = ethyl acetate and MeOH = Methanol, DMSO = Dimethylsulphoxide; CP = Ciprofloxacin.

Comparing the activities of thirteen extracts under screened in this study and the standard antibiotics, Ciprofloxacin had much higher activities than all plant extracts. The overall results showed the most susceptible strain was *Mycobacterium Indicus Pranii* which was inhibited most.

The inhibition of *M. Indicus Pranii* by most of plant extract when compared to *M. madagascariense* is well documented in literature (Magadula *et al.*, 2012; Erasto *et al.*, 2013)

CONCLUSION

The significant anti-mycobacterial and cytotoxicity activities of extracts of *M. oleifera* provide a good opportunity for further research and drug development. In particular, leave and seed extracts which showed high activities, hence the continuation of study on these plant extract is crucial to isolate, characterize and identify the bioactive compounds responsible for the observed pharmacological activities.

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