



International Journal of Life Sciences Biotechnology and Pharma Research





Research Paper

INVESTIGATION OF ANTIMYCOBACTERIAL AND CYTOTOXICITY ACTIVITY OF *BERSAMA ABYSSINICA FRESSEN* EXTRACTS FROM TANZANIA

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Bersama abyssinica is widely used for treatment of various infectious diseases in Africa. In Tanzania *Bersama abyssinica Fresen subsp. paullinioides* is locally used for treatment of various infectious diseases including Mycobacterial illness though the plant has never been investigated for antimycobacterial activity. Hence, this study was conducted to evaluate antimycobacterial and cytotoxicity activity of *Bersama abyssinica Fresen subsp. paullinioides* leaves, stem bark and root bark extracts. Leaves, stem bark and root bark were sequentially extracted with petroleum ether, ethyl acetate and methanol. Nine extracts were obtained and tested against the two fast growing Mycobacteria species namely; *Mycobacteria madagascariense* and *Mycobacterium indicus pranii* by using micro dilution method in which the Minimum Inhibitory Concentrations (MICs) were determined. The result showed all extracts exhibited antimycobacterial activity against *Mycobacterium madagascariense* and *Mycobacteria indicus pranii* with MIC ranging from 0.39 to 6.25 mg/mL and 0.19 to 6.25 mg/mL respectively. The brine shrimp lethality result obtained had the LC₅₀ value ranging from 30 to > 100 µg/mL indicating that extracts were mildly toxic to non toxic. The present results support the use of *Bersama abyssinica Fresen subsp. paullinioides* by local communities for management of Tuberculosis and suggest that the methanolic extracts could be a potential source of antimycobacterial agents. Based on the cytotoxicity analysis, *Bersama abyssinica* extracts pose no threat for human healthy and are considered as non toxic.

Keywords: *Bersama abyssinica* Fresen, Mycobacterium Tuberculosis, Cytotoxicity, Extracts

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* that mostly affects lungs termed as

pulmonary TB (Barreiro *et al.*, 2006), though it can affect other parts of the body as well known as extra pulmonary TB that require effective management to prevent serious effects

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(Bonifachich *et al.*, 2006). Tuberculosis account for about 1.4 million deaths and over 95% of these deaths occur in low- and middle-income (Basso *et al.*, 2007; Organization, 2013). For instance in Tanzania, tuberculosis is among the major public health problems where its cases has steadily increased from 11,753 in 1983 to about 60% of the total population infected in 2011 (Mtwangambate *et al.*, 2014). Tuberculosis spread in the air when TB patients expel bacteria through coughing or sneezing (Yang *et al.*, 2012) because Mycobacterial species release volatile compounds that expel to the environment quickly thus speed up the spread of disease (Nawrath *et al.*, 2012). Although a relatively small proportion of people infected with *Mycobacterium tuberculosis* develop TB but the probability of developing TB is much higher among people infected with the human immunodeficiency virus, HIV (Corbett *et al.*, 2003; Lawn *et al.*, 2010). Again it has been pointed out that TB is common among men than women, and affects mostly adults in the economically productive age groups particularly in mining area hence contributing to national and individual poverty (Marais *et al.*, 2006; Theobald *et al.*, 2006; Upton, 2008). So far many interventions have been made to manage TB but emergence of multi-drug resistance TB has undermined these efforts (Chonde *et al.*, 2010).

The multi-drug resistant tuberculosis is a global challenge that emerged since 1990's threatening lives of both developed and under developed world inhabitants although the rate is high in resource limited settings where diagnosis of latent TB is a major challenge (Abubakar *et al.*, 2013; Lawn and Wood, 2011). In 2010, the World Health Organization (WHO) estimated that there were 10 million new cases of TB and about 1.8 million deaths occur each year due to TB (Leung

et al., 2011). This is exacerbated by the emergence of drug resistant strains which proves difficult to cure with the standard anti-TB drugs namely isoniazid and rifampicin (Jain and Dixit, 2008; Wood and Iseman, 1993). This effect is compounded by number of factors such as poor diagnostic facilities, unregulated antibiotic access, lack of monotherapy TB drugs and poor implementation of the Direct Observed Treatment short-course program (DOTS) which is currently used (Pai *et al.*, 2010). Moreover, though TB can be curable with the kanamycin and amikacin, misuse and mismanagement of these drugs has led to the emergence of Extensive Drug Resistance Tuberculosis (XDR-TB) (Migliori *et al.*, 2008; Udwadia *et al.*, 2012) posing more challenges to the public because it takes longer to treat and not easily accessible especially in developing countries (Bruno, 2013) thus necessitates the search for alternative and more effective anti-TB agents (Gupta *et al.*, 2010; Jimenez Arellanes *et al.*, 2003). One of the promising sources is medicinal plants which are locally used for the treatment of mycobacterial infections. The dependency on the medicinal plants for treatment of microbial infectious including Mycobacterial infections has been pronounced in recent years (Chrian *et al.*, 2011; Dzoyem *et al.*, 2013; Erasto, 2012; Rahmatullah *et al.*, 2011).

Bersama abyssinica Fresen subsp. paullinioides (Melianthaceae) which is a medium sized evergreen tree distributed in Democratic Republic of Congo, Tanzania, Mozambique, Zambia, Zimbabwe, Angola, Nigeria, Ethiopia, Kenya, Sudan and Uganda (Jackson and Jethwa, 1973; Mikkelsen and Seberg, 2001) is used by local communities for the treatment of microbial infections including mycobacterial infections (Amit

et al., 2010). Based on its ethnomedical information, *B. abyssinica* extracts were evaluated antimycobacterial activity and results are reported in this paper.

MATERIALS AND METHODS

Solvents, Reagents and Growth Media

Methanol was bought from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands) and Dimethyl sulfoxide (DMSO) was purchased from RFCL Limited, Haryana, India whereas petroleum ether, ethyl acetate, chloroform was purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Middle Brook 7H9 Broth was supplied by Sigma-Aldrich® Co, whereas standard TB drugs; Isoniazid, Rifampicin and kanamycin were supplied by Macleods Pharmaceuticals Ltd. Atlanta Arcade, Marol Church Road, Andheri (E), Mumbai-400-059, India. The Brine shrimps eggs were purchased from Aquaculture innovations (Graham's town 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast.

Collection of Plant Materials and Extraction

The leaves stem bark and root bark of *Bersama abyssinica* were collected from Iloilo village, Rungwe district in Mbeya, Tanzania. Authentication was done by Mr. Ahmed Mndolwa, Research Assistant, Tanzania Forestry Research Institute (TAFORI) and voucher specimen (BANZ 0114) was kept at Nelson Mandela African Institution of Science and Technology.

The plant materials were air dried under shade and then pulverized into fine particles. The pulverized leaves (1000 g), stem bark (1000 g) and root bark (1000 g) were sequentially

macerated using petroleum ether, ethyl acetate and ethanol for 48 h twice for each solvent. The respective extracts were filtered through muslin cloth on a plug of glass wool in a glass column and solvents were evaporated in vacuum using a rotary evaporator and stored in refrigerator at -20°C.

Sub Culturing of Mycobacteria Strains

The *Bersama abyssinica* extracts were evaluated against *Mycobacteria madagascariense* (DSM 44641) and *Mycobacteria indicus pranii* (DSM 45239) and were supplied by DSMZ - The Germany Resource Centre for Biological Materials, Braunschweig, Germany. These strains were used as marker for determination of potential antituberculosis efficacy of extracts. *Mycobacterium madagascariense* (MM) and *Mycobacterium indicus pranii* (MIP) were sub-cultured in liquid media, Middle brook 7H9 broth base by measuring 0.64 g of Middle brook 7H9 broth base which was then suspended in 115 mL of distilled water in two separate Scotch bottles of 250 mL each. Thereafter, 0.5 mL of glycerol (AR) was added into each scotch bottle and the mixture was shaken to dissolve the broth completely then autoclaved at 121 for 15 min. The mixture was left to cool to 31 and 35°C under lamina flow, before being inoculated with *Mycobacterium madagascariense* (MM) and *Mycobacterium indicus pranii* (MIP) respectively. Thereafter MM and MIP were incubated at 31°C and 37°C.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations (MICs) of the extracts against two standard *Mycobacteria* were determined by two fold micro dilution method (Eloff, 1998), done in triplicate using 96 well

microtitre plates. The plates were first preloaded with 50 μ L of the broth media in each well followed by addition of 50 μ L of the extract (100 mg/mL) into the first wells of each row tested to make a total volume of 100 μ L in the first wells. After thorough mixing of first row of each plate, 50 μ L were drawn from each of the first row wells and added into the next row wells. The process was repeated down the columns to the last wells at the bottom whereas 50 μ L drawn from last columns were discarded. Thereafter, 50 μ L of *Mycobacteria madagascariense* (MM) and *Mycobacteria indicus pranii* (MIP) suspension of an approximate 0.5 Mac Farland standard turbidity were added making the final volume of 100 μ L in each well. The rows containing DMSO were used as negative control and the rows with broth and bacteria only were used to monitor bacterial growth whereas the rows containing isoniazid, rifampicin and kanamycin were used as a standard positive control drugs. The plates were therefore incubated at 32°C and 37°C for *Mycobacterium madagascariense* and *Mycobacterium indicus pranii* respectively for 24 h. Thereafter, minimum inhibitory concentrations were determined by addition of 30 μ L of 0.02% *p*-iodonitrotetrazolium (INT) chloride dye in each well followed by incubation for 1 h at 32°C and 37°C correspondingly. Mycobacterial augmentation was indicated by a change in pink color. The rows without color change into pick marked the activity of extracts and MICs were recorded.

Brine Shrimps Lethality Assay

Brine shrimp lethality assay was performed to determine the potential cytotoxicity effect of the extracts so as to recommend on the pharmacological use of the plant. The method described by author (Meyer *et al.*, 1982) was

adopted with minor modifications. The stock solutions of 40 mg/mL for each extract were prepared by dissolving 40 mg of extract in 1 mL of DMSO. Then concentrations of 240, 120, 80, 40, 24 and 8 μ g/mL were prepared by drawing different volumes from the stock solutions and then added into vials, containing ten brine shrimps larvae in 5 mL of artificial sea water that was prepared by dissolving 3.8 g of sea salt in 1000 mL of distilled water. Each level of concentration was tested in triplicate. The positive control vials contained brine shrimp larva and Cyclophosphamide while negative control contained brine shrimp, artificial sea water and DMSO only. The vials were incubated under light for 24 h, then the dead larvae were counted and the mean was 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 (Litchfield and Wilcoxon, 1949). The LC₅₀ value greater than 100 μ g/mL is considered as inactive extract whereas LC₅₀ values >30<100 μ g/mL were considered as less toxic .

RESULTS

Antimycobacterial Activity of Extracts

The Minimum Inhibition Concentration (MIC) of *Bersama abyssinica* extracts was determined for their antimycobacterial activity against two rapid growing *Mycobacteria* strains; *Mycobacteria madagascariense* (MM) and *Mycobacteria indicus pranii* (MIP). All extracts exhibited higher activity against the tested strains (MIC range of

Table 1: Antimycobacterial Activity of *Bersama abyssinica* Against MM and MIP

Minimum Inhibitory Concentration (MIC) in mg/ml			
Plant Part	Extracts	<i>Mycobacteria Madagascariense</i>	<i>Mycobacteria indicus pranii</i>
Leaves	Petroleum ether	3.125	6.25
	Ethyl acetate	0.781	3.125
	Methanol	0.19	0.19
Stem bark	Petroleum ether	6.25	6.25
	Ethyl acetate	0.781	6.25
	Methanol	0.19	0.78
Root bark	Petroleum ether	6.25	6.25
	Ethyl acetate	6.25	6.25
	Methanol	0.391	0.78
Controls	Isoniazid	12.5	12.5
	Rifampicin	0.19	0.19
	DMSO	0.00	0.00

0.19 – 6.25 mg/mL) as compared to the first line antimycobacterial drug isoniazid which had MIC value of 12.5 mg/mL against both strains (Table 1). These results are in agreement with previous studies which have reported the mycobacterial resistance against isoniazid and currently isoniazid is only administered in combination with other effective antimycobacterial drugs (Andries *et al.*, 2005; Jain and Dixit, 2008). Antimycobacterial activity displayed by rifampicin was comparable to leaf methanolic extract against both strains with MIC value of 0.19 mg/mL. The stem bark methanolic extract displayed the same activity against MM (0.19 mg/mL) but had lower activity against MIP (0.78 mg/mL). Methanolic root bark extract exhibited low antimycobacterial activity as compared to methanolic stem bark extract against MM with MIC value of 0.39 mg/mL but displayed the same

activity against MIP (0.78 mg/mL). Ethyl acetate leaf extract exhibited activity with MIC values of 0.78 and 3.25 mg/mL against MM and MIP respectively. Petroleum ether extracts had moderate activity against MM and MIP with MIC range of 3.125 – 6.25 mg/mL.

Brine Shrimp Lethality Results

The *Bersama abyssinica* leaf, stem bark and root bark was evaluated for lethality potential against brine shrimp larvae and results are indicated in Table 2. They exhibited fifty percent lethal dose (LC₅₀) values ranging from >2000 µg/mL to < 100 µg/mL. The stem bark methanolic extract exhibited the highest activity with LC₅₀ value of 29.64 µg/mL followed by (Moshi *et al.*, 2010) bark ethyl acetate extract (67.11 µg/mL). Other extracts had LC₅₀ higher than 100 µg/mL. Since the plant extracts with LC₅₀ values less than fifty and

Table 2: Lethality Assay of *Bersama abyssinica* Extracts Against Brine Shrimp Larvae

Plant Extract	Regression Equation	LC ₅₀ (µg/ml)	95% (CI)	R ²	100% Mortality
BALP	y=22.36logx-24.02	2043.5048	4953.2513	0.904	351974.8463
BALEA	y=40.60logx-21.85	58.8443	59.6777	0.962	1002.8397
BALM	y=65logx-62.68	54.1425	33.347	0.96	318.2506
BASP	y=34.22logx-35.96	325.0733	397.4584	0.94	9399.7248
BASEA	y=27.28logx-26.59	642.0241	4130.0337	0.988	43691.3933
BASM	y=49.42logx-22.74	29.6397	24.3868	0.977	304.5158
BARP	y=40.85logx-36.44	130.6274	138.9809	0.956	2187.8849
BAREA	y=42.09logx-26.89	67.1119	191.1826	0.95	1034.4996
BARM	y=44.12logx-48.69	172.5258	160.0786	0.909	2344.914
Cyclophosphamide	y=69.97logx-34.94	16.3662	10.299	0.995	84.8231

KEY: BALP=*Bersama abyssinica* extract leaves extract of petroleum ether, BALEA=*Bersama abyssinica* leaves extract of ethyl acetate, BALM=*Bersama abyssinica* of methanol, BASP=*Bersama abyssinica* stem bark extract of petroleum ether, BASEA=*Bersama abyssinica* stem bark extract of ethyl acetate, ,BASM=*Bersama abyssinica* stem bark extract of methanol, BARP=*Bersama abyssinica* root bark extract of petroleum ether, BAREA=*Bersama abyssinica* root bark extract of ethylacetate and BARM=*Bersama abyssinica* root bark extract of methanol.

greater than ten are regarded as moderately (Moshi *et al.*, 2010), the stem bark methanolic, leaf methanolic, leaf ethyl acetate and root bark ethyl acetate warrants further investigation for the development of antimycobacterial drugs. Cyclophosphamide a standard anticancer employed as a positive control had LC₅₀ value of 16.36 µg/mL

DISCUSSION

The result of the present study have demonstrated that *Bersama abyssinica* leaves, stem bark and root bark extracts possess antimycobacterial activity against *M. madagascariense* and *M. indicus pranii*. The methanolic extracts exhibited the highest antimycobacterial activity compared to rest of the extracts and the standard isoniazid. The minimum inhibition concentrations displayed by the methanolic extracts was comparable to that exhibited by rifampicin. Rifampicin is one of

the first line antimycobacterial drugs currently in clinical use and is more effective when used in combination with other drugs than alone (Ge *et al.*, 2010). Since the methanolic extracts had higher activity, it signifies that polar compounds from *B. abyssinica* are potential antimycobacterial drug templates.

The ethyl acetate leaf and stem bark extracts exhibited higher activity than the petroleum ether leaf and stem bark extracts indicating the antimycobacterial potential of moderate polar secondary metabolites present in the leaves and stem bark of *B. abyssinica*. Despite the fact that petroleum ether extracts had lower activity compared to methanolic and ethylacetate extracts, it was two times more potency than the standard isoniazid.

Reports on phytochemical studies on *Bersama abyssinica* have indicated the presence

of steroids, flavones, anthraquinones, phenolics with antitumor, anti HIV and antioxidant activities (Djemgou *et al.*, 2010; Mbaveng *et al.*, 2011; Taponjyou *et al.*, 2006). It is possible that these compounds might possess both antioxidant and antimycobacterial activity. Mycobacterium species are also well acknowledged to promote production of reactive oxygen species, which besides being an important part of the host defense against mycobacteria, their heightened production can lead to adverse health effects, predominantly in those with impaired antioxidant capacity, including HIV infected patients (Müller *et al.*, 2000; Reddy *et al.*, 2004). The discovery of antimycobacterial drug templates with antioxidant activities will be a valuable contribution in the fight against TB.

The *Bersama abyssinica* extracts were evaluated for the potential to induce toxicity to humans. Brine shrimp larvae were employed in this assay and the results revealed that extracts with high antimycobacterial potency were moderately toxic to brine shrimps. This suggests that antimycobacterial secondary metabolites from *Bersama abyssinica* might have moderate toxicity to humans and are therefore potential candidate for the development antimycobacterial drugs. Claims on the medicinal use of *Bersama abyssinica* for treatment respiratory infectious disease and other bacterial diseases by various communities in Africa and is therefore validated for the first time.

CONCLUSION

From this study it is concluded that the methanolic extracts *Bersama abyssinica* Fressen have high activities against MM and MIP at 0.039 and 0.78. mg/mL, respectively thus could be good source

of antimycobacterial agents. In addition to that, the Brine shrimp lethality results provide supportive baseline information for the medicinal use of *Bersama abyssinica* in treating infectious diseases in local community.

ACKNOWLEDGMENT

This work was financially supported by COSTECH through the Nelson Mandela African Institution of Science and Technology Scholarship. Mr. Abdul Kidukuli of Institute for Traditional Medicine of Muhimbili University of Health and Allied Sciences is acknowledged for technical assistance.

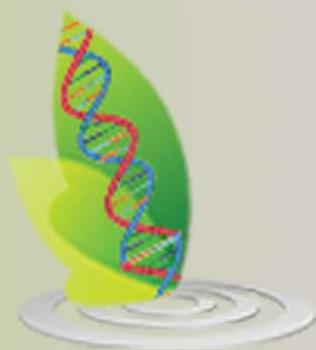
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