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Antituberculous, antimicrobial, cytotoxicity and phytochemical activity study of *Piliostigma thonningii* extract fractions

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World Health Organization studies have demonstrated that 80% of the world's population depends on medicinal plants for their primary health care. This has prompted increased efforts to the adoption and integration of herbal practices in health systems. This study sought to answer the question whether *Piliostigma thonningii* has antitubercular, antibacterial, antifungal and cytotoxic activity. Antimicrobial activity was investigated by disc diffusion and micro dilution techniques. Antituberculous activity was investigated using the BACTEC MGIT 960 system while cytotoxicity was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on Vero cells. Phytochemicals were profiled using standard chemical procedures. A major output of our study is the methanolic fractions which yielded the best antituberculous activity (minimum inhibitory concentration [MIC] of 12.5 µg/ml), the highest antibacterial activity with zones of inhibition of 20.3 mm and MIC of 31.25 µg/ml (*Staphylococcus aureus*), 18.3 mm and MIC of 62.5 µg/ml (Methicillin Resistant *S. Aureus*, MRSA), 14 mm and MIC of 125 µg/ml (*Escherichia coli*), 13.3 mm and MIC of 31.25 (*Shigella sonnei*) and 13 mm against (*Candida albicans*), all within the acceptable toxicity limit (CC₅₀ >500 µg/ml). The activity could be attributed to various phytochemicals that tested positive especially terpenoids. Important is its high activity against MRSA, *S. aureus*, *E. coli*, *S. sonnei*, *C. albicans* and *Mycobacterium tuberculosis* which are health challenge due to drug resistance and sources of community and nosocomial infections. To the best of our knowledge, this is the first report exploring the antituberculous activity of *P. thonningii* and thence a major addition in search of new safe antituberculous drug leads.

Key words: Antibacterial activity, cytotoxicity, antifungal activity, antibacterial activity, terpenoids, phytochemicals, *Piliostigma thonningii*, herbal medicine.

INTRODUCTION

There is an increased and concerted efforts to the adoption and integration of traditional medicine and

medical practices referred to as complementary or/and alternative medicine in both developing and developed

countries in their health system (World Health Organization [WHO], 2005). This is after studies by WHO have demonstrated that 80% of the world's population depends on medicinal plants for their primary health care (Mothana et al., 2008; Gupta et al., 2010).

Numerous studies have shown that medicinal plants are the oldest source of bioactive products with proven efficacy (Hemalatha et al., 2013; de Souza Nascimento et al., 2013; Yadav et al., 2010; Silva and Fernandes, 2010) serving as the basic components of several drugs (Rates, 2001), analgesics (Almeida et al., 2001), anesthetics (Dubey et al., 2013), antibiotics (Cowan, 1999), anti-cancers (Gonzales and Valerio, 2006; Prakash et al., 2013), anti-parasitics, anti-inflammatory (Shilpi et al., 2012), oral contraceptives (Ogbuwu et al., 2011; Umadevi et al., 2013) and diuretic drugs (Dutta et al., 2014) among others.

Piliostigma thonningii (Schum.) Milne-Redh locally known as *mukuura* in Mbeere, Embu County in Kenya has been used and reported for different and varied medicinal purposes. For example, it has been reported that in many African countries, various parts of the plant (root, bark, seed, and fruit) are used to treat wounds, ulcers, gastric and heart pain, gingivitis, and as an antipyretic (Silva et al., 1997; Maroyi, 2011, 2013). In addition, a cough remedy is prepared from the root bark (Akinpelu and Obuotor, 2000). Its roots mixed with roots of *Elephantorrhiza goetzei* are used as bilharzia (schistosomiasis) medicine (Maroyi, 2011). Certain compounds (alkaloids, flavonoids, saponins and tannins) isolated from some of its parts have been reported to bear anti-inflammatory, analgesic and antibacterial activities (Paiva et al., 2010; Akinpelu and Obuotor, 2000).

However, reported scientific work is rather incomprehensive, in relating the phytochemicals present in the antibacterial activities and cytotoxicity of the same extracts from any part of *P. thonningii*. For example, the reported studies deal distinctly in quantification of active substances (Babajide et al., 2010; Deshi et al., 2014) or antibacterial and anti-inflammatory activity (Etsuyankpa et al., 2013; Aderogba et al., 2006; Ibewuiké et al., 1997) or effects of ethanolic extracts on kidney function (Dasofunjo et al., 2012) and lipid profile (Ighodaro and Omole, 2012) in Wistar rats. Other studies independently show phytochemical evaluation, antibacterial activity (Tor-Anyiin and Anyam, 2013) and anti-malaria activity (Kwaji et al., 2010).

No literature was found on antitubercular activity of the plants extracts and therefore this study was designed to investigate anti-TB activity of the root extract. In addition, this study sought to investigate its antibacterial and anti-fungal activity, determine the phytochemicals present and

relate these findings with the observed activities and cytotoxicity to Vero cells.

MATERIALS AND METHODS

Plant

The plant used in this study was identified through ethnobotanical approach. The information of its use and preparation in Mbeere community, Kenya was gleaned from local herbalist and confirmed from documentation by Riley and Brokensha (1988) in The Mbeere in Kenya (ii), Botanical identity and use.. This plant is not an endangered species and it was collected in open community field and therefore no prior permission was required. The location for collection was around 0°46'27.0"S 37°40'54.9"E; -0.774156, 37.681908 of GPS co-ordinates. The identity was also confirmed by a Botanist at Egerton University where voucher specimen number NSN4 was deposited.

Plant extract preparation

Root samples were chopped into small pieces of 2 to 3 cm and air-dried in dark at room temperature (23±2°C) to constant weight. Using a mechanical grinder, the dried root specimens were ground to powder. The powder (50 g) was cold extracted in water with intermittent shaking to mimic the traditional local method of extraction and later lyophilized to obtain a dry powder. Methanol extraction was done by macerating 50 g in 200 ml of methanol for 48 h and then filtered using a filter paper (Whatmann 1) and the residue obtained was further re-extracted using similar amount of methanol. The two volumes of filtrate were pooled together and thereafter concentrated *in vacuo* using a rotary evaporator. Afterwards, the product was allowed to air dry and the yields recorded.

Fractionation of powdered root part of *P. thonningii* was done using different solvents of increasing polarity. The root powder (50 g) was macerated in 200 ml of Petro ether with intermittent shaking for 48 h (two days) after which they were filtered using Whatman No. 1 filter paper. The residue was further re-extracted using the same fresh solvent for 24 h and thereafter the filtrates pooled together. The resulting residue was air dried and further extracted with dichloromethane followed by ethyl acetate and lastly methanol using the same procedure carried out for petrol ether. Using a rotary evaporator, the solvent was removed from each filtrate under conditions of reduced temperature and pressure. The resulting dry extract was weighed and stored in air tight sample bottles at -20°C until the next use (Njeru et al., 2015).

Antibacterial activity

Culturing of micro organisms

One Gram positive: *Staphylococcus aureus* (ATCC 25923) strain and Methicillin Resistant *Staphylococcus aureus* strain (clinical isolate), five Gram negative: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (clinical isolate) and *Shigella sonnei* (clinical isolate) and two Fungi: *Candida albicans* (ATCC

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90028), *Cryptococcus neoformans* (ATCC 66031) and acid fast *Mycobacterium tuberculosis* strain H37Rv (ATCC 27294) were investigated for antimicrobial activity. These organisms were sourced from Kenya Medical Research Institute (KEMRI), Nairobi.

Disc diffusion test

The antibacterial activity was assayed by disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2007) and Mbaveng et al. (2008) with slight modifications. Fresh inoculum was prepared by suspending activated colonies in physiological saline water (0.85% NaCl). Using 0.5 McFarland turbidity standard, the bacteria and fungi suspensions were adjusted to 1.5×10^6 CFU/ml after which they were inoculated aseptically by swapping the surfaces of the Muller-Hinton agar (MHA) plates and Sabouraud dextrose agar (SDA) plates. Whatmann filter paper (No. 1) discs of 6 mm diameter were made by punching the paper, and the blank discs sterilized in the hot air oven at 160°C for 1 h. They were then saturated with 10 µl of various stock extract solution. The methanolic and water crude extracts stock solution was at 1.0 g/ml. For fractions, petro ether, dichloromethane, and methanol fractions stock solutions were made at 500 µg/ml, while ethyl acetate at 250 µg/ml. This afforded disc extract concentration of 1.0×10^4 µg/disc for water and methanol crude extracts, 5 µg/disc for petro ether, dichloromethane, and methanol fractions and 2.5 µg/disc for ethyl acetate. Three standard drugs were used as positive controls: Oxacillin 10 µg/disc (Oxoid Ltd, Tokyo-Japan) and Gentamycin 10 µg/disc (Oxoid Ltd, Tokyo-Japan) for Gram positive and Gram negative bacteria, respectively. Nystatin 100 µg/disc (Oxoid Ltd, Tokyo-Japan) was used as the standard drug for all fungi while discs loaded with 10 µl of DMSO was used as negative controls. Using a sterile forceps, the impregnated dry discs were carefully placed on the agar plates at equidistance points. A positive control as well as a negative control was incorporated in each plate and the plates incubated at 4°C for 2 h so as to allow the extract to diffuse into the media after which they were incubated at 37°C for 18 h. Antimicrobial activity was determined by measuring the size of the inhibition zone to the nearest mm and the results recorded. Extracts fractions that gave an inhibition zone of more than 10 mm were considered to be active (CLSI, 2007) and therefore their Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined (Mothana et al., 2008).

Determination of MIC and MBC

The MIC and MBC of the plant *P. thonningii* extracts was determined for all the organisms in triplicates using broth micro-dilution assay. The petro ether, dichloromethane, and methanol fractions stock solutions were made at 500 µg/ml while ethyl acetate at 250 µg/ml with dimethyl sulfoxide (DMSO). To 100 µl of nutrient broth in a sterile 96 well plate, 50 µl of varying plant concentration (petro ether, dichloromethane, and methanol fractions at 500 to 3.91 µg/ml while ethyl acetate at 250 to 1.95 µg/ml) was added followed by 50 µl of test organisms previously diluted to the equivalent of 0.5 McFarland standard. Addition of the test organisms was done in all the wells except for wells of column 11 which contained neat DMSO and broth, this served as control to check for purity. The adequacy of the media to support the growth of the test organism was evaluated by putting the broth and the test organism in wells of column 12. The plates were then covered with a sterile "cling-on" sealer and incubated for 24 h at 37°C. Bacterial growth was evaluated by addition of 40 µl of 0.2 mg/ml p-iodonitroterazolium chloride (INT, Sigma) to each well and incubated for 30 min. Growth of bacteria was detected by formation of a pink-red coloration while inhibition of growth was signaled by

persistence of a clear coloration. The lowest concentration that exhibited color change was considered as the MIC. MBC was determined by streaking a loopful of broth from wells that exhibited no color change onto sterile nutrient agar and Sabouraud dextrose agar for bacteria and fungi, respectively and thereafter incubated at 37°C for 24 h. The lowest concentration that exhibited no growth was considered as the MBC (Lai et al., 2010).

Antitubercular activity

Prior to its use, the *M. tuberculosis* was revived on Lowenstein Jensen (LJ) slants for 14 days at 37°C following standard procedures (Gupta et al., 2010; Mariita et al., 2010). The efficacy of the plant extracts against *M. tuberculosis* was carried out using the BACTEC MGIT 960 system (BD, New York-U.S.A). This is a fully automated, high volume, non-radiometric instrument that offers continuous monitoring of culture growth. The dry crude extract (water and methanolic) was first dissolved in DMSO to a final concentration of 1 g/ml for preliminary screening. Growth supplement (0.8 ml) containing a mixture of oleic acid, bovine albumen, dextrose and catalase (OADC) was added to five 7 ml BBL™ MGIT™ tube labeled growth control (GC), streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB) to provide essential substrates for rapid growth of *Mycobacteria*. 100 µl of BBL™ MGIT™ SIRE (streptomycin, isoniazid, rifampicin, ethambutol) prepared aseptically according to the manufacturers' instruction was added to corresponding labeled BBL™ MGIT™ tube followed by addition of 0.5 ml of 1% *Mycobacterium* suspension. *Mycobacterium* suspension was prepared by pipetting 0.1 ml Middlebrook 7H9 Broth containing *Mycobacterium* adjusted to 0.5 McFarland standard into 10 ml sterile saline aseptically. The BACTEC MGIT™ 960 system (BD, New York-U.S.A) was then loaded following the manufacturer's instructions and incubated at 37°C. Streptomycin at 1.0 µg/ml, isoniazid at 0.1 µg/ml, rifampicin at 1.0 µg/ml and ethambutol at 5.0 µg/ml served as the positive control whereas DMSO was used as a negative control. The procedure was repeated using plant water and methanolic crude extracts at 1.0 g/ml in place of SIRE. The process was also repeated with petro ether, dichloromethane, ethyl acetate and methanol solvent fractions. The fractions were tested at concentrations ranging from 50 to 6.25 µg/ml (petro ether, dichloromethane and methanol) or 25 to 3.125 µg/ml (ethyl acetate) to determine the MIC.

Cytotoxicity screening

MTT assay was used to determine the toxicity of the extracts obtained from the plant. This is a colorimetric assay hinged on the ability of mitochondrial enzyme (succinate dehydrogenase) to reduce yellow water soluble MTT to an insoluble colored substance, (formazan) that is spectrophotometrically measurable (Njeru et al., 2015). The level of formazan is directly proportional to the measure of cell viability because only metabolically active cells can reduce MTT. The test cell line used was Vero cells from African green Monkey Kidney cells (*Cercopithecus aethiops* epithelial cell line; ATCC CCL-81). The test cells were grown in growth media comprising of 100 ml DMEM, 10 ml fetal bovine serum (FBS), 1 ml penstrep, 1 ml amphotericin B, 1 ml L-glutamine. The test cells were incubated at 37°C in 5% CO₂ until they attained confluency after which they were passaged by adding 2 ml of 0.25% trypsin and further incubated at room temperature until the cell were detached. Growth media (6 ml) was introduced to the test cells to inactivate trypsin off action, while the cell crumps formed were broken gently by sucking and releasing the cell suspension using a pipette. 2 ml of the harvested cells were then transferred into a 50 ml vial and topped up to 50 ml mark using growth media. A cell suspension of 100 µl (1×10^5 cell/ml) was seeded into two rows of

wells in a 96-well micro-titer plate for one sample. The test cells were then incubated in 100 μ l growth media at 37°C and 5% CO₂ for 48 h to form a confluent monolayer. The growth medium was then aspirated off and replaced with 100 μ l of maintenance medium comprising of 100 ml DMEM, 2 ml fetal bovine serum (FBS), 1 ml penstrep, 1 ml amphotericin B, 1 ml L-glutamate and 0.1 ml gentamycin. Afterwards, cells were exposed to decreasing concentrations of respective plant extracts (from 250 to 0.24 μ g/ml for petro ether and dichloromethane fractions and 500 to 0.49 μ g/ml for ethyl acetate and methanolic fraction) and incubated at 37°C for 48 h. This was followed by a further incubation period of 4 h in 10 μ l of 5 mg/ml MTT solution after aspirating off the plant extracts. This was followed by addition of 100 μ l acidified isopropanol (0.04 N HCl in isopropanol). The well plate was gently shaken for 5 min to dissolve the formazan and then optical density measured using ELISA Scanning Multiwell Spectrophotometer (Multiskan Ex labssystems) at 562 nm using 690 nm as reference wave length. The last column containing medium without plant extracts was included to act as negative control. Cell viability (%) was calculated at each concentration as follows using the formula of Ngeny et al. (2013).

$$\text{Cell viability} = \frac{(\text{OD}_{\text{sample 562}} - \text{OD}_{\text{690}})}{\text{OD}_{\text{control 562}} - \text{OD}_{\text{690}}} \times 100$$

Phytochemical tests

Phytochemical tests were done to determine the class of compounds present in the active fractions that could be responsible for activity and/or cytotoxicity. They were identified by characteristic colour changes based on standard procedures according to Houghton and Raman (1998), Edeoga et al. (2005), Ngoci et al. (2011) and Somboro et al. (2011). The results were reported as either (+) for presence, and/or (-) for absence.

Alkaloids

Six to eight drops of Dragendorf reagent was mixed with 2 ml of the extract. Formation of brownish-red precipitate indicated the presence of alkaloids. The Dragendorf reagent was prepared by mixing two reagents: reagent 1 and reagent 2 in equal parts. Reagent 1 was made by dissolving 8.5 g of Bismuth subnitrate in a solution of 10 ml acetic acid and 40 ml of distilled water while as Reagent 2 was prepared by dissolving 8 g of potassium iodide in 20 ml of water (Harborne, 1973; Somboro et al., 2011).

Phenols

Phenols were detected using ferric ferichloride which was prepared by dissolving 0.1 g of ferric ferichloride in 10 ml of water. Equal volumes (2 ml) of both ferric ferichloride and the plant extract were mixed. Formation of a violet-blue color or greenish color was evidence that phenols is present (Harborne, 1973; Somboro et al., 2011).

Terpenoids

One gram of Vanillin was mixed with 100 ml of concentrated sulphuric acid after which 2 ml of the resultant solution was mixed with 2 ml of the plant extract. Formation of a blue-green ring or pink-purple coloration signified the presences of terpenoids (Harborne, 1973; Somboro et al., 2011).

Anthraquinones

0.5 ml of the plant extract was mixed with 0.5 ml of 10% methanolic potassium hydroxide. Red coloration indicated the presences of anthroquinones. 10% methanolic potassium hydroxide was prepared by dissolving 0.5 g of potassium hydroxide pellets in 50 ml of methanol (Harborne, 1973; Somboro et al., 2011).

Flavonoids

Five milliliters of dilute aqueous ammonia solution was added to a portion of the aqueous filtrate of the plant extract, followed by concentrated sulphuric acid. A positive test result was confirmed by the formation of a yellow coloration that disappeared instantly (Edeoga et al., 2005; Ngoci et al., 2011).

Statistical analysis

Ms Excel 2010 data sheets and Graphpad Prism version 6 were used to analyze the data. The data on cytotoxicity was expressed as a percentage of the untreated controls. CC₅₀ values, which is the concentration that kills 50% of the Vero cells, was determined by regression analysis. A particular fraction's extract was considered cytotoxic if it had CC₅₀ of less than 90 μ g/ml (Irungu et al., 2007). Furthermore, unpaired student's t-test was used to test for statistical significance in the differences between the treatments and the control in this study. A *p* value of less than 0.05 was considered to indicate statistical significance. Values were expressed as mean \pm standard error of mean (S.E.M).

RESULTS

Antibacterial, antifungal and antitubercular activity of water and methanolic crude extract

This study first sought to identify whether the plant bark extract had any general antibacterial activity by mimicking the indigenous extraction methods. Traditionally, the plant bark extract was prepared in water or in combination with alcoholic beverages. It was found out that both water and methanolic crude extract had general antimicrobial activity. The diameter of inhibition zones was in all cases higher than 10 mm (Table 1) and therefore this formed the basis for further fractionation.

The test was however performed at higher concentration and we needed to bring this down as well as determine MIC and MBC. Unexpectedly, both crude extract had no antituberculous activity, contrary to long held believe by members of Mbeere community that this plant can be used to treat tuberculosis. Both extract gave 400 GU which is the same as the one given by the negative control (Table 2). Therefore, the crude extract had a remarkable general antimicrobial activity but no antitubercular activity.

Antibacterial and antifungal activity of fractions

Further analysis on fractions demonstrated very weak activity on petro ether, dichloromethane and ethyl acetate fractions with all giving diameter of zones of inhibition of

Table 1. Antimicrobial activity results for the crude root extract.

Extract	Diameter of zone of inhibition (mm)		
	Organism		
	SA	EC	CA
WT	12.3±0.3	10±1.2	10.7±2.4
MOH	14.0±0.6	11.7±0.3	11.7±1.9
PC	24.0±1.3	22.0±0	16.3±0.9
NC	0	0	0

WT: Water crude extract at 1.0×10^4 µg; MOH: methanol crude extract at 1.0×10^4 µg; EC: *E. coli*; SA: *S. aureus*; CA: *C. albicans*; PC: positive control (Oxacillin 10 µg/disc for Gram positive, Gentamycin 10 µg/disc for Gram negative bacteria and Nystatin 100 µg/disc for fungi); NC: negative control (Discs loaded with 10 µl of DMSO); n=3; Values=mean±SEM.

Table 2. Antituberculous activity of crude extract.

Sample	Solvent	GU	R/S
<i>P. thoningii</i>	WT	400	R
	MOH	400	R
	SIRE	0	S
	NC	400	R

WT: Water crude extract at 1 g/ml; MOH: methanol crude extract at 1 g/ml; SIRE: positive control of streptomycin at 1.0 µg/ml, isoniazid at 0.5 µg/ml, rifampicin at 1.0 µg/ml and ethambutol at 5.0 µg/ml; GC: growth control; NC: negative control of media treated with DMSO; R: resistant; S: sensitive.

Table 3. Antimicrobial activity for fractions.

Fraction	Diameter of zone of inhibition in mm								
	Gram positive			Gram negative				Fungi	
	SA	MRSA	PA	EC	KP	SH	ST	CA	CR
PE	7.0±0.0	NT	0	7.0±0.0	0	0	0	7.0±0.0	NT
DCM	6.3±0.3	7.3±0.3	0	7.3±0.3	0	6.3±0.3	0	7.0±0.0	0
EA	0	0	0	0	0	0	0	0	0
MOH	20.3±0.3	18.3±0.3	0	14.0±0.6 ^a	7.0±0	13.3±0.3	8.0±0	13±0.6	NT
PC	33.7±0.3	24.3±0.3	23.7±0.6	15±0	15.7±0.3	19.7±0.6	21.3±0.3	16.3±0.3	20.3±0.3
NC	0	0	0	0	0	0	0	0	0

PE: Petro ether fraction at 5 µg/disc; DCM: dichloromethane fraction at 5 µg/disc; EA: ethyl acetate fraction at 2.5 µg/disc; MOH: methanol fraction at 5 µg/disc; PA: *P. aerogenosa*; EC: *E. coli*; SA: *S. aureus*; KP: *K. pneumoniae*; MRSA: *Methicillin Resistant S. aureus*; SH: *Shigella sonnei*; ST: *S. typhi*; CA: *C. albicans*; CR: *Cryptococcus*; PC: positive control (Oxacillin 10 µg/disc and Gentamycin 10 µg/disc for Gram positive and Gram negative bacteria respectively. Nystatin 100 µg/disc for fungi); NC: negative control (Discs loaded with 10 µl of DMSO); n=3; values= Mean±SEM. ^aIndicates there is no significant difference between the test fraction and standard control ($P>0.05$).

less than 10 mm. Therefore, these 3 fractions were not considered for MIC and MBC determination. Interestingly, methanolic extract had broad spectrum activity inhibiting the growth of Gram positive bacteria (the highest was 20.3 mm against *S. aureus*), Gram negative bacteria (the highest was 14.0 mm against *E. coli*) and fungi (the highest was 13 mm against *C. albicans*). Activity of methanolic fraction against *E. coli* (14.0) was remarkable

since there was no significant statistical difference in activity with that of positive control ($p>0.05$) (Table 3).

The MIC of methanolic fraction against 4 bacteria was less than 200 µg/ml. *S. aureus* and *S. sonnei* had MIC of 31.25 µg/ml; Methicillin resistant *S. aureus* had MIC of 62.5 µg/ml and *E. coli* had MIC of 125 µg/ml. However, all four had MBC >500 µg/ml and hence bacteriostatic in action (Table 4).

Table 4. MIC and MBC results for the Fractions.

Fraction	SA	MRSA	EC	SH
MIC ($\mu\text{g/ml}$)				
MOH	31.25	62.5	125	31.25
MBC ($\mu\text{g/ml}$)				
MOH	>500	>500	>500	>500

MOH: Methanol fraction; EC: *E. coli*; SA: *S. aureus*; MRSA: *Methicillin Resistant S. aureus*; SH: *Shigella. Sonnei*.

Table 5. Antituberculous results for fractions.

Plant	Antituberculous testing		GU	R/S	MIC ($\mu\text{g/ml}$)
	Fraction	Concentration $\mu\text{g/ml}$			
<i>P. thoningii</i>	PE	50	0	S	50
		25	400	R	
		12.5	400	R	
		NC	400	R	
		SIRE	0	S	
	DCM	50	400	R	>50
		25	400	R	
		12.5	400	R	
		NC	400	R	
		SIRE	0	S	
	EA	25	400	R	>25
		12.5	400	R	
		6.25	400	R	
		NC	400	R	
		SIRE	0	S	
	MOH	50	0	S	12.5
		25	0	S	
		12.5	0	S	
		NC	400	R	
		SIRE	0	S	

PE: Petro ether fraction; DCM: dichloromethane fraction; EA: ethyl acetate fraction; MOH: methanol fraction; SIRE: positive control of streptomycin at 1.0 $\mu\text{g/ml}$, isoniazid at 0.5 $\mu\text{g/ml}$, rifampicin at 1.0 $\mu\text{g/ml}$ and ethambutol at 5.0 $\mu\text{g/ml}$; GU: growth unit; NC: negative control of media treated with DMSO; R: resistant; S: sensitive.

Antituberculous, cytotoxicity activity and phytochemical profile of the fractions

When screening with crude water and methanolic extract revealed no antituberculous activity, we sought to investigate whether fractionation could improve on activity in some of the fractions. This is based on the knowledge that solvents of different polarities will retain certain compounds of equivalent polarity. It was also

hypothesized that different bioactive compound combinations could bear antagonistic effects on each other once lumped together in crude extract, but their separation could render certain fractions active against *M. tuberculosis* (MTB). Interestingly, methanolic extract had MIC of 12.5 $\mu\text{g/ml}$ and hence significant activity while petro ether fraction had MIC of 50 $\mu\text{g/ml}$, dichloromethane and ethyl acetate had MIC of >50 and 25 $\mu\text{g/ml}$ (Table 5). All the fractions had $\text{CC}_{50} > 90 \mu\text{g/ml}$

Table 6. Cytotoxicity results in $\mu\text{g/ml}$.

Fraction	CMOH	PE	DCM	EA	MOH
CC ₅₀ ($\mu\text{g/ml}$)	198.02	>250	110.82	>500	>500

CMOH: Crude methanolic extract; PE: petro ether fraction extract; DCM: dichloromethane fraction extract; EA: ethyl acetate fraction extract; MOH: methanol fraction extract; CC₅₀: Concentration that kills 50 % of the cells (Vero cells with CMOH and HEP-2 cells with fractions).

Table 7. Phytochemical results of fractions.

Extract Fraction	V-Ts	A-F	MK-A	D-A	F-P
Petro ether	-	-	-	-	-
Dichloromethane	+++	+	+	+	+
Ethyl acetate	+	+	-	-	+
Methanol	+	-	-	-	-

V-T: Vanillin test for terpenoids; A-F: ammonia test for flavonoids; MK-A: methanolic potassium hydroxide test for anthraquinones; D-A: Dragendorff test for alkaloids; F-P: ferric chloride test for phenols; -: absent phytochemicals; +: low concentration of phytochemicals; ++: medium concentration; +++: high concentration of phytochemicals.

and hence were all within the acceptable toxicity limit (Table 6). The varied activity in the extract could be attributed to various phytochemicals that tested positive. These include alkaloids, flavonoids, phenols, terpenoids and anthraquinones (Table 7).

DISCUSSION

Both crude extract and methanolic fraction extract had broad spectrum activity. They inhibited both Gram positive and Gram negative bacteria and fungi giving remarkable zones of inhibition. The activity against Gram positive was the highest. For example, methanolic fraction gave inhibition zone diameter of 20.3 mm and MIC of 31.25 $\mu\text{g/ml}$. This could be attributed to the cytoplasmic membrane of Gram-positive species which are simple lipid bilayer and hence not a barrier for most amphipathic compounds and can be readily traversed by antibacterials. In contrast, Gram-negative bacteria have evolved a sophisticated permeability barrier with an additional outer membrane comprising a highly hydrophilic lipopolysaccharide layer and this restricts penetration of hydrophobic and amphipathic compounds, which encompasses many drug compounds (Kim and Frederick, 2006). Difference in activity was also evidence among tested strains in both crude and fraction extracts. This could be due to genetic differences between different strains and this provides proof for the necessity of antibiogram prior to prescription as a precautionary measure in mitigating drug resistance development (Yimta et al., 2014).

Even though crude extract had no antituberculous

activity, fractionation gave us two fraction (petro ether and methanolic fraction) which remarkably inhibited growth of MTB with MIC of 50 and 12.5 $\mu\text{g/ml}$, respectively. Moreover, the general antibacterial and antifungal activity was higher with methanolic fraction with four bacteria having MIC of less than 200 $\mu\text{g/ml}$; the set threshold for herbal extract activity (Kueete, 2010). This may suggest that fractionation enhanced antitubercular activity, implying either that there is higher concentration of active principle better at certain level of polarity or possibility of antagonism of various active compounds when lumped together as in crude extracts thus diminishing the activity. Equally important to note is that both crude and fraction extracts had CC₅₀ >90 $\mu\text{g/ml}$ and therefore were within the acceptable toxicity limit. The bioactivity in *P. thonningii* bark sample can to some extent be attributed to the tested phytochemicals, especially terpenoids which previous studies have associated with antibacterial, antifungal and anti-tubercular activities. The mechanism of action of terpenoids is not entirely understood but is speculated to involve membrane disruption by the lipophilic compounds (Kueete, 2010).

Conclusion

A major output of the current study is the identification of the methanolic fractions which yielded the best antituberculous activity (MIC of 12.5 $\mu\text{g/ml}$) as well as the highest antibacterial activity (with zones of inhibition of 20.3 mm and MIC of 31.25 $\mu\text{g/ml}$ (*S. aureus*), 18.3 mm and MIC of 62.5 $\mu\text{g/ml}$ (MRSA), 14 mm and MIC of 125

µg/ml (*E. coli*) and 13.3 mm and MIC of 31.25 (*S. sonnei*) which was within the acceptable toxicity limit (CC₅₀ >500 µg/ml). For us the fraction provides the best candidates for further investigation due to its selective activity. Of particular relevance is its high activity against MRSA, *S. aureus*, *E. coli*, *S. sonnei*, *C. albicans* and MTB which are currently posing great public health challenge due to drug resistance development and as major sources of community and hospital based infections. To the best of our knowledge, this is the first report exploring the antituberculous activity of *P. thonningii* and thence a major output in search of new safe drug leads to mitigate the global tuberculosis threat.

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Conflicts of Interest

Authors have not declare no conflict.

REFERENCES

- Aderogba MA, Okoh EK, Okeke IN, Olajide AO, Ongundaini AO (2006). Antimicrobial and Anti-inflammatory effects of *Piliostigma reticulatum* Leaf extract. *Int. J. Pharm.* 2(1):70-74.
- Akinpelu DA, Obuotor EM (2000). Antibacterial activity of *Piliostigma thonningii* stem bark. *Fitoterapia* 71(4):442-443.
- Almeida RN, Navarro DS, Barbosa-Filho JM (2001). Plants with central analgesic activity. *Phytomedicine* 8(4):310-322.
- Babajide JM, Bello OQ, Babajide SO (2010). Quantitative determination of active substances (preservatives) in *Piliostigma thonningii* and *Khaya ivorensis* leaves and subsequent transfer in treated dry-yam. *Afr. J. Food Sci.* 4(6):382-388.
- Clinical and Laboratory Standards Institute (CLSI) (2007). Methods for determining bactericidal activity of antimicrobial agents, Tentative standard M26-T. Wayne: National Committee for Clinical Laboratory Standards.
- Cowan MM (1999). Plant products as anti-microbial agents. *Clin. Microbiol. Rev.* 12:564-582.
- Dasofunjo K, Nwodo FOC, Ipav SS Barminas ZL (2012). Effect of the ethanolic extract of *Piliostigma thonningii* leaves on kidney function indices and haematological parameters of male albino wistar rats. *J. Nat. Prod. Plant Resour.* 2(6):670-674.
- de Souza Nascimento S, DeSantana JM, Nampo FK, Ribeiro ÉAN, da Silva DL, Araújo-Júnior JX, da Silva Almeida JRG, Bonjardim LR, de Souza AA, Quintans-Júnior LJ (2013). Efficacy and Safety of Medicinal Plants or Related Natural Products for Fibromyalgia: A Systematic Review. *Evid. Based Complement Alternat. Med.* 2013:149468.
- Deshi SN, Wonang DL, Dongs IS (2014). Exploration of *Piliostigma Thonningii* (Schum). A tropical tree for possible utilization as a plant-derived pesticide. *Acad. J. Interdiscip. Stud.* 3(7):108-110.
- Dubey S, Maiti S, Singh M, Saraf SA, Saha S (2013). Phytochemistry, Pharmacology and Toxicology of *Spilanthes acmella*: A Review. *Adv. Pharmacol. Sci.* 2013:423750.
- Dutta KN, Chetia P, Lahkar S, Das S (2014). Herbal Plants Used as Diuretics: A comprehensive Review. *J. Pharm. Chem. Biol. Sci.* 2(1):27-32
- Edeoga HO, Okwu DE, Mbaebie BO (2005). Phytochemical constituents of some Nigerian medicinal plants. *Afr. J. Biotechnol.* 4(7):685-688.
- Etsuyankpa MB, Ndamitso MM, Suleman MA, Tijani JO, Idris S, Shaba EY, Mohammed A (2013). The antibacterial activity and some trace elemental compositions of the extracts of *Piliostigma thonningii*, a widely used ethnomedicinal tree in Minna, Nigeria. *Afr. J. Microbiol. Res.* 7(48):5411-5416.
- Gonzales GF, Valerio LG (2006). Medicinal plants from Peru: a review of plants as potential agents against cancer. *Anticancer Agents Med. Chem.* 6(5):429-44.
- Gupta K, Shukla C, Bisht G R, Kumar S, Thakur RL (2010). Detection of anti-tuberculosis activity in some folklore plants by radiometric BACTEC assay. *Lett. Appl. Microbiol.* 52:33-40.
- Harborne JB (1973). *Phytochemical methods* London. Chapman and Hall Limited.
- Hemalatha M, Thirumalai T, Saranya R, Elumalai EK, David E (2013). A review on antimicrobial efficacy of some traditional medicinal plants in Tamilnadu. *J. Acute Dis.* 2(2):99-105.
- Houghton PJ, Raman A (1998). In *Laboratory Hand Book for the Fractionation of Natural 1998; Extracts* (1st ed) London. Chapman and Hall pp. 1-153.
- Ibewuik JC, Ogungbamila FO, Ogundaini AO, Okeke IN, Bohlin L (1997). Antiinflammatory and Antibacterial Activities of C-methylflavonols from *Piliostigma thonningii*. *Phytother. Res.* 11:281-284.
- Ighodaro OM, Omole JO (2012). Effects of Nigerian *Piliostigma thonningii* Species Leaf Extract on Lipid Profile in Wistar Rats. *ISRN Pharmacol.* 2012:387942.
- Irungu B, Rukunga MG, Muthaura CN (2007). *In vitro* antiparasitodal and cytotoxicity activities of 14 medicinal plants from Kenya. *South Afr. J. Bot.* 73(2):204-207.
- Kim L, Frederick MA (2006). Prospects for plant-derived antibacterials. *Nat. Biotechnol.* 24(12):1504-1507.
- Kuete V (2010). Potential of Cameroonian Plants and Derived Products against Microbial Infections: A Review. *Planta Med.* 76:1479-1491.
- Kwaji A, Bassi PU, Aoill M, Nneji CM, Ademowo G (2010). Preliminary studies on *Piliostigma thonningii* Schum leaf extract: Phytochemical screening and *in vitro* antimalarial activity. *Afr. J. Microbiol. Res.* 4(9):735-739.
- Lai HY, Lim YY, Kim KH (2010). *Blechnum orientale* Linn - a fern with potential as antioxidant anticancer and antibacterial agent. *BMC Complement. Altern. Med.* 10:15.
- Mariita RM, Ogol CK, Oguge NO (2010). Antitubercular and phytochemical investigation of methanol extracts of medicinal plants used by the Samburu community in Kenya. *Trop. J. Pharm. Res.* 9:379-385.
- Maroyi A (2011). Ethnobotanical study of medicinal plants used by people in Nhema communal area, Zimbabwe. *J. Ethnopharmacol.* 136:347-354.
- Maroyi A (2013). Traditional use of medicinal plants in south-central Zimbabwe: review and perspectives. *J. Ethnobiol. Ethnomed.* 9:31.
- Mbaveng A, Ngameni B, Kuete V, Simo I, Ambassa P, Roy R (2008). Antimicrobial activity of the crude extracts and five flavonoids from the twigs of *Dorstenia barteri* (Moraceae). *J. Ethnopharmacol.* 116(3):483-489.
- Mothana RA, Abdo SA, Hasson S, Althawab FM (2008). Antimicrobial Antioxidant and Cytotoxic Activities and Phytochemical Screening of Some Yemeni Medicinal Plants. *Evid. Based Complement. Altern. Med.* 7(3):323-30.
- Ngeny LC, Magiri E, Mutai C, Mwikwabe N, Bii C (2013). Antimicrobial properties and toxicity of *Hagenia abyssinica* (Bruce) J.F. Gmel *Fuerstia africana* T.C.E Fries *Asparagus racemosus* (Willd) and *Ekebergia capensis* Sparrm. *Afr. J. Pharmacol. Ther.* 2(3):76-82.
- Ngoci SN, Mwendia CM, Mwaniki CG (2011). Phytochemical and cytotoxicity testing of *Indigofera lupatana* Baker F. *J. Anim. Plant Sci.* 2(1):1364-1373.

- Njeru SN, Obonyo MA, Onsarigo SN, Ngari SM (2015). Bioactivity of *Cissampelos pareira* medicinal plant against *Mycobacterium tuberculosis*. *J. Pharmacogn. Phytochem.* 3(6):167-173.
- Ogbuewu IP, Unamba-Oparah IC, Odoemenam VU, Etuk IF, Okoli IC (2011). The potentiality of medicinal plants as the source of new contraceptive principles in males. *N. Am. J. Med. Sci.* 3(6):255-63.
- Paiva PMG, Gomes FS, Napoleão TH, Sá RA, Correia MTS, Coelho LC (2010). Antimicrobial activity of secondary metabolites and lectins from plants. *Curr. Res. Technol. Educ. Topics Appl. Microbiol. Microbial Biotechnol.* 1:396-406.
- Prakash O, Kumar A, Kumar P (2013). A jeet Anticancer Potential of Plants and Natural Products: A Review. *Am. J. Pharmacol. Sci.* 1(6):104-115.
- Rates SMK (2001). Plants as source of drugs. *Toxicol.* 39(5):603-613.
- Riley BW, Brokensha DX (1988). In *The Mbeere in Kenya*; (ii) Botanical identity and use. University press of America, USA 2: pp. 76-77.
- Shilpi JA, Islam ME, Billah M, Islam KMD, Sabrin F, Uddin SJ, Nahar L, Sarker SD (2012). Antinociceptive Anti-Inflammatory and Antipyretic Activity of Mangrove Plants: A Mini Review. *Adv. Pharmacol. Sci.* 2012:576086.
- Silva NCC, Fernandes J (2010). A. Biological properties of medicinal plants: a review of their antimicrobial activity. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 16(3):402-413.
- Silva O, Barbosa S, Diniz A, Valdeira ML, Gomes E (1997). Plant extracts antiviral activity against Herpes simplex virus type 1 and African swine fever virus. *Pharm. Biol.* 35(1):12-16.
- Somboro AA, Patel K, Diallo D, Sidibe L, Chalchat JL, Figueredo G, Ducki S, Troin Y, Halar P (2011). An ethnobotanical and phytochemical study of the African medicinal plant *Guiera senegalensis* J F Gmel. *J. Med. Plant. Res.* 5(9):1639-1651.
- Tor-Anyiin TA, Anyam JV (2013). Phytochemical evaluation and antibacterial activity: A comparison of various extracts from some Nigerian trees Peak. *J. Med. Plant. Res.* 1(2):13-18.
- Umadevi M, Sampath KPK, Bhowmik D, Duraivel S (2013). Medicinal Plants with Potential Antifertility Activity. *J. Med. Plant. Stud.* 1(1):26-33.
- World Health Organization (WHO) (2005). National policy on traditional medicine and regulation of herbal medicines. Report of a WHO global survey.
- Yadav U, Hugo A, Boon EK, Saroj Y, Krishna KS (2010). Indigenous use and bio-efficacy of medicinal plants in the Rasuwa District Central Nepal. *J. Ethnobiol. Ethnomed.* 6(3):2010.
- Yimta F, Simplicie RM, Francos N, Sidor S, Dieu J, Rooger J (2014). Amicrobial activity of methanol Extract and Fractions from *Kalanchoe crenata*, *Terminalia avicennioides* and *Sarcocephalus latifolius*. *Pharmacologia* 199-204.