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Brine shrimp toxicity and in *vitro* antimicrobial activity of *Piliostigma thonningii* (Schum.) Milne-Redh. [Leguminosae-Caesalpinioideae] from Kenya and Malawi against some pathogens of human and veterinary importance

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Many microorganisms are responsible for causing serious diseases of bacterial origin. Development of drug resistance in animal and human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants. The present study reports on the antimicrobial and brine shrimp lethality of *Piliostigma thonningii* leaves collected from two geographical regions, Kenya and Malawi. Both aqueous as well as organic extracts from leaves of *P. thonningii* were screened for antibacterial activity against bacteria of human and veterinary importance using agar well diffusion and evaluated for acute toxicity using brine shrimp bioassay. Except for chloroform extract of *P. thonningii* from Malawi, all of the plant extracts demonstrated remarkable antibacterial activity against the five test bacteria at concentrations tested (250 µg/ml) in agar well diffusion method. In brine shrimp bioassay, all the crude extracts from Kenya and Malawi exhibited varying degrees of toxicity against *Artemia salina* larvae. Nevertheless, further evaluation of the *in vivo* toxicity and *in vivo* antibacterial activity of the crude plant extracts should be carried out.

Key words: *Piliostigma Thonningii*, brine shrimp bioassay, antibacterial activity, crude plant extract, Kenya, Malawi.

INTRODUCTION

In developing countries, diseases of bacterial origin is a serious problem, presenting a serious public issue of a significant segment of the population as uncovered by either private or health care systems. In economic crisis, high cost of industrialised medicine, inefficient public access to medical and pharmaceutical care, in addition to the side effects caused by synthetic drugs are some of the factors contributing to central role of medicinal plants in health care systems (Susan et al., 2007). The rapid development of multi-drug resistant strains of bacteria has increased the occurrence of bacterial infections that cannot be treated with conventional antimicrobial agents (Sieradski et al., 1999; Alanis, 2005). Because of the aforementioned reasons, lots of efforts are being made to discover new antimicrobial agents from various sources such as micro-organisms, animals and plants (Tomoko et al., 2002). Plants comprise the largest component of the diverse therapeutic elements of traditional health care practices both in humans and animals. Nearly all cultures and civilizations from ancient times to the present day

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have used herbal medicines which are antimicrobial sources to cure infections (Lino and Deogracious, 2006). Plant derived drugs against bacterial pathogens represent a vast untapped source of medicines and is effective in the treatment of infectious diseases, while, simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Parekh et al., 2005). Herbs are invaluable source of modern drugs, with more than 50% of modern drugs being derived from plants (Newman et al., 2005).

World Health Organization (WHO) has compiled an inventory of more than 20,000 species of medicinal plants which are used for a variety of applications (Buckingham, 1996; Hoffmann et al., 1993). Some of these species have been tested for antimicrobial properties, while majority are not yet evaluated. Documentation of toxicity and antimicrobial properties of medicinal plants is necessary in order to build a comprehensive database from which it may be possible to search for new leads in drug development when the need arises. On the other hand, the development of drug resistance in pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants. Screening of medicinal plants for phytochemicals and antibacterial activities is important for finding potential new compounds for therapeutic use.

Piliostigma thonningii (Schum.) Milne-Redh. [Leguminosae-Caesalpinioideae] is a plant used for medicinal purposes in many African countries. Different parts of the plant have been used traditionally for the treatment of various diseases in humans and animals (Djuma, 2003). Its roots and twigs have been used in treatment of dysentery, wounds, respiratory ailments, snake bites, hookworms and skin diseases (Asuzu and Onu, 1994). The leaves of *P. thonningii* are used to treat wounds, chronic ulcers, diarrhoea, toothache and gingivitis, cough, and bronchitis (Watt and Breyer-Brandwijk, 1962). The bark, leaves or root extracts are taken as cough medicine, whereas the leaf extract as menorrhagia medicine. Alkaloids, flavonoids, saponins and tannins have already been isolated from the leaves of *P. thonningii.* The leaves from the tree posess antibacterial, antimicrobial and antioxidant activities (Alfred Maroyi, 2013). The dry leaf powder has been reported to contain alkaloids, saponins, flavonoids and tannins (Ighodaro et al., 2012). Besides this carbohydrates, glycosides, flavonoids, tannins, saponins, balsams, volatile oil and terpenes have also been isolated from the leaves of *P. thonningii* (Egharevba et al., 2010). Few studies have been done to evaluate the pharmacological activity and safety of the leaves of *P. thonningii* despite their rich phytochemical composition.

Despite this medicinal usefulness, little information is available regarding the comparative antimicrobial and brine shrimp lethality effects of *P. thonningii*. The current study was therefore designed to investigate the antimicrobial activity and acute toxicity of crude extracts from leaves of *P. thonningii* collected from two different phytogeographical zones, Kenya and Malawi.

MATERIALS AND METHODS

Collection of plant materials

The fresh leaves of *P. thonningii* were collected from Bunda College of Agriculture forest located thirty kilometres South West of the City of Lilongwe in Malawi and Imenti North District in Meru County in Kenya during December, 2010 based on ethno pharmacological use according to interviews with local communities and traditional health practitioners. Consent to collect the plant material was given by the study communities. The taxonomic identities of the plants were confirmed by Mr. Musembi J.K of the Department of Land Resource Management and Agricultural Technology (LARMAT), College of Agriculture and Veterinary Medicine, University of Nairobi. The voucher specimen numbers “WMD, 01” and “WMD, 02” from Kenya and Malawi, respectively were deposited in the Herbarium of LARMAT, University of Nairobi.

Preparation of the extracts

The method described by quality control for medicinal plant materials was employed according to WHO (1998). In this method, the leaves were cleaned with distilled water to remove dust, external pollutants, sediments, additives, insecticides and parasites, air dried at room temperature and ground into fine powder using a laboratory hammer mill with a one millimetre sieve pore size. Aqueous extraction was performed by soaking 100 g of dry powder of the *P. thonningii* leaves in distilled water (1000 ml) and boiled at 60°C in a water bath for one hour. This was followed by filtration of the suspension through cotton wool plugged in a funnel and the filtrate was collected in bottles. The filtrate was further separated by centrifugation at 3,000 rpm for three minutes. The supernatant was decanted in falcon tubes and kept in a deep freezer for 24 h which was then lyophilized. The lyophilized dry powder was then collected in falcon tubes with screw tight covers and kept at -20°C until used.

Organic extracts were prepared by cold maceration. 100 g of ground plant material was dissolved in 1000 ml of solvent. It was incubated at room temperature for 48 h and stirred periodically. The sample was filtered using Whatman paper No.1 and the filtrate was concentrated *in vacuo* set at 40°C and then transferred into an oven set at 40°C for further evaporation of the solvents. The methanolic and [chloroformic and methanolic mixture (1:1)] crude extracts were further taken for lyophilisation so as to remove the water component from methanol and subsequently obtain a dry powdered material. Percentage yield of plant extracts (both aqueous and organic) were calculated. The dried plant extracts were collected in airtight falcon tubes and kept at - 4°C until used.

Preparation of test extracts

Samples of aqueous crude extracts of *P. thonningii* leaves were prepared by dissolving 0.1 g of the crude extract in 10 ml of distilled water making a stock solution of 10,000 µg/ml. Samples of organic crude extracts of *P. thonningii* leaves were prepared by dissolving 0.1 g in 1 ml of dimethyl sulphoxide (Sigma chemical CO., St. Louis, MO, USA) followed by subsequent dilution to lower concentration of dimethyl sulphoxide (DMSO), to < 1% to avoid carry over (solvent) effect (Dorin et al., 2001). The positive control drug, cyclophosphamide was prepared by dissolving 0.1 g of the crude extract in 10 ml of distilled water making a stock solution of 10,000 µg/ml. Cyclophosphamide was used in this experiment as positive control while dimethyl sulphoxide and distilled water were used as negative controls.

Hatching of brine shrimp

Brine shrimp eggs were incubated and hatched at 37°C in a shallow rectangular container (14 × 9 × 5 cm) containing 225 millilitres of artificial sea water which was prepared by dissolving 33 g of commercial salt mixture [consisting of: sodium chloride at a concentration of 24.6 g/l; potassium chloride at a concentration of 0.67 g/l; calcium chloride at a concentration of 1.36 g/l; magnesium sulphate at a concentration of 4.66 g/l and sodium bicarbonate at a concentration of 0.18 g/l]. The mixture had a pH of 8.0] in a litre of distilled water followed by filtration through Whatman filter paper No. 1. The container had two unequal compartments with 2 mm holes in between the two compartments. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. A few granules of yeast were added as source of energy for the *nauplii*. After 48 h, the phototrophic *nauplii* were collected by pipette from the lightened side, having been separated by the divider from the shells.

Brine shrimp bioassay

Ten larvae of brine shrimp (*Artemia salina* L.(Artemiidae) were transferred into test tubes using disposable pipettes. 5, 50, and 500 µl representing the three concentrations 10, 100 and 1000 µg/ml of the plant extract were transferred into test vials. Five replicates were used for each dose level per sample. Filtered brine solution at 3.3% was transferred to all test vials containing plant extract to make 5 ml volume. The vials were incubated for 24 h and percentage deaths were calculated at the various dose levels and the control. In cases where control deaths occurred, the data was corrected using Abbott's formula: Percentage death = [(Test – control / control)] × 100 (Abbotts, 1925).

Statistical analysis/LC_{50} determinations

Median lethal concentrations (LC_{50}s) were determined from the 24 h counts using probit analysis method described by Finney (Finney, 1971). In cases where data was insufficient for this technique, the dose response data were transformed into a straight line by means of a logit transformation (Hafner et al., 1977); the LC_{50} was derived from the best fit line obtained by linear regression analysis.

Agar well diffusion test and determination of antibacterial activity of *P. thonningii* crude extracts

Briefly, 1 ml of the test culture (10^7 CFU/ml) was inoculated into a sterile plate with 20 ml Muller Hinton molten agar and the plate was shaken for even spread and proper mixing of the organisms and agar. When the agar was solidified, six wells of 8 mm in diameter and 7 mm depth were made on the surface of the agar plates using a sterile borer. Stock solution of each plant extract was prepared at concentration of 250 mg/ml in the same solvent used for their extraction. Each of the four wells out of the six wells was filled with 0.350 ml of the plant extract. The fifth and the sixth wells were filled with 0.350 ml of distilled water and 13.13 µg equivalent of gentamycin in 0.350 ml of distilled water as negative and positive control, respectively. The plates were then incubated at 37°C for 24 h, the zones of inhibition were measured with a calliper and the ruler in millimetre, and the results were tabulated according to Yitbareck Habtamu et al. (2010). The test was conducted in duplicate for all the five bacteria of veterinary importance. Mean inhibition zones and significant differences between means of aqueous and organic extracts of *P. thonningii* were calculated by Genstat 13th Edition.

The antibacterial activity was tested using agar well diffusion method according to Lino and Deogracious (2006) and Sahm and Washington (1990). Aqueous, methanol, chloroform and chloroform + methanol crude extracts of *P. thonningii* from Kenya and Malawi were screened against five bacteria [(4 standard bacteria and 1 clinical isolate (*Streptococcus agalactiae*)] using agar well diffusion. The test bacteria which included *Bacillus cereus, Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus agalactiae* isolate were all obtained from the department of Public Health Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Nairobi.

Statistical analysis

Data were analyzed using descriptive statistics. Means were calculated from three separate experiments. The results of the antimicrobial screening of the leaves were compared to a reference antibiotic, gentamycin.

RESULTS

Percentage yield of crude plant extracts

P. thonningii crude extracts showed variation in percentage yield in both aqueous and organic solvents. The highest yield was recorded for methanolic extract of Kenyan *P. thonningii* (12%) and the lowest yield was observed for the chloroformic extract of Malawian *P. thonningii* which was 1.2% (Table 1).

Brine shrimp toxicity bioassay

Results of the toxicity of different extracts against brine shrimp larvae are shown in Table 2. Aqueous extracts and organic extracts of the Kenyan *P. thonningii* displayed toxicity range of LC_{50} between 63 and 991.3 µg/ml while aqueous and organic extracts of the Malawian *P. thonningii* displayed toxicity range of LC_{50} between 128.4 and 540 µg/ml (Table 2). The results obtained have shown that the aqueous extracts of the Kenyan *P. thonningii* are more toxic than the chloroform + methanol extract with LC_{50} values of 63 and 991.3 µg/ml, respectively. Aqueous extract of the Kenyan *P. thonningii* was as toxic as that of the control drug, cyclophosphamide (LC_{50} value of 95 µg/ml) with an LC_{50} value of 63 µg/ml (Table 2).

Antibacterial screening

The test bacteria were screened for antibacterial activity using agar well diffusion method.

Agar well diffusion

The growth inhibition zones are tabulated in Table 3. The results indicated that out of 8 crude extracts of *P. thonningii* from Kenya and Malawi, 7 extracts had antibacterial activity at concentration of 250 µg/ml and

Table 1. Yield of leaf extracts of *P. thonningii* (%) using aqueous and organic extraction methods.

Voucher specimen No.	Initial sample weight (g)	Type of extraction	% yield (w/w)
WMD 01	100	Aqueous	9.4
WMD 02	100	Aqueous	9.4
WMD 01	100	Methanol	12.0
WMD 02	100	Methanol	8.3
WMD 01	100	Chloroform	3.1
WMD 02	100	Chloroform	1.2
WMD 01	100	Chloroform + Methanol	12.0
WMD 02	25	Chloroform + Methanol	3.9

WMD 01*: P. thonningii* from Kenya. *WMD 02:* P. thonningii *from Malawi.*

Table 2. LC_{50} values for *P. thonningii* screened against brine shrimp larvae (*A. salina* Leach).

	Parameter	Percentage death after 24 h			Value	
Zone	Extract Type	10 µg/ml	100 µg/ml	1000 µg/ml	LC_{50}	Remarks
KE	Aqueous	20	48	100	63.0	Toxic
	Methanol	8	36	100	109.6	Toxic
	Chloroform	16	42	84	121.4	Toxic
	Chloroform/methanol	38	44	50	991.3	Non-toxic
MA	Aqueous	12	24	100	128.4	Toxic
	Methanol	8	32	62	408.3	Toxic
	Chloroform	2	24	60	540.0	Toxic
	Chloroform/methanol	56	44	58	476.3	Toxic
Controls	Cyclophosphamide	20	52	80	95.0	Toxic
	Distilled water	0	0	0	0.0	Non-toxic
	DMSO	0	0	0	0.0	Non-toxic

KE = Kenya *P. thonningii* leaf extract; MA = Malawi *P. thonningii* leaf extract; DMSO = Dimethylsulphoxide

one crude extract was not effective against all the test bacteria. The largest zone (34 mm) of inhibition was observed for aqueous extract of the Kenyan *P. thonningii* against *B. cereus* while the Malawian *P. thonningii* recorded (30 mm) (Table 3). The smallest zone (14 mm) was observed for methanol extract of the Kenyan *P. thonningii* and Malawian *P. thonningii* against *E. coli*. For the methanol extract, the largest zone (33 mm) inhibition was observed in the Kenyan *P. thonningii* against *S. agalactiae e* while the Malawian *P. thonningii* recorded (28 mm) against the same bacteria (Table 3). For the chloroform extract, the largest zone (26 mm) of inhibition was observed in the Kenyan *P. thonningii* against *E. coli* while the Malawian *P. thonningii* recorded no activity against all tested bacteria.

DISCUSSION

The current study was designed to evaluate the safety of crude leaf extracts from *P. thonningii* collected from Kenya and Malawi using brine shrimp bioassay; and also to investigate the antibacterial activity using agar well diffusion technique. Brine shrimp bioassay is a simple method for natural product research. The procedure determines median lethal concentration values of active compounds and extracts in the brine medium. This method is rapid, reliable, inexpensive and convenient as an in-house bioassay tool. Babajide et al. (2010) considered LC_{50} values of < 10 µg/ml as very active, of < 700 µg/ml as active and of > 700 µg/ml as none active. Meyer et al. (1982) also considered an LC_{50} value of lower than 1000 µg/ml of an extract in brine shrimp lethality bioassay as toxic. The classification of toxicity in brine shrimp bioassay described by Padmaja et al. (2002) was used in the current study where LC_{50} > 1000 µg/ml was considered to be non toxic, LC_{50} (500 to 1000 ug/ml) was considered to be weakly toxic, LC_{50} (100 to 500 µg/ml) was considered to be moderately toxic and LC_{50} (0 to 100 µg/ml) was considered to be strongly toxic.

The results showed that aqueous and organic extracts of the Kenyan *P. thonningii* and Malawian *P. thonningii* displayed toxicity as follows: aqueous extract of Kenyan *P. thonningii* leaves was strongly toxic with LC_{50} of 63

Table 3. Antibacterial activity of aqueous and organic extracts of *P. thonningii* using agar well diffusion method.

Organisms	Mean zones of inhibition (mm)									
	Aqueous		Methanol		Chloroform		Chloroform/ Methanol		Controls	
	KE	MA	KE	MA	KE	MA	KE	MA	Gentamycin	Distilled water
E. coli	19±0.6	19±1.0	14±0.6	14±0.6	26±1.2	-	16±0.6	15±1.0	22±0.6	-
B. cereus	34±1.0	30±0.6	24±0.8	21±0.6	20±1.0	-	20±0.6	22±1.0	18±0.8	-
P. aeruginosa	26±1.2	29±0.8	23.5±0.6	24±0.8	19±0.6	-	22±0.6	24±1.2	20±1.0	-
S. aureus	28.5±0.8	31±1.2	33±0.6	28±0.6	-	-	26±1.0	28±1.2	32±1.2	-
S. agalactiae	30±0.6	31±1.2	26.5±0.8	23±0.6	26±1.2	-	25±0.6	26±0.8	16±1.0	-

KE= Kenya *P.thonningii* leaf extract; MA=Malawi *P. thonningii* leaf extract; (-) No zone of inhibition detected. Values are means ±SD from three replicate experiments.

µg/ml while aqueous Malawian *P. thonningii* was moderately active with LC_{50} of 121.4 µg/ml, methanol and chloroformic extracts of the Kenyan *P. thonningii* and Malawian *P. thonningii* were both moderately active with LC_{50} values of 109.6 and 403.3 µg/ml for methanol extracts and 121.4 and 476.3 µg/ml for chloroformic extracts, respectively. The chloroform + methanol extracts of the Kenyan *P. thonningii* was weakly toxic while Malawian *P. thonningii* was moderately active with LC_{50} of 991.3 and 476.3 µg/ml, respectively.

Observed differences in LC_{50} values in brine shrimp bioassay could be attributed to phytogeographical origins of the studied *P. thonningii* and also the reported carbohydrates, glycosides, flavonoids, tannins, saponins, balsams, volatile oil and terpenes in the leaves of the plant (Egharevba et al., 2010; Ighodaro et al., 2012; Maroyi, 2013). A study by Baratta et al. (1999) using pods of *P. thonningii* from Guinea showed that ethanol extract of the pods were toxic to brine shrimp with an LC_{50} of 26 µg/ml. Elsewhere, studies have shown that new compounds (2-phenoxychrome and C-methylflavonols) isolated from leaves of *P. thonningii* had virucidal activity (Ibewuike et al., 1996). These compounds could be responsible for the observed toxicity in brine shrimp bioassay.

All the extracts of *P. thonningii* from Kenya and Malawi showed antibacterial activity against the five test bacteria with the exception of chloroform extract from Malawi at the test concentration (250 µg/ml) suggesting that none of the reported bioactive compounds (Egharevba et al., 2010; Ighodaro et al., 2012; Maroyi, 2013) resides in the chloroform extract from Malawi. It is not uncommon phenomenon for certain extracts to show preferential activity against selected micro-organisms (Shail et al., 2008). Chloroform extracts of the Kenyan *P. thonningii* exhibited stronger activity to *E. coli* than gentamycin at the same test concentration (250 µg/ml). Aqueous extracts from Kenya and Malawi have shown to be more potent and efficacious than gentamycin against *B. cereus* and *S. Agalactae.* It has also been observed that higher zones of inhibition were recorded in Gram positive bacteria (*B. cereus, S. aureus* and *S. agalacitae*) and lower zones of inhibition were recorded in Gram negative bacteria (*E. coli* and *P. aeruginosa*). This is in agreement with earlier studies that established that gram positive bacteria are much more susceptible to drugs than gram negative bacteria (Cos et al., 2006). Ibewuike et al. (1997) observed that ethanolic leaf extract of *P. thonningii* had antibacterial activity against *S. aureus*, *E. coli*, *B. subtilis* and *P. aeruginosa.* Similarly, a concentration of 20 mg/ml of methanolic stem bark extract of *P. thonningii* showed antibacterial activity against *B. subtilis*, *Corynebacterium pyogenes*, *E. coli*, *Proteus vulgaris*, *Shigella dysenteriae* and *S. aureus* (Akinpelu and Obuotor, 2000). These reports are in line with the results obtained from the current study. It has also been shown that aqueous and organic extracts from Kenya and Malawi exhibited antimicrobial activity in agar diffusion method at the concentration tested (250 µg/ml). The study has also shown that aqueous and organic extracts of *P. thonningii* had activity in the brine lethality bioassay regardless of the phytogeographical origins. The differences that have been observed among the extraction methods of *P. thonningii* in recovery percentages and LC_{50} can be attributed to differences in two different geographical regions. However, it was established that there was no significant differences between the means of inhibition zones of aqueous Kenyan *P. thonningii* and aqueous Malawian *P. thonningii* on the five test bacteria ($p > 0.05$), no significant difference between the mean of inhibition zones of methanol extract of Kenyan *P. thonningii* and

methanol of Malawian *P. thonningii* on the five test bacteria ($p > 0.05$) and no significant difference between the means of inhibition zones of (Akinpelu and Obuotor, 2000) methanol/chloroform extract of Kenyan *P. thonningii* and methanol/chloroform extract of Malawian *P. thonningii* on the five test bacteria was observed. The *in vitro* findings are not always dependable, plants which are effective *in vitro* might not work when used *in vivo* while other extracts showing little or no effect *in vitro* might also be effective when evaluated in animals due to various factors that affect or favour the release of active principles in animal bodies (Gessler et al., 1995). Therefore, further detailed *in vitro* and *in vivo* evaluation of efficacy and safety of *P. thonningii* from both ecological zones should be carried out.

The fact that the aqueous extracts of *P. thonningii* leaves collected from both Kenya and Malawi exhibited higher growth inhibition activity against *B. cereus* than the positive control, gentamycin is interesting and lends support to the traditional use of this plant against bacterial infections, but *in vivo* tests are required to support this. After detailed *in vivo* antibacterial evaluation and thorough toxicological studies, this plant may find use as an antibacterial agent especially in rural communities where the conventional drugs are unaffordable or unavailable and the health facilities inaccessible.

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