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## Phytochemical screening and cytotoxicity of selected plants used as anthelmintics in Loitoktok Sub-County, Kenya

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### ABSTRACT

The practice of traditional medicine is as old as the human race, and plants are an important source of research and development of new drugs. Anthelmintic resistance in human and animal pathogenic helminthes has been spreading in prevalence and severity to a point where there is multi-drug resistance against the three major classes of anthelmintics. It has become a global phenomenon in gastrointestinal nematodes of farm animals, and hence the need for novel anthelmintic products. The objectives of this study were to carry out phytochemical screening and determine bioactivity of plants which are commonly used in the treatment and control of helminthosis in Loitoktok Sub-County of Kenya. The plant species (*Albizia anthelmintica*, *Myrsine africana*, *Embelia schimperi* and *Rapanea melanophloeos*) were selected based on their ethnopharmacological uses, as anthelmintics, by the traditional health practitioners. Phytochemicals were screened in aqueous and organic extracts using standard methods and cytotoxicity determined using the Brine shrimp lethality test. Phytochemicals detected in the extracts were, anthraquinones, flavonoids, glycosides, saponins, steroids, tannins and triterpenoids. Organic extracts were generally more cytotoxic than the aqueous extracts with median lethal dose (LC<sub>50</sub>) of 11 to 581 µg/ml and 149 to 1000 µg/ml respectively. It was concluded that some of the plants used as anthelmintic remedies in Loitoktok contain different types of phytochemicals which could be responsible for their cytotoxicity and anthelmintic properties. Further studies may be necessary to assess their potential as anthelmintics for possible drug development.

**Keywords:** Phytochemical screening, Cytotoxicity, Anthelmintic plants, Loitoktok.

### INTRODUCTION

Medicinal plants are the richest bio-resource of drugs of traditional system of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediaries and chemical entities for synthetic drugs<sup>[1]</sup>. Phytochemicals are non-nutritive plant secondary metabolites that have protective or disease preventive properties. Plants produce these chemicals to protect themselves but recent research demonstrates that many of these phytochemicals can protect humans and animals against diseases<sup>[2]</sup>.

Extraction methods used pharmaceutically involves the separation of bioactive portions of plant tissues from the inert components by using selective solvents. During extraction, solvents solubilize compounds with similar polarity resulting in relatively complex mixtures of metabolites such as alkaloids, glycosides, terpenoids, flavonoids and lignans<sup>[3]</sup>. These are in the forms of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such extracts have been popularly called galenicals, named after the famous Roman physician, Claudius Galenus of Peragon 129-200 A.D<sup>[4]</sup>.

The systematic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many laboratories. Scientific analysis of plant components follows a logical pathway. Plants are selected either randomly or by following leads from local healers in geographical areas where the plants are found<sup>[5]</sup>. Fresh or dried plant materials can be used for the extraction of phytochemicals. However, due to differences in the water contents at harvesting, plants are usually air dried to a constant weight before extraction. Plant materials can also be dried in the oven at about 40°C for 72 hours. Reduction of the particle size to increase the surface area of adsorption is also standard practice.

Successful determination of phytochemicals from plant materials is largely dependent on the type of solvent used in the extraction process. The factors affecting the choice of solvent are the quantity of phytochemicals to be extracted, rate of extraction, diversity of compounds to be extracted, ease of handling, toxicity of solvent in the bioassay system, and the potential health hazard of the extractants<sup>[6]</sup>.

Bioassays offer special advantage in the standardization and quality of heterogeneous herbal products. Physical analytical methods, such as chromatography, are by themselves not very useful for this purpose

as they are usually insensitive to the chemical complexities found in crude botanical extracts<sup>[7]</sup>. Most often a desired biological response is due to not one but a mixture of bioactive plant components and the relative proportion of single components can vary from batch to batch while the bioactivity still remains within tolerable levels. Thus, physical or chemical analysis of a single component in such a mixture may not be adequate and for practical application in health care, today's work in medicinal plant chemistry should include bioassays<sup>[7]</sup>.

The brine shrimp lethality test (BST) is a rapid general bioassay described by Meyer<sup>[8]</sup> and refined by McLaughlin<sup>[9]</sup>. This *in vivo* lethality test in a simple zoologic organism can be used as a convenient tool for screening and fractionation in the discovery and monitoring of natural products<sup>[7]</sup>. The eggs of brine shrimp, *Artemiasalina* (Leach), are readily available in pet shops at low cost and remain viable for years in a dry state. Upon being placed in sea water, the eggs hatch within 48 hours to provide large numbers of larvae (nauplii) for experimental use.

In this study, the crude aqueous and organic extracts of plants traditionally used as anthelmintics in Loitoktok Sub County were qualitatively screened for phytochemicals. The cytotoxicity of the crude aqueous and organic plant extracts were also determined using the Brine shrimp lethality test.

## MATERIALS AND METHODS

### Collection and preparation of the Plant materials

The plant samples used in the study were collected from Loitoktok Sub-County with the aid of the local traditional health practitioners and identified by taxonomists at the University of Nairobi herbarium, where voucher specimens were deposited. The plant species (*Albizia anthelmintica*, *Myrsine africana*, *Embelia schimperi* and *Rapanea melanophloeos*) were chosen based on their ethnopharmacological uses, as anthelmintics, by the traditional health practitioners. The information gathered included parts of the plant used, methods of preparation of anthelmintic remedies and routes of administration. The chopped stem bark of *Albizia anthelmintica*, and the fruits of the other species were air dried under shade and separately ground into fine powder using a laboratory mill.

### Preparation of crude plant extracts

The aqueous extract of each of the plants was prepared by soaking 100 grams of powdered plant material in 500 ml of distilled water, with regular stirring and shaking at least 3 times daily for 3 days. The material was then filtered through muslin gauze and the filtrate frozen for 24 hours before lyophilization. The lyophilized dry powder was then weighed and stored in airtight bottles at -20 °C until used. The organic solvent (hexane, ethyl acetate and ethanol) extraction was done on a fresh sample for each plant by soxhlet apparatus for 6 hours. The extract was then concentrated in vacuum by use of a rotavapor and further dried in an oven at 40 °C. The dry solid extracts were then weighed and stored at -20 °C in airtight bottle containers until utilized. The (methanol: chloroform 1:1) extraction was done by cold maceration for 72 hours, filtered and then concentrated by rotavapor followed by oven drying at 40 °C.

### Qualitative phytochemical screening

One gram of each of the dried extracts were dissolved in 100 ml of their mother solvents to obtain stock concentrations of 1% w/v. The reconstituted extracts thus obtained were subjected to phytochemical screening following standard methods<sup>[10,11]</sup>.

### Preparation of the plant extracts for cytotoxicity screening

Stock solutions of aqueous extracts (10, 000 µg/ml) were made in distilled deionized water. The organic extracts were dissolved in a little dimethyl sulphoxide (DMSO) and further diluted with distilled water to make up stock solutions of 10, 000 µg/ml. The DMSO concentration in the final test solution was always less than 1%, to avoid solvent toxicity. Test extracts at appropriate amounts (5, 50, and 500 µl for 10 µg/ml, 100 µg/ml and 1000 µg/ml, respectively) were

transferred into 10 ml vials and the volume topped up to 5ml using brine with 5 replicates at each dose level.

### Culture and harvesting of *Artemiasalina*

*Artemiasalina* cysts, batch number DE RP 33801, were purchased from JBL GmbH & Co.KG (Neuhofen, Germany) and the product was labeled as JBL ArtemioPur Brand. The cysts had been harvested from Great Salt Lake, Utah, USA and were zoo-geographically identified as *Artemia salina*. The *Artemia salina* eggs were incubated to hatch in a rectangular dish (14 cm x 9 cm x 5 cm) filled with 225 ml of a 3.3% w/v solution of artificial sea water. A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs (1.11 grams) and yeast (0.08827 grams) were sprinkled into the larger compartment which was darkened. The smaller compartment was illuminated by a tungsten filament light. After 48 hours, hatched *A. salina* larvae were ready for the tests. The phototropic nauplii were collected by pipette from the lighter side, having migrated through the pores on the divider leaving the shells on the darker compartment.

### Bioassay of *Artemia salina*

For cytotoxicity tests, ten *A. Salina* nauplii were transferred into each sample vial using 230 mm disposable glass Pasteur pipettes and filtered brine solution was added to top up to 5 ml. The nauplii were counted macroscopically in the stem of the pipette against a lighted background. A drop of dry yeast suspension (3 mg in 5 ml artificial sea water) was added as food to each vial. All the vials were maintained under illumination. The surviving nauplii were counted with the aid of a 3x magnifying glass, after 24 hours, and the percentage of deaths at the three dose levels and the control determined. In cases where control deaths occurred, the data was corrected using the formula by Abbott<sup>[12]</sup> as follows:

Percent (%) deaths = [(Test – control)/control] x 100. The surviving nauplii were then killed by the addition of 100 µl of 5% (v/v) phenol to each vial.

### Determination of LC<sub>50</sub>

The lethal concentration fifty (LC<sub>50</sub>), at 95% confidence interval and slope were determined from the 24 hour counts using the probit analysis method as described by Finney<sup>[13]</sup>. The cytotoxicity was classified weak when the LC<sub>50</sub> is between 500 and 1000 µg/ml, moderate when it was between 100 and 500 µg/ml and strong when it was between 0 and 100 µg/ml<sup>[8]</sup>.

## RESULTS

### Phytochemical composition

The yields and phytochemicals detected from the aqueous and organic extracts of three plants, commonly used in anthelmintic remedies in Loitoktok District are presented in Table 1. The yields of the aqueous extracts ranged from 8.1 to 17% w/w, while those of the organic solvents varied from 0.5 to 32.6% w/w. The phytochemicals found to be present were alkaloids, anthraquinones, flavonoids, glycosides, saponins, steroids, tannins and triterpenoids.

### Cytotoxic effects of the plant extracts

The LC<sub>50</sub> values for the aqueous extracts ranged from 149 to 616 µg/ml while those for the organic solvents ranged from 11 to 581 µg/ml (Tables 2 to 4).

## DISCUSSION

The phytochemical screening demonstrated the presence of constituents which are known to exhibit medicinal and physiological activity<sup>[14]</sup>. For example, phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids have anti-inflammatory effects<sup>[15-17]</sup>. Glycosides, flavonoids, tannins and alkaloids have hypoglycemic activities<sup>[18, 19]</sup>. Saponins have been reported to possess hypocholesterolemic and anti-diabetic properties<sup>[20]</sup>. The steroids and triterpenoids have been shown to have analgesic properties<sup>[21, 22]</sup>. Steroids and Saponins are also known to have central

nervous system activities [23]. Terpenes and tannins, especially condensed tannins, have been shown to have potent anthelmintic activity [24, 25].

**Table 1:** Phytochemicals in plants used in anthelmintic remedies in Loitoktok District

Phytochemicals screened	<i>Albizia anthelmintica</i> (bark)				<i>Embelia schimperi</i> (fruits)				<i>Myrsine africana</i> (fruits)			
	Aqua	Eth	Eth	Hex	Aqua	Eth	Eth	Hex	Aqua	Eth	Eth	Hex
Alkaloids	+	+	-	-	-	-	-	-	+	+	+	-
Anthraquinones	-	-	-	-	-	-	-	-	+	-	+	+
Flavone aglycone	-	-	-	-	+	-	-	-	+	-	+	+
Flavonoids	+	+	+	-	-	-	-	+	+	-	-	+
Glycosides	+	+	+	+	+	-	+	+	+	-	+	+
Reducing sugars	-	-	-	-	+	+	+	+	-	+	+	-
Saponins	+	+	+	-	-	-	-	-	-	-	-	-
Steroids	+	+	+	+	+	-	+	+	+	+	+	+
Tannins	-	-	-	-	-	+	-	-	+	+	+	-
Triterpenoids	+	+	+	+	+	+	+	+	+	+	+	+
Percentage yield	8.1	9.5	3.4	0.5	17	32.6	20.6	7.8	10.4	31.8	18.6	14.3

**Key:** Aqua = Water extract; Eth = Ethanol extract; Ethy = Ethyl acetate extract; Hex = Hexane extract; + = Presence; - = Absence.

**Table 2:** Brine shrimp lethality of crude aqueous plant extracts

Plant species	Plant part	Percent deaths at 24 hrs					Limits 95% CI (µg/ml)	Slope
		10 (µg/ml)	100 (µg/ml)	1000 (µg/ml)	LC <sub>50</sub> (µg/ml)			
<i>Albizia anthelmintica</i> Brongn	Stem bark	2	10	94	259	107-658	0.3729	
<i>Embelia schimperi</i> L.	Fruits	0	30	100	149	60-498	0.5686	
<i>Myrsine Africana</i> L.	Fruits	10	20	60	616	ND	0.7391	
<i>Rapanea melanophloeos</i> L.	Fruits	0	8	20	NA	ND	2.2706	

**Key:** ND = Not detectable; NA = Not active; CI = confidence interval; µg/ml = micrograms per millilitre; LC<sub>50</sub>= Median lethal dose

**Table 3:** Brine shrimp lethality of crude organic (Ethanol) plant extracts

Plant species	Plant part	Percent deaths at 24 hrs					Limits 95% CI (µg/ml)	Slope
		10 (µg/ml)	100 (µg/ml)	1000 (µg/ml)	LC <sub>50</sub> µg/ml			
<i>Albizia anthelmintica</i> Brongn	Stem bark	3	82	96	23	2-74	0.4909	
<i>Embelia schimperi</i> L.	Fruits	50	68	100	14	0-54	0.6294	
<i>Myrsine africana</i> L.	Fruits	6	46	76	178	51-947	0.4071	
<i>Rapanea melanophloeos</i> L.	Fruits	0	20	60	581	ND	0.5362	

**Key:** ND = Not detectable; NA = Not active; CI = confidence interval; µg/ml = micrograms per millilitre; LC<sub>50</sub>= Median lethal concentration

**Table 4:** Brine shrimp lethality of organic (CHCL3: MeOH, 1:1) crude plant extracts

Plant species	Plant part	Percent deaths at 24 hrs					Limits 95% CI (µg/ml)	Slope
		10 µg/ml	100 µg/ml	1000 µg/ml	LC <sub>50</sub> µg/ml			
<i>Albizia anthelmintica</i> Brongn	Stem bark	50	80	100	11	0-39	0.6307	
<i>Embelia schimperi</i> L.	Fruits	30	60	100	36	6-112	0.4036	
<i>Myrsine africana</i> L.	Fruits	22	64	100	42	11-118	0.3469	
<i>Rapanea melanophloeos</i> L.	Fruits	40	52	88	36	0-142	0.7919	

**Key:** ND = Not detectable; NA = Not active; CI = confidence interval; µg/ml = micrograms per millilitre; LC<sub>50</sub>= Median lethal dose

Tannins are polyphenolic compounds that have been reported to have anthelmintic properties [26-31]. The mechanisms by which tannins produce anthelmintic effect remain unclear. Actions similar to those of synthetic phenolic anthelmintics such as niclosamide, oxyclozanide and nitroxylin, which interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation, have been reported [32-35]. Other reports have suggested tannins' ability to bind free proteins in the gastrointestinal tract of host animals [28, 36, 37] or to glycoproteins on the cuticle of parasites leading to their death [38]. A form of terpene known as palasonin (terpene anhydride) was reported to produce anthelmintic activity by inhibiting glucose uptake and hence depleting the glycogen content of parasites [39]. Similarly, gentistein (a form of flavone aglycone) was reported to produce

anthelmintic effect which was linked to its activity on nitric oxide synthetase [40]. The anthelmintic effects of flavonoids have also been reported [41, 42]. Other studies have indicated that alkaloids and flavonoids also have anthelmintic properties [34, 35, 43].

All the five myrsinaceae (*Myrsine africana*, *Maesa lanceolata*, *Rapanea melanophloeos*, *Embelia schimperi* and *Embelia keniensis*) in Kenya are widely used across ethnic groups as anthelmintics and have been found to be rich in benzoquinones [44]. The bioactivity of isolated individual phytochemicals may not be in doubt; however, the better therapeutic effect of crude extracts may result from a combination of active principles in each plant [45, 46]. Lactones such as santonin have been shown to have a strong anthelmintic activity

against *Ascaris* and other nematode species of livestock and humans [47]. Alkaloids have also exhibited strong nematocidal activity against *Strongyloides ratti* and *Strongyloides venezuelensis*, two rat nematodes used as models for human nematodes [48]. The nematocidal activity of tannins has been reported as early as the 1960s [49], and more recently evidence on the anthelmintic properties of condensed tannins has been supported by a series of *in vitro* [28, 50-52] and *in vivo* studies [27, 53-55].

All the plant extracts demonstrated a dose-dependent bioactivity. In bioassay studies, the LC<sub>50</sub> values less than 1000 µg/ml (ppm) are considered significant [8, 56]. In this study, crude aqueous and organic plant extracts were evaluated for their brine shrimp lethality (cytotoxicity/bioactivity). Four plants, belonging to two families, frequently used in anthelmintic remedies in Loitokitok Sub County were evaluated. The aqueous extracts of three of them (*Albizia anthelmintica*, *Embelia schimperi* and *Myrsine africana*) had significant activity, suggesting the presence of bioactive compounds and further supporting the results of the phytochemical screening. The bioactivity of all the organic extracts was significant with most of them categorized as strongly bioactive. The exceptions were ethanolic extracts of *Myrsine africana* and *Rapanea melanophloeos* which were moderate. These results indicate that majority of the bioactive components in these plants are non-polar and merit further investigation. The current observation is in agreement with the findings of others who have shown organic extracts to be more toxic than aqueous extracts of the same plant species, in brine shrimp bioassays [57, 58].

The bioactivity of the extracts of the plants in this study is an indication of the presence of potent compounds and may explain some of their traditional uses. These could be of particular interest in relation to their unexplored efficacy and can be potential sources of chemically interesting and biologically important drug candidates.

Brine shrimp lethality test is a simple and cheap benchtop bioassay which detects a broad range of biological activities and a diversity of chemical structures. It is very useful in screening extracts, for bioactivity, in the drug discovery process [9]. Currently there is growing pressure from human rights' advocates to limit the use of higher animals for toxicological studies and since the brine shrimp are crustacean, and sensitive to a variety of substances, the BST is rapidly gaining value as a quick and simple test for predicting the bioactivity/toxicity of plant extracts and guiding phytochemical fractionation in the search for novel compounds useful in health care [9, 59, 60].

## CONCLUSION

Medicinal plants used in anthelmintic remedies in Loitokitok Sub County have bioactivity against brine shrimp larvae and that organic extracts are generally more potent than the aqueous extracts. Medicinal plants used in anthelmintic remedies in Loitokitok Sub County are rich in phytochemicals that could be responsible for their bioactivity and merit further analysis to isolate the actual active components and determine their mode of action with a view of discovering novel products for use in health care.

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