ORIGINAL ARTICLE

In vitro anthelmintic activity of three medicinal plants against *Haemonchus contortus*

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The development of anthelmintic resistance and the high cost of conventional anthelmintic drugs led to the evaluation of medicinal plants as an alternative source of anthelmintics. In the current study, *in-vitro* experiments were conducted to determine the possible anthelmintic effects of crude aqueous and hydroalcoholic extracts of the leaves of *Chenopodium ambrosioides, Lawsonia inermis* and seeds of *Jatropha curcas,* on eggs and adult *Haemonchus contortus.* Both extracts of *C. ambrosioides* and *J. curcas* inhibited the hatching of eggs at a concentration less than or equal to 2 mg/ml, while the effect of *L. inermis* was not dose-dependent and did not inhibit the hatching of eggs of *H. contortus,* significantly, at all tested concentrations. Based on their ED_{50}, the two most potent extracts using egg hatch assay were the hydroalcoholic extract of *C. ambrosioides* (0.09 mg/ml) and the aqueous extract of *J. curcas* (0.1 mg/ml) in a decreasing order of potency. With regard to the effect of extracts on the survival of adult parasites, extracts from *C. ambrosioides* have shown a moderate effect, while *J. curcas* and *L. inermis* have shown no statistically significant effect on the survival of adult parasites at the concentrations tested, and the few mortality cases recorded were not dose-dependent ($P < 0.05$). The overall findings of the present study have shown that *C. ambrosioides* and *J. curcas* contain possible anthelmintic compounds and further evaluation of these plants should be carried out.

Key words: Anthelmintic activity, *Chenopodium ambrosioides, Haemonchus contortus, Jatropha curcas, Lawsonia inermis*

INTRODUCTION

Helminthosis plays a crucial role in the small ruminant production leading to enormous economic losses particularly in areas where extensive grazing is practiced.[1] *Haemonchus contortus* is a highly pathogenic helminth parasite of small ruminants, which is capable of causing acute disease and high mortality in all age groups, and is one of the top 10 constraints of sheep and goat production in East Africa.[2]

Development of resistance to most of the commercially available anthelmintics became a severe problem worldwide.[3] Moreover, these drugs are unaffordable, inaccessible or inadequately available to the resource-poor farmers of the developing countries.[4] These factors paved the way for herbal remedies as alternative anthelmintics.[5] Evaluation of the activities of medicinal plants claimed for possessing the anthelmintic property is getting attention these days.[6-9] Screening and proper evaluation of the claimed medicinal plants could offer possible alternatives that may be both sustainable and environmentally acceptable.[7] In the current study, we have attempted to investigate three medicinal plants for their claimed anthelmintic activity.

Lawsonia inermis L. (Lythraceae) locally called "Henna" is an evergreen shrub or tree 2-7 m tall. It grows in alluvial soils along rivers or near water holes, from sea level to 1100 m above sea level in Ethiopia and throughout the old world tropics.[10] The leaves boiled together with *Allium sativum* L leaves are drenched and fed to sheep and goats for treatment of worm infection.[11] A similar preparation is also used for treatment of febrile conditions caused by infectious agents in Nigeria. The powdered leaves mixed with tea, produce a red dye used for coloring hair, beards and nails.[10] The filtrate of a handful of leaves soaked in a liter of water for 12-24 hours is used for treatment of trypanosomosis in camels. The paste formed from the pulverized leaves is also used for treatment of wounds.[12] In India its bark is reported to be useful in treatment of jaundice, enlargement of spleen and reported to have anti-inflammatory, antipyretic and analgesic effects.[13] It has also been proved to have a hepatoprotective effect. The bark extract was reported to have a broad-spectrum antifungal and antimicrobial activity.[14,15] The phytochemical investigations have shown the presence of β-sitosterolglucosides, flavonoids, quinonoids, naphthalene derivatives, luteolin, betulin, lupeol, garlic acid, coumarins, xanthones and phenolic glycosides.[16]

Jatropha curcas, L. (Euphorbiaceae) locally called "Sudan gullo" and "Ayderke" is a shrub or small tree 4.5 to 8 m high. It has a smooth bark and milky latex. It is cultivated as an ornamental plant and live fencing at an altitude

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of 450-1300 m asl. It is commonly found in southern part of Ethiopia. It was known to be native to tropical America later introduced to old world tropics where it is now widely cultivated and naturalized.[10] The roots, stems, leaves seeds and fruits of the plant have been widely used in traditional folk medicine in many parts of West Africa. The seeds have been used as purgative, anthelmintic and abortifacient, for treating ascites, gout and skin diseases.[10] Its seeds have also been reported to be effective against *Strongyloides papillosus* infection in goats.[12] The seeds are good sources of oil, which can be used as a substitute for diesel. They are also used in the manufacture of soap and cosmetics in various tropical countries.[17] In Ethiopia, a study by Dessisa[18] revealed that the fruit is commonly used as a purgative and anthelmintic in human patients, by traditional healers. The seed of some provenances of *J. curcas* is toxic to rats, mice and ruminants. Several cases of *J. curcas* poisoning in humans after accidental consumption of the seeds have been reported. Phorbolesters have been identified as the main toxic agent of *J. curcas*.[17] Extraction using 92% alcohol was reported to remove toxic and heat-stable factors and the residue was found to be non-toxic to rats.[19] The defatted product was found to contain 50 to 62% of protein. A high proportion of the trypsin inhibitor, lectin activities, antimetabolic metal-chelating agent, heat-stable factor and phytic acid, was isolated from the seed of *J. curcas*.[17]

Chenopodium ambrosioides L. (Chenopodiaceae) locally called "Amedmado" is a strongly aromatic herb, about 1.2 m high, sometimes with a woody base. It is a common weed of cultivated areas, often in seasonally wet sites, found at an altitude of 950-2500 m above sea level. The origin is supposed to be from America, but now it has spread throughout the tropics and subtropics.[20] Oil of *C. ambrosioides* has been used for many years to treat parasite infections in humans and animals in different parts of the world.[7] Its use has been discontinued after more effective and less toxic anthelmintics have become available. Recently, Ketzis *et al.*[21] reported ineffectiveness of this plant extract against *H. contortus* infection in goats. Ascaridole, a monoterpene essential oil is reported to be the principal active component found in the extract of *C. ambrosioides*, responsible for the anthelmintic activity. The other components include isoascaridole, p-cymene, limonene and α-terpinene. The level of the different compounds varies depending on the part of the plant collected and age of the plant.[22] The objectives of this study are to conduct the qualitative phytochemical screening of medicinal plants and to investigate the *in vitro* anthelmintic activity of plant extracts on the eggs and adult *H. contortus*.

MATERIALS AND METHODS

Collection of Plant Materials and Phytochemical Screening

The seeds of the plants were collected from their natural habitat. Sample of the plant species collected were identified by a plant taxonomist and specimens of *L. inermis, J. curcas* and *C. ambrosioides* were deposited with herbarium voucher numbers MG-012/05, MG-013/05 and MG-031/05 respectively at the Herbarium of Addis Ababa University, Biology Department. The garbled plants were air dried at room temperature, ground and kept in an amber colored bottle until they were processed. A list of the plant species used in the study, parts used, areas of collection and yield are shown in Table 1.

Extraction and phytochemical screening was conducted at the Drug Research Department of the Ethiopian Health and Nutrition Research Institute (EHNRI). Aqueous extraction was performed by soaking a weighed amount of the dry powder (50-100 g) in distilled water and shaking it for three hours with an electric shaker. The suspension was filtered through muslin gauze and the filtrate was kept in a deep freezer for 24 hours, which was then lyophilized. The lyophilized dry powder was collected in stoppered sample vials, weighed and kept in a desiccator, to avoid absorption of water, until they were used. Hydroalcoholic extraction was conducted by percolating 200-300 g of the dried and powdered plant material using 80% methanol. It was then filtered through Whatman filter paper No.1. The solvent was evaporated using a Rotar vapour and the extract was kept in stoppered sample vials at 4°C until they were used. Preliminary qualitative screenings for major secondary metabolites of the medicinal plants were conducted according to Debella.[23] The plant materials were screened for the presence of polyphenols, cyanogenic glycosides, saponins, phytosteroides and withanoids, phenolic glycosides, flavonoids, tannins, alkaloids and antraquinone glycosides.

Table 1: Species, herbarium voucher number, areas of collection and yield of extraction for the medicinal plants

Species (family)	Herbarium voucher no.	Area of collection	Parts used	Extract type	% yield (w/w)
L. inermis (Lythraceae)	MG-012/05	Awassa	Leaves	Aqueous	12.45
				Hydro-alcoholic	30.53
J. curcas (Euphorbiaceae)	MG-013/05	Gojeb	Seeds	Aqueous	14.77
				Hydro-alcoholic	4.48
C. ambrosioides (Chenopodiaceae)	MG-031/05	Addis Ababa	Leaves	Aqueous	12.67
				Hydro-alcoholic	4.38

Parasites

Adult female parasites of *H. contortus* were collected from the abomasums of infected sheep obtained from the Addis Ababa Abattoir. The worms were washed and crushed to liberate eggs. The eggs were then cultured in a glass jar filled with autoclaved sheep faeces for eight days at room temperature. At the end of the eighth day, the infective larvae were harvested by rinsing the side of the culture jar with a drop of water. About 3000 larvae were inoculated into two worm-free sheep that were kept indoors in a separate house, in the animal facilities of the AL-IPB, throughout the study period. These sheep served as *H. contortus* egg donors for the egg hatch assay trial.

Egg Hatch Assay

Collection of eggs from previously mentioned donor sheep and egg hatch assay (EHA) were conducted according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines.[24] Aqueous and hydroalcoholic extracts of the plants were used as the test treatment. Albendazole (99.8% pure standard reference) was used as the positive control, while untreated eggs in water were used as the negative control. About 200 eggs in 1.5 ml of water were placed in each test tube. Aqueous and hydroalcoholic extracts of plants at concentrations of 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml in a total volume of 2 ml was prepared together with water, containing eggs. Albendazole was dissolved in Dimethyl sulfoxide (DMSO) and diluted at the concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.0156 μg/ml. The test tubes were then covered and kept in an incubator at 27°C for 48 hours. The experiment was replicated six times for each concentration. Hatched larvae (dead or alive) and unhatched eggs were then counted under a dissecting microscope with ×40 magnification.

Effect of Plant Extracts on Adult Worms

Adult *H. contortus* were collected from the abomasum of sheep slaughtered at the Addis Ababa Abattoir. Immediately after slaughter, the abomasums were collected and transported to the laboratory. The parasites were then collected, washed and kept in phosphate buffered saline (PBS). The experiment was conducted according to Eguale *et al*.[9] Ten actively moving worms were placed in Petri dishes containing 8.0, 4.0, 2.0, 1.0, 0.5, and 0.25 mg/ml of aqueous and hydroalcoholic extracts of plants in PBS and PBS alone for the control group, in a total volume of 4 ml. Albendazole dissolved in DMSO and diluted in PBS at concentrations of 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml was used as the positive control. Three replications per each treatment concentration were employed. After 24 hours, the plant extracts and albendazole were washed away and the parasites suspended in PBS for 30 minutes for possible recovery of parasite motility. The number of motile (alive) and immotile (dead) worms were counted under the dissecting microscope, and recorded for each concentration. Death of worms was ascertained by the absence of motility for an observation period of 5-6 seconds. A mortality index was calculated as the number of dead worms divided by the total number of worms per Petri dish.

Statistical Analysis

Data from EHA were transformed by probit transformation against the logarithm of extract concentration. The extract concentration required to inhibit 50% (ED_{50}) egg hatching was calculated using probit analysis. Comparison of mean percentages of egg hatch inhibition and mortality of adult parasites at different concentrations with the control was performed by one-way ANOVA.

RESULTS

Extraction and Screening of Plant Materials

Variation in yield among different plant species in both aqueous and hydroalcoholic extracts was observed [Table 1]. The lowest yield was recorded for the hydroalcoholic extract of the seeds of *J. curcas* (4.48%) and the highest yield was for the hydroalcoholic extract of the leaves of *L. inermis* (30.53%).

The major secondary metabolites detected in all plants were polyphenols, whereas, glycosides (oligosaccharids) were detected in *L. inermis* and *C. ambrisoides*. Flavonoids, phytosteroides and withanoids were detected only in *L. inermis*, and saponin only in *C. ambrisoides* [Table 2].

Egg Hatch Assay

Both aqueous and hydroalcoholic extracts of *J. curcas* and *C. ambrosioides* induced significant egg hatching inhibition in a dose-dependent manner. Both extracts of *J. curcas* induced 100% inhibition at 2 mg/ml. The aqueous extract of *C. ambrosioides* required a maximum of 1 mg/ml, whereas, the hydroalcoholic extract required a maximum

Table 2: Secondary metabolites in parts of investigated plants

Plant speciest	*L. inermis*	*J. curcas*	*C. ambrisoides*
Polyphenols	+	+	+
Cyanogenic glycosides	–	–	–
Saponins	–	–	+
Phytosteroides and withanoides	+	–	–
Phenolic glycosides	–	–	–
Glycosides (Oligosaccharids)	+	–	+
Flavonoids	+	–	–
Tannins	–	–	–
Alkaloides	–	–	–
Antraquinone glycosides	–	–	–

+ = present; – = absent

concentration of 0.5 mg/ml, to induce 100% egg hatch inhibition. On the other hand, both extracts of *L. inermis* did not induce significant inhibition at all concentrations tested [Figure 1].

The effective doses required to induce 50% inhibition (ED_{50}) of egg hatching, calculated by probit analysis, are shown in Table 3. Although there was a difference in ED_{50}, between the extract types of the same plants, the difference was not statistically significant ($P = 0.05$). Based on ED_{50}, the most potent extracts were the hydroalcoholic extract of *C. ambrosioides* (0.09 mg/ml), followed by aqueous extracts of *J. curcas* (0.1 mg/ml).

In vitro Effects on Adult Parasites

After 24 hours of exposure of adult *H. contortus* to different concentrations of plant extracts, significant and dose-dependent reduction in motility/mortality was observed only for aqueous extracts of *C. ambrosioides* ($P < 0.05$). Both aqueous and hydroalcoholic extracts of *L. inermis* and *J. curcas* produced few mortality cases, which were not statistically significant ($P > 0.05$) compared to the death of worms recorded in the control group. Albendazole, on the other hand killed the parasites in a dose-dependent manner and all the worms were dead at a concentration of 0.5 mg/ ml within 24 hours [Figure 2].

Table 3: *In vitro* anthelmintic activity of tested extracts on *H. contortus* eggs after 48 hours

Tested material	Extract	ED_{50}(LCL-UCL)(mg/ml)
*Albendazole	-	0.04 (0.026-.051)
L. inermis	Aqueous	11.74 (3.41-51.65)
	Hydro-alcoholic	13.8(3.79-75.53)
J. curcas	Aqueous	0.1 (0.02-0.26)
	Hydro-alcoholic	0.23 (0.08-0.65)
C. ambrosioides	Aqueous	0.15(0.09-0.22)
	Hydro-alcoholic	0.09 (0.08-0.1)

Albendazole is a standard reference; *unit is in µg/ml; LCL- lower confidence limit; UCL-upper confidence limit

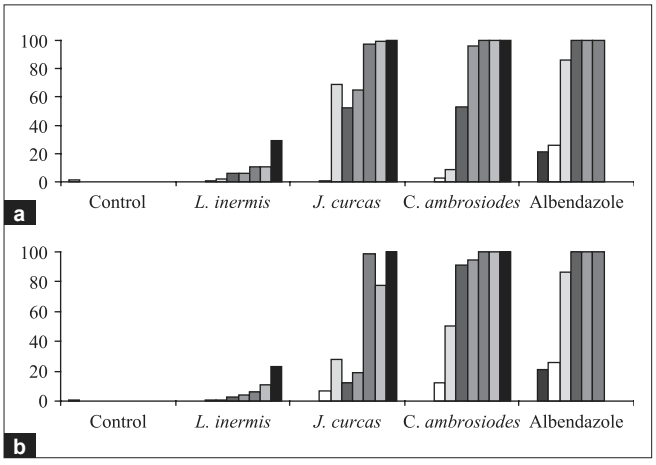

Figure 1: Mean percentage inhibition of egg hatching after 48 hours exposure of eggs of *H. contortus* to six increasing concentrations of Albendazole (0.0156, 0.03125, 0.0625, 0.125, 0.25, and 0.5 µg/ml,) and seven increasing concentrations of plant extracts (0.03125, 0.0625, 0.125, 0.25, 0.5, 1, and 2mg/ml), a) Aqueous extract; b) Hydro-alcoholic extract

DISCUSSION

Plant materials evaluated in the current study had been identified from various sources to serve as anthelmintic agents by traditional healers or farmers in different parts of Africa. Except for *C. ambrosioides*, our literature survey indicated no adequate prior scientific evaluation conducted for *J. curcas* and *L. inermis*. There were only putative reports of the traditional use of these plants for deworming purposes.

The presence of flavonoids in *L. inermis* is in agreement with the earlier works.[16] Dasgupta *et al.*[16] reported phenolic glycosides in *L. inermis*, however, it was not found in the current study. It might have been decomposed before extraction, due to the prolonged time after collection or improper collection. The dry leaves with unknown date of collection were purchased from the market. The lower egg hatch inhibition effect of *L. inermis*, in spite of reports of its traditional uses for anthelmintic purposes,[11,14] and detection of a higher number of secondary metabolites could be attributed to the difference in the method of preparation of the plant material. The other reasons for the lack of efficacy in the current study might be due to

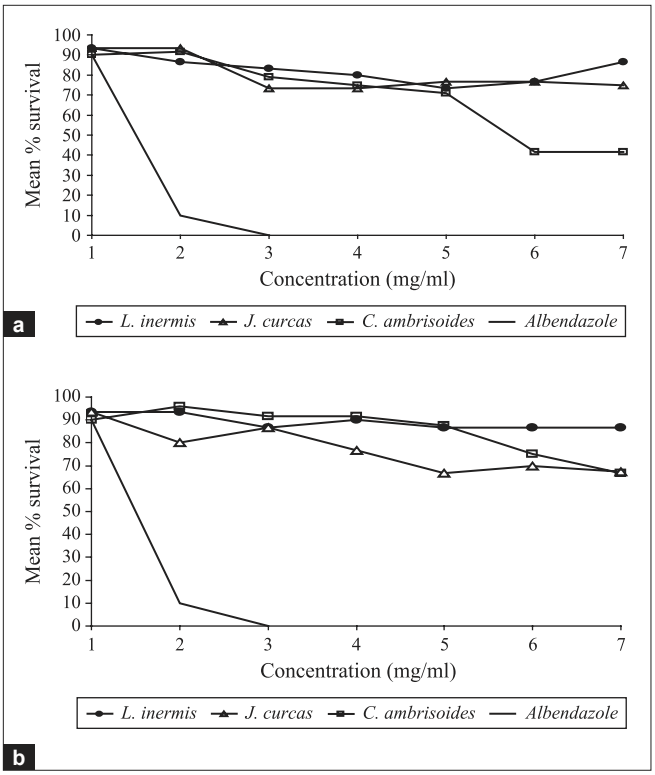

Figure 2: Mean percentage survival of adult *H. contortus* after 24 hours of exposure to different plant extracts and albendazole: (a) aqueous and (b) hydro-alcoholic extracts

the difference in localities and age of the plant, species of parasite tested[25] and/or the total absence of the real efficacy against *H. contortus*. The significant and dose-dependent egg hatching inhibition of the extracts of *J.* curcas is in agreement with the traditional uses of this plant reported previously.[11,18]

The oil of *C. ambrosioides* had been used for many years to treat parasite infections in humans and animals, in different parts of the world, before the emergence of more effective and less toxic modern anthelmintics.[7] A monoterpene (ascaridole) is believed to be the active principle in this plant.[21] Dry powder resulted in a 33- 36% reduction in mixed nematode parasite infections in sheep.[26] Short-term administration of oil or freshly ground plant material of *C. ambroisiodes* was ineffective in reducing adult *H. contortus* populations in goats, although oil of *C. ambroisiodes* at concentration of 3.3 µl/ml induced 100% egg hatch inhibition.[21] This finding was in agreement with the current finding, in which both aqueous and hydroalcoholic extracts of *C. ambrosioides* produced complete egg hatch inhibition at concentrations lower than 1 mg/ml. Long-term treatment of animals in a given farm may reduce hatchability of eggs excreted with faeces resulting in both reduced re- infection and lighter worm loads by decreasing pasture contamination.

Except for the aqueous extract of *C. ambriosoides*, none of them were effective against the adult parasite. The findings from the current work show that plant extracts effective against one developmental stage of a parasite may not be effective against the other.

In general, *C. ambrosioides* have revealed promising *in vitro* anthelmintic activity against eggs and adult *H. contortus*. Extracts from *J. curcas* and *L. inermis* have shown poor activity on survival of adult parasites at concentrations tested; however, both extracts of *J. curcas* have induced good egg hatching inhibition. The *in vitro* methods provide a means to rapidly screen for potential anthelmintic activities. However, due to the considerable variation in conditions encountered, *in vivo* like, metabolic biotransformation, interaction with feed materials and absorption, the results obtained by the *in vitro* method could not be extrapolated for *in vivo* activity. Therefore, the results should be ascertained by *in vivo* evaluation.

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