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Research Article

In vitro Anthelmintic Activity of Ethanolic Extracts of *Camellia sinensis* L. and *Albizia lebbeck* L. against *Haemonchus contortus*

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Authors' Contributions
SZ performed experimental work. AH designed and supervised the research. AQ reviewed the literature. MM complied the data and helped in manuscript writing. ML provided lab facilities and assisted in the experimental work.

Keywords
Camellia sinensis, *Albizia lebbeck*, *levamisole*, *Haemonchus contortus*, Anthelmintic activity, Adult Motility Assay.

Abstract | Helminths are familiar as a major problem to livestock production. Helminths cause multiple infections in animals of significant economic importance. *Haemonchus contortus,* (helminths worm), is highly infectious parasite of small ruminants, and is a major cause of diseases and significant mortality in various cattle, sheep, deer, buffalo and goat, etc. Various synthetic anthelmintics have been used, on wide scale, to control the helminths infections.But the parasite worms have developed multiple resistances against the synthetic anthelmintics. Hence, various biological controls, vaccines and local medicinal plants are being explored to control the parasitic worms. The aim of this study was to evaluate the anthelmintic potential of two local plants, *Albizia lebbeck* L. and *Camellia sinensis* L against *H. contortus* using Adult Motility Assay (AMA). Crude Ethanolic Extracts (CEE)of dried leaves of *A. lebbeck* L. and whole plant of *C. sinensis* L. were prepared by cold maceration method. The anthelmintic activity was analyzed by adult motility assay (AMA). Levamisole was used as positive control to compare the efficacy of CEE of both plants. Adult worms were treated with different concentrations i.e., 2, 4, 6 and 8 mg/ml of ethanolic extract and motility of the worms was examined after every two to eight hours. Both *A. lebbeck* and *C. sinensis* exhibited 88% and 95% mortality at 6 & 8 mg/ml after 8 hrs of treatment. In conclusion, the ethanolic extracts of *A. lebbeck* L. and *C. sinensis* L. showed *in vitro* anthelmintic activity against *H. contortus* at concentrations tested.

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Introduction

Livestock is an important pillar of Pakistan's economy. It increases commercial status of the rural poor. It is a main source of farming and supplement economy by generating job opportunities and enhancing house hold income in villages. Buffalos, goats and cows are being raised for variety of items such as meat, leather and milk production (Hassan *et al.,* 2011). Helminths are notorious for causing infections in cows, goats, buffalos and human beings.

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These infections cause serious diseases in livestock and hence lead to affect the economy of the country (Chaturvedi *et al.,* 2009). It is worked out previously that the sheep and goat industry of Faisalabad (Pakistan) has undergone a severe loss of Rs. 31.43 million in a year (Iqbal *et al.,* 2014).

It is reported that most of the intestinal infections are caused by nematodes of phylum Helminths (round worms) which are transmitted through soil to the animals (Sherwani *et al.,* 2013). Hemonchocosis is the most deleterious disease of small ruminants caused by *H.contortus* and *H. placei* in Pakistan. But *H. contortus* is predominantly the most infectious and key parasite of intestine of goats

and sheep (Maqsood *et al.,* 1996; Singh *et al.,* 1997; Asif *et al.,* 2008).These endoparasites feed on host blood and cause anemia, low productivity and may lead to death in heavy infections (Vatta *et al.,* 2001; Githigia *et al.,* 2001). Substantial financial losses occur due to these infections in livestock which is source of milk, meat, hides and wool all over the world (Nodtvedt *et al.,* 2002). The pathology of this parasite includes lowered (PCV) packed cell volume, hemoglobin level, blood cells like lymphocytes and erythrocytes etc. (Gibb *et al.,* 2005). Presently nematode worms have become resistant to the available anthelmintic drugs and consequently the rural community related with livestock is suffering from problems (Mortensen *et al.,* 2003; Saddiqe et al, 2013).

In Pakistan, helminth infections has caused a fiscal loss of Rs. 8800.09 million in 2011. Further, about 29% decrease in milk production has happened due to haemonchosis, which causes a loss of Rs.134062.39 million per year. It is further assessed that 27 % decrease in animal weight has occurred due to livestock infections which ultimately results in substantial loss of Rs. 40 million (Qamar, 2011).

Synthetic anthelmintics have been used for several years all over the globe to control and restrict the nematode infections. Nevertheless, the risk of resistance, less availability and high cost of anthelmintics for developing countries like Pakistan have necessitated to develop other alternatives for control of infections (Waller, 1997). Various biological interventions and medicinal plants are being explored to control the livestock infections (Githiori, 2004).

The local medicinal plants have been used on wide scale in veterinary and human medicines. The leaves of *Albezia lebbeck* has been used as treatment against gastrointestinal infections in villages for centuries. Similarly, syrup of tea powder has been used against stomach disturbances in buffaloes, goats and sheep.Therefore, to replace the synthetic anthelmintics, the scientists are trying hard to discover new plants for anthelmintic source (Patel *et al.,* 2010). A variety of plant based anthelmintic extracts/formulations are now available in Pakistan to treat helminths (Akhtar *et al.,* 2000; Iqbal *et al.,* 2014).

C. sinensis L. is native plant of Indo-Pakistan known as green tea. It belongs to family Theaceae with many other species of economic importance.Its cultivation is prevalent in more than 30 countries and it isconsumed as beverage on a large scale (Katiyar and Mukhtar, 1996). Camellia possesses about 700 chemicals, most of them are associated with human health such as vitamins (C,E and K) and caffeine polysaccharides (Dwivedi *et al.,* 2010). It has high content of polyphenols which cure many diseases like osteoporosis and liver disease. The extracts of *C.sinensis* are used as therapeutic agents against bacterial and viral infections (Mukhtar and Ahmad, 2000). It is further reported that the extract of green tea has anthelmintic effect against parasite infections (Sherwani *et al.,* 2013).

Albizia lebbeck L. is known as Shirin and is popularly used as fodder for cattle in Pakistan. It is used for treating flu, lung problems, abdominal tumor, cough and inflammation. It has also anthelmintic activity against parasitic worms.It is used against nematode worms which live in intestine of sheep (Khan *et al.,* 2010).

The present study was carried out to evaluate the anthelmintic potential of *A.lebbeck* L. and *C.sinensis* L. against *H.contortus* affecting the livestock on large scale. The study will help in developing easily available anthelmintic drugs with minor side effects to support the livestock industry.

Materials and Methods

Plant material

Leaves of *A.lebbeck* L. were collected from the primary author's university and the whole plant of *C.sinensis* L. was purchased from market (Lahore, Pakistan). The leaf architecture along with other recognition techniques were used to identify the both plants (Hongfei *et al.,* 2012). Moreover, the identified plants were verified by the Herbarium Incharge, Department of Botany, PU Lahore, Pakistan. *C.sinensis* is abundantly available in the market as dried powder which was purchased for anthelmintic evaluation. *C.sinensis* powder and the leaves of *A. lebbeck* L. were dried in sun light, ground in fine powder and subsequently sealed in sterilized polythene bags which were stored at 4°C for a week to avoid contamination. The ingredients were kept inactive at lower temperature (4°C).

Extract preparation

Powdered plant material was soaked in cold ethanol at 25°C for 3 days as described previously. The extracts were prepared in ethanol because the aqueous extracts were week in their efficacy as compared to ethanolic ones (Jabbar *et al.,* 2007; Tariq *et al.,* 2009). Later on, the macerated material was filtered through a Whatman's filter paper. The filtrate was further processed at 46°C in a rotary evaporator to make a thick dark color crude extract (CEE). For use in experiments, the extracts were diluted in 1× phosphate buffered saline (PBS) and kept at 4°C. The worms can survive for 2-3 days in PBS alone. Levamisole, a synthetic drug (0.55mg) was used as a standard reference as reported previously (Nawaz *et al.,* 2014). To evaluate *in vitro* anthelmintic activity, Adult Motility Assay (AMA) was used as described by Singh *et al.* (1985). In this assay, at certain concentrations of extracts, first the worms become paralyzed and then dead.

Worms' collection

Adult *Haemonchus contortus* worms were used as test organism in AMA. The worms were obtained from abomasum of the goat from slaughter house. A sufficient number of worms (250-300) were collected from various goats in 1× PBS. The worms were identified by the entomologist of primary author's University. After 2-3 hours, they were subjected to the experimental condition.

Adult motility assay (AMA)

Dilutions of CEE at 2, 4, 6 and 8mg/ml were made in 1x PBS which was also used as negative control. The experiment was repeated three times with positive control (levamisole) and negative control (PBS). The worms (10) in triplicate were treated with each CEE concentration in separate petri dishes at room temperature. The assessment of dead worms was made by loss of all sorts of movements and flat appearance. The mortality of the worms was studied after every two hours up till 8 hrs.

Statistical analysis

Statistical analysis was performed with the CoStat version 6.303. Statistical significance of the data was assessed by the Least significant difference (LSD) to compare the means ($p<0.05$).

Results

On exposure to crude extracts of *A. lebbeck* L. and *C. sinensis* L. adult *H. contortus* showed mortality rate at various concentrations on different time points (2-8 hrs). Figure 1 shows that *A. lebbeck* induced 66%, 84%, 88% and 85% mortality at 2mg/ml, 4mg/ml, 6mg/ml and 8mg/ml respectively after 8hrs of treatment. *C. sinensis* constitutes 44% mortality at 2mg/ml, 74% at 4mg/ml, 86% at 6mg/ml and 95% at 8mg/ml after 8 hrs.At low concentrations, the extracts were not significantly effective but with the increase of concentration, the extracts caused significant mortality of worms. Although the same concentrations of both plant extracts were used but *C. sinensis* caused 44% mortality whereas *Albezia lebbeck* L. caused 66% mortality at 2mg/ml concentration. At 6mg/ml, both plants had similar mortality rate (88% and 86%) showing identical efficacy but *C. sinensis* showed 95% mortality as compared to *albezia lebbeck* L. which caused 85% mortality at 8mg/ml concentration. At higher concentration (8mg/ml) the efficacy of *C.sinensis* L. was significantly more than *Albezia lebbeck* L. It was observed that *Albezia lebbeck* L showed insignificant discrepancy in mortality rate (88% & 85%) at 6mg/ml and 8mg/ml concentrations. Our results showed *C. sinensis* L. showed 95% mortality which was comparable to levamisole (positive control) efficacy. Similarly, insignificant number of worms underwent death in negative control (1x PBS) showing that massive death rate of worms could only happen when they exposed to different concentrations of CEE and not merely by any desiccation or lack of nutrients.

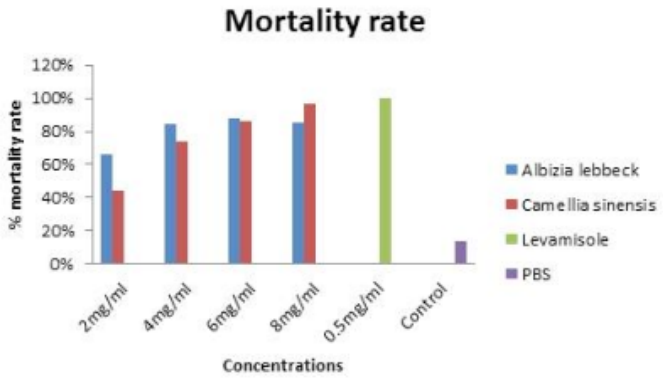

Figure 1: Anthelmintic activity of CEE of *Albizia lebbeck* and *Camellia sinensis* against *Haemonchus contortus*. The mortality of worms in negative and positive control has been shown.

Table 1: Mortality of Adult *Haemonchus contortus* treated with various dose concentrations of CEE of *A. lebbeck* and *C. sinensis* at 2,4, 6 and 8 hrs treatment.

	Time	Concentrations			
		2 mg/ml	4 mg/ml	6 mg/ml	8 mg/ml
Albizia lebbeck	2 hrs.	**a**1.00± 0.57**B**	**a**1.66± 0.66**AB**	**a**1.66± 0.33**A**	**a**2.00± 0.57**A**
	4 hrs.	**a**2.33± 0.33**AB**	**a**3.00± 0.57**A**	**a**1.66± 0.88**A**	**a**1.33± 0.33**A**
	6 hrs.	**a**4.00± 0.57**A**	**b**1.00± 0.57**B**	**ab**2.66± 0.33**A**	**b**1.00± 0.57**A**
	8 hrs.	**a**1.66± 0.66**B**	**a**3.00± 0.57**A**	**a**2.66± 0.33**A**	**a**2.33± 0.33**A**
Camellia sinensis	2 hrs.	**b**0.33± 0.33**C**	**ab**1.00± 0.57**B**	**ab**1.33± 0.33**B**	**a**2.00± 0.57**A**
	4 hrs.	**b**0.66± 0.33**BC**	**ab**2.00± 0.57**AB**	**ab**2.00± 0.57**AB**	**a**2.66± 0.88**AB**
	6 hrs.	**b**2.00± 0.57**AB**	**ab**2.66± 0.88**A**	**ab**2.33± 0.33**AB**	**b**1.99± 0.66**B**
	8 hrs.	**ab**2.33± 0.33**A**	**ab**1.66± 0.66**AB**	**a**3.00± 0.57**A**	**a**3.66± 0.33**A**

Values are expressed as mean ± SEM, (LSD test, $p<0.05$), ABCD means followed by the same letter in the column don't differ significantly, abcd means preceded by the same letter in the row don't differ significantly

Table 1 shows that the *in vitro* effect of CEE of *A. lebbeck* at 2mg/ml, the means effect was insignificant at all time points. At 4mg/ml, 6mg/ml, 8mg/ml the effect at 6hrs of treatment was significantly different while at 2, 4 and 8 hours, the effect was insignificant on mortality. Similarly, the extracts of C. *Sinensis* showed that at 2mg/ml, the effect after 8hrs of exposure was significant, while at other time points, the effect was insignificant. At 4mg/ml, the effect was insignificant at all time points. At 6mg/ml,

the effect was significant at 8hrs, while at other time points it was insignificant. At 8mg/ml, the effect was significant at 6hrs of treatment, while at other points, the effect was insignificant.

Discussion

In the present investigation, two plant extracts were used in vitro to treat the nematode parasites, the main cause of infection in livestock. The parasites are developing increasing resistance against the available synthetic drugs. The natural anthelmintic drugs derived from plants are stable and has diversity in terms of its structure and therefore, they produce new selection pressure/s for the nematode parasites. These parasites cannot be stopped to develop resistance due to selection process against new anthelmintics but the process of developing resistance can be delayed on account of standardized dose of alternative anthelmintics in replacement of conventional synthetic drugs (Tariq, 2015).

Our findings revealed that on exposure to leave extract of *A.lebbeck,* the larvae showed highest mortality rate at 6mg/ml dose after 8 hrs. The efficacy of this extract is little lower than the synthetic drug, levamisole, used as reference in this study. The efficacy of *A. lebbeck* rises with increase of dose and time due to anthelmintic agents. This study was supported by the results of Hussain *et al.* (2008) who reported that crude aqueous methanol extract of *A.lebbeck* leaves displayed time and dose dependent activity against nematode worms but this activity was effective at high doses. The study is further supported by the reports of Sujon *et al.* (2008) who showed that anthelmintic efficacy of ethanolic extract of pineapple leaves increased with increase of treatment time. Anthelmintic activity of indigenous plants had been evaluated against helminths of sheep as reported by many scientists (Sujon *et al.,* 2008; Tariq, 2015; Kumarasingha *et al.,* 2014). It is also documented that *A. lebbeck* contained strong antiparasitic agents which had been found effective against worms and hence substantiate their use as ethno-veterinary medicine (Khan *et al.,* 2010).

Present study reflected that extract of *C. sinensis* showed high mortality rate at 8mg/ml after 8 hrs. The anthelmintic efficacy of this plant is comparable to levamisole, a synthetic drug for nematode parasites. Our study is endorsed by Sherwani *et al.* (2013) who had evaluated the anthelmintic activity of *C.sinensis* aqueous extract at 25, 50 and 100 mg/ml against adult earthworm *Pheretimaposthuma*. The extract showed effective anthelminthic activity against the earthworms.

Taken together, the current study shows that both plants, *A.lebbeck* and *C.sinensis* possess anthelmintic potential at high doses. The ethanolic extracts of these plants can be evaluated against other Helminths parasites to have complete profile of anthelmintic potential. Moreover, the present study may help in replacing the synthetic anthelmintic with herbal ones to improve the control of worms.

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