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Evaluation of *In-Vitro* and *In-Vivo* Efficacy of *Hedera helix* Extracts against *Haemonchus contortus* in Sheep

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Key words
Haemonchus contortus, Hedera helix*, Larval development assay, Egg hatch assay, *In-vitro, In-vivo

ABSTRACT

Haemonchus contortus is an important nematode for small ruminants throughout the world. The commonly available drugs show unwanted side effects as well as resistance to different parasites, so medicinal plants have attracted attention due to their affordability and beneficial effects. In this study, the activity of *Hedera helix* extracts was investigated against *H. contortus* in sheep. In the in-vitro trial, the anthelminthic activity of methanolic and aqueous extracts of *Hedera helix* was checked on eggs and developed larvae of *H. contortus* at different doses (0.02, 0.05, 0.1, 0.15 0.2 mg). Aqueous and methanolic extract of *Hedera helix* was assessed for its *in-vivo* anthelmintic activity at doses of 1.13 and 2.25 gm/kg respectively in naturally infected sheep with *H. contortus*. ED50 of egg hatch assay (EHA) was studied with a dose of 0.103 for methanolic extract, 0.117 for aqueous extract, and 0.069 for albendazole. ED50 of LDA was seen at a dose rate of 0.035 for methanolic extract, 0.091 for aqueous extract, and 0.058 for albendazole. The *in-vivo* study of the methanolic extract showed better results than the aqueous extract in the fecal egg count reduction test (FECRT) at both dose rates. Post-treatment on days 4, 7, and 14 showed a significant ($p<0.05$) fecal egg count drop in the treated group of both extracts of *Hedera helix*. The efficacy of *Hedera helix* methanolic extract and aqueous extract at a dose rate of 2.25 gm was 67.6 and 44% respectively on day 14. The albendazole showed 75 % efficacy on day 14. It was observed that the surge of the *Hedera helix* dose rate potentially decreased the parasitic load.

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INTRODUCTION

Sheep and goats are one of the most important sources of animal protein throughout the world. In Pakistan, small ruminants are the most important source of meat and are used in ceremonial festivities throughout the country. However, very little attention is given to veterinary care and production improvement (Ijaz *et al.*, 2009). There

are a variety of gastrointestinal (GIT) parasites, which harbor sheep and goats. In small ruminants, *Haemonchus contortus* is a highly pathogenic parasite. It can cause severe illness and death in animals of all ages (Allonby and Urquhart, 1975; Arsenopoulos *et al*., 2021). Globally *H. contortus* is known for severe financial damage to small ruminants in tropical and developing countries. GIT nematode infection is one of the main sources of illness in small ruminants. It can also cause fatalities and impaired production in sheep and goats (Naeem *et al*., 2020). Every worm sucks about 30 to 50 µL blood/day (Arsenopoulos *et al*., 2021), and initiating prominent blood loss with a decrease of packed cell volume (PCV) (Angulo-Cubillán *et al*., 2007), anemia, and hypoproteinemia in ruminants (Chaudary *et al*., 2007). Anthelminthic resistance development is a severe problem worldwide (Kotze *et al*., 2020). That's why herbal medicine works as a substitute for synthetic anthelmintics (Fajimi and Taiwo, 2005).

Hedera helix L., also known as the English plant, is a perennial ornamental plant of the genus *Hedera. H. helix* plants usually grow in summer till late autumn with small and greenish-yellow flowers. The fruit ripens in winter and looks like small blackberries. *H. helix* naturally occurs in Asia, North America, and Western, Central, and Southern Europe (Lutsenko *et al*., 2010). The major chemical constituent of different parts of the plant contains triterpenoid saponins (Bedir *et al*., 2000). During the last decades, researchers are trying to form herbal extracts for the treatment and control of liver flukes. Plants comprise a different active material that has antibiotic-like activity these active ingredients are tannins, terpenoids, alkaloids, and flavonoids (Ghaffarifar *et al*., 2006). *H. helix* extracts have exhibited major anthelminthic action for liver flukes infestation (Eguale *et al*., 2007). Recent research has shown that *H. helix* hydro-alcoholic extracts have anthelmintic activity against *H. contortus* (Eguale *et al*., 2007; Mravčáková *et al*., 2019). The objective of this study was to evaluate the *in vitro* and *in vivo* efficacy of *H. helix* extract at different dose rates in comparison to albendazole.

MATERIALS AND METHODS

Collection of Hedera helix

The plant's mature fruit (seed) was collected from the local market (Akbari Mandi) in Lahore. The plant material was placed under the shade for about 25 days for air drying. For further drying, the material was spread on a plain surface and was kept for several days. After open-air drying, the seeds were shifted to a hot air oven at 37 °C for 3 days to attain constant weight for the next phase of the experiment. The seeds were then ground by using an electric blender machine for 5 min. The fine powder was packed in sealed plastic bags and was kept in the refrigerator (4 °C) for further use.

Preparation of aqueous extract

The plant aqueous extract was prepared using materials grounded through an electric shaker. The extraction was done by saturation of 100 grams of fine powdered material in 1 liter of distilled water taken in a flask of 1.5 liters. The flask containing distilled water and powder was placed on an electric shaker for continuous 3 h. After continuous 3 h of shaking the suspension was filtered through Whatman No. 1 filter paper (11µm). The final filter product was placed in a hot air oven at 37 °C for 5 days. After evaporation of water, the remaining dry powder was collected and placed in sealed plastic bags in the refrigerator for further use (Ahmad *et al*., 2014). The extract activity was checked *in-vitro* through egg hatch assay (EHA), Larval development assay (LDA), and *in-vivo* by fecal egg reduction test (FECRT).

Preparation of methanolic extracts

The alcoholic plant extract was prepared from the ground plant through the Soxhlet apparatus. About 50 gm of fine powder was soaked in half a liter of methanol. Tumble was made from filter paper for sample processing. The tumble was placed in the Soxhlet apparatus tube. The methanol-containing flask was fitted inside the Soxhlet apparatus and was heated up to 70 °C. The condensed vapors fall on plant material present in the tumble and draw active material from it and fall drop by drop into the flask. To get the proper extract, the process was repeated 8-10 times (Gigyani, 2015; Obame *et al*., 2013). After extraction, the methanol-containing active material was subjected to Rota vapor for separation. The collected material was kept in the refrigerator at 4 °C for further use. The extract activity was checked *in-vitro* through EHA, LDA, and *in-vivo* by fecal egg reduction test (FECRT).

Experimental animals

The present study was conducted using a small ruminant farm in Pattoki, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. The experimental animals were randomly selected from the farm. In this experiment, adult animals were selected irrespective of age and sex. The Lohi breed sheep were used in the experiment. A total of 84 naturally infected animals were screened, of which 30 sheep were positive for *H. contortus* infestation. Sheep having egg per gram (EPG) of more than 200 were included in the study. The animals used in the experiment belonged to the same breed, sex, and age.

In vitro *experiments*

In *in vitro* experiment, the efficacy of *H. helix* extract and albendazole were checked through EHA and LDA.

Egg hatch assay (EHA)

Egg collection and EHA were performed by using the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles *et al*., 1992). The collected eggs were washed several times with purified water. About 2 ml water containing 8 eggs was put into each test tube. Both extracts of *Hedera helix* (aqueous and methanolic) and albendazole were used at a dose rate of 0.02, 0.05, 0.1, 0.15, and 0.2 mg/ml on eggs placed in test tubes. The test tube containing eggs was covered and incubated for 2 days at 27 °C. After 2 days of incubation hatched larvae (dead or alive) and un-hatched eggs were calculated (Maingi *et al*., 1998). The calculation was made under a dissecting microscope at 40x magnification. The experiment was repeated three times for each concentration.

Larval development assay (LDA)

The eggs were collected from the sample through centrifugation and were properly washed with phosphate buffer saline. After collection, the eggs were incubated for 2 days for hatching. After hatching the larvae were reared for 6 days at room temperature (22-25 °C) in a phosphate buffer solution. In these six days of incubation, the larvae changed from L1 to the infective L3 stage. The L3 stage larvae were separated and taken in a petri dish. For each concentration, 10 larvae were separated for further process. After separation different doses of both extract *H. helix* and albendazole (0.02, 0.05, 0.1, 0.15, and 0.2 mg/ml) were applied for checking of larval development assay. After the application of drugs, the results were checked after 24 h. In the next step, the larvae were washed with a phosphate buffer solution. The motility of larvae was checked and counted as live and dead larvae were. The experiment was repeated three times for each concentration.

In vivo *experiments*

Grouping of animals for treatment

The positive animals were randomly placed in different groups. A total of six groups were made from positive animals and each group comprised five animals. Groups were labeled as A, B, C, D, E, and F. Fecal egg count (EPG) was done on day 0 of treatment. Sheep in group A were treated with Alba 10 Plus suspension (albendazole) (Drench) at a dose rate of 7.5 mg/kg body weight. The group B and C were treated with aq. extract of *H. helix* at 1.13 g/kg body weight and 2.25 g/kg body weight, respectively. Members of Group D and E were treated with methanolic extracts of *H. helix* at 1.13 g/kg and 2.25 g/kg body weight, respectively. Animals in Group F served as the untreated control group, while in another group the drug was orally administered to each animal.

Sample collection protocol

The fecal samples were directly taken from the rectum of infested animals by wearing latex gloves. The fecal collection was done on day 0 before treatment to calculate mean EPG (egg per gram) and on days 4th, 7th, and 14th after treatment. During fecal collection, animals were properly restrained by an attendant, and then samples were taken directly from the rectum through the lubricated finger (index and middle finger). The collected samples were placed in zip bags. The zip bags were marked with tag numbers and put in iceboxes for transportation. The fecal egg per gram (EPG) was determined by the McMaster method described by (Coles *et al*., 1992).

Statistical design

Data were expressed in Mean±SD and compared by two-way analysis of variance (ANOVA) to find a significant difference. Linear regression analysis (Probit analysis) was used for ED_{50} calculation. A significant level ≤ 0.05 was taken as statistically significant. The statistical analysis was done by using SPSS version 20.

Table I. *In-vitro* anthelminthic action of *H. helix* extracts and albendazole expressed in ED_{50} against egg hatching and larval developmenat of *H. contortus*.

Compound/ Extract	Extracts type	ED_{50}	
		Egg	Larvae
Albendazole		0.069	0.058
H. helix (mg/ml)	Aqueous	0.117	0.001
	Methanolic	0.10	0.035

RESULTS

Egg hatch assay

Maximum egg hatch inhibitions were seen at a high dose rate of 0.2 mg of all drugs. But the methanolic extract and albendazole were more potent than the aqueous extract. At this dose, the egg hatch inhibition of methanolic extract and albendazole was 87.5%, while the inhibition percentage of aqueous extract was 75% (Fig. 1A). The ED_{50} of aqueous, methanolic extracts and albendazole for egg hatch assay is given in Table I. The overall comparison of the EHA is presented in Figure 1A. There was a significant ($p<0.05$) difference for both extract and albendazole. In Figure 1A the overall activity of aqueous and methanolic extracts along with albendazole

are shown. The results exhibited maximum activity at a dose rate of 0.2 mg of methanolic extract and albendazole. The 50 % effectiveness was seen at about 0.1 mg of all used materials.

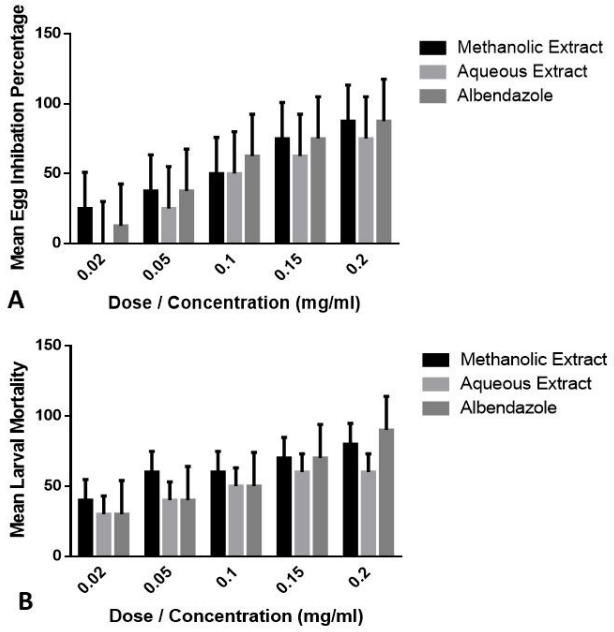

Fig. 1. *In-vitro* activity of methanolic, aqueous extract of *H. helix* and albendazole on egg inhibition (A) and larval mortality (B) of *H. contortus*.

Larval development assay

The maximum larval mortality was seen at a high dose rate of 0.2 mg of all drugs. Albendazole was the most potent drug with a mortality rate of 90%. The methanolic extract was at the 2nd number and the aqueous extract on the 3rd number with 80% and 60% mortality, respectively (Fig. 1B). The ED_{50} of aqueous, methanolic extracts and albendazole for larval development assay is given in Table II. The results were significant ($p<0.05$) for both extract and albendazole.

Figure 1B shows the activity of aqueous and methanolic extract along with albendazole at different concentrations. The maximum values were seen on 0.2 mg of all used materials. Albendazole was most potent at 0.2 mg showing 90% effectiveness. The methanolic extract was at 2nd number showing 80% results. The 50% effectiveness was seen at the 0.1 mg dose rate of all materials.

Fecal Egg count reduction test (FECRT) for H. helix *extracts and albendazole against* H. contortus

A significant reduction in egg per gram was noticed in methanolic extracts and albendazole ($p<0.05$) treated group in comparison with the untreated group. The efficacy of albendazole was maximum (75%) on day 14th. The effectiveness of aq. extract at amounts of 1.13 g/kg and 2.25g/kg on day 14th was 20.83 % and 44%, respectively. The maximum efficacy of methanolic extract at 1.13 g/kg and 2.25 g/kg body weight on day 14th was 46.42 % and 67.64%, respectively. The overall data before and after treatment of different groups is presented in (Table II). The ANOVA result shows that EPG before treatment between and within the group is non-significant ($P>0.05$). While the EPG after treatment at days 4, 7, and 14 between and within the group are significant ($P<0.05$).

DISCUSSION

This study was conducted to compare the anthelminthic activity of *Hedera helix* extracts and the reference drug albendazole. The aqueous and methanolic extracts of *H. helix* inhibited the egg hatch assay and larval development assay in the study conducted by (Eguale *et al*., 2007). There was little difference in the results of our study and the study reported by Eguale *et al*. (2007), probably due to dose differences or environmental conditions. *H. helix* aqueous and methanolic extract inhibition showed

Table II. Mean fecal EPG count pre-treatment and post-treatment of *H. helix* extracts and albendazole percent efficiency based on FECRT.

Treatment type	Dose (mg/Kg)	Pre-treatment		Post-treatment (% reduction)	
		Day 0	Day 4	Day 7	Day 14
Albendazole	7.5	800±126.49	500±70.71^{b} (37.5)	300±54.77^{b} (62.5)	200±31.62^{c} (75.00)
H. helix Aq. Ext	1.13	480±66.33	400±54.77^{b} (16.66)	380±37.41^{b} (20.83)	380±58.31^{b} (20.83)
	2.25	500±70.71	360±50.99^{b} (28.00)	320±58.31^{b} (36.00)	280±58.31^{bc} (44.00)
H. helix M. Ext	1.13	560± 74.83	360±24.49^{b} (35.71)	340±50.99^{b} (39.28)	300±31.62^{bc} (46.42)
	2.25	680 ± 58.31	440±40.00^{b} (35.29)	340±40.00^{b} (50.00)	220±20.00^{bc} (67.64)
Untreated control		640± 67.82	720±86.02^{a}	780±58.31^{a}	820±86.02^{a}

*Mean ± S.E; Superscript (a, b and c) shows the statistical significance (P<0.05) between the treatment group.

similar results with previously published studies. The *in-vitro* and *in-vivo* study was conducted to investigate the anthelmintic activity of crude and methanolic extracts of *N. tabacum* leaves. The methanolic extract was found to be more potent in EPG reduction than the aqueous extract (Iqbal *et al*., 2006). The AE efficiency was checked in the *in-vivo* study, and it was revealed that the EPG and parasitic load were decreased in naturally infected sheep (Cenci *et al*., 2007; Max, 2010).

The aqueous extract of *Annona senegalensis* at 7.1 mg/ml inhibited 11.5% of egg hatching (Alawa *et al*., 2003) and *Spigelia anthelmia* methanolic extract at a dose rate of 50 mg/ml inhibited 97.4% of egg hatching (Assis *et al*., 2003). There was an alteration in the ED50 value of both extracts of *H. helix* probably due to the presence of an active ingredient that has ovicidal activity at the same concentration of both extracts. In the current study, methanolic extract has higher activity than aqueous extract in an *in-vitro* assay against the parasite. The effect of hydroalcoholic extract of *Albizia gummifera*, *Croton macrostuchyus*, *Coriandium sativum*, *H. helix,* and *Ekebergia capensis* on the survival of the adult parasite was significantly higher as compared to their aqueous counterpart and other plants. The probable explanation for the good results of the hydroalcoholic extracts compared to the aq. extract on adult parasites might be due to the easy trans-cuticular fascination of the hydroalcoholic extract into the body of the parasite more than the aqueous extract. While the diverse chemical profiles of plant extracts are not known in general, hydroalcoholic extracts of the plant might contain some non-polar organic chemicals with a wide range of polarity than aqueous extract (Debella, 2002). The different kinds of extracts and the means of extraction also change the activity of the botanical compounds (Eloff, 1998). The findings in the current study match with the study conducted against *H. contortus* by using a high dose of *A. muricata* extract. This showed the efficacy of 84.91% and 89.08% in EHT and larval motility test respectively (Ferreira *et al*., 2013). The aqueous extract of *A. squamosa* L (sugar apple) caused egg hatch inhibition of 19.4% against GIT nematodes (Souza *et al*., 2008).

H. helix, validates the result of the plant extract on the fecundity of the parasite (Athanasiadou *et al*., 2001). In *in vivo* trial, the current study result of the methanolic extract is better than the aqueous extract. It matches with the result of (Eguale *et al*., 2007). There is a slight difference in the result as in our study albendazole shows 75% efficacy on day 14 whereas in the previous study, they found 100% efficacy. Whereases higher doses of methanolic extract were found to be more potent than the higher dose used in the previous study. This slight difference in result may be due to the environment or sheep breed. The *in vivo* study was conducted in sheep to check the effect of *A. squamosa* L (sugar apple) against *H. contortus*. In this study, a 40% reduction occurred in egg count (Vieira *et al*., 1999). Both aqueous and methanolic extract of *Nicotiana tabacum L* against GIT nematode. it is found that this extract has reduced power against the egg (Iqbal *et al*., 2006). The anthelminthic drug can reach the target site in the nematode parasite either by oral ingestion or by uptake/diffusion through the external surface. Though the study has shown that trans-cuticular diffusion is the common way of entry for non-nutrient and non-electrolyte substances in nematodes (Geary *et al*., 1999). The active ingredients found in plants (saponins, alkaloids, and flavonoids) are the main factor accountable for a broad range of therapeutic actions of most medicinal plants (Debella, 2002). The active principles that induced the observed anthelmintic action may be found in one or more of these classes of chemicals. The difference in the action of the extracts of the plant may be due to alteration in the amount of the active components accountable for the tested anthelmintic action resulting from the difference in solubility either in water or methanol.

CONCLUSIONSANDRECOMMENDATIONS

The evaluation of *H. helix* extracts *in-vitro and in-vivo* efficacy against *H. contortus* in sheep provides valuable insights into the potential use of these extracts as a natural parasite control treatment. According to the study's findings, *Hedera helix* extracts demonstrated significant activity against *H. contortus* in *in-vitro* tests. The extracts inhibited parasite mortality, egg hatching, larval development, and egg production. These findings suggest that *H. helix* extracts may have anthelmintic properties. We recommend that more research be done to investigate and evaluate the different fractions and uses of plants for therapeutic purposes, as well as the plant's potential toxicity in animals. This will provide a more complete understanding and will aid in development.

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IRB approval and ethics statement

Experimental observations on animal care and handling were conducted in accordance with the Guide for the use and care of laboratory animals/experimental animals of the University of Veterinary and Animal Sciences, Lahore, and were approved by institutional review board of the University of Veterinary and Animal Sciences, Lahore, laboratory animal/experimental animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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