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In vitro anthelmintic activity of crude extracts of ethnobotanically important wild plants of Sahiwal Division, Punjab, Pakistan against *Haemonchus contortus (Rudolphi 1803)*

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Key words: *Haemonchus contortus,* anthelmintic activity, ethnobotany, wild plants, Sahiwal Division.

Abstract

Our preceding ethnobotanical studies revealed that *Aerva* javanica, *Cistanche tubulosa, Cuscuta campestris, Heliotropium europaeum* and *Persicaria glabra* have been used in folk medicines in Sahiwal Division, Punjab, Pakistan. In the present study, different doses of the methanol, chloroform, n-hexane and aquous extracts of these plants were evaluated for their anthelmintic activity against the very devastating blood sucking macro-parasites of goats and sheep' *Haemonchus contortus*'. The serial dilution method was used to determine the most effective dose of the above samples to kill 100% of adult *Haemonchus contortus* under *in vitro* conditions. Methanolic, chloroform, n-hexane and aquous crude extracts of five plants were diluted to give four dilutions (10, 20, 50 and 100 mg /mL) each. Non-polar solvent-based extracts were found more effective than that of the polar solvent-based extracts. Chloroform extracts of *Cistanche tubulosa, Cuscuta campestris* and *Persicaria glabra* while methanolic extracts of *Aerva javanica* and *Cuscuta campestris* were found the most effective. Among all *Cuscuta campestris* demonstrated maximum efficacy providing 100% *H. Contortus* control within first hour of treatment however other four plants were found to have adequate anthelmintic activity. Helminthiasis is a common disease found in sheep and goats in Asia. Due to increased resistance in heliminths against chemical control, plant extracts are being preferred as an alternate remedy. All these plants particularly *Cuscuta campestris* warrants future investigations to identify and isolate the possible phytochemicals, responsible for its excellent anthelmintic activity.

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Introduction

Man has started utilizing plants and plant-based products for medication since the beginning of human civilization. The history of herbal medicine is almost as old as the life of cave dweller. The use of medicinal plants for various diseases of human and livestock is a common practice round the globe. Over centuries, cultures around the world have learned to use plants to fight illness and maintain health. Out of about 250,000 plant species, nearly 80,000 are reported as the medicinal plants. According to World Health Organization (WHO) more than 25% of the modern drugs currently being used in European countries have been obtained from plants. The medicinal value of a plant is due to the presence of secondary metabolites such as alkaloids, flavonoids, glucosides, tannins, gums, resins, essential oils and fatty acids etc. Such compounds produce definite pharmacological and physiological actions on human body (Blot *et al.*, 1993,Haraguchi *et al.*, 1999). Such phyto-metabolites are combating different diseases as antioxidant, antifungal, antibacterial, antidiabetic and anthelmintic. Different plant resources have different kinds of metabolites that are most suitable for specific ailments. Our medicinal plants are enriched with such compounds capable of combating against Helminthiasis. It is an important animal disease, inflicting heavy economic and production losses, enhancing mortality rate and weight losses especially in third world countries due to poor management practices and inadequate sanitation. Helminthiasis is being controlled through effective chemical control, adequate sanitation and improved management strategies globally. Unprecedented increases of resistance in heliminths against commercially available anthelmintic formulations (Vidyadhar *et al.,* 2010) led us to the proposal of screening various medicinal plants for their anthelmintic activity. Now the demand is increasing to produce more drugs from plant resources and such interests are being renewed in traditional medicines. This renewal of interest in plant-based drugs is a result of a famous belief that "green medicine" is more reliable and safer as compared to synthetic drugs (Kursat and Erecevit, 2009).

Proper screening and evaluation of anthelmintic efficacy of such medicinal plants can offer the promising, sustainable and environmentally acceptable alternatives of the currently available anthelmintic agents. *Haemonchus contortus* is a macro-parasite of small ruminants (mostly sheep and goats), causing hemorrhagic anaemia via blood sucking in abomasums of the host animal(Kiros*et al.*, 2016). These anthelmintic parasites are the multicellular organisms, having complete or partial organ system. Several of the drugs are being used to kill these worms by affecting their nervous system resulting in muscle paralysis or by affecting the uptake of glucose. Such medicines are administered orally for veterinary as well as for humans. To assess the *in vitro* and *in vivo* efficacy of these anthelmintics several trials had been conducted by different research groups. The present study was conducted to explore the *in vitro* anthelmintic activity of some selected indigenous wild plants of Sahiwal Division, Punjab, Pakistan. As Sahiwal division consists of old cities, their inhabitants are good representatives of old civilizations, preserving their own traditions, heritage, moral and ethnic values and plant resources. There was a need to explore this local ethnic knowledge about their local wild plants.The said Division lies between the coordinate values as;30°39′52″ North latitude and 73°6′30″ East longitude / 30.66444°N, 73.10833°E and about 560 ft AMSL altitude level. It consists of three districts; Sahiwal, Pakpattan and Okara. This research area has subtropical, continental type climate and alluvium soil laid by three rivers; Bias, Ravi and Satluj (Ali, 2010).

Materials and methods

Plant Material used

Ethnobotanical data about the indigenous plants of Sahiwal Division was collected and documented elsewhere (Ali *et al.*, unpublished), which revealed numerous plants being used as nematicidal therapy for animals by local people in the study area. Based upon those data five plants (Table.1) were selected to evaluate their anthelmintic activity.

Samples were collected from various locations of Sahiwal division, dried, identified by plant taxonomist and deposited to the Dr. Sultan Ahmad Herbarium, Botany Department, GC University, Lahore, Pakistan as voucher specimens (Table.1).

Preparation of Extracts (macerates)

Fresh plant material after collection was washed with distilled water, blotted on autoclaved filter papers and air dried at room temperature for ten to fifteen days. The dried material was ground, filtered through muslin gauze and preserved in amber colour bottles for further use. Four solvents (I) n-Hexane, (II) Chloroform, (III) methanol and (IV) distilled water were used for extraction. Dried and powdered plant material (200 grams each) was used for extraction. The extract was concentrated using Soxhlet's apparatus and yield of each extract was calculated on the basis of dry weight. Here after, the dried filtrate was termed as 'macerate'. As mentioned above each plant material was extracted in four solvents so there was a total 20 extracts which were tagged with respect to plant tag and solvent number. For example A-I means extract of plant-A (*Aerva javanica*) in solvent No. I (n-Hexane). Similarly B-III means extract of plant-B (*Cistanche tubulosa*) in solvent No. III (methanol) and so on.

Test organisms

Adult parasites *Haemonchus contortus* were collected by dissecting abomasums of freshly slaughtered infected sheep/goat. Abomasums were collected and transported to lab immediately. For collection of parasites, abomasums were washed with saline solution (NaCl 0.9% solution) to discard maximum filthiness and kept in NaCl 0.9% solution for further examination. The collected adult organisms were identified by Department of Zoology, GC University, Lahore. The identified adults were then subjected to *in vitro* anthelmintic studies.

Preparation of Piperazine citrate 10mg/mL

Solution of $3C_4H_{10}N_2.2C_6H_8O_7$ (Piperazine citrate) was prepared by dissolving 0.6g Piperazine citrate in 20-30 mL of distilled water with continuous stirring and making up the final volume (60mL) by pouring additional autoclaved water. It was used as positive control.

Preparation of macerate dilutions

Four dilutions of plant macerate (10, 20, 50 and 100 mg/mL) were formulated by weighing appropriate amount of powdered macerate and dissolving in required volume of distilled water.

Measuring Anthelmintic activity

Anthelmintic activity of plant macerates was investigated following Fasiuddin and Campbell (2000) and Egualeet *al.*(2007) with slight modifications. Four different concentrations (10, 20, 50 and 100 mg/mL) of plant macerates were employed to assess their anthelmintic potential against roundworm *Haemonchus contortus*. We devised an extensive experiment comprising of two control groups (negative and positive controls) and four experimental groups depending upon the solvent used for extraction. Negative control contained 0.9% saline while 10 mg/mL of Piperazine citrate was used as positive control. Each experimental group was further divided into five sub-groups depending upon the plant macerate so for each solvent there were five macerates. Hence, we had twenty sub-groups. Then, four concentrations (10 mg, 20 mg, 40 mg, and 100 mg of plant macerate per mL of solvent) were prepared. In this way, we end up with 80 experimental treatments (dilutions). Each dilution was assigned a distinct tag as shown in Table 2.Five actively moving same-sized parasites were placed in a petridish and dipped in 10mL of liquid (two controls or 80 experimental treatments).The experiment was done in triplicate for each treatment. Parasites were observed after each twenty minutes for mortality. After each observation at room temperature the inactive parasites were shifted to 0.9% saline for possible recovery. The number of motile (alive) and immotile (dead) worms were counted under dissecting microscope and recorded for each concentration. Death of worms was ascertained by absence of motility even after agitation.

Statistical Analysis

A mortality index was depicted by the number of dead worms out of the total of five worms per petridish.

Efficacy of plant extracts causing mortality was analysed by calculating the means and standard deviation of the means. Moreover ED_{50} was calculated after Eguale *et al.*(2007), by probit analysis. The results of ED_{50} were displayed by graph. All these analyses were done using Microsoft Excel.

Results

Mortality index, which is actually the count of total number of dead worms showed anthelmintic efficacy of plant extracts. It exhibited very encouraging results which can be applied in the field of veterinary healthcare.

Table 1. Plants selected for their anthelmintic activity assay**.**

Sr. No.	Plant name / Family	Voucher No.	Plant Tag	Part used
01	*Aerva javanica* (Burm. f.) Juss./ Amaranthaceae	GC.Herb. Bot. 2909	A	Whole plant
02	*Cistanche tubulosa* (Schrenk) Hook. f./ Orobanchaceae	GC.Herb. Bot. 2910	B	Whole plant
03	*Cuscuta campestris* Yuncker / Cuscutaceae	GC.Herb. Bot. 2911	C	Whole plant
04	*Heliotropium europaeum* L. / Boraginaceae	GC.Herb. Bot. 2912	D	Whole plant
05	*Persicaria glabra* (Willd.) M. Gomes / Polygonaceae	GC.Herb. Bot. 2913	E	Whole plant

Plant wise comparison of anthelmintic efficacy

The comparison of plant extracts with respect to their anthelmintic efficacy revealed a dose-dependent as well as time-dependent significant increase in mortality index in Table 2. However, plant-C (*Cuscuta campestris*) was found to be the most potent anthelmintic remedy for goats and sheep because its extract @ 100mg/mL killed 100% worms within first hour of treatment as depicted in Table 2.

It is remarkable that extract of plant-C was the most effective of all treatments. However, keeping in view its lower dose it exhibited 5 times lesser activity than that of the commercially used anthelmintic agents Piperazine citrate. After 2 hours, extracts of plant-A, B, C and E at their highest concentration (100mg/mL) killed 100% worms however extract of plant-C killed 100% worms at 50mg/mL. As these plant extracts had shown maximum inhibition at higher concentration, finding after Kamaraj and Rahuman (2011) was positive that our experimental plants contain possible anthelmintic compounds.

ED_{50} wise comparison of anthelmintic efficacy

The comparison of ED_{50} of different plant extracts revealed a solvent as well as time-dependent significant increase in mortality index (Table 2).

Plant-A & B was found to be the most potent as their extract were able to kill 100% worms at a dose 20mg/mL. Regarding ED_{50} efficacy of these plant extracts was 50% to that of standard Piperazine citrate which showed 100% mortality at 20mg/mL.

Solvent wise comparison of anthelmintic efficacy

Solvent used for extraction may also affect the efficacy of a plant extract. Hence, solvent wise comparison revealed that methanolic extract to be the more effective followed by chloroform and water.

Discussion

In vitro tests to evaluate the efficacy of plants extracts by calculating the motility index of adult worms (Hounzangbe-Adote *et al.*, 2005) is extensively used in veterinary parasitology for prospecting novel anthelmintic agents (Costa *et al.*, 2002; Vasconcelos *et al.*, 2007). Results obtained through in vitro evaluation of candidate anthelmintic agents have paved the way towards developing novel deworming strategies. Such endeavours have proved to be a rational and applied approach by virtue of time as well as cost effectiveness. On the other hand, numerous factors have rendered in vivo assessment of anthelmintic therapies problematic.

Table 2. Mortality index of *Haemonchus contortus* caused by plant extracts (expressed as Mean±S.D).

Extract Tag	Died within 1st hour Mean±S.D	Died within 2nd hour Mean±S.D	Died within 3rd hour Mean±S.D	Died within 4th hour Mean±S.D
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Standard	2.67±0.58	4.67±0.58	5.00±0.00	5.00±0.00
A-I/100	3.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00
A-I/50	2.67±0.58	4.67±0.58	5.00±0.00	5.00±0.00
A-I/20	2.00±0.00	2.67±0.58	3.67±0.58	4.00±0.00
A-I/10	0.67±0.58	2.33±0.58	3.00±0.00	3.67±0.58
B-I/100	3.67±0.58	4.67±0.58	5.00±0.00	5.00±0.00
B-I/50	2.67±0.58	3.67±0.58	4.67±0.58	5.00±0.00
B-I/20	2.33±0.58	3.33±0.58	4.00±1.00	3.33±0.58
B-I/10	1.67±0.58	2.00±1.00	2.67±0.58	3.67±0.58
C-I/100	1.67±0.58	2.67±0.58	3.67±0.58	4.67±0.58
C-I/50	0.33±0.58	0.67±0.58	1.67±0.58	2.67±0.58
C-I/20	0.00±0.00	0.33±0.58	1.33±0.58	1.67±0.58
C-I/10	0.00±0.00	0.00±0.00	0.67±0.58	1.33±0.58
D-I/100	2.67±0.58	4.00±1.00	4.67±0.58	5.00±0.00
D-I/50	2.00±0.00	3.67±0.58	4.33±0.58	4.67±0.58
D-I/20	1.67±0.58	2.67±0.58	3.67±0.58	4.33±0.58
D-I/10	0.67±0.58	2.33±0.58	3.33±0.58	4.00±1.00
E-I/100	3.00±1.00	4.33±0.58	5.00±0.00	5.00±0.00
E-I/50	2.33±0.58	3.67±0.58	4.67±0.58	5.00±0.00
E-I/20	1.33±0.58	2.33±0.58	3.00±1.00	3.67±0.58
E-I/10	1.00±0.00	2.00±1.00	2.33±0.58	2.67±0.58
A-II/100	3.33±0.58	4.33±0.58	5.00±0.00	5.00±0.00
A-II/50	2.33±0.58	3.00±1.00	4.33±0.58	4.67±0.58
A-II/20	1.67±0.58	2.67±0.58	3.67±0.58	4.33±0.58
A-II/10	1.33±0.58	1.67±0.58	2.67±0.58	4.00±1.00
B-II/100	4.67±0.58	5.00±0.00	5.00±0.00	5.00±0.00
B-II/50	4.33±0.58	4.67±0.58	5.00±0.00	5.00±0.00
B-II/20	3.33±0.58	4.33±0.58	4.67±0.58	5.00±0.00
B-II/10	2.33±0.58	2.67±0.58	3.33±0.58	4.00±1.00
C-II/100	4.67±0.58	5.00±0.00	5.00±0.00	5.00±0.00
C-II/50	4.33±0.58	4.67±0.58	5.00±0.00	5.00±0.00
C-II/20	2.67±0.58	3.33±0.58	4.33±0.58	4.67±0.58
C-II/10	2.33±0.58	2.67±0.58	3.67±0.58	4.33±0.58
D-II/100	0.33±0.58	0.67±0.58	1.33±0.58	1.67±0.58
D-II/50	0.00±0.00	0.33±0.58	1.00±0.00	1.33±0.58
D-II/20	0.00±0.00	0.00±0.00	0.33±0.58	0.67±0.58
D-II/10	0.00±0.00	0.00±0.00	0.00±0.00	0.33±0.58
E-II/100	4.67±0.58	5.00±0.00	5.00±0.00	5.00±0.00
E-II/50	4.33±0.58	4.67±0.58	5.00±0.00	5.00±0.00
E-II/20	3.67±0.58	4.00±0.00	4.33±0.58	4.67±0.58
E-II/10	2.33±0.58	2.67±0.58	3.67±0.58	4.00±0.00
A-III/100	4.67±0.58	5.00±0.00	5.00±0.00	5.00±0.00
A-III/50	4.33±0.58	4.67±0.58	5.00±0.00	5.00±0.00
A-III/20	3.33±0.58	4.33±0.58	4.67±0.58	5.00±0.00
A-III/10	2.33±0.58	2.67±0.58	3.67±0.58	4.00±0.00
B-III/100	3.33±0.58	4.33±0.58	5.00±0.00	5.00±0.00

B-III/50	2.33±0.58	3.67±0.58	4.67±0.58	5.00±0.00
B-III/20	2.00±1.00	2.67±0.58	4.00±1.00	4.33±0.58
B-III/10	0.67±0.58	1.67±0.58	2.33±0.58	3.33±0.58
C-III/100	5.00±0.00	5.00±0.00	5.00±0.00	5.00±0.00
C-III/50	4.33±0.58	5.00±0.00	5.00±0.00	5.00±0.00
C-III/20	3.33±0.58	4.00±1.00	4.33±0.58	4.67±0.58
C-III/10	2.33±0.58	2.67±0.58	3.33±0.58	4.00±1.00
D-III/100	0.67±0.58	1.67±0.58	2.33±0.58	3.67±0.58
D-III/50	0.33±0.58	0.67±0.58	1.67±0.58	2.67±0.58
D-III/20	0.00±0.00	0.33±0.58	1.33±0.58	2.33±0.58
D-III/10	0.00±0.00	0.00±0.00	0.67±0.58	1.33±0.58
E-III/100	2.67±0.58	4.33±0.58	5.00±0.00	5.00±0.00
E-III/50	2.33±0.58	2.67±0.58	4.00±1.00	4.33±0.58
E-III/20	0.67±0.58	1.67±0.58	2.67±0.58	3.67±0.58
E-III/10	0.33±0.58	1.00±0.00	2.33±0.58	2.67±0.58
A-IV/100	3.00±1.00	4.00±1.00	4.33±0.58	5.00±0.00
A-IV/50	2.33±0.58	3.33±0.58	4.33±0.58	5.00±0.00
A-IV/20	0.67±0.58	2.33±0.58	3.00±1.00	3.33±0.58
A-IV/10	0.33±0.58	1.33±0.58	2.33±0.58	2.67±0.58
B-IV/100	1.67±0.58	2.67±0.58	4.67±0.58	5.00±0.00
B-IV/50	0.67±0.58	1.67±0.58	2.67±0.58	4.33±0.58
B- IV /20	0.33±0.58	1.33±0.58	2.33±0.58	4.00±1.00
B- IV /10	0.00±0.00	0.67±0.58	1.67±0.58	3.00±1.00
C- IV /100	2.67±0.58	4.00±1.00	4.67±0.58	5.00±0.00
C- IV /50	2.33±0.58	3.33±0.58	4.00±1.00	4.33±0.58
C- IV /20	0.67±0.58	1.67±0.58	2.67±0.58	4.00±1.00
C- IV /10	0.33±0.58	1.33±0.58	2.33±0.58	3.33±0.58
D- IV /100	3.33±0.58	4.00±1.00	4.67±0.58	5.00±0.00
D- IV /50	2.67±0.58	3.67±0.58	4.33±0.58	4.67±0.58
D- IV /20	2.33±0.58	3.00±1.00	4.00±1.00	4.33±0.58
D- IV /10	1.00±1.00	1.67±0.58	3.00±1.00	4.00±1.00
E- IV /100	0.33±0.58	0.67±0.58	1.67±0.58	2.00±0.00
E- IV /50	0.00±0.00	0.33±0.58	1.00±0.00	1.33±0.58
E- IV /20	0.00±0.00	0.00±0.00	0.67±0.58	1.00±1.00
E- IV /10	0.00±0.00	0.00±0.00	0.33±0.58	0.67±0.58

Such factors include low bioavailability of active ingredients through transcuticular trafficking to reach helminth's body fluids (Egualee*t al.*, 2007), susceptibility of compounds to digestive conditions and interactions of compounds with gut flora in the rumen of animals (Chagas and Vieira, 2007,Peneluc *et al.*, 2009). Such factors are necessary to be taken into account while testing and screening of novel anthelmintic therapies (Ferreira *et al.*, 2013).Efficacy of anthelmintic agents can be ranked according to the guidelines devised by (Powers *et al.*, 1982). Any anthelmintic agent causing more than 90% worm mortality is considered effective while agents showing 80-90% mortality can be ranked as moderately effective.

Hence, the five plants extracts used in present studies especially in higher dilutions can be ranked as effective anthelmintic agents. However, extract to extract differences were there as plant-C (*Cuscuta campestris*) was found to be the most effective anthelmintic agent (Fig. 1) causing 100% worms mortality within first hour of treatment while other plant extracts took longer time period. The difference in the results of different plant extracts may be due to the type of solvent used for extraction, origin of the plant material, stage of development at harvesting, environmental factors at drying and storage time and place of harvesting (Badar *et al.*, 2011).

The data presented in the table-2 lead to the conclusion that different degree of helminthiasis of different extracts are due to the level of tannins present in the compounds. These are the secondary metabolites, occurring in different plants, having anthelmintic property. Tannins are the polyphenolic compounds, involved in energy generation by uncoupling oxidative phosphorylation, or binds to glycoprotein on the cuticle of parasite, causing death (Thompson and Geary, 1995).

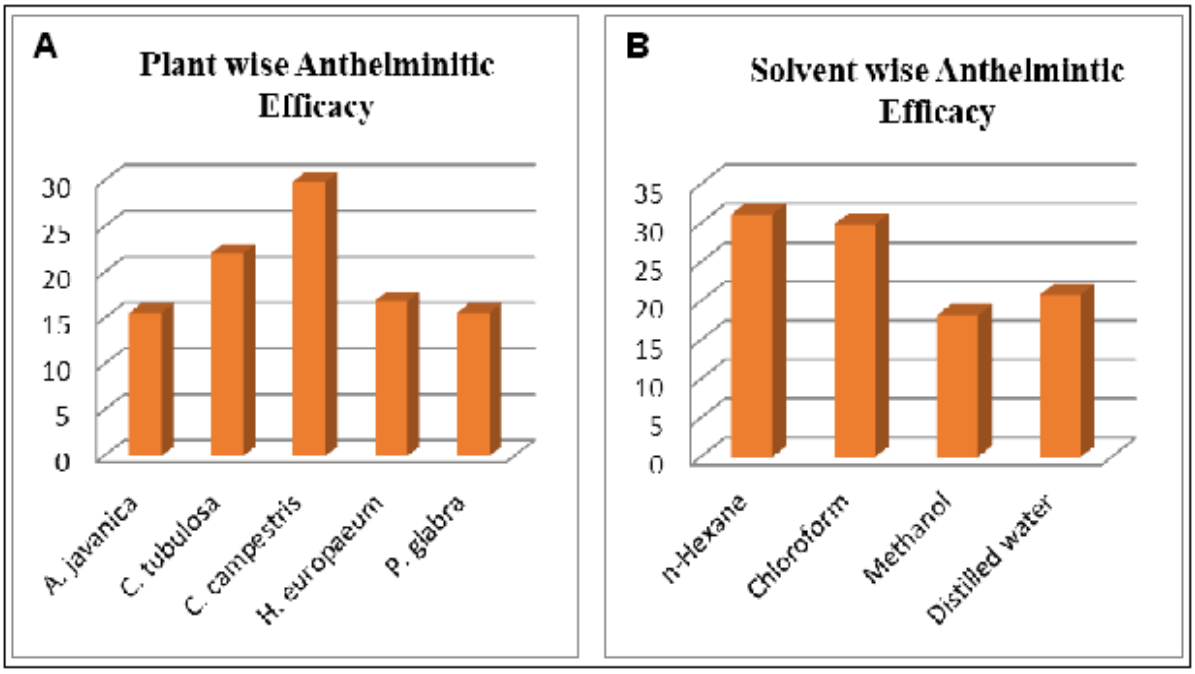

Fig. 1. A glance of Anthelmintic Efficacy. (A) shows the maximum anthelmintic activity of C. campestris in a plant wise comparison, (B) solvent wise comparison revealed n–hexane and chloroform as the best extraction solvents.

Extracellular matrix (ECM) of nematode, enriched with collagen provides cuticle that forms exoskeleton. Collagen is a kind of protein, modified by a range of co and post translational modifications, prior to assembly in to higher complex order. Very complex ordered reactions occurring between the cuticle of nematode and tannins of plant extracts, results in the loss of flexibility and toughness in the skin, this makes the worm immobile and non-functional, leading to paralysis and followed by death (Vidyadhar *et al.*, 2010).

Conclusions

It is concluded that anthelmintic activity was best observed in non-polar solvents than in polar solvents and *C. Campestris* exhibited maximum while other four plants were found to have moderate anthelmintic activity.

Out of twenty plant extracts only four plant extracts i.e. C-I, D-II, D-III and E-IV representing n-Hexane extract of *C. campestris*, chloroform extract and methanol extract of *H. europaeum* and aquous extract of *P. glabra* appeared to be suitable anthelmintic agents for goats and sheep in Pakistan while other sixteen plant extracts shown low anthelmintic efficacy. In future it is recommended to identify and isolate the possible phytochemicals, responsible for anthelmintic activity in order to develop plant-based anthelmintic therapies for small ruminants.

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