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FULL LENGTH ARTICLE

In vitro and *in vivo* anthelmintic activities of *Iris kashmiriana* Linn.

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Abstract In search of a natural antiparasitic, *in vitro* and *in vivo* anthelmintic activities of methanol and aqueous extracts of *Iris kashmiriana* Linn. rhizome were tested against gastrointestinal nematodes of sheep. A worm motility inhibition assay was used for *in vitro* study and a faecal egg count reduction assay was used for *in vivo* study. Crude aqueous extracts of *I. kashmiriana* exhibited greater anthelmintic activity against *Haemonchus contortus* than crude methanolic extract ($P < 0.05$). The aqueous extracts of *I. kashmiriana* resulted in a mean worm motility inhibition of 85.0%, while methanolic extracts resulted in a mean worm motility inhibition of 100.0%. The *in vivo* anthelmintic activity of aqueous and methanolic extracts of *I. kashmiriana* in sheep naturally infected with mixed gastrointestinal nematodes species demonstrated a maximum (70.27%) egg count reduction in sheep treated with aqueous extracts at 2 g kg^{-1} body weight on day 15 after treatment, closely followed by methanolic extracts at 1 g kg^{-1} body weight on day 15 after treatment (33.17% egg count reduction) while as lethal concentration (LC_{50}) values of aqueous and methanolic extracts of *I. kashmiriana* on adult worms of gastrointestinal nematodes of sheep *H. contortus* are 18.50 mg/ml and 16.66 mg/ml respectively. Thus aqueous extracts exhibited greater anthelmintic activity under both *in vitro* and *in vivo* conditions; this could be due to the presence of water soluble active ingredients in *I. kashmiriana*. From the present study it can be suggested that *I. kashmiriana* rhizome exhibited significant anthelmintic activity against gastrointestinal nematodes of sheep and has the potential to contribute to the control of gastrointestinal nematode parasites of small ruminants.

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1. Introduction

Gastrointestinal parasitism is a significant obstacle in the breeding of sheep, goat and other ruminants (Anderson and May, 1979; Daszak et al., 2000; Pessoa et al., 2002). Parasitism, especially by helminth species, impairs health by causing lack of appetite, diarrhoea, anaemia and, in severe cases, death (Athanasiadou and Kyriazakis, 2004). Synthetic anthelmintics have been used throughout the world for decades to minimize the losses caused by helminth infection. However, anthelmintic resistance in nematodes has become a major practical problem in many countries (Varady and Corba, 1999). Parasite resistance increases costs, reduces production efficiency along with the risk of contamination of the animal products (Waller, 1994; Dewanjee et al., 2007; Saddiqi et al., 2010) and increases the risk of environmental contamination (Hammond et al., 1997). Frequent use, increased dosage and increased application rate all correlate with declining effectiveness of synthetic anthelmintics (Donald, 1994). These disadvantages have stimulated a search for alternative control methods such as the use of traditional medicinal plants. Screening and proper evaluation of medicinal plants could reveal bioactive compounds that may be sustainable and environmentally acceptable (Eguale et al., 2007; Nisa et al., 2013).

Even today, plants play an important role in the health care of about 80% of the world population and is estimated that more than half of the drugs under clinical use at present owe their origin to plants (Sarin, 1996). Plants are utilized as therapeutic agents since time immemorial in both organized (Ayurveda, Unani) and unorganized (folk, tribal and native) forms (Girach et al., 2003). Recently, there has been an increasing interest in ethnomedical and ethnoveterinary practices around the world, especially as they pertain to the use of medicinal plants in treating various ailments (Bizimenyera et al., 2006). For acceptance of medicinal plants into scientific veterinary medicine, it is necessary that their effectiveness and safety be evaluated and confirmed through *in vitro* and *in vivo* testings (Rates, 2001).

Considering the vast potentiality of plants as sources of anthelmintic agents the present study was undertaken to screen the *in vitro* and *in vivo* anthelmintic activities of *Iris kashmiriana* commonly used in ethno-veterinary medicine by many tribes of Kashmir valley (Kaul, 1997; Khan et al., 2004). There is, however, no published scientific evidence for the anthelmintic and/or antimicrobial effects of *I. kashmiriana*.

2. Materials and methods

All animal proceedings were approved by the Ethical Committee of University of Kashmir (Number: F(Ethical Com. Animal) KU/2012/419.

2.1. Collection of plant materials

I. kashmiriana Foster (Iridaceae), locally called mazarmund, is a small herb with long stem, slender rhizome, short perianth tube and flowers white with darker blotches, found on open grassy slopes at 2400–3300 feet (730–1000 m) above sea level. The plant material was collected from Ganderbal, Kashmir (34°17′04″N, 75°13′46″E Altitude 10,068 ft), during May–August 2011. The mature plant at peak of flowering was collected in polythene bags and was processed by standard technique adopted by KASH (Kashmir University Herbarium). The plant was identified and authenticated by Plant Taxonomist Prof. Irshad Ahmad Nawchoo, Department of Botany, University of Kashmir, Srinagar, India. A voucher specimen (voucher No. 1701) was deposited in KASH (Kashmir University Herbarium). The collected plants were processed for shade drying at the environmental temperatures (25–30 °C) in a well-ventilated room (Drying room at Centre of Research for Development, University of Kashmir, Srinagar). The dried plant parts were milled to a fine powder using an electric stainless steel blender. The powdered plant material was stored in an airtight container/cellophane bags at 4 °C until extraction.

2.2. Preparations of extracts

Methanolic extracts were prepared by dissolving 200 g of the powdered plant material in a conical glass percolator to which 1000 ml (Qualigens) of methanol was added. The plant material was allowed to macerate for 16 h at room temperature and the percolate was collected by filtering through non-absorbent cotton wool. The process of maceration/percolation was repeated three times. The combined filtrate was evaporated in a vacuum rotary evaporator (R-201, Shanghai Shenshen) under reduced pressure of 22–26 mmHg at 40 °C. The final crude methanol extract (8.43 g) extract was scrapped off and transferred to a container and kept airtight for storage at 4 °C until further use.

Aqueous extracts were prepared by dissolving 200 g of the powdered plant material in 500 ml of distilled water in a glass percolator. It was allowed to macerate for 24 h at room temperature and the brew was filtered using Whatman #1 filter paper. The process of percolation was repeated three times. The combined filtrate was evaporated in a vacuum rotary evaporator (R-201, Shanghai Shenshen) under reduced pressure of 22–26 mmHg at 40 °C. The final crude aqueous extract (6.5 g) was scrapped off and transferred to a container and kept airtight for storage at 4 °C until further use.

2.3. In vitro anthelmintic activity by adult motility assay (AMA)

In vitro anthelmintic activity of the plant materials was evaluated by exposing the adult *Haemonchus contortus* worms to aqueous and methanolic extracts of *I. kashmiriana* as described in our earlier work (Lone et al., 2012). Adult live and motile *H. contortus* nematodes were collected from the gastrointestinal tract of slaughtered sheep at a local abattoir. Briefly, a minimum of 20 female *H. contortus* worms was exposed in 3 replicates to each of the treatments in separate petri dishes at room temperature (25–30 °C) and two petri dishes were also set for controls (Levamisole 0.5 mg ml^{-1} positive and for 0.95% phosphate buffer saline as negative control). The inhibition of motility and/or mortality of the worms kept in the above treatments was used as a criterion for anthelmintic activity. The motility was observed after 0, 1, 2, 5 and 8 h intervals and post-treatment revival of motility (if any) was observed by keeping the treated worms in the lukewarm fresh PBS for 30 min. The number of worms found dead at 8 h

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post-treatment to aqueous and methanolic extracts of *I. kashmiriana* was compared to the control group and percentage mortality was calculated by applying formula:

$$\text{Percentage mortality} = \%\ \text{test mortality} - \%\ \text{control mortality}\ /100 - \%\ \text{control mortality}\ \times 100$$

2.4. In vivo experiment

A total of 18 Kashmir Marino sheep of both sexes (1 year of age) weighing 18–25 kg having naturally acquired gastrointestinal (GI) nematode infection were selected from the local sheep farm of Sindhbal, Ganderbal, Kashmir. The sheep were pre-adapted to the pen conditions for 20 days prior to the start of the study. Water, hay and feed were provided regularly to the study animals. The study continued for a period of 20 days post-treatments. Before the start of the study, the animals were confirmed positive with an infection of mixed gastrointestinal (GI) nematodes by faecal examination using the standard parasitological procedures applicable to detection of nematode eggs in sheep faeces (Soulsby, 1982). Faecal samples were cultured to cultivate the L_3 larvae and identified for dependable diagnosis of mixed gastrointestinal (GI) nematode infection in sheep as per the methods of Coles et al. (2006). The sheep (n = 18) used for experiment were randomly divided into 6 treatment groups of three animals each on the basis of faecal egg counts (mean ± S.E. of eggs per gram of faeces) and assigned to different treatments as given below:

- *Group I:* Treated with single dose of crude methanolic extract (CME) @ 1.0 g kg^{-1} body weight (bw).
- *Group II:* Treated with crude aqueous extract (CAE) @ 1.0 g kg^{-1} body weight (bw).
- *Group III:* Treated with single dose of crude methanolic extract (CME) @ 2 g kg^{-1} body weight (bw).
- *Group IV:* Treated with single dose of aqueous extract (CAE) @ 2 g kg^{-1} body weight (bw).
- *Group V:* Treated with single dose of Levamisole @ 7.5 mg kg^{-1} body weight (bw) as positive control.
- *Group VI:* Untreated control.

Each group was isolated from other groups and no physical contact was possible between sheep from different treatment groups; anthelmintic effectiveness was assessed as per the guidelines of World Association for the Advancement of Veterinary Parasitology (WAAVP) (Wood et al., 1995).

2.5. Faecal egg count reduction test

To determine the faecal egg count reductions of gastrointestinal (GI) nematodes in sheep, faecal samples of each animal in the respective treatment groups were collected directly from the rectum in the morning, starting from day 0 and at days 5, 10 and 15 post-treatment (PT). The faecal samples were homogenized so that the eggs were uniformly distributed throughout the faeces prior to counting. The total numbers of nematode eggs (faecal egg counts) were determined using Mac Master Egg counting technique (Soulsby, 1982), with each egg counted representing 50 eggs per gram of faeces. Faecal egg count per cent reduction (FECR%) was calculated using the formula as described by Lone et al. (2012):

$$\text{FECR}\ \% = \frac{(\text{Pre-treatment egg count per gram}) - (\text{Post-treatment egg count per gram})}{(\text{pre-treatment egg count per gram})} \times 100$$

2.6. Statistical analyses

The data of AMA, faecal egg counts and zone of inhibition were presented as mean ± standard error of mean (Lone et al., 2012). The faecal egg count reduction (FECR%) was determined by the method described by Coles et al. (1992). The data from adult mortality test, FECRT and zone of inhibition from various treatments among different days were compared using the analysis of variance (ANOVA), Duncans test by using SPSS 17.0 program for windows.

3. Results

3.1. *In vitro* anthelmintic activity

Crude aqueous extracts of *I. kashmiriana* exhibited greater anthelmintic activity against *H. contortus* than crude methanolic extract ($P < 0.05$). The aqueous extract of *I. kashmiriana* exhibited highest worm mortality (100%) while the methanolic extract resulted (85%) mortality after 6 h exposure at 50 mg/ml concentration. There was 100% mortality of worms in Levamisole (used as a reference drug) within 4 h post-exposure and no mortality of worms was observed in PBS (Table 1).

The effective dose (ED_{50}) or lethal concentration (LC_{50}) values of methanolic and aqueous extracts of *I. kashmiriana* on adult worms of gastrointestinal nematodes of sheep *H. contortus* are 18.50 mg/ml and 16.66 mg/ml respectively.

3.2. In vivo anthelmintic activity by Faecal egg count reduction test

The mean eggs per gram counts (EPG) and percentage reduction in faecal egg counts of sheep treated with different doses of the aqueous and methanolic extracts of *I. kashmiriana* and commercial anthelmintic drug (Levamisole) are presented in Table 2. The results revealed that a gradual reduction in per cent faecal egg counts (% FEC) of naturally infected animals with mixed species of gastrointestinal nematodes was treated with both the aqueous and methanolic extracts and

Table 1 *In vitro* anthelmintic efficacy of different extracts of *Iris kashmiriana* on *Haemonchus contortus* of sheep.

Treatment	Conc. (mg/ml)	Mean ± SEM of number of *Haemonchus contortus* worms showing motility (per cent mortality)					
		0 h	1 h	2 h	4 h	6 h	Fresh PBS for 30 min
Crude methanolic extract	50.00	20 ± 0.0	8 ± 0.0 (60.00)	7 ± 1.2 (65.00)	4 ± 0.3 (80.00)	3.00 ± 0.0 (85.00)	3.00 ± 0.0 (85.00)
	25.00	20 ± 0.0	14 ± 1.2 (30.00)	11 ± 0.3 (45.00)	7 ± 0.0 (65.00)	5 ± 0.0 (75.00)	5 ± 0.0 (75.00)
	12.50	20 ± 0.0	16 ± 0.9 (20.00)	12 ± 0.6 (40.00)	9 ± 0.9 (55.00)	8 ± 0.1 (60.00)	8 ± 0.1 (60.00)
Crude aqueous extract	50.0	20 ± 0.0	4.66 ± 0.8 (76.7)	3.33 ± 0.3 (83.35)	2.00 ± 0.0 (90.00)	0.00 ± 0.0 (100.00)	0.00 ± 0.0 (100.00)
	25.00	20 ± 0.0	11.33 ± 0.8 (43.35)	8.33 ± 0.8 (58.35)	6 ± 0.3 (70.00)	2 ± 0.0 (90.00)	2 ± 0.0 (90.00)
	12.50	20 ± 0.0	13.33 ± 0.8 (33.35)	12 ± 0.0 (40.00)	9.33 ± 0.3 (53.35)	5.66 ± 0.3 (71.7)	5.66 ± 0.3 (71.7)
Levamisole (positive control)	0.55mg/ml	20 ± 0.0	6.00 ± 0.4 (70.0)	2.00 ± 0.1 (90.0)	0.00 ± 0.0 (100.00)	0.00 ± 0.0 (100.00)	0.00 ± 0.0 (100.00)
PBS (negative control)	0.9%	20 ± 0.0	20 ± 0.0	20 ± 0.0	20 ± 0.0	20 ± 0.00	20 ± 0.00

SEM, standard error of mean; PBS, phosphate-buffer saline.

Table 2 Mean faecal egg counts and percentage reduction in egg counts for *I. kashmiriana* extracts-treated sheep compared with untreated controls.

Treatment groups	Mean ± SEM of eggs per gram of faeces pre- and post-treatment(per cent reduction in egg count)			
	Pre-treatment	Post treatment		
	0 day	5th day	10th day	15th day
Group I. Aqueous extract of *I. kashmiriana* 1 g/kg bw	1462.50 ± 71.8[a]	925.0 ± 59.5[b] (36.71)	813.3 ± 44.41[c] (44.38)	691.7 ± 56.00[d] (52.70)
Group II. Methanolic extract of *I. kashmiriana* 1 g/kg bw	993.2 ± 31.71[a]	807.3 ± 23.71[b] (18.71)	680.0 ± 21.24[c] (31.530)	710 ± 27.64[d] (28.51)
Group III. Aqueous extract of *I. kashmiriana* 2 g/kg bw	1099.00 ± 40.6[a]	612.3 ± 34.33[b] (44.28)	412.7 ± 26.49[c] (62.44)	326.7 ± 19.65[d] (70.27)
Group IV. Methanolic extract of *I. Kashmiriana* 2 g/kg bw	957.3 ± 36.49[a]	754.3 ± 42.60[b] (21.20)	651.7 ± 51.85[c] (31.92)	639.7 ± 33.17[d] (33.17)
Group V. Levamisole 7.5 mg/kg bw	880.4 ± 19.03[a]	60.8 ± 22.37[b] (93.09)	31.6 ± 20.46[c] (96.41)	12.6 ± 26.25[d] (98.56)

SEM, standard error of mean; bw, body weight; different letters indicate significantly different values ($P < 0.05$).

was significant ($p < 0.5$) on day 15 post treatment. The maximum reduction (70.27%) in faecal egg counts was recorded for aqueous extract followed by methanolic extract of *I. kashmiriana* (33.17%) @ 2 mg/kg body weight at day 15 post-treatment.

4. Discussion

Pharmaceutical and scientific communities have recently received the attention of the medicinal plants, as the herbal remedies prepared from the whole plant are generally safe with fewer side effects if used in the proper therapeutic dosages (Hanrahan, 2001). In view of the apparently conspicuous effect of both the extracts of *I. kashmiriana* on inhibition of motility of the *H. contortus* worms, the mortality of the worms was much faster in levamasole treatment than in crude aqueous extract and crude methanolic extract treatments. We found that methanolic extracts showed good *in vitro* and *in vivo* anthelmintic activities and this could be due to the presence of a higher concentration of the water soluble active molecule(s) in the extract. The total action of the extracts is a sum of the activities of their constituents (Rates, 2001). *In vitro* studies showed highest mortality (100%) of worms was found at 50 mg ml^{-1} concentration of aqueous extract than methanolic extracts of same concentration. Tariq et al. (2008) suggested that crude aqueous and ethanolic extracts of *I. hookeriana* exhibit significant *in vitro* anthelmintic activity against *Trichuris ovis* of sheep, and have the potential to contribute to the control of gastrointestinal nematode parasites of sheep. These observations are also in agreement with the findings of various earlier workers who tested anthelmintic efficacy of various plant species against *H. contortus* (Ketzis et al., 2002;

Bizimenyera et al., 2006; Hordegen et al., 2006; Tariq et al., 2008). Similar results 98.00% mortality of worms have been observed in our previous findings on evaluating aqueous and methanolic extracts of *Euphorbia helioscopia* (Lone et al., 2012, 2013).

We found that aqueous extracts showed highest per cent faecal egg count reduction (70.00%) in *in vivo* and this could be due to the presence of a higher concentration of the alcohol-soluble active molecule(s) in the extract. A parallel *in vivo* study to ours though using a different *Iris* species had demonstrated efficacy against *H. contortus* parasite (Eguale et al., 2007). Khare (2007) has demonstrated that dried *I. kashmiriana* is effective against round and hookworms. Ascaridole, an active constituent of the oil of same genus, is highly active against roundworms, hookworms and amoebic dysentery and intestinal infections (Khare, 2007).

5. Conclusion

The discovery of a potent remedy from plant origin will be a great advancement in anthelmintic, antimicrobial infection therapies. These results suggest that the plant extracts possess compounds with anthelmintic properties that can be further explored for antimicrobial activity. This anthelmintic study of the plant extracts demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of these plants in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases. Further work is needed to isolate the active principle from the plant extracts and to carry out pharmaceutical studies.

Conflict of interest

The authors declare no conflict of interests. Equipment brands, chemicals, and other trade names are mentioned here solely for the convenience of the reader and imply no endorsement by the authors.

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