

Anthelmintic Activities of Aqueous and Methanol Extracts of *Prunella vulgaris* L.

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Abstract

The increasing anthelmintic resistance, drug residues in animal products and high cost of conventional anthelmintics has created an interest in studying medicinal plants as an alternative source of anthelmintics. The aqueous and methanolic extracts of *Prunella vulgaris* was evaluated for anthelmintic efficacy against gastrointestinal nematodes of sheep under both *in vitro* and *in vivo* conditions using worm motility inhibition assay, egg hatch assay and faecal egg count reduction percent (FECR%) assay respectively. Crude methanol extract and crude aqueous extract of *P. vulgaris* resulted in mean percentage mortality of 94.44%, as observed after the worms were put in lukewarm PBS for 30 min after exposure to different treatments ($p < 0.01$). Crude methanol extract ($LC_{50} = 2.48$ mg/mL) had higher inhibitory effects compared to that of crude aqueous extract ($LC_{50} = 3.36$ mg/mL) on egg hatching, exhibiting higher ovicidal activity. *In vivo*, maximum reduction (92.86%) in faecal egg counts was recorded for crude methanol extract, followed by crude aqueous extract (80.34%) with dose 2 g/kg of body weight at day 15 post-treatment. The results of the present study suggest that *P. vulgaris* extracts are promising alternatives to the commercially available anthelmintics for the treatment of sheep nematodes.

Keywords: Anthelmintic; *Prunella vulgaris*; *Haemonchus contortus*; Aqueous extracts; Methanolic extracts

Introduction

Gastrointestinal parasitism is a significant obstacle in the breeding of sheep, goat and other ruminants [1-3]. Parasitism, especially by helminth species, impairs health by causing lack of appetite, diarrhea, anemia and, in severe cases, death [4]. Synthetic anthelmintics have been used throughout world for decades to minimize the losses caused by helminth infection. However, parasite resistance increases costs, reduces production efficiency along with the risk of contamination of the animal products [5-7] and increases the risk of environmental contamination [8]. 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 [9,10].

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 [11] and about 80% of the world population relies on plant origin drugs [12]. 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* testing [13].

P. vulgaris L. belonging to family Labiatae, is commonly known as self-heal and is locally known as 'Kalaveath' in Kashmir valley of India. The herb has tremendous medicinal importance and is a single traditional composite unani medicine of sore throat, common cold and head ache is without this herb [14]. The aqueous extract of this herb is recently used in clinical treatment of herpetic keratitis [15]. The herb has great medicinal value and is used as hypotensive, antibiotic, antiseptic, antirheumatic, antipyretic, antibacterial, antioxidant, diuretic and vermifuge [16,17]. Chemical analysis of *P. vulgaris* has shown that it is rich in Lupeol, stigma sterol, beta-sitosterol, pentacyclic triterpenoids and sterol glucosides being obtained from the unsaponifiable fraction from the leaves as saponifiable fraction gave lauric, stearic, palmitic, myristic, oleic and linoleic acids.

The above-mentioned reports of medicinal uses of *P. vulgaris* accompanied with the claims of traditional healers for their anthelmintic

activity [14,18] made the basis of designing this study for *in vitro* and *in vivo* scientific validation of these novel herbs in Kashmir, revealing their anthelmintic activity in combating nematode infections in sheep by local livestock owners and veterinarians.

Materials and Methods

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

Collection of plant materials

The present plant material *Prunella vulgaris* (Local name *Kalaveath*) is frequently seen throughout the Kashmir valley in areas of high altitude – wastelands, agricultural lands and on open mountainous slopes, etc. The plant material was collected from Sonamerg area of district Ganderbal, Kashmir (34° 17' 04" N, 75° 13' 46" E Altitude 10068 ft) during June- August 2010. 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. 1603) 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. The dried plant parts (leaves, stem and flowers) 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.

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Preparations of methanolic extracts

Methanolic extracts were prepared by dissolving 500 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.

Preparation of aqueous extracts

The air dried and coarsely powdered plant material (500 g) was extracted with 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.

In vitro experiment

Collection of worms and recovery of eggs: Collection of worms and recovery of eggs was done according to the method described by Jabbar et al. [19] and Lone et al. [20]. Briefly, mature live *H. contortus* female worms were collected from the abomasum of freshly slaughtered sheep then washed and finally suspended in a bottle containing lukewarm phosphate buffer saline (PBS) (pH 7.2) for further use in adult worm motility assays. For egg hatch test (EHT), some of collected worms were triturated in pestle and mortar and the suspension was filtered through sieve of a 100-mesh (150 µm pore size). The suspension that passed through the sieve was collected and washed through another size 400 mesh sieve (38 µm pore size) with warm water. The material left on the sieve was back washed and transferred into Clayton Lane tubes. Filtrate was centrifuged in Clayton Lane tubes for 2 minutes at about 300×g and supernatant was discarded. Tubes were agitated to loosen the sediment and then saturated sodium chloride solution was added until a meniscus formed above the tube. A cover slip was placed and sample re-centrifuged for 2 min at about 130 × g. Cover slip was plucked off carefully from tubes and eggs were washed off into a conical glass centrifuge tube. Tubes were filled with water and centrifuged for 2 min at about 300 × g. Supernatant was decanted and eggs were re-suspended in water. The eggs were then washed thrice in distilled water and adjusted to 500 eggs mL⁻¹ using Mac Master Egg counting technique [21] for further use in egg hatch test (EHT).

Adult Motility Assay (AMA): *In vitro* anthelmintic activity of the plant material was evaluated by exposing the adult *H. contortus* to aqueous and methanolic extracts of *P. vulgaris* as described in our earlier work [22]. Twenty adult live and motile female *H. contortus* worms were immediately transferred to the petri dishes containing plant extracts 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) all the petri dishes were kept for incubation (at 37°C, 100% relative humidity in a 5% CO₂/air mixture). Three repetitions with three replicates for each extract concentration and for each control were performed. The inhibition of motility and/or mortality of the worms kept in the above treatments were 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 post-treatment to aqueous and methanolic extracts of *P. vulgaris* were compared to the control group and percentage mortality was calculated by applying formula:

$$\text{Percent mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

Egg Hatch Assay (EHA): Egg hatch assay was conducted according to procedure described by Coles et al. [23]. Approximately, 50 eggs were collected per tube; each tube contained 1 mL of phosphate buffer saline (PBS) and 1 mL of increasing concentrations of plant extracts (75, 150, 300, 600, 1200 and 2400 µg/ml) prepared with phosphate buffer saline (PBS). In addition, levamisole (0.125 mg/mL) was used as positive control and phosphate buffer saline (PBS) as negative controls. The tubes were covered, and the eggs were incubated for 48 h at temperature of 27°C. Thereafter, the number of the first stage larvae (L₁) present per tube was counted using a dissecting microscope. All the eggs and first-stage larvae in three replicates of treatment and control plates were counted. An inhibition percent (%) of egg hatching was calculated for each extract concentration using the following modified formula of Coles et al.:

$$\text{inhibition}(\%) = 100 \times \frac{1 - X1}{X2}$$

Where X1 is the number of eggs hatched in test extracts, and X2 is the respective number in phosphate buffer saline (PBS) control.

In vivo experiment

Animals and experimental design: A total 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 [21]. Faecal samples were cultured to cultivate the L₃ larvae and identified for dependable diagnosis of mixed gastrointestinal (GI) nematode infection in sheep as per the methods of Coles et al. [22]. 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 single dose of 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) [23]; revised by Wood et al. [24].

Laboratory procedure: 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 [21]; with each egg counted representing 50 eggs per gram of faeces. Faecal egg count percent reduction (FECR %) was calculated using the formula as described by Lone et al. [25]:

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

Statistical analyses

The lethal concentration 50 (LC_{50}) of extract concentration required to prevent 50% hatching of eggs (in case of egg hatch test) was calculated from the linear regression (for $y=0$ on the probit scale) by Probit analysis. The data of AMA, fecal egg counts and larval counts was presented as mean \pm standard error of mean [25]. The fecal egg count reduction (FECR) was determined by the method described by Coles et al. [26]. The data from adult mortality test, FECRT and larval counts from various treatments among different days were analyzed ANOVA and compared by the Duncans test ($P<0.05$) using the by SPSS 17.0 program.

Results

In vitro anthelmintic activity

The anthelmintic activities of different concentrations (ranging from 12.50 mg/mL to 50 mg/mL) of crude aqueous and methanolic extracts of *P. vulgaris* were determined against adult *H. contortus* and recorded as percent mortality (Table 1). Worm mortality varied significantly ($p<0.01$) among concentrations and test extracts and among exposure periods. Control mortality was significantly lower ($p<0.01$) than that recorded for any of the test concentrations. Mortality increased over time (post-exposure) and due to ascending concentrations. After 8th h exposure the highest tested concentration of 50 mg/mL, resulted in 75% mortality, being the highest value. The CME and CAE of *P. vulgaris* resulted in mean percentage mortality of 94.44%, as observed after the worms were put in lukewarm PBS for 30 min after exposure to different treatments. Further, there was 100% mortality of worms in levamisole (used as a reference drug) within 4 h PE. There was no mortality of worms kept in PBS till 8 h PE.

Egg hatch test

Egg hatch Inhibition for *H. contortus* exposed to methanolic extracts of *Prunella vulgaris* was less compared to levamisole. Crude methanolic extract ($LC_{50}=2.48$ mg/mL) was found to has higher inhibitory effects compared to that of aqueous extract ($LC_{50}=3.36$ mg/mL) on egg hatching. Correlation of regression of the data revealed a dose dependent response of extracts for aerial parts. The regression values and correlation of coefficient of extracts and levamisole (positive control) were $y=-0.0001x+6.4026$, $R^2=0.7454$ and $y=-0.2159x+6.2447$, $R^2=0.775$ (Table 2). However, ovicidal activities of *P. vulgaris* plant was lower than that of positive control.

In vivo anthelmintic activity

Faecal egg count reduction test: For nematodes, levamisole at the recommended dosage resulted in 98.56% reduction in mean faecal egg counts, whereas for methanolic extract of *P. vulgaris* at the dose levels of 1 g/kg bw and 2 g/kg bw resulted in faecal egg counts reduction of 81.47% and 92.86%, respectively (Table 3). All groups had positive reduction in mean faecal egg counts compared to the negative control after day 15. In the control group treated with aqueous extract of *P. vulgaris*, mean undifferentiated faecal egg count values were fairly steady throughout the period of observation, with arithmetic means varying from 1212.5 ± 4.3 on day 0 (Pretreatment) post-treatment to 480.0 ± 14.4 on day 15 post-treatment.

Discussion

Pharmaceutical and scientific communities have recently received the attention of the medicinal plants being used as herbal remedies and shown them to be generally safe with fewer side effects if used in the proper therapeutic dosages [27]. Various phytochemical constituents viz., alkaloids, saponins, tanins and phenolic acids have been isolated from *P. vulgaris* [28] which either in isolation or in combination may be responsible for the anthelmintic activity [29,30]. Evidence in support of the direct effect of condensed tannin was provided by Niezen et al. [31] and Molan et al. [32] who demonstrated that the condensed tannins extracted from *L. pedunculatus*, *L. corniculatus*, *H. coronarium*, and *O. viciifolia* forages reduced the rate of larval development (eggs to L_3 larvae) by 91%, reduced the number of eggs hatching by 34%, and decreased the mobility of L_3 larvae by 30%. A number of showed that reductions in egg excretion disappeared when CT-administration was stopped [33-35], thereby suggesting that CTs temporally reduced the female worm fecundity. In addition, the presence of phenolic compounds indicates the role of antimicrobial agents [36]. This might be the reason that traditional healers in Kashmir prescribe steam inhalation of this herb to clear phlegm from chest and brain cavity, hence reducing chest infections and head ache.

The present data clearly show that *P. vulgaris* is effective against *H. contortus* as is evident from FECR rate and percent mortality of *H. contortus*. On comparing the methanolic and aqueous extracts, highest FECR rate (92.86%) was found by administration of methanolic extract at day 15 and (80.34%) in aqueous extract on the same day. This result could be explained by the fact that the methanolic extracts possess several chemical compounds, and many of them could display ovicidal action. The present study reveals that methanolic extracts had good *in vitro* and *in vivo* anthelmintic activity and this could be due to the presence of a higher concentration of the alcohol-soluble active molecule(s) in the extract, also revealed by Iqbal et al. [30]. The latter further also opined that Neem (*Azadirachta indica*, Meliaceae) crude methanolic seed extract moderately reduced EPG in sheep infected with *Haemonchus* and *Trichostrongylus*, while feeding fresh leaves to infected sheep significantly reduced worm burden caused by *Haemonchus*, *Trichostrongylus*, and *Oesophagostomum*, but showed no effect for EPG reduction as suggested by Chandrawathani et al. [37]. The total action of the extracts is a sum of the activities of their constituents [13]. It has been speculated that the anthelmintic activity of aqueous extracts of *Prunella vulgaris* against *H. contortus* may be due to presence of excessive phenolic compounds, as reported by Adedapo et al. [38]. They also maintained that aqueous extract of *Euphorbia hirta* contained excessive phenolic compounds when used to treat dogs infected with *Ancylostomacanthum*, *Toxocaracanis*, *Dipylidiumcaninum* and *Echinococcus*. Moreover, as is evident from the experiments, *P. vulgaris* was able to kill *H. contortus* in both *in vitro* and *in vivo* studies. *In vitro* studies showed highest nematode motility (100%) in the higher concentrations of methanolic extract (50 mg mL⁻¹) rather

Treatment	Mean ± SEM of number of <i>Haemonchus contortus</i> worms showing percent motility (percent mortality)				
	0 h	1 h	2 h	5 h	8 h
Crude aqueous extract 50 mg/mL	20 ± 00	8 ± 1.2(60.00)	5 ± 0.0(75.00)	3 ± 1.9(85.50)	0 ± 0.1(100.00)
Crude aqueous extract 25 mg/mL	20 ± 00	10 ± 0.9(50.00)	8 ± 0.6(60.00)	7 ± 0.9(65.50)	3 ± 0.5(85.00)
Crude aqueous extract 12.5 mg/mL	20 ± 00	11 ± 1.2(45.50)	9 ± 1.3(55.00)	7 ± 1.8(65.00)	5 ± 0.5(75.00)
Crude methanolic extract 50 mg/mL	20 ± 00	6 ± 1.2(70.00)	3.50 ± 0.9(85.00)	20 ± 00(100.00)	20 ± 00(100.00)
Crude methanolic extract 25 mg/mL	20 ± 00	8 ± 1.0(60.00)	6 ± 0.3(70.00)	4 ± 0.9(80.00)	1 ± 0.7(95.00)
Crude methanolic extract 12.5 mg/mL	20 ± 00	9 ± 0.9(55.00)	8 ± 0.3(60.00)	6 ± 1.2(70.50)	2 ± 1.7(80.50)
Levamisole (positive control) 0.55 mg/mL	20 ± 00	3 ± 00(64.00)	20 ± 00(100.00)	20 ± 00(100.00)	20 ± 00(100.00)
PBS (negative control) 0.9%	20 ± 00	20 ± 00	20 ± 00	20 ± 00	18 ± 00

Table 1: *In vitro* anthelmintic efficacy of crude extracts of *Prunella vulgaris* L. on *Haemonchus contortus*.

Treatment	LC ₅₀	Regression values and correlation of regression
Methanolic extract of <i>P. vulgaris</i>	2.48	y=-0.0001x+6.4026, R ² =0.7454
Aqueous extract of <i>P. vulgaris</i>	3.36	y=-0.0003x+6.7332, R ² =0.9136
Levamisole	1.88	y=-0.2159x+5.2447, R ² =0.6475

Table 2: Regression values and correlation of regression of the effect of different extracts of *Prunella vulgaris* L. on egg hatching of *Haemonchus contortus*.

Treatment	Fraction	Mean ± SEM of eggs per gram of faeces pre- and post-treatment (percent reduction in egg count)			
		Pre-treatment Day 0	Post-treatment		
			Day 5	Day 10	Day 15
Group I and II <i>P. vulgaris</i> 1 g/kg bw	Methanol	1450.0±35.4 ^a	610.0 ± 20.4 ^b (57.93)	544.0 ± 20.4 ^c (62.48)	268.6 ± 16.6 ^d (81.47)
	Aqueous	1212.5±4.3 ^a	910.5±3.6 ^b (24.87)	793.50 ± 1.4 ^c (34.52)	480.0 ± 14.4 ^d (60.39)
Group III and IV <i>P. vulgaris</i> 2 g/kg bw	Methanol	742.6±2.92 ^a	282.6±1.7 ^b (61.94)	226.3 ± 10.05 ^b (69.52)	53.0 ± 2.5 ^d (92.86)
	Aqueous	1287.00±7 ^a	760.00 ± 2.6 ^b (40.94)	610.00 ± 1.7 ^c (52.60)	252.90 ± 2.4 ^d (80.34)
Group V Levamisole (Positive control)	-	880.4±10.0 ^a	60.8 ± 12.37 ^b (93.09)	31.6 ± 20.46 ^c (96.41)	12.6 ± 0.25 ^d (98.56)
Group VI Untreated (Negative control)	-	682.6 ± 4.95 ^a	697.6 ± 2.92 ^b	705.0 ± 2.79 ^c	742.6 ± 3.93 ^d

SEM=Standard error of mean; bw=Body weight; Different letters indicate significantly different values (P<0.05)

Table 3: Mean faecal egg counts and percentage reduction in egg counts for *Prunella vulgaris* L. extracts-treated sheep compared with untreated controls.

than in aqueous extracts of the same concentration. It is possible that tannins contained in the extracts of *Prunella vulgaris* produce similar effects causing death of *H. contortus* worms in *in vitro* studies, a fact also maintained by Kane et al. [29] and Tariq et al. [39]. Larvicidal and ovicidal effects of some plants have also been reported earlier against *H. contortus* eggs and larvae [11,25,40-43]. Amarante et al. [44] found that species of GI nematodes resistant to articular anthelmintic drugs showed higher ED₅₀/LC₅₀ value than susceptible species. The present study, however, confirmed the anthelmintic activity of *P. vulgaris* against nematode parasites. As per the World Association for Advancement of Veterinary Parasitology (WAAVP) guidelines, a FEC reduction of 99% or higher is considered to be highly efficacious, whereas an 80% or around is just adequate [45]. While considering these guidelines it can be inferred that *P. vulgaris* do possess adequate anthelmintic activities.

Conclusions

From the above results, it is concluded that *P. vulgaris* used, traditionally to treat intestinal worm infections, showed significant anthelmintic activity [46]. The experimental evidence obtained in case of *H. contortus* could provide a rationale for the traditional use of this plant as anthelmintic. Moreover, quality controlled extracts or possibly isolated bioactive compounds could be a promising alternative to conventional anthelmintics for the control of GIP in small ruminants in future [47]. However, the mechanisms of their effectiveness still remain to be tested *in vivo*. For this purpose, safety and toxicity studies must be conducted *in vivo* to determine the minimum non-lethal concentrations needed for the treatment of nematode infections [48].

Conflict of Interest Statement

The authors declare no conflicts of interest. 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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