Evaluation of Anthelmintic and Antimicrobial Activity of the Methanolic Extracts of *Nepeta cataria*

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Abstract: A worm motility inhibition assay was used for in vitro study and a faecal egg count reduction assay used for an in vivo study. The in vitro study revealed anthelmintic effects of crude methanolic extracts of *Nepeta cataria* (MENC) on live *Haemonchus contortus* worms ($P > 0.05$) as evident from their paralysis and/or death at 8 h after exposure. The in vivo anthelmintic activity of the extracts in sheep naturally infected with mixed species of gastrointestinal nematodes demonstrated a maximum (73.69%) egg count reduction in sheep treated with methanolic extracts at 2 g kg^{-1} body weight on day 15 after treatment. Various concentrations ranging from 100-500 mg/ml of the extract were subjected to screen the antimicrobial potential of the herb by disc diffusion methods against some selected animal pathogenic bacterial and fungal strains like *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, *Pasteurella multocida*, *Klebsiella pneumonia, Aspergillus flavus* and *Candida albicans*. The extract was found to inhibit the growth of all the bacterial and fungal test organisms, showing maximum inhibitory effect against *S. aureus*, *P. multocida* and *E. coli* while as mild inhibitory effect was observed against *A. flavus* among the selected strains. The effect produced by the different extract concentrations was comparable with the standard antibacterial agent Streptomycin sulphate and with the standard antifungal agent Nystatin, which were used as effective positive control in the study. From the present study it can be concluded that the leaves of the herb exhibit significant anthelmintic against gastrointestinal nematodes of sheep and has the potential to contribute to the control of gastrointestinal bacteria, fungi and nematode parasites of small ruminants.

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Introduction

Medicinal plants represent a rich source of antimicrobial and anthelmintic agents. They are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava *et al.*, 1996). A wide range of medicinal plant parts is used for extraction of raw drugs with varied medicinal properties.

Nematode infections of gastrointestinal tract adversely affect productivity of small ruminants all over the world especially in tropical and sub-tropical countries. Options of using synthetic anthelmintcs are decreasing due to development of resistance in gastrointestinal nematodes of small ruminants against several families of drenches (Waller, 1994; Siddiqi *et al.,* 2010). This global problem has created interest in researches on alternates to the use of synthetic chemicals for the control of nematodes (Waller, 1999). In this regard, traditionally used ethnobotanicals with anthelmintic properties are considered among the novel approaches particularly in temperate and tropical countries (Akhtar *et al.,* 2000). Majority of the ethnoveterinary medicine surveys and validation studies indicate much wider and effective use of plants as anthelmintics compared with other diseases/ conditions (Jabbar *et al.,* 2007; Hussain *et al.*, 2008; Al-Shaibani *et al.,* 2009; Deeba *et al.,* 2009 and Sindhu *et al.,* 2010).

Considering the vast potentiality of plants as sources of antimicrobial and anthelmintic drugs the present study was undertaken to screen the potential of methanolic extracts of *Nepeta cataria* for antimicrobial and anthelmintic activity.

Materials And MethodS

Plant material

The plant material *Nepeta cataria* an annual or perennial herbs locally called as *Gandh soi* (Lamiaceae) was collected from Sonamarg (34° 17' 04" N, 75° 13' 46" E Altitude 12068 ft), Kashmir and the taxonomic identification was made by Dr. Irshad A. Nawchoo, Department of Botany, University of Kashmir, Srinagar, India. The voucher specimen was numbered and kept in our research laboratory for further reference.

Extraction of plant material

Plant materials were washed with distilled water, dried in shade, grinded to fine powder and stored in airtight container at room temperature in the dark until used [Tetyana *et al*., 2002]. The powdered samples were subjected to extraction using methanol following the method of Nair *et al*., (2005)

Test organism

The antimicrobial activity of Methanolic Extract of *Nepeta cataria* (MENC) was assayed against some bacterial and fungal strains which include *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, *Pasteurella multocida*, *Klebsiella pneumonia, Aspergillus flavus* and *Candida albicans*.

Anti-bacterial Activity: Different concentrations of the MENC prepared, were tested by the disc diffusion method [Anonymous, 1996] for the antibacterial activity against five strains of bacteria *E. coli, P. aeruginosa, S. aureus*, *P. multocida* and *K. pneumonia*. The test microorganisms were seeded into respective medium by spread plate method with the 24h cultures of bacteria grown in nutrient broth. After solidification the filter paper discs (5 mm in diameter) impregnated with the extracts were placed on test organism-seeded plates. Streptomycin sulphate (200µg/ml) was used as positive control, the antibacterial assay plates were incubated at 37°C for 24h and the diameters of the inhibition zones were measured in mm.

Antifungal Activity: The antifungal activity was tested by disc diffusion method [Taylor *et al*., 2005] on Sabourd's agar plates inoculated with 10 day old fungal culture by point inoculation. Filter paper discs (5 mm in diameter) impregnated with different concentrations of the extract was placed on test organism seeded plates. Nystatin (200µg/ml) was used as positive control. The activity was determined after 72 h of incubation at 30°C and the diameters of the inhibition zones were measured in mm.

In vitro experiment for

For the evaluation of anthelmintic activity of the extract under in vitro conditions against adult *H. contortus*, the worm motility inhibition assay was adopted. Mature *H. contortus* worms were collected from the abomasa of freshly slaughtered sheep. The worms were washed and suspended in phosphate buffered saline (PBS) and the transported to the laboratory. MENC in 0.5% dimethyl sulphoxide (DMSO) were tested at 25 mg ml^{-1}. PBS (0.9%) was the negative control. Ten worms were exposed to each of the treatments at controlled temperature (37±1 ^{o}C). Three replicates were performed for each treatment. Inhibition of worm motility was the rationale for anthelmintic activity. The time required for paralysis or complete inactivity and mortality was recorded at 0, 1, 2, 5 and 8 h intervals. After 8 h the parasites resuspended in luke warm PBS (0.9%) for 30 minutes to test the revival of the worm motility. Percent worm motility inhibition (% WMI) was determined according to Rabel *et al*., (1994) by the following formula:

$$\%WMI = 151\frac{\text{number of mobile worms in negative control Petri dish} - \text{number of mobile worms in treatment Petri dish}}{\text{number of mobile worms in negative cotrol Petridish}} X\,100$$

The mortality index was calculated by the following formula

$$\text{Mortality Index (MI)} - \frac{\text{Total number of mobile worms (Death)}}{\text{Total number of worms per Petridish}} \text{ x } 100$$

In vivo experiment

For the evaluation of anthelmintic activity of the extract under in vivo conditions the faecal egg count reduction (FECR) assay in sheep harbouring a naturally acquired GI nematode infection was adopted. The animals were pre-adapted to the pen conditions for 18 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 15 days post-treatments. Before the start of the study, the animals were confirmed positive with an infection of mixed 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 L3 larvae and identified for dependable diagnosis of mixed GI nematode infection in sheep as per the methods of Coles *et al*., (2006) and MAFF (1986). The animals used for the study were randomly divided into two treatment groups of one animal each on the basis of faecal egg counts (mean ± S.E. of eggs per gram of faeces) and assigned to different treatments which were administered orally using a syringe. Group 1 received a single dose of MENC at 1.0 g kg^{-1} body weight (BW). Group 2 received no treatment and served as negative control. Each group was isolated from other groups and no physical contact was possible between animals from different treatment groups.

Laboratory procedure

To determine the faecal egg count reductions of 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 Stoll's technique (Soulsby, 1982); with each egg counted representing 50 eggs per gram of faeces. Faecal egg count percent reduction (FECR %) was calculated using the following formula:

$$FECR\% = 198 \frac{(\text{pre} - \text{treatment egg count per gram} - \text{Post} - \text{treatment egg count per gram})}{(\text{pre} - \text{treatment egg count per gram})} x\ 100$$

Results

In vivo anthelmintic activity

The in vivo anthelmintic activity (in terms of reduction of nematode egg output) of MENC in naturally infected sheep with mixed species of GI nematodes demonstrated significant anthelmintic activity. In vivo anthelmintic activity of Methanolic Extract of *Nepeta cataria* demonstrated a maximum faecal egg count reduction (FECR) of 73.69% in sheep treated on day 15 post treatment (PT), table 1. A progressive decline in the faecal egg count in all treated animals with extracts was observed from day 5 to day 15 PT.

Table 1. Mean faecal egg counts and percentage reduction in egg counts for MENC extracts-treated sheep compared with untreated controls.

Parasite	Mean ± SEM of eggs per gram of faeces pre- and post-treatment			
	Pre-treatment Day 0	Post-treatment		
		Day 5	Day 10	Day 15
Group I Methanolic extract of *Nepeta cataria* 2g kg^{-1}body weight	918.4 ± 5.03	770.8±22.37 (14.22)	501.6 ±20.46 (44.94)	261.6 ± 1.43 (73.69)
Group II (Levomisol 7.5mg kg^{-1} body weight)	1056.4 ±26.48	71.8 ± 11.62 (93.20)	43.4 ± 6.95 (95.89)	23.6 ± 3.64 (97.76)
Group III Untreated (Controll)	742.6 ±3.93	705.0 ±2.79 (5.06)	697.6 ±2.92 (6.05)	682.6 ±4.95 (8.07)

Figures in parentheses indicate faecal egg count percentage reduction (FECR %).

In vitro anthelmintic activity

The extract demonstrated the time dependent anthelmintic activity against *H. contortus* has revealed from the inhibition of motility and/or death of the worms after treatment (table 2). It resulted in mean percentage worm motility inhibition (%WMI) of 94.44%, as observed after the worms were put in lukewarm PBS for 30 minutes after exposure to different treatments. The mean mortality index (MI) of the MENC was 0.95. The worms that were exposed to Levamisole were found to be paralysed and/or dead at 5 h (100% mortality or paralysis). The result of in vitro anthelmintic activity of MENC compared with Levamisole was statistically non-significant, and was statistically significant compared with PBS.

Table 2. In vitro anthelmintic efficacy of crude methanolic extracts of MENC on *Haemonchus contortus* of sheep

Treatment	Mean ± SEM of number of *Haemonchus contortus* worms showing motility up to 8 h exposure				
	0 h	1 h	2 h	5 h	8 h
Levamisole 0.5 mg	10.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
MENC 50 mg	10.00±0.0	1.00±0.6	0.00±0.0	0.00±0.0	0.00±0.0
MENC 25 mg	10.00±0.0	2.33±0.7	0.67±0.7	0.00±0.0	0.00±0.0
MENC 12.5 mg	10.00±0.0	7.33±2.2	3.33±0.9	1.00±0.6	0.00±0.0
PBS	10.00±0.0	10.00±0.0	10.00±0.0	10.00±0.0	8 ± 0.00

SEM, standard error of mean; PBS, phosphate-buffered saline; CME, crude methanolic extract

Antimicrobial Activity:

Results obtained in the present study (table. 1) revealed that the Methanolic Extracts of *Napeta cataria* (MENC) possess potential antibacterial activity as compared to its antifungal activity. When tested by disc diffusion method the MENC showed significant activity against *S. aureus*, *P. multocida* and *E. coli*. The overall highest antibacterial activity of 28.24±0.14 mm recorded in *P. multocida* was measured at a concentration of 500mg/ml and least inhibition zone diameter, 3.66±0.57 mm was recorded for *S. aureus* at a concentration of 100mg/ml MENC, the only positive result for its lowest concentration. The Antifungal activity of the extract was observed at the concentration of 200mg/ml and above with maximum inhibition zone of 15±0.88 mm against *A. flavus* at a concentration of 500mg/ml and minimum inhibition zone of 5.03±0.011 mm against *C. albicans* at a concentration of 300mg/ml.

Table. 3. Antimicrobial activity of MENC in terms of zone of inhibition (mm)

Test organism	Zone of Inhibition in mm					Streptomycin sulphate (200 µg / ml)	Nystatin (200 µg / ml)
	Concentration of MENC in mg/ml						
	100	200	300	400	500		
E. coli	-	8.50±0.09	12±0.33	18.67±0.16	20±1.20	22.13±0.25	NPC
P.aeruginosa	-	7.03±0.11	8±0.57	11±0.33	14±0.33	15±0.33	NPC
S. aureus	3.66±0.57	9.00±0.06	16±0.57	20.0±0.11	28.24±0.14	22±0.57	NPC
P. multocida	-	-	10±0.33	16±0.57	22.13±0.25	28.24±0.14	NPC
K.pneumoniae	-	3.80±0.16	8.50±0.09	13±0.57	16±0.57	18±1.20	NPC
A. flavus	-	-	-	9±0.33	15±0.88	NPC	22±0.57
C. albicans	-	-	5.03±0.011	11±0.57	15±0.66	NPC	18.67±0.16

Values are mean inhibition zone (mm) ± S.D of three replicates; NPC= Not taken as Positive Control

Discussion

***In vitro* and *in vivo* anthelmintic activity:**

For the effective chemotherapy and strategic chemprophylaxis of Haemonchosis, a safe drug is required with high activity against all stages of *H.contortus*. Hence the development of newer, safe, curative and economical drug has remained an active area of research. *Nepeta cataria* exhibited antihelminthic activity against *H. contortus* as is evident from larval mortality of worms. A wide variation, however, was recorded in the antihelminthic effects among different concentrations as far as the intensity and dose dependent effects were concerned. The main route of acquisition of broad-spectrum antihelminthics by nematodes appears to be via trans-cuticular diffusion as proposed to oral ingestion (Ho *et al.,* 1994; Sims *et al.,* 1996). We found that MENC showed good In vitro anthelmintic activity and this could be due to the presence of a higher concentration of the alcohol-soluble active molecule(s) in the extract.

The In vivo antihelminthic activity of MENC which demonstrated a maximum faecal egg count reduction (FECR) is in agreement with Akhtar and Javed (1991), Keyyu *et al.* (2003), Itty *et al.* (1997), Waller *et al,* (2001), Maqbooi *et al* (2004), Githiori *et al* (2005, 2006) and Stafford *et al* (2007) against ovine Gastrointestinal helminths.

Results of the present study suggest that the extract exhibited significant in vitro anthelmintic activity against *H. contortus* of sheep, and has the potential to contribute to the control of gastrointestinal nematode parasites of sheep. Iqbal *et al.* (2005) reported higher in vitro anthelmintic activity in crude aqueous extracts as compared to crude methanol extracts and crude powder against *H. contortus*, which they considered as an indication of presence of water soluble active ingredients in *Calotropis procera* latex. Iqbal *et al.* (2006) also revealed significant in vitro anthelmintic activity of crude aqueous extracts and crude methanol extracts of *Swertia chirata* against *H. contortus.* Garg and Atal (1963) also reported in vitro paralysis of *Oesophagostomum columbianum* and *Bunostomum trigonocephalum* 4 and 6 h, respectively, after treatment with 1% calotropin isolated from the latex of *C. procera*. Al-Qarawi *et al*. (2001) observed a concentration-dependent larvicidal activity against *H. contortus* in vitro within 20 min of application of *C. procera* latex. Khobragade *et al.* (1994) found that crude aqueous extracts of *Allium sativum* caused complete cessation of motility (death) of *B. trigonocephalum* after 6, 12, 16 and 20 h of exposure in concentrations of 200, 100, 50, and 25mg/ml respectively.

In conclusion, the results obtained from the present study on *Nepeta cataria* against gastrointestinal nematodes of sheep suggest an alternative to the use of commercially available anthelmintics for the treatment of gastrointestinal nematodiasis in sheep. There is a need to undertake detailed phytochemical and pharmacological studies of the anthelmintic activity of *Nepeta cataria*. Research is recommended for anthelmintic studies on experimentally induced infections of particular species of gastrointestinal nematodes.

Antimicrobial Activity:

The presence of antifungal and antimicrobial substances in the plants is well established as they have provided a source of inspiration for novel drug compounds as plant derived medicines have made significant contribution towards human health. Phytomedicine have been used for the treatment of diseases as in done in cases of Unani and Ayervedic system of medicines, a natural blueprint for the development of new drugs. The development of new and useful chemotherapeutic agents requires the raw materials in the form of potentially useful compounds provided by plants. Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants. (Alim *et al*., 2009; Behera and Misra 2005; Bylka *et al*., 2004; Ghalem and Mohamed, 2008; Govindarajan *et al*., 2006; Kumaraswamy *et al*., 2002; Palombo and Semple, 2001; Reddy, 2010; Samy and Ignacimuthu, 2000; Singh *et al*., 2011; Stepanovic *et al*., 2003; Viji and Murugesan, 2010) The present investigations revealed that, the different concentrations of the MENC proved to be effective and are concentration dependent against different strains of bacteria and fungi tested. The antimicrobial activity of the MENC may be related to the monoterpenoid component *i.e.* nepetalactone present in the extracts confirmed by a study. (Zenasni *et al*., 2008) The antimicrobial effects of the extracts could be explained by disturbance of the permeability barrier of the bacterial membrane structure (Cowan, 1999). Indeed recent findings revealed that tea tree oil damages the cell membrane structure of *E. coli*, *S. aureus* and *Candida albicans*. (Cox *et al*., 2000) Such a phenomenon is due to the penetration of monoterpenes through the cell wall and cell membrane. In fact, monoterpenes are lipophilic, and may induce the expansion of cell membranes, increases fluidity, destroy the membrane structure and inhibit membrane embedded enzymes. (Cox *et al*., 2000 and 2001)

The lack of antibacterial activity in some of the concentrations of the extract is not surprising as a number of plant extracts have been found ineffective against certain test organisms at lower concentrations and may be attributed to the presence of lesser amounts of the antimicrobial compounds. Medicinal plant can be poisonous if wrong plant parts or wrong concentrations are used. (Frhone, 1999) Some compounds from plants may be toxic in higher doses. Tussilagone isolated from *Tussilago farfara* is a potent cardiovascular and respiratory stimulant but it has LD_{50} in mice 28.9 mg/kg. (Li and Wang, 1988) Much of the exploration and utilization of natural product as antimicrobial arise from microbial sources.

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