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# *IN VITRO* ANTHELMINTIC EFFECT OF ETHANOLIC AND AQUEOUS EXTRACTS OF *CALOTROPIS PROCERA*, *AZADIRACHTA INDICA* AND *PUNICA GRANATUM* ON *TRICHURIS GLOBULOSA*, AN INTESTINAL NEMATODE OF SHEEP

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**ABSTRACT**
Indigenous system of herbal therapy is becoming an increasingly attractive approach to control parasitic infection particularly in developing countries. Among parasites, helminth infections are responsible for considerable losses to the livestock industry of marginal farmers. The aim of the present study was to evaluate anthelmintic effect of both ethanolic and aqueous extracts of *Calotropis procera* flowers, *Azadirachta indica* leaves and *Punica granatum* fruit peel in comparison with albendazole on *Trichuris globulosa* a nematode, parasitizing the large intestine of sheep, through *in vitro* studies by the worm motility inhibition assay. LC-50 values were determined to be 4.21 mg/ml ± 1.26 and 4.77 mg/ ml ± 1.00 for *C. procera*, 5.85 mg/ml ± 1.38 and 7.49 mg/ml ±1.98 for *A. indica*, 9.56 mg/ml ± 2.78 and 8.75mg/ml ± 2.00 for *P. granatum*, ethanolic and aqueous extracts respectively, whereas it was 10.73µg/ ml ± 2.88 for albendazole. The mean mortality index (MI) was 0.83 and 0.33 for *C. procera*, 0.66 and 0.5 for both *A. indica* and *P. granatum*, ethanolic and aqueous extracts respectively whereas for albendazole it was 1.0. Percent mean worm motility inhibition (%WMI) was observed to be between 45.8 and 100 % for different extracts after the worms were given lukewarm PBS treatment for 30 min after exposure to different treatments for 3 h. It is concluded that all the three plants possess significant anthelmintic activity and could be potential alternative for treating cases of helminth infection in ruminants.

**KEYWORDS** Anthelmintic *Calotropis procera*, *Azadirachta indica*, *Punica granatum Trichuris globulosa*, nematode.

## INTRODUCTION

Parasitic helminths affect animals and man, causing considerable hardship and stunted growth. Most diseases caused by helminths are of a chronic, debilitating nature; they probably cause more morbidity and greater economic and social deprivation among humans and animals than any single group of parasites. The prevalence of helminth diseases is very high, especially during the wet season when infection is as high as 100% in cattle. Such high infection rates prevent them from attaining optimum productivity, especially under the traditional husbandry system. The major control strategy adopted against helminth parasites is the use of anthelmintics. However, the high cost of modern anthelmintics has limited the effective control of these parasites. Although the use of alternate drugs has also been advocated as a measure to avoid the development of resistant strains of helminth parasites, and as a means of reducing the cost of controlling helminthic diseases, the emergence of resistant strains of pathogenic helminth has stimulated the desire to search for additional chemotherapeutic agents that might allow more efficient control of helminth parasites.[1] A practical solution to this is to develop effective drugs from reasonably less expensive and available raw materials. This can rationally be approached through the study of indigenous traditional plant remedies.

*Calotropis procera* (Ait.) R.Br. (giant milkweed) belong to the family Asclepiadaceae, locally known as "aak" is being used as herbal medicine by people living in desert areas. Extracts from this plant have been found to possess various pharmacological activities. The flowers as well as latex of the plant have been claimed to be useful as an anthelmintic.[2]

*Azadirachta indica* (Meliaceae), whose common name is neem, has been investigated in the control of gastrointestinal nematodes of ruminants, but its real efficacy is still not well clarified scientifically. In cattle, the consumption of dried leaves caused a reduction in the number of eggs per gram of feaces.[3]

*Punica granatum* Linn. (Pomegranate) commonly known as anar is an ancient fruit with great medicinal importance related to Punicaceae family.The fruits of *P. granatum* have been used to treat acidosis, dysentery, microbial infections, diarrhoea, helminthiasis, haemorrhage, and respiratory pathologies.[4]

The crude ethanolic and aqueous extract of *C. procera* flower, *A. indica* leaf and *P. granatum* fruit peel have shown anthelmintic effect on soft bodied trematode parasite of sheep, *Gastrothylax indicus*[5] and also altered the activities of several tegumental[6,7,8] and lipogenic enzymes.[9,10]

The present study was undertaken to find the unexplored anthelmintic potential of crude ethanolic and aqueous extract of *C. procera* flower, *A. indica* leaf and *P. granatum* fruit peel in comparison with albendazole on *Trichuris globulosa* a nematode, parasitizing the large intestine of sheep.

## MATERIALS AND METHODS

### Plant material

Flowers of aak (*Calotropis procera*), leaves of neem (*Azadirachta indica*) and fruit peel of anar (*Punica granatum*) were collected from in and around Chandigarh. The plant materials were identified in Department of Botany, Panjab University, Chandigarh with Voucher numbers- aak 4830, neem 5028 and anar 8583.

### Preparation of extracts

Flowers of *C. procera*, fresh leaves of *A. indica* and fruit peel of *P. granatum* were washed thoroughly, shade dried and grounded by motor driven grinder into powder form. Both ethanolic and aqueous plant extracts were prepared.[2] Ethanolic flower extract of *C. procera* (EFECP), leaf extract of *A. indica* (ELEAI) and fruit peel extract of *P. granatum* (EFPEPG) were exhaustively extracted by mixing 80 gm of powdered plant material and adding approximately 300 ml of ethanol in a soxhlet apparatus. Aqueous extracts were prepared by dissolving 100 gm of powdered plant material mixed with 500 ml of distilled water in1 L flask and boiled for 4–6 h in water bath. It was allowed to macerate at room temperature for 24 h and the brew was filtered through muslin gauze and Whatman filter paper No.1. Both ethanolic and aqueous extracts of plant materials were evaporated in Rota evaporator to give crude ethanolic and aqueous extracts. The extracts were scraped off and transferred to screw capped vials at 4 $^{o}$C until used. Phytochemical analysis of extracts revealed the presence of phenols, alkaloids, saponins, tannins, flavonoids, steroids and triterpenoids.[5]

### *In vitro* anthelmintic activity of extracts

Worm motility inhibition assay was employed for the evaluation of anthelmintic activity of crude ethanolic and aqueous extracts (AFECP, ALEAI, AFPEPG) of all the three plant materials (aak flower, Neem leaf and Anar fruit peel) under *in vitro* conditions.[11] The *in vitro* anthelmintic activity was carried out on adult *T. globulosa* worms to determine the inhibitory effect of extracts on adult worms. Mature *T. globulosa* were collected from the large intestine of sheep/goat procured from local slaughter house. The worms were washed in phosphate buffered saline (PBS pH 7.2) and finally suspended in PBS. The study was conducted at four different dilutions of all the extracts viz., 0.781,1.562,3.125 and 6.25 mg/ml prepared in PBS. The crude aqueous extracts were diluted in PBS,whereas,crude ethanolic extracts in 1 % DMSO in PBS. Albendazole dissolved in 1 % DMSO and diluted in PBS at concentrations of 2,5,10,20 µg/ml and PBS alone served as postitive and negative control respectively. There were four replicates for each treatment concentration. Six vigorously motile worms were placed in each petri dish containing 4 ml of solution and observations were made at 0,1,2,3,4 h intervals. After exposure to different treatments, the worms were put in lukewarm PBS for 30 min in order to confirm mortality.

(i) LC-50 values of each plant extract and positive control were calculated by biostat software.

(ii) Percentage worm motility inhibition (%WMI) was determined using formula.[12]

$$\%WMI=\frac{number\ of\ mobile\ worms\ in\ negative\ control-number\ of\ mobile\ worms\ in\ treated\ group}{number\ of\ mobile\ worms\ in\ negative\ control}\ X\ 100$$

(iii) The mortality index (MI) was calculated by following formula:

$$MI=\frac{\text{Total number of immobile worms ( dead )}}{\text{total number of worms per petri dish}}$$

### Statistical analysis

Experimental results are expressed as mean ± S.D. All measurements were replicated four times. The data was analysed by analysis of variance ($p< 0.05$).

## RESULTS

Anthelmintic activity of both ethanolic and aqueous extracts of *Calotropis procera* flowers, *Azadirachta indica* leaves and *Punica granatum* fruit peel in comparison with albendazole was evaluated through *in vitro* studies by the worm motility inhibition assay in *Trichuris globulosa* (Table 1 and 2 ).

**Table 1: *In vitro* anthelmintic activity of ethanolic extracts of *C.procera, A.indica* and *P.granatum* on *T.globulosa*.**

| Time(h) | Dosing | | | | |
|---|---|---|---|---|---|
| | EFECP 3.125(mg/ml) | ELEAI 3.125(mg/ml) | EFPEPG 6.25 (mg/ml) | Albendazole 5.0µg/ml | Control (PBS) |
| | Worms showing motility (%mortality) | | | | |
| 0 | 6±0.0 | 6±0.0 | 6±0.0 | 6±0.0 | 6±0.0 |
| 1 | 4.25±0.5(29.1)** | 5.25±0.5(12.5) | 5.75±0.45(4.1) | 3.66±0.57(39.0)** | 6±0.0 |
| 2 | 3.5±0.5(41.6)** | 3.75±0.5(37.5)* | 4.25±0.5(29.1)* | 2.33±0.57(61.1)*** | 6±0.0 |
| 3 | 1.25±0.5(79.1)*** | 2.25±0.5(62.5)** | 2.25±0.45(62.5)*** | 0.0±0.0(100) | 6±0.0 |
| PBS For 30 min | 1.25±0.5 | 2.25±0.5 | 2.25±0.45 | 0±0.00 | 6±0.0 |

All values represent mean±S.D, n=4. Each replica (n) had 6 worms.
*** p<0.0005 Extremely significant, ** p<0.0005 very significant, * p<0.0005 significant

**Table 2: *In vitro* anthelmintic activity of aqueous extracts of *C.procera, A.indica* and *P.granatum* on*T.globulosa.***

| Time(h) | Dosing | | | | |
|---|---|---|---|---|---|
| | AFECP 3.125(mg/ml) | ALEAI 3.125(mg/ml) | AFPEPG 6.25 (mg/ml) | Albendazole 5.0µg/ml | Control (PBS) |
| | Worms showing motility (%mortality) | | | | |
| 0 | 6±0.0 | 6±0.0 | 6±0.0 | 6±0.0 | 6±0.0 |
| 1 | 5.25±0. 5(12.5) | 5.5±0.5(8.3) | 5.25±0.51(12.5) | 3.66±0.57(39.0)** | 6±0.0 |
| 2 | 4.25±0.5(29.1)** | 4.5±0.5(25)** | 4.75±0.5(20.8)** | 2.33±0.57(61.1)*** | 6±0.0 |
| 3 | 2.25±0.5(62.5)** | 3.25±0.5(45.8)** | 2.75±0.5(54.1)** | 0.0±0.0 | 6±0.0 |
| PBS For 30 min | 2.25±0.5 | 3.25±0.5 | 2.75±0.5 | 0±0.00 | 6±0.0 |

All values represent mean±S.D, n=4. Each replica (n) had 6 worms
*** p<0.0005 Extremely significant, ** p<0.0005 very significant

***Calotropis procera***

EFECP and AFECP demonstrated dose dependent and time dependent (p<0.0005) anthelmintic activity against *T. globulosa* as revealed from the inhibition of motility and/or death of the worm after treatment. Time taken for cessation of motility for EFECP and AFECP on adult *T. globulosa* at 0.781,1.562,3.125,6.25 mg/ml concentration has been depicted in histogram (Fig 1).

LC-50 value for EFECP was 4.21 mg/ml± 1.26 and for AFECP was 4.77 mg/ml± 1.00. EFECP resulted in mean %WMI of 79% while the AFECP of 62.5% as observed after the worms were put in lukewarm PBS for 30 min after exposure to different treatments for 3 h. The mean MI of EFECP and AFECP was 0.83 and 0.33 respectively. It was found to be dose dependent irrespective of solvent used when compared with negative control.

However comparing the EFECP and AFECP with albendazole, a conventional anthelmintic drug commonly used in study area positive control, a significant difference was observed. EFECP was found to be more potent than the AFECP as better mortality was seen in ethanolic extract with 3 h exposure.

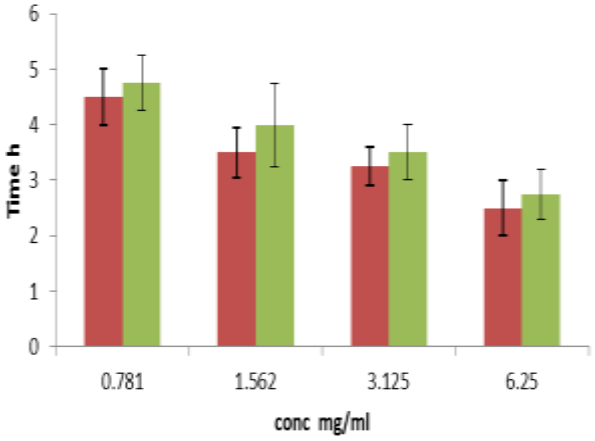


**Fig 1: Histogram showing dose dependent and time dependent response of different concentrations (0.781- 6.25 mg/ml) of EFECP (dark-red) and AFECP (light -green) *in vitro* on *T.globulosa.***

***Azadirachta indica***

ELEAI and ALEAI showed dose dependent and time dependent (p<0.0005) anthelmintic activity against *T. globulosa* as observed from the inhibition of motility and/or death of the worm after treatment . Dose dependent mortality was observed at 0.781, 1.562, 3.125, 6.25 mg/ml concentrations for both ELEAI and ALEAI on adult *T. globulosa* as shown in fig 2 LC-50 for ELEAI and ALEAI was 5.85 mg/ml± 1.38 and 7.49 mg/ml± 1.98 respectively. Both ELEAI and ALEAI caused mean %WMI of 62.5% and 45.8% as observed after the worms were given lukewarm PBS treatment for

30 min after exposure to different treatments. The mean MI for both ELEAI and ALEAI was found to be 0.66 and 0.5 for *T. globulosa*. The result of *in vitro* anthelmintic activity of ELEAI and ALEAI compared with PBS was statistically significant ($p<0.0005$). Both the extracts i.e. ELEAI and ALEAI were found to be equally potent.

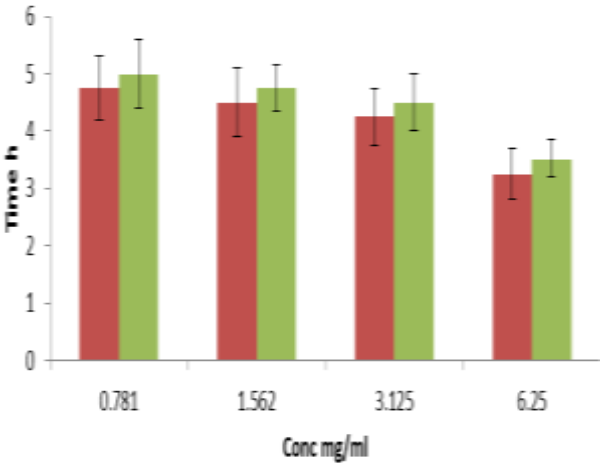


**Fig 2: Histogram showing dose dependent and time dependent response of different concentrations (0.781- 6.25 mg/ml) of ELEAI(dark) and ALEAI(light) *in vitro* on *T.globulosa.***

***Punica granatum***

The extracts (EFPEPG and AFPEPG) produced worm motility inhibition that was dose dependent and time dependent when compared to the negative control. Time taken for cessation of motility for EFPEPG and AFPEPG on adult *T. globulosa* at 0.781,1.562,3.125,6.25 mg/ml concentration has been depicted in histogram (Fig 3). LC-50 for EFPEPG was 9.56 mg/ml± 2.78 and for AFPEPG it was 8.75 mg/ml± 2.00. Mean %WMI was found to be 62.5% and 54.1% with EFPEPG and AFPEPG respectively. EFPEPG and AFPEPG resulted in mean MI of 0.66 and 0.50 respectively.

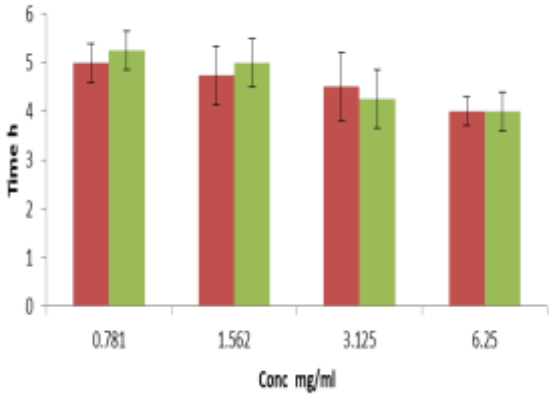


**Fig 3: Histogram showing dose dependent and time dependent response of different concentrations (0.781-6.25 mg/ml) of EFPEPG (dark) and AFPEPG (light) *in vitro* on *T.globulosa*.**

**Albendazole**

Paralyzing effect of albendazole was much faster and worms showed a complete loss of movement/motility in albendazole. Time taken for cessation of motility on adult *T. globulosa* at 2.0, 5.0, 10.0, 20.0 µg/ml concentration has been depicted in histogram (Fig 4). LC-50 value determined for albendazole was 10.73µg/ml± 2.88 which is quite low in comparison to all the extracts. Mean %WMI for albendazole was 100% .Mean MI for albendazole was found to be 1.0. The anthelmintic effect of albendazole and all the plant extracts remained static because no revival of motility was seen in lukewarm PBS after 3 h exposure to different treatments in all the replicas. The survival time of negative control worms (PBS) was longer and did not show any normal mortality.

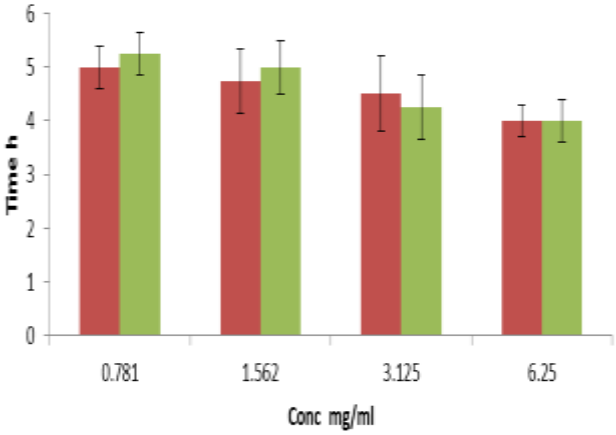


**Fig 4: Histogram showing anthelmintic activity of albendazole (2-20 µg/ml) *in vitro* on *T.globulosa.*1,2,3,4 showing 2,5,10,20 µg/ml conc respectively.**

## DISCUSSION

*In vitro* trials confirmed the potential of extracts of all the test plants to control the common sheep nematode *T. globulosa*. Albendazole and ethanolic extracts of all the three test plants showed significant *in vitro* anthelmintic activity against *T. globulosa* worms in term of worm motility. Based on the findings of earlier studies, the active principles in medicinal plants responsible for the anthelmintic activity are non-polar compounds which do not dissolve in water, therefore, water extracts of medicinal plant have shown lower anthelmintic activity than volatile solvents (ethanol, methanol, hydro-alcohol, acetone) extracts.[11,13,14,15] Greater anthelmintic activity of ethanolic extract of *Coriandrum sativum* against *Haemonchus contortus* was attributed to its lipid soluble nature which enhances rapid trans-cuticular absorption of active ingredient into the body of the worms.[13]

LC-50 of ethanolic and aqueous crude extracts of *C. procera*, *A.indica* and *P. granatum* varied with the

solvent used in extraction of active ingredients with EFECP and ELEAI being the most potent despite comparable efficacies. This would probably be related to the different chemical ingredients extracted in different solvents and to the source of parasites and previous exposure to the plants.[16] Anthelmintic efficacy of *Achillea millifolium* on *H. contortus* was observed and LC-50 for aqueous and ethanolic extracts was of 0.05 and 0.11 mg/ml respectively.[17]

Our studies revealed time dependent anthelmintic activity of ethanolic and aqueous extracts of *C. procera* flower, *A. indica* leaf and *P. granatum* fruit peel against *T. globulosa*. Increasing motility inhibition with increasing concentration of extract could be due to saturation of target receptors in agreement with earlier observations.[17] It is reported that receptors get saturated with increasing dose of active ingredient that increases with incubation period.[18] It is likely that at higher concentration all binding receptors on the worms were occupied thus leading to hyperpolarisation of membranes, limiting excitation and impulse transmission causing flaccid paralysis of worm muscle, a similar observation made by Wasswa and Olila.[19] Roy et al.[20] reported destruction of worm's surface when studied the effect of *Potentilla fulgens* on *Gastrothylax crumenifer* histochemically. Some workers have seen concentration – dependent larvicidal activity against *H. contortus in vitro* with the application of *C. procera* latex.[21] Subhedar et al.[22] reported dose and time dependent anthelmintic activity of methanolic extracts of seeds and bark of *P. granatum*.

The anthelmintic properties of *C. procera, A. indica* and *P. granatum* crude ethanolic and aqueous extracts could be attributed to the variety of secondary metabolites present as reported earlier in our previous study.[5] It is possible that the parasite paralysis and/or death observed may have been attributed to secondary metabolites[23] like tannins, alkaloids and saponins. Furthermore, these components may work together in a synergism action. Earlier workers[15] confirmed the presence of synergism effects between the secondary metabolites of *Vernonia amygdalina* when used to control *H. contortus* larvae. Others[24] however, acknowledged that plant metabolites action may be additive, synergistic or antagonistic in manner acting at single or at multiple target sites. It is therefore likely that a number of compounds could have contributed to the anthelmintic activity observed in all the three plant extracts.

It is well documented that some anthelmintic drugs like benzimidazoles kill the parasites by binding to a specific building block, and prevent its incorporation into microtubules which are essential for energy metabolism.[25] Thus these compounds could have caused their effect through same mechanism. Paralysis of worm tissues makes them unable to feed leading to death as result of lack of energy. It is also likely that high amount of alkaloids present in the plants could have contributed to the paralysis and consequent death of the worm.

Tannins were shown to produce anthelmintic activities.[26] The nematicidal activity of tannin extracts has also been reported with evidence of anthelmintic properties of condensed tannins by series of *in vitro* studies[27] and *in vivo* studies[28]. Tannins are polyphenolic compounds[29] Some synthetic phenolic anthelmintics like niclosamide and oxyclozanide are shown to interfere with the energy production in helminth parasites by uncoupling oxidative phosphorylation.[30] It is possible that tannins contained in ethanol and aqueous extracts of all three plants produced similar effects. It was also suggested that tannins bind to free proteins in the gastrointestinal tract of the host animal[26] or glycoprotein on the cuticle of the parasite disturbing physiological function like motility, feed absorption and reproduction[31] or interference with morphology and proteolytic activity of microbes[32] and cause death. Tanins or their metabolites have a direct effect on the viability of preparasitic stages of helminths.[33] Other phytochemicals reported to have an anthelmintic effect include flavonoids and terpenoids.[34] Thin layer chromatography analysis revealed the presence of flavonoids in ethanolic extract of *C. procera* suggesting the flavonoids may be responsible for anthelmintic effect in the study. Saponins present in crude extracts could have caused feed refusal and starvation of the parasites leading to their death from lack of energy.

Factors unknown to us may influence the anthelmintic activity of this plant. For instance, the active component(s) contained in the plant may vary in relation to location, age, and stage of development of the plant and whether the plant is freshly harvested or preserved.[35]

This anthelmintic property represents a promising alternative solution to chemical treatments. This would be of great importance for peri-urban cattle produced in free-range or organic farming systems which will contribute in sustainable agriculture. Furthermore, the active principles in plants are diverse and stable natural compounds with low molecular weight which can prevent the occurrence of anthelmintic resistance.[14] Moreover, all the three plants were found to be safe for medicinal use since no cytotoxic activity was observed on HeLa cell line as reported in an earlier study.[5] In conclusion, further studies to isolate and reveal the active compound(s) contained in the crude extracts of all the plants and to establish the mechanism(s) of action are required.

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