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Anthelmintic potential of *Calotropis procera, Azadirachta indica* and *Punica granatum* against *Gastrothylax indicus*

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Abstract 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. Significant anthelmintic effects ($p < 0.0005$) were observed on live *Gastrothylax indicus* worm as evident from their mortality at 4 h post exposure to both ethanolic and aqueous extracts. Phytochemical analysis of extracts revealed the presence of phenols, alkaloids, saponins, tannins, flavonoids, steroids and triterpenoids. LC-50 values were determined to be 12.05 mg/ml ± 3.24 and 23.52 mg/ml ± 6.4 for *C. procera*, 24.37 mg/ml ± 4.11 and 21.02 mg/ml ± 4.6 for *A. indica*, 18.92 mg/ml ± 4.54 and 24.43 mg/ml ± 6.96 for *P. granatum* ethanolic and aqueous extracts respectively, whereas it was 29.23 µg/ml ± 4.51 for albendazole. The mean mortality index (MI) was 1.0 and 0.90 for *C. procera*, 0.90 for *A. indica* and 0.73 and 0.80 for *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 70 and 100 % for different extracts. Various concentrations (5–5000 µg/ml) of all the plant extracts and albendazole were used to detect their cytotoxic effects against HeLa cell line to determine CC-50 by MTT assay. CC-50 values, of all the plant extracts were determined to be >1000 µg/ml and for albendazole it was found to be >10 µM. All the three plants can be potential sources for novel anthelmintics.

Introduction

Major helminthic diseases of small ruminants (sheep/goat) include paramphistomiasis which cause considerable economic losses characterized by acute parasitic gastroenteritis, lowered meat and hair production leading to high mortality in host. Almost hundred percent of grazing sheep and goat harbour, light to medium level of these infections affecting the livelihood of marginal farmers (Perry et al. 2002; Raza et al. 2007).

There is a growing interest in the ethno-veterinary approach to examine the anthelmintic properties of plants traditionally used by local farmers in different parts of globe due to increasing development of anthelmintic resistance and limited availability of commercial drugs to the rural people as well as the high cost of such synthetic medicines (Kaplan 2004; Mali and Mehta 2008). The World Health Organisation (WHO) estimated that 80 % of the population of developing countries rely on traditional medicine mostly plant drugs, for their primary health care needs.

Calotropis procera known as aak, is used in ethno-veterinary medicine system as an expectorant, anthelmintic, laxative, purgative, anti-inflammatory and diuretic (Iqbal et al. 2005). Different parts as well as latex of *C. procera* have been reported to have emetic, purgative and anthelmintic effect in traditional medicine (Jain et al. 1996). *C. procera* flowers possess good anthelmintic activity against nematodes (Iqbal et al. 2005).

Azadirachta indica or neem plant is reported to have multiple medicinal applications including its use as an anti-inflammatory, antipyretic, analgesic, immuno-stimulant,

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hypoglycemic, antifungal and antibacterial (Rahman et al. 2011).

Punica granatum (anar) is another herbal anthelmintic drug widely used in ayurveda. The most famous usage worldwide has been a vermifugal or taenicidal agent i.e. a killer and expeller of intestinal worms (Subhedar et al. 2011). The anthelmintic activity may be chiefly due to alkaloids.

The current study was aimed for phytochemical screening of crude ethanolic and aqueous extracts of all the three plants (Aak flowers, Neem leaves and Anar fruit peel) along with the objective of evaluating the anthelmintic efficacy of these crude extracts against *G. indicus* (a trematode parasite of sheep/goat) by worm motility inhibition assay in vitro. The study contributes to the knowledge base of materia medica and strategies for sustainable animal health management and the well being of people whose livelihoods are livestock based industries.

Many plant extracts and their active principles have been described and utilized as therapeutic agents. There is considerable interest in determining the risks that these products may pose to health, because many of these plants contain compounds which are known to cause diseases or even death in animals and humans. Thus, an assessment of their cytotoxic potential is necessary to ensure a relatively safe use of medicinal plants (Surh and Ferguson 2003). Ethanolic and aqueous extracts of all the three plants were screened in vitro for cytotoxic activity on HeLa cell line.

Despite several reports on the traditional medicinal use of *C. procera*, *A. indica* and *P. granatum*, experimental reports on anthelmintic efficacy are limited or varied. Different studies employing different parasites and methods have revealed different efficacies. However, no previous work has reported anthelmintic potential of all the three plants against *Gastrothylax indicus.*

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 according to method of Iqbal et al. (2005). 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 in 1 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 °C until used.

Phytochemical screening of plant extracts

Phytochemical screening of plant extracts were carried out by employing standard procedures (Harbone 1983; Trease and Evans 1989; Bagewadi et al. 2012).

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 (Iqbal et al. 2004). The in vitro anthelmintic activity was carried out on adult *G. indicus* worms to determine the inhibitory effect of extracts on adult worms.

Mature *G. indicus* were collected from the rumen of sheep/goat procured from slaughter house. The worms were washed in phosphate buffered saline (PBS pH7.2) and finally suspended in PBS. The study was conducted at four different dilutions of all the extracts viz., 6.25, 12.5, 25, 50 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 20, 40, 60, 80 μg/ml and PBS alone served as postitive and negative control respectively. There were six replicates for each treatment concentration. Ten vigorously motile worms were placed in each petridish 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 according to Rabel et al. (1994) using formula.

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

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

$$MI = \frac{total\ number\ of\ immobile\ worms\ (dead)}{total\ number\ of\ worms\ per\ petridish}$$

Cell line and culture medium

HeLa (human cervical carcinoma) cell line obtained from NSSC, Pune, was used in this study. Cells were cultured in DMEM supplemented 10 % Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5 % CO_2 and 95 % air at 37 °C (Freshney 1994).

In vitro assay for cytotoxic activity

Cells were washed by PBS and harvested by tripsinization and were distributed in 96 well plates (5000 cells/well) and incubated under 5 % CO_2 and 95 % air at 37 °C for 24 h. The cells were treated with different concentrations of plants extracts including 10, 20, 50, 100, 200,500, 1000, 5000 µg/ml and for albendazole it was 1.0, 5.0 and 10 mM/ml. Dilution of stock solutions were made in culture medium yielding final extracts concentrations with a final DMSO concentration of 0.1 %. Control cells were incubated in culture medium only. All concentrations of plants extracts were in triplicates on the same cell batch.

MTT assay

Growth of tumoral cells quantitated by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide(MTT) to a blue formazan product (Mosmann 1983). At the end of 48 h incubation, the medium in each well was replaced by MTT solution, the plates were incubated for 2–4 h under 5 % CO_2 and 95 % air at 37 °C. MTT reagent was removed and the formazan crystals produced by viable cells were dissolved in 100 µl DMSO and gently shaken. The absorbance was then determined by ELISA reader at 492 nm. The percentage viability was calculated using following formula,

$\%$ cell viability $= [(A_t - A_b)/(A_c - A_b)] \times 100$

where, A_t = absorbance value of test compound, A_b = absorbance value of blank and A_c = absorbance value of control.

The effects of extracts were expressed by CC-50 values (the drug concentration reducing the absorbance of treated cells by 50 % with respect to untreated cells).

Statistical analysis

Experimental results are expressed as mean ± S.D. All measurements were replicated six times. The data was analysed by analysis of variance ($p < 0.05$). The CC-50 values were calculated from probit regression analysis.

Results

Phytochemical screening of chemical ingredients

The results of phytochemical screening of ethanolic and aqueous extracts of all the three plants (aak flower, neem leaf and anar fruit peel) have been summarized in Table 1. Chemical analysis revealed the presence of a number of secondary metabolites whose intensity varied with both plant and solvent used. Ethanolic extracts of all the three plants possessed triterpenoids while aqueous extracts had steroids with same intensity.

In vitro anthelmintic activity of ethanolic and aqueous extracts of plants

Calotropis procera

EFECP and AFECP demonstrated dose dependent and time dependent ($p < 0.0005$) anthelmintic activity against *G. indicus* as revealed from the inhibition of motility and/or death of the worm after treatment (Tables 2, 3; Fig. 1). Time taken for cessation of motility for EFECP and AFECP on adult *G. indicus* at 6.25, 12.5, 25, 50 mg/ml concentration has been depicted in histogram (Fig. 1). LC-50 value for EFECP was 12.05 mg/ml ± 3.24 and for AFECP was 23.52 mg/ml ± 6.4. EFECP resulted in mean %WMI of 100 % while the AFECP of 88.4 % as observed after the worms were put in lukewarm PBS for 30 min after exposure to different treatments for 4 h. The mean MI of EFECP and AFECP was 1.0 and 0.90 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

Table 1 Phytochemical screening of ethanolic (EFECP, ELEAI, EFPEPG) and aqueous (AFECP, ALEAI, AFPEPG) extracts of *C. procera A. indica* and *P. granatum*

Phytoconstituents/Tests	EFECP	AFECP	ELEAI	ALEAI	EFPEPG	AFPEPG
Alkaloids						
a. Mayer's test	++	++	−	−	−	−
b. Hager's test	++	++	−	−	+	−
c. Dragendroff's test	++	++	−	−	+	−
Phenols						
a. Bromine water test	++	++	+	+	+	+
b. Ferric chloride test	++	++	+	+	+	+
Saponins						
a. Frothing test	−	−	−	−	++	++
b. Emulsion test	−	−	−	−	++	++
Tannins						
a. Gelatin test	+	+	+	+	+	+
b. Ferric chloride test	+	+	+	+	+	+
Flavonoids						
a. Ammonium test	+	+	++	++	++	++
b. Alkaline reagent test	+	+	++	++	++	++
Steroids and triterpenoids						
Salkowski test						
Steroids	−	+	−	+	−	+
Triterpenoids	+	−	+	−	+	−

++ strongly present, + present, − not present

Table 2 In vitro anthelmintic activity of ethanolic extracts of *C. procera, A. indica* and *P. granatum* on *G. indicus*

Time (h)	Dosing				
	EFECP 6.25(mg/ml)	ELEAI 12. 5(mg/ml)	EFPEPG 12.5 (mg/ml)	Albendazole 20 µg/ml	Control (PBS)
	Worms showing motility (%mortality)				
0	10 ± 0.0	10 ± 0.0	10 ± 0.0	10 ± 0.0	10 ± 0.0
1	7.8 ± 0.40(20)[*]	7.8 ± 0.40(20)[*]	7.8 ± 0.51(20)[*]	7.8 ± 0.40(20)	10 ± 0.0
2	4.5 ± 0.54(50)[*]	5.0 ± 0.0(50)	6.8 ± 0.40(30)*	2.8 ± 0.40(70)	10 ± 0.0
3	2.8 ± 0.40(70)[*]	3.6 ± 0.51(60)[*]	6.0 ± 0.0(40)	1.0 ± 0.0(90)	10 ± 0.0
4	0 ± 0.0(100)	1.0 ± 0.0(89.6)	2.6 ± 0.51(68.9)[*]	0 ± 0.0(100)	9.6 ± 0.81
PBS for 30 min	0 ± 0.0	1.0 ± 0.0	2.6 ± 0.51	0 ± 0.00	9.6 ± 0.81

All values represent mean ± S.D, n = 6. Each replica (n) had 10 worms

* $p < 0.0005$ extremely significant

control, there was no significant difference ($p > 0.05$). EFECP was found to be more potent than the AFECP as 100 % mortality was seen in ethanolic extract with 4 h exposure (Tables 2, 3).

Azadirachta indica

ELEAI and ALEAI showed dose dependent and time dependent ($p < 0.0005$) anthelmintic activity against *G. indicus* as observed from the inhibition of motility and/or

death of the worm after treatment (Tables 2, 3; Fig. 2). Time taken for cessation of motility at 6.25, 12.5, 25, 50 mg/ml concentration have been shown in histogram (Fig. 2). LC-50 for ELEAI and ALEAI was 24.37 mg/ml ± 4.11 and 21.02 mg/ml ± 4.6 respectively. Both ELEAI and ALEAI caused mean %WMIof 89.6 % 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 0.90 for *G. indicus*. The result of in vitro anthelmintic activity of

Table 3 In vitro anthelmintic activity of aqueous extracts of *C. procera, A. indica* and *P. granatum* on *G. indicus*

Time (h)	Dosing				
	AFECP 6.25(mg/ml)	ALEAI 12. 5(mg/ml)	AFPEPG 12.5 (mg/ml)	Albendazole 20 µg/ml	Control (PBS)
	Worms showing motility (%mortality)				
0	10 ± 0.0	10 ± 0.0	10 ± 0.0	10 ± 0.0	10 ± 0.0
1	8 ± 0.00(20)*	6.6 ± 0.51(30)*	6.6 ± 0.51(30)*	7.8 ± 0.40(20)	10 ± 0.0
2	5.8 ± 0.40(40)*	5.0 ± 0.0(50)	5.0 ± 0. 0(50)	2.8 ± 0.40(70)	10 ± 0.0
3	3.6 ± 0.51(60)*	2.8 ± 0.40(70)*	2.8 ± 0.40(70)*	1.0 ± 0.0(90)	10 ± 0.0
4	1.0 ± 0.0(88.4)	1.0 ± 0.0(89.6)	2.0 ± 0.0(79.2)	0 ± 0.0(100)	9.6 ± 0.81
PBS For 30 min	1.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.0	0 ± 0.00	9.6 ± 0.81

All values represent mean ± S.D, n = 6. Each replica (n) had 10 worms

* $p < 0.0005$ extremely significant

Fig. 1 Histogram showing dose dependent and time dependent response of different concentrations (6.25–50 mg/ml) of EFECP and AFECP in vitro on *G. indicus*
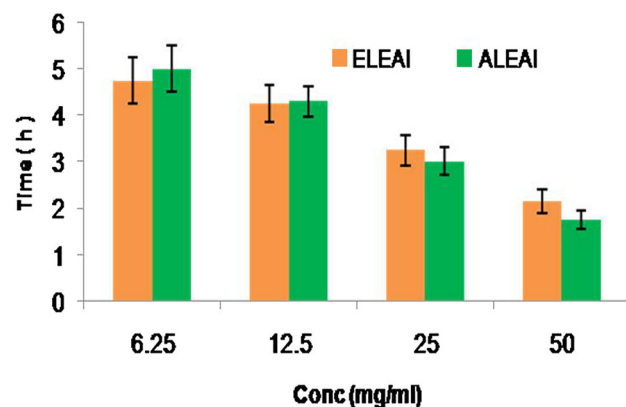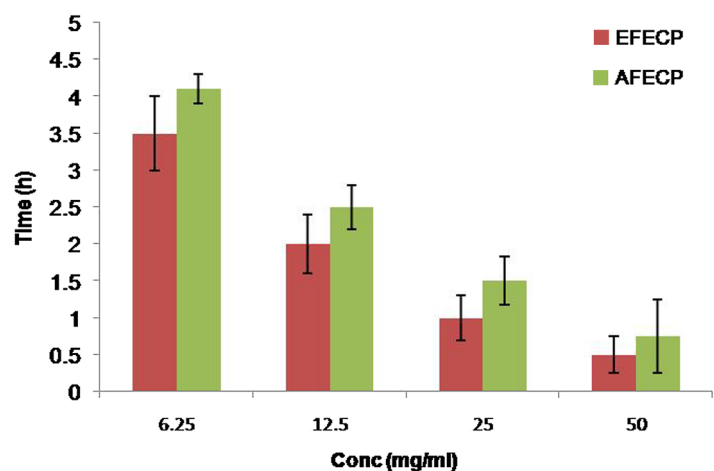
Fig. 2 Histogram showing dose dependent and time dependent response of different concentrations (6.25–50 mg/ml) of ELEAI and ALEAI in vitro on *G. indicus*

ELEAI and ALEAI compared with albendazole was statistically non significant ($p > 0.05$) and was statistically significant ($p < 0.0005$) compared with PBS. Both the extracts i.e. ELEAI and ALEAI were found to be equally potent.

Punica granatum

The extracts (EFPEPG and AFPEPG) produced worm motility inhibition that was dose dependent (Fig. 3) and time dependent (Tables 2, 3 Fig. 3) when compared to the negative control. Dose dependent mortality was observed at 6.25, 12.5, 25, 50 mg/ml concentrations for both EFPEPG and AFPEPG on adult *G. indicus* as shown in Fig. 3. LC-50 for EFPEPG was 18.92 mg/ml ± 4.54 and for AFPEPG it was 24.43 mg/ml ± 6.96. Mean %WMI was found to be 68.9 and 79.2 % with EFPEPG and AFPEPG respectively. EFPEPG and AFPEPG resulted in mean MI of 0.73 and 0.80 respectively.

Anthelmintic property of extracts compared with albendazole

Paralyzing effect of albendazole was much faster and worms showed a complete loss of movement/motility in albendazole. Mortality was observed at 3.25, 1.5, 0.5 h and instantly (0.08 h) at 20,40,60,80 µg/ml respectively for positive control i.e., albendazole which is quite effective when compared with both negative control and all the
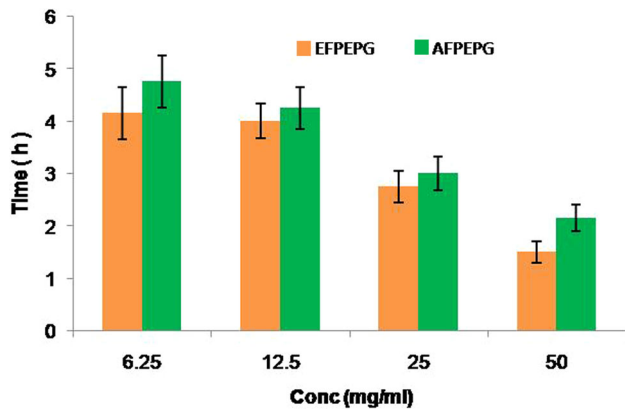
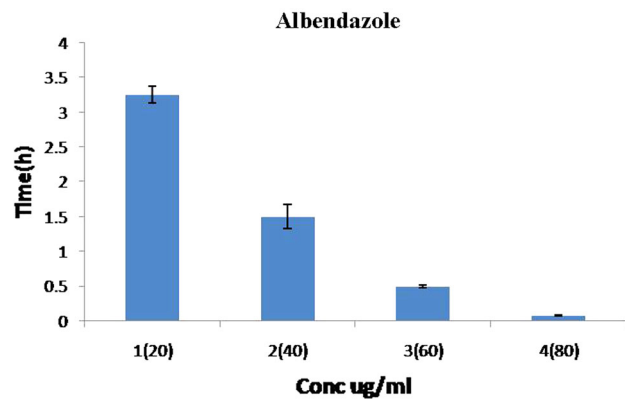**Fig. 3** Histogram showing dose dependent and time dependent response of different concentrations (6.25–50 mg/ml) of EFPEPG and AFPEPG in vitro on *G. indicus*

Fig. 4 Histogram showing anthelmintic activity of albendazole (20–80 μg/ml) in vitro on *G. indicus*

extracts (p < 0.0005) (Tables 2, 3; Fig. 4). LC-50 value determined for albendazole was 29.23 μg/ml ± 4.51 which is quite low in comparison to all the extracts. Mean %WMI for albendazole was 100 % little near with EFECP since time taken for 100 % mortality in EFECP was 4.0 h while in albendazole it was 3.25 h but quite high when compared to other extracts. 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 4 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; but after 4 h two worms died out of 10 in one replica out of 6 because of mechanical disturbance.

In vitro cytotoxicity assay of ethanolic and aqueous plant extracts

Cytotoxicity assay of all ethanolic and aqueous extracts (10–5000 μg) along with albendazole (1–10 μM) was

carried out against HeLa cell line to determine the CC-50 (50 % growth inhibition) by MTT assay. All the plant extracts were found to be non-toxic to HeLa cell lines even at higher concentrations. Cell viability for different extracts i.e. EFECP, AFECP, ELEAI, ALEAI, EFPEPG and AFPEPG were found to be 85.83, 85.33, 55.10, 52.40, 54.20 and 54.30 % respectively at concentration of 1000 μg/ml. The CC-50 of all the plant extracts was determined to be >1000 μg/ml. Cell viability for albendazole i.e. positive control was found to be 80.82 % at 10 μM (Table 4).

Discussion

Anthelmintic activity of different plant extracts on *G. indicus* reported in present study is in agreement with the findings of earlier workers on different helminth parasites (*Tariq* et al. 2008; Ghangale et al. 2009; Roy et al. 2010) 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 EFPEPG 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. Similar variation in potency and efficacy were observed by other workers (Costa et al. 2008; Suteky and Dwatmadji 2011), when they used different solvents for extraction of active ingredient and observed varying bioactivity results. Tariq et al. (2008) observed anthelmintic efficacy of *Achillea millifolium* on *Haemonchus contortus* and calculated LC-50 for aqueous and ethanolic extracts of 0.05 and 0.11 mg/ml respectively.

Increasing motility inhibition with increasing concentration of extract could be due to saturation of target receptors. Similar observations were made by Lullman and Morh (1993) who said that receptors get saturated with increasing dose of active ingredient that increases with incubation period. 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 (2006). Roy et al. (2010) reported destruction of worm's surface when studied the effect of *Potentilla fulgens* on *G. crumenifer* histochemically.

Iqbal et al. (2005) reported that aqueous and methanolic extracts of *C. procera* flower possessed time dependent anthelmintic activity on *H. contortus*, when studied in vitro. Our studies also revealed time dependent anthelmintic activity of ethanolic and aqueous extracts of *C. procera* flower, *A. indica* leaf and *P. granatum* fruit peel on *G.*

Table 4 CC-50 values of different plant extracts (EFECP, AFECP, ELEAI, ALEAI, EFPEPG, AFPEPG) and albendazole

Plant Extract	Conc. (µg/ml)	% Cell viability	CC-50 (µg/ml)
EFECP	1000	85.83 ± 2.69	>1000
AFECP	1000	85.33 ± 5.32	>1000
ELEAI	1000	55.10 ± 2.56	>1000
ALEAI	1000	52.40 ± 1.35	>1000
EFPEPG	1000	54.20 ± 3.69	>1000
AFPEPG	1000	54.30 ± 3.90	>1000
Albendazole	26.5/10 µM	80.82 ± 3.62	> 26.5/10 µM

All values represent ± SD of four independent experiments done in triplicate

indicus. Subhedar et al. (2011) 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. Preliminary phytochemical screening of plant extracts revealed presence of phenols, alkaloids, saponins, tannins, flavonoids, steroids and triterpenoids, whose intensity varied among the plants and also with the solvent of extract. It was also evident that *C. procera* was more potent than *A. indica* and *P. granatum*, an activity that could be attributed due to the strong presence of alkaloids and phenols.

It is possible that the parasite paralysis and/or death observed may have been attributed to secondary metabolites (Makut et al. 2008) like tannins, alkaloids and saponins. Briskin (2000) and Wynn and Fougere (2007) 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.

Barrowman et al. (1984) reported that benzimidazole anthelmintics act by interfering with the microtubules system in *Ascaris suum*. 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 *C. procera* plant could have contributed to the paralysis and consequent death of the worm.

Saponins present in crude extracts of *P. granatum* could have caused feed refusal and starvation of the parasites leading to their death from lack of energy.

The role of tannins in helminths control have been documented (Forbey et al. 2009; Kotze et al. 2009). The nematicidal activity of tannin extracts has also been reported with evidence of anthelmintic properties of condensed tannins by series of in vitro studies (Molan et al. 2003; Ademola and Idowu 2006) and in vivo studies

(Butter et al. 2001; Kotze et al. 2009). Chemically tannins are polyphenolic compounds (Bate-Smith 1962) and synthetic phenolic anthelmintics like niclosamide and oxyclozanide are said to interfere with the energy generation in helminth parasites by uncoupling oxidative phosphorylation (Martin 1997). 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 (Athanasiadou et al. 2001) or glycoprotein on the cuticle of the parasite disturbing physiological function like motility, feed absorption and reproduction (Thompson and Geary 1995; Githiori et al. 2006) or interference with morphology and proteolytic activity of microbes (Min et al. 2003; Waghorn and McNabb 2003) and cause death.

All the plant extracts (EFECP, AFECP, ELEAI, ALEAI, EFPEPG and AFPEPG) were found to be non-toxic to HeLa cell lines even at higher concentration in present studies. CC50 of all the plant extracts was determined to be >1000 µg/ml. Nemati et al. (2013) studied the cytotoxic properties of ethanolic extracts of some medicinal plants (*Consolida orientalis*, *Ferula assafoetida*, *Coronilla varia* and *Orobanche orientalis*) on HeLa cell line in vitro. The study evaluated that the extracts of *C. orientalis*, *F. assafoetida* and *C. varia* had potential cytotoxic activity on HeLa cell line but *O. orientalis* showed no significant growth inhibition.

Ranjit et al. (2012) reported percentage inhibition of 41.84 of HeLa cells with 512 µg/ml of ethanolic flower extract of *C. procera*. Manasathien et al. (2012) assayed the cytotoxic activity of fruit peel extract of *P. granatum* on Brine shrimp and determined CC-50 of 1205.98 and 1294.88 µg/ml with ethanolic and water extracts respectively. Sharma et al. (2014) reported EC-50 of ethanolic neem leaf extract for MCF-7 cells at 350 µg/ml after 72 h treatment whereas in HeLa cells it was found to be175 µg/ml in 48 h.

From the present in vitro study it can be suggested that both crude ethanolic and aqueous extracts of *C. procera*, *A. indica* and *P. granatum* exhibited significant in vitro

anthelmintic activity against *G. indicus* of sheep and have potential for use as an alternative anthelmintic agent for the control of gastrointestinal helminths of sheep and other ruminants. All the three plants were found to be safe for medicinal use since no cytotoxic activity was observed on HeLa cell line.

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