In vitro ovicidal assessment of methanol, ethyl acetate and chloroform extracts of *Annona squamosa* and *Chenopodium album* against caprine gastrointestinal nematodiosis

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Abstract The objective of this study was in vitro evaluation of the ovicidal efficacy of methanol, ethyl acetate and chloroform extracts of *Annona squamosa* (seeds) and *Chenopodium album* (whole plant) in comparison to albendazole against the GI nematodes of goats using egg hatch test. Eggs of GI nematodes were incubated at 27 °C in different extracts at concentration of 100–6.25 mg/ml for 2 days. Distilled water and albendazole were used as negative and positive controls, respectively. Percentage efficacy and ED₅₀ and ED₉₀ values were separately evaluated with upper and lower confidence limit by log probit analysis using SAS 9.2. The ED₅₀ and ED₉₀ values of methanolic, ethyl acetate and chloroform extracts of *A. squamosa* was calculated as 1.52 and 4.56; 2.48 and 10.73; 3.02 and 12.44 mg/ml, respectively against GI nematodes of goats. Similarly, the ED₅₀ and ED₉₀ values of methanolic, ethyl acetate and chloroform extracts of *C. album* was calculated as 3.86 and 7.14; 2.73 and 8.31; 4.41 and 20.11 mg/ml, respectively. This study shows that *C. album* and *A. squamosa* possess in vitro anthelmintic activities. The study also suggests further large scale pharmacological and toxicological studies for their safer use in veterinary medicine.

Keywords *Annona squamosa* · *Chenopodium album* · Egg hatch assay · Herbal anthelmintics

Introduction

Helminths are recognized as a major constraint to livestock production throughout the globe (Githori et al. 2004) owing to their adverse effects including retarded growth (Kochapakdee et al. 1995), lowered productivity (Perry and Randolph 1999), mortality (Sykes 1994) and high economic losses (Iqbal et al. 1993). Most of the parasite control programs are based upon a combination of chemotherapeutic control, grazing management, dietary management, biological control, vaccination and ethnoveterinary treatment (FAO 2002). Chemotherapeutic control practices have evolved a series of evils in the form of anthelmintic resistance (Chartier et al. 2001), chemical residues, toxicity problems, increased cost of treatment, non-adaptability of drugs and non-availability of the newer medicine in remote areas of the country. Options like, biological control, vaccine and traditional medicinal plants are being examined in different parts of the world. Screening and proper evaluation of the claimed medicinal plants could offer the possible alternatives that may both be sustainable and environmentally acceptable.

Chenopodium album (Syn: Lamb's-quarters, Fat-hen, Bathua) is a native of eastern Asia but often differ from European specimens. It is widely found in Africa, Australasia, North America, Oceania and almost everywhere in soils rich in nitrogen, especially on wasteland. Infusions and decoctions of the leaves, roots and inflorescences of the plant have been used for centuries as dietary condiments and as traditional anthelmintics by native peoples for the treatment of intestinal worms. Commercial preparations of oil of *Chenopodium* and its active constituent, ascaridol, have been and continue to be, used with considerable success in mass treatment campaigns as an anthelmintic (Kliks 1985).

Annona squamosa (Syn: Custard Apple, Sugar Apple, Sweet sop, Gandagaatra, Sitaa phala) is a native to South

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America and the West Indies and is now cultivated throughout India. The seeds of the plant are known to possess anthelmintic activity against *Haemonchus contortus*. A compound C37- trihydroxy adjacent bistetrahydrofuran acetogenin, present in the seeds, is responsible for inhibition of the egg hatching of gastrointestinal nematodes (Saha 2011). Besides, the leaves of the plant are used for removing lice from scalp by the natives.

Material and method

Collection and processing of plant material

Chenopodium album whole plant and seeds of *A. squamosa* were collected from the local market of Mathura. The plant and seeds were identified by Department of Botany, Babu Shrivnath Agrawal College, Mathura and the specimens were recorded for further references. The collected plant materials were shade dried and coarsely grinded with the electric grinder and stored in air tight containers.

Preparation of crude extracts

Three types of extracts of each *C. album* (whole plant) and *A. squamosa* (seeds) were prepared using Chloroform (Web chemicals), Ethyl Acetate (Qualigens) and Methanol (S.D Fine Chem. Ltd.) as solvents. Briefly, about 50 gm of the coarsely ground *C. album* (whole plant) and *A. squamosa* (seeds) were uniformly taken in a porous cellulose thimble and placed in soxhlet extractor (Perfit India) with 500 ml flask containing about 250 ml solvent at a temperature $40 \pm 5^\circ\text{C}$. The extraction was allowed to continue for 22 cycles. The extracts were then collected and kept in a rotatory and vacuum evaporator (Cyberlab) for evaporating the extra solvent. The percent yield of different extracts was calculated. The extracts of methanol are easily soluble in the distilled water so 100 mg of extract was dissolved in 1 ml of distilled water to get 100 mg/ml concentration, further four lower dilutions were prepared (100–6.25 mg/ml) by using two fold dilution. The extracts of chloroform and ethyl acetate did not dissolve in water so at first 100 mg of extracts were dissolved in 100 μl of DMSO (Rankem). Then 900 μl of water is added to make the final concentration to 100 mg/ml lower concentration were prepared by using twofold dilution of stock solution. The extracts were kept in air tight containers at 4°C to avoid loss of any volatile principles or/and activities for further studies.

Determination of extraction yield (% yield)

The yield (% w/w) from all the dried extracts was calculated as: $\text{Yield (\%)} = (W1 \times 100)/W2$.

where, W1 is the weight of the extract obtained after lyophilization of solvent, and W2 is the weight of the plant powder.

Phytochemical analysis of different crude extract

All the extracts in different solvents were tested for the presence of active principle such as carbohydrate, steroids, tannins, flavonoids, alkaloids, glycoside, fat and proteins using standard procedures (Debelu 2002).

Egg hatch test

The eggs of GI nematodes were recovered using the method described by Taylor et al. (2002) and World Association for the Advancement of Veterinary Parasitology, WAAVP (Coles et al. 1992). Goats from Damodarpura, Aurangabad, Bala-jipuram villages of Mathura district were used for the collection of eggs. Briefly, 10 g of faeces were collected directly from the rectum of goats and then, mixed and homogenized with tap water. The mixture was strained through strainer and centrifuged for 2 min at 2,000 rpm, supernatant was poured off and the tubes were agitated to loosen the sediment and saturated sodium chloride solution was then added in the tube until a meniscus was formed. Cover slips were put over the meniscus for and kept for 5 min and were then washed and eggs were collected. The different solvent extracts of the plant materials (*A. squamosa* seeds and *C. album* whole plant) were used as the active treatment. Albendazole at 0.125 mg/ml was used as positive control while untreated eggs in distilled water were used as negative control. The egg suspension with a concentration of 100 eggs/0.2 ml were distributed in 24-multiwell plate (50 μl per well) along with 50 μl of extract of 100–6.25 mg/ml concentration. Three replicates were made for each concentration. The plates were incubated under humidified conditions at ambient temperature (27°C) for 48 h and then, a drop of Lugol's iodine solution added to each well to stop further hatching. Hatched larvae and unhatched eggs were then counted under microscope.

Statistical analysis

The extract concentration required to inhibit 50 and 90 % (ED_{50} and ED_{90}) egg hatching was calculated by using log probit analysis by SAS programme (version 9.2).

Results

Extraction yield (% yield) of the various extracts

Variation in yield among the various extracts of the two plant species was observed (Table 1). The lowest yield was

Table 1 Percent (%) yield of extracts

Plant	Extract type	Weight of plant powder (g)	Weight of extract obtained (g)	% yield (w/w)
<i>Annona squamosa</i>	Methanol	91	39	42.85
	Ethyl acetate	109	45	41.28
	Chloroform	111	31	27.92
<i>Chenopodium album</i>	Methanol	81	24	29.62
	Ethyl acetate	102	23	22.54
	Chloroform	100	18	18

recorded for chloroform extract of *C. album* (18 %) and highest yield was for methanolic extract of *A. squamosa* 42.85 %.

Phytochemical analysis of different crude extract

The methanolic extract of *A. squamosa* was positive for alkaloids, carbohydrates, tannins and fat whereas only fat was detected in ethyl acetate extract of the plant. The fat and glycoside were present in chloroform extract. In *C. album*, tannins were present in all the three extracts and the glycoside was present only in methanolic extract (details are given in Table 2).

Egg hatch test

All the methanolic, ethyl acetate and chloroform extracts of *A. squamosa* and *C. album* induced significant egg hatch inhibition in a dose dependent manner. Methanol, ethyl acetate and chloroform extracts of *A. squamosa* at 100, 50 and 25 mg/ml concentration showed 100 % egg hatch inhibition. At 12.5 and 6.25 mg/ml concentrations, methanolic extract ($ED_{50} = 1.52$ and $ED_{90} = 4.56$ mg/ml) was 98 and 95.44 % effective in suppressing the hatching of eggs, respectively. For ethyl acetate extract ($ED_{50} = 2.48$ and $ED_{90} = 10.73$ mg/ml) at similar concentration 90.95

and 70.27 % egg hatch inhibition was observed. But chloroform extract ($ED_{50} = 3.02$ and $ED_{90} = 12.44$ mg/ml) at 12.5 and 6.25 mg/ml concentration showed 86.37 and 69.5 % ovicidal effects, respectively.

The ethyl acetate ($ED_{50} = 2.73$ and $ED_{90} = 8.31$ mg/ml) extract of *C. album* inhibited 100 % egg hatching at and above 25 mg/ml concentration. At 12.5 and 6.25 mg/ml concentration 96.8 and 94.26 % efficacy was observed. Methanolic extract ($ED_{50} = 3.86$ and $ED_{90} = 7.14$ mg/ml) of *C. album* reported 71.4, 83.6, 89.2, 99 and 100 % egg hatch inhibition at 6.25, 12.5, 25, 50 and 100 mg/ml concentrations, respectively. Chloroform extract ($ED_{50} = 4.41$ and $ED_{90} = 20.11$ mg/ml) at similar concentration inhibited 52.97, 75.8, 95.38, 99.09 and 100 % egg hatching respectively (details are given in Tables 3 and 4).

Discussion

The systematic work on secondary metabolite screening of *A. squamosa* and *C. album* is scanty and a very few handful of reports are available in India and abroad. The methanol and ethyl acetate extracts obtained from both the two plants in the current study have shown remarkable egg hatching inhibition as compared to some plants studied previously (Asase et al. 2005; Maciel et al. 2006; Kotze et al. 2009).

Souza et al. (2008), isolated C37-trihydroxy adjacent bistetrahydrofuran acetogenin compound from ethyl acetate extract of *A. squamosa* seeds, and found 100 % egg hatching inhibition in *H. contortus* at 25 mg/ml. Similar results are observed in the present study. The mechanism of action of *A. squamosa* yet not fully understood, yet the studies on the plants of Annonacea family have showed the presence of active substances with parasitocidal effects (Duret et al. 1998). Kamaraj et al. (2011) evaluated the anthelmintic efficacy of *A. squamosa* bark using ethyl acetate, acetone and methanol extracts against *H. contortus* and reported 94, 91 and 100 % egg hatch inhibition respectively, at 25 mg/ml concentration. The results of current study (ED_{50} of methanolic extract 1.52 mg/ml) are somewhat different with earlier work. The use of the different parts of *A. squamosa* used

Table 2 Chemical constituents in different crude plants extracts

Plants	Plant extract	Plant constituents							
		Alkaloids	Flavonoids	Fat	Carbohydrates	Steroids	Glycosides	Tannins	Protein
<i>Annona squamosa</i>	Methanol	+	—	+	+	—	—	+	—
	Ethylacetate	—	—	+	—	—	—	—	—
	Chloroform	—	—	+	—	—	+	—	—
<i>Chenopodium album</i>	Methanol	+	—	—	—	—	+	+	—
	Ethylacetate	—	—	—	+	—	—	+	—
	Chloroform	—	—	—	—	—	+	+	—

Table 3 Mean efficacy (percentage $\pm$ SD) of *Annona squamosa* and *Chenopodium album* extracts on GI nematodes on egg hatch inhibition

Concentration (mg/ml)	Mean efficacy (percentage $\pm$ SD)					
	<i>Annona squamosa</i>			<i>Chenopodium album</i>		
	Methanol	Ethylacetate	Chloroform	Methanol	Ethylacetate	Chloroform
100	100	100	100	100	100	100
50	100	100	100	99 $\pm$ 0.70	100	99.09 $\pm$ 0.64
25	100	100	100	89.2 $\pm$ 3.81	100	95.38 $\pm$ 1.37
12.5	98 $\pm$ 1.41	90.95 $\pm$ 2.63	86.37 $\pm$ 1.92	83.6 $\pm$ 4.56	96.8 $\pm$ 1.40	75.8 $\pm$ 5.60
6.25	95.44 $\pm$ 1.38	70.27 $\pm$ 4.08	69.5 $\pm$ 4.12	71.4 $\pm$ 4.09	94.26 $\pm$ 1.22	54.97 $\pm$ 2.53
Negative control (D_w)	10.88 $\pm$ 3.20	10.63 $\pm$ 2.48	10.55 $\pm$ 2.31	10.81 $\pm$ 3.27	10.61 $\pm$ 2.41	10.52 $\pm$ 2.37
Positive control (Albendazole @ 0.125 mg/ml)	100	100	100	100	100	100

Table 4 ED₅₀ and ED₉₀ of egg hatch test of two plant extract against GI nematodes

Plant	Extract type	ED ₅₀ $\pm$ SE	UCL–LCL	ED ₉₀ $\pm$ SE	UCL–LCL
<i>Annona squamosa</i>	Methanol	1.52 $\pm$ 0.096	2.3–0.46	4.56 $\pm$ 0.46	18.36–3.06
	Ethylacetate	2.48 $\pm$ 0.26	4.34–0.32	10.73 $\pm$ 0.09	1203–6.23
	Chloroform	3.02 $\pm$ 0.35	5.56–0.72	12.44 $\pm$ 1.02	98.19–7.16
<i>Chenopodium album</i>	Methanol	3.86 $\pm$ 0.48	7.14–0.44	26.24 $\pm$ 4.77	222.66–15
	Ethylacetate	2.73 $\pm$ 0.27	3.64–1.71	8.31 $\pm$ 0.92	15.35–6.1
	Chloroform	4.41 $\pm$ 0.55	5.62–2.78	20.11 $\pm$ 1.95	82.9–12.56

in both the studies might be a contributing factor in this regard. The present study indicates that seeds are more effective against GI nematodes.

Akhtar et al. (1999) found that the presence of glycosides in aerial parts of *C. album* which are responsible for anthelmintic property. Phytochemical analysis in the current study showed the presence of glycosides along with tannin and alkaloids in methanolic extract of *C. album* which is in partial agreement with the earlier study. These substances were not detected by the earlier workers. The difference in age of plant, stage and age of the plant along with locality from where the plant was collected might be the possible reasons for this variation. Egale and Giday (2009) determined the anthelmintic effects of aqueous and hydroalcoholic extracts of the leaves of *C. ambrosioides* through in vitro experiments on eggs of *H. contortus*. Jabbar et al. (2007) found the anthelmintic activity in whole plant crude aqueous and methanolic extracts of *C. album* on eggs and larvae of *Trichostrongylid* nematodes of sheep and reported that *C. album* exhibited ovicidal effect on eggs and larvae of parasites. The ED₅₀ values were lower than observed in present study and could be attributed to the different environmental conditions, age of plant used, harvesting, drying, processing, storage and genetic variation of plant species used in different study.

In conclusion, the investigation of chemical compounds from natural products is fundamentally important for the

development of new anthelmintic drugs, especially in view of the vast worldwide flora. Based on the results presented in this work, methanolic extract of *A. squamosa* is highly effective followed by ethyl acetate and chloroform along side ethyl acetate extract of *C. album* followed by methanol and ethyl acetate against caprine gastrointestinal nematodes. Classes of secondary metabolites, like alkaloids, flavonoids, etc. found in the current experiment, are considered the sources of chemical components responsible for wide therapeutic activities of several medicinal plants. The active principles that induced the observed anthelmintic activity might be found in one or more classes of chemicals. Further investigation on isolated fractions, at different dose levels and against different parasite species should be pursued. However, further research is required to study toxicity, mechanism of action, identification of phytochemicals and effectiveness of this plant in vivo before it can be recommended for use in animals.

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