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In vitro protoscolicidal efficacy appraisal of methanolic herbal extracts against hydatid cysts

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ABSTRACT

The present study aimed at an evaluation of the protoscolicidal potential of methanolic extracts of three herbs; *Ferula asafoetida* (dried latex), *Trachyspermum ammi* (fruits) and *Hippophae salicifolia* (leaves) at three different concentrations (10, 20 and 30 mg/mL) for 20, 40 and 60 minute treatment intervals, with respect to standard allopathic drug albendazole. The *in-vitro* viability of the protoscolices was assessed by the Eosin exclusion method. The slope of mortality and lethal concentration for 50% (LC_{50}) was determined from the regression graphs of the probit mortality of protoscolices plotted against log values of increasing concentrations of herbal extracts for 60 minute treatment intervals. The percentage of mortality caused by various extracts at concentrations ranging from 10-30 mg/mL varied from 29.54-97.76% with maximum mortality of 97.76, 97.30 and 81.91% recorded with methanolic extracts of *F. asafoetida, T. ammi* and *H. salicifolia*, respectively. Among all the extracts, the highest protoscolicidal activity was exhibited by *F. asafoetida* at 20 and 30 mg/mL concentrations in the 60 (97.16%) and 40/60 minutes (97.20% and 97.76%) treatments, respectively, and it showed a minimum LC_{50} value of 0.204, followed by *T. ammi* at 0.933. As compared to the standard allopathic drug, albendazole, the methanolic extracts of *F. asafoetida* and *T. ammi* exhibited better or comparable protoscolicidal activities.

Key words: cystic echinococcosis; herbs; lethal concentration; protoscolicidal activity

Introduction

Cystic echinococcosis is a severe, chronic, zoonotic disease distributed worldwide with immense public health significance (PENSEL et al., 2014). The site of predilection of adult tapeworms *Echinococcus granulosus* is the intestines of carnivores, most commonly dogs and other wild canids (GHOLAMI et al., 2013); whereas, the metacestodal larval stages establish themselves in a wide range of intermediate hosts, including sheep, goat, cattle, pigs, horses and humans (BUDKE et

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al., 2009). The intermediate hosts are infected by consumption of the eggs of the tapeworm with contaminated feed and water (MOAZENI et al., 2017). The blood circulation helps in dissemination of the liberated embryos (in the intestines) to the vital organs, including the lungs, liver etc., where hydatid cysts develop (MOAZENI et al., 2017). The life cycle completes when the definitive hosts (dogs and wild canids) are infected by consumption of infected viscera with fertile hydatid cysts (LARRIEU et al., 2001). The patient is subjected to surgery or different therapeutic treatments based upon the size, location and number of the hydatid cysts (NICOLAO et al., 2014).

Surgery is the preferred method for removal of intact hydatid cysts (GHOLAMI et al., 2013) but certain complications are associated with the technique, *i.e.* intra-operative spillage of protoscolices, leading to anaphylactic shock and reoccurrence, or multiple secondary infections (echinococcosis) (MORO and SCHANTZ, 2009). Various protoscolicidal agents, such as formalin, alcohol, hypertonic saline and povidone iodine, have been used in the past (KARAOGLANOGLU et al., 2011) but most of these agents have led to toxicity and severe hepato-billiary complications, along with fatal hyperthermia (ADAS et al., 2009; KARAOGLANOGLU et al., 2011). In inoperable cases, the only alternative remains chemotherapy using benzimidazole-methylcarbamate (BZ) compounds, such as albendazole (ABZ) and mebendazole. The application of BZ drugs cures approximately one-third of the patients, whereas regression of hydatid cysts has been recorded in 30-50% cases. On the other hand, 20-40% cases do not respond to the therapy (WALKER et al., 2004). Moreover, long term chemotherapy with BZ compounds has also led to certain adverse reactions in patients, including gastrointestinal disturbances, vomiting, eosinophilia, alopecia, leukopenia, elevation in transaminase levels and, most importantly, parasitic resistance (HEMPHILL and MULLER, 2009).

Considering the adverse side effects associated with surgery and chemotherapy, the search for new therapeutic alternatives with the least adverse effects and high efficacy (such as the use of traditional medicinal plants) has increased. *Ferula asafoetida* is known to have promising anthelmintic, antibiotic, antimicrobial, antifungal, anticancer, anti-diabetic and therapeutic properties due to the presence of polyphenolic compounds, such as terpenoids, sulphide derivatives, phenols and minerals (GUNDAMARAJU, 2013). Also, *Trachyspermum ammi*, a member of Apiaceae family, consists of 2-4% essential oils rich in monoterpes, such as thymol, γ-terpinene, p-cymene and is known to have anthelmintic, insecticidal and antiseptic properties (MOAZENI et al., 2012). *Hippophae salicifolia* contains tocopherols, sterols, flavinoids, carotenoids, lipids, ascorbic acid and tannins, and eventually exhibits biological and therapeutic properties, including anti-oxidant, anti-tumour, hepatoprotective and anti-inflammatory activities (SAIKIA and HANDIQUE, 2013). Hence, the present study was envisaged with the aim of identifying new protoscolicidal phyto-agents for the successful treatment of hydatidosis.

Materials and methods

Collection of protoscolices. The hydtid cysts were collected aseptically from the liver and lungs of infected sheep and goats, slaughtered in and around Palampur, Himachal Pradesh (North-Western Himalayan region), India. The intact cysts were then transferred (at 4˚C in ice box) to the Department of Veterinary Parasitology, DGCN College of Veterinary and Animal Sciences, Palampur (H.P.), India. The cysts were thoroughly cleaned with 0.9% phosphate buffered saline (pH 7.2). A small quantity of cyst fluid was aspirated (from all the cysts) with a syringe (10 mL), and was subjected for assessment of fertility. In the case of fertile cysts, the entire amount of cystic fluid was then aspirated and transferred into a beaker and left as such for 2 hours, in order to allow the protoscolices to settle down. The supernatant was then removed and the settled protoscolices were then washed 5 times with normal saline. The protoscolices were then counted under a microscope by taking a drop of the sediment on a microscopic slide. The viability status of the protoscolices was assessed with the Eosin exclusion method (SHAHNAZI et al., 2016). The cysts/ cystic fluid exhibiting more than 90% viability were considered for further experiments (SMYTH and BARRETT, 1980).

Procurement of plant material and methanolic extract preparation. *Ferula asafoetida* (dried latex) and *Trachyspermum ammi* (fruits) were procured from a local market, whereas the leaves of *Hippophae salicifolia* were procured from Kukumseri (Lahaul and Spiti) of Himachal Pradesh. The collected leaves were shade dried, and ground to obtain fine powder. Similarly, the fruits of *T. ammi* were also processed to obtain a fine powder. Fifty grams of powdered material for all species were weighed and mixed with 400 mL of absolute methanol (Analytical Reagent, Ranbaxy Laboratory Limited, S.A.S Nagar, Punjab, India) separately, and left for 24 hours in a 500 mL conical flask. The flasks were shaken at small intervals at room temperature and then placed in an electric tissue bath at a temperature of 40˚C. The content was then filtered through triple Whatman filter paper no. 1, and the filtrate was subsequently dried in a crucible in an electric tissue float bath. After extracting the dried filtrate, it was lyophilized and stored at 4˚C until further use.

In vitro protoscolicidal efficacy. In the present study, three different concentrations (10, 20 and 30 mg/mL) of each herb *i.e. F. asafoetida*, *T. ammi* and *H. salicifolia* was evaluated at 20, 40 and 60 minutes treatment intervals with respect to the standard allopathic drug albendazole, following the protocol of SHAHNAZI et al. (2016). Different concentrations were prepared by adding the required 10, 20 and 30 mg of lyophilized methanolic extracts per mL of normal saline (SHAHNAZI et al., 2016). Then, in each evaluation test tube, 2.5 mL of herbal concentration was added to 100 mL of cystic fluid containing around 1000 protoscolices, for periods of 20, 40 and 60 minutes at 37˚C. Following incubation, 10 mL normal saline was added to each tube and centrifuged for 1 min. at 3000 rpm. After removal of the supernatant, 0.1% aqueous eosin was added to the sediment and mixed gently. The supernatant was again removed after incubation for around 10-15 min. The sediment was then observed under a light microscope. The stained (dead) and unstained (live) protoscolices were then counted to evaluate the live and dead percentage, as well as mortality rate. Along with the test concentrations, a negative control group (containing 0.9% PBS) was also included to assure the accuracy of the test and for quality control. The test containing protoscolices treated with albendazole (10 µg/mL) was considered as the positive control. All the experiments were performed in triplicate (DALIMI et al., 2002).

Statistical analyses. Dose response data were analysed by the probit method (FINNEY, 1962) using GraphPad Prism 7 software. The slope of mortality and lethal concentration for 50% (LC_{50}) was determined from the regression graphs of probit mortality of protoscolices plotted against log values of increasing concentrations of herbal extracts for 60 minute treatment intervals.

Results and discussion

The present study is an effort to compare the efficacies of different herbal extracts used earlier (*T. ammi*) with newer ones (*F. asafoetida* and *H. salicifolia*) with respect to their lethal concentration (LC_{50}) values against *E. granulosus* protoscolices. Data on the slope [95% confidence limit (CL)], goodness of fit (R^2) and LC_{50} were determined at different concentrations for different time intervals and the results are presented in Table 1. The regression graph of the mean mortality of protoscolices plotted against the log values of progressively increasing concentrations of methanolic herbal extracts of *F. asafoetida*, *T. ammi* and *H. salicifolia* is shown in Figs 1-3, respectively.

Table 1. Dose response data of protoscolices against various herbal extracts at a 60 minute treatment interval

Herbal extract	Slope ± SE (95% CL)	R^2	LC_{50}
Ferula asafoetida	5.62 ± 0.44 (-0.03 to 11.28)	0.86	0.204
Trachyspermum ammi	5.04 ± 0.54 (-1.79 to 11.88)	0.88	0.933
Hippophae salicifolia	2.52 ± 0.29 (-1.25 to 6.30)	0.98	12.02

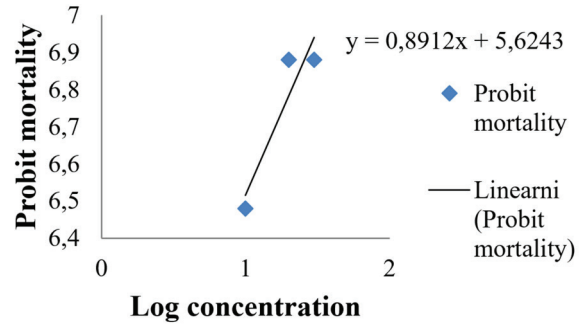

Fig. 1. Dose mortality curve of protoscolices against different concentrations of methanolic extracts of *Ferula asafoetida*

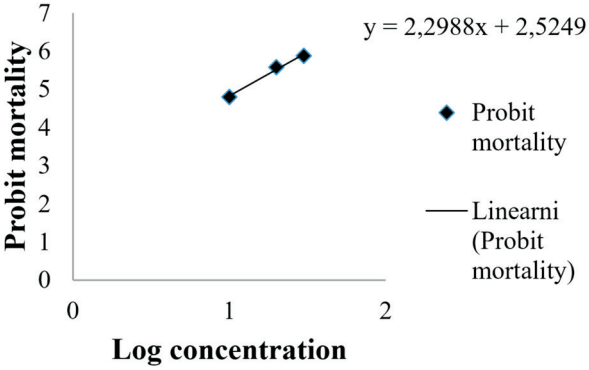

Fig. 2. Dose mortality curve of protoscolices against different concentrations of methanolic extracts of *Trachyspermum ammi*

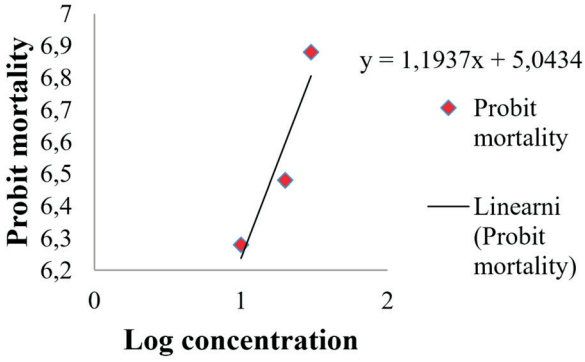

Fig. 3. Dose mortality curve of protoscolices against different concentrations of methanolic extracts of *Hippophae salicifolia*

From the regression equation, the LC_{50} values of *F. asafoetida*, *T. ammi* and *H. salicifolia* were calculated as 0.204, 0.933 and 12.02, respectively. The results indicated the minimum LC_{50} values for *F. asafoetida* and comparatively higher values for *H. salicifolia.* The lower LC_{50} values for *F. asafoetida* gum resin could easily be associated with its properties as an expectorant, laxative and vermifuge agent (MAHENDRA and BISHT, 2012). The anthelmintic activity of *F. asafoetida* could be implicated in the presence of polyphenolic compounds, which could have led to uncoupling of oxidative phosphorylation in the targeted parasites (GUNDAMARAJU, 2013). The protoscolicidal property of *T. ammi* could be associated with the presence of thymol (ROSTAMI et al., 2016), γ-terpinene, p-cymene (MOAZENI et al., 2012). The possible mechanism for thymol action could be asserted as the potentiation of ATPase activity, resulting in interference with the energy metabolism of parasites (ROSTAMI et al., 2016). The amphipathic and hydrophobic behaviour of thymol and p-cymene also results in alteration of the membrane permeability and leakage of intracellular material (MOAZENI et al., 2012). The results of the present study indicated the higher potential of methanolic extract of *F. asafoetida* as a protoscolicidal agent.

The efficacy of the herbal extracts can also be appreciated by observation of the collapsed germinal layers of the dead protoscolices with escaped materials (Fig. 4). However, the germinal layers of the live protoscolices were turgid and undamaged (Fig. 5). The negative control group (containing 0.9% PBS) of protoscolices, when examined at the initiation of the trial, showed turgidity and were motile. Only a few protoscolices were found damaged. The eosin exclusion method revealed 0.56-1.56% mortality rate. The mortality percentage caused by the different extracts at concentrations ranging from 10-30 mg/mL varied from 29.54-97.76% with maximum mortality of 97.76, 97.30 and 81.91% recorded against methanolic extracts of *F. asafoetida, T. ammi* and *H. salicifolia*, respectively (Table 2). *F. asafoetida* proved to be the most effective herbal extract, and depicted the highest protoscolicidal activity at 20 and 30 mg/mL concentrations for the 60 (97.16%) and 40/60 minute (97.20 & 97.76%) treatments, respectively.

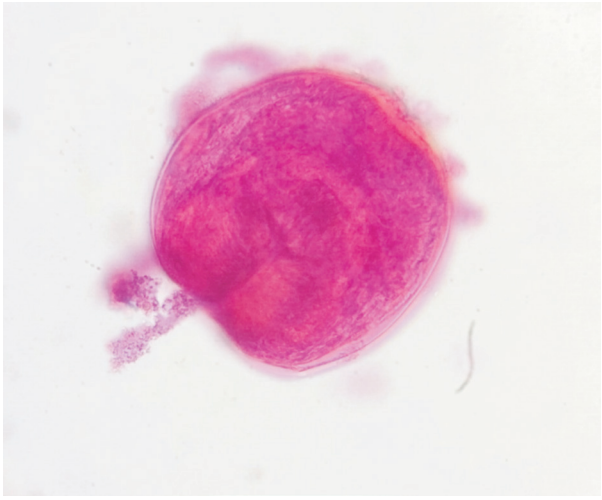

Fig. 4. Dead protoscolex after exposure to the herbal extract exhibiting damaged germinal membrane

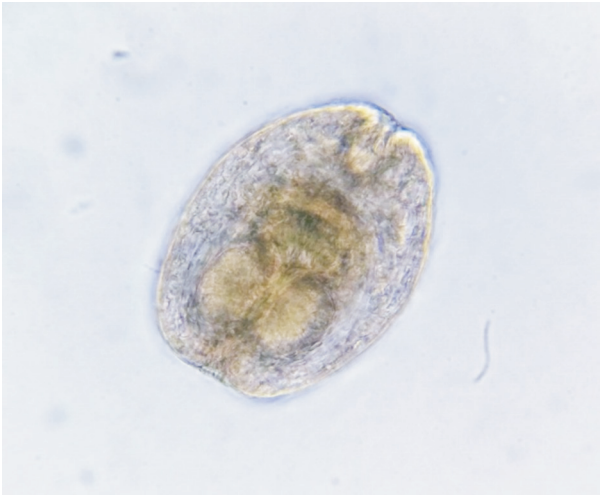

Fig. 5. Live protoscolex after exposure to the extract

Table 2. Protoscolicidal efficacies of various herbal extracts at various exposure intervals

Concentration	Exposure time (minutes)	Total protoscolices (Mean ± SD)	Live protoscolices (Mean ± SD)	Dead protoscolices (Mean ± SD)	Mortality rate (%)
0.9% PBS	20	412 ± 20.88	409.67 ± 19.39	2.33 ± 1.57	0.56
	40	361.33 ± 34.19	356.67 ± 31.89	4.67 ± 2.30	1.29
	60	363.67 ± 43.66	358 ± 42.33	5.67 ± 1.53	1.56
Albendazole	20	377 ± 52.16	49 ± 12	328 ± 51.12	87
	40	342 ± 39.95	29 ± 11.79	313 ± 50.16	91.52
	60	353.67 ± 85.41	12.33 ± 3.51	341.33 ± 88.46	96.51
FAM1 (10 mg/mL)	20	382.33 ± 26.08	101 ± 23.64	281.33 ± 25.48	73.58
	40	367.66 ± 61.23	68.33 ± 4.04	306 ± 49.79	83.23
	60	370.66 ± 25.79	23.33 ± 6.50	347.33 ± 32.19	93.70
FAM2 (20 mg/mL)	20	379.33 ± 59.77	86.67 ± 9.29	292.66 ± 67.85	77.15
	40	378.33 ± 69.61	46 ± 9.64	332.33 ± 63.70	87.84
	60	329 ± 17	9.33 ± 4.04	319.67 ± 17.47	97.16
FAM3 (30 mg/mL)	20	350 ± 51.64	65.67 ± 12.58	284.33 ± 49.90	81.23
	40	333.33 ± 39.51	9.33 ± 2.51	324 ± 38.97	97.20
	60	372 ± 49.73	8.33 ± 2.08	363.67 ± 50.84	97.76
TAM1 (10 mg/mL)	20	353 ± 27.62	98 ± 12.12	255 ± 24.57	72.24
	40	364.67 ± 42.72	74.67 ± 8.73	290 ± 34.07	79.52
	60	403 ± 23.81	38.33 ± 4.04	364.67 ± 23.96	90.49
TAM2 (20 mg/mL)	20	375.67 ± 33.50	82.33 ± 10.59	293.33 ± 28.18	78.08
	40	361.67 ± 37.68	44.67 ± 10.26	307 ± 32.51	87.29
	60	333 ± 51.39	21 ± 8	312 ± 58.79	93.69

*PBS - Phosphate Buffer saline, FAM- *Ferula asafoetida* methanolic extract, TAM - *Trachyspermum ammi* methanolic extract and HSM - *Hippophae salicifolia* methanolic extract

Table 2. Protoscolicidal efficacies of various herbal extracts at various exposure intervals (continued)

Concentration	Exposure time (minutes)	Total protoscolices (Mean ± SD)	Live protoscolices (Mean ± SD)	Dead protoscolices (Mean ± SD)	Mortality rate (%)
TAM3 (30 mg/mL)	20	258.33 ± 48.54	77.67 ± 7.09	180.66 ± 55.59	69.93
	40	345.33 ± 43	23.33 ± 5.13	322 ± 43.71	93.24
	60	346 ± 41.07	9.33 ± 2.08	336.67 ± 43.33	97.30
HSM1 (10 mg/mL)	20	343 ± 54.83	241.67 ± 43.43	101.33 ± 41.04	29.54
	40	404.33 ± 11.23	242.67 ± 44.76	161.66 ± 34.42	39.98
	60	317.33 ± 4.72	182.67 ± 12.74	134.66 ± 16.07	42.43
HSM2 (20 mg/mL)	20	381 ± 28.51	181.33 ± 30.67	199.67 ± 37.11	52.40
	40	392.66 ± 19.21	156.33 ± 23.67	236.33 ± 6.35	60.18
	60	332 ± 17.69	90 ± 20	242 ± 23.89	72.89
HSM3 (30 mg/mL)	20	380.66 ± 55.24	125.66 ± 26.67	255 ± 65.19	66.98
	40	341.33 ± 49.09	100.67 ± 19.33	240.66 ± 52.16	70.50
	60	372.33 ± 22.18	67.33 ± 16.67	305 ± 14.73	81.91

*PBS- Phosphate Buffer saline, FAM- *Ferula asafoetida* methanolic extract, TAM- *Trachyspermum ammi* methanolic extract and HSM- *Hippophae salicifolia* methanolic extract

On the other hand, albendazole exhibited mortality rates of 87-96.51% at different concentrations. *T. ammi* and *H. salicifolia* evinced 72.24-97.30 and 29.54-81.91% mortality rates at different concentrations, respectively. The observations of the present study pertaining to the efficacy of *T. ammi* are approximately in line with the findings of MOAZENI et al. (2012), who observed a 100% scolicidal effect of essential oils of *T. ammi*. The protoscolicidal efficacy of *T. ammi* could be ascribed to its thymol contents (50.07%) (MOAZENI et al., 2012), which hold antibacterial (GOUDARZI et al., 2011), insecticidal (PANDEY et al., 2009) and anti-fungal (NAGALAKSHMI et al., 2000) properties. Other herbal compounds (*Zataria multiflora*) with high thymol contents also exhibited scolidicidal activities with mortality rates as high as 100% in past studies (MOAZENI et al., 2017). On the other hand, the exact mechanism behind the higher efficacy of *F. asafoetida* is not known; however, essential oils of different *Ferula* species have been observed to exhibit cytotoxic effects against human tumour cell lines (MOLLAZADEH et al., 2010; MAZZIO and SOLIMAN, 2011). *F. asafoetida* has also been proved to hold significant anthelmintic (GUNDAMARAJU, 2013); anti-leishmanial and antiparasitic properties (BAFGAHI et al., 2014). These observations could be reckoned to be associated with the presence of polyphenolic (tannins) and disulphide compounds in *F. asafoetida.*

Conclusions

As compared to the standard allopathic drug, albendazole, the methanolic extracts of *F. asafoetida* and *T. ammi* exhibited better or comparable protoscolicidal activities. Being phyto-agents, the herbs manifest no side effects and could easily be chosen as a replacement for allopathic drugs. Further detailed studies (especially *in vivo* studies and stability studies inside the cysts) pertaining to these herbs against cystic echinococcosis are warranted.

Conflict of interest

Authors declare that they have no conflict of interest

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SAŽETAK

Cilj je istraživanja bila procjena protoskolicidnog potencijala metanolnih ekstrakata triju biljaka: *Ferula asafoetida* (sušena smola), *Trachyspermum ammi* (voće) i *Hippophae salicifolia* (lišće) u trima različitim koncentracijama (10, 20 i 30 mg/mL) u 20, 40 i 60-minutnim pokusnim intervalima u odnosu na standardni alopatski lijek albendazol. *In vitro* vitalnost protoskoleksa procijenjena je metodom isključivanja eozina. Krivulja pomora i smrtonosne koncentracije za 50 % (LC_{50}) određena je iz regresijskih grafikona mortaliteta protoskoleksa u odnosu na log vrijednosti rastućih koncentracija biljnih ekstrakata tijekom 60-minutnog intervala. Pomor uzrokovan različitim ekstraktima u koncentracijama od 10 do 30 mg/mL varirao je od 29,54 do 97,76 % s najvišim postotkom od 97,76, 97,30 i 81,91 % u slučaju metanolnih ekstrakata *F. asafoetida, T. ammi* i *H. salicifolia*. Od svih primijenjenih ekstrakata najvišu protoskolicidnu aktivnost imala je *F. asafoetida* u koncentracijama od 20 i 30 mg/mL tijekom tretmana od 60 (97,16 %) i 40/60 minuta (97,20 i 97,76%), te je pokazala minimalnu vrijednost LC_{50} od 0,204, praćenu s *T. ammi* od 0,933. Usporedbom sa standardnim antiparazitikom albendazolom, metanolni ekstrakti biljaka *F. asafoetida* i *T. ammi* ostvarili su bolji ili slični protoskolicidni učinak.

Ključne riječi: hidatidoza; biljke; smrtonosna koncentracija; protoskolicidna aktivnost