

Original papers

The *in vitro* effect of *Ferula asafoetida* and *Allium sativum* extracts on *Strongylus* spp.

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ABSTRACT. The high incidence of equine gastrointestinal worms and their increased resistance against anthelmintics has encouraged research into the effectiveness of rational phytotherapy. This study investigates the *in vitro* anti-parasitic effects of extracts of *Ferula asafoetida* and *Allium sativum*, two native plants that are widespread in Iran on *Strongylus* spp. larvae. Faecal samples were collected from horses, examined by routine parasitology methods and positive samples were used for future examination. After incubation, the third-stage larvae were harvested by the Baermann technique. A hydroalcoholic extract from the plants was used for the antiparasitic study, while tap water was used for controls. Trials for each concentration and control group were performed in three replicates. The results showed that during the first day of exposure, the hydroalcoholic extract of *F. asafoetida* at concentration of 10, 50 and 100 mg/ml killed over the 90% of the larvae, and *A. sativum* extract at concentration of 50 and 100 mg/ml killed over the 95% of larvae ($p < 0.05$). The results obtained from the bioassay showed that two plant extracts have a larvicidal effect on the *Strongylus* spp. larval stages compared with the control group.

Key words: anti-larval, equine, *Ferula asafoetida*, *Allium sativum*, *in vitro*

Introduction

Adult worms of *Strongylus* spp. live in the large intestine of horses and are commonly categorized as large and small strongyles [1,2]. Of the more than 100 species of internal parasites found in horses, almost half are strongyles [3]. *Strongylus* spp. is a ubiquitous and important nematode parasite of horses and related equidae [4]. They are frequently responsible not only for generally poor health, but also gastrointestinal dysfunctions, including colic and the potentially fatal condition of acute larval cyathostomosis [5]. Infective third-stage larvae (L₃) ingested from contaminated pasture penetrate the mucosa of the large intestine, moult to fourth-stage larvae (L₄) in the submucosa. In the case of *Strongylus vulgaris*, they then proceed to the root of the cranial mesenteric artery along the arterioles and arteries that supply the intestine, where they may

cause parasitic thrombosis and arthritis [6]. Larvae of the other two species may be found in various parts of the body, including the liver, pre-renal tissues, retroperitoneal tissues, and pancreas [7].

Over the past five decades, parasite control regimens involving frequent and regular anthelmintic treatment of all horses appear to have resulted in a decline in the prevalence and abundance of *S. vulgaris*, and the parasite is now considered rare in managed horse populations [8,9]; however, while the primary goal of the anthelmintic treatment regimens has been achieved, new challenges have arisen. Anthelmintic resistance in cyathostomin parasites is now being widely reported against benzimidazole-type drugs, as well as increasing levels of pyrantel resistance [6], and most recently, several publications have reported signs of ivermectin and moxidectin resistance [10]. This is further exacerbated by the world-wide

occurrence of ivermectin and moxidectin resistance reported in *Parascaris equorum* [6]. In the face of increasing levels of resistance against anti-parasite drugs and the damaging effects of these drugs themselves, alternative treatments are required. Anthelmintics derived from plants can be an alternative for the treatment of parasitic infections [11]. A good source of knowledge regarding the potential action of plant extracts on certain diseases and pests is constituted by research in the field of medicinal plants. Many researchers have considered the anti-cancer properties of *Allium sativum* (garlic), as well as its anti-parasite effect on *Trypanosoma* spp., *Entamoeba histolytica*, *Giardia lamblia*, *Leishmania major*, *Cryptosporidium* spp., *Plasmodium* spp., *Ascaris lumbricoides*, *Heterakis gallinae*, *Ascaridia galli*, *Haemonchus contortus*, *Nippostrongylus brasiliensis*, *Ascaris suum* [12–15].

Ferula asafoetida has traditionally been used for its anthelmintic properties in various countries such as Iran, China and Nepal, where it has been used for treating infection with intestinal parasites [16]. Some studies have examined the anti-parasitic properties of *F. asafoetida*, including activity against *Trichomonas vaginalis* and *Schistosoma mansoni* [17], and naturally-occurring plant products such as diterpenes, phenolics and sulfur-containing compounds are known to possess anti-*Leishmania* properties [18]. However, as no study has so far examined the activity of *F. asafoetida* against equine parasites, some aspects of this form of control remain unclear, and its anthelmintic activity warrants further investigation.

Ferula asafoetida is an Iranian endemic medicinal plant which has been used as a spice in food. For decades, it has also been used a traditional folk phytomedicine to treat various diseases, including intestinal parasites [19].

The aim of this study is to investigate the anti-parasitic effects of *F. asafoetida* and *A. sativum* plant extracts on *Strongylus* spp. larvae *in vitro*.

Materials and Methods

Preparation of parasites. In this study faecal samples were collected from infected horses around Urmia city, West Azerbaijan, Iran during November 2014. None of the animals had been given anthelmintic treatment for at least six months before they were sampled. Faecal sample were collected directly from the rectum of horses or immediately

after faecal excretion. The faecal samples were placed in plastic bags and sent to the Parasitology Laboratory, Faculty of Veterinary Medicine, Urmia University. The samples examined by routine parasitology methods and positive samples were used for future examination [20].

Larval culture. Culture were created for the detection of *Strongylus* larvae as follows: at least 100 grams of faeces mixed with autoclaved dry faecal samples. The sample was incubated for 7 days at 28°C, during which time more water was added if necessary. To prevent fungal growth, the samples were mixed daily using a spatula. After incubation, the third-stage larvae were harvested after 24 hours of sedimentation by the Baermann technique. All larvae were examined and identified by microscope using morphological criteria [21].

Preparation of hydroalcoholic extract. The plant *F. asafoetida* was collected in March 2014 from Sabzevar, Khorasan Razavi, Iran. The plant material was shade dried for several days. The plants were powdered with electric grinder. Whole plants were dried and ground in a hammer mill, then soaked in 70% ethanol and kept at room temperature for seven days. To complete the extraction process, the material was mixed twice daily. This procedure was repeated four times and combined ethanolic extract evaporated to dryness on a rotary evaporator at temperature between 45 and 50°C, resulting in the production of a deep dark brown semi-solid residue. The *F. asafoetida* hydroalcoholic extract (AAHE) was kept protected from light in a refrigerator at 4°C. *A. sativum* was purchased from local market of Urmia city, Iran, and same procedure was performed as for the preparation of the *F. asafoetida* hydroalcoholic extract [22,23].

Antiparasitic tests. Firstly, 1ml of larval suspension containing approximately 30 larvae was incubated at 25°C with 1 ml of hydroalcoholic extract of *F. asafoetida* at concentrations of 100, 50 and 10 mg/ml. In addition, 1 ml of larval suspension was also incubated at 25°C with 1ml of hydroalcoholic extract of *A. sativum* at concentrations of 100, 50, 10, 5, 2.5 and 1.25 mg/ml. These doses were selected according pilot study.

The survival rate of larvae was calculated 1, 2, 3, 24 and 48 hours after treatment. The parasites were removed from the incubator and the numbers of viable parasites were counted under a microscope: death was concluded when no movement was observed in larvae after stimulation. For control group, the specimens were only incubated in tap

Table 1. Mortality percentage of larvae with different concentrations of the *Allium sativum* hydroalcoholic extract after incubation periods

Treatment	% of dead larvae after incubation time					
	1(h)	2(h)	3(h)	24(h)	48(h)	
Control	35.50 ± 12.30	36.25 ± 11.10	32.20 ± 2.54	35.45 ± 3.88	45.55 ± 0.77	
Extract mg/ml	100	65.90 ± 6.64	74.70 ± 4.66	73.90 ± 5.37*	93.90 ± 0.84*	100.00 ± 0.0*
	50	56.50 ± 2.54	76.10 ± 3.95	81.55 ± 4.87*	97.55 ± 1.06*	97.05 ± 0.63*
	10	44.60 ± 7.63	70.15 ± 6.85	75.75 ± 1.06*	76.40 ± 2.82*	97.05 ± 4.17*
	5	41.00 ± 12.72	63.75 ± 7.99	71.70 ± 4.66*	82.95 ± 0.49*	90.60 ± 1.55*
	2.5	37.85 ± 15.20	59.25 ± 2.75	62.15 ± 2.05*	78.80 ± 2.40*	82.75 ± 8.13
	1.25	34.85 ± 5.86	51.65 ± 24.11	62.50 ± 4.94*	67.00 ± 2.82*	84.65 ± 5.02

Results are expressed as means ± standard deviation (SD). Data are means of three replications.

*Values in each column (hour) are significantly different in comparison with control group ($P \leq 0.05$).

water. Trials for each concentration and control group were performed in three replicates [24].

Statistical procedure. Data is expressed as means±standard deviation (SD). The percentage survival rate of larvae was calculated 1, 2, 3, 24 and 48 hours after treatment. The means of three replications was recorded for that time and defined as dependent variable for the statistical analysis of the data. Data obtained from the *in vitro* tests was analysed using ANOVA and compared by means of the Tukey’s test. At every time point, the results were compared with those of the control group. Statistical analyses were performed using the SPSS ver.19.0 statistic software package (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered significant [25].

Results

The results obtained from the bioassay showed that the two plant extracts had a larvicidal effect on larval stages of *Strongylus* spp. The worm larvae treated with the two plants extracts demonstrated a

significantly increased mortality rate compared with the control group.

The results showed that on the first day of exposure, the hydroalcoholic extract of *F. asafoetida* killed over the 90% of the larvae when administered at concentration of 10, 50 and 100 mg/ml. In addition, *A. sativum* extract killed over the 95% of the tested larvae in the first 24 hours when administered at concentration of 50 and 100 mg/ml, and killed 90% after 48 hours of exposure at 10 and 5 mg/ml (Table 1).

The results also showed that all extract concentrations of *A. sativum* examined were effective against the larval stage of *Strongylus* spp. three hours after treatment ($p < 0.05$) (Table 1), while the *F. asafoetida* extracts demonstrated the same results 48 hours after treatment ($p < 0.05$) (Table 2). The results also indicate that both the 100 mg/ml *A. sativum* extract and the 50 and 100mg/ml *F. asafoetida* extracts killed all the test larvae after 48-hour incubation.

Table 2. Mortality percentage of larvae with different concentrations of the *Ferula asafoetida* hydroalcoholic extract after incubation periods

Treatment	% of dead larvae after incubation time					
	1(h)	2(h)	3(h)	24(h)	48(h)	
Control	21.20 ± 1.96	29.00 ± 1.41	33.15 ± 1.62	36.15 ± 1.62	42.45 ± 6.01	
Extract mg/ml	100	28.2 ± 1.96	31.05 ± 1.41	46.25 ± 1.76	99.35 ± 4.17*	100.00 ± 0.0*
	50	34.10 ± 1.55	33.20 ± 1.96	42.20 ± 0.28	94.95 ± 1.20*	100.00 ± 0.0*
	10	27.05 ± 7.35	44.00 ± 1.41	43.35 ± 1.90	91.10 ± 2.54*	94.35 ± 2.89*

Explanation: see Table 1.

Discussion

Both of the tested extracts demonstrated dose-dependent anthelmintic activities, and the mortality rate increased significantly as the time of exposure of the larvae to the plant extracts increased. The activity of *A. sativum* ranged from a 100% mortality rate, demonstrated on day 2 of post-incubation with the 100mg/ml extract, to 84.65%, demonstrated by the lowest concentration of 1.25 mg/ml. Higher doses of *F. asafoetida* (50 and 100 mg/ml) were needed to cause 100% mortality after 48-hour exposure. The onset of an acceptable anthelmintic effect was rapid in both cases, being observed 24 hours after treatment.

Ajoene, an organosulfur compound derived from *A. sativum* is known to demonstrate significant antifungal, anti-trypanosomal and antiviral activities [10]. Ajoene may cause alterations in protein and lipid transport in the parasitic larva cell membranes, leading to irreversible damage to the parasites [12].

Many pharmacological properties, including antibacterial, antioxidant, antifungal, tranquilizing, carminative, gastrointestinal, antispasmodic, laxative, pain killing, diuretic, and disinfecting activities have been confirmed for *F. asafoetida* extract [26]. *F. asafoetida* consists of three main fractions; including resin (40–64%), containing ferulic acid and its esters, coumarins, sesquiterpenecoumarins and other terpenoids, gum (25%) and essential oil (10–17%) [27].

The results of the current study indicate that hydroalcoholic extracts of *F. asafoetida* and *A. sativum* have potential anthelmintic and larvicidal activities *in vitro*; however, further *in vivo* evaluation of the different parts and fractions is needed to make use of these plants for beneficial purposes. It is interesting to note that the extracts are combinations of many components and are not pure, so these primarily results only suggest the potency of these extracts. Nevertheless, our discovery that the plant extracts can be used as accessible source of natural anthelmintic from plants is rewarding, as it will lead to the development of a phytomedicine to act against parasite. These extracts have enormous therapeutic potential, as they can fulfil their purpose without any side effects that are often associated with synthetic compounds [28,29].

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