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IN VITRO EFFECT OF RAW EXTRACT OF SERBIAN GENOTYPE OF GARLIC (*ALLIUM SATIVUM* L.) ON EGGS OF GASTROINTESTINAL NEMATODES OF SHEEP*

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Summary: *Negative impact of gastrointestinal nematodes (GIN) on the productivity of grazing sheep has long been recognized. In the majority of sheep-rearing countries, their control has long relied on the intensive use of broad-spectrum anthelmintics, which led to the widespread development of anthelmintic resistance. The use of herbal devormers, such as garlic, has been suggested as one of the alternative ways of controlling parasitism in producing animals. The aim of the present study was to investigate the in vitro effect of raw garlic extract (RGE), prepared from a Serbian genotype of the plant, on the hatching of sheep GIN eggs. For this purpose, an egg hatch test was performed using three different concentrations of garlic extract. Significant, dose-dependent, ovicidal effect of RGE ($p=0.005$) was recorded. Extract concentrations of 16.7%, 33.3% and 66.7% (v/v) inhibited hatching of 68.1%, 76.7% and 92.6% (median values) of eggs, respectively. Significantly higher efficacy was achieved with the highest concentration than with the lower ones ($p<0.05$). In vivo confirmation of this effectiveness is necessary in order to evaluate practical use of extract under farming conditions.*

Key words*: garlic, ovicidal effect, in vitro, gastrointestinal nematodes, sheep.*

INTRODUCTION

Gastrointestinal nematode (GIN) parasites are known to have a significant impact on the productivity of grazing sheep, thus reducing overall income of sheep farmers (Miller et al., 2012; Papadopouloset al., 2012).

The use of broad-spectrum anthelmintics has long been the main approach to the control of GIN of sheep. As a consequence of their intensive use, widespread development of anthelmintic resistance occurred in three economically most important species of nematodes that infect small ruminants (*Haemonchus contortus*, *Teladorsagia circumcinta* and *Trichostrongylus* spp.) (Papadopouloset al., 2012). In Serbia, poor efficacy of ivermectin against *Trichostrongylus* spp. and *Nematodirus* spp. was recently found (Simin et al., 2014, Simin et al.,

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unpublished data), raising the question of the existence of anthelmintic resistance in sheep GIN in this country as well.

Therefore, in order to efficiently control small ruminant GIN in the future, as well as to fulfill the growing demand of public for food without drug residues (Charon, 2004**)**, current control schemes, that used to rely on the sole use of synthetic or semi-synthetic anthelmintics, may have to be replaced or complemented by inovative measures that are based on the use of herbal dewormers (Burke et al., 2009).

In addition to the variety of other plants with anthelmintic properties (Akhtar et al., 2000; Anthony et al., 2005; Guarrera,1999), garlic is traditionally used in some countries for the elimination of intestinal parasites of ruminants, dogs, cats, pigs, horses and donkeys (Guarrera,1999; Khan et al., 2010; Lans et al., 2007a; Lans et al., 2007b; Sutton and Haik, 1999). However, scientific data regarding anthelmintic activity of garlic metabolites are often ambiguous (Lalošević et al., 2013;Schelkleet al., 2013).

The aim of this study was to investigate the effect of raw garlic extract (RGE), prepared from a Serbian genotype of the plant, on hatching of eggs of sheep GIN *in vitro*.

MATERIALS AND METHODS

Experimental animals and sample collection. To collect GIN eggs, a flock of grazing Merinoland ewes naturally predominantly infected with *Haemonchus contortus* was visited. Faecal samples were taken from the rectum of 3 hoggets. Each sample was divided into 2 parts: one part was placed in a plastic bag to perform faecal egg counts (FEC, analytical sensitivity = 10 eggs per gram of feces (epg)) and coproculture (27°C for 7 days) (MAF, 1986), and the other part was packed in 100 mL plastic cup with screw top with addition of water and glass beads to obtain anaerobic conditions and prevent embryonation of eggs (Coles et al., 2006).

Preparation of egg suspension.The hogget with FEC value of 1850 epg was chosen as an egg donor. The eggs were extracted from anaerobically stored stock as described by (Coles et al., 2006). Number of eggs per milliliter of final suspension was quantified for the following trial.

Preparation of garlic extract. Raw garlic extract was obtained from Serbian garlic genotype (autumn garlic, *A. sativum* var. *vulgare*) one day prior start of experiment by mechanically pressing garlic bulbs. The bulbs were previously soaked in 96% ethanol for 5 min to achieve surface disinfection, and then washed in distilled water and left on filter paper at room temperature. After drying, the bulbs were squeezed in metal hand press; extract was filtered through sterile gauze to remove coarse bulb parts and stored at refrigerator at 4°C in sterile glass tubes until use.

Trial design. *In vitro* effect of RGE on sheep strongyle eggs was evaluated by simulation of egg hatch test (EHC) described by Coles et al. (2006) in 6-well tissue culture plates (Sarsted Inc., Newton, NC, USA). Three different concentrations of garlic extract were examined: 66.7%; 33.3% and 16.7% (v/v), with 6 replicates (one plate) for each concentration. One plate served as negative control. Approximately 160 strongyle eggs suspended in distilled water were added to each well. RGE and additional distilled water (when necessary) was added to total volume of 3 mL, respecting determined ratios. Plates were sealed with Parafilm "M"® (American National Can Co.,Chicago, IL, USA) and incubated at 25°C for 48 h. After that, 2 drops of Lugol's iodine was added to each well and all eggs and/or hatched larvae per well were enumerated using inverted microscope (CETI, Belgium) at 100x magnification.

Statistical analysis. For garlic concentrations, percentage of egg hatch inhibition (I (%)) for each of 6 tested wells was calculated according to following formula (Bizimenyeraet al., 2006):

$$I\ (\%) = 100(1 - P_{test}/P_{control})$$

where P_{test} is the number of eggs hatched (i.e. larval forms (L_1)) in test extracts, and $P_{control}$ is the respective numbers in distilled water control. Data were analyzed using Statgraphics Centurion 15.2.11.0. Median percentage of inhibition (%) was calculated for each concentration. For analysis of the effects of RGE on egg hatching, Kruskal-Wallis one way ANOVA (Petrie and Watson, 2006) was used. If there was significance, Mann-Whitney U test determined which garlic concentrations are significantly different. Significance level of 95% ($p < 0.05$) was used for all tests.

RESULTS

Coproculture revealed high level of *H. contortus* (85%) followed by *Trichostrongylus* spp. (10%) and *Oesophagostomum/Chabertia* (5%). The results of the EHT showed significant ovicidal effect of RGE ($p = 0.005$) on sheep GIN *in vitro*. Total number of eggs and hatched larvae for each garlic concentration and control are presented graphically (Figure 1). Increase of extract concentration (16.7%; 33.3% and 66.7% (v/v)) inhibited hatching of 68.1%, 76.7% and 92.6% (median values) of GIN eggs, respectively.

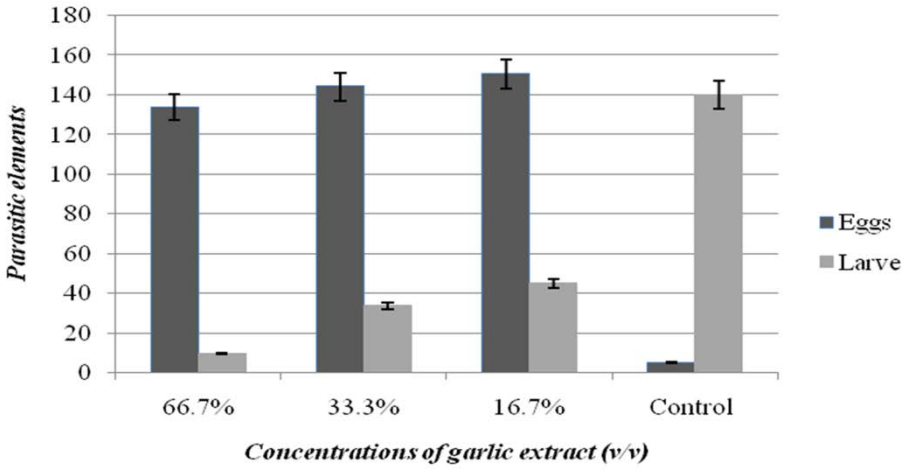

Figure 1.Mean (sd) number of eggs and hatched larvae given for each of three different RGE concentrations and control, used in the EHT. Short vertical lines that intersect columns indicate statistical error.

There was statistically significant difference between garlic concentrations, since fewer larvae were hatched in wells with the highest garlic dose (66.7%) compared to lower doses (16.7% and 33.3%; $p < 0.05$) (Table 1).

Table 1.Comparison of efficacy of different doses of raw garlic extract against sheep GIN *in vitro*

Comparison[a]	P-value	Significance[b]
Dose 1-Dose 2	0.045	yes
Dose 1-Dose 3	0.005	yes
Dose 2-Dose 3	0.128	no

[a] Doses 1, 2 and 3 correspond to 66.7%, 33.3% and 16.7 % (v/v) of raw garlic extract, respectively.
[b] Refers to statistically significant difference between efficacy of compared doses

DISCUSSION

In vitro tests for evaluation of novel anthelmintic agents are widely used in veterinary parasitology (Ferreira et al., 2013).

In the case of small ruminant parasites, modified EHT is often used to determine the effect of plant products against eggs of *H. contortus* or other trichostongylids (Githioriet al., 2006). Using this test, high level of experimental, mostly dose-dependent, inhibition on egg hatching (up to 100% of inhibition for highest concentrations) of major sheep GIN species was observed in the variety of plant extracts (e.g. Bizimenyera et al., 2006; Ferreira et al., 2013).

Results of the EHT from the present study suggest that raw extract of the domestic genotype of the garlic is capable of considerably impeding development and hatching of eggs of sheep GIN *in vitro*. Similarly to other plant extracts, there is also dose-dependent effect on egg hatching where the highest concentration caused mortality of over 90% of eggs. According to classification of efficacy of anthelmintic compounds proposed by Powers *et al.* (1982) (highly effective > 90%; moderately effective 80 to 90%; and low effectiveness 60 to 80), this performance may be considered as highly effective.

Ovicidal effect of garlic against helminthes was previously recorded by El Shenawy et al. (2008) by analyzing liver tissue egg counts and oogram pattern in *Shistosoma mansoni* infected mice treated with aqueous garlic extract

for 28 days (three times a week) and sacrificed at 49 days post infection. *In vitro* anthelmintic efficacy of garlic against other helminth life cycle stages has also been reported. Anthelmintic efficacy of garlic extract resulted in larvicidal (Ahmed et al., 2012) and adulticidal (Iqbal et al., 2001) effect on *H. contortus*. It was also effective against adult monogenean ectoparasite of fish, *Gyrodactylus turnbulli*, (Schelkleet al., 2013); paralytic effect on trematodes, *Gigantocotyle explanatum* (Singh et al., 2008) and *Fasciola gigantica* (Singh et al., 2009) as well as lethal effect on *Cotylophoron cotylophorum* (Radwan et al., 2012) was demonstrated. Protoscolices of cestode *Echinococcus granulosus* (Eskandarian, 2012; Moazeni and Nazer, 2010) and adult *Hymenolepis nana* (Omer chapbook et al., 2007) were inactivated with garlic extract; different concentrations of garlic oil caused mortality of common poultry nematodes, *Heterakis gallinae* and *Ascaridia galli*, (Singh and Nagaich, 2000).

However, it is well known that compounds or substances that are effective *in vitro* do not necessarily show same efficacy *in vivo* (Anassoriet al., 2011; Ferreira et al., 2013; Githioriet al., 2006). For example, high (Masamha et al., 2010; Noon, 2003) and low (Stricklandet al., 2009b) reduction of FEC in infected sheep was reported after treatment with water dilution of raw garlic juice and food pellets containing garlic, respectively. On the contrary, Burke et al. (2009), Strickland et al. (2009a), Worku et al. (2009) and Courter et al. (2012) found not only that garlic had no effect on percentage of egg reduction in small ruminant GIN but there was also evidence of low level of anti-nutritional properties of the 3.6% of garlic based diet (Stricklandet al., 2009a).

Discrepancies between the *in vitro* and *in vivo* efficacy of garlic against GIN could be explained by changes in the bioavailability and pharmacology of the active plant compound in different parts of the gastrointestinal tract as well as host-plant interactions (Ferreira et al., 2013; Stricklandet al., 2009a). The quantity and the nature of the host basal diet, the origin of garlic, the method of its processing and, in particular, its route of administration may also be other influencing factors (Anassoriet al., 2011). Finally, variations in the level of sulfuric compounds responsible for anti-parasitic effect of garlic (Anthony et al., 2005), that have been observed between different genotypes of garlic (Ghani, 2010), may too be responsible for success and failure of reduction of FEC in small ruminants from different studies.

Raw extract of the Serbian genotype of the garlic demonstrated both antimicrobial (Vlajić et al., 2014) and anthelminthic effect (current results) when tested *in vitro*. However, in order to obtain comparable results with other studies and to evaluate its practical use under farming conditions, *in vivo* confirmation of its efficacy against sheep GIN is necessary.

CONCLUSION

Raw extract of the Serbian genotype of garlic showed dose-dependent *in vitro* effect on development and hatching of sheep GIN eggs. Used in its highest concentration, extract exhibited high ovicidal efficacy. However, *in vivo* confirmation of this effectiveness is necessary in order to evaluate practical use of extract under farming conditions. If applicable, anthelmintic properties of garlic extract could be of great value in organic farming systems, particularly, as a part of an integrated approach designed to achieve sustainable parasite control in ruminant production systems.

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IN VITRO EFEKAT SIROVOG EKSTRAKTA SRPSKOG GENOTIPA BELOG LUKA (*ALLIUM SATIVUM* L.) NA JAJA GASTROINTESTINALNIH NEMATODA OVACA

Stanislav SIMIN, Slobodan VLAJIĆ, Vladislav SIMIN, Ljiljana KURUCA, Vesna LALOŠEVIĆ

Izvod: Negativan uticaj gastrointestinalnih nematoda (GIN) na produktivnost pašnih ovaca odavno je poznat. U većini zemalja koje se bave uzgojem ovaca, kontrola ovih parazita se dugo zasnivala na intenzivnoj upotrebi antihelmintika širokog spektra, što je rezultiralo razvojem široko rasprostranjene rezistencije na antihelmintike. Upotreba biljnih antiparazitika, popout belog luka, predložena je kao jedan od alternativnih načina kontrole parazita proizvodnih životinja. Cilj sprovedenog istraživanja bilo je ispitivanje *in vitro* efekta sirovog ekstrakta belog luka (RGE), pripremljenog od srpskog genotipa ove biljke, na izleganje jaja ovčijih GIN. Ogledom su obuhvaćene tri različite koncentracije ekstrakta belog luka, a ispitivanje je izvedeno pomoću tzv. „egg hatch" testa (testa izleganja jaja). Rezultati testa pokazali su značajan ovicidni efekat sirovog ekstrakta belog luka (p=0.005), koji je direktno zavisio od upotrebljene koncentracije ekstrakta. Koncentracije od 16.7%, 33.3% i 66.7% (v/v) inhibisale su izleganje 68.1%, 76.7% i 92.6% (srednje vrednosti) jaja. Značajno veća efikasnost (p<0,05) dobijena je upotrebom najviše koncentracije ekstrakta, u odnosu na ekstrakte najniže koncentracije. *In vivo* potvrda ove efikasnosti je, međutim, neophodna kako bi se mogla proceniti mogućnost praktične primene ovog ekstrakta u terenskim uslovima.

Ključne reči: beli luk, ovicidno delovanje, *in vitro*, gastrointestinalne nematode, ovce.

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