sciendo

DOI 10.2478/helm-2022-0023

HELMINTHOLOGIA, 59, 3: 265 – 274, 2022

Does *in vitro* and *in vivo* exposure to medicinal herbs cause structural cuticular changes in *Haemonchus contortus*?

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Article info

Received May 16, 2022
Accepted August 30, 2022

Summary

The health and production of small ruminants in constantly menaced by parasitic infections, especially those caused by the blood-sucking gastrointestinal nematode *Haemonchus contortus*. The aim of this study was to assess the structural cuticular changes in adult *H. contortus* induced by the use of extracts from local medicinal plants and to examine their ovicidal activity. Previous studies have confirmed the beneficial effect of herbal mixtures in preventing haemonchosis in lambs by lowering fecal egg production and improving immunocompetence. We exposed adult *H. contortus* to Herbmix (a mixture of medicinal plants) under *in vivo* and *in vitro* conditions for observation by scanning electron microscopy (SEM). For the *in vivo* observations, adult worms were isolated from the abomasa of experimentally infected lambs from a Herbmix group and a control group. Surface structure did not differ significantly between the exposed and control groups. The ovicidal activity of an aqueous Herbmix extract was assessed *in vitro*, establishing the inhibition of hatching with an ED_{50} of 6.52 mg/mL. Adult worms for *in vitro* examination were isolated from experimentally infected lambs and incubated in Herbmix aqueous extracts for 24 h. SEM observations indicated that none of the worms had prominent ultrastructural changes on their cuticles. This study suggests that previously demonstrated antiparasitic effects of medicinal plants did not negatively affect adult parasites by damaging their external structures.

Keywords: *Haemonchus contortus*; medicinal plants; herbal mixtures; antiparasitic effect; scanning electron microscopy

Introduction

Gastrointestinal nematodes are a large concern in agriculture, because they substantially reduce animal productivity and cause economic losses for breeders. The cost of helminth infections in ruminants in 18 countries (including 13 European countries) has been estimated at 1.8 billion €, where 81 % of the total cost was assigned to production losses and 19 % was assigned to treatment costs (Charlier *et al.,* 2020). The interest in studying the strategies for controlling nematode infections is thus constantly growing. The use of synthetic anthelmintics is still the preferred strategy, but it carries a risk of the development of anthelmintic resistance (Stear *et al.*, 2007). Synthetic anthelmintics also pose the risk of the presence of their residues in animal products for consumers and the environment (Jedziniak *et al.*, 2009; Biswas *et al.*, 2012). The blood-sucking nematode *Haemonchus contortus*, whose

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presence causes severe anemia and the loss of proteins, is the most harmful nematode in small ruminants and is most frequently connected with anthelmintic resistance (Besier *et al.*, 2016). The objective of most methods for controlling parasites is not to totally eliminate the parasites from the herd, but to maintain population at levels that will not affect the health of the host population (Selemon, 2018). Nematodes can be controlled by alternative ways, such as the use of nematophagous fungi, vaccination, rotational grazing or adjustment of feed rations (Molento *et al.*, 2013).

The use of plants in the treatment of human and animal diseases dates back to the beginnings of our species. In the first half of the 20th century, plants worldwide formed the basis of drugs in both human and veterinary medicine (Hammond *et al.*, 1997). Willcox *et al.* (2001) estimated that more than 30 % of drugs placed on the market are directly or indirectly of natural origin. Plants or plant components have been used for dehelminthization for several centuries (Waller *et al.*, 2001). More comprehensive research has been conducted over the last 10 – 15 years to evaluate the *in vitro* and *in vivo* activities of bioactive plants under experimental and field conditions (Sandoval-Castro *et al.*, 2012). Parallel *in vivo* and *in vitro* studies are recommended for finding promising herbal alternatives to synthetic antiparasitic drugs (Váradyová *et al.* 2018). Several studies have already confirmed the antiparasitic properties of plants and their secondary plant metabolites tannins or flavonoids (Spiegler *et al.*, 2017). The most important antiparasitic effect has been attributed to forage legumes containing condensed tannins, such as sainfoin (Heckendorn *et al.*, 2006; Komáromyová *et al.*, 2022), birdsfoot trefoil (Marley *et al.*, 2003), sulla (Tzamaloukas *et al.*, 2006) or sericea lespedeza (Dykes *et al.*, 2019). Extracts from various medicinal plants, however, also have anthelmintic properties (Carvalho *et al.*, 2012; Mravčáková *et al.*, 2019; Pinto *et al.*, 2019).

Changes in the ultrastructure of cuticles of parasites of small ruminants after exposure to tannin-rich materials were first described by Hoste *et al.* (2006). The effects of the consumption of individual plant species on the ultrastructural changes in nematodes have also been studied (Kommuru *et al.*, 2015; Desrues *et al.*, 2016; Martínez-Ortíz-de-Montellano *et al.*, 2013), but no evidence of the effect of herbal mixtures of multiple plant species has been found. Data on ultrastructural changes caused by blends of medicinal plants under *in vitro* conditions is also lacking, but some studies have nonetheless focused on individual plant species and their compounds (Ribeiro *et al.*, 2017; Adamu *et al.*, 2019; Cavalcante *et al.*, 2020).

The objective of our study was therefore to monitor the efficacy of a mixture of medicinal plants on superficial ultrastructural changes under *in vivo* and *in vitro* conditions and on the ovicidal activity of a herbal mixture against *H. contortus*. Individual plants contained in the herbal mixture (Herbmix) were chosen because of their previously described antiparasitic properties (Váradyová *et al.*, 2018) and their potential availability on pasture in temperate climatic zones.

Material and Methods

In vivo analysis

Adult parasites were obtained from lambs in a complex study evaluating the antiparasitic effect of Herbmix; the complete methodology of this *in vivo* experiment was previously described (Komáromyová *et al.*, 2021). Lambs of the Improved Valachian breed with the same age and similar body weight were supplemented with the "Herbmix" mixture of dry medicinal plants (100 g dry matter/d/animal) and an unsupplemented group was used as a control. After a period of acclimation, the lambs were orally infected on day 0 (D0) with approximately 5000 larvae of the MHCo1 strain of *H. contortus* susceptible to anthelmintics. The animals were re-infected on D49 and D77 with 3000 larvae of the same strain to simulate natural conditions on pasture. Lambs from the control group were fed hay containing no medicinal plants. Herbmix supplementation started on D0, continued throughout the experimental period and was terminated on D119 when the animals were slaughtered and necropsied. The lambs were euthanized 17 wk after infection following the rules of the European Commission (Council Regulation 1099/2009) for slaughtering procedures. Several dozen adult worms for observation by scanning electron microscopy (SEM) were randomly collected at necropsy from the abomasa of animals from the Herbmix and control groups.

Herbmix composition

The Herbmix contained nine medicinal plants: marshmallow (*Althaea officinalis*), butterbur (*Petasites hybridus*), elecampane (*Inula helenium*), ribwort plantain (*Plantago lanceolata*), rosemary (*Rosmarinus officinalis*), fennel (*Foeniculum vulgare*), goldenrod (*Solidago virgaurea*), fumitory (*Fumaria officinalis*) and hyssop (*Hyssopus officinalis*), complete composition of Herbmix was previously decribed by Komáromyová *et al.* (2021).

In vitro analyses

Egg hatch test

An egg hatch test was performed to determine the ovicidal activity of an aqueous extract prepared from ground Herbmix. The preparation of aqueous extracts has previously been described by Váradyová *et al.* (2018). *H. contortus* eggs used in the EHT were obtained from freshly rectally collected feces from experimentally infected lambs (control group). The methodologies for isolating eggs and for the EHT have previously been described by Váradyová *et al.* (2018). The final concentrations of Herbmix in the EHT obtained by serial dilution were 25, 12.5, 6.25, 3.125 and 1.563 mg/mL. We therefore calculated the median effective dose (ED_{50}), which is the concentration that inhibits hatching in 50 % of eggs.

In vitro exposure to the Herbmix extract

The methodology for exposing adult worms to Herbmix extracts was conducted as described by Martínez-Ortíz-de-Montellano *et al.* (2013), with slight modifications. Adult worms used for

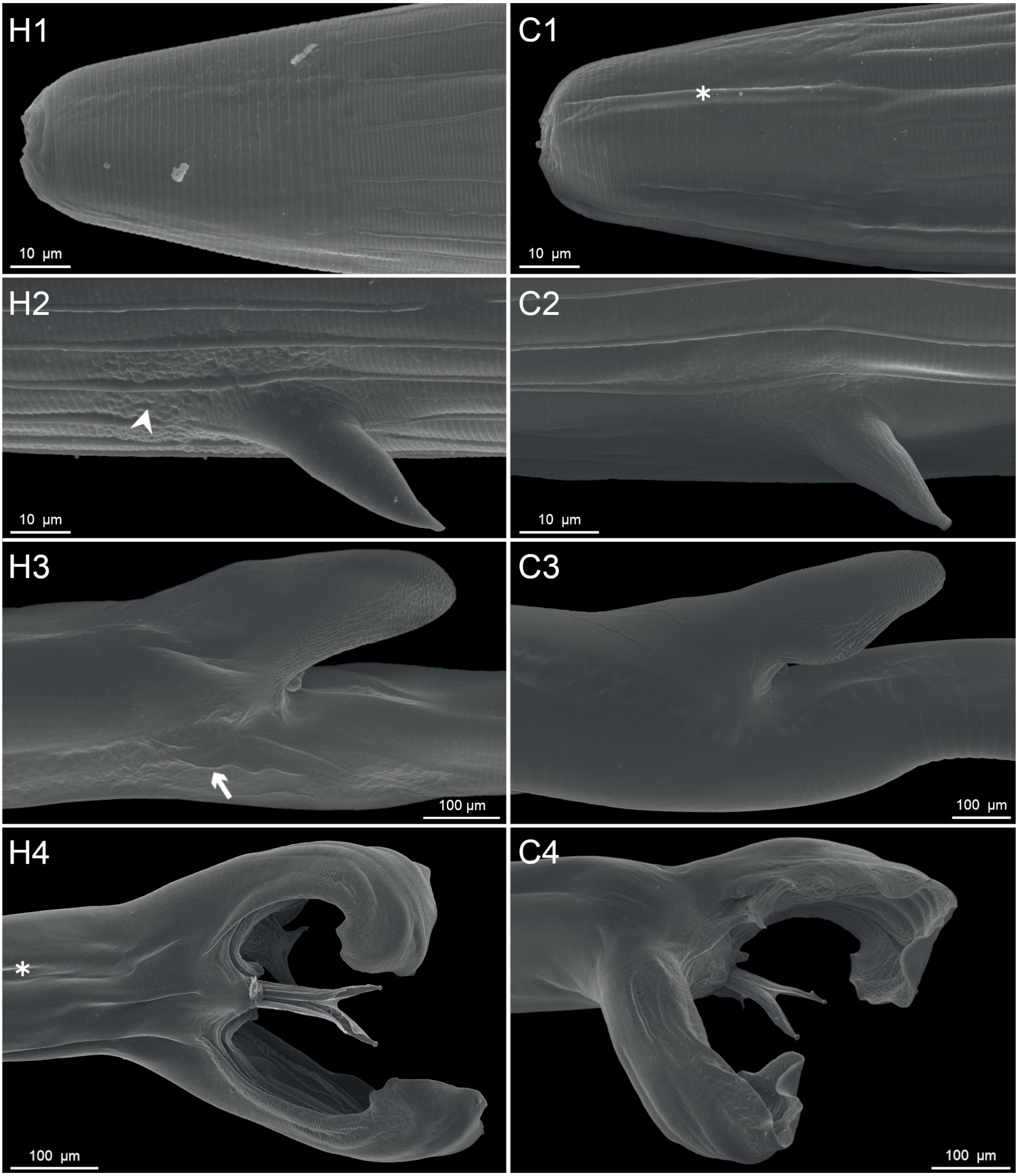

Fig. 1. Scanning electron photomicrographs of *Haemonchus contortus* from the *in vivo* experimental groups. H1–H4 depict specimens collected from lambs fed a diet enriched with a Herbmix extract, and C1–C4 depict specimens from the control animals. H1 and C1, cephalic end of the body, both females; H2 and C2, region near the cervical papillae, both females; H3 and C3, vulvar region of the body, both females; H4 and C4, posterior end with the copulatory bursa, both males. Note the cephalic end of the female body (C1) and the posterior part of the male body (H4), with several artificial folds (asterisk), small protuberances (arrowhead) in the region anterior to the base of a cervical papilla (H2) and weak ridges (arrow) on the cuticle in the vulvar region (H3).

the *in vitro* experiment were collected after slaughter from experimentally infected lambs from the control group (described in the section "*In vivo* analysis") that were fed hay containing no medicinal plants. Adult worms were recovered from the abomasum, washed with a physiological saline solution (0.9 % NaCl) and placed into a Petri dish containing the same solution to remove abomasal content. All procedures were performed at 37 °C. Female worms were then separately placed into 24-well plates containing either the Herbmix extract (25 mg/mL) for the treated group (n=12) or phosphate buffered saline (PBS) for the control group (n=12). All worms were incubated for 24 h and then fixed for scanning electron microscopy.

Scanning electron microscopy

Randomly chosen adults of both sexes from those collected from both the treated and control lambs, and all specimens in the *in vitro* experiment, were rinsed in saline, fixed in a hot 4 % formaldehyde solution and stored in 70 % non-denatured ethanol. All worms were then dehydrated using an ascending ethanol series (80-90-96-100 %; 15 min. at each concentration) and chemically dried in hexamethyldisilazane (Merck KGaA, Darmstadt, Germany). Dried specimens were transversely cut into three pieces. The anterior and posterior parts of their bodies were attached to aluminum SEM targets by double-sided tape, covered by gold in a JEOL JFC 1300 Auto fine coater and examined using a JEOL JSM 6510LA scanning electron microscope. The post-processing of the SEM photomicrographs included only the adjustment of brightness/contrast and blacking the background.

Ethical Approval and/or Informed Consent

This study was conducted following the guidelines of the Declaration of Helsinki and national legislation in the Slovak Republic (G.R. 377/2012; Law 39/2007) for the care and use of research animals. The experimental protocol was approved by the Ethical Committee of the Institute of Parasitology of the Slovak Academy of Sciences on 22 November 2020 (protocol code 2020/21).

Results

Scanning electron microscopic examination of *H. contortus* from the *in vivo* experiment

Scanning electron photomicrographs were acquired for six individuals (four females, two males) from each of the Herbmix and control groups. The ultrastructure of the body surface of the worms generally did not differ significantly between the groups. The cuticles were similarly smooth with straight, i.e. not sinuous, physiological longitudinal ridges (Fig. 1 H1, C1) throughout their bodies. The only observable differences were small protuberances around the bases of the cervical papillae and weak ridges in the vulvar area of the worms from the lambs fed the Herbmix-supplemented diet (Fig. 1 H2, H3), but which were also detected to some extent in the control group (Fig. 1 C2, C3). The surface ultrastructure of the copulatory bursae of males did not differ between the groups in a way, which could be attributed to an effect of the Herbmix. The cuticles of all worms, however, had many unnatural folds (e.g. Fig.1 C1, H4), which were considered to be artifacts of processing for SEM, because they occurred in both groups.

Egg Hatch Test

The effects of Herbmix in the EHT are presented in Table 1. Hatching was inhibited by 83 % at the highest concentration (25 mg/mL) of the Herbmix extract. Each concentration of Herbmix extract tested significantly negatively affected hatching compared to the control (p<0.001). The inhibition of hatching was dose-dependent (Fig. 2). ED_{50} and ED_{99} were 6.517 and 315.294 mg/mL, respectively.

Scanning electron microscopic examination of *H. contortus* from the *in vitro* experiment

Three worms fixed in the *in vitro* experiment were randomly chosen from each of the Herbmix and control (PBS) groups. SEM did not identify any obvious damage, which the Herbmix decoction could cause on their superficial structures. The cuticle at the cephalic ends of the worms in both groups was smooth, with the lack of any ruptures (Fig. 3 H1, C1). The physiological longitudinal ridges were slightly sinuous, and small protuberances occurred

Table 1. Hatching and ovicidal activity at the concentrations of the Herbmix aqueous extract tested in the EHT.

Herbmix concentration (mg/mL)	Ovicidal effect (%)	Hatching (%)	*p* value
25	83	17	**< 0.001**
12.5	71	29	**< 0.001**
6.25	45	54	**< 0.001**
3.125	34	66	**< 0.001**
1.563	15	85	**< 0.001**
Control	1	99	-

EHT – Egg Hatch Test

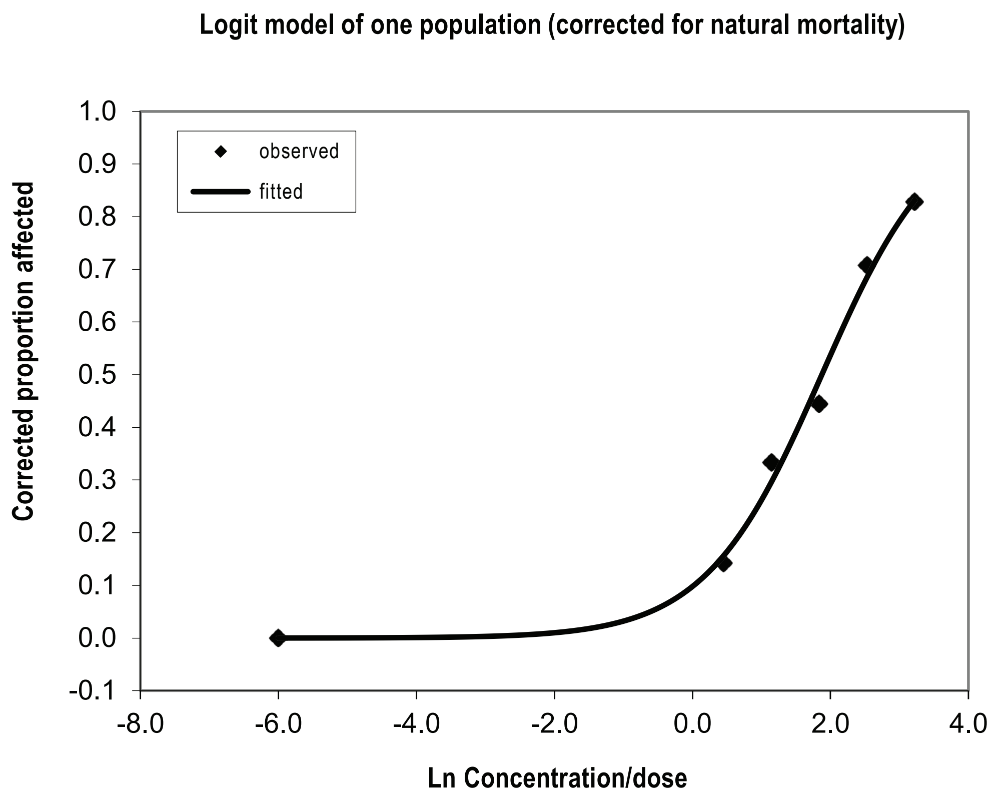

Fig. 2. Dose-response relationship of the Herbmix aqueous extract against *Haemonchus contortus* in the EHT.

around the bases of the cervical papillae, but these features were observed in both the treated and control groups (Fig. 3 H2, C2). The rough cuticle covered the vulvar flaps and lateral buttons of the worms from both groups, but it seems to be physiological appearance of these structures because of its presence also in control group, and weak transverse wrinkles were observed anterior and posterior to the vulvar flaps (Fig. 3 H3, C3). Similar to the *in vivo* group, all specimens, i.e. from both the treated and control groups, had deformed body surfaces, which were attributed to the preparation of the SEM samples (see artifacts in Fig. 3 C1, H2, H3, C3)

Discussion

The demand for organic animal production and for minimizing the levels of chemicals in animal products is growing with the popularization of ecofriendly lifestyles. The presence of parasites is usually an inherent part of intensive livestock production, and breeders and scientists are looking for ways to minimize the damage caused by parasitic infections. The available literature indicates that 458 studies have been published and 187 plant species have been monitored for their antiparasitic effects against *H. contortus* over the last 30 years (Ali *et al.*, 2021). Local and exotic plants in countries around the world are being explored for their activity against parasitic organisms under *in vivo* and/or *in vitro* conditions. Our studies are participating in the research for finding promising natural alternatives to synthetic anthelmintics. After the promising results obtained in our previous studies with herbal mixtures (Váradyová *et al.*, 2018b; Komáromyová *et al.*, 2021), we are focusing on possible ultrastructural changes to the adult stage of *H. contortus*. To our knowledge, this study is the first to examine the effects of herbal mixtures on ultrastructural changes in *H. contortus*.

The content of phytochemicals in plants and their extracts is the most important factor responsible for anthelmintic effects. Plant secondary metabolites have been discussed and contemplated as tools for the control of gastrointestinal nematodes in small ruminants (Hoste *et al.*, 2016). Herbmix has tested positive for tannins, saponins, alkaloids, terpenoids, flavonoids and steroids (Komáromyová *et al.*, 2021). The mechanism of action of condensed tannins against parasites, though, remains unexplained. What is known is that the surface structures (cuticle) of nematodes contain collagen proteins (Thompson & Geary, 1995). Tannin-binding proteins have been identified in the saliva of deer but not the saliva of cattle or sheep (Austin *et al.*, 1989), so tannins may be able to pass into the stomach or intestines unbound and interact there with parasites and their structures. Tannins can bind proteins or amino acids and modify their chemical properties to form soluble

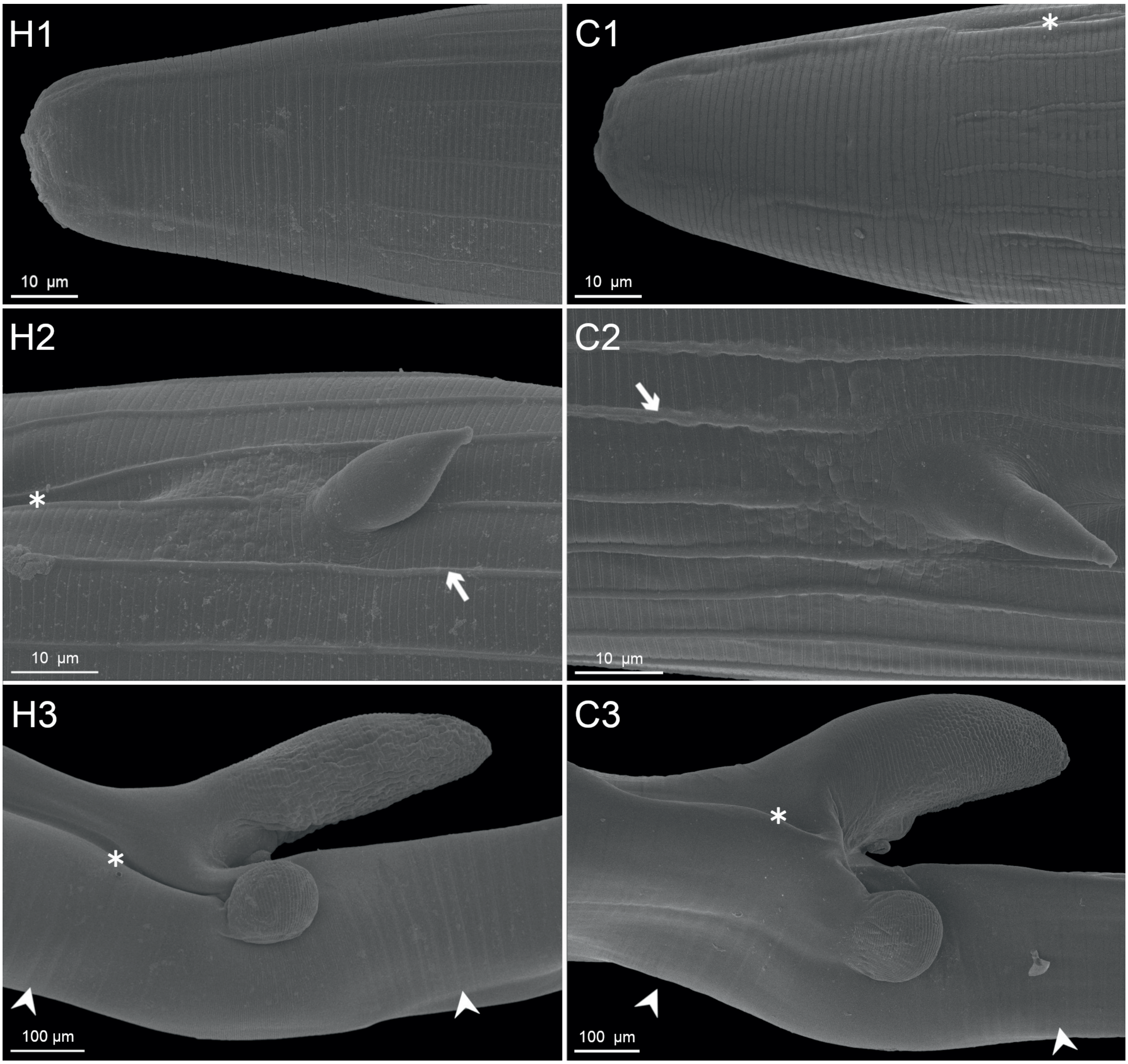

Fig. 3. Scanning electron photomicrographs of *Haemonchus contortus* from the *in vitro* experimental groups. H1–H3 depict specimens treated with the Herbmix extract diluted in PBS, and C1–C3 depict specimens from the control group kept in PBS. H1 and C1, cephalic end of the body; H2 and C2, region around a cervical papilla; H3 and C3, vulvar region with a lateral button. Note the slightly sinuous physiological longitudinal ridges (arrows), weak transverse wrinkles (arrowheads) and artifacts of sample preparation (asterisks).

complexes or precipitates, which can affect protein function or inhibit enzymatic activity (Rinaldi & Moio, 2020). Tannins have been hypothesized to bind to cuticular proteins and cause ultrastructural changes that can be seen by scanning electron microscopy (Hoste *et al.*, 2006).

In Figure 1 worms differed little between the Herbmix and control groups. Some differences were observed, but these were insufficient to confirm the effect of Herbmix on the superficial structures of *H. contortus*. Significant antiparasitic effects of Herbmix on fecal egg counts, live-weight gains and abomasal worm counts *in vivo*, however, have been reported (Komáromyová *et al.*, 2021). These results led us to hypothesize that antiparasitic effects could be achieved by improving the health of the host or by impairing internal structures of nematodes more than by directly harming the surface structures of adult parasites. Substances harmful to superficial structures may also not have been present. The concentrations of active plant substances in lambs after metabolic processes during digestion may also have been too low. Another study investigating the effects on structures of *H. contortus* in goats supplemented with tannin-rich sericea lespedeza, however, confirmed damage to the cuticular surface in a treated group (Kommuru *et al.*, 2015). *H. contortus* isolated from goats fed with tzalam or sainfoin also had signs of surface damage, e.g. ridges on the cuticle and aggregates around the buccal capsule, vulva and anus (Martínez-Ortíz-de-Montellano *et al.*, 2013). A similar study performed with sainfoin-fed calves infected with *Ostertagia ostertagi* and *Cooperia oncophora* found local cuticular damage (Desrues *et al.*, 2016). Similar studies with sheep have not yet been conducted.

In vitro tests are commonly used due to their low cost, quick outcome and possibility of testing purified compounds without interfering with metabolites or components present during digestion. Reproducing the same experiments *in vivo*, however, is difficult, mostly due to differences in dosage and absorption. Substances that are effective under *in vitro* conditions do not always have same effect under *in vivo* conditions due to differences in metabolism and bioactivity in the host organism (Ferreira *et al.*, 2013). A comparison of methanolic and aqueous extracts of the same plants confirmed differences in ovicidal activity in one plant (Váradyová *et al.*, 2018). *In vitro* tests are also usually performed with eggs or free-living larval stages rather than adults because of their viability outside their host (Athanasiadou & Kyriazakis, 2004). All these factors led us to conduct *in vivo* and *in vitro* tests and to include adult nematodes for assessing the efficacy of Herbmix.

One hypothesis of the possible mechanism of action of tannins is that they could have antiparasitic properties and could directly affect the biology of parasites (Hoste *et al.*, 2006). This hypothesis has been substantiated by many *in vitro* studies (Yoshihara *et al.*, 2014; Castañeda-Ramírez *et al.*, 2018; Silva Soares *et al.*, 2018) where direct effects of tannins were assumed. The ovicidal effects of the individual plants in Herbmix were previously examined, with the strongest ovicidal activity observed for *F. officinalis* and *A. officinalis* (Váradyová *et al.*, 2018a). The ovicidal effect of Herbmix in our study was obvious, but with a high ED_{99}, the ability to achieve the required concentration and therefore antiparasitic effect *in vivo* is uncertain.

In vitro studies with SEM and the effects of plants on adult nematodes always have complicated processes or chemical solvents behind the extraction of active plant substances (Martínez-Ortíz-de-Montellano *et al.*, 2013; Andre *et al.*, 2016; Adamu *et al.*, 2019; Cavalcante *et al.*, 2020). One study (Ribeiro *et al.*, 2017) used an aqueous extract of *Spigelia anthelmia* and a relatively simple process of decoction. This study found ultrastructural changes to the cuticle in the cephalic region, unlike in our study, even though the methods of extraction were very similar. The difference between findings may have been caused by the diverse plants examined. The accuracy of the method of extraction has been widely discussed, because plants cannot be similarly processed by the digestive tracts of animals (Mueller-Harvey *et al.*, 2018). Alternative therapies avoid the use of synthetic substances, so results confirming damaging effects on parasites *in vitro* are not adequate for dehelminthization in the ruminant husbandry. Aqueous extracts were also used in a study of *Acacia mearnsii* and *H. contortus*, where cuticular ruptures and wrinkling were observed, but the stock extract was commercially prepared (Yoshihara *et al.*, 2015). Structural cuticular changes that could inhibit the motility of worms were described in a study using aqueous extracts of bamboo leaves, although the method of extract preparation was not described (Widiarso *et al.*, 2021).

Resilience of parasites could be also influenced by their age. Ultrastructural changes to the cuticle and in the vulvar region after *in vitro* exposure to carvacrol and its derivates originating from essential oils of Lamiaceae species have been observed (Andre *et al.*, 2016). These authors used adult *H. contortus* collected 4 wk after the infection of lambs. Santos *et al.* (2014) established the prepatent period of *H. contortus* at between 18 and 22 d, but egg output peaked on D52 post-infection. These data suggest that the worms could be more resilient to external influences 7 wk after infection. Younger worms may generally be more sensitive to ultrastructural damage and external factors compared to our worms isolated 17 wk after infection, disregarding the type of plant extract examined. Exposure of adult *H. contortus* to an acetone extract of agrimol G (isolated from leaves of *Leucosidea sericeza*) for 3 h caused distinct surface disruptions (Adamu *et al.*, 2019). The worms in our study, however, did not have any signs of breakage, even though exposure time was 8-fold longer.

Conclusion

Changes in the surface structures of adult *H. contortus* after *in vivo* and *in vitro* contact with a herbal mixture were not observed under a scanning electron microscope. Aqueous extracts of Herbmix significantly inhibited hatching even at the lowest concentration. Together with our previous findings, this study suggests that Herbmix may be effective during the early developmental stages of *H. contortus* by directly damaging the internal structures of adult worms or by indirectly modulating immunity.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This research was funded by the Slovak Research and Development Agency (APVV 18-0131) of the Ministry of Education, Science, Research and Sport of the Slovak Republic. The authors are grateful to S. Spišáková for laboratory assistance.

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