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ANTHELMINTIC EFFICACY OF AGRO-INDUSTRIAL BY-PRODUCTS AGAINST GASTROINTESTINAL NEMATODES OF SHEEP: *IN VITRO TESTS*

ANTIHELMINTIČKA EFIKASNOST NUSPROIZVODA AGROINDUSTRIJE PROTIV GASTROINTESTINALNIH NEMATODA OVACA: *IN VITRO TESTOVI*

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ABSTRACT

Gastrointestinal nematodes (GINs) are ubiquitous in grazing small ruminants and cause significant costs due to production losses. Moreover, anthelmintic resistance (AR) is now widespread throughout Europe and poses a major threat to the sustainability of modern small ruminant livestock farming. Trichostrongylus colubriformis, an intestinal parasite of small ruminants, is the second nematode to Haemonchus contortus in terms of distribution and magnitude in several regions. The control of this helminth is traditionally achieved with the use of anthelmintic drugs, however due to regulations in organic farming and the rise in anthelmintic resistance (AR), alternatives are sought after. The aim of the present study was to evaluate the in vitro anthelmintic effects of olive (Olea Europaea), carob (Ceratonia siliqua), pomegranate juice (Punica granatum), wine (Vitis vibifera), citrus (Citrus Senensis), hazelnut (Corylys avellana) and tomato (Solanum lycopersicum) by-products extracts on T. colubriformis of sheep. Extracts of seven by-products were tested in vitro on two development stages of T. colubriformis (eggs and infective larvae) using the Egg Hatch Assay (EHA) and the Larval Exsheathment Inhibition Assay (LEIA). The egg hatching rate was measured after incubation with each by-product extract (concentrations: 150, 300, 600, 1200 μg/mL) for 48 h at 26 °C. Ensheathed infective larvae were incubated for 3h at 20 °C with each by-product extract (concentrations: 150, 300, 600, 1200 μg/mL). Artificial exsheathment was induced in vitro by adding sodium hypocloride solution (2%w/v) diluted in 1 to 300 in PBS to the larval suspension. The progress of exsheathment over time was measured by repeated observations at 20-min intervals for 60 min (Moreno-Gonzalo et al., 2013. Vet Parasitol, 197:235-43). Among the 7 extracts tested using the two in vitro tests (EHA and LEIA), those with the greatest anthelmintic potential against T. colubriformis were the by-product extracts of wine, pomegranate and hazelnut. In particular, the inhibition in the development of eggs using EHA was 2.3%-48.5% for wine, 6.1%-48.1% for hazelnut and 18.6%-42.4% for pomegranate by-product extracts. The inhibition of exsheathment using LEIA was 87.5%-100% for wine, 54.5%-98.7% for hazelnut and 62.3%-100% for pomegranate by-product extracts. Results showed that by-product extracts of wine, pomegranate and hazelnut exhibits in vitro anthelmintic activity, suggesting that, these by-products can also be an ally for T. colubriformis control in sheep.

Keywords: Gastrointestinal nematodes, *Trichostrongylus colubriformis*, agro-industrial by-products, *in vitro* tests, Egg Hatch Assay, Larval Exsheathment Inhibition Assay

SAŽETAK

Gastrointestinalne nematode (GIN) su sveprisutne u ispaši malih preživara i uzrokuju značajne troškove zbog gubitaka u proizvodnji. Štaviše, otpornost na anthelmintiku (AR) je danas rasprostranjena širom Evrope i predstavlja veliku pretnju održivosti savremenog uzgoja sitnih preživara. Trichostrongilus colubriformis, crevni parazit malih preživara, je druga nematoda posle Haemonchus contortus u smislu distribucije i veličine u nekoliko regiona. Kontrola ovog helminta se tradicionalno postiže upotrebom antihelmintičkih lekova, ali se zbog regulative u organskoj poljoprivredi i porasta anthelmintičke rezistencije (AR), traže alternative. Cilj ove studije bio je da se proceni in vitro anthelmintičko dejstvo masline (Olea Europaea), rogača (Ceratonia silikua), soka od nara (Punica granatum), vina (Vitis vibifera), citrusa (Citrus Senensis), lešnika (Corilis avellana).) i ekstrakti nusproizvoda paradajza (Solanum licopersicum) na T. colubriformis ovaca. Ekstrakti sedam nusproizvoda su testirani in vitro na dve razvojne faze T. colubriformis (jaja i infektivne larve) korišćenjem testa za izležavanje jaja (EHA) i testa inhibicije ekshubicije larve (LEIA). Brzina izleganja jaja je merena nakon inkubacije sa svakim ekstraktom nusproizvoda (koncentracije: 150, 300, 600, 1200 mg/mL) tokom 48 h na 26 °C. Infektivne larve u ovojnici su inkubirane 3 sata na 20 °C sa svakim ekstraktom nusproizvoda (koncentracije: 150, 300, 600, 1200 mg/mL). Veštačko odvajanje je indukovano in vitro dodavanjem rastvora natrijum hipoklorida (2% v/v) razblaženog u 1 do 300 u PBS u suspenziju larve. Napredak eksheathmenta tokom vremena meren je ponovljenim posmatranjima u intervalima od 20 minuta tokom 60 minuta (Moreno-Gonzalo et al., 2013. Vet Parasitol, 197:235-43). Među 7 ekstrakata testiranih korišćenjem dva in vitro testa (EHA i LEIA), oni sa najvećim anthelmintičkim potencijalom protiv T. colubriformis bili su ekstrakti nusproizvoda vina, nara i lešnika. Konkretno, inhibicija u razvoju jaja korišćenjem EHA bila je 2,3%-48,5% za vino, 6,1%-48,1% za lešnik i 18,6%-42,4% za ekstrakte nusproizvoda nara. Inhibicija uklanjanja ovojnice korišćenjem LEIA bila je 87,5% -100% za vino, 54,5% -98,7% za lešnik i 62,3% -100% za ekstrakte nusproizvoda nara. Rezultati su pokazali da ekstrakti nusproizvoda vina, nara i lešnika pokazuju in vitro anthelmintičku aktivnost, što sugeriše da ovi nusproizvodi takođe mogu biti saveznik za kontrolu T. colubriformis kod ovaca.

Ključne reči: gastrointestinalne nematode, *Trichostrongylus colubriformis*, poljoprivredni nusproizvodi, *in vitro* tests, Test leženja jaja, Test inhibicije izbacivanja larvi

INTRODUCTION

Gastrointestinal nematodes (GINs) are increasingly recognized as a significant threat to the future sustainability of keeping livestock for food production and leisure, especially for small ruminant farms (1). At a policy level, livestock significantly contributes to agricultural greenhouse gas (GHG) emissions, which may be exacerbated by GIN infections. GINs also threaten food security and the economic sustainability of livestock farming as they cause significant production losses in ruminants (2). Finally, the impact of subclinical and chronic infections remains an understudied but essential question in livestock helminth research (3).

In particular, Trichostrongylus colubriformis, a nematode of the Trichostrongylidae family which is frequently found in ruminants in many regions worldwide, in severe infections can cause enteritis with atrophy of large intestinal mucosa microvilli, formation of tunnels, and duodenal mucosa epithelium erosion, hyperplasia and crypt hypertrophy, leukocyte infiltration and high intestinal lumen serum protein exudation (4). As a consequence of this infection, the capacity for digestion and absorption of nutrients, including minerals, is compromised, leading to a reduction of skeletal growth, bone density and mineralization, resulting in reduced animal performance (4).

For several decades, the control of these helminth infections has mainly relied on the repeated use of chemical anthelmintics provided by pharmaceutical companies. However, the accelerating development of anthelmintic resistance (AR) in GIN populations and the extent of the phenomenon, including the rise of multi-resistant isolates, have been reported worldwide (5).

Moreover, the development of AR and other chemical drugs appears to be an inevitable process.

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Additionally, anthelmintics can be included among the "emerging pollutants" that have significantly risen in the last decades (6). Their ecotoxicological effects have raised concern in relation to environmental invertebrates, which can be irremediably damaged (7). At the same time, anthelmintics can indirectly affect human and animal health by reaching ground and surface waters and land resources or by accumulation in the food chain (6).

For these reasons, it is essential to develop innovative strategies in modern sheep and goat farming systems that are based on: i) the rational use of the drug as "targeted selective treatment (TST)" for animals clinically diagnosed with GINs to leave some parasite populations unexposed to anthelmintics (refugia), ii) the use resistant/resilient breeds to GIN infections, iii) the use of plants with anthelmintic properties and iv) the integration of sensitive diagnostic techniques able to early detect the onset of AR phenomenon.

Currently the most eco-sustainable solution is the use of plants with anthelmintic activity to GIN control. The aim of the present study was to evaluate the in vitro anthelmintic effects of olive (Olea Europaea), carob (Ceratonia siliqua), pomegranate (Punica granatum), wine (Vitis vibifera), citrus (Citrus Senensis), hazelnut (Corylys avellana) and tomato (Solanum lycopersicum) by-products extracts on T. colubriformis of sheep.

MATERIAL AND METHODS

The by-products used in this study were supplied by agro-industries present in southern Italy. Particularly olive, pomegranate, grape pomace, hazelnut and tomato by-products derive from agro-industries located in Campania region, instead carob and citrus by-products derive from agro-industries located in Sicily region.

Preparation of extracts

The method of extraction has been described previously (8). Briefly, 500 g of the whole waste were extracted with 2X3L of 70 :30 acetone : water (v/v) containing ascorbic acid (1 g/l) for 24 h. The acetone was removed under low pressure at a temperature < 35° C and the aqueous solution was washed 4 times with 500 ml of methylene chloride to remove chlorophyll and lipids. The solution was then concentrated under low pressure at 35 ° C. Finally, the extracts were frozen and lyophilized for 24 h to obtain a dry ground sample which was used in the *in vitro* biological assay.

In vitro assays

Third-stage larvae (L3) and eggs were obtained from feces of monospecifically infected sheep donor, with susceptible strains of *T. colubriformis*. L3 larvae were maintained in culture flasks for 2 months, at 4°C, before use in the Larval Exsheathment Inhibition Assay (LEIA), while eggs were collected on the day of the Egg Hatch Assay (EHA), and used up to 3 h after collection (9, 10).

The extracts were evaluated for the AH activity by using the *in vitro* Larval Exsheathment Inhibition Assay (LEIA) (11) and the EHA was conducted following the procedure suggested by (9, 10). Each assay was applied successively on a species of intestinal nematode (*T. colubriformis*). The two assays were performed with the same range of concentrations of 1200, 600, 300, and 150 µg/ml using Phosphate Buffered Saline (PBS) (0.1 M phosphate, 0.05 M NaCl, pH 7.2) as the solvent. For each concentration, including the negative and positive controls, assays were performed in four replicates.

For the EHA, twenty-four well culture plates were used for the *in vitro* anthelmintic test of the plant extracts against the eggs of *T. colubriformis*. The anthelmintic test against the eggs was evaluated separately for each plant extract.

In a 24 well culture plate, there were prepared 4 replicates of the same plant extract in the following concentrations: PBS control (without any plant extract), 150µg/ml, 300µg/ml, 600µg/ml, 1200µg/ml and TBZ (which is the golden standard for the egg hatch of the nematodes). The eggs and the larvae were counted for the four replicates and an average value was estimated, converted into percentage of inhibition of egg hatch.

For the LEIA, the third-stage larvae of *T. colubriformis* were produced from experimentally infected donor sheep. The same batch of 2 to 3-month-old larvae was used in the assays with different extracts. Firstly 1000 L3/ml solution were diluted at concentrations of 1200µg/ml, 600µg/ml, 300µg/ml and 150µg/ml plant extracts with 4 replicates in each concentration. Negative controls (L3 in PBS) were used as control of the assay. The ensheathed L3-larva were incubated for 3h at 20°C

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with the plant extracts before being resuspended at the concentrations of 1200µg/ml, 600µg/ml, 300µg/ml and 150µg/ml in phosphate buffer solution (PBS). After incubation, the larvae were washed and centrifugated, three times in PBS. After the incubation with the plant extracts, the larvae were submitted to the artificial process of exsheathment by contact with a sodium hypocloride solution (2%w/v) diluted in 1 to 400 in PBS.

The larvae kinetics and morphology were evaluated with microscopic observation and identification of exsheathed larvae at a magnification of x 200 by regular examination at 0, 20, 40 and 60 minutes of contact with the solution for exsheathment. Four replicates were run for each plant extract at the concentrations 1200µg/ml, 600µg/ml, 300µg/ml and 150µg/ml as long as the negative control to examine the changes in proportion of exsheathed larvae with time.

RESULTS

The highest ovicidal effect was obtained with grape and olive by-product extracts which had a same percentage of inhibition of egg hatch of 48.5%. The best results were also obtained with hazelnut (48.1% higher inhibition), tomato (43.2% higher inhibition) and pomegranate (42.4% higher inhibition) by-product extracts. The worst results were obtained with carob and citrus by-product extracts (< 16.3%).

Concerning larvicidal activity, the results obtained showed that the greatest anthelmintic potential against *T. colubriformis* were the by-product extracts of wine, pomegranate and hazelnut. Specifically, with pomegranate extract when the larvae were exposed to extract concentration of 1200 µg/ml and 600 µg/ml, the inhibition of exsheathment of L3 was 100%, furthermore, even at a concentration of 300 µg/ml only 15.8% of the larvae lost their sheath.

With the grape extract the inhibition of exsheathment using LEIA was 100% when larvae were exposed to extract concentration of 1200 µg/ml and 600 µg/ml, after 60 minutes, 89.6% at a concentration of 300 µg/ml and 87.5% at 150 µg/ml.

Hazelnut by-product extracts also gave excellent results because when the larvae were exposed to a

extract concentration of 1200 µg/ml, 600 µg/ml and 300 µg/ml, the lowest inhibition value was 98.2%.

Good results were also obtained with olive (81.7% higher inhibition), carob (90.0% higher inhibition) and tomato (100% higher inhibition) by-products.

The least encouraging results in this study were obtained with by-products deriving from citrus.

DISCUSSION

The use of extracts from plants has been extensively studied as an alternative to anthelmintics in the treatment of gastrointestinal parasites in sheep (12), as they may be sources of phytoconstituents, which display various biological proper- ties (13).

In the context of growing resistance to antihelmintic drugs, the potential of agro-industrial by-products against pathogens represents an alternative more sustainable than the use of synthetic drugs. Several *in vitro* studies have demonstrated the potential benefits arising from the inclusion of bioactive products in feed for ruminants and for the future use as nutraceuticals. Many of these studies have been performed on *T. colubriformis* (12).

The aim of the present study was to evaluate the *in vitro* efficacy of different agro-industrial by-products containing plant specialised metabolites (PSM), studying their potential bioactive.

Even if in a different way, all the products used have proven to have a certain anthelmintic activity on eggs and on L3 larvae of *T. colubriformis*, which was significant in some of the extracts (pomegranate extract, grape pomace extract, hazelnut extract), medium in others (olive extract, carob extract, tomato extract) and only in one extract was it shown to be low (citrus extract).

The different efficacy of the substances against GIN could be associated with qualitative and quantitative differences in phytochemical compounds. In particular, during the tests carried out, it was noted that the effectiveness of the products tested was dose-dependent and time-dependent.

Studies on the *in vitro* anthelmintic efficacy of pomegranate by-products (14), tested a watery macerate obtained from the fruits and their peels, obtaining a significant ovicidal activity (assessed with EHT) against GIN of sheep. These results are in agreement with the *in vitro* and *in vivo* studies conducted by Hassan et al. (2020) (15), who confirmed the effectiveness of *P. granatum* extract against *Haemonchus contortus* in goats and *Haemonchus* spp. and *Cooperia* spp. in cattle. These studies confirm the results obtained in this study.

An *in vitro* study by Soares et al. (2018) (16) evaluated ovicidal and larvicidal activity of a hydroalcoholic extract of grape, against gastrointestinal nematodes, on naturally infected sheep. The pomace extract showed a high ovicidal and larvicidal activity *in vitro* with LD50 values of 0.30 mg/ml for inhibition of egg hatching, 1.01 mg/ml for inhibition of development larval migration and 100% efficacy in all larval migration inhibition tests the concentrations evaluated. The present study also demonstrated that these by-products are one potential source of bioactive compounds with significant larvicidal properties *in vitro*.

As reported by Hoste et al. (2015) (17), has been compared in some studies the *in vitro* effectiveness of sainfoin and hazelnut extracts, thus allowing a comparison of IC50 values (18, 19). In the overall, lower IC50 values were obtained for hazelnuts, suggesting a higher anthelmintic activity than sainfoin, which is used as a reference among legume plants containing tannins. In another study the anthelmintic properties were confirmed *in vivo* (12). When goats experimentally infected with *H. contortus* and *T. colubriformis*, were fed hazelnut peels, was observed a significant reduction (>50%) in fecal nematode egg counts (FEC), related to a decrease in the fecundity of *Haemonchus* females (20). In a second phase, decreases were recorded in L3 larvae by 48% for *H. contortus* and 42% for *T. colubriformis*. In accordance with these studies, the results obtained in laboratory with the LEIA and EHA, have shown a high anthelmintic potential.

CONCLUSIONS

The present study aimed to investigate the antihelmintic potential of various agro-industrial by-products, in particular from the olive, wine, canning and fruit (citrus, carob, hazelnut and pomegranate). Despite being necessary to continue with further studies to confirm the results reached, it can be stated in the first instance that the extracts deriving from the by-products of winemaking, from the processing of hazelnuts and pomegranate have revealed themselves very effective against eggs and L3 larvae of *T. colubriformis*. The by-products follow of the processing of olives, tomatoes and carobs, which have shown an effectiveness that should not be underestimated, even if, to a lesser extent, compared to the previous ones. It follows that these substances could represent in future, a really promising alternative for them to be used as raw material to create new marketable products with activities anthelmintic, in order to achieve sustainable infection control parasitic.

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