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Original Research Article

In vitro effects of hydro-methanolic extract from *Gliricidia sepium* leaves on larvae of *Haemonchus contortus*

Key words

flavonoids;
flavonols;
larvae;
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tannins

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Abstract: The aim of this in vitro study was to evaluate the anthelmintic effects of extracts of *Gliricidia sepium* on sheathed and exsheathed larvae of *Haemonchus contortus*. Larvae of this parasite were incubated at 20–25 °C in hydro-methanolic extracts of leaves from this tropical tree at concentrations of 12.5, 25, 50, 100, and 200 mg/mL for 24, 48, or 72 h. Water and ivermectin were negative and positive controls, respectively. Total phenolic compounds of leaves of *G. sepium* were 6.4 ± 2.4 mg/g of dry matter. Other compounds identified in this leguminous tree by HPLC-mass spectrometry and that may be responsible for the anthelmintic effects observed were vanillin 4-sulfate, prodelphinidin p-coumaroyl glucose, kaempferol 3-o-glucosyl-rhamnosyl-glucoside, kaempferol-3-O-xylosyl rutinoside, p-coumaric acid, luteolin 7-rutinoside, isorhamnetin 3-glucoside-7-rhamnoside, and dihydro ferulic acid. At doses of 100 mg/mL mortality rate of sheathed and exsheathed *H. contortus* was 21.6 and 44.7%, respectively for 72 h of incubation. At 200 mg/mL, the hydro-methanolic extracts of *G. sepium* killed 61.5 and 93.8% of sheathed and exsheathed larvae, respectively, after 72 h of incubation. The effective concentration of the *G. sepium* extract for 50% sheathed and exsheathed larvae mortality (EC50) after 72 h of incubation was 74 mg/mL (CI = 46–100) and 68 mg/mL (CI= 32–100), respectively. The significant (P<0.001) ability to kill larvae compared to the negative controls, suggests in vitro anthelmintic properties of *G. sepium* against *H. contortus*.

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Introduction

One of the gastrointestinal nematodes with the highest prevalence in ruminants in the world is *Haemonchus contortus*, which is considered the most pathogenic parasite of small ruminants (1). This blood-sucking nematode affects young and adult ruminants either clinically or sub-clinically, decreasing the efficiency of digestion (2, 3), reducing the energy metabolism of maintenance and production, causing anemia due to the severe loss of blood (4). Additional effects of this nematode are a reduction of feed intake, feed conversion, weight loss, and often high mortality in young animals (5), which causes significant economic losses (6, 7). Infections with *H. contortus* are ubiquitous in grazing goats in a wide range of ecosystems and induce subclinical and clinical diseases resulting in clinical diseases and productivity loss (8).

Currently, gastrointestinal parasites control in livestock mainly relies on repeated treatments with anthelmintic drugs such as benzimidazoles and macrocyclic lactones (9). However, multiple-drug anthelmintic resistance in gastrointestinal nematodes of livestock is highly prevalent throughout the world (10, 11) and anthelmintic resistance is a growing issue (12, 13), and new nematicides are not likely to offer long-lasting solutions because resistance

anthelmintic drugs can develop rapidly. Also, with the use of anthelmintic drugs, there is a risk of accumulation of residues of these drugs in meat and milk, and could damage other beneficial organisms such as manure beetles (*Onthophagus landolti*) (14). For this reason, it is desirable to use plant extracts that possess inhibitory effects against free-living helminths which can replace synthetic anthelmintic compounds.

Gliricidia sepium is an adaptable, fast-growing tree tropical legume distributed worldwide, due to its extensive introduction across tropical regions of the world. It has a lot of uses such as a fodder tree (15), fuelwood (16), living fences (17), and atmospheric nitrogen fixation (18). This tree is an important forage crop in cut-and-carry systems but its use has been limited by palatability (19) and low feed intake (20) due to various secondary metabolites (21).

G. sepium has been shown to possess ovicidal (22) and larvicidal (23) activity against *Haemonchus contortus*, a gastrointestinal nematode with high prevalence and resistant features in goats (24, 25). due to its wide array of secondary compounds. Alternative methods for control of gastrointestinal parasites are needed not only because of the development of anthelmintic resistance but also because of a demand by consumers for avoidance of chemicals and drugs in farm animals, and reduced chemical residues in the environment and milk and meat. This necessity is echoed in higher demand by consumers for organic and grass-fed livestock products (26).

Even though secondary compounds of *G. sepium* have demonstrated activity against gastrointestinal nematodes, additional information is required to attune the use of this plant as an anthelmintic alternative. Therefore, this study aimed to evaluate the in vitro nematicidal effect of hydro-methanolic extracts of *G. sepium* against *H. contortus*.

Materials and methods

Ethics statement

This study was conducted according to ethical principles and guidelines for experiments on animals of the Autonomous Agrarian University Antonio Narro (protocol number 3001-2258).

Plant material and hydro-alcoholic extract

Ten kg of *G. sepium* leaves (7 weeks regrowth; 2.5 m above the ground) were collected from 10 trees in a tropical zone of southwest Mexico (97°33´ W, 16°18´ N) at an altitude of 370 m above sea level. Mean annual temperature is 25°C and the average annual precipitation is 1409 mm. Partially dehydrated leaves of *G. sepium* were dried using a forced-draft forage dryer for 48 h at 70 °C. Samples were ground with a Wiley mill to a particle size <355 µm. One hundred g of the resulting plant material were added to a solvent containing 210 mL methanol and 90 mL water for 48 h at room temperature. The supernatant was sieved through No. 3 Whatman filter paper. The extract was placed in a rotary evaporator (Yamato RE300 rotavaporator) at 100 °C to get rid of methanol. The water fraction was extracted by a lyophilizer (Labconco, Kansas City, MO, USA) and the extract was placed in glass vials and stored at 4 °C until analysis. The extract was reconstituted to concentrations of 12.5, 25, 50, 100, and 200 mg/mL using distilled water.

Determination of condensed and hydrolyzable tannins

The HCL-Butanol technique (27) was used to obtain condensed tannins (CT). Three mL of HCL-terbutanol was added to 0.5 mL of the extract, in triplicate and 0.1 mL of ferric reagent (HCL-NH_4Fe $(SO_4)_2$) was added. This was done in test tubes with screw cap (13 × 10 mm), which were placed in a water bath at 100 °C for 1 h. Subsequently, they were allowed to cool to room temperature. The reading of tubes was carried out with a spectrophotometer (absorbance at 460 nm). The concentration was calculated using the catechin as standard and the results were expressed as mg/g in catechin equivalents (mg/CE/g DM).

Hydrolyzable tannins (HT) were determined using the Folin Ciocalteu technique (28). Forty µL of the diluted extract were used in 360 µL of distilled water, and this was deposited in 16 × 50 tubes for each of the extracts in triplicate. Subsequently, 400 µL of the Folin-Ciocalteu reagent (Sigma Aldrich F9252) was added, the mixture was homogenized and left to rest for 5 min, then 400 µL of sodium carbonate ($NaCO_3$, 0.01 M) were added. Mixtures were stirred and left to rest for 5 min. Finally, 2000 µL of distilled water was added and the absorbance of 725 nm was read with the spectrophotometer, the concentration was calculated using the gallic acid standard and the results were expressed as mg of gallic acid equivalent per g of DM of the plant extract (mg/GAE/g DM).

Partial purification of metabolites

An aqueous extraction was carried out as previously described for leaves of the trees for column chromatography (29), to partially purify compounds of the extractions. For this procedure, a vertical glass column with a capacity of 150 mL was used. The packing was carried out with Amberlite XAD-16 (Sigma Aldrich), 25 mL of the extract were deposited inside the adsorbent. The column was activated, step by step, with distilled water and MeOH: H_2O (40:60), and elution with water was carried out to remove sugars, amino acids, organic acids, and low molecular weight phenols.

Finally, the purified concentrate of metabolites was eluted with ethanol. The liquid obtained was fractionated in Petri dishes and placed in an oven at 60 °C for 24 h. The

metabolites were recovered as a fine powder and placed in vials (1.5 mL Eppendorf ™), protected from light.

For the identification of the secondary metabolites, 1 mg of leaves of *G. sepium* was weighed out of this fine powder and dissolved in 1 mL of methanol. Then the samples were subjected to sonic vibration for 20 min (Branson Sonicator model 2510). The samples were then filtered using membranes of 0.45 µm and placed in 1.8 mL glass vials and 0.75 mL of methanol were added to each vial, which was also filtered with 0.45 µm membranes to obtain a dilution of 1:2 of each sample in vials. The components of the plant were detected with the ProStar Varian HPLC system (Spectra Lab Scientific Inc., Markham, Ontario Canada), with a three-phase pump, a model 410 autosampler, and a diode array UV-vis detector. The column used for the analysis was a Varian Pursuit XRs c18, 4.6 mm x 250 mm, with a flow of 1 mL/min and a volume injection of 10 µL per sample. The mobile phases of analysis were: A methanol (washing phase), B acetonitrile, and C acetic acid 3%, with the following elution gradients 0-10 min 100% C, 10-20 min 20% B 80% C, 20 -25 min 30% B 70% C, 25-26 min 60% B 40% C, 26-31 min 30% B 70% C, 31-40 100% C (30). The column was washed and reconditioned for mass spectrophotometry analysis, using Varian 500/MS equipment with ion trap, electrospray ionization (ESI), negative mode (MH-), capillary voltage of 90 V, and a mass range of 100-2000 m/z was used.

Larvae

Infective larvae of *H. contortus* were obtained from a previously worm-free 7-month-old lamb of approximately 30 kg which was infected orally with 350 *Haemonchus contortus* larvae (L3), per kg of live weight. The lamb was housed in a roofed pen and was fed a balanced commercial diet composed of concentrate and oat hay with free access to water. Coproparasitoscopic analyzes (McMaster chamber) were made weekly until day 21 post-infection when the parasite load was >800 eggs per g of feces. Feces were collected in plastic basins and were macerated. Then tap water was added to get a pasty consistency. Also, small polystyrene pellets were added to feces to enable homogenization and aeration of feces (31). The basin was sealed tightly to maintain a uniform relative humidity of 100% for 7 days to induce the hatching and exsheathing of larvae.

To get a suspension of larvae free of impurities, a filtering paper for cleaning the objectives of microscopes (Thomas Scientific, USA) was placed in a Baermann-funnel apparatus with tap water. Larvae descended using the optical lens paper and settled down on the bottom of the tube. The filtrated material was transferred into 50-mL plastic tubes and was kept at 4 °C for 1 h. Larvae were exposed to density gradients and centrifugation at 3500 rpm for 5 min. For this procedure, 4 mL of 40% sucrose was added to each of two 15-cm test tubes, then, with a Pasteur pipette, a pellet containing 2 mL of larvae was added slowly to the test tube and these tubes were centrifuged at 3500 rpm for 5 min. This process decanted fecal debris at the bottom of the tube. After centrifugation, a package of concentrated clean larvae (white ring) appeared on the surface of the suspension. These were collected and deposited in 10 mL tubes where they were washed three times with distilled water. This eliminated excess sucrose and maintained an appropriate osmotic balance for the survival of larvae. Cleaned larvae were placed in 30 mL cell cultures with 10 mL of clean water and were kept at 4 °C until quantification. Eleven 10 µL aliquots were used to count larvae on a slide under a microscope with a × 4 magnification. Larvae were placed in culture plates and kept at 4 °C. Sodium hypochlorite (0.186 µL) was used to remove the sheath of the infective larvae, and when larvae were observed to leave the sheath, they were washed with purified water to remove sodium hypochlorite.

Larval survival assay

The in vitro bioassays comparison (extract-larvae) was carried out on 96-well micro-titration plates. For each well, 50 µL of the extract was mixed with 12.5, 25, 50, 100, and 200, mg/mL with three replicates for each concentration and controls and in triplicate. A total of 110 larvae (either sheathed or exsheathed) were seeded into each well containing the extract. Then the plate was protected with a soaked paper towel to maintain moisture and then enclosed with aluminum foil. Survival determinations were made at 24, 48, and 72 h. For determination of survival, 11 10 µL aliquots were positioned on a slide and observed in a microscope with a × 10 magnification. The plates were incubated at 20 °C and 80% relative humidity for 24, 48, and 72 h in an oven. For the positive control, 50 µL of 1% ivermectin (Aranda Laboratories, Queretaro, Mexico) and 110 infective larvae contained in 50 µL per well were used.

The mortality of larvae at different extract concentrations was evaluated, and a percentage of larval mortality was calculated. After putting *H. contortus* in contact with the aqueous extract, motility was observed every 6 h using a magnifying glass. Adult worms' motility inhibition was evaluated as the following ratio: the number of immotile (dead) nematodes divided by the total number of initially mobile (alive) nematodes for each concentration or control. The death of larvae of *H. contortus* was determined by the lack of motility for five seconds.

Statistical analyses

The effect of concentration of hydro-methanolic extracts of *G. sepium* on larvae of *H. contortus* survival was analyzed using the GENMOD procedure of SAS (SAS Institute, Inc., Cary, NC, USA). The experimental units were the groups of larvae. For sheathed or exsheathed larvae the model included the effect of levels of extract, days of incubation, and simple interaction. For larvae survival, time of incubation

and the interaction treatment × time of incubation were non-significant (P>0.15) and thus, were excluded from the model, and data were pooled across extract levels. Differences among levels of extract were determined by the LSMEANS/DIFF option of SAS. The median lethal concentration (LC50) was analyzed by the probit method (PROC PROBIT of SAS). Models for the associations between extract level and larvae mortality were described using the CurveExpert software (CurveExpert Professional 2.5.6; Hyams Development, Huntsville, Alabama). P-values less than 0.05 were considered statistically significant.

Results

After lyophilization, the hydro-methanolic extract gave 6.8 g from 100 g of leaves of *G. sepium*. Total, condensed and hydrolyzable phenolic compounds obtained from leaves of *G. sepium* were 6.4 ± 2.4, 3.4 ± 1.2, and 3.1 ± 1.7 mg/g of dry matter, respectively (means ± SD). The compounds distinguished in the extracts from leaves of *G. sepium* by HPLC-mass spectrometry are presented in table 1. Proanthocyanidin and phenolic acid were found, as well as various flavonols and flavonoids.

The extracts of *G. sepium* exhibited a significant (P<0.001) sheated larvae mortality at concentrations of 12.5 and 25 mg/mL at 24 and 72 h post-incubation compared with the negative control (figure 1). Extract of *G. sepium* at concentrations of 50 and 100 mg/mL did not differ (P>0.05) between them in mortality rate of larvae but they were less effective (P<0.05) than lower extract concentrations. The larval mortality assay had a cubic dose-related mortality response for the sheathed larvae (Fig. 1) with 61.5 ± 20.4% mortality rate with the highest concentration (200 mg/mL) after 72 h of incubation.

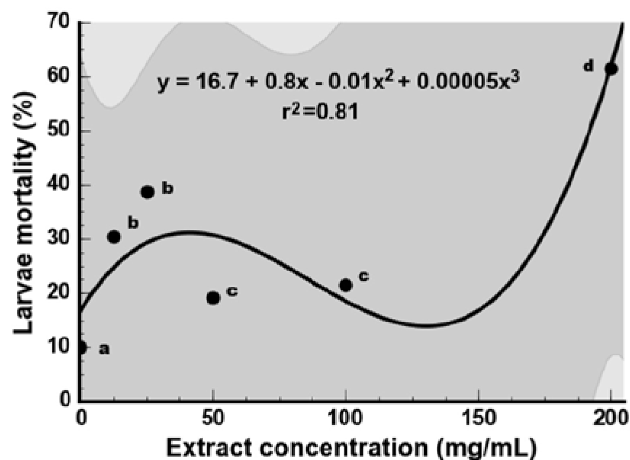

Figure 1: Sheathed larvae of *Haemonchus contortus* mortality assay concentration–response curve for hydro-methanolic extracts of *Gliricidia sepium*. Ivermectin was the positive control leading to 100% inhibition at concentration of 10 mg/mL used. The negative control consisting of plain water (value at 0 mg/mL extract concentration in the X axis) led to about 10% inhibition. Darker bands are 95% confidence intervals for predicted values. Lighter bands are 95% confidence intervals for actual values. Extract concentrations with different letters differ (*P*<0.05)

Regarding the exsheathed larvae, an extract concentration of 12.5 mg/mL did not alter larvae survival after incubation for 72 h (figure 2). Mortality rate of exsheathed larvae was less than 25% for 72 h incubation using 25, 50, and 100 mg/mL concentrations. The larval mortality assay had a quadratic dose-related mortality response in the sheathed larvae (Fig. 2) with 93.8 ± 2.9% mortality for a dilution of 200 mg/mL and incubation of 72 h.

Viability assay LC50 of *G. sepium* hydro-methanolic extract for sheathed and exsheathed larvae is shown in table 2. *G. sepium* had the highest LC50 (lowest toxicity) of 40 and 68 mg/mL for 48 and 72 h of incubation of exsheathed larvae

Table 1: Compounds identified by HPLC-mass spectrometry in the extracts of leaves of *Gliricidia sepium*

Retention time (min)	Mass/charge	Compound	Family
16.5	233	Vanillin 4-sulfate	Flavonoids
28.7	885	Prodelphinidin	Proanthocyanidins
29.6	325	p-Coumaroyl glucose	Phenolic acid
30.7	755	Kaempferol 3-o-glucosyl-rhamnosyl-glucoside	Flavonols
31.8	739	Kaempferol-3-O-xylosyl rutinoside	Flavonols
33.2	165	p-Coumaric acid	Phenolic acid
34	593	Luteolin 7-rutinoside	Flavonoids
35	623	Isorhamnetin 3-glucoside-7-rhamnoside	Flavonols
37	385	Dihydroferulic acid	Phenolic acid

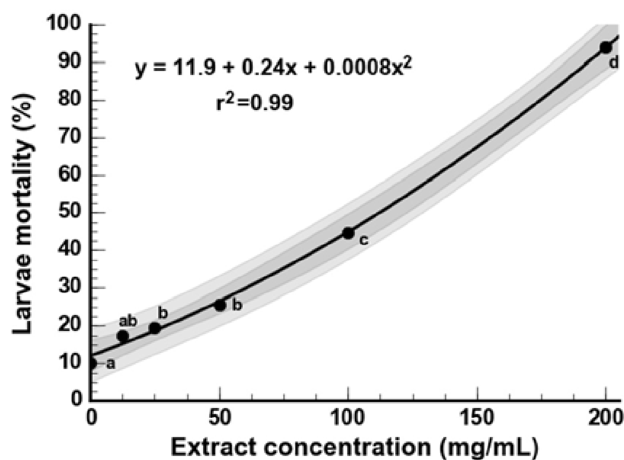

Figure 2: Exsheathed infective larvae of *Haemonchus contortus* mortality assay for different concentrations of hydro-methanolic extracts of *Gliricidia sepium*. Ivermectin was the positive control leading to 100% inhibition at concentration of 10 mg/mL used. The negative control consisting of plain water led to about 10% inhibition. Darker bands are 95% confidence intervals for predicted values. Lighter bands are 95% confidence intervals for actual values. Extract concentrations with different letters differ (P<0.05)

of *H. contortus*. Observations of the sheathed infective larvae of *H. contortus* subjected to the hydro-methanolic extract of *G. sepium* showed the presence of lesions on the nematode cuticle which were similar to those provoked by 1% ivermectin.

Discussion

In this study, extracts of leaves of *G. sepium* using hydro-methanolic solvents were examined for their anthelmintic activities. The phytochemical study showed that *G. sepium* was rich in flavonoids, flavonols but not total polyphenols. The phenolic contents of the analyzed extracts were similar to those previously reported (32). Also, the results of this study are consistent with previous results from other studies, which confirmed the high concentration of flavonoids and phenolic acid detected in *G. sepium* associated with anthelmintic activity in *G. sepium* (21).

In the present study, the in vitro model showed that hydro-methanolic extract from *G. sepium* had a dose-dependent effect on the sheathed and exsheated larvae of *H. contortus*, with the highest larvae mortality at 200 mg/mL. This concentration is much higher than the 1.25 mg/mL acetonic extract of *G. sepium* for eliminating >92% of L1 and L2 larvae of this blood-sucking nematode (33). Also, it was observed (34) that the percentage of exsheathment of *H. contortus* with 1200 µg/mL of *G. sepium* hydro-acetone extracts was 71.3%. A much lower concentration of ethanol extract of G. sepiun (40 mg/mL) than those used in the present study was adequate for in vitro larval exsheathment inhibition of *G. sepium* (21).

Efficiency of anthelmintic drugs adopted by the W.A.A.V.P. should inhibit larval motility by more than 90% (35). Thus, these in vitro results obtained with *G. sepium* extract against exsheathed infective larvae of *H. contortus* at the highest concentration, would classify the tested extract as effective. In the case of sheathed larvae of *H. contortus* the hydro-methanolic extract of *G. sepium* can be considered moderately effective against this nematode. It should be noted that the hydro-methanolic extract of *G. sepium* at its highest concentration exhibited an anthelmintic efficiency against exsheathed infective larvae of *H. contortus* close to that observed for the positive control (99.3%) in the nematode motility test. This result is important because it would reduce the chances of developing resistance of the exsheathed infective larvae of this nematode to the extract of *G. sepium* (36).

The mortality of exsheated and sheated larvae of *H. contortus* increased as the exposure period of the *G. sepium* extract went from 24 to 72 h. So, the nematode toxicity to *G. sepium* extract depends not only on the concentration of extracts but also on the exposure period. This is in line with previous studies in vitro where mortality rates of larvae of *H. contortus* increased with the increase in exposure time to plant extracts (37).

This leguminous tree undoubtedly contains nematotoxic constituents that cause the death of most of the exsheated larvae of this nematode. The chemicals contained in *G. sepium* either in a single form or in a combination induced mainly the death of exsheated larvae of *H. contortus*, which is in line with earlier studies that have shown that hydro-acetone and ethanolic extracts of this leguminous tree have ovicidal (21,22) and larval exsheathment inhibition (32).

It has been indirectly demonstrated (32) that tannins/polyphenolic compounds of *G. sepium* were involved in the anthelminthic activity of leaves from this tree. However, in the present study total polyphenols in the leaves of this legume were low (6.4 mg/g), thus other secondary metabolites of this leguminous tree other than polyphenols seem to be

Table 2: Viability assay (LC50) of *Gliricidia sepium* hydro-methanolic extract for sheathed and exsheathed larvae of *Haemonchus contortus*. Values are mg/g

Larvae	24 h	CI*	48 h	CI	72 h	CI
Sheathed	41	10-88	70	33-100	74	46-100
Exsheathed	23	1-81	40	8-94	68	32-100

*CI confidence interval

involved in the mortality of sheated and exsheated larvae of *H. contortus*. Flavonols contained in leaves of *G. sepium* could be involved in the death of this nematode because these chemical compounds have shown antimicrobial activity largely due to the ability of these compounds to hamper the cytoplasmic membrane function (38) and the nematicidal activity against *H. contortus* of flavonoids alone (39, 40) or in combination with condensed tannins (41) has been documented.

After exposure of sheated and exsheated larvae of *H. contortus* to extract of *G. sepium*, damages on the cuticle of this nematode were similar to those produced by ivermectin. Thus, the antiparasitic activity of the secondary compounds of *G. sepium* in larvae seems to be explained by their contact with the nematode's cuticle. However, visualization of the structural changes in the nematode by electron microscopy would be necessary to confirm this damage in this gastrointestinal parasite.

It is concluded that extract of leaves of *Gliricidia sepium* showed significant dose-dependent mortality of sheated and exsheated larvae of *H. contortus*. Flavonoids, flavonols, phenolic acid, and tannins apparently were responsible for the anthelmintic bioactivity of this plant. Since *G. sepium* is an important cultivated plant in tropical regions and toxicity has not been demonstrated for livestock, the leaves extract of this leguminous tree possesses in vitro larvicidal activity against *H. contortus*. However, purification will be necessary to isolate and purify the bioactive compounds from *G. sepium* extracts, to further evaluate their in vivo activities on gastrointestinal parasites, and to study the structural changes in the *H. contortus* by electron microscopy to determine the mode of action of the secondary compounds of *G. sepium* on this blood-sucking nematode.

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Conflict of interests. All authors declare that there are no actual or potential conflicts of interest between the authors and other people or organizations that could inappropriately bias their work.

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Author contributions. MM and JEG designed and drafted the manuscript. UMC carried out the statistical analysis. LG carried out the field and laboratory work. LAR and UMC revised the manuscript and reviewed the pertinent literature. Finally, all authors revised the manuscript and approved the submitted version.

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Učinki hidrometanolnega izvlečka listov *Gliricidia sepium* na ličinke *Haemonchus contortus in vitro*

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Izvleček: Namen te *in vitro* študije je bil oceniti protiglivične učinke izvlečkov *Gliricidia sepium* na ličinke *Haemonchus contortus* z ovojem in brez njega. Ličinke parazita so bile inkubirane 24, 48 ali 72 ur pri 20–25 °C v hidrometanolnih izvlečkih listov tega tropskega drevesa v koncentracijah 12,5, 25, 50, 100 in 200 mg/ml. Voda in ivermectin sta služila kot negativna in pozitivna kontrola. Skupne fenolne spojine v listih *G. sepium* so obsegale 6,4 ± 2,4 mg/g suhe snovi. Druge spojine, ki so bile v drevesu identificirane s HPLC-masno spektrometrijo in ki bi lahko bile odgovorne za opažene protiglivične učinke, so bile vanilin 4-sulfat, prodelfinidin p-kumaroil glukoza, kaempferol 3-O-glukozil-rimnozil-glukozid, kaempferol-3-O-ksilozil rutinozid, p-kumarna kislina, luteolin 7-rutinozid, izorhamnetin 3-glukozid-7-rimnozid in dihidro ferulinska kislina. Pri odmerkih 100 mg/ml in 72 urah inkubacije je bila stopnja smrtnosti pri *H. contortus* z ovojem 21,6 %, pri *H. contortus* brez ovoja pa 44,7 %. Pri odmerkih 200 mg/ml in 72 urah inkubacije so hidrometanolni izvlečki *G. sepium* uničili 61,5 % ličink z ovojem in 93,8 % ličink brez ovoja. Srednja efektivna koncentracija (EC50) izvlečka *G. sepium* za ličinke z ovojem je bila 74 mg/ml (CI = 46–100), za ličinke brez ovoja pa 68 mg/ml (CI = 32–100) po 72 urah inkubacije. Statistično značilna (P < 0,001) sposobnost uničenja ličink v primerjavi z negativno kontrolo kaže na protiglivične lastnosti *G. sepium* proti *H. contortus in vitro*.

Ključne besede: flavonoidi; flavonoli; ličinke; nematodi; tanini