



Chemical composition of various plant extracts and their *in vitro* efficacy in control of *Fasciola hepatica* eggs

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ABSTRACT: *Fasciolosis has been diagnosed in cattle, goats, sheep and horses in southern and southeastern Brazil. Effective alternative treatments are the targets of study. One promising alternative is the use of plant extracts. The aim of this study was to perform phytochemical analysis of extracts of Eugenia uniflora L., Harpagophytum procumbens, Psidium guajava L. and Stryphnodendron adstringens, and to evaluate the in vitro efficacy of these extracts on ovicidal activity in Fasciola hepatica. Plant extracts were analyzed for phytochemical properties. F. hepatica eggs were collected directly from the gallbladders of animals diagnosed as positive for fasciolosis on post mortem examination. One hundred eggs were incubated with 3 ml of each extract at concentrations of 0.10%, 0.25% and 0.50%, albendazole 0.50% (positive control) or tap water (negative control). To determine anti larval efficacy of each plant extract, hatched eggs were counted and the averages were used. Phytochemical analysis revealed the presence of phenolic compounds, tannins and terpenes in most extracts. E. uniflora L. extract was 100% effective at 0.10%, H. procumbens was effective at 0.25% and P. guajava L. and S. adstringens extracts were 100% effective at all concentrations tested. Taken together, the data suggested that ovicidal activity in F. hepatica is due to the presence of these bioactive compounds.*

Key words: *Eugenia uniflora L., Harpagophytum procumbens, Psidium guajava L., Stryphnodendron adstringens, ovicidal activity on Fasciola.*

Composição química de vários extratos vegetais e sua eficácia *in vitro* no controle de ovos de *Fasciola hepática*

RESUMO: *A fasciolose tem sido diagnosticada em bovinos, caprinos, ovinos e equinos no sul e sudeste do Brasil, sendo que tratamentos alternativos mais eficazes são alvos de estudo. Uma das alternativas promissoras é o uso de extratos vegetais no controle dessa e outras enfermidades. O objetivo deste estudo foi realizar a análise fitoquímica dos extratos de Eugenia uniflora L., Harpagophytum procumbens, Psidium guajava L. e Stryphnodendron adstringens, além de avaliar a eficácia in vitro desses extratos na atividade ovicida em Fasciola hepatica. Os extratos vegetais foram obtidos e analisados para determinação fitoquímica. Ovos de F. hepatica foram coletados diretamente das vesículas biliares de animais diagnosticados como positivos para fasciolose no exame post mortem. Cem ovos foram incubados com três mililitros de cada extrato nas concentrações de 0,10%, 0,25% e 0,50%, de albendazol a 0,50% (controle positivo) e água de torneira (controle negativo). Para determinar a eficácia de cada extrato vegetal os ovos eclodidos foram contados, e a média utilizada para os cálculos de eficácia. A análise fitoquímica revelou a presença de compostos fenólicos, taninos e terpenos na maioria dos extratos. O extrato de E. uniflora L. apresentou eficácia de 100% na concentração de 0,10%, o de H. procumbens a 0,25% e os extratos de P. guajava L. e S. adstringens apresentaram 100% de eficácia em todas as concentrações testadas. Assim, sugere-se que a atividade ovicida em F. hepatica seja devido à presença desses compostos bioativos.*

Palavras-chave: *Eugenia uniflora L., Harpagophytum procumbens, Psidium guajava L., Stryphnodendron adstringens, atividade ovicida sobre Fasciola.*

INTRODUCTION

Fasciolosis is a zoonosis caused by the liver fluke from *Fasciola* genus, thus generating substantial public health concern (MAS-COMA et

al., 2014). Its main impact is on veterinary science, especially in sheep and cattle breeding, as it causes substantial economic losses due to condemnation of livers and animal carcasses in addition to reduced milk productivity, contamination of livers, weight

loss and susceptibility to other diseases (SILVA et al., 2008).

OLIVEIRA & RESENDE (2017), in their study on historical-geographical tracking, point to *Fasciola hepatica* are present throughout Brazil in the coming years, saw its versatility of infection, in the presence of its intermediate host throughout the national territory and especially due to the transport of animals between the Brazilian regions. Thus, it is essential to control fasciolosis to minimize the negative impacts on the economy.

The main form of control of *F. hepatica* infection is the use of anthelmintics. Benzimidazole are known to prevent the hatching of flukes and roudworm eggs (ALVAREZ et al., 2009), among them the halogenated triclabendazole derivative is the most efficient in controlling *F. hepatica* as it targets adults, larvae and eggs (BORAY et al., 1983; BEESLEY et al., 2017). However, this overreliance on triclabendazole has inevitably resulted in the emergence of triclabendazole-resistance in liver fluke populations (BEESLEY et al., 2017). Besides that, triclabendazole is no longer available in Brazil due to low demand and high cost.

There are other drugs available in Brazil to treat animals infected with *F. hepatica* and most of them have report of resistance (ALVES & MARTINS, 2013). Thus, in addition to concerns about resistance, consumer demand for alternatives to synthetic products has spurred research on the use of medicinal plants in the worm control (TARIFA, 2001).

Another important matter is that secondary metabolites such as alkaloids, terpenes, tannins or flavonoids contained in crude plant extracts have been found related to parasitocidal activity (MERCADO et al., 2015). Hence, it is necessary to determine the chemical composition of the extracts because the validation of efficacy is an essential step for the correct use of products of plant origin as well as their active compounds (COSTA et al., 2002).

As environmental contamination by *F. hepatica* eggs is a major problem faced, more effective measures are needed to control this parasitosis. Thus, the aim of this study was to determine the phytochemical constituents of extracts of *Eugenia uniflora* L., *Harpagophytum procumbens*, *Psidium guajava* L. and *Stryphnodendron adstringens* and to evaluate the efficacy in ovicidal activity on *Fasciola hepatica* eggs.

MATERIALS AND METHODS

Preparation of ethanol plant extracts

The plant samples used were obtained from Pharmaceutical Production Laboratory of the Federal University of Espírito Santo. The plant collection was established by collecting native species from various locations in the states of Espírito Santo and São Paulo, Brazil. Exotic species that are not cultivated typically in Brazil were obtained from specialized suppliers (Table 1).

The plant materials were dried at 45 °C under forced air circulation (4–5 days) and were milled using knife mill (0.5 mm mesh size). The powdered-dried materials were extracted by maceration in analytical grade alcohol at RT, by soaking 100 g gram powder in 1L solvent. Each plant extract was prepared in triplicate. Solvent was filtered through Whatman cellulose filter paper and concentrated at 40 °C under reduced pressure (Laborota 4001, Heidolph). Aliquots of the extracts were partitioned between analytical grade ethyl acetate (2 × 400 mL) and deionized water (400 mL) to remove lipophilic pigments. Finally, the aqueous layer of each triplicate was collected and lyophilized (L101, Liotop).

Phytochemical analysis of plant extracts

Qualitative characterization of the chemical composition of plant samples was performed according to WAGNER (1984) and MATOS (2009), with minor modifications. The search for alkaloids

Table 1 – Plant species selected for *in vitro* evaluation of anti-helminthic activity against *F. hepatica* eggs.

Scientific name	Initials	Part	Collection	Code
<i>Eugenia uniflora</i>	EUL	Leaves	VIES ^a	VIES30550
<i>Harpagophytum procumbens</i>	HPR	Roots	Massaro ^b	7890529283225
<i>Psidium guajava</i>	PGL	Leaves	VIES ^a	VIES30552
<i>Stryphnodendron adstringens</i>	SAB	Barks	Massaro ^b	7898529281047

^aCentral Herbarium of the Federal University of Espírito Santo VIES, Jerônimo Monteiro-ES; ^bComercial Massaro, Ribeirão Preto-SP.

used the following reagents: Dragendorff (orange precipitate), Mayer (white precipitate), Bertrand (white precipitate), Bouchardat (brown precipitate), Sonnenschein (white precipitate), and Hager (yellow precipitate). For anthraquinones, the Bornträger reaction was used to observe the alkaline red color phase indicating positive reactions.

For cardiac glycosides, we used the Kedde reaction that reveals the presence of cardenolide rings as brown-reddish color solutions, in combination with a Legal reaction that gives a reddish color due to lactonic rings and the Keller-Kiliani reaction for free deoxy sugar analysis.

Coumarins were determined using the application of the extracts on paper filter, followed by addition of 10% KOH and observation under UV light. The occurrence of flavonoids was determined as proposed based on the combination of the reactions of Shinoda, Pew, and Taubock using 3% aluminum chloride under UV light (245 and 360 nm).

The presence of phenolic compounds was tested by adding drops of 5% FeCl₃ in all sample extract solutions (100 mg/ml); the formation of a dark precipitate indicated positive reactions. To verify the presence of saponins in the extracts, aqueous solutions of the extracts were prepared, and foaming of the solutions was analyzed.

Tannins were screened by using solutions of 2% gelatin, 10% lead acetate and 3% copper acetate that produce characteristic precipitates. Terpenic compounds were determined using the Liebermann-Buchard reaction, with the formation of blue-greenish or pinkish rings in the interface layer indicating steroidal or triterpenic nuclei, respectively.

The assays were performed in triplicate. As a positive control for the phytochemical tests, we used extracts from plants that had been previously reported to possess these compounds.

Evaluation of the ovicidal efficacy of plant extracts against F. hepatica eggs.

For this experiment, *F. hepatica* eggs were collected from the gallbladder of cattle diagnosed with fasciolosis on *post mortem* examination. The eggs, along with the bile fluid, were stored in glass vials and transported to the parasitology lab at veterinary Hospital of the Federal University of Espírito Santo (Hospital Veterinário da Universidade Federal do Espírito Santo, HOVET-UFES).

To evaluate the ovicidal activities, the nine crude extracts of plants were dissolved in 0.1%, 0.25% and 0.5% deionized water. 100 eggs were collected and transferred to 50-mL Falcon® tubes, and 3 mL of

the plant extracts were added in each tube. The assays were performed in triplicate. The positive and negative controls consisted of albendazole at 0.5% and tap water (FAIRWEATHER et al., 2012), respectively.

Falcon® tubes were wrapped in aluminum foil to prevent light exposure and were placed in a biochemical oxygen demand (BOD) chamber for 14 days at 25 °C. The samples were then exposed to 100 W incandescent light bulbs for 3 hours (FAIRWEATHER et al., 2012). Egg counts were performed once every hour during the 3-hour light exposure using a stereoscopic microscope at 2x magnification. At the end of the hatching analysis, the eggs were pipetted and deposited onto slides with water and covered with a coverslip for morphological analysis. Egg size, external wall integrity, miracidium formation and operculum opening were evaluated using light microscopy (Binocular Microscope Novel BM1000 LED) with the 40x and 100x objective lenses.

The efficacy, expressed as a percentage, was estimated for each extract using the following formula:

$$\text{Efficacy (\%)} = \frac{\text{mean negative control} - \text{mean of extracts treatment}}{\text{mean negative control}} \times 100$$

Extracts with a statistically significant difference between treatment and control groups with efficacy $\geq 90\%$ were considered effective (WOOD et al., 1995). Analysis of variance at $p < 0.05$ was performed, and means were compared using the Tukey's test at $p < 0.05$, using Assistat® online.

RESULTS AND DISCUSSION

The results of the phytochemical analysis of the extracts of four plants used in this study are summarized in table 2. Extract analysis of *E. uniflora* leaves (EUL) revealed the presence of phenolic compounds, tannins and terpenoids. Literature data suggested the presence of these and other metabolites of the genus *Eugenia*; FIUZA et al. (2008) observed the presence of tannins, steroids, triterpenes, heterosides, anthraquinones, saponins, flavonoids, sesquiterpenes, and phenolic compounds. The phytochemical analysis of *H. procumbens* root (HPR) extract revealed the presence of phenolic compounds and terpenoids. The literature suggested that *H. procumbens* roots are composed mainly by iridoids, main active compound of which is harpagoside, constituting 0.5% to 1.5% of the herbal drug (ROSA, 2007).

In our study, the analysis of *P. guajava* L. leaves (PGL) revealed the presence of phenolic compounds, including flavonoids and tannins, terpenoids. WANG et al. (2014) showed that *P.*

Table 2 – Phytochemical analysis of the plant species selected for *in vitro* evaluation of anti-helminthic activity against *F. hepatica* eggs.

Metabolite	Test performed	Results
Alkaloids	Dragendorff, Mayer, Bertrand, Bouchardat, Sonnenschein, Hager	None
Anthraquinones	Bornträger	None
Cardiac glycosides	Kedde, Legal, Pesez, Keller-killiani	None
Coumarins	KOH 10%	None
Flavonoids	AlCl ₃ , Pacheco, Shinoda, Pew; Taubouk	None
Phenolics	FeCl ₃	EUL, HPR, PGL, SAB
Saponins	Stable foam formation	None
Tannins	Gelatin 2%, Pb(AcO) ₂ , FeCl ₃ 1%, Cu(AcO) ₂	EUL ¹ , PGL ¹ , SAB ²
Terpenoids	Liebermann-Buchard	EUL, HPR, PGL, SAB

EUL: *E. uniflora* leaves; HPR: *H. procumbens* roots; PGL: *P. guajava* L. leaves; SAB: *S. adstringens* bark; ¹Hydrolysable Tannins, ²Condensed Tannins.

guajava L. leaves are composed of large amounts of phenolic compounds, including gallic acid, catechin, epicatechin, rutin, quercetin and monoheteroside derivatives, an essential oil composed of sesquiterpenes (54.9%) and low molecular weight volatile compounds, mainly hexanal aldehydes (65.9%). MARTINS et al. (1995) reported the presence of tannins in *P. guajava* L. that potentially prevent the penetration of tissues and mucous membranes by harmful agents that are responsible for the plant's anti-diarrheal properties.

Phytochemical analysis of *S. adstringens* bark (SAB) revealed the presence of phenolic compounds, flavonoids and tannins in the bark extracts and terpenoids in the leaf extracts. High molecular weight proanthocyanidins and other condensed tannins (flavan-3-ols, prodelfphinidins and prorobinetinidins) were identified in *S. adstringens* (MELLO et al., 1996a, 1996b, 1999). MACEDO et al. (2008) showed that *S. adstringens* leaves can be used as a source of phenolic compounds, mainly flavonoids and tannins. In leaves, these compounds deter herbivores and other pathogenic actors. *S. adstringens* is rich in tannins and other chemical classes, including flavonoids, terpenes, stilbenes, steroids and protease inhibitors (such as trypsin) that may be responsible for its anti-inflammatory activity and supposed antimicrobial activity (VASCONCELOS et al., 2004).

In the *in vitro* test to determine the efficacy of extracts on *F. hepatica* eggs, all plant extracts tested at concentrations of 0.1%, 0.25% and 0.5% had activities that were significantly different from the negative (water) and positive (albendazole) controls; all extracts inhibited larval

development or egg hatching (Table 3). According to POWERS et al. (1982), extracts with efficacies greater than or equal to 90% were highly effective and those with efficacies between 80% and 90% were moderately effective. However, WOOD et al. (1995) declared that efficacy of a product for nematodes, trematodes and cestodes, should be expressed for each genus/species (larvae/adults) as follows: highly effective (over 98%), effective (90%–98%), moderately effective (80%–89%) or insufficiently active (less than 80%).

In our study, the extracts of EUL 0.10%, HPR 0.25%, PGL and SAB at concentrations of 0.10%, 0.25% and 0.50% showed high efficacy. The 0.50% HPR extract was effective, while at the 0.25% concentration, it was moderately effective. For the extracts of EUL, HPR, PGL and SAB, there were no significant differences between the three tested concentrations, and the activities of these extracts were only significantly different from the negative control, showing that they were effective in preventing the hatching of *F. hepatica* miracidia.

In microscopic analysis of eggs, tap water did not prevent the hatching of miracidia from *F. hepatica* eggs, allowing the operculum to remain open. By contrast, albendazole, used as a positive control, prevented egg hatching, resulting in non-viable eggs, with no miracidium formation in their interior. The surface was not changed in any of the experimental conditions. The bark of *S. adstringens* allowed formation of miracidium; however, they did not hatch. In the literature we found no reports of the efficacy of these plant extracts on *F. hepatica* (Figure 1). MERCADO et al. (2015) reported that secondary metabolites such as alkaloids, terpenes, tannins and flavonoids in crude

Table 3 – Quantitative evaluation of hatchability and efficacy on *Fasciola hepatica* eggs after incubation with plant extracts within 3 hours of experiment.

Treatment	Concentration	-----Samples-----			Mean	Efficacy (%)
		S01	S02	S03		
EUL	0.10%	0	0	0	0	100
HPR	0.10%	10	12	18	13,33	88.20
HPR	0.25%	0	0	0	0	100
HPR	0.50%	2	22	0	8	92.92
PGL	0.10%	1	0	1	0.66	99.41
PGL	0.25%	0	0	0	0	100
PGL	0.50%	0	0	0	0	100
SAB	0.10%	0	0	0	0	100
SAB	0.25%	0	0	0	0	100
SAB	0.50%	0	0	0	0	100
Albendazole	0.50%	0	0	0	0	100
-----Tap water-----		116	102	121	113	0

*EUL: *E. uniflora* leaves; HPR: *H. procumbens* roots; PGL: *P. guajava* L. leaves; SAB: *S. adstringens* barks.

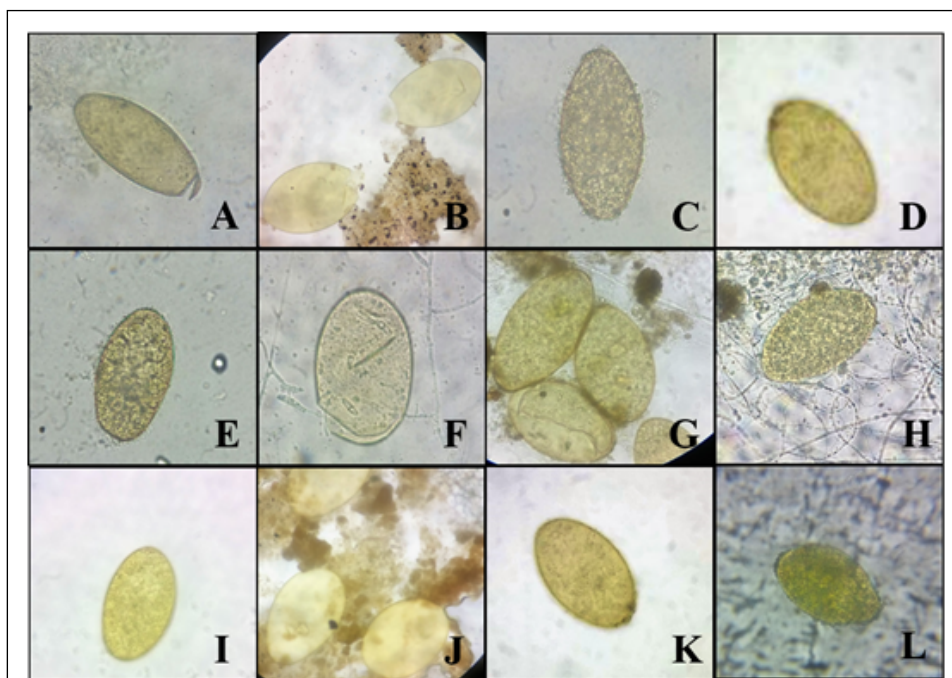


Figure 1 - Light micrographs of *Fasciola hepatica* eggs treated with different plant extracts. (A; B) *F. hepatica* egg exposed to water (negative control); (C) *F. hepatica* egg exposed to albendazole 0.50% (positive control); (D) *F. hepatica* egg exposed to 0.25% *E. uniflora* leaf extract; (E; F) *F. hepatica* egg exposed to *H. procumbens* leaf extracts at 0.10% and 0.50%, respectively; (G; H; I) *F. hepatica* egg exposed to *P. guajava* L. leaf extracts at 0.10%, 0.25% and 0.50%, respectively; (J; K; L) *F. hepatica* egg exposed to extracts of *S. adstringens* barks at 0.10%, 0.25% and 0.50%, respectively. Magnification: 400x.

plant extracts have anti-parasitic activity. Nevertheless, because these are not the only compounds produced by plants, the effects of other bioactive compounds cannot be ruled out. Therefore, determination of the chemical composition of extracts that exhibit antihelminthic activity is necessary.

In this study, all extracts were positive for tannins, suggesting that these compounds might be responsible for the biological activity that prevented miracidium hatching. It is also believed that the extracts that precluded miracidium hatching could have also altered morphology. A mechanism of tannin inhibition of miracidium hatching is binding of these compounds with proteins and other macromolecules. Given that tannins have astringent characteristics, they also display toxic properties. Another mechanism of tannin toxicity is binding with heavy metals. Biological systems, including microorganisms, require heavy metals as enzyme cofactors. For example, mice given phenolic-rich liquid as their fluid source showed decreased iron absorption (SCALBERT, 1991).

CONCLUSION

The tests performed with *F. hepatica* eggs revealed that *Eugenia uniflora*, *Harpagophytum procumbens*, *Psidium guajava* L. and *Stryphnodendron adstringens* exhibited high efficacy in the control of egg hatching, at the doses used. These extracts precluded miracidium hatching, and in some cases, precluded miracidium formation inside the egg. Phytochemical analysis revealed the presence of phenolic compounds, tannins and terpenoids in the extracts, with phenolic compounds and terpenoids being the predominant compounds in all plant extracts tested. The results suggested that these compounds could be responsible for the biological activity that prevented miracidium hatching.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

This study was approved by the Animal Use Ethics Committee of the Federal University of Espírito Santo under number 6102/2015.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

LTC, RAG and WDR performed data curation, formal analysis, developed the methodology and wrote the original draft. ABA performed writing, review and editing. IVFM and JAS performed the conceptualization, funding acquisition, project administration and supervision. All authors critically revised the manuscript and approved of the final version.

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