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In vitro Anthelmintic Activity of Aqueous Extracts from Edible Biomass of *Dichrostachys cinerea* (L) Wight et Arn.

Aida Yanet Borges Espinosa *, Mario Reinoso Pérez **, Ray Espinosa Ruiz ***, Eida Avello Oliver **

*Provincial Department of Animal Health, Ministry of Agriculture, Carretera Central No. 16, entre Segunda y Tercera, Santa Clara, Cuba.
**Marta Abreu Central University of Las Villas, Faculty of Agricultural Sciences, Department of Veterinary Medicine and Zootechnia. Carretera a Camajuaní, Km 5.5, Santa Clara, Cuba.
***Marta Abreu Central University of Las Villas, Faculty of Agricultural Sciences, Department of Agronomy. Carretera a Camajuaní, Km 5.5, Santa Clara, Cuba.
Correspondence: jdsanidad@dlgdir.vcl.minag.gob.cu

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ABSTRACT

Background: The utilization of edible biomass of *Dichrostachys cinerea* is an option for ruminant nutrition and the control of parasitosis in these animals. **Aim:** To evaluate *in vitro* anthelmintic activity of aqueous extracts from re-shoots and leaves of adult leguminous plants.
Methods: Three mother solutions were made from the plant material collected. Then the aqueous extracts were prepared by means of infusion, decoction, and crushing. Later, the aqueous extracts were diluted at different concentrations (infusion and 10% decoction, and crushing at 10, 15, and 20%), for a total of 15 treatments (five from each type of plant material). A total of four (two positive and two negative) controls were included. Soil worms were used as a biological model. A Petri dish was used in each treatment, which was filled with 10 ml of the extract and six worms. Time was measured (min) for occurrence of paralysis, and death of worms.
Results: The anthelmintic effect of the infusion and 10% decoction showed no significant differences in the times of death of worms. The preparation of extracts by crushing contributed to larger extraction of secondary metabolites, which are responsible for the anthelmintic activity demonstrated in this study.
Conclusions: All the extracts showed *in vitro* anthelmintic activity. The aqueous extract collected by crushing, and 20% dilution was the most effective of the three plant materials studied.
Key words: helminths, leaves, legumes, sicklebush, re-shoots (Source: *AIMS)*

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INTRODUCTION

Parasitosis caused by gastrointestinal nematodes in ruminants is a serious problem worldwide, since it affects the productivity of infested animals due to the negative impact they have on the growth rate, body condition, and fertility. Nematodes increase the susceptibility to diseases of different origins, and raise mortality, which cause significant economic losses in livestock production (Felice, 2015; Angel, Arrieta, and Fernández, 2015). This is a multy-etiological disease caused by the synergic action of various parasite genuses and species, so they are considered a parasitic complex affecting all organisms equally (Dorta-Contreras, 2007).

In the last four decades, highly effective antiparasitic synthetic molecules have been developed, with a wide scope and low residual effect (Dorta-Contreras, 2007). However, the treatment of this disease today is complicated due to commercial drug resistance, which calls for the need to use alternative ways to control resistant or multi resistant pathogens, one of which is the use of secondary metabolites from plants with antibacterial activity and/or against gastrointestinal nematodes (Hernández-Alvarado *et al*., 2018; Medina *et al*., 2014; Epe and Kaminsky, 2013; Satyajit and Lutfun, 2012). In this sense, there are various research studies about the nutraceutical potential of several plants, including *D. cinerea,* whose use is not spread in Cuba. Particularly, the effectiveness of this plant is said to control gastrointestinal nematodes (Arece, Roche, López, and Molina, 2012), so it is valid to assume that the utilization of edible biomass from this plant might be an effective alternative to control parasitosis in small ruminants.

Therefore, the aim of this paper is do evaluate the *in vitro* anthelmintic activity of aqueous extracts obtained through different methods, from leguminous plants, the leaves of adult plants, and previously dried and crushed re-shoots of *D. cinerea.*

MATERIALS AND METHODS

The research was done in the microbiology laboratory at the Faculty of Agricultural Sciences of Marta Abreu Central University of Las Villas. The plant material was collected from legumes, young re-shoots, and leaves of adult plants of *D. cinerea* established in areas of El Vaquerito Cooperative of Credits and Services, on the road to Camajuaní km 2.5, Santa Clara, province Villa Clara, Cuba. The collection period comprised April and May 2017.

Obtaining and preparing the plant material

The biomass collected was dehydrated in a stove at 45 °C for five days. The plant material was also placed and heated in the stove at 60 °C, for thirty minutes. Then it was ground in a blade micromill, at 3000 rpm. The plant material was crushed and sieved through a 3 mm mesh. Then it was stored in waterproof bottles until the assay.

Preparation of the aqueous extracts

The following sequential procedure was performed:

1. A mother solution was obtained from every portion of plant material, for which 40 g of legumes and 10 g of leaves from adult plants and young re-shoots. Distilled water was added at boiling temperature (100 ºC) to complete 100 mL.

2. The mother solution was left to rest for 30 minutes, then the aqueous extracts were obtained through infusion, decoction (10 minutes), and crushing.

3. Each extract was filtered using filter paper with a mid porosity; the pH was read with pHmeter METROM®, model 520, and a buffer of sodium bicarbonate was added to adjust the pH to neutral.

4. Finally, the extracts were diluted at different concentrations (infusion and 10% decoction, and crushing at 10, 15, and 20%), for a total of 15 treatments (five of each type of plant material). They were contrasted with four control treatments consisting of two commercial antiparasitic drugs with known effects -positive control- (1% Piperazine and 10% Levamisol)- with demonstrated anthelmintic activity, and two negative controls (nutricia solution of Prosser and Zimmerman, and distilled water).

Experimental protocol

The biological model used was the soil worm (*Lumbricus terrestris* Linnaeus, 1758), whose individuals were carefully collected from the culture medium, where they stayed in a temperature between 25 and 27 ºC, a relative humidity of 80%, neutral pH. The worms fed on rich organic matter. Later, they were placed in a container with distilled water for washing and selection, depending on their size, thus ensuring a uniform length, with differences below ±3.0 mm. In each treatment, including the controls, a 9.5 cm diameter Petri dish containing 10 mL of the extract and six worms (replicas) was used.

All the Petri dishes were taken to the parasitology lab at a stable temperature between 25 and 27 ºC.

The anthelmintic activity was determined according to the procedure reported by Ejiofor, Zaman, and Das (2017). Time was recorded in minutes required for paralysis and death of the worms. The paralysis criterion was the lack of normal motility, whereas death was determined five minutes after the detection of paralysis. Accordingly, the worms were placed in 25 mm diameter test tubes containing 10 mL distilled water, at 45 ºC, for 10 seconds, in order to stimulate and induce movements provided they were still alive. During the assay, a strict observation of changes in motility and alterations in the tegument of worms, both with the naked eye and the stethoscope, was maintained.

Statistical analysis

The primary data were tabulated with Microsoft Office Excel 2010, for later processing, with Statgraphics Centurion version XVI.I (Statistical Graphic Corp. USA, 2006). Before checking data normality (Kolmogorov-Smirnov test), and variance homogeneity (Cochran test), a multifactorial analysis of variance (multifactorial ANOVA) was performed, without interaction, to compare the effects of the extraction methods, the type of plant material, and the concentration of the extracts, for the paralysis and death times. The effects of the part of the plant on the extracts obtained through infusion and decoction were compared by the T-Student test.

Data statement

This study is part of the PhD research protocol of the main author of this paper, so the files containing the primary data and output of the statistical techniques used will only be sent following written request of the editors of the journal.

RESULTS AND DISCUSSION

Anthelmintic activity of the control solutions

The worms placed in the 1% Piperazine solution showed a flaccid paralysis 13 minutes after application. The death of 100% of worms took place at 41 minutes, on average. It coincided with the reports made by Flores (1996), who said that Piperazine acts on the myoneural junction or driving plate of worms, producing a flaccid paralysis. However, they disagree with the results reported by Hukkeri, Kalyani, Harpali, and Manui (1993), in a similar study, using worm species *Pheritima postuma*, since they found that the paralysis and death took effect at 40 and 60 minutes, respectively. This difference may be attributed to the experimental model used in each assay.

When exposing the worms to 10% levamisol (positive control), the worms were dead two minutes after the experiment, with a spastic paralysis. In all the cases, worm paralysis was preceded by a slight excitement characterized by contractions, wiggliness, stretching, and shrinking, to try and escape the medium. All this finally led to the death of the worm. Flores (1996) reported a similar behavior when he used conventional medication to treat helminthiasis.

In the negative controls (nutricia solution of Prosser and Zimmerman, and distilled water), the worms preserved their motility for over 48 hours, and no changes were observed in the tegument. The worms were grouped and responded to physical stimuli. Accordingly, deaths caused by other factors are discarded, except by the action of the substances evaluated.

Anthelmintic activity of aqueous extracts at 10%, obtained by infusion and decoction, respectively

Tables 1 and 2 show that by comparing the anthelmintic effect of infusion and 10% decoction, no significant differences were observed for death, though deaths did occur. This behavior shows that both extraction methods, regardless of the type of plant material, have very similar magnitudes of extracting the active principles responsible for vermifuge activity. These active principles are secondary metabolites, which are in charge of antiparasitic activity, as reported by Arece, Roche, López, and Molina (2012), by supplying aqueous extracts of *D. cinerea* to sheep. In that respect, triterpenes stand out due to their nematicidal potential (Rodés, Peña and Hermosilla, 2015), and tannins, which can bind to enzymes and alter microbial metabolism, and affect vital functions, motility, nutrition, and possibly, reproduction (Medina *et al*., 2014).

Several authors claim that the consumption of secondary metabolite-rich plants, especially the ones containing tannins and other phenolic compounds, is an alternative for the control of gastrointestinal nematodes in ovines and caprines (Hernández *et al.*, 2014), and equines (Chicaiza-Tisalema *et al.*, 2016). In that sense, Aguilera-Valle, Delgado-Martínez, and Salas-Romero (2015) suggest that *D. cinerea* may be effective in anthelmintic treatments of Albendazole-resistant cyathostome populations, though *in vivo* studies are necessary to confirm their anthelmintic properties, and evaluate the use for sustainable management of this parasitosis.

Table 1. Mean times (min) of paralysis of worms with the extracts obtained by infusion and decoction

Extract obtaining method	Parts of *D. cinerea*			SE±
	Re-shoots	Adult plant leaves	Legumes	
10% infusion	178.33^{bM}	132.00^{cM}	366.67^{aN}	9.07
10% decoction	118.67^{bN}	103.17^{bM}	394.00^{aM}	4.92
SE±	12.27	17.78	8.66	
Control solutions				
1% Piperazine	13			
10% Levamizole	0			

$^{a, b, c}$ unequal superindexes on the row differ for P < 0.05 (Tukey's test)
$^{M, N}$ unequal superindexes on the columns differ for P < 0.05 (Tukey's test)

Table 2 Mean times (min) of death of worms with the extracts obtained by infusion and decoction

Extract obtaining method	Parts of *D. cinerea*			SE±
	Re-shoots	Adult plant leaves	Legumes	
10% infusion	462.50^{aL}	309.17^{aL}	390.83^{aM}	26.52
10% decoction	360.17^{bM}	395.17^{abL}	457.00^{aL}	28.71
SE±	61.29	69.12	17.93	
Control solutions				
1% Piperazine	41			
10% Levamizole	2			

$^{a, b, c}$ unequal superindexes on the row differ for P < 0.05 (Tukey's test)
$^{L, N}$ unequal superindexes on the columns differ for P < 0.05 (Tukey's test)

Despite the previous, the treatments were less effective than 1% Piperazine.

Anthelmintic activity of aqueous extracts obtained by crushing at 10, 15, and 20%

Tables 3 and 4 show the results corresponding to the 10, 15, and 20% extracts obtained by crushing, whose biological activity is concentration dependent; the most effective ones were at 20 and 15%, respectively.

Table 3. Mean times (min) of paralysis of worms by extracts obtained by crushing at 10, 15, and 20%

Extract obtaining method	Parts of *D. cinerea*			SE±
	Re-shoots	Adult plant leaves	Legumes	
10% crushing	1996.41^{aL}	536.67^{bL}	185.82^{cL}	81.79
15% crushing	1180.83^{aM}	56.67^{cM}	196.67^{bL}	26.35
20% crushing	131.17^{bN}	30.67^{cM}	148.33^{aM}	3.60
SE±	27.84	81.26	4.33	
Control solutions				
1% Piperazine	13			
10% Levamizole	0			

$^{a, b, c}$ unequal superindexes on the row differ for P < 0.05 (Tukey's test)
$^{M, N}$ unequal superindexes on the columns differ for P < 0.05 (Tukey's test)

Table 4. Mean times (min) of death of worms by extracts obtained by crushing at 10, 15, and 20%

Extract obtaining method	Parts of *D. cinerea*			SE±
	Re-shoots	Adult plant leaves	Legumes	
10% crushing	2660.83^{aL}	1050.73^{bL}	221.67^{cM}	81.36
15% crushing	1924.00^{aM}	192.67^{bM}	240.00^{bL}	60.88
20% crushing	389.17^{aN}	110.33^{cM}	185.00^{bN}	15.21
SE±	98.33	29.66	2.95	
Control solutions				
1% Piperazine	41			
10% Levamizole	2			

$^{a, b, c}$ unequal superindexes on the row differ for P < 0.05 (Tukey's test)
$^{L, M, N}$ unequal superindexes on the columns differ for P < 0.05 (Tukey's test)

The preparation of extracts by crushing contributed to larger extraction of secondary metabolites, which are responsible for the anthelmintic activity demonstrated in this study.

The anthelmintic effect of the extracts compared in *in vitro* conditions has been demonstrated in this study. Their mode of action possibly owes to the presence of phenolic compounds, mainly tannins (Chicaiza-Tisalema *et al.*, 2016; Ferreira *et al*., 2015), which have the capacity to form complexes with parasite proteins (Alonso-Díaz, Torres-Acosta, Sandoval-Castro, and Hoste, 2010), thus affecting the biology and survival of nematodes. The existence of other secondary metabolites (alkaloids, terpentoides), along with the high contents of proteins and carbohydrates

in the leaves (Martín-Casas *et al*., 2017; Heuzé, Tran, and Giger-Reverdin, 2016; Vijayalakshmi, Periyanayagam, Kavitha, Akilandeshwar, 2013), provide *D. cinerea* with an invaluable nutraceutical potential for the nutrition and health of animals that consume it (Marius *et al*., 2018). Additionally, its use in production is an agroecological choice to control this invading exotic plant (Reinoso-Pérez, Joseau, and and Valdez, 2019), and to reduce methanogenesis in the rumen (Vélez-Terranova, Campos-Gaona, Sánchez-Guerrero, 2014), to improve the efficiency of fermentation, and reduce the emissions of this greenhouse gas into the atmosphere.

Studies conducted by Araújo-Alves *et al.* (2017) concluded that the protein fractions contained in the leaves, stems, and roots of *Spigelia anthelmia*control also intervene in the control of gastrointestinal nematodes. Consequently, it can be inferred that this is valid for the edible biomass of *D. cinerea.* In addition to it, the inedible fractions, like the crust and roots, contain a wide diversity of secondary metabolites (Rodés, Peña and Hermosilla, 2015), many of whom are responsible for the antiparasitic effects attributed to the plant.

CONCLUSIONS

All the extracts obtained from legumes, re-shoots, and leaves from adult plants of *D. cinerea* showed *in vitro* anthelmintic activity.

The effectiveness of the extracts obtained by crushing on worm survival was concentration dependent, the most effective at 15 and 20%, respectively.

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AUTHOR CONTRIBUTION

Conception and design of research: AYBE, MRP, RER, EAO; analysis and data interpretation, and redaction of the manuscript: AYBE, MRP.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.