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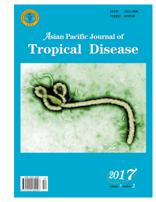

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In vitro anthelmintic activity and chemical composition of methanol extracts and fractions of *Croton paraguayensis* and *Vernonia brasiliana* against *Eisenia fetida*

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ABSTRACT

Objective: To evaluate the chemical composition and the anthelmintic activity of the methanol extracts and the acid and basic fractions of *Croton paraguayensis* (*C. paraguayensis*) and *Vernonia brasiliana* (*V. brasiliana*) against *Eisenia fetida*.
Methods: A preliminary phytochemical analysis was performed to assess the presence of groups of secondary metabolites. The plants were extracted with methanol to obtain the crude extracts. A differential pH extraction was performed to isolate basic compounds like alkaloids. The methanolic extracts and the fractions obtained were tested for anthelmintic activity against *Eisenia fetida*, using albendazole as positive control.
Results: The phytochemical test demonstrated the presence of alkaloids in the crude extracts and alkaline fractions, along with flavonoids, coumarins, steroids/triterpenes and tannins. The anthelmintic activity of the extracts and fractions of *C. paraguayensis* and *V. brasiliana* showed a statistically significant decrease of the times for paralysis and death compared to albendazole.
Conclusions: The methanolic extracts and fractions of *C. paraguayensis* and *V. brasiliana* contain compounds that possess anthelmintic activity. The isolation of the substances responsible for the biological effect described could result in the development of new drugs to treat helminth diseases.

1. Introduction

Infections due to helminths are among the most prevalent human diseases throughout the world and affect primarily large populations of developing countries, due to the lack of adequate sanitary systems and appropriate sources of drinking water[1]. Although these infections are not commonly fatal, they produce deficiencies in growth of children (physically and mentally) that adversely affect their school performance and can turn them vulnerable to other diseases[2].

The parasites also affect negatively the working performance of adults and promote the absence of children from school. The World Health Organization strategy to fight against these diseases is based on the administration of an antiparasitic drug like albendazole for the children in school age who live in areas with risk of infection.

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Albendazole is a broad-spectrum antiparasitic drug used for the treatment of most of the helminth infections and is useful against some protozoa. The main drawbacks of this drug are its adverse reactions, like abdominal pain, nausea, vomiting, diarrhea, headache, dizziness and vertigo[3]. On rare occasions, dermatological hypersensitivity reactions and alopecia had been reported. Albendazole can also produce mild to moderate elevation of hepatic transaminases and hepatotoxicity in rare cases[4]. The drug can also produce leucopenia, less frequently granulocytopenia and thrombocytopenia[5].

Those adverse reactions and the development of resistance of the parasites against the drug make the search necessary for substances that could be new drugs for the treatment of helminth diseases.

Medicinal plants are widely used by many people throughout the world to treat different illnesses, including those illnesses produced by helminths. In many cases, alkaloids have been isolated and those compounds confer a wide range of biological activities to the plants, one of which is anthelmintic[6,7].

The genus *Croton* belongs to Euphorbiaceae family and the genus *Vernonia* belongs to Asteraceae family. Alkaloids have been reported in both genera, which make them candidates for evaluation in search of new anthelmintic substances[8,9]. *Croton paraguayensis* (*C. paraguayensis*) Chodat and *Vernonia brasiliana* (L.) Druce (*V. brasiliana*) are species belonging to the aforementioned genera that grow in Paraguay and to the best of our knowledge are never evaluated for that activity.

According to these antecedents, we tested the anthelmintic activity of *C. paraguayensis* and *V. brasiliana* against *Eisenia fetida* (*E. fetida*), due to the anatomical and physiological similarities of these worms responsible for human infections[10]. We also determined the chemical composition of both plants by preliminary phytochemical analysis in order to establish groups of secondary metabolites present in them.

2. Materials and methods

2.1. Plant material

The plants were collected in the regions of Paraguarí and Cordillera, Paraguay, and identified by Rosa Degen, Germán González, Yenny González and Liz Britos from the Department of Botany, Faculty of Chemical Sciences. Voucher specimens of both plants were deposited in the Herbarium Faculty of Chemical Sciences for indexing purposes (Rosa Degen 4025 et Germán González, Yenny González for *V. brasiliana* and Rosa Degen 4198 et Germán González, Liz Britos for *C. paraguayensis*).

2.2. Reagents and chemicals

All chemicals were of analytical grade (E. Merck, Darmstadt, Germany), except the methanol used for the preparation of extracts, which was of high performance liquid chromatography grade (J.T. Baker, Center Valley, PA, USA). Albendazole (100% purity) was obtained from a local pharmaceutical company.

2.3. Preparation of the extracts

The aerial parts of *C. paraguayensis* and the rhizome of *V. brasiliana* were ground in a cutter mill to fine powder. The ground material (500 g) was poured into a beaker and methanol was added until all the powder was soaked. It was then submitted to an ultrasonic bath for 1 h and the process was repeated three times with an interval of 15 min. The entire procedure was repeated during 3 days. The extracts were then filtered through defatted cotton and the solvent was eliminated using a rotary evaporator to obtain the crude extracts.

2.4. Phytochemical preliminary test

The crude extracts were analyzed by the method of Sanabria-Galindo that used coloration and/or precipitation reactions along with thin-layer chromatography in order to reveal groups of secondary metabolites[11]. The analysis identified the presence of alkaloids, flavonoids, steroids and/or triterpenes, coumarins, tannins, saponins, naphthoquinones and/or anthraquinones, cardiotonic glycosides and sesquiterpenes lactones.

2.5. pH differential extraction

In order to isolate the alkaloids, 5 g crude extracts was treated with 5% HCl and heated at 60 °C in a water bath trying to dissolve most of the solid. The liquid was filtered and extracted with $CHCl_3$ and the organic and aqueous phases were separated. The process was repeated three times. The organic fractions were reunited, and after treatment with anhydrous Na_2SO_4, the solvent was eliminated using a rotary evaporator. This was labeled as the acid fraction.

The alkalinity of remainder aqueous fraction was taken to pH 8 with 20% NaOH and extracted with $CHCl_3$, as in the previous case. Finally, the alkaline aqueous phase was taken to pH 11 with 20% NaOH and extracted again with $CHCl_3$ three times. These two extracts were labeled as pH 8 and pH 11 fractions. The latest procedure was performed for separating compounds of different strengths as base. To confirm the presence of alkaloids, a small portion of the alkaline extracts was treated with the reagents of Dragendorff, Mayer, and Valser and with ammonium reineckate solution.

2.6. Anthelmintic assay

The anthelmintic assay was performed using the Californian red worm *E. fetida*, according to the method described by Pawar *et al.* with some modifications[10]. The worms were kindly provided by Dr. Alejandro Pino, from the experimental pisciculture farm of the Faculty of Veterinarian Sciences, National University of Asunción and were 4–6 cm long with a diameter of 0.2–0.4 cm.

To perform the assay, the crude extracts and the fractions of both plants were weighed and dissolved in sterile saline with 5% of dimethyl sulfoxide to obtain 10, 20 and 40 mg/mL concentrations for the crude extracts and 1 mg/mL concentrations for the acid fraction and for both basic fractions of every plant. Albendazole was used as positive control at 10 mg/mL for the analysis of the crude extracts and at 1 mg/mL for the test of the fractions. Three worms of approximately the same size (previously washed with sterile saline to eliminate dirt and other residues) were placed in Petri dishes and contacted with solutions of the crude extracts and the acid and basic fractions. An additional Petri plate was used for the positive control and sterile saline with 5% dimethyl sulfoxide was placed in another plate for the negative control. The time for paralysis and death (in minutes) was recorded. The worms were considered dead if they showed no movement, even when they were shaken vigorously, and their color faded. They shrank and did not regain their mobility when they were placed in sterile saline for at least 1 h. The assays were made in triplicate.

2.7. Statistical analysis

The data were processed according to the Shapiro-Wilk test to determine if they were normally distributed, followed by ANOVA and then by the Tukey method for relevant multiple comparisons using GraphPad Prism software version 6.0 for data analysis. P value < 0.05 was considered statistically significant.

3. Results

After the initial extraction procedure, 36.3 g methanolic extract was obtained from the aerial parts of *C. paraguayensis* (7.26% yield) and 16.9 g extract was obtained from the rhizome of *V. brasiliana* (3.38% yield).

The methanolic extracts were submitted to an acid-base extraction as described previously. The yields were 11.4% for the acid fraction, 0.6% for the pH 8 fraction and 1.58% for the pH 11 fraction of *C. paraguayensis* and 9%, 5% and 2.3% for the same fractions of *V. brasiliana*, respectively.

The results of the preliminary phytochemical analysis were summarized in Tables 1 and 2. Regarding the anthelmintic activity, the methanolic extract of *C. paraguayensis* showed statistically significant differences in the time of paralysis compared with the positive control at 10, 20 and 40 mg/mL. However, the time of paralysis between the concentrations of 10 and 20 mg/mL showed no statistically significant differences (Figure 1A).

Table 1

Preliminary qualitative phytochemical analysis of the methanolic extract of the aerial parts of *C. paraguayensis*.

Secondary metabolites	Results
Alkaloids	++
Flavonoids	+
Naphthoquinones and/or anthraquinones	–
Tannins	+++
Saponins	–
Steroids and/or triterpenes	++
Coumarins	++
Cardiotonic glycosides	–
Terpenic lactones	–

Identification comprised coloration and/or precipitation reactions along with thin-layer chromatography for detection of steroids, triterpenes and terpenic lactones.

+++: Highly present; ++: Moderately present; +: Low; –: Absent.

Table 2

Preliminary qualitative phytochemical analysis of the methanolic extract of the rhizome of *V. brasiliana*.

Secondary metabolites	Results
Alkaloids	++
Flavonoids	–
Naphthoquinones and/or anthraquinones	–
Tannins	+
Saponins	–
Steroids and / or triterpenes	++
Coumarins	–
Cardiotonic glycosides	–
Terpenic lactones	–

Identification comprised coloration and/or precipitation reactions along with thin-layer chromatography for detection of steroids, triterpenes and terpenic lactones.

+++: Highly present; ++: Moderately present; +: Low; –: Absent.

The same tendency was observed in the time for death, except that in this case, the concentrations of 20 and 40 mg/mL did not show statistically significant differences (Figure 1B).

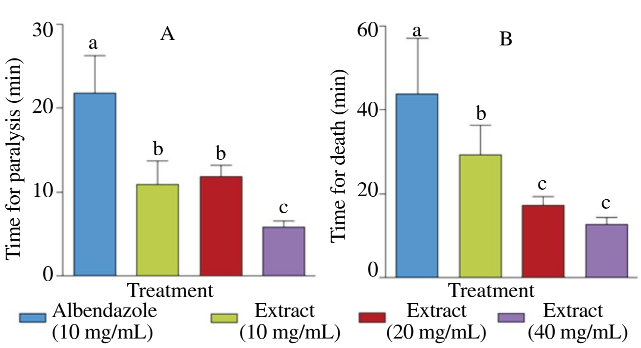

Figure 1. Anthelmintic activity of the crude extract of *C. paraguayensis* against *E. fetida*.

All values were expressed as mean ± SD and bars marked with different letters showed statistically significant differences with $P < 0.05$ (ANOVA, Tukey).

In the case of fractions, all of them showed significant differences in the time for paralysis and death compared to albendazole, due to their values lower than the control. The time for paralysis of the acid fraction and the pH 11 fraction were not significantly different, and were different in the time for death produced by the acid fraction and the pH 11 fraction as compared with the pH 8 fraction. Nevertheless, significant differences were found in the time for death when compared the acid fraction and the pH 11 fraction to each other (Figure 2A, B). The methanolic extract of *V. brasiliana* produced a significant decrease in the time for paralysis and the time for death of *E. fetida* as compared with albendazole. The effect was increased

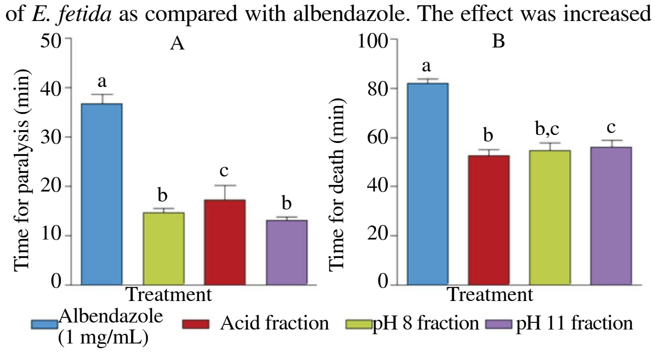

Figure 2. Anthelmintic activity of the acid and basic fractions of *C. paraguayensis* against *E. fetida*.

All values were expressed as mean ± SD and bars marked with different letters showed statistically significant differences with $P < 0.05$ (ANOVA, Tukey).

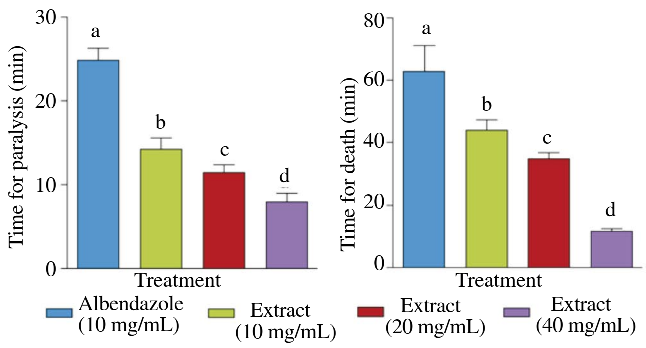

Figure 3. Anthelmintic activity of the crude extract of *V. brasiliana* against *E. fetida*.

All values were expressed as mean ± SD and bars marked with different letters showed statistically significant differences with $P < 0.05$ (ANOVA, Tukey).

Same decrease was observed for fractions, because the pH 11 fraction showed the highest activity in the case of the time for paralysis. Regarding the time for death, the basic fractions were the most active, but no significant differences were observed between them (Figure 4).

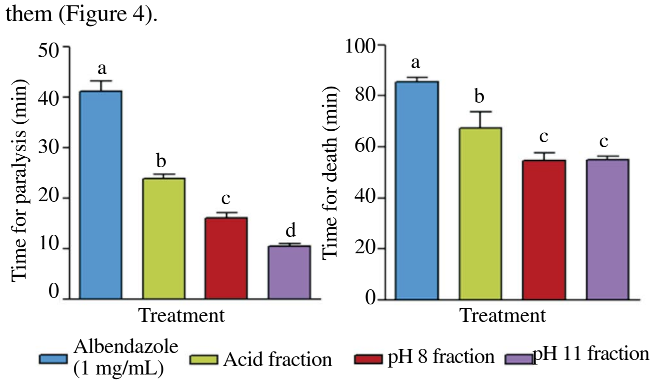

Figure 4. Anthelmintic activity of the acid and basic fractions of *V. brasiliana* against *E. fetida*.

All values were expressed as mean ± SD and bars marked with different letters showed statistically significant differences with $P < 0.05$ (ANOVA, Tukey).

4. Discussion

The preliminary phytochemical analysis of the aerial parts of

C. paraguayensis showed that alkaloids, flavonoids, steroids and/or triterpenes, tannins and coumarins were present. Regarding *V. brasiliana*, the analysis showed the presence of alkaloids, tannins and steroids and/or triterpenes.

The presence of alkaloids in both plants is important, given that many articles can be found in the literature that refer to the anthelmintic activity of these compounds[7,12,13].

The alkaloid content of *C. paraguayensis* was low, considering the yields obtained for the pH 8 and pH 11 fractions (0.6% and 1.58%, respectively). The amount of alkaloids from *V. brasiliana* was slightly higher (the yields of 5.0% and 2.3% for the pH 8 and pH 11 fractions, respectively).

According to the results obtained (Figure 1), the anthelmintic activity of the methanolic extract of *C. paraguayensis* was better than albendazole, which was used as positive control. The time for paralysis and death showed a tendency to decrease as concentration increased. However, regarding the time for death, the tendency to decrease as concentration rised was more evident than that in the case of the time for paralysis.

The anthelmintic activity of the fractions of *C. paraguayensis* was then better than albendazole. However, the results showed no markedly significant differences among the activity of the acid and basic fractions (Figure 2). This behavior may be due to the presence of various types of active components of acid or basic nature that move to the aqueous layer depending on the pH of the solution or because the compounds responsible for the activity are not sensitive to pH changes and are distributed similarly in acid or basic conditions. The isolation and evaluation of the individual compounds are necessary in order to identify the substances responsible for the effect observed.

Relative to *V. brasiliana*, the crude methanolic extract produced a significant decrease in the time of paralysis and death compared with the positive control and the effect increased along with concentration (Figure 3).

The results obtained showed that the fractions of *V. brasiliana* also produced a decrease in the time for paralysis and death compared with albendazole but as it is different with the fractions of *C. paraguayensis*, the effect increased along with the pH at which the compounds have been isolated, especially in the case of the time for paralysis (Figure 4). We can conclude that basic compounds are the most active and as their basicity increases, so does their activity. Regarding the time for death, the basic fractions were the most active, but no statistic significant differences were observed between them. As alkaloids have been detected in the chemical analysis, these compounds are mostly and certainly present in these fractions and are probably responsible for the activity observed. Other authors have evaluated species of the genus *Vernonia* and their findings showed that the plants possess alkaloids and showed anthelmintic activity, which was corroborated by our own work[14,15]. The isolation of the substances present mainly in the basic fractions in this case and the evaluation of their anthelmintic activity individually are then necessary.

One important point to consider is the fact that both crude extracts and fractions show better activity than albendazole spite of being a mixture of substances. Therefore, we think that the activity of the isolated compounds would be much better than the reference drug. This opens interesting possibilities for the development of lead compounds that could be transformed into drugs for the treatment of helminth diseases in the future.

In conclusion, the extracts and fractions of both *C. paraguayensis* and *V. brasiliana* contain compounds that possess anthelmintic activity. The compounds responsible are probably of acid and basic nature or not ionizable in the case of *C. paraguayensis* and eminently basic in the case of *V. brasiliana*, most probably alkaloids. Separation and evaluation of the activity of individual compounds are necessary. The plants could be source of substances that can be lead compounds for the development of new antiparasitic drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

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