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Chromatographic profiling and anthelmintic activity of solvent fractions of aerial parts of *Centratherum punctatum* Cass. against *Eisenia fetida*

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ABSTRACT

In this work, the chromatographic profile of the methanolic extract of *Centratherum punctatum* Cass. was determined by LC-MS. Also, the anthelmintic activity of the extract and its fractions was assayed against *Eisenia fetida*. Ultrasound-assisted maceration with methanol as solvent was used to obtain the crude extract. The extract was submitted to liquid–liquid partition with solvents (hexane, chloroform, ethyl acetate, and water) for the obtention of the fractions. The crude extract and the fractions were assayed for anthelmintic activity using *E. fetida* as a model, being albendazole the positive control. Hydroxycinnamic acid derivatives, flavonoids, and sesquiterpene lactones were identified by LC-MS. The methanolic extract and the fractions showed a significant decrease in the times of paralysis and death compared to albendazole. These results suggest that the methanolic extract of *C. punctatum* and its fractions contain substances with potential anthelmintic activity. The plant could be a source of molecules useful for the development of new anthelmintic drugs.

1. INTRODUCTION

Human infections by helminths are among the most widespread diseases in the world. In developing countries, mainly in tropical and subtropical ones, human infections cause serious health problems and contribute to malnutrition in children and deficiency in their mental development, mostly due to scarce adequate sanitary systems and lack of safe sources of drinking water [1].

Those diseases represent a public health concern for the sanitary authorities of these countries. According to the WHO, approximately 2 billion people suffer from soil-transmitted helminthiases around the globe. Among them, 820 million are children of the prescholar or scholar age. The damage that the parasites can produce varies and can comprise blood loss from the feeding of the parasite, mechanical damage to the intestinal mucosa, malabsorption, nutrient losses, and in some cases intestinal obstruction. The main areas affected are the Americas, sub-Saharan Africa, and Asia [2]. The WHO strategy to fight soil-transmitted helminthiases is the use of an anthelmintic drug for the children of scholar age that live in the affected areas [3].

The drugs used are benzimidazoles (albendazole and mebendazole), ivermectin, and pyrantel. From these, benzimidazoles have been more extensively used. Their efficacy varies depending on the worm type, drug, age, and intensity of the infection [4]. Some adverse effects have been described for the benzimidazoles and the other drugs. Also, the menace of development of resistance by the helminths is always present [5–7]. Therefore, the search for sources that can yield molecules to fight these diseases is needed. One of those sources is medicinal plants.

Plants have been used by almost all peoples on Earth since ancient times to treat illnesses that afflicted them. Among these, examples of the use of plants and extracts elaborated from them for the treatment of diseases caused by helminths can be found [8].

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Centratherum genus, belonging to the Asteraceae family, is widely distributed in the tropical regions of South and Central America, the West Indies, Australia, India, and the Philippines. Approximately 20 species are recognized for the genus, and it was first described by Alexandre Henri Gabriel of Cassini in 1817 [9]. The genus is attributed various properties such as anthelmintic, for the treatment of ulcers, diuretic, antioxidant, and anti-inflammatory [10,11].

Among the substances isolated from the plants of the *Centratherum* genus, sesquiterpene lactones are characteristic. These compounds have shown many biological activities, including anthelmintic, making the genus candidate for the isolation of molecules that could be used for new anthelmintic drugs development [12]. Besides sesquiterpenes, flavonoids and hydroxycinnamic acid derivatives were also identified [13].

With these antecedents, we decided to assay the anthelmintic activity of the methanolic extract of the aerial parts of *Centratherum punctatum* against *Eisenia fetida* and characterize its main constituents by liquid chromatography coupled to mass spectrometry (LC-MS).

2. MATERIALS AND METHODS

2.1. Plant Material

The aerial parts of *C. punctatum* Cass. were collected in the Paraguarí Department, Paraguay, South America, at 25°35′55″ S and 57°7′54″ W. The plant was identified by the Department of Botany of the Faculty of Chemical Sciences. For indexing purposes, a voucher specimen was deposited in the Herbarium FCQ of the Faculty of Chemical Sciences (R. Degen 4044).

2.2. Reagents and Chemicals

The chemicals were of analytical or LC-MS grade (E. Merck, Darmstadt, Germany). The methanol used for the preparation of extracts was of HPLC grade (JT Baker, Center Valley, PA). The reference drug albendazole (100% purity) was obtained from Jialing Medicine Industry Co., Ltd. (Changzhou, Jiangsu, China).

2.3. Preparation of the Methanolic Extract

The aerial parts of *C. punctatum* were ground to a fine powder in a cutter mill. The powder obtained (200 g) was macerated with methanol (1.5 L) with the assistance of an ultrasonic bath for 30 minutes three times at intervals of 15 minutes. The macerate was left to stand for 24 hours and then filtered through qualitative filter paper. The solvent was replaced, and the entire cycle was repeated for 3 days. Finally, the filtrates were reunited, and the solvent was evaporated using a rotary evaporator (RVO 400 SD, Boeco, Germany) to obtain the crude extract.

2.4. Fractionation of the Crude Extract

To obtain the fractions, the partial solubilization method was used. For this, 5 g of extract was suspended in water and subsequently submitted sequentially to liquid–liquid partition with different solvents, such as hexane, chloroform, ethyl acetate, and butanol. The extraction with each solvent was carried out three times. The solvents were evaporated *in vacuo* on a rotary evaporator, and the remaining aqueous fraction was lyophilized (Telstar LyoQuest, Japan). With this procedure, the compounds present in the crude extract were grouped according to their polarity.

2.5. Ultraperformance Liquid Chromatography Coupled with Tandem Mass Spectrometry (UPLC-ESI-MS/MS) Analyses

For this analysis, a Waters (Milford, MA) Acquity UPLC chromatograph was used coupled with a Xevo TQD QqQ-MS mass spectrometer as the detector. A Phenomenex KINETEX core-shell EVO-C18 (2.1 × 100 mm, 1.7 µm) column was used (flow rate 0.3 mL/minute). The temperature of the column was 40°C. The mobile phase was composed of methanol (MeOH) (A) and water (B), with 0.1% formic acid and 10 mM ammonium formate for both. Gradient mode was used for the elution according to the following program: 0–0.7 minute, 80%–80% B; 0.7–3.2 minutes, 80%–60% B; 3.2–7.6 minutes, 60%–20% B; 7.6–8.3 minutes, 20%–0% B; 8.3–10.4 minutes, 0%–80% B; and 10.4–13 minutes, 80%–80% B. The samples (5 mg/mL) were dissolved in LC-MS MeOH, filtered through 0.22 µm nylon syringe filters, and injected.

The characterization of the compounds was performed first using full scan mode (*m/z* 80–800, at scan time of 0.14 seconds) with ESI as an ionization source in negative and positive modes. The MS/MS analyses were carried out in daughter ion mode (scan time 0.1 seconds), selecting the major compounds for fragmentation. Argon was used as collision gas. Nitrogen was used as nebulizer gas and drying gas. The mass spectrometer conditions were as follows: electrospray capillary voltage was 4.0 kV, source temperature was 150°C, desolvation temperature was 500°C, cone gas flow was 50 L/hour, and desolvation gas flow was 900 L/hour. The cone voltage was set at 30 V, while the collision energy was optimized as follows: 20 V for hydroxycinnamic esters and 30 V for the rest. The system was controlled using Waters Masslynx V4.1 software.

2.6. Anthelmintic Assay

The anthelmintic activity was assayed using the Californian red worm *E. fetida* as a model, according to the method described by Cáceres *et al.* [14] due to its anatomical and physiological resemblances to human parasites. The worms were provided by the Phytochemistry Department hatchery and were 4–6 cm in length and 0.2–0.4 cm in diameter. The assays were carried out in triplicate.

2.7. Statistical Analysis

The data were processed using GraphPad Prism software v. 7.0 for Windows. A D'Agostino & Pearson test was performed first to determine if the values were normally distributed. For statistical significance, an ANOVA test and then the Tukey method for multiple comparisons were performed. A p-value < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Extraction Yield

The extraction procedure yielded 28.8 g of crude extract from the aerial parts of *C. punctatum* (14.4 %). Subsequently, the extract

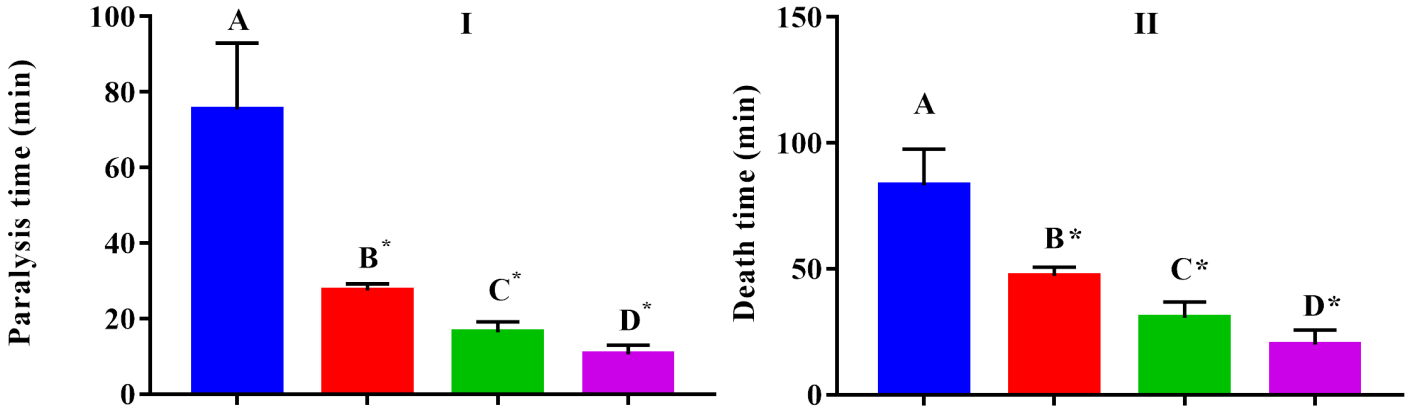

Figure 1. Time of paralysis (I) and time of death (II) of 10 (B), 20 (C) and 40 (D) mg/mL concentrations of the extract of *C. punctatum* against *E. foetida* compared with albendazole (A). Letters marked with an asterisc indicated statistical significant differences respect to the positive control.

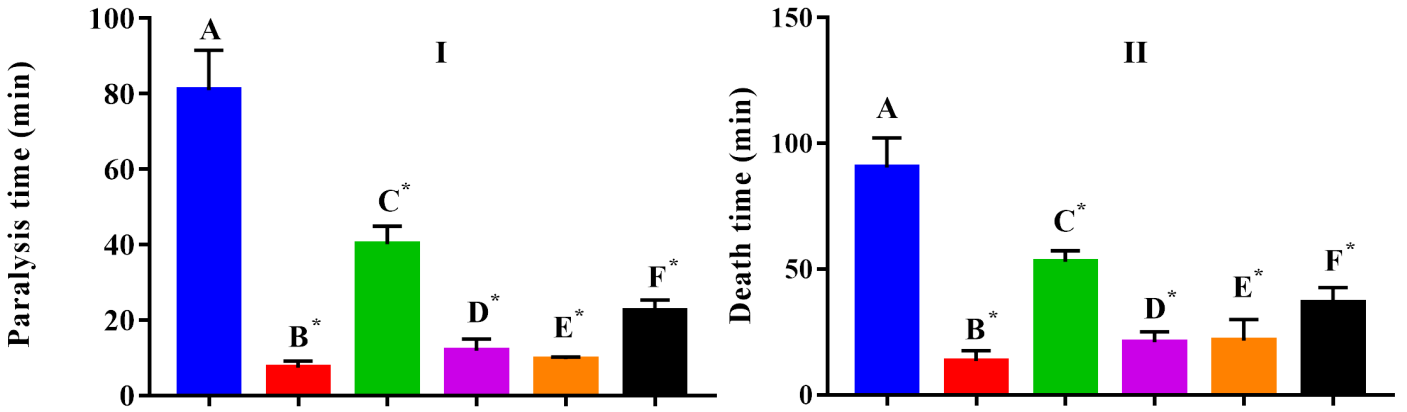

Figure 2. Time of paralysis (I) and time of death (II) of the ethyl acetate (B), hexane (C), chloroform (D), butanol (E) and aqueous (F) fractions of *C. punctatum* at 10 mg/mL against *E. foetida* compared with albendazole (A). Letters marked with an asterisc indicated statistical significant differences respect to the positive control.

was suspended in water and partitioned with solvents of increasing polarity. The yield of the procedure was as follows: 11.76% for the hexane fraction; 12.21% for the chloroform fraction; 5.37% for the ethyl acetate fraction; 20.90% for the butanol fraction; and 38.42% for the aqueous fraction.

3.2. Anthelmintic Activity

The methanolic extract of *C. punctatum* showed more activity than the positive control (albendazole) for both, time of paralysis and the time of death. The statistical analysis showed that significant differences existed for the three concentrations assayed (10, 20, and 40 mg/mL) compared with albendazole. The effect increased with the exposure to higher concentrations of the extract, indicating that the activity observed is concentration-dependent (Fig. 1). The activity observed cannot be attributable to the methanol used in the extract preparation because the solvent was evaporated to dryness before the assay. Furthermore, methanol is relatively nontoxic to the worms according to a previous study [15].

Regarding the anthelmintic activity of the fractions, all of them showed more activity than the positive control at 10 mg/mL (Fig. 2). The hexane fraction was the least active, while the ethyl acetate one was the most active. Nevertheless, regarding the time of paralysis, Tukey's *post-hoc* test showed no statistically significant differences among the ethyl acetate fraction, the chloroformic, and the butanol fractions. Concerning the time of death, the effect of the fractions was pretty similar to the observed for the paralysis time.

3.3. UPLC-ESI-MS/MS Analyses of *C. punctatum* Extracts

To identify the compounds present in the MeOH and the ethyl acetate (AcOEt) fraction that could be responsible for the anthelmintic activity, both were analyzed by UPLC-ESI-MS/MS. The ethyl acetate fraction was selected based on its lowest times of paralysis and death against *E. fetida*.

According to their fragmentation profiles, eighteen compounds were identified. To facilitate the discussion, they will be divided into three groups, namely, hydroxycinnamic derivatives, flavonoids, and sesquiterpene lactones. Their occurrence, retention time, and fragmentation profile are presented in Table 1. The SCAN mode chromatograms of the MeOH extract and the AcOEt fraction are shown in Figures 3 and 4.

Table 1. Identification of the main compounds in the methanolic (ME) and ethyl acetate (EA) extracts from *C. punctatum* through UPLC-ES-MS/MS.

Peak	Rt (minutes)	Molecular ion (*m/z*)	Polarity	MS/MS	Compound	ME	EA
1	0.91	341.34	Negative	178.98 (100), 112.85 (23)	Caffeoyl hexoside	X	
2	0.98	543.46	Positive	543.48 (100), 381.46 (25), 363.52 (10), 261.14 (5), 244.26 (5)	2′,3′-dihydroniveusin B hexoside	X	X
3	1.06–1.08	353.30	Negative	190.99 (100), 172.99 (50)	Caffeoylquinic acid	X	X
4	2.89–2.93	353.31	Negative	191.59 (100), 179.19 (10)	5-caffeoylquinic acid[a]	X	
5	4.24	408.39	Positive	373.03 (18), 275.40 (18), 257.02 (20), 229.48 (100), 211.24 (20)	Methyl-tetrahydro-hydroxy centratherin		X
6	4.90–5.57	515.51	Negative	353.37 (40), 179.44 (22), 161.44 (100)	Dicaffeoylquinic acid 1	X	X
7	5.40	393.45	Positive	393.75 (15), 275.17 (15), 257.38 (20), 229.02 (70), 201.09 (45), 185.38 (45), 55.00 (100)	Dihydro-hydroxy-centratherin 1	X	
8	5.41	361.41	Positive	247.82 (5), 239.02 (18), 229.05 (100), 211.47 (30), 201.43 (80), 183.61 (50)	Goyazensolide	X	
9	5.74	461.38	Negative	285.34 (100)	Kaempferol glucuronide	X	
10	5.38–5.99	515.51	Negative	353.16 (100), 179.34 (18), 173.62 (30)	Dicaffeoylquinic acid 2	X	X
11	5.75–6.08	375.40	Positive	275.21 (5), 257.38 (10), 239.28 (12), 229.24 (100), 211.32 (39), 201.41 (35), 183.36 (27)	Centratherin	X	X
12	6.07	431.21	Negative	268.55 (100)	Apigenin hexoside	X	X
13	6.08	393.00	Positive	276.47 (10), 248.151 (15), 239.73 (25), 230.09 (100), 183.08 (18)	Dihydro-hydroxy-centratherin 2	X	X
14	6.08	407.41	Positive	274.94 (35), 257.38 (20), 229.33 (35), 201.55 (25), 183.40 (40), 83.29 (100)	Methyl-dihydro-hydroxy centratherin		X
15	6.59–7.07	315.35/317.34	Negative/positive	299.15 (30), 271.32 (100), 255.12 (60), 243.37 (35)/317.24 (30), 302.35 (100), 273.99 (5), 228.59 (7)	Quercetin-3-methyl eter[b]	X	X
16	6.60–7.05	285.26	Negative	239.47 (10), 212.64 (20), 197.78 (40), 173.19 (40), 151.19 (100)	Luteolin	X	X
17	7.10	269.35	Negative	268.73 (60), 183.2 (100), 151.43 (50), 117.50 (45)	Apigenin		X
18	7.68–8.10	299.31	Negative	284.57 (100), 151.20 (18)	Chrysoeriol	X	X

[a] According to Clifford *et al.* [16].

[b] According to Gobbo-Netto *et al.* [17].

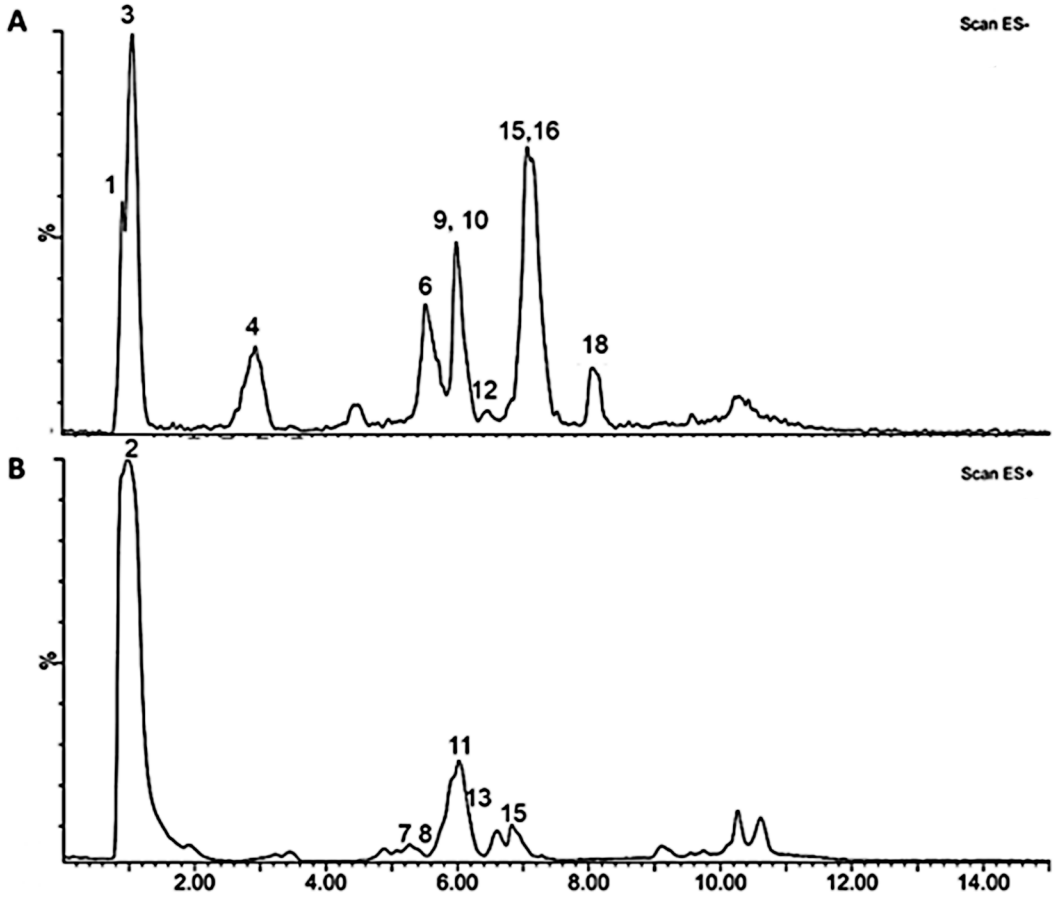

Figure 3. SCAN mode chromatogram in negative (A) and positive (B) ion mode of the MeOH extract of *C. punctatum.*

3.3.1. Hydroxycinnamic derivatives

Compound **1** was identified as caffeoyl hexoside, based on its molecular ion at *m/z* 341. A hexose loss (162 amu) affords a base peak at *m/z* 179, in agreement with caffeic acid. Peaks **3** and **4** showed a negative $[M - H]^-$ ion at *m/z* 353. The neutral loss of a caffeoyl moiety (162 amu) leading to the MS^2 base peak at *m/z* 191 suggested a quinic acid ion. Therefore, compound **3** was identified as caffeoylquinic acid and compound **4** as 5-caffeoylquinic acid (according to Clifford *et al.* [16] proposed scheme). Peaks **6** and **10** displayed a molecular ion at *m/z* 515 and a neutral loss of one caffeoyl moiety (162 amu), affording MS/MS fragments at *m/z* 353 and 179, in agreement with caffeoylquinic and caffeic acid ions, respectively. Then, compounds **6** and **10** were identified as dicaffeoylquinic acids 1 (**6**) and 2 (**10**), respectively. Several chlorogenic acids were reported for members of the Asteraceae family [13,17], in agreement with our results.

3.3.2. Flavonoids

Peak **9** showed a deprotonated $[M - H]^-$ ion at *m/z* 461 with a neutral loss of one glucuronate moiety (176 amu), leading to the base peak at *m/z* 285. This agrees with a kaempferol core. Then, compound **9** was identified as kaempferol glucuronide [18]. Peak **12** displayed a $[M - H]^-$ ion at *m/z* 431 and a neutral loss of one hexose (162 amu), leading to a base peak at *m/z* 269, suggesting an apigenin core. Thus, compound **12** was assigned as apigenin hexoside (**12**).

Among the aglycones, compound **15** was identified as quercetin-3-methyl ether (**15**) due to its $[M - H]^-$ ion at 315 and the MS/MS ions at *m/z* 299, 271, 255, and 243 [17]. This was further confirmed in positive ion mode, showing $[M + H]^+$ ion at *m/z* 317 and MS^2 ions at 303, 273, and 229. Peak **16** showed a molecular ion at *m/z* 285 and secondary fragment ions at *m/z* 239, 213, 197, 173, and 151, in agreement with luteolin aglycone [19]. Thus, this compound was identified as luteolin (**16**). Apigenin (**17**) and chrysoeriol (**18**) were also identified in our samples. The first (**16**) showed a negative $[M - H]^-$ ion at 269, yielding MS^2 fragments at *m/z* 183, 151, and 117, characteristic of an apigenin core [13], whereas compound **18** displayed a molecular ion at *m/z* 299, leading to the ions at *m/z* 284 and 151, suggesting a chrysoeriol core [13].

3.3.3. Sesquiterpene lactones

One heliangolide and six furanoheliangolides were identified. Among them, compound **2** showed a pseudomolecular ion at m/z 543 and a neutral loss of a hexose (162 amu), producing the MS/MS ions at *m/z* 381, 363, 261, and 244, suggesting a 2′,3′ -dihydroniveusin B core [20]. Therefore, **2** was identified as 2′,3′ -dihydroniveusin B hexoside.

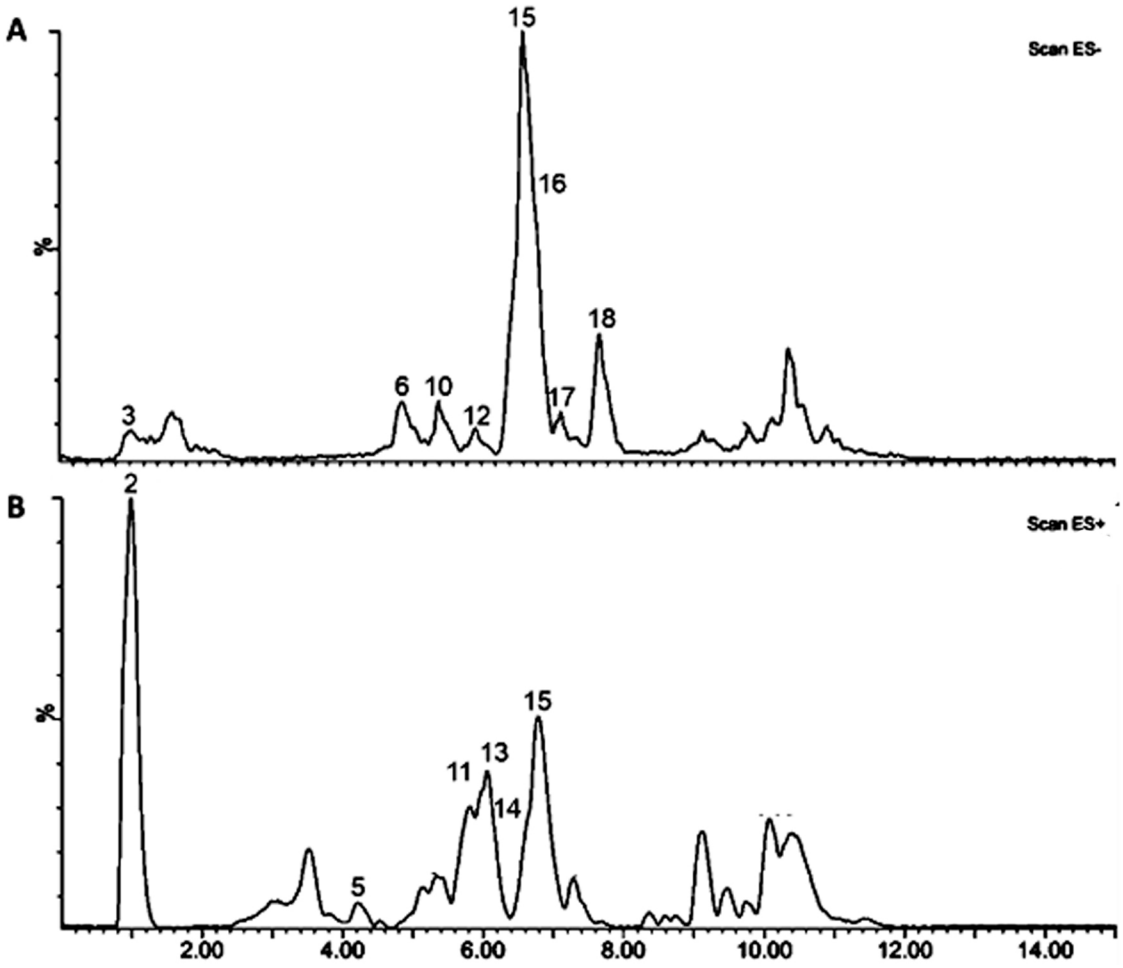

Figure 4. SCAN mode chromatogram of the AcOEt fraction of *C. punctatum.*

The other compounds were assigned as furanoheliangolides belonging to the goyasenzolide type, which are considered the most biologically active type of these compounds [21]. Compound **8** was identified as goyazensolide, based on the pseudomolecular ion of 361 amu and the characteristic MS^2 ions at *m/z* 247, 239, 229, 211, 201, and 183 [21,22]. The characteristics MS/MS ions at *m/z* 275, 257, 239, 211, 201, and 183 and the $[M + H]^+$ ion at *m/z* 375 allowed the identification of centratherin (**11**) [13,21].

Compounds **7** and **13** showed a molecular ion at *m/z* 393 that had 18 amu more than the centratherin molecular weight. Compound **7** showed the fragment ions at *m/z* 275, 257, 229, and 201, characteristics of centratherin [21], while compound **13** displayed ions of *m/z* 276, 248, 239, and 183, also diagnostic of centratherin. Therefore, compounds **7** and **13** were identified as dihydro-hydroxy-centratherin 1 and 2 isomers, respectively.

Peak **14** showed MS^2 ions at *m/z* 275, 257, 229, 201, 183, and 83, characteristic of centratherin [21], while the pseudomolecular ion at *m/z* 407 surpasses the protonated $[M + H]^+$ of compounds **7** and **13** by 14 amu (one methyl). Therefore, compound 19 was identified as methyl-dihydro-hydroxy centratherin. In addition, compound **5** was assigned as methyl-tetrahydro-hydroxy centratherin due to their fragment ions at *m/z* 275, 257, 229, and 211, characteristic of centratherin [21], and the molecular ion at *m/z* 409, two mass units over that of compound **14**.

The AcOEt fraction showed to be enriched in flavonoids and sesquiterpene lactones compared to the MeOH extract (Figs. 3 and 4). Of the three groups of compounds identified in our samples, the sesquiterpene lactones are the most probable candidates to be responsible for the anthelmintic activity observed. Various articles have demonstrated that these compounds are active against helminths. Recent evidence suggests that they may act synergistically against worms [12,23,24]. The polarity of the lactones can also explain the activity of the fractions since these compounds are soluble in chloroform, ethyl acetate, and butanol, and the Tukey *post-hoc* test showed no statistically significant differences among them [25–28]. Furthermore, the hydroxycinnamic derivatives and the flavonoids also showed anthelmintic activity; therefore, the isolation and assay of the individual compounds are necessary to clarify which are the compounds responsible for the effect observed [29–31].

4. CONCLUSION

The methanolic extract and the fractions of *C. punctatum* have shown anthelmintic activity against *E. fetida*. Their effect was higher than the reference drug albendazole. The ethyl acetate fraction showed the lowest paralysis and death times in the model employed. The chemical analysis of the crude extract and the most active fraction through UPLC-ESI-MS/MS allowed the identification of sesquiterpene lactones, flavonoids, and hydroxycinnamic derivatives that may be responsible for the observed anthelmintic activity. Isolation and test of the individual compounds are necessary to identify potential candidates to be new anthelmintic drugs.

5. ACKNOWLEDGMENT

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6. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

7. ETHICAL APPROVAL

Not applicable.

8. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to this work. MG made the extract preparation and fractionation and the anthelmintic assays. AB-E carried out the UPLC-MS/MS analysis and the identification of compounds. AC carried out the statistical analysis. NA analyzed and interpreted the data obtained, wrote the manuscript, and designed and supervised the work. All authors revised and approved the final manuscript.

9. PUBLISHER'S NOTE

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