ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 17:6 (2023) 1031-1045

records of natural products

Chemical Composition and Anthelmintic Activity of the Peruvian Endemic Species *Chuquiraga weberbaueri* "Amaro" on Sheep Fasciolosis

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(Received April 03, 2023; Revised September 08, 2023; Accepted September 10, 2023)

Abstract: *Chuquiraga weberbaueri* "amaro" (Asteraceae) is a plant species popularly used for the treatment of animal fasciolosis in Peru. However, biological and chemical studies of this species remain scarce. Hence, the objective of the present study was to evaluate the anthelmintic effect of the hydroethanolic and aqueous extracts of *C. weberbaueri* leaves on *Fasciola hepatica*, through a controlled and a therapeutic efficacy test in artificially infected sheep. In addition, the chemical composition of the extracts was evaluated using phytochemical screening and analysis by UHPLC-MS/MS. Among the evaluated extracts, the aqueous extract proved to be the most efficient and of low toxicity against *F. hepatica*, inhibiting about 70% of its oviposition, using a 100 mg/kg dose. The chemical study showed that the major constituents were phenolic derivatives, such as chlorogenic acid and ethyl caffeate, with the notable presence of flavonoids, saponins, and lactones. Therefore, it was possible to observe the antihelmintic effect of the *C. weberbaueri* on the *F. hepatica*, which suggests the use of this plant extract as a potential alternative in the sheep fasciolosis treatment.

Keywords: *Fasciola; Chuquiraga;* phytotherapy; bioactivity; phenolics.

1. Introduction

Fasciolosis is a very common parasitic disease of domestic animals throughout the world, caused by the trematode *Fasciola hepatica* [1]. The disease is especially important in domestic ruminants such as

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The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products November-December 2023 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.414.2304.2751
Available online: September 27, 2023

alpacas, cattle, and sheep [2-4], producing up to a 9% reduction in daily weight gain in livestock [5]. The adult parasites live in the bile ducts, generally causing a chronic disease process that produces digestive and nutritional disorders [6]. However, in addition to its high impact in veterinary medicine, fasciolosis as a human infection has been one of the most neglected diseases for decades even though it is commonly found in the liver and biliary system of humans [7] in endemic areas of the inter Andean valleys of Peru [8,9] and Bolivia [10-14].

F. hepatica has an indirect life cycle that requires an intermediate host of the Lymneaidae family. In the Cajamarca region, the snail species involved in the transmission have been identified as *Galba truncatula*, *Lymnaea neotropica*, and *Lymnaea schirazensis* [15,16]. After 8 to 10 weeks the adult parasite establishes itself in the bile ducts and start to produce eggs, which pass into the duodenum with the bile to be eventually eliminated in the feces [17]. Eggs complete their development outside the host in three weeks to several months depending on the climatic conditions, after which emerges the first larval stage called the miracidium. Then, the free-swimming ciliated miracidium invades the intermediate lymnaeid hosts where the parasite reproduces to give rise to sporocysts, rediae, and cercariae in turn [18]. The cercariae exit the soft tissues of the snail to form cystic shapes metacercariae, on herbage [18]. The definitive hosts (mainly herbivorous animals) become infected by ingesting plants contaminated with metacercariae [17]. Once in the small intestine, the new juvenile parasite is released, penetrates the intestinal wall, the peritoneum, the peritoneal cavity, and the hepatic capsule, and then moves through the hepatic parenchyma, feasting on hepatic cells, eventually seeking the bile ducts where it develops into an adult parasite in 3 to 4 months [19-21]. Humans, like animals are infected by ingesting vegetables or water contaminated with metacercariae [17,22-24].

The fasciolosis control relies on the use of anthelmintic chemicals being the triclabendazole one of the most used in the last decades [25]. In the region of Cajamarca, livestock are generally treated several times a year [26] without monitoring the efficacy of treatments. As a result, anthelmintic resistance has been reported in cattle from Cajamarca [27] as throughout the word [28-37]. In addition, the administration of the anthelmintic triclabendazole can contribute to the appearance of adverse effects such as abdominal pain associated to biliary colic [38,39]. Moreover, it generates the presence of residues in the food chain in countries where there are no adequate control measures of the presence of anthelmintic residues in the food chain [40-42]. In consequence it is necessary to search for new alternative drugs to treat fasciolosis, and in this context the use of plant extracts demonstrating anthelmintic activity may be an alternative to current commercial treatments. Plants contain a great diversity of phytochemicals, including flavonoids, phenolic compounds, tannins, saponins, terpenes, and terpenoids that may be active against a wide range of parasites [43,44]. The leaves of the *C. weberbaueri*, popularly known in Peru as "amaro", are used as an infusion and have been reported to be useful to control *F. hepatica* infestations, and some external parasites, in guinea pigs and cattle [45,46]. Another study carried out on guinea pigs reported a reduction in the number of adult *Fasciola* in the liver of guinea pigs [47].

The objective of this work was, therefore, to establish the phytochemical profile of *C. weberbaueri* leaf extract through UHPLC-PDA-MS/MS and HPLC-ESI-MRM/MS analysis and to evaluate its effectiveness against *F. hepatica*, and its possible use for sheep fasciolosis treatment.

2. Materials and Methods

2.1. Plant Material

Leaves of *C. weberbaueri* were collected from its habitat (Figure 1) in the marginal lands around San Antonio de La Pachachaca, adjacent to the hamlet of Totoracocha. Both locations are part of Chanta Alta town in the district of La Encañada, province of Celendín, department of Cajamarca. Plants were identified taking as reference the morphological characteristics of the plant and the traditional knowledge of the population. The rainy season and the months immediately afterwards are the most favorable for collecting specimens because it is easier to recognize the inflorescences of this plant genus at these times. Specimens of the plant have been submitted to the herbarium of the Universidad Nacional de Cajamarca (Herbarium Code CPUN: NY03746817) for full classification and registration.

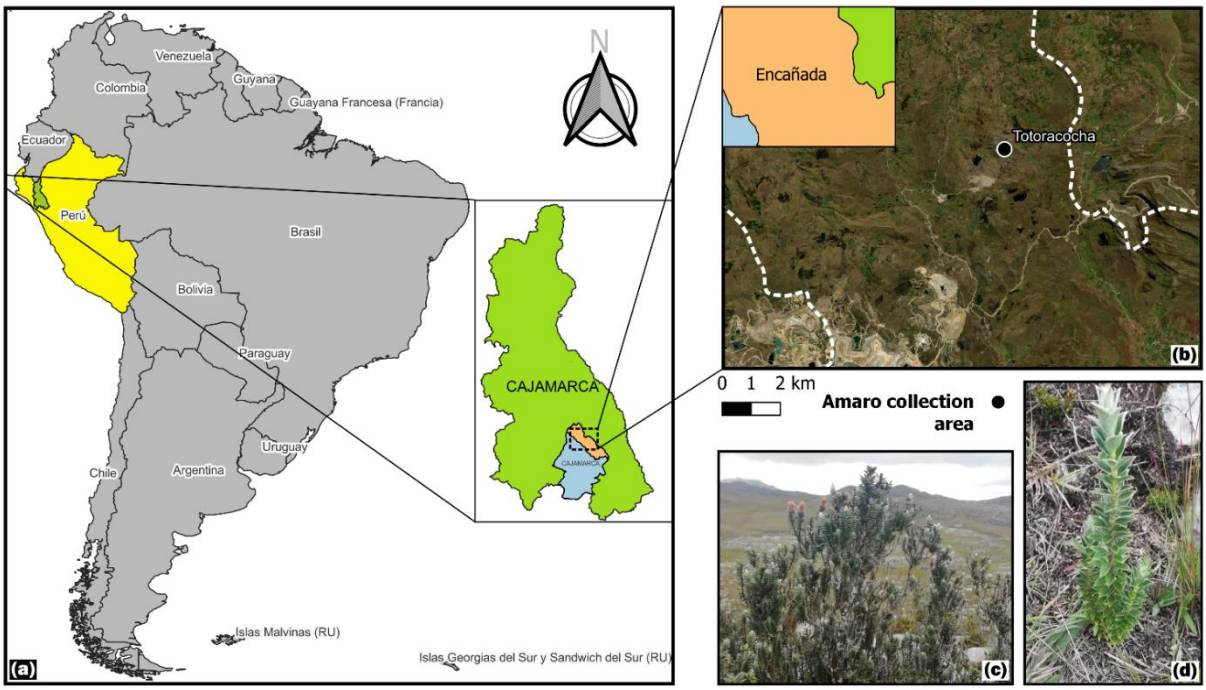

Figure 1. Natural area of the *Chuquiraga weberbaueri* "amaro" (a, b); mature specimen in its habitat (c); young specimen (d).

2.2. Preparation of Plant Extracts

The fresh leaves of *C. weberbaueri* were collected, dried and ground to obtain a hydroethanolic extract and aqueous extract, as follows. For the hydroethanolic extract, 350 g of leaves were crushed and weighed. Then, 3.5 L of ethanol 50% was added in the flask. The leaves were submitted to the maceration process for seven days, with moderate agitation in each day. After 7 days the mixture was filtered to obtain the liquid extract, which was subsequently dried under vacuum at 40 °C. The dry extract was then resuspended in distilled water and lyophilized at -80 °C at a pressure of 80 mTorr. The lyophilizate extract was stored at -4 °C [48-50]. For the aqueous extract, 15 g of crushed leaves were resuspended in 1L of distilled water. The mixture was then boiled for 5 minutes, with subsequent cooling and filtration. The filtered liquid (decoction) was concentrated under a vacuum at 40 °C, and frozen in an alcohol spin bath, before lyophilization, under the same condition as the hydroethanolic extract [48-50].

2.3. Preliminary Phytochemical Screening Methods

For the phytochemical screening, several tests were carried out for the identification of the chemical classes. Dragendorff, Mayer, and Wagner tests for alkaloids, the foam test for saponins, the Ballet test for lactones, the ferric chloride test for phenolics and tannins, the Bornträger test for quinones, and the Liebermann-Burchard test for terpenes, steroids, and terpenoids [51,52].

2.4. Chemical Identification by UHPLC-PDA-MS/MS

For each extract, solutions of 2 mg/mL were prepared in methanol. These solutions were filtered through a 0.22 µm PTFE syringe filter before passing through the UHPLC-PDA-MS/MS system (Waters Xevo TQ-XS**)**. Chromatographic separation was achieved on an ACQUITY HSS C18 column (100 × 2.1 mm, 1.7 µm). Formic acid 0.1% (v/v) in H_2O (A) and MeCN (B) mobile phases were used. The gradient conditions were as follows: 0.0-4.17 min 3-10% B; 4.17-6.25 min 10-15% B; 6.25-8.34 min 15% B; 8.34-

10.42 min 15-20% B; 10.42-14.49 min 20-25% B; 14.49-16.67 min 25-30% B; 14.49-18.76 min 30-50% B; 18.76-21.67 min 50-90% B; 21.67-25.0 min 90% B. The flow of the mobile phase was 300 µL/min, and the injection volume was 3 µL. The column temperature was kept at 40 °C. The PDA chromatograms were obtained at a range between 200 and 750 nm.

The triple quadrupole mass spectrometer was equipped with an electrospray ion (ESI) source operated in positive and negative ionization mode. The cone voltage was kept at 40 V. The drying temperature was set at 450 °C and the dry gas flow rate was set at 13 L/min. Nitrogen was used as the dry gas, fog gas, and collision gas. The collision energy was set at 30 eV. HRESIMS and MS/MS spectra were acquired in an *m/z* range of 50 to 2000 amu [53].

2.5. Quantification of Secondary Metabolites by UHPLC-ESI-MRM/MS

Chlorogenic acid, rutin and quercetin were quantified in the lyophilized extracts. Standards were prepared at 1 mg/mL in methanol for each substance, then diluted to 100, 50, 5, 1, 0.5, and 0.1 µg/mL to obtain the calibration curve for chlorogenic acid; diluted to 50, 5, 1, 0.5, 0.3, and 0.1 µg/mL to obtain the calibration curve for rutin; and diluted to 50, 10, 5, 1, 0.5, and 0.1 µg/mL to obtain the calibration curve for quercetin. Lyophilized extracts were prepared at 1 mg/mL in methanol. Both dilutions of the standards and the samples were filtered in 0.22 µm PTFE syringe filter before passing through the UHPLC-ESI-MRM/MS system (Waters Xevo TQ-XS). Chromatographic separation was achieved on an ACQUITY HSS C18 column (150 × 2.1 mm, 1.8 µm). Formic acid 0.1% (v/v) in H_2O (A) and MeCN (B) mobile phases were used. The gradient conditions were as follows: 0.00-9.00 min 10-22% B; 9.00-10.50 min 22-40% B; 10.50-11.00 min 40-10% B; 11.00-15.00 min 10% B for chlorogenic acid; and 0.00-7.50 min 10-22% B; 7.50-13.00 min 22-50% B; 13.00-17.00 min 50-90% B; 17.00-20.00 min 90-10% B for rutin and quercetin. The flow of the mobile phase was 300 µL/min, and the injection volume was 0.5 µL. The column temperature was kept at 40 °C.

The triple quadrupole mass spectrometer was equipped with an electrospray ion (ESI) source operated in positive ionization mode $[M+H]^+$. The cone voltage was kept at 30 V. The drying temperature was set at 480 °C and the dry gas flow rate was set at 2.5 L/min. Nitrogen was used as the dry gas, fog gas, and collision gas. The collision energy was set at 25 eV. Multiple reaction monitoring (MRM) was used for chlorogenic acid (parent ion *m/z* 355.1, daughter ion *m/z* 163), rutin (parent ion *m/z* 611.3, daughter ion *m/z* 303.1), and quercetin (parent ion *m/z* 303.1, daughter ion *m/z* 153).

Concentrations of chlorogenic acid, rutin, and quercetin were evaluated in triplicate. The values of the detection and quantification limits were considered as the minimum concentrations equivalent to three and six times the height of the background noise in the chromatogram, respectively. The equations of the line for each standard were as follows: chlorogenic acid ($y = 556625x - 4552.9$, r^2: 0.997; $y = 562968x + 11478.5$, r^2: 0.998; $y = 524147x - 33459.9$, r^2: 0.999), rutin ($y = 877034x + 45598.5$, r^2: 0.999; $y = 1043640x + 91038.9$, r^2: 0.995; $y = 838429x - 15613.6$, r^2: 0.936), quercetin ($y = 243505x + 1534.4$; r^2: 0.975; $y = 227933x - 1969.34$, r^2: 0.970; $y = 287785x - 20219.6$, r^2: 0.998).

2.6. Animals

Thirty-four sheep with similar characteristics were purchased from the livestock company SAIS Atahualpa Jerusalem SRL. All the animals were subjected to treatment with ivermectin (0.2 mg/kg body weight) and triclabendazole (10 mg/kg body weight) to remove any existing worm burden. Efficacy of treatments were confirmed after fifteen days by coprological examination. Subsequently, the animals were identified by ear tag and included in the efficacy tests. Ethical Committee approval for the animal experiments was obtained from the Universidad Peruana Cayetano Heredia, Peru, under the license number 027-03-19. Animal procedures and management were carried out in accordance with the Animal Welfare Policy of the Facultad de Ciencias Veterinarias of the Universidad Nacional de Cajamarca, Cajamarca, Perú.

2.7. Artificial Infection with Metacercariae of Fasciola hepatica

Previously, all the animals used in the efficacy tests were artificially infected with 250 *F. hepatica* metacercariae orally. Metacercariae of *F. hepatica* was recovered from artificially infected snails which were previously infected with miracidia obtained from *F. hepatica* eggs from gallbladders of naturally infected sheep [27]. In the 58th day post infection, some animals started excreting *F. hepatica* eggs in their feces determined by the Rapid Sedimentation technique [54]. In the 90th day post infection, all the animals were excreting eggs in their feces; then the animals were randomly distributed to be used in the Efficacy Therapeutic Test and the Controlled Efficacy Test.

2.8. Efficacy Tests Against Fasciola hepatica

Controlled and Therapeutic Efficacy tests were performed according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) [55].

2.8.1. Therapeutic Efficacy Test

Six experimental groups of three animals each and two control groups of four animals each were used. Groups I, II, and III received aqueous extract at doses of 25, 100, and 200 mg/kg, orally, twice a day, respectively, for five consecutive days. Groups IV, V, and VI received hydroethanolic extract in doses of 25, 100, and 200 mg/kg, orally, twice a day, respectively, for five consecutive days. Groups VII and VIII were the negative control group (without treatment) and the positive control group (with treatment) respectively, the latter treated with oxyclozanide at 15 mg/kg of body weight, orally as a single dose [55].

Fecal samples were collected from each animal in the treatment and control groups, from day 0 before the administration of the extracts and at 7-, 14-, and 21-days post-treatment. The eggs were counted by the Rapid Sedimentation technique [54] and the reduction in the number of eggs was calculated using the following formula [56]:

% Fecal Egg Number Reduction Test (FENR) = {[Egg count before treatment (epg) – Egg count post-treatment (epg)]/Egg count before treatment (epg)} x 100

For the statistical analysis of the groups, a Student´s t-test was used. A p-value of <0.05 was considered significant.

2.8.2. Controlled Efficacy Test

Two groups of four animals each were used. The experimental group was treated with the aqueous extract of *C. weberbaueri* at 100 mg/kg, orally, twice a day, for five consecutive days, while animals of the control group did not receive any treatment. The 100 mg/kg dose of the aqueous extract was chosen because it gave the best results in the Therapeutic Efficacy Test. Fourteen days after the end of the treatment period, animals of both groups were sacrificed, and the number of adult *F. hepatica* was counted in each animal's liver.

The efficacy of the treatment was determined by comparing the *F. hepatica* burdens in both groups of animals according to WAAVP guidelines [55]. The following formula was applied:

% Efficacy = {[Mean of F. hepatica in control group – Mean of F. hepatica in treated group]/Mean of F. hepatica in control group} x 100

The number of parasites is shown as the geometric mean and a Student´s t-test was used to compare means. $P < 0.05$ is considered significant.

2.9. Toxicity Study

Only animals treated with 100 mg/kg aqueous extract in the Therapeutic Efficacy Test were bled to evaluate the toxic effect of *C. weberbaueri* extracts. Liver markers were determined in blood samples. The liver markers, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct and indirect bilirubin, and alkaline phosphatase (ALP) were determined. The collection of blood samples was performed before the treatment regimen was initiated and then weekly until 21 days post-treatment. For the statistical analysis of the groups, a one-way or two-way ANOVA was used as appropriate. A *p*-value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Identification of Secondary Metabolites of the Plant Extracts

The yield of the hydroethanolic and aqueous extracts was 15.46% and 17.67%, respectively. The tests used for the phytochemical screening of the *C. weberbaueri* extracts identified different classes of secondary metabolites the most important being phenolic and lactonic compounds, in addition to saponins, alkaloids, and condensed tannins. The major compounds were identified or detected by subsequent analysis by UHPLC-PDA-MS/MS.

3.1.1. UHPLC-PDA-MS/MS Measurements

In the chemical analysis of the extracts by UHPLC-PDA-MS/MS (Table 1 and Figure 2), a total of 25 substances were identified and another four major substances from saponin and lactone classes were detected (Table 2). Most of the constituents identified are phenolic derivatives, including caffeic acid (*m/z* 179 [M-H]), ferulic acid (*m/z* 193 [M-H]), and coumaric acid (*m/z* 163 [M-H]) in free form or associated with carbohydrates or organic acids. In addition to these substances, conjugated flavonoids, such as miquelianin and kaempferol-3-*O*-glucose, and their aglycones, such as quercetin, kaempferol, and isorhamnetin, could also be identified. The saponins showed *m/z* 723, 839, and 765 [M-H], with triterpenoid nuclei of *m/z* 373, 503, and 519 amu, respectively, while the lactone glucoside showed a *m/z* 491 [M-H], presenting a nucleus with an *m/z* 329 amu (Table 2).

Table 1. Substances identified in the *C. weberbaueri* extracts by UHPLC-PDA-MS/MS analysis

N°	Substances	Rt (min)	[M-H]	Main fragments (*m/z*)	UV_{max} (nm)
1	Citric acid	1.59	191	111, 87	NI
2	Citramalic acid	1.90	147	129, 87	NI
3	2-Oxoadipic acid	2.17	159	87, 69	NI
4	4-*O*-arabinose-caffeic acid	5.63	311	179, 149, 135	326
5	4-*O*-rhamnosyl-caffeic acid	6.25	325	179, 163, 145	326
6	4-*O*-glucosyl-coumaric acid	7.50	325	325, 163	326
7	Chlorogenic acid	7.52	353	353, 191	327
8	Caffeic acid	8.16	179	133, 107, 89, 79	323
9	Caffeoyl-2-oxoglutaric acid	9.43	307	179, 145, 99	328
10	4-*O*-rhamnosyl-ferulic acid	10.33	339	193, 145, 134	327
11	Glucose-arabinose caffeate	11.75	473	293, 219, 179, 149	329
12	Arabinosyl ferulate	12.08	325	193, 178, 134	326
13	Rutin	12.50	609	343, 301, 300, 271, 179, 151	357
14	Miquelianin	12.82	477	477, 301	351
15	Dicaffeoylquinate	14.24	515	353, 285, 191, 179	329

16	Kaempferol 3-*O*-glucose	14.41	447	284, 255	356
17	Dicaffeoyl-2-oxoglutarate	14.78	487	325, 179, 163, 145	329
18	Dicaffeoylarabinose	14.99	473	293, 219, 179, 149	330
19	Caffeoyl-feruloyl-2-oxoglutarate	17.37	501	339, 307, 193, 179, 145	330
20	Caffeoyl-feruloyl-arabinose	17.80	487	219, 193, 179, 149, 135	330
21	Quercetin	18.64	301	179, 151, 107	367
22	Ethyl caffeate	18.91	207	179, 161, 133	325
23	Kaempferol-feruloyl-glucose	19.07	623	461, 447, 285	326
24	Isorhamnetin	20.18	315	300, 159	373
25	Kaempferol	20.71	287	211, 155, 141	357

Rt (min): Retention time (minutes); [M-H]: Negative quasi-molecular ion; NI: Not identified.

Table 2. Major saponins and lactones detected in the *C. weberbaueri* extracts by UHPLC-PDA-MS/MS analysis

N°	Detected compounds	Rt (min)	[M-H]	Main fragments (*m/z*)
D1	Terpenoid nucleum mass 373 + Rhamnose-Acetylglucose	19.76	723	681, 519, 373
D2	Lactone nucleum mass 329 + Glucose	20.08	491	329, 179, 161
D3	Terpenoid nucleum mass 503 + Pentose-Acetylglucose	20.41	839	797, 779, 635, 617, 503, 427, 293, 215
D4	Terpenoid nucleum mass 519 + Diacetylglucose	20.43	765	723, 681, 663, 645, 599, 519, 483, 439, 428, 343, 221

Rt (min): Retention time (minutes); [M-H]: Negative quasi-molecular ion.

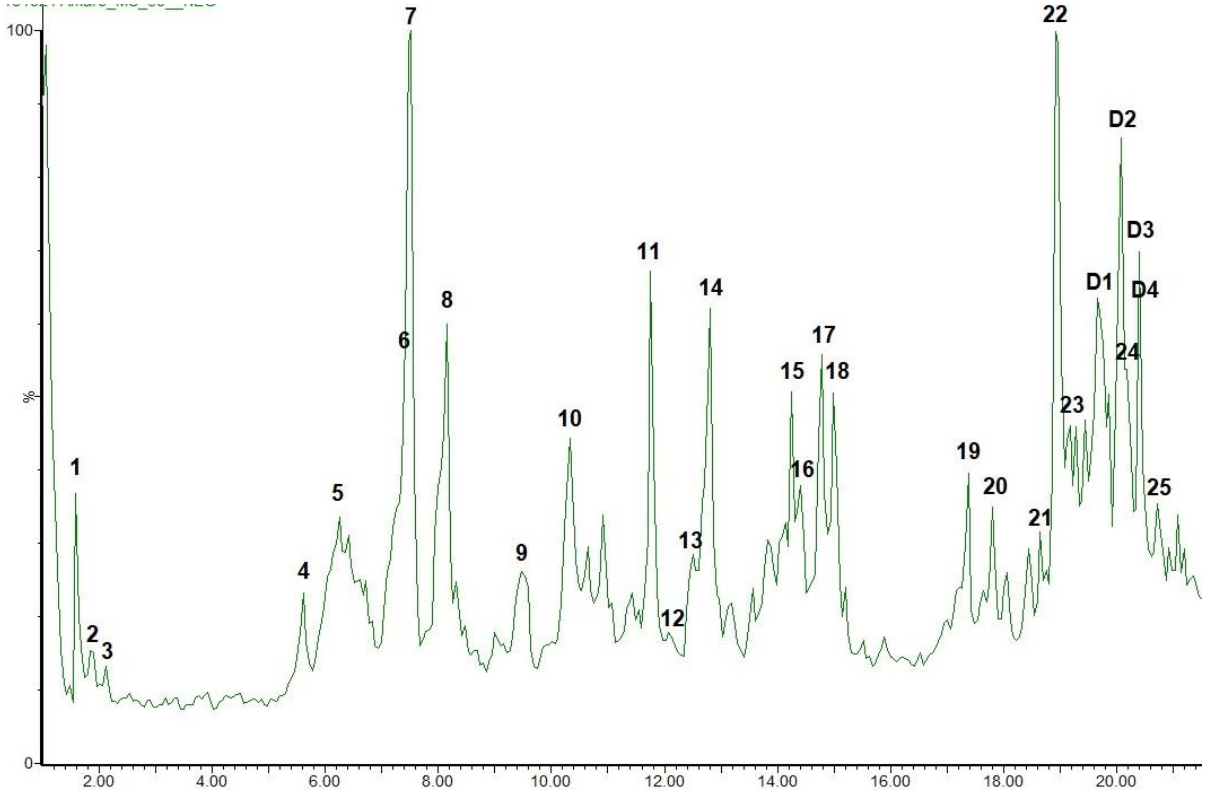

Figure 2. Chromatogram of the hydroethanolic extract from *C. weberbaueri* leaves

3.2. Quantification of Secondary Metabolites by UHPLC-ESI-MS/MS

Using ESI-MS/MS in positive ionization mode, 31.55 ± 0.90 mg of chlorogenic acid, 0.39 ± 0.21 mg of quercetin and 1.35 ± 0.28 mg of rutin for each gram of lyophilized hydroethanolic extract were quantified. Likewise, for each gram of lyophilized aqueous extract 1.62 ± 0.12, 0.80 ± 0.07, and 0.05 ± 0.03* mg of chlorogenic acid, rutin, and quercetin were quantified, respectively (Table 3).

Table 3. Quantification of chlorogenic acid, rutin, and quercetin in *C. werberbaueri* hydroethanolic and aqueous extracts

N°	Substances	DL (µg/mL)	QL (µg/mL)	Range (µg/mL)	Lyophilized aqueous extract (mg/g)	Lyophilized hydroethanolic extract (mg/g)
1	Chlorogenic acid	0.0005	0.001	100-0.1	1.62 ± 0.12	31.55 ± 0.90
2	Rutin	0.01	0.02	50-0.1	0.80 ± 0.07	1.35 ± 0.28
3	Quercetin	0.01	0.02	50-0.1	0.05 ± 0.03*	0.39 ± 0.21

*By extrapolation; DL: Detection limit; QL: Quantification limit

3.3. Efficacy Tests Against Fasciola hepatica

3.3.1. Efficacy Therapeutic Test

When the results of the Therapeutic Efficacy Test were analyzed, both aqueous extracts at doses of 100 and 200 mg/kg were significantly effective compared to the negative control group (Table 4). The efficacy of both extracts was not statistically different (69.69 and 70.09%), so the aqueous extract at the dose of 100 mg/kg was considered efficient in reducing the percentage of eggs in the sheep feces at the end of the 21 days. In the hydroethanolic extract, the most efficient dose was 200 mg/kg, showing a reduction percentage of *Fasciola* eggs of 57.01%.

Table 4. Therapeutic Efficacy Test of *C. weberbaueri* extracts at different doses (n = 3) expressed as the average in the number of eggs F. *hepatica* per 2 grams of feces (ep2g)

Groups	Average ep2g in each group				Percentage reduction in the number of eggs at 21 days
	Pre Treatment	7 days Post Treatment	14 days Post Treatment	21 days Post Treatment	
I – Aqueous Ext. 25 mg/kg	37	42	31	42	-13.51*
II - Aqueous Ext. 100 mg/kg	66	27	24	20	69.69[a]
III – Aqueous Ext. 200 mg/kg	107	32	89	32	70.09[a]
IV – Hydroetha. Ext. 25 mg/kg	67	63	46	60	10.44
V – Hydroetha. Ext. 100 mg/kg	36	26	26	31	13.88
VI – Hydroetha. Ext. 200 mg/kg	114	33	79	49	57.01
VII – Negative Control	30	23	33	91	-203.33*
VIII - Oxyclozanide Control	46	1	4	1	97.82

*Negative value indicates no reduction in the number of eggs conversely there was an increase in these numbers;
[a]Means are not significantly different from one another ($p>0.05$).

3.3.2. Controlled Efficacy Test

In the Controlled Efficacy Test, the group of sheep treated with the *C. weberbaueri* aqueous extract, at a 100 mg/kg dose for 5 consecutive days, showed a 51.79% of efficacy (62.25 ± 38.58 number of *F. hepatica*), when compared with the control group (117.25 ± 35.65) (Table 5).

Table 5. Efficacy Controlled Test of aqueous extract of *C. weberbaueri* at the dose of 100 mg/kg b.w. against *F. hepatica* in sheep

Control group		**Treated group 100 mg/kg for 5 days**	
Animal	**Number of adults *F. hepatica* in liver**	**Animal**	**Number of adults *F. hepatica* in liver**
1	89	1	20
2	85	2	80
3	139	3	78
4	156	4	71
Total	469	Total	249
Mean	117.25	Mean	62.25
Geometric mean	113.17[a]	Geometric mean	54.56[a]
		Efficacy %	51.79

[a]Means are not significantly different from one another (p>0.05).

3.4. Toxicity Study

The assays for the quantification of enzymes and liver markers did not indicate any significant toxicity of the aqueous extract after the third week of treatment.

Table 6. Evaluation of levels of liver enzymes after the treatment with aqueous extract of *C. weberbaueri* in the group of animals treated at 100 mg/kg b.w. (n = 3). Samples were taken before treatment and weekly three times.

Parameters	**Pretreatment**	**Post treatment W1**	**Post treatment W2**	**Post treatment W3**	**Reference Intervals**
Creatinine (mg/dL)	0.79 ± 0.08[a]	0.77 ± 0.04[a]	0.82 ± 0.13[a]	0.92 ± 0.14[a]	1.2 – 1.9 [81,82]
AST = Aspartate aminotransferase (U/L)	87.70 ± 42.52[b]	87.80 ± 25.51[b]	91.10 ± 44.61[b]	116 ± 83.82[b]	60 – 280 [81,82]
ALT = Alanine aminotransferase (U/L)	13.40 ± 10.64[c]	11.10 ± 1.15[c]	11.20 ± 2.88[c]	13.10 ± 7.34[c]	22 – 38 [82]
Total bilirrubin (mg/dL)	1.15 ± 1.17[d]	0.84 ± 0.01[d]	0.73 ± 0.11[d]	0.40 ± 0.10[d]	0.1 – 0.5 [81,82]
Direct bilirrubin (mg/dL)	0.44 ± 0.53[e]	0.30 ± 0.04[e]	0.26 ± 0.08[e]	0.11 ± 0.03[e]	0 – 0.27 [81,82]
Indirect bilirrubin (mg/dL)	0.71 ± 0.64[f]	0.54 ± 0.05[f]	0.47 ± 0.08[f]	0.29 ± 0.13[f]	0 – 0.12 [81]
Alkaline Phosphatase = ALP (U/L)	310 ± 82.94[g]	288 ± 49.52[g]	290 ± 69.35[g]	319 ± 33.15[g]	70 – 390 [82]

Means with a common superscript are not significantly different from one another (p>0.05).

Although the indirect bilirubin is above the reference interval, the administration of aqueous extract does not enhance the levels in comparison to the control group (Table 6), demonstrating the safety of the use of *C. weberbaueri* aqueous extract for the ovine fasciolosis treatment.

The use of natural plants and its secondary metabolites is well known for several years [57]. Remarkable results have been achieved with the use of natural or modified natural products, representing half of the drugs used in cancer, onchocerciasis (river blindness), and lymphatic filariasis [58,59]. Additionally, natural products constitute the basis for many new drugs as parent natural products or optimized derivatives [60].

There is a limited number of flukicidal drugs available for the treatment of fasciolosis, and the range progressively has been reduced due to the appearance of anthelmintic resistance [61]. The present study was aimed to demonstrate the efficacy of the *C. weberbaueri* extracts in ovine fasciolosis. In the therapeutic efficacy test, the aqueous extract administered at a dose of 100 mg/mL proved to be the most efficient, without demonstrating any significant toxicity. The Efficacy Tests in sheep demonstrated the effectiveness of both hydroethanolic and aqueous extracts in reducing the number of adult liver fasciolas and the number of eggs in the feces. Aminotransferase enzymes (ALT and AST) values were determined with the aim to evaluate any change due to toxicity of the *C. weberbaueri* aqueous extract, but not to evaluate liver enzyme changes occurring during the fluke migration through the liver parenchyma. As demonstrated in rabbits, aminotransferases did not show increase as flukes reach the adult stage in the bile ducts [62], only AST was detected in early stages on an experimental infection in cattle [63]. It is also known that ALT and AST enzymes are localized mainly in the cytoplasm of hepatocytes and have been recognized as markers of hepatocellular injury in rats, dogs, and non-human primates [64,65], whereas ALP and gamma glutamyl transferase (GGT) are the two most commonly used enzymatic marker of cholestasis in the clinical practice of small animal [64]. The absence of toxicity noted in the administration of the *C. weberbaueri* extract is a relevant advantage over the use of conventional anthelmintics, such as triclabendazole, since they demonstrate some liver toxicity, altering organ markers, including ALT [66]. Unlike some plant species, *C. weberbaueri* does not have hepatotoxic constituents, such as coumarins. Pyrrolizidinic alkaloids, other phytometabolites that causes hepatotoxicity, are not found in species of *Chuquiraga* genus [67,68]. Thus, *C. weberbaueri* aqueous extract is considered a safe alternative for the treatment of ovine fasciolosis. Moreover, the results of the controlled Efficacy Test gave a 51.79% of reduction in the number of adult fasciolas in the sheep livers.

Chemical analysis of the extracts by UHPLC-MS/MS identified the main phenolic compounds from *C. weberbaueri* as derivatives of the ferulic acid, the caffeic acid, and the coumaric acid, primarily associated with carbohydrates or organic acids, such as the quinic acid and 2-oxoglutaric acid. The profile of phenolic derivatives is described in the literature for species of the *Chuquiraga* genus [69]. In addition, the flavonoids kaempferol, quercetin, and isorhamnetin have also been identified for this genus, in both the aglycone and glycosylated forms [70]. Levels of quercetin, rutin and, mainly, chlorogenic acid were higher in hydroethanolic extract than in aqueous extract, that is because the first one tends to concentrate a greater number of substances such as flavonoids or phenolic genins [71]. The anti-nematode activity of phenolic acids and flavonoids is described in the literature, and they probably act through the inhibition of enzymes involved in the cellular respiratory cycle, causing suppression of oxidative phosphorylation, respiration, and movement in nematodes [72,73]. Moreover, the chemical analysis by UHPLC-MS/MS detected three saponins and a lactone as major compounds, which in turn belong to classes that have already been described in the genus, as well as alkaloids and tannins, also detected by the phytochemical screening [74,75]. Biological effects of saponins are normally described by their specific interaction with the cell membrane, causing dysfunction in cell permeability [76], in addition to specific toxic mechanisms for parasites, which includes membrane disruption, lipase inhibition, mitochondrial dysfunction, and cholesterol homeostasis alterations, caused by the genin moiety [77]. On the other hand, sesquiterpene lactones are known for their antiparasitic activity that seems to involve a Michael-addition reaction between the α-methylene functional group on the γ-lactone ring and nucleophiles targets such as thiol groups on cysteine-containing amino acids, causing cytotoxicity [78]. Furthermore, some alkaloids cause neurotoxicity in nematodes by interfering with the neurotransmitter release or by their agonist properties [79], whereas tannins demonstrate their anti-nematode action through the phenolic mechanisms (see above). In addition, tannins can bind to free protein in the gastrointestinal tract of the host animal or to

glycoprotein on the parasite's cuticle, causing its death [80]. Thus, a synergy between the different groups of secondary metabolites identified in the *C. weberbaueri* may be responsible for the effects observed in the biological tests performed in this study, as well as for the effects reported by the ethnomedicinal reports [45-47].

4. Conclusion

The study evaluated the anthelmintic activity of the hydroethanolic and aqueous extracts of the *C. weberbaueri* leaves, known as "amaro", on *F. hepatica*, so that they were able to reduce the oviposition of the parasite with doses of 100 and 200 mg/kg, with no significant toxicity for the host. In addition, the chemical analysis demonstrated the presence of phenolic acids, as well as the flavonoids and other constituents of the saponins and lactones classes. Thus, the study carried out in this work suggests the use of the species *C. weberbaueri* as a safe phytotherapeutic alternative in the treatment of ovine fasciolosis, corroborating several ethnomedicinal reports on the veterinary use of this species. In addition, the findings of the present study and their implications should be discussed in the broadest context possible and future research directions may also be highlighted.

Acknowledgments

The authors thank the Universidad Nacional de la Amazonia Peruana for the support in the publication of this research. Authors are grateful to Dr. John Claxton for providing critical review and English language revision assistance.

Declaration of competing interest

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

Funding

This work was funded by the Universidad Nacional de Cajamarca under the contract number 2871-2018-UNC, Canon Minero funds. The Universidad Nacional de Trujillo financed this publication, through a CANON MINERO 2023 fund.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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