In vitro antihelmintic effect of fifteen tropical plant extracts on excysted flukes of Fasciola hepatica

José Manuel Alvarez-Mercado¹, Froylán Ibarra-Velarde¹*, Miguel Ángel Alonso-Díaz², Yolanda Vera-Montenegro¹, José Guillermo Avila-Acevedo³ and Ana María García-Bores³

Abstract

Background: Fasciolosis due to Fasciola hepatica is the most important hepatic disease in veterinary medicine. Its relevance is important because of the major economical losses to the cattle industry such as: reduction in milk, meat and wool production; miscarriages, anemia, liver condemnation and occasionally deaths, are estimated in billions of dollars.

The emergence of fluke resistance due to over or under dosing of fasciolides as well as environmental damage produced by the chemicals eliminated in field have stimulated the need for alternative methods to control Fasciola hepatica. The aim of this study was to evaluate the in vitro anthelmintic effect of fifteen tropical plant extracts used in traditional Mexican medicine, on newly excysted flukes of Fasciola hepatica.

Results: The flukes were exposed in triplicate at 500, 250 and 125 mg/L to each extract. The efficacy was assessed as the mortality rate based on the number of live and dead flukes after 24, 48 and 72 h post-exposure. The plants with anthelmintic effect were evaluated once again with a concentration of 375 mg/L in order to confirm the results and to calculate lethal concentrations at 50%, 90% and 99% (LC⁵₀, LC⁹₀, and LC⁹⁹). Plant extracts of Lantana camara, Bocconia frutescens, Piper auritum, Artemisia mexicana and Cajanus cajan had an in vitro anthelmintic effect (P <0.05). The LC⁵₀, LC⁹₀ and LC⁹⁹ to A. mexicana, C. cajan and B. frutescens were 92.85, 210.44 and 410.04 mg/L, 382.73, 570.09 and 788.9 mg/L and 369.96, 529.94 and 710.34 mg/L, respectively.

Conclusion: It is concluded that five tropical plant extracts had promising anthelmintic effects against F. hepatica. Further studies on toxicity and in vivo biological evaluation in ruminant models might help to determine the anthelmintic potential of these plant extracts.

Keywords: Plant extracts, Fasciola hepatica, Anthelmintic activity, In vitro

Background

Fasciolosis caused by Fasciola hepatica has a worldwide distribution affecting cattle, sheep, goats, pigs, horses, rabbits and humans as well. It causes major economical losses to the cattle industry (estimated in billions of dollars) by decreasing milk and/or meat production, low reproductive efficiency, liver seizures in slaughterhouses, high costs to control parasitism and deaths [1,2].

The control of this disease has been based on the application of anthelmintics, but due to the development of resistance it seems that the efficacy of some chemical drugs has decreased [3,4]. The use of plants with anthelmintic activity may be an alternative to fluke control, given the great diversity of ecosystems. The opportunity of finding bioactive compounds with anti-fluke properties significantly increases because, secondary metabolites (SM) are the most important compounds as new alternatives for parasite control. Some SM such us alkaloids, saponins, skimmianins A and C, tannins, flavonoids, terpenes (mono, di and sesquiterpenes) have been shown to be active against a wide range of parasites [5].

* Correspondence: ibarraf@unam.mx
¹Departamento de Parasitología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México. Cd. Universitaria, C.P. 04510 México, DF, Mexico
Full list of author information is available at the end of the article

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Recent studies have reported the anthelmintic effect of plants such as *Artemisia mexicana*, *Mentha piperita*, *Achillea millefolium*, *Allium sativum*, *Piper nigrum*, and *Carica papaya* with parasiticial effects against *F. hepatica* [6-8].

Veracruz is the Mexican state with the highest livestock production in the country [9] and parasitic illnesses are the main threat to grazing bovines in this region. Because of the great diversity of ecosystems, the native vegetation of Veracruz has a wide variety of plant species (containing variable levels of SM) which potentially could be used as a fasciolicide. However, studies to evaluate the effect of plants with possible anthelmintic properties against *F. hepatica* in the area have been not carried out. The aim of the present study was to evaluate the anthelmintic effect of fifteen plants extracts from Veracruz, Mexico.

**Methods**

**Plant material**

Fresh leaves (700 g) of *Acacia cornigera* (2147 IZTA), *Acacia farnesiana* (2164 IZTA), *Artemisia absinthium* (2155 IZTA), *Artemisia mexicana* (2156 IZTA), *Bocconia frutescens* (2153 IZTA), *Cajanus cajan* (2164 IZTA), *Cordia spp*, *Hibiscus rosa-sinensis* (2149 IZTA), *Lantana camara* (2160 IZTA), *Leucaena diversifolia* (2169 IZTA), *Melia azedarach* (2161 IZTA), *Mentha sp* (2163 IZTA), *Ocimum basilicum* (2154 IZTA), *Piper auritum* (2156 IZTA), *Tecoma ambrosioides* (2157 IZTA) and *Teloxys ambrosioides* (2157 IZTA) were collected from villages in Veracruz, Mexico.

Prior to the beginning of this trial, samples of different plants were collected and identified by Dr. Edith López Villafranco of the IZTA Herbarium at the Facultad de Estudios Superiores Iztacala for the purpose of authenticating them. A voucher specimen was deposited in the IZTA herbarium for future reference (a reference number was assigned). The plants were chosen based on the traditional practices [10-12]; moreover reports of other authors [7,13-15] and interviews with local people have shown to be effective in finding remedies against other parasites.

**Extraction procedure**

Extraction procedures were undertaken in the phytochemistry laboratory of FES Iztacala and the evaluation of in vitro anthelmintic efficacy was carried out in the laboratory of experimental chemotherapy of the parasitology department, (FMVZ-UNAM).

The leaves of each plant (100 g) were dried in an oven for three days at 60°C, ground into powder and sequentially extracted with hexane, ethyl acetate and methanol. The extracts were filtered and successively concentrated. Each extract was concentrated under low pressure at low temperature and revolutions per minute (RPM) as follows: 1) hexane, at 60°C, 50 RPM, 2) ethyl acetate, at 78°C, 60 RPM and 3) methanol, at 65°C, 90 RPM using a

**Table 1 In vitro anti-fluke effectiveness of fifteen plant extracts**

| Plant extract                  | Reference control (%) | Untreated control (%) | Efficacy (%) |
|-------------------------------|-----------------------|-----------------------|--------------|
|                               | 125 mg/L | 250 mg/L | 500 mg/L | 125 mg/L | 250 mg/L | 500 mg/L |
| *A. cornigera*                | n = 10 | 100<sup>a</sup> | 100<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *C. cajan*                    | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *A. farnesiana*               | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *L. camara*                   | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *H. rosa-sinensis*            | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *B. frutescens*               | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 10 ± 0.1<sup>a</sup> | 100<sup>b</sup> | 100<sup>b</sup> |
| *M. azedarach*                | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 7 ± 0.11<sup>a</sup> | 7 ± 0.11<sup>a</sup> | 13 ± 0.11<sup>a</sup> |
| *L. diversifolia*             | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *C. spp*                      | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *C. ambrosioides*             | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *P. auritum*                  | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *M. sativa*                   | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *A. absinthium*               | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> | 0<sup>a</sup> |
| *O. basilicum*                | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 100<sup>b</sup> | 100<sup>b</sup> | 100<sup>b</sup> |
| *A. mexicana*                 | 100<sup>a</sup> | 100<sup>b</sup> | 0<sup>a</sup> | 100<sup>b</sup> | 100<sup>b</sup> | 100<sup>b</sup> |

<sup>a</sup>A different letter between columns indicates statistically significant differences. Significant at p < 0.05 level. Control—nil mortality.

<sup>b</sup>Average of three replicates ± standard deviation.

<sup>c</sup>Triclabendazole, average of three replicates ± standard deviation.

<sup>d</sup>Destilled water, average of three replicates ± standard deviation.
The plant extracts were kept in the dark at 4°C until tested.

**Bioassays**

To determine the antihelmintic effect of the 15 plant extracts on the mortality of excysted flukes a series of in vitro experiments were undertaken. Newly excysted flukes were obtained by the artificial excysment of *F. hepatica* metacercariae following the methodology described by Ibarra and Jenkins [18].

**Formulation of plant extracts for screening**

All compounds were formulated as follows: 500 mg of the compound were placed in a screw-capped 15 ml Eppendorf® tube to which 0.1 ml of methanol were added to dissolve the extract. Then two fold dilutions using distilled water were made to prepare concentrations of 500, 250 and 125 mg/L.

Plant extracts were placed in NUNC® culture dishes. Each well contained 1.6 mL of RPMI-1640® of the culture medium, 0.2 mL of solubilized extract and 0.2 mL containing 10 liver flukes. Four wells were used as untreated controls, three containing only a complete medium (RPMI-1640®), the last one containing a culture medium and 0.2 mL of methanol. In addition there were four more wells containing triclabendazole (SOFOREN®, Novartis) at a 10 and 50 mg/L, respectively. Each test remained incubated at 37°C for four days under a 5% CO₂ atmosphere; each experiment was replicated three times.

The plant extracts with in vitro anthelmintic efficacy higher than 80% were re-evaluated twice in order to confirm the results, and a concentration of 375 mg/L was added to calculate the lethal concentration to kill 50%, 90% and 99% of the flukes (LC₅₀, LC₉₀ and LC₉₉). All procedures were performed under aseptic conditions using a laminar flow hood.

**Test interpretation**

The flukes under study were examined at 24, 48 and 72 hours post-exposure. Activity was measured by comparing the survival of the treated flukes relative to those of the control group. At each evaluation time, these flukes without motility were considered as dead.

**Efficacy measurement**

The effectiveness of the plant extracts was assessed with the following formula [19]:

\[
\text{Efficacy(%) = } \frac{\text{No. of flukes alive in control group} - \text{No. of flukes alive in treated group}}{\text{No. of flukes alive in control group}} \times 100
\]

When an extract showed an in vitro efficacy greater than 80%, it was considered to possess fascioliscide activity.

### Table 2 Second assessment of anti-fluke effectiveness of five plant extracts

| Plant extract | Reference control (%)* | Untreated control (%)* | Efficacy (%) |
|---------------|-------------------------|------------------------|--------------|
|               | 10 mg/L                  | 50 mg/L                | 0 mg/L       | 125 mg/L | 250 mg/L | 500 mg/L | 500 mg/L |
| A. mexicana   | 100a                    | 100b                   | 0a           | 93 ± 0.06b | 100a     | 100a     | 100a     |
| B. frutescens | 100a                    | 100b                   | 0a           | 93 ± 0.06b | 100a     | 100a     |
| L. camara     | 100a                    | 100b                   | 0a           | 93 ± 0.06b | 100a     | 100a     |
| P. auritum    | 100a                    | 100b                   | 0a           | 93 ± 0.06b | 100a     | 100a     |
| C. cajan      | 100a                    | 100b                   | 0a           | 93 ± 0.06b | 100a     | 100a     |

* A different letter between columns indicates statistically significant differences. Significant at p < 0.05 level. Control—nil mortality.
* Average of three replicates ± standard deviation.
* Triclabendazole, average of three replicates ± standard deviation.
* Distilled water, average of three replicates ± standard deviation.

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![Figure 1](https://via.placeholder.com/150)

**Figure 1** Flukicide activity of plant extracts.  
- a. Untreated control flukes.  
- b. Flukes treated with *L. camara* extract 72 hrs post exposition. Dead flukes being severely affected in the tegument and internal organs.  
- c. Flukes treated with *A. mexicana* extract 72 hrs post exposition. Flukes showed no motility and internal changes.  
- d. Flukes treated with *P. auritum* extract 72 hrs post exposition. Flukes showed no motility and no internal changes.  
- e. Flukes treated with *C. cajan* extract 72 hrs post exposition. Flukes showed no motility and no internal changes.  
- f. Flukes treated with *B. frutescens* extract 72 hrs post exposition. Flukes showed motility, but presented internal changes and slightly affected tegument.
Phytochemical screening

The active extracts were subjected to phytochemical analysis to determine the presence of SM groups following standard published protocols [20,21].

Statistical analyses

A Kruskal-Wallis test, P <0.05 was used to determine significant differences [22] and a PROBIT test was performed with POLO PLUS [23] to determine the LC₅₀, LC₉₀ and LC₉₉ of the extracts that showed in vitro fascioliscide efficacy.

Results

Efficacy of the extracts

The flukes placed in the control wells remained alive and healthy throughout all the tests. From 15 plants evaluated (Table 1), five plant extracts at different dose levels effectively killed Fasciola hepatica (P <0.05). At a dose of 500 mg/L, C. cajan, L. camara and P. auritum had an efficacy of 100%, while B. frutescens and A. mexicana had a 100% efficacy at a dose of 125 mg/L.

The five extracts showing in vitro anthelmintic activity greater than 80% are indicated in Table 2. These were evaluated for a second time including a concentration of 375 mg/L to determine LC₅₀, LC₉₀ and LC₉₉ of the extracts that showed in vitro fascioliscide efficacy.

Figure 1 shows the flukicide activity before and after exposition with some plant extracts at 40×.

Phytochemical screening

Table 4 shows that most crude extracts contain MS such as alkaloids, phenolic compounds as well as coumarins, flavanones and flavonoids. Furthermore, sesquiterpene lactones, steroids, triterpenes and glycosides were also detected.

Discussion

Plant extracts currently represent a potential alternative for the effective control of fasciolosis in domestic ruminants. However, since this area has been explored only to a limited extent, there is a manifest need to carry out new research to determine their potential against F. hepatica.

Jeyathilakan et al. [24] evaluated on Fasciola gigantica adults the efficacy of ethno-medicinal plant aqueous extracts such as Allium sativum, Lawsonia inermis, and Opuntia ficus indica in vitro in comparison with Oxyclozanide with efficacies from 40 – 100%. Jeyathilakan et al.

Table 3 Lethal concentration estimates from plant extracts with anthelmintic efficacy in vitro

| Plant extract | LC₅₀ (mg/L) | LCL-UCL | LC₉₀ (mg/L) | LCL-UCL | LC₉₉ (mg/L) | LCL-UCL | SD  \( \chi² \) (df = 10) |
|---------------|------------|---------|------------|---------|------------|---------|--------------------------|
| A. mexicana   | 92.85      | 42.16-124.50 | 210.44 | 166.78-306.78 | 410.04 | 288.46-1135.26 | ±2.197 | 5.893 |
| C. cajan      | 382.73     | 327.13-444.12 | 570.09 | 479.89-908.48 | 788.9 | 603.92-1768.3 | ±3.653 | 15.258 |
| B. frutescens | 369.96     | 318.77-419.83 | 529.94 | 457.78-748.36 | 710.34 | 567.74-1298.47 | ±3.813 | 14.702 |

LC₅₀ — lethal concentration that kills 50% of the exposed flukes, LC₉₀ — lethal concentration that kills 90% of the exposed flukes, UCL: upper confidence limit; LCL: lower confidence limit, SD: standard deviation. \( \chi² \) — Chi-square; df: degree of freedom. Significant at p < 0.05 level.

Table 4 Results of phytochemical screening

| Colorimetric reaction | Plant extract | L. camara | B. frutescens | P. auritum | C. cajan | A. mexicana |
|-----------------------|---------------|-----------|---------------|------------|----------|------------|
| Phenolic compounds (FeCl₃) | ++ | + | ++ | ++ | + |
| Coumarins (UV)         | − | ++ | − | − | + |
| Flavanones (NH₃)       | + Yellow | − | + Yellow | + Yellow | + Yellow |
| Flavonoids (Shinoda)   | − | − | + Red | + Orange | + Red |
| Sesquiterpene lactones (Baljet) | + | − | + | + | + |
| Alkaloids (Meyer)      | +++ | +++ | +++ | +++ | +++ |
| Alkaloids (Dragendorff) | +++ | +++ | +++ | +++ | +++ |
| Steroids and triterpenoids (Liberman, Burchard) | ++ | + | + | + | + |
| Glycosides (α-naphtol) | − | − | − | − | + |

Symbology: −− negative; + weak positive; ++ positive; +++ strong positive.
Azadirachta indica in vivo and L. camara contributions extract. et al. BMC Veterinary Research extract had no toxicity in studies. For example, Ghanem et al. [28] reported A. mexicana [38], but not in – has an intrinsic anti-fluke ac-
L. camara B. frutescens F. hepatica [36], A. mexicana P. auritum (P <0.05). Recent studies have re-
known tox-
A. mexicana and P. auritum extract had an anthelmintic of effi-
L. camara livestock L. camara involves no B. frutescens [37], and anthelmintic activity in the P.
F. and C. cajanus extract a hepato protective effect on mice. Since with cultured
demonstrates great anthelmintic activity in continued
caused in the animals that consume this plant. If this plant
discard the effect of other bioactive compounds. Hence, it is necessary to determine the chemical composition of
activity (100%) whereas neem oil did not show any sig-
ificant effect. Their results indicated the potential for
developing herbal-based anthelmintics to control F. gigantica in livestock.

In this study, five plant extracts showed fasciolasicide activity: A. mexicana, B. frutescens, L. camara, P. auritum and C. cajan (P <0.05). Recent studies have reported that, at the same concentrations used in our study, A. mexicana extract had an anthelmintic of effi-
cacy 100% [19,26]. The latter findings show that at the
doses tested, A. mexicana has an intrinsic anti-fluke ac-
tivity; it also indicates that this extract may be an alter-
native to the chemical control of F. hepatica only after evaluation and in vivo toxicity studies. In this regard,
studies by Ibarra-Moreno et al. [27] in CD1 mice dem-
A. mexicana extract had no toxicity in renal or liver tissue.

To our knowledge, this is the first report of the anthel-
mintic effect of P. auritum, B. frutescens and C. cajan
against F. hepatica. Although these plants have not been
evaluated against trematodes before, they are found to
possess some interesting and additional positive charac-
teristics which deserve to be considered for future
in vivo studies. For example, Ghanem et al. [28] reported
a protective and an antioxidant effect in the plants of the Piperaceae family as well in P. auritum with cultured
hepatocytes of mice. In addition, Estrada et al. [29] men-
tion that acute toxicity tests show that the intake of ex-
tracts of different polarities of P. auritum involves no
health risks. Kundu et al. [30] have also found in the C.
cajan extract a hepato protective effect on mice. Since
there are no reported toxic effects of these plants, it is
possible to obtain a similar in vivo effect by direct ad-
ministration to ruminants.

Up to now there have been no reports of anti-fluke ef-
fectiveness for L. camara despite its well – known tox-
icity in cattle and sheep. This is the first report of
in vitro anthelmintic activity in the L. camara extract.
However, it is necessary to consider the undesirable ef-
facts such as photosensitivity and liver disorders that are
caused in the animals that consume this plant. If this plant
demonstrates great anthelmintic activity in continued
studies, there will be sufficient reason for further study in
order to identify the causal agents responsible for this tox-
icity. It is, therefore, convenient to find other species of
Lantana spp that have no toxicity reports [31].

Secondary metabolites such as alkaloids, terpenes, tan-
nins or flavonoids contained in crude plant extracts have
been related to parasiticidal activity [32-35]. Neverthe-
less, since these are not the only compounds that these
and other plant species possess, it would be wrong to

Conclusion Of the fifteen extracts tested, five showed promising
in vitro fasciolasicide efficacy, thus indicating that they
could possibly be strong candidates for further biological
and toxicological analyses aimed at demonstrating their
real potential for liver fluke control in ruminants.

Competing interests The authors of this manuscript have no financial or personal relationships
with other people or organizations that could inappropriately influence or bias the content of the paper.

Authors’ contributions FV, MAAD, YVM and JGAA contributed to conception and design of the
study. JMAM, AMGB were responsible for execution and data collection. JMAM and MAAD were primarily responsible for data analysis
and interpretation and all authors were involved in drafting the manuscript
critical reading, editing and final approval of the submitted version.

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Author details 1Departamento de Parasitología, Facultad de Medicina Veterinaria y
Zootecnia, Universidad Nacional Autónoma de México. Cd. Universitaria,
C.P. 04510 México, DF, Mexico. 2Centro de Enseñanza Investigación y
Extensión en Ganadería Tropical, Facultad de Medicina Veterinaria y
Zootecnia, Universidad Nacional Autónoma de México, Km. 5.5, Carretera
Federal Tlapacoyan-Martínez de la Torre, C.P. 93600 Veracruz, Mexico. 3Lab. de
Fitooquímica, UBIPRO, Facultad de Estudios Superiores Iztacala, UNAM,
Avenida de los Barrios 1, C.P. 54090 Edo. de México, Mexico.

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