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Obtaining the Minimum Lethal Dose against *Fasciola hepatica in Vitro* Using Plant Extract Hexanes with Fasciolicide Activity and Toxicity Evaluation on CD1 Male Mice

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ABSTRACT

Fascioliasis is a parasitic disease of worldwide distribution affecting mainly cattle and sheep. Its importance lies in the economic losses it produces in the livestock industry. Its control is carried out by using a chemical fasciolicide showing resistance problems and environmental contamination. Looking for an alternative control for this disease the present study was aimed at determining the hexane anti-*Fasciola hepatica* in the *in vitro* effect of some plant extracts and the minimum lethal dose of the mentioned extracts. All selected plants were tested *in vitro* at concentrations of 500, 250, 125 and 50 mg/L): *Achilleamillefolium* (plumajillo), *Artemisiaabsinthium* (wormwood), *Artemisia mexicana* (estafiate), *Castelatortuousa* (chaparroamargo), *Chenopodiumgraveolens* (epazote de zorrillo), *Gymnospermaglutinosum* (popote) *Justicia spicigera* (muicle), *Limpia critridora* (cedron), *Lippiagraveolens* (oregano), *Menthapiperita* (Mint), *Populus alba* (alamo) and *Thymusvulgaris* (thyme). Subsequently proceeded to perform a toxicity study with these fractions in CD1 male mice 10 - 13 weeks of age, forming groups of 3 - 5 animals they were administered a single oral dose being (5 mg/kg, 50 mg/kg, 500 mg/kg, 2500 mg/kg and 5000 mg/kg) and were kept under observation 20 days, later were sacrificed and a kidney and liver histology was performed, finding the safety of the extracts. To perform the toxicity study with these fractions, groups of five CD1 male-mice were formed, they were treated with oral doses of 5, 50, 500, 2500 and 5000 mg/kg, administered with a cannule. All mice were kept under observation for 20 days. Finally they were sacrificed to perform histology of the kidney and liver in search of possible side effects. Results show that none of the extracts exhibited that fasciolocide activity for mice CD1 even at the highest dose thereforefinding the safety of the extracts.

Keywords: *Fasciola hepatica*; Plant Extracts; *In Vitro*; Minimum Lethal Dose; Toxicity

1. Introduction

Fasciolosis is a parasitic disease of worldwide distribution [1]. It is due to the action of the liver fluke *Fasciola hepatica*, which affects primarily cattle and sheep and other animal species including humans [2]. Its importance lies in the resulting economic losses it produces in the livestock industry, which can be reduced through chemical treatment of livestock [3,4].

Over the years the private industry has tried to improve diagnostic methods and promote the production of more effective drugs with the purpose of obtaining a comprehensive control of this trematodosis [5,6]. However, since the parasites eventually develop resistance to these drugs, alternative methods such as the study of active substances of several plants with anthelmintic properties were considered essential to study [7].

Mexico is the fourth place worldwide in floristic diversity with more than 25,000 recorded species, of the 250,000 that exist in the world and there are estimated to be 30,000 more within the national-territory; the country's forest area comprises 73.3% of its territory. Moreover, there are over 6000 species of medicinal plants reported so far [8,9].

Based on ethnobotanical studies we have found different plants used in Mexican traditional medicine against dysentery, vomiting, vermifuge, nausea, poor absorption,

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diarrhea, indigestion, etc. [10,11]. From these plants we have processed hexane extracts which were previously tested an *in vitro* model against immature stages of *Fasciola hepatica*, yielding an efficiency of between 80% - 100% in the first 3 days at a concentration of 500 mg/Lt [12]. The following plants were selected for screening:

Achilleamillefolium (plumajillo)
Artemisia absinthium (wormwood)
Artemisia mexicana (estafiate)
Castela tortuosa (chaparro amargo)
Chenopodiumgraveolens (epazote de zorrillo)
Gymnospermaglutinosum (popote)
Justicia spicigera (muicle)
Limpia critridora (cedron)
Lippiagraveolens (oregano)
Menthapiperita (mint)
Populus alba (alamo)
Thymusvulgaris (thyme)

2. Materials and Methods

2.1. Preparation of Plant Extracts

The plant parts (leaves, flowers and stems) were extracted with a hexane solvent using a rota-evaporator. Then 3 distillations were performed every 3 - 4 days depending on the plant. The extracts were concentrated in different vials for later evaluation.

2.2. *In Vitro* Fasciolicide Evaluation of the Minimum Lethal Dose with Hexane Extracts

These evaluations were conducted in the Experimental Chemotherapy Laboratory, Department of Parasitology, College of Veterinary Medicine of the National Autonomous University of Mexico (**Figure 1**).

2.3. Preparation of Plant Extracts for Evaluation

5 mg of extract were placed in vials of 30 ml. Then the corresponding dilutions were prepared so as to obtain the concentrations required for the anti-*Fasciola hepatica* biological evaluation, these being (500 mg/L, 250 mg/L, 125 mg/L and 50 mg/L).

2.4. Operation of the Test for Screening

Culture dishes of 24 Nunc brand wells were used. For each well 1.6 ml of complete medium there was deposited 0.2 ml of a solubilized extract and 0.2 ml containing 10 newly excysted flukes per well. Each trial remained in incubation for 4 days at 37˚C under an atmosphere of 5% CO_2.

2.5. Interpretation of the Test

The flukes were examined carefully on days 1.2 and 3 using an inverted microscope at 40×. The activity of the extracts was assessed by a comparison of the surviving treated flukes with the untreated control flukes. All procedures were performed under sterile conditions using a laminar flow cabinet, as previously described by Rivera [13].

% Efficacy was assessed using the following formula [14]:

$$\frac{\text{No. of flukes in the control group} - \text{No. of flukes in the treated group}}{\text{No. of the flukes in the control group}} \times 100$$

2.6. Evaluation of Acute Toxicity in Mice CD1

CD1 male mice were formed in groups of 5 animals each. They were treated with different oral doses of the extracts previously selected. We use CD1 mice because they are strong, economical, easily produced and used in biological assays or in preliminary studies where the specific genotype is not important.

Extracts were prepared as a suspension/solution at concentrations of 500 mg/L, 250 mg/L, 125 mg/L and 50 mg/L, 2 - 5 µl absolute alcohol was used to dissolve the extract plus 995 - 998 µl of methyl cellulose as a vehicle. The suspension was administered with a cannule and the mice were under observation for 20 days (appetite, hair, motor activity, mucous etc.). The untreated control group (n = 6) dosed only with absolute alcohol + methyl cellulose was also observed in the same manner. Finally mice were sacrificed to carry out the histopathology of the

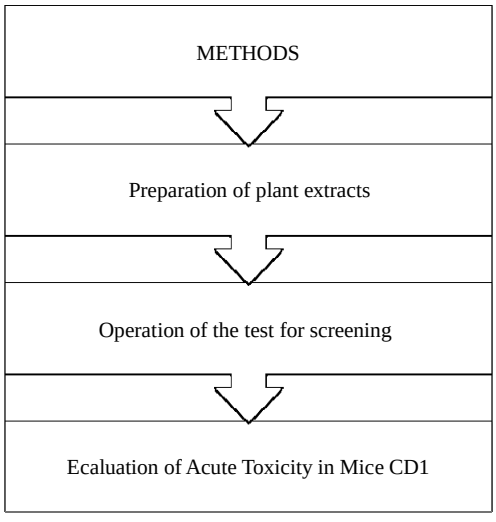

Figure 1. Methodology.

liver and kidney searching for possible toxic side effects.

3. Results and Discussion

Table 1 shows the *in vitro* fascioliscide activity of the hexanes plant extractshere is the scientific name, common name, number of flukes used, concentration used, and effectiveness for three days.

It is important to note only the hexane fraction was used in these trials because this fraction was shown to have the most effective in previous trials [15] and we can therefore assume that it is a metabolite or an active principle that is common in these plants that produce this fasciolicide effect. It was found, as shown in (**Table 2)** the minimum lethal dose of hexane extracts with fasciolicide activity in percent. Finally it is shown in (**Table 2**) the toxicity of hexane extracts in CD1 male mice for 20 days. In all *in vitro* tests were no controls with the same amount of solvent to solubilize the extracts and always remained alive for up to 7 days and in the same way the control miceremained healthy with methyl celluloseas a vehicle utilized.

Plants are a resource for obtaining new drugs and treatments for existing parasitic diseases in our country. [9,14,15] Ethnobotanical studies are very useful to locate

Table 1. *In vitro* fasciolicide activity of the hexanes plant extracts.

Plant	Total flukes	Concen-tration	1 Day efficacy	2 Day efficacy	3 Day efficacy	Part of the plant used
Achilleamillefolium (Plumajillo)	70	500 mg/L	100%	100%	100%	Leaf, flower stem
	70	250 mg/L	100%	100%	100%	
	70	125 mg/L	100%	100%	100%	
	40	50 mg/L	0%	0%	10%	
Artemisia absinthium (wormwood)	70	500 mg/L	98%	100%	100%	Leaf and stem
	70	250 mg/L	70%	100%	100%	
	40	125 mg/L	0%	0%	10%	
Artemisia mexicana (estafiate)	70	500 mg/L	94%	100%	100%	Leaf and stem
	70	250 mg/L	80%	100%	100%	
	70	125 mg/L	60%	100%	100%	
	40	50 mg/L	0%	0%	0%	
Castelatortuosa (chaparroamargo)	70	500 mg/L	100%	100%	100%	Leaf and stem
	60	250 mg/L	100%	100%	100%	
	40	125 mg/L	80%	100%	100%	
	40	50 mg/L	0%	0%	0%	
Chenopodiumgraveolens (*epazote de zorrillo*)	60	500 mg/L	98%	100%	100%	Leaf, flower and stem
	40	250 mg/L	0%	0%	10%	
	20	125 mg/L	0%	0%	0%	
Gymnospermaglutinosum (Popote)	60	500 mg/L	60%	100%	100%	Leaf and stem
	40	250 mg/L	0%	10%	20%	
	20	125 mg/L	0%	0%	0%	
Justicia spicigera (Muicle)	60	500 mg/L	100%	100%	100%	Leaf and stem
	70	250 mg/L	80%	100%	100%	
	40	125 mg/L	0%	0%	0%	
Limpiacritridora (*Cedrón*)	60	500 mg/L	70%	80%	88%	Leaf and stem
	60	250 mg/L	0%	0%	0%	
Lippiagraveolens (orégano)	60	500 mg/L	100%	100%	100%	Leaf and stem
	70	250 mg/L	80%	90%	100%	
	40	125 mg/L	0%	10%	10%	
Mentha piperita (Mint)	70	500 mg/L	100%	100%	100%	Leaf and stem
	70	250 mg/L	80%	100%	100%	
	70	125 mg/L	60%	100%	100%	
	40	50 mg/L	0%	0%	0%	
Populus alba (*Álamo*)	60	500 mg/L	100%	100%	100%	Leaf and stem
	40	250 mg/L	0%	20%	20%	
	20	125 mg/L	0%	0%	0%	
Thymus vulgaris (*thyme*)	70	500 mg/L	71%	88%	99%	Leaf, flower and stem
	60	250 mg/L	0%	0%	0%	
	20	125 mg/L	0%	0%	0%	

Table 2. Minimum lethal dose of hexane extracts with fasciolocide activity (*in vitro* model).

Extract hexanic	Minimum lethal dose *in vitro* (%)
Achilleamillefolium (plumajillo)	125 mg/L
Artemisia mexicana (estafiate)	125 mg/L
Castela tortuosa (chaparro amargo)	125 mg/L
Menthapiperita (mentha)	125 mg/L
Justicia spicigera (muicle)	250 mg/L
Lippiagraveolens (oregano)	250 mg/L
Artemisia absinthium (Ajenjo)	500 mg/L
Chenopodiumgraveolens (epazote de zorrillo)	500 mg/L
Gymnospermaglutinosum (Popote)	500 mg/L
Limpiacritridora (Cedron)	500 mg/L
Populus alba (alamo)	500 mg/L
Thymusvulgaris (tomillo)	500 mg/L

plants as possible prospects for the use of new antiparasitic drugs [10,14,16]. Various plants had previously been studied, obtained and processed to find several hexane extracts with fasciolicide activity in the *in vitro* model [15].

Thus in the present study we worked with these same extracts at different concentrations to find the minimum lethal dose *in vitro* since it was found that not all the hexane extracts had the same fasciolicide activity [15].

The results obtained showed that *Achilleamillefolium*, *Artemisia Mexicana*, *Castelatortuosa*, *Menthapiperita*, have a minimal lethal dose of 125 mg/L. *Justiciaspcigera* and *Lippiagraveolens* have a 250 mg/L activity. *Artemisia absinthium*, *Chenopodiumgraveolens*, *Gymnospermaglutinosum*, *Limpiacritridora*, *Populusalba* and *Thymus vulgaris*, have an activity of 500 mg/L respectively (**Tables 1**, **2**). These results were obtained by means of different repeated tests *in the vitro* model [17,18].

Then we proceeded to perform a toxicity study by forming various groups using different concentrations of the extracts above. It was found that none of the extracts led to the death of the treated mice. They were observed for 20 days noting their physical appearance and behavior, and it was observed that there was no difference between the control group and the treated group (**Table 3**) [19,20].

To ensure their safety the mice with the highest dose of extract (5000 mg/kg) were submitted to liver and kidney histopathology and no damage was found to any of the above mentioned organs [21].

Table 3. Toxicity of hexane extracts in CD1 male mice for 20 days.

Extract	Dose used	Animal number	State of the animal
Achilleamillefolium (Plumajillo)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Artemisia absinthium (Ajenjo)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Artemisia mexicana (Estafiate)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Castela tortuosa (Chaparro amargo)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Justicia spicigera (muicle)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Lippiagraveolens (Oregano)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Menthapiperita (Mint)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Populus alba (Álamo)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good
Thymusvulgaris (Tomillo)	0.5 mg/kg	3	Good
	5 mg/kg	3	Good
	50 mg/kg	5	Good
	500 mg/kg	5	Good
	5000 mg/kg	5	Good

Considering these promising results under *in vitro* conditions and being certain that they are not toxic to mammals we propose that these extracts on the definitive host of these parasites, viz., sheep and cattle be tested [21,22].

4. Conclusion

Our results are promising and suggest carrying tests *in*

vivo in the definitive host of *Fasciola hepatica* (cattle or sheep) and do a phytochemical study from plants showed more effective.

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