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Original Article

In Vitro Antischistosomal Activity of the *Argemone mexicana* Methanolic Extract and Its Main Component Berberine

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Abstract

Background: Schistosomiasis has been identified as a major public health problem in tropical countries. The present study aimed to investigate the schistosomicidal effects of the methanolic extract of *Argemone mexicana* L. and its active component, berberine against *Schistosoma mansoni* on in-vitro experiments.

Methods: *S. mansoni* adults were used. Various concentrations of the methanolic extract (10 - 200 µg/ml) and berberine (2.5 - 50 µM) were tested from 24 to 72 h. The viability of *S. mansoni* was confirmed with an invertoscope-microscope. Furthermore, cytotoxic (Hemolysis test), and antioxidant (DPPH radical scavenging assay) capacities were determined.

Results: The viability tests on *S. mansoni* showed that *A. mexicana* at 50 µg/mL is lethal at 48 h and berberine at 10 µM is lethal at 24 h. The hemolytic activity at 1,000 µg/mL was 2.9% for *A. mexicana* and 90.2% for berberine. The antioxidant capacities shown by *A. mexicana* and berberine, were EC_{50} 156.3 and 84.1 µg/mL, respectively.

Conclusion: The extract of *A. mexicana* and berberine demonstrated high antischistosomal activities in low concentration and short exposure time on the in-vitro model.

Introduction

Several clinical significant parasites can affect the human intestine, schistosomiasis is a neglected tropical disease caused by blood flukes of the Schistosoma genus, being *Schistosoma mansoni*, *S. haematobium,* and *S. japonicum* responsible for most human infections (1). More than 250 million people have been infected in more than 75 countries where the disease is endemic (2). In the absence of a vaccine, the first-line treatment for the control of schistosomiasis is praziquantel (PZQ) used since the 1970s in mass administration programs (3). That situation facilitated the development of resistant strains to PZQ, so there is a need to investigate new antischistosomal agents (4).

The use of plant-derived natural compounds is currently considered a promising approach in schistosomiasis treatment (5). The challenge is to obtain highly effective compounds, able to stimulate host-defense mechanisms and with no side effects. In this sense, natural products have the advantage of offering access to the development of new schistosomicidal compounds due to their well-documented transition to synthetic drugs in many other diseases (6).

Argemone mexicana L. (Papaveraceae) is a plant widely distributed in tropical countries. In traditional medicine, *A. mexicana* shows activity against *Plasmodium falciparum*, *Leishmania donovani* and *Trypanosoma brucei rhodesiense* (7,8). The presence of several alkaloids has been reported in this plant, being berberine its main component (9). Berberine is perhaps the constituent responsible for the biological activity of *A. mexicana* since its effectiveness has been attributed against L. *tropica* and *L. infantum* (10). Berberine causes morphological changes on Entamoeba histolytica and Giardia lamblia, inducing chromatin agglutination in the nucleus, formation of autophagic vacuoles and aggregates in the cytoplasm (11). Berberine has also been reported to possess activity against a variety of bacteria, fungi, viruses (12).

The aim of this work was focused on evaluating the antischistosomal activity of the methanolic extract of A. mexicana (Am), as well as the main component berberine.

Materials and Methods

Ethical statement

The procedures with animals comply with the European Union guidelines (European Directive 2010/63/CE) for the experimentation and use of laboratory animals. Accredited facilities at the USAL were used (Reg.No.PAE/SA/001). The Ethics Committee of the UANL approved the procedures (Reg.No.335) for the use of human blood.

Chemicals

Overall, 2,2-diphenyl-1-picrylhydrazyl (DPPH), penicillin, streptomycin sulfate, berberine, dimethylsulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), L-glutamine, absolute methanol (MeOH), praziquantel (PZQ), and quercetin were purchased from Sigma-Aldrich® (Merck, USA). RPMI-1640 culture medium and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Invitrogen, USA).

Plant material

The aerial part of *A. mexicana* was collected in Feb 2018 in the city of Monterrey (25°46'24.38"N & 100°20'59.28"W), México. The taxonomic identification of the plant was carried out at the Botany Laboratory of the School of Biological Science (Reg.029128).

Parasites

S. mansoni cercariae (strain-LE) were routinely maintained in *Biomphalaria glabrata* snails as an intermediate host, and in CD1 female mice (Charles River-Criffa. Spain), as the definitive

host. Forty-day old female mice, weighing 25 g, were infected with 150 cercariae by abdominal skin exposure (13). The mice were kept with food and water *ad-libitum* in an environment under an alternant cycle of 12 h light/12 h dark at 20 °C. After 8 wk, mice were sacrificed with a lethal dose of 60 mg/kg pentobarbital plus heparin (2 IU/mL) and then perfused with PBS and heparin (500 IU/L) to obtain couples of adult *S. mansoni* from the portal and mesenteric veins. The worms were washed in RPMI-1640 culture medium (pH 7.5), supplemented with 10% heat-inactivated (56 °C) FBS, L-glutamine (2mM), penicillin (100 IU/mL), and streptomycin (100 mg/mL) (14).

Preparation of the methanolic extract and phytochemical analysis

The dried plant (200 g) was ground and extracted by the maceration method with 500 mL of absolute-MeOH for 96 h, in-room temperature. The solvent was removed into a rotary evaporator and the extract was concentrated to dryness and stored at -20 °C, until testing. The functional groups present in the methanolic extract and the active component berberine had been previously determined in a publication previously published by our group in 2020 (15).

Activity against S. mansoni

The activity against *S. mansoni* in the adult stage was carried out using the technique previously described to obtain the worms (16). Stock solutions of Am or berberine (1 mg/mL) in DMSO (1% w/v) were prepared. The solutions were sterilized with membrane filters (0.22 µm). Serial dilutions were made from the stock solutions, with RPMI-1640 + 10% FSB. The concentrations of the solutions were 10 to 200 µg/mL for Am, and 2.5 to 50 µM for berberine in culture plates with a final volume of 3 mL (considering the constant culture media volume in each well) and applied to 24-well plates filled with a final volume of 3 mL culture medium. Nine worms were cultured per cavity at 37 ºC, 5% CO_2 and 95% humidity immediately after perfusion of animals to ensure vitality (17). Negative control (C-) wells contained adults incubated with 1% DMSO plus culture media while positive control (C+) wells contained worms incubated with 10 µM PZQ plus the culture media. All experiments were performed in triplicate and microplates were checked at 0, 24, 48, and 72 h using an invertoscope-microscope.

Cytotoxicity assay

Cytotoxicity was determined in a suspension of human erythrocytes by the hemolysis test. Serum was separated and EDTA (1.5 mg/mL blood) was added to wash out the erythrocytes, which mix and separate at 1,000 rpm (5 min/37 °C). The erythrocytes were centrifuged three times in pH 7.4 (10 mM PBS) phosphate buffer solution removing the supernatants. The erythrocytes obtained were used to prepare the 5% v/v red blood cell suspension in PBS). For the evaluation of Am and berberine cytotoxicity in erythrocytes, the prepared suspension was incubated with different concentrations of the extract, berberine and controls (50 to 1,000 µg/mL) for 30 min (37 °C), these were classified as treatments (Tr). The negative control was untreated erythrocytes (C-), positive control was distilled water to produce osmotic hemolysis (C+) (18). After incubation, treatments were centrifuged for 4 min at 13,000 rpm (4 °C), 200 µL were taken and placed in a 96-well microplate. Hemolysis was determined spectrophotometrically at 540 nm. The readings were recorded as absorbance (Abs) for each treatment minus the absorbance presented by the vehicle (AbsTr). The percentage of hemolysis was calculated with the formula:

$$\%\text{Hemolysis} = \frac{(\text{AbsTr}) - (\text{AbsC}-)}{(\text{AbsC}+) - (\text{AbsC}-)} \times 100$$

Antioxidant activity

It was evaluated by the DPPH radical scavenging method (19). Treatments (Tr) were

evaluated at concentrations of 20 to 2,500 µg/mL. DPPH was prepared at 125 µM in MeOH, 100 µL of each respective concentration was taken and 100 µL of DPPH was added. Samples remained protected from light for 30 minutes. A quercetin solution was used as C+ and as C- MeOH. Absorbance at 517 nm was measured and the percentage reduction was calculated as:

$$\%\text{Reduction} = \frac{(\text{AbsC}-) - (\text{AbsTr})}{(\text{AbsC}-)} \times 100$$

Statistical analysis

Statistical differences were calculated using analysis of variance (ANOVA) and the Tukey test to establish differences between groups. Mean inhibitory concentration (CI50) and mean effective concentration (EC50) were determined by the Probit test, with a 95% confidence interval. The analyses were performed with SPSS software, version 22.0 (Chicago, IL, USA). Differences were considered significant at $P<0.05$. All tests were performed in triplicate in at least three different tests.

Results

Phytochemical analysis

An extraction yield of 10.22% was obtained. Conventional phytochemical tests indicated the presence of unsaturations, quinones, triterpenes -sterols, phenols, saponins, flavonoids, carbohydrates, and alkaloids mainly. Finally, by HPLC-DAD and mass-spectrometry, the berberine alkaloid was determined as the most abundant component in the extract.

Activity against S. mansoni

Am and berberine can inhibit the viability of the worms and even separate couples. Both treatments produced an alteration of the integument, with a clear dose-response relationship since the percentage of viability decreases as the concentration of the extract or alkaloid increases (Table 1). In all treatments, at 0 h, all couples were viable and without integument release. The positive control showed 100% mortality after 4 h of incubation, while the negative control did not reveal a decrease in viability (Fig.1A).

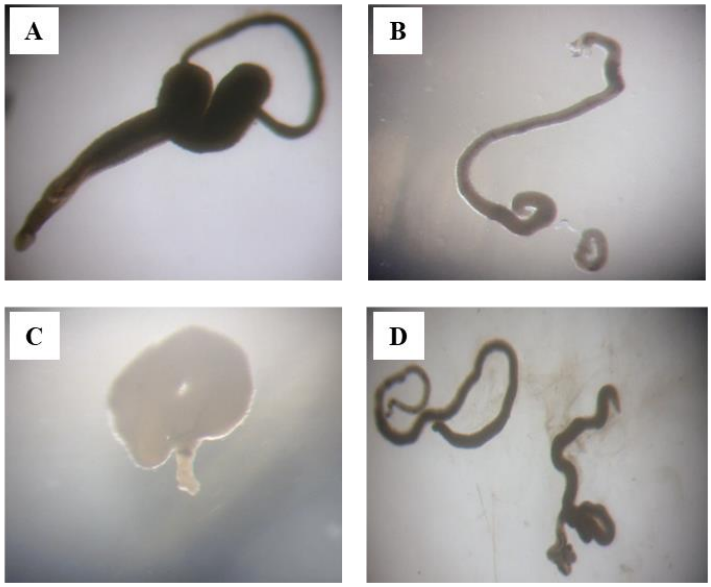

Fig. 1: In-vitro investigation of adult *S. mansoni* after 72 h of incubation. **A:** Negative control. **B:** Positive control (10 µM PZQ). **C:** A worm treated with 25 µg/mL Am. **D:** A worm treated with 10 µM berberine

Table 1: In-vitro effect of *Argemone mexicana* methanolic extract and berberine on adult *S. mansoni* couples

Variable		***Initial***	***24 h***				***48 h***				***72 h***			
Treatment	Concentration	Couples	S.C.	M%	T.D.	V%	S.C.	M%	T.D.	V%	S.C.	M%	T.D.	V%
Untreated (C-)		9	0	100	-	100	0	100	-	100	0	100	-	70.5
PZQ (C+)		9	9	0	++	6	9	0	+++	0.9	9	0	+++	0
Am	10	9	0	100	-	100	0	100	-	100	0	100	-	77
(µg/ml)	25	9	0	100	-	100	1	75	+	85	3	75	++	54*
	50	9	5*	75	++	69*	8**	50**	++	62*	8**	25**	+++	23**
	100	9	9**	25**	+++	15**	9**	0**	+++	0**	9**	0**	+++	0**
	200	9	9**	0**	+++	0**	9**	0**	+++	0**	9**	0**	+++	0**
ANOVA *p*			0.002	0.004		<0.001	0.004	0.005		0.001	0.004	0.001		<0.001
F			23.04	16.44		8.79	16.30	16.71		3.86	17.44	23.61		4.38
IC_{50} (µg/mL)						65.35				43.20				23.75
Berberine	2,5	9	0	100	-	86	1	100	-	77	3	75	-	41**
(µM)	5	9	6**	75	+	65*	8**	50**	+	46**	8**	25**	+	16**
	10	9	9**	0**	++	27**	9**	0**	++	11**	9**	0**	+++	2.6**
	25	9	9**	0**	+++	5.1**	9**	0**	+++	0**	9**	0**	+++	0**
	50	9	9**	0**	+++	0**	9**	0**	+++	0**	9**	0**	+++	0**
ANOVA *p*			0.018	<0.001		0.006	0.020	<0.001		0.002	0.109	0.001		<0.001
F			8.71	43.49		5.89	6.64	78.53		6.13	3.35	36.48		6.54
IC_{50} (µM)						8.76				4.82				1.68

S.C.: Separated couples. M%: Mean percentage of worm motility. T.D.: Tegument damage. V%: Viability percent. The tegument damage was classified as follows: Absent -, Present +, Abundant ++, and Very abundant +++. Statistical differences compared to untreated worm culture HDS Tukey post-hoc test **P* 0.05, ***P* 0.001

Am presented IC_{50} of 65.35, 43.20 and 23.75 µg/mL at 24, 48 and 72 h respectively. It was observed that at a 10 µg/mL concentration there were no significant changes, however, at 48 h and 72 h in concentrations of 50 µg/mL and 25 µg/mL (Fig.1C) respectively the effect is remarkable since at that time it was noted the detachment of all the pairs and an increase in the liberation of the integument. In concentrations of 100 and 200 µg/mL Am was effective after 24 h of incubation. Berberine presented IC_{50} of 8.76, 4.82 and 1.68 µM at 24, 48 and 72 h respectively, it also showed high effectiveness in 5 µM, with the pairs detaching at 48 h and being lethal at 48 h in the case of males and 72 h in the case of females (Fig.1D), in both cases, there is a detachment of the integument, which increases as the concentration of berberine increases.

Cytotoxicity of the methanolic extract and berberine

The percentage of cytotoxicity are shown in Table 2. At 1,000 µg/mL, Am had a hemolytic activity of 2.9%, which decreases as the concentration of the extract decreases (Table 2). For the lowest concentration of 50 µg/mL, the hemolysis presented in the erythrocytes was 0.1%. In the case of berberine, the hemolysis determined at 1,000 µg/mL was 90.2% and at 50 µg/mL it was 1.3%. Compared to C- which did not present hemolysis and C+ had 100% hemolysis, berberine presented a highly significant difference (P<0.001).

Table 2: Evaluation of the cytotoxicity of Am and berberine. Data are expressed as the mean ± SD (P<0.05) of the % of hemolysis plus standard error (SE). Different letters within the same column are significantly different analyzed via the Tukey test

Treatments	***Cytotoxicity***	
µg/mL	Am	Berberine
Distilled water (C+)	100 ± 0.0	100 ± 0.0
Untreated (C-)	0.0 ± 0.0	0.0 ± 0.0
50	0.1 ± 0.0^{a}**	1.3 ± 0.1^{a}**
100	0.3 ± 0.0^{a}**	2.8 ± 0.4^{b}**
200	0.9 ± 0.1^{b}**	11.8 ± 1.4^{c}*
400	1.2 ± 0.4^{b}**	23.8 ± 3.2^{d}*
600	1.6 ± 0.3^{b}**	55.9 ± 4.1^{e}
800	2.2 ± 0.4^{c}**	72.9 ± 2.3^{f}
1,000	2.9 ± 0.4^{d}**	90.2 ± 9.3^{g}
SE	0.4	13.6
ANOVA *p*	<0.001	<0.001
F	3.87	3.87

Statistical differences compared to positive control HDS Tukey post-hoc test *P 0.05, **P 0.001

Antioxidant capacity

The median effective concentration (EC_{50}) in which a treatment induces a median response after an exposure time showed, that the amount of sample necessary to decrease the DPPH concentration by 50%, for Am was 156.3 ± 6.5 µg/mL and for berberine 84.1 ± 7.4 µg/mL, however, the quercetin standard was more effective 1.03 ± 0.2 µg/mL.

Discussion

Interest in natural products for the treatment of different diseases, including schistosomiasis, is increasing, due to the scarce resources for their treatment, (20). The development of new drugs against this disease is required until an effective vaccine can be administered (21). The control of schistosomiasis is based on praziquantel which has many deficiencies (22). Many investigations have evaluated several substances from plant origin, such as berberine, abundant in *A. mexicana*, shown promising properties against *L. tropica*, *L. infantum*, *Trichomonas vaginalis,* and *E. histolytica* (15,23,24). However, no studies have been conducted against *S. mansoni*.

This research demonstrated that *A.mexicana* and its main component berberine, have a great capacity to inhibit the viability of *S. mansoni* in the adult stage. The effects of both were dependent on time and dose. The highest efficacy was observed in berberine at a concentration of 5 µM during an incubation period of 72 h, and from 10 µM at 24 h with effect in both sexes, combined to the separation of the couples. However, the effect was

much more pronounced against the male. Additionally, schistosomes exposed to both treatments showed mobility changes with atypical muscle contractions that became slower over time when treatment concentration increased and even parasite death was observed. Other researchers reported similar effects through in vitro tests, using compounds isolated from other plant species such as piplartine isolated from *Piper tuberculatum*, 8-hydroxyquinoline, and berberine chloride extracted from *Coptis chinensis* (13) evaluated against schistosomules, and adult worms. However, the mechanisms of action have not been clarified to date.

Previous studies have demonstrated the biological activity of berberine. Berberine from *B. vulgaris* significantly decreased the viability of *Echinococcus granulosus*, *L. tropica*, and *L. infantum* in in-vitro experiments (24,25). This may be because berberine inhibits the synthesis of nucleic acids and proteins, as well as the activity of telomerase (26). Plants containing alkaloids such as berberine are mainly related to the presence of quaternary nitrogen and the functions of oxygen necessary for the strong anti-plasmodic activity (27). The relationship between oxygenation and anti-plasmodic activity provides clues for possible molecular frameworks of synthesis and structure-activity relationship studies that could lead to the identification of new drugs (8). In-vitro studies indicate that berberine binds to tubulin inducing depolymerization of interphase and mitotic microtubules (28).

Concerning the tests with PZQ, males, and females, contracted without any movement at 4 h in addition to the detachment of the pairs and the detachment of the tegument. Previous studies indicated the effects of PZQ on nematodes, causing contractions when exposed to concentrations of 0.1 and 1 µg/mL, this is because PZQ causes a rapid influx of calcium followed by contraction, paralysis, and tegumental damage (29). PZQ caused severe muscle contractions and partial bend of the worms, as did berberine which caused paralysis and muscle contraction; however, the exact mechanisms underlying this effect are still unclear. In Am's case, it caused paralysis of the worms, but no contraction or curving. As the incubation time increased at higher doses of Am, the release of the tegument by the worms increased. In the case of berberine, it was observed from 5 µM, this is important because the tegument is involved in many functions that are of vital importance to *S. mansoni*, such as the absorption of nutrients (30). The tegument is the interface between the parasite and the host environment; therefore, it represents an important target for many drug actions. Different investigations evaluated natural compounds to evaluate the in-vitro and in-vivo activity and found alterations of the tegument surface as in our study, they were able to observe, induction of tegument damage in the immature and mature stages after in-vitro incubation, which depended on the dose and time, i.e. it intensified progressively as the incubation period and the concentration of treatments increased, and it was also possible to observe shrinkage, corrugation, unfolding and widening of the gynecophoral canal (31).

To evaluate the safety of the extracts, hemolysis tests were performed. As can be seen in Table 2, Am did not show high toxicity, being 2.9% at its highest concentration tested and presented almost no toxicity at the lowest concentration tested, this following Karimi et al. criteria (32), in which Am could be classified as a low toxicity, the probable hemolysis presented is related to mechanical stress at the time of agitation during the incubation time. In the case of berberine, hemolysis was proportional to the concentration, since as the dose was increased, hemolysis also increased. Berberine at 5 µM is effective against *S. mansoni* at 48 h, this dose is far below the lowest concentration evaluated in the cytotoxicity test, berberine stands out since in minimal doses it´s biological activity is very effective. These data are consistent with those previous-

ly reported (15). Therefore, at concentrations of interest, below 200 µg/mL (Table 2), Am and berberine were not found to be significantly cytotoxic ($P<0.001$). Berberine is not toxic, cytotoxic, or mutagenic at doses based on clinical situations, however, its side effects may result from high doses (33).

DPPH radical assays are considered representative among the various methods for evaluating the ability to capture free radicals, the antioxidant activity of plants varies according to the part of the plant used, its state of maturation, this is related to the phenolic content of the plant and the radical scavenging efficiency of DPPH will depend on this (34). The EC_{50} determined for Am and berberine was 156.3 and 84.1 µg/mL respectively, in the case of quercetin, it was 1.03 µg/mL. Based on the results Am and berberine, have a moderate capacity to capture free radicals compared to quercetin. These results are relevant because recent studies have shown that the combinations of antioxidant and anthelmintic activities could show antiparasitic synergistic effects (35). The ability to capture free radicals in plants of the Berberidaceae family has been reported due to some flavonoids present such as anthocyanins, which have multiple functions in plants such as protection under ultraviolet radiation (36).

Conclusion

The methanolic extract of *A. mexicana* and the principal compound berberina, showed high activity against *S. mansoni* in low concentration in the in-vitro model. However, in-vivo efficacy requires to be evaluated in an animal model.

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Conflict of interest

The authors declare that there is no conflict of interest.

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