



Evaluation of the Molluscicidal, Artemicidal and Cytotoxic Activities of the Lectin from *Opuntia ficus-indica* Cladodes (OfiL)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors GMSS, LPA and PMGP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EVP, LRSS, JSA, TGS, AMMAM and THN managed the analyses of the study. Authors GMSS, LPA, EVP and THN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: *Biomphalaria glabrata* is an intermediate host for the larvae of *Schistosoma mansoni*, which is distributed widely in the tropics. *B. glabrata* control is important to minimize the spread of schistosomiasis and natural compounds have been sought for use against this disease. The *Artemia salina* bench-top bioassay has been used to investigate the ecotoxicity of many natural compounds, and its results also correlate well with the *in vitro* cytotoxicity of natural compounds to tumor cells.

Aims: To evaluate deleterious effects of the *Opuntia ficus-indica* lectin (OfiL) on *B. glabrata*, *A. salina* and human cancer cell lines.

Methods: OfiL was isolated following a previously established protocol. The effects of OfiL on *B. glabrata* were investigated by determining survival of adults as well as development and hatching of embryos. The concentration required to kill 50% (LC₅₀) of *A. salina* nauplii was determined. The cytotoxicity was determined using the human cell lines Hep-2 (human larynx epidermoid carcinoma), NCI-H292 (human lung mucoepidermoid carcinoma) and K562 (chronic myelocytic leukemia).

Results: The development of most embryos (92.5–97.5%) treated with 1, 10 and 100 µg/mL of OfiL was found to be delayed, and dead (2.2–3.3%) and malformed (0.3–5.2%) embryos were also observed. OfiL did not kill *B. glabrata* adults, but a high percentage (30–45%) of the embryos generated by snails incubated with the lectin exhibited malformations. OfiL exhibited toxicity against *A. salina* (LC₅₀: 61.02 µg/mL) but did not display cytotoxicity against the tumor cell lines evaluated.

Conclusion: In conclusion, this study showed that OfiL can be a tool for schistosomiasis control that acts by impairing the viability of *B. glabrata* eggs and the fecundity of adult snails.

Keywords: *Cactus pear*; *lectin*; *Biomphalaria glabrata*; *Artemia salina*; *antineoplastic activity*.

1. INTRODUCTION

Schistosomiasis (bilharziasis) is a chronic disease caused by trematode worms (blood flukes) of the genus *Schistosoma* that affects at least 230 million people every year [1]. Snails in the genus *Biomphalaria* are intermediate hosts for the larvae of *Schistosoma mansoni*, and thus the reduction of snail populations in areas where the disease is endemic constitutes an important component of integrated strategies for schistosomiasis control. *Biomphalaria glabrata* is widely distributed in various parts of South America, Central America, and Africa, and has great importance to public health in Brazil [2,3].

Many chemicals, such as niclosamide, have been used to control *B. glabrata*, but environmental concerns have been raised over the use of most of them because they can affect non-target organisms, especially fishes [4,5]. The activities of molluscicidal preparations obtained from plants and lichens have previously been evaluated against *B. glabrata* snails and embryos [6–9]. The compounds present in these preparations usually have lower toxicity than synthetic compounds against nontarget organisms, but caution is still required in the use of some of them due to their potential environmental toxicity, for example that detected using the bench top *Artemia salina* assay [10].

Lectins are carbohydrate-binding proteins that interact with carbohydrates or glycoconjugates and have been shown to have toxic effects against fungi, bacteria, protozoans, and insects [11,12]. Lectins from the seeds of *Cratylia floribunda* and *Dioclea guianensis* were previously found to be promising molluscicidal compounds, since they killed *B. glabrata* snails at a median lethal dose (LC₅₀) of 13 and 12 µg/mL respectively [13]. The lectin from the rhizome of *Microgramma vacciniifolia* (MvRL) was also able to kill *B. glabrata* embryos and adults when tested at a dose of 100 µg/mL [6].

Artemia salina (brine shrimp) is a model organism that is frequently used to evaluate the likely toxicity of compounds in aquatic environments. An advantage of using *A. salina* as a test organism in toxicity bioassays is that it is extremely sensitive to many of the chemical substances discharged into aquatic environments [14]. The use of *A. salina* bioassays is recommended to evaluate the toxicity of pesticides, mycotoxins, stream pollutants, anesthetics, dinoflagellate and plant toxins, morphine-like compounds, and oils [15]. In addition, the effect of a given compound on *A. salina* can be considered indicative of its *in vitro* cytotoxicity against human solid tumor cell lines [16,17]. For example, the lectin from *Crataeva tapia* bark promoted *A. salina* mortality, with an

LC₅₀ of 71.73 µg/mL, and showed antitumor activity *in vivo* against sarcoma 180 cells in Swiss albino mice [18].

The cladodes of *Opuntia ficus-indica* (L.) Mill. (Cactaceae) have diuretic, antiulcer and wound healing activities [19–21]. A chitin binding lectin (OfiL) with a molecular mass of 8.4 kDa isolated from the cladodes of *O. ficus-indica* was previously found to have antifungal activity against the phytopathogens *Colletotrichum gloeosporioides*, *Fusarium decemcellulare*, *Fusarium lateritium*, *Fusarium oxysporum*, and *Fusarium solani*, and was also highly bioactive against the human pathogen *Candida albicans* [22]. OfiL also had deleterious effects on the insects *Nasutitermes corniger* [23] and *Sitophilus zeamais* [24].

This study reports the results of an assessment of the molluscicidal activity of OfiL against *B. glabrata* embryos and adults, as well as its toxicity against *A. salina* and possible antineoplastic activity against human cancer cell lines.

2. MATERIALS AND METHODS

2.1 Plant Material

Cladodes of *O. ficus-indica* were collected from different plant specimens at Limoeiro, Pernambuco, northeastern Brazil, with the authorization (38690) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio). The cladodes were dried at 28°C for 7 days, powdered, and stored at 4°C. Voucher specimens were deposited (access no. A03D92C) at the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen).

2.2 Adult Snails and Embryos of *B. glabrata*

Adult *B. glabrata* snails were reared at the *Departamento de Biofísica e Radiobiologia* of the *Universidade Federal de Pernambuco* (Recife, Brazil). The snails were kept at 25–30°C in plastic tanks (50 × 23 × 17 cm) containing filtered and dechlorinated water and aerators. Adult snails were reared with lettuce leaves provided as food. Transparent plastic strips were placed in the tanks to serve as substrates for oviposition.

2.3 *Artemia salina* Larvae

A. salina cysts (San Francisco Bay Brand, Inc., USA) were hatched at 25–30°C in natural seawater, the pH of which had been previously adjusted to 8.0–9.0 using Na₂CO₃ [25]. Cysts (25 mg) were placed in one of the two compartments of a container separated by a boundary plate. The compartment with the cysts was covered to keep the cysts in dark ambient conditions. The other compartment was illuminated to attract the phototropic newly hatched larvae to this compartment through perforations in the boundary plate. After 24 h, the brine shrimp larvae that had moved into the illuminated compartment were collected and used in the lethality assay.

2.4 Human Cancer Cells

Cultures of the cell lines K562 (chronic myelocytic leukemia), NCI-H292 (human lung mucoepidermoid carcinoma cells) and Hep-2 (human larynx epidermoid carcinoma cells) were obtained from the Instituto Adolfo Lutz (São Paulo, Brazil). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®, Life Technologies, USA), supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/mL streptomycin and maintained at 37°C with 5% CO₂.

2.5 Isolation of OfiL

OfiL was isolated according to the protocol described by Santana et al. [22]. Cladode powder (10 g) was homogenized (for 16 h at 4°C) in 50 mL of 0.15 M NaCl. After filtration through gauze followed by centrifugation (at 4000× g for 15 min), the crude extract (25 mg of protein) was loaded into a chitin column (7.5 × 1.5 cm) equilibrated with 0.15 M NaCl at a flow rate of 20 mL/h. The column was washed with the equilibrium solution until its absorbance was negligible. Afterwards, OfiL was eluted from the column with 1.0 M acetic acid and dialyzed (through a 3.5 kDa cut-off membrane) against 0.15 M NaCl for 6 h at 4°C for the elimination of the eluent.

2.6 Protein Concentration and Hemagglutinating Activity

The protein concentration was estimated according to the method of Lowry et al. [26] using bovine serum albumin (31–500 µg/mL) as a standard. Hemagglutinating activity (HA) was

assayed in microtiter plates (TPP-Techno Plastic Products) according to the method of Paiva and Coelho [27]. A glutaraldehyde-treated erythrocyte suspension (2.5% (v/v) in 0.15 M NaCl) was obtained following the method of Bing et al. [28]. HA was determined by mixing a twofold serial dilution of OfiL (50 μ L) with 0.15 M NaCl in microtiter plates. The erythrocyte suspension (50 μ L) was then added to each well and the plate was incubated at 27°C for 45 min. The HA was defined as the reciprocal value of the highest dilution of the sample that promoted full agglutination of the erythrocytes. The specific HA was defined as the ratio between the HA and the protein concentration. The HA of OfiL was also determined after the lectin was incubated for 45 min with 200 mM glucose before the addition of the erythrocyte suspension.

2.7 Molluscicidal Assays

The assay of the toxicity of OfiL against the embryos of *B. glabrata* was carried out according to the methods of Oliveira-Filho and Paumgarten [29]. Intact egg masses were observed under a stereomicroscope (Leica MZ6), and embryos in the blastula stage (0–15 h after spawning) were selected. Five egg masses containing approximately 100 eggs each were placed in Petri plates (90 x 15 mm) and exposed to 10 mL of OfiL (at doses of 1, 10, or 100 μ g/mL) or filtered tap water (control) for 24 h. Next, the egg masses were washed with tap water and placed in Petri plates containing filtered and dechlorinated water. The assay was maintained at 25 \pm 2°C until the embryos hatched. Mortality and the occurrence of abnormal development (malformations) of embryos was evaluated for 7 days. In addition, the embryo stages in the OfiL and control treatments were recorded, according to Albuquerque et al. [6], until all embryos in the control had hatched. Three independent experiments were performed, and all treatments were performed in triplicate.

The bioassay of the lectin's adulticidal activity against *B. glabrata* was carried out according to the protocol of the World Health Organization [30], following the recommendations of the Expert Committee on the Bilharzia. Ten healthy adult snails of uniform size (shell diameter of 10–13 mm) were immersed for 24 h in a glass jar containing 250 mL of OfiL (at doses of 1, 10, or 100 μ g/mL), cooper (II) carbonate (50 μ g/mL; positive control), or filtered tap water (negative control). After exposure, the adult snails were washed with tap water and then placed in filtered and dechlorinated water. The snails were

incubated at 25 \pm 2°C and fed lettuce leaves. Snail survival was evaluated daily for eight days to determine the percent survival (%) in each treatment. Snails were considered dead when they showed prolonged inactivity or underwent changes in their coloration patterns. The numbers of embryos generated by adult snails from the OfiL and control treatments, and the number of unviable embryos (dead and malformed embryos, as well as those with delayed development) from each treatment was recorded. The bioassay was performed in triplicate for each concentration, and three independent experiments were performed.

2.8 Artemia salina Lethality Assay

The artemicidal activity of OfiL was evaluated according to the method of Meyer et al. [15]. The assay was performed in test tubes containing groups of 12–15 *A. salina* larvae, which were exposed to solutions containing OfiL (10–100 μ g/mL) diluted in natural seawater to a final volume of 5 mL. After 24 h, the percent survival (%) was recorded in each treatment, and the concentration required to kill 50% of the larvae (LC_{50}) was determined. As a negative control, larvae were incubated in seawater. Three independent experiments were run, with each treatment performed in triplicate.

2.9 Cytotoxicity Assay

Adherent NCI-H292 and Hep-2 cells (10^5 cells/mL) or suspended K562 cells (0.3×10^6 cells/mL) were plated (100 μ L) in 96-well microtiter plates. After 24 h, 100 μ L of OfiL (6.25–50 μ g/mL) was added to each well, and the plates were incubated for 72 h at 37°C. Then, 100 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 5.0 mg/mL) was added to the well plates, and the viability of the tumor cells in them was estimated based on their ability to reduce the yellow tetrazolium to form a blue formazan product [31,32]. After 3 h, the formazan product was dissolved in dimethyl sulfoxide and the absorbance at 450 nm was measured using a microplate reader (Multiplate Reader Thermoplate). Negative control groups were also included, which corresponded to cells not treated with the lectin. Three independent assays were performed, with each treatment performed in triplicate.

2.10 Statistical Analyses

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows

(GraphPad Software, San Diego, California, USA), and all data were expressed as the mean \pm SD of all replicates for each treatment. Whether there were significant differences between the treatment groups used in the bioassays was determined by performing Student's *t*-tests (with a significance threshold of $p < 0.05$) using the Origin 6.0 program. The lethal concentrations required to kill 50% (LC₅₀) of *A. salina* larvae, as well as *B. glabrata* embryos and adult snails, were calculated by probit analysis with a reliability interval of 95% using the software StatPlus® 2006 (AnalystSoft, Canada). The concentration that inhibited 50% of tumor cell growth (IC₅₀) was also determined by performing a non-linear regression using the software SigmaPlot 11.0 (Systat, USA), with a reliability interval of 95%.

3. RESULTS AND DISCUSSION

Schistosomiasis occurs predominantly in tropical and sub-tropical areas, especially in poor communities without access to safe drinking water and adequate sanitation. In developing countries, population growth has aggravated these sanitation problems, leading to increased transmission of schistosomiasis [1]. This situation and the need for new alternative methods for controlling *B. glabrata*, stimulated the evaluation of the molluscicidal activity of OfiL in the present study.

The saline extract of *O. ficus-indica* cladodes, containing 13.3 mg/mL of protein, showed a specific hemagglutinating activity of 2.4. The OfiL isolated from this extract by chromatography on a chitin column showed a specific hemagglutinating activity of 40, which was inhibited by glucose. These data are in accordance with those reported by Santana et al. [22] and Paiva et al. [23].

The results obtained in the embryotoxicity assay are shown in Table 1. The main difference observed between the OfiL treatments and the control was the much higher number of unhatched embryos in the lectin treatments, although even embryos that were alive and free of malformations in the OfiL treatments exhibited delayed development. Although the numbers of malformed embryos were significantly higher than in the control in the OfiL treatments, and the number of these observed increased with the OfiL concentration, the frequency of malformations still did not exceed 5.2%. OfiL was more active against *B. glabrata* embryos than the

lectin from *Sebastiania jacobinensis* bark, which did not promote embryonic mortality and did not induce malformations when tests were previously performed at a concentration of 200 μ g/mL [33]. Zuzack and Tasca [34] demonstrated that cell surface glycoconjugates are involved in the development of mouse embryos, and that incubation with the lectins wheatgerm agglutinin, concanavalin A, *Lotus (Tetragonolobus) purpureas* agglutinin, and *Limulus polyphemus* agglutinin resulted in the retardation of cell division, and consequently delayed embryonic development.

OfiL did not promote the mortality of adult *B. glabrata* snails at concentrations of 1, 10 and 100 μ g/mL (Fig. 1A). The exposure of snails to cooper (II) carbonate (positive control) resulted in 100% mortality, and this result shows that the assay conditions in the present study were proper for the detection of molluscicidal activity. Lectins isolated from *Canavalia brasiliensis* (ConBr), *Cratylia floribunda* (CFL), *Dioclea guianensis* (Dgui), *Dioclea grandiflora* (DGL), and *Dioclea virgata* (Dvir) seeds were previously reported to be molluscicidal agents; among these, Dgui and CFL were the most promising molluscicidal compounds, since they killed snails with LC₅₀ values of 13 and 12 μ g/mL respectively [13].

There were also no significant differences between the number of embryos generated by snails from the control group and those exposed to OfiL (Fig. 1B). However, the embryos present in the egg masses generated by snails exposed to all concentrations of OfiL showed significantly more malformations (Fig. 1C). Among the embryos with abnormal development, exogastrulae (abnormal embryos that did not complete gastrulation and did not form normal neural structures) were detected, and unspecific malformations, which corresponded to teratomorphic embryos whose specific malformation could not be described well, occurred the most frequently. These results indicate that OfiL was able to persist in the bodies of adult snails, affecting the viability of the embryos they generated. In this way, despite it not having a direct molluscicidal effect, OfiL is still a potential tool for use in the control of schistosomiasis by preventing the spread of *B. glabrata*. The deleterious effects of lectins on the reproductive activities of invertebrates have been described previously. For example, Silva et al. [35] reported that larvae of *Aedes aegypti* exposed to lectin preparations from *Moringa*

oleifera seeds developed into adult females that produced eggs with reduced viability.

The brine shrimp lethality bioassay revealed that OfiL promoted the mortality of *A. salina* larvae in a dose dependent manner, with an LC₅₀ value of 61.02 µg/mL. The molluscicidal lectins CFL and Dgui also previously showed artemicidal activities, with LC₅₀ values of 4.75 and 5.21 µg/mL, respectively [13], meaning that these lectins were more toxic than OfiL. The toxicity of plant lectins against *A. salina* has been variable.

OfiL was less toxic against *A. salina* than the lectins from *M. oleifera* leaves (SLL-1, SLL-2, and SLL-3), which showed LC₅₀ values of 15.8, 17.78, and 14.12 µg/mL respectively [36]. The lectin from *Crataeva tapia* bark promoted *A. salina* mortality with an LC₅₀ of 71.73 µg/mL [18], a value close to that determined in the present study for OfiL. On the other hand, the lectin isolated from the bark of *Sebastiania jacobinensis* had very low toxicity against *A. salina*, with an LC₅₀ of 715.89 µg/mL, and was not toxic against *B. glabrata* embryos [33].

Table 1. Evaluation of embryotoxicity of *Opuntia ficus-indica* lectin (OfiL) against *Biomphalaria glabrata*

Treatments	Viable embryos	Unviable embryos		
		Delayed (%)	Malformed (%)	Dead (%)
OfiL				
1 µg/mL	0.0 a	97.52 ± 4.89 a	0.33 ± 0.57 a	2.16 ± 4.38 a
10 µg/mL	0.0 a	94.42 ± 3.52 a	2.29 ± 0.61 ^b	3.33 ± 3.06 a
100 µg/mL	0.0 a	92.47 ± 1.43 a	5.24 ± 1.45 ^c	2.29 ± 2.26 a
Negative control	99.31 ± 0.5 b	0.0 b	0.0 d	0.69 ± 0.5 b

Different letters indicate significant ($p < 0.05$) differences between the treatments

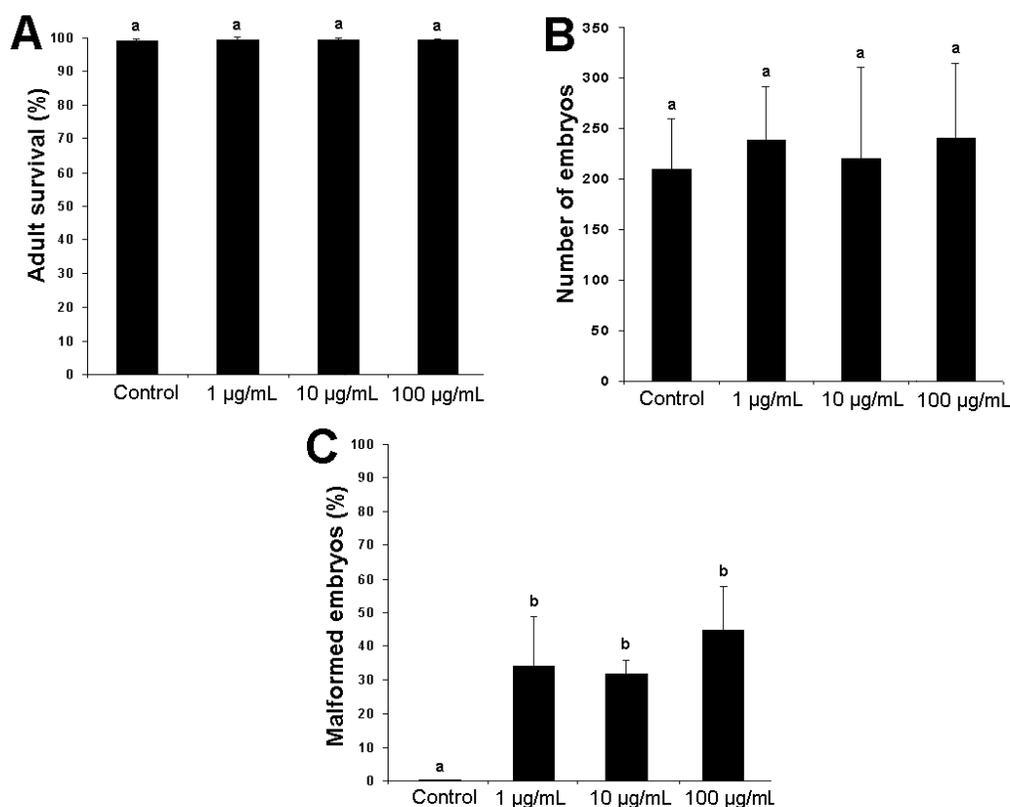


Fig. 1. Effects of *Opuntia ficus-indica* lectin (OfiL) on the survival of *Biomphalaria glabrata* adults (A), as well as on the number (B) and viability (C) of the eggs deposited by these snails
Different letters indicate significant ($p < 0.05$) differences among treatments

The artemicidal mechanisms of lectins remain unknown. However, Raineri [37] highlighted the fact that several structures in the body of *A. salina* larvae are composed of chitin, including the anterior parts, setae of the appendages, and joints, as well as portions of the digestive tract, such as the oesophagus and hindgut. These regions may constitute potential targets for interactions with chitin binding lectins, which is a plausible reason for the toxicity of OfiL against *A. salina* larvae.

The brine shrimp lethality bioassay is used as an indicator of cytotoxicity and a wide range of biological properties, such as antitumor activity [38]. Plant lectins have previously been reported as potential therapeutic agents due to their remarkable antitumor properties [39,40]. The artemicidal activity of OfiL stimulated its evaluation as an antitumor agent herein. However, OfiL did not exhibit cytotoxicity against all the tumor cell lines tested ($IC_{50} > 100 \mu\text{g/mL}$). Etoposide was used as a positive control, and exhibited IC_{50} values of 2.75, 6.1 and 4.48 $\mu\text{g/mL}$ against Hep-2, NCI-H292, and K562 cells, respectively.

4. CONCLUSION

OfiL could be a promising tool for use in the control of schistosomiasis because it delayed the development of *B. glabrata* embryos, and some eggs generated by *B. glabrata* snails treated with this lectin showed malformations, which would prevent the spread of this mollusk. OfiL was toxic against *A. salina* larvae but did not show potential for use as a chemotherapeutic agent against the three human cancer cell lines evaluated.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

Rabbit erythrocytes were collected following a protocol approved by the Ethics Committee on

Animal Experimentation of the *Universidade Federal de Pernambuco* (process no. 23076.033782/2015-70).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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