ECOSISTEMAS Y RECURSOS AGROPECUARIOS

Toxicological evaluation of three methods of applying *Rhizophora mangle* on *Galba cubensis* (Mollusca: Gastropoda: Lymnaeidae)

Evaluación toxicológica de tres métodos de aplicación de *Rhizophora mangle* sobre *Galba cubensis*: (Mollusca: Gastropoda: Lymnaeidae)

Luis José Rangel-Ruiz*, Juan Armando Arévalo-de la Cruz, Guillermo González-González, José Ángel Hernández-Ovando, Jaquelina Gamboa-Aguilar, Juan de Dios-Valdez Leal

[1] Laboratorio de Malacología. División Académica de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco. Carretera Villahermosa-Cárdenas Km. 0.5 entronque con Saloya, CP. 94250, Villahermosa, Tabasco, México Tel. Fax (993) 354-43 08. Autor de correspondencia: ljrangel@msn.com

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ABSTRACT. The molluscicidal efficacy of *Rhizophora mangle* on *Galba cubensis* was evaluated through three bioassays: two using aqueous extracts, one raw method extract (RME) and the other infusion method extract (IME), and direct application of powder (DAP). A completely randomized experimental design with three treatments, a control and five replicates was used. LD_{50} and LD_{90} were determined using Probit-Log software. RME was used at 500, 400, 333 and 289 g L^{-1} concentrations; IME at 50, 25, 12.5 and 6 g L^{-1} ; and DAP extract at 0.540, 0.270 and 0.135 g. IME residence time and toxicity at 50 g L^{-1} in the substrate were also determined. We recorded 96, 80, 76, and 59 % cumulative mortalities with RME at 500, 400, 333, and 286 g L^{-1} concentrations, respectively; LD_{50} was 261.4 g L^{-1} and LD_{90}, 442.79 g L^{-1} . For IME, it was 100, 94, 90 and 16 % for the concentrations of 50, 25, 12.5 and 6 g L^{-1} ; LDL50 was 8.57 g L^{-1} and LD_{90}, 15.83 g L^{-1} . Mortalities with DAP extract were 70, 58, and 22 % at the 0.540, 0.270 and 0.135 g concentrations respectively, none of which reached LD_{90} and only the 0.540 g concentration reached LD_{50}. IME residence and toxicity at 50 g L^{-1} decrease significantly after eight hours.
Key words: Molluscicidal, mangrove, *Galba Cubensis*

RESUMEN. La eficiencia molusquicida de *Rhizophora mangle* sobre *Galba cubensis* se evaluó mediante tres bioensayos; dos métodos de extracto acuoso: extracto por reposo en crudo (EMRC), extracto por infusión (EMI) y aplicación directa de polvo (ADP). Se utilizó un diseño completamente azar, con tres tratamientos, un testigo y cinco repeticiones. En la determinación de la DL50 y DL90 se utilizó el programa Probit-Log. Para el extracto EMRC se utilizaron las concentraciones de 500, 400, 333 y 289 g L^{-1}; para el EMI fue de 50, 25, 12.5 y 6 g L^{-1}; y para la ADP fue de 0.540, 0.270 y 0.135 g. Se determinó el tiempo de permanencia y toxicidad del EMI a 50 g L^{-1} en el sustrato. Las mortalidades acumuladas para el EMRP fueron del 96, 80, 76 y 59 % en las concentraciones de 500, 400, 333 y 286 g L^{-1}, la DL50 fue de 261.46 g L^{-1} y la DL90 de 442.79 g L^{-1}. Para el EMI fue del 100, 94, 90 y 16 % para las concentraciones de 50, 25, 12.5 y 6 g L^{-1}, la DL50 fue de 8.57 g L^{-1} y la DL90 de 15.83 g L^{-1}. Para la ADP del 70, 58 y 22 % en las concentraciones de 0.540, 0.270 y 0.135 g, solo la concentración de 0.540 g alcanzo la CL50. La permanencia y toxicidad del EMI a 50 g L^{-1} disminuye después de ocho horas.
Palabras clave: Molusquicida, mangle, *Galba cubensis*

INTRODUCTION

Hepatic distomatosis is a helminth disease distributed worldwide, affecting humans, livestock (cattle, sheep, goats) and other mammals (Olaechea 2004). To complete its life cycle, *Fasciola hepatica* needs two hosts: one intermediate, a snail of the family Lymnaeidae, and the other defini-

tive, a mammal (Cruz 2011, Ibarra *et al*. 2011). Distomatosis is one of the most important parasitic diseases which results in great economic losses to livestock farmers by causing low milk production and quality, reducing the growth rate and increasing reproductive disorders and mortality from infections (Milian 1986).

The intermediate host is the weakest link in the life cycle of fascioliasis and schistosomiasis, so the control of molluscs should be aimed at decreasing their populations (Zaid *et al*. 2013). To control the intermediate host, biological, ecological and chemical methods are used. The first introduces competitors or predators; the ecological one changes the conditions of the natural environment to prevent its development, and the chemical one uses inorganic and organic chemicals (Cruz 2011). Chemical molluscicides have the disadvantage of altering the structure of the environment, acting as biocides on flora and fauna (Rawani *et al*. 2014). The use of organic molluscicides for control purposes is important because it reduces environmental impact (Iannacone *et al*. 2008).

Globally, toxicity studies with plant extracts have been conducted in recent years to control the hosts of fascioliasis and schistosomiasis (Iannacone *et al*. 2013, Zaid *et al*. 2013). In particular for *Galba cubensis*, the molluscicidal activity of *Melia azedarach* (Piña *et al*. 1998) and *Momordica charantia* (Diéguez *et al*. 2012) has been successfully tested, resulting in snail mortality. In the state of Tabasco mangrove occupies 41 498.5 ha, of which 13 615.0 ha are *Rhizophora mangle* (Domínguez-Domínguez *et al*. 2011). Therefore, the aim of the study was to evaluate the molluscicidal efficacy of *Rhizophora mangle* against *Galba cubensis* as an alternative to reduce the livestock sector's economic losses caused by fascioliasis in the state of Tabasco.

MATERIALS AND METHODS

A total of 300 *Galba cubensis* adults, 4 to 6 mm in length, were collected from September to November 2011, at La Nueva Luz Ranch, located in the Jahuacapa ranchería at coordinates 17° 44' 25.76" NL and 92° 50' 25.25" WL in the municipality of Jalapa, Tabasco. Sediment and algae were also collected from the environment to make terrariums in 1 m^3 tubs to acclimatize the snails.

In addition, 7 kg of *Rhizophora mangle* leaves were collected in the town of Arroyo Polo in the municipality of Centla, Tabasco. To obtain the raw method extract (RME) at the 500 g L^{-1} concentration, 5 kg of fresh leaves were weighed, rinsed and liquefied in 10 L of water to obtain small particles of the plant material. The extract was steeped in 20 L plastic buckets for five days, after which the plant material was removed by filtering to obtain the aqueous extract without particles. Concentrations of 400, 333 and 286 g L^{-1} were obtained by dilutions. For the infusion method extract (IME), 1.5 kg of fresh leaves were dehydrated at a temperature of 60 $\pm$ 2 °C for 24 h and ground with a household mill. After that, 50 g of powder were taken and put in 1 L of water, then boiled for 10 min and left to cool at room temperature. It was then filtered and extract without plant residues was obtained for the 50 g L^{-1} concentration; the procedure was repeated in the same way to obtain the concentrations of 25, 12.5 and 6 g L^{-1} . For the direct application of powder (DAP), 0.135, 0.270 and 0.540 g of the preground material were weighed. In all *Rhizophora mangle* treatments, the pH, temperature and salinity were determined.

Toxicity testing of the RME and IME was performed under a completely randomized design with five treatments, four concentrations and a control, with five replicates, while for the DAP three concentrations were tested. For the RME, IME and DAP applications, 75 terrariums were prepared in 15 cm diameter plastic trays, to which 200 g of sterilized substrate were added. The substrate was saturated with dechlorinated water to simulate the habitat of snails; 10 snails were placed in each terrarium, and 3 ml of the extracts were applied to each snail with a sprayer, whereas the powder was applied directly.

Observations were made every 2 h to quantify snail mortality. To determine whether they were alive or dead, body movement in response to light and touch with a dissecting needle was

used as a reference. To establish significant differences between concentrations, the Kruskal-Wallis nonparametric test and the multiple range test were applied, with the Statgraphics Centurion XV statistical program. Probit-Log regression was used with the SPSS Statistics© Version 20 program to determine LD_{50} and LD_{90}, while the lethal times LT_{50} and LT_{90} for each concentration were considered as the time it takes for 50 and 90 % of the exposed snails to die.

To determine the residence time and toxicity loss of the *Rhizophora mangle* IME at 50 g L^{-1} on the substrate, four lots of five terrariums, each with 200 g of substrate saturated with dechlorinated water, were prepared. At the beginning 3 ml of the extract were applied with a sprayer to the four lots. At 0 h, 10 snails were placed in each of the first-lot terrariums; at 2 h the same number of snails was introduced into the second lot, at 4 h in the third lot, and at 6 h in the fourth lot. Mortality was determined every 2 h after the snails were introduced into each lot.

RESULTS

The mortality obtained by RME of *Rhizophora mangle* on *G. cubensis* had high toxicity. At 96 h the 500 g L^{-1} concentration had a cumulative mortality of 96 %, followed by the 400, 333 and 286 g L^{-1} concentrations with 80, 76 and 58 %, respectively, which were different from each other (P= 0.001), while the control treatment had no mortality (Figure 1). LT_{50} occurred at 3 h for the concentration of 500 g L^{-1} , at 14 h for 400 g L^{-1} , at 29 h for 333 g L^{-1} and at 74 h for 289 g L^{-1} . LT_{90} occurred with the 500 g L^{-1} concentration at 62 h. According to Probit-Log regression analysis, LD_{50} was obtained at the concentration of 261.46 g L^{-1} and LD_{90} at 442.79 g L^{-1} .

The cumulative mortality caused by the IME was 100, 94, 90 and 16 % for the concentrations of 50, 25, 12.5 and 6 g L^{-1} , respectively, while the control treatment showed 0.00 % mortality. The 6 g L^{-1} concentration was the only one that did not achieve LD_{50} or LD_{90}, which meant that there were no differences between this concentration and the others (Figure 2). The concentrations of 50, 25 and 12.5 g L^{-1} exceeded LT_{50} in 2 h and LT_{90} was only reached at the 50 and 25 g L^{-1} concentrations at 5 h. According to Probit-Log regression analysis, LD_{50} was obtained at a concentration of 8.57 g L^{-1} and LD_{90} at 15.83 g L^{-1} .

For the direct powder application, cumulative mortality at 96 h was 70, 58 and 22 %, for the concentrations of 0.540, 0.270 and 0.135 g, respectively, with the 0.135 g concentration showing significant (P= 0.0028) differences (Figure 3). At the end of the bioassay the control sample showed no mortality. LD_{50} was obtained by the 0.540 g concentration with a LT_{50} at 5 h after application and the 0.270 g concentration at 72 h. LD_{90} did not occur with any of the three concentrations. According to Probit-Log regression analysis, LD_{50} was obtained at a concentration of 0.270 g and LD_{90} at 1.079 g (Table 1).

Table 1. Physicochemical parameters of two extracts and powder of *Rhizophora mangle*.

pH	Temperature	^{o}C	Salinity ppm
RME	5.00	24.7	1.4
IME	4.93	24.5	1.4
DAP	5.06	25.2	1.2

IME residence time at 50 g L^{-1}

The toxic effect of IME at 50 g L^{-1} showed a decrease in *G. cubensis* mortality from time zero to 8 h. The highest cumulative mortality was in lot one with 48, 72, 82 and 92 % at 2, 4, 8 and 96 h; in lot two it was 14, 24 and 30 % at 2, 4 and 6 h, in lot three 2 and 4 % at 2 and 4 h, in lot 4 % at 2 h, and in the control group there was no mortality. Lot one and two with 2 and 4 h were statistically different from the other lots (P= 0.0000) (Figure 4).

DISCUSSION

The molluscicidal activity of substances extracted from plants is studied in order to reduce the costs resulting from applying chemical molluscicides, which are bioaccumulative and unspecific and alter the structure of the environment by acting as

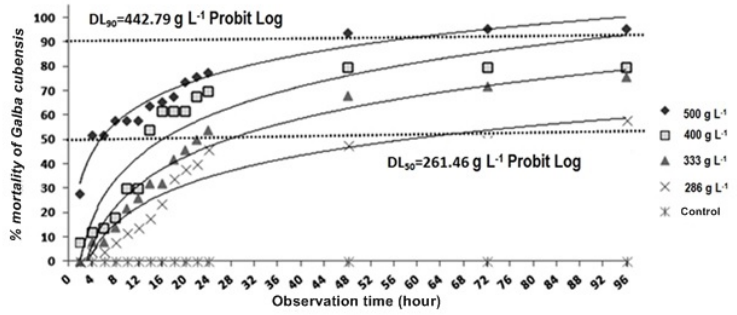

Figure 1. Mortality of *Galba cubensis* by *Rhizophora mangle* extract obtained by the raw extraction method, and determination of lethal doses LD_{50} and LD_{90}.

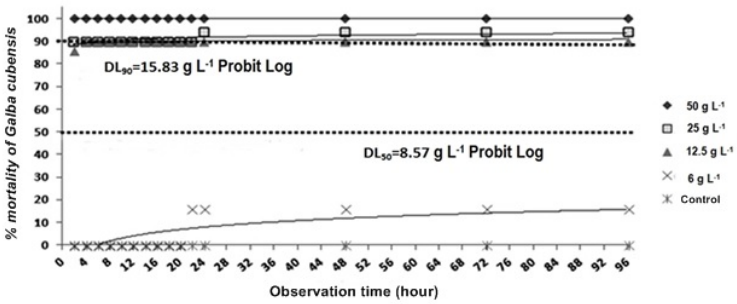

Figure 2. Mortality of *Galba cubensis* by *Rhizophora mangle* extract obtained by the infusion method, and determination of lethal doses LD_{50} and LD_{90}.

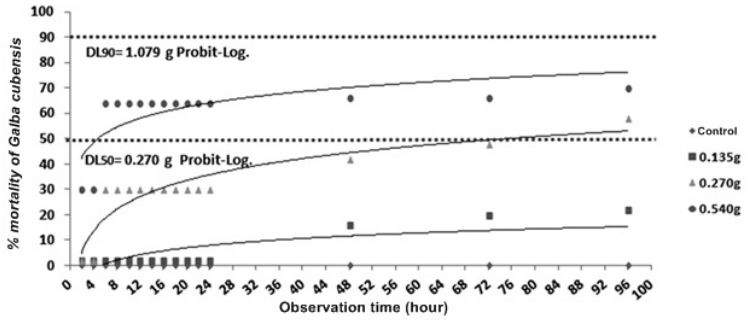

Figure 3. Mortality of *Galba cubensis* by direct application of powder of *Rhizophora mangle* and determination of lethal doses LD_{50} and LD_{90}.

biocides (Vasconcelos and Amorim 2003). Therefore, a molluscicide must have the following characteristics: the raw extract must be active at concentrations of less than 100 g L^{-1} for freshwater snails; the plant should grow abundantly in the environment where one wishes to use it, and have a high capacity for regeneration or replacement of the parts used for the extract; extraction should be easy and inexpensive and it must show low toxicity to other organisms (Marston *et al.* 1993). In the present study the molluscicide made with the infusion extract covers the above conditions, as it is toxic, affordable and sustainable.

The *Rhizophora mangle* extract was effi-

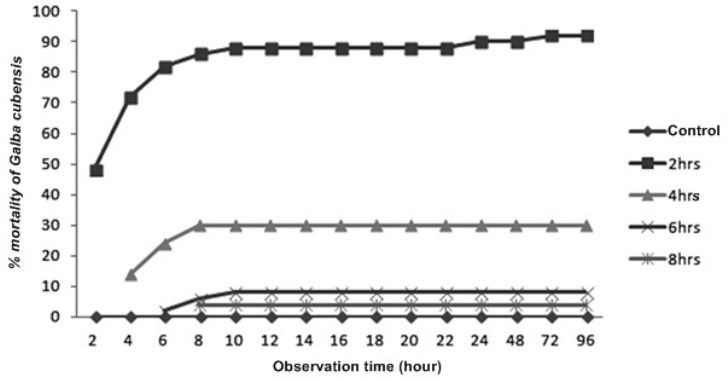

Figure 4. Mortality of *G. cubensis* at four introduction times after application of IME at 50 g L^{-1} of *Rhizophora mangle*.

cient due to its high toxicity at a low concentration and short exposure time to *G. cubensis*, an amphibious snail that dwells in the land-water interface and remains submerged for a few minutes (Rangel-Ruiz 1994). The above should be taken into account, since when applied in the field the extract is absorbed, dilutes in the substrate or evaporates quickly. The opposite is true when applied to aquatic snails, since exposure times can last up to 96 h (Chauhan and Singh 2011, Chen *et al*. 2012, Chen *et al*. 2012). This allows applying concentrations lower than those used in the present study.

LD_{90} at 15.83 g L^{-1} obtained with the IME is advisable for environmental conditions in which *G. cubensis* dwells, since under experimental conditions the phytochemical acts by contact with the snail and at reduced exposure times. The application of the extract in terrariums resembling their natural habitat allowed defining lethal doses close to those required in the field. This is important, since field experience indicates that concentrations have to be increased from two to four times the concentration obtained in the laboratory (Carrillo 2001).

Of the mangrove species recorded in the state of Tabasco, *Rhizophora mangle* is the most abundant and best distributed in lagoons and coastal rivers; using its leaves is appropriate because they are produced easily and quickly, and cutting can be selective so as not to damage the plant, as this plant is subject to protection by Mexican Official Standard NOM-059-2010-SEMARNAT (SEMARNAT 2010) as a category (A) threatened species. In addition, it is protected by NOM-022-SEMARNAT 2003 since it is considered to be in danger of disappearing in the short or medium term, and by article 60 TER of the General Wildlife Law, which prohibits removal, filling, transplanting, pruning or any other activity affecting mangroves or their habitat (DOF 2011). For this reason, for the extensive use of mangrove leaves, they will have to be obtained from special plantations such as Forest Management Units (Ley General del Desarrollo Forestal Sustentable 2013).

The active compounds of molluscicides can be obtained at higher concentrations by extraction methods using chemicals such as methanol, chloroform and acetone or by complex extraction techniques (Hassan *et al*. 2012, Otarigho and Morenikeji 2012). However, the use of aqueous extracts and simple extraction techniques are the best option when attempting to make a direct technology transfer. The high tannin content of *Rhizophora mangle* extracts may explain their molluscicidal efficacy, as tannins are their main components, of which 80 % are polymeric and the rest hydrolyzable (Sánchez *et al*. 2005). In addition to these, *Rhizophora mangle* extracts are highlighted by the presence of epicatechins, catechins, chlorogenic acid, gallic acid, ellagic acid, gallotannins, ellagitannins and condensed tannins with proven antiseptic, antibacterial and antifungal activity, for which reason they have been

used to obtain herbal medicines due to the plants medicinal properties (Pérez *et al*. 2011, Sánchez *et al*. 2005, Alemán 2011). The greater efficiency in the release of secondary metabolites in water was higher in the infusion method extract, which is attributed to the solubility and heat resistance (Pérez 2011).

The infusion method due to drying and milling reduces moisture and facilitates storage for prolonged periods, which prevents microbial contamination and interrupts degradation processes caused by enzymes or ferments, as well as oxidation and hydrolysis reactions that affect the quality of the plant material and the metabolites responsible for its biological activity (Acosta 2006, Álvarez *et al*. 2007, Pérez *et al*. 2011). Natural plant factors and technological processes can produce variations, which have not yet been evaluated, in the obtaining and molluscicidal efficacy of the aqueous extract of *Rhizophora mangle*. For example, tree age, growth location, soil, salinity, climatic variations and nature genetic factors may affect the content and performance of the active ingredients (Sánchez *et al*. 2005).

CONCLUSIONS

To control *Galba cubensis*, intermediate host of *Fasciola hepatica* in Tabasco, the use of aqueous extract of *Rhizophora mangle* obtained at a concentration of 50 g L^{-1} by infusion is recommended as it eliminates 96 % of the snail population under laboratory conditions. Further research is required to determine the active compound in the aqueous extract of *Rhizophora mangle* that produces the molluscicidal effect, and to perform the necessary toxicological tests.

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