



## Evaluation of embryotoxic and embryostatic effects of the aqueous extract of *Rhizophora mangle* and tannic acid on eggs and larvae of *Aedes aegypti*

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### ABSTRACT

*Rhizophora mangle* is an abundant plant in mangroves and tannic acid is a polyphenol produced by the secondary metabolism of plants. The aim of the study was to evaluate the embryotoxic and embryostatic effects of the aqueous extract of *R. mangle* and synthetic tannic acid on eggs and larvae of *Aedes aegypti*. *A. aegypti* eggs were exposed in duplicate at concentrations of 250, 500, 750 and 1000 µg/mL of extract and tannic acid for a period of 14 days. Mineral water was used as a negative control. The eggs were observed and counted in a stereomicroscope (1.2x). In all extract concentrations there was stimulation in hatching in relation to the control, but only in concentration of 750 mg/mL it was statistically significant. In tannic acid (250µg/ml) there was significant stimulus in hatching, but in 500, 750 and 1000 µg/mL there was significant inhibition. All concentrations of aqueous extract and tannic acid on larvae showed embryotoxic and embryostatic effects when compared to the control. The aqueous extract of *R. mangle* showed effect on hatching of *A. aegypti* eggs and synthetic tannic acid showed embryotoxic and embryostatic effects. On larvae, both the aqueous extract as tannic acid showed embryotoxic and embryostatic effects.

**Key words:** *Aedes aegypti*, embryostatic, embryotoxic, *Rhizophora mangle*, tannic acid.

### INTRODUCTION

*Aedes aegypti* is the mosquito that transmits dengue, ZIKA fever, chikungunya fever and yellow fever. It is a serious public health problem given

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the complications that cause those arboviruses. This insect has acquired great adaptability to human dwellings, accompanying people on their migrations across continents. *Aedes aegypti* reproduces in clean water reservoirs, strictly in the home environment, distancing itself just meters from human habitations; therefore it is not usually found in Brazilian rural areas, where houses appear

isolated (Donalisio and Freitas 2014, Vasconcelos 2015).

The female mosquitoes are responsible for the spread of disease since the oviposition process is dependent on blood repasts. Egg maturation or oogenesis starts after the blood repast. In *Aedes aegypti* complete ovariole maturation is necessarily related to digestion of one or more blood repasts. If there is no complete intake of blood, the development of oocytes will not pass the first stage (Carron et al. 2007).

One of the strategies to combat arboviruses is the elimination of the vector through insecticides. As a result of the continued use of these products came the resistant populations. Moreover, undesirable effects of such insecticides as remaining for long periods of time cause environmental impacts. These facts stimulated research on natural products. Several studies point to compounds of plant origin with larvicidal activity for use in vector control (Park et al. 2002, Silva et al. 2003, Rajeswary and Govindarajan 2014).

Plants have been evaluated as sources of natural insecticides against *A. aegypti*, and larvicidal bioassays have been conducted using third (L3) and fourth (L4) instars or comparing the effect of plant extracts on larval development of L1–L4. Various studies have addressed the possibility of using the embryo culture technique as an assay for embryotoxic potential of xenobiotic compounds. The electron microscopy of *A. aegypti* larvae transmission treated with aqueous extracts of *Derris urucu* and *Indigofera suffruticosa* showed histological changes in the intestine, and larval mortality was associated with damage to peritrophic matrix (Gusmão et al. 2002, Vieira et al. 2012). The peritrophic matrix of insects consists of proteins, glycoproteins, proteoglycans, and chitin (Nation 2016) and its integrity is important in the digestive process, as well as to protect against invasion by microorganisms and parasites (Lievin-Le and Servin et al. 2006). Plants have

been evaluated as natural insecticides sources and bioassays have been conducted using larvae in third (L3) and fourth (L4) stages or comparing plant extracts effect on larval development L1-L4 (Murugan et al. 2007).

*Rhizophora mangle* L is the most frequently plant found in the Brazilian mangrove (Chapman 1970, Silva et al. 2005). It belongs to Rhizophoraceae family and is known by the popular name mangrove or red mangrove. It is a plant whose leaves, stem, roots and fruits have varied applications in folk medicine (Coelho-Ferreira 2009) being used for treatment against gastric ulcers (De-Faria et al. 2012) as an antibacterial (Melchor et al. 2001), antioxidant (Sánchez et al. 2006), anti-inflammatory (Marrero et al. 2006), antidiarrheal (Wendel et al. 2013) and also in healing skin wounds (Fernandez et al. 2002).

Leaf and stem extracts of *R. mangle* L. exhibited insecticidal activity against *Cylas formicarius* performed by triterpenoids that act synergistically with each other and with other compounds that potentiate this effect, indicating its potential as a new source of insecticidal products of natural origin, which are better tolerated and of quicker degradation in the environment than those of synthetic origin (Williams 1999).

Tannins are among the major polyphenolic compounds of *R. mangle* L., including both polymeric and hydrolyzables tannins (Berenguer et al. 2006). Tannic acid is a hydrolysable tannin, produced by the secondary metabolism of plants and belongs to the large category of phenolic acids. It is found in many foods such as grapes, lentils, chocolate, red wine, beer, coffee, black tea and green tea. (Apud Genaro-Mattos 2009).

Studies have shown that tannic acid has embryotoxic activity on Culicidae larvae, but have not reported effects on eggs (David et al. 2000).

The need for research aimed at controlling the *Aedes aegypti* vector, arboviruses-causing, leads to research of compounds of synthetic and natural

origin in order to provide data for development of less toxic, more efficient and low-cost products in the fight against mosquitoes. Studies have addressed the possibility of using the embryo culture technique as a test for the embryotoxic potential of xenobiotics compounds (Murugan et al. 2007). Therefore, this study was developed in order to prove that the aqueous extract of *R. mangle* leaves and synthetic tannic acid have embryotoxic and embryostatic effects on eggs and larvae of *Aedes aegypti*.

## MATERIALS AND METHODS

### STUDY DESIGN

This study was developed by the Translational and Innovation Therapeutics Laboratory Research team of the Department of Histology and Embryology of the Federal University of Pernambuco (UFPE) Biosciences Center in partnership with the Laboratory of Biology of the Academic Unit of Serra Talhada of the Federal Rural University Pernambuco. This is an experimental study (*in vitro*) on embryotoxic and embryostatic effects of aqueous extract of *R. mangle* leaves and synthetic tannic acid in eggs and larvae of *A. aegypti*.

### VEGETABLE MATERIAL

*Rhizophora mangle* leaves were collected in the Vila Velha district, Itamaraca, State of Pernambuco, Brazil (latitude 7° 40' south and longitude 34° 50' west) with the permission of Pernambuco Company Control of Environmental Pollution (Companhia Pernambucana de Controle da Poluição Ambiental) and Water Resources Management (Administração de Recursos Hídricos) under CA DRFB N° 120/2014. The plant was identified by biologist Marlene Barbosa, curator of UFP Herbarium of the Biosciences Center (CCB) of the Federal University of Pernambuco. A voucher specimen of the plant material was deposited in properly listed Herbarium collection and cataloged under number

UFP. 69,655.

### AQUEOUS EXTRACT PREPARATION OF *R. mangle* AND TANNIC ACID

*R. mangle* leaves (500g) were weighed, crushed and subjected to extraction by infusion with distilled water (80° C). After lyophilization, the material was stored at 20°C. The dry residue (100mg) of aqueous extract was homogenized in 100ml distilled water and diluted in water in concentrations of 250, 500, 750, and 1000 µg/ml. Tannic acid was purchased commercially (Merck, Germany) and stored at room temperature and diluted in the same concentrations as the extract.

### EGG COLLECTION

Egg collection of natural populations of *Aedes aegypti* was performed by ovitraps installed in volunteer homes. The ovitraps were made with Polyethylene terephthalate bottles (PRBs) and painted black to attract mosquitoes. Within the same bottles 0.5 mg of biological larvicide (Bti) were placed to prevent from becoming a breeding ground. Cardboard rolls were used to substrate oviposition pieces.

### HATCHING

Before starting the experiments, the eggs were counted and washed to ensure no effect on the part of Bti. Then 10 eggs were placed in a disposable cup. For the experiment with larvae, the eggs were put for hatching in a 1% hay-water solution then divided in groups of 10 larvae in L1 stage.

### EMBRYOTOXIC AND EMBRYOSTATIC TEST

The experiments were carried out over 14 days. In the study, *Aedes aegypti* larvae and eggs were placed in 180ml disposable plastic cups containing the aqueous extract of *Rizophora mangle* and tannic acid solution. Each extract was used at four different concentrations: 250µl/ml, 500µl/ml,

750µl/ml and 1000µl/ml, and a control group. All treatments were performed in duplicate. The cups were identified by date, extract concentration and if there were larvae or eggs. Feeding was administered in all treatments whenever necessary to prevent death by starvation, CEDAN® for fish being used to feed. Daily observations every 24 hours were carried out with the aid of Motik® microscope-stereoscope in the microscopy laboratory UFRPE-Academic Unit of Serra Talhada. The hatchings and seedlings were duly noted and then transferred to an Excel® spreadsheet. The laboratory temperature was maintained at around 27°C.

#### STATISTICAL ANALYSIS

Data was analyzed descriptively by absolute and percentage frequencies and was analyzed inferentially using the Pearson's Chi-squared test or Fisher's exact test when the condition was not verified for using the chi-square test, or the Verisimilitude Reason test when it was not possible to obtain the results by Fisher's exact test. The margin of error used in the statistical tests was 5%. Data was entered in an Excel spreadsheet and the program used for the preparation of statistical calculations was the SPSS version 21.

#### RESULTS

In all concentrations of aqueous extract of *R. mangle*, there was stimulus in hatching in relation to the control, but only in the 750 mg/mL concentration was there statistically significant stimulus (Table I).

At the 250 µg/mL concentration of the synthetic tannic acid there was a significant stimulus in hatching, but at 500, 750 and 1000µg/mL there was a significant inhibition (Table II).

Figure 1 shows the frequency of hatching of *A. aegypti* eggs in 14 days relating the aqueous extract of *R. mangle* with synthetic tannic acid. The 500 µg/mL concentration of tannic acid showed a greater embryostatic effect considering that there

were no hatching eggs.

Regarding the larvae, all concentrations of aqueous extract and tannic acid showed statistically significant embryotoxic and embryostatic effects when compared to the control during the 14 days of the experiment (Tables III and IV).

#### DISCUSSION

The results of this study showed that aqueous extract of *R. mangle* showed a stimulating effect on hatching eggs and embryotoxic and embryostatic effects on *A. aegypti* larvae.

Studies reporting effects on the development of *A. Aegypti* with *Rhizophora mangle* were not found in literature, but plants from mangrove showed effects on eggs and larvae (Kabaru and Gichia 2001, Santana et al. 2013a). Extracts of the stem and *Rhizophora mucronata* pulp showed high toxicity against *A. Aegypti* larvae with values  $LC_{50}$  157.4 ppm for stem and 168.3 ppm for pulpa (Kabaru and Gichia 2001). According to Santana et al. (2013b), the aqueous extract of pneumatophore *Avicenia shaueriana* showed significant inhibitory effect on hatching *A. aegypti* eggs in the concentrations of 250, 500 and µg/mL.

The percentage of hatching was 22.5%, 35% and 22.5% respectively in the 1000 µg/mL concentration, there was no significant difference compared to the negative control. Study with aqueous extract of *C. erectus* leaves revealed that there was hatching in 55% for the negative control, and at concentrations of 250, 500, 750 and 1000 µg/mL after five days of exposure. The hatching percentage was 5%, 7.5%, 0% and 5%, respectively (Santana et al. 2013a).

The fact that it was observed in this study increase of hatching in the presence of aqueous extract of *R. mangle*, leads to a new perspective in relation to the factors that stimulate *A. aegypti* eggs to hatch. It is suggested that the stimulus caused by the aqueous extract of *R. mangle* is

**TABLE I**  
Assessment of embryotoxic and embryostatic effects of aqueous extract of *R. mangle* in eggs of *A. aegypti* over 14 days

Result	Group					TOTAL
	Control <sup>(A)</sup>	250 g/mL <sup>(A)</sup>	500 g/mL <sup>(AB)</sup>	750 g/mL <sup>(B)</sup>	1000 g/mL <sup>(A)</sup>	
Hatched	6	11	12	10	7	46
Hatched and died	1	-	2	7	1	11
Non-hatched	13	9	6	3	12	43
TOTAL	20	20	20	20	20	100

p = 0.003 by Fisher's exact test.

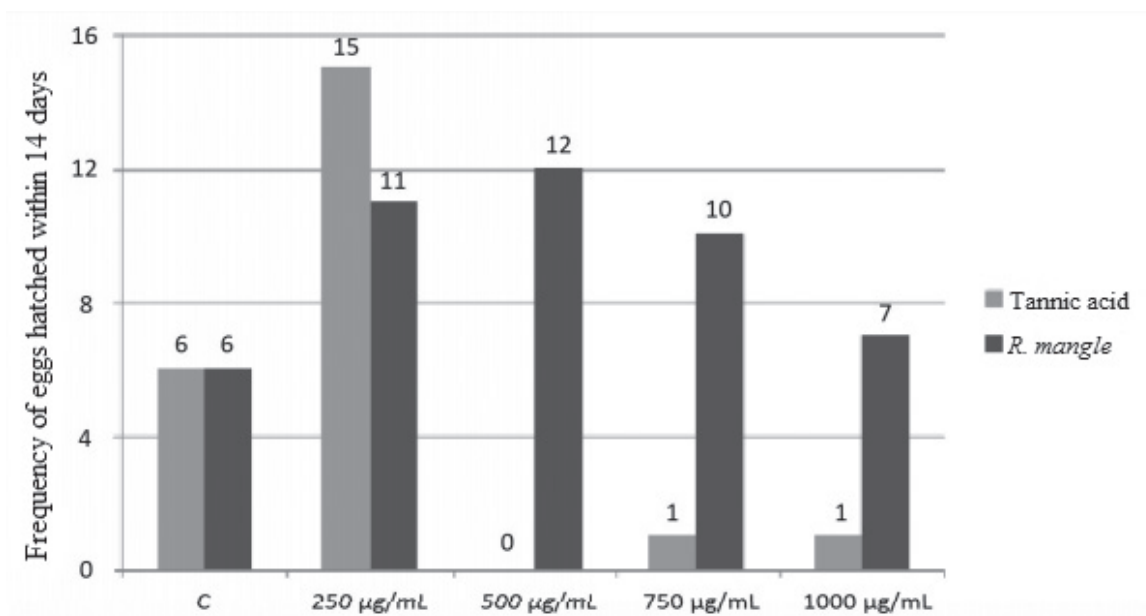
If between parentheses, they are distinct significant difference between the groups is proven.

**TABLE II**  
Evaluation of embryotoxic and embryostatic effects of synthetic tannic acid in *A. aegypti* eggs over 14 days

Result	Group					TOTAL
	Control <sup>(A)</sup>	250 g/mL <sup>(B)</sup>	500 g/mL <sup>(C)</sup>	750 g/mL <sup>(C)</sup>	1000 g/mL <sup>(C)</sup>	
Hatched	6	15	-	1	1	23
Hatched and died	1	3	-	1	1	6
Non-hatched	13	2	20	18	18	71
TOTAL	20	20	20	20	20	100

p = 0.001 by Fisher's exact test.

If between parentheses, they are distinct significant difference between the groups is proven.



**Figure 1** - Frequency of egg hatchability during 14 days on aqueous extract of *R. mangle* and tannic acid.

**TABLE III**  
Evaluation of embryotoxic and embryostatic effects of aqueous extract of *R. mangle* in *A. aegypti* larvae over 14 days

Result	Group					TOTAL
	Control <sup>(A)</sup>	250 g/mL <sup>(B)</sup>	500 g/mL <sup>(BC)</sup>	750 g/mL <sup>(BC)</sup>	1000 g/mL <sup>(C)</sup>	
L1	-	-	-	-	2	2
L2	-	-	-	1	2	3
L3	-	-	1	-	2	3
L4	5	4	6	2	2	19
Pupae	15	6	3	1	-	25
Dead	-	10	10	16	12	48
TOTAL	20	20	20	20	20	100

p < 0.001 by Verisimilitude Reason test with comparisons between groups pairs using Fisher's exact test.

If between parentheses, they are distinct significant difference between the groups is proven.

**TABLE IV**  
Evaluation of embryotoxic and embryostatic effects of synthetic tannic acid in *A. aegypti* larvae over 14 days

Result	Group					TOTAL
	Control <sup>(A)</sup>	250 g/mL <sup>(B)</sup>	500 g/mL <sup>(B)</sup>	750 g/mL <sup>(C)</sup>	1000 g/mL <sup>(C)</sup>	
L1	-	-	-	-	1	1
L2	-	-	-	1	2	3
L3	-	-	-	-	1	1
L4	5	1	3	-	-	9
Pupae	15	6	5	-	-	26
Dead	-	13	12	19	16	60
TOTAL	20	20	20	20	20	100

<0.001 by Verisimilitude Reason test with comparisons between groups pairs using Fisher's exact test.

If between parentheses, they are distinct significant difference between the groups is proven.

due to the antioxidant effects of polyphenols and its free radicals with their chelating properties (Haslam 1996). It has been shown that chlorogenic acid, which is present in the aqueous extract of *R. mangle*, has an antioxidant effect as high as DL-tocopherol (Gjullin et al. 1939) studied mosquito eggs (*Ae. Vexans* (Meigen) and *Ae. Aldrichi* Dyar and Knab [= *Ae. sticticus* (Meigen)]) in plant fermented infusion, observed a significant increase in hatching. The authors attributed this effect to the presence of amino acids, proteins and phosphate salts present in the plant.

In literature, there are quotations showing inhibition activity hatching of *A. aegypti* eggs from plant extracts. According to Vieira et al. (2012),

*Indigofera suffruticosa*, common plant of rural and arid regions (*agreste* and *sertão*) in Pernambuco, showed embryotoxic activity in relation to hatching *A. aegypti* eggs as well as repellent action in oviposition activity.

In this study, synthetic tannic acid at a concentration of 250 µg/mL, stimulated the hatching of *A. aegypti* eggs. In other concentrations tested, there were embryotoxic and embryostatic effects on both eggs as larvae.

In nature, the presence of tannic acid in low concentrations seems to provide chemical and nutritional conditions suitable for larval development similarly to that found in this study (Yadav 1997). Conversely at higher concentrations



tannins exhibit high toxicity.

Studies have shown that the major toxic effects caused by tannins in the mesenteron cells of larvae in the third instar of *A. aegypti* were: high cytoplasmic vacuolation, absence of cytoplasmic limits, apical vesicle formation with the release of cytoplasmic contents of the cells, increased intercellular space and detached cells from the basement membrane. These results are similar to those histopathological processes reported in insects, in response to a variety of biological toxic substances (Abed et al. 2007, Arruda et al. 2003, Barreto et al. 2006, Delphine et al. 1999, Gusmão et al. 2002, Rey et al. 1999).

Histopathological studies in which tannic acid is used against Diptera larvae demonstrated that changes first reached the anterior region of the midgut, progressing to the median and posterior regions (Rey et al. 1999). Molan et al. (2002) showed that tannin inhibited the development of eggs and *Trichostrongylus colubriformis* larvae in concentrations from 200 to 500 µg/mL. This result indicates that tannins have an inhibitory effect even in higher organisms in the evolutionary scale.

New insecticides of herbal origin discovered through ethnopharmacological studies have shown interesting results. Purification of tannins from *Rhizophora mangle* is underway, and further investigations may improve our understanding of possible development of natural products in *Aedes aegypti* control.

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