

Full Length Research Paper

Effects of volatile oils of the *Microlobius foetidus* on trypsin, chymotrypsin and acetylcholinesterase activities in *Aedes aegypti* (Diptera: Culicidae)

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Volatile oils of *Microlobius foetidus* were used for the evaluation of mortality, trypsin, chymotrypsin and acetylcholine inhibition and *Aedes aegypti* morphology. Bioassays were made with different concentrations (25, 50 and 100 µg/ml) and alterations in the gut of 4th stage larvae were observed. Volatile oils affected larvae in all stages, with 100% mortality for the 100 µg/ml concentration (LD₅₀ >33.02). The acute toxic unit (2.7 µg/ml), chronic toxic unit (32.68 µg/ml) and toxic load (2.7 µg/ml) confirm the sensibility of the 4th stage larvae. These larvae and 3rd presented a lesser trypsin (0.176 µmol/min), chymotrypsin (0.110 µmol/min) and acetylcholinesterase (0.172 µmol/min) synthesis. Larvae of the 4th stage also had their internal morphology observed, and the main alterations were discontinuity of the peritrophic epithelium, thickening of the peritrophic membrane, decrease/increase of the subjacent epithelium and decrease of endoperitrophic space were observed in the mid gut. The results show the repellent activity of the volatile oils of *M. foetidus*, with a retardation of overall growth, that are associated with the inhibition of the trypsin and chymotrypsin synthesis. Mortality and enzymatic inhibition in all developmental stages confirm the insecticide potential of *M. foetidus*.

Key words: Dengue fever, digestive system, biological control agents, natural products.

INTRODUCTION

Classic and hemorrhagic dengue epidemics have been reemerging over the last 25 years in tropical regions of the planet which present warm and humid climate, as well as favorable socio-environmental conditions for the proliferation of the mosquito-vector, *Aedes aegypti* (Silva et al., 2008; Who, 2009).

In Brazil, adults and young people are the most affected by the disease, since the introduction of the virus. Since 2006, there has been an increase in the number of

cases, in the number of severe forms, and of hospitalizations in children, mainly in the Northeastern Region of the country. In 2008, 585.769 cases were notified, and new epidemics occurred in various states of the country, configuring the worst national scenario of this disease yet regarding the total number of hospitalizations and deceases (Programa Nacional de Controle da Dengue, 2012).

These epidemics in Brazil also affect children of 1 to 10

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years of age, amounting to 50% of inpatients in the municipalities with a greater population quota, and 25% in smaller municipalities. In the year 2012, 266.285 cases of dengue were notified deaths (Programa Nacional de Controle da Dengue, 2012).

The control of *A. aegypti* is done with chemical synthetic insecticides with both larvicide and pesticide effects (Luna et al., 2004; Lima et al., 2006). However, its frequent use is costly, and may cause environmental pollution, damages to public health and the emergence of resistant mosquitoes (Oga, 2003; Braga and Valle, 2007). In view of this problematic, the use of bioactive natural products with insecticide potential surges as a viable alternative.

Microlobius foetidus (subsp. *paraguensis* (Benth.) M. Sousa et G. Andrade) belongs to the Fabaceae family, and is popularly known as pau-alho. The species has an arboreal constitution and can reach the height of 18 m. It occurs in the Pantanal region of the Brazilian state of Mato Grosso do Sul, in altered areas (pastures and roadsides), resprouting with great vigor after clear-cutting and burning. Due to the strong garlic aroma, it has potential for use as agriculture repellent and as a pioneer species, for the composition of heterogeneous reforestations destined to the recovery of the vegetation in degraded areas (Pott and Pott, 1994).

There are studies demonstrating the effects of vegetal extracts in larvae mortality by trypsin and chymotrypsin inhibition during larvae stages (Venâncio et al., 2009; Melo-Santos et al., 2010). These soluble enzymes are found in the intestinal lumen (Christofolletti et al., 2005) and its secretion occurs in the gut (Terra and Ferreira, 2005). In this regard, the middle portion of the gut of *A. aegypti* larvae contains great amounts of proteolytic enzymes that play a central role in the supply and replenishment of free aminoacids essential for normal larvae development (Michaud et al., 1995).

These enzymes are the most important enzymes for food digestion in *A. aegypti*, and take part in all phases of its biological cycle, being especially expressed in the larvae and pupa phases (Melo-Santos et al., 2010; Yang and Davies, 1971). Trypsin and chymotrypsin are synthesized by the intestinal epithelium cells and can cross the peritrophic membrane, reaching the endoperitrophic space, where the initial phase of digestion, consisting on the breaking of the ingested polymers to oligomers, occurs (Terra et al., 1996). These fragments are now capable of crossing the peritrophic matrix so as to be degraded to smaller oligomers or dimers (intermediate phase). Finally, these molecules are broken into monomers and absorbed (Terra et al., 1996; Tellam, 1996).

Taking into account that the digestive and absorptive processes take place in the middle portion of the gut, the spatial organization of the digestion rely on the relations between each of its compartments (cell, esoperitrophic and subperitrophic spaces) such as the subperitrophic epithelium, epithelial tissue and the different phases of digestion and corresponding enzymes (Terra et al., 1996).

Thus, our research was aimed at identifying the volatile oils present in the leaves of *M. foetidus*, and evaluating the larvicide activity of the different growth stages, by means of mortality assays, trypsin, chymotrypsin and acetylcholinesterase inhibition, and alterations in the gut morphology of 4th-stage larvae.

MATERIALS AND METHODS

Plant

Leaves from *M. foetidus* were collected in the Pantanal region of the Brazilian state of Mato Grosso do Sul state, under the coordinates 19°29'16.20" S; 57°02'35.50" W and a voucher specimen was placed in the Federal University of Mato Grosso do Sul (CGMS) and Botanical Garden of Curitiba (MBM) herbariums, under number 21739 and 334776, respectively. The leaves were stored in an iced polystyrene box and taken to the laboratory for the volatile oil extraction.

Volatile oils extraction

Fresh leaves of *M. foetidus* were submitted to various hydrodistillation processes during four hours in a Clevenger-type apparatus, followed by exhaustive extraction of the distilled with hexane.

The gas chromatography–mass spectrometry (GC-MS) analyses were performed in a Varian GC-MS-MS system equipped with Varian – 3900 gaseous chromatographer equipped with a ZB-5 capillary column, a 1077 injector, a CP-8410 automatic injector coupled with a Varian Saturn 2100 mass spectrometer operating with an electron impact of 70 eV, at the same analysis conditions of GC/FID.

The utilized fiber was of NiTi-ZrO₂-PDMS 35 µm, with 100 mg of leaf being used, with a 30-min extraction time and 40°C extraction temperature (Gebara et al., 2011). The analyses were performed in a gaseous chromatographer (GC 3900) coupled to the ion-trap (Saturn 2100) mass spectrometer, using a molten silica capillary column, VF-5 ms, measuring 30 m of length, internal diameter of 0.25 mm, film width of 0.25 µm and stationary phase with 5%-phenyl-95%-dimethylpolysiloxane of low bleed.

The chromatographic parameters used for the separation of the components were: Injector temperature: 250°C; Liner: Single gooseneck SPME liner for 1177 0.75 injector; Drag gas: Helium 99.999%. Outflow of drag gas in the column: constant of 1.0 minute, followed by a split rate of 50:1 for 15.0 min and of 20:1 for the remaining of the run. Temperature programming of the column oven: 50°C (2 min isotherm) and 50 to 250°C ramp with 3°C min⁻¹ heating. The ion-trap, manifold and transference line temperatures were 200, 50 and 250°C, respectively. 70 eV ionization energy was employed with mass scanning from 40 to 450 m/z.

The identification of the oil components was based in comparisons of the retention times, by means of determination and comparison of the Kovats retention indexes and mass spectrums obtained from the NBS/NIST library (NIST Mass Spectral Library, 2002) with the indexes described by Adams (1995). A n-alkane (C₈-C₃₂) homologous series was used to calculate the Kovats retention indexes.

Larvicidal assays

The larvicide activity of the volatile oil against *A. aegypti* was evaluated according to World Health Organization (1981), with some modifications. Eggs from *A. aegypti*, Rockefeller strain, were provided by Oswaldo Cruz Foundation - RJ; the insecticide activity

being used as a susceptibility standard for the *A. aegypti* species (Hartberg and Craig, 1970). For the eclosion of the eggs, they were put in a plastic tray and 500 ml of water lacking chlorine was added, and then taken to a BOD incubator at a temperature of $27 \pm 2^\circ\text{C}$ and relative humidity of $80 \pm 5\%$.

Larvae feeding was prepared with Fish's diet (Aldon Basic™, MEP 200 Complex) from the eclosion period to the 4th larvae stage, and solutions of volatile oils, in the concentration of 100 µg/ml, were prepared solubilizing the samples with 0.5% of dimethylsulphoxide (DMSO) and diluted in water lacking chloride in the concentrations of 100, 50 and 25 µg/ml for the assays.

Samples containing 15 1st, 2nd, 3rd and 4th stage larvae were put in plastic cups separately containing 5 ml of chlorine-free water. For each concentration, 45 larvae were used, in triplicate. A 0.5% DMSO aqueous solution was used in triplicate, as a negative control.

The protocol consists of the mortality response against the exposure in Diagnostic Concentration (DC) in the exposure to a concentration gradient (Multiple Concentrations – MC). The larvicide activity was evaluated after 24 h, by counting the number of dead larvae in each sample. Moribund larvae, unable to reach the water surface when touched, were considered dead (Who, 1981). The acute toxic unit (ATU), chronic toxic unit (CTU) and toxic load (TL) were calculated (Who, 1981).

The lethal concentration (LC_{50}) values, in µg/ml were determined using the Probit analysis method (Finney, 1971). For each evaluated sample, triplicates were used, and data were submitted to analysis of variance and when a difference was detected, the averages were compared by the Dunnet test, with 5% of probability.

Morphologic study of *A. aegypti* larvae

For the internal morphology evaluation, 4th stage larvae were selected, because they have more developed tissues. The collected larvae were immediately fixed in 2% glutaraldehyde, 2% paraformaldehyde, 3% saccharose in 0.1M Sodium Cacodylate Buffer pH 7.2 and stored at room temperature until the analysis were performed (Arruda et al., 2008). Glasses containing the larvae were prepared and photographed, using a digital camera (Sony), connected to a Zeiss inverted microscope (magnification of 40x).

Preparation of larvae homogenate

A. aegypti larvae homogenates, submitted to the extract and stearic acid by 6 h, were prepared according to Macedo et al. (1993), with some modifications. The gut of each larva was removed using a needle (8 mm length; 0.3 mm caliber) and immediately homogenized in tissue grinder with 1.0 ml of Tris/HCl Buffer 0.05 M (pH 8.0), and centrifuged in 17000 rpm during 20 min, 4°C . The supernatants were collected, and an additional 1.0 ml of Tris/HCl Buffer was added.

Protein concentration

The protein content in the larvae which were submitted to extracts and fractions was determined according to Lowry et al. (1951), using serum bovine albumin (31.25 to 500 µg/ml) as standard.

Acetylcholinesterase activity

For the acetylcholinesterase evaluation, 10 µl of the *A. aegypti* homogenates were incubated with 20 µl of acetylcholine, 0.062 µl of 0.25 mM DTNB for 3 min, at 25°C (Ellman et al., 1991). The increase in the absorbance was read at 405 nm (ϵ , $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Determination of trypsin and chymotrypsin content

The total trypsin activity was determined using the N-benzoyl-D, L-arginine-p-nitroanilide (BAPNA) as substrate. About 500 µl of the larvae homogenate were incubated in 0.05 M Tris-HCl buffer (pH 8.0), to a final volume of 500 µl for 10 min before the addition of 1.0 ml BAPNA substrate. The reaction was allowed to proceed at 37°C for 20 min then stopped by adding 30% acetic acid (v/v). The trypsin activity was read in a microplate reader, at 410 nm (ϵ , $10.0 \text{ mM}^{-1} \text{ cm}^{-1}$) in accordance with Silva et al. (2009).

Chymotrypsin activity was determined using N-succinyl-Ala-Ala-Pro-Phe-p Nitranilide (SAAP) as substrate. About 50 µl of the homogenates were incubated in 0.1 M Tris-HCl buffer (pH 8.0), to a final volume of 500 µl for 10 min before the addition of 1.0 ml SAAP substrate dissolved in pure dimethylsulfoxide (DMSO). The reaction was allowed to proceed at 37°C for 20 min, then stopped by adding 30% acetic acid (v/v). The absorbance was read at 405 nm (ϵ , $8.8 \text{ mM}^{-1} \text{ cm}^{-1}$) (Silva et al., 2009).

Statistical analysis

The values for lethal concentration (CL_{50}) in µg/ml were determined using the Probit analysis method (Fynnei, 1971). For each evaluated sample, triplicates were used, and data were submitted to analysis of variance and when a difference was detected, the averages were compared by Dunnet test, with 5% of probability.

RESULTS

The oil obtained by hydrodistillation had a yield of 0.84%; it has a yellow color and a characteristic odor. The solid-phase micro-extraction (SMPE) method was more efficient when considering the amount of identified substances and the time for chromatogram attainment (30 min), and may simplify considerably the sample preparing procedure by the hydrodistillation method. Furthermore, when combined with gas chromatography–mass spectrometry (GC/MS), it provides the adequate conditions of the optimization and identification of a greater number of volatile substances.

Eleven substances were identified: 1.2.4-trithiolane (31.8%), 1.3.5-trithiane (8.5%), 1.2.5-trithiepane (0.2%), 1.2.4.5-tetrathiane (7.0%), 1.2.3.4-tetrathiane (0.3%), 1.2.4.6-tetrathiepane (12.3%), 1.3.5.7.9-pentathiepane (1.2%), 1.2.5.6-tetrathiocane (0.4%), lenthionine (10.2%), hexathiepane (1.1%) and sulfur cyclic octaatomic (3.4%) (Table 1).

Based on this composition, these oils may be responsible for the poignant onion aroma, that is, exhaled from the plant, and that explains its popular name (garlic stick). Cyclic polysulfides have a restricted occurrence in nature, with few reports in plants. The presence of sulfur-containing substances in the oil composition turns it into quite a peculiar material regarding chemical composition. Antineoplastic activity of sulfur compounds has already been reported in the literature (Gmelin et al., 1981) and the presence of cyclic polysulfides such as 1.2.4-trithiolane and 2.4.6-tetrathiepane, have already been related in the brute extracts of *Parkia* species, exhibiting allelopathic activity.

Table 1. Chemical composition of the volatile sulfur compounds of *Microlobius foetidus* obtained by SPME (solid phase micro extraction).

| Compounds ^{a,b} | MF | RI ^c | SPME |
|--------------------------|---|-----------------|----------|
| 1,2,4-trithiolane | C ₂ H ₄ S ₃ | 1095 | 31.8±5.6 |
| 1,3,5-trithiane | C ₃ H ₆ S ₃ | 1249 | 8.5±1.2 |
| 1,2,5-trithiepane | C ₄ H ₈ S ₃ | 1300 | 0.2±0.0 |
| 1,2,4,5-tetrathiane | C ₂ H ₄ S ₄ | 1318 | 7.0±0.5 |
| 1,2,3,4-tetrathiane | C ₃ H ₆ S ₄ | 1354 | 0.3±0.2 |
| 1,2,4,6-tetrathiepane | C ₃ H ₆ S ₄ | 1488 | 12.3±2.8 |
| 1,3,5,7,9-pentathiepane | C ₅ H ₁₀ S ₅ | 1545 | 1.2±0.4 |
| 1,2,5,6-tetrathiocane | C ₄ H ₈ S ₄ | 1551 | 0.4±0.1 |
| Lenthionine | C ₂ H ₄ S ₅ | 1597 | 10.2±2.3 |
| Hexathiepane | CH ₂ S ₆ | 1685 | 1.1±0.5 |
| Cyclic octaatomic sulfur | S ₈ | 2006 | 3.4±0.3 |
| Total | | | 76.4% |

MF: molecular formula; ^aCompounds listed in order of elution from a ZB-5 column; ^bIdentification: RI, retention indices, GC-MS, gas chromatography-mass spectroscopy; ^cProgrammed temperature retention indices determined on apolar ZB-5 column (50 to 250°C; 3°C min⁻¹).

Table 2. Effect of different concentrations of the volatile oils of *M. foetidus* in the mortality and LD₅₀ on *A. aegypti* larvae, in all the development stages.

| Stage | Number of dead | | | | LD ₅₀ (µg/ml) | Confidence interval 95% |
|-------|----------------|---------|----------|-----------|--------------------------|-------------------------|
| | control | 25µg/ml | 50 µg/ml | 100 µg/ml | | |
| 1° | 2 | 36* | 51* | 60* | >45.68 | 36.72-25.96 |
| 2° | 1 | 33* | 48* | 60* | >51.69 | 41.31-28.57 |
| 3° | 3 | 31* | 43* | 60* | >58.42 | 42.002-28.78 |
| 4° | 2 | 30* | 58* | 60* | >33.02 | 44.31-29.88 |

Table 3. Effect of different concentrations of the volatile oils of *M. foetidus* in the acute toxic unit (ATU), chronic toxic unit (CTU) and toxic load (TL) on *A. aegypti* larvae, in all the development stages.

| Stage | ATU (µg/ml) | CTU (µg/ml) | TL (µg/ml) |
|-------|-------------|-------------|------------|
| 1° | 3.1 | 36.69 | 0.67 |
| 2° | 2.9 | 35.42 | 0.65 |
| 3° | 2.8 | 34.02 | 0.61 |
| 4° | 2.7 | 32.68 | 0.56 |

The different concentrations of the volatile oils led to *A. aegypti* larvae mortality in the different stages. The 25 µg/ml concentration affected larvae survival by >50% (30 dead individuals). Larvae in the 1st (51 dead individuals) and 4th (58 dead individuals) stages were more affected by the volatile oils activity, with LD₅₀ of >45.68 and >33.02 µg/ml, respectively. The greater concentration led to 100% larvae mortality in the different tested stages

(Table 2).

The used concentrations were toxic to all larvae stages. Although all concentrations were effective, larvae in the 4th stage were more sensible, and presented ATU, CTU and TL by 2.7, 32.68 and 0.56 µg/ml respectively, demonstrating high insecticide potential (Table 3). Alterations in the shifting of the larvae stages were also observed. There was a decrease in the shifting of the different stages as a function of the used concentrations.

Living larvae of the 4th stage submitted to volatile oil concentrations and water control were evaluated after 8 h with an inverted optical microscope. Larvae treated with the 25 µg/ml concentration presented a thickening of the peritrophic membrane and a less thickened subperitrophic epithelium. The endoperitrophic space also appeared leaner and clearer when compared to control, and the elongation of the region between the thorax and the cephalic capsule was observed (Figure 1B and C). In a similar way, the 50 µg/ml concentrations led to thickening and darkening of the peritrophic membrane, and thickening of the subperitrophic epithelium. Endoperitrophic space showed discontinuations in the tissue segments

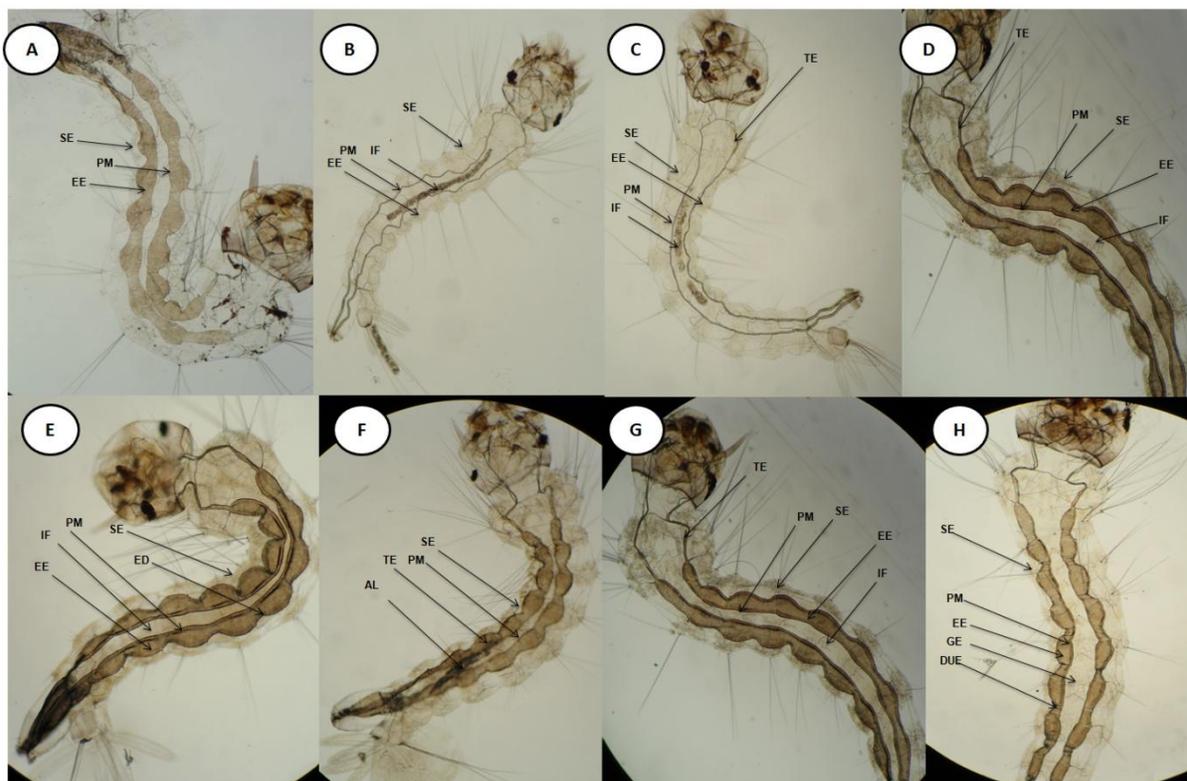


Figure 1. Photomicrography (40x, 900 µm) of the *A. aegypti* larvae on the 4th stage of development. A- control live, B and C- 25 µg/ml, D and E- 50 µg/ml, F G and H- 100 µg/ml. *Note = SE: subperitrophic epithelium, EE: endoperitrophic epithelium, PM: peritrophic membrane, IF: ingestion of food, TE: thorax extended.

and the region between the cephalic capsule and the thorax was also more elongated (Figure 1D and E). The same alterations were observed in the 100 µg/ml concentration (Figure 1F and G). The endoperitrophic space was also less thick, and an enlargement of the digestive tube in some larvae was also observed, and the non-continuous epithelium was also observed (Figure 1H).

The digestive enzyme activity decreased in larvae treated with different concentrations of the volatile oils of *M. foetidus* (Figure 2). Regarding trypsin activity, it was decreased in all development stages, and a lesser synthesis was verified in larvae of the 3rd (178 µmol/min) and 4th (176 µmol/min) stages that had been treated with the 100 µg/ml concentration (Figure 2A). Chymotrypsin activity was also decreased, and the least activity was observed with the 100 µg/ml concentration in larvae of the 4th development stage (0.11 µmol/min) (Figure 2B). The least trypsin and chymotrypsin synthesis observed in 4th stage larvae may be related to volatile oil ingestion, leading to intoxication and death.

Larvae treated with various concentrations of volatile oils showed a decrease in the acetylcholinesterase synthesis. Inhibitory effects were observed in all stages, with the lowest enzyme synthesis on the 100 µg/ml (0.256 µmol/min) concentration in the 4th larvae stage

(0.172 µmol/min) (Figure 3). The differences between effects of 3 concentrations were not significant (subgroup b).

DISCUSSION

The increase of mosquito resistance to chemical insecticides led to an increase in research on natural resources and biodegradable insecticides, aiming to minimize the environmental impact and find new substances that promote mortality, preventing the proliferation of resistant larvae due to rotation of insecticides such as organophosphates and pyrethroids in Brazil.

Although many conventional synergists have relatively low acute toxicities to mammals, there is evidence that pyrethroids show adverse health effects with prolonged exposure to humans (Horton et al., 2011).

Some plant extracts have already demonstrated consistent insecticide activity, and these potential phytoinsecticides could become safer alternatives for mosquito control, as a high degree of degradation in the environment is expected (Lima et al., 2013; Rajasekaran and Duraikannan, 2012). Although many conventional

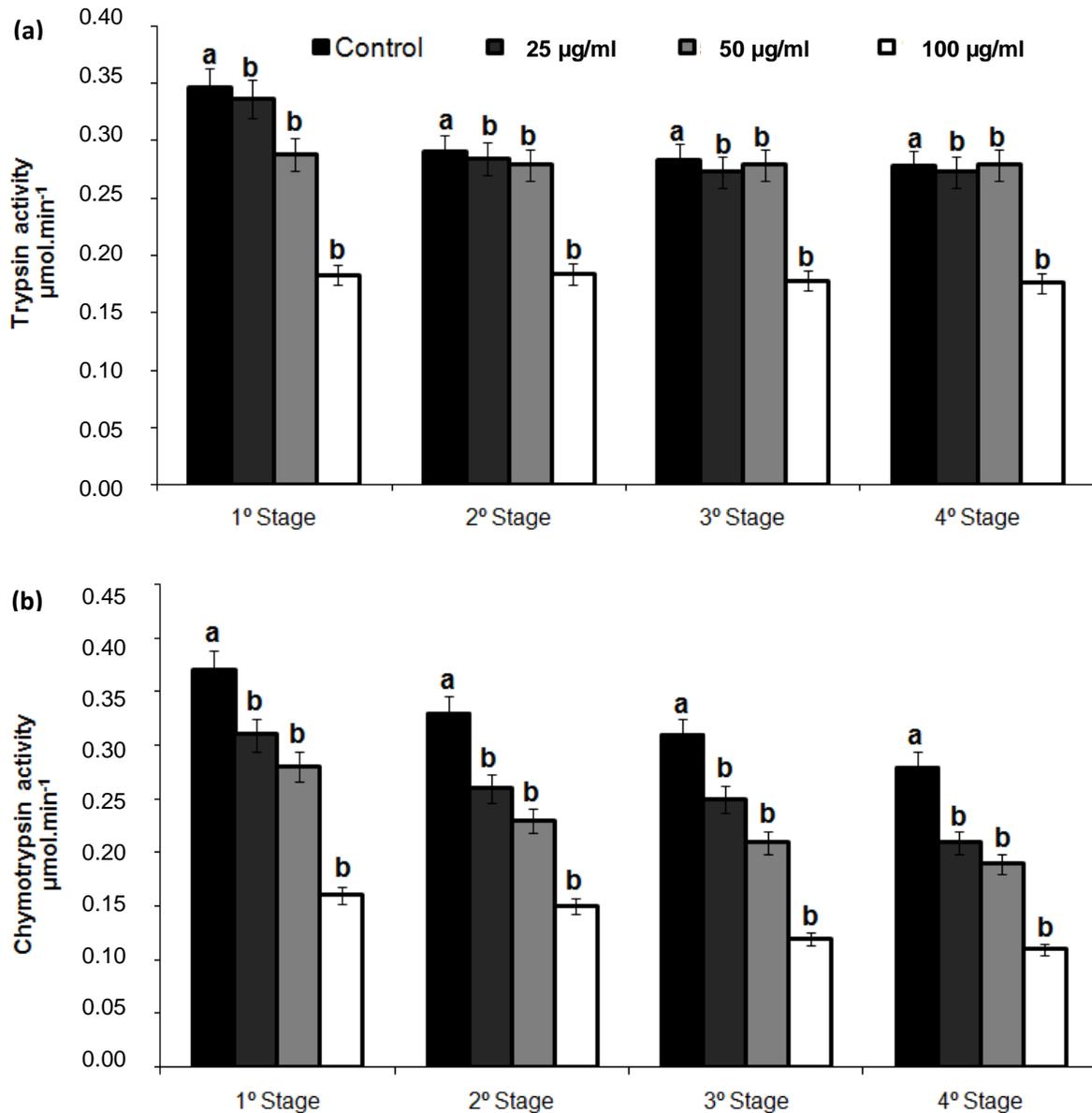


Figure 2. Effect of different concentrations of the volatile oils of *M. foetidus* on the trypsin and chymotrypsin activities in *A. aegypti* larvae (Mean \pm Standard deviation or Standard error). Means followed by the same letter do not differ statistically from the control group by Dunnet's test ($p < 0.05$).

synergists have relatively low acute toxicities to mammals, there is evidence that these chemicals show adverse health effects with prolonged exposure to humans (Horton et al., 2011).

The high larvae mortality in different stages exposed to the volatile oils of *M. foetidus* indicate its use as a natural insecticide. The alterations in internal morphology explain the high mortality rate arising from the intoxication and inhibition of trypsin and chymotrypsin.

The results showed significant repellent effect on larvae posture and general growth retardation on *A. aegypti* by medium containing aqueous of volatile oils. Few works

have reported larvicidal activities of plant extracts against *A. aegypti*, describing the changes in the PM (Vieira et al., 2012), although potent larvicidal extracts have been described, with IC₅₀ values less than 50 µg/ml (Lima et al., 2013).

The main biological functions of PM include the spatial organization of digestion, protection from ingested toxins, and serves as a physical barrier to pathogens (Zhong et al., 2012).

The PM not only plays important roles in facilitating food digestion and providing protection to the gut epithelium, but can also be a significant structural target

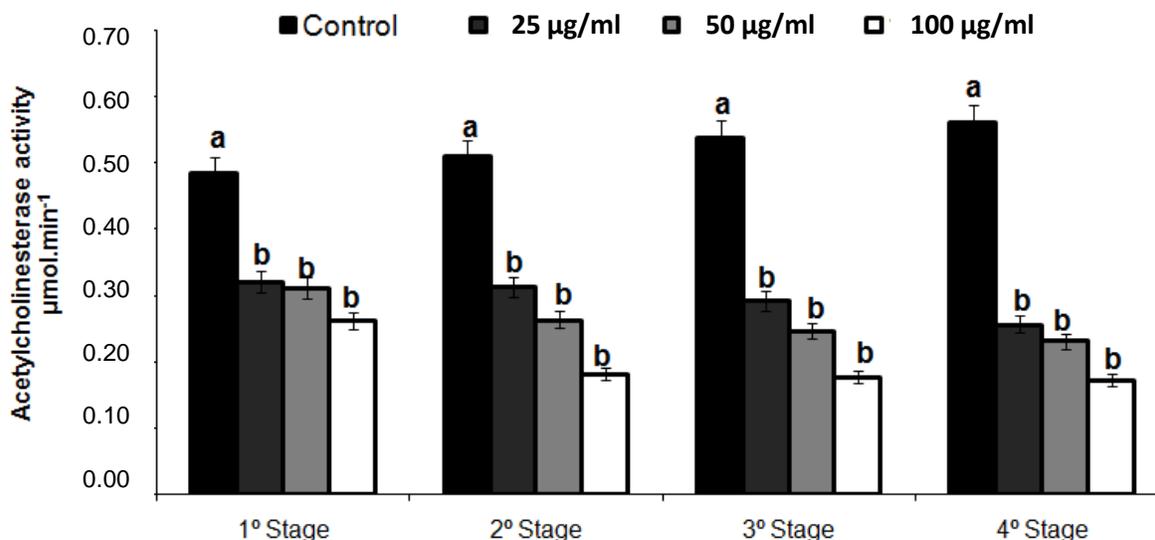


Figure 3. Effect of different concentrations of the volatile oils of *M. foetidus* on the acetylcholinesterase activity in *A. aegypti* larvae (Mean \pm Standard deviation or Standard error). Means followed by the same letter do not differ statistically from the control group by Dunnet's test ($p < 0.05$).

for insect control (Zhong et al., 2012). Trypsin and chymotrypsin may play various important roles in food digestion, immune defense and zymogen activation in insects (Ge et al., 2012). Despite several studies concerning adult *A. aegypti* digestive biochemistry and molecular biology, very few studies have been performed to elucidate the digestion in *A. aegypti* larvae. Trypsin-like and chymotrypsin-like activities are known in *A. aegypti* larvae (Mesquita-Rodrigues et al., 2011).

Enzymatic inhibitors, when added to the diet of insects, interfere with their digestive process by decreasing the assimilation of nutrients, leading to delayed development and mortality (Napoleão et al., 2012).

Zhang et al. (2010) reported what enzymes involved in the food digestion, it has to be secreted into the interspaces between the epithelium and peritrophic membrane or the lumen of the gut, where it digests the ingested food proteins, and the results showed that digestive enzymes were present not only in the epithelium of the anterior, middle and posterior midgut, but also in the lumen food residues of the anterior, middle and posterior midgut, as well as the feces of the larvae, suggesting that the protein was secreted into the lumen of the gut.

Current strategies based on the elimination of breeding sites and applications of chemical insecticides for larval and adult mosquito control have resulted in development of resistance without eliminating the constant risk of dengue epidemics (Lima et al., 2011). Thus new approaches are urgently needed. Interest on possible use of environment friendly natural products such as oils of plants or plant parts increased for vector control. Plant derived products have received increased attention from

scientists and more than 2000 plant species are already known to have insecticide properties (Pankaj and Anita, 2010; Kamaraj et al., 2011).

Vieira et al. (2012) reported that larvicidal activity of *Indigofera suffruticosa* had as main molecular targets: apoptosis, caspase 3 activation, DNA degeneration and mitotic catastrophe. Due to these actions, *I. suffruticosa* can impinge upon different conditions. Plausible infer that volatile oils of *M. foetidus* cause the same effects on larvae of *A. aegypti*, and these effects are associated with inhibition of gut enzymes.

Thus, the use of volatile oils for the control of insects is an alternative way to minimize the harmful effects of pesticides used to control the mosquito. Further, samples of locals where there has already been intensive use of pesticides by the control programs demonstrate that the larvae and mosquitoes became resistant, by mechanisms such as increase in the synthesis of acetylcholinesterase (Pinheiro and Tadei, 2002).

Volatile oils from the plants could be used in stagnant water bodies which are known to be the breeding grounds for mosquitoes. However, further studies on the active principals involved and their mode of action and field trials are usually needed to recommend any of these plant materials as an anti-larvicidal product used to combat and protect from mosquitoes in a control program.

Plant could be an alternative source for mosquito larvicides, because they constitute a potential source of bioactive chemicals and generally free from harmful effects. Use of these botanical derivatives in mosquito control instead of synthetic insecticides could reduce the cost and environmental pollution.

Taking this into consideration, the volatile oils of *M. foetidus* represent an alternative to the larvae control, as they acted not only as inhibitors of the synthesis of acetylcholine, but also as inhibitors of digestive enzymes such as trypsin and chymotrypsin, demonstrating its potential as physiologic pesticide.

The isolation of substances present in the volatile oils is underway, and new research on the isolated activity of these substances could improve our understanding on the popular indication of this plant as pesticide by the Pantanal population, and evolve to the development of formulations that could be used for the control of larvae and mosquitoes.

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