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How long is long enough? Decreasing effects in *Aedes aegypti* larval mortality by plant extracts over time

Quanto tempo é tempo suficiente? Redução dos efeitos na mortalidade larval de *Aedes aegypti* por extratos de plantas ao longo do tempo

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ABSTRACT

Aedes aegypti has overcome all kinds of mosquito control attempts over the last century. Strategies for population control resorts to the use of synthetic insecticides, which can lead to problems like human intoxication and environmental contamination. The effects of *Bacillus thuringiensis* var. *israelensis* (Bti), *Ilex paraguariensis* (yerba mate), and *Ilex theezans* (caúna herb) extracts against *A. aegypti* larvae were evaluated. The bioassays were conducted under controlled laboratory conditions of temperature (27 ± 3°C) and photoperiod (12 h). Hydroalcoholic extract of the leaves of *I. theezans* displayed better residual effect compared to the aqueous extract of *I. paraguariensis* fruits. The strongest residual effect of *I. theezans* was probably due to the presence of certain chemicals in its leaves, such as coumarins, hemolytic saponins, and cyanogenic glucosides, which were absent in *I. paraguariensis*. The results herein contributed to the prospection of natural insecticides and opened the possibility for subsequent studies on the use of plant extracts in field situations in a short-time scale.

Keywords: dengue; vector control; inseticide; entomology; mate herb.

RESUMO

Aedes aegypti superou todos os tipos de tentativas de controle do mosquito pelo homem no último século. Estratégias para controle populacional recorrem ao uso de inseticidas sintéticos, que podem levar a problemas como intoxicação humana e contaminação ambiental. Foram avaliados os efeitos de *Bacillus thuringiensis* var. *israelensis* (Bti), extratos de *Ilex paraguariensis* (erva-mate) e *Ilex theezans* (erva-caúna) contra a mortalidade de larvas de *A. aegypti*. Os bioensaios foram conduzidos sob condições laboratoriais controladas de temperatura (27 ± 3°C) e fotoperíodo (12 h). O extrato hidroalcoólico de folhas de *I. theezans* apresentou melhor efeito residual quando comparado ao extrato aquoso de frutos de *I. paraguariensis*. O efeito residual mais forte de *I. theezans* provavelmente ocorreu devido à presença de substâncias químicas em suas folhas, tais como cumarinas, saponinas hemolíticas e glicosídeos cianogênicos, ausentes em *I. paraguariensis*. Nossos resultados contribuíram para a prospecção de inseticidas naturais e abriram a possibilidade de estudos subsequentes sobre o uso de extratos vegetais em situações de campo em um curto espaço de tempo.

Palavras-chave: dengue; controle vetorial; inseticida; entomologia; erva-mate.

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Introduction

Over the last century, the mosquito *Aedes aegypti* (Linnaeus, 1762) has overcome all mosquito control attempts. *A. aegypti* females are well known by their capacity of naturally and/or under laboratory conditions replicate and transmit over 100 kinds of viruses (Weaver and Reisen, 2010). As an example, the viruses of Dengue, Chikungunya, Zika, and, most recently, the Mayaro (Weaver and Reisen, 2010) virus can be listed in Brazil, which represent a real threat to public health (Lopes et al., 2014). Therefore, its medical importance requires the population control of this species to reduce virus transmission and, consequently, its epidemic status. Although several chemical and natural products have been extensively used on attempts to reduce the population of adults and larvae (Liu, 2015; Apaire-Marchais et al., 2016), adequate mosquito control is not even close to become true, especially due to the genetic resistance selectivity because of the incorrect use of natural products and chemicals (Sun et al., 2019). As a consequence, the most effective disease prevention method still focus on targeting the mosquito population by eliminating mosquito breeding places (Brasil, 2002).

A very promising field for reducing the mosquito population is to focus on mosquito control strategies that target immature aquatic stages, when the insect is more vulnerable (Brasil, 2001; Crivelenti et al., 2010). For this purpose, the use of synthetic insecticides is well known for its efficacy, causing mosquito larval mortality (Busato et al., 2015; Govindarajan et al., 2018). However, those chemicals might affect humans, resulting in intoxication and environmental contamination, and affecting biodiversity (Busato et al., 2015; Tahir et al., 2015; Baskar et al., 2018; Govindarajan et al., 2018). Regarding the environment, the continuous use of synthetic insecticides may present undesirable effects, such as the long-term permanence in the environment, selection of resistant populations, and the appearance of new pests (Tahir et al., 2015; Baskar et al., 2018; Govindarajan et al., 2018). As to human health, the presence of such synthetic chemicals on the environment can cause neurological damage and is associated with a wide range of symptoms, with significant deficits in the nervous system function (Araújo et al., 2007).

Alternatively to the use of synthetic chemicals, biological control plays an important role on mosquito management (Zara et al., 2016; Coelho et al., 2017). The use of bacteria spores as a mosquito larvicide has stood out among the several components that are part of mosquito-integrated management programs (Zara et al., 2016). Over the last decade, the use of inactivated spores of the bacteria *Bacillus thuringiensis* var. *israelensis* (Bti), spread in the water of mosquito breeding places, has met the expected results, reaching mortality rates above 99% (Soares-da-Silva et al., 2017; Nakazawa et al., 2020). Additionally, during the last few years, plant-derived compounds have been extensively used as an alternative method for controlling mosquitoes, not only because this is a new insecticidal agent, but also because it has been described as being environmentally friendly (Gomes et al., 2016; Guarda et al., 2016; Knakiewicz et al., 2016; Rosa et al., 2016). The use of natural insecticides has some advantages over traditional synthetic products, because natural products are potentially less toxic to the environment. Environmentally-friendly compounds are less concentrated, have faster degradation, and are specific to certain insect groups, resulting in less occupational exposure and less environmental pollution (Krinski et al., 2014).

Ilex paraguariensis A. St.-Hil (Aquifoliaceae), known as *mate*, is an abundant plant, native of South America, 20 m tall, with a dense crown, and very branched (Souza, 2009). After processing, its leaves are traditionally used in a regional tea known as *mate* in Argentina, Brazil, Paraguay, and Uruguay (Souza, 2009). *I. paraguariensis* is commercially important due to the presence of caffeine and theobromine, both recognized as having a stimulant effect in the nervous and cardiocirculatory systems (Castaldelli et al., 2011). The described pharmacological activities for *I. paraguariensis* leaf extracts include antioxidant, hypolipidemic (Gao et al., 2013; Messina et al., 2015), and hypoglycemic effects (Conceição et al., 2017). Besides that, *Ilex theezans* Mart. Ex Reissek (Aquifoliaceae), popularly known as caúna-herb, is commonly found in Southern Brazil (Souza, 2009). It is well known due to the physiological characteristics of its leaves as an adulterant of *I. paraguariensis* (Athayde et al., 1999). It is an evergreen tree, early secondary or late secondary species (Souza, 2009), 20-m tall and 70-cm diameter, on average (Athayde et al., 1999). Both the *I. paraguariensis* fruit extract and the *I. theezans* hydroacoholic leaf extracts have larvicidal effect against *A. aegypti* larvae (Busato et al., 2015; Knakiewicz et al., 2016).

Some studies showed that *Ilex* spp. leaves and fruit extracts kill *A. aegypti* larvae within a 24-h observational time (Busato et al., 2015; Knakiewicz et al., 2016). However, there are no studies in the current literature evaluating the effects of time on the bioinsecticide lethal activity. How long is long enough for the bioinsecticide to maintain its ability to kill? (Resende and Gama, 2006; Santos et al., 2007; Guirado and Bicudo, 2009). In this context, the lethal residual effect of *B. thuringiensis* var. *israelensis,* and leaf and fruit extracts of *I. theezans*, and *I. paraguariensis*, respectively, against *A. aegypti* larvae were evaluated. Time would positively affect *A. aegypti* larvae survival due to decay of the lethal compounds, as a hypothesis, but the mortality caused by *I. theezans* was higher when compared to *I. paraguariensis*, due to the difference in physical and chemical characteristics.

Material and Methods

Animal source

The *A. aegypti* larvae used in this experiment were provided by Laboratório de Entomologia Ecológica (LABENT-Eco). A filter paper holding about 300 eggs was placed in a plastic tray (30 × 20 cm) holding 1 L of tap dechlorinated water. After hatching, the larvae were distributed among three plastic trays of equal size and fed with 2 g of fish food. The mosquito larvae were raised for about 4-5 days until reaching 3rd and 4th instars.

Plant source and extract preparation

Fruits and leaves were obtained from native trees located at the Marechal Bormann district (27°19'05"S; 52°65'11"W), Chapecó City (Santa Catarina State), in December 2016. Plant parts were dehydrated at room temperature (± 20°C), pulverized in a knife mill (Cielamb®, CE 430), and stored away from light and humidity. Plant extracts were prepared according to Busato et al. (2015) and Knakiewicz et al. (2016). Samples of 20 g of *I. paraguariensis* dehydrated fruits and *I. theezans* leaves were used. Both samples were extracted by turbolysis, using 200 mL of distilled and deionized water and a hydroalcoholic solution (90% ethanol; 200 mL) as solvent, respectively (ANVISA, 2019). The extracts were filtered in Büchner funnel, concentrated by rotavapor under reduced pressure, lyophilized, weighed, identified, and stored in a freezer at -20°C. Hydroalcoholic and aqueous extracts were prepared using *I. theezans* and *I. paraguariensis* leaves and fruits, respectively. Leaves at a concentration of 1,000µg/mL were used, and fruits were diluted to 2,000 µg/mL. *B. thuringiensis* var. *israelensis* (Bti), strain WG®, was used in a concentration of 0.004 g/L, the lethal dose specified by the manufacturer.

Experimental microcosms and design

Plastic cups of 300 mL with 100 mL of dechlorinated water plus the treatment proposed were adopted. In each individual sample, 20 3rd and 4th instar *A. aegypti* larvae were added. Each container was covered with a mosquito net held by a rubber elastic band. Tests for the effects of Bti spores; *I. theezans*, and *I. paraguariensis* leaves and fruits, respectively, were conducted; clean aged water (control) on the *A. aegypti* larval mortality after seven days of exposure was also performed (Nakazawa et al., 2020). Before running the mortality test, each experimental treatment aged from one to eight weeks. Each week was considered as one age block with each treatment replicated six times. With this experimental design, the independence of each set of treatments was assured. The aged treatments were used to test for larval survival in each experimental week. At the end of the 7th day, larval survival was recorded, with both pupae and emerged adults being considered as survivals. The experiment was performed for eight weeks (56 days) and carried out between April and May 2017 at the LABENT-Eco mosquito colony room, under controlled conditions of temperature and photoperiod (27 ± 3°C, 12h D:L).

Statistics

Since both negative (Bti) and positive (tap water) control survival rates were 0.16% and 100%, respectively, the data were analyzed in both ways, with (complete model) and without (simple model) these two categories. In order to evaluate differences in the percentage of larval mortality (response variable) between simple (*I. paraguariensis* and *I. theezans*) and the complete models (only water, Bti spores, *I. paraguariensis* and *I. theezans*), regarding week (1 to 8) and week-treatment interaction (explicative variables), we used factorial GLM, with binomial correct to quasi-binomial (link = logit, test = Chi-square) distributions (Crawley, 2007). All analyzed GLMs were corrected for cases of under- or overdispersion.

Differences among the categorical variables were assessed with a contrast analysis (Crawley, 2007). In this analysis (orthogonal), the dependent variables (different treatment and weeks) were ordered increasingly and tested pairwise (with the closest values); sequentially, adding to the model values with no differences and testing with the next values in a stepwise model simplification (for more details see also Chapter 9 of Crawley, 2007). All analyses were performed using the R program (Venables et al., 2019).

Results

The *A. aegypti* larval mortality was not affected by the water age (positive control). In contrast, the Bti resulted in the death of all the larvae until the age of seven weeks, with only 6.6% of larvae alive on the age of eight weeks (negative control). In this way, due to these extreme results in controls (0% of mortality in the positive control and 100% of mortality in the negative control), mortality data were analyzed only between treatments (*I. paraguariensis* and *I. theezans*).

Larval mortality was significantly different between treatments (*I. paraguariensis* and *I. theezans*), weeks (1 to 8), and interaction factors (week:treatment) for both GLMs models (with and without positive and negative controls; Table 1). The highest larval mortality was found in the Bti treatment (negative control), followed by *I. theezans, I. paraguariensis*, and positive control (Table 1; Figure 1A). In addition, the *I. theezans* hydroalcoholic leaf extract, regardless of extract's age, killed significantly more *A. aegypti* larvae than the aqueous *I. paraguariensis* fruit extract (Table 1; Figure 1B).

A positive relationship between the survival of the larvae and the plant extract age was observed. In general, both *I. paraguariensis* and *I. theezans* killed less (mainly after seven weeks) mosquito larvae as the plant extracts aged (Figure 2). A higher significant larval mortality was found in week 1, followed by weeks 2 and 3, weeks 4 and 6, week 5, and weeks 7 and 8 (Figures 1B and 1C).

Residual deviance (estimate of the variance of the tested variables) in GLM with positive and negative controls, showed that differences in all treatments (74%) was the main responsible for larval mortality, followed by extracts' age (18%; Table 1). On the other hand, residual deviance in GLM, without positive and negative controls, showed that differences between all weeks (68%) was the main responsible for larval mortality, followed by treatments (6%; Table 1).

Discussion

Transformation of the larvicide effect into food resource over time

I. theezans and *I. paraguariensis* extracts are promising against mosquito larvae (if applied and monitored in the first weeks). The potential of using these plant extracts as larvicides for *A. aegypti* may be an advantage, since they are natural extracts and do not leave toxic waste in the environment. These extracts are an abundant and accessible alternative in Southern Brazil, where *A. aegypti* infestation and dengue cases have been observed in the last decade (Busato et al., 2015). However, extracts' age should be considered; the main objective of the present study is not to discourage the use of such alternative method, but to warn about the importance of extract aging before using it for mosquito-control purposes. Better results may also be obtained with the development of additional studies, evaluating the larvicidal activity of pure compounds isolated from these plants. Furthermore, better results might be obtained by evaluating if there is a supporting effect of more than one active principle with larvicidal action against *A. aegypti*.

Plant extracts may degrade as time goes by, and these organic compounds with previous larvicidal activity may become food for *A. aegypti* larvae (explaining the residual deviance percentage in GLMs models). The transformation of larvicides into food probably took place, especially in those treatments with seven- and eight-weeks old plant extracts, which presented the highest survival rate. Therefore, the age of plant extract should be considered (Albeny-Simões et al., 2015).

Aedes aegypti larval mortality between plant extracts

Plant extract age plays an important role on mosquito larvae mortality (mainly with positive and negative controls). Despite the plant species, plant parts, and extraction method, mosquito larval mortality decreases with the aging of plant extracts. However, the extracts tested were highly efficient in the first weeks of the experiment (high mortality). The higher larval mortality found in the *I. theezans* extracts can be partially explained by the use of solvents during the extraction process (Lee and Houghton, 2005). The hydroalcoholic extraction method used to obtain the *I. theezans* extracts removed low polarity chemical constituents from plant tissues, and these molecules have a higher ability to penetrate mosquitoes' larvae cells and modify their metabolic activities.

On the other hand, aqueous extraction, used for the *I. paraguariensis* fruits, preferentially removes high-polarity chemical compounds, which are not able to easily penetrate such cells (Lee and Houghton, 2005). Moreover, the susceptibility of *A. aegypti* larvae to *I. theezans* may be explained by the presence of secondary metabolites of the coumarin class and absence of alkaloids when compared to *I. paraguariensis* (Valduga et al., 1997). Coumarins are part of the secondary metabolism of several plants, being well known for presenting insecticidal activities, acting as a repellent of adult insects, preventing oviposition, impairing feeding and growth, promoting morphogenetic and hormonal system alterations, sexual behavior changes, and adult sterilization, among other effects (Dietrich et al., 2011). Furthermore, *I. paraguariensis* has a higher content of caffeoyl derivatives and flavonoids than *I. theezans*. Flavonoids are recognized for their potent larvicidal activity, which may partially explain the results obtained herein (Filip et al., 2001; Garcez et al., 2013). In raw plant extracts, the active constituents are usually found in small concentrations (Krinski et al., 2014).

Larvae mortality and extract age of *Aedes aegypti*

In both *I. theezans* and *I. paraguariensis,* the mortality of *A. aegypti* larvae exposed to one-week plant extracts was 100%.

Table 1 – Generalized linear models (GLM), degrees of freedom (Df), Residual Deviance (total and in), and *p* values, comparing the percentage of *Aedes aegypti* larvae mortality after exposure to treatments (water control, *Bacillus thuringiensis israelensis* — Bti, hydroalcoholic dried leaves extract of *Ilex theezans*, and aqueous *Ilex paraguariensis* fruits extract), time (8 weeks) and interaction among treatments and weeks, under laboratory conditions.

GLM	Df	Resid. Dev.	Resid. Dev. %	Pr(>Chi)	Analysis of contrast
a. With positive and negative controls					
Treatments	3	132.9	74.1	< 0.001	Control < *Ilex paraguariensis* < *Ilex theezans* < Bti
Weeks	7	33.4	18.6	< 0.001	Week 8 = 7 < 5 < 4 = 6 < 3 = 2 < 1
Treatment: weeks	21	3.0	1.7	< 0.001	
Residual	160	10.0	5.6		
b. Without positive and negative controls					
Treatments	1	3.1	6.6	< 0.001	*Ilex paraguariensis* < *Ilex theezans*
Weeks	7	32.3	68.7	< 0.001	Week 8 = 7 < 5 < 4 = 6 < 3 = 2 < 1
Treatment:weeks	7	2.4	5.1	0.002	
Residual	80	9.2	19.6		

However, the plant extracts for both species reduced the mortality of mosquito larvae as the plant extracts aged. These results pointed out the need for carefully selecting the right age for an *Ilex* spp. plant extract before using it to control mosquito larvae.

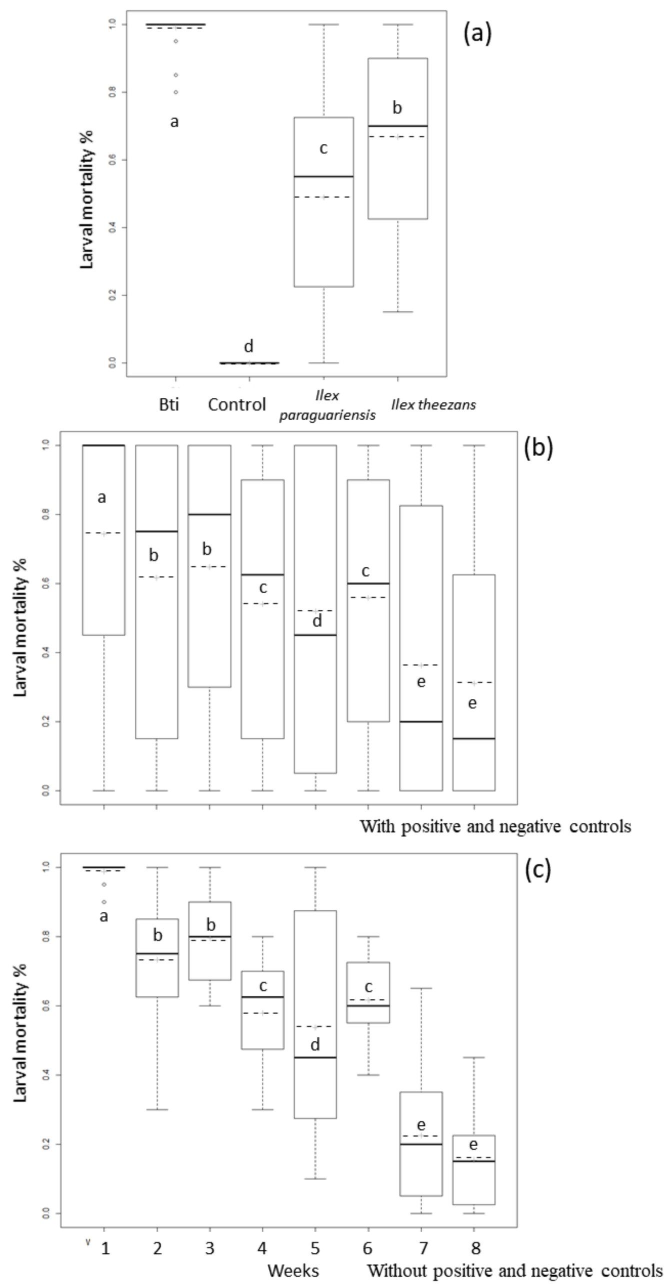

Figure 1 – (A) *Aedes aegypti* larvae mortality among treatments, (B) sample weeks with positive and negative controls, and (C) sample weeks without positive and negative controls among them*.
*Different letters ("a", "b", "c", "d", and "e") indicate significant differences. Boxes represent the quartiles, bold line represents the median, horizontal dashed line represents the mean, vertical dashed line represents the upper and lower limits, and circles represent the outliers.

This is especially true since a product is considered efficient for pest population control when it reduces individuals above 80%, otherwise resistance genes are selected (Jagadeesan et al., 2016).

In addition, besides the decaying of lethal chemical compounds which were toxic for mosquito larvae on the first week, as time goes by, the organic compounds present on the plant extracts may act as a useful food source for mosquito larvae. In this way, since organic compounds are well known for being an important component of several larval habitats, forming the basis of many food webs (Merritt et al., 1992; Moore et al., 2004), microorganisms such as bacteria play an important role in the cycling and breaking of large organic molecules (Sinsabaugh and Linkins, 1990). Therefore, microorganisms may act making them more easily absorbed by aquatic organisms, such as mosquito larvae, especially those belonging to the Culicidae family (Merritt et al., 1992). As a result, decomposing microbial communities present a relevant contribution to the diet of culicid larvae, being ingested with the organic remains over time (Merritt et al., 1992; Cochran-Stafira; Von Ende, 1998; Kaufman et al., 1999; Eisenberg et al., 2000).

The Bti of the present study resulted in 93.4% mortality in the eight-week solution. The residual effect described in the technical manual of the manufacturer is 30 days. Moreover, the values obtained herein were much higher than those described in the literature, with a lethality of 100% for 49 days and 6.6% of larval survival in up to 56 days. In this way, evaluating the effectiveness of the products that are already being used by state programs to control and combat *A. aegypti* could be performed. The use of the methodology without water renewal in the experiment resulted in a longer residual effect. Thus, not evaluating the effect of this renewal in reducing the residual effect of larvicidal substances (Pontes et al., 2005). Other studies in the field should encourage this water renewal by constantly emptying and replacing water, which would probably contribute to the reduction of the residual effect for all treatments tested.

Conclusions

The present study emphasized the need to implement alternative methods for vector control, since they represent a long-term risk. Furthermore, in the short term, it reported potential alternative pathways for mosquito-population control using natural products originated from the native flora. The plants presented a high larvicidal effect against *A. aegypti*, contributing to the maintenance of the quality of life and well-being of the population, since they are easily accessed by the local populations, reducing public expenditure with vector control and treatment of confirmed cases of dengue. Finally, time positively affected the survival of *A. aegypti* larvae due to the decay of lethal compounds from plant extracts, corroborating our first hypothesis. We also found that mortality by *B. thuringiensis* var. *israelensis* was constant throughout the experimental period and *A. aegypti* larvae survival was lower in the treatments with plant extracts than with *B. thuringiensis* var. *israelensis*. High mortality was observed in extracts of *I. theezans* compared to *I.*

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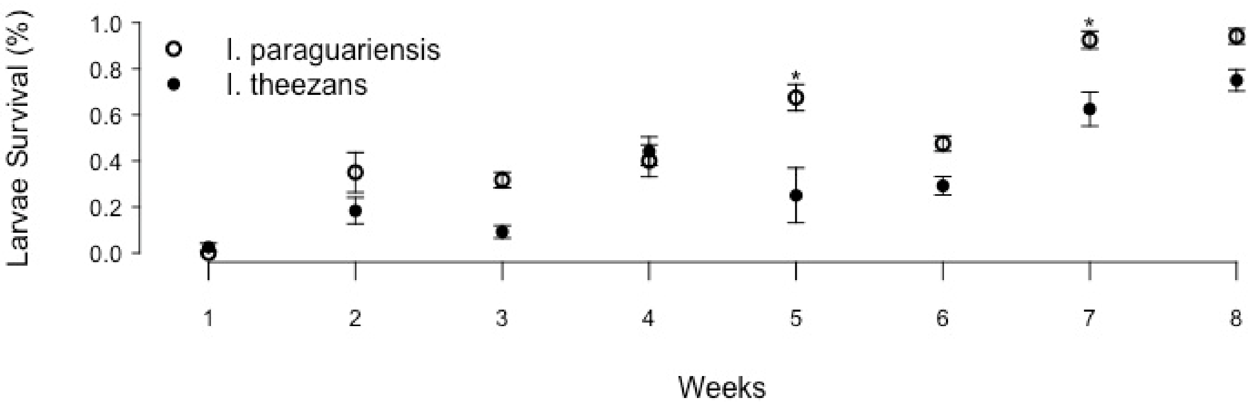

Figure 2 – *A. aegypti* larvae survival's mean as a function of plant extracts' age. Extracts' age are represented by weeks. The circles represent, aqueous *I. paraguariensis* fruits extract (open) and *I. theezans* hydroalcoholic leaves extract (closed).
*Significant statistical difference between *I. paraguariensis* and *I. theezans* affecting larvae survival.

paraguariensis, corroborating our second hypothesis. The strongest residual effect of *I. theezans* was probably due to the presence of chemicals on their leaves, such as coumarins, hemolytic saponins, and cyanogenic glucosides, which were absent in *I. paraguariensis*.

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Contribution of authors:

Cozzer, G.D.: Investigation, Data Curation, Writing – original draft. Rezende, R.S.: Software, Formal Analysis, Writing – review & editing. Lutinski, J.A.: Conceptualization, Methodology, Supervision, Writing – review & editing. Roman Júnior, W.A.: Methodology, Writing – review & editing. Busato, M.A.: Writing – review & editing. Simões, D.A.: Supervision, Formal Analysis, Funding Acquisition, Writing – review & editing.

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