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ACTIVITY OF HYDROALCOHOLIC EXTRACTS OF COMMONLY DISCARDED SEEDS OF THEOBROMA GRANDFLORUM AGAINST THE VECTOR OF ARBOVIRUSES *AEDES AEGYPTI*

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ABSTRACT

Arboviruses are still diseases considered a global threat to public health, being transmitted by the vector Aedes aegypti, belongs to the family Culicidae. They are hematophagous mosquitoes in which they adopt humans as amplification hosts to ensure the transmission of arboviruses. Aiming at an alternative proposal with the use of a plant source in vector control, the present study aims to evaluate the chemical composition and larvicidal activity of hydroalcoholic extracts of Seeds of Theobroma grandiflorum (Willd. ex Spreng). The extracts were prepared from maceration in extractor ethanol 70%PA solvents in hydromodulus 1:4/1:6/1:8 in 7 days with subsequent concentration in rotaevaporator. On the other, larvicidal activity achieved by the WHO method of Lethal Concentration 50% against Aedes aegypti larvae with statistics by Probit method. Toxicity to non-target organisms was verified by artemia saline test. Larvicidal activity with CL50 was evidenced ranging from 35.018 to 369.191 mg L-1 with a 95% confidence interval. It is noted that the extract T. grandiflorum exhibits high larvicidal potential and has no toxicity to target organisms. It is observed that the extract of T. grandiflorum is shown to be a promising and sustainable strategy in the fight and control of Aedes aegypti larvae.

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INTRODUCTION

Diseases caused by arboviruses are called arboviruses and characterized by a wide geographical distribution, causing asymptomatic infections or febrile diseases in humans in enzootic and urban cycles (Higuera *et al*., 2019). They continue to be promoters of great impact on public health and causing overload to health systems in the world (Queiroz *et al*, 2020). They are transmitted by the vector Aedes aegypti, belonging to the family Culicidae (Wu *et al*., 2019), they are hematophagous mosquitoes in which they adopt humans as amplification hosts to ensure the transmission of arboviruses in urban cycles (Huang *et al*., 2019). They are agents of serious diseases such as dengue, chikungunya fever, yellow fever and zika fever (Viana *et al*., 2018), considered epidemics that require urgency and the need for integrated control and preventive measures (Girard *et al*., 2020). They are diseases considered a global threat to public health, the region of the Americas, dengue is more common and of greater circulation, with a progressive growth in the number of cases since its introduction in 1980. In 2019 it reached 3.1 million reported cases, the highest record in recent years (WHO, 2020).

Among the main factors that show the emergence of these diseases worldwide, we can highlight climate change, ecosystem breakdown, accelerated urbanization, waste management and garbage accumulation, including automobile tires, plastics, cans and possible vector breeding sites (Zientara *et al,* 2020). Despite the advance of vaccines to prevent infection with arboviruses, they still demonstrate failures and risks to vaccinated individuals, whether from interaction between serotypes, post-vaccine exposure to the virus, decline of vaccine protection, amount of dose administered and risk of causing severity (Floresto & Fernandes, 2020). It is noteworthy that only the yellow fever vaccine is effective and safe for 10 years (Rothman, 2004). Prevention and control actions are also focused on vector management, ranging from treatment with synthetic insecticides, environmental interventions and social mobilization (Samuel *et al*., 2017). However, synthetic insecticides that are widely used contain synthetic pyrethroids and other organophosphate compounds that can cause toxic effects in humans, mosquito resistance-related mutations and ecosystem contamination (Takagi *et al*, 2020). It is observed that no strategy in isolation is effective in combating arboviruses, some resources allow to reduce the burden of the disease, such as access to clinical services, internal space sprays, eradication of vector breeding sites, diagnosis and laboratory surveillance (Samuel *et al*, 2017).

It is necessary to establish a relationship between environment and health, with the purpose of preventing health problems, understanding that the control of vectors such as Aedes aegypti in which they present high proliferation in environments with accelerated urbanization. (Almeida, Cota, Rodrigues, 2020). It is necessary to develop new vector control alternatives with different mechanisms of action. The ideal is one that is effective, ecologically safe, sustainable, low cost and contains low toxicity for mammals (Silva *et al*, 2017). It is known that plants generate secondary metabolites such as flavonoids, alkaloids and terpenoids as a strategy to protect against insects and are considered natural sources of insecticidal substances (Simões *et al*, 2010). With the proposal of incorporating the use of plant source as alternatives to control A. aegypti, the present study aims to evaluate the larvicidal activity of hydroalcoholic extracts of Seeds of Theobroma grandiflorum (Willd. ex Spreng), besides contributing to the reuse of by-products from the food industry.

METHODOLOGY

Botanical material: The fruits of Theobroma grandiflorum were collected at a property located in the Ipem Turu neighborhood, in the municipality of São Luis-MA in November 2020. These were dried in a greenhouse with air circulation at 40ºC and crushed in a knife mill with moderately thick powder granulometry. Exsicatas were produced in flowered branches and sent to the Attic Aherary Seabra, the Center for Biological and Health Sciences at the Federal University of Maranhão for confirmation of botanical recognition.

Obtaining hydroalcoholic extract: To obtain the hydroalcoholic extracts, the maceration technique was used using an ethanol extracting solvent 70% PA in hydromodules of 1:4, 1:6 and 1:8 (m/v), in 70% ethanol. The maceration will be carried out for 10 days, under agitation and filtration (Figure 1). The resulting extracts, observed in Figure 1, were concentrated in a rotaevaporator (Figure 2) and stored in a refrigerator at 4ºC in amber colored flasks (Matos, 2009). The extracts yields were calculated by gravimetric technique and the physicochemical parameters: density and refractive index were determined according to the techniques recommended by the Brazilian Pharmacopoeia (2019).

Phytochemical analysis: The extracts were subjected to chemical tests based on the methodology presented by Matos (2009). The tests performed were: Salkowsk test (steroids), Mayer test (alkaloids), flavonoids, glycosides, saponins test, keller kiliani test (cardiac glycosides), ferric chloride test (phenols) and lead acetate test (tannins). And the total phenolic content was quantified through the spectrophotometric method of Folin-Ciocalteau and the total flavonoid through the spectrophotometric method of complexation with aluminum (Lugasi *et al*., 1998; Oliveira *et al*., 2009).

Collection of Aedes aegypti eggs: The collection of Aedes aegypti eggs was carried out using traps called ovitraps (Figura 3), with the aid of Eucatex palettes. The Eucatex palettes were properly inspected, sanitized and dried before setting up the traps. The traps were installed at various points on the campus of the Universidade Federal do Maranhão, Recanto dos Vinhais (São Luís, MA) and Alemanha (São Luís, MA) under shelter from the sun and rain. The collected eggs were hatched in mineral water and fed until the stage where the experiments were carried out.

Larvicide activity against Aedes aegypti: The tests for larvicidal activity were carried out according to the adapted methodology proposed by Silva (2006). Initially, a 500 mg L-1 stock solution of each of the hydroalcoholic extracts was prepared and diluted in a 2% Tween 80 solution. From this solution, serial dilutions were prepared at concentrations 100-400 mg L-1. At each concentration, 10 larvae were added at the rate of 2 ml per larva. All tests were performed in triplicate and as negative control a solution made up of 2% Tween 80 was used, and as positive control, a solution of temephos (O,O,O',O'-tetramethyl O,O'-thiodi- p-phenylene bis (phosphorothioate) at 100 ppm, equivalent to the concentration used by the National Health Foundation (FUNASA) for the larvicide control of the vector, in addition to Novaluron (±-1-[3-chloro-4-(1-1-) 3-trifluro-2-trifluoromethoxyethoxy) phenyl-3-(2,6-diflurobenzoyl) urea at 0.02 mg L-1, dose adopted by the Ministry of Health, indicated by the WHO in the range of 0.01 to 0.05mg L-1. After 24 hours the count of live and dead was carried out, and larvae that did not react to touch after 24 hours of the beginning of the experiment were considered dead. To quantify the efficiency of the extracts, the Probit statistical test (Finney, 1952) was applied.

Toxicity assessment against *Artemia salina*

This test was performed according to the methodology described by Meyer *et al*. (1982). To assess the lethality of Artemia salina Leach, a stock saline solution of each hydroalcoholic extract was prepared at a concentration of 10,000 mg L-1 and 0.02 mg of Tween 80 (active tension). Aliquots of this were transferred to test tubes and supplemented with saline solution previously prepared up to 5 mL, obtaining in the end concentrations of 1000-10 mg L-1, respectively, where ten larvae in the nauplial stage were transferred to each of the tubes of rehearsal. For the blank, 5 mL of saline solution was used, for the positive control K2Cr2O7 and for the negative control 5 mL of a 4 mg L-1 solution of Tween 80. After 24 hours of exposure, the count of live larvae was performed, considering dead those that did not move during the observation or with the slight shaking of the flask. The criterion established by Dolabela (1997) was adopted to classify the toxicity of hydroalcoholic extracts, being considered highly toxic when LC50 ≤ 80 mg L-1, moderately toxic for 80 mg L-1 ≤ LC50 ≥ 250 mg L-1 and slightly toxic or non-toxic when LC50 ≥ 250 mg L-1. The statistical analysis of the data for the toxicity test was carried out according to the method of Reed&Muench (1938). The intersection point between the curves is the 50% Lethal Concentration (LC50), since at this point the number of surviving animals is equal to the number of dead animals (Colegate; Molyneux, 2007).

RESULTS AND DISCUSSION

Phytochemical parameters and phytochemical screening: The physicochemical parameters are important for determining aspects of biological applications and are presented in Table 1. The classes of secondary metabolites identified in the extracts obtained from Theobroma grandiflorum are shown in Table 2. It was found that the phytochemical screening of hydroalcoholic extracts of Theobroma grandiflorum showed high extraction power for the classes of metholithic compounds such as: steroids, alkaloids, flavonoids, glycosides, cardiac glycosides, phenols and tannins, presented in Table 2. A diversity of constituents is noted and can be considered an indication of different biological activities. It is noteworthy that the quality of the extraction is directly sensitive to the type of solvent adopted (Ferro, 2008). It is observed in the phytochemical screening of the present study in which ethanol was used as a solvent, one observed the heterogenicity of the metábolites. Oliveira (2016) points out that ethanol is a substance with amphiphilic character, which allows the extraction of both nonpolar and polar characteristics. The chemical compositions of the same plant species may diverge due to the characteristics inherent to the plant and the conditions in which it was cultivated (physiology, stage of development and environmental conditions) (Silva *et al*, 2021).

In a study conducted by Freitas *et al*. (2017), recorded 403.00 mg EAT g-1 of phenolic content in residues of the extraction of the retained pulp in the sieves of the pulp industry. In research conducted by Pérez-Mora *et al*. (2018) with the pulp juices of Theobroma grandflorum and obtained 226 mg EAT g-1 of the total phenols. In an analysis performed by Couto *et al*. (2020) in the pulp of Theobroma grandiflorum presented flavonoid content of 20.5 ± 3.0 mg EQ g-1. The flavonoid contents present in fruits may fluctuate from one period to the next, Araújo (2017) points out that extrinsic factors such as climatic conditions, degree of fruit maturation, crop period and colheiras directly influence the composition of tropical fruits.

Table 1. Physicochemical parameters of the hydroalcoholic extract of *Theobroma grandiflorum*

Parameter	Density (g mL^{-1})	Refractive Index (nD 25°)
EHTG1:8	0,9078	1,341
EHTG1:6	0,8934	1,349
EHTG1:4	0,92	1,344

Source: Author (2021).

Table 2. Classes of secondary metabolites identified in extracts obtained from the seeds of *Theobroma grandiflorum*

Hydroalcoholic extracts *Theobroma grandiflorum.*	1	2	3	4	5	6	7	8
EHTG1:8	+	+	+	+	+	-	+	+
EHTG1:6	+	+	+	+	+	-	+	+
EHTG1:4	+	+	+	+	+	-	+	+

Note: 1-Steroids,2-Alkaloids,3-Flavonoids,4-Glycosides, 5-Cardiac Glycosides, 6-Saponins, 7-Phenols,8-Tannins.

Table 3. Total phenolic content (mg EAT g^{-1}) quantified for extracts obtained from *Theobroma grandiflorum* seeds

Extract	Total phenolic content (mg EAT g^{-1})	Equation	R^2
EHTG1:8	236,55	0,05857x + 0,06	0,9998
EHTG1:6	283,422		
EHTG1:4	416,596		

Note: EHTG –*Theobroma grandiflorum* hydroalcoholic extract.

Table 4. Total flavonoid content (mg EQ g^{-1}) quantified for extracts obtained from *Theobroma grandflorum* seeds

Extract	Total flavonoid content (mg EQ g^{-1})	Equation	R^2
EHTG1:8	28,14	0,00332x +0,00058	0,9997
EHTG1:6	72,11		
EHTG1:4	15,19		

Source: Author (2021).

Table 5. Mortality *Artemia salina* Leach against the action of the hydroalcoholic extract of *Theobroma grandflorum* in 1:8, 1:6 and 1:4 hydromodule (m/v)

	Accumulated curve intersection log	LC_{50} mg L^{-1}	Classification
EHPT 1:8	2,93	851,1	Atoxic
EHPT 1:6	2,98	955,0	Atoxic
EHPT 1:4	2,99	977,2	Atoxic

Source: Author (2021).

Table 6. Mortality *Aedes aegypti* against the action of the hydroalcoholic extract of *Theobroma grandflorum* in 1:8, 1:6 and 1:4 hydromodule (m/v)

EHTG 1:8	Log C	%Mortality	LC_{50} (mg L^{-1}) 95% CI	X^2	σ	R^2
	2,00	20,0	369,191 (215,20-633,35)	0,507	0,652	0,930
	2,30	30,0				
	2,48	50,0				
	2,60	50,0				
	2,70	100,0				
EHTG 1:6	Log C	%Mortality	LC_{50}(mg L^{-1}) 95% CI	X^2	σ	R^2
	2,00	70,0	35,018 (15,39-79,66)	1,000	0,834	0,912
	2,30	80,0				
	2,48	90,0				
	2,60	90,0				
	2,70	90,0				
EHTG 1:4	Log C	%Mortality	LC_{50}(mg L^{-1}) 95% CI	X^2	σ	R^2
	2,00	50,0	113,337 (75,74-169,57)	0,954	0,420	0,851
	2,30	60,0				
	2,48	90,0				
	2,60	90,0				
	2,70	100,0				

Source: Author (2021).

According to the criterion adopted by Dolabela(1997) regarding plant extracts on A. salina for classification of the toxicity of natural products, it is considered a highly toxic product when CL50 ≤ 80 mg L-1, moderately toxic to 80 mg L-1 ≤ CL50 ≥ 250 mg L-1 and mildly toxic or nontoxic when CL50 ≥ 250 mg L-1. There were few studies with Seeds of Theobroma grandflorum in this way compared seed toxicities of other species. Wedd against nauplii from Arthemia sp and obtained as a result the value of 67.85 mg L-1, thus presenting toxicity.

Almeida *et al* (2021) evaluated extracts of Triplaris gardneriana Dantas *et al* (2020) found in ethanolextract of moringa oleiferseeds the value of 1783.40, being considered nontoxic. It is noteworthy that when there is no toxicity present in seed extracts, it represents that such extract does not present substances that cause damage to biological systems (Campos *et al*, 2016). It was proved that the extracts analyzed in the present study, in the three hydromodules of 1:4, 1:6 and 1:8 (m/v), in 70% ethanol is considered nontoxic, according to dolabela criterion (1997).

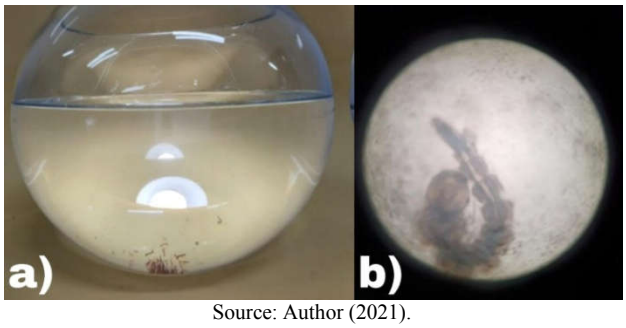
Source: Autor (2021).

Figure 1. Filtration processing using the vacuum system to separate the extract solid from the extracting solvent after 10 days of maceration

Source: Author (2021).

Figure 2. Rotaevaporation of the extracting solvent to concentrate the hydroalcoholic extract

Source: Author (2021).



Source: Author (2021).

Figure 4. Process of collection and recognition of Larvae of *A. aegypti*

Table 6 presents mortality data of Aedes aegypti larvae for extract action. Similar results were observed by Madhiyazhagan *et al*. (2020) in which they evaluated ethanolextracts of Momordica charantia seeds against *Aedes aegypti* and obtained LC50 between 51,820 and 336.137 mg L-1. However, in research conducted by Sogan *et al*. (2018) in which they determined the lethal concentration of Ricinus communis seeds in methanolextracts and obtained LC50 between 7.08 and 12,757 mg L-1. In a study conducted by Berhe *et al*. (2021) with powdered seeds diluted in Azadrichiata indica water, presented satisfactory results in a lethal concentration of 58 mg L-1. Thus, studies with seeds are relevant for the control and combat of the vector of arboviruses *A. aegypti.*

Conclusions

It can be observed that the hydroalcoholic extracts of Theobroma grandiflorum presented toxicity with larvae of A. aegypti being considered an alternative control of the vector *A. aegypti.* The application of larvicides of plant origin for mosquito control has been shown to be more effective, because it is used directly in the vector breeding sites and are less harmful to the environment. However, further studies are necessary for chemical characterization and isolation of the active constituents present in the hydroalcoholic extracts of *Theobroma grandiflorum* in order to develop a biocontrol product to minimize vectors of persistent arboviruses.

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