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Major Article

In vitro evaluation of the effect of botanical formulations used in the control of *Aedes aegypti* L. (Diptera: Culicidae) on liver enzymes.

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Abstract

Introduction: Dengue fever is a viral disease transmitted by the *Aedes aegypti* Linn. (1792) (Diptera: Culicidae) mosquito, which is endemic in several regions of Brazil. Alternative methods for the control of the vector include botanical insecticides, which offer advantages such as lower environmental contamination levels and less likelihood of resistant populations. Thus, in this study, the ability of botanical insecticide formulations to inhibit the activity of the liver enzymes serum cholinesterase and malate dehydrogenase was evaluated. **Methods:** Inhibition profiles were assessed using *in vitro* assays for cholinesterase and malate dehydrogenase activity and quantitated by ultraviolet-visible spectroscopy at 410nm to 340nm. **Results**: Insecticide products formulated from cashew nutshell liquid [A] and ricinoleic acid [B] showed cholinesterase activity levels of 6.26IU/mL and 6.61IU/mL, respectively, while the control level for cholinesterase was 5-12IU/mL. The products did not affect the level of 0.44IU/mL established for malate dehydrogenase, as the levels produced by [A] and [B] were 0.43IU/mL and 0.45IU/mL, respectively. **Conclusions**: Our findings show that *in vitro* testing of the formulated products at concentrations lethal to *A. aegypti* did not affect the activity of cholinesterase and malate dehydrogenase, indicating the safety of these products.

Keywords: Cytotoxicity. Botanical insecticide. Dengue fever.

INTRODUCTION

Vector surveillance programs for dengue fever are one strategy to reduce its incidence in Brazil. Chemical insecticides are the most common strategy to reduce mosquito populations, although other options are available. Given the low effectiveness and high costs of these programs, there is a need to review control strategies as the figures clearly show that dengue fever is becoming endemic in Brazil[1]. The course of infection may be influenced by migration and urbanization, making disease monitoring and surveillance challenging[2]. This scenario is compounded by the continuous use of chemical insecticides, which has caused the target insects to develop resistance to various compounds, leading to the need for development of other forms of control using new products that are less harmful to humans and to the environment[3].

Although resistance to insecticides can be seen as a process of rapid evolution within a population in response to selective pressure, it is pre-adaptive and is the result of random mutations whose mechanisms are related to the penetration rate of the insecticide into the vector's cuticle, its increased metabolic detoxification, and decreased sensitivity of the target site[4].

Among the various related mechanisms, plant-based compounds are the main sources of new molecules with the potential for interacting with biological systems, acting at different stages of development and presenting different mechanisms of action[5].

Plant-based products whose molecular complexity exerts lower selective pressure, leading to decreased resistance. Thus, the use of substances or formulations with differentiated activity are gaining ground in vector control when compared with conventional chemical insecticides, given the latter's toxicity and ability to cause changes in the target organism, their environmental impact, and their effect on human metabolism and the central nervous system[6].

Botanical insecticides elicit less resistance and also accumulate more slowly in the environment owing to their low potency and dosages. However, for their effective use among the general population, they must meet all safety and efficacy requirements established by Brazilian regulatory agencies. The establishment of clinical toxicity protocols serves to evaluate the safety and monitoring methods used for chemical control.

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This study involved an *in vitro* evaluation of the effect of botanical insecticides formulated from cashew nutshell liquid [A] and ricinoleic acid [B] on the activity of the liver enzymes serum cholinesterase (ChE) and malate dehydrogenase (MDH).

METHODS

The formulated products were diluted according to the lethal concentrations determined in larvicidal assays for *Aedes aegypti* Linn. (1792) (Diptera: Culicidae), using the determined LC_{90} dosages of 0.135mg/mL for liquid cashew nuts and 0.202mg/mL for ricinoleic acid as references[7]. The effects of botanical insecticides on the activity of liver enzymes were assessed as the ability inhibit ChE and increase MDH levels. The nicotinamide adenine dinucleotide (NAD) dehydrogenase disappearance rate correlates to the increase in MDH levels. Calibration curves indicated the linearity of each reaction between zero and the maximum absorbance of the enzyme at different concentrations, measured at the absorbance specific to each reaction, which was 410nm for ChE and 340nm for MDH in the ultraviolet range.

Serum cholinesterase activity

The action of products [A] and [B] on ChE activity was assessed by spectrophotometry[8] [9]. Serum cholinesterase hydrolyzes propionylthiocholine to release thiocholine, which reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) - a color reagent - to form a yellow compound that is absorbed at 410nm [Thermo Scientific ultraviolet (UV)-Vis, AquaMate®]. The substrate (propionylthiocholine) was added at a concentration of 0.43mol/L. The enzyme activity assay was performed in triplicate at controlled temperature (37ºC) using distilled water (4mL), DTNB (3mL), and 7.0IU/mL (20µl) of ChE. Product [A] was used at concentrations of 0.2, 0.8, and 1.5g/L and product [B] was used at concentrations of 0.035, 0.123, and 0.430g/L, corresponding to LC_{10}, LC_{50}, and LC_{90}. A positive control was prepared with substrate, DTNB, and enzyme, and the negative control was water and DTNB. Enzyme activity was inhibited using a solution of 0.5% quinidine sulfate (3mL), 30 sec after additional of the enzyme.

Calibration curves were constructed using ChE at concentrations of 1.875, 3.75, 7.0, 10.5, 14.0, and 17.5IU/mL. The straight line equation was y = 0.861x + 0.543, r^2= 0.9703 (**Figure 1**).

Transaminase activity

Nicotinamide adenine dinucleotide (NAD) dehydrogenase is an intermediate of most biochemical reactions, but it cannot be directly quantified. NAD dehydrogenase was therefore quantified in triplicate by UV-spectrophotometry at 340nm (Thermo Scientific UV-Vis, AquaMate®) following a coupling reaction with MDH at 37ºC[10].

For these assays, 1.5mL of enzyme reagent (containing MDH) and 0.5mL of a solution containing NAD were heated to 37ºC. After 3 min, 200µL of products [A] and [B] at a concentration of 1g/L was added, while water was added to the positive control. The reaction was stopped after 1 min by adding 1mL of 1 mol/L sodium oxalate solution. Calibration

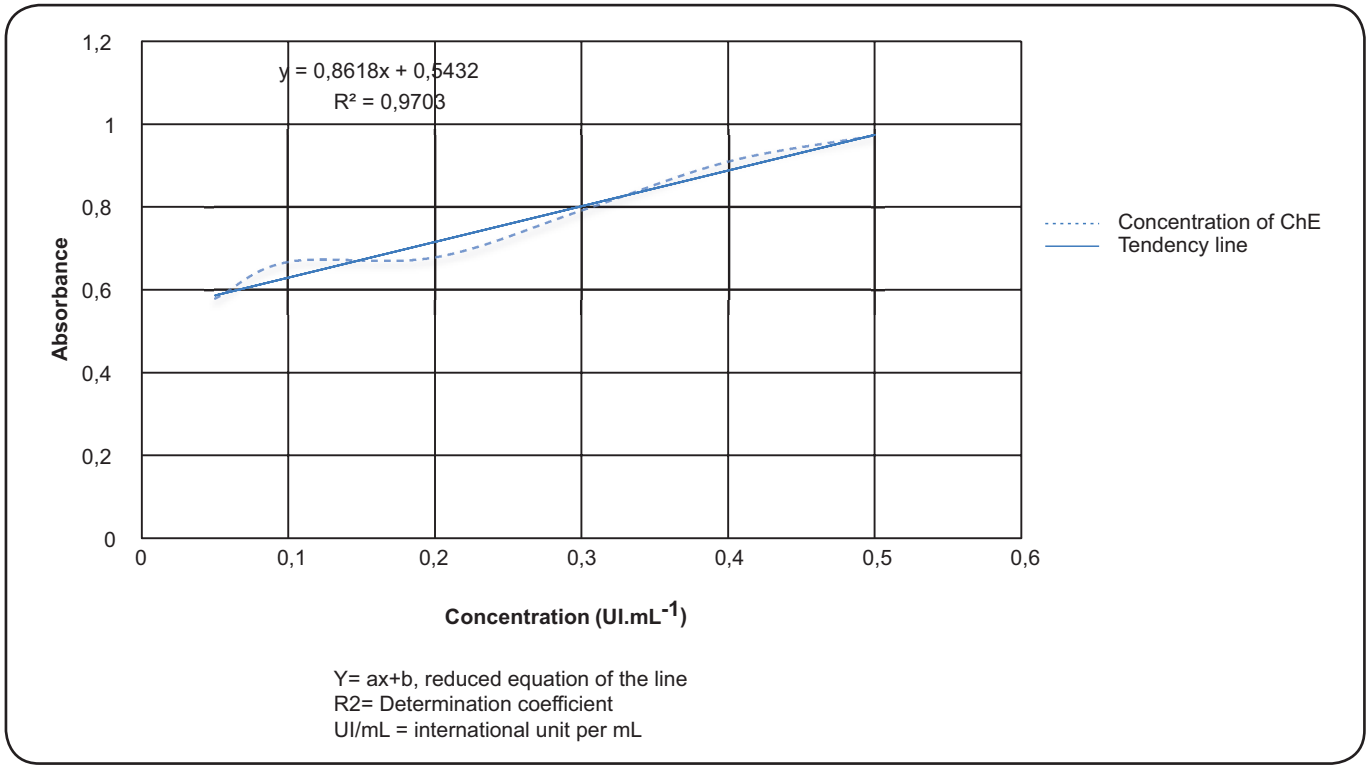

FIGURE 1. Graphical representation of the relationship between absorbance at 410nm and concentration of serum cholinesterase dilutions of a standard solution of 7IU/mL. **ChE:** serum cholinesterase.

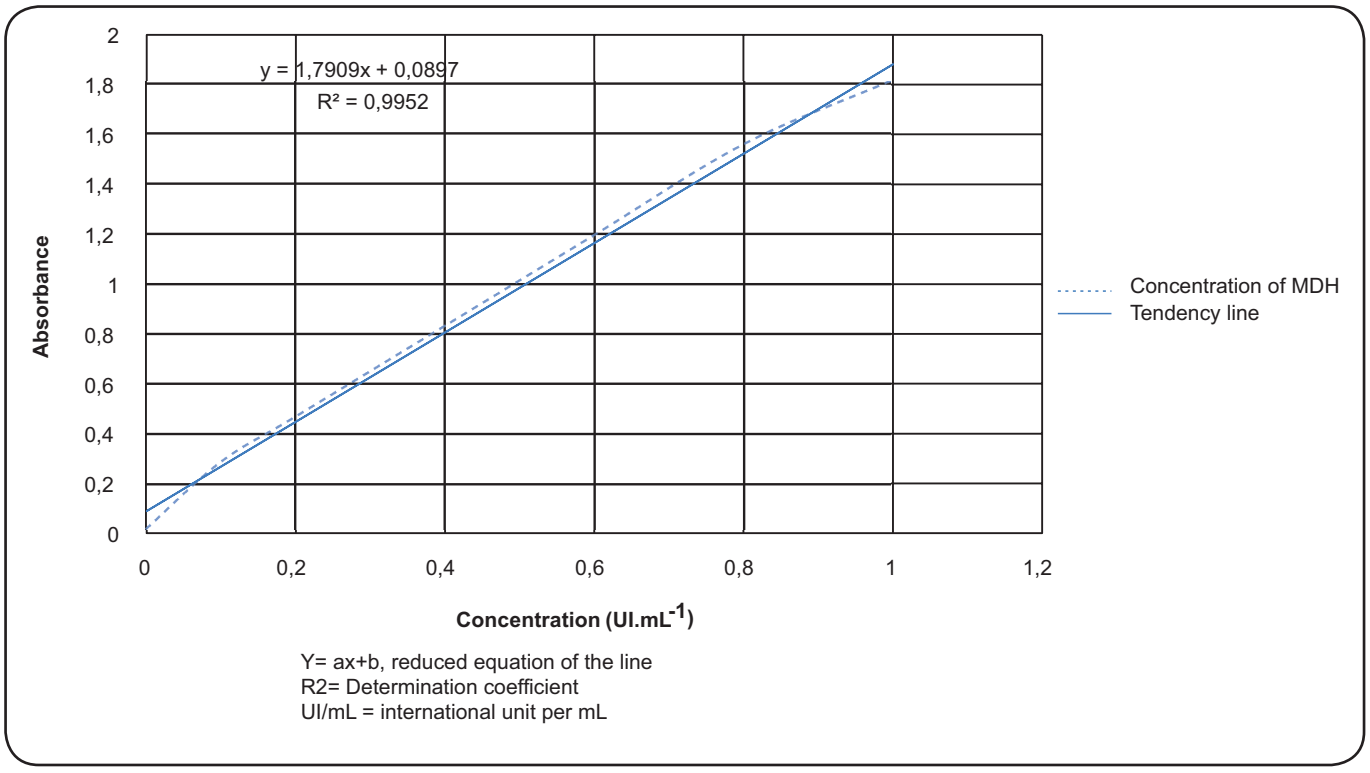

FIGURE 2. Graphical representation of the relationship between absorbance at 340nm and concentration of malate dehydrogenase dilutions of a standard solution of 0.48IU/mL. **MDH:** malate dehydrogenase.

curves were constructed using MDH at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1IU/mL. The straight line equation for MDH was y = 1.790x + 0.089 r^2= 0.9952 (**Figure 2**).

Sensitivity test

The sensitivity of enzyme activity is directly related to the color generated by the enzymatic reaction with the substrate in the presence of the color reagent in aqueous medium.

The sensitivity test was designed to assess and monitor the analytical variability of ChE and MDH activity with standard solutions of 7IU/mL and 0.48UI/mL, respectively.

Determination of enzyme inhibition and statistical analysis

Data on mean enzyme inhibition were analyzed using one-way analysis of variance (ANOVA) and Tukey's post-hoc test, at a significance level of 5%, using the Statistical Assistance (ASSISTAT) version 7.7 beta software program (ASSISTAT 2015)[11].

RESULTS

Sensitivity testing to ascertain the inhibition and stability of ChE *in vitro* indicated that different concentrations of the formulations did not inhibit enzyme activity at the significance level of the control (p-value < 0.01). None of the values below the most toxic level of insecticide affected enzyme activity, and lower initial values than control were observed at the lowest concentration established on the linearity curve (**Table 1**).

Formulations [A] and [B] did not reduce the enzyme activity of ChE, which fell within the normal levels of 5-12IU/mL, with a mean value of 7.08IU/mL.

For the catalytic concentration pertaining to the oxidation of MDH, it was found that the [A] and [B] formulations did not modify the predicted values for enzyme activity. A comparison of the positive control and tested sample values indicated that the average absorbance did not vary and that the values remained within the linear range, as shown in **Table 2**. ChE activity or NAD dehydrogenase were not affected by the insecticide formulations.

DISCUSSION

New products or formulations under study often pose serious threats to the ecosystem, not only because they suppress some metabolic mechanism of the target insect but also because of their cellular similarity to other organisms in the environment.

The dangers of and problems caused by the use of insecticides have long been known, as has the establishment of a model to explore the impact of these compounds on humans and the environment[12].

Earlier study have revealed high esterase activity associated with the survival of the L4 larvae of *A. aegypti*, even when exposed to isolated chemicals[13].This finding was observed in larvicidal studies in western Venezuela[14], where the high efficiency of the chemical insecticide Temefos was found to be inversely proportional to the expression of the enzymatic activity of transaminases and acetylcholinesterase.

TABLE 1

Determination of the inhibitory capacity of formulations [A] and [B] based on the analytical method for *in vitro* evaluation of serum cholinesterase at 410nm.

Variables	Positive control for ChE	Formulation [A]	Formulation [B]
ChE (IU/mL, ± SD)	8.36[a] (0.02)	6.26[b] (0.04)	6.61[b] (0.016)
Overall mean (IU/mL^{-1})	7.08		
Coefficient of variation (%)	3.86		

ChE: serum cholinesterase; **SD**: standard deviation. [a,b]Means sharing the same superscript letter are not significantly different from each other (Tukey's test at 5% significance). UI/mL = international unit per mL.

TABLE 2

Determination of the inhibitory capacity of formulations [A] and [B] based on of the analytical method for *in vitro* evaluation of malate dehydrogenase at 340nm.

Variables	Positive control for ChE	Formulation [A]	Formulation [B]
MDH (IU/mL, ± SD)	0.45[a] (0.11)	0.43[a] (0.069)	0.44[a] (0.018)
Overall mean (IU/mL)	0.45		
Coefficient of variation (%)	18.99		

[a]Means sharing the same superscript letter are not significantly different from each other (Tukey's test at 5% significance). UI/mL = international unit per mL.

Alterations in transaminase were also observed in the blister beetle, *Mylabris pustulata* (Thunberg, 1821), following exposure to sublethal doses of carbaryl, which inhibited enzyme levels after both short and long periods of treatment[15].

The level of toxicity of insecticides to humans or the environment will depend on the toxicity of the substance or compound, its exposure time or accumulation, and the quantity employed. However, it is difficult to establish a diagnosis in the case of exposure to multiple components with differentiated mechanisms of action[16].

It is common for insect species to use different resistance mechanisms to adapt to the pressure exerted by continuous use of an insecticide or to single molecules, and one mechanism of resistance that insects develop is the modification of the target protein of the insecticide, although the use of selective insecticides is a major factor in insect control[17].

Cholinesterases are enzymes that promote the hydrolysis of acetylcholine, which acts as a neurotransmitter responsible for the transmission of nerve impulses. In the human body, true cholinesterases are classified as those present in red blood cells, the lungs, spleen, neurons, and in the brain, while pseudocholinesterases are those present in the liver plasma, pancreas, and intestine. In humans, the inhibition of acetylcholine hydrolysis caused by the presence of anticholinesterase substances leads to disorders of muscarinic (in smooth muscle, cardiac fibers, and exocrine glands) and nicotinic activity (in skeletal muscles and autonomic ganglia)[18][19]. When poisoning by anticholinesterase agents occurs with inhibition exceeding 50% of the activity of the pool of cholinesterase, it is commonly accompanied by a severe acute cholinergic crisis, although stimulation of the parasympathetic nervous system with nicotinic receptor response is observed[20].

The insensitivity of ChE to the action of insecticides is a frequent mechanism of resistance in insects[21]; hence, a botanical insecticide that does not reduce or block sensitivity is of particular value because it may also reduce the likelihood of health risks to humans.

Enzymatic and immunohistochemical evidence indicates a high degree of similarity to homologues that participate in mitochondrial electron transport involved in nicotinamide adenine dinucleotide (NADH) proton translocation between insects, fungi, and mammals, related to energy metabolism. The energy obtained from the oxidation of organic molecules in the form of reduced cofactors, such as NADH, is used to trigger the phosphorylation of adenosine diphosphate, which is energetically unfavorable, supplying adenosine triphosphate (ATP), which acts in coupled processes[22][23]. Thus, the presence of an inhibitor of the electron transport chain will adversely affect the production of ATP.

Increased hepatobiliary enzyme levels are one of the most sensitive markers used for the detection of drug-induced liver disease, also known as hepatotoxicity indicator serum liver enzymes. These enzymes are alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase, and gamma-glutamyltransferase (γ-GT). These increases may indicate alterations ranging from adaptive biochemical and structural changes to morphological lesions, often with irreversible metabolic or cell damage. The risk is even greater when the substance is fat-soluble, enabling it to bind to plasma proteins until it reaches the liver, where it undergoes biotransformation into a water-soluble substance that is subsequently eliminated through biliary or renal excretion[24]. The results of MDH exposure to formulations [A] and [B], which are fat-dispersible substances, indicated that these products did not alter enzyme activity. Under the study conditions, the results also confirmed

that these products are safe for humans in the event of exposure, although they are highly toxic to the target insect.

The identification and quantification of certain changes in organic systems, based on biochemical or physiological cell signaling, can be used for the biological monitoring of populations exposed not only to environmental but also industrial agents[22]. The assessment of enzymatic activity as a bioindicator serves not only to monitor the degree of exposure but also enables clinical or toxicological diagnosis associated with an effect or dysfunction of the biological system under evaluation[25], as shown in previous studies involving rural workers exposed to organophosphates and carbamates[26].

In summary, our findings show that *in vitro* testing of the formulated products at concentrations lethal to *A. aegypti* did not affect the enzymatic activity of ChE and MDH, indicating the safety of these products. Furthermore, the *in vitro* enzymatic assays described here represent potential preliminary safety indicators for insecticide products as biochemical parameters are recognized as indicators of *in vivo* toxicity during assessment of the effects of exposure to these agents.

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Conflict of Interest

The authors declare that there is no conflicts of interest.

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