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Behavior of detoxifying enzymes of *Aedes aegypti* exposed to girgensohnine alkaloid analog and *Cymbopogon flexuosus* essential oil

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ABSTRACT

Because mosquito control depend on the use of commercial insecticides and resistance has been described in some of them, there is a need to explore new molecules no resistant. *In vivo* effects of girgensohnine analog 2-(3,4-dimethoxyphenyl)-2-(piperidin-1-yl)acetonitrile **DPPA** and *Cymbopogon flexuosus* essential oil **CFEO**, on the detoxifying enzymes acetylcholinesterase (AChE), glutathione-S-transferase (GST), nonspecific esterases (α- and β-), mixed function oxidases (MFO) and p-NPA esterases were evaluated on a Rockefeller (Rock) and wild *Aedes aegypti* population from Santander, Colombia (WSant). The action was tested after 24 h of exposure at concentrations of 20.10, 35.18 and 70.35 mg L^{-1} of **DPPA** and 18.45, 30.75 and 61.50 mg L^{-1} of **CFEO**, respectively. It was found that AChE activity of Rock and WSant was not influenced by the evaluated concentration of **DPPA** and **CFEO** ($p > 0.05$), while MFO activity was significantly affected by all **CFEO** concentrations in WSant ($p < 0.05$). GST, α- and β-esterase activities were affected in Rock exposed at the highest **CFEO** concentration, this concentration also modified β-esterases activity of WSant. **DPPA** and **CFEO** sublethal doses induced inhibition of AChE activity on untreated larvae homogenate from 12 to 20% and 18 to 26%, respectively. For untreated adult homogenate, the inhibition activity raised up to 14 to 27% for **DPPA** and 26 to 34% for **CFEO**. Elevated levels of detoxifying enzymes, found when **CFEO** was evaluated, showed a larval sensitivity not observed by the pure compound suggesting that **DPPA**, contrary to **CFEO**, was not recognized, transformed or eliminated by the evaluated detoxifying enzymes.

1. Introduction

The proliferation of *Aedes (Stegomyia) aegypti* (Linnaeus, 1762), main vector of dengue, chikungunya and zika virus, is a public health problem in tropical and subtropical regions around the world (Riaz et al., 2013). These diseases are endemic in Latin America, Africa, Southeast Asia, and Western Pacific and collectively infect 50–100 million people every year from the 2.5 billion that are living in areas where the diseases can be transmitted. Dengue hemorrhagic fever is responsible for approximately 25.000 deaths (Zhu and Lin, 2018; Huang et al., 2017; Yacoub and Wills, 2014). Even though several dengue vaccine candidates are in development, nowadays there are no treatments or vaccines commercially available for dengue disease, and therefore vector control activities are the only available methods to prevent the disease transmission (Scott, 2016; Schwartz et al., 2015; Villar et al., 2014). Control activities are based principally on the elimination of vector mosquitoes through the use of chemical insecticides. However, this method is threatened by increasing reports of *Ae. aegypti* resistance to common classes of insecticides including organochlorines (OC), organophosphates (OP), carbamates and pyrethroid (Macoris et al., 2014; Grisales et al., 2013; Ocampo et al., 2011; Polson et al., 2011; Georghiou, 1994).

Different studies have focused on the search for natural products as new alternative methods intended to control *Ae. aegypti* proliferation (Bakkali et al., 2008). Hundreds of essential oils obtained from plants have been screened as potential sources of insecticides (Tak et al., 2017; Grzybowski et al., 2013). Despite of xenobiotic properties of essential oils, their lack of selectivity and short life time in their effectiveness, have encouraged the isolation and evaluation of pure natural molecules such as alkaloids (Nerio et al., 2010; Silva et al., 2008). The scarcity of

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alkaloids and its limited availability in natural sources stimulates the search for synthetic methodologies that allow the production of these products with high yields (Angle and Breitenbucher, 1995).

Girgensohnine, a *N*-cianomethyl piperidine racemic metabolite extracted with a yield no > 0.05% from *Girgensohnia oppositiflora* (Pall.) Fenzl (Amaranthaceae), exhibited moderate *in vitro* anti-AChE properties (IC_{50} = 20.1 mg L^{-1}) (Vargas and Kouznetsov, 2013). According to a previously reported study, a new series of girgensohnine alkaloid analogs were designed and synthesized seeking to increase their biological activity (Mendoza et al., 2016; Carreño Otero et al., 2014). Biochemical studies, which include *in silico* ADMETox properties calculations and *in vitro* AChE inhibition, showed that the evaluated molecules could be considered as acetylcholinesterase inhibitor (AChEI) with micromolar IC_{50} values ranging from 10.3–15.0 mg L^{-1}. Because of AChE inhibitors affect the natural transmission of nerve impulses in the insect central nervous system, *in vivo* insecticidal activity was evaluated. The most active AChEI α-aminonitrile against *Ae. aegypti* larvae showed a good larvicidal activity at concentrations < 140 mg L^{-1}, but the behavior of its detoxifying mechanisms is still unknown (Walker et al., 2012).

The discovery of how a xenobiotic can modify different target sites in insects, for example new molecules that affecting AChE normal activity, plays a key role in the generation of new insecticides. Besides AChE, nonspecific esterases (α- and β-), mixed function oxidases (MFO) and glutathione S-transferases (GST) are also enzymes that help the organisms to transform or/and eliminate endogenous and exogenous compounds (Polson et al., 2011; Mukherjee et al., 2007). These phase I enzymes can recognize environmental xenobiotics and transform them by oxidation, reduction or hydrolysis. Occasionally, phase I products become substrates for phase II enzymes. Phase II enzymes catalyze the conjugation of xenobiotic to a hydrophilic compound, turning it into a less toxic metabolite that can be eliminated more easily than the precursor molecule (Hemingway and Ranson, 2000).

Mixed function oxidases (P-450 monooxygenases, phase I enzymes) are located in the smooth endoplasmic reticulum and are the most important enzymes involved in the metabolism of pyrethroids in *Aedes*, *Anopheles* and *Culex* genera (Ocampo et al., 2011; Melo-Santos et al., 2010; Schuler, 1996). MFO has low specificity and high functional versatility what is reflected in the different types of processes than can be catalyzed. *p*-NPA, α- and β- esterases bind to xenobiotics sequestering them rather than rapidly metabolizing them. In mosquitoes, esterases are the primary mechanisms involved in OP, pyrethroids and carbamate resistance, *e.g.* an elevated esterase activity has been associated with temephos resistance in *Ae. aegypti* from Venezuela (Cui et al., 2007; Fernandez et al., 2005). Glutathione S–transferase is a phase II family of detoxifying enzymes that catalyze the nucleophilic attack of reduced glutathione (GSH) on the electrophilic centers of lipophilic compounds such as OP and OC (Rebechi et al., 2014; Boelsterli, 2003). The products of this combination are usually less toxic and more soluble in water, hence are more easily excreted from cells (Crane et al., 2002). In toxicology studies an increase of GST activity is used as a marker of organic contamination in freshwater invertebrates (Lumjuan et al., 2011; Domingues et al., 2007, 2010; Che-Mendoza et al., 2009; Kostaropoulos et al., 2001; Hemingway, 1992; Grant and Matsumura, 1989). Acetylcholinesterase (AChE), a serine esterase found in nerve synapses, is the target site of the OP and carbamate insecticides, both inhibit this enzyme resulting in a general nervous system failure (Rebechi et al., 2014; Walker et al., 2012). Decrease in sensitivity to these insecticides is due to mutations in AChE (Weill et al., 2003; Hemingway and Ranson, 2000).

Although commercial insecticides have been previously evaluated in *Ae. aegypti* populations, new alternatives, as synthetic natural analogs, have not been extensively investigated. Furthermore, fewer reports include wild populations evaluations and the behavior of their detoxification mechanism remains unknown. The objective of this study was to investigate the activity of detoxifying enzymes in two populations of *Ae. aegypti* larvae, Rockefeller reference (Rock) and Santander wild (WSant) and to study how larvae respond when are treated for 24 h with different concentrations of two AChE inhibitors with reported larvicidal activity.

2. Materials and methods

2.1. Ae. aegypti collection

Ae. aegypti eggs were used from UIS-CINTROP Rockefeller colony established in an insectary. Larvae were reared on plastic trays (35.5 cm × 21.5 cm × 6.5 cm) with approx. 3.000 mL of dechlorinated water under 25 ± 5 °C; 75–80% humidity and 12:12 (light–dark) photoperiod. Late 3rd to early 4th stage larvae were used in experiments. Rockefeller adults were reared on breeding cages under the same insectary conditions. An established wild colony of *Ae. aegypti*, originated from a sampling carried out with a total of 120 Ovitraps OV installed at Universidad Industrial de Santander, Bucaramanga-Colombia in February 2016, was reared at the insectarium of Centro de Investigaciones en Enfermedades Tropicales at the same University. Obtained eggs were allowed to hatch and the obtained larvae were used to establish the Santander wild colony under the same conditions of Rockefeller colony.

2.2. Active molecules

2.2.1. General procedure for the synthesis of α-aminonitrile derivative

The girgensohnine analog 2-(3,4-dimethoxyphenyl)-2-(piperidin-1-yl) acetonitrile **DPPA**, was synthesized using a modified Strecker reaction (Fig. 1). An equimolar mixture of the corresponding 3,4-dimethoxybenzaldehyde (1 mmol) and piperidine (1.1 mmol), NaCN (1.5 mmol) and silica sulfuric acid (SSA) catalyst (1:1 by weight) in acetonitrile were mixed at room temperature for 16 h. Synthesized compound **DPPA**, obtained as white crystals (mp. 60–62 °C), was structurally characterized by instrumental techniques IR, GC–MS, ^{1}H and ^{13}C NMR.

2.2.1.1. 2-(3,4-Dimethoxyphenyl)-2-(piperidin-1-yl)acetonitrile DPPA. IR (KBr): 2229 ν(C ≡ N), 1250 ν(N—C) cm^{-1}. ^{1}H NMR (DMSO-d_6,400 MHz) δ (ppm): 6.21–6.12 (3H, m, 2,5,6-H_{Ar}), 4.38 (1H, s, 10-H), 2.95 (6H, s, 3,4-OCH_3), 1.72–1.54 (4H, m, 2,6-H_{Pip}), 0.79–0.64 (4H, m, 3,5-H_{Pip}), 0.59 (2H, d, J = 5.2 Hz, 4-H_{Pip}). ^{13}C NMR (100 MHz) δ (ppm): 149.9 (4-C_{Ar}), 149.0 (3-C_{Ar}), 126.8 (1-C_{Ar}), 120. 8 (6-C_{Ar}), 117.1 (− CN), 112.4 (2-C_{Ar}), 112.0 (5-C_{Ar}), 62.1 (10-C), 56. 4 (3,4-OCH_3), 51.1 (2,6-C_{Pip}), 26.2 (3,5-C_{Pip}), 24.6 (4-C_{Pip}). GC–MS (70 eV): t_R = 20.7 min, *m/z* = (260, M^{+-}, 6), 177 (30), 176 (100),

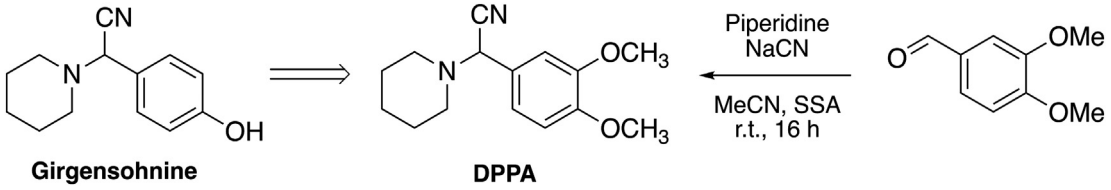

Fig. 1. Chemical structures of natural alkaloid, its analog DPPA and synthetic methodology.