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# Effects of a cyanobacterial extract containing anatoxin-a(s) on the cardiac rhythm of *Leurolestes circunvagans*

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**Abstract:** This work presents the effects of an anatoxin-a(s) containing extract on a cockroach semi-isolated heart preparation and the results supporting the extract's biological activity on acetylcholinesterase (purified from eel). The presence of the toxin in cyanobacterial strains *Anabaena spiroides* (ITEP-024, ITEP-025 and ITEP-026) isolated from the Tapacurá reservoir in Pernambuco, Brazil, was confirmed by means of liquid chromatography coupled to an ion-trap mass spectrometer. The anticholinesterase activity was assessed biochemically by the Ellman test and was confirmed by measuring the cockroach's heart rate. The concentration of the extract containing the tested anatoxin-a(s) (antx-a(s)) (10, 16 and 100 $\mu g.\mu L^{-1}$) inhibited the eel acetylcholinesterase (AChE) by more than 90%. The cockroach cardiac frequency increased by a maximum of about 20% within 29 min after the addition of $2.5x10^3$ µg of extract containing antx-a(s).$g^{-1}$ bw (n=9, $p<0.05$). Our results strongly indicate that antx-a(s) is capable of exerting biological effects on cockroach, indicating that more research might be conducted to determine its role in the environment, especially on insects.

*Article*

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## Introduction

Cyanobacteria are ancient, ubiquitous photosynthetic microorganisms that play an important role in the oxygenation of the terrestrial atmosphere. Cyanobacteria may produce a broad of compounds as pigments which have not been detected in eukaryotic algae and toxins (Sigaud-Kutner et al., 2002; Pinto et al., 2003; Guaratini et al., 2005; Guaratini et al., 2009; Aráoz et al., 2010). Nevertheless, these organisms raise public-health concerns, due to their ability to form blooms in the freshwater environment worldwide and to produce a diversity of toxins, called cyanotoxins (Sivonen & Jones, 1999; Hummert et al., 2001; Bittencourt-Oliveiraet al., 2005; Frias et al., 2006; Kujbida et al., 2006; Dorr et al., 2010).

Anatoxin-a(s) (antx-a(s)) is a cyanobacterial neurotoxin structurally unrelated to the other neurotoxic alkaloids also produced by cyanobacteria. Its mechanism of action is the irreversible inhibition of acetylcholinesterase (Mahmood & Carmichael, 1986). Antx-a(s) acts by a similar mechanism as the organophosphates and carbamate insecticides, such as paraoxon, physostigmine and pyridostigmine (Cook et al., 1988), and the chemical-warfare agent sarin (Pita et al., 2003). An important characteristic of antx-a(s) is that it is unable to cross the blood-brain barrier, being a peripheral cholinesterase inhibitor (Cook et al., 1988; Cook et al., 1989). Matsunaga et al. (1989) characterized this toxin through the positive Fast Atom Bombardment Mass Spectrometry (FABMS) and Nuclear Magnetic Resonance (NMR) techniques as a unique *N*-hydroxyguanidine methyl phosphate ester with a molecular mass of 252 Da. Antx-a(s) was originally characterized from the strain *Anabaena flos-aquae* Bréb. Ex Bornet & Flahault NRC 525-17 (Carmichael et al., 1977; Carmichael & Gorham, 1978). Since then, other cyanobacterial strains within the *Anabaena* genus, such as *Anabaena lemmermanii* P. Richter and *A. spiroides* Klebahn, have also been reported as antx-a(s)-producers (Molica et al., 2005; Onodera et al., 1997). Among the countries where antx-a(s) has already been identified are Canada (Carmichael et al., 1977), the United States (Mahmood et al., 1988), Denmark (Onodera et al., 1997), and Brazil (Molica et al., 2005). This low level of incidence, when compared with that of other cyanotoxins, such as microcystins, could be explained by antx-a(s)'s instability in an alkaline medium (Matsunaga et al., 1989; Dorr et al., 2010). However, the main problem related to antx-

a(s) monitoring is the lack of a commercially available analytical standard or reference material. Therefore, the development of a reliable and specific method for its detection has been proposed (Dorr et al., 2010).

Data on the biological activity of antx-a(s) in arthropods are nonexistent. Several researchers, however, have used bioassays in cockroach hearts to evaluate the possible effects on arthropods of venoms, pharmacological agents, neurotransmitters and organophosphate insecticides (Miller & Metcalf, 1968; Schaefer & Miles, 1970; Majori et al., 1972; Klowden et al., 1992). Indeed, acetylcholinesterase (AChE, E.C. 3.1.1.7) has been found to be the predominant esterase for acetylcholine hydrolysis in the cholinergic synapses of both vertebrates and invertebrates (Nachmansohn & Rothenberg, 1945; Augustinsson et al., 1961; Corbett, 1974; Panda & Sahu, 2004).

The aim of this work was to investigate the presence of antx-a(s) in *A. spiroides* extracts using spectrometric analyses and by monitoring the effect of this toxin on semi-isolated heart preparations from *Leurolestes circunvagans*.

## Materials and Methods

### *Chemicals*

Electric-eel AChE (type V-S lyophilized powder, 480 $U.mg^{-1}$ solid, 530 $U.mg^{-1}$ protein), acetylthiocholine iodide (ATCI), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), bovine serum albumin (BSA), ammonium formate and formic acid were obtained from Sigma-Aldrich (St. Louis, USA). Merck (Darmstadt, Germany) supplied HPLC-grade ethanol, methanol, acetonitrile and acetic acid. All other chemicals were of analytical grade. Throughout the AChE assay, 50 mM Tris-HCl buffer, pH 8.0 was used as a buffer unless otherwise stated. The lyophilized enzyme was prepared in the buffer to obtain a 1 $U.mL^{-1}$ stock solution. The enzyme stock solution was stored at -80 ºC. Enzyme working solutions were dissolved in buffer containing 0.1% BSA. DTNB was dissolved in buffer containing 0.1 M NaCl and 0.02 M $MgCl_2$. ATCI was dissolved in ultra-pure water.

### *Preparation of extracts containing anatoxin-a(s)*

The antx-a(s) producer cyanobacterial strains ITEP-024, ITEP-025 and ITEP-026 were isolated from the Tapacurá freshwater reservoir in Pernambuco-Brazil by Molica et al. (2005). The three strains were previously confirmed as antx-a(s) producers by LC-MS/MS (Dorr et al., 2010). They were cultivated in laboratory conditions in ASM1 medium using a light/dark photoperiod of 12:12 h and an irradiance of 66560 $E.m^{-2}.s^{-1}$ (Gorham et al., 1964). A pool of the lyophilized, culture-harvested material was extracted according to the method proposed by Henriksen et al. (1997) with some modifications. Briefly, 100 mg of the lyophilized cyanobacterial cells were resuspended in 5 mL of ethanol/acetic acid 0.1 M (20:80), sonicated for 3 min in ice and centrifuged at 6300 *g* for 15 min. The supernatant was applied to a pre-conditioned (5 mL of methanol followed by 5 mL of ethanol/acetic acid 0.1 M 20:80) C18 cartridge (Sep-Pack, Waters, MA, USA) to retain the pigments. Eluates were dried in an SPD121P Speed Vac system (Thermo Scientific, MA, USA) and resuspended in 1 mL of acidified methanol. One aliquot of the pre-purified extract was used for the LC-MS analysis, and another was dried under $N_2$ flow and resuspended in saline solution (NaCl 0.15 M) for the AChE assay.

### *LC-MS characterization*

Chromatographic and mass spectrometric analysis conditions were set according to the method proposed by Dorr et al. (2010). An ion-trap mass spectrometer (Esquire HCT, Bruker Daltonics, MA, USA) equipped with an electrospray source and coupled to a Shimadzu Prominence liquid chromatograph (Shimadzu Co., Japan) was used for sample analysis. Separation was conducted under gradient conditions in a ZIC-HILIC column (150x2.0 mm, 5 µm, SeQuant, Umeå, Sweden) using a mixture of (A) water containing 5 mM ammonium formate and 0.05% formic acid and (B) acetonitrile. The flow rate of 0.2 $mL.min^{-1}$ was split into a 1:4 ratio before entering the mass spectrometer source, which was operated in the positive-ion mode.

### *Microplate assay for AChE activity*

The *in vitro* inhibition of AChE was evaluated according to the assays described by Ellman et al. (1961) and Ingkaninan et al. (2003) with some modifications. In brief, 162.5 µL of TRIS-HCl buffer pH 8.0, 12.5 µL of 30 mM DTNB, 25 µL of 0.28 mM $U.mL^{-1}$AChE and 25 µL of inhibitor (0.5; 2.5; 5; 10; 16 and 100 $\mu g.\mu L^{-1}$ of antx-a(s) containing extract) were pre-incubated for 5 min at 37 ºC; 25 µL of 15 mM ATCI was later added to the wells. The microplate was then read at 405 nm every 30 s for 2 min using a microplate reader (Power Wavex340, Bio-Tek Instruments, USA). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared with that of the assay using buffer instead of extract. Inhibitory activity was calculated by subtracting the percentage of enzyme activity from 100. Every experiment was performed in duplicate. The antx-a(s)-extract concentrations were chosen according to the dose tested in the cockroach bioassay.

*Semi-isolated cockroach heart preparation*

A semi-isolated cockroach heart bioassay was used to evaluate the biological effects of antx-a(s) containing extract (Baumann & Gersch, 1982; Klowden et al., 1992). *L. circunvagans* adult cockroaches were anesthetized with ether until immobile and placed ventral side up. The lateral margins of the abdomen were cut along each side, and the ventral abdominal body wall was peeled up to expose the viscera. The viscera were carefully moved aside to expose the heart, which was still contracting while attached to the dorsal body wall. The heart preparations were washed by bathing them in drops of insect saline solution (0.15 mM NaCl) at room temperature (21-24 °C). After 5 min of heart beat stabilization, 200 µL of antx-a(s)-containing extract ($2.5x10^2$, $1.25x10^3$, $2.5x10^3$, $5x10^3$ and $8x10^3$ $\mu g.g^{-1}$ bw) were added to the exposed heart. The mean $beats.min^{-1}$ in the first 5 min was taken as a reference. Heartbeat frequency was monitored for 30 min under a stereoscopic microscope. Nine cockroaches were used for each group. In the control group, only saline solution was added.

*Statistical analysis*

The results are expressed as the mean±SE as appropriate. Statistical comparisons were performed using the Student's unpaired t-test or with ANOVA/MANOVA for repeated measures. A significance value of $p<0.05$ was used.

## Results and Discussion

*LC-MS anatoxin-a(s) characterization*

Although antx-a(s) is one of the most potent neurotoxins produced by cyanobacteria that may be present in polluted water, it is not included in the Guidelines Values for Drinking Water Quality from the World Health Organization. Its low stability in an alkaline medium and the lack of commercially available analytical standards hamper the assessment of antx-a(s). However, some strategies have been developed to improve the evaluation of the toxicological potential of environmental samples with the intent of measuring exposure to antx-a(s).

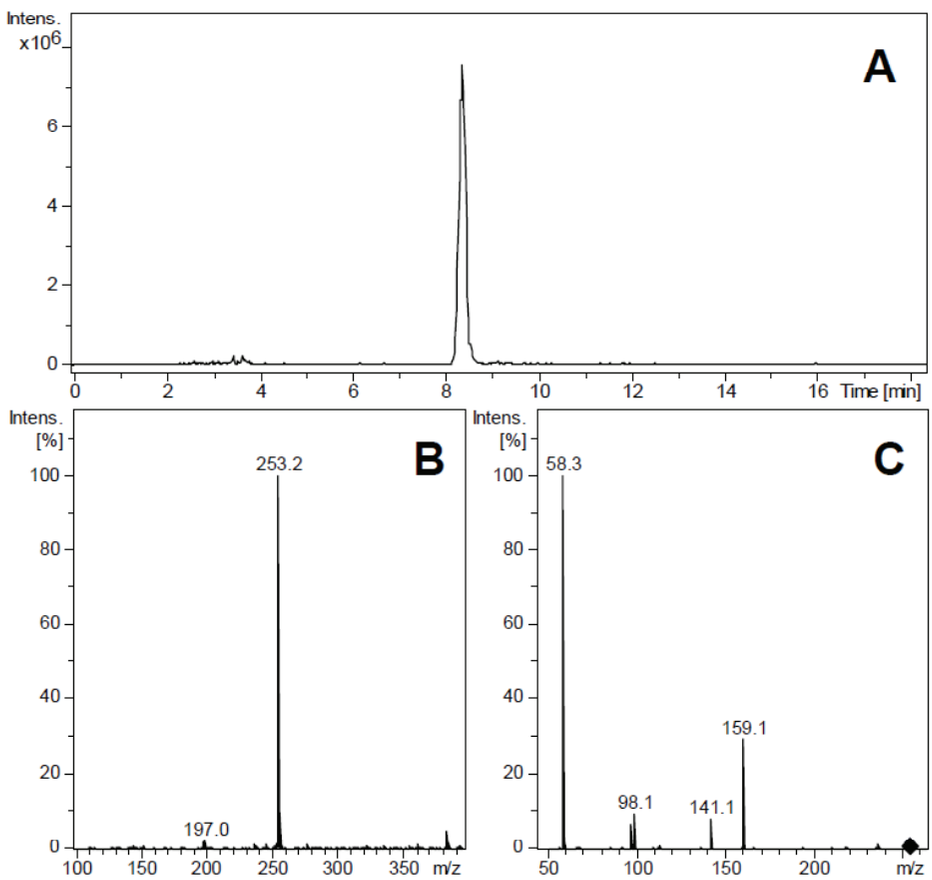


**Figure 1.** Identification of antx-a(s) in an *Anabaena spiroides* extract using LC-MS/MS. (A) Extracted ion chromatogram of *m/z* 253, the $(M+H)^+$ ion of antx-a(s); (B) MS Scan spectrum; (C) MS/MS fragmentation spectrum of *m/z* 253,exhibiting the characteristic product ions of antx-a(s) with *m/z* 159.1, 141.1, 98.1 and 58.3.

Villatte et al. (2002) and Devic et al. (2002) developed electrode biosensors using acetylcholinesterase enzymes from various organisms and a mutated enzyme from *Drosophila melanogaster* Meigen 1830, respectively, to detect antx-a(s).

Several researchers have been employing the HPLC-UV method proposed by Mahmood & Carmichael (1986) to purify and quantify antx-a(s). In assessing this chromatography method, at least two considerations can be noted. Because antx-a(s) is very polar (CLog P=-1,7), its retention in a cyanopropyl chromatographic column (most commonly used and reported for this toxin) can vary significantly. In fact, the same authors mentioned above observed a completely different retention level for antx-a(s) using the same chromatography conditions (Mahmood et al., 1988). Moreover, the lack of chromophore groups in antx-a(s) makes its detection by UV difficult because many matrix impurities may absorb in the same wavelength where antx-a(s) is detected (220 and 230 nm). Indeed, James et al. (1998) criticized the use of low wavelength values for the monitoring of anatoxins. For these reasons, we decided to employ an alternative extraction procedure that was monitored by an LC-MS method proposed by Dorr et al. (2010). Panel A of Figure 1 exhibits an extracted ion chromatogram of *m/z* 253, which is the protonated ion ($[M+H]^+$) of antx-a(s). A sharp peak is clearly evident, and the scan spectrum with the characteristic ion is presented in Panel B. Panel C depicts the MS/MS spectrum obtained after collision-induced dissociation of the ion at *m/z* 253. According to Dorr et al. (2010) the few characteristic fragment ions revealed in this panel confirm the presence of antx-a(s) in the extract. Therefore, measurable levels of antx-a(s) were confirmed in the extracts prepared with cultures of the ITEP 24, 25 and 26 cyanobacterial strains.

In vitro *acetylcholinesterase assay*

As previously mentioned, the mechanism of action of antx-a(s) is related to cholinesterase inhibition. Therefore, the rate of hydrolysis of acetylthiocholine by eel AChE was evaluated *in vitro* by incubating the antx-a(s) containing extract with the eel AChE enzyme. The eel AChE was chosen because of the extremely high activity values found in *Electrophorus electricus* Linnaeus 1766 (Toutant, 1989). Figure 2 shows the relationship between eel AChE activity and antx-a(s) containing extract solutions. Interestingly, the lowest tested concentration of antx-a(s) containing extract still caused significant AChE inhibition (approximately 80%). These results corroborate the previous report of Mahmood & Carmichael (1987), which found that four different antx-a(s) concentrations (0.16 to 0.80 $\mu g.mL^{-1}$) inhibited 0.25 U of eel AChE. In that study, the two higher concentrations tested (specifically, $4.8x10^{-4}$ and $8x10^{-3}$ $\mu g.\mu L^{-1}$) produced levels of AChE inhibition ranging from 75-90%, which is close to the percentage of inhibition we obtained with concentrations ranging from 0.5 to 10 $\mu g.\mu L^{-1}$. However, at the same time, no significant difference in the percentage of AChE inhibition was observed in the overall range of extract concentrations tested. This strong inhibition of AChE, even for the lowest antx-a(s) concentration we tested, could be explained by the binding of antx-a(s) with the catalytic site of AChE. The attraction between the enzyme and acetylcholine takes place through an electrostatic interaction between the positive charge of the quaternary ammonium of the substrate (i.e., acetylcholine) and the negative charge(s) of the anionic subsite (Toutant, 1989). While acetylcholine hydrolysis occurs through an acetylation-deacetylation cycle, antx-a(s) occupies both the anionic and the estearic subsites and phosphorylates the enzyme. The adduct formed with the serine hydroxyl group does also not undergo induced hydrolysis by reactivators. Therefore, like some synthetic organophosphates, antx-a(s) causes AChE to degrade rapidly (Hyde & Carmichael, 1991). According to our results, based on 2.5-10 $\mu g.\mu L^{-1}$ of antx-a(s) containing extracts, the interaction of antx-a(s) with the AChE catalytic sites leads to a high degree of inhibition regardless of the concentrations used in this study (see Figure 2).

Following the biochemical determination of the biological effect of antx-a(s), a further investigation of its biological activity was conducted using semi-isolated preparations of the cockroach heart.

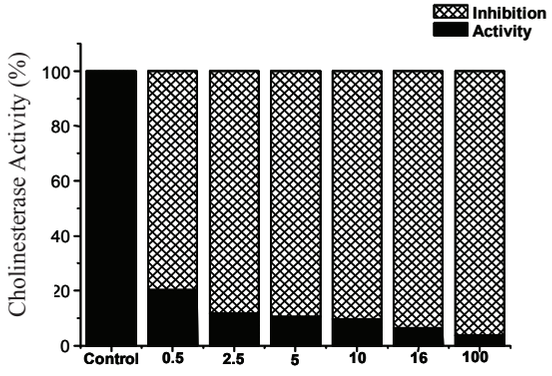


**Figure 2.** *In vitro* AChE activity/inhibition after 5 min of incubation with antx-a(s) containing extract. The rate of ATCI hydrolysis was measured spectrophotometrically for 3 min at 405 nm. The tested antx-a(s) containing extract concentrations were 0.5, 2.5, 5, 10, 16 and 100 $\mu g.\mu L^{-1}$.

*Effects of cyanobacterial extract containing anatoxin-a(s) on semi-isolated cockroach hearts*

Figure 3 shows the effects of various doses of antx-a(s) containing extract on cockroach heartbeats. Specifically, after treatment with several doses, there was a gradual increase in the frequency of beats by increasing the doses to $1.25x10^3$ μg.g$^{-1}$. When a dose of $2.5x10^3$ μg.g$^{-1}$ was added to the preparation, a maximum increase of 18±3% was seen at 29 min (with averages of 67 and 80 heartbeats.min$^{-1}$ for the control group and the group treated with $2.5x10^3$ μg.g$^{-1}$, respectively; n=9, $p<0.05$) (Figure 3). At higher doses, the positive chronotropic effect diminished, but the heart rate remained above control levels after 30 min (Figure 3). It is also noteworthy that, in some animals, when the addition of a concentration of $8x10^3$ μg.g$^{-1}$ led to a cardiac arrest after 15 min of monitoring. In the semi-isolated cockroach heart, the addition of anticholinesterase drugs or acetylcholine may increase or decrease the frequency of beats, depending on the concentration tested (Fox, 1981). In our set of experiments, we observed both increases and decreases in cardiac rhythm caused by the optimal dose of extract containing antx-a(s) ($2.5x10^3$ μg.g$^{-1}$, n=9, $p<0.05$). Thus, the inhibitory effects on AChE activity found in the biochemical tests can be correlated with the cardio modulatory effects after being inhibited by antx-a(s) containing extract. Nevertheless, the decrease in the effect of the antx-a(s) extract at higher doses may be a result of acetylcholine accumulation in the cockroach cardiovascular system. Indeed, in experiments, this hypothesis is supported by the cardiac arrest observed in some cockroaches during treatment with higher concentrations of extract containing antx-a(s).

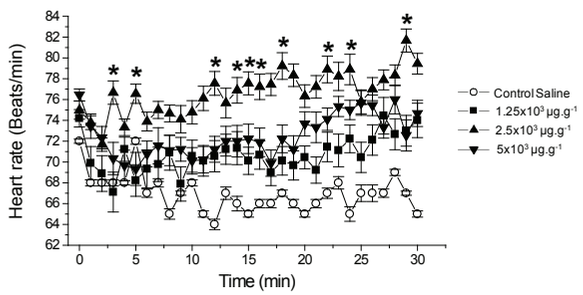


**Figure 3**. Effect of various doses of anatoxin-a(s)-containing extract (μg.g of cockroach$^{-1}$) on the actual cockroach heart rate over the course of 30 min. Each point represents the mean±SE of nine hearts for each dose tested. *Significance is defined as $p<0.05$.

In cockroaches, acetylcholine binds to the cholinergic receptors present not only in the heart but also in the nerves of the paired cardiac system (Collins & Miller, 1977; Fox, 1981). Discrepancies regarding the effect of acetylcholine on the rate of the cockroach heartbeat have been noted in the literature. Although some authors have demonstrated that concentrations ranging from $10^{-3}$ to $10^{-7}$ M can increase the heart rate (Naidu, 1953; Collins & Miller, 1977), others have suggested that acetylcholine (at concentrations between $10^{-2}$ and $10^{-6}$ M) can slow the heart rate (Richards, 1963; Schaefer & Miles, 1970). Consequently, the response obtained with $2.5x10^3$ μg.g$^{-1}$ of antx-a(s) containing extract (18% increase) is similar to the results described by Collins & Miller (1977). It is also important to consider the slight differences in the kinetics and phylogeny of AChE between vertebrates and invertebrates (Kim et al., 2006).

The inhibition of insect AChE is toxicologically significant, as this process is the main mechanism of action of organophosphates and carbamates, which are commonly used as pest management agents (Siegfried & Scott, 1990). Indeed, studies with insect acetylcholinesterase have mainly been undertaken to investigate insecticide resistance (Matsumura & Madhukar, 1980). Regarding this subject, Revuelta et al. (2009) employed spectrophotometric and zymogram measurements to demonstrate the existence of two AChE genes in *Blattella germanica* Linnaeus 1767, *Bgace1* and *Bgace2*. The BgAChE1 (*ace1*) proteins usually represent 65-75% of the total active AChE in the *B. germanica* nerve tissue, which is the main target of organophosphate insecticides in cockroaches. Considering the use of praguicides in agriculture and in insect control may represent a potential danger, the search for new insecticides that might act on a specific residue of the insect AChE, be free of resistance and be less toxic for mammals, birds and fish is still in progress (Badea et al., 2006, Polsinelli et al., 2010). The effects of antx-a(s) in the environment should be further investigated, as there are few reports about its biological activity in different species, such as insects. Only a couple of published toxicological reports have confirmed the effect of antx-a(s) in dogs and birds (Mahmood et al., 1988). Also, considering that antx-a(s) is a natural organophosphate that cannot cross the blood-brain barrier; this compound should be subjected to further study for possible insecticide activity.

This work presents confirmatory information about the effect of a natural organophosphate on the semi-isolated cockroach heart, suggesting that antx-a(s) may play an important role in insects control and can be considered an alternative source for the development of novel selective insecticide molecules.

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