NEUROTOXIC EFFECT INDUCED BY
Prasiola crispa ANTARCTIC-ALGAE
METHANOLIC EXTRACT IN COCKROACHES

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Abstract: In this work we have demonstrated the direct neurotoxic effect of Prasiola crispa methanolic extract at cockroach
neuromuscular junctions. At in vivo cockroach neuromuscular preparations the extract induced a progressive and irreversible
neuromuscular blockade, which was preceded by an increase in the muscular tonus with the appearance of spontaneous twitches.
The previous application of chloral hydrate, an n-methyl-d-aspartate receptor blocker, increased the time to blockade of the muscle
twitches by 50%, whereas it extinguished both the previous increase of tonus and the appearance of spontaneous twitches, before
the onset of neuromuscular blockade. Together, these results suggest that P. crispa extract induced an insecticide activity by acting
mainly at insect neuromuscular junctions. Therefore, the NMDA receptors must be the target of this pharmacological interaction.
A further phytochemical and pharmacological investigation will increase the knowledge about the cellular and molecular aspects
of P. crispa insecticide effect.

Keywords: Prasiola crispa, Antarctic Algae, NeuROTOXIC Eff ect, Cockroaches, Neuromuscular Junctions

Introduction
From the biological point of view, the co-evolution between
plants and insects are recognized (Ryan & Byrne, 1988)
and may explain the selection of a broad spectrum of
secondary metabolites used as defense (i.e. one or a few
plant chemicals antagonistic to insects) (Bown et al., 2006).
Nowadays, there is a major motivation to promote research
on botanical insecticides with the low environmental cost
and minimal ecological impact, and a demand for healthier
products. Cockroaches are primitive pest insects, and
most of their functional systems are fairly unspecialized.
The cockroach nervous system can be used not only to
understand the physiological aspects between insect and
natural compounds, but also as an important model of
biomedical research (Stankiewicz et al., 2012).

The thalloid green alga Prasiola crispa is abundant on the
upper shores of Antarctica, often near to and on penguin
benches. In this habitat, the alga is exposed to a number of
stressful conditions, which may give rise to the development
of multiple survival strategies, including the production of
different secondary metabolites (Harwood & Gushina, 2009;
Pereira et al., 2009). The studies targeting P. crispa biological
activity are scarce, and indeed, our group was the pioneer
in investigating the inherent insecticide activity of this alga
(Posser et al., 2010). Despite the wealth of literature on the
ecological and pesticidal effects, few studies of plant extracts
have considered their mechanism of action. Therefore, to
the best of our knowledge, the present study is likely to be
the first on investigating the cellular aspects involved in the
action insecticide actions of Antarctic algae in cockroaches. The aim of this work was to investigate the mechanism involved in the insecticide effect of *Prasiola crispa* methanolic extract, especially those related to the cellular interactions at insect neuromuscular junctions.

**Materials and Methods**

**Animals**
Cockroaches (*Leurolestes circunvagans*) were reared with water and food *ad libitum*, at controlled temperature (22-25°C).

**Reagents**
All chemicals and reagents used were of the highest purity and were obtained from Sigma, Aldrich, Merck or BioRad.

**Plant material**
*Prasiola crispa* (Lightfoot) Kützing (1843) was collected in the ice-free areas near Arctowski Polish Base Region, Admiralty Bay, King George Island (61º 50' - 62º 15' S and 57º 30' - 59º 00' W), Antarctica. The *Prasiola crispa* methanolic extract (PCME) was produced as described elsewhere (Posser et al., 2010). The extracts where dissolved in 100% dimethyl sulfoxide (DMSO) previously to animal administration.

**In vivo Cockroach Metathoracic Coxal-Adductor Nerve-Muscle Preparation**
We used the *in vivo* cockroach metathoracic coxal-adductor nerve-muscle preparation as biological assay (Full & Stokes, 2008). The animals were previously anesthetized by chilling (-5°C), and fixed ventral side up in Perspex plate recovered with Styrofoam by means of entomologic needles. Isometric recordings were made using a computer-based software model AQDAD (AVS Instruments, São Carlos, SP, Brazil) with a 1g force transducer model AECAD (AVS Instruments). Supramaximal electrical stimuli (5 ms, 0.5 Hz) was delivered at nerve 5 by insertion of a bipolar Ag/AgCl electrode, coupled to a stimulator (AVS Instruments), during 120 min. Drugs (40 µL) were injected with a Hamilton syringe at the third abdominal segment.

**Statistical analysis**
The results were presented as mean ± S.E. The ANOVA/MANOVA, was used for significance (*p* < 0.05). The non-parametric Student “t” test was used as a *post hoc*

**Results**
The administration of DMSO alone did not interfere with the muscle strength (*n* = 6) during 120 min recordings. The injection of PCME (100, 200, 400 and 800 µg/g of animal weight) induced a dose and time-dependent neuromuscular blockade in 120 min recordings. When the minimum dose of 100 µg/g was assayed there was 80 ± 5% blockage of twitch in 120 min recordings (*n* = 6, *p* < 0.05). The injection of (800 µg/g) PCME induced 100% blockade of twitch tension in 10 min (*n* = 6, *p* < 0.05). In all doses tested, before the onset of extract neurotoxic effect, there was a previous increase of muscular tonus followed by a blockade of the twitches. The appearance of spontaneous post-twitches, during the first 30 min recordings was also noticed. The addition of Chloral hydrate (40 µg/g), a n-methyl-d-aspartate receptor antagonist, 15 min previously to PCME (400 µg/g), increased the time required to 50% blockade of the twitches (from 70 ± 2 min to 110 ± 3 min, *n* = 5, *p* ≤ 0.05). In this protocol, there was also an inhibition of the tonus increasing effect.

**Discussion**
In this work we have dealt with the cellular aspects of the insecticide activity induced by *Prasiola crispa* methanolic extract. In this respect, we have confirmed this latter effect and, in addition, we observed a direct interaction of the algae biocompounds at the cockroach neuromuscular junctions.

A number of plants have been ascribed as natural insecticides and their active constituents isolated and characterized (Pavela, 2005). Such plants, synthesize several chemical constituents which act by inhibiting insect growth, feeding and by altering other important physiological parameters (Viegas Junior, 2003). In cockroaches, the neuromuscular transmission releases glutamate as chemical neurotransmitter to promote the muscle twitch by activation of the insect n-methyl-d-aspartate (NMDA) receptor (Peoples & Weight, 1998). According to our results, PCME induced a progressive inhibitory effect of insect muscle twitch tension that resulted in an irreversible paralysis of neuromuscular activity. Neurotoxic insecticides act in different manners in the insect nervous system. Natural and synthetic piretroids for example, act by causing a persistent activation of muscle sodium channels, inducing an irreversible and lethal depolarizing effect.
(Soderlund, 2012). Others, like insect venoms, prefer the central nervous system as target, inducing a decrease in the release of the neurotransmitter dopamine to cause lethargy and motion weakness (Weisel-Eichler et al., 1999). In the case of PCME, it seems that the algae biocompounds are activating the NMDA receptors at the insect end plate. The first clue for this theory comes from the observation that chloral hydrate, an NMDA blocker, is able to reduce the potency of the neuromuscular blockade induced by the algae extract. Therefore, having followed this treatment, no increase in muscular tonus was seen, indicating that NMDA receptors were not activated. In addition, the spontaneous twitches were extinguished after chloral hydrate, which demonstrates that the depolarizing action of PCME is caused possibly by the activation of NMDA receptors. The results are of notorious importance because it reinforces the potential biotechnological application of Prasiola crispa. To note, NMDA-receptor subunits are particularly involved in several CNS pathologies including acute and chronic pain, stroke, head trauma, drug-induced dyskinesias, and dementia in Alzheimer’s disease and Parkinson’s disease (Dingledine et al., 1999). The evidence that P. crispa possess secondary metabolites that interact with NMDA receptors not only demonstrates the cockroach

Figure 1. Effect of Prasiola crispa methanolic extract (PCME) on the in vivo cockroach metathoracic coxal-adductor nerve-muscle preparation. Panel A, shows the graph of the effect of different concentrations of P. crispa extract on 120 min muscle twitch tension recordings. Panel B shows the increase of muscular tonus and the followed appearance of spontaneous twitches. In C, the graphical expression of the time required to 50% blockade of twitches before and after chloral hydrate (40 µg/µl). In Panel D, complete inhibition of neuromuscular twitches by PCME (40 µg/g) after 5 min recordings. *significance at p ≤ 0.05; • increase of muscular tonus; † spontaneous twitches; ‡ complete neuromuscular blockade.
neuromuscular models as suitable for investigating the mechanism of insecticide interactions, as well as, showed its yield potential for the development of NMDA-receptor-selective therapeutic agents.

Conclusion

Prasiola crispa methanolic extract induces neuromuscular blockade in Leurolestes circunvagans. The latter effect is probably evoked by activation of insect NMDA receptors at the motor end plate. The results also confirm the presence of potent insecticide chemical constituents in Prasiola crispa extract as future candidates for the development of NMDA-receptor-selective therapeutic agents.

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