Redox imbalance mediates entomotoxic effects of the conifer Araucaria angustifolia in Anticarsia gemmatalis velvetbean caterpillar

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Redox imbalance mediates entomotoxic effects of the conifer Araucaria angustifolia in Anticarsia gemmatalis velvetbean caterpillar

Cátia dos Santos Branco1, Tiago Selau Rodrigues1, Émilin Dreher de Lima1, Lúcia Rosane Bertholdo-Vargas2, Neiva Monteiro Barros2 and Mirian Salvador1*

Abstract: The velvetbean caterpillar, Anticarsia gemmatalis is one of the most important pests of soybean crops in tropical America. By feeding on leaves, significant defoliation occurs resulting in reduced photosynthetic capacity required for plants' maintenance and growth, which subsequently can lead to crop losses and reduced agricultural productivity. Many studies have sought to look for compounds that have insecticidal effects. One class of compounds is phenolics, which are produced by plants and have been found to influence the behavior and development of defoliators, representing an important alternative approach to many synthetic insecticides. Particularly, Araucaria angustifolia is a plant rich in polyphenols, which are compounds able to alter cellular dynamics through modulating redox status. In this study, A. angustifolia extract (AAE) was added to the artificial diet of A. gemmatalis. The results demonstrated that AAE was able to reduce larval viability by inducing morphological changes and a delay in the insect's development. In addition, AAE was found to induce oxidative damage to lipids and proteins, as well as increased nitric oxide levels in A. gemmatalis larvae. AAE treatments also decreased the antioxidant defense systems, leading to a redox imbalance. The reduction in viability in A. gemmatalis was positively correlated with oxidative markers, suggesting that redox imbalance can lead to larvae's death. These results suggest that AAE possess...
insecticidal potential through the mechanisms of action of altering cellular redox state. Though further studies are required to confirm this, our study nevertheless contributes to a better understanding of AAE’s mechanisms of action as potential biopesticides in pest management, opening new perspectives on the development of compounds with insecticidal action.

Subjects: Agriculture & Environmental Sciences; Biochemistry; Biotechnology; Environmental Studies & Management

Keywords: biological control; lepidopteran; velvetbean caterpillar; polyphenols

1. Introduction

The Lepidoptera order covers a broad and diverse group of insects around the world, including species that are crop pests. Among them, the velvetbean caterpillar, Anticarsia gemmatalis (Hübner, 1818) (Lepidoptera: Erebidae) is the main insect pest that requires control measures in soybean crops, especially in Brazil, which is the world’s second-largest producer of soybeans, behind the United States of America (USDA, 2015). Attack of A. gemmatalis on soybean crops causes defoliation, which in turn compromises the quality and yields by altering the filling of the grains and pods of the plant (Levy, Falleiros, Moscardi, & Gregório, 2011). Considering the importance of soybean as one of the most cultivated grains in the world, (Ray, Mueller, West, & Foley, 2013) pest control actions and management are therefore crucial.

Currently, the most common intervention for A. gemmatalis control in soybean agriculture is the employment of synthetic insecticides (Bernardi et al., 2012). However, the continuous intensive use of chemical pesticides may contribute to an increase in pest resistance leading to failure to pest control (Sarfraz, Keddie, & Dosdall, 2005). Considering the diverse problems associated with the massive use of these chemicals in agriculture and in environment, the biotechnology field can contribute to the discovery and development of new alternative pest control strategies (Penman, 1994). Some natural opponents including viruses (Baculovirus anticarsia) (Braconi et al., 2014; Piubelli, Hoffmann-Campo, Moscardi, Miyakubo, & de Oliveira, 2006; Piubelli, Moscardi, & Hoffmann-Campo, 2009), entomopathogenic fungi (Bertholdo-Vargas et al., 2009), and bacteria (Fiuza, Schünemann, Pinto, & Zanettini, 2012; Gobatto et al., 2010) have been used for biological control of pest populations of A. gemmatalis; however, they are not completely effective, prompting more studies to investigate better pest management strategies for agronomical applications.

The great diversity in plant kingdom represents an important source of molecules with potential entomotoxic value. Araucaria angustifolia (Bert. O. Kuntze) belongs to the Araucariaceae family and is found in South America. It is a dioecious species that contains male and female reproductive organs carried on distinct strobilus. The female strobilus consists of seeds (the edible part of A. angustifolia) and bracts, which are undeveloped seeds naturally discarded into the environment that contains high levels of bioactive compounds, particularly polyphenols (Branco et al., 2015; Michelon et al., 2012; Souza et al., 2014). Plants naturally produce phenolic compounds in order to provide self-defense against pests. Some of these phenolic compounds have been shown to present entomotoxic effects on defoliating insects, including A. gemmatalis (Batista Pereira et al., 2002; Gazzoni, Hülsmeyer, & Hoffmann-Campo, 1997; Hoffmann-Campo, Ramos Neto, Oliveira, & Oliveira, 2006; Piubelli et al., 2006; Salvador et al., 2010) and S. frugiperda (Batista Pereira et al., 2002).

The potential of natural compounds must be taken into account when defining the best pest control approach. In this context, the aim of this study was to investigate possible entomotoxic effects induced by A. angustifolia extract (AAE) when administered in an artificial diet to soybean caterpillar, A. gemmatalis. In order to understand the possible entomotoxic mechanisms induced by AAE, oxidative damage to lipids and proteins, nitric oxide levels, and antioxidant defenses systems were evaluated.
2. Materials and methods

2.1. Araucaria angustifolia extract
Mature female strobili of *A. angustifolia* were collected in Caxias do Sul, Rio Grande do Sul (29°9′34.90″S, 51°8′45.34″W); Ibama n° 02001.001127/2013-94, Brazil. Voucher specimens were identified by the Herbarium of the University of Caxias do Sul, Rio Grande do Sul, Brazil (HUCS 40710/40711). Bracts were manually separated from the pine, and the extract was obtained using 5 g of bracts in 100 mL of distilled water, under reflux (100°C) for 15 min, filtered in Millipore equipment (pore size, 0.45 μm; SFGS 047LS, Millipore Corp.) and then lyophilized (LIOBRAS model L-101) under vacuum pressure to yield a powder. To perform the assays, AAE was solubilized in distilled water immediately before added to artificial diet. The chemical characterization of AAE was already described by our research group (Branco et al., 2015; Michelon et al., 2012; Souza et al., 2014) and the presence of several polyphenols, including isoflavones and bioflavonoids was confirmed (Figure 1).

2.2. Insects
The insects (*A. gemmatalis*) used in this study were from colonies kept at the Laboratory of Control of Pests, Institute of Biotechnology, University of Caxias do Sul, Brazil. The insects were reared on artificial diet (Greene, Leppla, & Dickerson, 1976), maintained in an acclimatized chamber at 27 ± 1°C; 50 ± 10% relative humidity; and a 14:10 light/dark photoperiod.

2.3. Treatments and experimental design
Thirty larvae (third instars) of *A. gemmatalis* were used in each group, which included three AAE treatments (1.25, 5, and 10 mg/mL AAE) and one control group. The larvae were transferred to individual cups, and every two days, the insects were fed on artificial diet of 1.25, 5, and 10 mg/mL AAE. The AAE concentrations were defined from previous tests. Insects served as control were fed with artificial diet without AAE. The diet was cut with a stainless steel spatula, previously cleaned with 70% alcohol, and individually offered to each caterpillar, in cubes around 1 cm³, during the daily maintenance activities. Diets were consumed by the insects within two days and were replaced until the insects reach the pre-pupal stage. All the insect groups were evaluated every two days for weight gain. Rates of larvae and pupae mortality and malformation of larvae and pupae (morphological alterations) were evaluated. Oxidative and nitrosative stress, as well as enzymatic and non-enzymatic antioxidant cellular defenses were evaluated on the fifth day of the treatment.

2.4. Lipid and protein oxidative damage
Larvae of each treatment were homogenized (five insects per mL) in ice-cold 50 mM phosphate potassium buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and centrifuged at 1,500 × g at 4°C for 5 min. The supernatant was used for the assays. To determine lipid damage, aliquots (100 μL) of the supernatant were mixed with 100 μL of the color reagent (1% thiobarbituric acid (TBA), 50 mM sodium hydroxide (NaOH), 0.1 mM butylated hydroxytoluene (BHT)) and 50 μL 7% (v/v) phosphoric acid. The mixture was placed in a boiling water bath for 15 min. After cooling, 1.5 mL of n-buthanol was added to the mixture followed by centrifugation for 5 min at 1,600 × g. The absorbance of supernatant was measured at 532 nm, and the results were expressed in nmol/mg of protein (Hermes-Lima & Storey, 1995). Oxidative damage to proteins was measured by quantifying the carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). Two hundred μL of DNPH (10 mM) or 200 μL of hydrochloric acid (HCl) (2 M) for blank was added to 50 μL of the samples. The reaction mixture was incubated in the dark for 30 min and vortexed every 10 min. Next, 250 μL of 20% trichloroacetic acid (TCA) was added and centrifuged at 1,500 × g for 8 min. The supernatant was discarded and the pellet was washed three times with ethanol-ethyl acetate (1:1) to remove the free reagent. Samples were centrifuged and pellets were redissolved in 1,000 μL of urea solution (8 M) at 37°C for 15 min. Absorbance was read at 365 nm, and results were expressed as nmol DNPH/mg protein.
2.5. Nitric oxide levels

To evaluate the possible nitrosative stress induced by AAE in A. gemmatalis larvae, nitric oxide (NO) levels were determined as nitrite (NO$_2^-$) production, using the Griess reaction-based method (Green, Tannenbaum, & Goldman, 1981). Larvae of each treatment were homogenized (five insects per mL) in ice-cold 50 mM phosphate potassium buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and centrifuged at 1,500 \times g at 4°C for 5 min. For the assay, 50 μL of supernatants was reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine and 1%...
sulfanilamide in 5% H₃PO₄) for 10 min at room temperature, and the absorbance was read at 550 nm. Sodium nitroprusside was used as the standard. The results were expressed as nmol of nitrite per mg of protein.

2.6. Superoxide dismutase and catalase activities
After treatments, third instar *A. gemmatalis* were homogenized (five insects per mL) in ice-cold 50 mM phosphate potassium buffer containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (protease inhibitor). The homogenate was centrifuged at 1,600 × g at 4°C for 30 min, and the supernatants were used for both enzymatic assays. Superoxide dismutase (Sod) activity was measured by the inhibition of self-catalytic adrenochrome formation rate at 480 nm, in a reaction medium containing 1 mmol/L adrenaline (pH 2.0) and 50 mmol/L glycine (pH 10.2) at 30°C for 3 min (Bannister & Calabrese, 1987). Results were expressed as USod (units of enzyme activity)/mg protein. One unit is defined as the amount of enzyme that inhibits the rate of adrenochrome formation by 50%. Catalase (Cat) activity was measured by rate of H₂O₂ decomposition at 30°C for 1 min in 240 nm (Aebi, 1984). Results were expressed as UCat/mg of protein. One unit is defined as the amount of enzyme that decomposes 1 mmol of H₂O₂ in 1 min at pH 7.4. All absorbance were measured in a microplate reader (Victor-X3, multilabel counter, Perkin Elmer, Finland).

2.7. Protein sulfhydryl content
This assay is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB), whose absorption is determined spectrophotometrically at 412 nm (Aksenov & Markesbery, 2001). The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were expressed as mmol of DTNB/mg of protein.

2.8. Protein content determination
Larvae total protein content was quantified by the method of Bradford, using the bovine serum albumin as standard (Bradford, 1976).

2.9. Statistical analysis
Results were expressed as mean ± standard deviation (SD) obtained from two independent experiments. The Kolmogorov–Smirnov test was used to assess the parametric distribution of data. Statistical significance was evaluated using a one-way analysis of variance (ANOVA) and Duncan’s post hoc test. Relationships between the continuous variables were assessed using Pearson’s correlation coefficient. Significance was accepted at *p* ≤ 0.05. All statistical analyses were performed using the SPSS 21.0 software (SPSS Inc., Chicago, IL).

3. Results
In this study, we started the experiments with third instars *A. gemmatalis*, which received an artificial diet containing 1.25, 5, and 10 mg/mL AAE for 10 days. The diet was offered until the insects reached the pre-pupation stage, which occurred between 8th and 10th days. In view of this, the parameter weight gain was evaluated until the 6th day. It was observed that larvae fed with AAE had a reduced weight in all tested concentrations when compared to control group, even before the 4th day (Figure 2). Moreover, higher concentrations of AAE (5 and 10 mg/mL) were able to induce a significant decrease in weight compared to those fed with 1.25 mg/mL AAE on the 4th and 6th days.

In addition to weight changes, AAE-treated *A. gemmatalis* showed significant altered morphology, mainly with 5 and 10 mg/mL treatments (Figure 3), along with malformation of pupae and low emergence of adults (data not shown). Among AAE-treated larvae, mortality at the larval stage reached to 10.0, 96.7, and 99.0% with 1.25, 5, and 10 mg/mL AAE, respectively (Figure 4(A)). In comparison to pupal mortality, 26.6% of mortality with 1.25 mg/mL AAE was observed, while 99% of pupae mortality was observed at both concentrations of 5 and 10 mg/mL AAE (Figure 4(B)).
For evaluation of redox status, we analyzed cellular extracts of larvae fed with artificial diet, in the presence or absence of AAE on the fifth day of treatment. AAE treatments (5 and 10 mg/mL) induced an increase in oxidative damage to both lipids and proteins. In addition, nitric oxide levels were significantly increased in all AAE concentrations tested when compared to the control (Table 1), indicating that AAE is able to induce oxidative and nitrosative stress. This condition, along with low antioxidant defenses systems, is very dangerous to cells. For this reason, we next investigated the activity of the antioxidant enzymes, superoxide dismutase, and catalase, as well as the non-enzymatic cellular defense, sulfhydryl protein content in \textit{A. gemmatalis} larvae. Our results showed that the activities of Sod and Cat enzymes, and the protein sulfhydryl levels were significantly decreased at all treatment concentrations of AAE (Table 2), indicating a depletion in antioxidant defenses.

Pearson’s correlation analysis between \textit{A. gemmatalis} viability (at the larval stage or pupal) and redox status markers was performed and is shown in Table 3. Viability was positively correlated with both superoxide dismutase ($r = 0.933; p < 0.05$) and catalase activities ($r = 0.756; p = 0.030$), and with sulfhydryl content ($r = 0.904; p = 0.002$); however, it was negatively correlated with oxidative damage to lipids ($r = -0.940; p = 0.001$) and proteins ($r = -0.966; p = 0.001$); and nitric oxide...
production ($r = -0.746; p = 0.034$). Moreover, negative correlations were found significant between oxidative damage to biomolecules and nitric oxide levels with antioxidant defenses (Table 3).

### 4. Discussion

Natural products constitute an important source of phytochemical agents with several biological activities, including entomotoxic activity (Céspedes, Calderón, Lina, & Aranda, 2000; Macedo, Oliveira, & Oliveira, 2015; Miyazawa, Wada, & Kameoka, 2001). The use of these compounds offers numerous advantages over conventional synthetic pesticides used in crops, and therefore could
potentially prevent problems associated with insecticidal resistance, reduction in predatory and parasitoid insects, and also toxicity to other animals and to environment (Duke et al., 2003).

Phenolic compounds found in plants cover a chemically diverse and widespread group of defensive molecules that ranges from simple phenolics to complex polymers (Del Rio et al., 2012; Quideau, Deffieux, Douat-Cassous, & Pouységuy, 2011). These secondary metabolites participate in defense mechanisms against ultraviolet radiation and plant pathogens (Appel, 1993; Whitehill, Rigsby, Cipollini, Herms, & Bonello, 2014). Although polyphenols are well known by its antioxidant effects, they can become pro-oxidant at high doses, leading to the formation of reactive oxygen species (ROS) and inducing oxidative stress (Halliwell, 2007, 2008; Procházková, Boušová, & Wilhelmová, 2011). Thus, phenolic compounds can be toxic to herbivorous insects (Appel, 1993; War, Paulraj, War, & Ignacimuthu, 2011a, 2011b).

The endemic conifer, A. angustifolia (Bert. O. Kuntze), contains phenolic compounds of different classes including flavonoids and non-flavonoids. Previously, we investigated the entomotoxic effect of an aqueous extract obtained from the sterile seeds of AAE on velvetbean caterpillar A. gemmatalis cellular state. We found that this extract was able to induce oxidative and genotoxic damage in larvae of A. gemmatalis; however, it was unable to induce larval mortality (Branco et al., 2014). Considering that phenolic compounds are molecules that may be oxidized by different conditions, such as luminosity exposition, heating, and humidity level, we believe that the absence of significant effect on the viability of A. gemmatalis could be attributed to the experimental design performed by us. Therefore, in the present study, we added the Araucaria extract powder on the insect’s artificial diet in order to maintain its stability, while avoiding possible food interferences. We observed that AAE was able to induce a significant reduction in weight of A. gemmatalis, as well as alter the larval morphology, deform the structure of pupas, and decrease emergence of adults. Besides these actions, we found that AAE administration caused significant reduction in A. gemmatalis viability, both at larval and pupal stages. At larval stage, the lethal concentration (LC50) found was at 3.5 mg/mL AAE, i.e. the lethal concentration that is needed to reduce 50% of the individuals of a population. On the other hand, the LC50 was 2.3 mg/mL during the pupal stage, indicating that these insects are more sensitive to AAE at this particular stage of development. These results are consistent with a study performed by Batista Pereira et al. (2002) who demonstrated that flavonoid astrilin was able to induce significant toxicity during the pupal stage of A. gemmatalis and S. frugiperda. The data found in our study indicate that concentrations around 3.0 mg/mL are most promising for future tests aimed at evaluating the toxic effects of AAE on the biology and development of A. gemmatalis.

### Table 3. Pearson correlations between A. gemmatalis viability, lipid and protein oxidative damage, nitric oxide levels, and antioxidant defense systems

|                      | Viability | Lipid damage | Protein damage | Nitric oxide | Sod activity | Cat activity | Sulphhydryl |
|----------------------|-----------|--------------|----------------|--------------|--------------|--------------|-------------|
| Viability            |           | –0.940**     | –0.966**       | –0.746*      | 0.933**      | 0.756*       | 0.904**     |
| Lipid damage         | –0.940**  | –            | 0.970**        | 0.702        | –0.849**     | –0.699       | –0.871**    |
| Protein damage       | –0.966**  | 0.970**      | –              | 0.706        | –0.885**     | –0.744*      | –0.875**    |
| Nitric oxide         | –0.746*   | 0.702        | 0.706          | –            | –0.889**     | –0.865**     | –0.921**    |
| Sod activity         | 0.933**   | –0.849**     | –0.885**       | –0.889**     | –            | 0.853**      | 0.986**     |
| Cat activity         | 0.756*    | –0.699       | –0.744*        | –0.865**     | 0.853**      | –            | 0.854**     |
| Sulphhydryl          | 0.904**   | –0.871**     | –0.875**       | –0.921**     | 0.986**      | 0.854**      | –           |

Notes: Sod: superoxide dismutase; Cat: catalase.
*Statistically significant for p ≤ 0.05.
**Statistically significant for p ≤ 0.01.
In order to evaluate the entomotoxic mechanisms of action of AAE, we decided to investigate levels of oxidative stress, as well as the possible modulation of the antioxidant defense systems in third instars A. gemmatalis. The AAE treatments induced an increase in oxidative damage to both lipids and proteins, as well as an elevated production of nitric oxide, indicating high index of oxidative stress and cellular damage. Oxidative damage to biomolecules such as lipid peroxidation and protein modifications is involved in several events that subsequently may lead to cell death (Cobb & Cole, 2015). Cellular nitrosative stress may also occur through nitrosylation of proteins and lipids through the incorporation of nitric oxide and its derivatives (O’Donnell et al., 1999). Nitric oxide (NO) is a ubiquitous and water-soluble molecule, which plays key role in various physiological and pathological processes in mammals (Korde Choudhari, Chaudhary, Bagde, Gadball, & Joshi, 2013). In invertebrates, including insects, NO production has been shown to play a key role in inducing cellular response of these organisms against pathogens and other stress conditions (Faraldo, Só-Nunes, Del Bel, Faccioni, & Lello, 2005; Foley & O’Farrell, 2003; Gourdon, Guérin, Torreilles, & Roch, 2001; Nappi & Ottaviani, 2000). Moreover, NO plays a critical role in the initiation of insect metamorphosis, as observed during Drosophila development (Caceres et al., 2011), indicating that this molecule is associated with important biological processes regarding metabolism and behavior (Yamanaka & O’Connor, 2011). In our study, the increment in NO levels induced by AAE may be understood as the insect’s response to toxicity as a result of polyphenol metabolism.

To counteract oxidative stress, eukaryotic cells possess an antioxidant defense system that controls ROS generation and levels. However, when ROS levels exceed the capacity of antioxidant defense systems, cellular oxidative stress occurs (Halliwell, 2007, 2008; Halliwell & Gutteridge, 1995). In our study, we demonstrated increased oxidative stress, as the activities of Sod and Cat antioxidant enzymes were found significantly depleted after treatments. This suggests that AAE is inducing the generation of high levels of superoxide anion radical (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), contributing to cellular redox imbalance in A. gemmatalis. Sod enzyme is important for the detoxification of O$_2^-$ into H$_2$O$_2$, which is subsequently dismutated by Cat enzyme. In addition to the reduction of Sod and Cat activity, we demonstrated for the first time that AAE was able to reduce the levels of protein sulfhydryl, an important non-enzymatic antioxidant defense marker, in A. gemmatalis larvae. Superoxide anion and hydrogen peroxide are thought to be byproducts of aerobic respiration generated by electron transport chain of the mitochondria (Bae, Oh, Rhee, & Yoo, 2011). Though mitochondria are traditionally known for producing energy for cellular survival, they also play an important role in regulating other cellular mechanisms such as apoptosis, an essential dynamic process that maintains the stability of the internal environment and controls the development of multicellular organisms, including insects (Huang, Lv, Hu, & Zhong, 2013). Taking into account the link between redox imbalance and apoptosis, our results suggests that AAE-induced ROS generation, mainly by O$_2^-$ formation observed in this study, may be responsible for the reduction in insect viability, leading to cell death possibly via mitochondrial regulation of apoptosis signaling. However, further studies are needed in order to confirm this hypothesis.

In summary, our data demonstrated that the entomotoxic mechanisms presented by AAE are associated with redox imbalance in A. gemmatalis larvae, leading to reduction in viability and deleterious changes in its development. As well, our data suggest that the natural extract of A. angustifolia presents a potential to be used as a biological control agent for the management of velvetbean caterpillar A. gemmatalis in soybean crops.

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**Competing interests**
The authors declare no competing interests.

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