Lethal and Sublethal Toxicities of Annona sylvatica (Magnoliales: Annonaceae) Extracts to Zabrotes subfasciatus (Coleoptera: Chrysomelidae: Bruchinae)

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Lethal and sublethal toxicities of *Annona sylvatica* (Magnoliales: Annonaceae) extracts to *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae: Bruchinae)

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Abstract

Plant secondary metabolites comprise a diverse range of compounds (allelochemicals) that affect insect–plant interactions; many function in plant defense against herbivory. Thus, allelochemicals constitute an important source of insecticidal molecules that potentially can be used in different forms in integrated pest management programs. The objective of this study was to evaluate the bioactivity of ethanolic extracts and partially purified fractions of these extracts obtained from the leaves, branches, and seeds of *Annona sylvatica* A. St.-Hil. (Magnoliales: Annonaceae), a native Brazilian species, against *Zabrotes subfasciatus* (Boheman) (Coleoptera: Chrysomelidae: Bruchinae). In the screening assay, the ethanolic extract of *A. sylvatica* seeds was the most promising treatment, causing lethal (LC_{50} = 753.47 and 701.06 mg kg^{-1} for males and females, respectively) and sublethal effects, mainly oviposition deterrence (EC_{50} = 438.70 mg kg^{-1}). On the other hand, ethanolic extracts prepared from branches and leaves caused only sublethal effects including mainly oviposition deterrence (EC_{50} = 1,168.90 and 1,010.70 mg kg^{-1}, respectively) and reduction in number of offspring. Based on these results, the extracts were submitted to liquid–liquid partitioning, and their fractions were tested against *Z. subfasciatus* to verify their bioactivity. Overall, the results of the fraction bioassays showed evidence of synergistic interactions among compounds of different chemical classes and polarities. Chemical analyses of active fractions revealed the presence of triglycerides, alkaloids, and acetogenins in the seed fractions; alkaloids, lignans, and long-chain fatty acid ethyl esters in the branch fractions; and glycosides, flavonoids, terpenoids, and long-chain fatty acid ethyl esters in the leaf fractions. Thus, *A. sylvatica* is an interesting and potentially important source of structurally diverse grain-protective compounds.

Key Words: Mexican bean weevil; bioactivity; allelochemicals; acetogenins; alkaloids; lignans

Resumo

Metabólitos secundários de plantas compreendem uma gama diversificada de compostos (aleloquímicos) que, entre outras funções, atuam na regulação das interações inseto-planta e, muitos deles, atuam na defesa da planya contra a herbivoria. Assim, aleloquímicos constituem uma importante fonte de moléculas inseticidas que podem ser utilizadas em programas de manejo integrado de pragas (MIP) em diferentes formas. Portanto, o objetivo deste estudo foi avaliar a bioatividade de extratos etanólicos e frações parcialmente purificadas desses extratos obtidos de folhas, de ramos e de sementes de *Annona sylvatica* A.St.-Hil. (Magnoliales: Annonaceae), uma espécie nativa do Brasil, sobre *Zabrotes subfasciatus* (Boheman) (Coleoptera: Chrysomelidae: Bruchinae). Na triagem inicial, o extrato etanólico de sementes foi o tratamento mais promissor, causando efeitos letal (CL_{50} = 753,47 e 701,06 mg kg^{-1}, respectivamente, para machos e fêmeas) e subletais, especialmente deterrência de oviposição (EC_{50} = 438,70 mg kg^{-1}). Por outro lado, os extratos etanólicos preparados a partir de ramos e de folhas causaram apenas efeitos subletais, principalmente deterrência de oviposição (EC_{50} = 1168,90 e 1010,70 mg kg^{-1}, respectivamente) e efeito na progênie. Com base nesses resultados, os extratos foram submetidos à partição líquido-líquido e as frações obtidas testadas sobre *Z. subfasciatus* para verificar sua bioatividade. De um modo geral, os resultados dos bioensaios com as frações mostraram evidências de sinergismo entre compostos de diferentes classes químicas e polaridades. As análises químicas mostraram a presença de triglicerídeos, alcaloides e acetogeninas nas frações ativas do extrato de sementes; alcaloides, lignanas e ésteres etílicos de ácidos graxos de cadeia longa nas frações ativas do extrato de ramos; e glicosídeos, flavonoides, terpenoides e ésteres etílicos de ácidos graxos de cadeia longa nas frações ativas do extrato de folhas. Assim, *A. sylvatica* é uma fonte interessante de compostos estruturalmente diversificados com propriedades protetoras de grãos.

Palavras Chave: caruncho-do-feijão; bioatividade; aleloquímicos; acetogeninas; alcaloides; lignanas

The development of sustainable practices that can be incorporated into food production systems currently constitutes one of the greatest challenges for the scientific community (Prasifka & Gray 2012). In Brazil, this is even more evident when taking into consideration that current pest management programs of major agricultural commodities are based on the systematic and routine application of synthetic

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pesticides instead of an integrated pest management approach, which takes into account the economic, environmental, and social aspects of pest control interventions (Norris et al. 2003).

Recently, with the decreased availability of synthetic molecules with insecticidal action from the market of compounds with broad-spectrum action, interest in botanical insecticides as an alternative method for insect pest control has been revived (Dayan et al. 2009). The advantages of botanical insecticides compared with conventional insecticides are related to their lower mammalian toxicity and decreased health risk to applicators and their rapid degradability, which reduces residues in the environment and in the treated products (Isman 2006). Additionally, they generally contain various biologically active compounds capable of synergistic interactions that may reduce the selection of resistant pest populations in the field (Akhtar & Isman 2013). Owing to the ease of obtaining botanical derivatives (as plant powders, crude extracts, or oils), these botanical derivatives have been used for various purposes in the management of insect pests of stored grains in many countries, especially Latin America, Africa, and Asia (Isman 2008). Another important aspect of research on botanical derivatives with insecticidal potential is the discovery of model prototypes that can be used in the synthesis of new insecticides, especially those with different modes/mechanisms of action and less impact on the environment and human health (Regnault-Roger et al. 2012).

Several phytochemicals with insect-associated biological properties are known; these chemicals can act as feeding and oviposition deterrents, repellents, attractants, growth inhibitors, and insecticides (Al Lawati et al. 2002). Brazilian scientists are in a prime position to identify such active phytochemicals, as plant genetic diversity is higher in Brazil than in all other countries, with more than 55,000 vascular plant species catalogued in Brazil (Simões & Schenkel 2002).

Among botanical families from tropical regions, plants of the family Annonaceae are one of the most promising sources of bioactive molecules because of the wide variety of biologically active secondary metabolites produced by this family (Leboeuf et al. 1980; Bermejo et al. 2005). The acetogenins, a chemically diverse class of compounds found in some genera of Annonaceae (Alali et al. 1998), are promising compounds that act against insect pests; thus, they may be developed as new grain protectants (Ribeiro et al. 2013).

The tree species *Annona sylvatica* A. St.-Hil. (Magnoliales: Annonaceae), previously assigned to the genus *Rollinia*, is native to south-central Brazil (Lobão et al. 2005). It produces edible fruits with considerable health benefits due to the presence of phenolic compounds, antioxidants, carotenoids, and vitamin C (Pereira et al. 2013), making this species common in domestic orchards. However, despite its relative abundance, not much is known about *A. sylvatica* from a phytochemical point of view, especially concerning the bioactive potential of its secondary metabolites. To date, sylvaticin, an acetogenin that can be isolated from hexane extract of *A. sylvatica* fruit, is the only allelochemical reported to have effects on insects. Its action has been shown on *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) and *Acalymma vittata* F. (Coleoptera: Chrysomelidae) (Mikolajczak et al. 1990).

The aim of this study was to evaluate the bioactivity of extracts and semi-purified fractions from different parts (branches, leaves, and seeds) of *A. sylvatica* on the Mexican bean weevil *Zabrotes subfasciatus* (Boheman) (Coleoptera: Chrysomelidae: Bruchinae). *Zabrotes subfasciatus* is considered to be the main insect pest of stored beans (*Phaseolus vulgaris* L.; Fabales: Fabaceae), a staple dietary vegetable in many developing countries such as Brazil. In addition to the immeasurable loss of grain quality, the direct losses caused by this pest species were estimated to be 35% in Mexico, Central America, and Panama and between 7 and 15% in Brazil (Van Schoonhoven & Cardona 1982). Despite the relative effectiveness of chemical control of *Z. subfascia-*

tus, chemical control has some limitations that are related mainly to the cost of insecticides, which hinders their availability to small-farm owners, and the limited number of active ingredients of synthetic insecticides available on the Brazilian market.

Materials and Methods

PLANT SAMPLES AND PREPARATION OF CRUDE EXTRACTS

The plant parts used in the study (branches, leaves, and seeds) were collected on 23 Mar 2011 from specimens of *A. sylvatica* grown in a domestic orchard located in Erval Seco municipality, RS State, Brazil (27°25'41.8"S, 53°34'11.2"W; 466 m asl). A voucher specimen, previously identified by Dr. Renato Mello-Silva (Institute of Biosciences, University of Sao Paulo, Brazil), was deposited in the ESA herbarium in Piracicaba, SP, Brazil (record number 121205).

For preparing the extracts, the collected plant structures were dried in an oven at 40 °C for 48 to 72 h. Next, the structures were ground in a knife mill, and the resulting branch (100 g), leaf (100 g), or seed (100 g) powders were stored separately in sealed glass containers. The organic extracts were prepared by allowing each plant powder to steep in ethanol (1:5 w/v) in a hermetically sealed flask for 3 d. The samples were then filtered through filter paper; this procedure was repeated 3 times. The ethanol in the filtered solution was removed in a rotary evaporator at 50 °C at a vacuum of −600 mm Hg. After completing the evaporation of the solvent in an aerated chamber, the yield was determined by weighing the extracts from each *A. sylvatica* plant part, i.e., 5.01, 7.18, and 11.00% (w/w) for the branches, leaves, and seeds, respectively.

BIOASSAYS

All bioassays were conducted in a temperature-controlled room at 25 ± 2 °C and 60 ± 10% relative humidity with a 14:10 h L:D photoperiod and a mean illuminance of 200 lux. As the substrate for performing the tests, we used shelled bean seeds (*P. vulgaris* 'Bolinha') that we obtained in the Piracicaba (SP, Brazil) trade and that we manually selected.

For the crude extract application on the surface of the bean seeds, a microatomizer coupled to a vacuum pump was used. This microatomizer was adjusted to provide a pressure of 0.5 kg cm^{-2} to deliver a spray volume of 30 L t^{-1} (30 mL per kg of seeds) according to conditions defined in previous studies (Ribeiro 2010). After spraying, the bean seeds were transferred to a plastic bag with a capacity of 2 L and were stirred manually for 1 min. Preliminary tests were performed to evaluate the homogeneity of the application and to verify the possible effects of the solvents used for extract solubilization on *Z. subfasciatus*. To identify promising bioactive extracts with activity against *Z. subfasciatus*, bioassays were conducted to assess the lethal and sublethal effects (acute and chronic toxicity) of the extracts.

EVALUATION OF INSECTICIDAL ACTIVITY

In this bioassay, Petri dishes (6 cm diameter × 2 cm height) containing 10 g of beans were used. These sample units were treated separately with ethanolic extracts of branches, leaves, or seeds at a concentration of 1,500 mg kg^{-1} (1.5 g of extract per kg of bean seeds), which was determined based on previous studies (Ribeiro et al. 2013). For the control, the bean seeds were treated with only the solvent solution (acetone:methanol 1:1 [v/v]) used in the suspension of the extracts. Ten replicates per treatment were used, and each sample unit was infested with 5 weevil pairs aged between 0 and 24 h that were

derived from a population maintained under laboratory conditions for approx. 15 generations. Adult survival was assessed on the 5th day after infestation. Insects with legs completely extended that showed no reaction to contact with a thin brush for 1 min of observation were considered dead.

ASSESSMENT OF SUBLETHAL EFFECTS

The same experimental arrangement used in the previous test was used to evaluate the sublethal effects of the crude extracts. For this assessment, the adults were removed after 5 d of infestation, and the eggs on each bean seed surface were counted with the aid of a stereo-microscope. Then, the sampling units were maintained under the climatic conditions mentioned previously. At 60 d after the initial infestation, the adults that emerged were separated by gender and counted. At this time, the percentage of damaged seeds (with holes) in each sample was determined through visual assessment of each seed. Similar to the previous bioassay, 10 replicates per treatment were used.

CONCENTRATION–RESPONSE CURVES OF THE PROMISING EXTRACTS

The extracts that showed the most promising results were tested for LC_{50} and LC_{90} estimations, which correspond to the concentrations required to kill 50 and 90%, respectively, of the weevil population. Here, preliminary tests were performed using these extracts to determine the basic concentrations that caused 95% adult mortality and a mortality rate similar to the control. Based on these data, a range of concentrations (100 to 2,500 mg kg^{-1} [mg of extract per kg of seeds]) were set to determine the LC_{50} values for both males and females, and this was accomplished by applying the formula proposed by Finney (1971). Concentration–response curves were constructed to estimate the EC_{50} (the effective concentration required to reduce the number of eggs laid per sample by 50% [oviposition deterrence]). The same procedures described previously were used.

LIQUID–LIQUID PARTITIONING OF THE SELECTED CRUDE EXTRACTS

Based on the results from the bioassays described previously, the most promising extracts were selected and subjected to liquid–liquid partitioning. Specifically, the selected extracts were solubilized separately in methanol:water (hydro–methanol fraction; 1:3 [v/v] for the leaf and branch extracts and 8:2 [v/v] for the seed extracts) and partitioned in a separatory funnel, using hexane for the seed extract and organic solvents of increasing polarity (hexane, dichloromethane, and ethyl acetate) for the leaf and branch extracts. The fractions were concentrated on a rotary evaporator at 50 °C at a vacuum of −600 mm Hg. The yields were determined using the mass of the respective extracts as the basis. The seed fractions yielded 83.93 and 16.07% (w/w) for the hexane and hydro–methanol, respectively; leaf fractions yielded 41.76, 3.88, 5.98, and 48.38% for the hexane, dichloromethane, ethyl acetate, and hydro–methanol, respectively; and branch fractions yielded 16.59, 8.54, 6.87, and 68.00% for the hexane, dichloromethane, ethyl acetate, and hydro–methanol, respectively.

ASSESSMENT OF THE BIOACTIVITY OF THE OBTAINED FRACTIONS

Each of the obtained fractions (phases) was tested to evaluate its lethal and sublethal effects on *Z. subfasciatus*. In this assessment, the sample units (10 g of beans) were treated with these fractions using

the respective LC_{50} estimated for the crude extract, and the same experimental procedures described previously for the screening assay were adopted. For cases where LC_{50} could not be estimated (> tested range for extracts of leaves and branches), the fractions were tested at the same concentration that was used in the bioassays with the crude extracts (1,500 mg kg^{-1}). In these tests, the same variables and experimental procedures adopted in the previous tests were used, and for each treatment, 10 replicates containing 5 pairs of *Z. subfasciatus*, aged between 0 and 24 h, were used.

CHEMICAL ANALYSIS OF BIOACTIVE FRACTIONS

To identify the class of compounds present in the bioactive fractions, hydrogen nuclear magnetic resonance (^{1}H NMR) was performed with a BRUKER DRX 400 instrument operating at 400 MHz for ^{1}H nucleus (9.4 Tesla) and the deuterated solvents CDCl$_3$ and CD$_3$OD.

The chemical profiles of the bioactive fractions were analyzed using thin-layer chromatography (TLC). The TLC analyses were carried out by testing the proportions of solvents of different polarities with the aim of identifying the best mobile phase for the separation of the components present in the samples including hexane:dichloromethane 1:1 (v/v), hexane:acetone 8:2, 7:3, and 1:1 (v/v), dichloromethane:acetone 7:3 and 1:1 (v/v), dichloromethane (100%), and acetone (100%). For the detection of the spots of the constituents present in each sample, the following tools were used: ultraviolet light (254 and 365 nm), vanillin sulfuric solution (general developer), ethanolic solution of 1% aluminum chloride (for detection of flavonoids [Jácome et al. 2010]), Dragendorff reagent (specific to alkaloids [Sherma 2000]), and Kedde reagent (indicating the presence of the g-lactone-α,b-unsaturated subunit present in acetogenins [Caloprisco et al. 2002]).

DATA ANALYSES

Generalized linear models (GLMs) belonging to the exponential family of distributions (Nelder & Wedderbum 1972) were used to assess the biological variables of *Z. subfasciatus* exposed to the extracts and fractions of *A. sylvatica*. The quality of the fit was verified using a half-normal probability graph with a simulation envelope (Hinde & Demétrio 1998). When significant differences were observed between treatments, multiple comparisons (Tukey test, $P < 0.05$) were performed using the *glht* function of the multicomp package with adjusted P values. These analyses were performed using the statistical software R, version 2.15.1 (R Development Core Team 2012).

To estimate the lethal concentrations (LC_{50} and LC_{90}), we used a binomial model with a complementary log–log link function (gompit model), using the Probit Procedure of SAS software, version 9.2 (SAS Institute 2013). To estimate the average effective concentration (EC_{50}), i.e., the concentration required to reduce the number of eggs per sample by 50%, we employed a non-linear logistic model using the Nlin Procedure of SAS software version 9.2 (SAS Institute 2013). Finally, the mean lethal time (LT_{50}) was estimated using the method proposed by Throne et al. (1995) for the Probit analysis of correlated data.

Results

BIOLOGICAL ACTIVITY OF ETHANOLIC CRUDE EXTRACTS

The ethanolic extract from *A. sylvatica* seeds was the only treatment that caused significant mortality of *Z. subfasciatus* adults (Table 1). There was no significant difference in mortality between the sexes (LC_{50} for females: 701.06 mg kg^{-1} [CI 95%: 664.85–727.67], $\chi^2 = 2.21$, df = 5; LC_{50} for males: 753.47 mg kg^{-1} [CI 95%: 636.53–804.31], $\chi^2 = 10.36$, df

Table 1. Mortality (mean ± se) of *Zabrotes subfasciatus* adults exposed to samples of bean seeds treated with crude ethanolic extracts from different *Annona sylvatica* parts (at 1,500 mg kg^{-1}) after they had been pulverized and steeped for 5 d in ethanol.

	Mortality (%)[a]		
A. sylvatica plant parts	Males	Females	Total
Leaves	4.00 ± 2.66 b	8.00 ± 4.42 b	6.00 ± 2.66 b
Branches	4.00 ± 4.00 b	4.00 ± 2.66 b	4.00 ± 2.21 b
Seeds	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
Control (acetone:methanol 1:1 [v/v])	2.00 ± 2.00 b	2.00 ± 2.00 b	2.00 ± 2.00 b
F value	63.49	75.15	145.61
P value	< 0.0001	< 0.0001	< 0.0001

[a]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-binomial distribution followed by Tukey's post hoc test, $P < 0.05$).

= 5). Moreover, the average lethal time LT_{50} did not differ between the sexes (males: 27.59 h [CI 95%: 24.89–30.10], $\chi^2 = 6.44$, df = 8; females: 28.81 h [CI 95%: 25.98–31.48], $\chi^2 = 7.80$, df = 8).

The ethanolic extracts prepared from the 3 plant parts caused significant sublethal effects (Table 2), mainly a large reduction in the number of eggs per sample (EC_{50}: 1,010.70 mg kg^{-1} [CI 95%: 643.10–1,378.40], 1,168.90 mg kg^{-1} [CI 95%: 832.80–1,505.90], and 438.70 mg kg^{-1} [CI 95%: 403.80–473.60] for the ethanolic extracts from the leaves, branches, and seeds, respectively). These effects were accordingly reflected in the F_1 progeny size and the percentage of damaged seeds, i.e., variables that can significantly influence the population dynamics of pest species and the level of degradation of seed quality. However, the extracts had no effect on the egg-to-adult viability or the sex ratio of the F_1 progeny (Table 2), demonstrating that the bioactive compounds in ethanolic extracts had no effect on the embryonic and post-embryonic development of *Z. subfasciatus*.

BIOLOGICAL ACTIVITIES OF THE FRACTIONS OBTAINED FROM ETHANOLIC EXTRACTS

None of the fractions originating from the ethanolic extracts prepared from the branches and leaves of *A. sylvatica* caused significant acute toxicity in adult *Z. subfasciatus* (Table 3). However, the hexane and hydro–methanol fractions of the crude seed extract caused significant adult mortality, and there was no difference in mortality ($P > 0.05$) between the sexes (Table 3). Based on the mortality levels caused by the partially purified fraction in comparison with the ethanolic extract (Table 3), it was possible to infer that the insecticidal activity of the crude seed extract was due to the interaction among compounds with low and high polarity.

The hexane and ethyl acetate fractions from the partitioning of the leaf ethanolic extract and the dichloromethane and hexane fractions from the partitioning of the branch ethanolic extract of *A. sylvatica* (es-

pecially the hexane fraction, in both cases) caused a significant reduction in the number of eggs per sample and the number of F_1 progeny, and in the damage caused to the treated bean samples (Table 4). Moreover, the hydro–methanol and hexane fractions from the partitioning of the ethanolic extract of *A. sylvatica* seeds significantly affected all parameters (Table 4). However, there was no difference in effects between these 2 fractions, except for the percentage of damaged seeds, where the hydro–methanol fraction reduced the damage caused to the treated bean samples to a much greater extent than the hexane fraction of the ethanolic seed extract.

CHEMICAL ANALYSIS OF BIOACTIVE FRACTIONS

The analysis of the hydro–methanol fraction of the seeds using TLC (hexane:acetone 7:3 [v/v]) reacted positively with Kedde reagent (spots with a reddish color), as brown spots appeared after the use of sulfuric vanillin solution, indicating the presence of acetogenins. The analysis using Dragendorff reagent revealed orange spots at the base of the analytical plate, indicating the presence of polar alkaloids in this fraction, and absorption occurred at 365 nm after visualization using UV light (confirming the presence of compounds with a high conjugation of π bonds, observed in the alkaloids). This fraction was analyzed using ^{1}H NMR, which allowed the identification of the major classes of compounds present in this fraction as acetogenins. Thus, d_H 7,28, d_H 5,05, and d_H 1,38 refer to the lactone α,b-unsaturated unit, signals at the region of d_H 4,42 to d_H 3,08 are related to the oxymethine hydrogens of their substituents, and signals at d_H 2,49 and d_H 2,37 refer to diastereotopic hydrogens adjacent to the lactone ring (Cortes et al. 1993; Colman-Saizarbitoria et al. 1995).

The hexane fraction of the ethanolic seed extract, present as an oil at room temperature, was similarly analyzed using TLC (hexane:dichloromethane 1:1 [v/v]) and developed using a vanillin/sulfuric acid solution; the appearance of blue spots indicated the pres-

Table 2. Sublethal effects (mean ± SE) of crude ethanolic extracts from different parts of *Annona sylvatica* at 1,500 mg kg^{-1} on *Zabrotes subfasciatus*.

	No. eggs/sample[a]	F_1 progeny[a]			Viability (%) (egg–adult)[b]	Sex ratio[c]	Grains damaged (%)[b]
A. sylvatica plant parts		Males	Females	Total			
Leaves	11.90 ± 2.71 c	5.00 ± 1.08 c	5.10 ± 1.28 c	10.10 ± 2.33 c	85.60 ± 7.52	0.47 ± 0.04 a	21.11 ± 3.73 c
Branches	26.60 ± 3.66 b	10.00 ± 2.02 b	11.60 ± 1.79 b	21.60 ± 3.46 b	77.61 ± 3.72	0.55 ± 0.04 a	36.43 ± 4.53 b
Seeds	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d	0.00 ± 0.00 d	—	—	0.00 ± 0.00 d
Control (acetone:methanol 1:1 [v/v])	83.70 ± 5.02 a	37.90 ± 2.03 a	36.90 ± 2.96 a	74.80 ± 4.63 a	89.30 ± 0.84	0.49 ± 0.01 a	90.60 ± 1.87 a
	F: 72.18	*F*: 67.62	*F*: 54.77	*F*: 70.25	*F*: 2.62[ns]	χ^2: 53.54	*F*: 89.48
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.0918$	$P = 0.0455$	$P < 0.0001$

[a]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-Poisson distribution followed by Tukey's post hoc test, $P < 0.05$).
[b]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-binomial distribution followed by Tukey's post hoc test, $P < 0.05$).
[c]Sex ratio can be inferred from the female fraction data, which are presented here.
[ns]Non-significant ($P > 0.05$).

Table 3. Mortality (mean ± SE) of *Zabrotes subfasciatus* adults exposed to samples of bean seeds treated with fractions prepared by liquid–liquid partitioning from ethanolic extracts of different *Annona sylvatica* parts after they had been steeped for 5 d in ethanol.

Fractions	Mortality (%)[a]		
	Males	Females	Total
A. sylvatica leaves			
Hexane	4.00 ± 2.67	6.00 ± 4.27	5.00 ± 3.07
Dichloromethane	6.00 ± 3.05	6.00 ± 4.20	6.00 ± 3.09
Ethylacetate	4.00 ± 2.67	8.00 ± 4.42	6.00 ± 2.21
Hydro–methanol	2.00 ± 2.00	8.00 ± 3.27	4.00 ± 1.63
Control (acetone)	8.00 ± 4.42	8.00 ± 4.42	8.00 ± 3.59
Control (methanol)	10.00 ± 4.47	8.00 ± 3.27	9.00 ± 2.77
F value	0.81[ns]	0.07[ns]	0.33[ns]
P value	0.5479	0.9968	0.8911
A. sylvatica branches			
Hexane	24.00 ± 5.81 a	30.00 ± 5.37 a	27.00 ± 4.95
Dichloromethane	22.00 ± 10.08 a	14.00 ± 6.70 a	18.00 ± 7.27
Ethylacetate	12.00 ± 3.26 a	12.00 ± 4.42 a	12.00 ± 3.26
Hydro–methanol	2.00 ± 2.00 a	28.00 ± 8.54 a	15.00 ± 4.53
Control (acetone)	8.00 ± 4.42 a	8.00 ± 4.42 a	8.00 ± 3.59
Control (methanol)	10.00 ± 4.47 a	8.00 ± 3.27 a	9.00 ± 2.77
F value	2.62	2.78	2.21[ns]
P value	0.0342	0.0263	0.0668
A. sylvatica seeds			
Hexane	30.00 ± 9.45 a	6.00 ± 2.89 ab	15.00 ± 5.96 a
Hydro–methanol	44.00 ± 12.22 a	18.00 ± 3.59 a	31.00 ± 6.57 a
Control (acetone:methanol 1:1 [v/v])	2.00 ± 2.00 b	4.00 ± 2.67 b	3.00 ± 1.53 b
F value	16.32	4.40	17.11
P value	< 0.0001	0.0221	< 0.0001

[a]Means followed by different letters within columns (each plant part) indicate significant differences between treatments (GLM with a quasi-binomial distribution followed by Tukey's post hoc test, $P < 0.05$);
[ns]Non-significant ($P > 0.05$).

ence of triglycerides. These compounds are the major constituents of vegetable seed oils, which are also classified as triacylglycerols, wherein each functional ester group contains a saturated or unsaturated hydrocarbon chain (Fernandes et al. 2002). The presence of these compounds in this fraction was confirmed by the analysis of signals in the ^{1}H NMR spectrum, which presented a signal for this class of compound as observed by Colzato et al. (2008).

The dichloromethane fraction of the branches was eluted in dichloromethane:acetone 7:3 (v/v), and after the staining of the chromatographic plate with Dragendorff reagent, orange spots that interacted strongly with the silica (retention factor: intermediate to high) could be visualized, indicating the presence of alkaloids as constituents of this fraction. This evidence was confirmed by the analysis of the signals observed in the ^{1}H NMR spectrum, showing the characteristic signs of lignans and alkaloids as observed in the literature (Tantisewie et al. 1989; Biavatti et al. 2001; Pinheiro et al. 2009).

The ethyl acetate fraction of the leaves showed a strong interaction with the stationary phase of the chromatographic plate. This fraction was eluted with acetone:methanol 9:1 (v/v) and showed brown spots after staining with vanillin/sulfuric acid and a methanolic solution of aluminum chloride (1%). Yellow spots were observed after UV (365 nm) analysis, indicating the presence of flavonoids. The ^{1}H NMR spectrum showed signals with different values of chemical shifts, which indicated the presence of terpenoids and glycosyl-flavonoids in this fraction as suggested by Ibrahim et al. (2007), Silva et al. (2012), and Somanawat et al. (2012).

Due to the non-polar nature of the hexane fractions from the leaves and branches, their chromatographic profiles were analyzed using TLC and ^{1}H NMR. The TLC analysis allowed the comparison of the chemical profiles of both fractions, which were characterized by the presence of spots with the same retention factor for both samples (indicating the similarity of compounds present in both fractions) after elution with hexane:acetone 8:2 (v/v) and staining with vanillin/sulfuric acid solution. The analysis of the ^{1}H NMR spectrum showed signals of chemical shifts that were characteristic of furofuranlignans, as described by Biavatti et al. (2001). Additionally, signals similar to those described for the hexane fraction of seeds were observed, which indicated the presence of triacylglycerols (Colzato et al. 2008).

Discussion

Plants synthesize and accumulate large amounts of allelochemicals, and concentrate a wide variety of them in their seeds as an evolutionary strategy to ensure the survival of the progeny and the success of the species (Wink 2003). In an evolutionary context, herbivorous insects have constituted major selective pressure and are the main targets of plant secondary metabolism. In addition to the large accumulation in seeds of allelochemicals with insecticidal properties (triglycerides, acetogenins, and alkaloids), *A. sylvatica* synthesizes and accumulates a wide variety of allelochemicals in other plant parts (e.g., long-chain fatty acid ethyl esters, lignans, flavonoids, and terpenoids in

Table 4. Sublethal effects (mean ± sE) on *Zabrotes subfasciatus* of fractions prepared by liquid–liquid partitioning from ethanolic extracts of different parts of *Annona sylvatica* at 1,500 mg kg^{-1}.

Fraction	No. eggs/ sample[a]	F[1] progeny[a]			Viability (%) (egg–adult)[b]	Sex ratio[c]	Grains damaged (%)[b]
		Males	Females	Total			
			A. sylvatica leaves				
Hexane	1.20 ± 0.72 c	0.60 ± 0.43 d	0.60 ± 0.34 c	1.20 ± 0.73 c	100.00 ± 0,00[d]	0.50 ± 0.00[d]	3.20 ± 1.80 d
Dichloromethane	79.60 ± 8.96 a	30.40 ± 3.78 b	34.10 ± 3.77 a	64.50 ± 6.92 a	82.15 ± 2.21	0.53 ± 0.03	66.75 ± 4.64 b
Ethyl acetate	27.90 ± 7.32 b	9.40 ± 2.53 c	11.30 ± 3.36 b	20.70 ± 5.77 b	72.08 ± 4.82	0.42 ± 0.08	32.17 ± 7.73 c
Hydro–methanol	100.40 ± 6.70 a	39.50 ± 2.10 ab	40.20 ± 2.39 a	79.70 ± 4.12 a	77.27 ± 1.69	0.50 ± 0.01	92.32 ± 1.60 a
Control (acetone)	103.50 ± 7.84 a	44.80 ± 3.96 a	42.30 ± 2.84 a	87.10 ± 6.26 a	84.40 ± 1.32	0.49 ± 0.01	93.16 ± 1.93 a
Control (methanol)	82.30 ± 4.90 a	36.90 ± 2.10 ab	36.00 ± 2.84 a	72.90 ± 4.59 a	88.35 ± 2.07	0.48 ± 0.01	89.02 ± 2.79 a
	F: 48.03	F: 48.26	F: 42.78	F: 50.92	F: 2.14^{ns}	χ^2: 42.32^{ns}	F: 62.61
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.0916$	$P = 0.3424$	$P < 0.0001$
			A. sylvatica branches				
Hexane	15.80 ± 4.44 d	5.00 ± 1.48 c	7.40 ± 2.31 a	12.40 ± 3.47 d	81.91 ± 3.99	0.61 ± 0.09[d]	22.13 ± 4.82 d
Dichloromethane	37.90 ± 7.03 c	13.80 ± 2.75 b	16.70 ± 2.60 b	30.50 ± 5.17 c	84.26 ± 3.65	0.55 ± 0.03	49.28 ± 6.75 c
Ethyl acetate	83.90 ± 5.87 ab	37.90 ± 3.26 a	36.30 ± 3.13 a	74.20 ± 5.81 ab	88.01 ± 1.50	0.49 ± 0.02	86.23 ± 2.76 ab
Hydro–methanol	61.80 ± 9.25 bc	30.20 ± 4.00 a	27.00 ± 4.46 ab	57.20 ± 8.31 b	92.72 ± 1.78	0.45 ± 0.02	68.99 ± 5.67 b
Control (acetone)	103.50 ± 7.84 a	44.80 ± 3.96 a	42.30 ± 2.84 a	87.10 ± 6.26 a	84.40 ± 1.32	0.49 ± 0.01	93.16 ± 1.93 a
Control (methanol)	82.30 ± 4.90 ab	36.90 ± 2.10 a	36.00 ± 2.84 a	72.90 ± 4.59 ab	88.35 ± 2.07	0.48 ± 0.01	89.02 ± 2.79 a
	F: 21.94	F: 27.49	F: 17.64	F: 24.97	F: 0.58^{ns}	χ^2: 35.48^{ns}	F: 32.59
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.6749$	$P = 0.3026$	$P < 0.0001$
			A. sylvatica seeds				
Hexane	21.50 ± 4.11 b	3.50 ± 0.49 b	5.00 ± 0.70 b	8.50 ± 1.00 b	37.89 ± 3.73 b	0.53 ± 0.04[d]	62.07 ± 9.78 b
Hydro–methanol	11.60 ± 4.57 b	1.30 ± 0.64 b	1.60 ± 0.74 b	2.90 ± 1.33 b	15.93 ± 4.67 b	0.36 ± 0.13[d]	7.55 ± 3.02 c
Control (acetone:methanol 1:1 [v/v])	72.10 ± 4.86 a	23.10 ± 2.18 a	22.20 ± 2.12 a	45.30 ± 3.87 a	62.52 ± 2.84 a	0.49 ± 0.02	95.00 ± 1.63 a
	F: 29.45	F: 78.41	F: 59.73	F: 79.12	F: 27.05	—	F: 49.32
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	—	$P < 0.0001$

[a]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-Poisson distribution followed by Tukey's post hoc test, $P < 0.05$).
[b]Means followed by different letters within columns indicate significant differences between treatments (GLM with a quasi-binomial distribution followed by Tukey's post hoc test, $P < 0.05$).
[c]Sex ratio can be inferred from the female fraction data, which are presented here.
[d]Not included in the analysis due to a small sample unit.

the leaves; alkaloids, lignans, and long-chain fatty acid ethyl esters in the branches) that are capable of causing potent effects on *Z. subfasciatus*, an important pest of stored beans. Thus, extracts from this native species of Brazilian Annonaceae are an interesting potential source of structurally diverse grain-protective compounds. The observed differences between the bioactivities of the various fractions of the ethanolic extracts obtained from different plant parts (leaves, branches, and seeds) are most likely associated with changes in their chemical profiles and may not be specifically due to the effect of the concentration of the same active compounds.

Knowledge of interactions between chemical compounds assists in the formulation of insecticides that contain synthetic molecules that are analogous to natural compounds, and in the formulation of botanical insecticides that contain several active ingredients. Based on such knowledge, antagonistic interactions can be avoided and synergistic interactions may be optimized. Therefore, the use of crude extracts may be more efficient than the use of isolated compounds. In relation to the activity observed for the seed extract of *A. sylvatica*, triglycerides may hypothetically act as an adjunct to the acetogenins present in the crude extract. Alternatively, the triglycerides alone may cause biological effects. It is noteworthy that due to the predominant presence of triglycerides, it was not possible to identify other compounds in the hexane fraction; thus, it is uncertain whether the insecticidal effect of the hexane fraction was due to the action of triglycerides or of minor compounds belonging to other chemical classes.

Many oil products are used as adjuvants in the application of pesticides. They may act as a vehicle that helps the active ingredient come into contact with the target (kidney bean) and achieve better coverage of its surface, thereby assisting the principal compound in exerting its insecticidal action. Therefore, it is possible that triglycerides only help the active ingredients to express their insecticidal effect. However, if triglycerides promote the mortality of exposed insects, this may be due to asphyxia caused by the blocking of weevil spiracles (Hewlett 1947). By contrast, if the insecticidal effect is due to the presence of minor chemical compounds or compounds derived from the breakdown of triglycerides, then in the latter case, fatty acids probably are responsible for this bioactivity. A study conducted by Ramsewak et al. (2001) supports this hypothesis, as these authors identified 5 compounds in the hexane extract of the seeds of *Dirca palustris* L. (Malvales: Thymelaeaceae). Three triglycerides (1,3-dilinoleoyl-2-olein; 1,3-dioleoyl-2-linolein; 1,2,3-trilinolein) showed no bioactivity, but the other 2 compounds (oleic acid and linoleic acid) isolated from the methanol fraction of the hexane extract of the seeds promoted larval mortality of the 4th instar of *Aedes aegypti* (L.) (Diptera: Culicidae) and reduced the growth of *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), *Lymantria dispar* L. (Lepidoptera: Lymantriidae), *Orgyia leucostigma* (Smith) (Lepidoptera: Lymantriidae), and *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae) larvae, likely due to the phagodeterrent action of these compounds. These results demonstrate that fatty acids may exert bioactivity on the insects, which was also reported by Fatope et al. (2000).

Similarly, Annonaceae species have the same fatty acids in their seeds. Egydio & Santos (2011) observed little variation in the fatty acids of various species of *Annona* (*A. crassiflora*, *A. coriacea*, *A. montana*, *A. cherimola*, *A. squamosa* ´ 'Pink Mammoth' and *A. cherimola* ´ *A. squamosa* 'Gefner'), and they found palmitic, stearic, oleic, and linoleic acids to be the major components in all species. Chemical characterization of seed fatty acids from *A. sylvatica* showed the presence of palmitic (20.84%), stearic (4.26%), oleic (54.41%), and linoleic acids (20.49%) (Andrade et al. 2012). Annonaceae species, including *A. sylvatica*, produce certain fatty acids with insecticidal effects, as reported by Peñaflor et al. (2006). Moreover, in the current study, the hexane extract of the seeds of *A. sylvatica* drastically decreased the oviposition of *Z. subfasciatus* females. Thus, some fatty acids present in *Annona* seeds may deter oviposition. Indeed, Peñaflor et al. (2006) demonstrated that fatty acids that have between 5 and 9 carbon atoms in their chains are capable of affecting the behavior of insects; more specifically, these fatty acids can repel workers of *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae).

During the evaluation of the experiments investigating the effect of exposure to the hydro–methanol fraction of the *A. sylvatica* seed extract, a decrease in muscle coordination of the beetles was observed, and this symptom is characteristic of the effect promoted by acetogenins (González-Coloma et al. 2002). The acetogenins (C-35/C-37) are derived from long-chain fatty acid (C-32/C-34) units that combine with 2-propanol, and they have a wide range of biological effects, including insecticidal activity (Alali et al. 1999). Mikolajczak et al. (1990) reported that the insecticidal action of the hexane extract of *A. sylvatica* fruit on *O. nubilalis* and *A. vittata* was due to the presence of sylvaticin, an acetogenin. Acetogenins act by interrupting energy production in the mitochondria through the inhibition of complex I (NADH: ubiquinone oxidoreductase) of the electron transport system and NADH oxidase at the plasma membrane (González-Coloma et al. 2002).

The presence of several active principles that act at different sites by exerting effects on the physiology and behavior of insects potentially reduces the number of resistant pest populations (Rattan 2010), which is an important advantage of botanical insecticides. Alali et al. (1999) showed that 6 acetogenins exhibited insecticidal effects that were equivalent to or greater than the effects of 5 commercial products (synthetic amidinohydrazone [hydramethylnon], carbamate [propoxur, bendiocarb], organophosphate [chlorpyrifos], and pyrethroid [cypermethrin] insecticides) that were used in baits to control populations of *Blattella germanica* (L.) (Blattodea: Ectobiidae). This aspect is of great importance for the management of stored-grain pests because several populations are resistant to the currently registered synthetic insecticides (Pimentel et al. 2010; Boyer et al. 2012) that consist largely of phosphines for fumigation and pyrethroids and organophosphates for preventive control. This observation encourages the advancement of research on new active principles in plant metabolism. Although perspectives regarding the use of insecticidal products have gradually changed, the use of diverse natural compounds with potential insecticidal value still remains a novelty.

Research on secondary metabolites present in the seeds of *A. sylvatica* requires innovation and demonstrations of the enormous potentials of these compounds. These metabolites constitute a source of prototypes for the development of synthetic analogues and a source of new biopesticides that may be useful in management programs for pests that affect stored grain.

Acknowledgments

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