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Searching for promising sources of grain protectors in extracts from Neotropical Annonaceae

[Búsqueda de fuentes prometedoras de protectores de granos em extractos de Anonaceas Neotropicales]

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Abstract: To investigate potential sources of novel grain protector compounds against *Sitophilus zeamais* (Coleoptera: Curculionidae), which is an important insect pest of stored cereals, this study evaluated the bioactivity of ethanolic extracts (66) prepared from 29 species belonging to 11 different genera of Neotropical Annonaceae. A screening assay demonstrated that the most pronounced bioactive effects on *S. zeamais* were caused by ethanolic extracts from *Annona montana*, *A. mucosa*, *A. muricata*, and *A. sylvatica* seeds, causing the death of all weevils exposed, almost complete inhibition of the F1 progeny and a drastic reduction in grain losses. Furthermore, the ethanolic extracts obtained from the leaves of *A. montana*, *A. mucosa*, *A. muricata*, and *Duguetia lanceolata*, especially *A. montana* and *A. mucosa*, demonstrated significant bioactive effects on the studied variables; however, the activity levels were less pronounced than in the seed extracts, and the response was dependent on the concentration used. This study is the first to report the activity of secondary metabolites from *D. lanceolata* on insects as well as the action of *A. sylvatica* on pests associated with stored grains.

Keywords: Allelochemicals, acetogenins, bioactivity, *Sitophilus zeamais*, stored cereals.

Resumen: Para investigar las posibles fuentes de nuevos compuestos protectores de granos contra *Sitophilus zeamais* (Coleoptera: Curculionidae), una importante plaga de los cereales almacenados, este estudio evaluó la bioactividad de los extractos etanólicos (66) preparados a partir de 29 especies pertenecientes a 11 géneros distintos de Anonaceas Neotropicales. Un ensayo de selección demostró que los efectos bioactivos más relevantes sobre *S. zeamais* fueron causados por los extractos etanólicos de las semillas de *Annona montana*, de *A. mucosa*, de *A. muricata* y de *A. sylvatica*, que causaron la muerte de todos los gorgojos expuestos, la inhibición parcial de la progenie F1 y una drástica reducción de las pérdidas de grano. Además, los extractos etanólicos obtenidos de las hojas de *A. montana*, de *A. mucosa*, de *A. muricata* y de *Duguetia lanceolata*, especialmente de *A. montana* y de *A. mucosa*, demostraron efectos bioactivos significativos sobre las variables estudiadas. Sin embargo, los niveles de bioactividad fueron menores en comparación con los extractos de semillas, y la respuesta fue dependiente de la concentración utilizada. Este estudio es el primer relato sobre la actividad de los metabolitos secundarios de *D. lanceolata* sobre insectos, así como la acción de *A. sylvatica* sobre plagas asociadas a los granos almacenados..

Palabras clave: Aleloquímicos, acetogeninas, bioactividad, *Sitophilus zeamais*, cereales almacenados.

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INTRODUCTION

The large diversity of secondary metabolites in plants (allelochemicals) originates from a long evolutionary process that relies on the relationships between the plants and their competitors (natural enemies). Plants develop these compounds mainly as a defense mechanism (Wink, 2003). The study of allelochemicals has not only increased the knowledge of the processes involved in the interactions between plants and other factors in the environment but has also led to the discovery of important bioactive molecules of great interest for humankind (Ramesha *et al*., 2011). Among other functions, these bioactive molecules are used as model-prototypes for the development of new drugs (Miller, 2011) and new products for the protection of agricultural crops and stored commodities (Cantrell *et al*., 2012).

The structural and functional diversity of allelochemicals is a key factor for the survival and evolutionary success of plant species inhabiting an environment with an abundance of natural enemies. Therefore, the tropical flora, with its unique biodiversity, is a promising natural reservoir of bioactive substances (Valli *et al*., 2012). In this context, Brazil exhibits enormous potential for the development of novel active substances based on natural products because the country has the highest plant genetic diversity in the world, with more than 55,000 catalogued species (Simões & Schenkel, 2002). However, to date, this potential has not been well exploited.

Among the botanical families that occur in the Neotropical regions, Annonaceae is the main family of the order Magnoliales (APG III, 2009) and is one of the most specious families of angiosperms comprising 135 genera and approximately 2,500 species (Chatrou *et al*., 2004). Annonaceae exhibits a pantropical distribution with 40 genera and 900 species in the Neotropical region. In Brazil, this family is represented by 29 genera, of which 1 are endemic, and 386 species, and a large proportion of this richness is found in the Amazon Rain Forest and Atlantic Forest (Maas *et al*., 2013).

Despite the lack of studies, a large number of diverse chemical compounds present in the different structures of Annonaceae plants have been isolated. Alkaloids, acetogenins, diterpenes and flavonoids are the main chemical groups in extracts from the bark, branches, leaves, fruits and seeds of Annonaceae (Lebouef *et al*., 1982; Chang *et al*., 1998; Kotkar *et al*., 2001). Among these classes, acetogenins are conspicuous because of the vast array of biological activities they exhibit. Acetogenins are a series of natural products (C-35/C-37) derived from long-chain fatty acids (C-32/C-34) combined with a 2-propanol unit (Alali *et al*., 1999).

Our previous studies (Ribeiro, 2010; Ribeiro *et al*., 2013) demonstrated a promising grain-protectant effect in seed extracts from two species of *Annona*, which are characterized by a complex mixture of acetogenins and alkaloids. This observation motivated additional biomonitoring investigations in other Annonaceae species in order to explore more comprehensively the richness of allelochemicals in this plant family and the species diversity of the Brazilian flora. These studies prompted our research program to search for allelochemicals with activities against pest species of stored grains, which is an essential component of current stored grain integrated pest management programs (IPM).

This study evaluated the bioactivity of ethanolic extracts (66) of different structures from 29 Annonaceae species (7.5% of all Brazilian species) belonging to 11 different genera (*Anaxagorea*, *Annona*, *Duguetia*, *Ephedranthus*, *Guatteria*, *Hornschuchia*, *Oxandra*, *Porcelia*, *Pseudoxandra*, *Unonopsis*, and *Xylopia*) against *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), which is an important pest of stored cereals under tropical conditions. In addition, the fungicidal and antiaflatoxigenic activities of the promising extracts were evaluated *in vitro* against the isolate CCT7638 of *Aspergillus flavus* Link (Ascomycota: Eurotiales: Trichocomaceae), a producer of aflatoxin B_1, in order to better characterize the potential of these extracts as grain protectors.

MATERIAL AND METHODS

Species sampling and plant extract preparation

The collection data for the plant species used in the study, which were obtained from different locations in the south and southeast regions of Brazil, are shown in Table 1. In total, 29 species of Annonaceae belonging to 11 genera were collected and were investigated.

For the extract preparation, the plant structures were collected and were dehydrated in an oven at 40º C for 48 to 72 hours. Subsequently, the materials were separately milled in a knife mill to obtain a powder of each plant structure, which were stored separately in sealed glass containers until use. The organic extracts were obtained by maceration in ethanol solvent (1:5, w v^{-1}). For this step, the plant powder was maintained in the solvent for 3 days after which it was filtered through filter paper. The remaining residual cake was placed back in the ethanol solvent, and this process was repeated 4 times. The solvent remaining in the filtered solution was eliminated in a rotary evaporator at 50º C and -600 mm Hg pressure. After the solvent was evaporated in the airflow chamber, the extraction yield of each structure for all species was determined.

Bioassays

The bioassays were performed in a climate-controlled room at 25 ± 2º C, 60 ± 10% relative humidity, a photoperiod of 14 hours and a mean luminosity of 200 lux. Whole corn grains were used as the substrate for the assays. The corn grains were manually selected from the hybrid AG 1051 (yellow dent, semi-hard) from crops developed without insecticides. The experimental design used for the tests was completely randomized.

A microatomizer coupled to a pneumatic pump and adjusted to a pressure of 0.5 kgf cm^{-2} with a spray volume of 30 L t^{-1} was used for the application of the treatments. After spraying, the grains/extract mixture was manually placed in 2-liter plastic bags, which were lightly shaken for 1 minute.

Screening for the identification of promising extracts

To identify extracts with bioactivity against *S. zeamais*, bioassays were performed to verify the insecticidal activity and sublethal effects, which were assessed by evaluating the number of insects emerged (F1 progeny) and the damage to the treated samples. As a large number of extracts was obtained, they were divided into 5 groups in order to be tested. The groups were established according to the similarity of collection dates and plant structures available for each species. The extracts were assayed at concentration of 3,000 mg kg-1 (mg of extract kg-1 of corn), which were defined based on previous studies (Ribeiro, 2010).

Evaluation of insecticidal activity

For this bioassay, corn samples (10 g, in Petri dishes measuring 6-cm in diameter × 2-cm high) were treated separately with the respective extracts. The growth substrate treated with the solvent [acetone:methanol solution (1:1, v v-1)] was used as the control. Preliminary assays were performed to evaluate the possible effects of the solution used for the resuspension of the extracts on *S. zeamais*. Next, each Petri dish was infested with 20 adult *S. zeamais* (aged 10 to 20 days) from both sexes, and 10 replicates per treatment were performed. The adult survival was evaluated on day 10 after infestation. The insect was considered dead when its extremities were completely distended and it exhibited no reaction to contact with a paintbrush for 1 minute.

Evaluation of F1 progeny and damages

The same sampling units used for the insecticidal assay were used in this bioassay. The grains were treated with the respective extracts and were infested with 20 adults from both sexes (aged 10 to 20 days). After 10 days of infestation, the adults were removed, and the sampling units were kept under the climate conditions previously described. As before, 10 replicates per treatment were performed.

At 60 days after the initial infestation, the number of emerged adults in each dish was counted. The damages caused by the feeding of the *S. zeamais* were determined through the visual verification of the percentage of damaged or perforated grains in each sample. In addition, the grain weight loss (%) was estimated based on the equation proposed by Adams and Schulten (1976).

Table 1
Species and structures of Annonaceae used in the study and the respective collection data.

Species	Plant structures	Local of collection	Data of collection	Voucher (Herbarium)[1]
Anaxagorea dolichocarpa Sprague & Sandwith	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º08'04,0" S; 40º03'24,5" W; elevation: 33 m)	17/11/2011	Lopes 361 (CVRD, ESA, SPF)
Annona acutiflora Mart.	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º09'05,4" S; 40º04'02,4" W; elevation: 70 m)	16/11/2011	Lopes 144 (CVRD, ESA, SPF)
Annona cacans Warm.	Leaves, branches, and seeds	IAC/APTA, Jundiaí, SP, Brazil (23º06'56,6" S; 46º55'58,3" W; elevation: 599 m)	04/03/2011	Ribeiro 17 (ESA)
Annona dolabripetala Raddi	Leaves and branches	Botanical Garden of São Paulo, São Paulo, SP, Brazil (23º32'18,2" S; 46º36'44,5" W; elevation: 745 m)	07/06/2011	Ribeiro 18 (ESA)
Annona emarginata (Schltdl.) H.Rainer	Leaves and branches	IAC/APTA, Jundiaí, SP, Brazil (23º06'53,4" S; 46º56'07,2" W; elevation: 616 m)	04/03/2011	Ribeiro 16 (ESA)
Annona montana Macfad.	Leaves, branches, and seeds	ESALQ/USP Campus, Piracicaba, SP, Brazil (22º42'28,2" S; 47º37'59.4" W; elevation: 537 m)	21/03/2011	Ribeiro 3 (ESA)
Annona mucosa Jacq.	Leaves, branches, and seeds	ESALQ/USP Campus, Piracicaba, SP, Brazil (22º42'28,5" S; 47º37'59.6" W; elevation: 534 m)	17/03/2011	Ribeiro 4 (ESA)
Annona muricata L.	Leaves, branches, and seeds	ESALQ/USP Campus, Piracicaba, SP, Brazil (22º42'25,4" S; 47º37'43,9" W; elevation: 576 m)	12/04/2011	Ribeiro 12 (ESA)
Annona reticulata L.	Leaves and branches	ESALQ/USP Campus, Piracicaba, SP, Brazil (22º42'51,4" S; 47º37'38,8" W; elevation: 548 m)	01/03/2011	Ribeiro 11 (ESA)
Annona sp. 1	Leaves and branches	São Luís Farmer, Descalvado, SP, Brazil ($21^0$52'58,0" S; $47^0$40'38,0" W; elevation: 679 m)	02/04/2011	Ribeiro 13 (ESA)
Annona sp. 2	Leaves and branches	Frutas Raras Farmer, Rio Claro, SP, Brazil (23º06'53,4" S; 46º56'07,2" W; elevation: 716 m)	02/04/2011	Ribeiro 14 (ESA)
Annona sylvatica A.St.-Hil.	Leaves, branches, and seeds	Ribeiro Small Farmer, Erval Seco, RS, Brazil (27º25'41,8" S; 53º34'11,2" W; elevation: 466 m)	25/04/2011	Ribeiro 10 (ESA)
Duguetia lanceolata A.St. Hil.	Leaves, branches, and seeds	ESALQ/USP Campus, Piracicaba, SP, Brazil (22º42'41,5" S; 47º38'0,2" W; elevation: 556 m)	23/03/2011	Ribeiro 9 (ESA)
Ephedranthus dimerus J.C.Lopes, Chatrou & Mello-Silva (1)	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º09'05,5" S; 40º04'00,1" W; elevation: 67 m)	17/11/2011	Lopes 145 (CVRD, ESA, SPF, WAG)

Ephedranthus dimerus J.C.Lopes, Chatrou & Mello-Silva (2)	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º10'19,2" S; 40º01'08,7" W; elevation: 48 m)	17/11/2011	Lopes 362 (CVRD, ESA, SPF, WAG)
Guatteria australis A. St.-Hil.	Leaves and branches	Vale Natural Reserve,Linhares, ES, Brazil (19º08'28,5" S; 40º04'05,7" W; elevation: 18 m)	17/11/2011	Lopes 153 (CVRD, ESA, SPF)
Guatteria ferruginea A. St.-Hil.	Leaves and branches	Santa Lúcia Biological Station, Santa Tereza, ES, Brazil (19º58'02,5" S; 40º32'15,5" W; elevation: 694 m)	13/11/2011	Lopes 348 (ESA)
Guatteria sellowiana Schltdl.	Leaves and branches	Santa Lúcia Biological Station, Santa Tereza, ES, Brazil (19º58'02,5" S; 40º32'15,5" W; elevation: 694 m)	13/11/2011	Lopes 345 (ESA)
Guatteria villosissima A. St.-Hil.	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º09'25,6" S; 40º31'43,6" W; elevation: 636 m)	16/11/2011	Lopes 146 (ESA)
Hornschuchia bryotrophe Nees	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º07'56,4" S; 40º05'05,7" W; elevation: 58 m)	17/11/2011	Lopes 111 (ESA)
Hornschuchia citriodora D.M. Johnson	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º07'57,8" S; 40º05'05,9" W; elevation: 48 m)	17/11/2011	Lopes 110 (ESA)
Hornschuchia myrtillus Nees	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º11'13,3" S; 39º54'49,6" W; elevation: 44 m)	17/11/2011	Lopes 364 (CVRD)
Oxandra martiana (Schltdl.) R E. Fr.	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º11'30,8" S; 39º57'09,3" W; elevation: 31 m)	17/11/2011	Lopes 363 (CVRD, ESA, SPF)
Porcelia macrocarpa (Warm.) R.E. Fries	Leaves and branches	Botanical Garden of São Paulo, São Paulo, SP, Brazil (23º33'55,8" S; 46º36'08,3" W; elevation: 752 m)	07/06/2011	Eiten 791 (SP)
Pseudoxandra spiritus-sancti Maas	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º04'44,8" S; 39º53'19,5" W; elevation: 20 m)	16/11/2011	Lopes 317 (CVRD, SPF)
Unonopsis sanctae-teresae Maas & Westra	Leaves and branches	Goiapaba-açu Road, Santa Teresa, ES, Brazil (19º54'50,5" S; 40º31'59,1" W; elevation: 840 m)	14/11/2011	Lopes 355 (ESA)
Xylopia brasiliensis Spreng.	Leaves and branches	Augusto Ruschi Biological Reserve, Santa Tereza, ES, Brazil (19º54'27,1" S; 40º33'02,0" W; elevation: 805 m)	14/11/2011	Lopes 351 (ESA)
Xylopia decorticans D.M. Johnson & Lobão	Leaves and branches	Augusto Ruschi Biological Reserve, Santa Tereza, ES, Brazil (19º54'28,5" S; 40º32'57,3" W; elevation: 807 m)	14/11/2011	Lopes 352 (ESA)
Xylopia frutescens Aubl.	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil	16/11/2011	Lopes 359 (ESA)

		(19º09'23,4" S; 39º59'30,3" W; elevation: 30 m)		
Xylopia laevigata (Mart.) R.E. Fr.	Leaves and branches	Vale Natural Reserve, Linhares, ES, Brazil (19º05'01,2" S; 39º53'04,8" W; elevation: 22 m)	16/11/2011	Lopes 316 (ESA)

[1] CVRD (Vale Natural Reserve Herbarium, Linhares, ES, Brazil); ESA ("Luiz de Queiroz" College of Agriculture Herbarium, Piracicaba, SP, Brazil); SP (Botanical Institut of São Paulo Herbarium, São Paulo, Brazil); SPF (University of São Paulo Herbarium, São Paulo, SP, Brazil); WAG (Wageningen University Herbarium, Wageningen, Netherlands).

Concentration-response curves of active extracts

The extracts demonstrating the most promising results were bioassayed for the estimation of the LC_{50} and LC_{90}, corresponding to the concentration necessary to kill 50% and 90% of the population of weevils, respectively. For these estimations, preliminary tests were performed to determine the baseline concentrations that caused the death of 95% of the adults and a mortality rate similar to that in the control. Based on these results, the test concentrations (5-7 concentrations; range: 50 - 4,000 mg kg-1) were established using the formula proposed by Finney (1971). The remaining experimental procedures were the same as those used in the initial screening, in which the mortality was assessed 10 days after the infestation of the sample units.

Estimation of average lethal time (LT_{50}) of the promising extracts

For each selected extract, the time required to kill 50% of the weevil population (LT_{50}) was estimated based on the LC_{90} value determined in the previous bioassay. The same procedures described in the screening assay were used for this bioassay; however, the evaluation of weevil mortality was performed every 24 hours for 10 days.

Fungicidal and antiaflatoxigenic effects of the most promising extracts

The antifungal and antiaflatoxigenic activities (against isolate CCT7638 of *A. flavus*, a producer of AFB_1) of the most promising extracts were evaluated using a method termed *poison food* (Alvarez-Castellanos *et al*., 2001). This technique is based on the observation of the growth of fungal mycelium in YES (yeast extract saccharose) culture media using 1,000 mg L^{-1} of the respective extracts (final concentration) dissolved in 5 mL solvent solution [acetone: water, (1:3, v v^{-1})], incorporated by manual agitation in unfused culture media (temperature approximately 45º C). The extract + medium (10 mL) were added to each Petri dish (6.5-cm diameter), and 10 dishes were used per treatment. The solvent solution (acetone: water) was included as a control, and water was used as the negative control.

The fungus was inoculated following the solidification of the media as follows: the central area of the Petri dish was perforated using a Stanley knife previously immersed in a conidia solution. After incubation of the fungal colonies for 11 days in PDA (potato, dextrose and agar) media, the spore suspension was prepared by scraping the media surface using a Drigalski spatula followed by immersion in 50 mL of an aqueous solution (47.5 mL of distilled water + 2.5 mL of dimethyl sulfoxide). The amount of conidia in the solution was standardized to contain 2 to 9 × 10^5 conidia mL^{-1}, measured using a Neubauer chamber. The inoculated dishes were sealed with plastic film and were incubated upside down at 25 ± 2º C and a scotophase of 24 hours.

The radial mycelial growth was evaluated at 48, 96, 144 and 192 hours after the fungal inoculation. The evaluation consisted of measuring the diameter of each colony in 2 directions at a straight angle using a caliper. Based on the arithmetic mean of the 2 measurements, the percentage inhibition (P.I.) of the treatments relative to the control was calculated using the following equation:

$$P.I. = [(Control - Treatment)/Control]100$$

The production of aflatoxin (B_1) by isolate CCT7638 of *A. flavus* grown in culture media containing the respective treatments was assessed

using the thin-layer chromatography (TLC) technique. The culture media from 5 Petri dishes randomly chosen from each treatment was transferred to a 50-mL Falcon tube using Stanley knives and spatulas. The weight of the transferred material was recorded, and the material was subjected to an aflatoxin extraction process.

For the extraction of aflatoxins from the media, 14 mL of distilled water and 18 mL of analysis-grade methanol were added to the tube. The content was vortexed for 1 minute. A 5-mL aliquot of the extract was transferred to an amber vial, and the solution volume was evaporated entirely using a sample concentrator with airflow at 45º C. The dried material was redissolved in 200 µL of toluene: acetonitrile (9:1) and agitated for 30 seconds using ultrasound. The presence and quantification of aflatoxins in the extract were performed in aluminum chromatoplates with silica gel. The development of the chromatography plates was performed in vats containing 5 mL of the elution solution composed of ether:methanol:water (96:3:1). The presence of aflatoxins in the samples was verified by comparison to the AFB_1 standard. The standard AFB_1 solution was prepared based on the Sigma-Aldrich standard (Sigma AF-1), and the concentration was determined according to methodology 971.22 found in the American Official Analytical Chemistry (2006). For this assay, the concentration of aflatoxins in the samples was compared with 3-, 4- and 6-µL aliquots of the AFB_1 standard. The calculation of contamination was performed according to the following equation:

$$AF = \left[(YSV)/(XW)\right]$$

Where: AF= aflatoxin content (AFB_1); Y = standard concentration in µg mL^{-1}; S = µL of the standard toxin with fluorescence equivalent to the sample; V = extract final volume (sample) in µL; X = extract initial volume (sample) in µL; W = sample weight, in grams, in the final extract.

Data analysis

Generalized linear models (GLM) (Nelder & Wedderburn, 1972) with quasi-binomial distributions were used for the analysis of the proportions of mortality and damaged grains, whereas GLM with quasi-Poisson distributions was used for the analysis of emerged insect numbers. GLM with Gaussian distribution was used for the analysis of *A. flavus* vegetative growth and aflatoxin production. In all cases, the goodness-of-fit was determined using a half-normal probability plot with a simulated envelope (Hinde & Demétrio, 1998). When a significant difference was observed between the treatments, multiples comparisons (Tukey's test, $P < 0.05$) were performed using the glht function of the multicomp package with adjustment of *P* values.

Multivariate analyses were performed to determine the grouping of the crude extracts of Annonaceae based on the variables analyzed in the screening assay. The mean Euclidian distance was used as a measurement of similarity, and the UPGMA (*unweighted pair-group average*) method was used as a clustering strategy. The relationship between the variables analyzed was determined using Spearman's nonparametric analysis ($P = 0.05$). The analyses were performed using the software "R", version 2.15.1 (R Development Core Team, 2012).

A binomial model with a complementary log-log link function (gompit model) was used to estimate the lethal concentrations (LC_{50} and LC_{90}), using the *Probit Procedure* in the software SAS version 9.2 (SAS Institute, 2011). Finally, the mean lethal time (LT_{50}) was estimated using the method proposed by Throne *et al.* (1995) for Probit analysis of correlated data.

RESULTS

Selection of the promising crude extracts

Of the 66 tested extracts at 3,000 mg kg^{-1}, the most pronounced bioactive effects on *S. zeamais* were caused by the ethanolic extracts from the *A. montana*, *A. mucosa*, *A. muricata* and *A. sylvatica* seeds (Table 2). These extracts produced complete mortality of the exposed weevils, almost total inhibition of the F_1 progeny and a drastic reduction in damage to the treated samples. The ethanolic extracts from the *A. montana*, *A. mucosa*, *A. muricata* and *D. lanceolata* leaves, especially the *A. montana* and *A. mucosa* leaves, exhibited significant bioactive effects; however, compared with the seed extracts, the effects were at lower levels and exhibited a concentration-dependent response.

Table 2

Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* (Coleoptera: Curculionidae) in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (3,000 mg kg^{-1})*.

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative (%)[4]
Group A[2]						
Annona cacans	Leaves	0.50±0.50 c	51.00±5.12 a	86.98±6.99 a	10.87±0.87	96.71
	Branches	0.00±0.00	54.20±2.64 a	89.09±1.54 a	11.13±0.19	99.02
	Seeds	14.50±2.03 c	41.50±4.11 ab	76.20±3.01 a	9.52±0.37	84.70
Annona montana	Leaves	77.50±5.73 a	8.40±3.35 cd	17.71±6.90 c	2.21±0.86	19.66
	Branches	1.50±0.76 c	46.90±2.24 a	82.01±2.57 a	10.25±0.32	91.19
	Seeds	100.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Annona mucosa	Leaves	83.00±5.33 a	6.10±1.79 d	13.54±3.84 c	1.69±0.48	15.04
	Branches	0.00±0.00	59.90±3.92 a	92.22±2.68 a	11.52±0.33	102.49
	Seeds	100.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Annona muricata	Leaves	34.00±6.27 b	25.90±4.61 bc	48.31±7.50 b	6.03±0.93	53.65
	Branches	1.50±0.76 c	47.40±2.74 a	86.38±2.81 a	10.79±0.35	96.00
	Seeds	100.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Annona sylvatica	Leaves	19.50±4.18 b	41.40±4.32 ab	74.67±4.49 ab	9.36±0.56	83.27
	Branches	0.00±0.00	46.70±2.84 a	83.39±3.30 a	10.42±0.41	92.70
	Seeds	100.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00
Duguetia lanceolata	Leaves	37.50±4.60 b	17.00±2.74 c	42.35±6.13 b	5.29±0.76	47.06
	Branches	1.00±0.66 c	51.04±2.71 a	87.58±2.21 a	10.94±0.27	97.33
	Seeds	0.50±0.50 c	51.00±2.40 a	89.23±1.93 a	11.15±0.24	99.20
Control (acetone:methanol, 1:1 (v/v))	--	0.00±0.00	51.40±3.72 a	89.97±3.10 a	11.24±0.38	--
F		57.24	24.24	25.94		
P value		< 0.0001	< 0.0001	< 0.0001		
Group B[2]						
Annona dolabripetala	Leaves	4.50±1.74 ab	28.50±2.70 c	61.90±3.74 d	7.73±0.46	70.98
	Branches	0.00±0.00	57.80±2.07 ab	88.44±2.41 abc	11.05±0.30	101.47
Annona emarginata	Leaves	0.00±0.00	46.90±2.79 b	84.49±3.65 abc	10.56±0.45	96.97
	Branches	0.00±0.00	55.1±1.64 ab	95.10±0.48 a	11.88±0.06	109.09
Annona reticulata	Leaves	5.00±2.23 ab	43.10±3.98 b	82.08±4.19 abc	10.26±0.52	94.21
	Branches	3.00±1.10 ab	44.50±4.43 b	79.71±6.60 c	9.96±0.82	91.46
Annona sp.1	Leaves	0.50±0.50 b	47.30±3.26 b	81.03±3.23 bc	10.12±0.40	92.93
	Branches	0.00±0.00	68.40±3.47 a	94.35±1.10 abc	11.79±0.13	108.26
Annona sp. 2	Leaves	16.50±4.15 a	27.90±3.32 c	58.57±4.56 d	7.32±0.57	67.22
	Branches	0.00±0.00	54.80±2.41 ab	88.75±1.63 abc	11.09±0.20	101.84
Porcelia macrocarpa	Leaves	0.00±0.00	68.30±1.66 a	94.72±1.31 ab	11.84±0.16	108.72
	Branches	0.50±0.50 b	53.20±3.00 ab	91.78±1.80 abc	11.47±0.22	105.33
Control (acetone:methanol, 1:1 (v/v))	--	2.00±1.52 ab	51.00±2.48 b	87.17±2.64 abc	10.89±0.33	--
F		7.23	16.29	12.38		
P value		<0.0001	<0.0001	<0.0001		

Group C[2]						
Annona acutiflora	Leaves	7.00±2.26	27.70±2.57	65.84±3.45	8.23±0.43	94.93
	Branches	4.50±1.38	37.50±3.27	81.37±3.97	10.17±0.49	117.30
Guatteria australis	Leaves	6.00±2.76	32.30±3.50	74.80±3.58	9.35±0.44	107.84
	Branches	6.50±1.50	32.90±3.90	72.72±4.75	9.09±0.59	104.84
Guatteria ferruginea	Leaves	9.00±2.66	25.00±3.09	62.16±3.28	7.77±0.41	89.62
	Branches	8.50±2.24	30.00±1.61	74.67±3.30	9.33±0.41	107.61
Guatteria sellowiana	Leaves	7.50±3.00	33.90±4.37	73.82±6.04	9.22±0.75	106.34
	Branches	2.00±0.81	35.70±2.64	78.21±2.73	9.77±0.34	112.69
Guatteria villosissima	Leaves	3.50±1.50	30.50±3.00	69.52±5.72	8.69±0.71	100.23
	Branches	2.00±1.10	38.20±2.45	77.57±2.81	9.69±0.35	111.76
Unonopsis sanctae-teresae	Leaves	4.00±1.45	32.70±2.13	74.51±3.49	9.31±0.43	107.38
	Branches	3.00±1.10	28.60±2.85	67.41±5.18	8.42±0.64	97.12
Control (acetone:methanol, 1:1 (v/v))	--	3.00±1.33	29.00±2.60	69.37±5.17	8.67±0.64	100.00
F		1.81 ns	1.66 ns	1.65 ns		
P value		0.0540	0.0834	0.0868		
Group D[2]						
Oxandra martiana	Leaves	0.00±0.00	46.80±2.96 ab	87.98±3.45	10.99±0.43	101.85
	Branches	2.50±0.83	45.20±4.18 ab	85.78±3.58	10.72±0.44	99.35
Pseudoxandra spiritus-sancti	Leaves	3.00±1.52	38.00±1.69 b	83.21±2.14	10.40±0.26	96.39
	Branches	4.00±2.33	41.90±3.91ab	80.60±5.03	10.07±0.62	93.33
Xylopia brasiliensis	Leaves	0.00±0.00	48.70±2.72 ab	90.20±2.94	11.27±0.36	104.45
	Branches	1.00±0.66	49.80±2.48 ab	90.01±1.74	11.25±0.21	104.26
Xylopia decorticans	Leaves	0.50±0.50	44.30±2.81 ab	86.21±2.79	10.77±0.34	99.81
	Branches	0.50±0.50	41.80±2.90 ab	86.16±2.79	10.77±0.34	99.81
Xylopia frutescens	Leaves	0.00±0.00	44.20±4.76 ab	83.65±4.44	10.45±0.55	96.85
	Branches	0.50±0.50	56.40±4.83 a	89.72±3.66	11.21±0.45	103.89
Xylopia laevigata	Leaves	1.00±0.66	38.50±1.63 b	81.53±2.50	10.19±0.31	94.44
	Branches	0.50±0.50	45.60±2.71 ab	88.24±2.48	11.03±0.31	102.22
Control (acetone:methanol, 1:1 (v/v))	--	0.50±0.50	41.50±2.73 ab	86.35±2.85	10.79±0.35	100.00
F		1.78 ns	2.31	0.94 ns		
P value		0.8097	0.0110	0.5079		
Group E[2]						
Anaxagorea dolichocarpa	Leaves	9.50±3.11 a	35.60±3.46 ab	75.23±3.50 ab	9.40±0.43	97.11
	Branches	4.50±1.74 a	47.60±4.59 a	85.66±3.35 ab	10.70±0.41	110.54
Ephedranthus dimerus 1	Leaves	1.50±1.06 a	39.50±4.48 ab	77.55±6.33 ab	9.69±0.79	100.10
	Branches	0.50±0.50 a	34.40±3.21 ab	79.98±4.41 ab	9.99±0.55	103.20
Ephedranthus dimerus 2	Leaves	8.50±2.98 a	32.40±4.90 ab	71.11±8.13 ab	8.88±1.01	91.74
	Branches	1.00±0.66 a	45.50±2.68 a	89.06±2.52 a	11.13±0.31	114.98
Hornschuchia bryotrophe	Leaves	7.50±2.81 a	23.80±2.36 b	60.89±5.75 b	7.61±0.71	78.62
	Branches	3.00±0.81 a	38.80±4.92 ab	77.94±5.48 ab	9.74±0.68	100.62
Hornschuchia citriodora	Leaves	8.00±3.81 a	44.00±4.48 a	85.62±4.46 ab	10.70±0.55	110.54
	Branches	1.00±0.66 a	34.60±4.07 ab	74.66±4.40 ab	9.33±0.55	96.38
Hornschuchia myrtillus	Leaves	12.00±5.22 a	27.88±4.96 ab	62.60±6.92 b	7.82±0.66	80.79

	Branches	2.00±1.10 a	48.80±2.96 a	87.45±2.07 a	10.93±.025	112.91
Control (acetone:methanol, 1:1 (v/v))	--	1.00±0.66 a	37.40±5.89 ab	77.48±6.17 ab	9.68±0.77	100.00
F		3.72	3.09	2.84		
P value		0.0115	0.0108	0.0190		

[1]Means followed by different letters in the columns containing each tested group extracts indicate significant differences between treatments (GLM with quasi-binomial distribution followed by Tukey's *post hoc* test, $P<0.05$); [2]Means followed by different letters in the columns containing each tested group extracts indicate significant differences between treatments (GLM with quasi-Poisson distribution followed by Tukey's *post hoc* test, $P<0.05$); [3]Calculated using the formula proposed by Adams and Schulten (1976); [4]Calculated based on the relative comparison of the treatment (extract) with its respective control; *Applied using a spray volume of 30 L t^{-1}; [ns]: Not significant ($P>0.05$)

The hierarchical grouping analysis using the data from the variables analyzed in the screening bioassays indicated the formation of 3 groups (Figure 1). The first group comprised the ethanolic extracts from the *A. montana*, *A. mucosa*, *A. muricata* and *A. sylvatica* seeds, as well as extracts from the *A. montana* and *A. mucosa* leaves, which demonstrated the most pronounced lethal and sublethal effects. The second group comprised the ethanolic extracts from *D. lanceolata* and *A. muricata* leaves, species that demonstrated less pronounced bioactive effects (lethal and sublethal). The third group encompassed the controls and extracts that did not demonstrate bioactivity against the targeted pest.

Independently of the concentration tested, the adult mortality was inversely correlated with the other tested variables [F_1 progeny ($r = -0.59$; $P < 0.0001$) and % damaged grains($r = -0.58$; $P < 0.0001$)]. Although mortality was the variable with the highest weight in the separation between treatments, based on the Spearman's correlation coefficients, one cannot discard the small oviposition- and/or feeding-deterrent action of the extracts from the respective Annonaceae species.

Estimation of lethal concentrations and average lethal time of the selected extracts

The extract prepared from the *A. mucosa* seeds demonstrated the lowest LC_{50} and LC_{90} values (288.33 and 505.47 mg kg^{-1}, respectively) (Table 3). These values were significantly different from the values for the other active extracts based on the comparison of the estimated confidence intervals ($P < 0.05$). In general, the extracts from the seeds of different bioactive *Annona* species demonstrated the lowest values compared with the active extracts from leaves. On the other hand, the average lethal time (varying between 82.06 and 94.85 hours) did not demonstrate great differences between the treatments (Table 4), showing a slower activity of the active extracts, which is probably related with the mechanisms of action of the active compounds.

Fungicidal and antiaflatoxigenic activity of the most promising extracts

Overall, the ethanolic extracts tested (1,000 mg kg^{-1}), which were selected based their activity on *S. zeamais*, did not significantly inhibit the vegetative growth of the isolate CCT7638 of *A. flavus* and did not affect the production of AFB_1 after 192 hours of incubation (Table 5). However, the extract from the *A. sylvatica* seeds reduced the initial growth rate (fungistatic effect) of the radial mycelial growth after 48 hours of incubation. Based on the results, the toxicity of the solvent used for extract solubilization (acetone) in *A. flavus* was verified. Although the concentration was low (25%), caution is recommended when using this organic solvent in these types of bioassays.

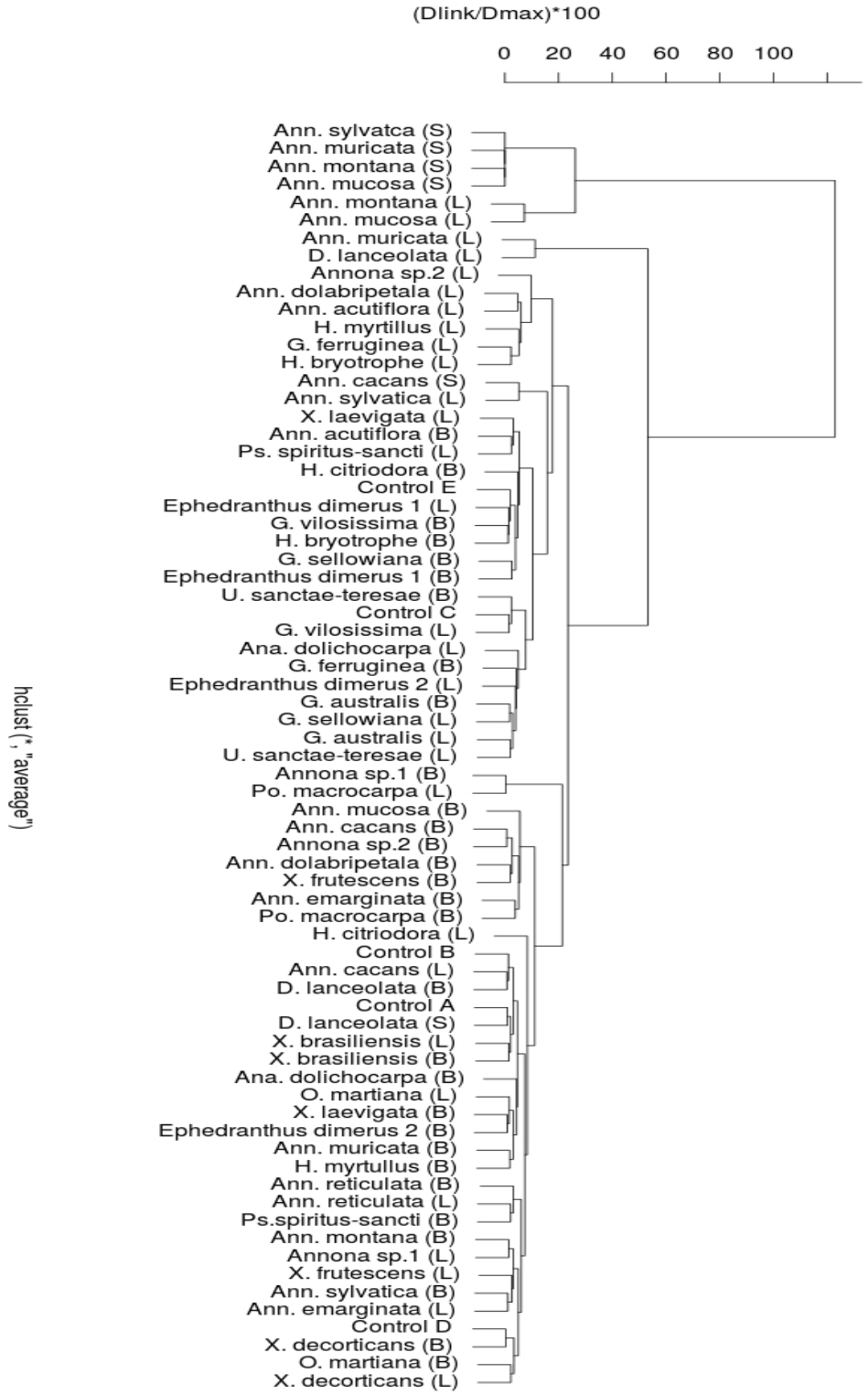

Figure 1
Dendrogram obtained from Cluster analysis based on bioactivity similarity of ethanolic extracts from Annonaceae on *Sitophilus zeamais* (Coleoptera: Curculionidae) [mean Euclidian distance as dissimilarity measurement and UPGMA (*Unweighted Pair Group* Method) as a clustering strategy method] at 3,000 mg kg^{-1}.

Table 3
Estimation of LC_{50} and LC_{90} (in mg kg^{-1}*) and confidence interval of ethanolic extracts from Annonaceae for *Sitophilus zeamais* (Coleoptera: Curculionidae) adults after 10 days of exposure in treated maize samples (10 g).

Species (structures)	n [1]	*Slope* ± SE (valor de *P*)	LC_{50} (CI) [2]	LC_{90} (CI) [2]	χ^{2} [(3)]	d.f. [4]	h.[5]
Annona montana (leaves)	1,400	4.86±0.29 (*P*<0.0001)	1,851.00 (1,758.00 –1,942.00)	3,270.00 (3,075.00 –3,516.00)	3.75	4	0.94
Annona montana (seeds)	1,200	7.09±0.42 (*P*<0.0001)	621.70 (557.11–677.44)	942.45 (858.96–1,071.91)	5.55	3	1.85
Annona mucosa (leaves)	1,400	5.74±0.46 (*P*<0.0001)	1,972.00 (1,847.00–2,080.00)	3,190.00 (3,026.00–3,405.00)	1.56	4	0.82
Annona mucosa (seeds)	1,600	4.92±0.33 (*P* <0.0001)	288.33 (267.29–307.21)	505.47 (478.58–537.75)	4.71	5	0.94
Annona muricata (seeds)	1,600	6.35±0.37 (*P* <0.0001)	384.94 (364.47–403.75)	594.76 (569.35–624.19)	4.88	5	0.98
Annona muricata (leaves)	--	--	>3,000	--	--	--	--
Annona sylvatica (seeds)	1,200	6.32±0.75 (*P* <0.0001)	554.48 (471.11–617.90)	858.58 (799.49–918.24)	3.07	3	0.37
Duguetia lanceolata (leaves)	--	--	>3,000	--	--	--	--

[1] n: number of tested insects; [2] CI: 95% confidence interval; [3] χ^2: calculated chi-squared value; [4] d.f.: degrees of freedom; [5] h.: heterogeneity factor; * Applied using a spray volume of 30 L t^{-1}; [--] Not determined.

Table 4
Estimation of average lethal time (LT_{50}, in hours) and confidence interval of ethanolic extracts from Annonaceae for *Sitophilus zeamais* (Coleoptera: Curculionidae) adults.

Species (structures)	n [1]	*Slope* ± SE	LT_{50} (CI) [2]	χ^{2} [(3)]	d.f. [4]	h.[5]
Annona montana (leaves)	400	8.30±0.41	88.76 (83.39–94.34)	18.19	7	2.60
Annona montana (seeds)	400	7.08±0.30	86.67 (82.12–91.08)	15.39	8	1.98
Annona mucosa (leaves)	400	5.98±0.24	94.85 (88.89–100.68)	13.67	7	1.95
Annona mucosa (seeds)	400	6.31±0.18	86.13 (84.04–88.17)	7.60	8	0.95
Annona muricata (seeds)	400	5.61±0.22	90.42 (84.32–96.23)	19.14	8	2.39
Annona sylvatica (seeds)	400	7.18±0.23	82.06 (78.81–85.21)	16.69	8	2.09

[1] n: number of tested insects; [2] CI: 95% confidence interval; [3] χ^2: calculated chi-squared value; [4] d.f.: degrees of freedom; [5] h.: heterogeneity factor.

Table 5
Radial growth (mean ± standard error) of isolate CCT7638 of *Aspergillus flavus* colonies and production of aflatoxin (AFB_1) in YES (yeast extract saccharose) culture media containing ethanolic seed extracts from different Annonaceae (1,000 mg L^{-1}).

Extracts / Incubation time	Diameter of colonies (mm) [1]								Production of
	48 hours	P.I. (%)[2]	96 hours	P.I. (%)[2]	144 hours	P.I. (%)[2]	192 hours	P.I. (%)[2]	AFB_1 (ppm mm^{-2}) [1]
Annona montana	10.30±0.88 b	+27.16	37.90±2.02 b	+22.65	50.80±2.09 ab	+ 25.43	54.77±1.24 ab	+ 10.65	42.99±5.21
Annona mucosa	9.30±1.22 bc	+14.81	33.30±3.26 b	+ 7.77	44.20±3.56 bc	+ 9.16	53.40±1.97 ab	+ 7.88	41.91±7.32
Annona muricata	7.30±0.20 c	- 9.87	29.00±0.83 bc	- 6.15	43.00±2.05 bc	+ 6.17	53.80±1.07 ab	+ 8.69	42.23±3.94
Annona sylvatica	4.80±0.23 d	- 40.74	25.40±0.91 c	- 17.80	38.50±1.98 c	- 4.94	52.00±1.71 ab	+ 5.05	40.82±6.77
Control (acetone:water, 1:3 (v/v))	8.10±0.83 bc	--	30.90±2.71 bc	--	40.50±2.85 c	--	49.50±2.66 b	--	38.85±5.11
Negative control (water)	15.60±0.25 a	--	46.30±1.29 a	--	54.10±1.34 a	--	55.95±0.05 a	--	43.92±4.49
F	37.662		18.21		8.0269		2.4677		0.6521^{ns}
P value	<0.0001		<0.0001		<0.0001		0.04378		0.6439

[1] Means followed by different letters, in the columns, indicate significant differences between treatments (GLM with Gaussian distribution followed by Tukey's *post hoc* test, *P*<0.05)
[2] P.I.: Percentage of inhibition;
[ns]: Not significant (*P*>0.05).

DISCUSSION

Our study provides important information regarding promising sources of compounds to be used as grain protectors in the preventative management of Coleoptera pest species in stored cereals. Annonaceae is one of the most diverse and abundant plant family in Neotropical forests (Chatrou *et al*., 2004; Maas *et al*., 2011a; Maas *et al*., 2011b) and has been shown to exhibit secondary metabolites with great chemical diversity and promising biological activities (Lebouef *et al*., 1982; Zafra-Polo *et al*., 1998; Colom *et al*., 2010); however, this study represents the most comprehensive screening study performed to date.

Despite the variations in soil-climate conditions in the sampling sites, which could have influenced the chemical profiles of the extracts, and the differences in the sampling effort for the different plant structures, genus *Annona* seeds were identified as the main sites of accumulation of compounds with activity against insects. Therefore, our results are consistent with other reports in the literature (Leatemia & Isman, 2004; Llanos *et al*., 2008; Seffrin *et al*., 2010; Grzybowski *et al*., 2013; Ribeiro *et al*., 2013).

Concerning the action on insects, this study is the first to report the activity of compounds derived from *A. sylvatica* (formerly *Rollinia sylvatica*) on pests associated with stored grains. To date, a small number of secondary compounds from *A. sylvatica*, native to the center-south region of Brazil (Lorenzi *et al*., 2005), were isolated and evaluated for their bioactive potential. Consistent with our results, Mikolajczak *et al.* (1990) demonstrated the oral toxicity of a hexanic extract from *A. sylvatica* fruits against *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) larvae. After successive fractionation, the compound sylvaticin (the only acetogenin reported from this species to date) was isolated and shown to exhibit a series of biological activities, including the protection of cantaloupe plants against *Acalymma*

vittata Barber (Coleoptera: Chrysomelidae). Recently, Formagio *et al.* (2013) demonstrated the anti-inflammatory and anti-carcinogenic properties of essential oils of *A. sylvatica* leaves, which are mostly composed of a combination of oxygenated sesquiterpenes.

A number of reports in the literature ratify the putative toxicological effects (acute or chronic) of derivative compounds from the remaining species of *Annona* for medically and agriculturally relevant pests. Therefore, compounds from *A. muricata* demonstrate activities against *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) (Trindade *et al*., 2011), *Bactericera cockerelli* (Sulc) (Hemiptera: Triozidae) (Flores-Davila *et al*., 2011), *Anastrepha ludens* (Loew) (Diptera: Tephritidae) (Gonzalez-Esquinca *et al*., 2012) and *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) (Grzybowski *et al*., 2013). Acetogenins isolated from *A. montana* seeds demonstrate toxicity for *Oncopeltus fasciatus* (Dallas) (Hemiptera: Lygaeidae) (Colom *et al*., 2008) and insecticidal and anti-feeding activities for *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) larvae (Blessing *et al*., 2010). Furthermore, the extract obtained through the decoction of *A. mucosa* seeds has a repellent effect on *Acromyrmex octospinosus* (Forel) (Hymenoptera: Formicidae) workers (Boulogne *et al*., 2012).

Using *S. zeamais* as a model, Llanos et al. (2008) demonstrated the efficient control of adults and the complete inhibition of the F_1 progeny using extracts (at concentrations higher than 2,500 ppm) from *A. muricata* seeds using hexane and ethyl acetate as solvents. In previous studies, we demonstrated that *A. montana* (Ribeiro, 2010) and *A. mucosa* (Ribeiro *et al*., 2013) seed extracts in both hexane and dichloromethane solvents caused promising bioactive effects on *S. zeamais*. In contrast to the solvents used in this study, the extractions in the previous study were performed using organic solvents at gradients of increasing polarity in all cases. Given the changes in the chemical profiles of the derivatives obtained using different techniques and/or solvents, this difference can partially explain the differences in bioactive concentrations observed between the studies. Meanwhile, this study demonstrates the possibility of extracting active principles of these species using a "green solvent" that is naturally biodegradable and produced from renewable sources.

A large number of compounds of diverse chemical natures in several structures of the genus *Annona* have been isolated in a number of phytochemical studies (Lebouef *et al*., 1982; Chang *et al*., 1998; Kotkar *et al*. 2001). Among the compounds, acetogenins stand out because of their structural abundance and the wide array of biological activities they exhibit, such as powerful insecticidal and acaricidal activities (Alali *et al*., 1999; Colom *et al*., 2008, 2010). Acetogenins are potent inhibitors of complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial electron-transport system and of the enzyme NADH:oxidase in the cell membrane of target arthropods (Lewis *et al*., 1993). According to Bermejo et al. (2005), the majority of acetogenins in Annonaceae have been isolated from the seeds and stems of the genera *Annona, Anomianthus, Asimina, Desepalum, Goniothalamus, Rollinia* [now *Annona* (Rainer, 2007)], *Polyalthia, Porcelia, Uvaria* and *Xylopia*. However, the relative content of the derivatives originating from the different genera and the structure-activity relationship of the acetogenins from different structures remain to be investigated.

Based on the estimated lethal time (in the LC_{90} estimated for each selected extract), the slower action of the active compounds was confirmed. The symptomatology of the contaminated insects was characterized by the inactivity, locomotive instability and food avoidance, followed by the collapse, paralysis, and slow death by respiratory insufficiency. These signs are typical of the action of compounds that inhibit mitochondrial respiration, such as rotenone and piericidin (Ware and Whitacre, 2004.). Therefore, based on these findings and previous analysis (Ribeiro *et al*., 2013), it is possible to hypothesize that the biological activity of the extracts from *A. sylvatica* seeds and *A. montana*, *A. mucosa* and *A. muricata* seeds and leaves are because of the presence of acetogenins.

Despite the effects were less expressive compared with those observed for the extracts of the genus *Annona*, this study is the first to report the activity of *D. lanceolata* derivatives on pest insects, a plant species that has not been studied from a phytochemical point of view. However, a number of pharmacological properties, such as antiprotozoal (Tempone *et al*., 2005), antinociceptive and anti-

inflammatory activities (Sousa *et al*., 2008), of crude ethanolic extracts and/or the isoquinoline alkaloid-rich fraction from *D. lanceolata* leaves have been reported in the literature and corroborate the potential observed in this study.

To better elucidate the potential protectant effect on grains, the antifungal and antiaflatoxigenic activities of the most promising extracts (regarding the action on *S. zeamais*) were evaluated using the *A. flavus* isolate. Although the fungal toxicity of the crude extracts and acetogenins isolated from the genus *Annona* species were reported previously for a number of plant species (Dang et al., 2011) and human pathogens (Ahmad and Sultana, 2003; Lima *et al*., 2011), the extracts evaluated did not exert pronounced effects on *A. flavus.* Hypothetically, the lack of fungicidal effect could be because of evolutionary selection in *A. flavus* species adapted to coexist with these secondary metabolites. Corroborating this hypothesis, Okwulehie and Alfred (2010) demonstrated that *A. flavus* is a species that deteriorates *A. muricata* fruits in Nigeria. However, because the insects are the main agent of dispersion of fungal spores in the grain mass, the adequate control of pest insect species provided by the respective extracts can decrease the incidence of *A. flavus* and consequently the aflatoxin levels in the stored grains.

Despite the preliminary nature of the data in this study, it can be concluded that ethanolic extracts from *A. sylvatica* seeds, *D. lanceolata* leaves and *A. montana, A. mucosa* and *A. muricata* seeds and leaves exert bioactive effects on *S. zeamais*. Accordingly, bio-guided studies are being conducted in order to purify, isolate and characterize the compounds responsible for the observed bioactivity. Additionally, it will be possible to evaluate the potential use of these compounds as model-molecules or biorational compounds in integrated management programs of the stored pests. However, this study provides a scientific basis for the rational utilization of Annonaceae species with potential use to humans, mainly as a homemade tool for stored grain protection.

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