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Exploring genetic biodiversity: secondary metabolites from Neotropical Annonaceae as a potential source of new pesticides

Leandro do Prado Ribeiro

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

Piracicaba
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“I offer this thesis to my family due to their support, understanding, and affection in every step of this challenge.”

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[...]
Nunca deixe que lhe digam
Que não vale a pena acreditar no sonho que se tem
Ou que seus planos nunca vão dar certo
Ou que você nunca vai ser alguém
Tem gente que machuca os outros
Tem gente que não sabe amar
Mas eu sei que um dia a gente aprende
Se você quiser alguém em quem confiar
Confie em si mesmo
QUEM ACREDITA SEMPRE ALCANÇA!

"Mais uma vez – Renato Russo"
(In Portuguese)

SUMMARY

RESUMO

Explorando a biodiversidade genética: metabólitos secundários de anonáceas neotropicais como uma fonte potencial de novos pesticidas

Visando investigar potenciais fontes de novos protetores de grãos, este estudo avaliou, primeiramente, a bioatividade de extratos etanólicos (66) obtidos de 29 espécies pertencentes a 11 diferentes gêneros de anonáceas neotropicais sobre o gorgulho-do-milho *Sitophilus zeamais* (Coleoptera: Curculionidae). A triagem inicial demonstrou que os efeitos (agudos e crônicos) mais pronunciados sobre *S. zeamais* foram causados pelos extratos de sementes de *Annona montana*, de *A. mucosa*, de *A. muricata* e de *A. sylvatica*, seguidos pelos extratos de folhas de *A. montana*, de *A. mucosa*, de *A. muricata* e de *Duguetia lanceolata*. No entanto, os extratos mais ativos (sementes) não afetaram o crescimento vegetativo e a produção de aflatoxinas de um isolado de *Aspergillus flavus* (Ascomycota: Trichocomaceae). Fracionamentos biomonitorados foram então realizados a fim de isolar, purificar e caracterizar o(s) composto(s) ativo(s) majoritário(s) dos extratos mais promissores, utilizando-se, para isso, o gorgulho-do-milho como bioindicador. Por meio de diferentes procedimentos cromatográficos, foram isolados nove compostos: cinco acetogeninas, três esteroides e um composto aromático. As acetogeninas roliniastatina-1 e ACG4 (determinação estrutural em andamento), o composto aromático 2,4,5-trimetoxiestireno e os esteroides campesterol, estigmasterol e sitosterol [testados em mistura (8,44 + 12,37 + 79,19%, respectivamente)] mostraram promissoras propriedades protetoras de grãos. Em geral, os resultados obtidos indicaram que compostos de diferentes naturezas químicas têm efeito sinérgico sobre a atividade biológica dos extratos brutos. No segundo estudo, foi avaliada a toxicidade aguda e crônica dos extratos selecionados de sementes de *Annona* (*A. montana*, *A. mucosa*, *A. muricata* e *A. sylvatica*) e de um bioinseticida comercial à base de acetogeninas (Anosom® 1EC) sobre a lagarta-mede-palmo *Trichoplusia ni* (Lepidoptera: Noctuidae) e sobre o pulgão-verde *Myzus persicae* (Hemiptera: Aphididae). Em laboratório, os extratos de *A. mucosa* e de *A. sylvatica* e o bioinseticida Anosom® foram especialmente ativos através da administração oral e tópica. Em casa de vegetação, um extrato formulado de *A. mucosa* e Anosom® foram altamente eficazes contra larvas de terceiro ínstar de *T. ni*, com eficácia comparável ao de um inseticida comercial à base de piretrinas (Insect Spray®) utilizado como controle positivo. Similar aos resultados com *T. ni*, o extrato de *A. mucosa* apresentou a maior atividade aficida, tanto em bioensaios em laboratório quanto em casa de vegetação. No terceiro estudo, a atividade acaricida [sobre o ácaro-purpúreo-dos-citros *Panonychus citri* (Acari: Tetranychidae)] do extrato de sementes de *A. mucosa* (mais ativo) foi avaliada em bioensaios laboratoriais. O extrato de *A. mucosa* apresentou eficácia superior aos acaricidas/inseticidas comerciais de origem natural [Anosom® 1EC (anonina), Derisom® 2EC (karanjina) e Azamax® 1.2EC (azadiractina + 3-tigloilazadiractol)] e similar a um acaricida sintético [Envidor® 24SC (espirodiclofeno)]. Finalmente, foi avaliada a compatibilidade do extrato de sementes de *A. mucosa* com três espécies de fungos entomopatogênicos (*Beauveria bassiana*, *Isaria fumosorosea* e *Metarhizium anisopliae*). De modo geral, o extrato de *A. mucosa* foi compatível com as três espécies quando testado nas concentrações preconizadas para o controle das espécies-praga alvo. Assim, este estudo fornece importantes subsídios para o uso de derivados de anonáceas neotropicais como um componente útil para os programas de manejo integrado de pragas (MIP).

Palavras-chave: Aleloquímicos; Inseticidas botânicos; Acetogeninas; Fracionamentos biomonitorados; Técnicas cromatográficas

ABSTRACT

Exploring genetic biodiversity: secondary metabolites from Neotropical Annonaceae as potential source of new pesticides

To investigate potential sources of novel grain protectors, this study evaluated, firstly, the bioactivity of ethanolic extracts (66) prepared from 29 species belonging to 11 different genera of Neotropical Annonaceae against the maize weevil *Sitophilus zeamais* (Coleoptera: Curculionidae). A screening assay demonstrated that the most pronounced effects (acute and chronic) on *S. zeamais* were caused by extracts from the *Annona montana*, *A. mucosa*, *A. muricata* and *A. sylvatica* seeds, and, to a lesser extent, by extracts prepared from leaves of *A. montana*, *A. mucosa*, *A. muricata*, and *Duguetia lanceolata*. However, the most active extracts (from seeds) did not affect fungal growth and aflatoxin production of *Aspergillus flavus* (Ascomycota: Trichocomaceae). Using the maize weevil as bioindicator, bioguided fractionations were then conducted in order to isolate, purify and characterize the possible active compound(s) from the most interesting extracts. By means of different chromatographic procedures, nine compounds (five acetogenins, three steroids, and one aromatic compound) were isolated. The acetogenins rolliniastatin-1 and ACG4 (structural determination in progress) and the aromatic compound 2,4,5-trimethoxystyrene as well as the steroids campesterol, stigmasterol, and sitosterol [tested in mixture (8.44 + 12.37 + 79.19%, respectively)] showed promising grain protective properties. Furthermore, the obtained results indicate that compounds from different chemical natures have a synergistic effect on the overall biological activity of the crude extracts. In a second study, the acute and chronic toxicity of selected ethanolic seed extracts from *Annona* species (*A. montana*, *A. mucosa*, *A. muricata*, and *A. sylvatica*) and an acetogenin-based commercial bioinsecticide (Anosom® 1EC) were investigated against the cabbage looper *Trichoplusia ni* (Lepidoptera: Noctuidae) and the green peach aphid *Myzus persicae* (Hemiptera: Aphididae). In the laboratory, extracts of *A. mucosa* and *A. sylvatica* as well as Anosom® were especially active through oral and topical administration. A greenhouse trial showed that a formulated *A. mucosa* extract and Anosom® were highly effective (>98% mortality) against third instar of *T. ni* larvae, and comparable to a pyrethrin-based commercial insecticide (Insect Spray®) used as a positive control. Similar to results with *T. ni*, *A. mucosa* extract showed the greatest aphicidal either in laboratory or greenhouse bioassays. In a third study, the acaricidal activity [against the citrus red mite *Panonychus citri* (Acari: Tetranychidae)] of the ethanolic extract from *A. mucosa* seeds (most active) was investigated. In laboratory tests, it exhibited levels of activity superior to commercial acaricides/insecticides of natural origin [Anosom® 1EC (annonin), Derisom® 2EC (karanjin), and Azamax® 1.2EC (azadirachtin + 3-tigloylazadirachtol)] and similar to a synthetic acaricide [Envidor® 24 SC (spirodiclofen)]. Finally, the compatibility of *A. mucosa* seed extract with three entomopathogenic fungi species (*Beauveria bassiana*, *Isaria fumosorosea* and *Metarhizium anisopliae*) was assessed. In overall, it was compatible with the three entomopathogenic fungi species when tested at recommended concentrations for target pest species control. Therefore, this study argues for the use of derivatives from Neotropical Annonaceae as a useful component in the framework of integrated pest management (IPM) programs.

Keywords: Alellochemicals; Botanical insecticides; Acetogenins; Bioguided fractionations; Chromatographic techniques

1 INTRODUCTION

To offer to the domestic and international markets food with quality and free from chemical contaminants, ensuring competitiveness and environmental sustainability during the production process is the greatest challenge of the primary sector in Brazil. However, this challenge also extends to the Brazilian scientific community, responsible for the generation and validation of technologies capable of promoting changes in productive systems in order to create conditions to adapt to new market trends.

Among the actions required to overcome such challenges, the development of biorational methods of pest management becomes a strategic point. Species of arthropod pests represent throughout the production chain, in most cultures, a considerable percentage of production costs because they attack in various developmental stages, both in the field and in storage, causing huge economic losses. In addition, there are immeasurable negative implications in environmental and social frameworks resulting from the constant use of synthetic insecticides in the eradication of pest species and protection of agricultural crops (KOGAN, 1998).

In general, the success of traditional agricultural practices depends on the use of chemicals for insect pests control (FORIM et al., 2010). However, the chemical control by means of synthetic insecticides has been facing significant restrictions. Currently, the issue concerns the permanence of residues in grains, due to the active ingredients used, as well as their inefficiency in controlling pest species because of occurence of resistant populations to several molecules (RIBEIRO et al., 2003; PIMENTEL et al., 2009; SANTOS et al., 2011). This scenario is aggravated by the limited number of insecticides registered in Brazil that hinders the alternation of active ingredients (BOYER; ZHANG; LEMPERIERE, 2012), one of the strategies for the management of resistance. Therefore, new substances (natural or synthetic) with improved features are necessary for the effective pest control in the field and in the grain storage.

The study of defense mechanisms of plants comprise an important approach for the selection of new (bio)insecticides that meet the requirements of effectiveness, safety and selectivity [essential precepts of the integrated pest management (IPM) and of economic entomology] (VIEGAS JUNIOR, 2003). Plant-derived compounds show great potential for the management of populations of insect pests, both through

homemade preparations for direct use in the field and in the development of botanical insecticides (non-synthetic), as well as templates molecules for the synthesis of new synthetic insecticides (VENDRAMIM; CASTIGLIONI, 2000; ISMAN, 2006; CANTRELL; DAYAN; DUKE, 2012).

Brazil has the largest plant genetic diversity in the world with over 55,000 catalogued plant species (SIMÕES; SCHENKEL, 2002) and has a huge potential for the development of new products based on natural compounds. Moreover, bioprospecting studies carried out with diverse flora species constitute a strategic action for the generation of differentiated products with high-added value, such as agricultural (bio)pesticides. In addition, the medicinal, economic and ecological importance of native species, as well as the risk of extinction by human predatory action, has supported the studies of these plants for their preservation and sustainable use (SOUZA et al., 2008).

The Insecticidal Plants Laboratory at the Department of Entomology and Acarology at “Luiz de Queiroz” College of Agriculture/University of São Paulo (ESALQ/USP), member of the National Science and Technology Institute for Biorational Control of Pest Insects (INCT-CBIP), based at the Department of Chemistry at the Federal University of São Carlos (UFSCar), has been conducting studies searching for substances in species from Brazilian flora in order to be used in the IPM of pest species of importance to Brazilian agriculture.

In some previous studies (RIBEIRO, 2010; RIBEIRO et al., 2013), we reported the promising grain protective properties of derivates from two species of Annonaceae (*Annona montana* and *A. mucosa*) against the maize weevil *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera: Curculionidae), major pest species of stored corn (CERUTTI; LAZZARI, 2005). These results led to further bioguided studies of such species to isolate the compounds responsible for the bioactivity observed, as well as with other species and/or genera, exploring more comprehensively the rich availability of compounds in this family (LEBOEUF et al., 1980) and the diversity of species available in the Brazilian flora.

Annonaceae is the main family of the order Magnoliales (THE ANGIOSPERM PHYLOGENY GROUP, 2009) and is one of the largest among the Angiosperms, with 135 genera and 2,500 described species (CHATROU; RAINER; MAAS, 2004). Despite the great diversity of species of Annonaceae, it is one of the families of tropical plants less studied from the chemical point of view. The few studies already

carried out showed large amounts of compounds of diverse chemical nature in different structures and species (LEBOEUF et al., 1980; CHANG et al., 1998; KOTKAR et al., 2001). Moreover, extracts and isolated compounds of Annonaceae showed potential biological activity, including promising insecticidal properties (ALALI; LIU; McLAUGHLIN, 1999).

Among the classes of bioactive secondary metabolites of Annonaceae are the annonaceous acetogenins, which feature a range of biological activities, including anthelmintic, antimalarial, antimicrobial, antiprotozoarian, and pesticidal activities, in addition to being toxic to tumor cells (OCAMPO; OCAMPO, 2006). The acetogeninas comprise a series of natural products (C-35/C-37) derived from long-chain fatty acids (C-32/C-34) combined with a unit of 2-propanol (ALALI; XIAO-XI; MCLAUGHLIN, 1999), which are found only in the Annonaceae family (JOHNSON, 2000).

Aiming to identify potential sources of new grain protectors, this study investigated first the bioactivity of ethanolic extracts prepared from different structures of distinct species of Neotropical Annonaceae against the maize weevil. Additionally, antiaflatoxigenic and antifungal actions of most promising extracts were evaluated on the isolate CCT7638 of *Aspergillus flavus* Link, 1809 (Ascomycota: Trichocomaceae), a producer of aflatoxin B_1. Afterwards, bioguided fractionations were conducted to isolate and characterize the main active compounds from selected extracts. To this end, the maize weevil was used again as bioindicator.

In a second step, the acute and chronic effects of selected extracts were investigated on two important insect pest species of *Brassica* crops (Brassicaceae): the cabbage looper *Trichoplusia ni* (Hübner, 1803) (Lepidoptera: Noctuidae) and the green peach aphid *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae), in tests in laboratory and greenhouse (with formulated extract). Subsequently, the acaricidal activity of the most promising extract was evaluated on the citrus red mite *Panonychus citri* (McGregor, 1916) (Acari: Tetranychidae) in comparison with commercial acaricides/ insecticides of natural and synthetic origin. Finally, the compatibility of this extract with three species of entomopathogenic fungi, which are important biological control agents used in Brazil, was also verified.

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2 SEARCHING FOR POTENTIAL SOURCES OF GRAIN PROTECTORS IN EXTRACTS FROM NEOTROPICAL ANNONACEAE: SCREENING AND BIOGUIDED FRACTIONATIONS

Abstract

The Annonaceae family is a potential source of insecticidal substances due to the large number of species with diversified chemical compounds in the different structures. To investigate potential sources of novel grain protector compounds against the maize weevil *Sitophilus zeamais* (Coleoptera: Curculionidae), which is a primary insect pest of stored cereals, this study evaluated the bioactivity of ethanolic extracts 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 the *A. montana*, *A. mucosa*, *A. muricata* and *A. sylvatica* seeds, causing the death of all weevils exposed (LC_{50}: 621.70, 280.06, 363.14 and 565.64 mg kg^{-1}, respectively), almost complete inhibition of the F_1 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. Additionally, the antifungal and antiaflatoxigenic activities of the most promising extracts against the isolate CCT7638 of *Aspergillus flavus* (Ascomycota: Trichocomaceae), a producer of aflatoxin B1, were investigated. However, the selected extracts did not affect fungal growth and aflatoxin production. By means of different chromatographic procedures, nine compounds (five acetogenins, three steroids, and one aromatic compound) were isolated. The acetogenins rolliniastatin-1 and ACG4 (structural determination in progress) and the aromatic compound 2,4,5-trimethoxystyrene as well as the steroids campesterol, stigmasterol, and sitosterol (tested in mixture) showed promising grain protective properties. Furthermore, the obtained results indicate that compounds from different chemical natures have a synergistic effect on the overall biological activity of crude extracts. Therefore, compounds produced by the secondary metabolism of Neotropical Annonaceae are potential sources of new grain protectors.

Keywords: Allelochemicals; *Sitophilus zeamais*; *Aspergillus flavus*; Acetogenins; Chromatographic procedures

2.1 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 (ALMEIDA; AMORIM; ALBUQUERQUE, 2011; MILLER, 2011; CRAGG; NEWMAN, 2013) and new products for the protection of agricultural crops and stored commodities (ISMAN, 2006; CANTRELL; DAYAN; DUKE, 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 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; RAINER; MAAS, 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 (LEBOEUF et al., 1980; 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; LIU; McLAUGHLIN, 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 with these species and 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, 1855 (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,1809 (Ascomycota), a producer of aflatoxin B_1, in order to better characterize the potential of these extracts as grain protectors. In the next step, bioguided fractionations were conducted in order to isolate and chacacterize the active compound(s) from the most promising crude extracts.

2.2 Development

2.2.1 Material and methods

2.2.1.1 Species sampling and plant extract preparation (Step 1)

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 2.1. In total, 29 species of Annonaceae belonging to 11 genera, corresponding to 7.5% of Brazilian species, were collected and were investigated.

For the extract preparation, the plant structures were collected and were dehydrated in an oven at 40°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 was stored separately in sealed glass containers until use.

The organic extracts were obtained by maceration in ethanol solvent (1:5, w/v). 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 50°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.

2.2.1.2 Bioassays

The bioassays were performed in a climate-controlled room at 25±2^{o}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.

Table 2.1 - Species and structures of Annonaceae used in the study and the respective collection data

(continues)

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)

Table 2.1 - Species and structures of Annonaceae used in the study and the respective collection data

(conclusion)

Species	Plant structures	Local of collection	Data of collection	Voucher (Herbarium)[1]
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 Biol. 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 Biol. 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 (19º09'23,4" S; 39º59'30,3" W; elevation: 30 m)	16/11/2011	Lopes 359 (ESA)
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)

2.2.1.2.1 Screening for the identification of promising extracts (Step 2)

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 (F_1 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 concentrations of 1,500 and 3,000 mg kg^{-1} (mg of extract kg^{-1} of corn), which were defined based on previous studies (RIBEIRO, 2010).

2.2.1.2.1.1 Evaluation of insecticidal activity

For this bioassay, corn samples (10 g, in Petri dishes measuring 6-cm in diameter x 2-cm high) were treated separately with the respective extracts. The growth substrate treated with the solvent [acetone:methanol solution (1:1, v/v)] 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 repetitions 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.

2.2.1.2.1.2 Evaluation of F_1 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 repetitions 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):

$$Lw(\%) = (Ndg / Ntg)100C$$

where Lw = weight loss (%); Ndg= number of damaged grains; Ntg= total number of grains; C= 0.125 if the maize is stored as loose grains or on the cob without bracts and C= 0.222 if the maize is stored on the cob with bracts.

2.2.1.2.2 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 were established using the formula proposed by Finney (1971). The procedures described in section 2.2.1.2.1.1 were used in this bioassay. 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.

2.2.1.2.3 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.

2.2.1.2.4 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 methodology termed *poison food* (ALVAREZ-CASTELLANOS; BISHOP; PASCUAL-VILLALOBOS, 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)], incorporated by manual agitation in unfused culture media (temperature approximately 45°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 x 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±2^oC 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:

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

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 45°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 (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.

2.2.1.3 Bioguided fractionations of active crude extracts (step 3)

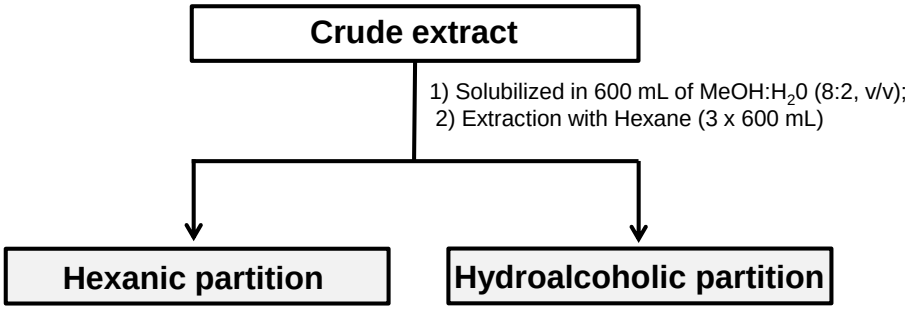
2.2.1.3.1 Partitioning of promising extracts

Based on the results from our screening assay (activity on *S. zeamais*), the most promising crude extracts were selected and subjected to chromatographic procedures in order to isolate, purify and characterize the possible active compound(s). For that purpose, the liquid-liquid partitioning technique was used first to separate the partitions (phases) with different chemical affinities (partition chromatography). Then, the selected extracts were solubilized in a mixture of methanol and water (volume ratio of 1:3 and 8:2 for the leaves and seeds extracts, respectively) and subjected to partitioning with a separating funnel and organic solvents in increasing polarity, according to the procedure described in Figure 2.1. The partitions obtained were then concentrated in a rotary evaporator under the same temperature and pressure conditions used to produce the crude extracts. The yield assessment of each partition was performed taking into account the masses of its respective crude extracts.

Afterwards, the partitions were submitted to bioassays (separately by plant species) to assess their insecticidal activity to *S. zeamais* adults. For this, corn samples (10 g) were treated and for each phase, the same experimental procedure described in the item 2.2.1.2.1.1 was adopted. The concentration used in the bioassays with partitions prepared from seed extracts were LC_{50} estimated in the previous bioassays. In turn, the partitions prepared from leaf extracts were tested at concentration 1,500 mg kg^{-1}, the same concentration used in the bioassay with crude extracts, once LC_{50} values were higher than 1,500 mg kg^{-1}. This criterion was adopted to use a smaller amount of each partition and, thus, preserving the mass available for subsequent fractionation procedures.

The mortality of exposed insects was assessed at 10^{th} day. For each treatment, 10 repetitions were used, and in each repetition, 20 adult weevils, not sexed and aged between 10- 20 days were exposed.

A) Extracts prepared from seeds



B) Extracts prepared from leaves

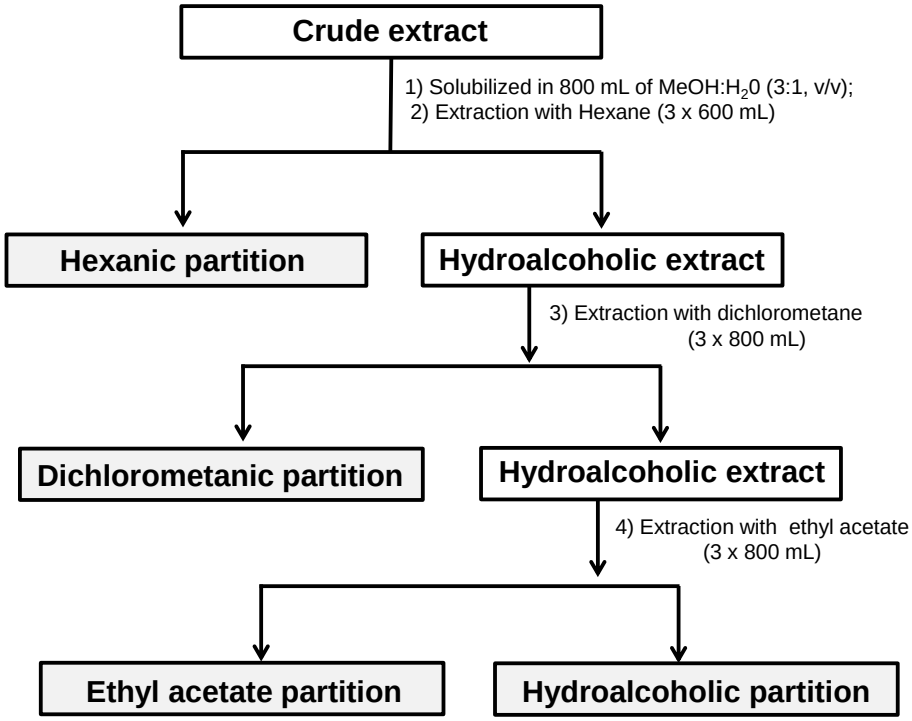


Figure 2.1 - Procedure used in the liquid-liquid partition of the selected extracts

2.2.1.3.1.1 Chemical profile of active partitions

In order to verify the major classes of compounds as well as the differences and similarities in the chemical profiles, the bioactive partitions were analyzed in ^{1}H NMR in a Bruker AVANCE^{TM} Nanobay III 400 MHz instrument using the deuterated solvents $CDCl_3$ and DMSO-d_6.

2.2.1.3.2 Fractionations of bioactive partitions

Based on the results from our bioassays and in the chemical profiles of the active partitions, the phases that caused the most promising activities against *S. zeamais* and showed the major differences in their chemical profiles were selected for the next steps of the fractionation processes.

2.2.1.3.2.1 Hydroalcoholic partition from *Annona mucosa* seed extract

Firstly, the hydroalcoholic partition from ethanolic extract prepared from *A. mucosa* seeds was fractionated by adsorption chromatography using silica gel as stationary phase and solvents in gradient elution (hexane, dichloromethane, ethyl acetate, and methanol). The obtained fractions were analyzed in thin layer chromatography (TLC) and ^{1}H NMR. The fractions were grouped by means of chemical similarities based on the results of these analyses. This procedure resulted in six fractions, which were bioassayed with *S. zeamais* adults.

For this bioassay, the same experimental procedure described in item 2.2.1.2.1.1 was used. The concentration used was 57.66 mg kg^{-1}, corresponding to 1/5 of the LC_{50} estimated for the respective crude extract.

2.2.1.3.2.2 Hydroalcoholic partition from *Annona sylvatica* seed extract

The hydroalcoholic partition prepared from ethanolic extract of *A. sylvatica* seeds was subjected to a first fractionation by size-exclusion chromatography, using Sephadex LH-20 as a stationary phase and an isocratic elution system [MeOH: DCM (7:3, v/v)]. This procedure resulted in 38 fractions grouped into six groups according to their similarities in TLC and ^{1}H NMR analyzes. Due to the improved separation of the constituents (verified in the TLC analysis), we selected the group “ASHi 4” for further purification processes using silica gel column chromatography. The initial mobile phase for elution was hexane:acetone (9:1, v/v) with an increasing gradient of methanol (from 10% to 100%).

In this procedure, 31 fractions were collected, which were then placed into 7 groups by means of similarities in the TLC cromatoplates. The group “ASHi 04/04” showed a dark-brown spot in the TLC (developer solution: vanillin sulfuric) as well as a positive result (reddish spots) in Kedde reagent, which indicated the presence of α, β- unsaturated lactone ring present in the acetogenin structure. The ^{1}H NMR analysis of this fraction showed a high concentration of acetogenins due to the presence of a characteristic set of signals of this class of compounds, such as in δ_H 7.28; δ_H 5.05 and δ_H 1.38, related to α, β- unsaturated lactone unit, signals in shifts δ_H 4.42- 3.08, referring to hydrogens of oxymetinic carbons, and signals in δ_H 2.49 and δ_H 2.37, associated to diastereotopic hydrogens adjacent to a lactone ring.

In addition, the obtained purified fraction (rich in acetogenins) was then bioassayed with the maize weevil in order to assess its insecticidal activity. The concentration used was 55.45 mg kg^{-1}, corresponding to 1/10 of LC_{50} estimated for the respective ethanolic crude extract.

2.2.1.3.2.3 Hexanic partition from *Duguetia lanceolata* leaf extract

The hexanic partition from ethanolic extract prepared from *D. lanceolata* leaves was fractionated by vacuum column chromatography using silica gel (70-230 mesh) and solvents of increasing polarity: hexane, dichloromethane, ethyl acetate, methanol and methanol:water (1:1, v/v). Ten fractions were obtained and analyzed by TLC and ^{1}H NMR. According to similarities detected in these analyses, they were grouped in 7 fractions, which were assayed again with our model insect.

The bioassay procedure, unit samples, and number of repetitions were the same used in the bioassay described in item 2.2.1.2.1.1. The concentration used was 750 mg kg^{-1}, corresponding to the 1/2 of the concentration used in the bioassay with the respective ethanolic crude extract.

2.2.1.3.3 Purification, isolation, and characterization of the major compound(s) from the most active fractions

2.2.1.3.3.1 Major acetogenin from the most active fraction of *Annona mucosa*

The purification and isolation of the major acetogenin of the most active fraction obtained from *A. mucosa* ethanolic seed extract were performed using different chromatographic techniques: by adsorption chromatography in an analytical thin layer (silica gel 60 F_{254} aluminum cromatoplates with 0.2 mm of thickness), by adsorption collumn chromatography (using silica gel 70-230 mesh and 230-400 mesh as stationary phases), and by high performance liquid chromatography (HPLC). For this procedure, we used a chromatograph (model 1200, Agilent Technologies) equipped with a G1311A quaternary pump, a G1322A degasser, a G1329A autosampler, and a G1314B UV detector. The equipment was connected to a G1369A interface, and the chromatograms were recorded using the EZCHrom Elite software. The stationary phase used was the reverse Luna C-18 in analytic (10 µm, 25.0 x 0.46 cm) and preparative (10 µm, 25.0 x 1.0 cm) mode and with a recycle valve and "*loop*" of 200 µL.

The identification of the isolated compound was performed by spectroscopic techniques [one-dimensional (^{1}H NMR and ^{13}C NMR) and two-dimensional (COSY, HSQC, HMBC) nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR)] and by mass spectrometry. For these analyses, the spectrometers BRUKER AVANCE^{TM} III NanoBay 9,4T (400 MHz for ^{1}H NMR and 100 MHZ for ^{13}C NMR) and MICROMASS QUATTRO LC were used in method of direct insertion and in electron capture ionization mode (negative).

In order to determine the insecticidal activity and to assess its effect on F_1 progeny of *S. zeamais* and the damage to corn samples, the isolated acetogenin was tested at concentration of 57.66 mg kg^{-1} (corresponding to 1/5 of LC_{50} estimated for the respective crude extract).The experimental procedures, sampling units, number of repetitions, and variables were the same used in the initial screening assay. As a positive control, a diatomaceous earth-based commercial formulation (Insect®, Bernardo Química SA, São Paulo, SP) was used at concentration of 1,000 mg kg^{-1}, which is the recommended rate for *S. zeamais* control in stored corn in Brazil (AGROFIT, 2014).

2.2.1.3.3.2 Major acetogenins from the purified fraction of *Annona sylvatica*

The same procedures (with minor modifications) and equipment described in item 2.2.1.3.3.1 were used in the purification and isolation of the acetogenins from purified fraction of *A. sylvatica.* Structural determination of these acetogenins is in progress in the Natural Products Laboratory at UFSCar.

The grain protectant action of the isolated acetogenins (at 27.72 mg kg^{-1}, corresponding to 1/20 of LC_{50} estimated for the respective crude extract) was assessed against *S. zeamais* using the same procedures, sampling units, number of repetitions, and variables described in the initial screening assay. For comparison purposes, Insect® (at 1,000 mg kg-1) was used as a positive control.

2.2.1.3.3.3 Major compounds from the most active fraction of *Duguetia lanceolata*

The major compounds of the most active fraction of the hexanic extract from *D. lanceolata* leaves were purified by flash collumn cromatography (silica gel 230-240 mesh) and in a Sephadex LH-20 column.

The identification of the isolated compounds was performed by spectroscopic techniques (^{1}H and ^{13}C NMR) and by gas chromatography coupled to mass spectrometry (GC-MS) and also by comparison with data from the literature. The same equipment described above was used.

The grain protectant action of the isolated acetogenins (at 75 mg kg^{-1}, corresponding to 1/20 of the concentration used in the screening assay) was assessed against *S. zeamais* using the same procedures, sampling units, number of repetitions, and variables described in the initial screening assay. Similar to the previous tests, Insecto® (at 1,000 mg kg^{-1}) was used as a positive control.

2.2.1.4 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 (DEMÉTRIO; HINDE, 1997; 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.

2.2.2 Results and discussion

2.2.2.1 Extraction yield (Step 1)

Based on the assessment of yield in organic extract obtained by means of maceration process in ethanol, we verified a strong variation [0.99 – 24.52% (w/w), Table 2.2] between the different species and/or structures of the studied Annonaceae. In general, the highest yields were obtained from the leaf and seed extracts in comparison with the branch extracts, except for *Pseudoxandra spiritus-sancti*, in which branches showed higher yield when compared with leaves. The high content of oil stored in Annonaceae seeds (AMADOR et al., 2007; BRANCO et al., 2010, RIBEIRO, 2010; EGYDIO; SANTOS, 2011) and the large amount of chlorophyll in the leaves, which is widely extracted by ethanol (polarity: 5.2), are hypotheses that can explain such results. Therefore, the highest yields (above 20%) were obtained from leaves of *Porcelia macrocarpa* (24.52%), *Xylopia laevigata* (22.71%), and *A. cacans* (20.62%) as well as from seeds of *A. muricata* (21.10%) and *A. montana* (20.23%) (Table 2.2).

Table 2.2 - Yield of organic extract obtained from the maceration process of different structures and/or Annonaceae species in ethanol at a 1:5 (p/v) ratio

(continues)

Species	Plant structure	Material weight (g)	Yield[11] (g)	Yield[11] (%)
Anaxagorea dolichocarpa	Leaves	100.00	7.26	7.26
	Branches	100.00	1.57	1.57
Annona acutiflora	Leaves	100.00	8.53	8.53
	Branches	100.00	5.50	5.50
Annona cacans	Leaves	200.00	41.24	20.62
	Branches	200.00	10.07	5.03
	Seeds	105.00	15.88	15.09
Annona dolabripetala	Leaves	100.00	12.45	12.45
	Branches	100.00	4.40	4.40
Annona emarginata	Leaves	200.00	33.28	16.64
	Branches	200.00	13.03	6.51
Annona montana	Leaves	100.00	11.79	11.79
	Branches	100.00	3.59	3.59
	Seeds	95.60	19.34	20.23
Annona mucosa	Leaves	100.00	9.96	9.96
	Branches	100.00	0.99	0.99
	Seeds	106.21	19.95	18.79
Annona muricata	Leaves	100.00	14.09	14.09
	Branches	100.00	6.04	6.04
	Seeds	104.50	22.05	21.10
Annona reticulata	Leaves	200.00	19.84	9.92
	Branches	200.00	6.66	3.33
Annona sylvatica	Leaves	100.00	7.18	7.18
	Branches	100.00	5.01	5.01
	Seeds	100.00	11.00	11.00
Annona sp.1	Leaves	100.00	12.33	12.33
	Branches	100.00	6.68	6.68
Annona sp.2	Leaves	100.00	14.17	14.17
	Branches	100.00	4.72	4.72
Duguetia lanceolata	Leaves	100.00	17.32	17.32
	Branches	100.00	6.27	6.27
	Seeds	106.00	10.31	9.27
Ephedranthus dimerus (1)	Leaves	100.00	11.74	11.74
	Branches	100.00	5.15	5.15
Ephedranthus dimerus (2)	Leaves	100.00	10.21	10.21
	Branches	100.00	7.17	7.17
Guatteria australis	Leaves	100.00	12.54	12.54
	Branches	100.00	9.93	9.93
Guatteria ferruginea	Leaves	100.00	9.93	9.93
	Branches	100.00	3.45	3.45
Guatteria sellowiana	Leaves	100.00	8.43	8.43
	Branches	100.00	6.20	6.20
Guatteria vilosissima	Leaves	100.00	17.01	17.01
	Branches	100.00	4.95	4.95
Hornschuchia bryotrophe	Leaves	100.00	10.53	10.53
	Branches	100.00	5.05	5.05
Hornschuchia citriodora	Leaves	100.00	10.05	10.05
	Branches	100.00	4.09	4.09

Table 2.2 - Yield of organic extract obtained from the maceration process of different structures and/or Annonaceae species in ethanol at a 1:5 (p/v) ratio

(conclusion)

Species	Plant structure	Material weight (g)	Yield[1]	
			(g)	(%)
Hornschuchia citriodora	Leaves	100.00	10.05	10.05
	Branches	100.00	4.09	4.09
Hornschuchia myrtillus	Leaves	100.00	12.41	12.41
	Branches	100.00	3.16	3.16
Oxandra martiana	Leaves	100.00	9.84	9.84
	Branches	100.00	7.63	7.63
Porcelia macrocarpa	Leaves	100.00	24.52	24.52
	Branches	100.00	10.50	10.50
Pseudoxandra spiritus-sancti	Leaves	100.00	6.53	6.53
	Branches	100.00	10.08	10.08
Unonopsis sanctae-teresae	Leaves	100.00	15.22	15.22
	Branches	100.00	13.75	13.75
Xylopia brasiliensis	Leaves	100.00	17.19	17.19
	Branches	100.00	8.18	8.18
Xylopia decorticans	Leaves	100.00	12.65	12.65
	Branches	10000	5.48	5.48
Xylopia frutescens	Leaves	100.00	11.01	11.01
	Branches	100.00	8.42	8.42
Xylopia laevigata	Leaves	100.00	22.71	22.71
	Branches	100.00	13.56	13.56

[1] Obtained from the quantity (g) of the plant material ground using a knife mill (plant powder)

In obtaining plant extracts, several variables can influence the processes and extraction yields. These aspects are mainly associated to the natural genetic variability (SHIDU et al., 2004; LEATEMIA; ISMAN, 2004; FORIM et al., 2010), which is corroborated by the results obtained in our study with the different species of Annonaceae, and by intraspecific occurrence of chemotypes (TAVARES et al., 2005). Climatic and temporal variations in different culture environments can also affect the plant secondary metabolism and the amount of produced allelochemicals as well as the chemical profile of the extracts. As the secondary metabolism is closely related to the defense mechanism of plants, which is genetically controlled by a limited number of genes (BOTTIGNON, 2009), the occurrence of phenotypic plasticity in various species is commonly observed. This situation leads to significant changes in the composition and yield of their derivatives (FONTANEL; TABATA, 1987) and consequently significantly impact on its biological activities.

Although the extraction yield is an important aspect in the direct application of plant extracts as biopesticides in the form of homemade preparations, the relative concentration of potential active constituents in these extracts is the primary factor to be considered because it has a direct influence on the concentrations necessary to

obtain certain biological activities of interest (RIBEIRO, 2010). Thus, to evaluate the potentiality of a particular derivative, extraction yields should be analyzed together with toxicological data (lethal doses or concentrations) obtained in bioassays.

2.2.2.2 Selection of the promising crude extracts (Step 2)

Of the tested extracts (66), 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 (Tables 2.3 and 2.4). 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, even at the lowest concentration tested (1,500 mg kg^{-1}). Therefore, these extracts can be used as potential grain protectors. The ethanolic extracts from the *A. montana*, *A. mucosa, A. muricata* and *D. laceolata* 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.

At the highest concentration tested (3,000 mg kg^{-1}), the hierarchical grouping analysis using the data from the variables analyzed in the screening bioassays indicated the formation of 3 groups (Figure 2.2). 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. The same group compositions were found at the lowest concentration tested (1,500 mg kg^{-1}) (Figure 2.2), with the exception of extracts from the leaves of *D. lanceolata*, which were included in the non-active treatments together with the controls. Therefore, the concentrations of the extracts did not influence greatly the distinction between the most promising extracts. Hypothetically, this observation suggests the predominance of qualitative variation in the chemical profiles of the extracts (and the possible interactions of compounds from different classes) originating from different species and/or structures of Annonaceae, a plant family known to exhibit highly diverse secondary metabolites.

Independent of the concentration tested, the adult mortality was inversely correlated (Figure 2.3) with the other tested variables (F_1 progeny and % damaged grains). 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.

Table 2.3 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (1,500 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(continues)

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative[4]
Group A[1]						
Annona cacans	Leaves	8.00±3.17 c	27.70±5.64 d	71.41±5.38 bc	8.92±0.67	91.49
	Branches	1.50±1.06 c	38.90±3.21 ab	79.09±4.09 abc	9.88±0.51	101.33
	Seeds	11.50±3.94 c	34.20±3.25 bc	71.41±5.38 bc	8.92±0.67	91.49
Annona montana	Leaves	65.00±6.54 a	7.00±1.50 e	19.55±5.76 ef	2.44±0.72	25.03
	Branches	3.50±1.30 c	40.30±3.45 ab	77.16±4.05 abc	9.64±0.50	98.87
	Seeds	100.00±0.00 **	0.50±0.22 e	1.21±0.62 f	0.15±0.07	1.54
Annona mucosa	Leaves	30.50±6.60 b	29.70±3.66 cd	59.10±7.10 cd	7.38±0.88	75.69
	Branches	2.00±0.81 c	47.60±3.47 ab	81.52±5.07 ab	10.19±0.63	104.51
	Seeds	100.00±0.00 **	0.00±0.00 **	0.00±0.00 **	0.00±0.00	0.00
Annona muricata	Leaves	17.00±5.12 bc	17.30±3.94 d	37.21±7.55 de	4.65±0.94	47.69
	Branches	1.50±0.76 c	51.40±2.61 ab	88.24±1.61 ab	11.03±0.20	113.13
	Seeds	100.00±0.00 **	0.30±0.15 e	0.40±0.16 f	0.10±0.04	1.03
Annona sylvatica	Leaves	4.00±1.63 c	43.40±3.27 ab	78.36±4.06 abc	9.79±0.50	100.41
	Branches	1.50±1.50 c	38.50±4.97 ab	70.36±6.98 bc	8.79±0.87	90.15
	Seeds	100.00±0.00 **	0.40±0.22 e	0.83±0.45 f	0.10±0.05	1.03
Duguetia lanceolata	Leaves	4.00±1.24 c	36.20±2.54 bc	74.32±4.61 abc	9.29±0.57	95.28
	Branches	0.00±0.00 **	55.70±4.21 a	93.18±1.90 a	11.64±0.23	119.38
	Seeds	1.50±1.50 c	51.80±3.81 ab	88.09±2.75 ab	11.01±0.34	112.92
Control (acetone:methanol, 1:1 (v/v))	--	1.50±0.76 c	38.90±3.25 ab	78.04±4.35 abc	9.75±0.54	100.00
F		21.21	44.86	39.80		
p value		<0.0001	<0.0001	<0.0001		
Group B[1]						
Annona dolabripetala	Leaves	2.50±1.53	33.00±3.51 b	72.31±5.84 b	9.03±0.73	81.35
	Branches	0.50±0.50	39.20±2.45 ab	81.41±2.38 ab	10.17±0.29	91.62
Annona emarginata	Leaves	2.00±0.81	41.90±2.56 ab	85.90±2.93 ab	10.73±0.36	96.67
	Branches	1.00±0.66	38.80±2.39 ab	80.76±2.99 ab	10.09±0.37	90.90
Annona reticulata	Leaves	0.00±0.00 **	45.00±2.39 ab	86.18±2.59 ab	10.77±0.32	97.03
	Branches	1.00±0.66	47.10±1.74 ab	87.68±1.69 ab	10.96±0.21	98.74

Table 2.3 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (1,500 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(continuation)

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative[4]
Annona sp. 1	Leaves	0.00±0.00 **	39.40±3.77 ab	79.37±2.62 ab	9.92±0.32	89.37
	Branches	1.00±1.00	49.00±3.31 a	84.77±3.74 ab	10.59±0.46	95.41
Annona sp. 2	Leaves	1.00±0.66	41.10±2.99 ab	82.68±3.61 ab	10.33±0.45	93.06
	Branches	1.00±0.66	46.10±5.28 ab	83.03±4.04 ab	10.37±0.50	93.42
Porcelia macrocarpa	Leaves	2.50±1.11	43.50±2.54 ab	87.95±2.52 ab	10.99±0.31	99.01
	Branches	0.00±0.00 **	46.90±3.07 ab	89.26±3.02 a	11.15±0.37	100.45
Control (acetone:methanol, 1:1 (v/v))	--	0.00±0.00 **	45.50±2.64 ab	88.83±2.49 ab	11.10±0.31	100.00
F		0.67^{ns}	2.10	2.05		
p value		0.7128	0.0218	0.0254		
Group $C_{[1]}$						
Annona acutiflora	Leaves	1.00±0.66	48.20±3.19 ab	89.79±3.24	11.22±0.40	116.63
	Branches	2.50±1.34	32.00±2.47 bc	78.60±3.42	9.82±0.42	102.08
Guatteria australis	Leaves	2.50±1.11	31.60±2.92 bc	73.95±4.47	9.24±0.55	96.05
	Branches	2.50±0.83	36.00±2.70 abc	79.62±2.83	9.95±0.35	103.43
Guatteria ferruginea	Leaves	8.00±4.42	26.80±4.22 c	69.37±7.41	8.67±0.92	90.12
	Branches	5.00±1.82	34.30±4.30 abc	72.55±4.49	9.06±0.56	94.18
Guatteria sellowiana	Leaves	2.50±1.11	31.60±2.79 bc	73.68±3.76	9.21±0.47	95.74
	Branches	3.00±1.10	40.10±4.01 abc	82.26±5.06	10.28±0.63	106.86
Guatteria villosissima	Leaves	1.00±0.66	49.60±4.86 a	89.29±2.76	11.16±0.34	116.01
	Branches	4.00±1.63	35.90±3.17 abc	74.37±5.31	9.29±0.66	96.57
Unonopsis sanctae-teresae	Leaves	3.00±1.52	36.40±4.00 abc	78.49±5.67	9.81±0.70	101.97
	Branches	2.00±1.10	38.80±2.61abc	82.44±3.51	10.30±0.43	107.07
Control (acetone:methanol, 1:1 (v/v))	--	3.00±1.10	32.90±2.41 abc	76.97±4.15	9.62±0.51	100.00
F		1.38^{ns}	3.36	2.06^{ns}		
p value		0.1837	0.0003	0.0251		

Table 2.3 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (1,500 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(continuation)

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative[4]
Group $D_{[1]}$						
Oxandra martiana	Leaves	0.50±0.50	48.00±3.57 ab	90.89±2.19	11.36±0,27	114.06
	Branches	0.00±0.00 **	48.90±3.51 ab	90.51±2.96	11.31±0.37	113.55
Pseudoxandra spiritus-sancti	Leaves	2.00±0.81	43.70±3.15 ab	84.44±2.75	10.55±0.34	105.92
	Branches	1.00±0.66	45.10±4.96 ab	86.66±3.68	10.83±0.46	108.73
Xylopia brasiliensis	Leaves	1.50±0.76	55.50±4.25 a	92.31±2.44	11.53±0.30	115.76
	Branches	1.00±0.66	45.60±2.62 ab	85.98±2.85	10.74±0.35	107.83
Xylopia decorticans	Leaves	0.00±0.00 **	51.30±3.76 ab	89.77±2.37	11.22±0.29	112.65
	Branches	0.00±0.00 **	46.50±3.22 ab	90.12±2.53	11.26±0.31	113.05
Xylopia frutescens	Leaves	0.50±0.50	50.30±2.85 ab	92.38±2.14	11.54±0.26	115.86
	Branches	0.50±0.50	53.30±4.54 ab	92.16±2.92	11.52±0.36	115.66
Xylopia laevigata	Leaves	1.00±0.66	50.90±2.06 ab	91.49±1.63	11.43±0.20	114.76
	Branches	0.50±0.50	40.60±3.91 ab	83.75±4.13	10.46±0.51	105.02
Control (acetone:methanol, 1:1 (v/v))	--	0.00±0.00 **	37.60±3.33 b	79.72±3.33	9.96±0.42	100.00
F		0.61^{ns}	1.97	1.83^{ns}		
p value		0.7688	0.0325	0.0508		
Group $E_{[1]}$						
Anaxagorea dolichocarpa	Leaves	0.00±0.00 **	53.50±4.87	91.62±3.10 ab	11.45±0.38	97.36
	Branches	2.00±1.10	51.70±3.10	89.76±2.45 ab	11.22±0.30	95.41
Ephedranthus dimerus (1)	Leaves	0.50±0.50	59.90±2.18	97.94±0.62 a	12.24±0.07	104.08
	Branches	1.50±1.06	52.50±3.92	95.37±1.34 ab	11.92±0.16	101.36
Ephedranthus dimerus (2)	Leaves	0.50±0.50	60.80±4.52	95.89±1.43 ab	11.98±0.18	101.87
	Branches	2.50±1.53	47.70±1.39	93.89±0.90 ab	11.73±0.11	99.74
Hornschuchia bryotrophe	Leaves	4.50±3.02	50.50±2.98	94.57±1.50 ab	11.82±0.18	100.51
	Branches	1.00±0.66	54.70±5.57	91.83±2.58 ab	11.47±0.32	97.53
Hornschuchia citriodora	Leaves	3.00±1.69	51.00±4.38	92.07±2.21 ab	11.50±0.27	97.79
	Branches	0.50±0.50	52.20±4.57	90.20±1.86 ab	11.27±0.23	95.83

Table 2.3 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (1,500 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(conclusion)

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative[4]
Hornschuchia myrtillus	Leaves	1.00±1.00	51.60±3.17	92.70±2.05 ab	11.58±0.25	98.47
	Branches	4.00±1.79	42.80±3.87	87.91±2.40 a	10.98±0.30	93.37
Control (acetone:methanol, 1:1 (v/v))	--	0.50±0.50	51.50±2.92	94.04±1.49 ab	11.76±0.18	100.00
F		1.3559^{ns}	1.46^{ns}	2.34		
p value		0.2045	0.1475	0.0100		

[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};
** Not included in the analysis (null variance);
^{ns}: Not significant ($p>0.05$).

Table 2.4 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (3,000 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(continues)

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

Table 2.4 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (3,000 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(continuation)

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative (%)[4]
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		

Table 2.4 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (3,000 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(continuation)

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative (%)[4]
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

Table 2.4 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with ethanolic extracts from different species and/or structures of Annonaceae (3,000 mg kg^{-1})*. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

(conclusion)

Species	Plant structures	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight losses	
					Total (%)[3]	Relative (%)[4]
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};
** Not included in the analysis (null variance);
^{ns}: Not significant ($p>0.05$).

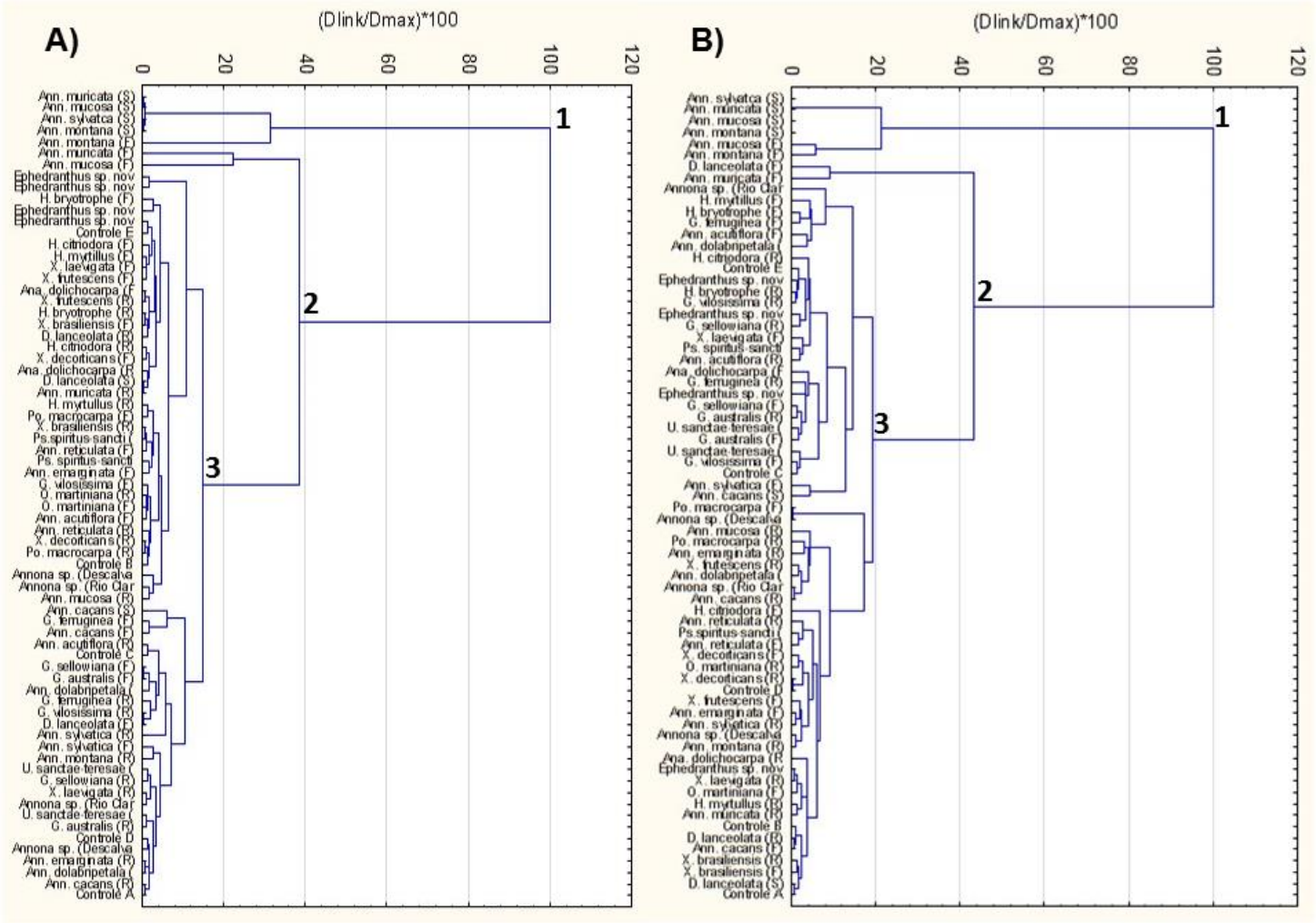


Figure 2.2 - Dendrogram obtained from Cluster analysis based on bioactivity similarity of ethanolic extracts from Annonaceae on *Sitophilus zeamais* (mean Euclidian distance as dissimilarity measurement and UPGMA (*Unweighted Pair Group* Method) as a clustering strategy method) at A) 1,500 mg kg^{-1} and B) 3,000 mg kg^{-1}

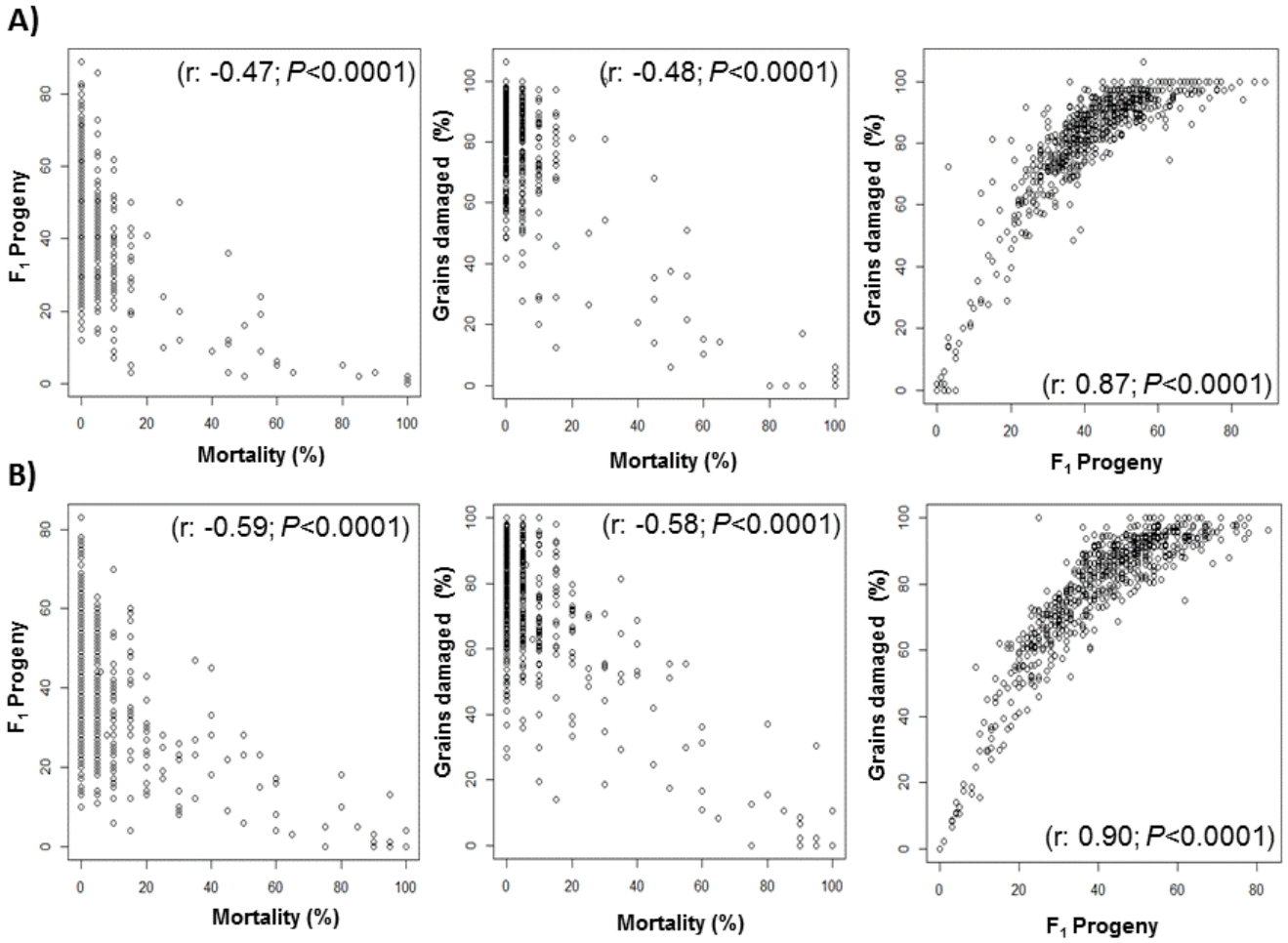


Figure 2.3 - Spearman's correlation coefficient (r) between the analyzed variables on the screening bioassay at: A) 1,500 mg kg^{-1} and B) 3,000 mg kg^{-1}

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

The lethal concentrations (LC_{50} and LC_{90}) and the average lethal times (LT_{50}) estimated using the toxicological assays for each selected extract are shown in Tables 2.5 and 2.6, respectively. 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). These values were significantly different from the values for the other extracts based on the comparison of the estimated confidence intervals (p<0.05) (Table 2.5). In general, the extracts from the seeds of different bioactive *Annona* species demonstrated the lowest values compared with the active extracts from leaves. This observation suggests that there is a greater accumulation of active substance in the seeds of species from this genus, a strategy evolutionarily selected to guarantee the survival of the progeny.

Moreover, the average lethal time (varying between 82.06 and 94.85 hours) did not demonstrate great differences between the treatments (Table 2.6). The values reflect slower activity of the active extracts compared with neurotoxins, which may preclude the possible activity of the active compounds on the nervous system.

2.2.2.4 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 2.7). However, the extract from the *A. sylvatica* seeds reduced the initial velocity (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.

Table 2.5 - Estimation of LC_{50} and LC_{90} (in mg kg^{-1}*) and confidence interval of ethanolic extracts from Annonaceae for *Sitophilus zeamais* adults after 10 days of exposure in treated maize samples (10 g). Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

Species (structures)	n [1]	*Slope* ± SE (*p* value)	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 2.6 - Estimation of average lethal time (LT_{50}, in hours) and confidence interval of ethanolic extracts from Annonaceae for *Sitophilus zeamais* adults. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux

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 2.7 - 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}). Temp.: 25±2°C; R.U.: 60±10%; scotophase: 24 h

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 ($p>0.05$).

Currently, new insecticide compounds of improved quality are necessary for the integrated pest management (IPM) of stored grains. The identification of new compounds is lacking because of the limited number of efficient management tools and active principles registered for chemical control (BOYER; ZHANG; LEMPERIERE, 2012), a method that has an increasingly restricted efficiency because of resistant populations (PEREIRA et al., 2009; ROSSI; COSIMI; LONI, 2010; BRAGA et al., 2011; OPIT et al., 2012). This situation has significantly increased postharvest losses. Given this scenario, this 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, 2011b) and has been shown to exhibit secondary metabolites with great chemical diversity and promising biological activities (LEBOEUF et al., 1980; 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; ARANGO; GIRALDO, 2008; SEFFRIN et al., 2010; GRZYBOWSKI et al., 2013; RIBEIRO et al., 2013). Secondary metabolites play a vital role in the plant defense against herbivory (WINK, 2003; MITHOFER; BOLAND, 2012; HAGG; ZAGROBELNY; BAK, 2013; NEILSON et al., 2013); therefore, the expression of allelochemicals of a determined structural type with similar ecological functions can reflect specific adaptations and life strategies developed for a given phylogenetic scenario. This scenario could have led to the establishment of common metabolic pathways because of exposure to the same selective pressures (WINK, 2003). Therefore, similarities in the chemical composition of the extracts, especially at the chemical class level, are consistent with the results of this study regarding the biological activities on *S. zeamais*. Given this assumption, during the evolutionary process, insect herbivory could have been a key factor for the establishment of the chemical defense of *Annona* species seeds; hypothetically, this could have

guaranteed the evolutionary success of the genus, which is one of the most abundant of the Annonaceae family (MAAS et al., 2011b).

Based on the previous hypotheses and the screening results, it is evident that the use of precepts of phylogenetic rationale can significantly improve the efficiency of bioprospecting studies (RAMESHA et al., 2011). Bioprospecting is a contemporary approach comprised of multiple objectives that cover not only the conservation of biodiversity and management of resources but also economic development (BEATTIE et al., 2011), all of which are essential links of sustainability.

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; ARAUJO; KURTZ, 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* 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, 1947 (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 (hinesol, z-caryophyllene, ɑ-maaliene, ɣ-gurjunene, silphiperfol-5-en-3-ol, ledol, cubecol-1-epi and muurola-3,5-diene).

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* (L., 1758) (TRINDADE et al., 2011), *Bactericera cockerelli* (Sulc, 1909) (FLORES-DAVILA et al., 2011), *Anastrepha ludens* Loew, 1873 (GONZALES-ESQUINCA et al., 2012) and *Aedes aegypti* (L., 1762) (GRZYBOWSKI et al., 2013). Acetogenins isolated from *A. montana* seeds demonstrate toxicity for *Oncopeltus fasciatus* (Dallas, 1852) (COLOM et al., 2008) and insecticidal and anti-feeding activities for *Spodoptera frugiperda* (J.E. Smith, 1797) larvae (BLESSING et al., 2010). Furthermore, the extract obtained through the

decoction of *A. mucosa* seeds has a repellent effect on *Acromyrmex octospinosus* (Forel, 1899) workers (BOULOGNE et al., 2012).

Using *S. zeamais* as a model, Llanos, Arango and Giraldo (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 (LEBOEUF et al., 1980; 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; LIU; McLAUGHLIN, 1999; COLOM et al., 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; WHITACRE, 2004). Therefore, based on these findings and previous analysis (RIBEIRO et al., 2013), it is possible to conclude 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 species that has not been studied from a phytochemical point of view. However, a number of pharmacological properties, such as antiprotozoal (FISCHER et al., 2004; TEMPONE et al., 2005), antinociceptive and anti-inflammatory activities (SOUZA 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 et al., 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. This process may contribute indirectly to the control of this microorganism that deteriorates stored grains.

Despite the preliminary nature of the data in this step, 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. Moreover, our results represent an important argument for the conservation of the flora from different biomes, an important challenge for megadiverse countries such as Brazil.

2.2.2.5 Partitioning of promising extracts: yield and insecticidal activity (Step 3)

2.2.2.5.1 Yield

The yield values of different phases obtained are detailed in Table 2.8. For the leaf extracts, the highest values were recorded in hexane and hydroalcoholic partitions, except for *A. muricata* that showed the highest yield in the ethyl acetate phase. For seed extracts, however, the highest yields were obtained with hexane phases, regardless of the species. This fact is probably associated with the high oil content in the seed extracts, which was mostly concentrated in the hexane partition. However, the procedure adopted allowed to separate a large part of the oily phase and facilitated the subsequent use of separation techniques in chromatographic columns of silica.

Table 2.8 – Yield of partitions from ethanolic extracts selected of different species and/or structures of Annonaceae

Species (plant part)	Weight of extract (g)	Phases	Yield[1]	
			(g)	(%)
Annona montana (leaves)	200.00	Hexane	86.13	43.06
		Dichloromethane	27.86	13.93
		Ethyl acetate	7.24	3.62
		Hydroalcoholic	34.57	17.28
Annona montana (seeds)	110.00	Hexane	61.86	56.27
		Hydroalcoholic	45.26	41.14
Annona mucosa (leaves)	152.30	Hexane	37.7	24.75
		Dichloromethane	15.27	10.03
		Ethyl acetate	9.31	6.11
		Hydroalcoholic	53.20	34.93
Annona mucosa (seeds)	105.00	Hexane	66.90	63.71
		Hydroalcoholic	29.20	27.81
Annona muricata (leaves)	8.48	Hexane	1.05	12.38
		Dichloromethane	1.44	16.98
		Ethyl acetate	2.33	27.48
		Hydroalcoholic	0.90	10.61
Annona muricata (seeds)	6.50	Hexane	3.59	55.23
		Hydroalcoholic	2.50	38.46
Annona sylvatica (seeds)	57.53	Hexane	44.10	76.65
		Hydroalcoholic	9.22	16.03
Duguetia lanceolata (leaves)	83.16	Hexane	17.35	20.87
		Dichloromethane	7.39	8.89
		Ethyl acetate	7.85	9.44
		Hydroalcoholic	36.21	43.54

[1] Obtained from the quantity (g) of the crude extract subjected to liquid-liquid partitioning

2.2.2.5.2 Insecticidal activity of obtained partitions

The hexane partitions from ethanolic leaf extracts of *A. montana* and *D. lanceolata*, as well as the phases in hexane and dichloromethane of *A. mucosa* leaf extract (mainly the first mentioned) were the ones that caused significant mortality of *S. zeamais* adults (Figure 2.3). This feature shows evidence of a more apolar nature of the active compound(s) in the extract from these plant species. On the other hand, for *A. muricata*, the dichloromethane phase (intermediate polarity) showed the most pronounced insecticidal effect, differing significantly from hexane and ethyl acetate phases, which also caused significant mortality of *S. zeamais* adults (Figure 2.3).

The hexane and hydroalcoholic phases of all selected extracts from seeds caused mortality rates different from those of the control (Figure 2.4). Despite the promising insecticidal activity, there was no significant difference between both partitions of seed extracts of *A. muricata* and *A. mucosa*, while the hydroalcoholic phase of the extract of *A. sylvatica* caused mortality higher than the hexane phase,

and the inverse occurred in fractions of seed extracts of *A. montana*, where the hexane phase caused higher mortality (Figure 2.4).

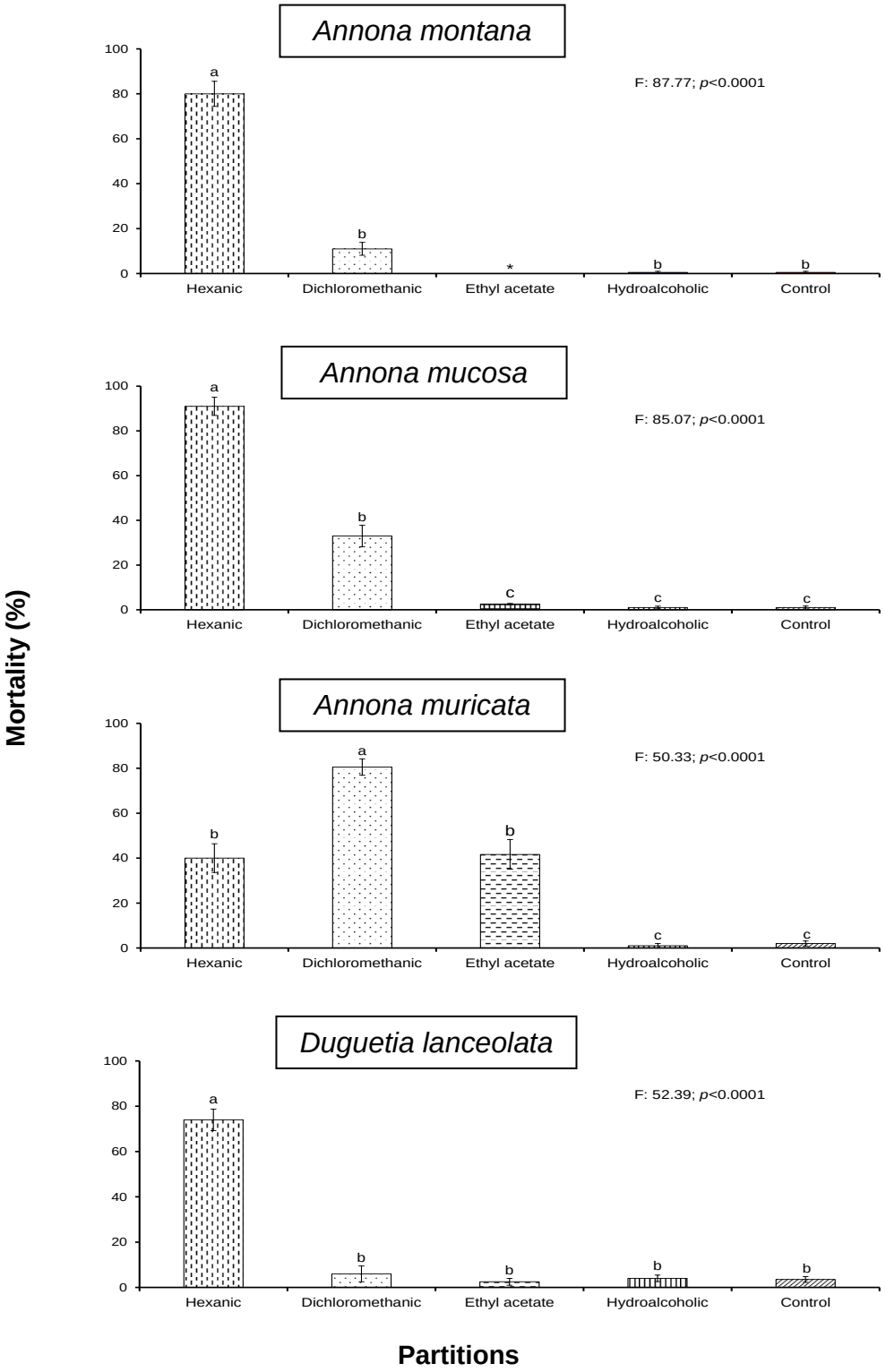


Figure 2.4 - Mortality on the 10th day after infestation (± standard error) of *Sitophilus zeamais* adults exposed to corn samples (10 g) treated with partitions of ethanolic leaf extracts from different Annonaceae (at 1,500 mg kg^{-1}). Temp.: 25±2°C; RH: 60±10%; photophase: 14 h; average luminosity: 200 lux. Bars followed by different letters in each figure indicate significant differences among the treatments (GLM with quasi-binomial distributions, followed by a *post hoc* Tukey test, $p<0.05$)

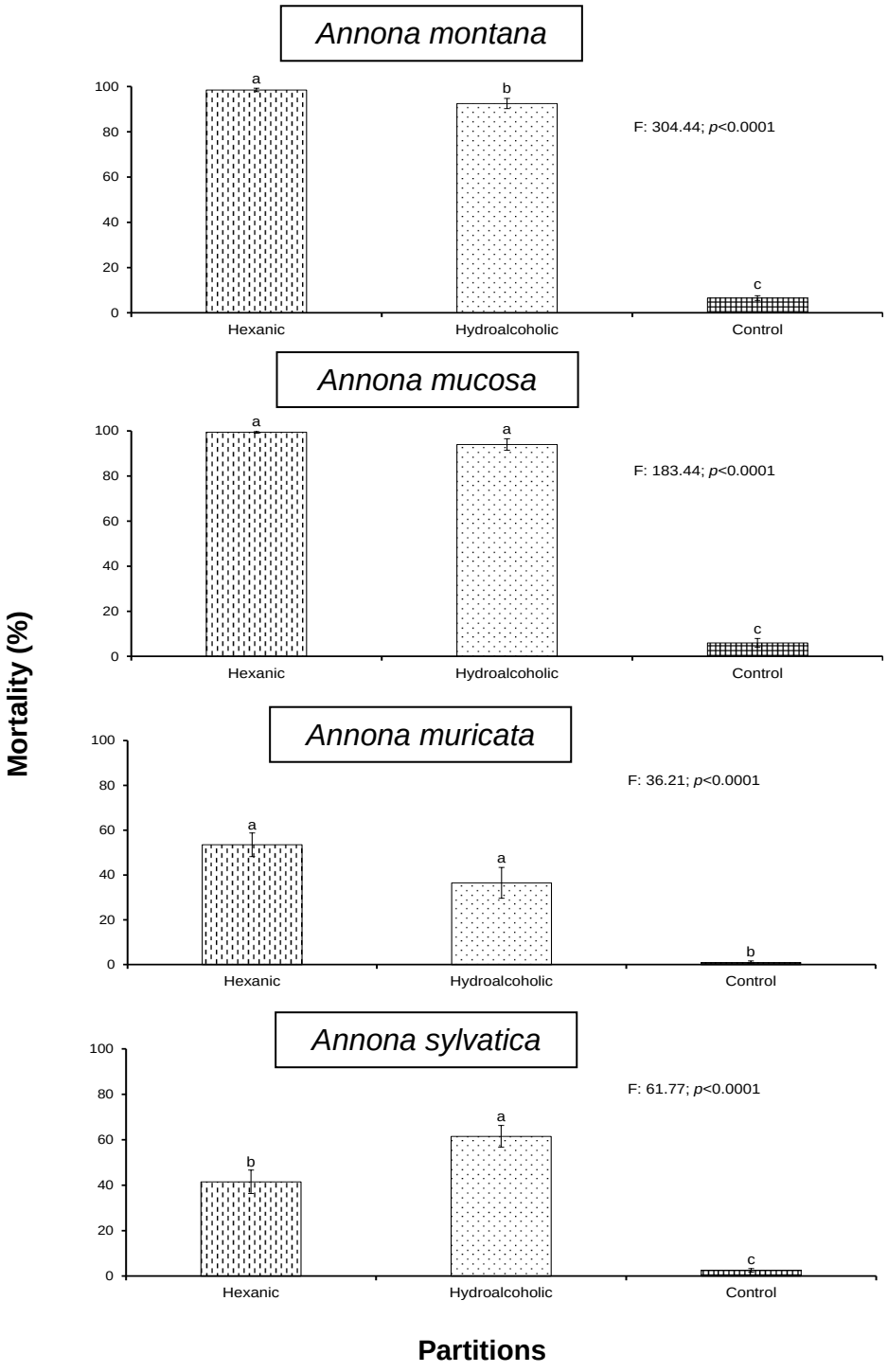


Figure 2.5 - Mortality on the 10th day after infestation (± standard error) of *Sitophilus zeamais* adults exposed to corn samples (10 g) treated with partitions of ethanolic seed extracts from different Annonaceae (at 1,500 mg kg^{-1}). Temp.: 25±2°C; RH: 60±10%; photophase: 14 h; average luminosity: 200 lux. Bars followed by different letters in each figure indicate significant differences among the treatments (GLM with quasi-binomial distributions, followed by a *post hoc* Tukey test, $p<0.05$)

2.2.2.5.3 Chemical profile of active fractions

The ^{1}H NMR spectra in the hexane phases of the ethanolic seed extract of *A. mucosa*, *A. montana*, *A. sylvatica* and *A. muricata* (Figures 2.8, 2.10, 2.12 and 2.14) showed that the triglycerides class was majority in the composition of these samples. When a class is majority in a sample, the intensity of signals for these compounds is significantly higher when compared to signals referring to substances of other classes, making it difficult to visualize and confirm the presence of other “minority” classes.

Triglycerides (general structure shown in Figure 2.6) are triacylglycerols where each group contains a hydrocarbon chain (saturated or not saturated). The ^{1}H NMR analysis showed the presence of oxymethine hydrogens and α and β-carboxylic ester groups as well as the presence of alkyl hydrogen chain saturated or not. Alkyl chains are evidenced by the observation of signals in the range between δ_H 0.78 and 1.23 ppm that refer to methyl and methylene groups, respectively. Signals between δ_H 5.18 and 5.30 ppm, which are characteristic of double hydrogen bonds, and in the range between δ_H 1.91 and 1.99 ppm, referring to methylene groups (CH_2) neighbors to the double bonds indicate the presence of fatty acids containing insaturations in the alkyl chain lengths. Glycerol units are confirmed by observing signals in δ_H 2.68 and 2.22 ppm, characteristic of hydrogens α and β-carboxylic, respectively, and still in the range between δ_H 4.04 and 4.25 ppm that refer to hydrogens of carbons oxygenated of glycerol.

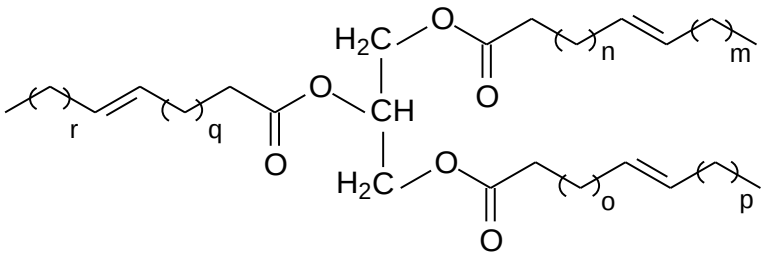


Figure 2.6 – General structure of triglycerides

The ^{1}H NMR spectra of hydroalcoholic partitions of ethanolic seed extracts of *A. montana*, *A. mucosa*, *A. sylvatica*, and *A. muricata* (Figures 2.9, 2.11, 2.13, and 2.15) showed the majority presence of acetogenins (general structure shown in Figure 2.7) in the samples analyzed. Overall, this was indicated by the presence of the following signals in the spectra: hydrogens of the lactonic ring unsaturation in chemical shift δ_H 7.40 ppm; several signals around δ_H 5.11-5.41 ppm, which are

characteristic of hydrogens of saturated carbon of the lactonic ring; signals around δ_H 3.35-3.84 ppm, which are characteristic of hydrogens of methylene groups (CH) of the alkyl chain bonded directly to hydroxyl groups or to epoxide groups; signals in the δ_H 2.01-2.52 ppm; which are characteristic of α-lactonic and/or α-carbonyl hydrogens, in the case of molecules with ketone groups in alkyl chains; signals in the range δ_H 1.4-1.7 ppm, which are related to various groups CH_2 that occur along the alkyl chains and signals in δ_H 0.9 ppm, which are characteristic of terminal methylene of long alkyl chains.

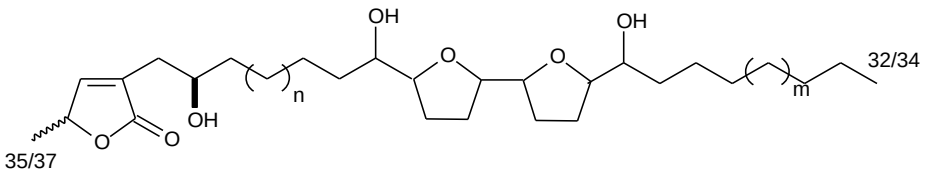


Figure 2.7 - General structure of acetogenins

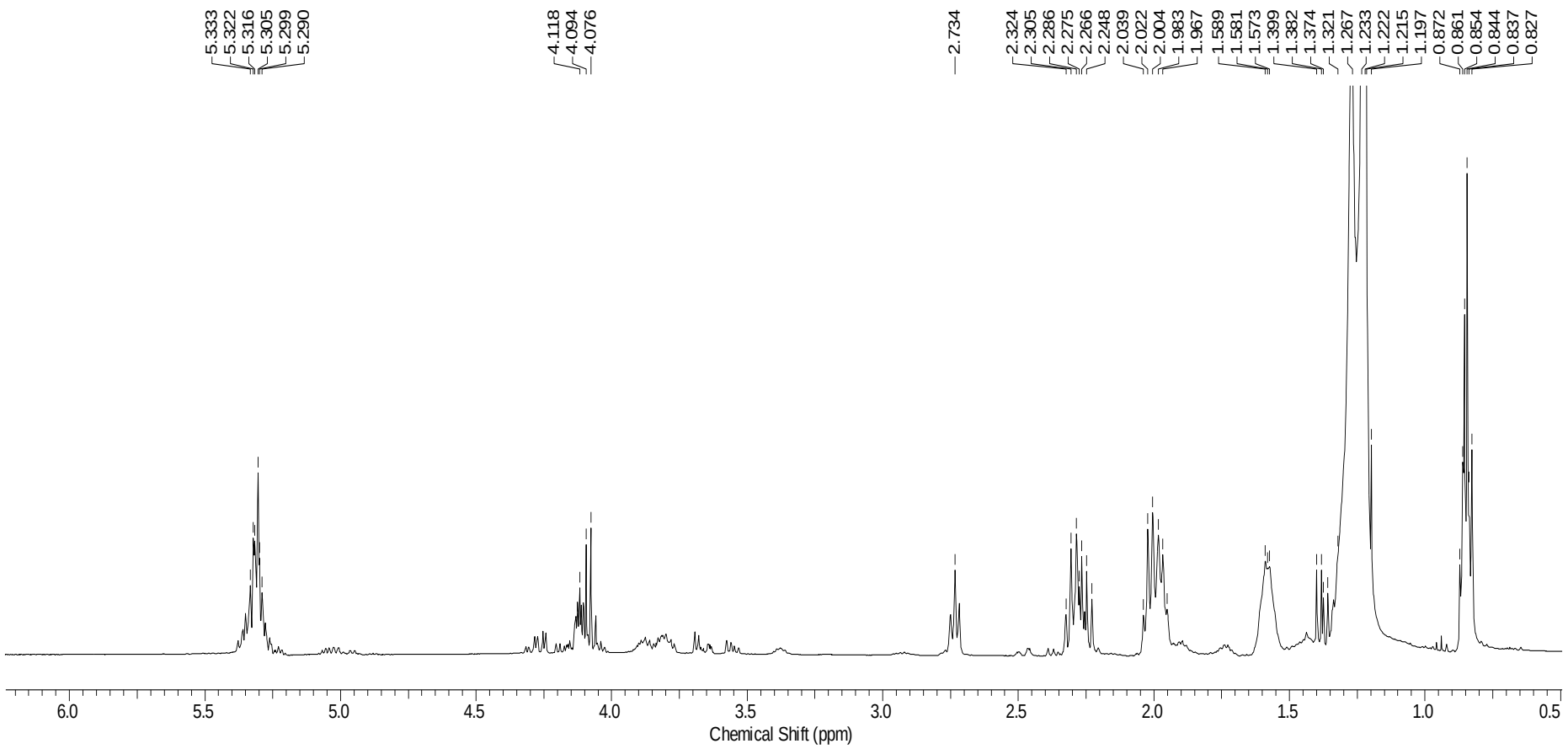


Figure 2.8 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the seeds of *Annona mucosa* in ethanol ($CDCl_3$, 400 MHz)

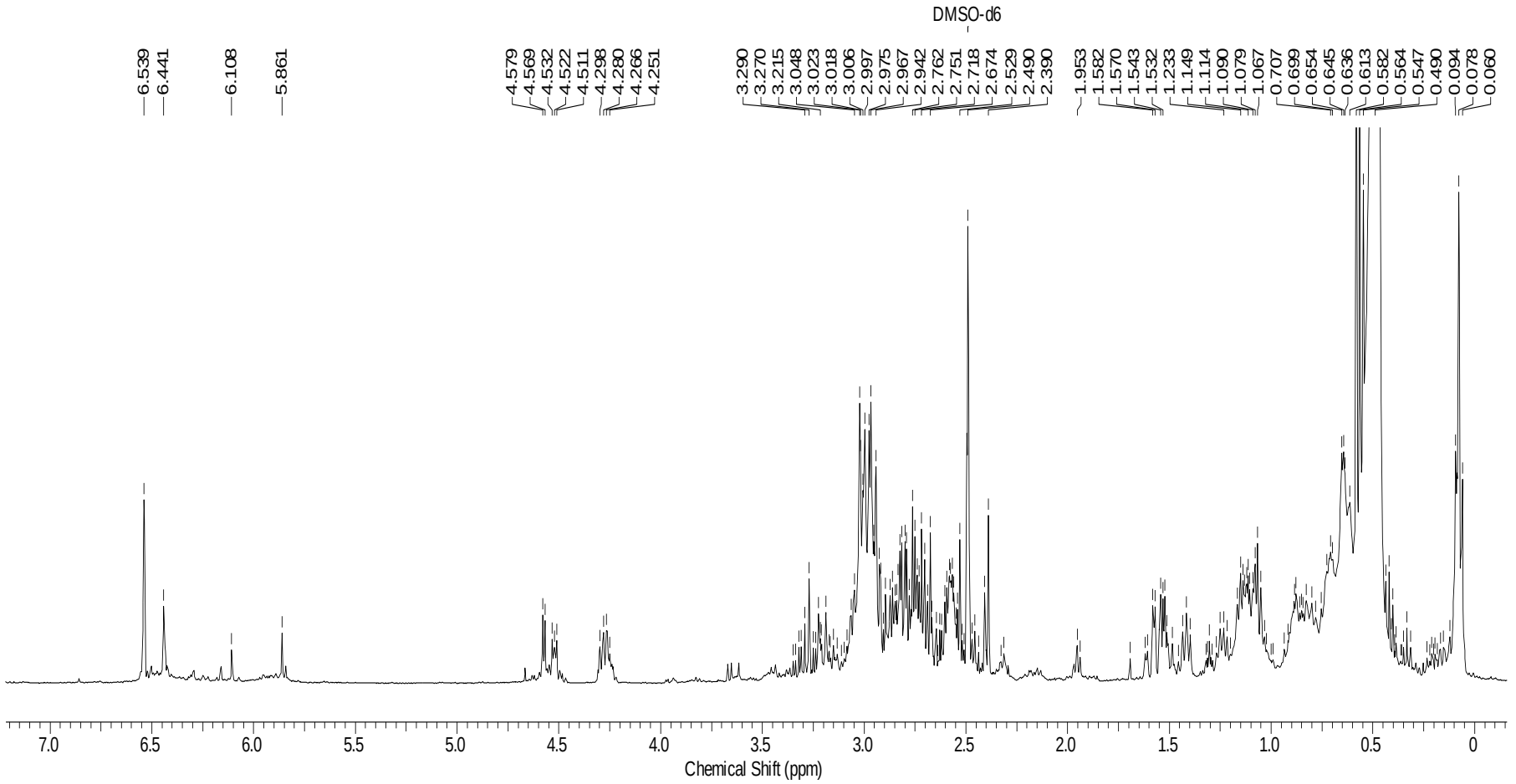


Figure 2.9 - ^{1}H NMR spectrum of the hydroalcoholic partition of the extract prepared from the seeds of *Annona mucosa* in ethanol (DMSO-d_6, 400 MHz)

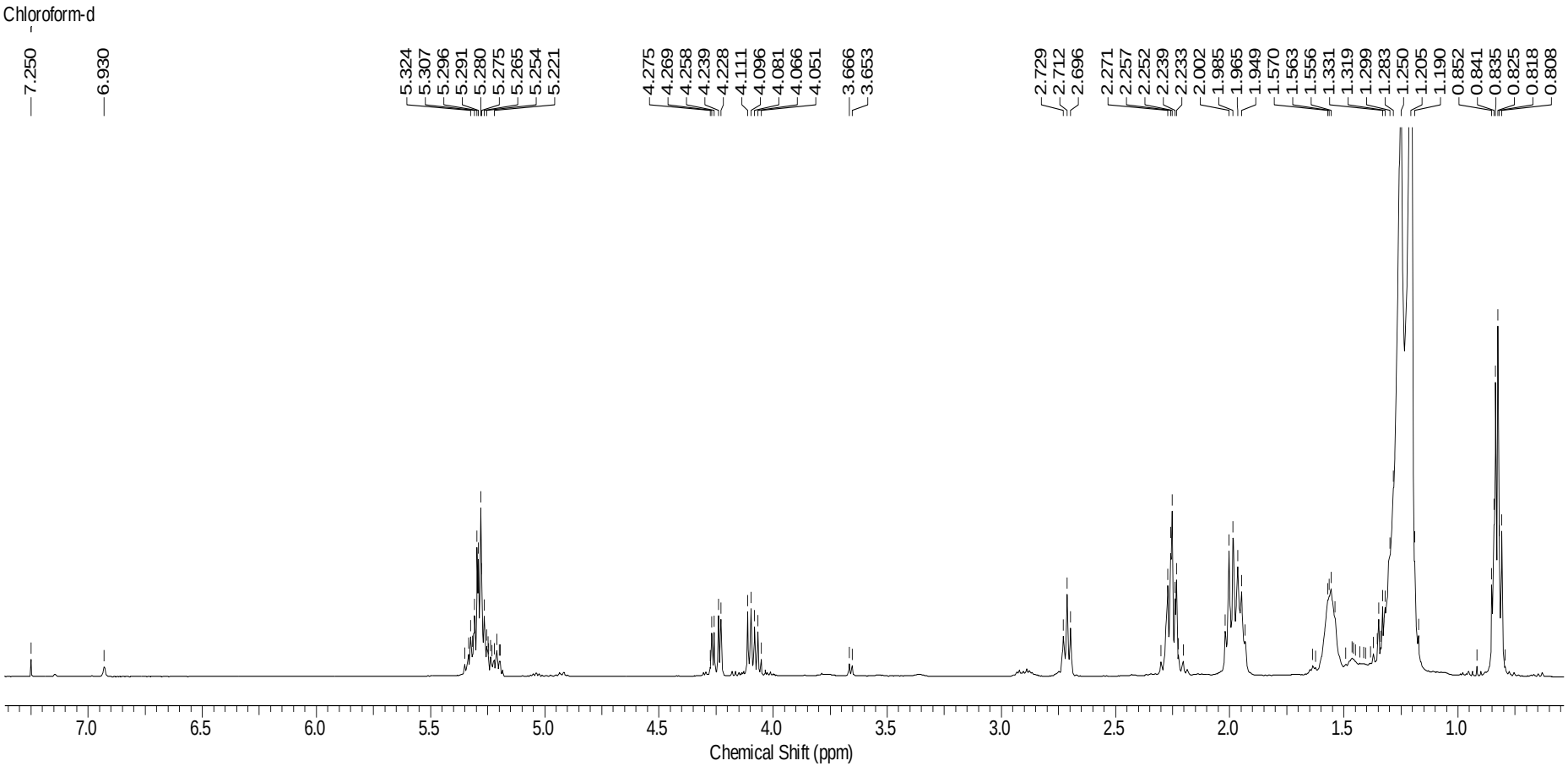


Figure 2.10 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the seeds of *Annona montana* in ethanol ($CDCl_3$, 400 MHz)

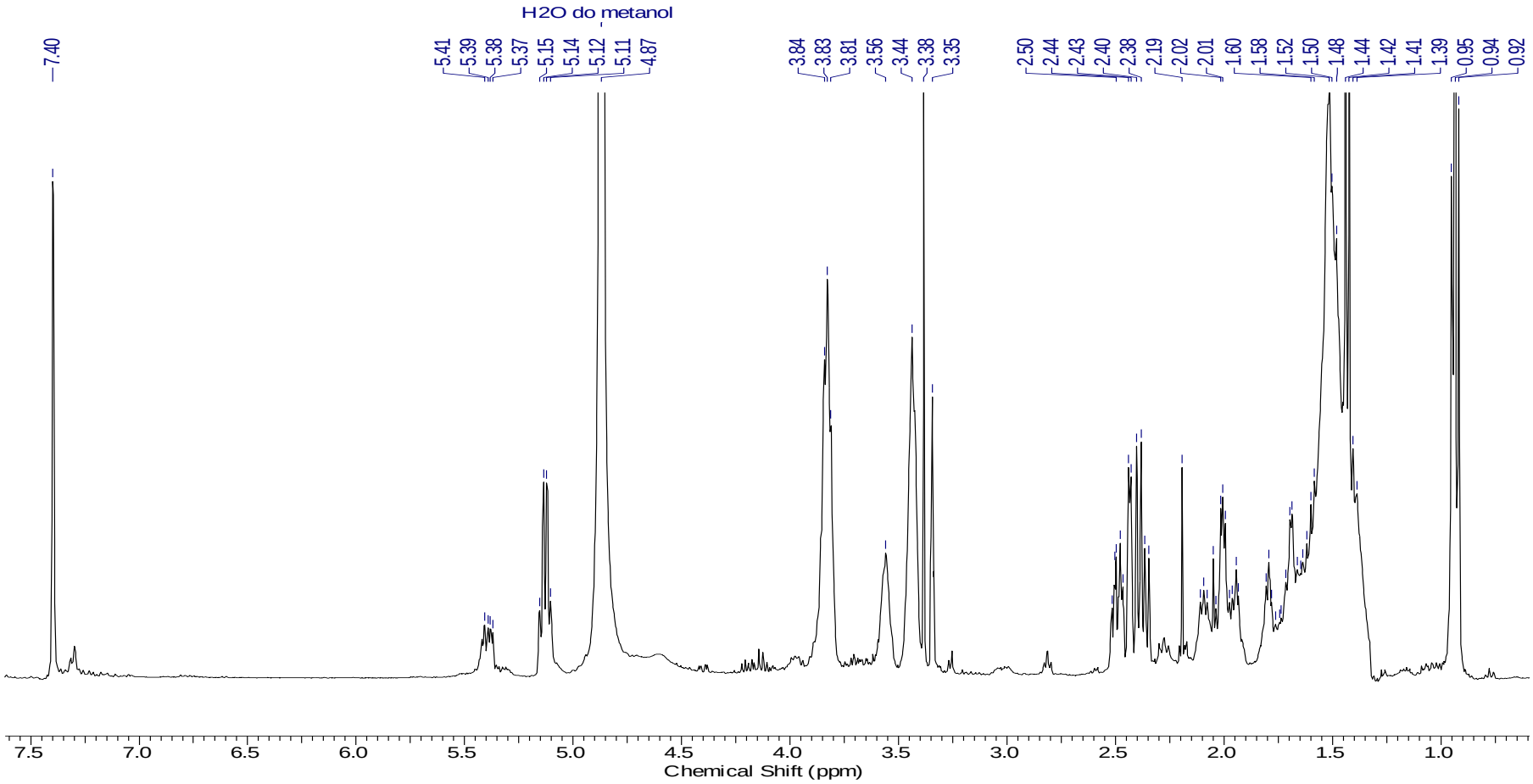


Figure 2.11 - ^{1}H NMR spectrum of the hydroalcoholic partition of the extract prepared from the seeds of *Annona montana* in ethanol (DMSO-d_6, 400 MHz)

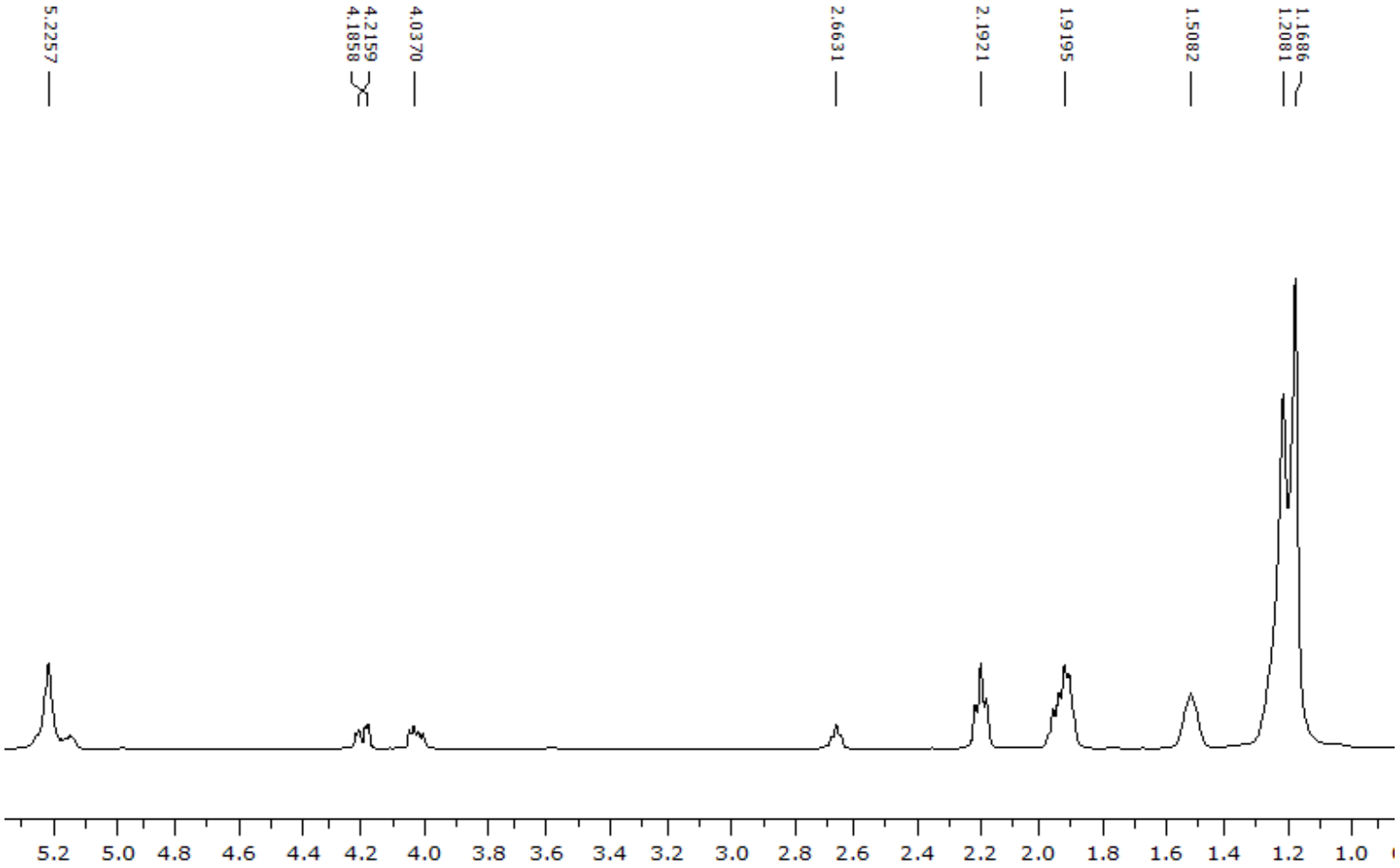


Figure 2.12 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the seeds of *Annona sylvatica* in ethanol ($CDCl_3$, 400 MHz)

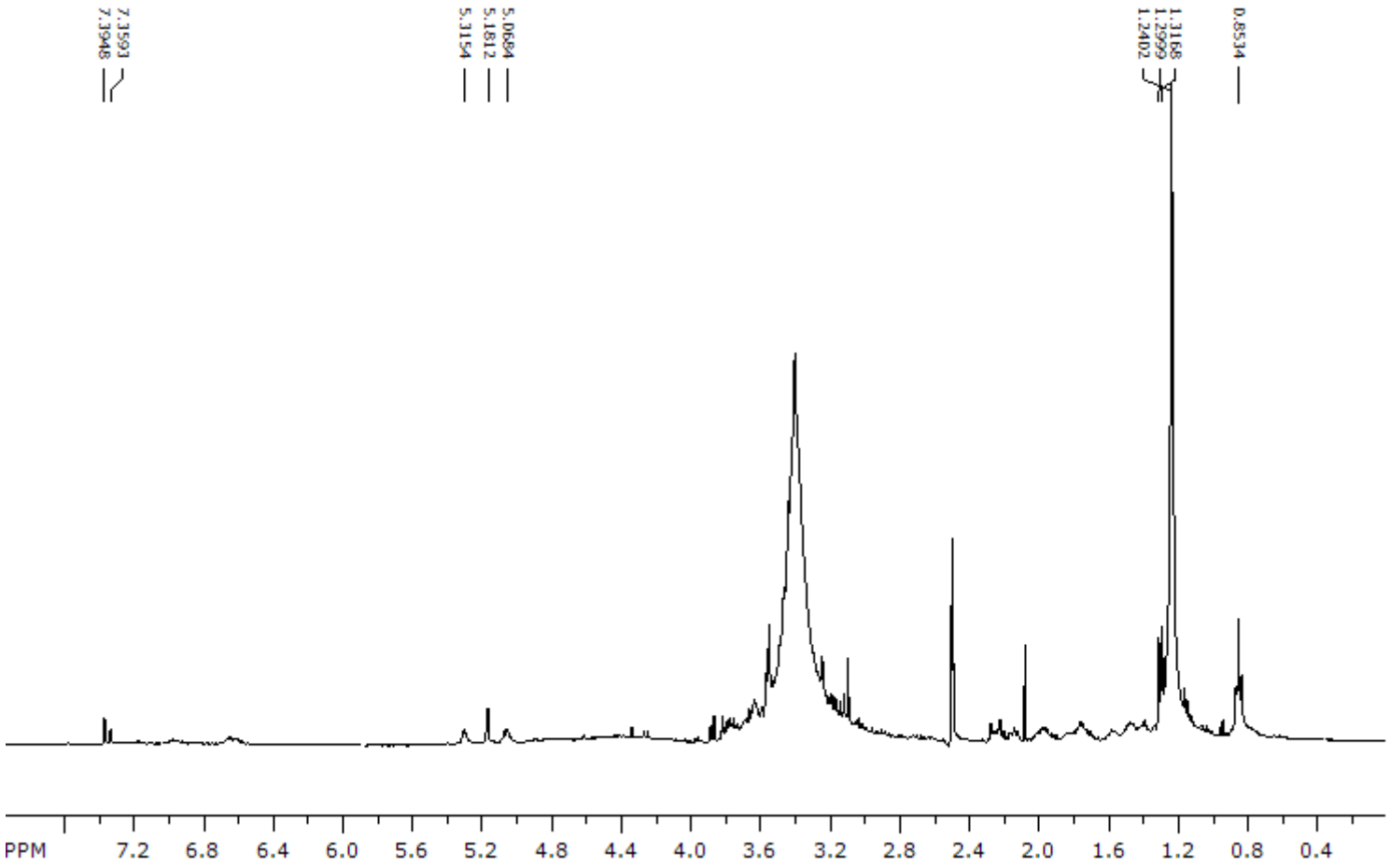


Figure 2.13 - ^{1}H NMR spectrum of the hydroalcoholic partition of the extract prepared from the seeds of *Annona sylvatica* in ethanol (DMSO-d_6, 400 MHz)

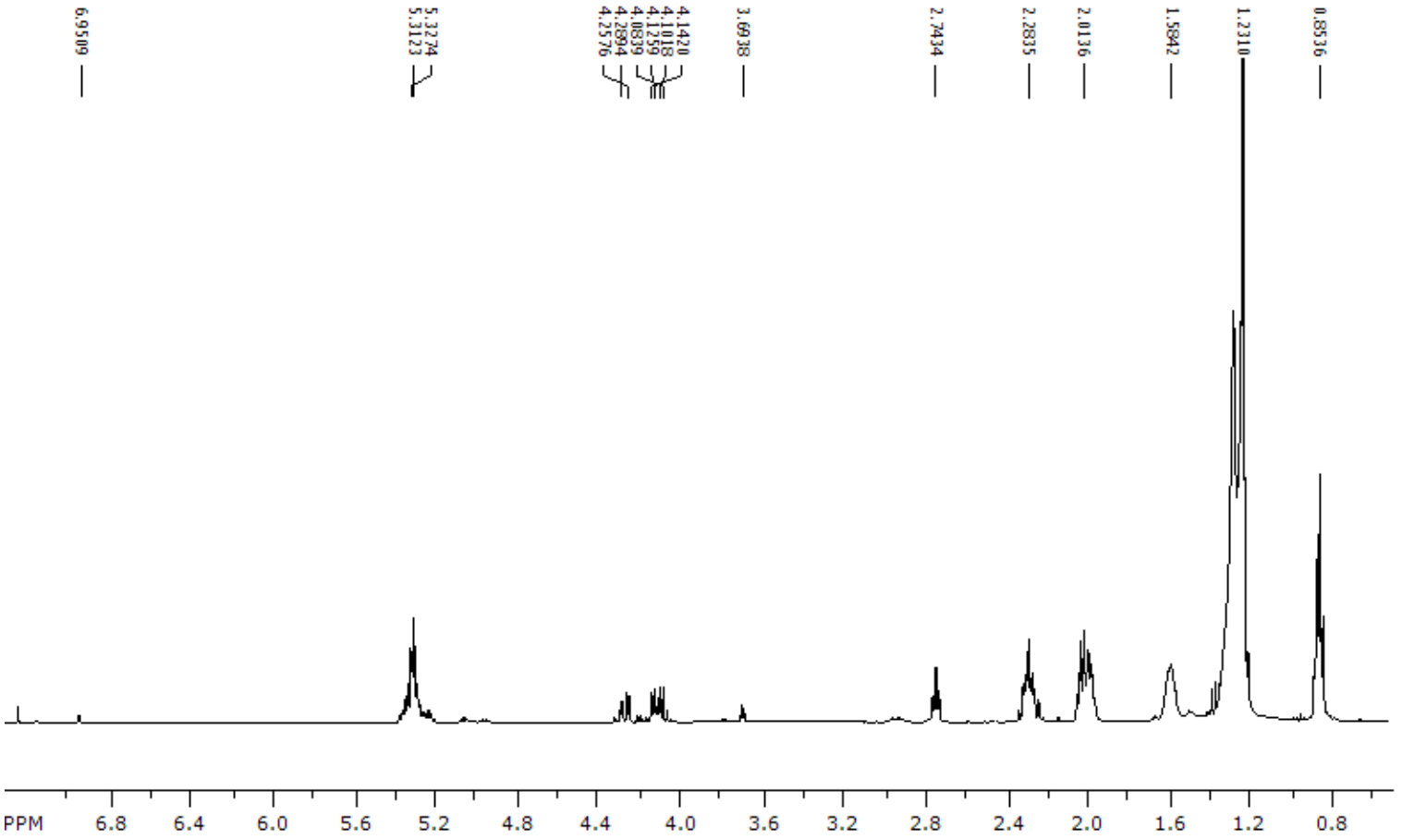


Figure 2.14 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the seeds of *Annona muricata* in ethanol ($CDCl_3$, 400 MHz)

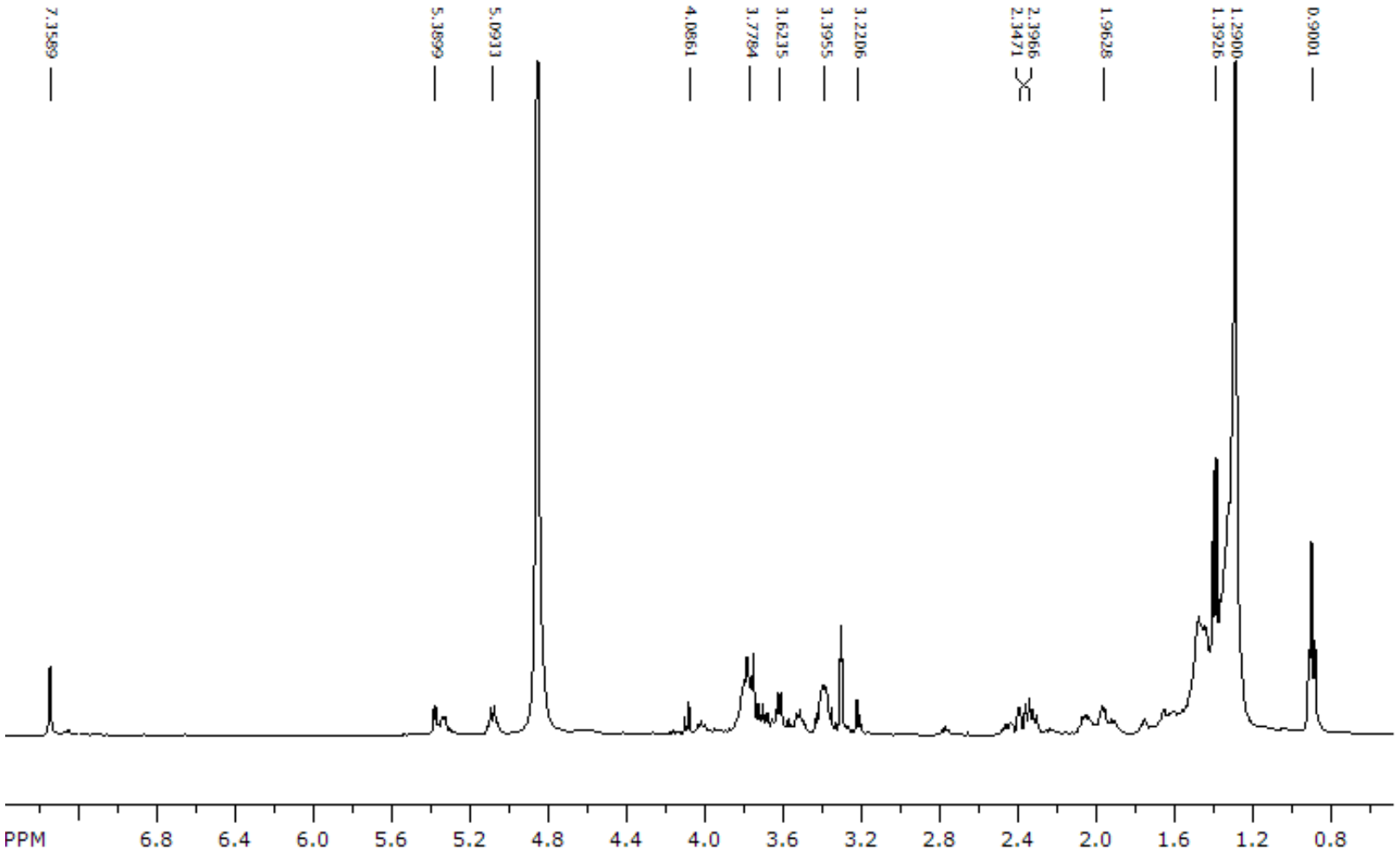


Figure 2.15 - ^{1}H NMR spectrum of the hydroalcoholic partition of the extract prepared from the seeds of *Annona muricata* in ethanol (DMSO-d_6, 400 MHz)

The active phases from selected leaf extracts are composed, in general, of mixtures that are more complex. The spectrum for hexane fractions of ethanolic extracts of *A. mucosa*, *A. montana*, and *A. muricata* (Figures 2.20, 2.21, and 2.22) showed very similar chemical profiles, allowing to observe signals indicative of the presence of acetogenins. In addition, there were indications of the presence of lignans, due to the occurrence of signals in the region around aromatic δ_H 6.5 ppm, doublets in δ_H 4 ppm and multiplet in δ_H 2.7 ppm, besides signals characteristic of metoxyl groups with shifts between δ_H 3.1 and 3.9 ppm.

There was also the presence of signals in the aromatic δ_H 8.0 ppm, which may be indicative of the presence of flavonoids and alkaloids. Because it is the spectrum of a little purified partition, it is not possible to state which classes of compounds are found, since many of them have signals in the same region, in addition to signals in many regions, and these signals are not well resolved because of the complexity and diversity of the compounds found.

The dichloromethanic partition of the ethanolic leaf extract of *A. muricata* showed signals in the region of acetogenins (Figure 2.23). In turn, the fraction in ethyl acetate of ethanolic leaf extract of *A. muricata* (Figure 2.24) showed similarities with the previous one, which may indicate the presence of acetogenins quite hydroxylated in the sample.

It is possible to observe in the ^{1}H NMR spectra of leaf hexane partition of *D. lanceolata* (Figure 2.25), the presence of characteristic signals of steroids, such as signals in δ_H 0.66 and 2.70 for the methynic protons, methylene and methyl, which indicate the existence of a steroidal skeleton. Moreover, such spectrum indicates the presence of alkaloids isoquinoline, aporphine and oxoaporphine (general structures shown in Figures 2.16, 2.17 and 2.18, respectively) due mainly to the presence of intense signals in the range of δ_H 3.70-4.20, which can be related to hydrogens of the O-Me group present in the structures. Signals in the δ_H 6.15-6.40 range may refer to hydrogens of replaced aromatic rings.

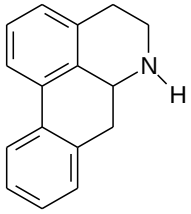
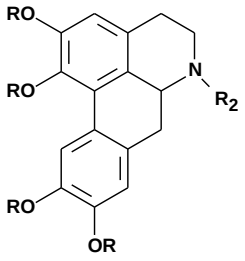
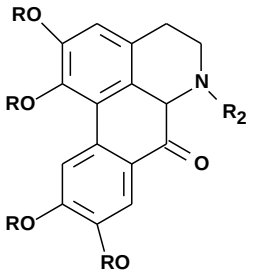


Figura 2.16 - General structure of isoquinoline alkaloids



R e R_2= H ou CH_3

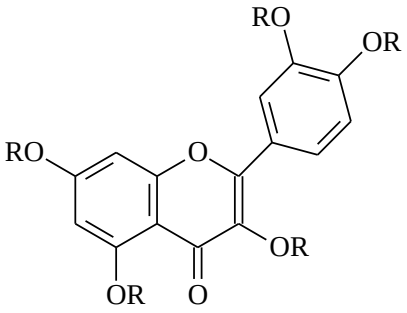
Figure 2.17 - General structure of aporphine alkaloids



R e R_2= H ou CH_3

Figure 2.18 - General structure of oxoaporphine alkaloids

Another class of substance found in the hexane partition of ethanolic leaf extract of *D. lanceolata* is the substituted flavonoids (general structure shown in Figure 2.19). They have characteristic signals in the δ_H 3.70-4.20 range; which can be related to hydrogens of the group O-Me and signals in the δ_H 6.15-6.40 range, which may be related to hydrogens of replaced aromatic rings.



R = H ou CH_3

Figure 2.19 – General structure of flavonoids

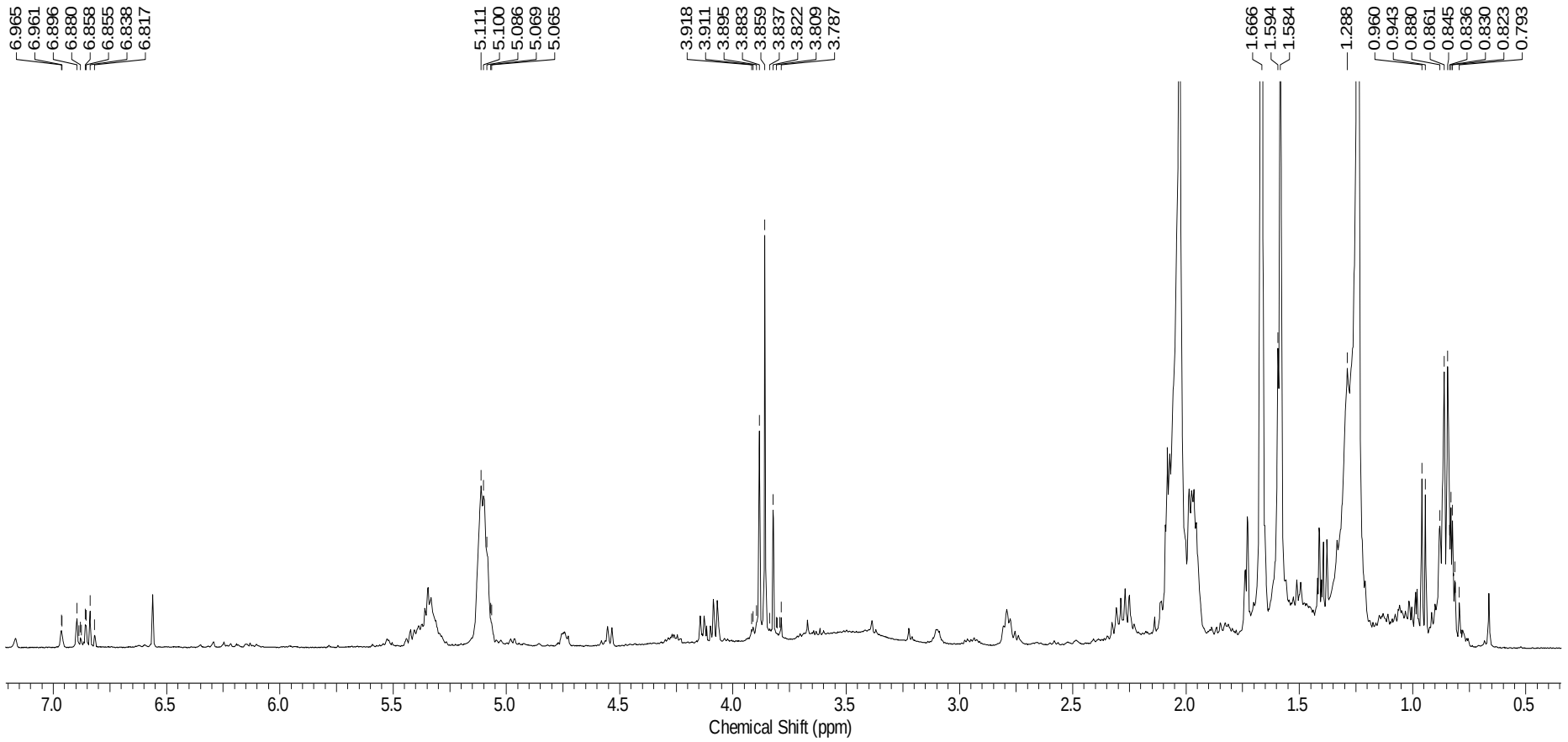


Figure 2.20 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the leaves of *Annona mucosa* in ethanol ($CDCl_3$, 400 MHz)

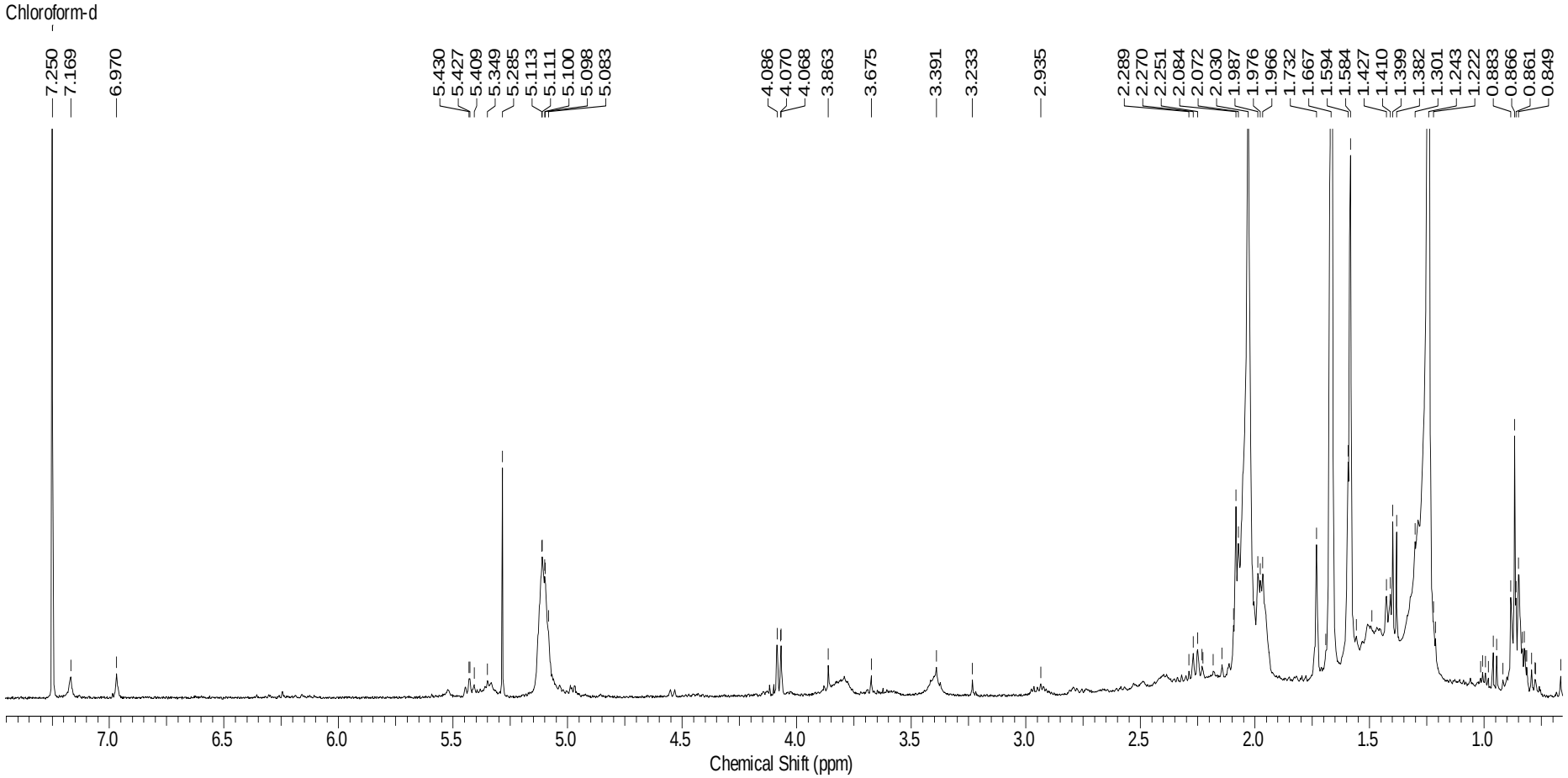


Figure 2.21 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the leaves of *Annona montana* in ethanol ($CDCl_3$, 400 MHz)

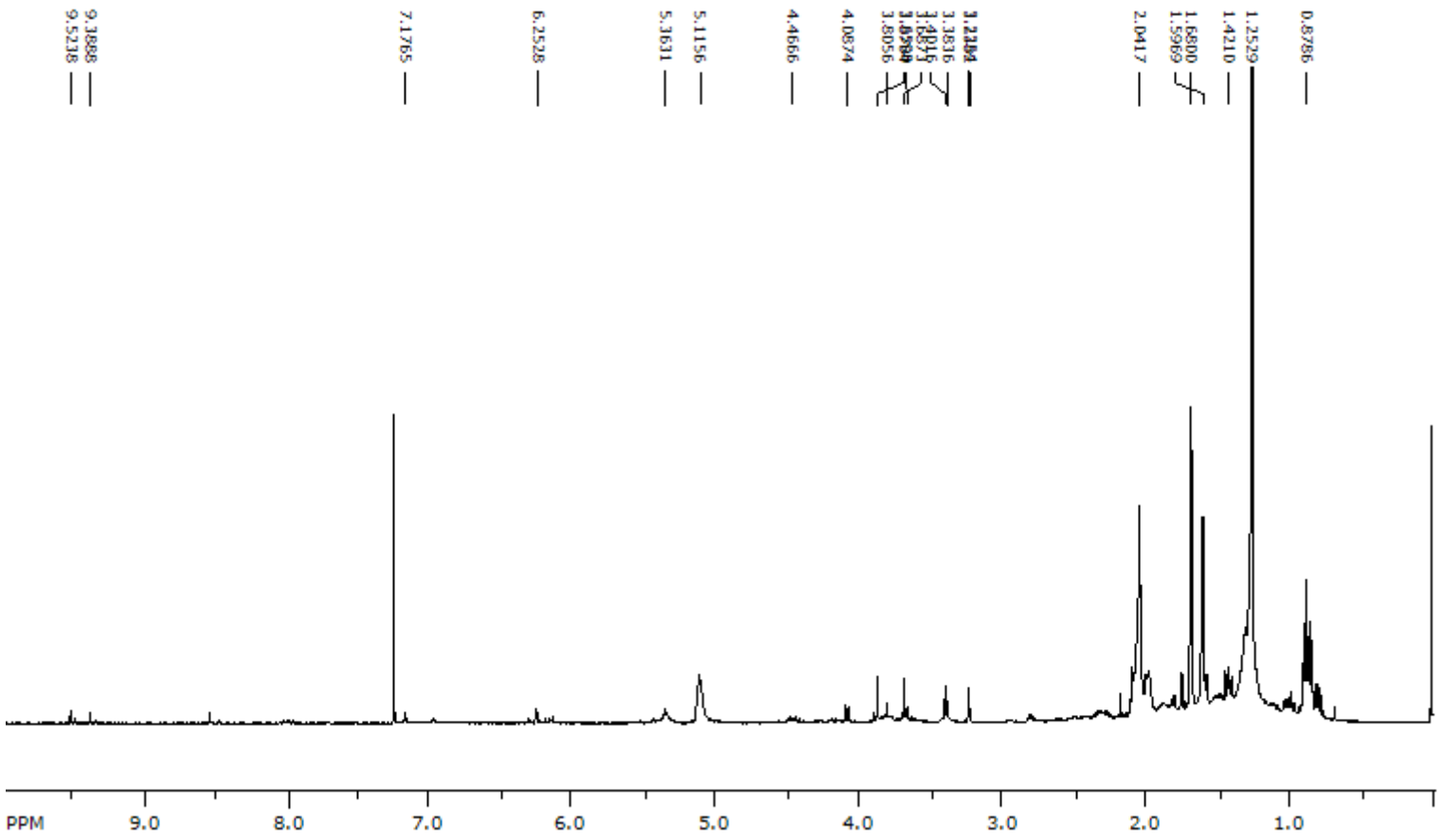


Figure 2.22 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the leaves of *Annona muricata* in ethanol ($CDCl_3$, 400 MHz)

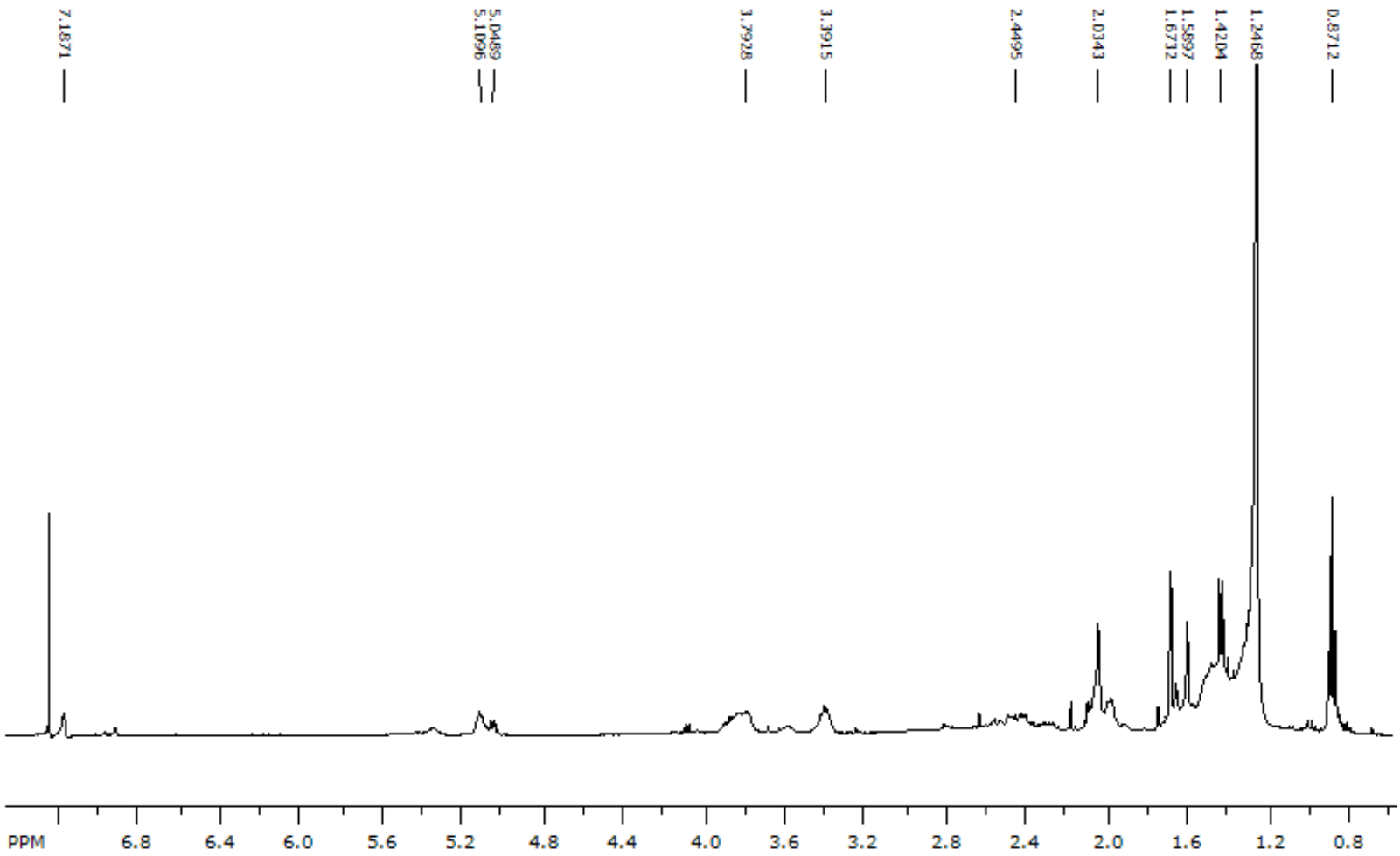


Figure 2.23 - ^{1}H NMR spectrum of the dichloromethane partition of the extract prepared from the leaves of *Annona muricata* in ethanol ($CDCl_3$, 400 MHz)

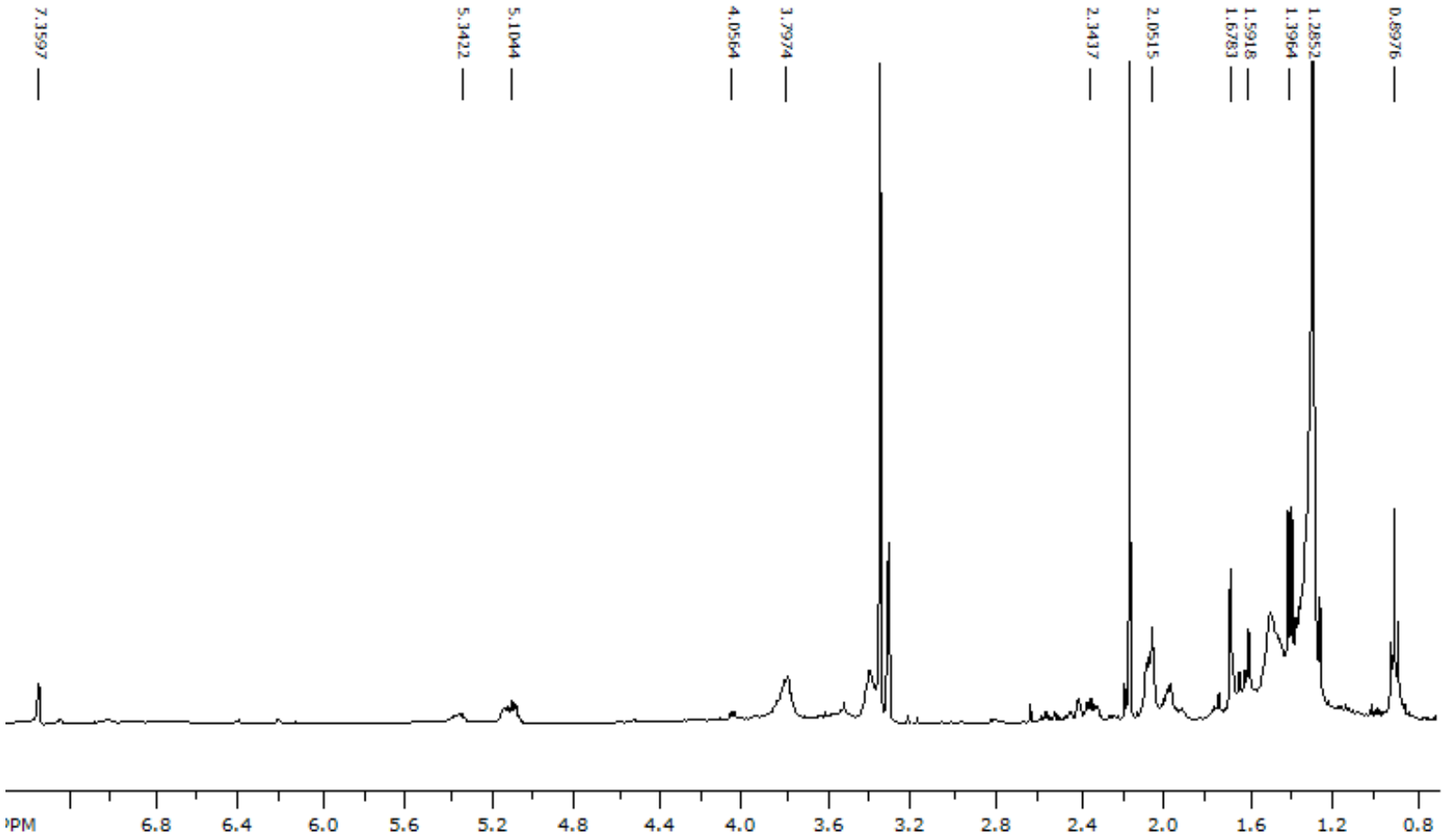


Figure 2.24 - ^{1}H NMR spectrum of the ethyl acetate partition of the extract prepared from the leaves of *Annona muricata* in ethanol (DMSO-$_{d6}$, 400 MHz)

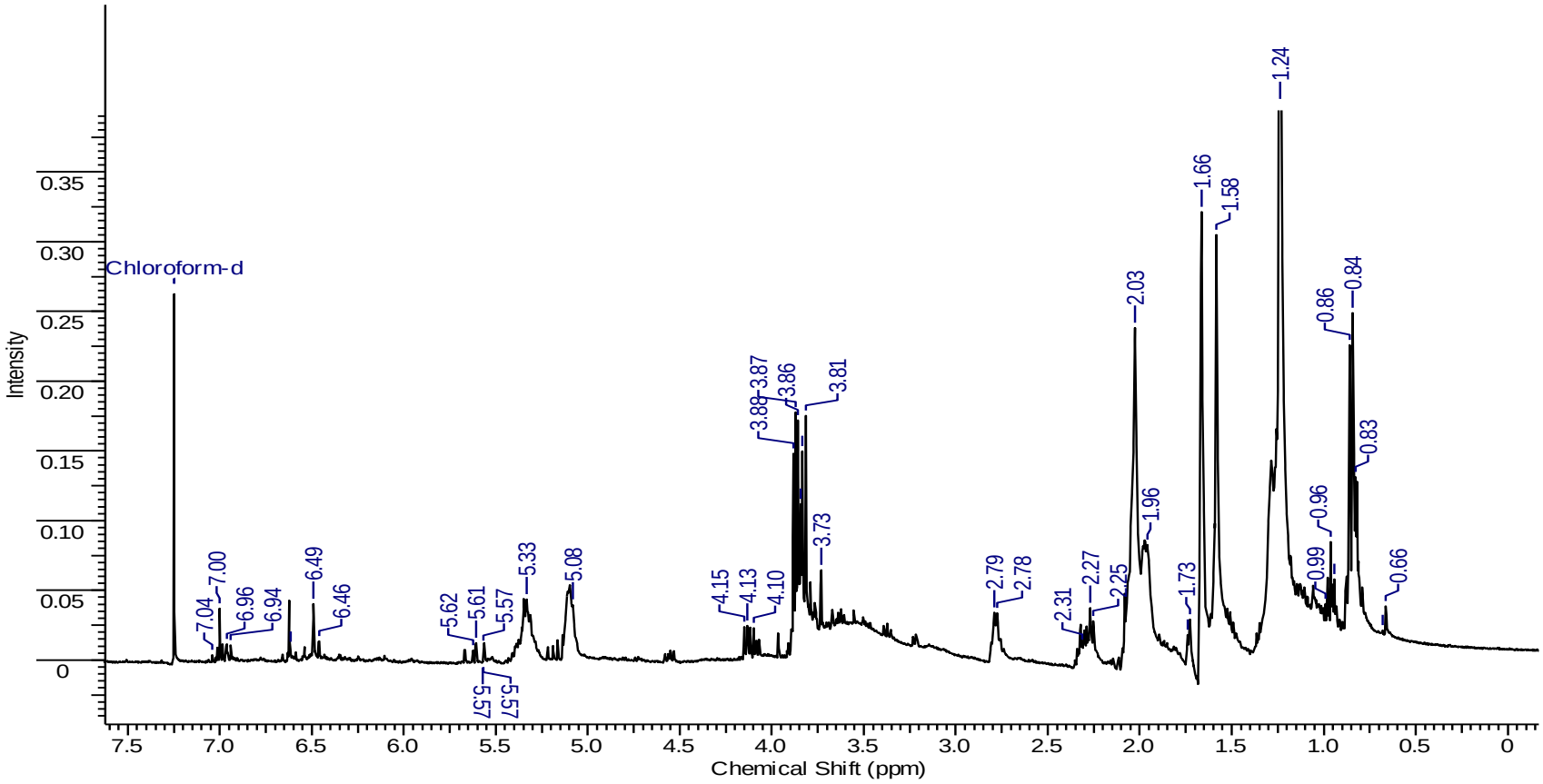


Figure 2.25 - ^{1}H NMR spectrum of the hexane partition of the extract prepared from the leaves of *Duguetia lanceolata* in ethanol ($CDCl_3$, 400 MHz)

2.2.2.6 Insecticidal activity of fractions obtained from the most active partitions

2.2.2.6.1 Fractions from hydroalcoholic partition of ethanolic extract of *Annona mucosa* seeds

At the concentration of 57.66 mg kg^{-1} (=1/5 of the estimated LC_{50} for its crude extract), fractions 4 and 5 from hydroalcoholic partition of an ethanolic extract of *A. mucosa* seeds (especially the first mentioned) caused significant mortality of *S. zeamais* adults, via residual contact (Figure 2.26).

Based on the ^{1}H NMR analyses, signals with chemical shifts characteristic of the presence of acetogenins and triglycerides were identified in both fractions, which may be interacting synergistically to cause toxicity to maize weevil. Afterwards, fraction 4 was selected because of its significant toxicity to *S. zeamais* (88.0%), which differed significantly from that observed in fraction 5 (28.1%), as well as for the similarities in chemical profiles of both active fractions observed in the ^{1}H NMR spectra.

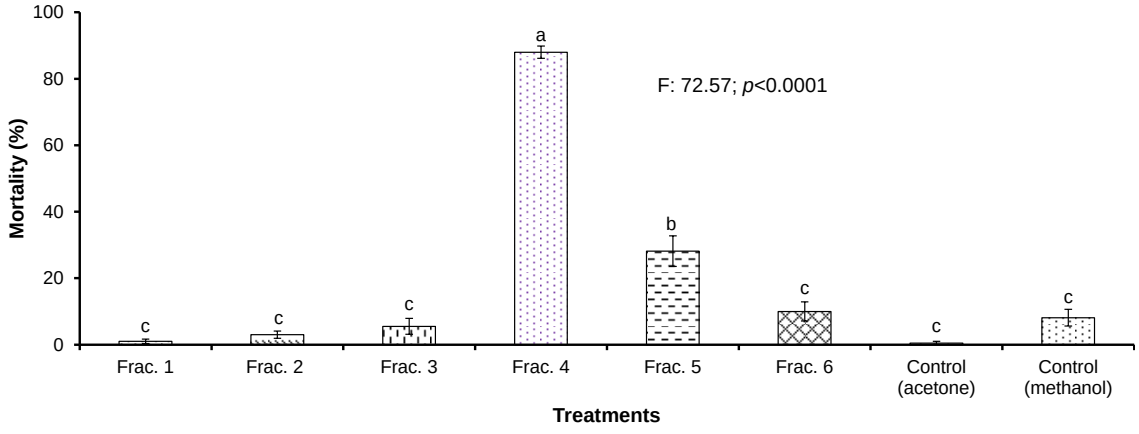


Figure 2.26 - Mortality on the 10th day after infestation (± standard error) of *Sitophilus zeamais* adults exposed to corn samples (10 g) treated with different fractions from hydroalcoholic partition of an ethanolic extract of *Annona mucosa* seeds. Temp.: 25±2°C; R.H.: 60±10%; photophase: 14 h; average luminosity: 200 lux. Bars followed by different letters indicate significant differences among the treatments (GLM with quasi-binomial distributions, followed by a *post hoc* Tukey test, $p<0.05$)

2.2.2.6.2 Purified fraction (rich in acetogenins) from hydroalcoholic partition of an ethanolic extract of *Annona sylvatica* seeds

The acetogenin-rich fraction, which was purified from the hydroalcoholic partition of an ethanolic extract of *A. sylvatica* seeds, caused significant mortality (75.8%) of *S. zeamais* adults when sprayed in corn samples (residual contact) at a concentration of 55.45 mg kg^{-1} (=1/10 of the estimated LC_{50} for its crude extract) (Figure 2.27). Because of the promising insecticidal action against *S. zeamais*, this fraction was subjected to chromatographic procedures to isolate the majority acetogenin(s) in the sample.

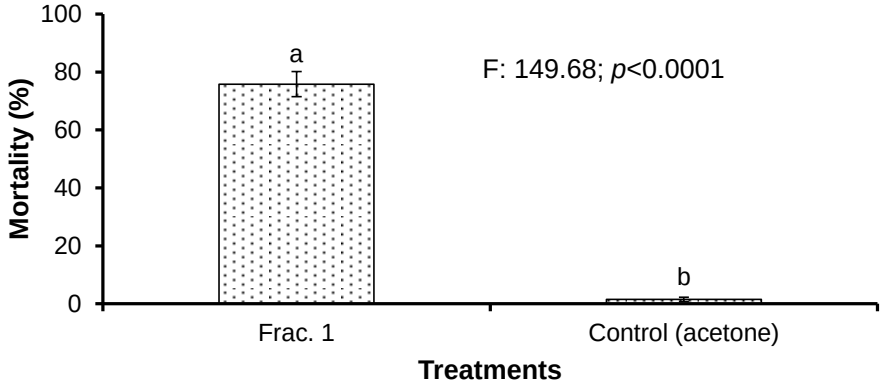


Figure 2.27 - Mortality on the 10th day after infestation (± standard error) of *Sitophilus zeamais* adults exposed to corn samples (10 g) treated with a purified fraction (rich in acetogenins) from hydroalcoholic partition of an ethanolic extract of *Annona sylvatica* seeds. Temp.: 25±2°C; RH.: 60±10%; photophase: 14 h; average luminosity: 200 lux. Bars followed by different letters indicate significant differences among the treatments (GLM with quasi-binomial distributions, followed by a *post hoc* Tukey test, $p<0.05$)

2.2.2.6.3 Fractions from hexane partition of an ethanolic extract of *Duguetia lanceolata* leaves

At the concentration of 750 mg kg^{-1}, fractions 2 and 6 from hexane partition of an ethanolic extract of *D. lanceolata* leaves (mainly fraction 2) caused significant mortality of *S. zeamais* adults, via residual contact (Figure 2.28). The ^{1}H NMR analysis indicated the presence of steroids and aromatic compounds in fraction 2 and isoquinoline alkaloids in fraction 6, which may be related to the insecticidal action against the maize weevil. Based on the same criteria used previously, fraction 2 was selected for further study.

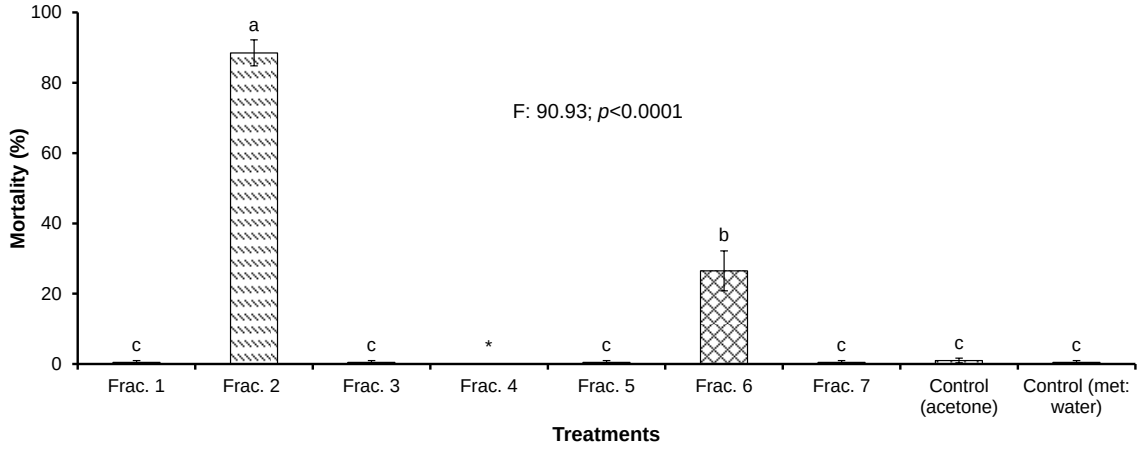


Figure 2.28 - Mortality on the 10th day after infestation (± standard error) of *Sitophilus zeamais* adults exposed to corn samples (10 g) treated with different fractions from hexane partition of an ethanolic extract of *Annona mucosa* leaves. Temp.: 25±2°C; RH: 60±10%; photophase: 14 h; average luminosity: 200 lux. Bars followed by different letters indicate significant differences among the treatments (GLM with quasi-binomial distributions, followed by a *post hoc* Tukey test, $p<0.05$). * Not included in the analysis (null variance)

2.2.2.7 Grain protective properties of isolate compounds from the most active fractions

2.2.2.7.1 Majority acetogenin from the most active fraction of *Annona mucosa*

Based on the chromatographic procedures used, the acetogenin bis-tetrahydrofuran rolliniastatin-1 (structure shown in Figure 2.29) was isolated and identified as the major component of the most active fraction (4) from hydroalcoholic partition prepared of an ethanolic extract of *A. mucosa* seeds. Although this acetogenin was already isolated in phytochemical studies conducted with seeds of *A. mucosa* (PETTIT et al., 1987) or even of structures of other species of Annonaceae [*Rollinia membranacea* (SAEZ et al., 1993), *Ophrypetalum odoratum* (SUNG'HWA et al., 1999), *R. emarginata* (FÉVRIER et al., 1999); *R. leptopetala* (ARIAGA et al., 2008) and *R. ocidentalis* (TOLOSA et al., 2012)], this was the first time that it was isolated in a bioguided study using an insect as bioindicator.

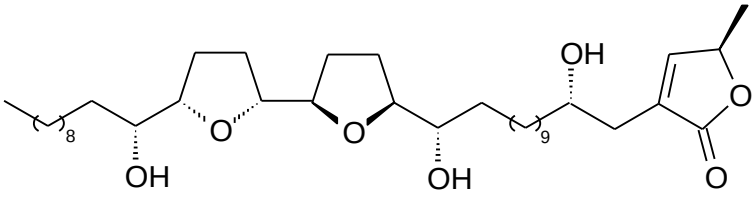


Figure 2.29 – Chemical structure of acetogenin rolliniastatin-1, majority compound of the most active fraction (4) from hydroalcoholic partition of an ethanolic extract of *Annona mucosa* seeds

At the concentration of 57.66 mg kg^{-1}, rolliniastatin-1 caused significant mortality (51.11%) of *S. zeamais* adults (Table 2.9). Although the acute toxicity level was lower than the formulation based on diatomaceous earth (Insecto®) used as positive control, this acetogenin also caused pronounced reduction in F_1 progeny and in the damage to the treated samples (Table 2.9 and Figure 2.30), and these effects are statistically similar to those caused by the positive control.

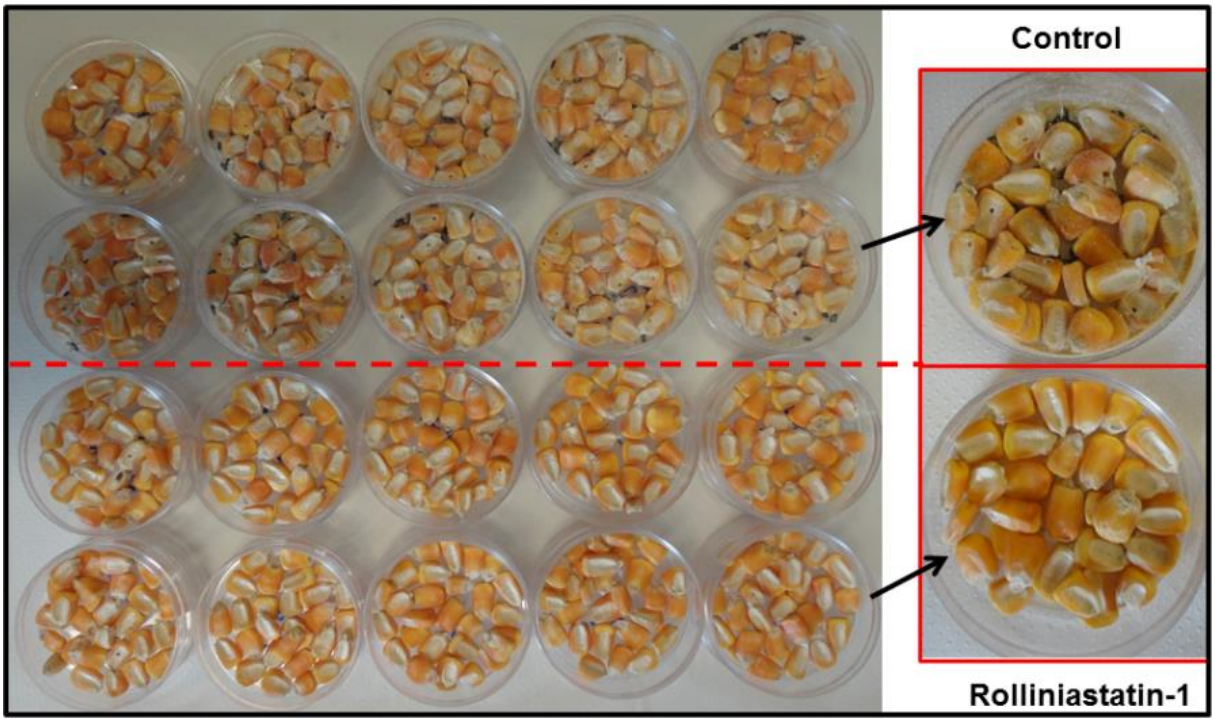


Figure 2.30 - Overview of experimental units (Petri dishes) treated and untreated with acetogenin rolliniastatin-1, after 60 days of infestation of corn grain samples (10 g) with *Sitophilus zeamais* adults

Table 2.9 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with acetogenin rolliniastatin-1 (57.66 mg kg^{-1}*) isolated of the most active fraction (4) prepared from hydroalcoholic partition of an ethanolic extract of *Annona mucosa* (Annonaceae) seeds. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux. Note: The formulation based on diatomaceous earth (Insecto®, at 1,000 mg kg^{-1}) was used as a positive control

Treatments	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight loss	
				Total (%)[3]	Relative[4]
Rolliniastatin-1	51.11±5.40 b	1.60±0.90 b	9.46±4.49 b	1.17	14.94
Insecto® (positive control)	99.5±0.50 a	0.10±0.10 b	0.36±0.36 b	0.04	0.57
Acetone (negative control)	0.50±0.50 c	18.50±1.86 a	62.49±4.47 a	7.85	100.00
F	194.11	59.592	56.905	--	--
p value	< 0.0001	< 0.0001	< 0.0001	--	--

[1]Means followed by different letters in the columns 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 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 with its respective control;
*Applied using a spray volume of 30 L t^{-1}.

2.2.2.7.2 Majority acetogenins from purified fraction of *Annona sylvatica*

From the purified fraction from hydroalcoholic partition of an ethanolic extract of *A. sylvatica* seeds, four majority acetogenins (ACG1, ACG2, ACG3, and ACG4) were isolated that were undergoing the structural determination phase and that, possibly, were acetogenins that had never been published or that will be described for the first time in *A. sylvatica*.

Based on the results obtained in the bioassay conducted with the maize weevil, acetogenins ACG4 was the only that caused mortality of adults different from that of control (Table 2.10). Even if it caused mortality far below that observed with the application of the commercial formulation based on diatomaceous earth (Insecto®) used as positive control, this acetogenin significantly reduced the number of emerged insects (F_1 progeny) and the damage to the treated samples, similar to that observed in the positive control, confirming the potential of this compound as a grain protector. Acetogenin ACG3 also caused significant sublethal effects, with reduction of F_1 progeny and damage to the treated samples. This fact reveals evidence of synergy effect between the different isolated acetogenins, which may be contributing significantly to the overall insecticidal activity of crude extract (ethanolic) against the maize weevil.

Table 2.10 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with acetogenins (27.72 mg kg^{-1}*) isolated of the purified fraction prepared from hydroalcoholic partition of an ethanolic extract of *Annona sylvatica* (Annonaceae) seeds. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux. Note: The formulation based on diatomaceous earth (Insecto®, at 1,000 mg kg^{-1}) was used as a positive control

Compounds	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight loss	
				Total (%)[3]	Relative[4]
ACG1	3.50±1.30 c	18.20±1.61 ab	62.97±3.34 ab	7.86	78.52
ACG2	1.00±1.00 c	22.33±1.68 a	75.70±5.34 a	9.46	94.51
ACG3	5.50±2.16 c	14.30±1.97 b	53.41±6.37 b	6.69	66.83
ACG4	30.50±4.62 b	4.20±1.38 c	18.17±4.77 c	2.24	22.38
Insecto® (positive control)	99.00±0.66 a	0.30±0.21 d	1.55±1.21 c	0.18	1.80
Acetone (negative control)	0.50±0.50 c	26.88±2.15 a	80.05±5.25 a	10.01	100.00
F	117.22	45.904	37.688	--	--
p value	< 0.0001	< 0.0001	< 0.0001	--	--

[1]Means followed by different letters in the columns 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 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 with its respective control;
*Applied using a spray volume of 30 L t^{-1}.

2.2.2.7.3 Majority compounds from the most active fraction of *Duguetia lanceolata*

Figure 2.31 shows the chemical structures of the steroids campesterol, stigmasterol and sitosterol (purified in mixture (8.44 + 12.37 + 79.19%, respectively) and of the aromatic compound 2,4,5-trimethoxystyrene, which were identified as major components of the most active fraction (2) from hexane partition of an ethanolic extract of *D. lanceolata* leaves. Although already detected in derived from other genera of *Duguetia* (WANG et al., 1988; MATOS et al., 2006; SILVA et al., 2007), such compounds had not yet been described in this species of Annonaceae.

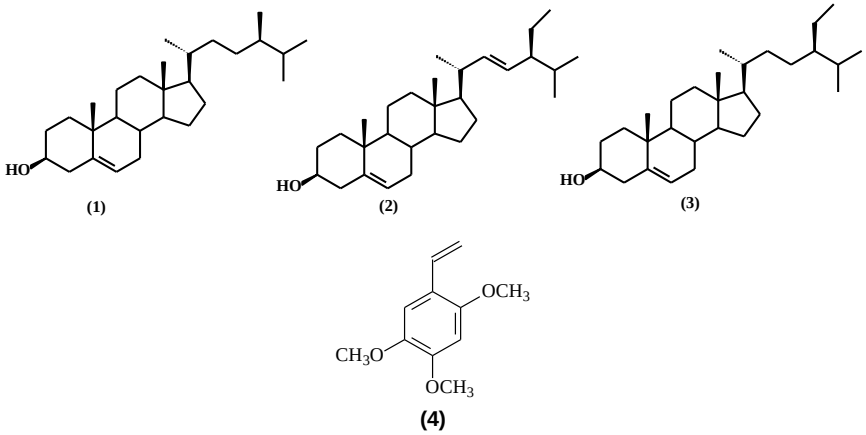


Figure 2.31 – Chemical structures of the steroids campesterol (1), stigmasterol (2), and sitosterol (3), and the aromatic compound 2,4,5-trimethoxystyrene (4), majority compounds isolated from the most active fraction from hexane partition of an ethanolic extract of *Duguetia lanceolata* leaves

At concentration of 75 mg kg^{-1}, the mixture of steroids (campesterol, stigmasterol and sitosterol) and the aromatic compound 2,4,5-trimethoxystyrene did not cause significant mortality of adults of *S. zeamais* (Table 2.11). These four compounds, however, reduced significantly F_1 progeny and damage to the treated samples without causing significant differences between both treatments. These results also indicate a synergy effect between these two chemical compounds classes.

Despite differences at the concentrations used, the grain protector effect provided by these compounds against the maize weevil was lower than that observed in the positive control. In this case, higher concentrations of these compounds are required for a more effective grain protection.

Table 2.11 - Mortality (mean ± standard error) on day 10, number of emerged insects (F_1 progeny) and damage after 60 days of infestation with *Sitophilus zeamais* in corn samples (10 g) treated with compounds (75 mg kg^{-1}*) isolated of the most active fraction (2) prepared from hexanic partition of an ethanolic extract of *Duguetia lanceolata* (Annonaceae) leaves. Temp.: 25±2°C; R.H.: 60±10%; Photophase: 14 h.; Mean luminosity: 200 lux. Note: The formulation based on diatomaceous earth (Insecto®, at 1,000 mg kg^{-1}) was used as a positive control

Compounds	Mortality (%)[1]	No. of emerged insects[2]	% Grains damaged[1]	Grain weight loss	
				Total (%)[3]	Relative[4]
Aromatic compound (2,4,5-trimethoxystyrene)	6.00±1.87	15.60±2.31 b	53.03±8.12 b	6.41	60.24
Mixture of steroids [(campesterol (8.44%) + stigmasterol (12.37%) + sitosterol(79.19%)]	3.00±2.00	17.20±2.35 b	52.71±4.73 b	7.17	67.39
Insecto® (positive control)	100.00±0.00*	0.20±0.20 c	1.33±1.33 c	0.09	0.85
Acetone (negative control)	1.00±1.00	29.40±3.38 a	85.10±4.72 a	10.64	100.00
F	2.0686 ^{ns}	41.288	35.058	--	--
p value	0.1691	< 0.0001	< 0.0001	--	--

[1]Means followed by different letters in the columns 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 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 with its respective control;
*Applied using a spray volume of 30 L t^{-1}; ** Not included in the analysis (null variance).

Overall, our results showed a promising grain protective properties provided by acetogenins isolated from *A. mucosa* (rolliniastatin-1) and *A. sylvatica* (especially ACG4), a previously unknown potential use for this class of natural compounds. Some studies already published confirm the entomotoxic action of acetogenin rolliniastatin-1 for some species of insect pests of importance to agriculture, causing both acute and chronic effects. In this context, González-Coloma et al. (2002) found a significant adult mortality of *Leptinotarsa decemlineata* Say, 1824 (Coleoptera: Chrysomelidae) exposed to rolliniastatin-1 (topical contact) as well as the growth inhibition of *S. littoralis* larvae. When incorporated into the diet of artificial neonate larvae of *S. frugiperda*, rolliniastatin-1 caused mortality rates of 90 and 100%, respectively at concentrations of 50 and 100 ppm (TOLOSA et al., 2012). Février et al. (1999) reported the tripanocidal action of rolliniastatin-1, however, isolated from *R. emarginata*.

On the other hand, only the acetogenin silvaticin had been, until then, isolated from *A. sylvatica* (MIKOLAJCZAK et al., 1990). These authors also found that silvaticin causes an effective protection for cantaloupe plants from harmful action of *A. vittata* Mikolajczak et al. (1990). The aromatic compound 2,4,5-trimethoxystyrene, isolated from *Pachypodanthium staudtii* (Annonaceae), caused significant adult mortality (LD_{50}: 0.07 mg kg^{-1}) and completely inhibited the F_1 progeny of *Acanthoscelides obtectus* Say, 1831 (Coleoptera: Bruchidae) (KOONA; BOUDA, 2006), which was not observed in our study on maize weevil even applied at higher concentrations.

In addition to the acute toxicity, our results showed the promising transgenerational effects of the active acetogenins isolated from *A. mucosa* and *A. sylvatica*, as well as the steroids and the aromatic compound isolated from *D. lanceolata*. Hypothetically, such effect may be caused by the deterrent action of feeding and/or oviposition or even the action of these compounds on embryonic and/or post-embryonic development of *S. zeamais*. In this case, the reduction of energy metabolism caused by the inhibition of enzymes involved in the mitochondrial electron transport system (GONZÁLEZ-COLOMA et al., 2002) may be the key factor for the occurrence of chronic (transgenerational) effects observed against the maize weevil.

In addition to the aspects related to agronomic effectiveness of these compounds, it is necessary a careful assessment of their possible effects on non-target organisms (especially mammals) and their behavior in the environment. In a preliminary approach, González-Coloma et al. (2002) found that cells in the ovary of a mammal (Chinese hamster) were less sensitive (~400 times) to acetogenin rolliniastatin-1 than *S. frugiperda* cells (Sf9), although enzymatic and immunochemical studies revealed a high similarity between the enzymes involved in cellular respiration of insects, mammals and fungi (LÜMMEN, 1998). However, structure-dependent membrane factors and the metabolic capacity of inactivation of acetogenins in different groups are aspects that may provide different sensitivity levels, which should be further investigated.

Considering all fractioning stages of the performed bioguided studies and the activity level obtained in the each stage of purification, our results clearly showed the occurrence of the synergism between compounds of both the same and distinct chemical classes. Corroborating our results, some studies (JIANG et al., 2009; SINGH et al., 2009; AKHTAR et al., 2012) demonstrated that minority constituents ("inactive") have synergistic effect on the major "active" constituents and that, although they are not individually active, their presence is necessary to achieve the overall biological activity of a given derivative. Synergistic effect of natural compounds were found in acute toxicity and behavioral effects of insect pests (deterrence and/or repellency) (AKHTAR; ISMAN, 2013). This strategy was possibly selected in the evolution course of plant species in order to reduce the possibility of adaptation of herbivores against allelochemicals synthesized by them (WINK, 2003).

Aiming at the development of botanical insecticides based on seeds of Annonaceae species, an abundant residue in fruit processing industries in Brazil (RIBEIRO et al., 2013), our results indicate that the use of little purified derivatives (crude extracts or partitions) are an effective measure (from the agronomic viewpoint) and efficient (from the economic viewpoint). However, further studies should be carried out to investigate the optimization of extraction methods of each derivative, which should take into account economical factor, applicability at industrial scale as well as the adequacy to the principles of environmental sustainability and green chemistry.

Because of their possible mode of action and potential grain protector properties observed in the present study, synthesis and semi-synthesis trials may be

conducted taking into consideration the chemical structure of acetogenins isolated from *A. mucosa* and *A. sylvatica* seeds. In case of success, such studies could lead to the development of a synthetic insecticide with a new mode/mechanism of action, broadening the range of active ingredients in the market, contributing significantly to the management of insect populations resistant to neurotoxins (RIBEIRO et al., 2003; PARKER et al., 2009; ARAÚJO et al., 2011; BRAGA et al., 2011) widely used in the preventive control of coleopterous pests of stored grain in Brazil. In turn, semi-synthesis studies can contribute to the understanding of the structure-activity relationship of these compounds, aspect of significant relevance for the development of more powerful and selective synthetic derivatives.

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3 COMPARATIVE BIOACTIVITY OF SELECTED SEED EXTRACTS FROM BRAZILIAN *Annona* SPECIES AND AN ACETOGENIN-BASED COMMERCIAL BIOINSECTICIDE AGAINST *Brassica* INSECT PESTS

Abstract

The acute and chronic toxicity of ethanolic seed extracts from selected Brazilian *Annona* species (*Annona montana*, *A. mucosa*, *A. muricata*, and *A. sylvatica*) and an acetogenin-based commercial bioinsecticide (Anosom®) were investigated against the cabbage looper *Trichoplusia ni* (Lepidoptera: Noctuidae) and the green peach aphid *Myzus persicae* (Hemiptera: Aphididae). In the laboratory, extracts of *Annona mucosa* and *A. sylvatica* as well as Anosom® were especially active through oral and topical administration. A greenhouse trial showed that a formulated *A. mucosa* extract and Anosom® were highly effective (>98% mortality) against third instar *T. ni* larvae, and comparable to a pyrethrin-based commercial insecticide (Insect Spray®) used as a positive control. Similar to results with *T. ni*, *A. mucosa* extract showed the greatest aphicidal activity followed by *A. sylvatica* extract and Anosom®. In another greenhouse trial, aphid population reduction from the formulated *A. mucosa* extract was superior to that provided by other treatments including the positive control. Though inferior to the *A. mucosa* extract, the acetogenin-based commercial insecticide (Anosom®) and *A. sylvatica* extract also reduced aphid populations in a manner comparable to the positive control. Botanical insecticides based on these Annonaceae derivatives could be useful in the framework of *Brassica* IPM in Brazil and elsewhere, especially for organic production.

Keywords: Annonaceae; Botanical insecticide; Acetogenins, Larval growth inhibitors; *Trichoplusia ni*; *Myzus persicae*

3.1 Introduction

Organic farming, especially horticultural production, represents one important approach to moving agriculture in a more sustainable direction, one that can provide added value to farmers in niche markets, enhance food security in developing countries, and deliver a range of ecosystem services to the public in general (GRANATSEIN; KIRBY; WILLER, 2010). Because of these factors and of the increasingly awareness of consumers, organic systems of food production, as well as other low-input systems, have expanded significantly worldwide in recent years (WILLER; KILCHER, 2009). However, technical barriers to insect pest management have often limited the feasibility and consolidation of this sector (RIBEIRO et al., 2012) mainly due to the limited number of tools and techniques which are compatible and available for integrated pest management (IPM) in these systems of food production (ZEHNDER et al., 2007).

Botanical insecticides can play an important role in IPM programs in low-input systems, mainly in small farming in developing countries (ISMAN, 2008). Plants produce, by means of their secondary metabolism, a diverse range of chemical compounds (allelochemicals) that mediate insect-plant interactions, mostly in defense against herbivory (WINK, 2003). These allelochemicals can constitute an important source of natural insecticides that can be utilized in IPM programs either as homemade insecticides from locally available species or as commercial formulations of biopesticides (ISMAN, 2006; REGNAULT-ROGER; VINCENT; ARNASON, 2012). Moreover, allelochemicalls can lead to development of new synthetic insecticides, a strategy already used to produce some of the major classes of insecticides in current use (CANTRELL; DAYAN; DUKE, 2012).

Among tropical plant families, the Annonaceae has shown great potential as a source of biopesticides (LEBOEUF et al., 1980; CHANG et al., 1998; KOTKAR et al., 2001), mostly attributable to the acetogenins, a class of natural compounds limited to some genera of the Annonaceae (ALALI; LIU; McLAUGHLIN, 1999). Acetogenins are potent inhibitors of complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial electron transport systems and of the NADH:oxidase in the plasma membrane (GONZÁLEZ-COLOMA et al., 2002). These compounds also induce apoptosis (programmed cell death) likely as a result of the deprivation of ATP (TORMO et al., 1999), which is reflected in potent bioactive effects including promising insecticidal and/or acaricidal activities (CÓLOM et al., 2008).

In order to obtain new insecticidal compounds for use in pest management in stored grains, our current screening program of bioactive allelochemicalls from Neotropical Annonaceae verified promising bioactivities of ethanolic extracts from seeds of four Brazilian *Annona* species (*Annona montana*, *A. mucosa*, *A. muricata* and *A. sylvatica*) against the maize weevil *Sitophilus zeamais* (RIBEIRO et al., submitted paper). In light of these findings, these ethanolic extracts are being investigated for their potential as important tools for management of insect pest species as homemade insecticides or in the production of new commercial botanical insecticides. In addition to isolation of actives principles and assessment of their bioactivity to non-target organisms, it is important to assess their bioactivity on other economically important insect pests of agriculture to determine their spectra of action, including pest species that occur in organic farming, a growing niche of market.

Brassica crops (Brassicaceae) are extensively grown in organic systems

throughout the Western Hemisphere. Both the cabbage looper *Trichoplusia ni* (Hübner, 1803) (Lepidoptera: Noctuidae) and the green peach aphid *Myzus Persicae* (Sulzer, 1776) (Hemiptera: Aphididae) are important widespread insect pest species of *Brassica* crops growing under greenhouse and field conditions in both tropical and temperate regions. Previous studies have shown the potential of crude extracts from *A. squamosa* seeds in the control of the cabbage looper in laboratory tests (LEATEMIA; ISMAN, 2004a, 2004b; SEFFRIN et al., 2010) and in the protection of cabbage plants in the greenhouse (LEATEMIA; ISMAN, 2004c; SEFFRIN et al., 2010). Recently, a bioinsecticide based on a botanical extract of *A. squamosa* (Anosom®) (AgriLife SOM Phytopharma Ltda., Hyderabad, Andhra Pradesh State, India) was registered in India, providing an important alternative for pest control in organic crops on larger scales. It contains the acetogenin annonin as main active ingredient and is formulated as a 1% emulsifiable concentrate (10,000 ppm). Although fewer studies of acetogenins have been conducted using sucking insects, the acetogenin annonin (= squamocin), which was isolated from *A. glabra* seeds, caused strong toxicity to *M. persicae* and it did not show mutagenicity (GUADAÑO et al., 2000), an important parameter to assess the environmental safety of xenobiotic compounds. These findings provide the impetus for additional investigations to detected new sources of botanical insecticides for use in the protection of *Brassica* crops.

Objectives of the present study are to evaluate the insecticidal and sublethal effects of seed extracts from selected Brazilian *Annona* species (*A. montana*, *A. mucosa*, *A. muricata* and *A. sylvatica*) and an acetogenin-based commercial bioinsecticide against *T. ni*. In addition, the most promising extracts, selected based on their effects on *T. ni*, were tested against the green peach aphid in order to better characterize the potential of these annonaceous derivatives as *Brassica* protectants. For both insect pests, bioassays were conducted under laboratory and greenhouse conditions. In greenhouse tests, the efficacy of the selected Annonaceae derivatives was compared to that of a pyrethrin-based commercial bioinsectide (positive control).

3.2 Development

3.2.1 Material and methods

3.2.1.1 *Annona* crude seed extracts: sources and preparation

Information about the *Annona* species used in the study is shown in Table 3.1. Voucher specimens of these species, previously identified by Prof. Dr. Renato Mello-Silva {Department of Botany, Biosciences Institute/University of São Paulo (IB/USP)}, were deposited in the herbarium of the Department of Biological Sciences at "Luiz de Queiroz" College of Agriculture/University of São Paulo, in Piracicaba municipality, São Paulo State, Brazil.

Table 3.1 - *Annona* species used in the study: collection data

Species	Local of collection	Date of collection	Number of voucher
Annona montana Macfadyen	Piracicaba municipality, SP State, Brazil (22º42'28.2" S; 47º37'59.4" W; Elevation: 537 m)	21/03/11	121203
Annona mucosa Jacquin	Piracicaba municipality, SP State, Brazil (22º42'28.5" S; 47º37'59.6" W; Elevation: 534 m)	17/03/11	120985
Annona muricata Linnaeus	Piracicaba municipality, SP State, Brazil (22º42'25.4" S; 47º37'43.9" W; Elevation: 576 m)	12/04/11	121892
Annona sylvatica A. St.-Hill	Erval Seco municipality, RS State, Brazil (27º25'41.8" S; 53º34'11.2" W; Elevation: 466 m)	25/04/11	121205

To prepare the extracts, seeds collected from ripe fruits were dried in an oven at 38°C for 48 h. Subsequently, they were milled to a powder in a knife mill. Powders were stored in sealed glass containers and kept at ~ -10^{o}C until use. Organic extracts were obtained by cold maceration using ethanol as a solvent (5:1, v/w), with the seeds powders held in ethanol for three days, after filtering through paper. This process was repeated three times. Remaining solvent was removed *in vacuo* at 50^{o}C. After complete evaporation of the solvent in an air flow chamber, the extraction yield for each species was determined.

3.2.1.2 Test insects

T. ni eggs were obtained from Insect Production Services Company (Sault Ste. Marie, Ontario, Canada) and larvae were reared on an artificial diet as previously described (AKHTAR; ISMAN, 2004). Colonies of green peach aphid (*M. persicae*)

were maintained on cabbage plants grown in the Horticulture Greenhouse at the University of British Columbia (UBC).

3.2.1.3 Screening of *Annona* seed extracts in the laboratory

Insecticidal activity of the extracts was determined through oral and contact toxicity bioassays as was their effect on larval development against cabbage looper larvae. Concentrations used were determined based on previous studies (LEATEMIA; ISMAN, 2004 a, 2004b, 2004c; SEFFRIN et al., 2010).

3.2.1.3.1 Oral toxicity bioassay

Oral toxicity was assessed by adding each extract to an artificial diet, according to the procedure described previously (AKHTAR; ISMAN, 2004; SEFFRIN et al., 2010). A 2 mL solution of each extract was added to 7 g of dry artificial diet (No. 9795, Bio-Serv Inc., Frenchtown, New Jersey, United States) supplemented with finely ground alfalfa, to improve acceptability, and vitamins (No. 8045, Bio-Serv Inc.). Following evaporation of the solvent, the diet was mixed with a boiling agar solution (1 g of agar + 32 mL of water) to produce 40 g of treated diet with the extract at a concentration of 1,000 mg kg^{-1} fwt.

Each treated diet was cut into equal sized pieces (~1 g) and placed into individual compartments of a plastic assay tray. A cabbage looper neonate larva (<24 h old) was placed into each compartment (n=40). Controls were treated with solvent alone {acetone:methanol, 2:1 (v/v) for the extracts and distilled water for the bioinsecticide}. Mortality of larvae was assessed at day 10. LC_{50} and LC_{90} values (concentrations causing 50 and 90% mortalities, respectively) were determined for the active treatments.

3.2.1.3.2 Contact toxicity bioassay

The contact toxicity of each treatment was evaluated in third instar *T. ni* larvae by means of topical application and contact with a treated substrate (filter paper).

a) Topical application: Groups of ten 3rd instar *T. ni* larvae were placed in plastic Petri dishes (9 cm diameter) lined with filter paper. A 1µl aliquot of solution (=100 µg $larva^{-1}$ of extract) was applied to the dorsal surface of each larva using a microsyringe (Hamilton Company, Reno, Nevada, USA). Controls were treated with solvents alone. Treated or control larvae were introduced into Petri dishes each

containing a piece (~ 2 g) of artificial diet. Six replicates of 10 larvae each were used per treatment (n=60).

Insect mortality was assessed at 24, 48, and 72 hours. Larvae were considered dead if they did not move following stimulation with a forcep. LD_{50} and LD_{90} values (doses causing 50% and 90% mortalities, respectively) were calculated for the active treatments.

b) Contact with the contaminated substrate (filter paper): Filter paper discs (9 cm diameter) were placed on insect pins attached to a polystyrene base to prevent loss of solution (RIBEIRO, 2010). Each disc received an aliquot of 1 mL of solution at a concentration adjusted to provide an equivalent dose of 1,000 µg cm^{-2}. Control discs were treated with solvents alone as described previously.

After ~20 minutes of application (time required for evaporation of the solvent), the treated filter paper discs were placed into Petri dishes (9 cm diameter). Group of 10 3rd instar larvae were introduced in each dish with a piece of artificial diet (~ 2 g). For each treatment, five discs were used (n=50). Larval mortality was assessed after 24, 48, and 72 hours. LC_{50} and LC_{90} values (concentrations causing 50 and 90% mortality, respectively) were calculated for the active treatments.

3.2.1.3.3 Growth inhibition

Surviving insects in the oral toxicity bioassay were weighed on day 10. EC_{50} values (concentration causing 50% larval weight reduction) were calculated for the active treatments.

3.2.1.4 Aphicidal activity of selected seed extracts (laboratory test)

The most active extracts based on bioassays with the cabbage looper were assessed for aphicidal activity against green peach aphids, *M. persicae,* for demographic effects (STARK; BANKS, 2003). Potted (300 mL) cabbage plants (*Brassica oleracea* var. *capitata* 'hybrid Stonehead'), 40-45 days old (5-6 leaves), were infested with 15 apterous adult female aphids using a thin brush. Infested plants were kept in growth chambers at a temperature of 25±2^{o}C and a photoperiod of 16:8 (L: D) for colony (population) establishment for a period of 5 days.

After the colonization period, numbers of aphids on each plant were counted. Aqueous solutions of selected extracts emulsified with 5 g L^{-1} of Tween 80® were sprayed, by means of a manual atomizer, to the point of runoff. The concentration

used (2,500 mg L^{-1}) was determined based on efficacy against *T. ni* larvae. Six plants were used for each treatment.

After five days (120 hours), numbers of live aphids per plant were counted and compared with pretreatment numbers. In addition, the instantaneous population growth rate (*ri*) was calculated based on Stark and Banks (2003) using the formula:

$$ri = \ln (Nf/Ni)/\Delta t$$

where Nf = final number of individuals in the populations, Ni = initial number of individuals in the population, and Δt = time (days) of observation post treatment. Positive values of *ri* indicate a population increase during the observation period, and negative values of *ri* indicate a population decrease; values of *ri* = 0 indicate no numerical change in the population.

3.2.1.5 Cabbage plant protection in the greenhouse

In order to determine the efficacy of selected extracts at 2,500 mg L^{-1} for protection of cabbage plants, experiments were conducted in a horticultural greenhouse under grow lights (16:8; L:D period). Potted cabbage plants as described above were used. A commercial bioinsecticide - Insect Spray® (Schultz Bridgeton Co., New Jersey, United States) containing 200 ppm pyrethrins as the active ingredient was used as a positive control.

***Trichoplusia ni* test:** Each cabbage plant (n=10) was infested with eight 3rd instar *T. ni* larvae. After 24 hours, the number of remaining larva on each plant was counted. Each plant was then sprayed with an aqueous solution of extract containing 5 g L^{-1} of Tween 80®, to the point of runoff, by means of a manual atomizer. After air-drying, each plant in its pot was covered with a perforated acetate bag, and the bag was closed using twist ties. After five days (120 hours), the number of live larvae on each plant was counted and the surviving larvae weighed.

***Myzus persicae* test:** The same procedure was used in the greenhouse as that described above for the lab bioassay. However, each plant in its pot was covered with a perforated acetate bag, closed using twist ties, in order to exclude parasitoids and predators of aphids existing in the greenhouse.

3.2.1.6 Data analyses

All bioassays were conducted using a completely randomized design. Generalized linear models (GLM) (NELDER; WEDDERBURN, 1972) of the quasi-binomial and Gaussian distributions were used for data analysis of the mortality rates and larval weight, respectively. GLM with quasi-Poisson distributions was used for the analysis of aphid counts. In all of cases, the fit of the GLM was determined by using the half-normal probability plot with a simulation envelope (HINDE; DEMÉTRIO, 1998). When significant differences were found among treatments, multiple comparisons (Tukey test, $p<0.05$) via the glht function in the multicomp package with adjusted *p* values was performed. All of these analyses were carried out using "R" statistical software, version 2.15.1 (R DEVELOPMENT CORE TEAM, 2012). Proportional mortality data from the greenhouse experiments were corrected using the Henderson and Tilton (1955) formula.

To obtain lethal concentrations/doses (LC_{50} and LC_{90} or LD_{50} and LD_{90}), mortality data were fitted with a binomial model using the log-log (gompit) complement connection function in PROC PROBIT – SAS software version 9.3 (SAS INSTITUTE, 2013). To determine effective concentration (EC_{50}) and respective confidence intervals (95%), non-linear logistical models adapted from Sims et al. (1996) were performed using the JMP (SAS) software version 10 (SAS Institute, 2013).

3.2.2 Results and discussion

3.2.2.1 Screening of *Annona* extracts in the laboratory

The oral toxicity bioassay indicated that the seed extract of *A. mucosa* (at 1,000 mg kg^{-1}) was the most active against *T. ni* larvae (Table 3.2) resulting in high mortality (LC_{50}: 328.86 mg kg^{-1}, Table 3) and strong growth inhibition (EC_{50}: 114.71 mg kg^{-1}) in a concentration dependent manner. Although somewhat less active, the extract of *A. sylvatica* and the acetogenin-based commercial insecticide (Anosom®) also caused significant acute toxicity (LC_{50}: 690.11 and 700.37 mg kg^{-1}, respectively, Table 3.3) and growth inhibition (EC_{50}: 254.62 and 223.37 mg kg^{-1}, respectively) of *T. ni* larvae, and were equitoxic based on their confidence intervals (Table 3.3). Seed extracts of *A. montana* and *A. muricata* (EC_{50}: 2,758.30 and 3,159.88 mg kg^{-1},

respectively) were ~ 24 and 28-fold (respectively) less active than the highly active extract of *A. mucosa* in reducing growth of *T. ni* larvae.

However, *A. mucosa* and *A. sylvatica* seed extracts were equitoxic via topical application in 3rd instar *T. ni* larvae (Table 3.2), both extracts exhibiting dose and time responses (Table 3.4). Regardless of exposure time, these crude extracts were ~ 1 to 4-fold more active than the bioinsecticide Anosom®, which also exhibited a strong dose response (Table 4). However, Anosom® was ~ 2 to 8-fold (at least) more active than *A. montana* and *A. muricata* seed extracts. None of the extracts caused significant mortality (at 1,000 µg cm^{-2}) of 3rd instar *T. ni* larvae through contact with the contaminated substrate (filter paper) (Table 3.2).

Table 3.2 - Mortality (± SE) of *Trichoplusia ni* larvae exposed to *Annona* seed extracts and an acetogenin-based commercial bioinsecticide (Anosom®) by three methods of application

Treatments	Oral toxicity	Contact toxicity	
	Mixed in artificial diet (1,000 mg kg^{-1})	Topical application (100 µg $larva^{-1}$)	Contaminated substrate (1,000 µg cm^{-2})
Annona montana ethanolic seed extract	7.50±5.00 c	40.00±5.77 b	13.33±6.66
Annona mucosa ethanolic seed extract	97.50±2.50 a	96.63±3.33 a	16.66±3.33
Annona muricata ethanolic seed extract	7.50±5.00 c	23.33±3.33 bc	16.66±3.33
Annona sylvatica ethanolic seed extract	60.00±6.12 b	93.33±6.66 a	10.00±0.00
Anosom®	65.00±10.54 b	56.66±6.66 b	13.33±6.66
Control (Acetone:methanol, 2:1,(v/v))	15.00±6,12 c	3.33±3.33 c	3.33±3.33
Control (water)	12.50±5.59 c	6.66±3.33 c	3.33±3.33
F	24.85	24.982	1.6544^{ns}
p value	<0.0001	<0.0001	0.2048

[1]Means followed by different letters within a column indicate significant differences between treatments (GLM with quasi-binomial distribution followed by Tukey's *post hoc* test, $p<0.05$);
^{ns}: Not significant ($p>0.05$).

Table 3.3 - Lethal concentrations (LC_{50} and LC_{90} in mg kg^{-1}) and respective confidence intervals of different *Annona* seed extracts and an acetogenin-based commercial bioinsecticide (Anosom®) to *Trichoplusia ni* larvae though dietary exposure

Treatments	n [1]	Slope (± SE)	χ^2 [2]	df [3]	h [4]	LC_{50} (CI) [5]	LC_{90} (CI) [5]	Toxicity index [6]
Annona montana ethanolic seed extract	140	--	--		--	> 4,000	--	< 17.51
Annona mucosa ethanolic seed extract	140	3.83±0.89 (*p*<0.0001)	2.56	4	0.64	328.86 (219.53 – 421.42)	664.43 (515.30 – 1,044.00)	212.97
Annona muricata ethanolic seed extract	140	--	--		--	> 4,000	--	< 17.51
Annona sylvatica ethanolic seed extract	140	2.84±0.44 (*p*<0.0001)	2.79	4	0.70	690.11 (546.89 – 848.00)	1,945.77 (1,470.61 – 3,099.57)	101.49
Anosom ®	140	1.89±0.22 (*p*<0.0001)	2.90	4	0.72	700.37 (554.42 – 919.24)	3,340.59 (2,202.48 – 6,311.77)	100.00

[1] n: number of tested insects;
[2] χ^2: calculated chi-squared value;
[3] d.f.: degrees of freedom;
[4] d.f.: Heterogeneity factor;
[5] CI: 95% confidence interval;
[6] Toxicity index (Sun, 1950): (LC_{50} of commercial bioinsecticide (standard)/LC_{50} of extract) x 100.

Table 3.4 - Lethal doses (LD_{50} and LD_{90} in µg larva^{-1}) and respective confidence intervals of different *Annona* seed extracts and an acetogenin-based commercial bioinsecticide (Anosom®) to *Trichoplusia ni* larvae via topical application

Treatments	n [1]	Slope (± SE)	χ^{2} [2]	df [3]	h [4]	LD_{50} (CI) [5]	LD_{90} (CI) [5]	Toxicity index [6]
Annona montana ethanolic seed extract	240	1.50±0.30 (*p*=0.0005)	2.72	3	0.90	167.44 (46.36 – 287.71)	836.65 (687.31 – 1372)	24.03
Annona mucosa ethanolic seed extract	240	1.76±0.32 (*p*<0.0001)	2.87	4	0.72	12.61 (6.32 – 18.72)	60.76 (42.92 – 103.34)	319.11
Annona muricata ethanolic seed extract	210	2.11±0.54 (*p*=0.0008)	0.80	3	0.27	430.78 (263.00 – 617.23)	1,734.23 (1,056.78 – 6,340.86)	9.34
Annona sylvatica ethanolic seed extract	240	1.04±0.19 (*p*<0.0001)	1.15	4	0.29	7.39 (2.33 – 13.85)	103.07 (60.57 – 241.51)	544.52
Anosom ®	240	1.43±0.28 (*p*<0.0001)	5.26	4	1.31	40.24 (18.06 – 63.24)	279.03 (178.49 – 610,62)	100.00

[1] n: number of tested insects;
[2] χ^{2}: calculated chi-squared value;
[3] d.f.: degrees of freedom;
[4] d.f.: Heterogeneity factor;
[5] CI: 95% confidence interval;
[6] Toxicity index (Sun, 1950): (LC_{50} of commercial bioinsecticide (standard)/ LC_{50} of extract) x 100.

3.2.2.2 Cabbage plant protective effects of formulated extracts (greenhouse trial)

Both the seed extract of *A. mucosa* and the acetogenin-based bioinsecticide (Anosom®) produced >90% population reduction of 3rd instar *T. ni* larvae (Table 3.5) on intact cabbage plants in the greenhouse. These treatments were as active as the pyrethrin-based commercial insecticide (Insect Spray®) used as a positive control (Table 3.5, Figure 3.1).

Table 3.5 - Mortality of *Trichoplusia ni* larvae 120 hours after spraying with an aqueous emulsions of different *Annona* seed extracts, an acetogenin-based commercial bioinsecticide (Anosom®), and a pyrethrin-based commercial bioinsecticide (Insect Spray®, positive control) in a greenhouse trial (at 2,500 mg L^{-1})

Treatments	Mortality (%)[1]	C.E.[2]
Annona mucosa ethanolic seed extract	98.00±2.00 a	98.48
Annona sylvatica ethanolic seed extract	51.67±6.31 b	54.34
Anosom®	93.98±2.61 a	92.74
Insect Spray® (positive control)	98.88±1.11 a	98.13
Control (methanol:water, 1:10,(v/v) + Tween 80®, 0,5%)	1.42±1.42 c	--
Control (water)	2.77±1.89 c	--
F	76.889	--
p value	<0.0001	--

[1] Means followed by different letters in the column indicate significant differences among the treatments (GLM with quasi-binomial distribution, followed by a *post hoc* Tukey test, $p<0.05$);
[2] C.E.: control efficacy calculated by means of Henderson and Tilton (1955) formula.

Although the *A. sylvatica* extract was significantly less toxic (54.3% mortality) than the *A. mucosa* extract, it produced a significant weight reduction among surviving larvae compared with the control (GLM with Gaussian distribution, F=16.67, $p<0.0001$), confirming its growth inhibitory effect in both the lab and the greenhouse. Despite the differences in the efficacy of the different *Annona* extracts in the greenhouse, none of the formulated extracts produced any phytotoxicity in the cabbage plants.

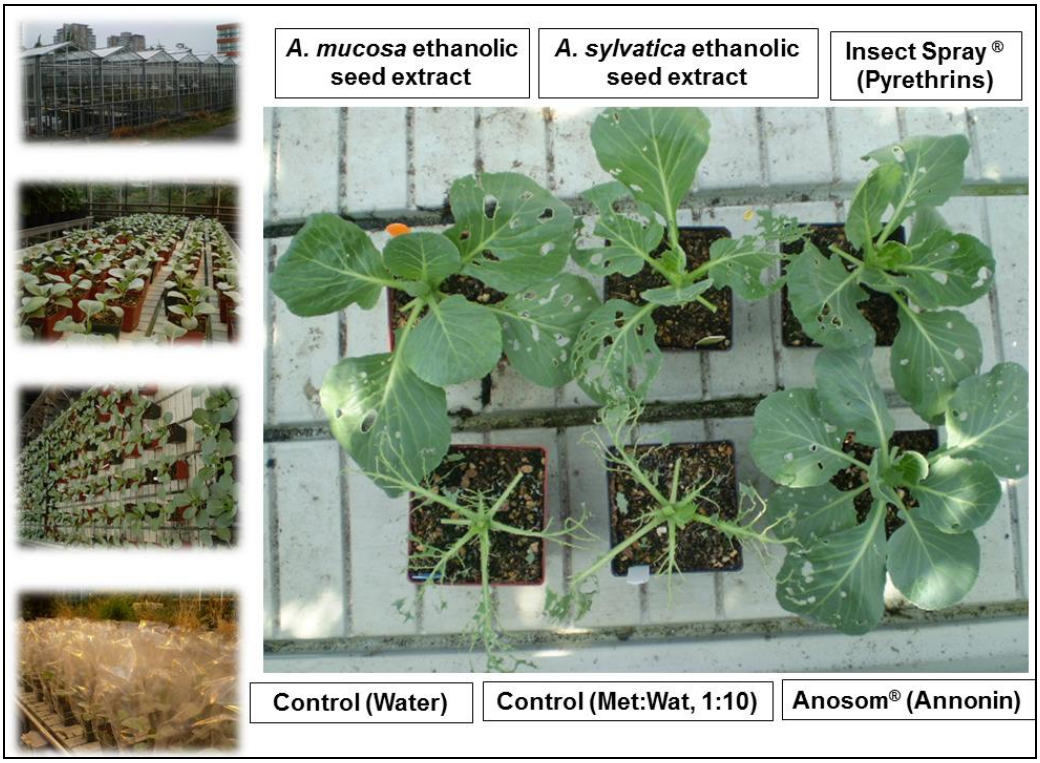

Figure 3.1 - Overview of the greenhouse trial (left) and damage of *Trichoplusia ni* larvae on cabbage plants treated with different botanical insecticides (right)

3.2.2.3 Aphicidal activity of formulated extracts (laboratory and greenhouse trials)

In both laboratory and greenhouse tests, the assessment of green peach aphid (*M. persicae*) infestation on cabbage plants prior to treatment indicated homogeneous populations (no significant differences in average no. of aphids per plant (p>0.05, Table 3.6). In both tests, all the *Annona* extracts (at 2,500 mg L^{-1}) caused strong reduction in *M. persicae* populations at 120 hours post-treatment and good efficacy (Table 3.6).

As with *T. ni* larvae, the seed extract of *A. mucosa* showed the greatest aphicidal activity (Table 3.6). In the laboratory bioassay, the *A. mucosa* extract was significantly more active than the other treatments whereas in the greenhouse test it was equitoxic to the *A. sylvatica* seed extract, but superior to the acetogenin-based bioinsecticide and the pyrethrin- based insecticide spray (positive control)(Table 3.6).

Based on the values of instantaneous population growth rate (*ri*), of aphids in both the greenhouse (-0.40160) and laboratory (-0.8046) bioassays, the *A. mucosa* seed extract produced the most pronounced reductions in aphid populations (Table 3.6) compared to the other treatments. In the greenhouse test, the reduction in aphid

population was significantly greater than that with the pyrethrin-based commercial bioinsecticide used as a positive control. Although *A. sylvatica* seed extract was significantly less active than the *A. mucosa* seed extract, it was equitoxic to Anosom® and the positive control (Table 3.6).

Table 3.6 - Means (± SE) of number of *Myzus persicae*/cabbage plant before and after spraying with aqueous emulsions of selected *Annona* seed extracts and an acetogenin-based commercial bioinsecticide (Anosom®), in laboratory and greenhouse tests. Note: In the greenhouse trial, a pyrethrin-based commercial bioinsecticide (Insect Spray®) was included as a positive control

Treatments	Number of aphids/plant [1]		C.E. (%)[2]	Instantaneous rate of population growth (*ri*) [3]
	Initial	Post-treatment (120 hours)		
Laboratory test				
Annona mucosa ethanolic seed extract	74.83±7.81	10.5±1.99 c	95.53	- 0.40±0.04 c
Annona sylvatica ethanolic seed extract	78.16±5.00	40.83±5.54 b	83.36	- 0.14±0.03 b
Anosom®	77.66±8.51	42.33±8.87 b	79.78	- 0.16±0.05 b
Control (methanol:water, 1:10,(v/v) + Tween 80®, 0,5%)	71.60±5.04	224.80±20.79 a	--	0.23±0.01 a
Control (water)	83.83±10.94	226.00±37.62 a	--	0.19±0.02 a
F	0.3129 [ns]	47.854	--	54.58
p value	0.8665	<0.0001	--	<0.0001
Greenhouse test				
Annona mucosa ethanolic seed extract	97.14±13.97	1.85±0.40 c	99.57	- 0.80±0.02 c
Annona sylvatica ethanolic seed extract	94.57±13.83	22.28±7.09 bc	94.74	- 0.36±0.09 b
Anosom®	100.28±14.78	40.71±6.98 b	91.41	- 0.18±0.03 b
Insect Spray® (positive control)	90.71±13.97	36.57±7.86 b	91.47	- 0.21± 0.05 b
Control (methanol:water, 1:10,(v/v) + Tween 80®, 0,5%)	103.28±15.52	462.71±58.80 a	--	0.30±0.01 a
Control (water)	105.85±15.39	500.28±75.53 a	--	0.30±0.02 a
F	0.1738 [ns]	80.50		80.79
p value	0.9707	<0.0001		<0.0001

[1] Means followed by different letters within the column indicate significant differences among the treatments (GLM with quasi-Poisson distribution, followed by a *post hoc* Tukey test, $p<0.05$);
[2] C.E.: control efficacy calculated by means of Henderson and Tilton (1955) formula;
[3] Means followed by different letters within the column indicate significant differences among the treatments (GLM with Gaussian distribution, followed by a *post hoc* Tukey test, $p<0.05$).

Results from our laboratory and greenhouse tests show that an ethanolic extract prepared from *A. mucosa* seeds is the most promising treatment among the selected seed extracts from Brazilian *Annona* species. This crude extract has strong insecticidal activities, comparable or superior in efficacy to a bioinsecticide (our positive control) based on pyrethrins, the most widely used botanical insecticide (ISMAN, 2006). Therefore, it is the main candidate for development of a botanical insecticide for protection of cole crops in Brazil. Corroborating these findings, our previous study had shown promising protection of stored grain (against *S. zeamais*) with hexanoic and dichlromethanoic extracts (LC_{50}: 110.28 and 149.79 mg kg^{-1}, respectively) from seeds of this plant species, likely owing to their abundant content of acetogenins (RIBEIRO et al., 2013). *A. mucosa* derivatives also can be used for protection of crops in greenhouses, constituting an important alternative for integrated pest management in organic production, a system in need of efficient tools (ZEHNDER et al., 2007). Because of their mechanism of action (inhibitors of the mitochondrial electron transport chain), acetogenin-based insecticides could constitute an important tool for the management of insecticide-resistant *T. ni* and *M. persicae* populations, an increasingly worldwide problem (MAZZONI; CRAVEDI, 2002; LIU et al., 2003; MARGARITOPOULOS et al., 2007, JANMAAT et al., 2008; SLATER et al., 2012; FUENTES-CONTRERAS et al., 2013).

We also showed that the extract prepared from *A. sylvatica* seeds caused strong bioactivity in both pest species, although to a lesser extent. Due to the great abundance of this species in the south-central region of Brazil [the center of origin of this species, (LORENZI; ARAUJO; KURTZ, 2005)], it could be used as a homemade tool for *Brassica* IPM in smallholdings, mainly in association with other techniques (e.g., natural and/or applied biological control). To date, a small number of secondary compounds from *A. sylvatica* have been isolated and their bioactivity evaluated. Our results concur with those of Mikolajczak et al. (1990) who demonstrated oral toxicity of a hexanoic extract from *A. sylvatica* fruits against *Ostrinia nubilalis* (Hübner, 1796) larvae. After successive fractionation, the compound sylvaticin (the only acetogenin reported from this species to date) was isolated and shown to provide protection to cantaloupe plants against the beetle *Acalymma vittata* Barber, 1947. Although our laboratory results showed that an ethanolic seed extract from *A. sylvatica* was comparable or superior in bioactivity to an acetogenin-based commercial insecticide

(Anosom®), this crude extract did not provide satisfactory control (< 80% mortality) of 3rd instar *T. ni* larvae in the greenhouse trial.

Some published studies have shown the potential of *A. squamosa* seed derivatives in the control of *Brassica* pests, including *Crocidolomia pavonana* (F., 1794) (DADANG; PRIJONG, 2009), *Plutella xylostella* (L., 1758) (SINCHAISRI; CHUNGSAMARNYART, 1991; LEATEMIA; ISMAN, 2004b, 2004c; DADANG; PRIJONG, 2009), *T. ni* (LEATEMIA; ISMAN, 2004a, 2004c; SEFFRIN et al., 2010) and *Spodoptera litura* (F., 1775) (LEATEMIA; ISMAN, 2004a). In light of these findings, we included the recently registered (in India) acetogenin-based bioinsecticide (Anosom®) in our study. Our results indicate that this standardized bioinsecticide has potential for use in the *Brassica* IPM (at 2,500 mg L^{-1}), as an alternative tool in large scale cropping. Regarding the remaining species, *A. montana* and *A. muricata* were significantly less effective than Anosom® and only caused growth inhibition and contact toxicity by means of topical application to *T. ni* larvae. Similarly, Prijono, Gani and Syahputra (1997) showed that acetonic seed extracts of *A. squamosa* were about 30-fold more active than those of *A. muricata* against *C. binotalis*. Moreover, Leatemia and Isman (2004a) found that the ethanolic extracts prepared from *A. squamosa* seeds collected in Indonesia were 20-fold more active than *A. muricata* seed extract against *S. litura*. Interestingly, *A. muricata* (“soursop”) and *A. montana* (“mountain soursop”) are morphologically close but their respective acetogenin profiles are not well documented. Unfortunately it is difficult to quantitate individual acetogenins or total acetogenins in crude extracts of *Annona* seeds using conventional analytical methods. Therefore we cannot determine if the acetogenins in *A. mucosa* are especially active against insects, or simply more abundant. What we have been able to show is that our crude extract of *A. mucosa* is as or more efficacious than the commercial preparation (Anosom®) based on a seed extract of *A. squamosa*, and reputed to contain 1% annonin as the active ingredient.

Based on our results obtained under laboratory and greenhouse conditions, we conclude that ethanolic extracts prepared from *A. mucosa* and *A. sylvatica* seeds have promising bioactivity against *T. ni* and *M. persicae*, with potential utility for *Brassica* IPM especially in organic systems. These *Annona* seed extracts, especially those prepared from *A. mucosa* seeds are excellent sources of biorational compounds that could be used to produce bioinsecticides. For this purpose, the waste (seeds) from fruit processing industries could serve as an inexpensive and

readily available source of biomass. Consequently, these extracts are being chemically investigated and monitored using bioassays to isolate and characterize the compounds responsible for the observed bioactivity. Additionally, the effects of these crude extracts against beneficial insects (natural enemies and pollinators) are being investigated.

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4 COMPARATIVE TOXICITY OF AN ACETOGENIN-BASED EXTRACT AND COMMERCIAL PESTICIDES AGAINST CITRUS RED MITE

Abstract

Acetogenins, a class of natural compounds produced by some Annonaceae species, are potent inhibitors of mitochondrial electron transport systems. Although the cellular respiration processes are an important biochemical site for the acaricidal action of compounds, few studies have been performed to assess the bioactivity of acetogenin-based biopesticides on spider mites, mainly against species that occur in orchards. Therefore, this study aimed to evaluate, using residual contact bioassays, the bioactivity of an ethanolic extract from *Annona mucosa* seeds (ESAM) (Annonaceae), a homemade biopesticide which was previously characterized by its high concentration of acetogenins, against the citrus red mite *Panonychus citri* (Acari: Tetranychidae), an important pest of the Brazilian citriculture. ESAM caused both high mortality (LC_{50} = 7,295; 4,662; 3,463; and 2,608 mg L^{-1}, after 48, 72, 96, and 120 hours of exposure, respectively) and significant reduction in the egg production per female (EC_{50} = 3.194,80 mg L^{-1}). However, there was no effect on the *P. citri* female fertility. In addition, the ESAM efficacy (in terms of its LC_{90}) was compared with commercial acaricides/insecticides (at its recommended rate) of both natural [Anosom® 1 EC (annonin), Derisom® 2 EC (karanjin), and Azamax® 1.2 EC (azadirachtin + 3-tigloylazadirachtol)] and synthetic origin [Envidor® 24 SC (spirodiclofen)]. Based on all of the analyzed variables, the ESAM exhibited levels of activity superior to other botanical commercial acaricides and similar to spirodiclofen. Thus, our results indicate that ESAM may constitute a biorational acaricide for citrus red mite integrated pest management in Brazilian citrus orchards, particularly for local use.

Keywords: Allelochemicals; Annonaceae; Bioactivity; *Panonychus citri*

4.1 Introduction

Mites (Arthropoda: Acari) are responsible for significant economic losses due to their ability to colonize plants, animals, and humans. Mites inflict damage by the transmission of diseases, by triggering allergic reactions, and by parasitic effects that affect the normal growth and development of domestic animals and cultivated plants (FLAMINI, 2006). Among the species of mites that feed on plants, the citrus red mite, *Panonychus citri* (McGregor, 1916) (Acari: Tetranychidae), is widely considered one of the most important pest species due to its wide geographic distribution and voracious appetite and may infest more than 110 species of host plants, although these mites prefer plants of the genus *Citrus* (PAN et al., 2006; FADAMIRO et al., 2013; MIGEON; DORKELD, 2013).

Although *P. citri* is still considered a secondary pest of the Brazilian citrus culture (a cultivation with significant economic value within the country), its populations have increased significantly each year due to the large number of applications of broad-spectrum insecticides, especially pyrethroids and neonicotinoids, used for pest control in commercial plantations (MORAES; FLECHTMANN, 2008; YAMAMOTO; ZANARDI, 2013). These chemicals affect the natural balance of the agroecosystem because of the low susceptibility of phytophagous mites to insecticides, the increased female fecundity, and the mortality of biological control agents (GOTTWALD et al., 2002; SILVA et al., 2006; GERSON et al., 2007; SZCZEPANIEC et al., 2011). Therefore, an increased frequency of acaricide applications resulting from these population outbreaks and an increased frequency of individuals resistant to the main active ingredients have commonly been observed (HU et al., 2010; DÖKER; KAZAK, 2012) - a scenario that increases production costs and reduces the efficiency of the system management and environmental sustainability.

By contrast, crude extracts or compounds derived from the secondary metabolism of plants (allelochemicals) are being investigated as an alternative for controlling mites of agricultural, veterinary, and public health importance [see the reviews by Flamini (2003 and 2006)]. In the agricultural sector, such studies are focused on obtaining biologically rational management tools (especially for use in organic production systems) and detecting template molecules for synthesizing new acaricides/insecticides acting on various molecular targets (ISMAN, 2006; CANTRELL; DAYAN; DUKE, 2012), which is an important aspect of managing populations resistant to the currently used neurotoxins. Compounds that interfere with the cellular respiration processes of arthropod pests are an important source of pesticidal substances (LÜMMEN, 1998). Recently, many pesticides (e.g., pyridazine, pyrimidine, pyrazole, quinazoline, naphthoquinone, pyrroles, and thiourea) with actions in different processes of mitochondrial respiration (DEKEYSER, 2005) were released on the market, confirming the potential of this biochemical site as a target for acaricide action.

Acetogenins, a class of natural compounds (C-35/C-37) that are derived from long chain fatty acids (C-32/C-34) combined with a unit of 2-propanol (ALALI; LIU; McLAUGHLIN, 1999), are potent inhibitors of complex I (NADH:ubiquinone oxidoreductase) of mitochondrial electron transport (MET) system and the enzyme

NADH:oxidase of the cellular plasma membrane of target arthropods. This class therefore presents promising biological effects, including potent acaricidal/insecticidal effects (OCAMPO; OCAMPO, 2006). Despite the potential of this class of compounds, which are found exclusively in some genera of the Annonaceae (BERMEJO et al., 2005), few studies have been conducted to evaluate the bioactivity of derived from Annonaceae on mite species of economic importance for agricultural crops, especially fruit orchards. Thus, the objective of this study was first to evaluate the acute and chronic effects of an ethanolic extract prepared from the seeds of *Annona mucosa* Jacq. (Annonaceae), previously selected in our screening (RIBEIRO et al., submitted paper) and characterized by a high concentration of acetogenins (RIBEIRO et al., 2013), on the citrus red mite *P. citri*. The extract's effectiveness was then compared with that of commercially available natural and synthetic acaricides.

4.2 Development

4.2.1 Material and methods

4.2.1.1 Mites

The *P. citri* females used in the assays were obtained from stock reared under laboratory conditions (temperature: 25 ± 2°C, relative humidity (RH): 60 ± 10% and photoperiod of 14 L:10 D hours) for at least 30 generations. The population was maintained on leaves of the "Valencia" sweet orange [*Citrus sinensis* (L.) Osbeck] placed on a foam layer moistened with distilled water. The leaves were surrounded with moistened strips of absorbent cotton to prevent the escape of mites and to maintain leaf turgor.

The laboratory population was established by collecting specimens of *P. citri* from citrus plants occurring in an orchard located on the campus of the "Luiz de Queiroz" College of Agriculture (ESALQ/USP), in Piracicaba, São Paulo State, Brazil.

4.2.1.2 Ethanolic extract: source and preparation method

The seeds of *A. mucosa* used in the crude extract preparation were obtained from ripe fruits collected on March 17, 2011, from a specimen grown on the ESALQ/USP campus, in Piracicaba, São Paulo State, Brazil (22º42'28.5" S, 47º37'59.6" W; altitude: 534 m). An exsiccate of the specimen, previously identified

by Heimo Rainer, Ph.D. (Department of Systematics and Evolution of Higher Plants, University of Vienna), was deposited in the ESA Herbarium of the Department of Biosciences ESALQ/USP, in Piracicaba, SP, Brazil, under registration number 120985.

For the preparation of extracts, the seeds were dried in an oven at 40°C for a period of 48 to 72 hours and were subsequently crushed in a knife mill. The vegetable powder obtained was stored in sealed glass containers and kept refrigerated until use. The organic extracts were obtained using the technique of maceration in ethanol (in the ratio 1:5, w/v). For this purpose, the vegetable powder was kept at rest in the solvent for a period of three days and was immediately filtered through filter paper. The resultant cake was resubmitted to the ethanol solvent, and the process was repeated four times. The remaining solvent in the filtered solution was eliminated in a rotary evaporator at 50ºC and -600 mmHg. After complete evaporation of the solvent in an airflow chamber, the extraction yield was determined.

4.2.1.3 Bioassays

All bioassays were conducted in a temperature-controlled room (temperature of 25±2°C, RH of 60±10%, and photoperiod of 14L:10D hours) using a completely randomized design.

Arenas were used as bioassay experimental units (Figure 4.1). These arenas were made with 5-cm-diameter (19.6 cm^2) cotton discs arranged on the upper surface of ‘Valencia’ sweet orange leaves placed in 29.0 x 21.0 x 6.0 cm (length, width, and height) plastic trays containing a base layer of foam moistened with distilled water.

Figure 2 - Arenas (experimental units) used in the bioassays

4.2.1.3.1 Acaricidal activity via residual contact

The acaricidal activity of the ethanolic extract from *A. mucosa* seeds was assessed against newly emerged (<48 hours) *P. citri* females using a residual contact bioassay. To this end, 'Valencia' sweet orange leaves were used as a substrate for treatment application and mite feeding during the evaluation of the bioassay.

The ethanolic crude extract of *A. mucosa* seeds solubilized in a solution of acetone: methanol (1:1, v/v), was assessed initially at concentrations of 625, 1,250, 2,500, 5,000, and 10,000 mg L^{-1}, as well as a control solution consisting only of the solution used to solubilize the extract. For the treatment application, the leaves were immersed in the solutions for 10 seconds and then maintained on filter paper in a climate-controlled room (temperature: 25±2°C and RH: 60±10%) for solvent evaporation. Subsequently, arenas (experimental units) were prepared according to the procedure described above and were immediately infested with 10 females each. For each concentration, five arenas (repetitions) were used, and the bioassay was repeated thrice over time (n = 3 x 50).

Evaluations of the mortality of females exposed to the residues were performed every 24 hours for a period of five days. Females were considered dead when they had no reaction to the touch of a fine brush. The mites that were trapped in the cotton barrier were excluded from the analysis. Next, the lethal concentrations

(LC_{50} and LC_{90}) and mean lethal time (LT_{50}) were estimated with their respective confidence intervals.

4.2.1.3.2 Effects on fecundity and fertility

The action of the ethanolic extract from *A. mucosa* seeds on the fecundity and fertility of *P. citri* females was evaluated under laboratory conditions, using the same concentrations and experimental procedures as the acute toxicity test (section 2.3.1). In each experimental unit, 10 newly emerged (<48 hours) *P. citri* females were exposed to the residues with three replications of the bioassay over time (n = 3 x 50), and for each treatment level, five repetitions were used.

The number of eggs laid on “Valencia” sweet orange leaves treated with different concentrations of the extract was counted every 24 hours for a period of five days. Fecundity was determined by the sum of the average number of eggs laid by females during the trial period. The mean number of eggs was calculated using the ratio of the number of eggs laid and the number of live females every 24 hours. Based on these values, the effective concentration EC_{50} was estimated (i.e., the concentration that inhibits by 50% the number of eggs laid per live female). Fertility was determined from the percentage of hatched larvae after seven days of incubation of the eggs under the same environmental conditions as described previously.

4.2.1.3.3 Bioactivity comparison

The lethal and sublethal effects of the ethanolic extract from the seeds of *A. mucosa*, at the respective LC_{90} determined in the previous bioassay, were compared with some botanical acaricides/insecticides available in the market, in addition to a synthetic acaricide widely used in Brazilian orchards for managing *P. citri*. The products and evaluated concentrations are detailed in Table 4.1. The solvents for solubilizing the treatments [water and acetone: methanol (1:1, v/v)] were employed as controls. Due to their rapid degradation, the levels of the limonoids azadirachtin and 3-tigloylazadirachtol (formerly azadirachtin A and B) in the biopesticide Azamax® 1.2 EC were quantified simultaneously using the procedures and techniques described by Forim et al. (2010), from product samples collected at the time of use.

Similarly to the previous bioassays, after applying the treatments and drying the residues, arenas were prepared, and 10 newly emerged (<48 hours) females

were transferred to each experimental unit (arena). The other experimental procedures and variables analyzed were the same as described in sections 4.2.1.3.1 and 4.2.1.3.2.

Table 4.1 - Acaricides tested on *Panonychus citri* using the residual contact bioassay

Treatment	Description	Concentration tested	Manufacturer
Anosom® 1 EC	Biopesticide based on extracts from *Annona squamosa* L. and *Annona reticulata* L., with the acetogenin annonin as the main active ingredient (10,000 mg L^{-1})	2 mL L^{-1} (Recommended by the manufacturer)	Agrilife Biosolutions, Ltd. (Hyderabad, Andhra Pradesh, India)
Azamax® 1.2 EC	Limonoid-based biopesticide [azadirachtin (6220.15 mg L^{-1}) + 3-tigloylazadirachtol (2596 mg L^{-1}) [1]] extracted and purified from *Azadirachta indica* L. seeds	2.5 mL L^{-1} (Recommended by the manufacturer)*	UPL Brasil, Ltd. (Campinas, São Paulo State, Brazil)
Derisom® 2 EC	Bioacaricide based on extracts from the seeds of *Pongamia glabra* Vent/ *Derris indica* (Lam.) Bennett, with the furanoflavonol karanjin as the main active ingredient (20,000 mg L^{-1})	2 mL L^{-1} (Recommended by the manufacturer)	Agrilife Biosolutions, Ltd. (Hyderabad, Andhra Pradesh, India)
Envidor® 24 SC	Spirodiclofen (synthetic acaricide)	0.2 mL L^{-1} (Recommended by the manufacturer)**	Bayer CropScience S.A. (São Paulo, São Paulo State, Brazil)
ESAM	Ethanolic extract of de *Annona mucosa* Jacq seeds containing a complex mixture of acetogenins (RIBEIRO et al., 2013)	LC_{90} previously estimated	--

* Concentration recorded for use in citrus orchards aiming to control *Diaphorina citri* (AGROFIT, 2013);
** Concentration recorded for use in citrus orchards aiming to control *Panonychus citri* (AGROFIT, 2013);
[1] Respective limonoid levels in the product used were quantified simultaneously using the procedure described by Forim et al. (2010).

4.2.1.4 Data analysis

Generalized linear models (NELDER; WEDDERBURN, 1972) with quasi-binomial, quasi-Poisson and binomial distributions were used for data analysis of mortality ratios, the number of eggs laid, and the hatching rate, respectively. The model fit was assessed using half-normal probability plots with simulated envelopes (HINDE; DEMÉTRIO, 1998). When significant differences between treatments were found, multiple comparisons (Tukey test, $p<0.05$) were performed using the function glht in the package multicomp with *p* values adjusted for treatments with qualitative levels, whereas non-linear regressions were employed for comparing treatments with quantitative levels. All analyses were performed with the statistical software "R", version 2.15.1 (R DEVELOPMENT CORE TEAM, 2012).

To estimate the lethal concentrations (LC_{50} and LC_{90}), a binomial model was employed with a log-log complementary function (gompit model), using the Probit Procedure of the SAS software version 9.2 (SAS INSTITUTE, 2011). To estimate the mean lethal time (LT_{50}), the method proposed by Throne et al. (1995) for the Probit analysis of correlated data was used. To estimate the average effective concentration (EC_{50}), i.e., the concentration required to reduce by 50% the number of eggs laid per *P. citri* female, a nonlinear logistic model adapted from Sims et al. (1996) was employed using the *Nlim* Procedure of the SAS software version 9.2 (SAS INSTITUTE, 2011).

4.2.2 Results and discussion

Significant mortality was found in adult *P. citri* females exposed to residual contact with the ethanolic extract of *A. mucosa* seeds, and the activity level was dependent on both the concentration and the exposure time (LC_{50} = 7.295, 4.662, 3.463, and 2.608 mg L^{-1} after 48, 72, 96, and 120 hours of exposure, respectively) (Table 4.2). Likewise, the mean lethal time (LT_{50}) estimated varied according to the concentration, being 132.82, 74.92, and 31.77 hours, at concentrations of 2,500, 5,000, and 10,000 mg L^{-1}, respectively (Table 4.3).

Table 4.2 - Estimation of the LC_{50} and LC_{90} (mg L^{-1}) and confidence intervals of the ethanolic extract from *Annona mucosa* seeds against *Panonychus citri* at different exposure times

Exposure time (hours)	n [1]	*Slope* ± SE (*p* value)	CL_{50} (CI) [2]	CL_{90} (CI) [2]	χ^{2} [(3)]	d.f. [4]	h. [5]
48	777	3.49 ± 0.52 (*p*< 0.0001)	7,295 (6,384 – 8,239)	16,094 (13,213 – 22,492)	4.06	3	1.35
72	774	3.32 ± 0.30 (*p*< 0.0001)	4,662 (4,127 – 5,188)	10,704 (9,382 – 12,679)	4.18	3	1.39
96	771	2.93 ± 0.41 (*p*< 0.0001)	3,463 (2,138 – 4,619)	8,900 (6,574 – 15,677)	6.89	3	2.29
120	735	2.78 ± 0.37 (*p*< 0.0001)	2,608 (1,555 – 3,554)	7,050 (5,150 – 12,140)	6.42	3	2.14

[1] n: number of insects tested;
[2] CI: confidence interval at a 95% error probability;
[(3)] χ^{2}: Pearson´s chi-square test value;
[4] d.f.: degrees of freedom;
[5] h.: heterogeneity factor.

Table 4.3 - Estimation of the LT_{50} (in hours) and confidence interval of the ethanolic extract from *Annona mucosa* seeds against *Panonychus citri* at different concentrations

Concentration (mg L^{-1})	n [1]	*Slope* ± SE	LT_{50} (CI) [2]	χ^{2} [(3)]	d.f. [4]	h. [5]
2,500	150	2.10 ± 0.35	132.82 (103.97 – 213.75)	3.22	3	1.07
5,000	150	2.53 ± 0.24	74.92 (68.07 – 83.29)	0.81	3	0.27
10,000	150	3.58 ± 0.27	31.77 (28.45 – 34.85)	1.51	3	0.50

[1] n: number of insects tested;
[2] CI: confidence interval at 95% error probability;
[(3)] χ^{2}: Pearson´s chi-square test value;
[4] d.f.: degrees of freedom;
[5] h.: heterogeneity factor.

In addition to the acute toxicity, the extract from *A. mucosa* seeds also caused sublethal effects on *P. citri*, as demonstrated by the reduction (which was directly proportional to the concentration) in the number of eggs laid by females that survived after five days of exposure to the residues [EC50 = 3194.80 mg L^{-1} (95% CI: 2017.8 to 4371.7)] (Figure 4.2). However, there was no effect of this extract on the fertility of *P. citri* females (GLM with quasi-binomial distribution, F = 2.3479, *p*= 0.0893) (Figure 4.2).

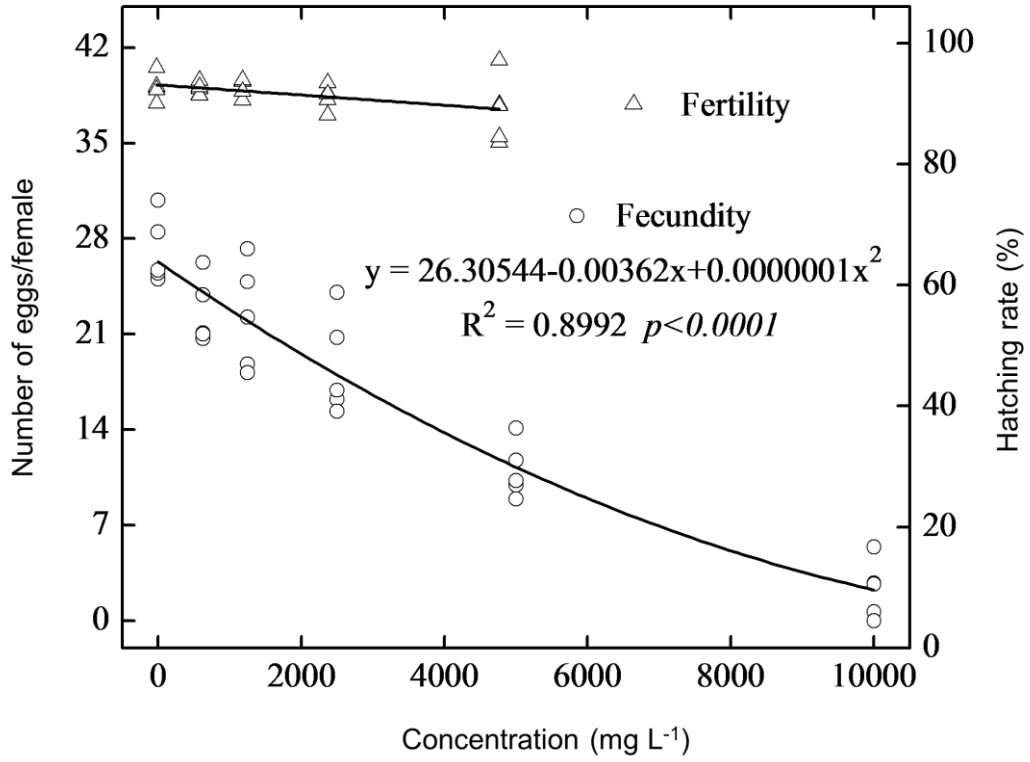

Figure 4.2 - Fecundity and fertility of *Panonychus citri* females kept for five days in arenas treated with different concentrations of an ethanolic extract from *Annona mucosa* seeds

Based on the promising acute and chronic effects of the *A. mucosa* seed extract, its effectiveness was compared with commercial acaricides/insecticides of natural and synthetic origin. Regardless of the time of exposure, the lethal effect caused by the seed extract of *A. mucosa* did not differ significantly from that caused by the biopesticide Azamax® 1.2 EC (azadirachtin + 3-tigloylazadirachtol) (Table 4.4). Both treatments differed significantly from the other products tested, either those of natural origin based on the acetogenin annonin (Anosom® 1 EC) and the furanoflavonol karanjin (Derisom® 2 EC) or the synthetic acaricide based on spirodiclofen (Envidor® 24 SC) (Table 4.4). *P. citri* mortality induced by Anosom® 1 EC and Envidor® 24 SC did not differ from the control treatment.

Regarding sublethal effects, the *A. mucosa* seed extract caused the greatest reduction in *P. citri* fecundity (Table 4.4), differing significantly from the other treatments. Although less pronounced, Azamax® 1.2 EC, Envidor® 24 SC, and Derisom® 2 EC (especially the first two products) also significantly reduced the number of eggs laid per female, compared with the control. However, a significant

effect on the hatching rate (fertility) was found only with the synthetic acaricide, with the activity level being extremely pronounced and sufficient to influence the population dynamics of the pest, which proves the product’s effectiveness.

Table 4.4 - Cumulative mortality (%), fecundity, and fertility of *Panonychus citri* females kept for five days in arenas treated with different acaricides/insecticides

Treatment	Mortality (%) – Exposure time (hours)[1]					Fecundity[2]	Fertility[3]
	24	48	72	96	120	(N^{o}. eggs/female)	[hatching rate (%)]
ESAM*	48.31±3.38 a	80.33±3.86 a	94.44±2.27 a	95.56±3.24 a	97.78±2.22 a	1.70±0.44 e	0.00±0.00**
Azamax® 12 EC	29.88±7.99 ab	60.53±5.12 a	74.27±5.95 a	90.29±2.67 a	96.59±2.33 a	4.23±1.22 d	88.89±8.61 a
Anosom® 1 EC	10.00±1.58 c	17.50±5.95 bc	28.75±4.73 bc	33.75±6.25 bc	38.16±8.14 bc	22.71±1.78 ab	95.74±1.17 a
Derisom®2 EC	18.16±2.96 b	33.21±4.76 b	35.26±5.91 b	46.32±5.35 b	54.47±3.02 b	16.99±1.24 b	88.43±2.85 a
Envidor® 240 SC	2.00±1.22 c	5.00±2.24 c	10.00±5.80 cd	21.11±3.60 c	23.22±4.48 c	9.71±0.65 c	1.05±1.05 b
Control (acet:met., 1:1)	2.00±1.22 c	2.50±2.50 c	2.50±2.50 d	6.53±3.85 cd	7.84±3.29 cd	22.27±0.50 ab	93.13±0.87 a
Control (water)	4.00±1.87 c	4.00±1.87 c	4.00±1.87 d	4.00±1.87 d	6.45±3.91 d	23.29±0.97 a	94.70±1.72 a
	F=21.976 *p*<0.0001	F=40.739 *p*<0.0001	F=32.216 *p*<0.0001	F=53.184 *p*<0.0001	F=52.149 *p*<0.0001	F=62.648 *p*<0.0001	χ^2 = 20.775 *p* = <0.0001

[1]Means followed by the same letter in columns do not differ significantly (GLM with a quasi-binomial distribution followed by the Tukey post hoc test, $p<0.05$);
[2]Means followed by the same letter in columns do not differ significantly (GLM with a quasi-Poisson distribution followed by the Tukey post hoc test, $p<0.05$);
[3]Means followed by the same letter in columns do not differ significantly (GLM with a quasi-binomial distribution followed by the Tukey post hoc test, $p<0.05$);
* ESAM: Extracts obtained from *Annona mucosa* seeds;
** Not analyzed due to the small sample size.

Short life cycle, high biotic potential, and specialized reproduction are characteristics that enable the phytophagous mites (especially from the family Tetranychidae) to rapidly evolve resistance to the main commercial acaricides (DEKEYSER, 2005; VAN LEEUWEN et al., 2010). Given this scenario, a constant search for new compounds with acaricidal activity against different molecular targets is necessary, providing conditions for implementing a system of rotating active ingredients (especially as an alternative to neurotoxins) and thereby enabling the management of strategies against resistant populations (DENHOLM; ROWLAND, 1992). Although activity against insects (Arthropoda) has already been reported in our previous study (RIBEIRO et al., 2013), the results presented here demonstrate, for the first time, promising acute and chronic effects of the ethanolic extract from *A. mucosa* seeds (with a high concentration of acetogenins) on a species of mite pests. Thus, our study indicates the potential of these compounds as acaricides, which may be used after isolation and structural characterization (stages in development) as template molecules for synthesizing new commercial acaricides. Alternatively, this extract may constitute a pre-commercial and/or domestic (local use) tool for the low-cost management of *P. citri* in small areas, especially in domestic or agroecological orchards. Because of the ease of preparation, standard commercial formulations may be prepared, in this case facilitating application in larger-scale orchards. For this purpose, the waste (seeds) from Brazilian fruit-processing industries constitutes an important source of raw materials (SEFFRIN et al., 2010; RIBEIRO et al., 2013).

Considering all of the variables analyzed and the possible consequences of acute and chronic effects on the population dynamics of *P. citri*, it was established that the *A. mucosa* seed extract exhibits activity levels similar to that of the synthetic acaricide spirodiclofen (Envidor® 24 SC) and greater than those of the other commercial biopesticides tested. Although still poorly explored, some studies available in the literature have demonstrated the acaricide potential of allelochemicals from the Annonaceae, which often present activity levels equivalent or even superior to those of commercial acaricides used as positive controls. For example, based on comparing the effective concentration (EC_{50}), Raynaudi et al. (2000) found that the dichloromethane extract of a bark from *Uvaria pauci-ovulata* (EC_{50}: 0.028 g m^{-2}) presents a more promising activity against *Dermatophagoides pteronyssinus* Trouessart, 1897 (Acariformes: Pyroglyphidae) than that of the commercial active ingredient benzyl benzoate (EC_{50}: 0.06 g m^{-2}). By contrast, the oil

obtained by pressing *A. squamosa* seeds (concentrations ≥ 0.125%) had an efficacy similar to that of commercial etoxazole-based acaricides (0.03%) in controlling *Tetranychus kanzawai* Kishida, 1927 (Acari: Tetranychidae) (LIN et al., 2009).

In general, products made from the crude extracts of plants cause a range of effects on mites, such as mortality (different stages), repellence, feeding and oviposition deterrence, and growth regulation (PAN et al., 2006; WEI et al. 2011; RODRÍGUEZ et al., 2011; GHADERI et al., 2013; BERNARDI et al., 2013). In addition to the acute toxicity, the *A. mucosa* seed extract was also observed to cause a significant concentration-dependent reduction in the fecundity of *P. citri* females. This effect is attributed to the interference with energy metabolism (ATP synthesis) by the acetogenins in the extract because of the inhibitory action on the mitochondrial electron transport system (PERRY et al., 1998). Oviposition deterrence was also an effect observed by Kalakumar et al. (2000), who evaluated the action of the oil (rich in acetogenins) from *A. squamosa* seeds on *Boophilus microplus* (Cannestrini, 1888), *Hyalomma anatolicum* (Koch, 1844), and *Rhipicephalus haemaphysaloides* Supino, 1897 (Acari: Ixodidae) females. As demonstrated in the present study, botanical derivatives generally consist of more than one active substance that act synergistically on the target species (AKHTAR; ISMAN, 2013) and produce different biological effects (acute and chronic). These effects may contribute to the maintenance of the *P. citri* populations at levels below the economic damage threshold under field conditions (VENDRAMIM; CASTIGLIONI, 2000). Additionally, greater compatibility with biological control agents and action at biochemical multiple sites are characteristics of botanical derivatives that can considerably retard the evolution of resistance (FENG; ISMAN, 1995; RATTAN, 2010).

Regarding the action of the other products tested, Envidor® 24 SC (an acaricide widely used in the management of *P. citri* in Brazil) caused chronic effects, especially on the fecundity and fertility that ensure its effectiveness in controlling *P. citri* while not causing severe acute effects in adult females. These effects are due to its mechanism of action involving the inhibition of lipid biosynthesis by blocking acetyl-coenzyme A carboxylase, which is involved in the formation of important fatty acids during mite vitellogenesis (BRETSCHNEIDER et al., 2003). Although not assessed in our study, spirodiclofen also has an ovicidal action, especially in early embryonic development (REIS et al., 2005). Azamax® 1.2 EC is a product registered for managing *Diaphorina citri* Kuwayama, 1908 (Hemiptera: Liviidae) in Brazil,

displays a satisfactory efficacy in controlling *P. citri* and thus can be extended to the control of this species. Due to the selectivity for predatory mites (Phytoseiidae) found in previous studies (BERNARDI et al., 2013; SCHLESENER et al., 2013), Azamax® 1.2 EC may constitute an important component in the integrated management of tetranychid mites in citrus orchards.

Despite the high content of acetogenins (including, among others, 10,000 mg L^{-1} of annonin), the biopesticide Anosom® 1 EC (recently registered in India) had a low efficacy against *P. citri*. This reduced efficacy is attributed to the higher tolerance of this mite for the active components of the product. Variation in the response of different mite species to Annonaceae derivatives has been observed in previous studies. Ohsawa et al. (1991) found that the seed extract of *A. glabra* and the acetogenin squamocin (= annonin) were ineffective in controlling *T. urticae*, whereas the same acetogenin, isolated from *Uvaria pauci-ovulata*, was active against *D. pteronyssinus* (RAYNAUD et al., 2000). According to Sakunwarin, Chandrapatya and Visetson (2004), S-transferases of *Tetranychus truncatus* Ehara, 1956 (Acari: Tetranychidae) play an important role in degrading secondary metabolites found in *A. squamosa* seeds and in establishing a greater tolerance of tetranychid mites to such natural compounds. However, the differential toxicity of acetogenins against mites demonstrates a possible compatibility with biological control, especially with species of predatory mites (Phytoseiidae) that are abundantly present in citrus agroecosystems - an aspect to be investigated in future studies. In addition to the tolerance of the target species induced by differences in enzymatic constitution, changes in the chemical profiles of the derivatives obtained from different species and/or their extraction methods are factors that can significantly alter the biological response (RIBEIRO et al., 2013).

The bioacaricide Derisom® 2 EC (registered in India), an extract obtained from seeds of *Pongamia glabra* Vent / *Derris indica* (Lam.) Bennett, caused mortality in *P. citri* females (54.47% after 120 hours of exposure) and oviposition deterrence below the levels observed in studies with other species of tetranychid mites, such as *Tetranychus* spp. (KUMAR et al., 2007) and *Oligonychus coffeae* (Nietner, 1861) (ROOBAKKUMAR et al., 2010). The same hypotheses proposed earlier may also explain the variations in the biological responses of different species to the product.

In addition to the action on the biochemical and physiological functions of the target species, environmental safety and toxicological aspects of acetogenins derived

from the ethanolic extract of *A. mucosa* seeds should be thoroughly investigated prior to their recommendation. After this prerequisite is met, the effects of the *A. mucosa* seed extract should be validated under field conditions, and if successful, the extract may constitute an important tool for use in integrated pest management systems, allowing a reduction in the use of synthetic acaricides that usually affect the natural balance of agroecosystems. Despite the commercial availability of products based on secondary compounds from the Annonaceae in oriental countries, the diversity of bioactive allelochemicals produced by Annonaceae species found abundantly in the Neotropics is a vast richness still unexplored. Thus, the potential of derivatives from *A. mucosa*, a species native to Brazil, for developing new products to manage pest mites of economic importance is demonstrated for the first time in the present study.

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5 COMPATIBILITY OF AN ACETOGENIN-BASED BIOINSECTICIDE WITH THREE ENTOMOPATHOGENIC FUNGAL SPECIES

Abstract

Despite the promising acute and chronic toxicity reported for different arthropod pests and the recent registration of commercial acetogenin-based bioinsecticide formulations, we are unaware of any study yet published that examines the compatibility of these botanical derivatives with entomopathogenic fungi, which are important natural and applied biological control agents. Thus, this study investigated the compatibility of *Annona mucosa* (Annonaceae) ethanolic seed extract (AMES), an effective bioinsecticide previously characterized by its high acetogenin content, with three entomopathogenic fungal species, *Beauveria bassiana* (ESALQ-PL63 isolate), *Isaria fumosorosea* (ESALQ-1296 isolate), and *Metarhizium anisopliae* (ESALQ-E9 isolate), at different concentrations and in different bioassays. The effects of the botanical insecticide varied according to the fungal isolate exposed, the biological variable analyzed, and the concentration used. However, based on the compatibility index adopted, AMES was classified as compatible with the three fungal species, except at the highest concentration tested (8,000 mg L^{-1}) for *M. anisopliae*, and it was therefore classified as moderately toxic in this case. Thus, when used under recommended concentrations, the combination of AMES with mycoinsecticides based on entomopathogenic fungi is an important tool in arthropod pest management, especially for organic or ecologically based food production systems.

Keywords: Botanical insecticides; *Annona mucosa*; *Beauveria bassiana*; *Isaria fumosorosea*; *Metarhizium anisopliae*

5.1 Introduction

Entomopathogenic fungi are important agents in regulating the populations of arthropod pests of agricultural (CHARNLEY, 1997; SHAH; PELL, 2003; LI et al., 2010), medical (SCHOLTE et al., 2004; KNOLS et al., 2010; SINGH; PRAKASH, 2013), and veterinary importance (KAAYA; HASSAN, 2000; FERNANDES et al., 2012). The fungi interfere with pest species populations mainly by naturally occurring as epizootics (conservative biological control), an important factor for ecosystem balance, and via commercial mycoinsecticide formulation applications (inundative biological control). Mycoinsecticides are increasingly used as an important tool for integrated pest management (IPM) programs in the phytosanitary management of agricultural crops, especially in organic farming systems (ZEHNDER et al., 2007). However, successful management strategies using entomopathogenic fungi require a deep understanding of how environmental factors affect pathogen/host dynamics and

of the possible interactions among different agronomic practices used in crop management (RIBEIRO et al., 2012).

Recently, the removal of synthetic active ingredients from the market, the increased demand by consumer markets, and the expansion of alternative "low-input" farming systems have caused a marked increase in botanical insecticide use for crop protection in many countries. In Brazil, this scenario, together with the recent enactment of specific legislation for registering products used in organic farming [Decree no. 6,913/2009 (BRASIL, 2009)], has catalyzed bioprospecting studies aimed at detecting new sources of raw material for botanical pesticide development, a potential market that is still underexploited in Brazil, in light of the country's vast plant biodiversity.

Among the classes of bioactive secondary metabolites (allelochemicals) synthesized by Neotropical plant species, annonaceous acetogenins, natural compounds (C-35/C-37) derived from long-chain fatty acids (C-32/C-34) combined with a 2-propanol unit, stand out because of their diverse bioactivities. Among these activities, their potent insecticidal/acaricidal action, resulting from the ability to inhibit complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial electron transport systems and plasma membrane enzyme NADH:oxidase (ALALI et al., 1999; GONZÁLEZ-COLOMA et al., 2002), is notable.

Given this potential, our current screening program, aimed at obtaining bioactive allelochemicals from Neotropical Annonaceae, found promising insecticidal/insectistatic effects of *A. mucosa* ethanolic seed extract (AMES) on pest species of stored grains (RIBEIRO, 2010; RIBEIRO et al., 2013) and other species important for tropical fruit crops, horticultural species, and other commodities (cereals and oilseeds) in laboratory tests with crude extracts and in protected and field crops with formulated extracts (RIBEIRO et al., *in press*). Subsequently, biomonitored fractionations indicated that the acute and chronic toxicity of this botanical derivative is due to the synergistic interaction of structurally diverse acetogenins (unpublished data).

Despite the promising acute and chronic effects on pest species of agricultural importance already reported in studies available in the scientific literature (LEATEMIA; ISMAN, 2004; COLOM et al., 2008; BLESSING et al., 2010; RIBEIRO et al., 2013) and the recent availability of acetogenin-based formulations on the market in Eastern countries (Anosom®, Bio Rakshak®, AnonaCin®), we are unaware of any

study yet published that sought to evaluate the compatibility of Annonaceae derivatives with entomopathogenic fungi, a key issue to be considered within IPM programs. Given this context, this study investigated the compatibility of AMES with three entomopathogenic fungal species, *Beauveria bassiana* (ESALQ-PL63 isolate), *Metharizium anisopliae* (ESALQ-E9 isolate), and *Isaria fumosorosea* (ESALQ-1296 isolate), which are important microbial control agents under Brazilian conditions. Currently, isolates of the first two species cited are being used in the production of commercial mycoinsecticides registered in Brazil (AGROFIT, 2014). In turn, the *I. fumosorosea* isolate has been showing potential for use in citrus pest management (CONCESCHI et al., 2013) in our studies, and it could potentially be used in the development of a new formulation for the Brazilian market.

5.2 Development

5.2.1 Material and methods

5.2.1.1 Bioinsecticide source and preparation method

The *A. mucosa* seeds used in preparing the crude extract were obtained from mature fruit collected on 17 March 2011 from specimens grown on the “Luiz de Queiroz” College of Agriculture/University of São Paulo campus, in Piracicaba, São Paulo, Brazil (22º42’28.5” S; 47º37’59.6” W; altitude: 534 m). One voucher specimen, previously identified by Dr. Heimo Rainer (Department of Systematics and Evolution of Higher Plants, University of Vienna), is deposited in the ESA herbarium of the Department of Biological Sciences at ESALQ/USP, in Piracicaba, SP, under registration number 120985.

To prepare the extract, the seeds were dried in an oven at 40°C for 48 to 72 hours, and subsequently ground in a knife mill. The plant powder was stored in hermetically sealed glass containers and refrigerated until use. The organic extract was obtained using an ethanol solvent soaking technique (in a 1:5 powder:ethanol ratio, w/v). For this procedure, the plant powder was maintained in the solvent for three days and was then immediately filtered through filter paper. The resulting solid material was resubjected to ethanol, and the entire process was repeated four times. The solvent remaining in the filtered solution was eliminated in a rotary evaporator at

50ºC and -600 mmHg pressure. The extract yield was determined after complete evaporation of the solvent under a laminar air flow chamber.

5.2.1.2 Obtaining and propagating the entomopathogenic fungi

The isolates used in this study (ESALQ-PL63 - *Beauveria bassiana*, ESALQ-E9 - *Metarhizium anisopliae*, and ESALQ-1296 - *Isaria fumosorosea*) were obtained from the Pathogen Bank of the Laboratory of Insect Pathology and Microbial Control at ESALQ/USP, in Piracicaba, SP, Brazil. Additional information on the isolate origin and use in commercial mycoinsecticide formulations available on the Brazilian market is detailed in Table 5.1.

Table 5.1 - Entomopathogenic fungi used in the bioassays of compatibility with *Annona mucosa* (Annonaceae) ethanolic seed extract - AMES

Species	Isolate	Original host	Sampling location	Commercial brands[1]
Beauveria bassiana	ESALQ-PL 63	*Atta* sp.	Piracicaba, SP	Boveril®
Isaria fumosorosea	ESALQ-1296	*Bemisia tabaci* biotype B	Jaboticabal, SP	--
Metarhizium anisopliae	ESALQ-E9	*Mahanarva posticata*	Boca da Mata, AL	Metarril®

[1]Commercial formulations registered in Brazil (AGROFIT, 2014) based on the respective isolates.

Before using the isolates in the bioassays, they were propagated on Difco® PDA culture medium (Becton-Dickinson Company, Franklin Lakes, New Jersey, USA) with 5 g L^{-1} of pentabiotic (Fort Dodge Saúde Animal Ltda.) and incubated in a growth chamber at 25±1^{o}C and a 12 hour photoperiod.

5.2.1.3 Bioassays

All bioassays were conducted in a climate chamber (at 25±1^{o}C and a 12 hour photoperiod) under a completely randomized design. The concentrations used in the bioassays (0, 250, 500, 1,000, 2,000, 4,000, and 8,000 mg L^{-1}) were defined based on the lethal concentrations (LC_{90}) estimated in our previous studies on controlling different arthropod pest species of agricultural importance with different feeding habits. Subconcentrations and superconcentrations were used to determine the thresholds compatible with the aforementioned fungal isolates.

5.2.1.3.1 Estimating vegetative growth

Different AMES concentrations were incorporated into Difco® PDA culture medium with pentabiotic (5 g L^{-1}), which was added while the medium was still liquid (at approximately 45°C). For this procedure, 10 mL of culture medium was transferred into Petri dishes (90 mm diameter), with 10 Petri dishes per treatment level and two replicates (n: 2x10). The controls included an acetone:water solution (1:3) used to solubilize the extract and one negative control comprised solely of deionized water.

After the culture media solidified, they were inoculated with the fungi by monosporic isolation. For this, the conidia were disaggregated from the Petri dishes by adding 20 mL of 0.01% Tween 80® adhesive spreader solution, and after serial dilution, the conidia were inoculated on Petri dishes (90 mm diameter) to obtain approximately 50 conidia per Petri dish. Next, the Petri dishes were incubated in a growth chamber (at 25±1^{o}C and a 12 hour photoperiod) for 24 hours for conidia germination. The germinated conidia were observed under a stereoscopic microscope, and with the aid of a sterile needle, one spore was individually transferred to the center of each Petri dish from the different treatment levels.

Colony diameters were measured after 2, 4, 6, 8, and 10 days of incubation for *B. bassiana* and *I. fumosorosea* and after 3, 5, 7, and 10 days of incubation for *M. anisopliae* (which had slower initial growth). Colony measurements were taken vertically and horizontally and the diameter comprised the mean of the two values. Subsequently, colony areas were calculated on the tenth incubation day, and the radial growth velocity (RGV, cm day^{-1}) was determined based on the data obtained on each measurement day.

5.2.1.3.2 Estimating conidial production

Conidial production (conidiogenesis) was estimated from five randomly selected colonies (n=2x5) grown in PDA culture medium under the different treatment levels after ten days of incubation. To estimate this parameter, the selected colonies were cut out and individually transferred into test tubes containing 10 mL of sterile distilled water and 0.01% Tween 80®. The conidia were removed from the discs by individually mixing the test tubes with a vortex mixer and an ultrasound apparatus for one minute. Next, the samples were counted in a Neubauer chamber after serial

dilutions of the conidia suspensions were performed, which allowed counting under an optical microscope (400 x).

5.2.1.3.3 Evaluating conidia germination

The effect of AMES on conidial viability of the three fungal species isolates was evaluated under the two following conditions:

Pre-contact toxicity: The conidia germinated on PDA culture medium in the absence of the product were disaggregated from the Petri dishes by adding 20 mL of Tween 80® adhesive spreader solution (0.01%). The fungal suspensions were quantified in a Neubauer chamber, and the concentration was standardized to 10^6 conidia mL^{-1} in a 50 mL volume, to which different extract concentrations were added. The suspensions were then homogenized with a Vortex® apparatus and incubated for 120 minutes at 25°C.

After exposing the conidia to the product, one 150 µL aliquot was placed in the centers of Rodac® Petri dishes containing 5 mL of Difco® PDA culture medium plus 5 g L^{-1} of pentabiotic and 10 µL L^{-1} of Derosal® (Bayer S.A., São Paulo, SP). Next, the Petri dishes with the fungal isolates were incubated in a growth chamber (at 25±1^oC and a 12 hour photoperiod) for 24 hours. After this period, the proportions of germinated and ungerminated conidia were counted under an optical microscope (400 x). Seven Petri dishes were used per treatment level, with two replicates (n=2x7) per level. The controls included an acetone:water solution (1:3) used in solubilizing the extract and sterile distilled water alone (negative control).

Post-contact toxicity: Conidial germination of the fungal species grown on culture medium containing the different botanical insecticide concentrations was also evaluated. For this procedure, the suspensions employed to estimate conidial production in the previous bioassay were used. Thus, the samples were adjusted to a concentration of 10^6 conidia mL^{-1}, and the viability was determined following the same procedures described for the pre-contact toxicity bioassay.

5.2.1.4 Calculating botanical insecticide and entomopathogenic fungi compatibility

The compatibilities of the botanical insecticide with the three entomopathogenic fungal species isolates at different concentrations were calculated

using the biological index formula proposed by Rossi-Zalaf et al. (2008), according to the following equation:

$$BI : [47 * (VG) + 43 * (SPO) + 10 * GERM] / 100$$

where:

BI: biological index;

VG: percentage of vegetative growth compared to the control;

SPO: percentage of sporulation compared to the control;

GERM: percentage of conidia germination compared to the control.

The effect of the botanical insecticide on the fungi was classified based on the following thresholds: toxic (BI between 0 and 41), moderately toxic (BI between 42 and 66), and compatible (BI greater than 66).

5.2.1.4 Data analysis

Generalized linear models (GLMs) belonging to the exponential family of distributions (NELDER; WEDDERBURN, 1972) were used to analyze the data. Fit quality was assessed using half-normal probability plots with envelope simulation (HINDE; DEMÉTRIO, 1998). Whenever there was a significant difference between the treatments, linear and non-linear regression analyses were used to determine the concentration-response relationships for the variables studied, and the fit quality was subsequently assessed. All analyses were performed using the "R" statistical software, version 2.13.1 (R DEVELOPMENT CORE TEAM, 2011).

The radial growth rate (RGR, cm day^{-1}) of each colony was calculated based on the slopes of the linear regressions fitted to the data obtained on the different measurements days (CABANILLAS; JONES, 2009).

5.2.2 Results and discussion

The acetone:water (1:3) solution had no significant effect (p>0.05) on any of the biological variables when the values obtained were compared with those of the negative control (sterile distilled water), independent of the entomopathogenic fungal species. Thus, because of the results observed and the polarity of the extract's

constituents, the acetone:water solution appeared adequate for use in solubilization, as it did not interfere with the variables analyzed.

***Vegetative growth*:** Incorporating AMES into the culture medium led to a significant decrease in the mean colony area of the isolates (Figure 5.1 and 5.2) [GLM with Gaussian distribution: F=61.44, $p<0.0001$ (*B. bassiana*); F=9.99, $p<0.0001$ (*I. fumosorosea*); F= 0.02, $p<0.0001$ (*M. anisopliae*)] and the radial growth rate (Figure 5.3) [GLM with Gaussian distribution: F=46.31, $p<0.0001$ (*B. bassiana*); F=10.10, $p=0.0002$ (*I. fumorosea*); F=10.28, $p<0.0001$ (*M. anisopliae*)] after ten days of incubation for the three species studied. For both variables, an exponential model was used to describe the effect of incorporating the extract at different concentrations into the culture medium (Figures 5.1 and 5.3, respectively).

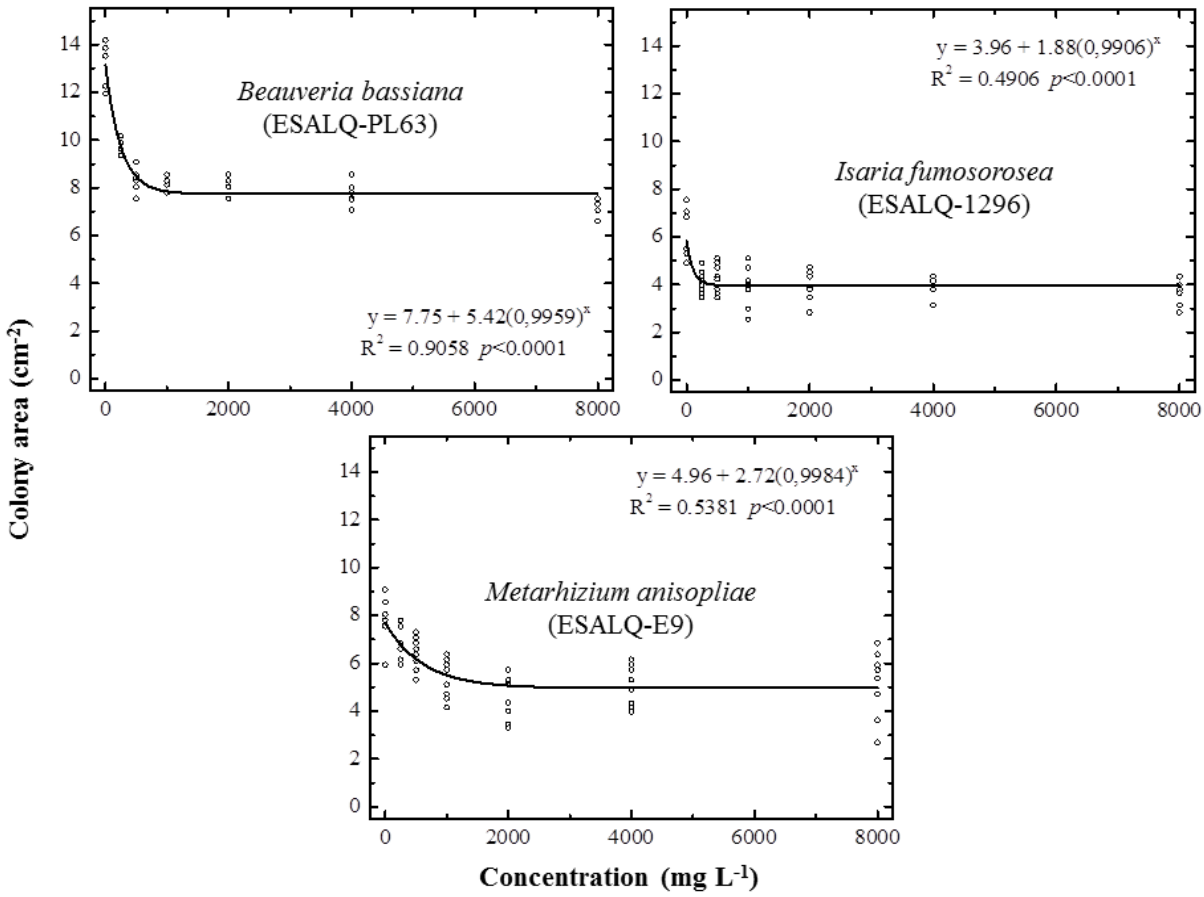

Figure 5.1 - Colony areas (cm^2) of isolates of three entomopathogenic fungal species in culture medium containing different concentrations of *Annona mucosa* (Annonaceae) ethanolic seed extract - AMES after ten days of incubation. Temperature: 25±2ºC; photoperiod: 12 hours

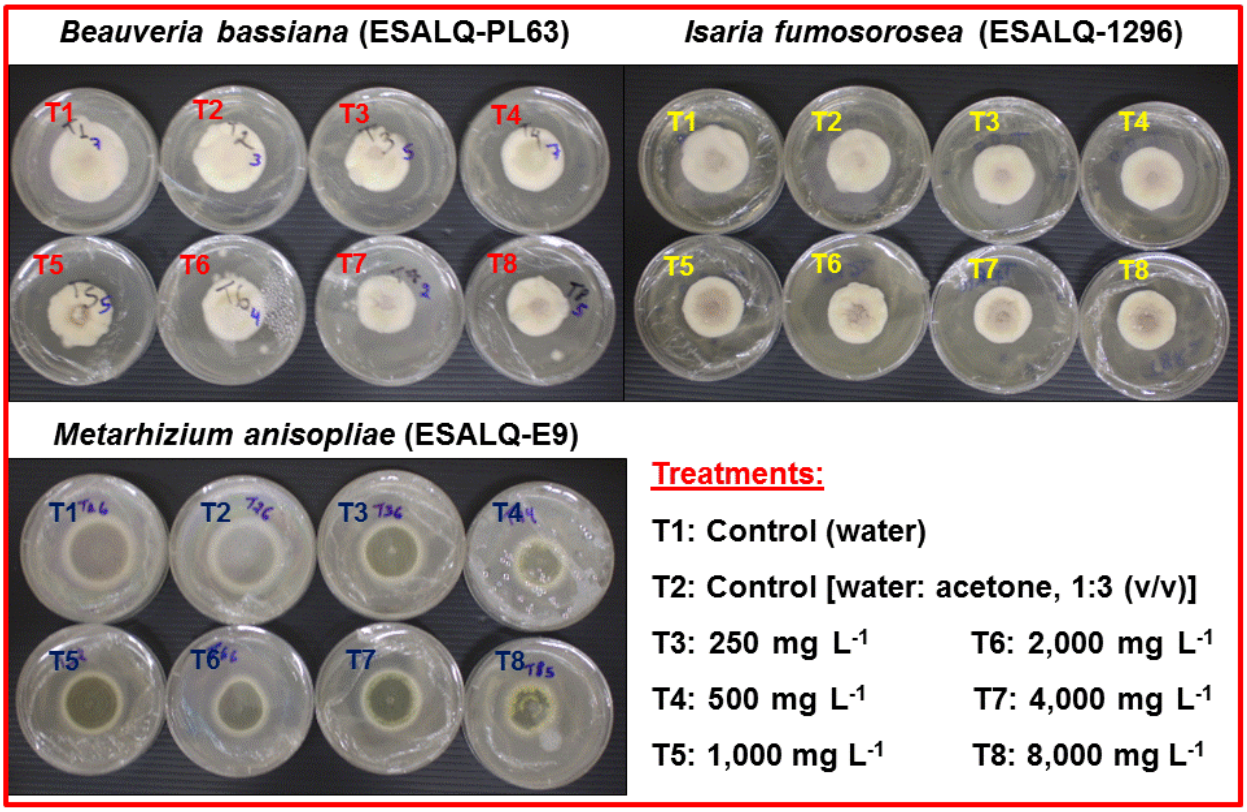

Figure 5.2 – Colonies of isolates of three entomopathogenic fungal species in culture medium containing different concentrations of *Annona mucosa* (Annonaceae) ethanolic seed extract - AMES after ten days of incubation. Temperature: 25±2ºC; photoperiod: 12 hours

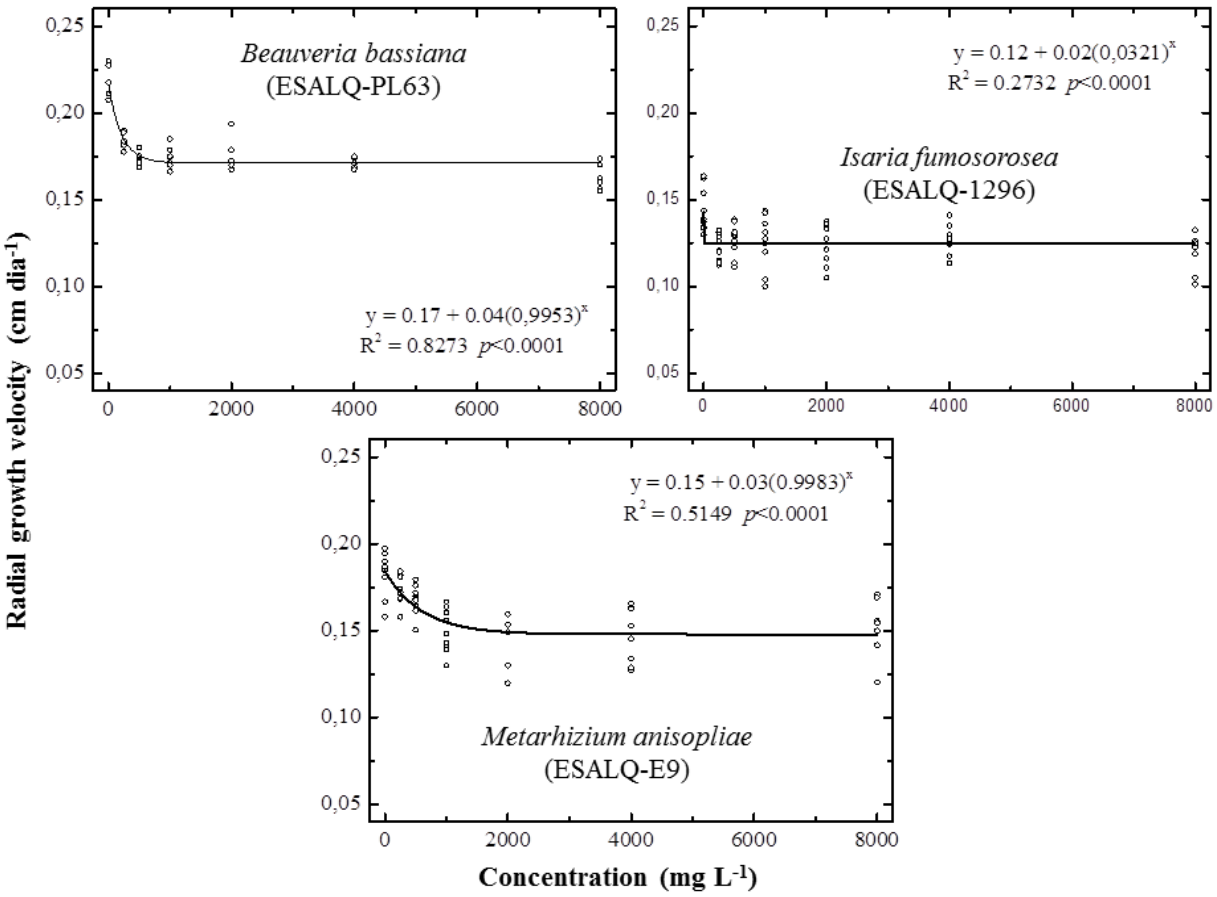

Figure 5.3 - Radial growth velocity (cm day^{-1}) of isolates of three entomopathogenic fungal species in culture medium containing different concentrations of *Annona mucosa* (Annonaceae) ethanolic seed extract - AMES after ten days of incubation. Temperature: 25±2ºC; photoperiod: 12 hours

***Conidiogenesis*:** When estimating the number of conidia per unit area (cm^2) produced by the colonies grown in culture medium at the different treatment levels, the different species isolates behaved differently (Figure 5.4). For all concentrations used, AMES did not inhibit sporulation in the *B. bassiana* isolate (ESALQ-PL 63), (GLM with quasi-Poisson distribution: F=2.00, *p*=0.0986). However, the significantly reduced sporulation observed in the *M. anisopliae* isolate was concentration-dependent (GLM with quasi-Poisson distribution: F=4.13, *p*=00043). In contrast, AMES stimulated sporulation in the *I. fumosorosea* isolate (GLM with quasi-Poisson distribution: F=4.31, *p*=0.0033), with conidiogenesis tending to increase with increased product concentration in the culture medium at the lowest concentrations but reaching a plateau within the range tested and showing a reduced increase at the highest concentrations (4,000 and 8,000 mg L^{-1}). For both species (*M. anisopliae* and

I. fumosorosea), a third-degree polynomial model was fitted to describe conidiogenesis behavior at different treatment levels (Figure 5.4).

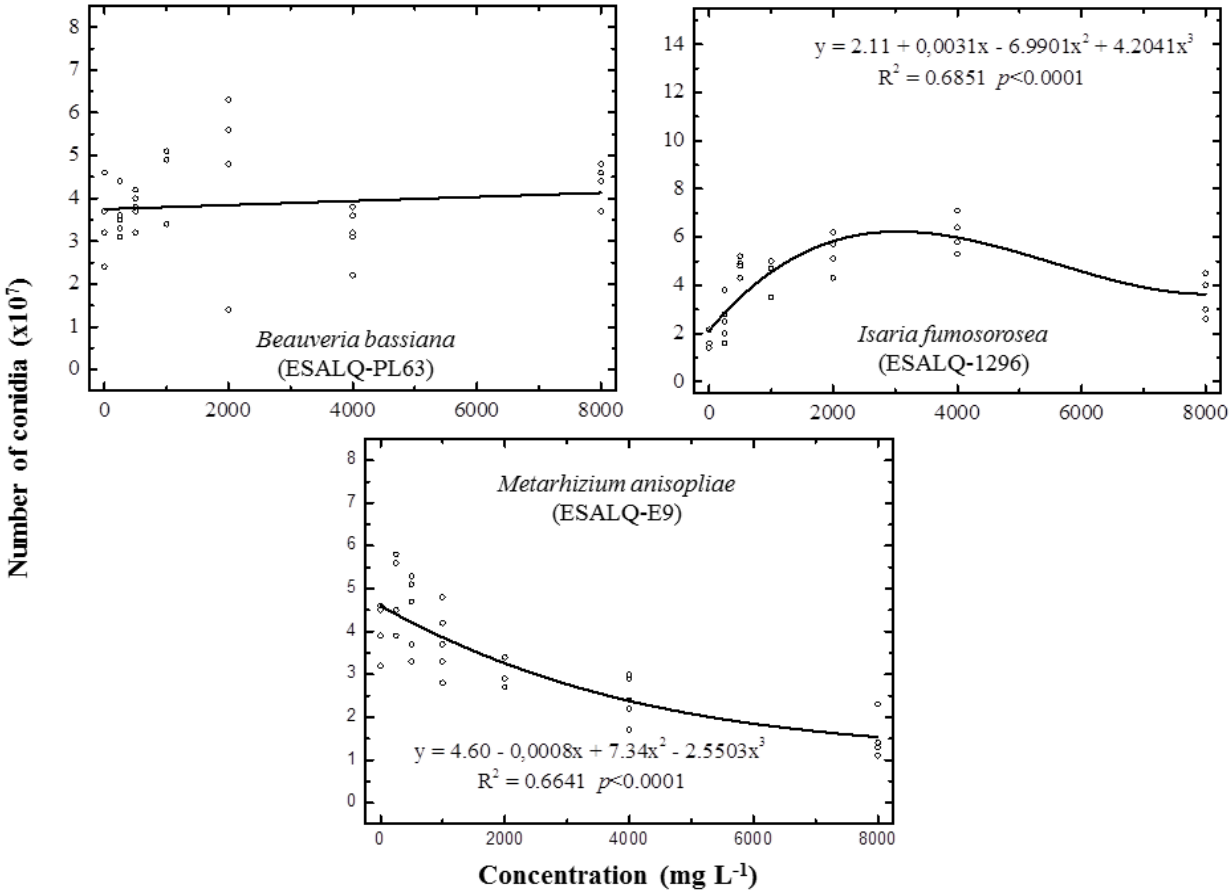

Figure 5.4 - Number of conidia (x 10^7) produced by isolates of three entomopathogenic fungal species in culture medium containing different concentrations of *Annona mucosa* (Annonaceae) ethanolic seed extract - AMES after ten days of incubation. Temperature: 25±2ºC; photoperiod: 12 hours

***Conidial germination*:** The AMES did not affect conidial viability of the three entomopathogenic fungal species isolates [GLM with quasibinomial distribution: F=1.78, *p*=0.1257 (*B. bassiana*); F=0.34, *p*=0.9095 (*I. fumorosea*); F=0.61, *p*=0.7198 (*M. anisopliae*)], when exposed to solutions containing the extract at concentrations of up to 8,000 mg L^{-1} for two hours (pre-contact toxicity) (Figure 5.5).

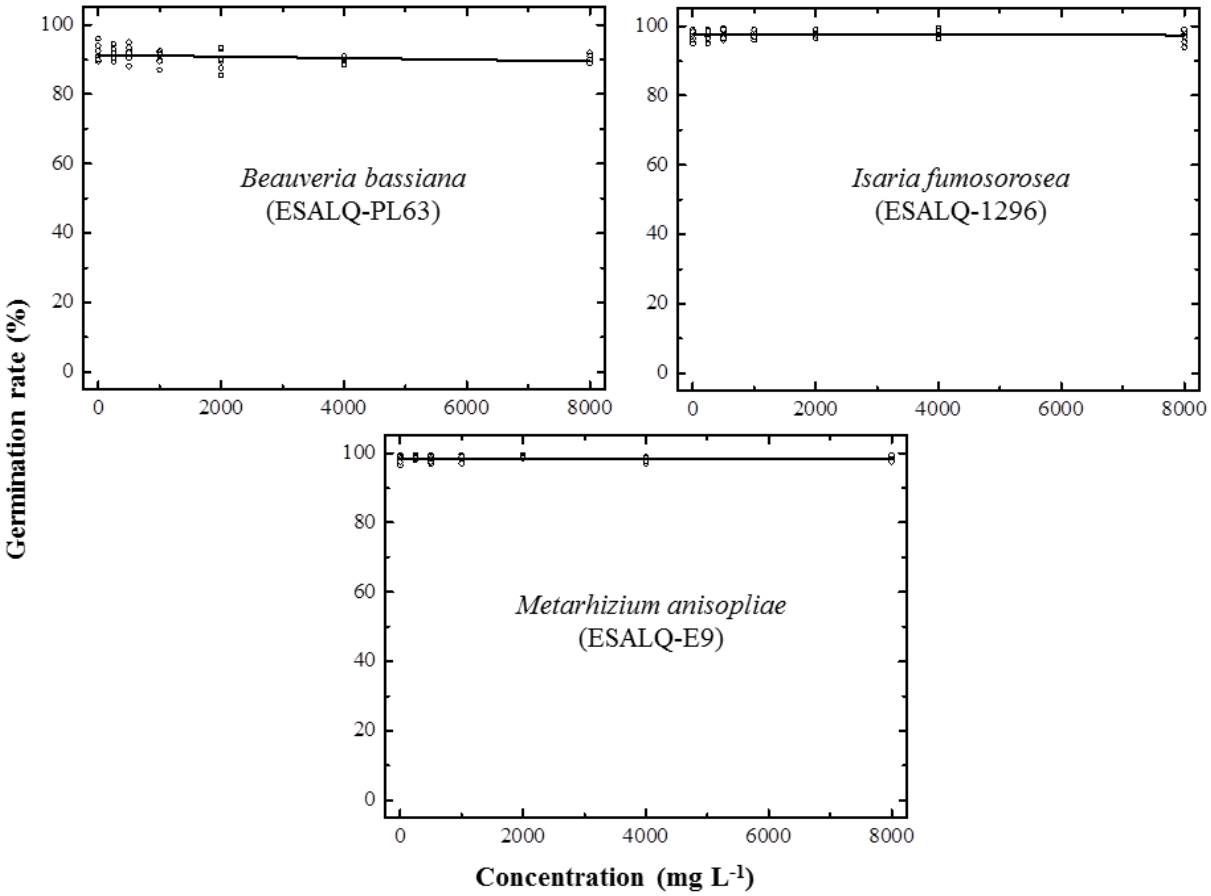

Figure 5.5 - Conidial germination (%) in isolates of three entomopathogenic fungal species exposed for two hours (pre-contact toxicity) to solutions containing different concentrations of *Annona mucosa* (Annonaceae) ethanolic seed extract - AMES. Temperature: 25±2ºC; photoperiod: 12 hours

Similarly, conidia from the three species isolates grown in culture media at the different treatment levels (post-contact toxicity) exhibited similar viabilities compared to the control [GLM with quasibinomial distribution: F=0.737, *p*=0.6242 (*B. bassiana*); F=1.5519, *p*=0.1982 (*I. fumorosea*); F=0.4063, *p*=0.8685 (*M. anisopliae*)] (Figure 5.6).

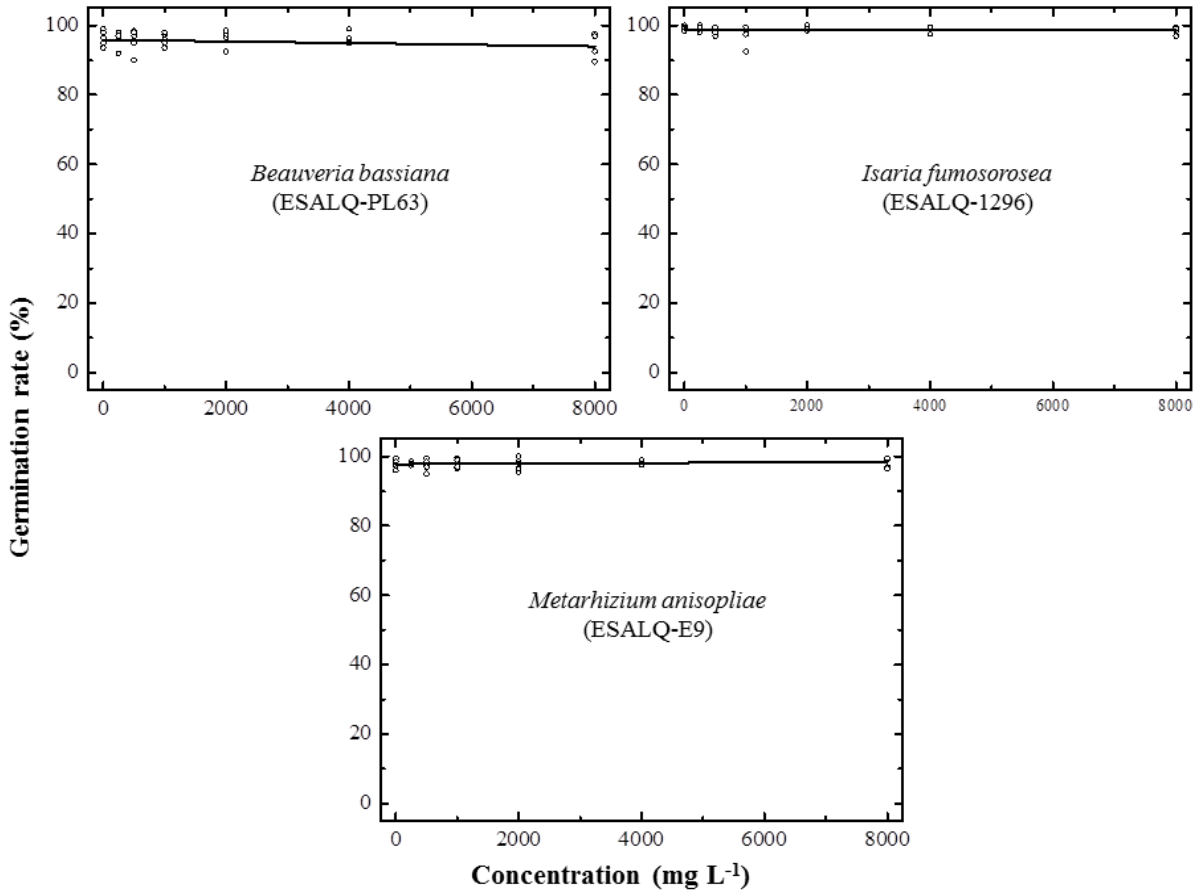

Figure 5.6 - Conidial germination (%) of isolates of three entomopathogenic fungal species grown in culture medium (post-contact toxicity) containing different concentrations of *Annona mucosa* (Annonaceae) seed ethanol extract - AMES. Temperature: 25±2ºC; photoperiod: 12 hours

Based on the compatibility index (ROSSI-ZALAF et al., 2008), AMES was compatible with the three entomopathogenic fungal species isolates (Table 5.2), except for *M. anisopliae* at the highest concentration tested (8,000 ppm), and it was thus classified as moderately toxic in this case. In general, and considering all of the variables analyzed, the *M. anisopliae* isolate (ESALQ-E9) was more sensitive to the extract's components, especially to the deleterious effect on sporulation.

Table 5.2 - *In vitro* toxicity classification of the *Annona mucosa* (Annonaceae) ethanolic seed extract – AMES for isolates of three entomopathogenic fungal species. Temperature: 25±2ºC; photoperiod: 12 hours

Species (isolates)	Concentration (ppm)	BI*	Classification**
Beauveria bassiana (ESALQ-PL63)	250	92.72	Compatible
	500	90.99	Compatible
	1,000	95.34	Compatible
	2,000	106.10	Compatible
	4,000	80.52	Compatible
	8,000	96.87	Compatible
Isaria fumosorosea (ESALQ-1296)	250	111.45	Compatible
	500	155.56	Compatible
	1,000	131.56	Compatible
	2,000	112.54	Compatible
	4,000	128.46	Compatible
	8,000	93.43	Compatible
Metarhizium anisopliae (ESALQ-E9)	250	95.99	Compatible
	500	96.86	Compatible
	1,000	84.96	Compatible
	2,000	74.15	Compatible
	4,000	69.19	Compatible
	8,000	64.83	Moderately toxic

*BI: Biological index proposed by Rossi-Zalaf et al. (2008);
**Classification: BI between 0 and 41 = toxic; BI between 42 and 66 = moderately toxic, and BI higher than 66 = compatible.

Our results indicate that AMES is compatible, at the concentrations recommended for managing the target arthropod pest species (< 4,000 ppm), with the three fungal species studied, which constitute important microbial control agents in Brazilian agroecosystems. In contrast to our results, some studies conducted with the aim of developing new biofungicides reported the toxicity of derivatives of *A. squamosa* or of acetogenins isolated from extracts prepared from its different structures to different fungal species that cause diseases in humans and crops, as well as mycotoxin-producing species in stored food (GOPALAKRISHNAN et al., 2010; DANG et al., 2011; VIDYASAGAR; SHIVAKUMAR, 2012). However, the following factors may explain the differences observed: variations in the chemical

profiles of the acetogenins (with their marked structure-activity relationship perculiarities) present in the derivatives of the different species, the use of different solvents and/or extraction methods (with their different selectivity/extraction capacity levels), and the different tolerances of fungal species to the active components. Corroborating the finding results, we also did not observe significant inhibition of vegetative growth in the CCT7638 *Aspergillus flavus* isolate or an effect on its aflatoxin (AFB_1) production in our previous study (RIBEIRO et al., submitted paper).

According to Alves and Lecuona (1998), the toxicity of pesticides to entomopathogens varies according to the fungal species, the isolate, the active ingredient and its mode of action, the product's formula, and the recommended dose/concentration. In our study, the effects of the botanical insecticide on the entomopathogenic fungi *B. bassiana* (ESALQ-PL63 isolate), *I. fumosorosea* (ESALQ-1296 isolate), and *M. anisopliae* (ESALQ-E9 isolate) depended on the fungal species exposed, the biological variable analyzed, and the concentration used.

Conidial germination is the first step in fungal infection in the insect host. Thus, if a chemical product confers reduced or inhibited conidial germination, it can reduce the efficiency of the fungi in controlling pests and establishing epizootics in the field. In the present study, we showed that conidial germination of the three fungal species isolates remained unaffected when they were exposed to concentrations of up to 8,000 mg L^{-1} for two hours (pre-contact toxicity), a scenario that simulates direct contact of the conidia in a liquid spray mixture. This result indicates the possibility of combining these two techniques (entomophatogens and bioinsecticides), which must be better examined in semi-field and field studies. Nevertheless, high germination rates correlate positively with virulence (ALTRE et al., 1999; HASSAN et al., 1989; RANGEL; ALSTON; ROBERTS, 2008). Faria et al. (2010) defined vigorous or high-vigor conidia as those that rapidly germinate within 24 hours of incubation, and these conidia may play an important role in successful microbial control. Conversely, conidia that germinate after 24 hours of incubation are considered weak or low-vigor and may consequently cause low mortality of the target insect. Regardless of the bioinsecticide concentration used, a high conidial germination rate (> 90%) was observed in the three entomopathogenic fungal species isolates after 24 hours of incubation. Thus, it is possible to infer that AMES does not affect vigor and, consequently, the ability to penetrate the host's cuticle, ensuring efficient microbial

control even when the two techniques (entomopathogens and bioinsecticides) are combined.

Entomopathogenic fungal mycelia grow within the insect, and thus there is small chance that they would be negatively affected by a chemical product at this phase (JARONSKI, 2010). However, vegetative growth and conidiogenesis are important steps for the development of secondary infections from epizootics in the field and for the fungus to persist in the environment (SCHUMACHER; POEHLING, 2012). In the present study, reduced vegetative growth (fungistatic effect) was observed in the three fungal species in the presence of botanical insecticide in the culture medium. However, the fact that a chemical product reduces the mycelial growth of an entomopathogenic fungus is not necessarily indicative of a significant reduction in conidial sporulation and germination (ZIMMERMANN, 1975). In our study, independent of the fungal species, the conidia produced in culture medium (post-contact toxicity) under different bioinsecticide concentrations also exhibited high viability (>95%). However, we observed reduced conidiogenesis (conidia cm^{-2} of colony) in *M. anisopliae*, which was proportional to the reduced mycelial growth. In contrast, conidiogenesis in the *B. bassiana* isolate remained unaffected by the different AMES concentrations, whereas for *I. fumosorosea*, the pattern depended on the concentration used, exhibiting points of stimulated and reduced conidiogenesis within the concentration range used. In general, the *M. anisopliae* isolate was most sensitive to the bioinsecticide, especially in terms of conidiogenesis, which affected its classification based on the compatibility index.

In light of the results obtained, it is possible to conclude that AMES combined with entomopathogenic fungi constitutes an important tool for arthropod pest management in organic and/or ecological food production systems that lack alternative management techniques. Additionally, the compatibility with biological control agents and the possibility of preparing AMES bioinsecticide from plant matter available on the farm itself [see preparation method in Ribeiro et al. (2013)] are characteristics that may significantly contribute to improved economic and environmental viability in agricultural production in developing countries, which are key links for sustainability.

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