SHAIENE COSTA MORENO

BIOACTIVITY OF NEOTROPICAL PLANT COMPOUNDS TO AGRICULTURAL AND VEGETABLE PESTS AND SELECTIVITY TO NON-TARGET INSECTS

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Entomologia, área de concentração em Entomologia Agrícola para a obtenção do título de Doutor.

Orientador

Dr. Geraldo Andrade Carvalho

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APROVADA em 04 de abril de 2011.

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RESUMO

A demanda por novos produtos para o manejo de pragas é crescente. Os riscos ambientais do uso indiscriminado de pesticidas sintéticos para controle de pragas agrícolas são evidentes e vêm sendo amplamente discutidos. Consequentemente, os pesticidas naturais, especialmente os de origem vegetal, são considerados alternativas promissoras. Este trabalho teve como objetivo avaliar o efeito de plantas sobre importantes pragas agrícolas e alguns insetos não-alvo. Inicialmente realizou-se uma seleção de plantas bioativas, avaliando-se extratos hexânicos e etanólicos de 23 plantas. O extrato hexânico da planta *Acmella oleracea* (L.) R.K. Jansen foi o que apresentou maior atividade inseticida, sendo selecionado para isolamento e identificação de compostos bioativos. Foram isolados três alkamidas do extrato hêxanico de *A. oleracea*: spilanthol, (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide and (*R*,*E*)-*N*-(2-methylbutyl)undeca-2-en-8,10-diynamide. Avaliou-se então a atividade inseticida desses compostos sobre *Tuta absoluta* Meyr. (Lepidoptera: Gelechiidae), *Ascia monuste* Latr. (Lepidoptera*:* Pieridae), *Diaphania hyalinata* L. (Lepidoptera: Crambidae), e *Plutella xylostella* L. (Lepidoptera: Plutellidae), e a seletividade sobre o predador *Solenopsis saevissima* Smith (Hymenoptera: Formicidae) e sobre o polinizador *Tetragonisca angustula* Latr. (Hymenoptera: Apidae: Meliponinae). Os efeitos de extratos e compostos presentes em *A. oleracea* também foram avaliados sobre os pulgões *Myzus persicae* Sulz. e *Lipaphis erysimi* Kalt. (Hemiptera: Aphididae), e sobre o parasitóide *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae) e o predador *Orius insidiosus* Say (Hemiptera: Anthocoridae). O extrato de *A. oleracea* e as alkamidas avaliadas aprentaram elevada atividade inseticida sobre os insetos pragas e foram seletivos aos insetos não-alvo. Também foi objetivo deste trabalho avaliar o efeito letal e comportamental de extratos de plantas sobre operários das formigas cortadeiras *Atta sexdens rubropilosa* Forel, *Atta laevigata* Smith e *Acromyrmex subterraneus molestans* Santschi (Hymenoptera: Formicidae). Todos os extratos testados apresentaram efeito inseticida sobre as formigas e o extrato de *A. oleracea* foi o mais tóxico para todas as espécies, além de não apresentar efeito no comportamento de caminhamento das formigas.

Palavras-chave: Inseticidas botânicos. Metabólitos secundários de plantas. Controle de pragas.

ABSTRACT

The demand for new products to control pests is growing. The number of environmental issues stemming from the use of synthetic pesticides to control agricultural pests is also increasing. Accordingly, natural pesticides, particularly those of plant origin, are now considered to be promising alternatives. This study aimed to evaluate the effects of plants on important agricultural pests and several non-target insects. An initial bioassay screening with hexane and ethanol extracts from 23 plants was performed. The hexane extract from *Acmella oleracea* (L.) R.K. Jansen exhibited the highest activity of all extracts, and the structure of its bioactive compounds was identified. The following three alkamides were isolated from the hexane extract of the aerial parts of *A. oleracea*: spilanthol, (*E*)-*N*-isobutylundeca-2-en-8,10-diynamide and (*R*,*E*)-*N*-(2-methylbutyl)undeca-2-en-8,10-diynamide. We analyzed the insecticidal activity of these compounds on *Tuta absoluta* Meyr. (Lepidoptera: Gelechiidae), *Ascia monuste* Latr. (Lepidoptera*:* Pieridae), *Diaphania hyalinata* L. (Lepidoptera: Crambidae), *Plutella xylostella* L. (Lepidoptera: Plutellidae), the predator *Solenopsis saevissima* Smith (Hymenoptera: Formicidae) and the pollinator *Tetragonisca angustula* Latr. (Hymenoptera: Apidae: Meliponinae). We also evaluated the effects of extracts and compounds present in *A. oleracea* on the aphids *Myzus persicae* (Sulz.) and *Lipaphis erysimi* (Kalt.) (Hemiptera: Aphididae), the aphid parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae), and the predator *Orius insidiosus* (Say) (Hemiptera: Anthocoridae). The extracts and alkamides of *A. oleracea* showed high insecticidal activity against pest insects and were selective to non-target insects. Another objective of this research was to assess the lethal and behavioral effects of plant extracts on the leaf-cutting ants *Atta sexdens rubropilosa* Forel, *Atta laevigata* Smith and *Acromyrmex subterraneus molestans* Santschi (Hymenoptera: Formicidae). All extracts showed some insecticidal effect on the ants, and the *A. oleracea* extract was the most toxic to all ant species studied. No extract affected the walking behavior of the ants.

Keywords: Botanical insecticides. Plant secondary metabolites. Pest control.

SUMARY

PART ONE

1 GENERAL INTRODUCTION

A variety of factors affect crop production. Insect pests account for approximately 30% of agricultural production losses. To avoid such losses, producers use control methods including behavioral, biological, genetic, cultural and chemical approaches. Among these methods, the application of insecticides is the most common, and it offers distinct advantages, such as high efficiency, low costs and ease of use (SODERLUND, 1995).

Botanical insecticides are one type of insecticide used in pest control. The use of botanical pesticides is not new and dates back to ancient times when it was one of the main methods of pest control. However, after the Second World War, the advent of synthetic organic pesticides and the emergence of molecules such as HCH, DDT, aldrin, dieldrin and chlordane (LAGUNES; RODRÍGUEZ, 1992; VIEGAS JR., 2003) quickly led to the replacement of botanical compounds (CASIDA; QUISTAD, 1998; FLINT; VAN DEN BOSCH, 1981; THACKER, 2002). Now, the excessive use of these synthetic products is seriously affecting the environment. In addition to occupational, food and public health hazards, the indiscriminate use of insecticides can reduce the population of beneficial insects, contribute to the resurgence and outbreak of pests and decrease the effectiveness of insecticides through the selection of resistant populations (CAMPANHOLA, 1990; GUEDES, 1999; KAY; COLLINS, 1987; MALTBY, 1999; SIQUEIRA, 2000).

Although synthetic insecticides (e.g., chlorinated hydrocarbons, organophosphates and pyrethroids) have been an important part of pest management strategies for years, their disadvantages and risks are now apparent. As a result, there is a continuous search for less hazardous alternatives to

conventional synthetic insecticides (MARICONI, 1981; VIEIRA et al., 2001). Ideally, insecticides should reduce pest populations, be target-specific (i.e., kill pests but not other organisms), break down quickly and have a low level of toxicity for humans and other mammals.

Studying natural products of plant origin can help to discover more specific and less persistent pesticides. Plants, as organisms that co-evolved with insects, are natural sources of insecticidal substances. These compounds are products of secondary metabolism and are likely related to defense mechanisms (CATEHOUSE, 2002; MANN, 1995). Plant secondary metabolites play an important role in plant–insect interactions, and therefore, such compounds may have insecticidal, hormonal or antifeedant effects on insects.

Natural pest control products are advantageous because they are not persistent in the environment, rapidly control pests and have a low level of toxicity for natural enemies and humans. However, the biological activity of a compound cannot be associated only with the mortality that it causes. Sublethal effects, which are not usually considered, may be equally or more important than lethal effects. Most investigations are based on the acute lethal action triggered by compounds, but the effects of natural products on insects are variable and may be toxic, repellent, cause sterility, modify behavior and development, or reduce feeding activity (ARNASON et al., 1990; BELL et al., 1990).

Assessments of population growth, reproductive capacity and behavioral parameters need to be further evaluated to determine the biological activity of compounds that have the potential for use in insect pest management. Extreme cases, such as leaf-cutting ants, deserve mention. The use of bait to control ants is based on a behavioral response in which worker ants carry the bait to the nest, where it then expresses its control potential.

As mentioned, concern about the use of synthetic insecticides has stimulated interest in the study of plants that possess insecticidal properties.

Plants such as *Azadirachta indica*, *Trichilia pallida*, *Melia azadarach*, *Chrysanthemum cinerariaefolium*, *Chrysanthemum cineum*, *Lonchocarpus* spp., *Derris* spp., *Schoenocaulon officinale*, *Ryania speciosa*, *Nicotiana tabacum*, *Citrus* spp., *Piper* spp., *Allium sativum*, *Eucaliptus citriodora*, *Lycopersicon* spp. and *Manihot esculenta* have been identified as toxic to insect pests. (NAIR, 1994; ISMAN, 2006).

In addition to these identified plants, various plants are anecdotally known to have insecticidal properties, and these are important in the search for products with biological activity. However, these plants must be further studied to confirm and characterize their effects.

Brazilian biomes have a great potential to provide a source of natural compounds with pesticidal properties because of their abundance and diversity of plant species. However, much remains to be discovered about this resource. It has been estimated that the chemical compositions of only 8% of Brazilian plants have been studied, and the number of plants that have had their biological properties characterized is small (SIMÕES, 2003). Meanwhile, several animal and plant species have become extinct. Further, flora and fauna are constantly smuggled by transnational organized crime networks for research purposes and to create patented goods. These factors increase the need to conduct research at sites of origin, especially on the pesticidal aspects of plants, which are not analyzed when research is focused on developing drugs.

In this context, studies that aim to select plants that contain large amounts of compounds with insecticidal properties are extremely important to the management of pests. Additionally, the toxicity of new substances, their effects on the biology and behavior of insects, and their selectivity to natural enemies should be studied.

This study was undertaken with the following objectives: i) to screen plants with insecticide activity against important agricultural pests; ii) to

evaluate the biological activity of compounds present in *Acmella oleracea* (L.) R.K. Jansen, the plant selected in the bioassay screening process, against *Tuta absoluta* Meyr. (Lepidoptera: Gelechiidae), *Ascia monuste* Latr. (Lepidoptera*:* Pieridae), *Diaphania hyalinata* L. (Lepidoptera: Crambidae), and *Plutella xylostella* L. (Lepidoptera: Plutellidae), and to evaluate the selectivity of these compounds to the predator *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) and to the pollinator *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae: Meliponinae); iii) to assess the effects of extracts of Amazonian plants on the survival of the leaf-cutting ants *Atta sexdens rubropilosa* Forel, *Atta laevigata* Smith and *Acromyrmex subterraneus molestans* Santschi (Hymenoptera: Formicidae) and to assess their effect on the mobility of these species; and iv) to evaluate the toxicity of *A. oleracea* extracts in the aphids *Myzus persicae* Sulz. and *Lipaphis erysimi* Kalt. (Hemiptera: Aphididae), the aphid parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae), and the predator *Orius insidiosus* Say (Hemiptera: Anthocoridae).

2 THEORETICAL BACKGROUND

2.1 Plant secondary metabolism: a source of bioactive compounds

The beneficial actions of plant materials typically result from combinations of secondary products in the plant. Multiple chemical compounds often act together through additive or synergistic action at single or multiple target sites associated with a physiological process (BRISKIN, 2000). The bioactivity of plants is unique to particular plant species or groups, according to the concept that combinations of secondary products in a particular plant species are often taxonomically distinct (DIXON, 1999; WINK, 1999). This is in contrast to primary products, such as carbohydrates, lipids, proteins, heme chlorophyll, and nucleic acids, that are common to all plant species and are involved in the primary metabolic processes of building and maintaining plant cells (KAUFMAN et al., 1999; WINK, 1999). Although secondary products have historically been defined as chemicals that do not appear to have a vital biochemical role in the process of building and maintaining plant cells, current research has shown that these chemicals play a pivotal role in the ecophysiology of plants. In this respect, secondary products can have a defensive role against herbivory, pathogen attack, and interplant competition or an attractant role toward beneficial organisms such as pollinators or symbionts (DIXON, 1999; KAUFMAN et al., 1999; WINK; SCHIMMER, 1999).

Plant secondary products can also exhibit protective properties in relation to abiotic stresses such as those associated with changes in temperature, water status, light levels, ultraviolet (UV) exposure, and mineral nutrients. Furthermore, recent research has indicated potential roles of secondary products at the cellular level as plant growth regulators and modulators of gene expression and in signal transduction (KAUFMAN et al., 1999).

To promote plant survival, the structures of secondary products have evolved to interact with molecular targets that affect the cells, tissues, and physiological functions of other competing organisms. In this respect, some plant secondary products may resemble endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters (e.g., those of the central nervous system or endocrine system) (DIXON, 1999; KAUFMAN et al., 1999). Wink (1999) referred to this development of structural similarity between plant secondary products and the endogenous substances of other organisms as "evolutionary molecular modeling".

Secondary metabolites are numerous and widespread, especially in higher plants. The total number of plant secondary metabolites for which structures have been elucidated is around 50,000, and this is likely only a small fraction of all plant secondary metabolites. Because fewer than 20% of all plants have been studied in depth, it is likely that the number of secondary metabolites in the plant kingdom exceeds 100,000 (SCHWAB, 2003; WINK, 2007).

Toxic secondary metabolites are present in plants at low concentrations, generally less than 2% of dry matter. The amount of secondary compounds in an organism is the result of an equilibrium among synthesis, storage, and degradation. Regulation of secondary metabolism is complex. The onset of secondary metabolism is linked to the developmental stage of an organism and, often, to morphological and cytological changes (MAKKAR; SIDDHURAJU; BECKER, 2007).

The chemical structures of secondary plant products are more complex than those of primary products. This is partially explained by the fact that many, though not all, secondary products are derived from amino acids or nucleotides. Most of the compounds found in plants belong to a limited number of families of substances. Minor chemical modifications, such as methylations,

hydroxylations, and intercalations with metal ions, produce a wide spectrum of functionally distinct substances (SEIGLER, 2002).

Interest in plant secondary metabolites has increased dramatically in recent years because of their diverse effects, including antioxidant, antiviral, antibacterial, anticancer and pesticidal effects. The search for new active compounds has been termed bioprospecting, the search for biological gold. Understanding the physiology, biochemistry, and ecology of secondary metabolism is essential to exploit bioactive plant chemicals in a rational way in medicine and agriculture.

2.2 The classes of secondary metabolites

Although the structures of secondary metabolites may seem to be bewilderingly diverse, the majority of these compounds belong to one of a limited number of families, each of which has particular structural characteristics based on the ways in which they are biosynthesized (HANSON, 2003).

Secondary metabolites can be divided into two groups: those without nitrogen and those with nitrogen in their structures (WINK, 2007). Nitrogen-containing compounds include alkaloids, amines, nonprotein amino acids, cyanogenic glycosides, glucosinolates, alkamides, protease inhibitor peptides and lectins. Nitrogen-free compounds include terpenoids (e.g., mono-, sesqui-, di-, tri- and tetraterpenes), polyketides, phenolics (e.g., flavonoids, tannins and lignans), and polyacetylenes.

Currently, there are 17 classes of secondary metabolites. Table 1 gives an overview of the known classes and number of structures that belong to each class.

Table 1 Structural types of secondary metabolites and known structures.

Class	Number of structures
With nitrogen	
Alkaloids	29,000
Non-protein amino acids	700
Amines	100
Cyanogenic glycosides	60
Glucosinolates	100
Alkamides	150
Lectins, peptides	800
Without nitrogen	
Monoterpenes (including iridoids)	2,500
Sesquiterpenes	5,000
Diterpenes	2,500
Triterpenes, steroids, saponins	5,000
Tetraterpenes	500
Phenylpropanoids, coumarins, lignans	2,000
Flavonoids, tannins	4,000
Polyacetylenes, fatty acids, waxes	1,500
Polyketides (anthraquinones)	750
Carbohydrates	200

Source: Adapted from Wink (2007).

2.3 Chemical ecology

Many plant secondary metabolites were originally investigated because of their value as medicines, perfumes or foods. However, beginning in the latter part of the 20th century, an increasing amount of attention has been directed at the biological functions of natural products and their ecological roles in regulating interactions between organisms. Developments in instrumental methods have enabled the detection and identification of very small amounts of materials as well as the observation of their effects, particularly on insects.

Natural products often have an ecological role in regulating the interactions between plants, microorganisms, insects and animals. Secondary metabolites can protect plants against herbivores, microbes, or competing plants. Some secondary metabolites also function as signal compounds to attract pollinating or seed-dispersing animals. Because of their ecological role, secondary plant substances can be classified as 'allelochemics', which are defined as 'non-nutritional chemicals produced by an individual of one species that affect the growth, health, behavior, or population biology of another species (WHITTAKER, 1970).

Several plant-insect relationships are determined by the presence of secondary metabolites. The production of chemicals that are capable of deterring insect pests by toxic activity is an important survival strategy for plants. These compounds may be either deterrents or attractants. Plants have chemical defense systems to avoid attack from phytophagous insects, and plant products can act as insecticides, repellents and antifeedants against insect.

Structurally, such toxins are usually non-volatile compounds as a result of their molecular weight or hydrophilicity. If they accumulate in the tissue of healthy plants prior to insect attack, they are considered to be constitutive (STAMP, 2003; WITTSTOCK; GERSHENZON, 2002). Alternatively, they may only be present, or present in much higher concentrations, after plants have encountered attack or after exposure to natural plant- or insect-derived defense activators. In this case, they are considered to be induced toxicants (WALLING, 2000). Induction provides economic advantages to the plant because metabolic energy is diverted from primary metabolism to produce toxins. In addition, insect herbivores are less likely to develop resistance to induced defense products because they are not frequently exposed to them. However, the balance between these two strategies may depend on the likelihood that a plant will come under attack. Plants that encounter more frequent attacks by pests may be

forced to rely more heavily on constitutive rather than induced defenses, despite the greater energetic cost to the plant (MCKEY, 1979).

The distribution of defense metabolites is often restricted both spatially and temporally, and plant organs associated with survival or reproduction tend to contain the highest concentrations of constitutive defense metabolites (WITTSTOCK; GERSHENZON, 2002). They may be developmentally regulated, being present at the highest concentrations when the plant is young and less able to protect itself against predators, or they may be concentrated around the region of contact with the invader.

2.4 Botanical insecticides

Botanical insecticides, occasionally referred to as "botanicals", are derived from plants. Many botanical insecticides have been used for hundreds of years but have recently been displaced by synthetic insecticides. The use of botanical insecticides in agriculture in China, Egypt, Greece, and India dates back at least two millennia (THACKER, 2002; WARE, 1883). Nicotine (from *Nicotiana tabacum*), piretrins (from *Tanacetum cinerariifolium*) and rotenone (from *Derris* and *Lonchocarpus*) are examples of plant compounds that were used long ago to control agricultural pests. In Europe and North America, the documented use of botanicals extends back more than 150 years, predating discoveries of the major classes of synthetic chemical insecticides (e.g., organochlorines, organophosphates, carbamates, and pyrethroids) in the mid-1930s to 1950s. Synthetic insecticides have effectively displaced botanicals from their important role in agriculture and relegated them to an essentially trivial position in the marketplace of crop protectants. However, recent history shows that the overzealous use of synthetic insecticides has led to numerous problems unforeseen at the time of their introduction: the disruption of natural biological

control and pollination; the acute and chronic poisoning of applicators, farm workers, and even consumers; the destruction of fish, birds, and other wildlife; extensive groundwater contamination that is potentially threatening to human and environmental health; and the evolution of resistance to pesticides in pest populations.

In response to these events, many countries have reassessed the risks of using synthetic insecticides and banned products from use in agriculture, especially those developed before 1980. Consequently, there is an increased impetus to discover and develop alternative pest management products, including insecticides derived from plants.

2.4.1 Chemical synergisms in the ecological function of secondary products and the benefits of botanical insecticides

In contrast to synthetic insecticides based on single chemicals, many phytochemicals exert their toxic effects through several chemical compounds that act additively or synergistically at single or multiple target sites associated with a physiological process (PESSARAKLI, 2001). This synergistic or additive effect can promote pesticidal effectiveness without the problematic effects associated with the predominance of a single xenobiotic compound. For example, with the use of multiple compounds, insects are less likely to develop resistance to insecticides.

The additive or synergistic interaction of multiple chemicals probably originated in the functional role of secondary products in promoting plant survival (WINK; SCHIMMER, 1999). The additive or synergistic effects of a mixture of chemicals at multiple target sites not only ensures effectiveness against a wide range of herbivores or pathogens but also decreases the chance

that these organisms will develop resistance or adaptive responses (KAUFMAN, 1999; WINK, 1999).

2.4.2 The potential of using botanical insecticides for the control of agricultural pests

The use of insecticides against pest insects is one of the main pest management tools available in agriculture (COOPER; DOBSON, 2007; EDWARDS-JONES, 2008), but attitudes and behaviors regarding the use of these compounds are steadily changing as safety demands increase (MATSUMURA, 2004; MATTHEWS, 2008). New compounds have been developed to answer such demands (NAUEN; BRETSCHNEIDER, 2002; NICHOLSON, 2007), but the concern remains about their overuse and their effects as pollutants.

Among the newly developed and used pesticides, biopesticides or biorational pesticides have received a considerable amount of attention (ISMAN, 2006; ROSELL et al., 2008). Natural products have had and continue to have value as components of crop protection strategies, both as *per se* insecticides and as chemical backbones for the synthesis of new insecticidal molecules (COATS, 1994; KIDD, 2000). Phytochemicals are an attractive alternative to the currently used synthetic insecticides because they constitute a rich source of bioactive molecules (WINK, 1993). They are usually active against a limited number of specific target pests, biodegrade into non-toxic compounds, and are, therefore, potentially useful in integrated pest management programs. Accordingly, recent efforts have been directed toward the discovery of secondary metabolites that could be used as commercial insecticides or lead compounds (GULERIA; TIKU, 2009).

Overall, botanical insecticides can be used in the following ways to control pests:

- As a powder product prepared “in natura” and as aqueous or alcoholic extracts (GUERRA, 1985; SANTOS et al., 1988).
- In commercial and semi-commercial concentrated formulations (MORDUE; BLACKELL, 1993).
- As purified and isolated pure compounds obtained from plant extracts. (NAIR, 1994).
- As a source of molecules for the synthesis of novel agrochemicals with desirable characteristics (CUTLER, 1988; HEDIN et al., 1994; ISMAN, 1989; NAIR, 1994).
- Incorporated into the genetic material of crops by genetic engineering, thus minimizing the damage caused by insect pests, microorganisms and weeds (CUTLER, 1988; ELANOVICH, 1988).

2.4.3 Advantages of botanical insecticides

Many compounds with diverse chemical structures and different modes of action are classified as botanical insecticides. Therefore, presenting a detailed list of advantages or disadvantages that apply to all compounds in this category is difficult. General advantages shared by most of these compounds include the following (CLOYD, 2004; WEINZIERL; HENN, 1994):

- Rapid degradation. Botanicals and insecticidal soaps degrade rapidly in sunlight, air, and moisture and are readily broken down by detoxification enzymes. This is important because rapid breakdown means less persistence in the environment and reduced risks to nontarget organisms. Many botanicals may be applied to food crops shortly before harvest without leaving excessive residues.

- Rapid action. Botanicals and soaps act quickly to stop feeding by pest insects. Although they may not cause death for hours or days, they often cause immediate paralysis or cessation of feeding.
- Low mammalian toxicity. Most botanicals and insecticidal soaps have low to moderate mammalian toxicity.
- Selectivity. Although most botanicals have a broad spectrum of activity in standard laboratory tests, in the field, their rapid degradation and the action of some as stomach poisons makes them more selective in some instances for plant-feeding pest insects and less harmful to beneficial insects.
- Low toxicity to plants. Most botanicals are not phytotoxic (toxic to plants). Insecticidal soaps and nicotine sulfate, however, may be toxic to some ornamentals.

2.4.4 Disadvantages of botanical insecticides

Natural insecticides are generally less stable than synthetic materials and degrade quickly in the environment, meaning that they are also less potent and have shorter residual periods than their synthetic counterparts (KÜHNE, 2008). Therefore, satisfactory arthropod pest management can only be achieved if insecticide use is integrated with other strategies, including the timing of applications to minimize harmful effects on beneficial organisms.

One obstacle to the commercialization of new insecticides made of natural substances is the requirement of a large marketing base to cover the high costs associated with marketing approval (KÜHNE, 2008). Botanicals also tend to be more expensive than synthetics, and some are not as widely available. Furthermore, there are three major barriers to the commercialization of botanical insecticides: the sustainability of the botanical resource, the standardization of chemically complex extracts, and regulatory approval. Other drawbacks or

limitations include the slowness of their action and the lack of residual action for most botanicals (ISMAN, 2006).

2.4.5 Current botanical insecticides in use

Currently, there are three major types of botanical insecticides used for pest control, pyrethrins, rotenone and azadiractins, and three others in limited use, ryania, nicotine, and sabadilla. Other plant extracts and oils (e.g., garlic oil and Capsicum oleoresin) have limited regional uses in various countries (ISMAN, 2006).

Pyrethrins

Pyrethrins refer to the insecticidal compounds that occur in pyrethrum. Pyrethrum is an oleoresin extracted from the dried flowers of the pyrethrum daisy, *Tanacetum cinerariaefolium* (Asteraceae), and is considered as the archetypical natural insecticide (GLYNNE-JONES, 2001). Pyrethrins include three esters of chrysanthemic acid and three esters of pyrethric acid. Among the six esters, those incorporating the alcohol pyrethrolone, namely pyrethrins I and II, are the most abundant and account for most of the compound's insecticidal activity. Most insects are highly susceptible to low concentrations of pyrethrins. Pyrethrins are extremely fast acting, and their insecticidal action is characterized by a rapid knockdown effect, hyperactivity and convulsions. These symptoms are a result of the neurotoxic action of the pyrethrins, which block voltage-gated sodium channels in nerve axons. As such, the mechanism of action in pyrethrins is qualitatively similar to that of DDT and many synthetic organochlorine insecticides. Pyrethrins are especially labile in the presence of the UV component of sunlight, a fact that has greatly limited their use outdoors. They

are the predominant botanical in use, accounting for approximately 80% of the global botanical insecticide market (ISMAN, 2006).

Pyrethrins are effective as broad spectrum insecticides and are used to control pests such as aphids, whiteflies, stinkbugs, and mites (COX, 2002). They are available as dusts, sprays, and aerosols and may be mixed with synthetic pesticides or other botanicals.

Rotenone

Rotenone is one of several isoflavonoids produced in the roots or rhizomes of the tropical legumes *Derris*, *Lonchocarpus*, and *Tephrosia*. As an insecticide, rotenone has been used for more than 150 years (SHEPARD, 1951; ISMAN, 2006). It is widely used in gardens and to a lesser extent on pets. Rotenone is a powerful inhibitor of cellular respiration, the process that converts nutrient compounds into energy at the cellular level. In insects, rotenone exerts its toxic effects primarily on nerve and muscle cells, causing rapid cessation of feeding (KLAASSEN; WATKINS, 2003; TADA-OIKAWA, 2003). Death occurs several hours to a few days after exposure. Rotenone is extremely toxic to fish, and is often used as a piscicide in water management programs.

Rotenone is effective against a wide range of insects and has a short residual life. It is not toxic to honeybees, but it does kill some beneficial insects (WEINZIERL; HENN, 1994). It is registered for use against a number of chewing insects on many vegetables and some fruits.

Azadirachtins

The azadirachtins belong to a group of tetranortriterpenoids that exhibit a variety of biological activities. This class of chemicals, extracted from the seeds of the neem tree, *Azadirachta indica* (Meliaceae), has generated a considerable amount of excitement with respect to insect control and safety to

mammals. The numerous reported effects include repellency, feeding deterrency and interference with growth, development, and reproduction (GULERIA; TIKU, 2009).

Research has shown that azadirachtins can control more than 400 species of insects, including many key crop pests, and has proven to be one of the most promising plant ingredients for integrated pest management. Azadirachtins effectively control common pests such as thrips, whiteflies, leaf folders, bollworms, aphids, jassids, pod borers, fruit borers, stem borers, leafhoppers and caterpillars (MARTINEZ; EMDEN, 2001).

Ryania

The stems and roots of the South American plant *Ryania speciosa* (Flacourtiaceae) yield the alkaloid ryania, which is much more stable than rotenone and nicotine but not as potent as other botanical insecticides (WARE, 1892). Ryanodine was originally isolated as the active principle, but eleven ryanoids have been identified with different insecticidal activities. The mechanism of action primarily affects the Ca^{2+} release channel in muscle, and ryanodine acts as a muscular poison by blocking the conversion of ADP to ATP in striated muscles (GULERIA; TIKU, 2009).

The acute and chronic oral toxicity of ryania in mammals is moderate. It is generally not harmful to most natural enemies, but it may be toxic to certain predatory mites. The residual activity of ryania is longer than that of most other botanicals. It has been used commercially in fruit and vegetable production against caterpillars, including European corn borers and corn earworms, and thrips (WEINZIERL; HENN, 1994).

Nicotine

Five different families of plants produce nicotinoids that are potent insecticides in situ or when extracted from the leaves. Tobacco, *Nicotiana tabacum* (Solanaceae), is the primary source, and its use has been widespread for more than a century. Nicotine constitutes between 2 and 8 percent of dried tobacco leaves. Insecticidal formulations generally contain nicotine in the form of 40 percent nicotine sulfate (ISMAN, 2006).

In both insects and mammals, nicotine is an extremely fast-acting nerve toxin. It competes with acetylcholine, the major neurotransmitter, by bonding to acetylcholine receptors at nerve synapses and causing uncontrolled nerve firing. This disruption of normal nerve impulse activity results in the rapid failure of body systems that depend on nervous input for proper functioning. In insects, the action of nicotine is fairly selective, and only certain types of insects are affected. It is used in greenhouses and gardens as a fumigant and contact poison to control soft-bodied sucking pests such as aphids, thrips, and mites (GULERIA; TIKU, 2009).

Despite the fact that smokers regularly inhale small quantities of nicotine in tobacco smoke, nicotine in pure form is extremely toxic to mammals and is considered a Class I (most dangerous) poison. Nicotine has been responsible for numerous poisonings and accidental deaths because of its rapid penetration of both skin and mucous membranes and because it is used in a concentrated form (WEINZIERL; HENN, 1994).

Sabadilla

Sabadilla is a botanical insecticide obtained from the seeds of the tropical lily *Schoenocaulon officinale*. When sabadilla seeds are aged, heated, or treated with alkali, several insecticidal alkaloids are formed or activated. Alkaloids are physiologically active compounds that occur naturally in many

plants. The alkaloids in sabadilla are known collectively as veratrine or as the veratrine alkaloids. The mode of action of these alkaloids is remarkably similar to that of the pyrethrins, despite their lack of structural similarity (DENAC, 2000; ISMAN, 2006; ZLOTKIN, 1999). Sabadilla is a broad-spectrum contact poison, but has some activity as a stomach poison. Baits, dusts or sprays may be used in organic fruit and vegetable production against squash bugs, harlequin bugs, thrips, caterpillars, leaf hoppers, and stink bugs. These alkaloids break down rapidly in sunlight and air, leaving no harmful residues. However, it is highly toxic to honeybees, and should only be used when bees are not present (ISMAN, 2006).

2.4.6 Prospects of botanical insecticides

According to Isman (2006), in industrialized countries, it is difficult to imagine botanicals playing a more prominent role than they currently play, except in organic food production. In conventional agriculture, botanicals face tremendous competition from the newest generation of reduced risk synthetic insecticides, such as the neonicotinoids.

The benefits of botanical insecticides can be best realized in developing countries, where farmers may not be able to afford synthetic insecticides and plants and plant derivatives are traditionally used for crop protection. Even if synthetic insecticides are affordable, limited literacy rates and a lack of protective equipment result in thousands of accidental poisonings annually (FORGET; GOODMAN; VILLIERS, 1993).

Pest control efficacy is only one factor in the adoption of botanicals. The logistics of production, preparation, and use can affect the uptake of botanicals (MORSE et al., 2002). It may be time to refocus the attention of the research

community toward the development and application of known botanicals, while continuing to screen more plants and isolate novel bioactive substances.

3 CONCLUSION

The crude hexane extract of *A. oleracea* showed high insecticidal activity and can be used to control *T. absoluta*, *A. monuste*, *D. hyalinata* and *P. xylostela,* in organic or conventional crops. Quantification of LD_{50} values of isolated compounds showed that alkamides could serve as potent insecticides for *T. absoluta*, *A. monuste*, *D. hyalinata* and *P. xylostela* control programs. The spilanthol was the main alkamide active isolated. This alkamide is the most promising as it had the highest insecticidal activity and was selective to non-target organisms.

All extracts showed some insecticidal effect on the leaf-cutting ants *A. sexdens rubropilosa*, *A. laevigata* and *A. subterraneus molestans*, and the *A. oleracea* extract was the most toxic to all ant species studied. No extract affected the walking behavior of the ants.

The results of this study demonstrate the substantial effects of ethanol extracts of *A. oleracea* against *M. persicae* and *L. erysimi* under laboratory conditions and verify the extract selectivity to natural enemies, suggesting its potential in controlling this insect pest under field conditions.

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PART TWO – ARTICLES

ARTICLE 1

BIOACTIVITY OF COMPOUNDS FROM *Acmella oleracea* AGAINST *Tuta absoluta* (MEYRICK) (LEPIDOPTERA: GELECHIIDAE) AND SELECTIVITY TO TWO NON-TARGET SPECIES

This article was written in accordance with the standards of Pest Management Science, for which it was submitted and accepted in March 2011.

Bioactivity of compounds from *Acmella oleracea* against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) and selectivity to two non-target species

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Abstract

BACKGROUND: Tropical plants are recognized sources of bioactive compounds that can be used for pest control. Our objective was to evaluate the biological activity of compounds present in *Acmella oleracea* (L.) R.K. Jansen (Asteracea) against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), which is the main pest of tomato crops in Latin America. We were also interested in the selectivity of these compounds to the predator *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) and to the pollinator *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae: Meliponinae).

RESULTS: A bioassay screening with hexane and ethanol extracts from 23 plants was performed. The hexane extract of *A. oleracea* was the most active of the extracts and was selected for further study. The following three alkamides were isolated from the hexane extract of the aerial parts of *A. oleracea*: spilanthol, undeca-2*E*-en-8,10-diynoic acid isobutylamide and (2*E*)-*N*-(2-

methylbutyl)-2-undecene-8,10-diynamide. All of the isolated compounds showed insecticidal activity with spilanthol being the most active (LD_{50} = 0.13 μg mg^{-1}) against *T. absoluta*. The alkamides were selective to both beneficial species studied.

CONCLUSION: The crude hexane extract of *A. oleracea* showed high insecticidal activity and can be used to control *T. absoluta* in organic or conventional crops. Quantification of LD_{50} values of isolated compounds against *T. absoluta* showed that alkamides could serve as potent insecticides for *T. absoluta* control programs. The spilanthol was the main alkamide active isolated. This alkamide is the most promising as it had the highest insecticidal activity and was selective to non-target organisms.

Keywords: botanical pesticide; insect control; secondary metabolites; bioactive alkamides, tomato leafminer

1 INTRODUCTION

The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a neotropical oligophagous insect that attacks solanaceous crops.[1] Since the 1960s, it has become one of the key pests of tomato crops in most South American countries.[2] Recently, *T. absoluta* has also become a serious threat to the tomato production in the Mediterranean region.[3] Following the detection in the Spanish tomato-growing area at the end of 2006, *T. absoluta* spread quickly to other European and northern African countries.[4,5] The larvae attack tomato plants during all growth stages, producing large galleries in their leaves, burrowing stalks, apical buds, green and ripe fruits.

Chemical control has been the main method of control used against *T. absoluta*. Horticultural growers have attempted to decrease the damage caused by *T. absoluta* by applying insecticides more than two times a week during a

single cultivation period.[2] *T. absoluta* control is a challenge due to the nature of the damage it causes and its rapid ability to develop resistance toward conventional insecticides.[1,6] Thus, there is an urgent need to develop safe alternatives to conventional insecticides for the protection of tomato plants against *T. absoluta*.

The use of eco-friendly and easily biodegradable plant products with natural insecticidal activity has increased in recent years. To control pests without disturbing the environment, natural products have been screened for potential sources of insecticides. Plant materials with insecticidal properties have been used to kill insects throughout the world for generations. These plants are considered an alternative to conventional pesticides because of their low toxicity to warm-blooded mammals as well as their high volatility. Botanical insecticides may be safer for the environment than synthetic insecticides, and they are usually easily processed and used by farmers and small industries.[7]

Tropical plants are recognized sources of bioactive compounds, but less than 1% have been chemically investigated.[8] They can be used for pest control as plant extracts, horticultural oils or as a source of molecules for pesticide synthesis such as pyrethroids and neonicotinoids.

Acmella oleracea (L.) R.K. Jansen is an annual plant of the family Asteraceae (Compositae) originating in tropics of Brazil. The distribution covers tropical and subtropical areas around the world, and it is known in English as toothache plant or paracress and in Portuguese as jambú.[9] The inflorescence is composed of yellow flowers, and the leaves have a pungent flavor accompanied by tingling and numbness. The plant has been used in cooking and in popular medicine, mainly for stammering, toothache, stomatitis and throat complaints.

The plant contains alkamides including spilanthol, which is the principal pungent compound. This chemical compound is known for having several chemical and pharmaceutical applications. It has shown anti-inflammatory,

antibacterial, antifungal, diuretic, sialagogic and larvicidal properties.[9] The activity of *A. oleracea* has been studied extensively. However, only few studies have assessed the insecticidal activity of compounds from this plant. [10-13] Furthermore, the majority of these studies have focused on human health pests such as insect vectors of pathogens.[10-13] These studies show that compounds of *A. oleracea* have high insecticidal activity and that the potential use of this plant species for management of agricultural pests requires further investigation. [10-13]

New compounds should provide selectivity to non-targets species, especially predators and pollinators, in addition to efficiency against insect pests. Attack by natural enemies are the most frequent source of mortality for phytophagous arthropods in agroecosystems and the conservation of these organisms is an essential component in Integrated Pest Management (IPM).[14] Furthermore, pollination is central for successful reproduction in most plants. Thus, pollinators should be preserved because they support the maintenance of biodiversity in the ecosystems they inhabit and because they are known as keystone species in many terrestrial habitats.[15] In agroecosystems, there are many species that are part of these groups, including the predator *Solenopsis saevissima* (Smith) (Hymenoptera: Formicidae) and the pollinator *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae: Meliponinae). The predation by *S. saevissima* has played an important role in reducing pest insects in agricultural systems. Way & Khoo[16] cited species of the genus *Solenopsis* as important agents of biological control in the tropics and subtropics. *T. angustula* is one of the most common stingless bees in the neotropical region. Stingless bees are generalist foragers and are efficient native pollinators of the American flora.[15]

Considering the potential of tropical plant species for pest control and the importance of *T. absoluta*, the aims of this study were to screen plants with insecticide activity to *T. absoluta*. The goal was to isolate, identify and assess the bioactivity of insecticide compounds present in the bioactive plant against

this key insect pest of tomato crops. Furthermore, we wanted to investigate the selectivity of these compounds to the beneficial insects *S. saevissima* and *T. angustula*.

2 MATERIALS AND METHODS

2.1 Insects

The bioassays were performed with second-instar larvae of *T. absoluta* and adults of *S. saevissima* and *T. angustula*. Larvae of *T. absoluta* were obtained from a laboratory rearing located at the campus of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais State, Brazil. Adults of *S. saevissima* and *T. angustula* were collected from nests located around the campus of the UFV.

2.2 Plant screening

2.2.1 Plant extracts preparation

Table 1 describes the plants that were used for extraction and toxicity bioassays. The plants were chosen based on available literature, popular or indigenous knowledge and chemotaxonomy. The plant material was identified in the botanical park of the Federal University of Acre.

Samples of 1.0 kg from the canopy of each plant species were collected in Rio Branco, AC, Brazil (plants of the Amazon Biome) and in Viçosa, MG, Brazil (plants of the Cerrado and of general occurrence). Each sample was lyophilized and the dried material was crushed and placed in a 2 L Erlenmeyer flask, with enough hexane to submerge the plant material. After 48 hours, the solvent was removed under filtration. Ethanol extraction was performed by grinding the samples with the solvent and waiting for 48 hours. The hexane and ethanol extracts were concentrated under low pressure and reduced temperature (45-50°C) using a rotary evaporator. The yield for each extract is shown in Table 1. The plant extracts were stored at low temperature for subsequent bioassays.

2.2.2 Screening bioassay

A set of screening bioassays was performed to identify the bioactive plant extracts to *T. absoluta*. The stored extracts were diluted with acetone to a dose of 10 μg mg^{-1} body mass. The average weight was obtained by measuring the mass of ten groups containing ten insects each, on an analytical balance. The experimental design was completely randomized with six replications. Each experimental unit consisted of a glass petri dish (9.5 cm x 2.0 cm) containing ten insects.

The bioassays were conducted by topical application. For each insect a 10 µl Hamilton micro syringe was used to add 0.5 µL of a solution of the test extract. In a control experiment under the same conditions, 0.5 µL of hexane was applied on each insect.

After the application, the insects were kept in individual Petri dishes containing tomato leaflets (cv. Santa Clara) as food. The Petri dishes were placed in an incubator at 25 ± 0.5°C, 75 ± 5% relative humidity, with a photoperiod of 12 h. The mortality counts were made after 6, 12 and 24 hours of treatment. Mortality included dead individuals as well as those without movements. Mortality data were subjected to analysis of variance and the averages were compared by the Scott-Knott grouping analysis test ($P < 0.05$).

2.3 Bioactivity of compounds from *A. oleracea*

2.3.1 Extract preparation of A. oleracea

The hexane extract of *A. oleracea*, which showed the highest insecticidal activity in the screening bioassay, was selected for isolation and structure elucidation of its bioactive compounds. A total of 2.0 kg of dried and powdered aerial parts of *A. oleracea* was used for this purpose. The solvent (hexane) was changed every two days for 45 days. The extraction continued until the solvent

was colorless. The filtered extract obtained was concentrated in a rotary vacuum evaporator under low pressure and reduced temperature (45-50°C).

2.3.2 Isolation and structural elucidation of bioactive compounds

Fractionation of the hexane extract (28 g) was performed by column chromatography (Silica Gel 60, 70-230 mesh) using hexane with increasing amounts of ethyl acetate and finally with methanol as the eluting solvents. Thin layer chromatography (TLC, Silica gel 60 F254 0.25 mm) was used to identify fractions containing similar compounds. The TLC spots were detected under UV (254 and 365 nm) as well as by heating the plates to 100ºC after spraying with phosphomolybdic acid/ethylic alcohol. Eight fractions (A-H) were collected and subjected to bioassay with *T. absoluta* using the same methods as described in section 2.2.2. The most toxic fractions were purified by preparative TLC of Silica gel 60 F254, Merck (20 x 20 cm plates, 0.75 mm adsorbent). The IR spectra of isolated compounds were recorded on KBr in an infrared spectrometer Paragon 1000 FTIR (Perkin Elmer, Wellesley, MA, USA) from 600 to 4000 cm^{-1}. GC-MS was conducted with a Shimadzu QP5050A gas chromatograph-mass spectrometer. To identify the isolated compounds, ^{1}H NMR and ^{13}C NMR were recorded in a Varian Mercury 300 spectrometer (Varian Inc., Palo Alto, CA, USA) using $CDCl_3$ as a solvent and TMS as an internal standard.

2.3.3 Dose-mortality bioassays

The isolated compounds and the hexane extract of *A. oleracea* were subject to toxicity bioassays against *T. absoluta*, *S. saevissima* and *T. angustula.* The insecticidal activity of neem (*Azadirachta indica* A. Juss) seed kernel hexane extract and of permethrin (92.2% purity, Syngenta), a synthetic derivative of the natural pyrethrins recommended for *T. absoluta* control, were also evaluated and used as positive controls. The experimental design was completely randomized

with six replications. Each experimental unit consisted of a glass Petri dish (9.5 cm x 2.0 cm) containing ten insects. The average weight of each insect species was obtained by measuring the mass of ten groups containing ten insects each, on an analytical balance.

Initially, four doses of each compound were tested to identify the range of concentrations that would provide mortalities greater than zero and less than 100%. Once the range of concentration was defined, other doses were tested for each compound. The number of doses used to obtain the dose-mortality curves varied from five to eight.

Bioassays were conducted by topical application. For each insect a 10 µl Hamilton micro syringe was used to apply 0.5 µL of a solution of the test compound, dissolved in acetone. In a control experiment, carried out under the same conditions, 0.5 µL of acetone was applied to each insect.

After application, the insects were kept in individual Petri dishes containing the appropriate food. *T. absoluta* were fed tomato leaflets (cv. Santa Clara) while *S. saevissima* and *T. angustula* both received a mixture of honey (50%) and pure water (50%). The mixture of honey and water were supplied in plastic containers that were 1.5 cm in diameter and 1.0 cm high.

The Petri dishes were placed in an incubator at 25 ± 0.5°C, 75 ± 5% relative humidity, with a photoperiod of 12 h. The mortality counts were made after 24 h. Mortality included both dead individuals and those that were no longer moving. Dose-mortality data were subjected to probit analysis using SAS software (PROC PROBIT; SAS) to estimate dose-mortality curves.[17] We accepted curves which had probabilities greater than 0.05 by the χ^2 test.[18]

2.3.4 Risk assessment to non-target insects

To determine the magnitude of selectivity of the compounds to the beneficial insects, we calculated the selectivity ratio using the formula $S_LR_{50} = LD_{50}$ of the

insecticide for the beneficial insect per LD_{50} of the insecticide for *T. absoluta*. Values of 1 and <1 indicate that the chemical is non-selective to the beneficial insect. Values >1 indicate that the chemical is selective and/or harmless to the beneficial insect.[19] Using the dose-mortality curves, mortalities caused to beneficial insects by the doses of the compounds that caused 80% mortality in *T. absoluta* were also estimated.

3 RESULTS

3.1 Bioactivity of plant extracts (plant screening)

The hexane extract from aerial parts of *A. oleracea* exhibited the highest activity of all extracts, causing 100.0 ± 0.0% (N = 60) mortality ± standard error (SE) in *T. absoluta* after six hours of exposure. The mortality caused by the solvents was zero (0.0%) in all of the bioassays (Table 2).

The ethanol extract of *A. oleracea* also showed high activity (88.6% mortality) against *T. absoluta*, and was the second most active extract. The hexane and ethanol extracts of the remainder of the plants tested showed low insecticidal activity toward *T. absoluta* (Table 2).

On the basis of these results, the hexane extract of *A. oleracea* was selected for isolation and structure elucidation of its bioactive compounds.

3.2 Isolation and structural elucidation of compounds from *A. oleracea*

To obtain bioactive compounds, hexane extract (28 g) was fractionated by a bioactivity guided fractionation approach and eight fractions, A-H, were obtained. The eight fractional groups were evaluated for their insecticide activity against *T. absoluta* larvae. Fractions F and G eluted with hexane-ethyl acetate (1:1) were biologically active, causing 100% mortality 6 hours after administration of a dose of 10 μg mg^{-1} body mass. The remainder of the fractions (A, B, C, D, E and H) caused mortalities less than 40%.

The bioactive fraction F was purified by preparative TLC (hexane-ethyl acetate, 6:1) to yield the following three major bands: **I** (725 mg, Rf 0.65), **II** (56 mg, Rf 0.45), and **III** (21 mg, Rf 0.25). Band **I** was biologically active and was further purified by preparative TLC (hexane-ethyl acetate, 1:2) to give compounds **1** and **2** (320 and 210 mg, respectively). The bioactive fraction G was purified by preparative TLC (hexane-ethyl acetate, 3:1) to yield compound **3** (27 mg).

Compound **1**, (2*E*,6*Z*,8*E*)-deca-2,6,8-trienoic acid N-isobutyl amide or spilanthol (Fig. 1), was isolated as a colorless oil. The IR spectrum showed the presence of a secondary amide group (3340, 1636, and 1550 cm^{-1}), a double bond conjugated to an amide carbonyl group (1678 cm^{-1}), and a conjugated diene group with the *Z*, *E* or *E*, *Z* configuration (987 and 953 cm^{-1}). The MS spectrum had the molecular ion peak at m/z 221, which indicates the molecular formula $C_{14}H_{23}NO$. GC-EIMS 70 eV, m/z (rel. int.): 221 [M]+ (20), 206 (3), 141 (100), 126 (23), 98 (23), 81 (87), 69 (10), 53 (10). The ^{13}C NMR ($CDCl_3$) and the ^{1}H NMR ($CDCl_3$) spectra showed the spilanthic acid (Tables 3 and 4). On the amine moiety, the typical signals at δ 3.15 (2H, t, H-1'), 1.78 (1H, m, H-2'), and 0.93 (6H, d, H-3', 4') in ^{1}H NMR and δ 46.9 (C-1'), 28.6 (C-2') and 20.1 (C-3',4') in the ^{13}C NMR indicated the presence of a isobutylamino group. All of the spectral data were in agreement with those of spilanthol (**1**) in literature.[20]

Compound **2** was isolated as a colorless crystal. In the ^{1}H NMR spectrum characteristic signals at δ 3.18 (dd, H-l'), 1.79 (m, H-2') and 0.91 (d, H-3' and H-4') indicated the isobutylamide moiety. This compound was identified as undeca-2*E*-en-8,10-diynoic acid isobutylamide (Fig. 1) by comparing its ^{1}H NMR spectral data (Table 3) with published values.[21] The ^{13}C NMR spectrum (Table 4) was consistent with the published data.[10]

Compound **3**, (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (Fig. 1), was isolated as a colorless oil. The IR spectrum presented absorption bands

attributable to a triple bond (2225 cm^{-1}) in addition to a secondary amide group (3299, 1627 and 1554 cm^{-1}) and a double bond conjugated with an amide carbonyl group (1669 cm^{-1}). The ^{1}H NMR spectrum revealed signals at δ 5.77 (d, J = 15 Hz) and 6.80 (dt, J = 15 Hz and 7 Hz) which have been attributed to olefinic protons, H-2 and H-3, respectively (Table 3). On the amine moiety, a pair of 1H ddq signals at δ 1.17 and 1.41 are attributed to methylene protons of C-3' and a pair of ddd signals at δ 3.14 and 3.27 are attributed to methylene protons of C-1' due to the presence of asymmetric carbon at C-2' (Table 3). The ^{13}C NMR spectrum gave rise to 16 carbon signals. Five carbon signals at δ 45.2, 35.1, 27.1, 11.3 and 17.2 confirmed a 2-methylbutylamine moiety (Table 4). The ^{13}C NMR and ^{1}H NMR signals correspond well with the literature.[20]

3.3 Bioactivity of isolated compounds

The dose-mortality results from insecticide application in larvae of *T. absoluta* showed low χ^2 and high *P* values (<7.7 and >0.103 respectively), indicating the suitability of the probit model for fitting the dose-response curves and consequently obtaining estimates of the mortality parameters LD_{50} and LD_{80} (Table 5).

Compound **1** (spilanthol) exhibited the highest toxicity to *T. absoluta*, with the lowest LD_{50}. Furthermore, the spilanthol (**1**) was approximately five times more toxic than permethrin and approximately 321 times more potent than *A. indica* extract (Table 5).

The compounds undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**) showed insecticidal activity similar to the commercial insecticide permethrin. In comparison with the extract of *A. indica*, compounds **2** and **3** were about 62 and 52 times more toxic to *T. absoluta*, respectively (Table 5).

The *A. oleracea* extract was less toxic than the isolated compounds, but it showed good insecticidal activity. It was 23-fold more toxic than the neem extract (Table 5).

3.4 Selectivity of isolated compounds

The dose needed to kill 50% of the test population (LD_{50}) was determined for the beneficial insects (Table 6) and used to calculate selectivity ratios of the insecticides for the two beneficial insects (Table 7). The *A. oleracea* extract and the compounds **1**, **2** and **3** were selective to the predator *S. saevissima* and the pollinator *T. angustula* relative to *T. absoluta*, with a selectivity ratio (S_LR_{50}) greater than 1.0 (Table 7). For the *A. oleracea* extract, the doses that caused 50% mortality of *T. absoluta* larvae were 36% and 39% lower than the doses that caused the same mortality to *S. saevissima* and *T. angustula*, respectively. The estimated mortality of *S. saevissima* and *T angustula* by the LD_{80} of this extract to *T. absoluta* were 56% and 55%, respectively (Table 7).

For compounds **1**, **2** and **3**, the doses that caused 50% mortality of *T. absoluta* larvae were 38%, 39% and 64% lower than the doses that caused the same mortality to *S. saevissima*, respectively. Furthermore, the doses were 169%, 37% and 35% lower than the doses that caused the same mortality to *T. angustula*, respectively. The estimated mortality of *S. saevissima* and *T angustula* by the LD_{80} of these compounds to *T. absoluta* ranged from 55% to 68%. However, the LD_{50} of permethrin for *T. absoluta* was 15.4-fold and 2,366.7-fold higher than the LD_{50} for *S. saevissima* and *T. angustula*, respectively. These results indicate that permethrin is harmful to the beneficial insects. The estimated mortality of *S. saevissima* and *T. angustula* by the LD_{80} of this insecticide to *T. absoluta* was 100% (Table 7).

Based on the S_LR_{50}, the neem extract was selective to *S. saevissima*. However, the DL_{80} of neem extract to *T. absoluta* caused a mortality of 84% and 98% to *S. saevissima* and *T. angustula*, respectively (Table 7).

4 DISCUSSION

The plant species showing higher insecticide activity in our study was the toothache plant *A. oleracea.* Furthermore, the activity was higher in the hexane extract than in the ethanol extract.

The bioactivity of *A. oleracea* is due to alkamides present in the plant. The main active amide in the plant is an isobutylamide, (2*E*, 6*Z*, 8*E*)-deca-2,6,8-trienoic acid, commonly known as spilanthol.[22,23] These alkamides have a pungent effect and have been studied for various purposes. The flowers and leaves of *A. oleracea* are used in cooking and in popular medicine, mainly as an analgesic for toothache. The spilanthol is known for having several chemical and pharmaceutical applications in addition to the analgesic for toothache already mentioned. It is used for the treatment of aphtha and herpes, for stomatitis and infections in the throat, in treatment of tuberculosis, as a synagogue, as a fungistat and fungicide against *Aspergillus* spp., as an antimutagenic agent and as a cicatrizant.[24-26]

The results also showed that the alkamides evaluated in this study have the potential to control arthropods of agricultural importance. Three alkamides were identified in the bioactive fractions of the hexane extract of *A. oleracea* (spilanthol, undeca-2*E*-en-8,10-diynoic acid isobutylamide and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide). This study evaluated the effect of *A. oleracea* on *T. absoluta*, an important pest of tomato in the world. The results showed that all of the compounds isolated had high insecticidal activity, which was at least as toxic as permethrin, a pyrethroid recommended for control of *T. absoluta*. Furthermore, the compounds were far more toxic than the neem

extract. The high efficiency of these compounds combined with the ready availability from natural sources and the friendlier environmental footprint makes this plant an excellent candidate as a future natural insecticide.

The results from this study showed the alkamides **1**, **2** and **3** were selective to *S. saevissima* and *T. angustula.* The tolerance of beneficial insects to alkamides could be related to lower rates of insecticide penetration through the integument, higher rates of insecticide break down, and relative insensitivity of the target site in beneficial insects compared with *T. absoluta*.[27,28]

Penetration rates of insecticides in the insect integument are associated with the physicochemical properties of the insecticide and the insect cuticle, including cuticle thickness and biochemical composition.[29-31] Soft-bodied insects such as *T. absoluta* have a thinner cuticle than *S. saevissima* and *P. sylveirae,* which supports this hypothesis. The selectivity of alkamides may also be associated with higher rates of metabolization in beneficial insects by detoxification enzymes such as P450-dependent monooxigenases. These enzymes transform lipophilic xenobiotics into polar metabolites that are then excreted.[18] This hypothesis is based on the high lipophilic character of these alkamides (spilanthol has a log Pow of 3.4 and it is practically insoluble in water, 18.63 mg/L).[32]

The selectivity provided by alkamides to *S. saevissima* and *T. angustula*, suggests that the use of these compounds to control *T. absoluta* present a low risk to these beneficial insects. Furthermore, the results from this study showed that all compounds had a lower toxicity than permethrin (insecticide already used to control *T. absoluta*) to all non-target species studied. This finding indicates that the alkamides are less harmful to the beneficial insects. Thus, to preserve the predaceous and the pollinator investigated in this study, the use of these compounds for pest control can be recommended as a strategy to manage these beneficial insects.

Physiological selectivity is based on the use of insecticides that are more toxic to the target pest than the natural enemies and should always be considered when controlling pests. Furthermore, the principles of ecological selectivity should also be considered.[33,34] The ecological selectivity is related to the different methods of applying insecticides as a means to minimize exposure of natural enemies to the insecticide.[33] It is of utmost importance to use selective insecticides to preserve the beneficial species in the ecosystem, and it is necessary to resort to strategies that will enable the achievement of ecological selectivity even if it is not possible. With the ecological selectivity, an insecticide can be applied with a methodology designed to make it selective. The low stability of botanical pesticides and consequent rapid degradation in the environment is a characteristic that favors ecological selectivity, because it reduces the exposure time of beneficial organisms to toxic compounds.

The mechanism of action of active alkamidas found in *A. oleracea* has not yet been determined. It appears to affect the nervous system as evident by abnormal movement such as uncoordinated muscular activity. This effect suggests that the compounds disturb nerve conduction somewhere. The analgesic activity of spilanthol in humans has been attributed to an increased GABA release in the temporal cerebral cortex,[35] while other bioactive alkylamides are acting on voltage-gated sodium channels.[36] The mortality after short exposure to the compounds indicate that alkamides greatly disturb the ongoing processes of histolysis of larval tissues. Saraf and Dixit[11] observed rapid mortality of pupae of *Aedes aegypti* Linn, *Anopheles culicifacies* Giles and *Culex quinquefasciatus* Say (Diptera: Culicidae) when exposed to spilanthol. These results suggest that spilanthol interferes in histolysis and histogenesis processes. Further research is needed to address this question.

Overall, the results of this research indicate that the *A. oleracea* extract is the most promising among the plant extracts studied. The active alkamides

from *A. oleracea* can be a potential alternative for controlling *T. absoluta* and should be studied further for other agricultural pests. All compounds presented high insecticidal activity for the insect pest *T absoluta* and selectivity for beneficial insects *S. saevissima* and *T. angustula*. Given the vital need for environmentally friendly chemicals that represent new insecticide groups with novel mechanisms of action, low persistence in the field and low toxicity to mammals and non-target species, the feasibility and impacts of using natural chemicals in pest management programs require further attention. We must remember, however, that the biological activity of a chemical is a function of its structure rather than its origin. The biological properties of a chemical depend on its structure and the way in which the chemical is used. Bioactive alkamides from *A. oleracea* have been found harmless to the majority of vertebrates and lethal to invertebrates.[37,38] Because *A. oleracea* is widely used as both food and folk medicine in their region of origin, it is assumed that the toxicity to humans is extremely low. However, the actual risks of using these natural products should be identified. Therefore, to assess the feasibility and impacts of using the *A. oleracea* alkamides in agriculture more research on the effects on humans and the environment should be performed.

5 CONCLUSION

The hexane extract of *A. oleracea* showed high insecticidal activity and can be used to control *T. absoluta* in organic or conventional crops. Quantification of LD_{50} values of isolated alkamides of *A. oleracea* against *T. absoluta* showed that alkamides could serve as potent insecticides for *T. absoluta* control programs. The spilanthol was the main alkamide active isolated. This alkamide is the most promising as it had the highest insecticidal activity and was selective to non-target organisms. Therefore, spilanthol and the others alkamides isolated are potential pest management tools likely to have they insecticide activity improved

through organic synthesis guided by studies of quantitative structure-activity relationship.

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Table 1. Identification of plants used in screening bioassays (scientific name and family) and yield of hexane and ethanol extracts obtained from 1.0 kg of the plants aerial parts

Nº	Scientific name	Family	Yield (g)	
			Hexane extract	Ethanol extract
	Plants of the Amazon Biome			
1	*Acmella oleracea* L.	Asteraceae	10.74	15.04
2	*Banara guianensis* Aubl.	Flacourtiaceae	22.74	26.54
3	*Banara nitida* Spruce ex Benth.	Flacourtiaceae	4.2	10.49
4	*Clavija weberbaueri* Mez.	Theophrastaceae	5.42	35.88
5	*Copaifera duckei* Dwyer	Caesalpinioideae	7.42	18.44
6	*Eugenia egensis* DC.	Myrtaceae	7.42	18.44
7	*Mayna parvifolia* Sleumer	Flacourtiaceae	16.95	9.23
8	*Piper aduncum* L.	Piperaceae	7.6	11.58
9	*Piper augustum* Rudge	Piperaceae	7.86	21.33
10	*Ryania speciosa* Vahl.	Flacourtiaceae	8.96	77.34
11	*Siparuna amazônica* Mart. ex A. DC.	Monimiaceae	10.43	36.3
	Plant of the Cerrado Biome			
12	*Curatela americana* L.	Dilleniaceae	13.26	19.87
	Plants of general occurrence			
13	*Ageratum conyzoides* L.	Asteraceae	12.00	25.24
14	*Allamanda cathartica* L.	Apocynaceae	5.31	4.51
15	*Argemone mexicana* L.	Papaveraceae	5.98	6.48
16	*Artemisia vulgaris* L.	Asteraceae	4.47	6.81
17	*Bauhinia variegate* L.	Caesalpinioideae	11.48	32.02
18	*Bougainvillea glabra* Choisy	Nyctaginaceae	7.56	9.63
19	*Calendula officinalis* L.	Asteraceae	4.30	5.81
20	*Chenopodium ambrosioides* L.	Chenopodiaceae	4.75	6.38
21	*Coriandrum sativum* L.	Apiaceae	5.59	7.52
22	*Spathodea campanulata* P. Beauv.	Bignoniaceae	12.70	28.76
23	*Tropaeolum majus* L.	Tropaeolaceae	6.32	7.21

Table 2. Contact toxicity of plant extracts at concentration of 10 μg of extract per mg of insect against *Tuta absoluta* 6, 12 and 24 hours after topical application

Plants	Mean percent mortality[a]					
	6 hours after topical exposure		12 hours after topical exposure		24 hours after topical exposure	
	Ethanol extract	Hexane extract	Ethanol extract	Hexane extract	Ethanol extract	Hexane extract
Acmella oleracea	88.3 (± 1.5) Ab	100.0 (± 0.0) Aa	88.3 (± 1.5) Ab	100.0 (± 0.0) Aa	88.3 (± 1.5) Ab	100.0 (± 0.0) Aa
Ageratum conyzoides	26.7 (± 1.9) Bb	45.0 (± 2.0) Ba	35.0 (± 3.1) Bb	48.3 (± 2.8) Ca	35.0 (± 3.1) Bb	51.7 (± 3.1) Ca
Allamanda cathartica	21.7 (± 2.8) Ba	18.3 (± 2.1) Da	21.7 (± 2.8) Ba	23.3 (± 1.9) Ea	25.0 (± 3.4) Ba	23.3 (± 3.3) Ca
Argemone mexicana	25.0 (± 3.1) Ba	20.0 (± 2.4) Da	25.0 (± 3.1) Bb	33.3 (± 2.8) Ea	28.3 (± 3.1) Bb	41.7 (± 3.1) Ca
Artemisia vulgaris	5.0 (± 2.0) Da	6.7 (± 1.9) Ea	13.3 (± 1.9) Ba	8.3 (± 1.7) Gb	15.0 (± 2.2) Ca	8.3 (± 1.7) Eb
Banara guianensis	26.7 (± 1.9) Ba	28.3 (± 2.8) Ca	36.7 (± 3.3) Ba	33.3 (± 3.6) Ea	36.7 (± 3.3) Ba	41.7 (± 4.0) Ca
Banara nitida	16.7 (± 3.7) Ca	13.3 (± 2.9) Ea	23.3 (± 3.3) Ba	18.3 (± 1.7) Fa	23.3 (± 3.3) Ba	18.3 (± 1.7) Da
Bauhinia variegate	6.7 (± 3.1) Da	8.3 (± 2.8) Ea	11.7 (± 1.7) Ba	15.0 (± 2.2) Fa	11.7 (± 1.7) Ca	15.0 (± 2.2) Da
Bougainvillea glabra	25.0 (± 3.1) Bb	42.5 (± 2.5) Ba	33.3 (± 2.1) Bb	42.5 (± 2.5) Da	33.3 (± 2.1) Bb	51.7 (± 3.1) Ca
Calendula officinalis	13.3 (± 1.9) Ca	6.7 (± 1.9) Eb	13.3 (± 1.9) Ba	15.0 (± 2.2) Fa	13.3 (± 3.3) Ca	15.0 (± 2.2) Da
Chenopodium ambrosioides	15.0 (± 2.0) Ca	16.7 (±3.3) Da	16.7 (± 3.3) Ba	16.7 (±3.3) Fa	16.7 (± 3.3) Ca	21.7 (± 3.1) Ca
Clavija weberbaueri	25.0 (± 2.2) Ba	28.3 (± 2.8) Ca	25.0 (± 2.2) Bb	36.7 (± 3.3) Ea	25.0 (± 2.2) Bb	36.7 (± 3.3) Ca
Copaifera duckei	36.7 (± 3.3) Bb	50.0 (± 4.1) Ba	36.7 (± 3.3) Bb	63.3 (± 3.3) Ba	36.7 (± 3.3) Bb	63.3 (± 3.3) BA
Coriandrum sativum	18.3 (± 1.7) Cb	41.7 (± 2.8) Ba	21.7 (± 1.7) Bb	48.3 (± 2.8) Ca	21.7 (± 1.7) Bb	66.7 (± 3.3) BA
Curatela americana	31.7 (± 2.8) Ba	30.0 (± 3.3) Ca	41.7 (± 3.1) Ba	30.0 (± 3.3) Eb	41.7 (± 3.1) Bb	48.3 (± 1.7) Ca
Eugenia egensis	30.0 (± 2.6) Ba	16.7 (± 1.9) Db	30.0 (± 2.6) Ba	28.3 (± 2.8) Ea	30.0 (± 2.6) Ba	36.7 (± 2.1) Ca
Mayna parvifolia	26.7 (± 3.3) Ba	20.0 (± 2.4) Da	26.7 (± 3.3) Ba	30.0 (± 3.6) Ea	26.7 (± 3.3) Ba	30.0 (± 3.6) Ca
Piper *aduncum*	33.3 (± 2.1) Ba	35.0 (± 2.2) Ca	33.3 (± 2.1) Ba	35.0 (± 2.2) Ea	33.3 (± 2.1) Ba	35.0 (± 2.2) Ca
Piper augustum	35.0 (± 2.2) Ba	18.3 (± 1.5) Db	35.0 (± 2.2) Ba	25.0 (± 2.2) Eb	35.0 (± 2.2) Ba	25.0 (± 2.2) Cb
Ryania speciosa	15.6 (± 3.9) Cb	33.3 (± 3.8) Ca	18.3 (± 1.7) Bb	33.3 (± 3.8) Ea	18.3 (± 1.5) Cb	33.3 (± 3.8) Ca
Siparuna amazônica	16.7 (± 1.9) Ca	20.0 (± 2.4) Da	25.0 (± 3.1) Ba	28.3 (± 3.1) Ea	26.7 (± 3.0) Ba	28.3 (± 3.1) Ca
Spathodea campanulata	3.3 (± 1.9) Db	13.3 (± 1.9) Ea	6.7 (± 3.3) Cb	26.7 (± 1.9) Ea	6.7 (± 3.3) Db	26.7 (± 1.9) Ca
Tropaeolum majus	31.7 (± 3.1) Ba	33.3 (± 2.1) Ca	31.7 (± 3.1) Ba	33.3 (± 2.1) Ea	31.7 (± 3.1) Ba	33.3 (± 2.1) Ca
Control[b]	0.0 (± 0.0) Ea	0.0 (± 0.0) Fa	0.0 (± 0.0) Da	0.0 (± 0.0) Fa	0.0 (± 0.0) Ea	0.0 (± 0.0) Fa

[a] Means followed by the same lower-case letter in a row (for comparation between ethanol and hexane extracts) or by the same upper-case letter in a column are not significantly different by the Scott-Knott grouping analysis test at $P > 0.05$. [b] Only acetone was used in the control.

Table 3. ^{1}H NMR spectral data for compounds 1-3 (δ values, 300 MHz, $CDCl_3$)

No.	**1**	**2**	**3**
H-2	5.79 br. d (15)	5.76 dt (15; 1)	2.77 br. d (15)
3	6.83 dt (15; 7)	6.79 dt (15; 7)	6.80 dt (15; 7)
4	2.23-2.35 m	2.24 m	2.20 m
5	2.23-2.35 m	1.60 m	1.57 m
6	5.26 dt (11; 7)	1.60 m	1.57 m
7	5.97 dd (11; 11)	2.24 m	2.27 m
8	6.29 br. dd (11; 15)		
9	5.70 dq (15; 7)		
10	1.78 d (7)		
11		1.95 t (1)	1.96 br. s
$1'_a$	3.15 dd (7; 6)	3.18 dd (7; 7)	3.14 ddd (14; 7; 6)
$1'_b$			3.27 ddd (14; 7; 6)
2’	1.78 m	1.79 m	1.57 m
$3'_a$	0.93 d (7)	0.91 d (7)	1.17 ddq (14; 7; 7)
$3'_b$			1.41 ddq (14; 7; 7)
4’	0.93 d (7)	0.91 d (7)	0.91 t (7)
5’			0.91 d (7)
H-N	5.47 br. s	5.45 br. s	5.38 br. s

Table 4. ^{13}C NMR spectral data for compounds 1-3 (δ values, 100 MHz, $CDCl_3$)

No.	**1**	**2**	**3**
C-1	166	165.9	165.9
2	124.2	124.0	124.3
3	143.5	143.8	143.6
4	32.1	31.3	31.3
5	26.4	27.4	27.4
6	127.7	27.2	27.5
7	129.5	18.9	18.9
8	126.7	77.9	77.9
9	130.0	65.0	65.0
10	18.3	68.4	68.5
11		64.7	64.7
1’	46.9	46.9	45.2
2’	28.6	28.6	35.1
3’	20.1	20.1	27.1
4’	20.1	20.1	11.3
5’			17.2

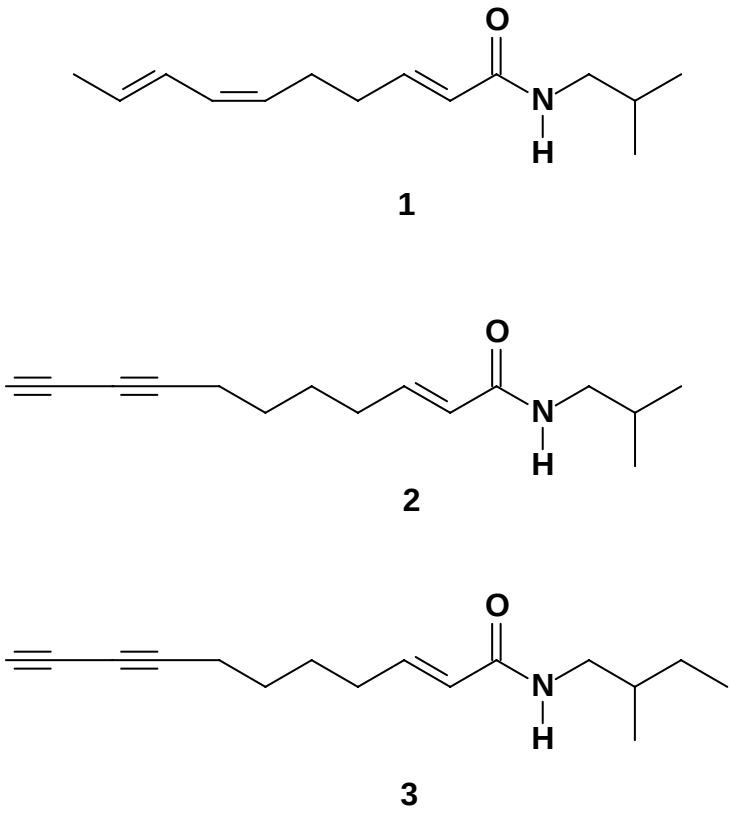

Fig. 1. Structure of the three alkamides isolated from *Acmella oleracea*: spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**).

Table 5. Contact toxicity of *Acmella oleracea* hexane extract and of spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**) extracted from aerial parts of *A. oleracea* against *Tuta absoluta*, 24 hours after topical application

Treatments	N[a]	LD_{50} (μg mg^{-1}) (95% FL)[b]	LD_{80} (μg mg^{-1}) (95% FL)[b]	Slope ± SE	χ^2	*P* value
Acmella oleracea extract	420	1.83 (1.43 - 2.04)	2.94 (2.50 - 3.83)	1.00 ± 0.06	4.50	0.480
Compound **1**	600	0.13 (0.09 - 0.16)	0.56 (0.41 - 0.84)	0.39 ± 0.01	1.69	0.998
Compound **2**	420	0.49 (0.39 - 0.62)	1.34 (1.06 - 1.79)	0.53 ± 0.03	2.01	0.987
Compound **3**	540	0.81 (0.46 - 1.18)	1.76 (1.21 - 3.55)	0.51 ± 0.04	1.06	0.998
Azadirachta indica extract[c]	420	41.73 (37.90 - 46.99)	98.19 (86.78-114.90)	2.21 ± 0.09	6.27	0.370
Permethrin[c]	360	0.71 (0.38 - 1.08)	2.57 (1.60 - 4.72)	0.63 ± 0.04	7.70	0.103

[a] Number of insects tested.
[b] Lethal dose with 95% fiducial limits (FL).
[c] Positive control.

Table 6. Contact toxicity of *Acmella oleracea* hexane extract and of spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**) extracted from aerial parts of *A. oleracea* against *Solenopsis saevissima* and *Tetragonisca angustula*, 24 hours after topical application

Treatments	N[a]	LD_{50} (μg mg^{-1}) (95% FL)[b]	LD_{80} (μg mg^{-1}) (95% FL)[b]	Slope ± SE	χ^2	*P* value
			Solenopsis saevissima			
Acmella oleracea extract	420	2.48 (2.14 - 2.84)	5.47 (4.71 - 6.54)	0.68 ± 0.03	3.75	0.967
Compound **1**	420	0.18 (0.13 - 0.23)	1.12 (0.73 - 2.24)	0.44 ± 0.03	6.02	0.450
Compound **2**	360	0.67 (0.54 - 0.82)	2.22 (1.78 - 2,88)	0.47 ± 0.03	6.37	0.278
Compound **3**	420	1.33 (1.06 - 1.63)	4.40 (3.53 -5.70)	0.47 ± 0.03	6.12	0.354
Azadirachta indica extract[c]	360	72.65 (68.47 - 76.63)	92.50 (87.39 - 99.10)	2.76 ± 0.25	1.42	0.999
Permethrin[c]	300	0.046 (0.029 - 0.073)	1.458 (0.682 - 4.453)	0.28 ± 0.02	4.69	0.450
			Tetragonisca angustula			
Acmella oleracea extract	420	2.55 (2.22 - 2.86)	4.28 (3.82 - 4.90)	1.08 ± 0.07	3.62	0.608
Compound **1**	420	0.35 (0.29 - 0.42)	0.81 (0.67 - 1.01)	0.68 ± 0.02	1.62	0.998
Compound **2**	360	0.67 (0.48 - 0.86)	2.02 (1.55 - 2.91)	0.55 ± 0.05	7.49	0.112
Compound **3**	420	1.10 (0.89 - 1.33)	2.26 (2.16 - 3.47)	0.58 ± 0.03	1.81	0.994
Azadirachta indica extract[c]	360	29.52 (25.23 - 34.15)	71.04 (59.62 - 88.92)	0.68 ± 0.05	6.93	0.399
Permethrin[c,]	300	0.0003 (0.0002 - 0.0005)	0.002 (0.001 - 0.004)	0.40 ± 0.02	4.95	0.427

[a] Number of insects tested.
[b] Lethal dose with 95% fiducial limits (FL).
[c] Positive control.

Table 7. Risk assessment of *Acmella oleracea* hexane extract and of spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**) extracted from aerial parts of *A. oleracea* on adults of *Solenopsis saevissima* and *Tetragonisca angustula*

Treatments	S_LR_{50}[a]	Category of the insecticide	Mortality (%)[b]
	Solenopsis saevissima		
Acmella oleracea extract	1.36	Selective	56
Compound **1**	1.38	Selective	68
Compound **2**	1.39	Selective	57
Compound **3**	1.64	Selective	55
Azadirachta indica extract[c]	1.74	Selective	84
Permethrin[c]	0.00042	Non-selective	100
	Tetragonisca angustula		
Acmella oleracea extract	1.39	Selective	55
Compound **1**	2.69	Selective	58
Compound **2**	1.37	Selective	62
Compound **3**	1.35	Selective	59
Azadirachta indica extract[c]	0.70	Non-selective	98
Permethrin[c]	0.065	Non-selective	100

[a] A selectivity ratio at the LD_{50} (S_LR_{50}) of >1 indicate that the insecticide is selective (more toxic to the pest than to the natural enemy).
[b] Mortality estimated by the LD_{80} (lethal concentration for 80% of populations) of compounds to *Tuta absoluta*.
[c] Positive control.

ARTICLE 2

EFFICACY OF COMPOUNDS FROM *Acmella oleracea* AGAINST LEPIDOPTERAN VEGETABLE PESTS AND SELECTIVITY TO BENEFICIAL SPECIES

This article was written in accordance with the standards of Applied Entomology and Zoology.

Efficacy of compounds from *Acmella oleracea* against lepidopteran vegetable pests and selectivity to beneficial species

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Abstract

Tropical plants are recognized sources of bioactive compounds that can be used for pest control. Our objective was to evaluate the biological activity of compounds present in *Acmella oleracea* (L.) R.K. Jansen against the Lepidoptera pests of vegetable crops *Ascia monuste* Latr. (Lepidoptera*:* Pieridae), *Diaphania hyalinata* L. (Lepidoptera: Crambidae) and *Plutella xylostella* L. (Lepidoptera: Plutellidae). We were also interested in accessing the selectivity of these compounds to the predator *Solenopsis saevissima* Smith (Hymenoptera: Formicidae) and to the pollinator *Tetragonisca angustula* Latr. (Hymenoptera: Apidae: Meliponinae). First, the insecticide activity of hexane and ethanol extracts from 23 plants was evaluated against the insect pests. The hexane extract of *A. oleracea* was the most active of the extracts and was selected for further study. The following three alkamides were isolated from the hexane extract of the aerial parts of *A. oleracea*: spilanthol, undeca-2*E*-en-8,10-diynoic acid isobutylamide and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide. All of the isolated compounds showed insecticidal activity with

spilanthol being the most active against the lepidopterous pest. The alkamides were selective to both beneficial species studied. Quantification of LD_{50} values of alkamides against *A. monuste*, *D. hyalinata* and *P. xylostella* showed that alkamides could serve as potent insecticides for control programs of these pests.

Keywords: botanical pesticide, secondary metabolites, *Ascia monuste*, *Diaphania hyalinata*, *Plutella xylostella*

INTRODUCTION

In recent years, the use of synthetic organic insecticides in crop pest control programs around the world has resulted in damage to the environment, pest resurgence, pest resistance to insecticides, and lethal effects on non-target organisms (Kamaraj et al., 2008). Due these problems, news chemicals, including plant extracts, are being tested as pest control agents. Many plant species produce noxious chemicals to kill or otherwise inhibit insect feeding activity. Most of these secondary metabolites are very potent and often very specific. It has been found that herbal extracts are one safer alternative method of control since they have low human toxicity and a high degree of biodegradation, reduce the risk of adverse ecological effects, and do not induce pesticide resistance (Promsiri et al., 2006).

Acmella oleracea (L.) R.K. Jansen is an annual herb belonging to family Asteraceae (Compositae) and commonly known as paracress or toothache plant (Phrutivorapongkul at al., 2008). It is native to tropical America, but with a distribution ranging in tropical and subtropical areas around the world. The inflorescenses and the leaves of this species have a pungent flavor accompanied by tingling and numbness, and have been used in cooking or in popular medicine (Ramsewak et al., 1999; Sharma et al., 2010). In the Brasilian state of Para it is a fundamental part of the traditional cooking, taking part in typical dishes such as “tacacá” and “pato no tucupi”.

The plant contains alkamides that are quite known for having several chemical-pharmaceutical applications. It has shown analgesic, antibiotic, diuretic and anti-inflammatory activities. It is common spice, has been administered as traditional folk medicine for years to cure toothache, stammering and stomatitis. The anti-ageing proprieties of an *A. oleracea* extract also have been evaluated and clinically proved (Sharma et al., 2010).

Despite several studies about the activity of *A. oleracea*, few have assessed the insecticide activity of compounds from this plant, and these efforts have focused on human health pests, like insect vectors of pathogens (Ramsewak et al., 1999; Saraf and Dixit, 2002; Amer and Mehlhorn, 2006; Pandey et al., 2007). These studies show that compounds of *A. oleracea* have high insecticidal activity. Since, the potential use of this plant species for management of agricultural pests requires further investigation.

The insects *Ascia monuste* Latr. (Lepidoptera*:* Pieridae), *Diaphania hyalinata* L. (Lepidoptera: Crambidae) and *Plutella xylostella* L. (Lepidoptera: Plutellidae) represent an important order of insect species with economic importance in horticultural crops, the order Lepidoptera. The kale leafworm, *A. monuste*, and the diamondback moth, *P. xylostella*, are key pests of Brassica crops (kale, cabbage, cauliflower, broccolis, mustard and radish) with a cosmopolitan distribution (Vendramim and Martins, 1982; Talekar and Shelton, 1993). The melonworm, *D. hyalinata*, is a destructive insect pest of Cucurbitaceae which is found in most countries of the Americas (Picanço et al., 2000). Chemical control of these pests has not only led to various forms of resistance (Eigenbrodeet al., 1990; Luogen et al., 2005), but also adverse effects such as outbreaks of secondary pest infestations because of the elimination of their natural enemies (Hajek, 2004) and production loss due to an insect pollinator decline (Gallai et al., 2009).

Considering the potential of plant species as a source of potential insecticides and the importance of these lepidopteran pests in horticultural crops, our objective was evaluate the biological activity of compounds present in *A. oleracea* against *A. monuste*, *D. hyalinata* and *P. xylostella*. Furthermore, as compounds used in pest control should be selective to non-target species, we want to evaluate the selectivity of these compounds to the predator *Solenopsis saevissima* Smith (Hymenoptera: Formicidae) and the pollinator *Tetragonisca angustula* Latr. (Hymenoptera: Apidae: Meliponinae). They are beneficial insects often found in horticultural crops and must be preserved to maintain balance and biodiversity in the agroecosystems.

MATERIALS AND METHODS

Insects and plant material

The bioassays were performed with second-instar larvae of *T. absoluta*, *D. hyalinata* and *A. monuste*. The insects were obtained from a laboratory rearing located at the Universidade Federal de Viçosa (UFV), Viçosa, MG, Brazil.

The following plants were subject to extraction and toxicity bioassays: *Acmella oleracea* L., *Ageratum conyzoides* L., *Allamanda cathartica* L., *Argemone mexicana* L., *Artemisia vulgaris* L., *Banara guianensis* Aubl., *Banara nitida* Spruce ex Benth., *Bauhinia variegate* L., *Bougainvillea glabra* Choisy, *Calendula officinalis* L., *Chenopodium ambrosioides* L., *Clavija weberbaueri* Mez., *Copaifera duckei* Dwyer, *Coriandrum sativum* L., *Curatela americana* L., *Eugenia egensis* DC., *Mayna parvifolia* Sleumer, *Piper aduncum* L., *Piper augustum* Rudge, *Ryania speciosa* Vahl., *Siparuna amazônica* Mart. ex A. DC., *Spathodea campanulata* P. Beauv. and *Tropaeolum majus* L.. These plants were chosen based on available literature, popular or indigenous knowledge and

chemotaxonomy. The plant material was identified in the botanical park of the Federal University of Acre.

Samples of 1.0 kg from the canopy of each plant species were collected in Rio Branco, AC, Brazil and in Viçosa, MG, Brazil. Each sample was lyophilized and the dried material was crushed and placed in a 2 L Erlenmeyer flask, with enough hexane to submerge the plant material. After 48 hours, the solvent was removed under filtration. Ethanol extraction was performed by grinding the samples with the solvent and waiting for 48 hours. The hexane and ethanol extracts were concentrated under low pressure and reduced temperature (45-50°C). The plant extracts were stored at low temperature for subsequent bioassays.

The hexane extract of paracress (*A. oleracea*) was selected for isolation and structure elucidation of its bioactive compounds. A total of 2.0 kg of dried and powdered aerial parts of *A. oleracea* was used for this purpose. The solvent (i.e., hexane) was changed every two days for 45 days. The extraction continued until the solvent was colorless. The filtered extract obtained was concentrated in a rotary vacuum evaporator under low pressure and reduced temperature (45-50°C).

Screening bioassay

A set of screening bioassays was performed to identify the bioactive plant extracts to *A. monuste*, *D. hyalinata* and *P. xylostella*. The stored extracts were diluted with acetone as solvent to a dose of 10 μg mg^{-1} of fresh body mass. The average weight was obtained by measuring the mass of ten groups containing ten insects each, on an analytical balance. The experimental design was completely randomized with six replications. Each experimental unit consisted of a glass Petri dish (9.5 cm x 2.0 cm) containing ten insects.

The bioassays were conducted by topical application. For each insect was applied, via a 10 µl Hamilton micro syringe, 0.5 µL of a solution of the test extract. In a control experiment under the same conditions, 0.5 µL of acetone was applied on each insect.

After application, the insects were kept in individual Petri dishes containing food. The foods supplied were: disks of cabbage for *A. monuste* and *P. xylostella*, and disks of cucumber leaf for *D. hyalinata*. The Petri dishes were placed in an incubator at 25 ± 0.5°C, 75 ± 5% relative humidity, with a photoperiod of 12 h. The mortality counts were made after 24 hours of treatment Mortality included dead individuals as well as those without movements.

Isolation and structural elucidation of compounds from paracress

Fractionation of the paracress hexane extract (28 g) was performed by column chromatography (Silica Gel 60, 70-230 mesh) using hexane with increasing amounts of ethyl acetate and finally with methanol as the eluting solvents. Thin layer chromatography (TLC, Silica gel 60 F254 0.25 mm) was used to identify fractions containing similar compounds. The TLC spots were detected under UV (254 and 365 nm) as well as by heating the plates to 100ºC after spraying with phosphomolybdic acid/ethylic alcohol. Eight fractions (A-H) were collected and subjected to bioassay with to *A. monuste*, *D. hyalinata* and *P. xylostella* using the same methods as described in the previous section. The most toxic fractions were purified by preparative TLC of Silica gel 60 F254, Merck (20 x 20 cm plates, 0.75 mm adsorbent). The IR spectra of isolated compounds were recorded on KBr in an infrared spectrometer Paragon 1000 FTIR (Perkin Elmer, Wellesley, MA, USA) from 600 to 4000 cm^{-1}. To identify the isolated compounds, NMR spectra were recorded on a Varian Mercury 300 MHz spectrometer. Tetramethylsilane ($SiMe_4$) was used as internal standard ($\delta = 0$) and deuterated chloroform ($CDCl_3$) as solvent.

Bioactivity of compounds from paracress

The isolated compounds and the paracress hexane extract were subject to toxicity bioassays against *A. monuste*, *D. hyalinata*, *P. xylostella*, *S. saevissima* and *T. angustula.* The insecticidal activity of neem (*Azadirachta indica* A. Juss) seed kernel hexane extract and of permethrin (92.2% purity, Syngenta), a synthetic derivative of the natural pyrethrins were also evaluated and used as positive controls. The experimental design was completely randomized with six replications. Each experimental unit consisted of a glass Petri dish (9.5 cm x 2.0 cm) containing ten insects. The average weight of each insect species was obtained by measuring the mass of ten groups containing ten insects each, on an analytical balance.

Initially, four doses of each compound were tested to identify the range of concentrations that would provide mortalities greater than zero and less than 100%. Once the range of concentration was defined, other doses were tested for each compound. The number of doses used to obtain the dose-mortality curves varied from five to eight.

Bioassays were conducted by topical application. For each insect a 10 µl Hamilton micro syringe was used to apply 0.5 µL of a solution of the test compound, dissolved in acetone. In a control experiment, carried out under the same conditions, 0.5 µL of acetone was applied to each insect. After application, the insects were kept in individual Petri dishes containing the appropriate food. The following foods were supplied: disks of cabbage for *A. monuste* and *P. xylostella*, and disks of cucumber leaf for *D. hyalinata*. *S. saevissima* and *T. angustula* both received a mixture of honey (50%) and pure water (50%). The mixture of honey and water were supplied in plastic containers that were 1.5 cm in diameter and 1.0 cm high.

The Petri dishes were placed in an incubator at 25 ± 0.5°C, 75 ± 5% relative humidity, with a photoperiod of 12 h. The mortality counts were made after 24 h. Mortality included both dead individuals and those that were no longer moving.

Statistical analysis

Mortality data of screening bioassay were subjected to analysis of variance and the averages were compared by the Scott-Knott grouping analysis test ($P < 0.05$).

Dose-mortality data of bioactive plant compounds were subjected to probit analysis using SAS software (PROC PROBIT; SAS) to estimate dose-mortality curves (SAS Institute, 2002). We accepted curves which had probabilities greater than 0.05 by the χ^2 test (Young and Young, 1998).

To determine which insecticide was more toxic to a particular species, we calculated the toxicity ratio for each insecticide (T_XR_{50} = LD_{50} of the least toxic insecticide per LD_{50} of the insecticide). The toxicity ratio indicates how many times an insecticide is more potent (i.e. toxic) than the least toxic insecticide for a given insect population under test.

Because all insecticides exhibited the higher LD_{50} values for *P. xylostella*, we used it as reference and calculated the susceptibility ratio (S_CR_{50}) of *A. monuste* and *D. hyalinata* relative to *P. xylostela* for each one of the insecticides. The formula used was S_CR_{50} = LD_{50} of the insecticide for *P. xylostella* per LD_{50} of the insecticide for *A. monuste* or *D. hyalinata*.

To determine the magnitude of selectivity of the compounds to the beneficial insects, we calculated the selectivity ratio using the formula S_LR_{50} = LD_{50} of the insecticide for the beneficial insect per LD_{50} of the insecticide for the insect pest. Values of 1 and <1 indicate that the chemical is non-selective to the

beneficial insect. Values >1 indicate that the chemical is selective and/or harmless to the beneficial insect (Bacci et al., 2009).

RESULTS

Bioactivity of plant extracts

The hexane extract from aerial parts of paracress exhibited the highest activity of all extracts, causing 100.0 ± 0.0% (N = 60) mortality ± standard error (SE) after 24 hours of exposure. It was significantly different from mortalities caused by the other extracts tested and from the controls to *A. monuste*, *D. hyalinata* and *P. xylostella*. The mortality caused by the solvents was zero (0.0%) in all of the bioassays (Table 2).

The ethanol extract of paracress showed considerable activity (>70% mortality) against *A. monuste*, *D. hyalinata* and *P. xylostella*, and was the second most active extract. The hexane and ethanol extracts of the remainder of the plants tested showed low insecticidal activity toward the lepidopteran pests (Table 2). On the basis of high activity of the paracress hexane extract, it was selected for isolation and structure elucidation of its bioactive compounds.

Isolation and structural elucidation of compounds from paracress

The hexane extract of paracress (28 g) was fractionated by a bioactivity guided fractionation approach and eight fractions, A-H, were obtained. The eight fractional groups were evaluated for their insecticide activity against the lepidopterous pests tested. Fractions F and G eluted with hexane-ethyl acetate (1:1) were biologically active, causing 100% mortality 6 hours after administration of a dose of 10 μg mg^{-1} body mass. The remainder of the fractions (A, B, C, D, E and H) caused mortalities less than 30%.

The bioactive fraction F was purified by preparative TLC (hexane-ethyl acetate, 6:1) to yield the following three major bands: **I** (825 mg, Rf 0.65), **II** (56

mg, Rf 0.45), and **III** (21 mg, Rf 0.25). Band **I** was biologically active and was further purified by preparative TLC (hexane-ethyl acetate, 1:2) to give compounds **1** and **2** (320 and 210 mg, respectively). The bioactive fraction G was purified by preparative TLC (hexane-ethyl acetate, 3:1) to yield compound **3** (27 mg).

Compound **1**, (2*E*,6*Z*,8*E*)-deca-2,6,8-trienoic acid N-isobutyl amide or spilanthol (Fig. 1), was isolated as a colorless oil. The IR spectrum showed the presence of a secondary amide group (3340, 1636, and 1550 cm^{-1}), a double bond conjugated to an amide carbonyl group (1678 cm^{-1}), and a conjugated diene group with the *Z*, *E* or *E*, *Z* configuration (987 and 953 cm^{-1}). The MS spectrum had the molecular ion peak at m/z 221, which indicates the molecular formula $C_{14}H_{23}NO$. The ^{13}C NMR ($CDCl_3$) and the ^{1}H NMR ($CDCl_3$) spectra showed the spilanthic acid (Table 2). On the amine moiety, the typical signals at δ 3.15 (2H, t, H-1'), 1.78 (1H, m, H-2'), and 0.93 (6H, d, H-3', 4') in ^{1}H NMR and δ 46.9 (C-1'), 28.6 (C-2') and 20.1 (C-3',4') in the ^{13}C NMR indicated the presence of a isobutylamino group. All of the spectral data were in agreement with those of spilanthol in literature (Nakatani and Nagashima, 1992).

Compound **2** was isolated as a colorless crystal. In the ^{1}H NMR spectrum characteristic signals at δ 3.18 (dd, H-l'), 1.79 (m, H-2') and 0.91 (d, H-3' and H-4') indicated the isobutylamide moiety. This compound was identified as undeca-2*E*-en-8,10-diynoic acid isobutylamide (Fig. 1) by comparing its ^{1}H NMR spectral data (Table 2) with published values (Bauer et al., 1989). The ^{13}C NMR spectrum (Table 2) was consistent with published data (Ramsewak, 1999).

Compound **3**, (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (Fig. 1), was isolated as a colorless oil. The IR spectrum presented absorption bands attributable to a triple bond (2225 cm^{-1}) in addition to a secondary amide group (3299, 1627 and 1554 cm^{-1}) and a double bond conjugated with an amide

carbonyl group (1669 cm^{-1}). The ^{1}H NMR spectrum revealed signals at δ 5.77 (d, J = 15 Hz) and 6.80 (dt, J = 15 Hz and 7 Hz) which have been attributed to olefinic protons, H-2 and H-3, respectively (Table 2). On the amine moiety, a pair of 1H ddq signals at δ 1.17 and 1.41 are attributed to methylene protons of C-3' and a pair of ddd signals at δ 3.14 and 3.27 are attributed to methylene protons of C-1' due to the presence of asymmetric carbon at C-2' (Table 2). The ^{13}C NMR spectrum gave rise to 16 carbon signals. Five carbon signals at δ 45.2, 35.1, 27.1, 11.3 and 17.2 confirmed a 2-methylbutylamine moiety (Table 4). The ^{13}C NMR and ^{1}H NMR signals correspond well with the literature (Nakatani and Nagashima, 1992).

Bioactivity of isolated compounds to lepidopterous pests

For several compounds, the slopes of dose-mortality curves generated for *P. xylostella* were slightly steeper than those observed for the other lepidopterous pests (Fig. 2). Thus, *P. xylostella* tended to respond more homogeneously to the insecticides than *A. monuste* and *D. hyalinata*.

The dose-mortality results from insecticide application in second-instar larvae of *A. monuste*, *D. hyalinata* and *P. xylostella* showed low χ^2 and high *P* values (<7.62 and >0.113 respectively), indicating the suitability of the probit model for fitting the dose-response curves and consequently obtaining estimates of the mortality parameter LD_{50} (Table 3).

Compound **1** (spilanthol) exhibited the highest toxicity to the lepidopterous pests, with the lowest LD_{50}. The spilanthol was about 3 to 5 times more toxic than the commercial insecticide permethrin to lepidopteran pests, and approximately 250, 421 and 286 times more potent than neem extract to *A. monuste*, *D. hyalinata* and *P. xylostella*, respectively (Fig. 3).

For *A. monuste* and *P. xylostella*, the compound undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) showed insecticidal activity similar to

permethrin. However, the compound **2** was more toxic than permethrin to *D. hyalinata*. In comparison with the neem extract, the compound **2** was about 109, 158 and 97 times more toxic to *A. monuste*, *D. hyalinata* and *P. xylostella*, respectively (Fig. 3).

The compound (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**) showed toxicity similar to that of permethrin against *D. hyalinata* and slightly lower than permethrin against *A. monuste* and *P. xylostella*. Compared with the neem extract, the compound **3** were about 54, 58 and 56 times more toxic to *A. monuste*, *D. hyalinata* and *P. xylostella*, respectively (Fig. 3).

The *A. oleracea* extract was less toxic than the isolated compounds, but it showed good insecticidal activity. It was 9.6- to 11.7-fold more toxic than neem extract to the lepidopterous pests (Fig. 3).

Susceptibility of lepidopterous pests to isolated compounds

In our bioassays, *P. xylostela* was the less susceptible insect to all compounds as indicated by highest LD_{50} values for this species relative to the other pests (Table 3). As a result, we used it as reference to calculate the susceptibility ratio, a measure of the relative susceptibility of the insect pests to the toxic substances (Fig. 4). Susceptibility ratios for *A. monuste* relative to *P. xylostela* varied between 1.05 and 1.35. Based on the confidence limits there were no differences in susceptibility of *A. monuste* and *P. xylostella* to the paracress extract and permethrin. Likewise for *D. hyalinata*, susceptibility ratios varied between 1.11 and 2.25. According to the confidence limits, there was no difference in susceptibility of *D. hyalinata* and *P. xylostella* to permethrin. The differential susceptibility between the species was greatest for the compound **2** and **3** (Fig. 4).

Selectivity of isolated compounds

The dose needed to kill 50% of the test population (LD_{50}) was determined for the beneficial insects (Table 4) and utilized to calculate selectivity ratios of the insecticides for the two beneficial insects (Fig. 5). The *A. oleracea* extract and the compounds **1**, **2** and **3** were selective to the predator *S. saevissima* and the pollinator *T. angustula* relative to *A. monuste*, *D. hyalinata* and *P. xylostella*, with a selectivity ratio (S_LR_{50}) greater than 1.0 (Fig. 5).

The neem extract also showed some selectivity to *S. saevissima* as LD_{50} values for *A. monuste, D. hyalinata* and *P. xylostella* (Fig. 5) were about 1.71, 1.91 and 1.42-fold lower that those for *S. saevissima*. On the other hand, the LD_{50} of neem extract for *A. monuste, D. hyalinata* and *P. xylostella* was higher than the LD_{50} of neem extract for *T. angustula* (Fig. 5), indicating that it was harmful to the pollinator.

For permethrin, the LD_{50} for *A. monuste, D. hyalinata* and *P. xylostella* was 11.74, 10.22 and 13.26-fold higher than the LD_{50} for *S. saevissima*, and 1800, 1556 and 2033-fold higher than the LD_{50} for *T. angustula*, respectively. These results indicate that permethrin is harmful to the beneficial insects.

DISCUSSION

The only plant species showing insecticide activity in our study was the paracress plant *A. oleracea.* This plant is attributed with immense medicinal (Ramsewak et al., 1999; Wu et al., 2008), antimicrobial (Fabry et al., 1996, 1998; Rai et al., 2004) and insecticidal properties (Pendse et al., 1945; Jondiko, 1986; Ramsewak et al., 1999) because of the presence of several bioactive compounds, which includes Spilanthol, the main active compound, and a group of other isobutylamides. The lethal effect of *A. oleracea* extracts has been reported for several insect vectors of diseases such as *Aedes aegypti* Linn, *Anopheles stephensi* Liston, *Anopheles culicifacies* Giles and *Culex quinquefasciatus* Say (Diptera: Culicidae) (Pendse et al., 1945; Jondiko, 1986;

Ramsewak et al., 1999; Saraf and Dixit, 2002; Amer and Mehlhorn, 2006; Pandey et al., 2007). However, there is no thorough study on the effect of this plant on insect pests of agricultural crops.

At the end of chemical investigation of paracress extract performed in our study, three different compounds were identified: spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**). The results showed that all of the compounds isolated had high insecticidal activity, which were often as or more toxic than permethrin to *A. monuste, D. hyalinata* and *P. xylostella*, a pyrethroid recommended for control of these lepidopterous pest. Furthermore, the compounds were far more toxic than the neem extract. The high efficiency of these compounds combined with the ready availability from natural sources and the friendlier environmental footprint makes this plant an excellent candidate as a future natural insecticide.

The low insecticidal activity of neem extract may be a consequence of the mode of action of azadirachtin. Several studies reported that the high lethality caused by azadirachtin takes longer to occur. This delayed mortality by azadirachtin may equivocally suggesthig inactivity or higher safety, but delayed mortality does not imply need of extended exposure, which is particularly true for insect growth regulators such as azadirachtin, where the insecticidal action takes longer to occur. The need of an extended assessment period to evaluate the mortality by azadirachtin has been recently stressed (Medina et al., 2009). Such need is further justified based on the growth regulator activity of azadirachtin interfering with the transport and release of neurosecretory peptides, which is regarded as its main mode of action, aided by its antifeedant activity (Mordue et al., 2005).

In contrast with the extract of neen, which showed low effect (high DL_{50}) at 24 h evaluation time, the paracress extract and the alkamides showed high toxicity (low DL_{50}) after short exposure to the compounds. The mechanism

of action of active alkamidas found in *A. oleracea* has not yet been determined. Saraf and Dixit (2002) investigated the mortality of *A. culicifacies*, *C. quinquefasciatus* and *A. aegypti* when exposed to spilanthol and observed abnormal movement like jerks, spinning and uncoordinated muscular activity, suggesting that the drug disturbs nerve conduction somewhere. In addition, they observed the mortality of pupae in short span of time upon exposure to the drug which indicates that spilanthol greatly disturbs the ongoing processes of histolysis and histogenesis.

Plutella xylostela was the most tolerant among the pests. The higher tolerance to *P. xylostella* is possibly due to the fact that this insect is little susceptible to many insecticides. There are even many populations resistant to insecticides (Mohan and Gujar, 2003; Vickers et al., 2004; Li et al., 2006). However, compounds **1** and **2** are presented as an alternative to control this pest, since these substances were highly toxic to *P. xylostella*.

The results from this study showed the alkamides **1**, **2** and **3** were selective to *S. saevissima* and *T. angustula.* The mechanisms of selectivity are varied. But in general, selectivity results from three main aspects: reduced insecticide penetration, increased metabolism of the insecticide (by esterases, mixed function oxidases, or glutiathione transferases) in the non-target species body, and relative insensitivity of the target site in beneficial insects compared with the pests (Guedes, 1999).

Insecticide impact on non-target species, such as insect predators and pollinators, is an ever-growing concern in agriculture. The selectivity provided by alkamides to *S. saevissima* and *T. angustula*, suggests that the use of these compounds to control the lepidopterous pest present a low risk to these beneficial insects. Furthermore, the results from this study showed that all compounds had a lower toxicity than permethrin (insecticide already used to control the lepidopterous pest) to all non-target species studied. This finding

indicates that the alkamides are less harmful to the beneficial insects. Thus, the use of these compounds for pest control can be recommended as a strategy to manage these beneficial insects. Besides the isolated alkamidas show physiological selectivity in favor of beneficial insects, the low stability of botanical pesticides and consequent rapid degradation in the environment is a characteristic that favors ecological selectivity, because it reduces the exposure time of beneficial organisms to toxic compounds (Ripper et al., 1951).

In summary, the paracress extract and the bioactives alkamides showed a high potencial to control *A. monuste, D. hyalinata* and *P. xylostella* because while they were highly toxic to lepidopterous pest, they were safe to beneficial insects evaluated. An important point should be emphasized here. The notion that natural compounds are safer than synthetic compounds to non-target species was refuted in many studies (Qi et al., 2001; Medina et al., 2004; Venzon et al., 2007; Cordeiro et al, 2010), which proves that bioinsecticides should not be exempted from risk assessment. Thus, the impact of these products on other important non-target organisms should be further assessed when considering potential insecticide use in agriculture. Nevertheless, as *A. oleracea* is widely used as both food and folk medicine in their region of origin, it is assumed that the toxicity to humans is extremely low, which makes it an attractive alternative to control agricultural pests. Besides the possibility of using paracress crude extract or semi-commercial products, the alkamides are potential pest management tools likely to have their insecticide activity improved through organic synthesis guided by studies of quantitative structure-activity relationship.

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Table 1. Contact toxicity of plant extracts at concentration of 10 µg of extract per mg of insect against *Diaphania hyalinata*, *Ascia monuste* and *Plutella xylostella*, 24 hour after topical application

Plant	Mean percent mortality[1]					
	Ascia monuste		*Diaphania hyalinata*		*Plutella xylostella*	
	Ethanol extract	Hexane extract	Ethanol extract	Hexane extract	Ethanol extract	Hexane extract
Acmella oleracea	80.0 ± 2.6 Ab	100.0 ± 0.0 Aa	86.7 ± 2.1 Ab	100.0 ± 0.0 Aa	73.3 ± 3.3 Ab	100.0 ± 0.0 Aa
Ageratum conyzoides	23.3 ± 2.1 Cb	31.7 ± 1.7 Da	21.7 ± 3.1 Cb	33.3 ± 2.1 Ca	16.6 ± 2.1 Bb	31.7 ± 1.7 Ca
Allamanda cathartica	35.0 ± 2.2 Bb	55.0 ± 2.2 Ca	40.0 ± 2.6 Bb	63.3 ± 3.3 Ba	28.3 ± 1.7 Bb	41.7 ± 1.7 Ba
Argemone mexicana	20.0 ± 0.0 Cb	30.0 ± 2.6 Da	26.7 ± 2.1 Cb	35.0 ± 2.2 Ca	20.0 ± 2.6 Bb	33.3 ± 2.1 Ca
Artemisia vulgaris	13.3 ± 2.1 Da	16.7 ± 2.1 Ea	8.3 ± 1.6 Ca	11.7 ± 3.1 Da	11.7 ± 3.1 Ba	15.0 ± 2.2 Ca
Banara guianensis	21.7 ± 1.7 Ca	18.3 ± 1.7 Ea	18.3 ± 1.6 Ca	16.7 ± 2.1 Da	16.6 ± 2.1 Bb	23.3 ± 2.1 Ca
Banara nitida	15.0 ± 2.2 Db	20.0 ± 0.0 Ea	13.3 ± 2.1 Cb	21.7 ± 1.6 Da	8.3 ± 1.7 Bb	36.7 ± 2.1 Ca
Bauhinia variegate	8.3 ± 1.7 Db	18.3 ± 1.7 Ea	10.0 ± 2.6 Cb	20.0 ± 2.6 Da	15.0 ± 2.2 Ba	16.7 ± 2.1 Ca
Bougainvillea glabra	30.0 ± 2.6 Ba	31.7 ± 1.7 Da	28.3 ± 3.1 Ca	26.7 ± 2.1 Ca	23.3 ± 2.1 Ba	20.0 ± 2.6 Ca
Calendula officinalis	23.3 ± 2.1 Ca	25.0 ± 2.2 Ea	26.7 ± 2.1 Ca	31.7 ± 3.1 Ca	18.3 ± 1.6 Bb	35.0 ± 2.2 Ca
Chenopodium ambrosioides	18.3 ± 1.7 Ca	18.3 ± 1.7 Ea	21.7 ± 3.1 Ca	20.0 ± 2.6 Da	16.7 ± 2.1 Ba	10.0 ± 0.0 Da
Clavija weberbaueri	35.0 ± 2.2 Ba	40.0 ± 2.6 Da	38.3 ± 2.1 Bb	46.7 ± 1.6 Ca	31.7 ± 3.1 Bb	41.7 ± 1.7 Ba
Copaifera duckei	30.0 ± 0.0 Bb	38.3 ± 1.7 Da	38.3 ± 1.6 Ba	41.7 ± 3.1 Ca	25.0 ± 2.2 Bb	33.3 ± 2.1 Ca
Coriandrum sativum	11.7 ± 3.1 Db	20.0 ± 2.6 Ea	16.7 ± 2.1 Ca	21.7 ± 1.6 Da	10.0 ± 0.0 Bb	20.0 ± 2.6 Ca
Curatela americana	38.3 ± 3.1 Ba	46.7 ± 2.1 Da	43.3 ± 2.1 Bb	55.0 ± 2.2 Ca	36.7 ± 2.1 Bb	43.3 ± 2.1 Ba
Eugenia egensis	28.3 ± 2.1 Ba	35.0 ± 2.2 Da	38.3 ± 1.6 Ba	36.7 ± 3.3 Ca	28.3 ± 3.1 Ba	30.0 ± 0.0 Ca
Mayna parvifolia	23.3 ± 2.1 Cb	36.7 ± 3.3 Da	26.7 ± 3.3 Cb	35.0 ± 2.2 Ca	16.7 ± 2.1 Ba	21.7 ± 1.7 Ca
Piper aduncum	30.0 ± 0.0 Ba	33.3 ± 2.1 Da	30.0 ± 2.6 Ca	31.7 ± 1.7 Ca	25.0 ± 2.2 Ba	23.3 ± 2.1 Ca
Piper augustum	21.7 ± 1.7 Cb	40.0 ± 2.6 Da	33.3 ± 2.1 Cb	43.3 ± 2.1 Ca	30.0 ± 2.6 Ba	35.0 ± 2.2 Ca
Ryania speciosa	10.0 ± 0.0 Db	23.3 ± 2.2 Ea	18.3 ± 3.1 Cb	30.0 ± 0.0 Ca	16.7 ± 2.1 Ba	18.3 ± 1.7 Ca
Siparuna amazônica	13.3 ± 2.1 Db	25.0 ± 2.2 Ea	16.7 ± 2.1 Cb	28.3 ± 3.1 Ca	11.7 ± 3.1 Bb	26.7 ± 3.3 Ca
Spathodea campanulata	0.0 ± 0.0 Ea	3.3 ± 2.1 Fa	3.3 ± 3.3 Da	5.0 ± 2.2 Da	0.0 ± 0.0 Ca	3.3 ± 2.1 Ea
Tropaeolum majus	38.3 ± 1.7 Bb	63.3 ± 3.3 Ba	31.7 ± 1.6 Cb	51.7 ± 3.1 Ca	26.7 ± 3.3 Bb	43.3 ± 2.1 Ba
Control[2]	0.0 ± 0.0 Ea	0.0 ± 0.0 Fa	0.0 ± 0.0 Da	0.0 ± 0.0 Da	0.0 ± 0.0 Ca	0.0 ± 0.0 Ea

[1]Means followed by the same lower-case letter in a row (for comparation between ethanol and hexane extracts) or by the same upper-case letter in a column are not significantly different by the Scott-Knott grouping analysis test at $P > 0.05$. [2]Only acetone was used in the control.

Table 2. ^{1}H NMR (δ values, 300 MHz, $CDCl_3$) and ^{13}C NMR ((δ values, 100 MHz, $CDCl_3$) spectral data for compounds 1-3

Atom	**1**		**2**		**3**	
	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
C-1	166		165.9		165.9	
C-2	124.2	5.79 br. d (15)	124.0	5.76 dt (15; 1)	124.3	2.77 br. d (15)
C-3	143.5	6.83 dt (15; 7)	143.8	6.79 dt (15; 7)	143.6	6.80 dt (15; 7)
C-4	32.1	2.23-2.35 m	31.3	2.24 m	31.3	2.20 m
C-5	26.4	2.23-2.35 m	27.4	1.60 m	27.4	1.57 m
C-6	127.7	5.26 dt (11; 7)	27.2	1.60 m	27.5	1.57 m
C-7	129.5	5.97 dd (11; 11)	18.9	2.24 m	18.9	2.27 m
C-8	126.7	6.29 br. dd (11;	77.9		77.9	
C-9	130.0	5.70 dq (15; 7)	65.0		65.0	
C-10	18.3	1.78 d (7)	68.4		68.5	
C-11			64.7	1.95 t (1)	64.7	1.96 br. s
C-1’	46.9	3.15 dd (7; 6)	46.9	3.18 dd (7; 7)	45.2	3.14 ddd (14; 7; 6)
						3.27 ddd (14; 7; 6)
C-2’	28.6	1.78 m	28.6	1.79 m	35.1	1.57 m
C-3’	20.1	0.93 d (7)	20.1	0.91 d (7)	27.1	1.17 ddq (14; 7; 7)
						1.41 ddq (14; 7; 7)
C-4’	20.1	0.93 d (7)	20.1	0.91 d (7)	11.3	0.91 t (7)
C-5’					17.2	0.91 d (7)
N		5.47 br. s		5.45 br. s		5.38 br. s

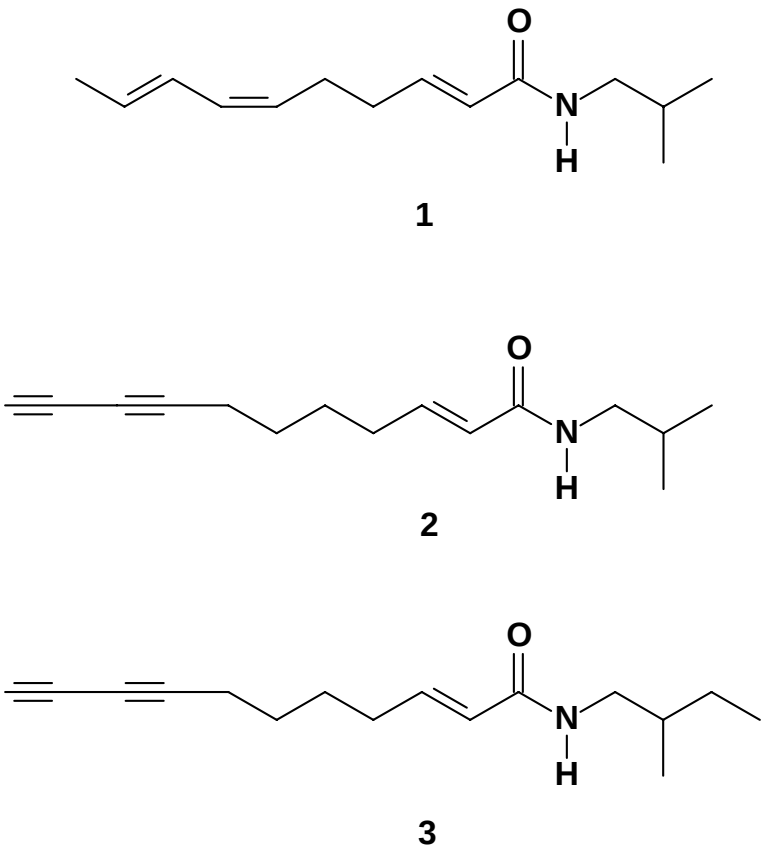

Fig. 1. Structure of the three alkamides isolated from *Acmella oleracea*: spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8.10-diynamide (**3**).

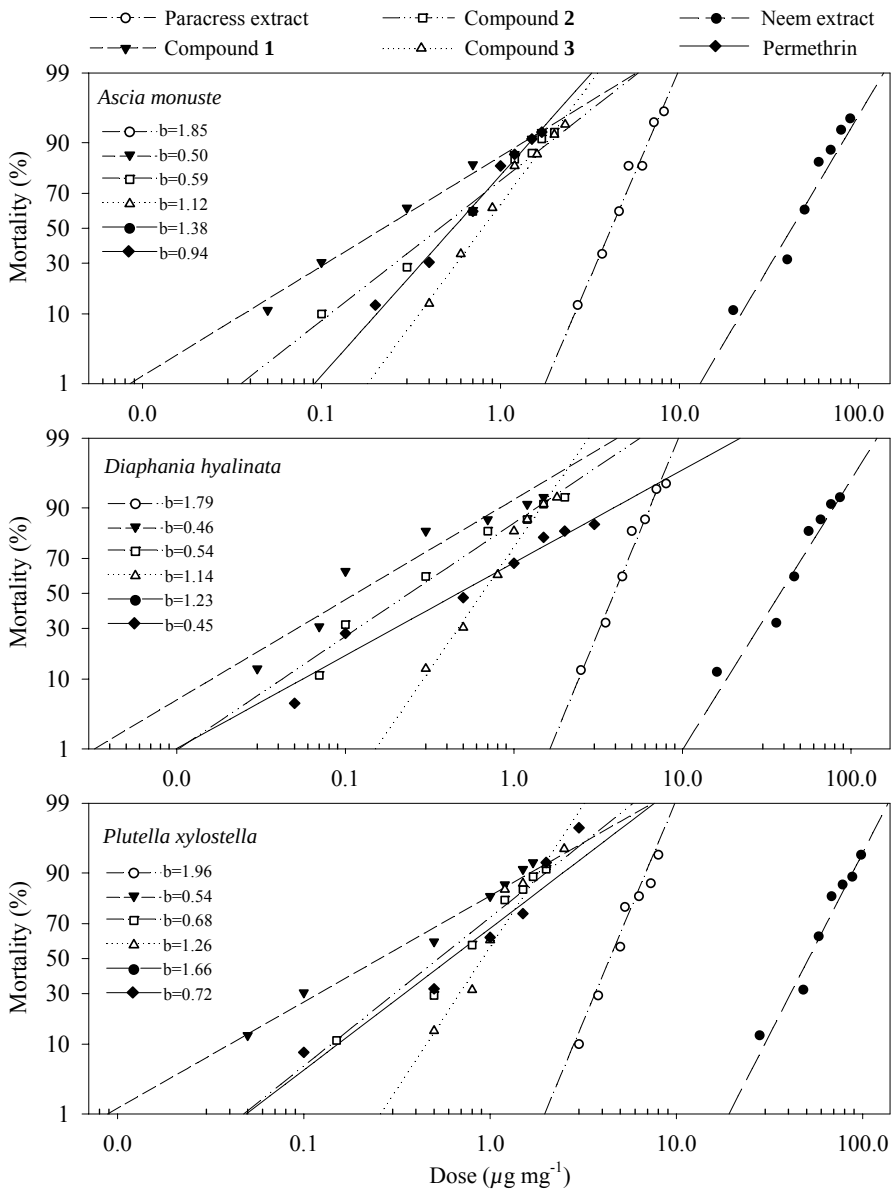

Fig. 2. Dose-mortality regression lines of paracress hexane extract and of spilanthol (**1**), undeca-2E-en-8.10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8.10-diynamide (**3**) tested against second-instar larvae of *Diaphania hyalinata*, *Ascia monuste* and *Plutella xylostella*. Neem extract and Permethrin were used as positive controls.

Table 3. Results of probit analysis on mortality of *Diaphania hyalinata*, *Ascia monuste* and *Plutella xylostella*, 24 hour after topical application of paracress hexane extract and of spilanthol (**1**), undeca-2E-en-8.10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8.10-diynamide (**3**) extracted from aerial parts of paracress

Treatments	N[1]	LD_{50} (μg mg^{-1}) (95% FL)[2]	χ^2	*P* value
		Ascia monuste		
Paracress extract	420	4.17 (3.85 - 4.46)	3.35	0.917
Compound **1**	420	0.17 (0.13 - 0.24)	1.27	0.982
Compound **2**	420	0.39 (0.30 - 0.49)	1.58	0.965
Compound **3**	420	0.79 (0.69 - 0.89)	2.99	0.957
Neem extract[3]	420	42.51 (37.77 - 46.76)	3.87	0.822
Permethrin[3,4]	420	0.54 (0.46 - 0.63)	2.69	0.979
		Diaphania hyalinata		
Paracress extract	420	3.94 (3.62 - 4.22)	3.63	0.871
Compound **1**	420	0.09 (0.08 - 0.12)	3.58	0.777
Compound **2**	420	0.24 (0.18 - 0.30)	2.90	0.966
Compound **3**	420	0.65 (0.56 - 0.73)	2.86	0.968
Neem extract[3]	420	37.95 (33.18 - 42.24)	4.08	0.771
Permethrin[3,4]	420	0.47 (0.34 - 0.62)	4.03	0.545
		Plutella xylostella		
Paracress extract	420	4.39 (4.07 - 4.68)	3.60	0.875
Compound **1**	420	0.18 (0.13 - 0.23)	6.02	0.198
Compound **2**	420	0.53 (0.41 - 0.64)	3.96	0.586
Compound **3**	420	0.91 (0.81 - 1.00)	4.10	0.765
Neem extract[3]	420	51.28 (46.55 - 55.48)	3.62	0.871
Permethrin[3]	360	0.61 (0.47 - 0.73)	7.62	0.113

[1] Number of insects tested.
[2] Lethal dose with 95% fiducial limits (FL).
[3] Positive control.

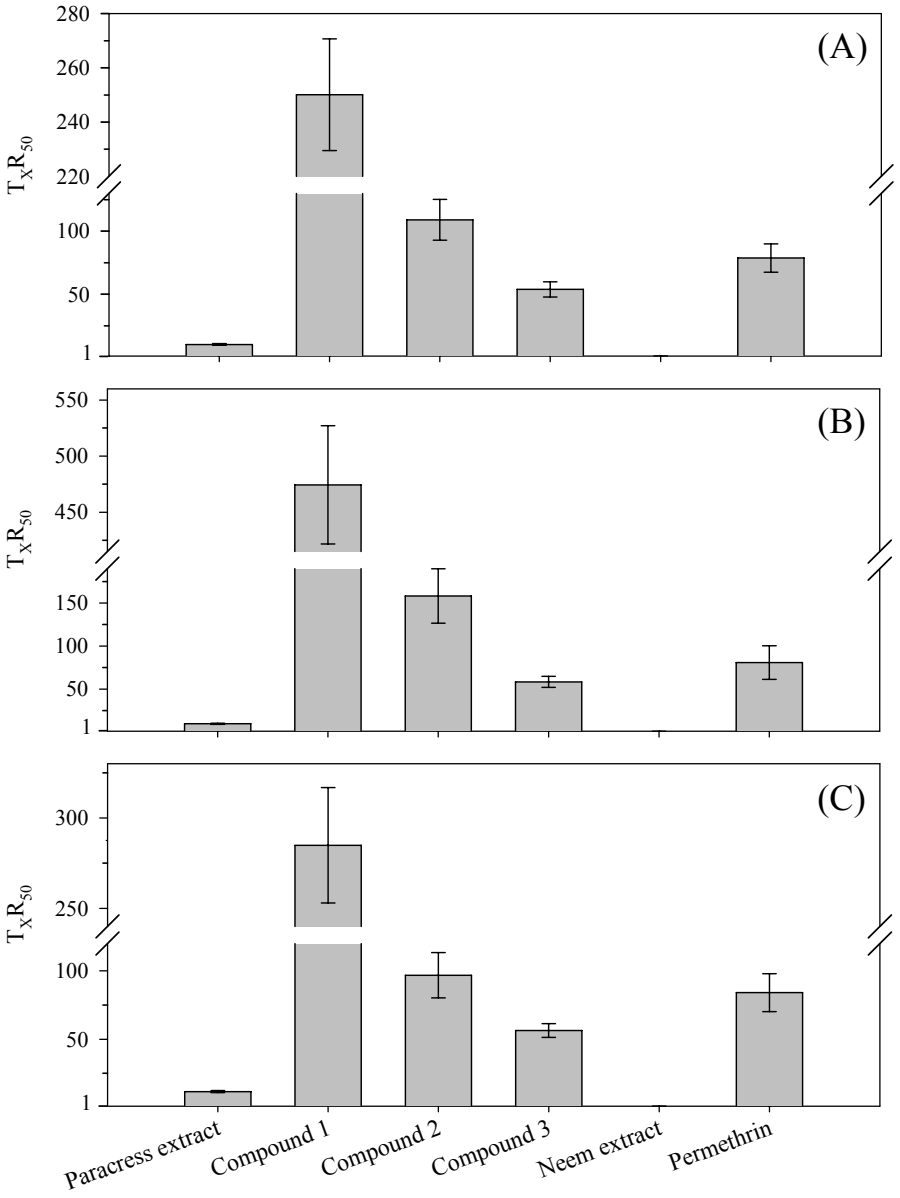

Fig. 3. Toxicity ratio at the LD_{50} (T_XR_{50}) of paracress hexane extract and of spilanthol (**1**), undeca-2E-en-8.10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8.10-diynamide (**3**) tested against second-instar larvae *Ascia monuste* (A), *Diaphania hyalinata* (B) and *Plutella xylostella* (C). Brackets indicate 95% confidence limits. Neem extract and Permethrin were used as positive controls.

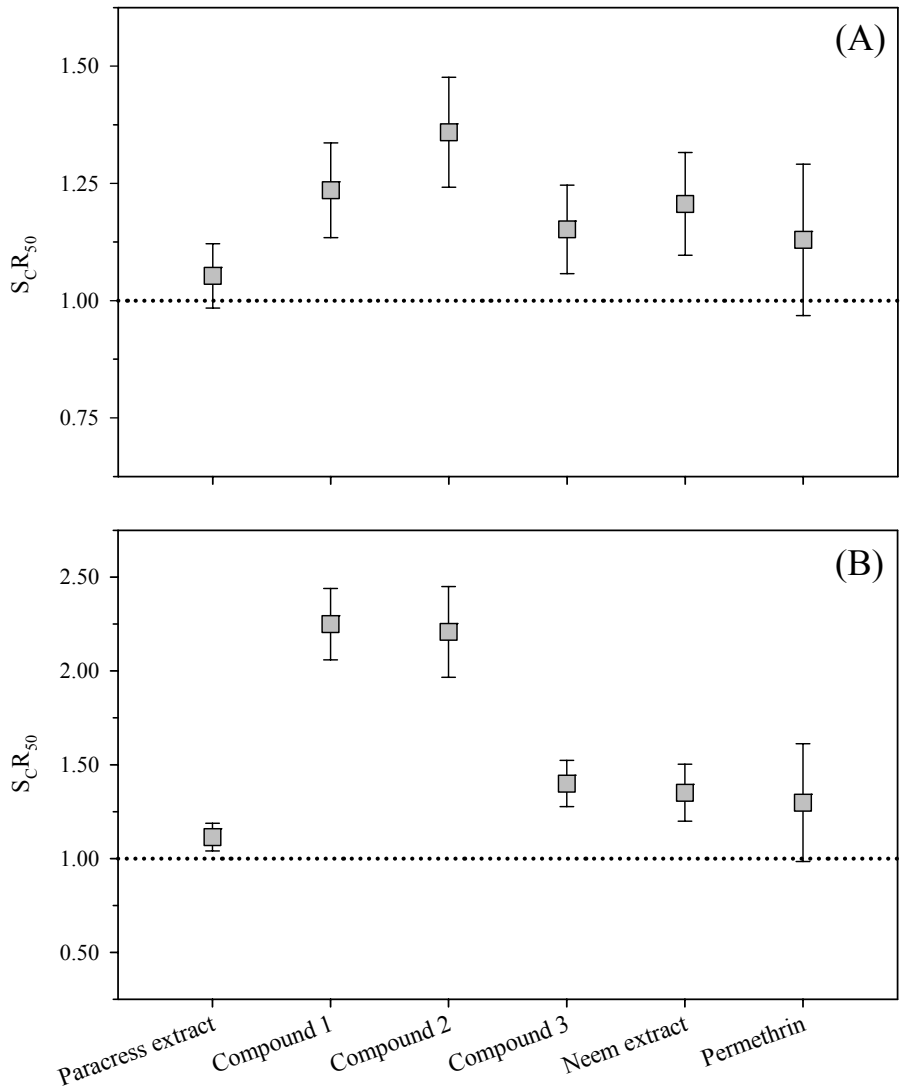

Fig. 4. Susceptibility ratio at the LD_{50} (T_LR_{50}) of *Ascia monuste* (A) and *Diaphania hyalinata* (B) relative to *Plutella xylostella* (least sensitive species). Brackets indicate the 95 confidence limits. The LD values for the species being compared were considered significantly different ($P < 0.05$) if the confidence limits on the susceptibility ratio did not include the value 1.

Table 4. Contact toxicity of paracress hexane extract and of spilanthol (**1**), undeca-2E-en-8.10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8.10-diynamide (**3**) extracted from aerial parts of paracress plant against *Solenopsis saevissima* and *Tetragonisca angustula*, 24 hours after topical application

Treatments	N[1]	LD_{50} ($\mu g\ mg^{-1}$) (95% FL)[2]	Slope ± SE	χ^2	*P* value
		Solenopsis saevissima			
Paracress extract	420	5.66 (5.05 - 6.24)	0.97 ± 0.04	2.58	0.999
Compound **1**	420	0.21 (0.16 - 0.27)	0.46 ± 0.03	1.34	0.976
Compound **2**	360	0.67 (0.54 - 0.82)	0.47 ± 0.03	6.37	0.278
Compound **3**	420	1.33 (1.06 - 1.63)	0.47 ± 0.03	6.12	0.354
Neem extract[3]	360	72.65 (68.47 - 76.63)	2.76 ± 0.25	1.42	0.999
Permethrin[3]	300	0.046 (0.029 - 0.073)	0.28 ± 0.02	4.69	0.450
		Tetragonisca angustula			
Paracress extract	420	5.34 (4.61 - 5.97)	0.83 ± 0.03	3.95	0.997
Compound **1**	420	0.35 (0.29 - 0.42)	0.68 ± 0.02	1.62	0.998
Compound **2**	360	0.66 (0.49 - 0.85)	0.38 ± 0.02	7.49	0.112
Compound **3**	420	1.10 (0.89 - 1.33)	0.58 ± 0.03	1.81	0.994
Neem extract[3]	360	29.52 (25.23 - 34.15)	0.68 ± 0.05	6.93	0.399
Permethrin[3]	300	0.0003 (0.0002 - 0.0005)	0.40 ± 0.02	4.95	0.427

[1] Number of insects tested.
[2] Lethal dose with 95% fiducial limits (FL).
[3] Positive control.

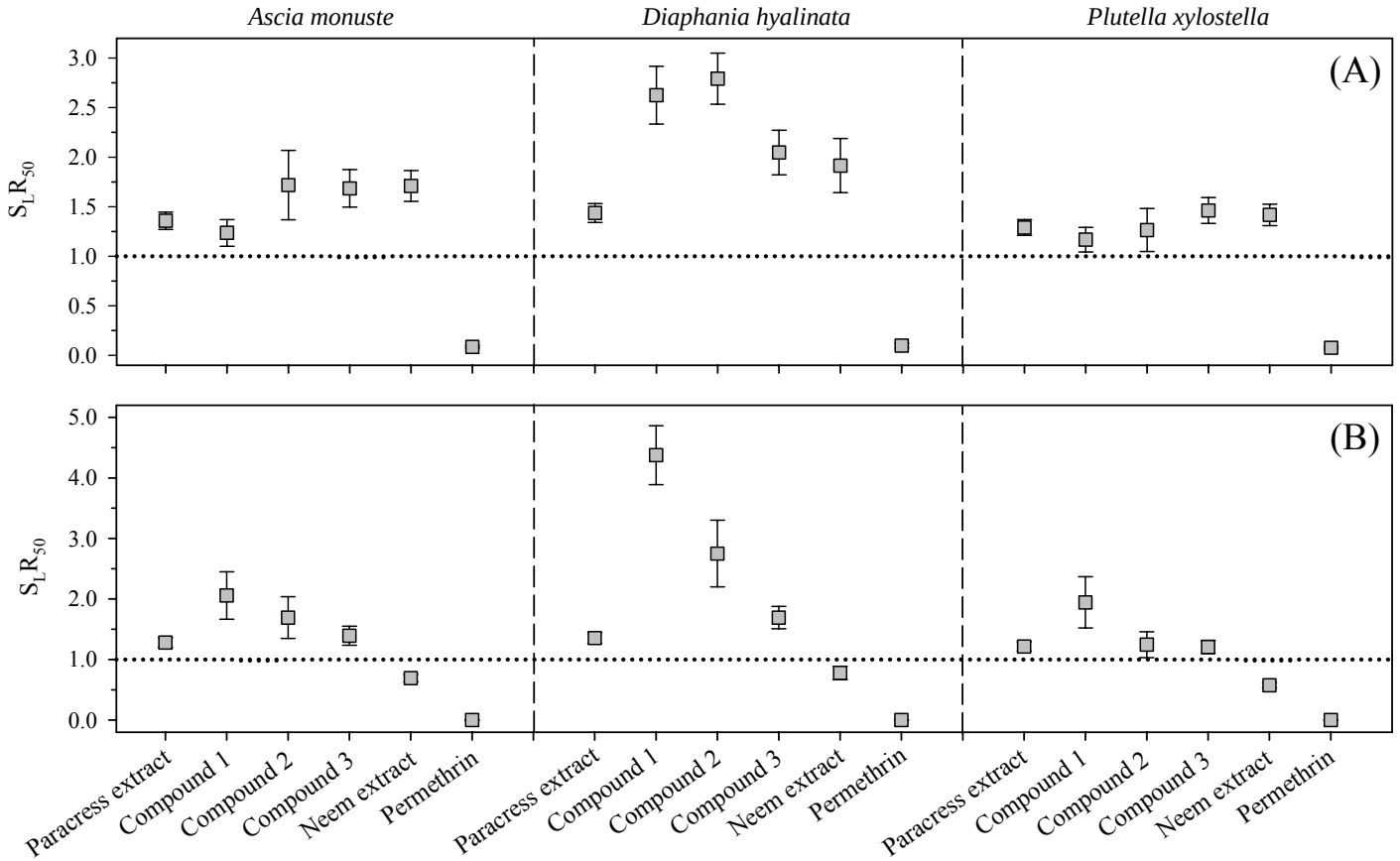

Fig. 5. Selectivity ratio at the LD_{50} (SLR_{50}) to adults of *Solenopsis saevissima* (A) and *Tetragonisca angustula* (B) relative to second-instar larvae of *Ascia monuste*, *Diaphania hyalinata*, *Plutella xylostella*. Brackets represent the 95 confidence limits. A selectivity ratio >1 indicate that the insecticide is selective (more toxic to the pest than to the natural enemy).

ARTICLE 3

LETHAL AND BEHAVIORAL EFFECTS OF AMAZON PLANT EXTRACTS ON LEAF-CUTTING ANT WORKERS

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Lethal and Behavioral Effects of Amazon Plant Extracts on Leaf-Cutting Ant Workers

by

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ABSTRACT

Leaf-cutting ants are economically important pests in neotropical agricultural and forestry ecosystems. The present study aimed to assess the effect of extracts of six amazon plants (*Banara guianensis*, *Clavija weberbaueri*, *Mayna parvifolia*, *Ryania speciosa*, *Acmella oleracea* and *Siparuna amazonica*) on survival of the leaf-cutting ant workers *Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans*, and also their effect on the mobility of these species. All of these extracts had some insecticidal effect on the ants and *A. oleracea* extract was the most toxic to all ant species studied. For *A. laevigata* the extract of *M. parvifolia* also was highly toxic. Only the extract of *R. speciosa* had an effect on walking behavior of ants, reducing the total distance moved and the walking velocity.

Keywords: leaf-cutting ants, plant extracts, plant toxicity, walking behavior.

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INTRODUCTION

Ants are dominant social insects in most terrestrial ecosystems. True leaf-cutting ants, *Atta* spp. and *Acromyrmex* spp. (Hymenoptera: Formicidae), are dominant herbivores in the neotropical region. Ants of these genera caused severe damage to agriculture and forests because they cut fresh plants, including flowers, fruits, leaves and twigs to cultivate their symbiont fungus. Moreover, in addition to direct crop damage, their impacts on agriculture include loss of land surface and destruction of farm roads because of their large colonies, leading to accidents involving machinery and livestock (Hölldobler & Wilson 1990, Zanetti *et al*. 2003).

The main control method used against these pests are insecticides applied in different formulations such as dry powders, granulated baits, thermo-fogging and liquefied gases. Historically, leaf-cutting ants were successfully controlled by the application of synthetic organic insecticides formulated as granulated baits (Boaretto & Forti 1997, Della Lucia & Araújo 2000). Although many insecticides have been tested as baits against leaf cutting ants, the market was dominated by those with dodecachlor. In recent years it was shown that this compound can seriously pollute the environment so it has been banned and the new baits for these pests have either sulfluramid or chlorpirifos (Zanuncio 1999). However these compounds also may cause damage to the environment, reaching not only target pests but also non-target species including man. Thus, other control strategies with higher specificity and less aggressiveness toward the environment should be investigated (Morini *et al*. 2005).

Among potentially cost-effective and environmental friendly alternatives to synthetic insecticides are the botanical extracts, which can be toxic to workers of leaf-cutting ants, to their fungi or to both. Plants such as neem (*Azadirachta indica*), sesame (*Sesasum indicum*), castor bean (*Ricinus*

communis), common rue (*Ruta graveolens*) jack bean (*Cannavalia ensiformis*), black sage (*Cordia verbenacea*) billygoat weed (*Ageratum conyzoides*), peppermint (*Mentha piperita*) and spotted gum (*Eucalyptus maculata*) have been tested for their control, but their efficiency is relatively low compared to synthetic organic insecticides (Hebling *et al*. 1996, Takahashi-Del-Bianco 2002, Bueno *et al*. 2004, Marinho *et al*. 2006, Ribeiro *et al*. 2008). For this reason, more research should be conducted to test plant extracts and demonstrate possible applications for future use in leaf-cutting ant control.

The singular focus of most toxicological studies is survival/mortality estimates. But there are other factors that warrant closer attention. Insecticides used to control ants as baits must be lethal at low concentrations and have slow action. Furthermore, to be effective in control, the compound must not modify the walking behavior of ants, which could reduce the effectiveness of control by preventing the ants return to the nest (Boaretto & Forti 1997). Thus, the study of the effect of these products in the behavior of ants is as important as the study of their toxicity.

Therefore, our study had the objective of evaluating the effect of extracts of six amazon plants (*Banara guianensis*, *Clavija weberbaueri*, *Mayna parvifolia*, *Ryania speciosa*, *Acmella oleracea* and *Siparuna amazonica*) on survival of the leaf-cutting ant workers *Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans*, and also their effect on the walking behavior of these species.

MATERIALS AND METHODS

Insect species and plant material

The bioassays were performed with workers of *A. sexdens rubropilosa*, *A. laevigata* and *A. subterraneus molestans*. The workers were caught in

colonies located around the Campus of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais State, Brazil.

The following plants were subject to extraction and bioassays: *Banara guianensis* Aubl., *Clavija weberbaueri* Mez., *Mayna parvifolia* Sleumer, *Ryania speciosa* Vahl., *Acmella oleracea* L. and *Siparuna amazonica* Mart. ex A. DC. These plants were chosen based on popular knowledge of natives from the Amazon.

Samples of 500 g of the canopy of each plant species were collected from the Amazon rain forest, state of Acre, Brazil. Each sample was placed in 1L erlenmeyer flask, with enough hexane to submerge the plant material. The solvent was removed under filtration after 48 hours. The hexane extracts were concentrated under low pressure and reduced temperature (45-50°C). The plant extracts were stored at low temperature for subsequent bioassays.

Toxicity bioassays

The stored extracts were diluted with acetone as solvent to a concentration of 5 mg mL^{-1}. The experimental design was completely randomized with five replications. Each experimental unit consisted of a glass petri dish (9.5 cm x 2.0 cm) containing ten insects.

Bioassays were conducted by topical application. For each individual insect was applied on the thoracic tergite, via a 10 µl Hamilton micro syringe, 1.0 µL of a solution of the test extract. To achieve this, each ant was immobilized by using a tweezer while they received the solution on their pronotum. In a control experiment, carried out under the same conditions, 1.0 µL of acetone was applied on each insect.

After application, the insects were kept in individual Petri dishes containing honey + water at proportions of 1:1 and pure water. The honey and water were supplied in plastic containers of 1.5 cm diameter and 1.0 cm high.

The Petri dishes were placed in an incubator at 25 ± 0.5 °C, 75 ± 5% relative humidity and 12 hours photophase. The mortality counts were made after 1, 3, 6, 12, 24, 48 and 72 hours of treatment. Mortality included dead individuals and those without movements.

Behavioral bioassays

Behavioral bioassays were carried out in glass arenas (3.0 cm high × 15 cm inner diameter) containing filter paper fully sprayed with extracts diluted in hexane (control treatments were sprayed with hexane).

The inner walls of each arena were covered with Teflon® PTFE (DuPont) to prevent insects from escaping. A single insect was placed in each arena (always at the center of the arena). Twenty arenas (i.e. independent replicates) with individual insects were used for each treatment in the behavioral bioassay, and no insect mortality was observed within the 10 min exposure (trial duration) used for the bioassays.

The movement of each insect within the arena during 10 min was recorded using a Canon® NTSC video camcorder (XL1 3CCD; Canon USA, Lake Success, NY) equipped with a 16x video lens (zoom XL 5.5–88mm) and digitally transferred to a computer for subsequent analysis using the software Studio version 9 (Pinnacle Systems, Mountain View, CA). The movement of the insects was recorded for each arena using the software EthoVision Pro 3.0 (Noldus Information Technology, Sterling, VA). EthoVision detected the insect's position using the subtraction method after applying an erosion and dilation filter.

Average movement parameters were calculated for the treatments to determine differences in ant response to extract-sprayed surfaces. The parameters calculated were total distance moved (cm) and velocity (cm s^{-1}).

Data analysis

Mortality data and the results of behavioral bioassay were submitted to analysis of variance and their averages were compared using the Scott-Knott test at $p<0.05$ (Scott & Knott 1974). Regression analyses were also used to determine time-mortality curves for the concentrations and extracts used. The confidence intervals were calculated at 95% of probability to verify the differences among the curves over the time.

RESULTS AND DISCUSSION

Insecticide toxicity: time–mortality responses

The plant extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, *R. speciosa*, *A. oleracea* and *S. amazonica* had an insecticidal effect against *A. sexdens rubropilosa*, *A. laevigata* and *A. subterraneus molestans* workers, since significantly higher mortality of treated ants occurred when compared to that of control ants (Fig. 1).

Among the plant extracts tested, the extract of *A. oleracea* at the concentration of 5 mg mL^{-1} exhibited the highest toxicity to all leaf-cutter species, causing 100% mortality at 70, 56 and 60 hours after application on *A. laevigata*, *A. sexdens rubropilosa* and *A. subterraneus molestans* workers respectively (Fig. 1, Table 1). Ribeiro e*t al.* (2008), evaluating the toxic effect of four hexane extracts (*Ruta graveolens, Cordia verbenaceae, Mentha piperita* and *Ageratum conyzoides*) when applied on workers of *A. sexdens rubropilosa* and *A. subterraneus molestans* obtained lower mortalities (> 40%) at the same concentration and with similar methodology. In this study, mortality reached 100% only for concentrations of 50 and 100 mg mL^{-1}., depending on the plant extract. This indicates that extract of *A. aleracea* had significantly higher mortality and can be further used to control leaf-cutting ants.

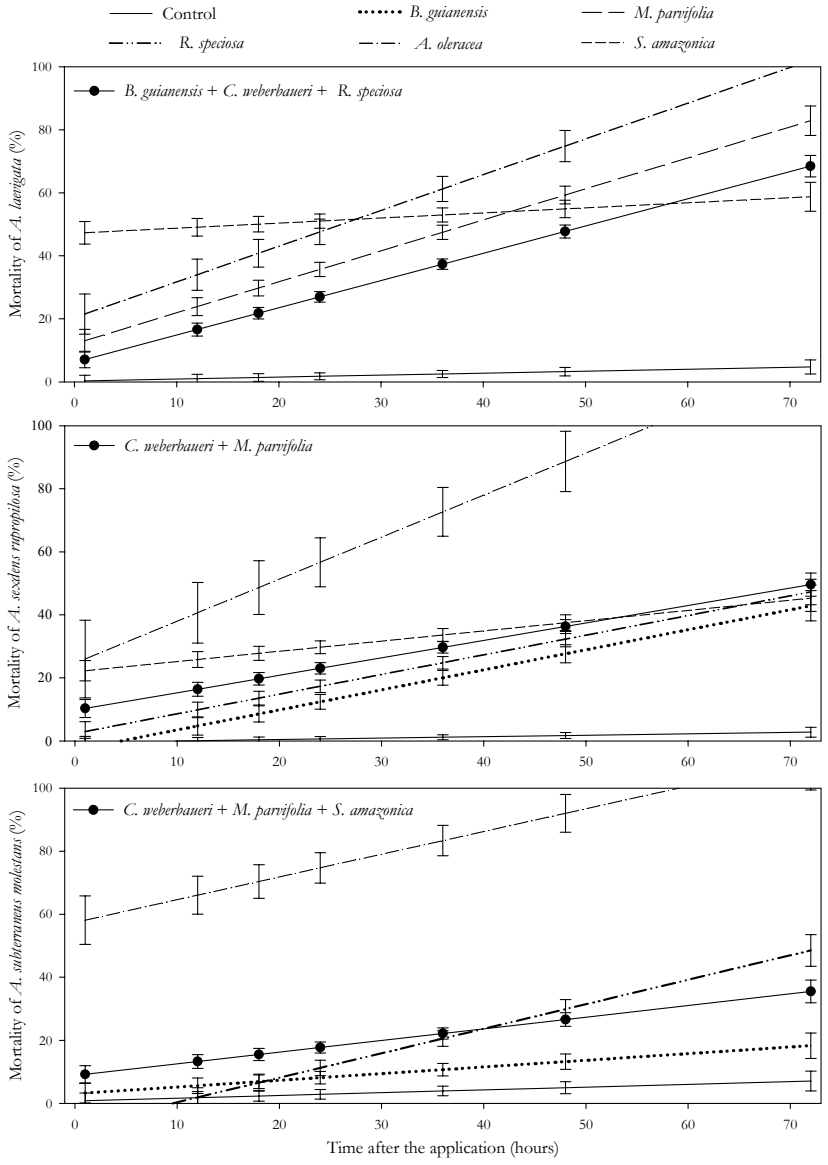

Fig. 1. Mortality (%) of workers of *Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans* caused by extracts of six plant species and control, applied topically at the concentration of 5 mg mL^{-1}. Vertical bars indicate confidence interval (CI) at 95%. Regressions represented by a continuous line with black circles indicate that the curves do not differ among themselves at a 95% CI and were therefore grouped together. Information related to the regressions is found in Table 1

Table 1. Data of regression analysis for plant extracts applied topically on workers of *Atta laevigata*, *Atta sexdens rubropilosa* and *Acromyrmex subterraneus molestans*.

Extracts	Regression equations*	R^2	F-values	Probability
	Atta sexdens rupropilosa			
B. guianensis	y = -2.88 + 0.64x	0.83	$F_{1,34} = 164.77$	<0.0001
C. weberbaueri *M. parvifolia*	y = 9.75 + 0.55x	0.75	$F_{1,69} = 201.50$	<0.0001
R. speciosa	y = 2.35 + 0.62x	0.87	$F_{1,34} = 219.98$	<0.0001
S. amazonica	y = 21.94 + 0.32x	0.69	$F_{1,34} = 55.92$	<0.0001
A. oleracea	y = 24.65 + 1.33x	0.66	$F_{1,34} = 65.23$	<0.0001
Control	y = -0.44 + 0.04x	0.12	$F_{1,55} = 7.24$	0.01
	Atta laevigata			
B. guianensis *R. speciosa* *C. weberbaueri*	y = 6.24 + 0.86x	0.85	$F_{1,104} = 572.35$	<0.0001
M. parvifolia	y = 12.08 + 0.98x	0.93	$F_{1,34} = 414.81$	<0.0001
S. amazonica	y = 47.15 + 0.16x	0.26	$F_{1,34} = 11.30$	0.002
A. oleracea	y = 20.39 + 1.13x	0.84	$F_{1,34} = 176.59$	<0.0001
Control	y = 0.27 + 0.06x	0.11	$F_{1,55} = 6.91$	0.01
	Acromyrmex subterraneus molestans			
B. guianensis	y = 3.05 + 0.21x	0.43	$F_{1,34} = 25.39$	<0.0001
C. weberbaueri *M. parvifolia* *S. amazonica*	y = 8.83 + 0.37x	0.48	$F_{1,104} = 93.66$	<0.0001
R. speciosa	y = -7.43 + 0.78x	0.87	$F_{1,34} = 221.15$	<0.0001
A. oleracea	y = 57.41 + 0.72x	0.60	$F_{1,34} = 49.10$	<0.0001
Control	y = 0.75 + 0.09x	0.12	$F_{1,55} = 7.01$	0.01

*Mortality curves that did not differ among themselves by the 95% CI were combined into a single curve.

For *A. sexdens rubropilosa* only *A. oleracea* extract showed considerable insecticidal effect. The other extracts showed significantly higher mortality than control, but mortality caused by them did not reach 50%. The mortality caused by *C. weberbaueri* and *M. parvifolia* was the same and both differed from the control immediately after application (Fig 1, Table 1).

As far as *A. laevigata* is concerned we can say that in addition to the extract of *A. oleracea*, the extract of *M. parvifolia* also showed high insecticidal effects. For this extract, the mortality reached 82% 72 hours after treatment. The extracts of *B. guianensis*, *C. weberbaueri* and *R. speciosa* led to the same mortality in *A. laevigata* (68% at 72 hours after application) and differed from control immediately after application. The mortality caused by *S. amazonica* was 58% at 72 hours after application. Unlike the other extracts, *S. amazonica* extract caused worker mortality in a short amount of time (47% mortality at 1 hour after application). The other extracts caused less than 20% mortality at 1 hour after treatment (Fig 1, Table 1).

For *A. subterraneus molestans*, the fastest response was achieved with *A. oleracea* extract, which caused about 60% mortality right after application and 100% mortality 59 hours after application. This was followed by extract of *R. speciosa*, which showed 50% mortality 72 hours after application. The extracts of *C. weberbaueri*, *M. parvifolia* and *S. amazonica* did not differ among themselves and mortality caused by them was less than 40%. *B. guianensis* extract was the least potent (mortality > 20%) against *A. subterraneus molestans* (Fig. 1, Table 1).

Extracts that cause worker mortality in a short amount of time, such as that of *S. amazonica* to *A. laevigata* and *A. oleracea* to *A. subterraneus molestans* are more appropriate for ant control methods that use direct application, such as thermo fogging. On the other hand, if the purpose is to utilize these extracts for bait manufacture, the ideal extract is one that slowly

promotes ant death. In this way, worker ants live long enough to take the baits back to the nest and feed it to the colony and queen (Della Lucia & Araújo 2000).

The chemical compounds responsible for the insecticidal effect in the extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, and *S. amazonica* have not been chemically isolated yet but are liposoluble substances, since they were extracted with hexane, a non-polar solvent.

The powdered stemwood of *R. speciosa* is a botanical insecticide known as Ryania (Kuna & Heal 1948). The stem extract contains several structurally related ryanoids, including: ryanodine, 10-(*O*-methyl)-ryanodine, 9,21-dehydroryanodine, and ryanodol. The most toxic and abundant compounds in the extract are ryanodine and 9,21-dehydroryanodine, and thus, they account for virtually all of the insecticidal activity (Rogers *et al*. 1948). Ryania is highly toxic to the fruit moth (*Grapholita molesta*), coddling moth (*Cydia pomonella*), corn earworm (*Heliothis zea*), European corn borer (*Ostrinia nubilalis*) and citrus thrips (*Scirtothrips citri*), but its efficiency in controlling ants had not yet been investigated (Leslie 1989, Jefferies *et al*. 1992, Kamrin 1997). In our study, the extract of *R. speciosa* showed moderate toxicity at concentration of 5 mg mL-1, but the effect in higher concentrations should be further analyzed in prospective studies.

For *A. oleracea* there are several studies of isolation and identification of bioactive compounds. Spilanthol, considered to be one of the most active constituents, was obtained from *A. oleracea* in 1903 (Gerber 1903). This compound is the main constituent of *S. oleracea*, which has been administered as a traditional folk medicine for years to cure toothaches, stammering, and stomatitis. Previous studies have demonstrated its diuretic, antibacterial, and anti-inflammatory activities (Wu *et al*. 2008), besides presenting insecticidal activity (Phrutivorapongkul *et al*. 2008). Since the isolation of spilanthol, a

number of other *N*-isobutylamides such as 2*E*-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, 2*E*,7*Z*-*N*-isobutyl-2,7-tri-decadiene-10,12-diynamide and 7*Z*-*N*-isobutyl-7-tride-cene-10,12-diynamide, undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acid isobutylamide. have been reported (Nakatani & Nagashima 1992, Ramsewak *et al*. 1999, Saraf & Dixit 2002). Nevertheless, there are few reports concerning the investigation of insecticidal activity of these compounds and most studies are related to the control of insect vectors of disease (Saraf & Dixit 2002, Phrutivorapongkul 2008).

The results indicate that the *A. oleracea* extract is most promising among the plant extracts studied and should be further investigated as an alternative for control of the leaf-cutting ants. The extract of *M. parvifolia* can also be an alternative to control *A. laevigata*, which already had mortality greater than 80% to this specie.

We observed that *A. laevigata* was the most susceptible insect to the plant extracts, suggesting that different leaf-cutting ant species are differently affected by exposure to the same plant extract. Results found by Ribeiro et al. (2008) with *A. laevigata* and *A. subterraneus subterraneus*, using *A. conyzoides*, *M. piperita*, *C. verbenaceae* and *R. graveolens* extracts also showed that distinct species of leaf-cutting ants responded differently to plant extracts. Therefore it becomes very important to determine which extract is the most effective against each ant species as well as the optimum concentration to be utilized. Thus, the plant extracts that showed low toxicity at the concentration used in this bioassay should be studied at higher concentrations.

Walking behavior

Tracks representative of the typical walking behavior of ants species on arenas sprayed with extracts are shown in Fig. 2.

Only the hexane extract of *R. speciosa* affect the walking behavior of ants species. The walking behavior of all the three ants on arenas sprayed with extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, *A. oleracea* and *Siparuna amazonica* was similar to the control. These amazon plant extracts did not affect the total distance moved or velocity of *A. laevigata, A. sexdens rubropilosa* and *A. subterraneus molestans*. Already the extract of *R. speciosa* reduced the distance moved and velocity of the three species (Fig. 3).

The reduced mobility observed with *R. speciosa* may result from neurotoxic activity of Ryanodine, the main chemical compound responsible for the Ryania insecticidal effect. Ryanodine induces paralysis in insects by causing a sustained contracture of skeletal muscle without depolarizing the muscle membrane. A number of studies have confirmed that ryanodine can irreversibly activate the calcium release channel in the sarcoplasmic reticulum. The irreversible activation of this calcium channel floods the muscle fibers with calcium, inducing the sustained contraction of skeletal muscle and paralysis observed in ryanodine poisoning (Bloomquist 1996).

The sublethal behavioral effects of insecticides are also relevant for leaf-cutting ants management because the species are expected to remain exposed to sublethal concentrations of these compounds during loading of the bait or as a consequence of insecticide degradation. The behavioral effects may modify the foraging activity of workers and they can not take the baits back to the nest (Boaretto & Forti 1997). So, insecticides that modify the workers behavior are not indicated for use as bait and should be used in different formulation. The results of the present study indicate that the extracts of *B. guianensis*, *C. weberbaueri*, *M. parvifolia*, *A. oleracea* and *S. amazonica* did not present behavioral effects to the ants. Therefore, the application of these extracts for leaf-cutting ants control in the form of baits may be feasible, since the extracts did not interfere in the behavior of ants.

In summary, *A. oleracea* extract in the concentration of 5 mg mL^{-1} was efficient against all ant species, and the extract of *M. parvifolia* was effective only to *A. laevigata*. The extract of *R. speciosa* was the only one that affected the walking behavior of ants, and may not be efficient for use in baits.

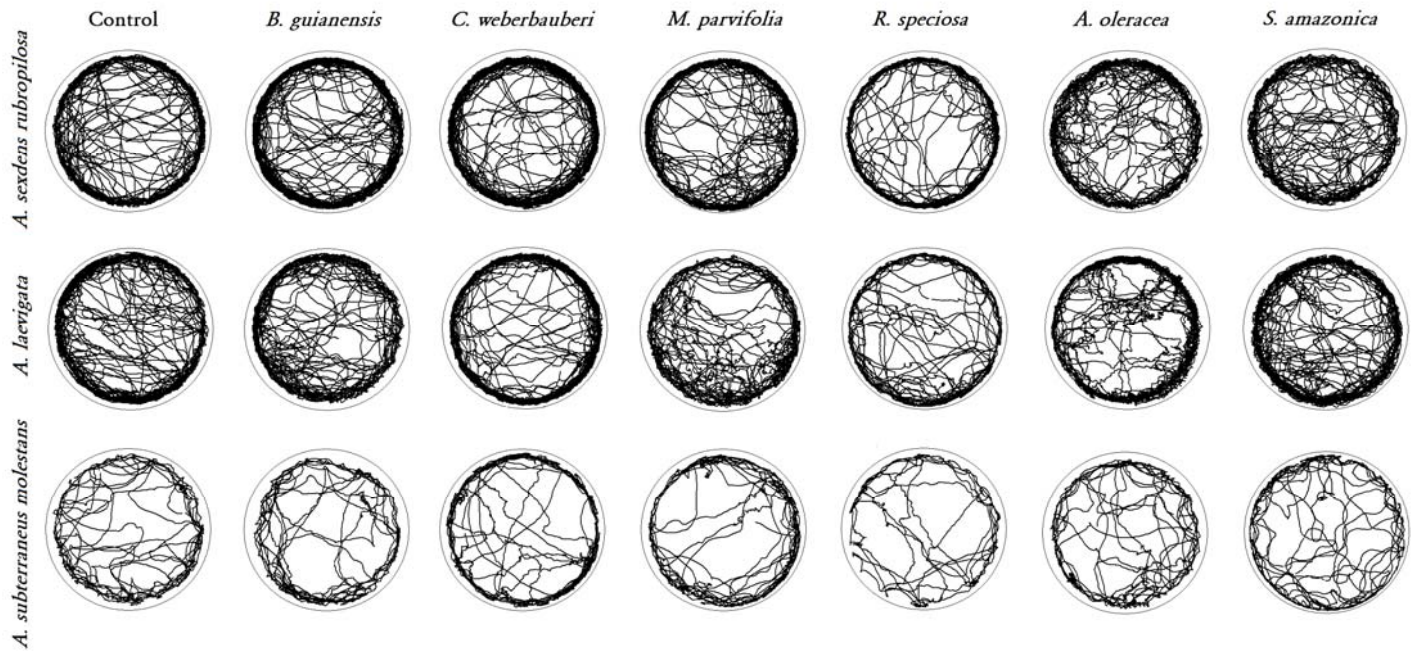

Fig. 2. Representative tracks showing the movement of individual ants from three species of leaf-cutting ants (*Atta sexdens rubropilosa*, *Atta laevigata*, and *Acromyrmex subterraneus molestans*) over a 10min period on arenas fully sprayed with solvent or plant extracts.

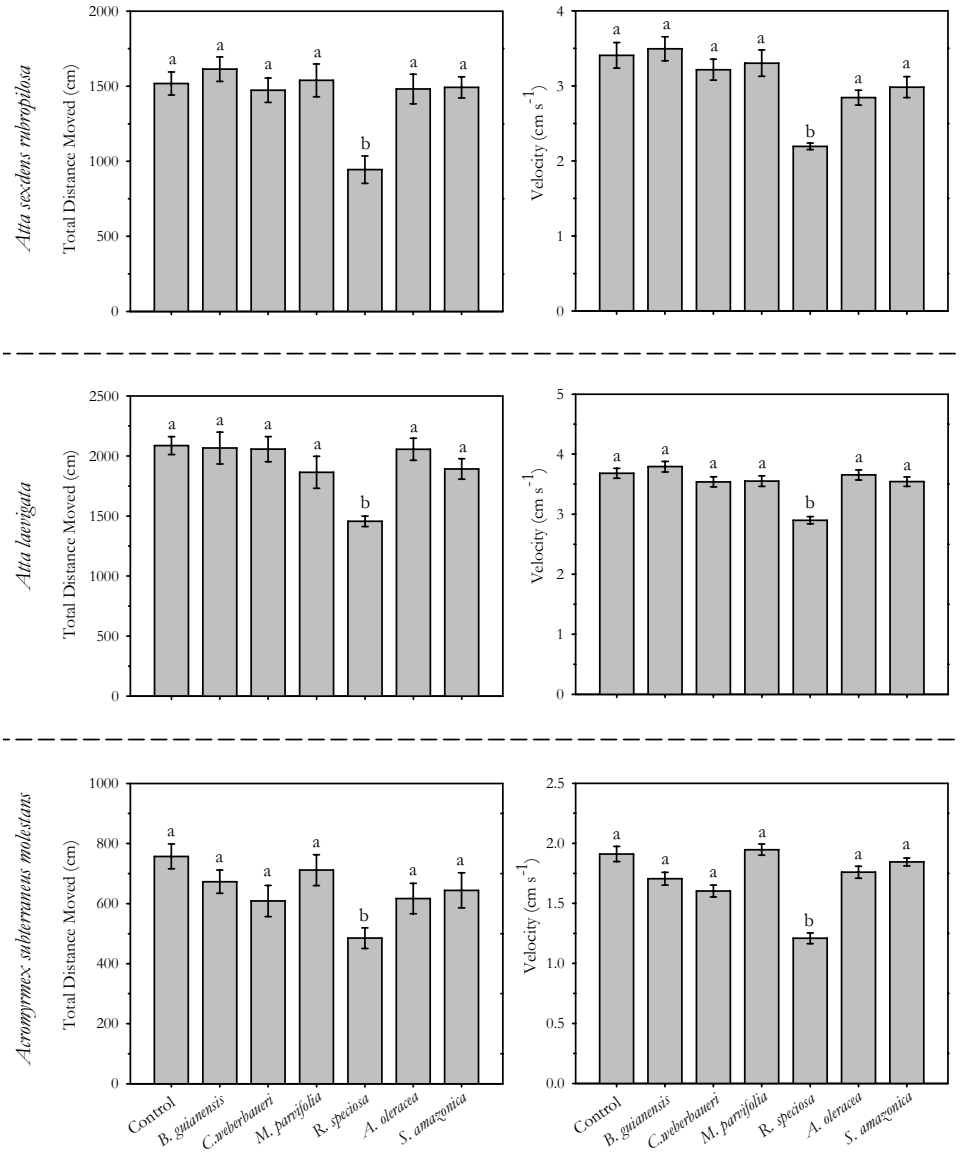

Fig. 3. Total distance moved and velocity (± standard error) by three species of leaf-cutting ants (*Atta sexdens rubropilosa*, *Atta laevigata* and *Acromyrmex subterraneus molestans*) exposed to arenas fully sprayed with either solvent or plant extracts over a 10min period. Histogram bars with the same letter do not significantly differ by Scott-Knott test ($P < 0.05$).

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ARTICLE 4

EFFECTS OF *Acmella oleracea* EXTRACTS ON THE APHIDS *Myzus persicae* AND *Lipaphis erysimi* AND ON TWO NATURAL ENEMIES

This article was written in accordance with the standards of Journal of Applied Entomology.

Effects of *Acmella oleracea* extracts on the aphids *Myzus persicae* and *Lipaphis erysimi* and on two natural enemies

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Abstract

This study was conducted to evaluate the toxicity of *Acmella oleracea* extracts to *Myzus persicae* (Sulz.), *Lipaphis erysimi* (Kalt.) (Hemiptera: Aphididae), the aphid parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae), and the predator *Orius insidiosus* (Say) (Hemiptera: Anthocoridae). We evaluated aqueous and ethanol extracts from *A. oleracea*. Only the ethanol extract was effective against all aphid species, and it may provide an alternative for the control of these insect pests. This extract produced a 90% mortality rate in both natural enemies in less than 70 h and significantly reduced the fecundity of the two species of aphids. In addition, the natural enemies showed no lethal response to this extract. In an attempt to identify bioactive compounds in the ethanol extract, three alkamides, spilanthol, undeca-2*E*-en-8,10-diynoic acid isobutylamide and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide, were isolated from the *A. oleracea* extract and tested on *L. erysimi*. All of the isolated alkamides showed high insecticidal activity against *L. erysimi*, indicating that they contributed to the observed activity of the extract. The results of this study demonstrate the substantial effects of ethanol extracts of *A. oleracea* against *M.*

persicae and *L. erysimi* under laboratory conditions and verify the extract's selectivity to natural enemies, suggesting its potential in controlling this insect pest under field conditions.

Keywords: botanical insecticide, foliar treatment, green peach aphid, mustard aphid, *Diaeretiella rapae*, *Orius insidiosus*

1 Introduction

Aphids (Hemiptera: Aphididae) are one of the main groups of agricultural pests. These insects are important pests of brassica crops, such as kale (*Brassica oleracea* var. *acephala*), cabbage (*Brassica oleracea* var. *botrytis*), broccoli (*B. oleracea* var. *italica*), cress (*Lepidium ruderale* L.) and mustard (*Brassica juncea* L.). The main aphids that attack Brassica crops include the species *Myzus persicae* (Sulz.) and *Lipaphis erysimi* (Kalt.). These insects suck sap from plants, introduce toxins and transmit viruses. The most visible symptoms infestation by these pests are curling and chlorosis of leaves and shoots (Blackman and Eastop 2000; Collier and Finch 2007).

The mustard aphid, *L. erysimi*, is distributed across the world and attacks both leaves and terminal stems and flowers of various species of Brassica. This aphid acts as a vector of many pathogenic viruses and is an important pest of mustard in India and China (Collier and Finch 2007).

These pests have mainly been controlled by applications of synthetic insecticides. However, in recent years, the use of plant-derived compounds as natural insecticides has received a significant amount of attention, as farming systems are developed to have reduced environmental impacts (Isman 2000). The search for new solutions to control insect pests in agriculture is currently influenced by the following concerns: the banning of synthetic insecticide use in organic crops; the public perception that natural compounds are better; plant-

based bio-pesticides are generally regarded as Safe (GRAS) and have low regulatory registration requirements; and the reliance on extracts versus pure compounds.

Several studies have demonstrated the insecticidal activity of plants extracts against aphids and others agricultural pests. The most widely evaluated plants are the neem (*Azadirachta indica* A. Juss) and chinaberry (*Melia azedarach* L.) plants (Chen et al. 1996; Basedow et al. 2002; Gajmer et al. 2002). Plants such as neem have produced excellent results, and commercial products derived from neem are already available in the market. Neem extracts have been tested on aphids with encouraging results (Stark and Rangus 1994; Lowery and Isman 1995; Tang et al. 2002). In addition, pepper extracts and naturally obtained pyrethrin were shown to cause mortality in aphids (Edelson et al. 2002). However, many other plant species, especially those from tropical regions, could be used as botanical insecticides, and their properties have been reported in the scientific literature (Quignard et al. 2003; Shaalan et al. 2005).

Acmella oleracea, commonly referred to as jambu, is a typical herb from northern Brazil, where it is used in the local cuisine. In folk medicine, it is employed as an analgesic for toothaches and throat and mouth complaints. In industry, jambu extracts have been used in oral care products and food as a flavor and a refresher. These extracts have also been used in cosmetics compositions as anti-ageing ingredients. The activity of *A. oleracea* has been studied extensively; however, only a limited number of studies have assessed its insecticidal activity. Nevertheless, the studies were related to the control of insect disease vectors (Saraf & Dixit 2002; Phrutivorapongkul 2008). Thus, studies to investigate the effects of this plant against agricultural pests are needed.

The majority of studies on botanical extracts have used extracts that were made in laboratories using solvents and procedures that prevent the

production of these extracts by farmers. In this research, however, priority was given to extracts that could be easily produced by farmers. Furthermore, in addition to studying the efficiency of crude extracts, if the specific identities of bioactive compounds are ascertained, then these compounds could be chemically synthesized and serve as lead compounds for the generation of more selective or more potent analogues of interest to the agro-chemical industry.

Given this context, our objective was to evaluate the effects of *A. oleracea* extracts on the aphids *M. persicae* and *L. erysimi* and to purify the active principles in the bioactive extracts. We were also interested in the selectivity of the extracts in the aphid parasitoid *Diaeretiella rapae* McIntosh (Hymenoptera: Braconidae) and the predator *Orius insidiosus* Say (Hemiptera: Anthocoridae) because a substance used to control pests should not select non-target species, such as natural enemies, while efficiently controlling insect pests.

2 Materials and Methods

2.1 Plant extract preparation

Samples of 20 g of the canopy of *A. oleracea* were collected from the campus of the Federal University of Viçosa (UFV), Minas Gerais, Brazil, where this plant is permanently cultivated. Each sample was placed in a 1-L Erlenmeyer flask with 200 mL of solvent (ethanol or deionized water). The extracts were removed via filtration after 48 hours. Subsequently, 100 mL of the filtered extracts was diluted in 900 mL of deionized water (i.e., 100 mL of filtered extract per liter of solution) and used immediately.

2.2 Insect rearing

The aphids *L. erysimi* and *M. persicae* were obtained from the Laboratory of Integrated Pest Management at UFV. To begin the laboratory rearing of aphids, infested kale leaves were collected in Viçosa, MG. Adult females from these

colonies were transferred with a brush to clean kale leaves, the petioles of which were immersed in 100-mL containers filled with water. These leaves were then placed inside wooden cages lined with organza (50 x 50 x 50 cm), and separate cages were used for each aphid species. To prevent parasites and fungi infestations, females were removed 24 hours after the transfer, and all but the first instars were removed from the leaves.

Every three days, new kale leaves were added to the cages. These leaves were placed flush against the aphid-infested leaves to facilitate travel to the new leaves. The following day, the yellow leaves were removed after the remaining aphids were transferred with the aid of a brush to the leaves placed on the previous day.

The parasitoid *D. rapae* also were obtained from a population reared in the laboratory. To the establishment of massal rearing, mummies of *M. persicae* parasited by this parasitoid species were collected in brassica crops and placed inside wooden cages closed with organza contain kale leaves infested *M. persicae* (from massal rearing). These leaves were with their petioles immersed in 100 mL containers filled with water. Every three days, new leaves of kale infested with aphids were added to the cages.

The predator *O. insidiosus* were collected from vegetable crops near the Campus of the UFV.

2.3 Experimental procedure

Four sets of bioassays were conducted. The first was a bioassay to determine the effects of *A. oleracea* extracts on the biology and survival of *M. persicae* and *L. erysimi*. The second bioassay was conducted to establish dose-mortality curves of the most active plant extract for *M. persicae* and *L. erysimi*. The third bioassay investigated the effects of the most active plant extract on the natural enemies *D. rapae* and *O. insidiosus*. In the fourth set of bioassays, two N-

isobutylamides isolated from *A. oleracea* were tested on aphids to determine their roles in the observed insecticidal activity.

2.3.1 Effect of *A. oleracea* extracts on the biology and survival of aphids

The experiments regarding the effects of *A. oleracea* extracts on *M. persicae* and *L. erysimi* were conducted on neonate nymphs. For this purpose, apterous adults gave birth on kale leaves, the petioles of which were immersed in 100-mL containers filled with water to prevent wilting. After 24 hours, adult aphids were removed, and ten newborn nymphs were kept on each leaf.

The experimental design was completely randomized with six replications. Each experimental unit consisted of a kale leaf containing ten nymphs. The kale leaves and aphids were sprayed until run-off with the *A. oleracea* extracts. In a control experiment with the same conditions, plants and insects were sprayed with deionized water. The leaves were stored in a climate cabinet at 25 ± 1°C, 65 ± 5% relative humidity (RH), and 12 hours photophase. Observations regarding aphid survival, development and adult fecundity were taken at 12 hours intervals.

2.3.2 Time–mortality bioassays

The first instar nymphs of each species were subjected to time–mortality bioassays with the *A. oleracea* ethanol extract. This experiment was conducted because of the high mortality rates caused by this extract. We chose the probit analysis to complement the analysis of survival and to confirm the results because it does not depend on time. The experimental unit consisted of a kale leaf containing ten nymphs. Each leaf had its petiole immersed in a 100-mL container of water. Six independent replicates were used for each combination of insect species, extracts (or water) and length of exposure. The kale leaves and aphids were sprayed until run-off with the *A. oleracea* ethanol extract. In the

control treatment, plants and insects were sprayed with deionized water in a procedure identical to that of the extract application. The leaves containing insects were placed in a climate cabinet at 25 ± 1°C, 65 ± 5% RH and 12 hours photophase. Mortality assessments were conducted at regular and independent exposure intervals (i.e., with separate replicates at each time interval) that had been pre-established after preliminary tests.

2.3.3 Effect of *A. oleracea* extract on natural enemies

These bioassays were performed using *D. rapae* and *O. insidiosus* adults. The experimental design was completely randomized with six replications. Each experimental unit consisted of a kale leaf containing ten insects. The leaves were sprayed until run-off with the *A. oleracea* ethanol extract or deionized water. We assessed only the ethanol extract of *A. oleracea* because it was the only one that showed high insecticidal activity against the aphids.

Treated leaves were dried at room temperature for 2 hours and placed on the bottom of glass Petri dishes (90 x 20 mm). The Petri dishes were covered with organza, which was tied with a rubber band to prevent insects from escaping. Adults were transferred to Petri dishes using aspirators. The Petri dishes were maintained at 25 ± 1°C and a relative humidity of 65 ± 5%. Mortality, defined as immobility upon stimulation with a fine camel-hair brush, was recorded 12, 24 and 48 hours after treatment.

2.3.4 Bioassay with compounds from *A. oleracea*

This bioassay was conducted to evaluate the insecticidal activity of compounds in *A. oleracea* against the aphids and to determine if the isolated compounds were responsible for the insecticidal activity of the *A. oleracea* extracts. This test was performed only with the aphid *L. erysimi* because the small size of the *M.*

persicae nymphs did not allow topical applications, which were necessary because only a small amount of the compounds was used.

To isolate compounds from *A. oleracea*, 2.00 kg of fresh *A. oleracea* material was processed for extraction. The extract obtained was concentrated under a low pressure and reduced temperature (<50°C). Three compounds were isolated by fractioning the *A. oleracea* extract with silica gel 60 (70-230 Mesh) column chromatography. Hexane, with increasing portions of ethyl acetate and methanol, was used as the eluent. Thin layer chromatography (TLC, Silica gel 60 F254 0.25 mm) was used to identify fractions containing similar compounds.

The experimental design was completely randomized with six replications. Each experimental unit consisted of a glass Petri dish (9.5 cm x 2.0 cm) containing ten insects. The average weight of insects was obtained by measuring, on an analytical balance, the mass of ten groups containing 10 insects each.

Bioassays were conducted by topical application. For each individual insect, 0.5 μL of a solution of the test compound dissolved in acetone at doses of 1 and 5 μg of compound per mg of insect was applied via a 10-μl Hamilton micro syringe. In the control experiment, which was conducted under the same conditions, 0.5 μL of acetone was applied to each insect.

After application, the insects were kept in individual Petri dishes containing disks of kale leaves as food. The Petri dishes were placed in an incubator at 25 ± 0.5 °C, 65 ± 5% RH and 12 hours photophase. The mortality counts were made after 12, 24 and 48 h, and they included dead individuals and those that did not move.

2.4 Statistical analyses

The data on aphid survival were subjected to a survival analysis using the non-parametric procedure LIFETEST and by stratifying the survival differences among insecticides (SAS Institute, 2001). This procedure allows the estimation of survival curves obtained through Kaplan–Meier estimators generated from the proportion of aphids that survive from the beginning until the end of an experiment. Data of nymphal duration and adult fecundity were analyzed by analysis of variance (ANOVA), and the averages were compared with the Tukey test at $P < 0.05$ using the SAEG software (SAEG, 2001). The data were not transformed prior to analysis.

Time-mortality data were subjected to probit analysis using the SAS software (PROC PROBIT; SAS) to obtain times for 50% (LT_{50}) and 95% (LT_{95}) mortality. The selectivity ratio for each insecticide was obtained by dividing the LT_{50} or LT_{95} value of *M. persicae* by the corresponding LT estimate for *L. erysimi*. The 95% confidence limits of these estimates were calculated, and the LT values were considered to be significantly different ($P < 0.05$) if the confidence limits on the selectivity ratio did not include the value 1.

Mortality data of natural enemies were subjected to ANOVA tests at $p > 0.05$. Data of aphid mortality were subjected to ANOVA and the averages were compared by the Scott-Knott test ($P < 0.05$) using the SAEG software (SAEG, 2001).

3 Results

3.1 Survival and biological characteristics of aphids

The survival analysis of aphids exposed to *A. oleracea* extracts indicated significant differences among treatments for both *M. persicae* (Log-rank test, χ^2 = 104.51, d.f. = 2, P < 0.0001) and *L. erysimi* (Log-rank test, χ^2 = 138.79, d.f. = 2, P < 0.0001). Survival rates were above 75% for insects not exposed to insecticides, while the *A. oleracea* ethanol extract led to a 90% mortality rate in both natural enemies in less than 70 h. The *A. oleracea* aqueous extract led to an 80% mortality rate in *L. erysimi* and a 60% mortality rate in *M. persicae*, but these effects only occurred after more than 200 h (Fig. 1). Such differences were reflected in the mean survival times (LT_{50}) observed for each extract, with the aqueous extract leading to higher LT_{50} values (Fig. 2). The mean survival time was not estimated for the control treatment because of the low observed mortality rate (<25%).

The effects of extract application on the duration of nymphal development was not significant in comparison with the control treatment for *M. persicae* and *L. erysimi*. The average nymphal durations of *M. persicae* and *L. erysimi* were 6.8 ± 0.2 (SE) days and 6.2 ± 0.12 (SE) days, respectively. In contrast, the effects of the *A. oleracea* extracts on the fecundity of *M. persicae* and *L. erysimi* were significant (Fig. 3). The fecundity (number of nymphs per female) of *M. persicae* and *L. erysimi* was reduced when the ethanol extract of *A. oleracea* was applied. On average, compared to the control, the ethanol extract reduced the fecundity by 46 and 65% in *M. persicae* and *L. erysimi*, respectively. Conversely, the aqueous extract only reduced the fecundity of *L. erysimi* by 36% and did not affect the fecundity of *M. persicae* (Fig. 3).

3.2 Time–mortality responses: extract toxicity

Due to the high mortality of nymphs caused by the ethanol extract of *A. oleracea*, probit bioassays were conducted. The time–mortality results from exposing nymphs of the two aphid species to the extract show low χ^2 and high *P* values (< 7.7 and > 0.3, respectively), indicating the suitability of the probit model for fitting the time–response curves and consequently obtaining estimates of the mortality parameters LT_{50} and LT_{95} (Table 1).

The ethanol extract caused more rapid mortality (LT_{95} = 24.9 h) in *L. erysimi* than in *M. persicae* (LT_{95} = 57 h). Thus, *M. persicae* (2.69× at LT_{50}) was slightly more tolerant to the extract than *L. erysimi* (2.30× at LT_{95}) (Table 1). Furthermore, the time–mortality response curves for *M. persicae* had gentler slopes than those for *L. erysimi*, indicating more heterogeneous responses to insecticides among individuals of the former species (Table 1).

3.3 Effect of *A. oleracea* ethanol extract on *D. rapae* and *O. insidiosus*

The *A. oleracea* ethanol extract did not significantly affect the mortality of the parasitoid *D. rapae* (F = 2.5, d.f. = 1, P = 0.14493) or the predator *O. insidiosus* (F = 1.0, d.f. = 1, P = 0.34089). The mortality caused by the *A. oleracea* ethanol extract was equal to the mortality caused by the control group (not exposed to extract) at all evaluation times (Table 2).

3.4 Toxicity of compounds from *A. oleracea*

To obtain bioactive compounds, the *A. oleracea* extract was fractionated by a bioactivity-guided fractionation approach, and eight fractions, A-H, were obtained. *Three alkamides were* isolated from the active fractions. Compounds **1** and **2** were isolated from fraction F eluted with hexane-ethyl acetate (8:l). Compound **3** was isolated from fraction G eluted with hexane-ethyl acetate (6:l).

Compound **1** (320 mg) was isolated as a colorless oil. This compound was identified by its ^{1}H NMR and ^{13}C NMR spectra and confirmed as

(*2E,6Z,8E*)-*deca-2,6,8-trienoic acid N-isobutyl amide or spilanthol* (Fig. 4). All spectral data were in agreement with those of spilanthol (**1**) in the literature (Nakatani and Nagashima 1992). The ^{13}C NMR ($CDCl_3$) and the ^{1}H NMR ($CDCl_3$) spectra indicated spilanthic acid (deca-2*E*,6*Z*,8*E*-trienoic acid). Regarding the amine moiety, the typical signals at δ 3.15 (2H, t, H-1'), 1.78 (1H, m, H-2'), and 0.93 (6H, d, H-3', 4') in the ^{1}H NMR and δ 46.9 (C-1'), 28.6 (C-2') and 20.1 (C-3',4') in the ^{13}C NMR indicated the presence of an isobutylamino group.

Compound **2** was isolated as a colorless crystal. In the ^{1}H NMR spectra, characteristic signals at δ 3.18 (dd, H-l'), 1.80 (m, H-2') and 0.93 (d, H-3' and H-4') indicated the isobutylamide moiety. This compound was identified as undeca-2*E*-en-8,10-diynoic acid isobutylamide (Fig. 4) by comparing its ^{1}H NMR spectral data (Table 3) with published values (Bauer et al. 1989). The ^{13}C NMR spectrum was consistent with published data (Ramsewak et al. 1999*).*

Compound **3**, (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (Fig. 4), was isolated as a colorless oil. The IR spectrum presented absorption bands attributable to a triple bond (2225 cm^{-1}), a secondary amide group (3299, 1627 and 1554 cm^{-1}) and a double bond conjugated with an amide carbonyl group (1669 cm^{-1}). The ^{1}H NMR spectrum revealed signals at δ 5.77 (d, J = 15 Hz) and 6.80 (dt, J = 15 Hz and 7 Hz), which have been attributed to olefinic protons H-2 and H-3, respectively. Regarding the amine moiety, a pair of ^{1}H ddq signals at δ 1.17 and 1.41 were attributed to methylene protons of C-3', and a pair of ddd signals at δ 3.14 and 3.27 were attributed to methylene protons of C-1' due to the presence of asymmetric carbon at C-2'. The ^{13}C NMR spectrum showed 16 carbon signals. Five carbon signals at δ 45.2, 35.1, 27.1, 11.3 and 17.2 confirmed a 2-methylbutylamine moiety. The ^{13}C NMR and ^{1}H NMR signals correspond well with the literature (Nakatani and Nagashima 1992).

The bioassay with the three identified compounds showed significant insecticide activity for all compounds against *L. erysimi.* The effectiveness against *L. erysimi* differed among the compounds ($P < 0.05$). This difference was most evident at a dose of 1 μg mg^{-1} body mass (Table 3).

When alkamides were applied at a dose of 1 μg mg^{-1}, compound **1** caused the highest mortality rates at all evaluation times, with mean values exceeding 70% 12 and 24 hours after application and 80% 48 hours after application. Compounds **2** and **3** were significantly less effective than compound **1** at all three evaluation times (Table 3).

At a dose of 5 μg mg^{-1} body mass, compounds **1**, **2** and **3** caused 100% mortality 12 hours after application (Table 3).

4 Discussion

This study identified a bioactive extract that was effective against *M. persicae* and *L. erysimi*. The ethanol extract of *A. oleracea* effectively controlled all aphid species tested and may be an alternative for the control of these insect pests, especially in organic farming systems in which synthetic pesticides are prohibited.

The ethanol extract of *A. oleracea* caused high mortality rates in both *M. persicae* and *L. erysimi*, indicating acute toxicity against aphids. Furthermore, application of this extract significantly reduced the fecundity of two species of aphids, which could substantially lower of the number of aphids. Significant adverse effects of plant extracts on aphid fecundity have been reported with the use of nettle extract on *M. persicae* (Gaspari at al. 2007) and the use of neem extract on the aphids *Brevicoryne brassicae* (L.) (Opender 1998) and *Toxoptera citricida* (Kirklady) (Tang et al 2002).

The aqueous extract showed significantly higher mortality rates than the control, but it only produced high mortality levels (80% to *L. erysimi* and 60% to

M. persicae) above 200 hours after treatment. This extract did not substantially affect the fecundity of aphids. The low activity of the aqueous extract was explained by the small amount of bioactive compounds in this extract. Earlier biochemical analyses of the *Acmella* spp. revealed that the majority of secondary metabolites in these plants consist of a group of alkamides and their respective isomers (Ramsewak et al. 1999; Crouch et al. 2005; Ley et al. 2006). Based on the chemical nature of alkamides, both polar (methanol, ethanol and chloroform) and non-polar solvents (hexane, petroleum ether and diethyl-ether) have been used for extraction and isolation. However, these alkamides were practically insoluble in water, and consequently, water is not likely to efficiently extract the bioactive alkamides.

Based on the high mortality rates caused by the ethanol extract of *A. oleracea*, time–mortality bioassays were conducted. The results clearly show that the ethanol extract was effective against *M. persicae* and *L. erysimi*. These aphid species exhibited differential tolerances to the ethanol extract, although the magnitude of the differences was moderate. *M. persicae* was slightly more tolerant than *L. erysimi*. In addition, *M. persicae* consistently exhibited heterogeneous responses to various insecticides, suggesting a higher individual variability and, therefore, a higher risk of selection for insecticide resistance than in *L. erysimi*. The higher tolerance of *M. persicae* to ethanol extract of *A. oleracea* may be due to its generalist feeding habits, which exposes the species to a larger number of plant secondary metabolites. As a result, this species showed more tolerance to the various compounds than the monophagous and oligophagous species. Insects' ability to overcome toxic allelochemicals is involved in ecological discrimination and diversification (Berenbaum 2002). Such a key adaptation is mediated through various detoxifying mechanisms and coupled with processes to excrete allelochemicals; this enables radiation and the colonization of novel ecological niches by tolerant taxa (Johnson 1999).

The toxicity of the ethanol extract to natural aphid enemies was also evaluated. The *A. oleracea* ethanol extract, prepared in the same concentration as that used for aphids, was not toxic to the aphid parasitoid *D. rapae* or the predator *O. insidiosus*. Therefore, this extract could be used in integrated pest management (IPM) systems and in organic agriculture to manage *M. persicae* and *L. erysimi* populations because it is effective against aphids and is relatively nontoxic to natural enemies. Our results provide practical information to improve IPM systems in brassicas through the use of botanical insecticides. Conservation biological control is an important component of integrated pest management and can be achieved with the use of selective insecticides, thus enabling the integration of chemical and biological methods to suppress pest populations in agricultural systems. The availability of new alternatives that control *M. persicae* and *L. erysimi* and are selective to natural enemies is important for the development of sound IPM systems in brassica crops.

As mentioned, previous studies have shown that the bioactivity of *A. oleracea* is due to the presence of alkamides (Gokhale at al. 1945; Ramsewak et al. 1999). In this study, three alkamides were isolated from an *A. oleracea* extract and tested on *L. erysimi*. The results show that all of the isolated alkamides had high insecticidal activity against *L. erysimi*, thus proving the bioactivity of the isolated alkamides and indicating that they contribute to the observed activity of the extract. Some studies have shown that in addition to the alkamides isolated in this study, *A. oleracea* has other active alkamides (Nakatani and Nagashima 1992; Ramsewak et al. 1999); however, spilanthol is considered to be the main active alkamide of this plant, which is consistent with our results.

In addition to the possibility of developing commercial formulations based on extracts of *A. oleracea*, the ease of preparation of the ethanol extract will allow farmers to perform the extraction process on their farms at low cost,

which is important for the sustainability of agricultural systems. The bioactive alkamides could also be used as lead compounds in the generation of novel pesticides such as pyrethroids (synthetic derivatives of pyrethrins) and neonicotinoids (synthetic derivatives of nicotine).

In conclusion, the ethanol extract of *A. oleracea* could be used as a botanical insecticide in IPM programs. The observed effects show that *A. oleracea* is a source of biologically active compounds, such as alkamides, that are potentially efficient insecticides. Because *A. oleracea* is eaten by humans and provides a source of natural compounds for various pharmacological activities, its bioactive compounds are expected to be of low or no hazard to human beings or other animals. Consequently, the possibility of using these natural products to control insects in crops should be investigated further.

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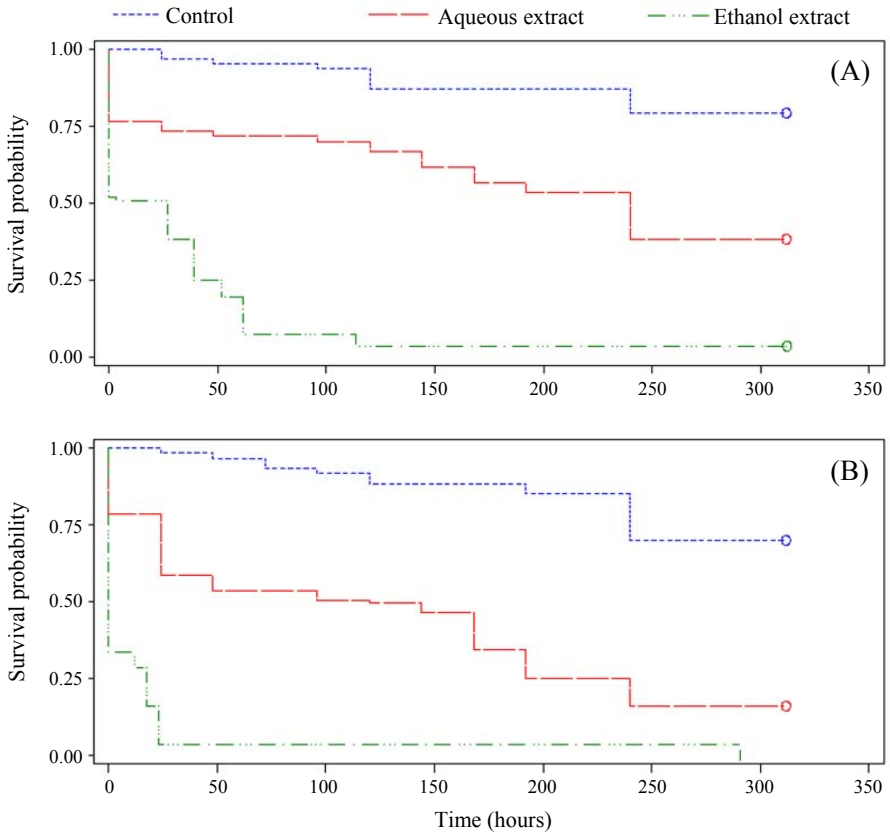

Fig. 1. Estimated survival curves (product-limit survivor function estimates) of two aphid species, *Mysus persicae* (A) and *Lipaphis erysimi* (B), exposed to *Acmella oleracea* aqueous (long-dashed line) and ethanol (dot-dashed line) extracts, and to deionized water (dotted line).

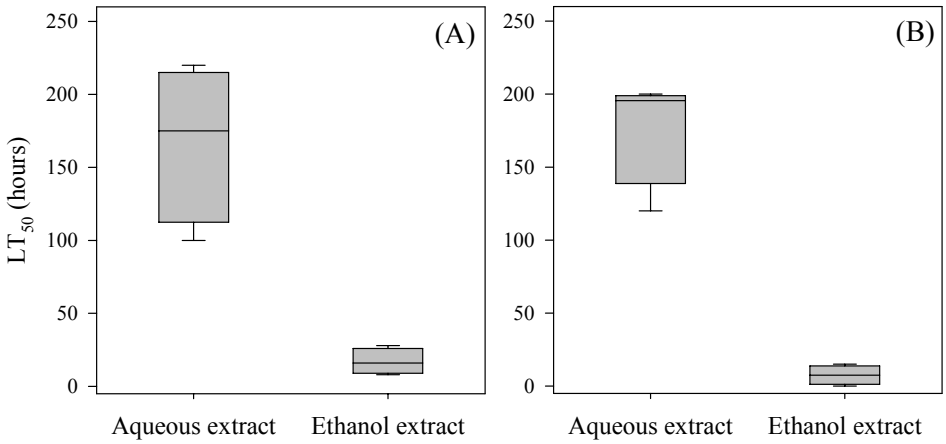

Fig. 2. Mean survival time of larvae of two aphid species, *Myzus persicae* (A) and *Lipaphis erysimi* (B), exposed to *Acmella oleracea* aqueous and ethanol extracts. The box plots indicate the median and dispersion (lower and upper quartiles) of the mean survival times (LT_{50}).

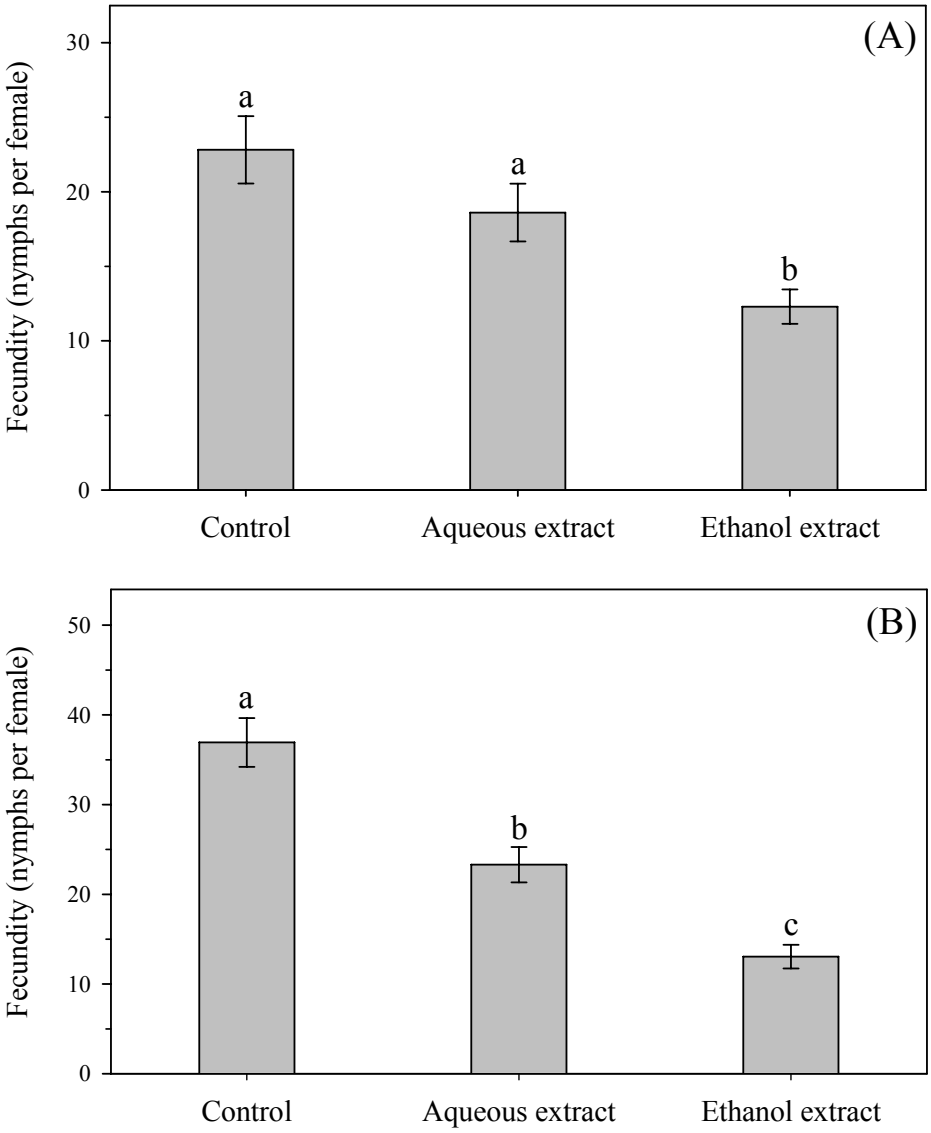

Fig. 3. Fecundity (nymphs per female) (mean ± SE) of *Myzus persicae* (A) and *Lipaphis erysimi* (B) on kale plants when sprayed with the *Acmella oleracea* aqueous and ethanol extracts and with deionized water (control treatment), at 25°C. Brackets indicate Standard Error. Histogram bars with the same letter do not differ significantly by Tukey test ($P < 0.05$).

Table 1. Susceptibilities of two aphid species, *Myzus persicae* and *Lipaphis erysimi*, to ethanol extract of *Acmella oleracea*

Aphids	N[a]	Slope ± SE	LT_{50} (h) (95% FL)[b]	Differential selectivity ratio (95% CL)	LT_{95} (h) (95% FL)[b]	Differential selectivity ratio (95% CL)	χ^2	*P*
M. persicae	420	0.20 ± 0.04	8.85 (5.30 - 12.40)	2.69	57.05 (45.58 - 68.05)	2.30	7.606	0.302
L. erysimi	420	0.33 ± 0.03	3.29 (0.41 - 8.32)		24.85 (18.01 - 36.23)		6.530	0.450

[a] Number of insects tested.
[b] Lethal time with 95% fiducial limits (FL).

Table 2. Contact toxicity of *Acmella oleracea* ethanol extract to the natural enemies *Diaeretiella rapae* and *Orius insidiosus*

Teatments	Mean percent mortality*			
	12-hour exposure	24-hour exposure	48-hour exposure	72-hour exposure
		Diaeretiella rapae		
A. oleracea ethanol extract	3.33 ± 2.11 a	3.33 ± 2.11 a	3.33 ± 2.11 a	3.33 ± 2.11 a
Control	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
		Orius insidiosus		
A. oleracea ethanol extract	1.67 ± 1.67 a	1.67 ± 1.67 a	1.67 ± 1.67 a	1.67 ± 1.67 a
Control[§]	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a

*Means followed by the same lower-case letter in a column are not significantly different by the F-test at $P > 0.05$. [§] Only water was used in the control.

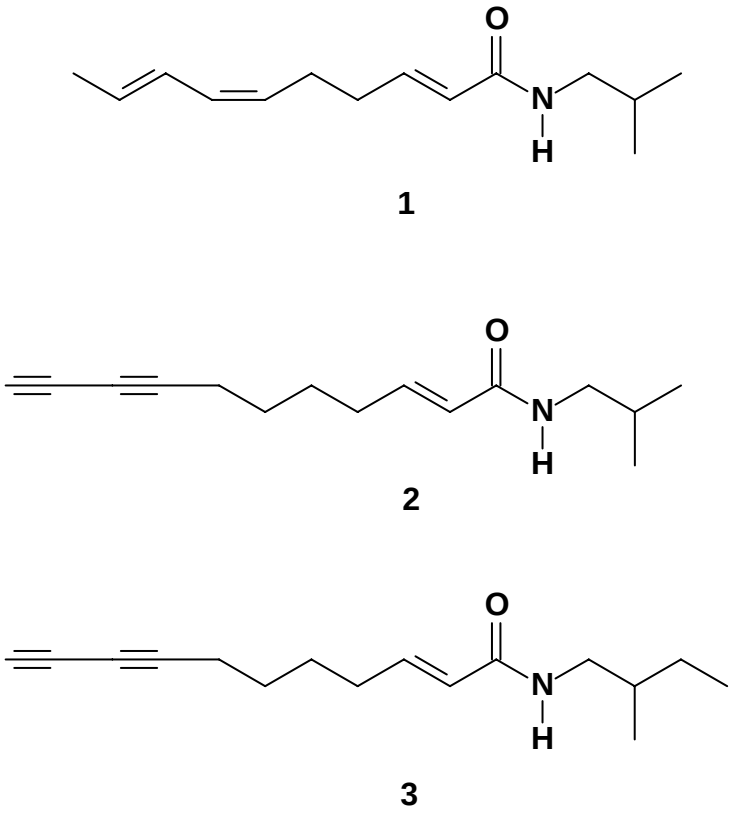

Fig. 4. Structure of the three alkamides isolated from *Acmella oleracea*: spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8.10-diynamide (**3**).

Table 3. Contact toxicity of Spilanthol (**1**), undeca-2*E*-en-8,10-diynoic acid isobutylamide (**2**) and (2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide (**3**) to *Lipaphis erysimi* at concentrations of 1 and 5 μg of compound per mg of insect, 12, 24 and 48 hours after application

Treatment	Mean percent mortality*					
	12 h after topical application		24 h after topical application		48 h after topical application	
	1 μg mg $^{-1}$	5 μg mg $^{-1}$	1 μg mg $^{-1}$	5 μg mg $^{-1}$	1 μg mg $^{-1}$	5 μg mg $^{-1}$
1	75.0 ± 6.19 a	100.0 ± 0.0 a	76.67 ± 6.15 a	100.0 ± 0.0 a	83.33 ± 3.42 a	100.0 ± 0.0 a
2	60.0 ± 5.77 b	100.0 ± 0.0 a	63.33 ± 3.33 b	100.0 ± 0.0 a	68.33 ± 4.01 b	100.0 ± 0.0 a
3	36.67 ± 4.22 c	100.0 ± 0.0 a	51.67 ± 4.01 c	100.0 ± 0.0 a	55.0 ± 3.42 c	100.0 ± 0.0 a
Control[§]	0.00 ± 0.00 d	0.00 ± 0.00 c	1.67 ± 1.67 d	1.67 ± 1.67 b	3.33 ± 2.11 d	3.33 ± 2.11 b

***** Means followed by the same lower-case in a column are not significantly different by the Scott-Knott groupment analysis test at $P > 0.05$. [§] Only ketone was used in the control.