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DISCOVERY, ISOLATION AND CHARACTERIZATION OF NOVEL BIOMOLECULES WITH NEMATICIDAL ACTIVITY

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

Degree of

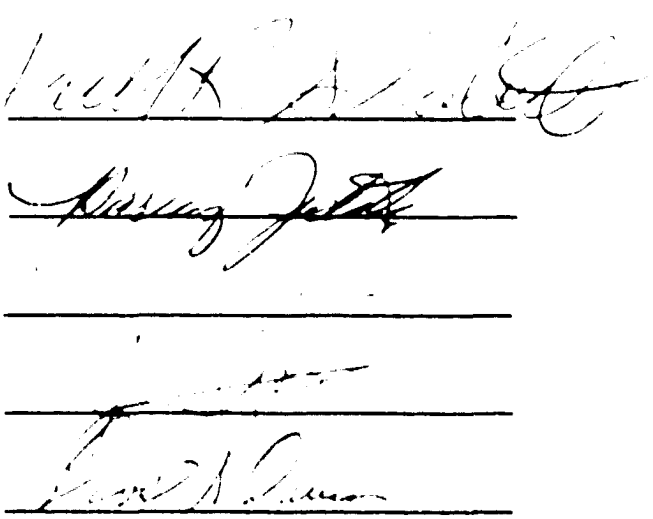
Doctor of Philosophy

By
LILY GAVILANO
Norman, Oklahoma
2000

DISCOVERY, ISOLATION AND CHARACTERIZATION OF NOVEL BIOMOLECULES WITH NEMATICIDAL ACTIVITY

A Dissertation APPROVED FOR THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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List of Abbreviations

BAPNA	N-benzoyl-DL-arginine p-nitroanilide
BBI	Bowman-Birk inhibitor
BCA	Bicinchoninic acid
β-ME	Beta-mercaptoethanol
BSA	Bovine serum albumin
BTNA	N-benzoyl-L-tyrosine p-nitroanilide
CBB	Coomassie Brilliant Blue
CTI	*Cassia* trypsin inhibitor
Da	Dalton
DMSO	Dimethyl sulfoxide
ECB	European corn borer
EDTA	Ethylene diamine tetraacetate
FMN	Flavin mononucleotide
FPLC	Fast protein liquid chromatography
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing electrophoresis
KDa	Kilodalton
LMW	Low molecular weight
MALDI-TOF	Matrix assisted laser desorption ionization time-of-flight
MTI	*Mucuna* trypsin inhibitor
MWCO	Molecular weight cutoff
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PVP	Polyvinylpyrrolidone
RBC	Red blood cells
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAC	Trypsin affinity chromatography
TEMED	N-tetramethylethylenediamine
TI	Trypsin inhibitor
TLC	Thin layer chromatography
TLCK	Tosyl lysyl chloromethyl ketone
TPCK	Tosyl phenylalanyl chloromethyl ketone

ABSTRACT

The purpose of this research was to discover novel biological substances with nematicidal activity. Plants contain a variety of bioactive natural products. Many of them may play a role in plant defense. Plants also contain bioactive proteins involved in defense. Two groups of plants were tested for nematicidal activity. The nematicidal activity found in the first group of plants was attributed to the presence of secondary metabolites, while the activity found in the second group was apparently due to the presence of active proteins. Aqueous extracts of *Solidago missouriensis*, *Ambrosia psilostachya* and *Lespedeza stuevei*, native plants of the United States, were tested for activity against *M. incognita* juveniles. *Solidago* and *Ambrosia* reduced gall formation in tomato infected with *M. incognita* in greenhouse experiments. In laboratory experiments, extracts of *Solidago* exerted a nematistatic effect. *Lespedeza* inhibited nematode motility temporarily. Of the three, only *A. psilostachya* crude extract exhibited nematicidal activity, causing 100% mortality at a concentration of 20 mg/ml. Bioactivity-directed TLC fractionation of *Ambrosia* extract resulted in the isolation of three nematicidal secondary metabolites. The structures of two of these compounds, parthenin and coronopilin, were determined by NMR and mass spectrometry. Parthenin and coronopilin, are known

sesquiterpenes and these compounds were reported previously to possess antitumor, antibacterial, antifungal, molluscicidal and insect antifeedant activities. However, this is the first report of their nematicidal activity. We proposed a model for the mode-of-action of parthenin in which the parthenin reacts with essential sulfhydryl groups of proteins. To test this hypothesis, parthenin was mixed with cysteine and then tested for nematicidal activity. Indeed, the nematicidal effect of parthenin was reduced 95% by mixing the compound with equimolar concentrations of cysteine. This suggests that the lethal effect of parthenin on nematodes is due to reaction with sulfhydryl groups present in essential proteins.

Bioactive defense-related proteins include proteins such as protease inhibitors. Trypsin inhibitors have exhibited antiinsect, antifungal and nematicidal activities. The gene for trypsin inhibitor may protect plants from different pests and pathogens when expressed in transgenic plants. For this reason, we are involved in the isolation of novel trypsin inhibitors. Earlier studies indicated that seeds of *Cassia fruticosa* and *Mucuna holdii*, two tropical species, possess trypsin inhibitory activity. Trypsin inhibitors were isolated from both species by affinity chromatography. Isoforms of trypsin inhibitors were further purified by anion exchange chromatography.

Three trypsin inhibitors were purified from *Cassia*. Five trypsin inhibitors were detected in *Mucuna* seed extract and these inhibitors exhibited high thermostability. Based on SDS-PAGE, the molecular weight of the purified trypsin inhibitors from *Cassia* was 17, 700 Da and inhibitors from *Mucuna* were found to possess a molecular weight around 9,000 Da. Their molecular weight was also determined by MALDI-TOF laser mass spectrometry. MALDI-TOF mass analysis indicated that the molecular weights of *Cassia* and *Mucuna* inhibitors were 21,500 and 8,000 Da, respectively. Bioassays of trypsin inhibitors using a *Caenorhabditis elegans* feeding bioassay indicated that the trypsin inhibitors from both *Cassia* and *Mucuna* were capable of causing nematode mortality under the conditions of the bioassay.

Both, sesquiterpene lactones isolated from *Ambrosia* and trypsin inhibitors isolated from *Cassia* and from *Mucuna* are new potential tools to engineer transgenic plants with resistance to plant parasitic nematodes.

CHAPTER I : Introduction

Biotechnology has been practiced successfully to solve several problems in agriculture in the last decade. However, control of pests like nematodes and insects is still one of the major problems facing agriculture today. Parasitic nematodes and insects spread plant diseases and destroy entire crops in association with bacteria, viruses and fungi. Nematodes are distributed worldwide (Platt, 1994; Sasser and Freckman, 1987) and they can parasitize all living organisms including humans (Daub *et al.*, 2000), livestock (Emery *et al.*, 1993), insects (Garcia del Pino, 1996), plants (Trudgill, 1997) and other organisms.

Plant-parasitic nematodes are potentially serious pests of crops that are important for human nutrition such as wheat (Johnson *et al.*, 2000), beans (Saka, 1990) and potato (Gonzalez and Phillips, 1996). Nematodes are a major problem worldwide. Many species of nematodes can infect plants but the most damaging ones are those which are highly specialized, with limited host ranges. *Meloidogyne*, root-knot nematode, is one of the most devastating species. Even though a few *Meloidogyne* species have a broad host range (*M. incognita* and *M. javanica*), most species of *Meloidogyne*

are highly specific. For example, *Meloidogyne pinus* is restricted to *Pinus* spp. (Trudgill, 1997). Like fungal pathogens and insects the most damaging species of nematodes, such as *Meloidogyne*, exist as more than one race or pathotype and have genes for virulence which are matched by genes for resistance in host species (Janssen *et al.*, 1991).

Plant parasitic nematodes cause an estimated 100 billion dollars (Taylor and Sasser, 1978) in crop losses annually worldwide. Phytoparasitic nematodes are especially important in the developing nations (Greco and Crozzoli, 1995; Doucet and De Doucet, 1997) where crop yields may be severely reduced by nematode attack. Nematodes infest the staple crops such as corn, soybean and rice. The earth's population depends upon these crops as their primary sources of food, fiber and economic well-being. Many phytoparasitic nematodes can be effectively controlled using fumigant and non-fumigant chemical nematicides (Evans, 1973). The high cost of applying chemical pesticides, however, prohibits their widespread use on crops around the world, especially in developing nations (Bourne, 1999). In addition, chemical nematicides may have potentially hazardous effects on the environment and humanity causing acute and chronic health

risks through direct exposure, contamination of ground water sources, air pollution and food residues contaminated by chemicals.

For this reason, alternative strategies for nematode control are sought. In order to combat parasitic nematodes, farmers around the world are effectively using chemical pesticides. However, chemical pesticides commonly used have a dramatic effect on non-target organisms, killing natural enemies of pests and pathogens. Many pests and pathogens are developing resistance to chemical control. Nematode recovery is possible after short exposure to concentrations as high as 1000 ppm of ethoprophos (Brown, 1987). As a result, the amount of pesticides used has to be increased forming a vicious circle – the farmers increase the amount of pesticides applied producing more resistant insects and plant-parasitic nematodes that will require the application of more and more chemical pesticides and, thereby, increase the cost of pest control. Pesticides are absorbed by soil and carried away by underwater flows contaminating the environment (Jones and Back, 1983). Fumigants such as methyl bromide reduce the ozone layer and could contribute to the greenhouse effect that is affecting the earth's weather (Karliner *et al.*, 1997). Many of the chemicals that are currently used are under review by the Environmental Protection

Agency due to their toxicity. Additional research is needed to replace traditional chemical pesticides with safer pesticides.

Biological control represents one of the alternatives to solve this problem. Biological control uses natural enemies or their products to eliminate a target organism. This method plays an important role in assuring a safe and affordable food supply for the future. There is a need however to identify new biocontrol agents. Newer concepts of biological control include the use of compounds isolated from various plants and microorganisms including bacteria and fungi that inhibit growth and development of pests and pathogens. Crude extracts of these materials are suitable for use in low input agricultural systems found in many of the developing nations.

Plants are a huge source of active substances that can be used as biological agents to control pests and pathogens. Several of these substances have been used successfully to control nematodes (Bauske *et al.*, 1994). Besides nematicidal and insecticidal properties, many biopesticides exhibit anticarcinogenic effects (Mosin *et al.*, 1999). Efforts are under way to combat plant parasitic nematodes using biological control agents. Because of the high cost and potential hazards of synthetic nematicides, interest in

the development and production of safe biological nematicides has increased over the last 20 years (Feldmesser *et al.*, 1985).

The long-term goal of the present study is to understand the mode of action of active proteins, both the ones that act directly against pests and the proteins involved in biosynthesis of phytoalexins with nematicidal activity. This knowledge will allow us to develop an environmentally safe method to control parasitic nematodes. The isolation of novel active proteins will allow us to identify the genes responsible for protein expression. The identification of required genes will permit us to manipulate and transfer the gene into target crops to increase plant resistance to pests and pathogens.

Immediate objectives of this study were to:

- Examine the effects of aqueous extracts from *Lespedeza, Solidago* and *Ambrosia*, perennial herbs found in Norman, OK on *M. incognita* juveniles under greenhouse and laboratory conditions.
- Test extracts of the tropical plants *Mucuna holdii* and *Cassia fruticosa* for trypsin inhibitory and nematicidal activity.

- Isolate and characterize the molecules with nematicidal activity from plant extracts.

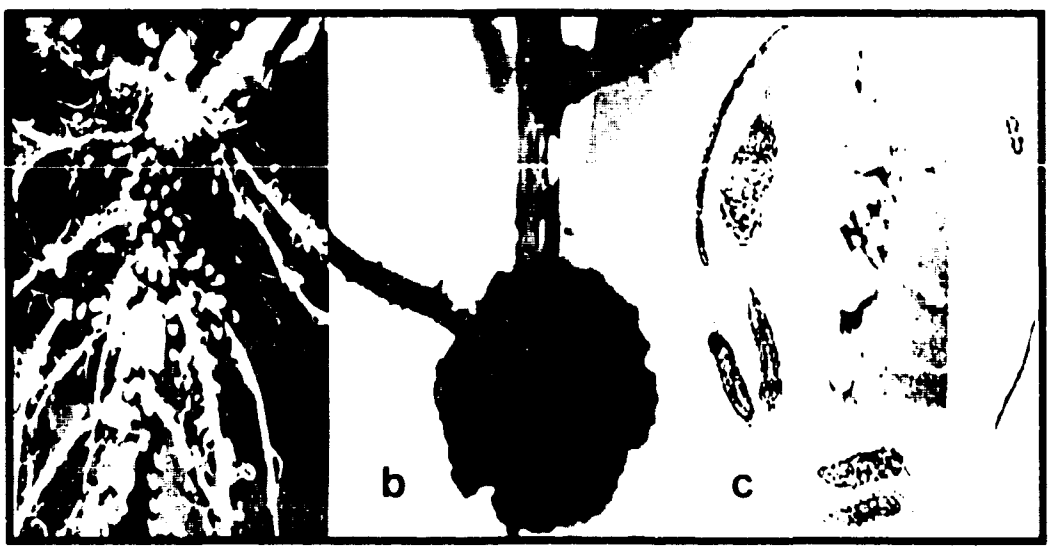
1.1 Pest Problems

Plant parasitic nematodes and insects are among the most important problems in agriculture production, causing 40 % losses in crop yields (Chavez, 1996). Attack by insects and plant parasitic nematodes causes wounds which allow the entrance of bacterial, viral, and fungal pathogens increasing the loss in yield and producing plants that are commercially unacceptable (Thomas, *et al.* 1995b). For instance, European corn borer, *Ostrinia nubilalis* (Hübner), affects production of around 200 plants including *Zea mays* L. (Hedin *et al.*, 1994), the preferred host, and other important crops such as sorghum, cotton, potato, beans, tomato, pepper, etc. In the United States, this pest costs farmers more than 1 billion dollars annually in yield loss.

Significant crop losses are also caused by plant parasitic nematodes. Plant parasitic nematodes cause annual crop damage estimated at 100 billion dollars worldwide (Sasser and Freckman, 1987). The root-knot nematode, *Meloidogyne* is considered to be the most devastating plant parasitic

nematode, parasitizing more than 300 different crops worldwide. One important characteristic of *Meloidogyne* infection is root galling (Taylor and Sasser, 1978). These root galls are tumor-like deformations that seriously affect roots preventing their normal uptake of water and nutrients from the soil and, thereby, weakening the whole plant. Galls caused by root-knot nematodes are different from nodules or tumors caused by bacteria as shown in Figure 1.1.

In order to control nematodes, it is important to understand their physiology. For this reason, many scientists are investigating gene regulation and gene expression during nematode infection. When feeding, the nematode injects esophageal secretions into the plant cell, resulting in the development of specialized multinucleate feeding cells called giant cells (Hussey, 1989). Giant cells act as nutrient sinks creating a permanent feeding site for the nematode (Hussey, 1985). These giant cells undergo rapid nuclear divisions without cytokinesis, resulting in multiple, enlarged, lobed nuclei.



A N_2-fixing Root Nodules induced by *Rhizobium*

B Crown Gall caused by *Agrobacterium*

C Galls formed by *M. incognita*

Fig. 1.1. Comparison of plant roots infected by nematodes and bacteria.

Farmers use chemical nematicides effectively to control *Meloidogyne* nematodes. However, nematodes are developing resistance against these chemicals and it is necessary to increase the rates of application of nematicides, increasing at the same time the price. Furthermore, these kinds of products contaminate the environment because most of them are not easily biodegradable being a risk for human health (Arcury and Quandt, 1998). Chlorinated naphthalenes were found to cause cirrhosis (Ward *et al.*, 1996), and also a rare and fatal disease in persons subjected to occupational exposure. Dieldrin, aldrin and endrin are considered to be among the most toxic poisonous of all the synthetic pesticides in the world (Fisher, 1999).

1.2 Pest Management

Farmers use a wide range of pest control techniques such as crop rotation, pheromones, chemical pesticides, biological agents and genetically engineered plants. Traditional chemical pesticides have failed to provide long-term solutions to pest problems. Regulatory organizations such as the EPA and USDA are making efforts to replace some chemical pesticides such as methyl bromide that are reducing the ozone layer (Karliner *et al.*, 1997). Many others are under review due to their proven toxicity.

Pheromones are very effective, but the high cost of their production make them unavailable to most farmers.

A safe and effective alternative is offered by biological control using natural enemies and biological products such as secondary metabolites or active proteins that can be engineered by means of plant genetic engineering. Potential biological controls include predatory nematodes, nematophagous microorganisms, as well as plants with nematicidal or nematistatic properties. In the latter case, it is possible to control nematodes by interplanting or rotating the growth of plants producing nematicidal/nematistatic factors or by applying residues or extracts of plants expressing these factors.

Plants have evolved a broad array of defense mechanisms involved in disease resistance (Enyedi *et al.*, 1992). Several plants and their active compounds have been reported with fungicidal (Grace, 1989), bactericidal (Shelef, 1980; Huhtanen, 1980; Cuong *et al.*, 1994), insecticidal (Duke and DuCellier, 1993) and nematicidal (Jatala *et al.*, 1995) activity. Some plant extracts inhibit hatching of *Meloidogyne incognita* (Bhatti, 1988).

1.3 Preliminary Results

Jatala *et al.* (1995) reported on the nematicidal activity of a number of tropical plants from Peru active against root-knot nematode based on results of a laboratory bioassay. Based on their results, they suggested that some of the plants tested could be used as organic amendments to reduce the population of parasitic nematodes in the soil. Plant products represent a good alternative not only because they could be inexpensive but also they may be safer to the environment.

CHAPTER II : Nematicidal and Insecticidal Natural Products and Proteins

2.1 Introduction

Plants contain a variety of useful pesticidal factors active against nematodes and insects. Because of their activity against pests, some plants are being used as soil amendments with very good results (Akhtar and Alam, 1990; Akhtar and Alam, 1992; Bansode and Kurundkar, 1989; Salawu, 1992, Tiyagi *et al.*, 1991). Secondary metabolites with nematicidal activity have been isolated from several plants (See Table 2.2) offering new tools to control plant parasitic nematodes. Alternatively, if the active compound is a protein, it is possible to transfer the appropriate gene to produce this active compound in another crop of interest by means of genetic engineering. Plant secondary metabolites and plant protease inhibitors are especially emphasized as biological control alternatives in this chapter.

2.2 Bioactive Secondary Metabolites

Bioactive secondary metabolites have various important roles in plants. Many of the bioactive compounds are produced as a response to infection

or under stress conditions. Antimicrobial secondary metabolites produced by microorganisms are known as antibiotics and antibiotics of plant origin are called phytoalexins. Phytoalexins are small organic molecules that are produced by plants in response to pest and pathogen attack. They can also be produced when plants are under stress caused by abiotic agents such as heavy metals. Phytoalexins can be produced by *de novo* synthesis from a distant precursor. The production of the required phytoalexin from a distant precursor usually takes several steps. Alternatively, the phytoalexin can be released from a pre-existing phytoalexin precursor.

There are many reports about plant secondary metabolites, their identification and characterization. Several active secondary metabolites have been assayed against insects (Casida and Quistad, 1998) and microorganisms (Jawad *et al.*, 1985). However, more work is needed to find nematicidal activity. Many secondary metabolites with activity against insects and nematodes have been isolated from microorganisms and they have been well characterized. The application of secondary metabolites of bacterial or fungal origin to control insects and nematodes has been successfully applied in pilot experiments under laboratory and greenhouse

conditions. However, before they can be applied in the field there are still major problems to be solved.

Some secondary metabolites isolated from different microorganisms are presented in Table 2.1.

Table. 2.1 Secondary Metabolites from Microorganisms, Active Against Nematodes and Insects. This table is a modification of the original one published by Copping (1996).

Compound name	**Source**	**Activity***	**Reference**	
Avermectins	*Streptomyces avermitilis*	I, N	Babu	1988
Tetranactin	*Streptomyces aureus*	I	Ando *et al.*	1971
Alanosine	*Streptomyces alanosinicus*	I	Matsumoto *et al.*	1984
Milbemycin	*Streptomyces hygroscopicus*	I	Mishima	1983
Altemicidin	*Streptomyces sioyansis*	I	Takahashi *et al.*	1989
Bafilomycins	*Streptomyces* sp.	I	Hensens *et al.*	1983
Terehazolin	*Micromonospora* sp.	I	Takeuchi *et al.*	1958
Decenedioic acid	*Pleurotus oestreatus*	I, N	Kwok *et al.*	1992

* I, insect; N, nematode

Secondary metabolites may be isolated by TLC or by HPLC (Figure 2.1). Some natural products with nematicidal activity isolated from plants are shown in Table 2.2.

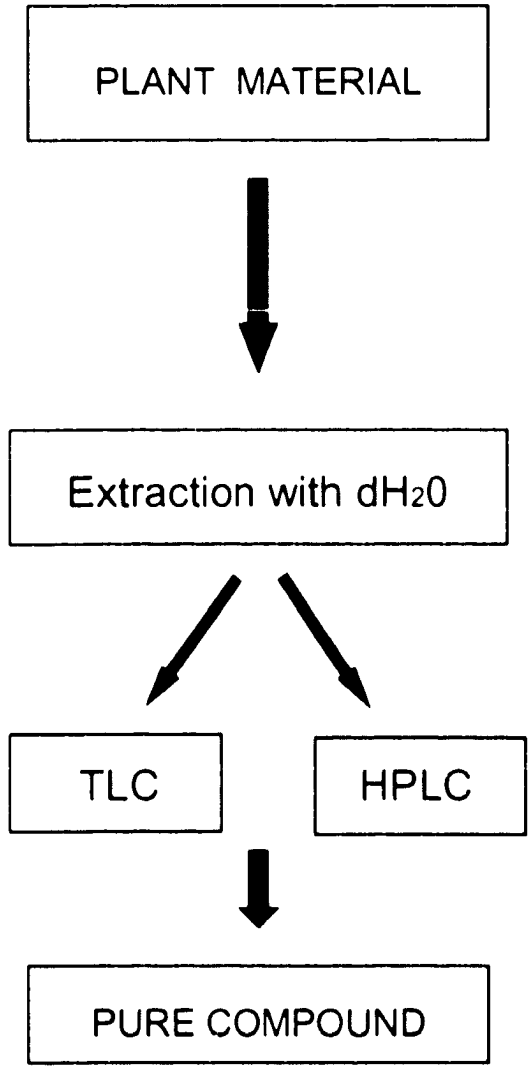


Fig. 2.1. Scheme for isolation of secondary metabolites from *Ambrosia*. Separation of active compounds was monitored using the nematode bioassay and analytical TLC.

Table 2.2. Secondary Metabolites with Nematicidal Activity Isolated from Plants.

Compound name	Source	Reference	
Acaciaside A, and B	*Acacia auriculiformis*	Roy *et al.*	1993
Asparagusic acid	*Asparagus officinalis*	Takasugi *et al.*	1975
Atropine	*Datura stramonium*	Uhlenbrock and Bijloo	1959
Bocconine	*Bocconia cordata*	Rohde	1972
Chelerythrine	*Bocconia cordata*	Rohde	1972
Cystine	*Sophora flavescens*	Matsuda *et al.*	1991
Diallyl disulphide	*Allium sativum*	Nath *et al.*	1982
Eugenol	*Ocimum sanctum*	Chatterjee *et al.*	1982
Linoleic acid	*Lawsonia inermis*	Badami and Patil	1975
Medicarpin	*Tavernniera abyssinica*	Stadler *et al.*	1994
Nicotine	*Datura stramonium*	Uhlenbrock and Bijloo	1959
Nimbidin	*Azardirachta indica*	Khan	1974
Odoracin, odoratrin	*Daphne odora*	Munakata	1978
Palmitic acid	*Lawsonia inermis*	Badami and Patil	1975
Sanguinarine	*Bocconia cordata*	Rohde	1972
Scopalamine	*Datura stramonium*	Uhlenbrock and Bijloo	1959
α-Terthienyl	*Tagetes erecta*	Uhlenbrock and Bijloo	1959
Thionimone	*Azardirachta indica*	Khan	1974
Wadelectone	*Eclipta alba*	Prasad and Rao	1979

The host defense response that involve production of isoflavonoid phytoalexins is initiated by signals called elicitors. These elicitors can be cell wall fragments or other agents of host or pathogen origin such as fatty acids (Koga *et al.*, 1998). These agents can initiate the synthesis of phytoalexins or phenolic compounds in higher plant tissues. Cooper and

Rao (1992) reported the biosynthesis of plant phenolics associated with the host responses to physiological stress factors such as light, growth regulators, nutritional deficiency, wounding and infection with various microorganisms.

Plants produce secondary metabolites with different activities such as terpenes, flavonoids, alkaloids, etc. Terpene and its derivatives are an important group of bioactive secondary metabolites. Terpenes are defined as naturally ocurring substances made up of repeating isoprene (C_5H_8) units. Several derivatives of isoprenes such as sesquiterpenes and triterpenes are phytoalexins (Fig. 2.2). Terpenes are called isoprenoids based on their isoprene precursor. According to Haagen-Smit (1948) terpenes are classified as follows:

Hemiterpenes	C_5H_8
Monoterpenes	$C_{10}H_{16}$
Sesquiterpenes	$C_{15}H_{24}$
Diterpenes	$C_{20}H_{32}$
Triterpenes	$C_{30}H_{48}$
Polyterpenes	$(C_5H_8)_n$, $(n \geq 56)$

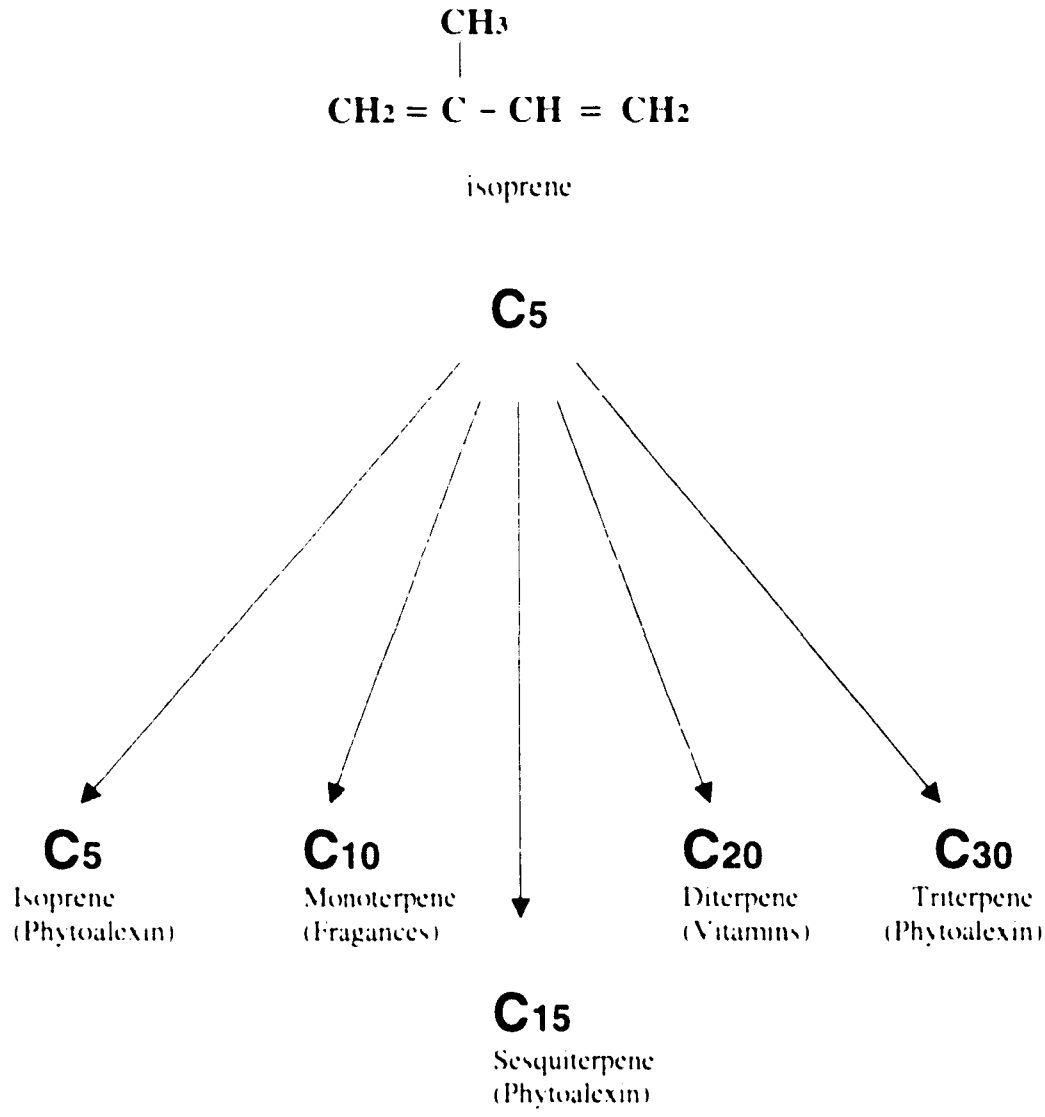


Figure 2.2 Plant Terpenoids

Sesquiterpene lactones constitute an important group of terpenes and represent one of the largest classes of plant products with over 4000 known compounds (Song *et al.*, 1995). This group is of interest because they account for the bitter principles of various herbal remedies. Many of these are reported to possess antiparasitic activity. The presence of sesquiterpenes with potential therapeutic activity was noted from observations of the behavior of sick Mahale chimpanzees (Koshimizu *et al.*, 1994). Apparently these animals "treat" parasite-related diseases by ingesting the pith of *Vernonia amygdalina*. Healthy chimpanzees usually do not consume the pith of this plant in their daily diet. After analysis of the pith of this plant Koshimizu found that the pith contained the largest amount of these so-called bitter principles. Subsequently, Koshimizu isolated two sesquiterpene lactones, vernodalin and vernonioside B, from the pith. These purified lactones exhibited a variety of activities including antiparasitic activity.

Phytoalexins produced after infection of the plants by pathogenic organisms may also be derived from flavonoids (Bailey and Mansfield, 1982; Dixon *et al.*, 1995). Isoflavonoids are commonly released as root exudates in members of the leguminosae such as soybean, alfalfa, pea and

chickpea (Grady *et al.*, 1995). Pterocarpans such as medicarpin and glyceollin are found in the roots of *Glycine* and *Medicago*. The isoflavonoid medicarpin has been reported to possess nematicidal activity (Stadler *et al.*, 1994). Isoflavonoids play various roles in plants including induction of the nodulation process in nitrogen-fixing association and prevention of pathogen infection (Edwards *et al.*, 1995). Flavonoids are commonly found in the form of O-glycosides in nature. Glycosylation makes them less reactive and more water soluble, allowing for storage of these molecules in the plant vacuole. Approximately 75% of the daidzein and genistein appear to be glycosylated (Kosslak *et al.*, 1987). Over the few last years, the production of active isoflavonoids such as medicarpin is being modified by means of molecular biology and biotechnology techniques (Dixon *et al.*,1995).

Besides terpenoids and isoflavonoids, plants also produce phenolic compounds in response to insect or nematode attack. Insects and nematodes cause wounding of the host plant. Plant wounds secrete a variety of chemicals including phenolic compounds that are recognized by some pathogens (Lee *et al.*, 1996). The recognition and attachment of signal molecules released by the host and the pathogen triggers a series of

important reactions specific in every resistance response or susceptible plant response.

Tagetes has exhibited nematicidal activity and these active principles were attributed to the accumulation of thiophenes (Bolhmann *et al.*, 1973; Sutfeld, 1982; Norton *et al.*, 1985; Ketel, 1987). Thiophene and its derivatives exhibit strong biocidal activity (Uhlenbrock and Bijloo, 1959; Chan *et al.*, 1975; Arnason *et al.*, 1986; Hudson *et al.*, 1986). Uhlenbrock and Bijloo reported that the nematicidal activity of *Tagetes* was due to alpha-terthienyl. However, it was suggested that nematicidal compounds other than alpha-terthienyl are produced in hairy root cultures of *Tagetes* induced by *Agrobacterium rhizogenes* (Kyo *et al.*, 1990).

2.3 Biologically Active Proteins

Plants produce various active proteins as a response to pest and pathogen attack such as proteinase inhibitors, hydrolytic enzymes (e.g. chitinases) and phytohemagglutinins. Most of the phytohemagglutinins are carbohydrate-containing proteins, while others may contain lipid (Rüdiger, 1998). Most of them cause clumping of red blood cells. Proteases play key

roles in the synthesis and degradation of macromolecules in every living organism and they also play an important role in the control of pests.

2.3.1 *Plant Protease Inhibitors*

Protease inhibitors play various roles in the synthesis and elimination of cell products (Annis, 1997). Inhibitors also play a role in the defense against pathogen invasion (Ryan, 1990). The presence of protease inhibitors have been reported in bacteria (Mahadik *et al.*, 1972), yeast (Cabib and Farkas, 1971), and many higher plants and animals (Vogel *et al.*, 1968). Protease inhibitors usually do not clump human red blood cells (Janzen *et al.*, 1986).

Chen (1998) reviewed the structure and characteristics of plant trypsin inhibitors and classified them into seven groups: 1) Kunitz serine protease inhibitor group; 2) Bowman-Birk serine protease inhibitor group; 3) squash inhibitor group; 4) α-amylase trypsin inhibitor group; 5) potato trypsin inhibitor group I; 6) potato trypsin inhibitor group II; and 7) Brassica trypsin inhibitor group. A short summary of this classification is presented in Table 2.3.

Table 2.3 Classification and Properties of Plant Trypsin Inhibitors.

Group	Source	Size [Da]	Disulfide bridges
1. Kunitz inhibitors	Leguminosae	21,000	2
2. Bowman-Birk inhibitors	Leguminosae	8,000	7
3. Squash inhibitors	Cucurbitaceae	5,000	3
4. α-Amylase trypsin inhibitors	Gramineae	12,000	5
5. Potato trypsin inhibitors I	Solanaceae	41,000	1
6. Potato trypsin inhibitors II	Solanaceae	23,000	5
7. Brassica trypsin inhibitors	Cruciferae	7,000	4

Trypsin inhibitors have been classified into 7 groups. This table shows only some of the more relevant characteristics.

Among the plant inhibitors, Bowman-Birk inhibitors (BBI) and Kunitz inhibitors are the best characterized. BBIs are smalls proteins with molecular weights around 8,000 Da and 7 disulphide bridges. BBIs are generally acid stable and resist high temperatures (>90 °C) for several minutes. They are found mainly in the Leguminosae such as soybean, peanut, etc. The structures of some BBIs have been determined using X-ray crystallography (Suzuki *et al.*, 1987). A typical structure of a Bowman-Birk trypsin inhibitor and a synthetic analog are shown in Figure 2.3.

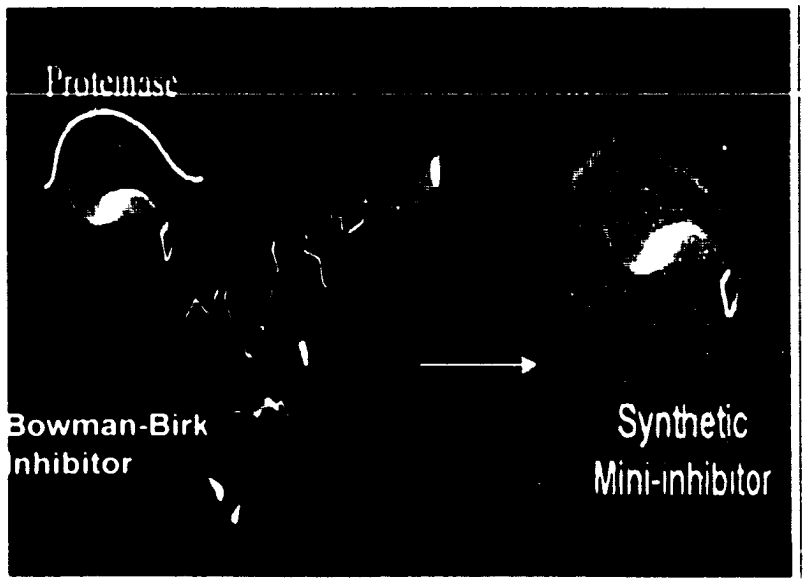


Fig. 2.3. Structure of Bowman-Birk Trypsin Inhibitor and Synthetic Inhibitor
Source: http: www.ch.ic.ac.uk leatherbarrow 1 Template.htm

Kunitz inhibitors are bigger than BBIs (Laskowski and Kato, 1980; Tanaka *et al.*, 1997) having a molecular weight of approximately 22,500 Da. These inhibitors are potent inhibitors of trypsin but they are readily destroyed by heating (Kunitz, 1945). Kunitz isolated for the first time inhibitors from soybean with the characteristics stated above and these inhibitors were called Kunitz inhibitor in honor of their discoverer. After many years, the primary structure of soybean Kunitz inhibitor was elucidated by Koide *et al.* (1973) and its three-dimensional structure was elucidated by Sweet *et al.* (1974).

2.3.2 *Physiological Importance of Protease Inhibitors*

Proteolytic enzymes present in the digestive system of insects and nematodes play a very important role in their nutrition and survival. Resistant plants produce many inhibitors of these proteases. These inhibitors may be expressed constitutively in storage organs or overexpressed during pathogen attack (Joshi *et al.*, 1998; Ryan *et al.*, 1974). The presence of these inhibitors may repel or deter attack by insects and nematodes. Usually nematodes, insects, and pathogens release proteases (Robertson *et al.*, 1999; Lam *et al.*, 2000) that damage the structure of host cells. Some plant inhibitors such as the precursor of barley

trypsin α-amylase inhibitor are synthesized on membrane-bound polysomes (Garcia-Olmedo *et al.*, 1987). This allows for rapid synthesis of inhibitors in response to pathogen infection or insect attack.

Trypsin is a protease commonly found in insects and nematodes (Dasgupta and Ganguly, 1975; Lam *et al.*, 2000). High levels of trypsin-like protease activity in gut extracts from *Spodoptera frugiperda* has been reported as well as their inactivation by barley trypsin inhibitors (Alfonso *et al.*, 1997). The accumulation of inhibitors started in the wound site caused by insects extended to other plant parts. Results of some experiments have shown that protease inhibitor induction in the tomato reduces the grazing by larvae of the armyworm *Spodoptera littoralis* after 48 hr mainly on the young leaves. Because of their physiological importance in digestion, the inhibition of trypsin and chymotrypsin may be useful in the control of insects and nematodes.

2.4 Plant Genetic Engineering

In the last two decades scientists discovered that genes can be exchanged or transferred among animals, plants, bacteria and other organisms by means of recombinant DNA techniques. Regardless of which method is used, the

general process of transferring DNA from one organism to another is called genetic engineering. Almost any desirable trait found in nature can, in principle, be transferred into any chosen organism using recombinant DNA methods.

For centuries farmers have used genetic selection of seeds from plants with resistance to pests and pathogens to use in the next year's crop. However, this traditional selection process to obtain a plant resistant to major diseases may take decades in many cases. During the last decade, the advances in biotechnology and molecular biology provide methods to introduce genetic modification in a very short period of time. These methods include microinjection, biolistic processes, pollen-mediated transformation and the so called "natural" way of DNA transfer using bacteria that colonize plants (Zambryski, 1988; Gheysen, 1987). *Agrobacterium*-mediated gene transfer has been the method of choice for introducing new economically important traits into plants such as insect resistance (Vaeck *et al.*, 1987). Petunia and tobacco (Müller *et al.*, 1987) were the first plants to be genetically transformed.

Corn (Bourguet *et al.*, 2000), cotton (Sachs *et al.*, 1998), sweet potatoes

(Newell *et al.*, 1995) and other crops have been genetically engineered to express a gene from *Bacillus thuringiensis* (Bt) that encodes for a protein toxic to certain detrimental insects such as *Heliothis* caterpillars. If the active compound is a phytoalexin, its manipulation by genetic engineering is more complex. A deep knowledge and understanding of the pathway of phytoalexin synthesis is required. Over the last few decades many efforts to manipulate phytoalexin production have been attempted. For the study of a required phytoalexin, it is important to use infected plants that produce more phytoalexins as well as the enzymes involved in their synthesis (Paiva *et al.*, 1991) than uninfected plants (Edwards and Strange, 1991). The characterization of enzymes involved in the synthetic pathway of a target phytoalexin is essential. For instance, enzymes involved in the pathway of medicarpin have been well characterized (Dewick and Martin, 1979) in order to manipulate the synthesis of medicarpin in some crops.

2.4.1 *Importance of Plant Genetic Engineering*

Plant biotechnology and genetic engineering are revolutionizing conventional techniques to improve the quality of crops. Transgenic plants that can tolerate herbicides (Yanchinski, 1995), resist insects (Shelton *et al.*, 2000) or viruses (Xu *et al.*, 1998), or produce modified fruit or flowers

are being grown and tested. Copies of genes for these traits have been transferred to the plants from other unrelated plants, bacteria or viruses. Corn plants that produce an insecticidal protein Cry1Ab (Pilcher *et al.*, 1997) to resist the European corn borer *Ostrinia nubilalis* and tomatoes with delayed ripening are examples of transgenic plants available commercially. The bacterium *Bacillus thuringiensis*, commonly known as Bt produces proteins that are lethal to certain insects with alkaline digestive tracts. Genetic techniques make it possible to isolate the gene that codes for Bt proteins lethal to insects and to transfer the gene into crop plants (Sachs *et al.*, 1998). Genetic engineering has been used in this way to control many of the pests and pathogens that affect important crops around the world. However, the expressed protein may affect non-target organisms. For example, Monarch butterflies were reported to be affected by a Bt protein (Cry 1) that was being expressed in pollen of transgenic corn (Hileman, 1999; Nabhan, 1999).

There is concern about transgenic plants, not without reason, since some expressed proteins could affect other living organisms. It is very important to take this fact into consideration and to look for the best and the safest alternative for everyone. It is important to look for proteins that could be

used mainly as repellents to certain target pathogens or used to reduce the pest population without selecting for resistance while minimizing the economic impact. Usually, pests and pathogens are naturally controlled since pathogenesis is a rare situation and it is not the rule. However, host plant resistance can be enhanced by means of genetic transfer to express protease inhibitors (PIs) that are normally present in legume seeds and could be safer than chemical pesticides for the human health because of the anticarcinogenic properties of PIs (Calkins and Sloane, 1995).

2.4.2 *Transgenic Plants and Their Potential Use for Nematode Control*

Over the last fifty years, nematodes have been controlled mainly by the application of chemical nematicides (Melton, 1995). Much research effort has been devoted to the development of new nematicides and new methods of application. In the last few decades it has become possible to transfer genes to produce active proteins that affect nematodes by means of genetic engineering.

Defense against nematodes is a useful property of some plants which allows them to survive in nature. Plants defend themselves from nematode attack by producing active proteins (Vain *et al.*, 1998) and active secondary

metabolites (Kimura *et al.*, 1981) such as phytoalexins. Most phytoalexins are formed by complex biosynthetic pathways involving several enzymatic steps. Plants usually have common precursors for the synthesis of different phytoalexins. Manipulation of pathways to produce a required phytoalexin by molecular techniques may be possible if the relevant genes can be isolated and transferred to the genome of the desired host.

Another method to control nematodes is by disrupting their eggs by degrading the chitin present in the nematode egg shell (Cohen, 1993). Some scientists are looking for genes responsible for the production of chitinase. This enzyme breaks down chitin disrupting the normal development of embryos. The transfer of these genes into target plants might increase their ability to protect themselves from phytoparasitic nematodes.

Inhibitors of proteases are very important in plant resistance. Proteases released by nematodes destroy the plant wall and inner membranes weakening plant cells allowing the entrance of nematode or insect larvae with a progressive structural damage (Rosso *et al.*, 1996). However, plants have evolved their defense system by producing protease inhibitors. These

plant protease inhibitor genes could be used to increase resistance in different crops in a short time by means of genetic engineering. Protease inhibitors have many useful and interesting characteristics such as small size and stability. Besides, the interesting feature of having reactive sites with hypervariability allows their manipulation for different purposes. The hypervariability of reactive sites is characteristic of protein inhibitors which retain their activity upon replacement of their reactive site residue by another residue.

Nematodes have cysteine proteases (Lilley *et al.*, 1996) in their digestive system in contrast to mammals, which do not have these enzymes in their guts. For this reason, the expression of cysteine protease inhibitor genes in transgenic plants may provide protection against nematode invasion. Urwin *et al.* (1998) observed that dual proteinase inhibitor constructs enhanced plant resistance to nematodes when compared to individual gene constructs. In this case, Urwin fused two distinct proteinase inhibitor genes for a cysteine and a serine proteinase inhibitor and expressed them in *Arabidopsis thaliana*. The combination of both proteinase inhibitor genes showed an additive effect against plant parasitic nematodes. Protease inhibitor expression in transgenic plants was obtained in tobacco and these

transgenic plants showed activity against insects and nematodes (Thomas *et al.*, 1995a).

CHAPTER III : Search for Nematicidal Natural Products

3.1 Introduction

Plants such as weeds and tropical plant species are a vast source of defense-related molecules, which include secondary metabolites and active proteins. Some weedy species could provide us with an inexpensive source of active substances. Several weeds such as *Tagetes* are being used successfully to control nematodes like *Meloidogyne incognita* (Queneherve, 1998). There are many reports about plant extracts with nematicidal activity. However, very few nematicidal molecules have been isolated and identified. There is a need to identify novel biomolecules with nematicidal activity that may be an alternative for chemical nematicides that are under review by the EPA because of their proven toxicity.

When searching for natural products as an alternative to replace expensive chemical pesticides, it is important to take into consideration that every healthy plant including weeds is a possible source of active compounds. There are several reports confirming that the presence of some weeds frequently reduces pest populations (Risch *et al.*, 1983). The greater the diversity of plants, the greater the possibility of finding novel molecules

with pesticidal activity. Biodiversity seems to be an important factor when searching for novel natural products (Shuler, 1994). The greater the complexity of the biological community, the greater is its stability. The importance of biodiversity can be represented in a pyramid. Baker and Cook (1974) made a good comparison of the stability of one organism as a pyramid balanced on its apex. In contrast, a complex association has greater stability resembling the pyramid resting on its base.

Biological control is playing an important role in providing a safe and affordable food supply for the future. During the last 20 years, interest in the development and production of economical, biodegradable nematicides has increased enormously (Duke, 1990). As an economical alternative many farmers are using different plants not only against nematodes but also against other pests and pathogens (Flint, 1988; Benner, 1993).

In our study we determined the effectiveness of extracts of several plants native to Oklahoma against the root-knot nematode *Meloidogyne incognita*. Plants were chosen based on their healthy appearance. Experiments were conducted under laboratory and greenhouse conditions to demostrate the effect of *Ambrosia psilostachya, Solidago missouriensis, Lespedeza stuevei*

and *Tagetes patula* extracts against *M. incognita* juveniles. The first part of this research focused on the evaluation of aqueous extracts from the plants mentioned above.

Ambrosia psilostachya is known as western ragweed. *Ambrosia* is a coarse, unattractive annual herb. The leaves are very dissected and the flowers are green and inconspicuous. A poultice of the crushed plant has been used to treat poison sumac symptoms. It has been used to treat gonorrhea, diarrhea and other intestinal disturbances. In Mexico, it is believed to be useful for treating intestinal worms and reducing fever (Block *et al.*, 1994).

Prairie goldenrod, *Solidago missouriensis* (Asteraceae), is a common perennial herb found on road-sides and in abandoned fields throughout the United States. A related species, *Solidago altissima* is attacked by about one hundred species of insect hervibores, many of which are quite common (Maddox and Root, 1987; 1990). Extracts of this species are active against many organisms (Bosio, 1990). Many North American goldenrods have long been used for teas, foods, and medicines by the Amerindians. Although Prairie goldenrod (*Solidago missouriensis*) is not mentioned in

this regard, most likely this species exhibits some of these useful properties.

Lespedeza is a perennial herb that can be infected by the soybean cyst nematode, *Heterodera glycines* (Kim, 1989) and other plant parasitic nematodes. In a former study (Bernard, 1997), *Lespedeza* (bush clover) was a susceptible host and was infected by *Meloidogyne trifoliophila*.

Besides the mentioned above activities, we did not find scientific reports about the activity of *Solidago*, *Lespedeza* and *Ambrosia* against nematodes. In our study of natural products, we used *Tagetes* as a positive control because of its well known activity against *Meloidogyne incognita* (Akhtar and Alam, 1990; Kyo *et al.*, 1990). The experiment of adding plant extracts to tomato seedlings infected with nematodes confirmed that the presence of plant extracts may lead to a reduction in infection and gall formation when added as a soil ammendment.

3.2 Materials and Methods

Living Material:

- Nematodes: Second stage *M. incognita* (juveniles). Nematodes were mantained on tomato plants under greenhouse conditions.
- Seeds: Rutgers tomato harvested on 11/19/92.
- Plants: *Tagetes* (marigold); *Solidago missouriensis* (golden rod); *Ambrosia psilostachya* (western ragweed); and *Lespedeza stuevei* (bush clover) were collected from roadsides in the Norman, OK area during the fall of 1995 (September and October). *Tagetes patula* (marigold) was provided by the University of Oklahoma greenhouse.

Other Material:

Peter's fertilizer 15:16:17 (N:P:K) was purchased from Horticultural Products Company, Grace-Sierra, Milpitas. Dialysis membranes MWCO 3500 were from Spectra/Por. Twenty-four well microtiter plates for nematode bioassays were obtained from Becton Dickinson & Company, Sparks, Maryland 21152.

Two types of experiments were carried out to observe the effect of plant extracts on *Meloidogyne*. One experiment was carried out under

greenhouse conditions where tomato seedlings were inoculated with nematodes and treated with plant extracts. A second experiment was performed under laboratory conditions where nematode juveniles were exposed directly to plant extracts and observed under the stereoscope for three days.

3.2.1 *Greenhouse Experiments*

3.2.1.1 *Growth of Tomato Seedlings:*

The seeds of a root-knot nematode susceptible tomato (*Lycopersicon esculentum*) cultivar Rutgers were sown in a tray with sterile soil in the greenhouse. One week after sowing the batch of seeds, 30 seedlings were transplanted, one per 15-cm pot. Tomato plants were fertilized with a solution of 15-16-17 (N:P:K) once a week. Plants were grown under greenhouse conditions with 16 h supplemental lighting.

3.2.1.2 *Nematode Culture:*

Nematodes were maintained on tomato plants under greenhouse conditions. The second stage juveniles of *M. incognita* were obtained from the egg masses attached to the tomato roots. For optimal nematode hatching, the

egg masses were placed in 2-cm deep chlorine-free water considering that only about 20% of the eggs extracted with NaOCl yield infective juveniles (Hussey and Barker, 1973) and hatched. The juveniles were separated from egg impurities and washed using the Baerman method (Townshend, 1963). The latter consists of placing egg masses in a funnel covered by a tissue paper filled with water. Nematode juveniles pass through the tissue paper and are collected in a container placed under the funnel, while impurities remain on the paper. After collecting juveniles, they were counted under the stereoscope to determine the concentration of nematodes. These infective juveniles were used in the laboratory bioassay and in the greenhouse experiments.

3.2.1.3. *Preparation of Plant Extracts:*

Plant samples of *Ambrosia*, *Solidago* and *Lespedeza* were collected during the fall along roadside areas of Norman, OK. Stems and roots were discarded and only leaves were used to prepare plant extracts. Plant material was air dried for 3-5 days. Dried plant leaves were ground in a portable Wiley Mill to pass through a 20 mesh screen. A total of 50 grams of each plant material was suspended in 500 ml of distilled water and allowed to seep in the refrigerator at 4 °C for 24 hr. The aqueous extracts

were filtered through Whatman No. 4 MM filter paper. After filtration, the extracts were centrifuged at 5,000 g for 10 minutes using a Sorvall SS-34 rotor. The supernatant fluid from each extract was concentrated approximately five fold using dialysis membranes (MWCO 3500) exposed to a continuous air flow for 24 hr. Concentrated extracts were kept at 4 °C. These plant extracts were used in the next step (inoculation of seedlings).

3.2.1.4. *Inoculation of Seedlings* :

One-week-old tomato seedlings (*Lycopersicon esculentum* cv. Rutgers) were transplanted to individual 15-cm clay pots. After two weeks 20 ml of concentrated plant extract from *Ambrosia*, *Tagetes*, *Solidago*, or *Lespedeza* were added to the pots with the tomato seedlings. Two hours after each concentrated plant extract was added to the soil mix, 1 ml of *Meloidogyne incognita* suspension containing approximately 300 juveniles/ml was added to each pot. Tomato plants inoculated only with nematodes and tomato plants without plant extract and without nematodes were used as positive and negative controls. Five replicates of each treatment were included (Figure 3.1). Tomato plants were watered daily with distilled water and were fertilized with Peter's fertilizer N:P:K (15:16:17) once a week. The tomato plants were harvested 8 weeks after inoculation. The top from each

plant was removed using pruning shears and the fresh weight of each shoot was recorded for each treatment. After the roots were cleaned of adhering soil, the roots were blotted with tissue paper to remove excess water and the fresh weights of each replicate of each treatment were recorded. Finally, the number of galls were counted and recorded. The galling index used is described in Table 3.1

Table 3.1. Galling Index for *Meloidogyne* spp.

Galling Index	% of total root system galled
0	0
1	10
2	20
3	30
4	40
5	50
6	60
7	70
8	80
9	90
10	100

The level of root galling found on each root system was rated according to Barker (1978).

Fig. 3.1. Experimental treatments of nematode-infected tomato seedlings. Plants were treated with 20 ml of *Lespedeza*, *Solidago*, *Tagetes*, and *Ambrosia* extracts.

3.2.2 *Experimental Design for in vitro Nematode Assays*:

The effect of plant extracts was tested against plant nematodes under laboratory conditions. The *in vitro* nematode assay was carried out by placing juveniles of *Meloidogyne incognita* or *Caenorhabditis elegans* in a microtitre plate containing different dilutions of plant extracts as described below. Nematodes were observed under the stereoscope for three days. Nematistatic and nematicidal activity were determined for each extract. For the latter, the fluid surrounding the nematodes was carefully removed with a Pasteur pipet and the nematodes were washed five times for 2 hr with distilled water to remove the plant extracts. Nematode survival was measured before and after removal of the extracts and reported as percent survival of the nematodes. If nematodes resumed their movement after eliminating plant extracts the activity was considered to be nematistatic.

The formula to calculate nematicidal or nematistatic effect used was:

$$\% \text{ Nematode mortality} = \frac{Nd}{Nt} \times 100$$

Nd refers to the number of dead nematodes and Nt is the total number of nematodes per well.

3.2.2.1 *Meloidogyne Bioassay:*

Aliquots of 100 µl of distilled water containing 50 juvenile nematodes were transferred to a FALCON 24-well tissue culture plate. Dried extracts of

Ambrosia, *Solidago*, *Tagetes* or *Lespedeza* were suspended in distilled water. Water and extract were added to each well to give concentrations of 5, 10, and of 25 mg/ml in a final volume of 500 µl per well. The number of nematodes that were alive was recorded at the beginning of the experiment. Nematodes were observed under the stereoscope after 24, 48, and 72 hr and nematicidal and nematistatic effects were calculated as described above according to Jatala *et al.* (1995). A nematode suspension without plant extract was used as a control.

3.2.2.2 *Effect of Extracts of Ambrosia on M. incognita and C. elegans*

The Extract of *Ambrosia* [60 mg/ml] was treated with *Escherichia coli* to see if nematicidal compounds present in the plant extract could be inactivated by bacteria upon which *C. elegans* feed. The effect of *Ambrosia* extract treated with bacteria on *M. incognita* and on *C. elegans* was tested as follows. One milliliter of plant extract was inoculated with 50 µl of bacterial suspension prepared as follows one colony of bacteria used to feed *C. elegans* was resuspended in 50 µl of distilled water. The mixture containing extract and bacteria was incubated overnight at 25 °C with shaking. The next day the extract was transferred into an Eppendorf tube and centrifuged for 2 min. in a microcentrifuge. The supernatant fluid was

transferred to another Eppendorf tube and placed in a boiling water for 4 min. to kill bacteria. This supernatant of the *Ambrosia* extract was used for the bioassay. Approximately 200 nematodes of *M. incognita* and of *C. elegans* were placed in each well of a microtitre plate and *Ambrosia* extract was added to a final concentration of 30 mg/ml in a volume of 300 µl per well. *M. incognita* and *C. elegans* were observed after 24 and 48 hr. and nematicidal and nematistatic effect was calculated as described in 3.2.2.

3.3 Results and Discussion

Plants species were collected in Norman, OK based on their vigorous growth. The effects of aqueous extracts of *Ambrosia, Lespedeza, Solidago,* and *Tagetes* on *M. incognita* nematodes was evaluated *in vivo*, under greenhouse conditions where the root-galling caused by nematodes in tomato plants was evaluated. In parallel, the effects of plant extracts on nematodes was also evaluated *in vitro*, where juveniles were exposed to plant extracts in microtitre plates and their mortality was recorded as described previously. Plant extracts before and after dialysis were used in preliminary laboratory experiments. All plant extracts lost their activity after dialysis suggesting the presence of active secondary metabolites. Results obtained under greenhouse and under laboratory conditions were

positively correlated. *Solidago* and *Tagetes* exhibited nematistatic activity and only *Ambrosia* showed nematicidal activity. Details of results are given in the next sections.

3.3.1 *Greenhouse Experiments*

The extent of *Meloidogyne incognita* infection may be measured by observing the relative extent to which the roots are galled. Barker (1978, 1981) proposed a rating scale to measure nematode infection based on root-galling. This galling index is useful in the evaluation of nematicide efficacy and to correlate estimates of yield losses with the extent of nematode infection. Yield losses increase linearly as the root gall index increases.

The purpose of this experiment was to evaluate the effect of plant extracts of *Ambrosia*, *Solidago*, *Tagetes* and *Lespedeza* on root galling of tomato plants infected with *M. incognita* under greenhouse conditions. Tomato plants were grown in pots to which plant extracts were applied. Positive and negative controls were included and each treatment was replicated five times. Results of these studies are presented in Table 3.2 . Shoot dry weights were not affected by inoculation with nematodes or by the addition of extracts. In contrast, root dry weights were reduced in plants inoculated

with *M. incognita* larvae by approximately 25% when compared to non-infected control plants. In infected plants treated with any of the three plant extracts, exposure to the extracts had little effect on shoot or root growth when compared to the plants infected with nematodes alone.

Table 3.2. Effect of Plant Extracts on Tomato Plants Infected with Nematodes.

	Weight [g]				
SAMPLE	**Stem**	**Root system**	**Whole plant**	**No of Galls**	**Std. Dev.**
Control	49	19	66	0	-
A. psilostachya	49	12	61	11	8
L. stuevei	51	12	63	209	64
S. missouriensis	59	13	71	52	23
T. patula	56	12	68	119	41
Only nematode	52	15	68	274	27

All values are the mean of five replicas per plant. Each pot was inoculated with approximately 300 juveniles of *M. incognita*. Std. Dev is the standard deviation in the number of galls per plant. Control was not inoculated.

Gall formation on the other hand was dramatically affected by the application of the plant extracts (Table 3.2, 3.3). Addition of extracts of *Lespedeza* to the soil of inoculated plants reduced gall formation slightly. Application of extracts of *Tagetes* to the soil decreased gall formation by

57% (*Tagetes* is not shown in the Figure 3.2) when compared to plants infected with nematodes without plant extracts (Table 3.2).

Table 3.3. Galling Index of Tomato Plants Treated with Different Plant Extracts

SAMPLE	Galling index
Control	0
Ambrosia psilostachya	1
Lespedeza stuevei	7
Solidago missouriensis	1
Tagetes patula	4
Only nematodes	10

The most effective extract was the one from *Ambrosia*, followed by the extract of *Solidago* (Figure 3.2). *Solidago* and *Ambrosia* extracts reduced gall formation by 81% and 96%, respectively. These results were consistent with results of *ex situ* bioassays. Analysis using the root-galling index yielded similar results. These results suggest that these plants, especially *Ambrosia*, *Solidago* and *Tagetes* contain low molecular weight compound(s) that exhibit a nematoxic effect on *M. incognita*.

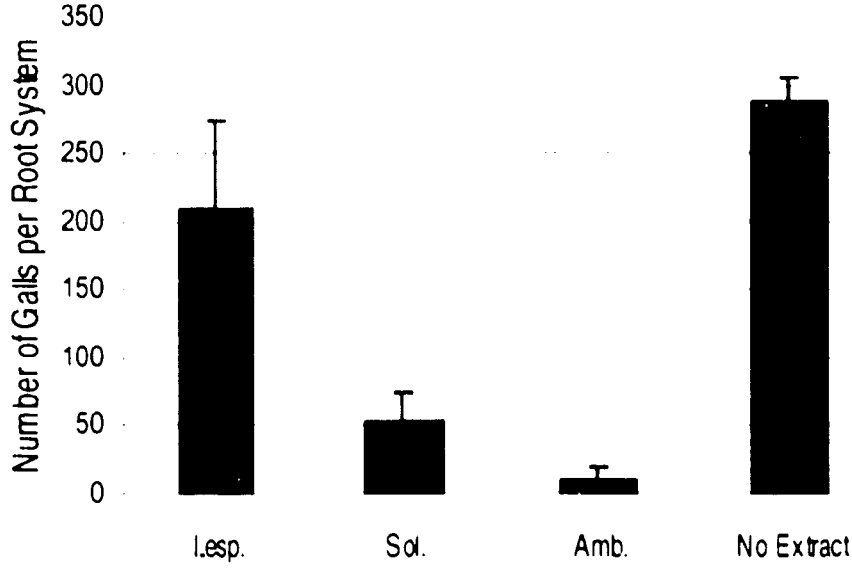


Fig. 3.2. Effect of plant extracts on root gall formation. Crude aqueous extracts (20 ml) of *Lespedeza* (Lesp.), *Solidago* (Sol.) and *Ambrosia* (Amb.) were applied to tomato plants grown in pots in the greenhouse. All plants were inoculated with approximately 300 nematodes. Five replicates were included per treatment. The number of galls per plant was measured after two months. The error bars represent the standard deviation.

3.3.2 *Laboratory Experiments*

Extracts of *Lespedeza* inhibited nematode motility for a short period of time but nematodes resumed their activity even in the presence of the plant extract. *Solidago* extract exerted a nematistatic effect and all nematodes resumed their activity only after removal of the plant extract. Only *Ambrosia* extract exerted a nematicidal effect on *M. incognita*. One hundred percent of the nematodes were dead after exposure to *Ambrosia* extracts at concentrations of 20 mg/ml. Nematodes did not recover even after washing 5 times with distilled water. The apparent mortalities of *M. incognita* juveniles exposed to the three plant extracts are presented in Fig. 3.3. The nematode bodies straightened and lacked movement which suggested that the *Ambrosia*, *Tagetes* and *Solidago* extract may have a lethal effect on the worms. Apparent mortality increased with increasing concentrations of the three cited plant extracts after 72 hr. All four plant extracts inhibited nematode motility 48 hr after inoculation. Exposure of *M. incognita* to the highest concentration (25 mg/ml) of *Solidago* extract for 72 hr. paralyzed 100 % of the nematodes. Under the same conditions, extracts of *Tagetes* paralyzed 89% of the nematodes (not shown in the Figure).

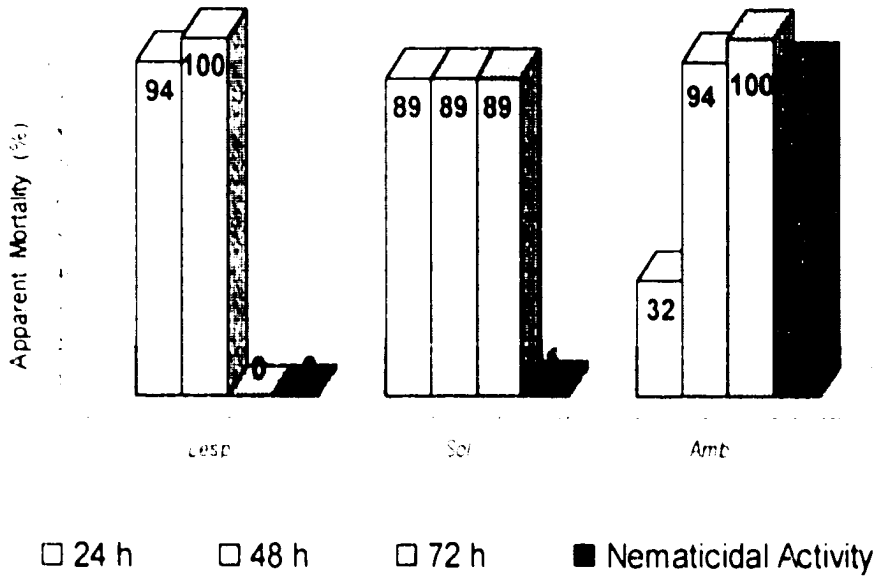


Fig. 3.3. Effect of plant extracts on nematodes in bioassay. The apparent mortality of nematodes after 24, 48 and 72 hr. exposure to plant extracts. The concentration of each extract used in the final bioassay was 25 mg of dried extract/ml. Approximately 80 *M. incognita* juveniles were used per well. Percent of paralyzed nematodes were counted as apparent mortality.

To determine whether the extracts were truly nematicidal or merely nemastatic, the extracts were removed and the worms were washed five times with distilled water. After washing nematodes exposed to extracts of *Solidago* and *Tagetes*, all the individuals resumed normal movement at all concentrations suggesting that extracts of *Solidago* and *Tagetes* exerted a nematistatic effect on *M. incognita*. The effects of the highest concentrations of extracts of *Lespedeza* were substantially different. Nematodes treated with 25 mg/ml of *Lespedeza* extracts appeared dead after exposure to the extract for 24 hr. These worms remained inactive at 48 hr after inoculation but resumed activity at 72 hr. Nematodes exposed to lower concentrations of the *Lespedeza* extracts resumed their movement after 24 hr. These observations suggest that the effector molecule(s) in the *Lespedeza* extracts was either not stable or was metabolized. All the plant extracts lost their activity after dialysis (data not shown) using membranes with a molecular weight cut-off of 3,500 Daltons.

In order to have an idea of the toxicity of *Ambrosia* extract on free-living soil nematodes or other non-target organisms we decided to test the extract on *C. elegans*, a free-living bacterial-feeding nematode. Many studies of *C. elegans* have been done. Its life cycle is very short and can be cultured *in*

vitro to provide a good model for higher animals. *C. elegans* usually feed on *Escherichia coli* which could inactivate the active principle of *Ambrosia* extract. So, bacteria that are a food source for *C. elegans* were mixed with *Ambrosia* extract and incubated overnight. After incubating overnight, bacteria were inactivated by placing the plant extract in boiling water for several minutes and then were discarded by centrifuging the extract at 14,000 rpm for 5 min. in a microcentrifuge. The effect of bacteria-treated *Ambrosia* extract on *M. incognita* and *C. elegans* was tested and the extract was found to have a good nematicidal activity. This result suggested that these bacteria did not inactivate the active compounds present in the plant extract. The activity remained the same after the bacterial treatment and after boiling the extract for 4 minutes (Figure 3.4). The percentage of mortality for *Meloidogyne* was 71 % and 42 % for *C. elegans* when a 30 mg/ml concentration of *Ambrosia* extract was used. This variance of mortality in *M. incognita* when compared to assays with untreated extract may be due to the different number of nematodes used in both assays. Results suggest that the active material(s) are most likely low molecular weight secondary metabolites. The mortality of *C. elegans* was less than the mortality of *M. incognita* at the same concentration of the plant extract.

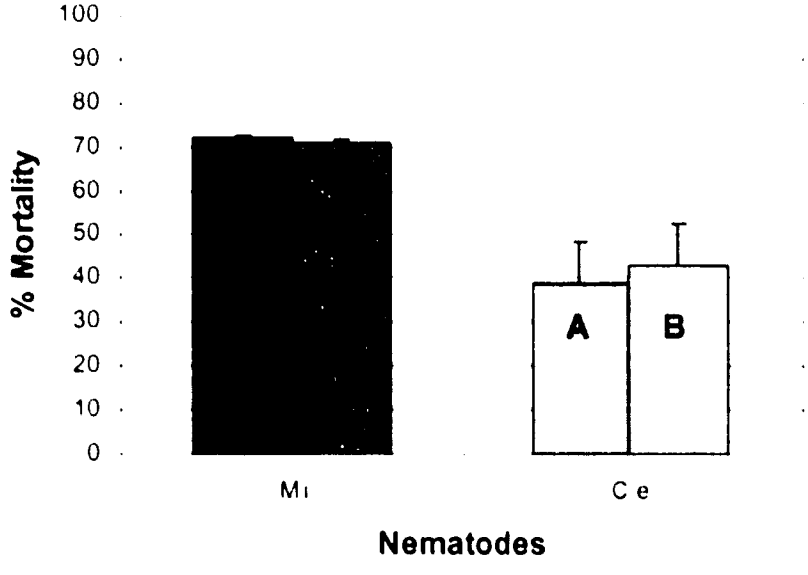


Fig. 3.4. Effect of *Ambrosia* extract on *Caenorhabditis elegans* and *Meloidogyne incognita*. Treatments: A, non-boiled; B, boiled for 4 min. A concentration of 30 mg ml was used for each sample and 200 nematodes were used per well. There were three replicates per treatment. The number of dead nematodes was recorded after 48 h. The bars represent the standard deviation.

CONCLUSIONS

Solidago and *Ambrosia* reduced gall formation by 81% and 96% on tomato roots inoculated with *M. incognita*. These results were positively correlated with bioassays in the laboratory. *Ambrosia* caused 91% nematode mortality while *Solidago* caused 89% nematistatic effect under laboratory conditions. *Lespedeza* showed a temporary nematistatic effect (24 - 48h). This suggests that the active agent was either not stable or was metabolized. Nematodes resumed their activity only after removal of the plant extract. Only *Ambrosia* exhibited nematicidal activity. The activity of *Ambrosia* caused 72% *M. incognita* mortality after treating with bacteria and this extract remained the same after boiling the extract for several minutes. The nematicidal activity was lost after dialysis suggesting the presence of secondary metabolites.

CHAPTER IV: Isolation and Characterization of Secondary Metabolites from *Ambrosia psilostachya*

4.1 Introduction:

Extracts of *Solidago*, *Lespedeza*, *Tagetes* and *Ambrosia* all showed activity against root-knot nematodes. However, only *Ambrosia* extracts exhibited a nematicidal effect. There are several reports about nematistatic principles found in Compositae (Prakash and Rao, 1996) but there is none for *Ambrosia*. The name *Ambrosia* is derived from the Latin term for " food of the gods " or " immortal " (Boulos *et al.*, 1994). The name " immortal " is very fitting because attempts to eradicate this plant have met with little success. The medicinal properties of this plant are atributted to the bitter substance sesquiterpene lactone. Sesquiterpene lactones are characteristic constituents of the Compositae and they are associated with anti-tumor, anti-microbial, allergenic and phytotoxic activity (Rodriguez *et al.*, 1976). *Ambrosia* was reported to cause allergic contact dermatitis (Mitchell, 1971) in some people. However, tea prepared with *Ambrosia* is still used to treat gonorrhea, diarrhea and other intestinal disturbances. In Mexico, extracts of *Ambrosia* are used to treat intestinal worms and to reduce fever (Block *et al.*, 1994). In Egypt, the infusion of *Ambrosia maritima* is used to expel

renal stones (Boulos *et al.*, 1994; Salam *et al.*, 1984). The isolation, identification and characterization of active compounds with nematicidal activity from aqueous extracts of *Ambrosia psilostachya* (Figure 4.1) are presented in this chapter.

4.2 Materials and Methods :

Analytical and preparative TLC plates with fluorescent indicator and organic solvents such as acetone, HPLC-grade methanol, chloroform and dichloromethane were obtained from Fisher Scientific (1801 Gateway Blvd. Richardson, Dallas, TX 75080-3750). Hy-Soy powder was purchased from Quest International (Sheffield Products, P.O. Box 630, Norwich, New York 13815) and yeast extract was from Difco (Lincoln Park, New Jersey 07035). U-bottom ELISA plates for agglutination assays were purchased from Becton Dickinson & Company, 2 Bridgewater Lane, Lincoln Park, New Jersey 07035. Human, rabbit and porcine red blood cells were purchased from Sigma, Chemical Co., St. Louis, MO.

4.2.1 *Nematode Culture*

Nematodes *Meloidogyne incognita* was maintained under greenhouse conditions on tomato (*Lycopersicon esculentum* cv. Rutgers) plants.

Fig. 4.1 Photograph of *Ambrosia psilostachya*. This plant was collected in Norman, OK. Secondary metabolites with nematicidal activity against *Meloidogyne incognita* were isolated from dried leaves of this plant.

Egg masses of *M. incognita* were picked from infected tomato roots and placed in a Petri dish with distilled water. After two or three days, second-stage juveniles (J2) of *M. incognita* were obtained from these egg masses. Juveniles were transferred to a small beaker with a Pasteur pipet. *C. elegans* nematodes were cultured in a bacterial-free medium according to Tomlinson (1962). Bacterial-free cultures were made according to Hocker (1997) and stored at – 40 °C. The composition and preparation of the culture medium is described herein.

The medium was prepared by mixing 1 part of heated liver extract (HLE) and 9 parts of autoclaved HySoy-Yeast Extract (HS-YE). HLE was filter-sterilized before adding to the media. HS-YE: Composed of 40 g of Hy-Soy Powder and 10 g of yeast extract per liter of deionized water. HLE: (Heated Liver Extract): One to two pounds of frozen beef liver was purchased from a local market. The liver was thawed and rinsed with water then cut into 1 inch squares and drained. The liver was stored at 4 °C for approximately 24 hr so auto-lysis could take place. Liver was placed in an equal volume of water and homogenized in a blender until all of the large clumps had been disrupted. The homogenate was filtered through two layers of Miracloth and held on ice until all of the liver had been processed.

The homogenate (600 ml) was placed into a one-liter beaker and heated in a 60 °C water bath to a final temperature between 52-53 °C. The extract was maintained at this temperature for 6 min. The beaker containing the heated extract was then placed into ice-water until all of the liver homogenate was prepared. The homogenate was placed into screw capped centrifuge tubes and centrifuged at 37,000 to 39,000 g for 30 min. The supernatant was collected and filtered through two layers of Miracloth. The extract was prefiltered through a 90 mm, 1 micron Gelman Science 61664 type A/E glass fiber in a Buchner vacuum filter apparatus. Subsequently, the filtrate was filter-sterilized using a Nalgene 125-0045 sterilization filter unit.

4.2.2 *Thin Layer Chromatography (TLC)*

The plant extract preparation is described in 3.2.1.3 section. The plant extract from *Ambrosia* was lyophilized and resuspended in methanol at a final concentration of 400 mg/ml. This sample was used for analytical and preparative separations.

4.2.2.1 *Detection of active compounds by analytical TLC*

Separation of active compounds was performed using 3 cm x 7 cm analytical TLC plates (Fisher Scientific. Cat. No. 05-713-162). The solvent system used was acetone: methanol: chloroform (3:4:91; v/v/v). Organic compounds were detected using Liebermann reagent composed of acetic anhydride: ethanol: sulphuric acid [$(CH_3CO)_2O$: CH_3CH_2OH: H_2SO_4 (1:8:1; v/v/v)]. The preparation of the Liebermann spray must be done with caution especially when mixing H_2SO_4 and EtOH. Liebermann reagent was prepared in an extraction chamber. A beaker containing the ethanol was placed in an ice bath while H_2SO_4 was added very carefully drop by drop with stirring.

After chromatographic separation, plates were sprayed with Liebermann reagent and heated with a hair dryer for two min. or until the spots were revealed. After heating the stained compounds were observed under long (365 nm) and short wavelength (254 nm) UV light.

4.2.2.2 *Separation of active molecules by preparative TLC*

Lyophilized aqueous extract (3 g) from *Ambrosia* was resuspended in 15 ml of methanol : ethanol (1:1; v/v) overnight. The supernatant was

evaporated, weighed and resuspended in 500 µl of methanol. This extract was used for TLC separation. Preparative TLC silica gel plates, RP-18W, 20x20 cm, 2000 µm with fluorescent indicator (Fisher Scientific, Cat. No. M5717-7) were used. The plates were developed in a standard chamber with acetone-chloroform-methanol (3:91:4; v/v/v). After chromatography, plates were air dried and observed under UV (at 254 nm and at 365 nm) light. The silica containing each fraction was scraped off the plate, pulverized and resuspended over night in 40 ml of methanol : chloroform (1:1; v/v). After filtration through a Whatman No. 1 filter, the solvent was evaporated in a rotary evaporator. The sediment from each fraction was resuspended in a minimal volume of methanol (500 µl) and an aliquot was used for analytical TLC. Pure active compounds were analyzed to determine their structure. Sesquiterpene lactones appeared as brown spots under short wavelength light. In analytical TLC they were visualized using the Liebermann spray reagent. The structures of the isolated molecules were elucidated by NMR, COSY, and by MALDI-TOF laser mass spectrometry.

4.2.3 *Separation of Active Molecules by HPLC*

Components in the *Ambrosia* extracts were separated by HPLC on a 10 ODS, 250 x 10 mm column (Phenomenex:Prodigy, 2320 W. 205th St. Torrance, California 90501). A guard column containing the same resin was placed in front of the main column. Active compounds prepurified by TLC were dissolved in methanol to a final concentration of 2.5 mg/ml and 50 µl was loaded onto the HPLC column. Active compounds were eluted with a stepwise gradient of water and methanol as follows:

Time [min]	**Flow rate**	**Time duration**	**% H_2O**	**% MeOH**
0	6	0.5	80	20
0.5	7	30	65	35
30.5	5	3.5	80	20

Eluted fractions of 10 ml each were mixed with 10 volumes of pure methanol and dried using a rotary evaporator. One milligram of each HPLC separated compound was taken and resuspended in 100 µl of pure methanol for TLC analysis. Three milligrams of each separated compound was resuspended in 150 µl of distilled water for nematode assay. Aliquots of 50 µl of distilled water containing 50 juveniles of *M. incognita* were placed on a flat-bottom ELISA plate and 50 µl of each compound was added per well

to a final concentration of 10 mg/ml. Three replicates per sample were prepared. Nematodes were observed after 24, 48 and 72 hr under the stereomicroscope. Living and dead nematodes were counted and recorded. Active fractions were analyzed by TLC on silica analytical plates (3x7 cm) and isolated compounds were detected by heating the plate after spraying it with Liebermann reagent.

4.2.4 *NMR Analysis of Isolated Secondary Metabolites*

Isolated compounds were resuspended in HPLC-grade methanol and subjected to analytical TLC before NMR analysis. All 1D and 2D-NMR spectra were measured on a Varian VXR500 spectrometer. Methanol-d_4 was used as a solvent and chemical reference. Proton ^{1}H and carbon ^{13}C spectra were analyzed.

4.2.5 *Nematode Respiration Assay*

Respiration is an important physiological parameter in the life of all living organisms. Nematode respiration rate may be affected as a consequence of the inhibitory activity of nematoxic compounds. The purpose of this experiment was to observe the effect of the nematicidal compound parthenin isolated from *Ambrosia* on the respiration rate of nematodes

Meloidogyne incognita and *C. elegans*. Nematode respiration was measured using an oxygen monitor (Model 55, Yellow Springs Instrument Co., Ohio 45387) to observe the effect of *Ambrosia* extract and parthenin on the oxygen uptake of nematodes. *C. elegans* was cultivated in a medium free of bacteria in order to avoid interferences with bacterial respiration. *Meloidogyne* juveniles were resuspended in distilled water. A 5 ml volume containing approximately 30,000 nematodes were used to measure the oxygen uptake. *Ambrosia* total extract was added to a final concentration of 20 mg/ml and parthenin to a final concentration of 5 mg/ml.

4.2.6 *Agglutination Assay*

Rabbit, pig, and human A red blood cells were used in the agglutination assays (lectin assay). Aliquots of 50 µl of phosphate buffered saline PBS were added to a U-bottom ELISA plate. Parthenin resuspended in distilled water was added to final dilutions of 0.15, 0.3, 0.6, 1.25, 2.5 and 5 mg/ml. Finally, 100 µl of diluted red blood cells were added to each well (see appendix). Samples were incubated for 30 min. at room temperature. Results of the agglutination assay were observed on a light box and recorded. PBS was used as a control.

4.2.7 *Insect Bioassay*

The effect of *Ambrosia* total extract and its active principle, parthenin, on insect larvae growth was tested for several Lepidopteran insects. Eggs of *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera frugiperda* or *Trichoplusia ni* were incubated at 23 °C for 48 hr. or until the larvae hatched. Only *Ostrinia nubilalis* (European corn borer, ECB) eggs were incubated at 28 °C until larvae hatched (72 hr.). The Stoneville insect diet was prepared by mixing several ingredients as described in Appendix II, or the diet was provided by Pioneer Hi-Bred International, Inc. (Johnston, IA). Stoneville insect diet was prepared by mixing 153.4 g of powdered dry ingredients and 2.5 ml mold inhibitor [propionic acid : phosphoric acid : water (70:7:90; v/v/v)] with 900 ml boiling distilled water in a blender. This hot liquid diet was aliquoted into a plastic rearing tray (0.5 ml per well). After the medium solidified, 80 µl of parthenin [10 mg/ml] resuspended in distilled water was added to a final concentration of 1.6 mg/ml. Crude extract of *Ambrosia* was used as a positive control and 80 µl of this extract was added to a final concentration of 3.2 mg/ml. The negative control was prepared by adding 80 µl of distilled water to the diet. Eight replicates were prepared for each sample. The diet was left for two hr. in a laminar flow hood until all of the added samples were completely

absorbed. Two neonate larvae were placed onto each well of the artificial diet. Trays were covered with mylar film and incubated at 28 °C for 5 days. At the end of this time, the larvae were examined and larvae weight and insect mortality were recorded for each treatment.

4.2.8 *Effect of Cysteine on Nematicidal Activity*

In order to know if parthenin and coronopilin reacted with sulfhydryl groups, commonly present in proteins, both parthenin and coronopilin were mixed with cysteine before starting the nematode assay. The reduction in the nematicidal activity of parthenin and coronopilin in the presence of cysteine would indicate a reaction with cysteine. The effect of cysteine on the nematicidal activity of parthenin and coronopilin was observed by mixing different concentrations of cysteine with a solution containing 10 mM parthenin or 10 mM coronopilin at the beginning of the nematode assay. The assay was prepared in a ELISA plate containing 50 µl of distilled water and approximately 50 *M. incognita* juveniles per well. Parthenin and coronopilin were added separately to a final concentration of 10 mM. Immediately after adding parthenin and coronopilin, cysteine was added to final concentrations of 0.1, 1, 5 or 10 mM. Controls with each concentration of cysteine, 10 mM parthenin alone, 10 mM coronopilin

alone, and with just distilled water were prepared. Four replicates of each sample were prepared. The effect of cysteine on nematodes treated with parthenin and coronopilin was observed under a stereomicroscope after 24, 48, and 72 hr. Dead and living nematodes were counted and recorded.

4.2.9 *HPLC Analysis of the Cysteine-parthenin Adduct*

Sesquiterpene lactones parthenin and coronopilin from *Ambrosia psilostachya* were analysed by HPLC. Studies of several sesquiterpenes have demostrated that their activity with sulfhydryl-containing compounds is due to the presence of the α-methylenebutyrolactone unit (Heptinstall *et al.*, 1987; Groenewegen *et al.*, 1986; Pieman *et al.*, 1979; Pieman *et al.*, 1981b). Since parthenin and coronopilin possess this α-methylene unit, it is possible that their nematicidal activity could be due to their reaction with enzymes and other cysteine containing proteins. This hypothetical mechanism of action was confirmed by analysing the formation of reaction adducts between parthenin and cysteine by HPLC. Twenty five µl of 0.02 M cysteine was mixed with 25 µl of 0.01 M parthenin and incubated for 0, 30 and 60 min. prior to HPLC analysis. The elution of the adduct was

monitored at 215 nm. Parameters described in 4.2.3 were used for the HPLC separation.

4.3 RESULTS

4.3.1 *Isolation and Properties of Parthenin and Coronopilin*

After lyophilization, a total of 10.2 g of dry material was extracted from 50 g of powdered *Ambrosia psilostachya* resuspended in 500 ml of distilled water. A parallel methanolic extraction was performed to compare the quantity of active compound extracted and 6 g of extract was obtained from 50 g after evaporating the methanol. Aliquots (212 mg) of aqueous and methanolic extracts were resuspended in 400 µl of methanol for preparative thin layer chromatography.

Natural products were partially purified using 2 mm silica preparative TLC plates (Fisher Scientific. Cat. No. M5717-7) and a $CHCl_3$: MeOH (96 : 4; v/v) solvent system. Bands observed under UV light at 254 nm wavelength appeared to be blue in color. At 365 nm, the same bands appeared to be brown in color. Bands were scrapped from the plate, powdered and resuspended in 40 ml of MeOH : CH_2Cl_2 (1 : 1; v/v) and extracted

overnight. Then, each extract was filtered to remove silica using Whatman No. 1 filter paper and evaporated to dryness using a rotary evaporator. Fractions corresponding to each band were tested for nematicidal activity (Table 4.1).

Table 4.1. Effects of TLC Fractions of *Ambrosia* Extracts on Nematodes

Rf	Methanolic extract		Aqueous extract	
	% *M.i.* Mortality	Yield (%)	% *M.i.* Mortality	Yield (%)
0.36	-		-	
0.29	-		82	0.5
0.25	5		85	0.9
0.19	96	0.76	13	
0.13	98	0.76	-	
0.07	93	0.28	-	
0.01	8		-	

Solvent System : CHCl3 : MeOH (96 : 4; v/v). Yield was based on initial ground plant material. Methanolic fractions with Rfs 0.19 and 0.13 seemed to be similar to aqueous fractions with Rf 0.29 and 0.25. Difference in Rf may be due to the fact that methanolic extract contained more compounds with lower polarity that affected the migration of compounds with higher polarity.

Three active compounds with Rf values of 0.07, 0.13, and 0.19 (compounds III, II and I, respectively) were isolated from the methanolic extract. Isolated fractions were resuspended in methanol and analyzed on small analytical TLC plates (Fisher Scientific, Cat. No. 05-713-162) to

determine their purity. The active compound with Rf 0.07 found in the methanolic extract was very unstable even though its activity against nematodes was high (93 % nematode mortality). Compounds with Rf 0.13 (II) and 0.19 (I) were similar to compounds isolated from aqueous extracts with Rf 0.25 and 0.29. The aqueous extract gave only two active compounds with Rf 0.25, and 0.29, with a yield of 9 g/kg and 5 g/kg of parthenin and coronopilin respectively but they were not pure. The crystals were resuspended in a minimum volume of MeOH (1 ml) for further purification. The actual bands had to be further purified for NMR analysis.

4.3.1.1. *TLC Purification of Parthenin and Coronopilin*

Aqueous extract gave a better separation, however two or three compounds were still present in each active fraction. An alternative TLC method using silica plates (Fisher Scientific. Cat. No. 05-713-314) containing an inert preadsorbent band (150 Å, PLKSF), which provide a better separation was used to obtain pure compounds. Aqueous lyophilized *Ambrosia* extract (250 mg) was resuspended in 20 ml of methanol:ethanol (1:1; v/v) over night. The supernatant was filtered under vacuum and the solvent was evaporated with a rotary evaporator. The sediment (500 mg) was resuspended in 2 ml of methanol : ethanol (1:1) and applied on 4 TLC

plates (1 mm thickness, 150 Å). A solvent system composed of acetone:MeOH:$CHCl_3$ (3:4:91; v/v/v) was used. After chromatography, plates were dried in an extraction chamber and then observed with UV light. Three brown bands were observed under short wavelength UV light (254 nm) (compounds I, II and III). Bands were scrapped from the plate and pulverized. Each fraction was resuspended in 50 ml of MeOH:$CHCl_3$ (1:1; v/v). Then, the supernatant was filtered through Whatman No. 1 filter paper and the solvent was evaporated in a rotary evaporator.

TLC fractions were tested for activity using the nematode assay. Two compounds (I and II) with nematicidal activity were further analyzed by TLC on a microplate (3 x 7 cm) using a solvent system composed of acetone : methanol : chloroform (3:4:91). Separated compounds were detected by heating plates after spraying plates with Liebermann reagent. Active compounds gave single spots when using several solvent systems indicating that they were relatively pure. Five milligrams of each active compound were subjected to NMR analysis to determine their structure and they were found to be coronopilin and parthenin, a third molecule with activity was not identified due to its instability. In the TLC plate sprayed with Liebermann compound I (coronopilin) gave a yellow color and

compound II (parthenin) and the compound III gave a purple color that changed to brown after a few minutes. When observed under UV (365 nm) compound I appeared light blue in color and compound II and the compound III gave a brown color under 254 nm light (short wavelength UV) in TLC plates with fluorescent indicator.

Isolated active compounds were in crystal form, compound I (11.3 mg) with a Rf = 0.31 gave white crystals and compound II (10.8 mg) with a Rf = 0.27 gave pale yellow crystals. The compound III gave white crystals (17.6 mg) and had a Rf = 0.19 (Figure 4.2). The Rfs of isolated compounds were different to the active compounds isolated earlier because the TLC plates used for purification had an inert band that allowed a better separation and also a different solvent system was used. The addition of acetone in the solvent system seemed to increase resolution of the active compounds. The yield of compound I was 2 g/kg, 2 g/kg for compound II, and 4 g/kg for the compound III of the initial ground plant material, respectively.

A higher yield of parthenin and compound I was obtained with a small variation of the described method.

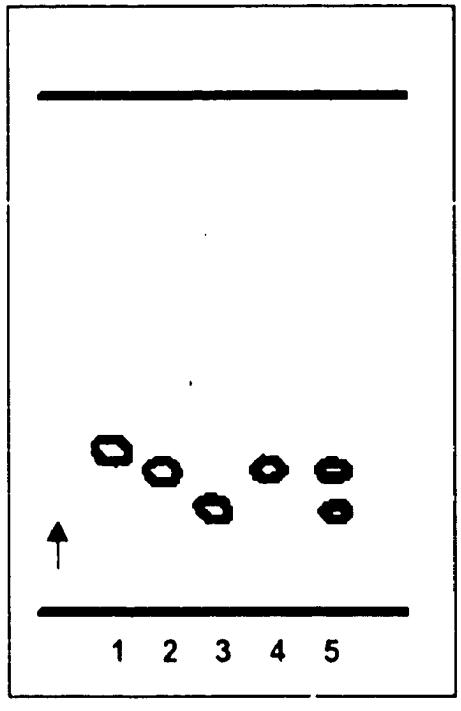


Figure 4.2. TLC Patterns of Active Molecules Isolated from *Ambrosia*. Lane 1, is compound I (coronopilin); lane 2, and 4, compound II (parthenin); lane 3, compound III; lane 5, mixture of compound II and III. Analytical detection of active compounds was made by staining the silica plate with Liebermann spray . Compound I gave a yellow color, compound II and III gave a purple-brown color. When observed under UV, compound I showed a light blue color at 365 nm, compound II and III showed a brown color at 254 nm wavelenght.

Instead of resuspending the aqueous lyophilized extract in MeOH:EtOH, a minimal volume of pure methanol was used. Compounds were separated by TLC using the solvent system acetone:methanol:chloroform (2:4:94; v/v/v). With the variation of this method, only compound I and compound II were visible. However, their yield was higher with a final yield of 6 g/kg for compound I and 12 g/kg for compound II of the initial ground material. The high content of these active compounds suggests that they could be involved in several esential roles in *Ambrosia*. The ratio of compound II to compound I in *Ambrosia psilostachya* was always 2 : 1 when using this method for their isolation.

Several solvent systems were used for analytical TLC separations of isolated compounds to confirm their purity. TLC isolated compounds were dried under nitrogen and each of them was tested for biological activity using the *M. incognita* nematode assay. Purified active compounds were subjected to NMR and mass spectrometry to elucidate their structures.

4.3.1.2 *NMR profiles of Parthenin and Coronopilin*

Two alternative methods (TLC, HPLC) were used to isolate active compounds from *Ambrosia* in pure form for NMR analysis. After NMR and laser mass spectral analysis isolated molecules were identified as

sesquiterpene lactones. The elucidation of structures was performed by Schmitz and Li in the Dept. of Chemistry, University of Oklahoma. The structure of compound II was elucidated by ^{13}C NMR (Figure 4.3) and H-H COSY (Figure 4.4). The spectral results were consistent with the conclusion that compound II was parthenin. Analysis of the molecular mass by mass spectrometry gave a molecular mass of 262. The latter supports the structure shown in Figure 4.5. The figure shows parthenin forming an adduct with sodium giving an apparent MW of 282.

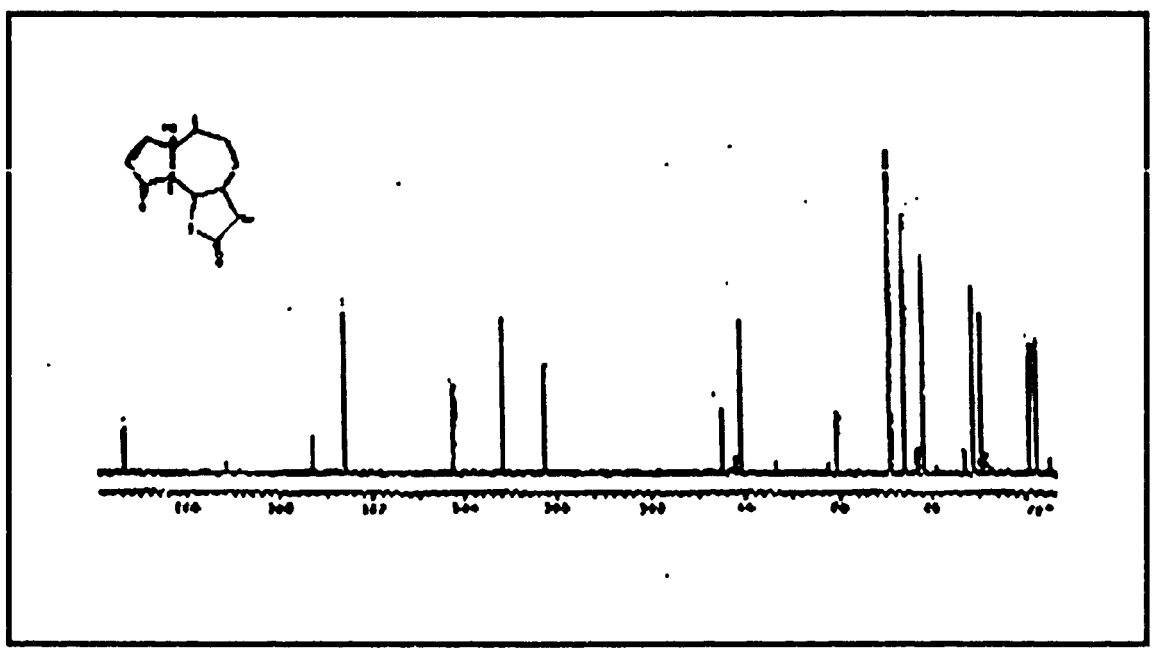


Fig. 4.3. ^{13}C - NMR Spectrum of Parthenin in CD_3OD

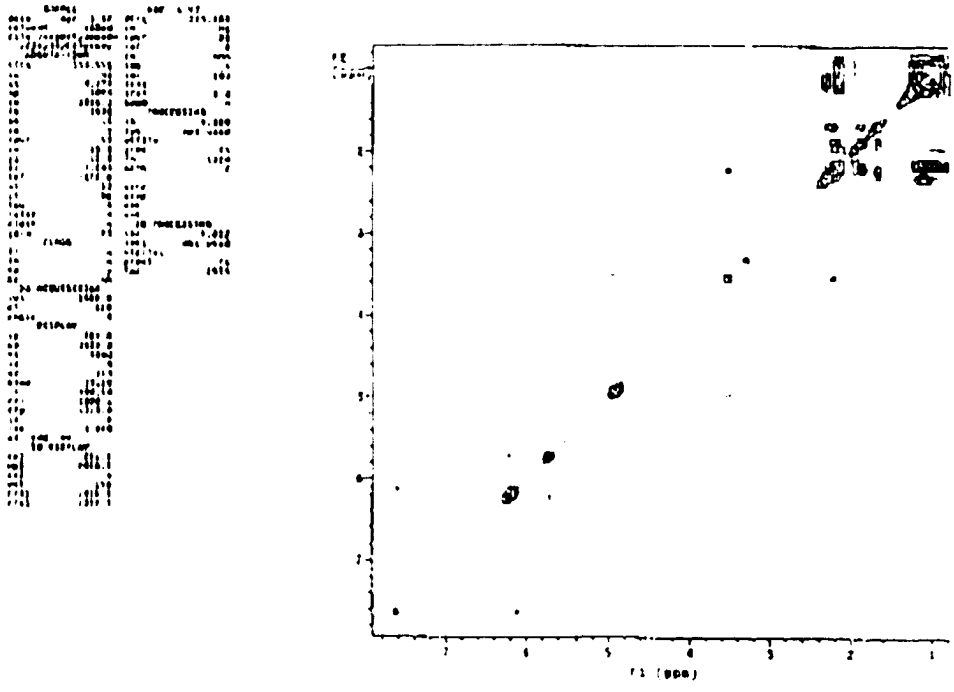


Fig. 4.4. H-H COSY Spectrum of Parthenin

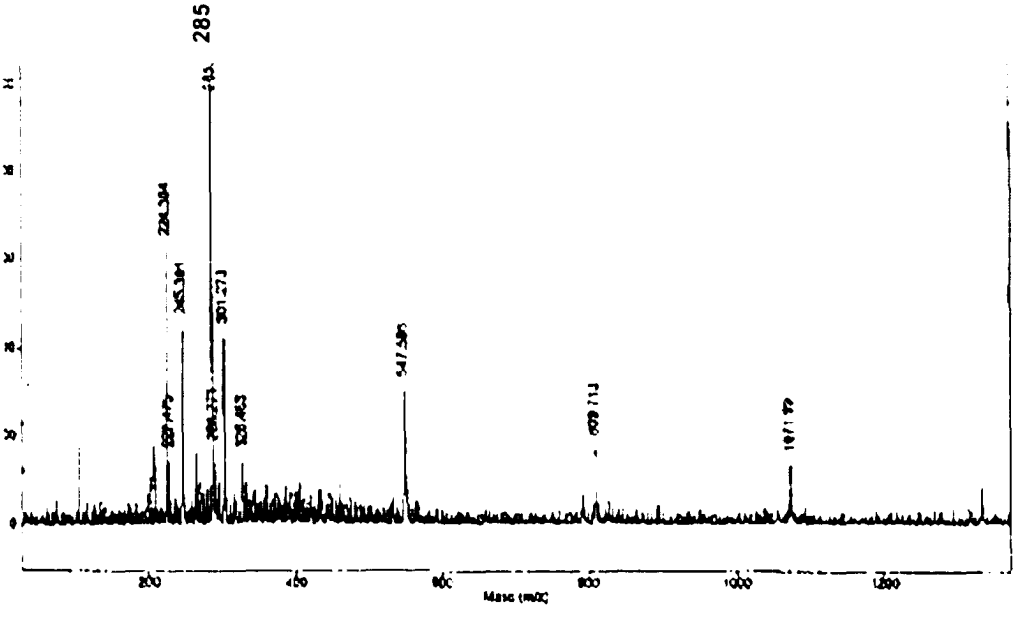
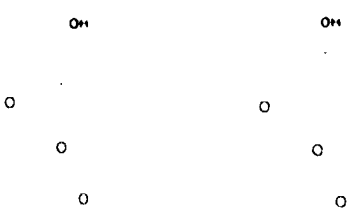


Fig. 4.5. Laser Mass Spectrum of Parthenin. The molecule forms an adduct with Na (MW 23) giving an apparent MW of 285. The MW of parthenin is 262. Dimers (2 molecules of parthenin + Na), trimers, and tetramers were detected with apparent MWs of 547, 809 and 1071, respectively.

Similarly, the structure of compound I was elucidated by 2D NMR, H-C COSY (Figure 4.6). These results indicate that compound I is coronopilin.

Parthenin has a stereoisomer, hymenin. However, both stereoisomers never have been found together in a single plant (Picman *et al.*, 1981ab). In order to confirm the structure and the stereochemistry of parthenin, NMR and TLC patterns were compared with those of authentic samples of parthenin provided by N. Fischer and they were found to be identical. The structure of parthenin and coronopilin are very similar. The only difference is the double bond present in parthenin.



parthenin coronopilin

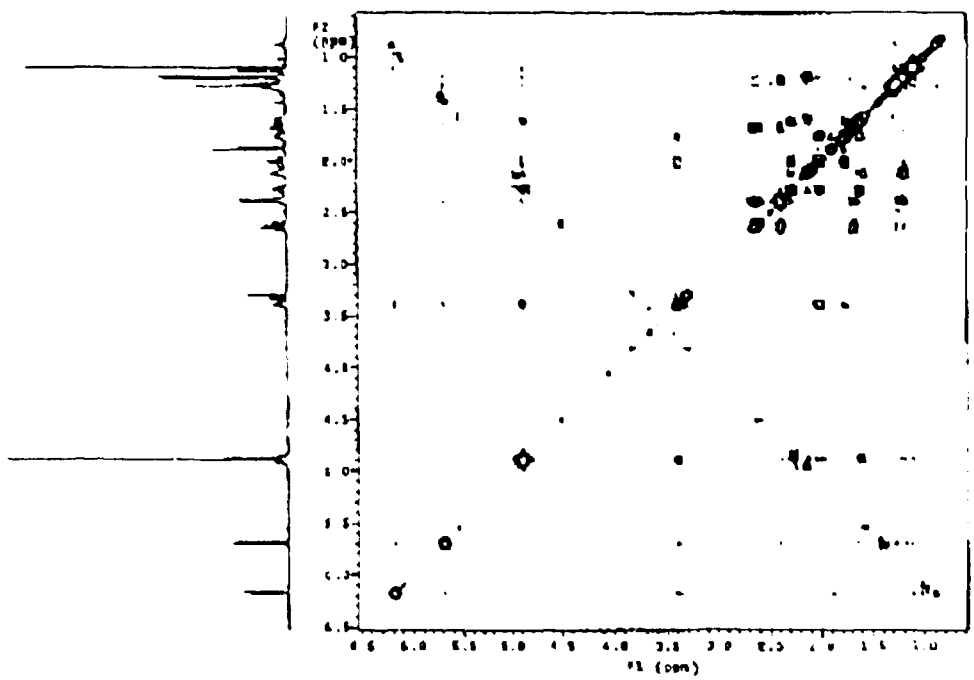


Fig. 4.6. 2D NMR Spectrum of Coronopilin

4.3.1.3 *HPLC Isolation of Parthenin and Coronopilin*

Several solvents were tested for optimal separation of parthenin and coronopilin (data not shown) by HPLC. The UV detector was set at 215 nm. Fifty µl of a methanolic mixture (50 mg/ml) containing parthenin and coronopilin was injected. Parthenin was eluted with a retention time (Rt) of 24 min. and coronopilin had a Rt of 25 min. (Figure 4.7). In order to confirm the activity of parthenin and coronopilin separated by HPLC, a nematode bioassay was performed.

A concentration of 10 mg/ml of parthenin and coronopilin was used in the bioassay with *Meloidogyne* juveniles. Three replicates of each sample were prepared. Distilled water was used as a control. The effects of each compound on nematode survival was observed after 72 hr. Living and dead nematodes were counted and recorded as follows:

Table 4.2. Nematicidal Effect of Parthenin and Coronopilin Isolated by HPLC.

Coronopilin	% Mortality	Parthenin	% Mortality	Control	% Mortality
6 (45)	88	1 (51)	98	(0) 54	0
7 (39)	85	0 (52)	100	(0) 52	0
9 (47)	84	0 (44)	100	(0) 49	0

Juveniles of *M. incognita* nematodes were tested with HPLC fractions. Approximately 50 nematodes were used per well. Living and (dead) nematodes were recorded. The three rows are replicates of each fraction.

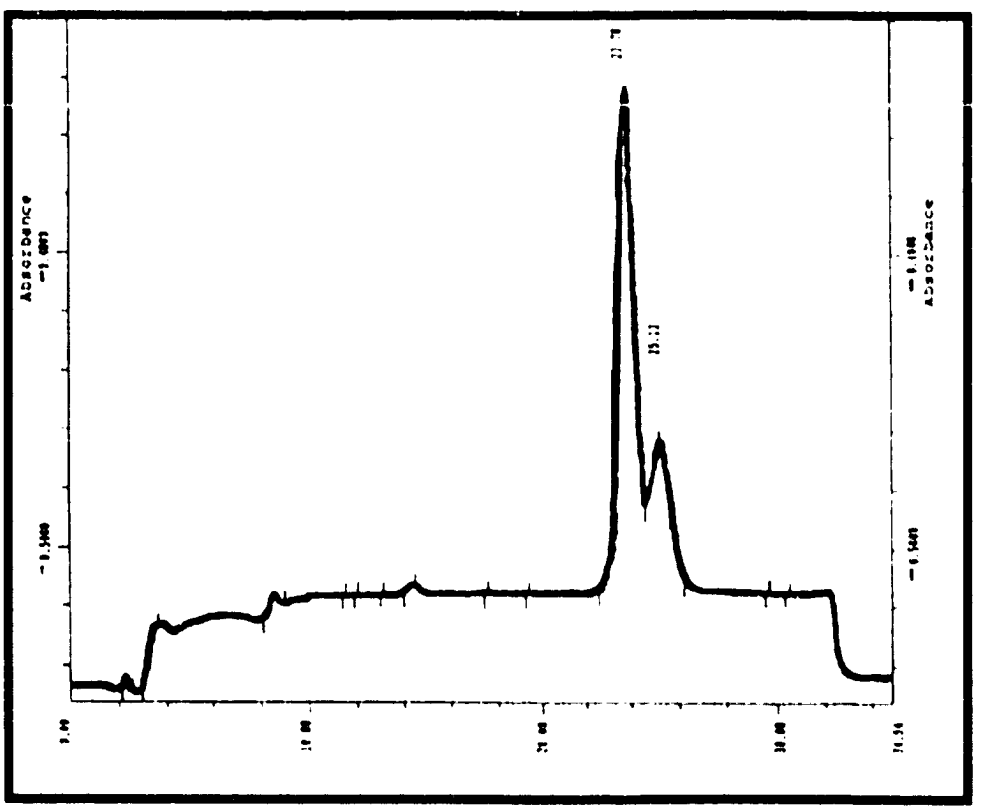
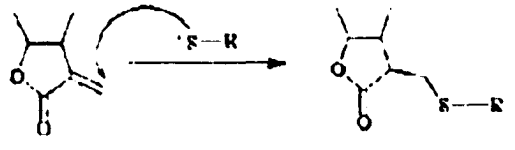


Fig. 4.7 HPLC Separation of Parthenin and Coronopilin. Parthenin was eluted with a Rt of 24 min. and coronopilin with a Rt of 25.4 min. A reverse-phase 10 µ ODS 250x10 mm column was used for the separation. Parthenin and coronopilin were eluted with a stepwise gradient of water:methanol (65:35; v v). Fifty µl of each compound resuspended in methanol was loaded onto the column.

4.3.2 *Biological Activity of Parthenin and Coronopilin*

Parthenin and coronopilin are pseudoguaianolides that belong to a large group of sesquiterpenoid tricyclic lactones. Sesquiterpene lactones are reported to possess several activities with anticarcinogenic activity being the most important (Lee *et al.*, 1975; Imakura *et al.*, 1980; Woynarowski and Konopa, 1981). The activity of sesquiterpene lactones is attributed to their α-methylene-δ-lactone group (Macias *et al.*, 1992). This functional group represents a reactive receptor site for biological nucleophiles, especially thiol and amino groups. A typical reaction of methylene with small molecules or proteins containing a sulfhydryl group is represented as follows:



In our study the activity of parthenin and coronopilin was tested against the plant parasitic nematodes *M. incognita* and against some commercially important insect larvae.

4.3.2.1 *Effect of Different Concentrations of Parthenin and Coronopilin on Meloidogyne incognita.*

Two active natural products from *Ambrosia*, parthenin and coronopilin, were tested against nematodes at different concentrations (1, 2, 5 and 10 mM). Nematodes (50 per well) in 100 µl of distilled water were placed into individual wells of a 96-well microtitre plate. Four replicated samples were used for each treatment. The nematodes were observed under a stereomicroscope after 24, 48 and 72 hours. The nematistatic and nematicidal effects were measured according to Jatala *et al.* (1995). Effects of parthenin and coronopilin at concentrations of 1 or 2 mM were not experimentally distinguishable. However, high mortality was observed when using 5 mM and 10 mM concentrations of parthenin or coronopilin as shown in Figure 4.8. Coronopilin showed higher toxicity than parthenin at 5 mM. However, a concentration of 10 mM caused nearly 100 % mortality for both compounds.

4.3.2.2. *Insect Bioassay*

The effect of total *Ambrosia* extract was tested against *Heliocoverpa zea*, *Trichoplusia ni*, *Spodoptera frugiperda* and *Heliothis virescens* larvae.

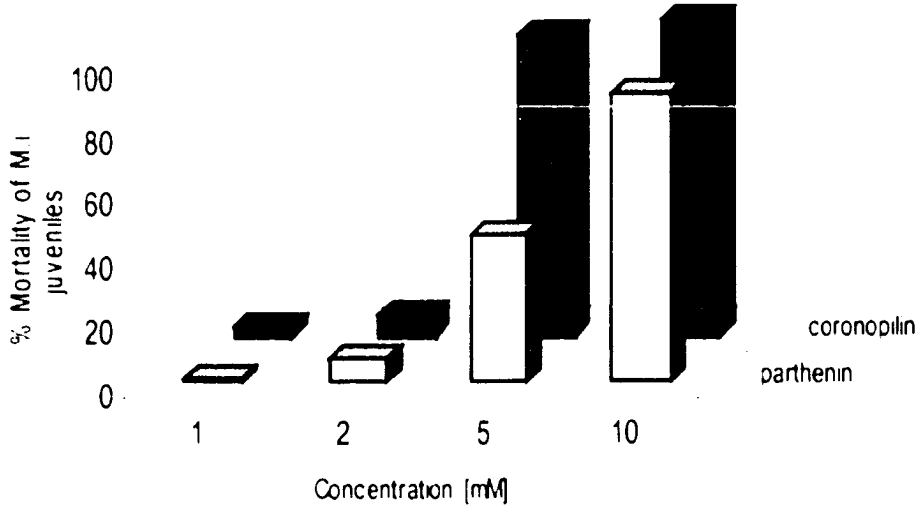


Fig. 4.8. Effect of Different Concentrations of Parthenin and Coronopilin on *M. incognita*. Juveniles were tested with different concentrations of parthenin and coronopilin (1, 2, 5, and 10 mM). Fifty nematodes per well were used. Three replicates of each sample were used. Standard deviations for 1, 2, 5 and 10 mM of parthenin were 0, 2, 5 and 3 and for coronopilin were 1, 1, 1, 0, respectively.

A final concentration of 2 mg/ml was obtained by mixing 100 µl of *Ambrosia* extract [20 mg/ml] with 1 ml of Stoneville diet. When the artificial medium was dry, two insect larvae were placed on the diet surface in each of eight wells. Insects were incubated at 28 °C for 5 days. At this time, larvae were weighed and larval mortality was recorded.

Ambrosia extract exhibited strong antifeedant properties. Larval weight of insects on diets containing crude extract was reduced approximately 50 % when compared to the control. Larvae of *H. zea, T. ni, S. frugiperda, H. virescens* weighed 54 %, 55 %, 47 %, and 64 % compared to the control, respectively (Figure 4.9 a). Similarly, the effect of purified parthenin on insect growth was tested. Neonate larvae of *H. zea, T. ni, Ostrinia nubilalis*, and *S. frugiperda* were used in this bioassay. Four neonate larvae were placed in each well of the artificial Stoneville diet containing 1.6 mg/ml of parthenin. Only *H. zea* larvae were placed 1 per well because of their cannibalism. Sixteen larvae were used for each treatment. The greatest anti-insect effect of parthenin was noticed in assays with *Ostrinia* (ECB). In this case larval weight was reduced by 96 % compared to the control (Figure 4.9 b).

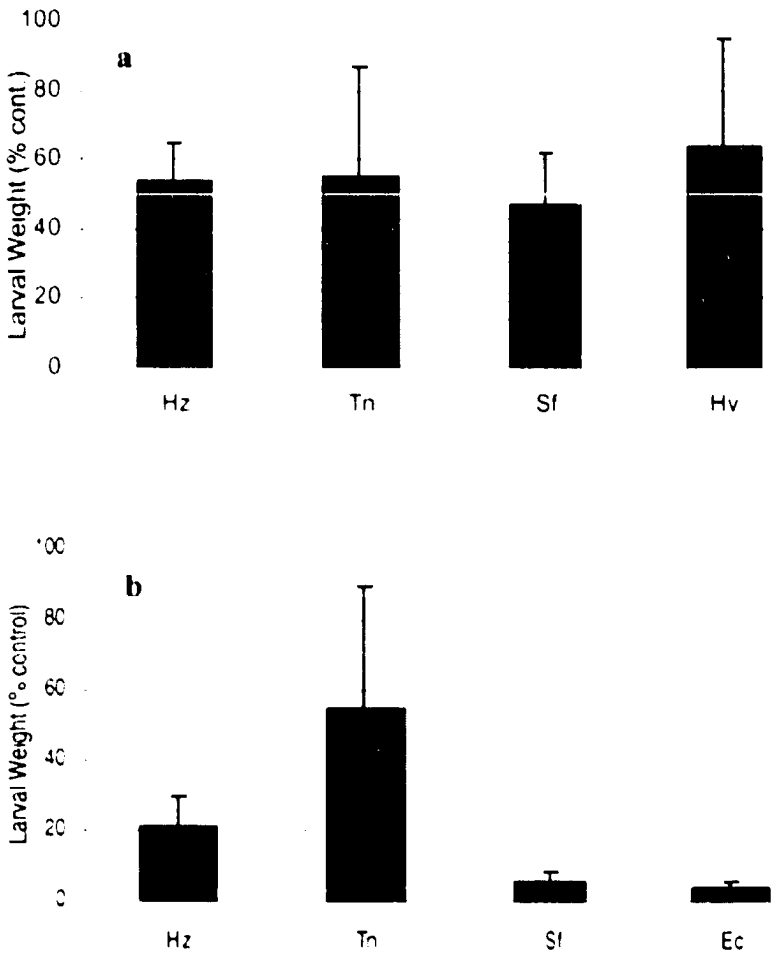


Fig. 4.9. Effect of Crude *Ambrosia* Extract and Parthenin on Insects. Panel a: Effect of *Ambrosia* Total Extract on Insects and Panel b: Effect of Parthenin on Insect Growth (*Heliothis zea*, Hz; *Trichoplusia ni*, Tn; *Spodoptera frugiperda*, Sf; *Heliothis virescens*, Hv; and *Ostrinia nubilalis* [European corn borer], Ec). Bars represent the standard deviation.

4.3.3 *Studies on The Mode of Action of Parthenin and Coronopilin*

Penetration of nematode cuticle is an important prerequisite for the natural compounds to cause nematode death or paralysis. The effectiveness of several nematicides is related to the penetration of the nematode cuticle and their immediate action. Measurement of the rate of respiration (oxygen uptake) is an important indicator of the activity of nematicidal compounds. In order to determine whether parthenin penetrated the nematode cuticle rapidly, causing a change in the rate of respiration, oxygen uptake by *M. incognita* and *C. elegans* was measured in this study.

4.3.3.1 *Respiration Assay*

Plant nematode respiration is affected by many environmental factors. High concentrations of CO_2 seems to be an important factor attracting nematodes to plant roots. In some experiments of nematode respiration using *Ditylenchus pratylenchus* and *Anguina* spp. the rate of respiration was observed for several days in a solution containing glucose (Bhatt and Rohde, 1970). In our experiment the effect of parthenin on *M. incognita* and on *C. elegans* oxygen uptake was observed for a few hours. For our purpose we did not use glucose in order to avoid bacterial contamination and related respiration.

Respiration measurements were performed with an oxygen electrode using juveniles (30,000) resuspended in distilled water and *C. elegans* respiration was measured in a bacteria-free medium to avoid interference due to bacterial respiration. *Ambrosia* total extract was added to a final concentration of 20 mg/ml. Oxygen concentration in the cuvette was reduced by 61 % when just the extract was added. This fact suggests that the extract may contain several molecules which react with oxygen or components of the extract may "poison" the electrode surface. When parthenin resuspended in distilled water was added to a final concentration of 5 mg/ml, the rate of oxygen comsumption by *M. incognita* increased rapidly during the first 10 minutes after addition of parthenin and then it was non detectable (Figure 4.10). The effect of parthenin on respiration was more visible when using *C. elegans* nematodes (Figure 4.11). Parthenin had also a stimulatory effect on nematodes suggesting a rapid penetration through the nematode cuticle.

Based on these results, it seems that the sesquiterpene lactone parthenin penetrated the cuticle of the nematode rapidly reaching endodermic cells, intoxicating the whole nematode system, causing paralysis and death.

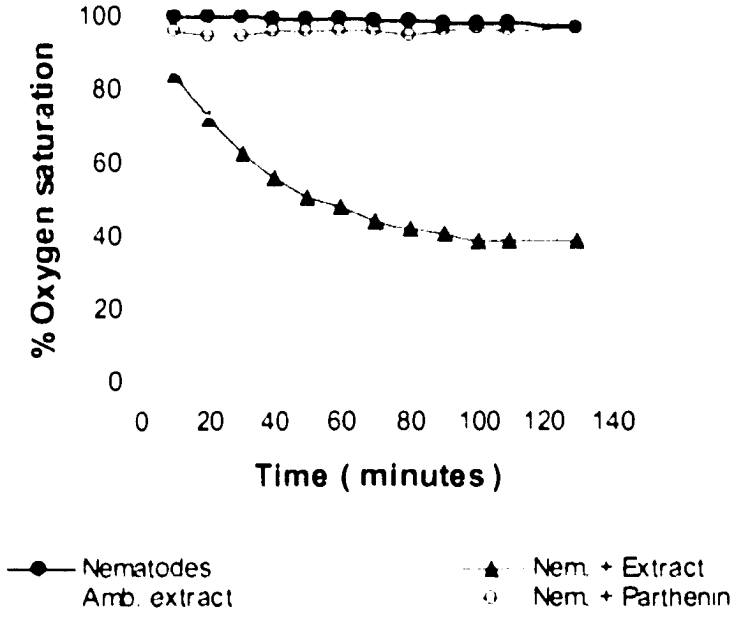


Fig. 4.10 *Meloidogyne incognita* Respiration Assay. Concentrations of 20 mg ml of total extract and 5 mg ml of parthenin were used in the assay. Oxygen uptake was measured for 30,000 *M. incognita* juveniles in dH_2O. Just two replicates with similar results were performed.

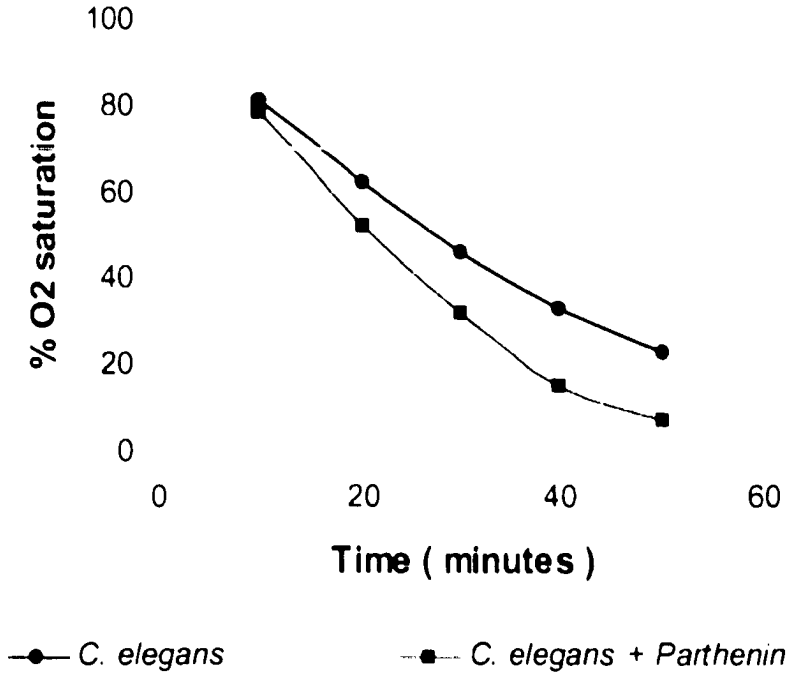


Fig. 4.11. *Caenorhabditis elegans* Respiration Assay. Total extract concentration was 20 mg ml and parthenin concentration used was 5 mg ml. Approximately 30,000 nematodes in bacteria-free medium was used in the assay. Two replicates with similar results were performed.

4.3.3.2 *Comparison of Agglutination Assay and Nematode Bioassay*

Some studies have suggested that the anti-aggregation (agglutination) activity of several sesquiterpene lactones are due to the reaction with sulfhydryl groups of platelets (Heptinstall *et al.*, 1987). The agglutination effect of parthenin was observed using three kinds of red blood cells (rabbit, pig, and human RBC). The agglutination of rabbit RBC was higher than pig and human RBC agglutination when treated with parthenin. The minimum concentration of parthenin that gave a positive result for all three kinds of RBC was 2.5 mg/ml. These results were positively correlated with the results of the nematode assay. The minimal concentration of parthenin that caused 8% nematode mortality was 0.6 mg/ml. The same parthenin concentration gave a positive reaction in the agglutination assay where a fuzzy ring was observed. Similarly, a concentration of 5 mg/ ml of parthenin caused a 100 % mortality of nematodes and this concentration gave a very positive agglutination reaction. Similar results to parthenin were obtained with coronopilin (data not shown). The agglutination test was performed in a plate with round bottom wells to determine serum titers. In the round-bottom cells, RBCs settle to the bottom in a tight button. But when Abs cause hemagglutination, the RBCs form clumps along the side of the well.

Positive samples were observed according to the following parameters:

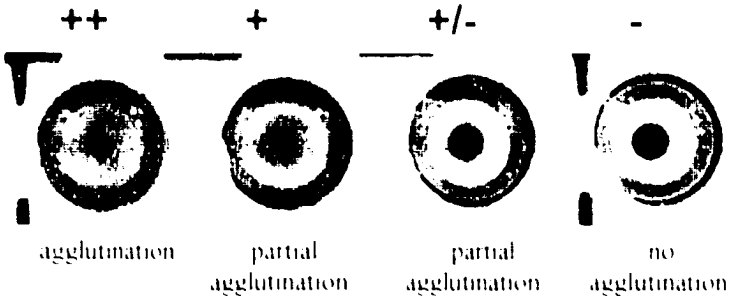


Table 4.3. Effect of Parthenin on Agglutination of Red Blood Cells and on Nematodes

AGGLUTINATION ASSAY						
Red Blood Cells	Parthenin Concentration (mg ml)					
	0.15	0.30	0.60	1.25	2.5	5.0
Rabbit	-	-	+/-	+	+	++
Control	-	-	-	-	-	-
Pig	-	-	-	-	+/-	+/-
Control	-	-	-	-	-	-
Human	-	-	-	-	+/-	+/-
Control	-	-	-	-	-	-

NEMATODE ASSAY						
Nematodes	Parthenin Concentration (mg ml)					
	0.15	0.30	0.60	1.25	2.5	5.0
% Mortality	0	1	8	53	90	100

Parthenin was resuspended in distilled water. Agglutination assay was performed according to Schubert protocol of lectin assay developed in Costa Rica (Appendix I). Approximately 60 juveniles of *M. incognita* nematodes were used per well. Two replicates of each sample were used. Nematode mortality was recorded after 72 hr.

4.3.3.3 *Effect of Cysteine on Nematicidal Activity.*

Several concentrations (0.1, 1, 5, 10 mM) of cysteine alone were tested to determine whether cysteine had any effect on nematodes. These concentrations had no adverse effects on nematodes. Then, samples with each of these cysteine concentrations were mixed separately with 10 mM parthenin or 10 mM coronopilin. The nematicidal effect of parthenin was reduced from 95 % to 5 % and coronopilin was reduced from 83 % to 2 % after mixing with 10 mM cysteine (Fig. 4.12). The reactivity between parthenin, coronopilin and cysteine seems to be very high since the reduction in nematicidal activity of parthenin or coronopilin was more than 90 % when mixing equimolar quantities of each of them with cysteine.

4.3.3.4 *HPLC Analysis of Cysteine-Parthenin Adduct*

The nematicidal activity of sesquiterpene lactones, parthenin and coronopilin isolated from *Ambrosia psilostachya* was suggested to be due to their immediate reaction with cysteine-containing proteins that play a key role for nematode survival. This hypothesis of action was supported by HPLC analysis of the formation of a parthenin-cysteine adduct after mixing cysteine with parthenin.

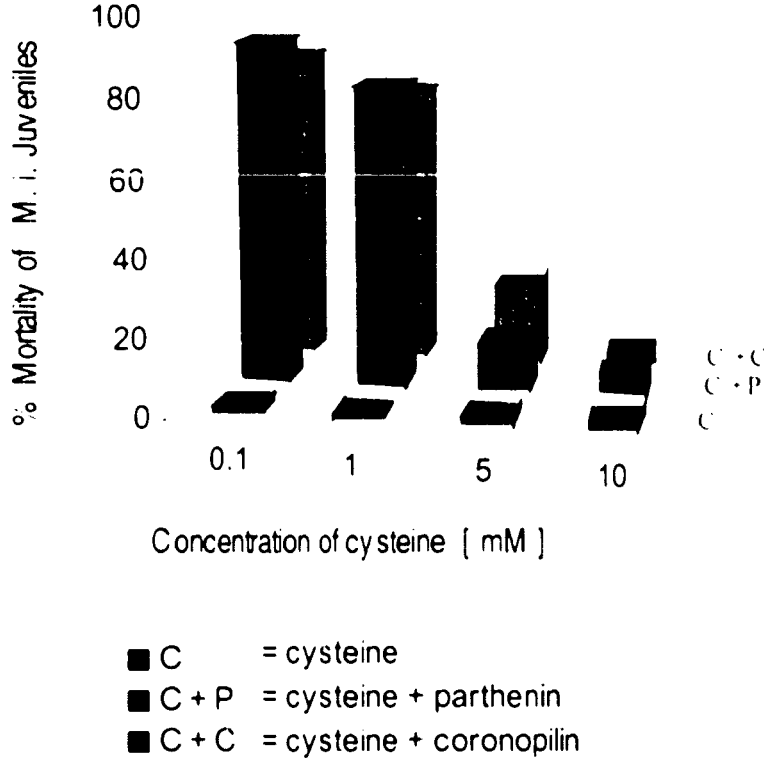


Fig. 4.12. Effect of Cysteine on Nematicidal Activity of Parthenin and Coronopilin. Nematodes were incubated with varying concentrations of the amino acid cysteine with or without 10 mM parthenin or coronopilin. Nematode mortality was recorded after 72 hr. of treatment. The values presented are the average from 4 replicates with approximately 50 nematodes well according to the standard bioassay described above. Standard deviations for 0.1, 1, 5 and 10 mM of cysteine, coronopilin-cysteine and parthenin-cysteine were 2, 0.6, 1, 1; 6, 8, 4, 2 and 4, 5, 3, 3, respectively.

Cysteine (0.02 M) and parthenin (0.01 M) were mixed and incubated at room temperature. Samples were injected into an HPLC after 30 min and 60 min of incubation. A sample containing only parthenin (peak 1) was injected as a control with a Rt = 25.2 min. A new peak with a retention time Rt = 7.8 min. (peak 2) appeared after mixing parthenin with cysteine. The appearance of a new peak after mixing cysteine and parthenin and the reduction in size of the parthenin peak suggests that cysteine reacts to form an adduct with parthenin. Figure 4.13 shows the HPLC profile of the mixture.

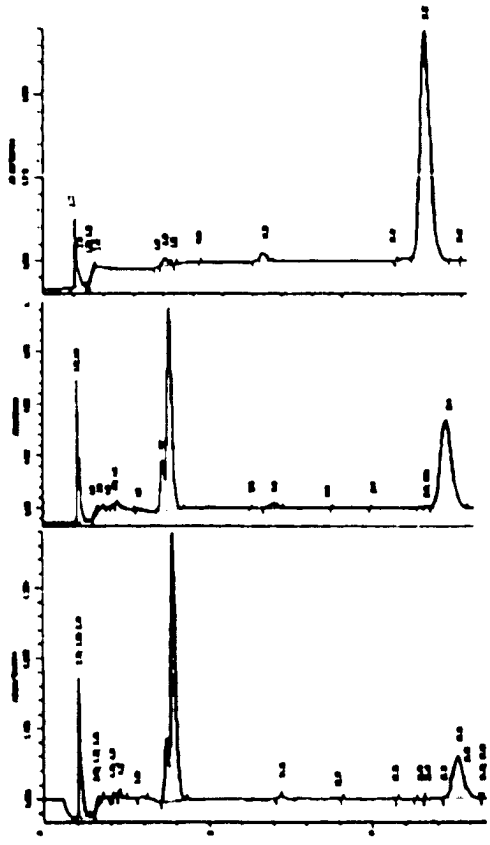
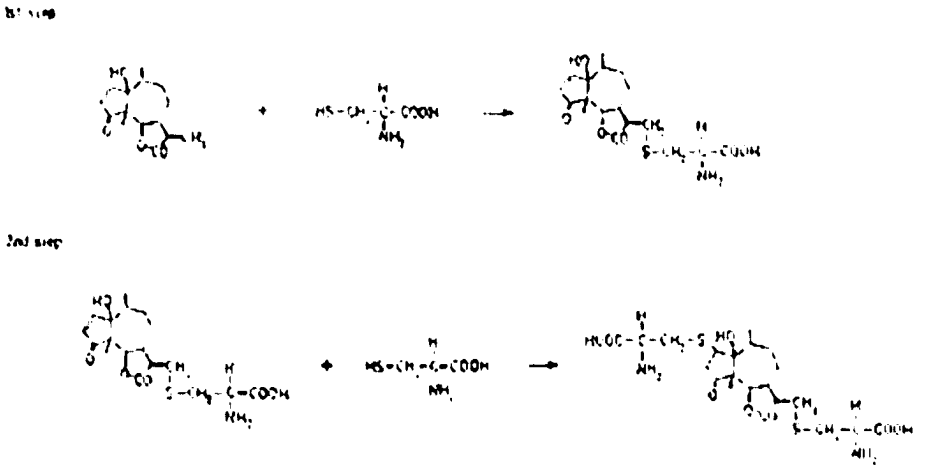


Fig. 4.13. HPLC Profiles of the Adduct Between Cysteine and Parthenin. Concentrations of 0.02 M cysteine and 0.01 parthenin were mixed and incubated for three different times (0, 30, 60 min) for HPLC analysis. a) Mixture at 0 min. b) Mixture incubated for 30 min. c) Mixture incubated for 60 min. 25 µl of each sample, cysteine and parthenin were used in this experiment.

4.3.4 *Proposed Mode of Action of Parthenin*

Results observed in HPLC profiles and nematode bioassay with the equimolar mixture of parthenin-cysteine suggest the formation of an adduct between these two molecules. It has been confirmed that sesquiterpene lactones such as parthenolide bind and form adducts with cysteine through the exocyclic methylene group (Hay *et al.*, 1994). It is possible that a similar reaction occurs *in vivo* with cysteine-containing proteins or with other reactive groups. This proposed mechanism of reaction between parthenin and cysteine is supported by Pieman (1981ab). The activated methylene group of sesquiterpene lactones is susceptible to nucleophilic attack via Michael addition (Groenewegen *et al.*, 1986). The following scheme describes a proposed mechanism of parthenin reaction with proteins.



4.4 Discussion

Many of the traditional pesticides such as methyl bromide, dichloropropene, and dibromoethane used to control plant parasitic nematodes are under review by the Environmental Protection Agency (EPA) (Brown and Kerry, 1987). There is a general interest in replacing these synthetic pesticides with inexpensive and safe biological nematicides to insure a safe and adequate food supply for the future. To date, although there are many reports showing nematicidal or nematistatic activities in various plants, very few of the actual molecules responsible for activity have been identified and characterized.

Our results show that parthenin and coronopilin, sesquiterpene lactones isolated from *Ambrosia*, have nematicidal activity. Besides, parthenin and coronopilin, *Ambrosia* sp. contain a number of other sesquiterpene lactones that are used for taxonomic identification (Miller *et al.*, 1968). Sesquiterpenes can be detected by analytical TLC. However, it is impossible to distinguish parthenin and coronopilin with vanillin reagent because their Rfs are very close. For this reason we used Liebermann spray that proved to clearly distinguish between the two compounds. Other populations or species of *Ambrosia* may have or not have the same

terpenes. Potter and Mabry in 1972 reported that for 20 populations of *A. psilostachya* only 5 populations contained parthenin and coronopilin.

Parthenin and coronopilin increased nematode mortality at concentrations of 5 mg/ml or greater. Parthenin stimulated nematode respiration immediately after adding this compound to the media containing nematodes. The immediate response to parthenin suggests that parthenin penetrates through the cuticle. The reduction of the nematicidal effect when adding cysteine suggests that the lethal effect of parthenin and coronopilin on nematodes may be due to an interference with sulfhydryl groups present in essential enzymes and proteins. It is probable that *in vivo* parthenin reacts with esential cysteine-containing proteins following the mechanism proposed by Picman.

Biological activities such as antitumor activity of parthenin and coronopilin have been investigated by many authors (Heathcock *et al.*, 1982; Isman and Rodriguez, 1983; Sharma and Bhutani, 1988) but nothing has been reported about their nematicidal properties. These sesquiterpenes are smaller and simpler than many other nematicides. This makes them interesting for further investigation.

4.5 Conclusions

- Three nematicidal secondary metabolites were isolated using bioactivity-directed TLC fractionation. The structures of two of them, parthenin and coronopilin, were elucidated by NMR and by mass spectrometry. The structure of the third one that exhibited the best activity could not be elucidated because of its high apparent reactivity with oxygen, which caused the chemical alteration of this compound.
- Parthenin and coronopilin are known molecules and their antitumor, antibacterial, fungicidal, molluscicidal and insect antifeedant activities have been reported (Heathcock *et al.*, 1982; Isman and Rodriguez, 1983; Sharma and Bhutani, 1988). However, this is the first report of their nematicidal activity.
- Parthenin stimulated respiration in *M. incognita* and *C. elegans*. The immediate response of nematodes suggests that parthenin penetrates through the cuticle rapidly.
- The nematicidal effect of parthenin was reduced 95% by mixing the compound with equimolar concentrations of cysteine. This suggests that the lethal effect of parthenin on nematodes may be due, at least in part to reaction with sulfhydryl groups present in essential enzymes and proteins or other reactive groups.

CHAPTER V: Isolation and Partial Characterization of Trypsin Inhibitors from *Cassia fruticosa* and *Mucuna holdii*

5.1 Introduction

Different species of *Mucuna* and *Cassia* contain biologically-active compounds and are commonly used as food by many native peoples. *Mucuna* species are commonly known as velvetbean (Udedibie and Carlini, 1998). Members of this genus have been reported to be immune to root-knot caused by nematodes (Buckles, 1995). *Mucuna* is cultivated in several countries such as India, Sumatra, Mexico, Nigeria, Ghana, and the United States (Siddhuraju *et al.*, 1996). In Mexico this plant is known as nescafé and teas made from this plant are consumed as a daily beverage (Buckles, 1995). It was reported that plants of *Mucuna deeringiana* possesses factors that reduced the gall index and infection of tomato by *M. incognita* (Kloepper *et al.*, 1991).

Two secondary metabolites with nematicidal effects against *M. incognita* juveniles were isolated from *Mucuna aterrima* leaves and stems. These nematicidal natural products were triacontyl tetracosanate and triacontanol

(Nogueira *et al.*, 1996). There are several reports about *Mucuna pruriens* (L.) DC. (Fabaceae) cv. "Nescafé" ("Nescao"). Toasted ground seeds of this plant are used as a coffee substitute. Plants of this genus are believed to possess anodyne, antidotal, aphrodisiac, diuretic, nervine, resolvent, rubefacient and vermifuge properties and the plants are used to treat anasarca, asthma, cancer, cholera, cough, diarrhea, dogbite, dropsy, diabetes, insanity, mumps, Parkinson, ringworm, snakebite, fever, sores, syphilis, tumors and worms (Akhtar *et al.*, 1990; Iauk, 1993; Houghton and Skari, 1994; Manyam and Sánchez-Ramos, 1999). Some Indian tribes consume the hairs of the pods for their vermifugal effect (Vasudeva and Shanpru, 1981).

In our study, we used seeds from *M. holdii* as a source of novel trypsin inhibitors. So far, there are no reports of the antitryptic activity of this plant. The goal of this work was to isolate the proteins responsible for trypsin inhibition. There are many reports about the safety of consuming *Mucuna*. For example, Janardhanan (1982) reported that the consumption of seeds from *Mucuna utilis* is safe to many tribals. Udedibie and Carlini (1998) observed that proteins isolated from seeds of *M. pruriens* failed to agglutinate human red blood cells. This fact could explain why this plant is

used as an anti-inflammatory agent. Trypsin inhibitors contained in seeds are sometimes considered as antinutritional factors in plants. However, they can be inactivated by heating or reaction with reducing sugars at 37 °C and at 50 °C (Kato and Matsuda, 1997). Since sugars are main components in daily food, consumption of trypsin inhibitors in their raw form is usually safe.

Another plant used as a source of trypsin inhibitors was *Cassia fruticosa*. Its seeds were used as well to isolate trypsin inhibitors. Several authors have reported the presence of different biological activities present in *Cassia* species. Besides its biological activities, it is interesting to mention that *Cassia* is known to be a holy plant. Moses received instructions to use this plant with other selected plants in the making of holy compounds especially composed for the Lord (King, 1941).

C. occidentalis roots have been reported to possess antibacterial activity, mainly against *Salmonella typhi* (Perez and Anesini, 1994). Besides its antibacterial activity, *C. occidentalis* possesses anti-inflammatory activity (Kuo *et al.*, 1996). *C. abbreviata* has been reported to possess antimalarial activity (Connelly *et al.*, 1996). Extracts of *Cassia* have also shown anti-

insect activity. For example, *C. auriculata* was found to have antifeedant properties against Hadda *Henosepilachna vigintioctopunctata* (Sreedevi *et al.*, 1993). Some *Cassia* species have been found to possess activity against plant parasitic nematodes. *C. fasciculata* has been used in crop rotation to control *Meloidogyne* nematodes with positive results (Rodriguez-Kabana and Canullo, 1992). Another example is *C. fistula*. Aqueous extract of flowers of this plant exhibit nematicidal effects on *M. incognita* (Sharma and Trivedi, 1994).

Schubert has found insect antifeedant activity in the tropical plants, *Cassia fruticosa* and *Mucuna holdii* collected from the rainforests of Costa Rica and suggested that this activity might be due at least in part to trypsin inhibitors present in these plants. The presence of trypsin inhibitor activity was confirmed in both plant extracts as a part of this work. Trypsin inhibitors were purified by affinity chromatography, ion exchange chromatography and these inhibitors were partially characterized.

5.2 Materials

Trypsin (TPCK treated) from bovine pancreas (Cat. No. T-1426), α-Chymotrypsin (TLCK treated) (Cat. No. C-3142), N-benzoyl-L-tyrosine p-

nitroanilide (BTNA) (Cat. No. B-6760), N-benzoyl-DL-arginine p-nitroanilide (BAPNA) (Cat. No. B-4875), polyvinylpolypyrrolidone (insoluble PVP, Cat. No. P-6755), potassium phosphate, NaCl, betha-mercaptoethanol (β-ME), tricine, flavin mononucleotide, crocein scarlet, bromophenol blue, trypsin-chymotrypsin inhibitor (BBI, Cat. No. T-9777), N-acetyl-dl-phenylalanine-β-naphtyl ester, and tetrazotized ortho-dianiside were purchased from Sigma Chemical Co. St. Louis, Missouri. CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech, Uppsala, Sweden. Tris-hydroxymethyl-aminomethane (Tris) and sodium dodecyl sulphate (SDS) were purchased from Research Organics Inc., Cleveland, Ohio. Centricon tubes with 10,000 MWCO were purchased from Amicon, Millipore Corporation, Bedford, MA. Acrylamide, N-tetramethylethylene-diamine (TEMED), ampholytes for isoelectric focusing (Bio-Lyte 8/10; 3/5; 5/8), protein markers and ammonium persulfate (APS) were purchased from Bio-Rad Laboratories, Richmond, California. 96-well flat bottom microtitre plates were purchased from Becton Dickinson & Company, Lincoln Park, New Jersey. N,N-dimethylformamide, Commassie Brilliant Blue (CBB-G-250) and other reagent grade chemicals were purchased from Fisher Scientific, Fair Lawn, New Jersey. Miracloth was purchased from Calbiochem, La Jolla, California.

5.3 Methods

5.3.1 *Preparation of Plant Crude Extract*

Seeds of *Cassia* (3.8 g) and *Mucuna* (15.7 g) were homogenized in 5 volumes of 10 mM sodium phosphate buffer, pH 7.5, containing 0.1 g insoluble PVP per g seed weight. The homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged at 15,000 rpm for 15 min. at 4 °C. The supernatant fluid was removed and filtered again through Miracloth. This crude extract was used for trypsin inhibitor assays and nematode bioassays. Both extracts appeared brownish color.

5.3.2 *Dialysis*

In both samples the crude extract was dialyzed against 4 liters of 10 mM sodium phosphate buffer, pH 7.5, using Spectra/Por dialysis membranes with a 3,500 MWCO. The buffer was changed five times every 5 hours. After dialysis, the extracts were centrifuged at 15,000 rpm for 15 min. The volume of the supernatant was measured and this material was saved as crude dialyzed extract. The pellet was discarded. This supernatant was used for assay and for further purification by trypsin affinity chromatography.

5.3.3 *Affinity Chromatography*

This method is based on the reversible interaction between molecules and it is a powerful tool for purifying proteins. Trypsin inhibitors were isolated from crude dialyzed seed extracts based on their binding to trypsin. The trypsin affinity column (TAC) was prepared as described in Appendix I.

The TAC column was equilibrated with 10 mM Na phosphate buffer, pH 7.5. A 15-ml column with a diameter of 1.5 cm was used. Before loading the column with the sample, 10 ml of each extract was diluted twice with the same buffer and centrifuged at 10,000 rpm for 10 min. After loading the sample, the column was washed with 7 volumes of 10 mM Na phosphate buffer to remove the unbound proteins. The trypsin inhibitors were eluted step-wise using the following solutions:

1) 0.5 M NaOAc + 0.5 M NaCl, pH 7.0 50 ml

2) 0.5 M NaOAc + 0.5 M NaCl, pH 5.0 50 ml

3) 0.5 M NaOAc + 0.5 M NaCl, pH 3.0 70 ml

4) 0.5 M NaOAc + 0.5 M NaCl, pH 2.2 70 ml

A total of 80 3-ml fractions were collected. Protein elution was monitored by measuring the absorbance at 280 nm. Active fractions were identified using the spectrophotometric trypsin inhibition assay and by the trypsin in-gel assay. After affinity separation, active fractions were pooled, dialyzed against 10 mM Tris, pH 8.0, and further purified by anion exchange chromatography on a Pharmacia Resource Q (RSQ) column using a Pharmacia FPLC system.

5.3.4 *Ion-exchange Chromatography (FPLC)*

Partially purified trypsin inhibitors were further separated by Fast Protein Liquid Chromatography (FPLC), using a RSQ anion-exchange column. The column was equilibrated with 10 mM Tris, pH 8.0. Before injecting 2 ml of the active fraction from the affinity column, the sample was centrifuged at 10,000 rpm for 5 min. in a microcentrifuge at room temperature.

Several elution programs with different gradients (data not shown) were tested to optimize the separation of active proteins from *Cassia* and *Mucuna*. The programs with the best separation for each sample are summarized in the following tables:

Table 5.1. FPLC Program to Separate Trypsin Inhibitors from *C. fruticosa*

Volume [ml]	Time duration	% 10 mM Tris	% 10 mM Tris + 1M NaCl
0	5	100	0
5	5	92	7.7
10	25	92	8
35	2	88	12
37	2	0	100
39	4	0	100
43	1	100	0
44	3	100	0

Table 5.2. FPLC Program to Separate Trypsin Inhibitors from *Mucuna*

Volume [ml]	Time duration	% 10 mM Tris	% 10 mM Tris + 1M NaCl
0	5	100	0
5	15	98	2
20	10	95	5
30	2	95	5
32	3	0	100
35	5	0	100
40	2	100	0
42	3	100	0

The flow rate used to separate *Cassia* trypsin inhibitors was 1 ml/min and 0.75 ml/min for *Mucuna*. Around twenty fractions of 2 ml each were eluted from the column using a NaCl gradient from 0 to 1 M in Tris buffer. Aliquots of fractions were used for the in-gel assay and the

spectrophotometric trypsin assay to identify the active fractions. Fractions with activity were pooled and dialyzed against 10 mM Tris, pH 8.0. Aliquots of 2 µl and 10 µl of active fractions were used to determine the protein concentration by the BCA assay. Finally, purified active fractions were dialyzed against distilled water and aliquots were taken for IEF and MALDI-TOF mass analysis. All *Cassia* and *Mucuna* samples were stored frozen at –20 °C.

5.3.5 *SDS-PAGE*

SDS-PAGE was used during the trypsin inhibitor isolation to assess the purity and to estimate the molecular weight of trypsin inhibitors. Gels of 15% acrylamide were prepared according to Laemmli (1970) using 20x20 cm gels in a vertical chamber. Samples were run with a low constant voltage of 80 V to avoid overheating and inactivation of trypsin inhibitors. Gels were run for approximately 4 hours. Samples with and without β-ME and with and without heating were run. Parallel gels were run at the same time, one for staining with Coomassie Brilliant Blue (CBB) followed by silver staining and the second one for the in-gel activity assay.

5.3.6 *Tricine-SDS PAGE*

Tricine-SDS polyacrylamide gel electrophoresis was necessary to separate peptides from *Mucuna* FPLC purified fractions. Small proteins (<20,000 Da) are separated more effectively on a 16.5 % gradient Tricine-SDS gel according to Schagger and von Jagow (1987) (see Appendix I for details). The estimated subunit molecular weight of the trypsin inhibitors was determined under native and denatured conditions. Proteins was detected by staining with Coomassie Brilliant Blue R-250 and silver staining. Parallel gels were run for trypsin in-gel activity assays. Solutions and gel preparation for tricine-SDS electrophoresis are described in Appendix II.

Twenty microliter (2 µg) of each FPLC purified *Mucuna* fraction was mixed with 6 µl of 4X sample buffer and incubated for 30 min. at 40 °C. The gel was run at 90 V for 16 h at room temperature.

5.3.7 *Native PAGE*

Under non-denaturing conditions, proteins are separated in an electric field on the basis of both charge and mass. If the protein consists of multiple sub-units of different molecular sizes, purity is confirmed by detecting a single band after gel electrophoresis under non-denaturing conditions.

The native PAGE was prepared according to Laemmli (1970), with 15% acrylamide without using SDS. The running and the sample buffer were prepared without SDS and without β-ME as well. Two µg of each sample was loaded on the gel. A lower voltage was used (approx. 70 V) during the separation process.

5.3.8 *IEF-PAGE*

Homogeneity and presence of possible isoinhibitors was determined by isoelectric focusing. Samples were electrophoresed on thin layer polyacrylamide gels containing ampholytes. The migration distances of the pI markers from the anode were measured and plotted against the pI of the markers. The migration distance of the stained trypsin inhibitor bands from the anode was measured and their isoelectric point (pI) was calculated by extrapolation from the standard curve.

A BioRad mini-IEF apparatus was used for determination of the isoelectric points of isolated inhibitors. The IEF gel was prepared according to the Bio-Rad protocol. The composition of the gel follows: 1 ml acrylamide solution (25% acrylamide; 3% bisacrylamide), 1 ml glycerol, 0.5 ml Bio-lyte 8/10 and deionized H_2O to a final volume of 5 ml. Finally, 5 µl of 10%

ammonium persulfate, 50 µl of 0.1% FMN, and 5 µl TEMED were added. After mixing all the ingredients, this gel solution was poured into a horizontal chamber and was polymerized for about 30 min. One µg of each sample resuspended in distilled water was loaded in the center of the IEF gel. The gel was run at 100 and 200 volts for 15 min each. Finally, the voltage was increased to 450 volts and the gel was run for 1 hour. Proteins were first stained with crocein scarlet and then silver stained. A parallel IEF gel was stained to detect trypsin inhibitors using the in-gel assay.

5.3.9 *In-gel Activity Assay*

In-gel activity assay was performed according to Uriel and Berges (1968). After electrophoretic separation, the gel was washed with 0.1 M potassium phosphate buffer, pH 7.0. Then, the gel was incubated in 150 ml 0.1 M K-phosphate buffer, pH 7.0, containing 50 mg of bovine trypsin at room temp for 30 min. with gently shaking. The trypsin solution was discarded and substrate solution containing 20 mg acetyl-dl-phenylalanine-b-naphtyl ester dissolved in 10 ml of N'N-dimethylformamide and 100 ml 0.05 M potassium phosphate buffer, pH 7.0 containing 40 mg tetrazotized ortho-dianiside was added to the gel. The gel was incubated with gentle shaking until the activity bands were detected. After approximately two hours of

incubation the reaction was stopped by discarding the substrate solution and adding a solution with 2% acetic acid containing 4% glycerol. The gel was transferred to a plastic bag and sealed.

5.3.10 *Trypsin Inhibition Assay*

Assays were carried out using a Beckman DU7500 diode array spectrophotometer. Data were recorded at 410 nm with a 30-second interval for 5 min. using disposable cuvettes. The assay was linear for the 5 min. period. The reaction mixture was prepared by adding the following to each cuvette: 1 ml of 0.1 M Tris buffer, pH 8.0, containing 5 µl of trypsin [5 mg/ml]. Next, 20 µl of the protein sample was added and incubated for 5 min. at room temperature. After preincubation, 40 µl of the substrate p-BAPNA in DMSO [40 mg/ml] was added to a final concentration of 4 mM. After mixing, the reaction mixture was incubated for another five min. before recording the change in absorbance. Different concentrations (0.4 – 12 µg/ml) of inhibitor were used to determine the amount of inhibitor needed to reduce the rate reaction by 50% (50% inhibition, I_{50}). Three replicates of each reaction were performed and values were expressed as percent of inhibition compared to the rate for the control.

5.3.11 *Nematode Assay*

The effect of *Mucuna* and *Cassia* trypsin inhibitors on nematodes was tested using the *C. elegans* bioassay. Nematodes were cultivated in a bacteria-free liquid medium according to the method described in 4.3.1. Fifty microliters of medium containing approximately 20 nematodes were added to a ELISA microtitre plate. FPLC purified proteins from *Mucuna* and *Cassia* were resuspended in distilled water and a volume of each was added to each well to give a final concentration of 13 µg/ml. Nematodes were observed under the stereoscope after 24 h. Dead and living nematodes were counted and recorded. Data was expressed as percentage of mortality.

5.3.12 *Protein Determination (BCA)*

It is important to calculate the amount of protein in the sample to determine specific activities. The quantity of a protein is commonly determined by colorimetric methods such as Bradford or Lowry. Both methods include the generation of a standard curve of a standard protein, usually bovine serum albumin (BSA). In our experiments we preferred to use the bicinchoninic acid (BCA) method. This method is accurate and has fewer interferences. The concentration of purified trypsin inhibitors and protein concentration of total extracts were measured according to the Pierce Micro BCA assay

protocol (PIERCE, Rockford, Illinois). A set of protein standards was prepared using BSA (from 0.5-20 µg/ml). Dilutions of 100x and 500x were prepared for each sample. After adding 1 ml of working reagent, the samples were incubated at 60 °C for 1 hr. Then the samples were cooled to room temperature for 5 min. Finally, the absorbance at 562 nm was measured. A standard curve was prepared by plotting the net absorbance vs. known protein concentrations. Concentrations of unknown protein samples were determined by extrapolation using the BSA standard curve.

5.3.13 *Characterization of Trypsin Inhibitors from Mucuna and from Cassia*

A partial characterization of trypsin inhibitors from *Mucuna* and from *Cassia* was performed. Purity, molecular weight and isoelectric point were determined for the purified trypsin inhibitors. Electrophoretic methods were used to determine the purity and their molecular weight was determined using SDS PAGE and MALDI-TOF mass analysis.

5.3.14 *Determination of Purity of the Isolated Proteins*

The purity of trypsin inhibitors was determined by gel electrophoresis under non-denaturing conditions (see 5.3.7). A single band is usually observed when the protein is pure. The purity of proteins alternatively can be confirmed by IEF-PAGE separating proteins according to their isoelectric point.

5.3.15 *Determination of Molecular Weight (MW)*

Another important characteristic of isolated proteins is their MW. Molecular size of isolated enzymes can be made by comparing the elution volume of an unknown protein from a conventional gel-filtration or high performance size-exclusion column (HP-SEC) to the elution volumes of standard proteins. The molecular weight of isolated proteins can be determined by SDS-PAGE. However, there are many factors that may interfere with this analysis. Because of the small amount of purified trypsin inhibitors obtained, the molecular weight was determined by MALDI-TOF mass spectrometry, a method that requires only nanograms of protein. In the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) technique, the sample is mixed with an excess (~10,000 fold) of a matrix substance such as sinapinnic acid. The mixture is allowed to dry and

form a crystalline deposit that is irradiated with a short pulse from a UV laser. So, the matrix volatilizes and takes sample with it into gas phase. The ions are accelerated by a strong electric field (20 kV) toward the detector. Since all ions have the same charge, their velocities are related to the mass.

The quaternary structure of the inhibitor (subunit composition) was determined by electrophoresis under denaturing conditions with SDS and β-ME, as described in 5.3.5. The quaternary structure was deduced by comparing the molecular size of the "native" protein and the molecular size of the denatured protein.

5.3.16 *Determination of the Isoelectric Point*

Gels with different pH gradients were used: 3-10, 5-8, 3-5 to determine the isoelectric point of purified inhibitors from *Cassia* and *Mucuna*. Four µl of each protein sample resuspended in distilled water was loaded in the middle of the gel. Two µl of the standard pI markers from Pharmacia High pI Calibration Kit (Cat. No. 17-0473-01) was loaded in each run.

5.3.17 *Chymotrypsin Assay*

Ten ml of 0.1 M Tris, pH 8.0, was mixed with chymotrypsin to a final concentration of 50 µg/ml. After mixing the buffer and the enzyme; aliquots of 0.5 ml buffer were transferred to 6 spectrophotometric cuvettes. Then, 20 µl of trypsin inhibitor from *Mucuna* or from *Cassia* (MTI-4 and CTI-1) were added to each cuvette and the mixture was incubated at room temperature for 5 min. The substrate used was N-benzoyl-L-tyrosine p-nitroanilide (BTNA) (5 mg in 5 ml of acetone and 5 ml of 0.2 M Tris, pH 8.0). To each cuvette, 0.5 ml of substrate was added and the reaction was incubated at room temperature for 5 min. The rate of reaction was measured continuously at 410 nm.

5.4 Results and Discussion

5.4.1 *Plant Extraction and Dialysis*

Plant extracts were prepared from *Mucuna* seeds (15.7 g) and *Cassia* seeds (31.6 g). Seed coats were peeled off and seeds were homogenized according to the method described in 5.3.1. Seeds were homogenized with a Brinkman polytron and extracts were filtered and dialyzed. Final extract volumes of 42.5 ml for *Mucuna* and 82.5 ml for *Cassia* were obtained.

Extracts of *Mucuna* and *Cassia* were tested for their trypsin inhibitory activity (Figure 5.1). To determine if the activity was due to a proteinaceous inhibitor, extracts were autoclaved for 30 minutes. Both extracts, from *Mucuna* and from *Cassia*, showed trypsin inhibition activity that was lost after autoclaving suggesting that a proteinaceous inhibitor was responsible for this activity.

Aliquots (20 µg) of both extracts, boiled (3 min.) and non-boiled, were loaded onto a 15 % SDS-PAGE gel. Trypsin inhibitor was detected using the in-gel assay. These samples were not treated with β-ME to avoid denaturation or loss of activity. More than one band corresponding to trypsin inhibitor was detected in extracts of *Cassia* using the in-gel activity assay suggesting the presence of isoinhibitors (Figure 5.2). This activity was completely lost after heating the sample (*Cassia* extract) for a few minutes in boiling water.

Mucuna trypsin inhibitors conserved their activity after heating for a few minutes as observed in the gel but this activity was lost after autoclaving for 30 min. The *Mucuna* sample of total extract showed one band in the in-gel activity gel.

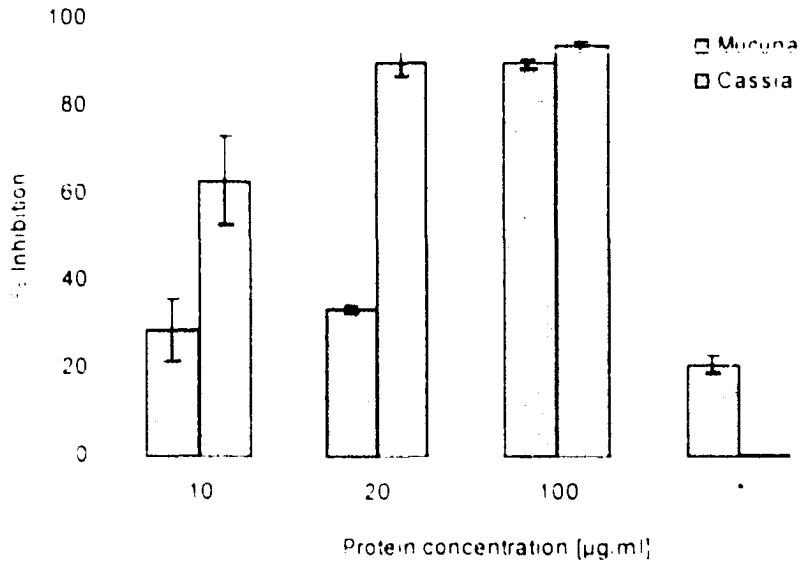


Fig. 5.1. Presence of Trypsin Inhibitors in Extracts of *Mucuna* and *Cassia*. Five µl of trypsin [5 mg ml] was added to 0.1 M Tris, pH 8. The reaction was initiated with the addition of the substrate BAPNA to a final concentration of 4 mM. The rate of reaction was determined by measuring the change in absorbance at 410 nm. Three different protein concentrations (10, 20, 100 µg ml) of *Mucuna* and *Cassia* were tested. * Crude dialyzed extract and samples autoclaved for 30 minutes were tested to determine the thermostability of trypsin inhibitors. Error bars represent the standard deviation

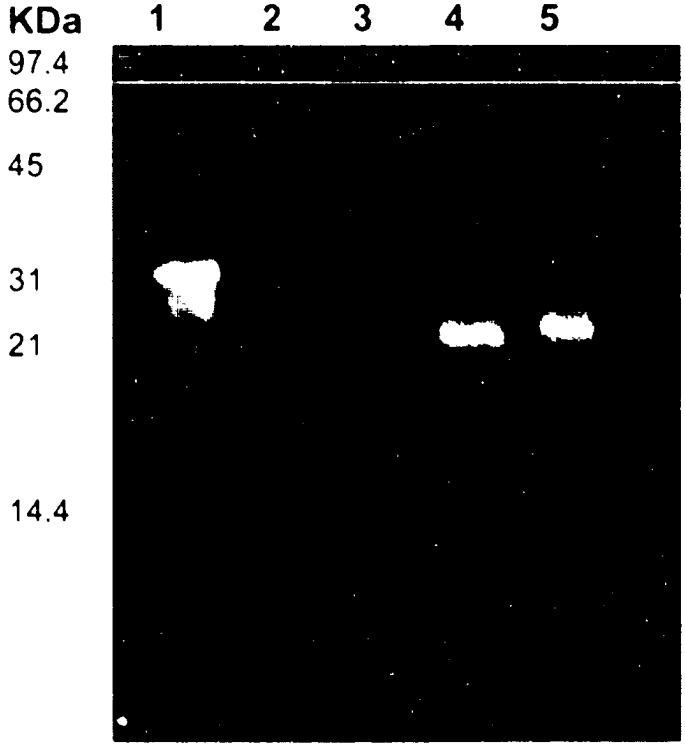


Fig. 5.2 In-gel Trypsin Inhibitor Activity Assay of *Mucuna* and *Cassia* Extracts. About 20 µg of each dialysed extract was loaded per lane. Lane 1, *Cassia* extract; lane 2, *Cassia* boiled for 3 min.; lane 3, Bio-Rad LMW marker; lane 4, *Mucuna* extract; lane 5, *Mucuna* ext. boiled 3 min. A 15 % SDS gel was used. The apparent change in size of sample 5 could be due to a change in protein conformation after boiling the sample.

5.4.2 *Affinity Chromatography*

Trypsin was attached to CNBr-activated Sepharose 4B resin by washing several times with coupling buffer and 0.1 M NaOAc, pH 4 (see Appendix I). Affinity chromatography with trypsin attached to the resin was performed according to the method described in 5.3.3. Dialyzed crude *Mucuna* and *Cassia* extracts in 10 mM sodium phosphate buffer, pH 7.5, were loaded onto a trypsin affinity column equilibrated with the same buffer. Unbound proteins were removed by washing the column several times with 5 volumes of 10 mM sodium phosphate buffer, pH 7.5. A flow rate of 1 ml/min was used for *Cassia* and the same for *Mucuna*. Then, a stepwise gradient of 0.5M NaOAc + 0.5M NaCl of decreasing pH (7 → 2) was used to elute bound inhibitors. Trypsin inhibitors bound to the column were eluted with the acetate buffer at pH 3. The elution profile for *Cassia* *Mucuna* are shown in Figure 5.3 and 5.4, respectively. Active fractions (fractions 46, 47) were dialyzed 6x against 10 mM Tris, pH 8.0 using Spectra/Por MWCO 3500 membranes. All of the dialyzed affinity purified fractions were subjected to trypsin inhibition assay to find which fractions had activity. Absorbance was measured at 280 nm to monitor protein elution.

The efficacy of the affinity column can be observed in both chromatograms where the peak of protein absorbance correlates with the activity peak in both cases.

Fractions from the affinity column were analyzed electrophoretically. Two parallel gels were run each time, one for silver staining and the other one for the in-gel activity assay. *Cassia* and *Mucuna* PAGE gels showed more than one band with activity using the in-gel activity stain suggesting the presence of isoforms. Trypsin inhibitors were present at very low concentrations. Ten affinity columns of each plant extract were run under the same conditions to have sufficient amount of trypsin inhibitor for further bioassays. Then, active fractions were pooled and dialyzed against 10 mM Tris, pH 8.0 for further purification using ion-exchange chromatography.

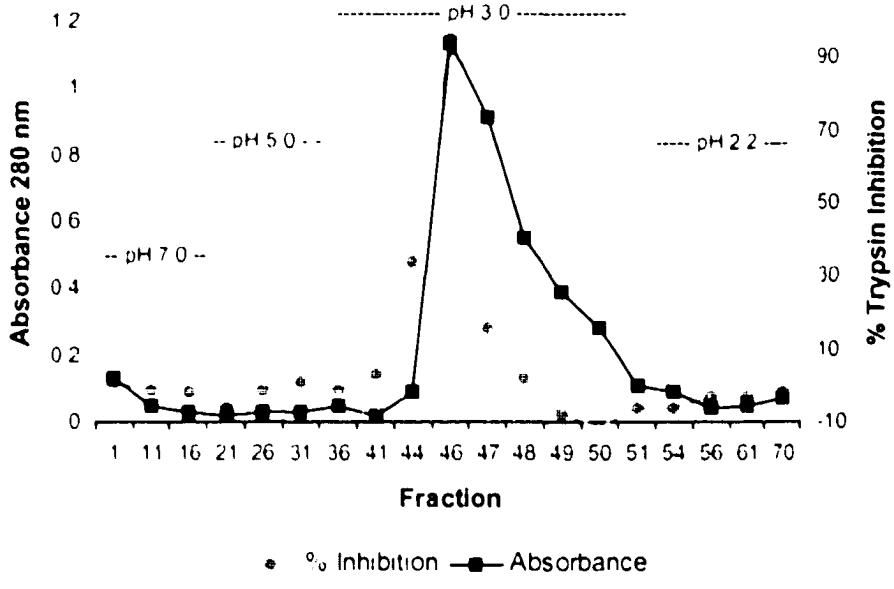


Figure 5.3. Separation of Trypsin Inhibitors from *Cassia* by Trypsin Affinity Chromatography. The flow rate used was 1 ml min and 70 3-ml fractions were collected using a stepwise gradient of acetate buffer (pH 7 to pH 2.2). The absorbance of each fraction at 280 nm was measured to monitor protein elution. Trypsin inhibitors were eluted with acetate buffer pH 3. The spectrophotometric trypsin inhibition assay was performed using 20 µl of each fraction.

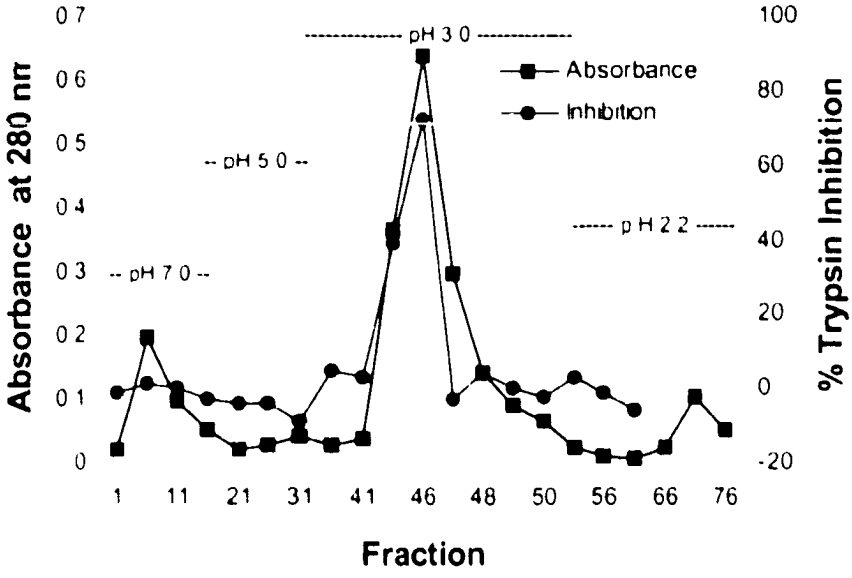


Figure 5.4. Separation of Trypsin Inhibitors from *Mucuna* by Trypsin Affinity Chromatography. The flow rate used was 1 ml min and 80 3-ml fractions were collected using a stepwise gradient of acetate buffer (pH 7 to pH 2.2). Trypsin inhibitors were eluted with the acetate buffer pH 3. The absorbance of each fraction was measured at 280 nm to monitor protein elution. Trypsin inhibition was performed using the standard assay with 20 µl of each fraction.

5.4.3 *FPLC Ion-exchange Chromatography*

Affinity purified active fractions dialyzed against 10 mM Tris, pH 8.0, were centrifuged in a microcentrifuge at 10,000 rpm for 5 min. and then the supernatant was injected into the FPLC system using 2 ml per injection. In order to optimize the protein separation several NaCl gradients were evaluated (data not shown). The best gradients used for separating *Cassia* and *Mucuna* trypsin inhibitors are described in Tables 5.1 and 5.2. After every chromatographic run, all fractions were concentrated at least five fold using Centricon tubes (10,000 Da) and subjected to electrophoretic analysis to determine their activity.

For the best separation *Cassia* trypsin inhibitors were eluted using a gradient of 0.07 – 0.08 M NaCl. Using these conditions for elution, two main peaks (1 and 2) of activity were observed (Figure 5.5). FPLC profiles of *Mucuna* showed several active peaks (Figure 5.6). Active proteins were best separated with a gradient of 0.02 M – 0.05 M NaCl. Five absorbance peaks (# 2 – 6) inhibited trypsin. Active FPLC-fractions from each plant were pooled and dialyzed in separate containers against distilled water 6 times. Finally, FPLC fractions were concentrated using Centricon concentrations tubes (10,000 Da).

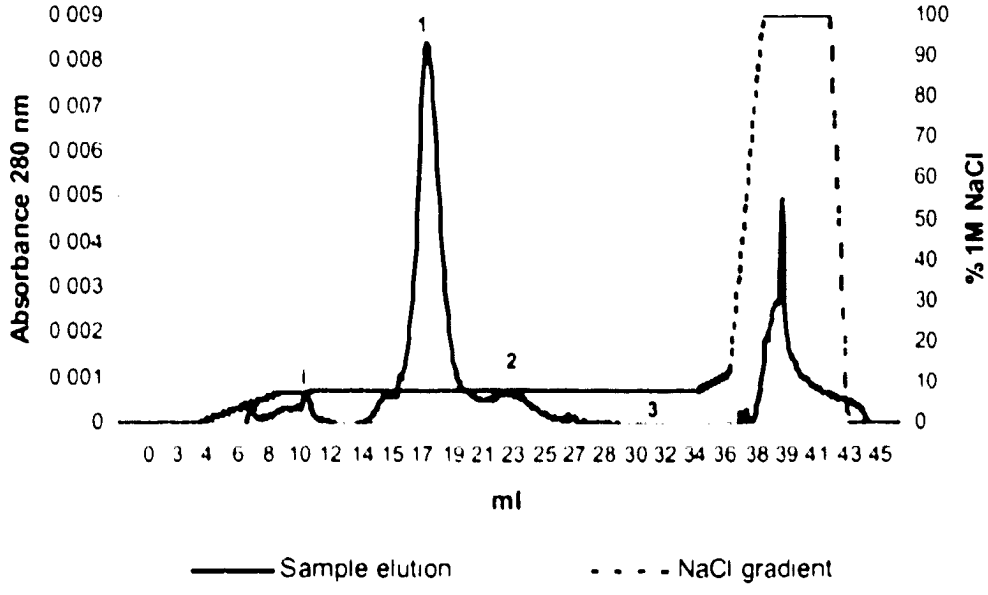


Fig. 5.5. Separation of Trypsin Isoinhibitors from Affinity Purified Fractions from *Cassia* Extracts by Ion-exchange Chromatography. The RSQ anion-exchange column was equilibrated with 10 mM Tris, pH 8.0. Three fractions containing trypsin inhibitors (peaks 1, 2, 3) were eluted with a gradient of 0.077 M - 0.080 M NaCl. Peak 1 did not show any trypsin inhibitor activity but showed some nematicidal activity. Peak 3 did show trypsin inhibitor activity even though the small peak does not always appear.

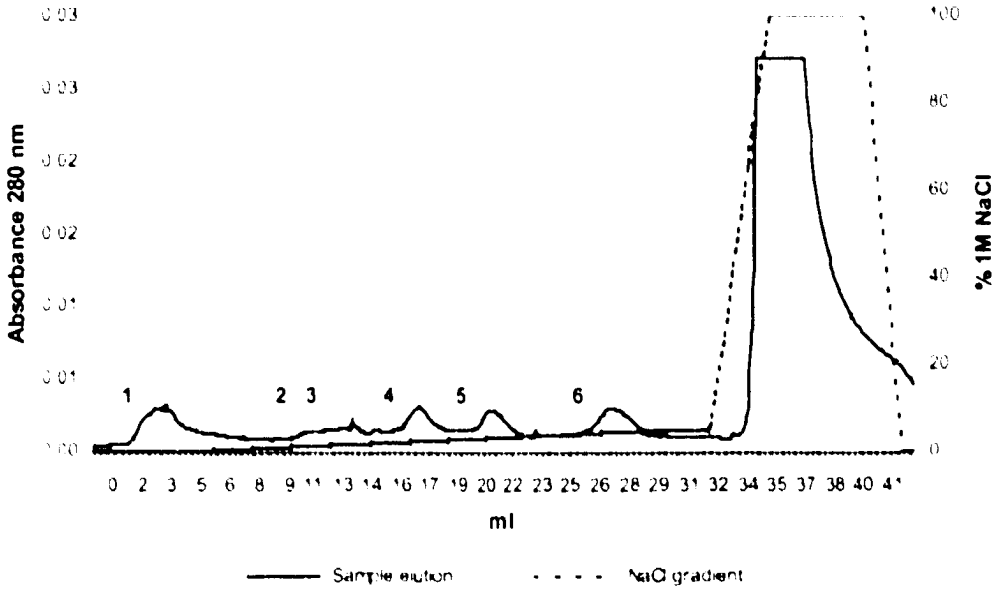


Fig. 5.6 Separation of Trypsin Isoinhibitors from Affinity Purified Fractions from *Mucuna* Extracts by Ion-exchange Chromatography. The anion-exchange column was equilibrated with 10 mM Tris, pH 8.0. Five fractions (peaks 2 - 6) containing trypsin inhibitors were eluted with a gradient of 0.02 M - 0.05 M NaCl.

The concentration of each active fraction was determined using the BCA method. The yield of isolated inhibitors were:

CTI-1	5.7 mg/100 g fresh material
CTI-2	1.6 mg/100 g fresh material
CTI-3	0.6 mg/100 g fresh material
MTI-1	1.15 mg/100 g fresh material
MTI-2	1.4 mg/100 g fresh material
MTI-3	1.6 mg/100 g fresh material
MTI-4	1.6 mg/100 g fresh material
MTI-5	1.6 mg/100 g fresh material

5.4.4 *SDS-PAGE Analysis of Protein*

Trypsin inhibitors from *Cassia* were electrophoresed under denaturing conditions in a 15% SDS gel. Samples were treated with SDS, β-mercaptoethanol, and boiled for 4 min. to disrupt disulfhyd bridges. A low molecular weight protein standard was used. The affinity purified fraction showed two main bands, one of them corresponding to the trypsin inhibitors with a molecular weight of 17.7 kDa and the other one had a lower weight with a estimated MW of 16 kDa that was another form of inhibitor (CTI-2) separated by ion exchange column. Lanes number 4 and 5

which corresponded to the two main peaks, showed just one band with a molecular weight of 17.7 kDa (Figure 5.7).

Mucuna FPLC fractions with trypsin inhibition activity were treated similarly to *Cassia* samples with SDS and β-ME and these samples were run under the same conditions. Only peaks with trypsin inhibitory activity (2 - 6) showed silver stained bands in the gel. FPLC purified fractions showed only one band. Peaks 2, 3, 4, 5 showed a MW of approximately 9,500 Da and peak number 6 showed a molecular weight of 8,000 Da (Figure 5.8).

Mucuna purified inhibitors were analyzed by SDS PAGE. However, because of their small size it was necessary to separate them using a tricine SDS gel system. Both SDS gels, from *Cassia* and *Mucuna* samples, were stained first with Coomassie Blue and then with silver.

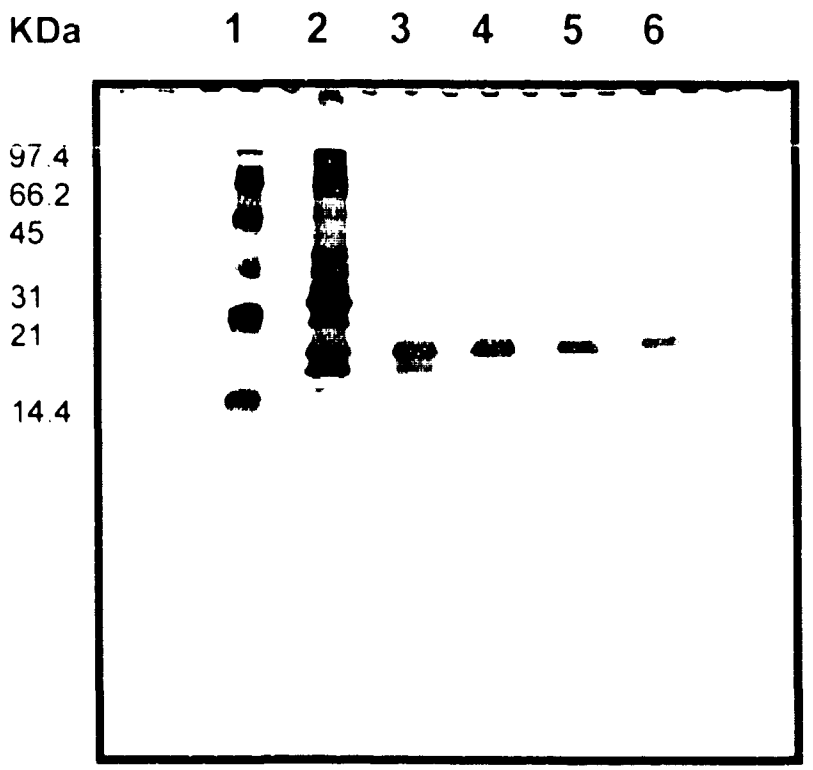


Fig. 5.7 SDS-PAGE Gel (15%) of RSQ Fractions from *Cassia fruticosa*. Gel was stained first with Coomassie Brilliant Blue and then silver stained. Lane 1, Bio-Rad LMW marker; lane 2, total extract; lane 3, affinity purified active fraction; lane 4 - 6, active FPLC purified fractions with a MW of 17,700 Da. Samples were treated with β-ME and boiled for 3 minutes prior to loading on the gel. Lanes 2, 3, 4, 5 and 6 were loaded with 10 µg, 1 µg, 1 µg, 0.5 µg and 0.2 µg.

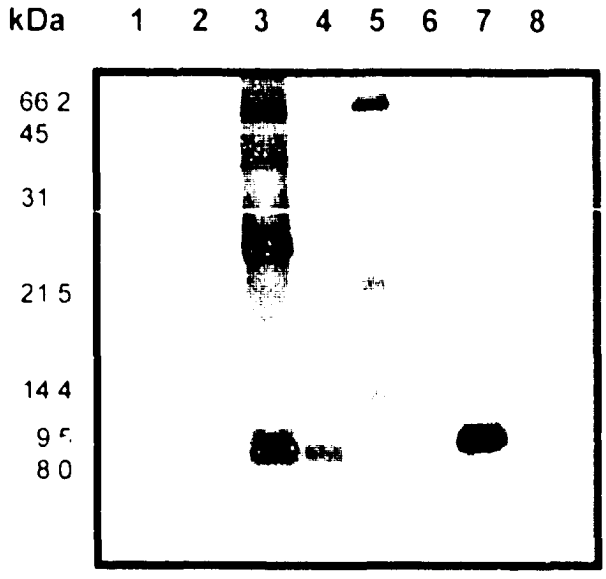


Fig. 5.8 SDS-PAGE Gel (15%) of RSQ Fractions from *Mucuna holdii*. Lanes correspond to 1, 2, 6, 7, 8 purified trypsin inhibitors; lane 3, total extract; lane 4, affinity purified active fraction; lane 5, Bio-Rad LMW marker. The gel was silver stained. The gel was loaded with 14 µg of total extract, 3 µg of sample 4, and 7, and 1 µg for the rest of samples.

5.4.5 *Tricine-SDS PAGE*

Only *Mucuna* active fractions were run on the Tricine-SDS PAGE system because of their small size (< 10 kDa). The gel was prepared according to the procedure described in 5.3.6. An ultra low molecular weight marker was used (Bio-Rad - Cat. No 161-0326, 1.4 – 26.6 kDa). The affinity purified fraction showed several bands that did not appear when running only SDS-PAGE. However, FPLC fractions corresponding to peaks 2, 3 and 5 showed just one band confirming the purity of these trypsin inhibitors with a molecular weight of approximately 13,000 Daltons (Figure 5.9). The *Mucuna* inhibitors showed a higher MW on the tricine-SDS gel than on the Tris-glycine SDS gel.

5.4.6 *Native PAGE*

Electrophoretic analysis of *Cassia* fractions under non-denaturing conditions showed that trypsin inhibitors were relatively pure, one band per peak. The purity of *Cassia* trypsin inhibitors can be observed in silver stained gels and in gels stained for activity (Figure 5.10). Most of the isolated inhibitors (CTI-1, CTI-2, MTI-1, MTI-2 and MTI-4) were apparently pure based on results of SDS gels and IEF gels.

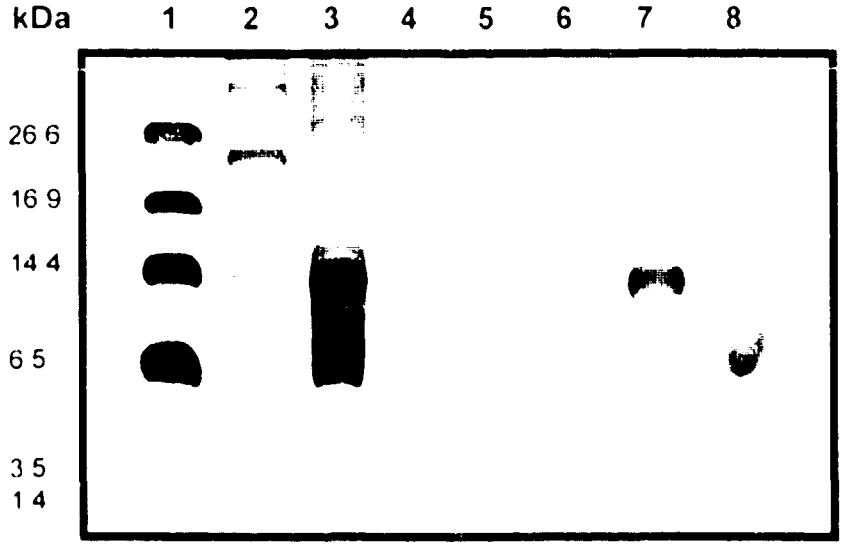


Fig. 5.9 Tricine-SDS PAGE of Purified Trypsin Inhibitors from *Mucuna holdii*. Lane 1, Bio-Rad Ultra low molecular weight markers; lane 2, crude dialyzed extract; lane 3, affinity purified active fraction; lane 4, RSQ peak 2; lane 5, RSQ peak 3; lane 6, RSQ peak 4; lane 7, RSQ peak 5; lane 8, RSQ peak 6. Ten µg of total extract, 3 µg of affinity purified active fraction and 0.6 µg of each purified trypsin inhibitor were loaded on the gel. The gel was first stained with Commassie Blue followed by silver staining.

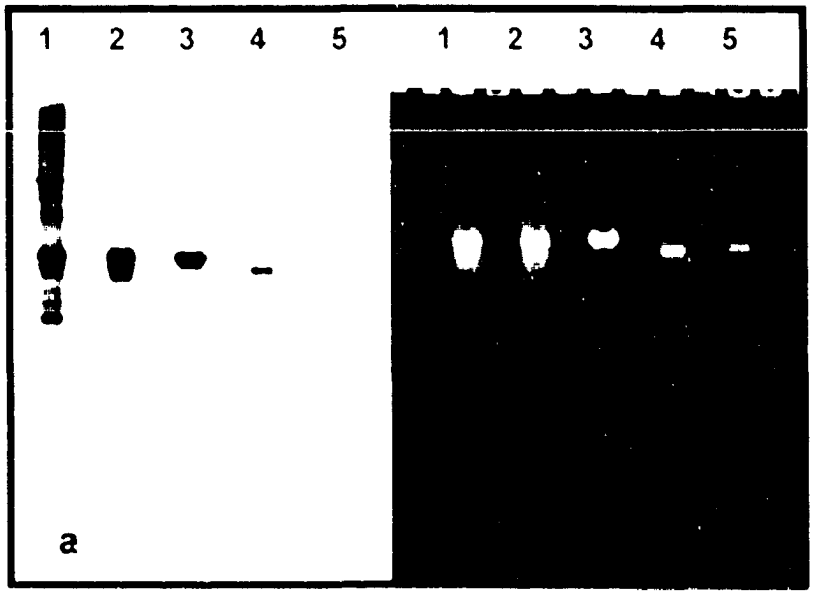


Figure 5.10. Native PAGE (15%) of Purified Trypsin Inhibitors from *Cassia fruticosa* . Lane 1, crude dialyzed extract; lane 2, affinity active fraction; lanes 3 - 5, RSQ purified fractions from peaks 1, 2 and 3. a) Silver stained gel; b) In-gel activity assay. Twenty µg of crude extract, 2 µg of affinity purified fraction, 1.5 µg of *Cassia* trypsin inhibitor (CTI) 1, 0.5 µg of CTI2 and 0.2 µg of CTI3 were loaded into each lane. Fraction of peak i did not show any trypsin inhibitory activity.

Mucuna FPLC fractions were subjected to electrophoresis under nondenaturing conditions using a 15% native gel. A parallel gel was run for the in-gel activity assay. *Mucuna* proteins showed only one band suggesting that they were relatively pure. Five peaks (fractions 2 - 6) showed trypsin inhibitory activity (Figure 5.11). Trypsin inhibitors MTI-1, MTI-2 and MTI-5 were weakly stained because of their relatively low concentrations and therefore the activity bands were faint. The peak number 1 from the FPLC A_{280} profile of *Mucuna* did not show any trypsin inhibitory activity. Likewise, there were no protein bands detected in the gel after silver staining. Interestingly, this fraction had the best nematicidal activity against *C. elegans*. The identity of the active component will require further study.

5.4.7 *IEF of Trypsin Inhibitors*

Cassia trypsin inhibitors, peaks 1 and 2, were resuspended in distilled water and subjected to IEF in a gel with a pH 3 - 5 range. The pI for both trypsin inhibitors was approximately 5.0 (Figure 5.12). The gel was stained first with a staining solution containing 27 % isopropanol, 0.04 % of CBB R-250, 10 % acetic acid, 0.5 % $CuSO_4$, 0.05 % crocein scarlet 4B.

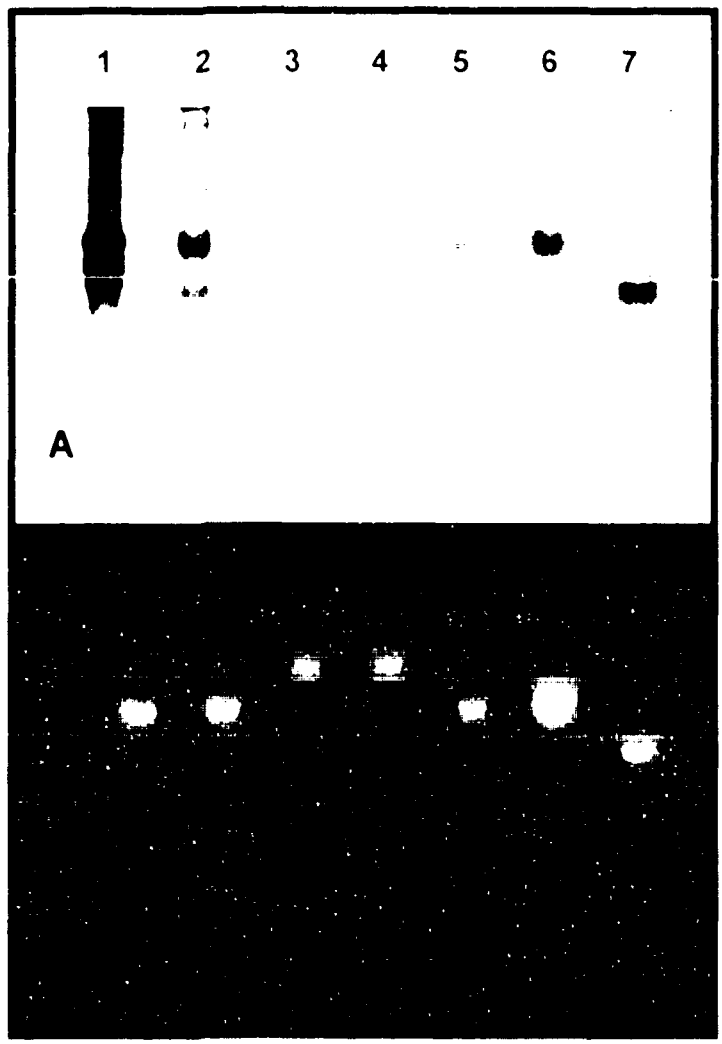


Fig. 5.11 Native PAGE (15%) of Purified Trypsin Inhibitors from *Mucuna holdii*. Lane 1, crude dialyzed extract; lane 2, affinity active fraction; lanes 3 - 7, RSQ purified fractions from peaks 2, 3, 4, 5 and 6. Peak 1 did not inhibit trypsin. A. Silver stained gel. B. Trypsin activity gel. One μg of each sample was loaded into each lane. All isoinhibitors were not detected in the crude and affinity fractions apparently due to their low concentration.

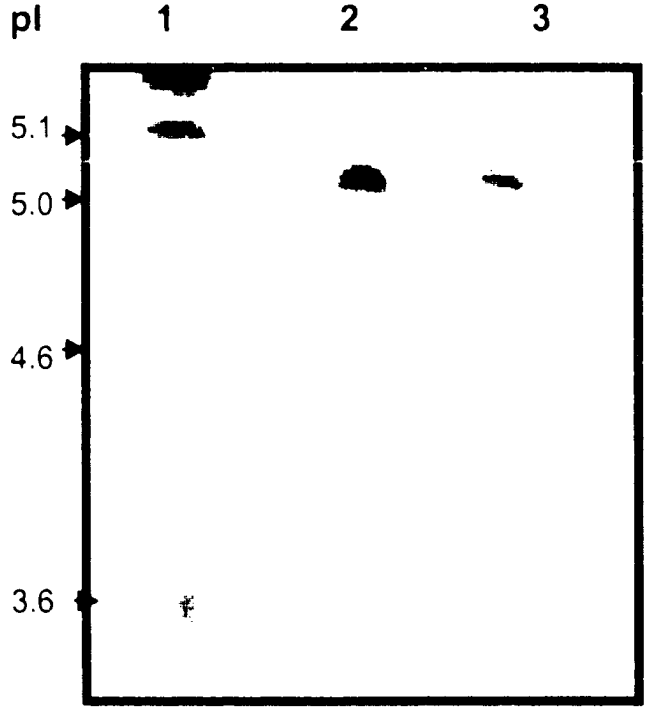


Fig. 5.12 Determination of pI Values for Trypsin Inhibitors Isolated from *Cassia fruticosa* by IEF. Lane 1, Bio-Rad markers; lane 2, RSQ peak 1; lane 3, RSQ peak 2. The gel was silver stained. IEF was performed with an ampholyte of 3 to 5 pH range.

The gel was destained by washing the gel several times with a solution of 12% isopropanol, 7 % acetic acid, 0.5% $CuSO_4$ (v/v/w). Finally, the gel was silver stained to confirm the purity of the samples.

Mucuna trypsin inhibitors were subjected to IEF under similar conditions, but the best pH range for the gel was 5 – 8. The main problem with *Mucuna* trypsin inhibitors was the fact that most of the trypsin inhibitors diffused out of the gel during the staining and destaining process. This is probably because of their small size (~ 8 000 Da). Most of them seemed to have a similar isoelectric point of around 5.4 (Figure 5.13). The pIs of trypsin inhibitors from different plant species are given in the Table 5.3.

Table 5.3. Isoelectric Point of Trypsin Inhibitors from Different Plant Species.

Common Name	Genus	pI	Reference
Buckwheat	*Fagopyrum esculentum*	4.6 - 5.6	Kiyohara & Iwasaka (1985)
Beans	*Phaseolus vulgaris*	4 - 5	Rayas-Duarte *et al.* (1992)
Orchid Tree	*Bauhinia variegata*	4.8 - 5.2	Di Ciero *et al.* (1998)
Jackbean	*Canavalia lineata*	4.5	Terada *et al.* (1994)
Love-lies-bleeding	*Amaranthus caudatus*	7.8 - 8.3	Hejgaard *et al.* (1994)
Zucchini	*Cucurbita pepo*	5.6	Leluk *et al.* (1983)
Squash	*Cucurbita maxima*	8.3	Leluk *et al.* (1983)
Potato	*Solanum tuberosum*	6.9	Rouleau & Lamy (1975)
Black-eyed pea	*Vigna sinensis*	6.5	Gennis & Cantor (1976)

5.4.8 *Caenorhabditis elegans Assay*

The effect of *Cassia* FPLC purified proteins on nematode mortality/ survival were tested using *C. elegans* (Figure 5.14). A protein concentration of 13 µg/ml per well was used for the assay. Each well contained around 20 nematodes resuspended in diluted bacteria-free medium. Nematodes were observed after 24 hr. under a stereomicroscope. Four FPLC purified proteins were tested: Ci, CTI-1, CTI-2, and CTI-3 that corresponded to peaks i, 1, 2 and 3 (See Figure 5.5). CTI-1 showed the highest activity causing 50% nematode mortality. CTI-1 also had the highest trypsin inhibition activity. CTI-2 did not exhibit any nematicidal effect at the concentrations used in the assays and CTI-3 caused only low (18 %) mortality.

Even though trypsin inhibitor activity was not detected in Ci, this fraction caused 29 % nematode mortality suggesting the presence of another active protein but not a trypsin inhibitor. The nature of this activity is not known and will require further investigation.

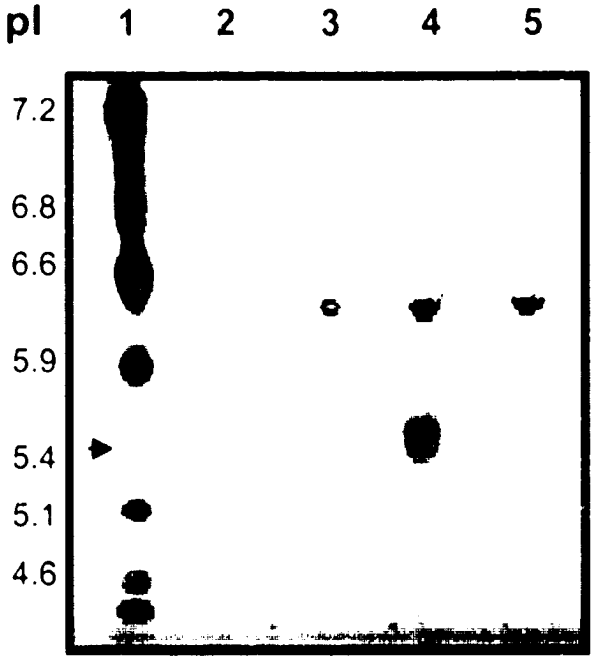


Fig. 5.13 Determination of pI Values of Trypsin Inhibitors Isolated from *Mucuna* by IEF. Sample 4 corresponded to peak number 4 in the FPLC chromatogram. 0.5 µg of each sample was loaded on the gel. The bands of the other samples (1, 2, 3 and 5) were washed off when staining. Gel was silver stained. Ampholytes of 5 to 8 pH range were used.

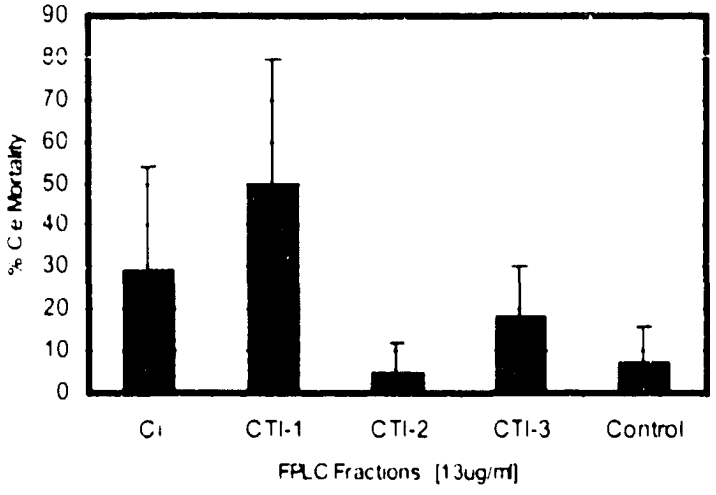


Fig. 5.14 Effect of *Cassia* trypsin inhibitors on *C. elegans*. The assay was performed in a ELISA plate containing around 20 nematodes per well. Protein was added to a final concentration of 13 µg ml. Four replicates were used per sample. Ci does not have trypsin inhibitor activity. CTI-1 and CTI-2, and CTI-3 are trypsin inhibitors and correspond to peaks 1, 2 and 3 (Fig. 5.5). The total number of nematodes used (20) was considered as 100 %. Dead and live nematodes were counted and recorded after 48 hr. Error bars represent the standard deviation

Mucuna trypsin inhibitors were also tested using *C. elegans* under similar conditions to *Cassia*. Six isolated proteins were tested (M1, MTI-1, MTI-2, MTI-3, MTI-4, and MTI-5) corresponding to peaks (1-6) in the FPLC chromatogram (See Fig. 5.6). M1 was the only fraction without trypsin inhibitory activity, however, this fraction exhibited the highest nematicidal activity causing 59 % mortality. MTI-2, and MTI-3 showed a lower activity (25 and 29% nematode mortality, respectively), when using a protein concentration of 13 µg/ml. M1 showed almost the same percentage of mortality (60%) with a higher concentration of protein 40 µg/ml. Mortality caused by MTI-3 increased from 29% to 62% with the higher concentration (Figure 5.15). MTI-1, MTI-4, and MTI-5 did not show any nematicidal activity against *C. elegans* at the concentration of 13 µg/ml and higher concentrations were not tested because of the limited amount of material available. A protein band was not detected in M1 even though this fraction gave a positive reaction for protein using the BCA protein determination. It is possible that this material is a peptide or other lower molecular weight compound.

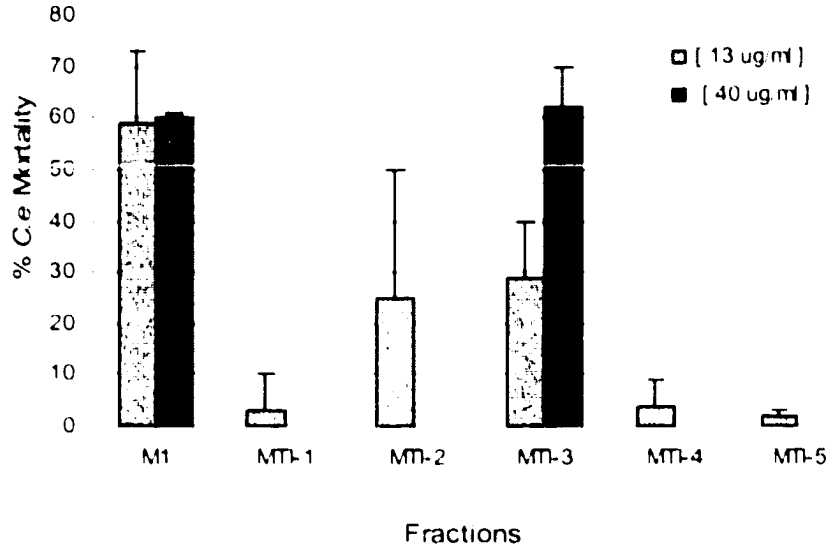


Fig. 5.15 Effect of *Mucuna* trypsin inhibitors on *C. elegans*. The assay was performed in a ELISA plate containing around 20 nematodes per well. Protein was added to a final concentration of 13 µg ml. Only sample 1 and 4 were tested with a higher concentration of 40 µg ml. Four replicates were made per sample. M1 is the only isolated protein that did not show trypsin inhibitor activity in the spectrophotometric assay. The error bars represent the standard deviations.

5.4.9 *Characterization*

Trypsin inhibitors from *Cassia* and from *Mucuna* were partially characterized by determining their purity, quaternary structure, molecular weight, isoelectric point and their specificity for trypsin and chymotrypsin.

5.4.9.1 *Purity*

The purity of both *Cassia* and *Mucuna* trypsin inhibitors was assessed by native PAGE. Each of them showed just one band in the native gel after silver staining and parallel staining in the activity gel. CTI-1 and CTI-2 fractions from *Cassia* as well as *Mucuna* fractions showed one band on SDS gels. The same trypsin inhibitors when separated on an IEF gel showed just one band confirming their purity.

5.4.9.2 *Quaternary Structure*

Samples were treated with SDS, β-mercaptoethanol and heated in boiling water for 4 min. to determine the number of subunits present in each protein. Most of inhibitors showed just one band on SDS gels, native gels and IEF gels. The MW according SDS gel run were to MW calculated by MALDI-TOF analysis (See Table 5.4) than the MW on tricine gels. Trypsin inhibitors from *Cassia* showed a MW slightly higher than 30 kDa

on SDS gel (15 %) and after treating these inhibitors with SDS and β ME they showed a MW of 17.7 kDa suggesting that *Cassia* inhibitors could be forming dimers.

Inhibitors from *Mucuna* showed a MW higher than 21 kDa on SDS gel (15 %) but denatured proteins showed a MW of 9 kDa. After analyzing these inhibitors by MALDI TOF MTI 1, MTI 4 and MTI 5 showed a MW of 8 kDa and MTI 2 and MTI 3 showed a MW of 5 kDa. These results suggest that some *Mucuna* inhibitors may exist as trimers or tetramers.

5.4.9.3 *Molecular Weight Determination*

The molecular weight of purified trypsin inhibitors was determined by SDS PAGE, by Tricine SDS PAGE and by MALDI TOF laser mass spectrometry. MALDI was the fastest and most accurate method used for determining the MW of isolated inhibitors. Small aliquots (1 µl) of each trypsin inhibitor resuspended in deionized water were used for laser mass spectrometry. The MALDI TOF analysis for *Cassia* inhibitors is presented in Figure 5.16.

A small peptide or other factor (peak M1) without detectable trypsin inhibitory activity was isolated from *Mucuna*. This component corresponds with the first absorbance peak that appeared in the FPLC chromatogram. This fraction was also sensitive to BCA analysis. These results suggested the presence of a protein or a peptide. This compound was the only one that was not detected on silver stained SDS gels. The fact that this peptide did not appear in the SDS-PAGE gel could be because it has some molecule attached and does not react with silver stain, making the peptide inaccesible to silver staining reagents. Alternatively, the peptide part is so small that after treatment with β-ME and SDS it could not be retained in the gel and was washed away during the run. After analysis by laser mass spectrometry, a molecule with MW of 7460 was detected (Figure 5.17).

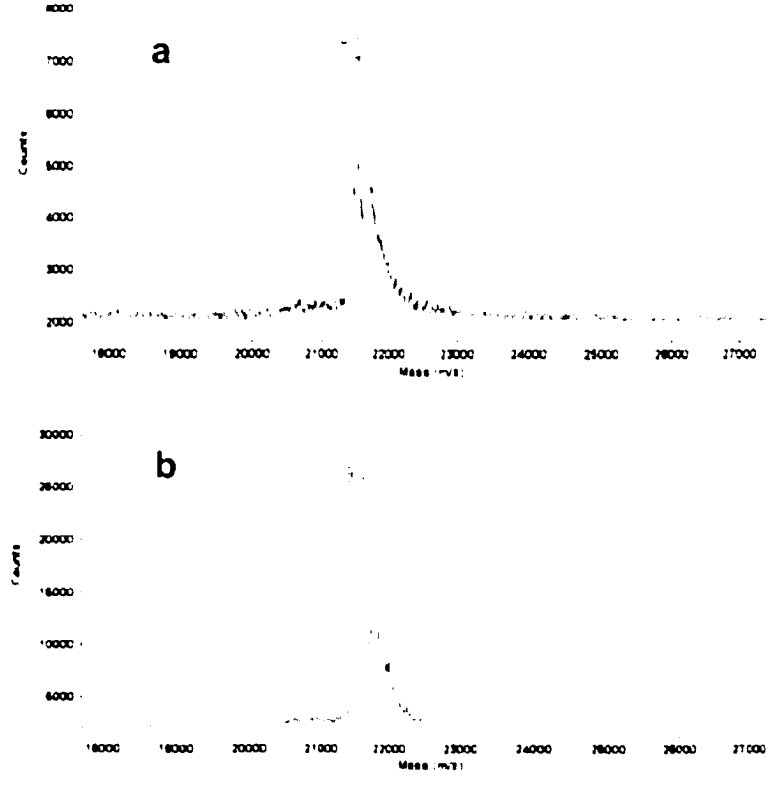


Fig. 5.16 MALDI-TOF Mass Analysis of Trypsin Inhibitors from *Cassia fruticosa*. Panel a corresponds to FPLC peak 2 and panel b corresponds to FPLC peak 1.

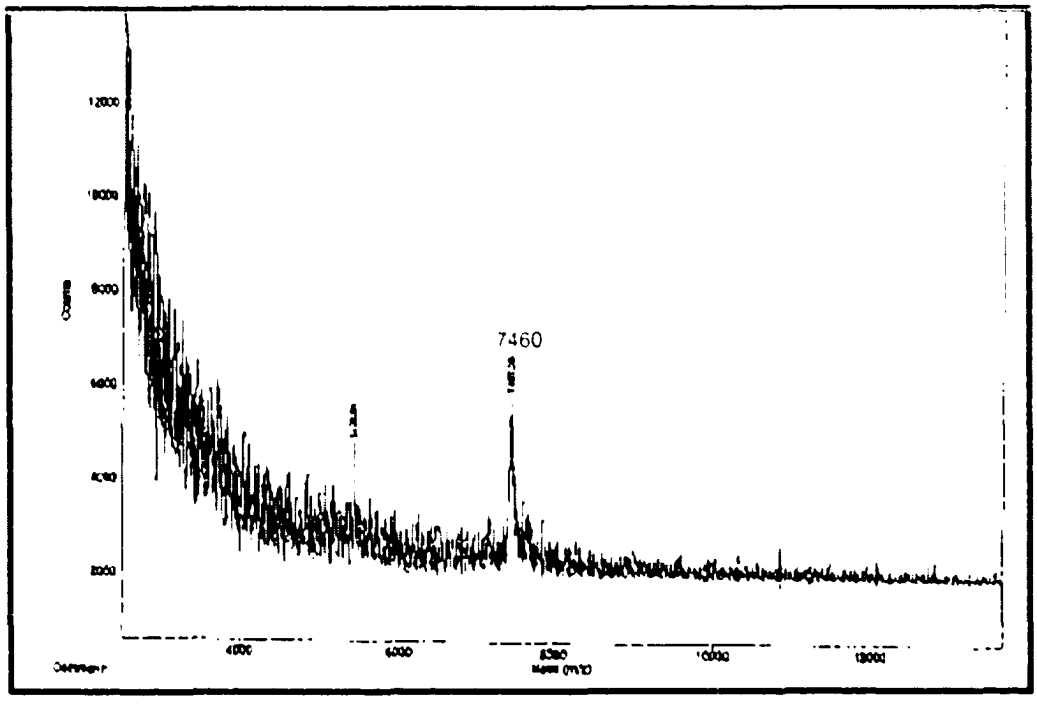


Fig. 5.17 MALDI-TOF Mass Profile of the FPLC Purified Nematicidal Peptide (peak 1) from *Mucuna holdii*. This active peptide showed a MW of 7,460 Da

The MWs of trypsin inhibitors (MTI-1-MTI-5) from *Mucuna* were divided in two subgroups based on their size determined by MALDI-TOF mass analysis: MTI-2 and MTI-3 with MWs of 5440 and 5485 are shown in Figure 5.18. The second sub-group of inhibitors (MTI-1, MTI-4 and MTI-5) with MWs of 8167, 8358, and 8378 Da, respectively, are presented in Figure 5.19. A summary of the MW data obtained using gels and mass spectrometry is given in the following table:

Table 5.4. Estimated Molecular Weights [kDa] of *Cassia* and *Mucuna* Trypsin Inhibitors

Inhibitors	CTI-1	CTI-2	CTI-3	MTI-1	MTI-2	MTI-3	MTI-4	MTI-5
SDS PAGE	17.7	17.7	10.9 17.7	9.5	9.5	8.0 9.5	9.5	8.0
Tricine SDS	ND	ND	ND	13	13	6.2 13	13	12.2 8.0
MALDI TOF	21.77	21.69	ND	8.17	5.4	5.5	8.4	8.4

ND: not determined

There was a marked difference in the molecular weight of these two groups of inhibitors. *Cassia* inhibitors have properties similar to those of Kunitz inhibitors, i.e. relative size and sensitivity to heating. *Mucuna* inhibitors resemble Bowman – Birk inhibitors because of their small size (~ 8,000 Da) and stability to heating.

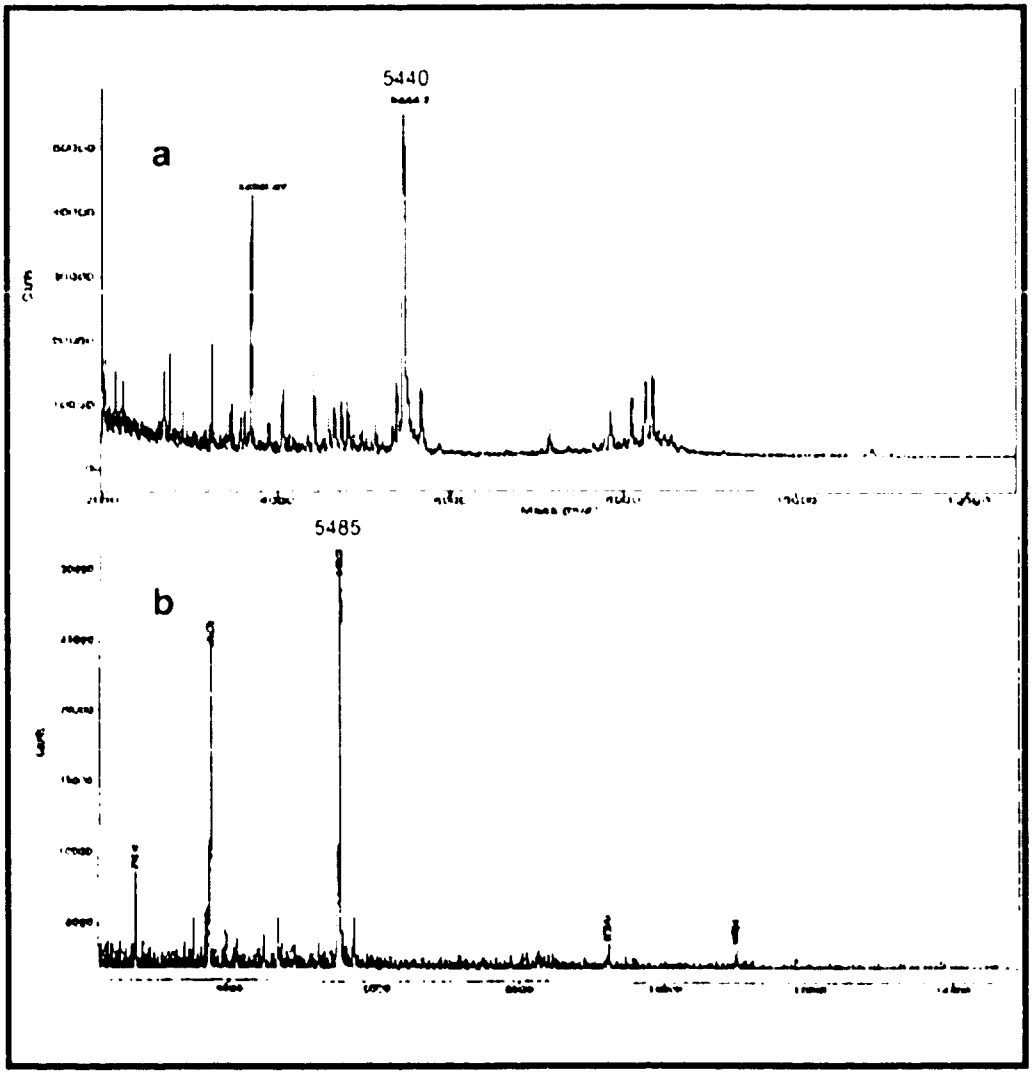


Fig. 5.18. MALDI-TOF Mass Analysis of Trypsin Inhibitors from *Mucuna*. Panel a: Trypsin inhibitor that corresponds to peak 3 (MTI-2) in the FPLC profile showing a MW of 5,440 Da. Panel b: Trypsin inhibitor (5,485 Da) that corresponds to peak 4 (MTI-3) in the FPLC profile.

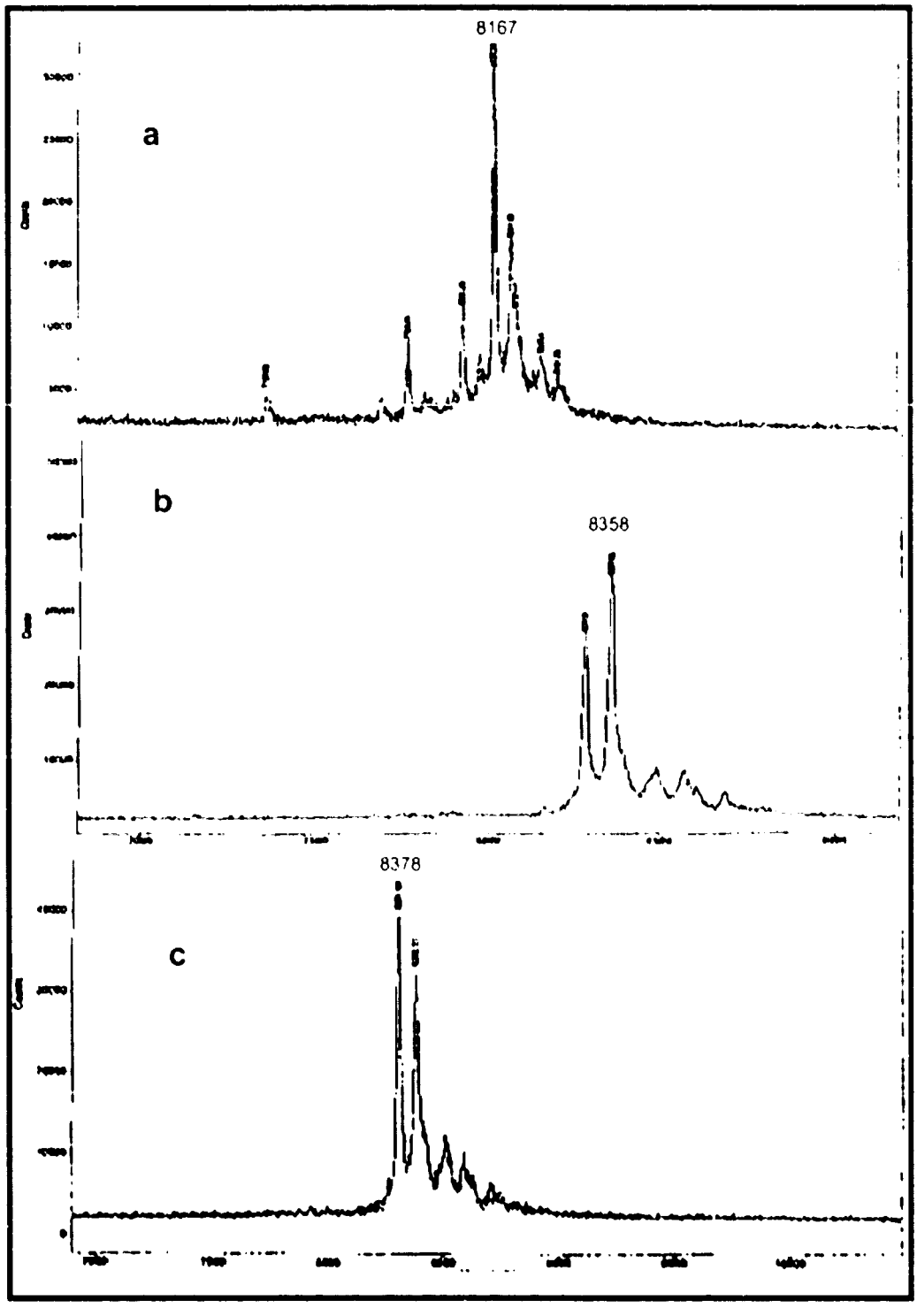


Fig. 5.19. MALDI-TOF mass analysis of trypsin inhibitors from *Mucuna*. Profiles from FPLC peaks: 2 (MTI-1); 5 (MTI-4); and 6 (MTI-5) for a, b, and c, respectively.

Tricine-SDS PAGE gave MWs (13,000 Da) for *Mucuna* inhibitors with a difference from MWs determined by laser mass spectrometry (8,000 Da) of 5 kDa.

After sequencing and determining the primary structure of these small inhibitors it is possible to isolate the gene responsible encoding each of them and to introduce the best one into crops to enhance their resistance to pests and pathogens. The small size of these peptides will facilitate gene manipulation and expression in crop plants. For example a trypsin inhibitor of 8,000 Da will have approximately 218 bp gene. This small gene is easier to sequence, amplify by PCR or be detected in small DNA libraries. Besides, small proteins expressed in transgenic plants are more probably to be consumed by target plant parasitic nematodes.

5.4.9.4 *Isoelectric Point*

The pIs for both *Cassia* and *Mucuna* trypsin inhibitors were in the acidic range. All *Cassia* inhibitors showed similar pIs of about 5.0 and the pIs for most of *Mucuna* inhibitors were about 5.4.

5.4.9.5 *Inhibition of Chymotrypsin by Purified Trypsin Inhibitors from Cassia and Mucuna.*

Trypsin inhibitors from *Cassia* (CTI-1) and from *Mucuna* (MTI-4) were tested with chymotrypsin to determine their specificity. CTI-1 and MTI-4 correspond to peak 1 and peak 5 in FPLC profiles of *Cassia* and *Mucuna*, respectively. The chymotrypsin assay used is described in 5.4.4. Commercial trypsin-chymotrypsin inhibitor BBI was used as a positive control. Inhibitors were tested at a final concentration of 6 µg/ml. Neither CTI-1 nor MTI-4 were effective inhibitors of chymotrypsin. CTI-1 and MTI-4 reduced chymotrypsin activity by 10 % and 18 %, respectively; while BBI reduced chymotrypsin activity by 55 %. In contrast, inhibition of trypsin by CTI-1, MTI-4 and BBI were 96 %, 46 % and 92 % (Fig. 5.20).

5.4.9.6 *Determination of I_{50} of Cassia and Mucuna Inhibitors*

The values for I_{50} were obtained from a linear plot of % trypsin inhibition versus the amount of inhibitor. Three replicates of each sample was performed. The amount of *Cassia* and *Mucuna* inhibitors needed to get 50 % inhibition of trypsin was 1.4 µg/ml and 3.6 µg/ml (Figures 5.21 and 5.22).

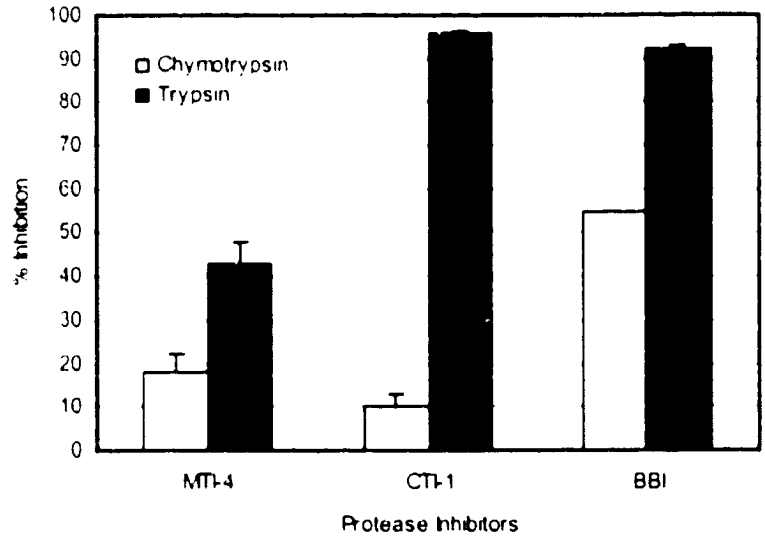


Fig. 5.20. Comparison of chymotrypsin and trypsin inhibitory activity of proteinase inhibitors isolated from *Mucuna* and *Cassia*. Bowman-Birk inhibitor was used as a positive control. Six µg of each inhibitor was used in the assay. Error bars represent the standard deviation.

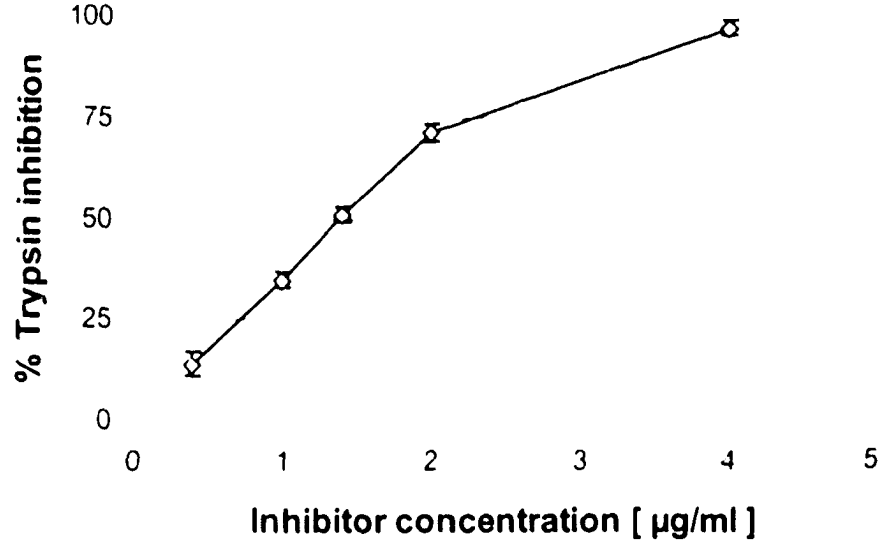


Fig. 5.21. Determination of I_{50} of *Cassia* Inhibitor.
Trypsin inhibition (% of control) in each reaction was plotted against the concentration of the inhibitor (affinity purified fraction). The amount of *Cassia* inhibitor needed to get 50 % inhibition of trypsin (TI_{50}) was 1.4 µg ml.

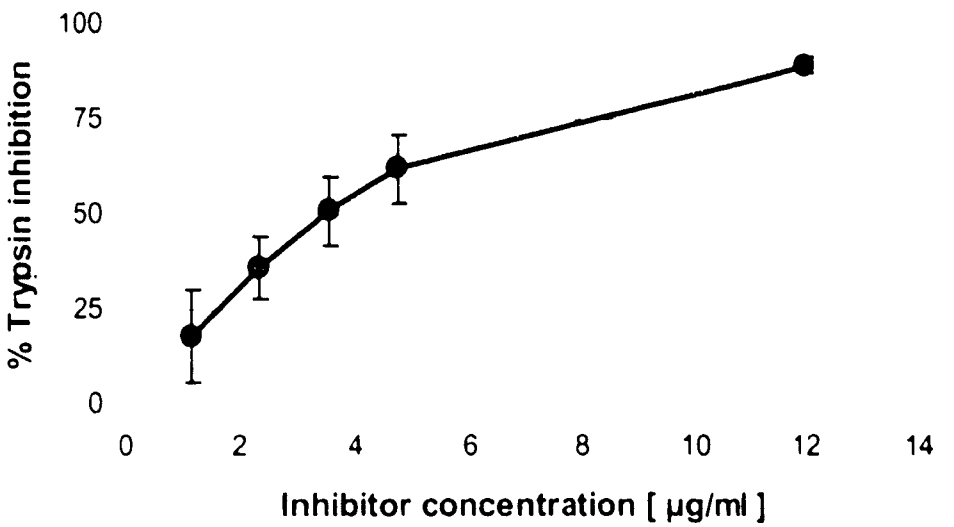


Fig. 5.22. Determination of I_{50} of *Mucuna* Inhibitor.
Trypsin inhibition (% of control) in each reaction was plotted against the concentration of the inhibitor (affinity purified fraction). The amount of *Mucuna* inhibitor needed to get 50 % inhibition of trypsin (TI_{50}) was 3.6 µg ml.

5.4.10 *Discussion*

The isolated inhibitors have several advantages to be used in plant genetic engineering. One of them is their small size and another is their inactivation by heat. Both *Cassia* and *Mucuna* were inactivated after autoclaving for 30 min. Consumption of *Mucuna* and *Cassia* seeds has proven to be safe. The elucidation of their primary and secondary structure will make them potent tools to engineer nematode resistance in several crops.

Isolated trypsin inhibitors (affinity-purified fractions) from *Mucuna* and *Cassia* resemble Kunitz and Bowman inhibitors (Steiner and Frattali, 1969)(Table 5.5).

Table 5.5. Comparison of Some Properties of *Cassia* and *Mucuna* Trypsin Inhibitors with the Activity of Kunitz and Bowman-Birk Trypsin Inhibitors.

Protease Inhibitor	Specific Activity [a]	MW [kDa]	I_{50} [b]	Thermostability [c]
Cassia TI	1	21	1.4	-
Mucuna TI	1.2	8	3.6	+
Kunitz TI	1	21	ND	-
Bowman-Birk TI	2	8	ND	+

[a] Specific activity measured as micromoles of substrate hydrolyzed per minute per milligram of protein [U/mg]. [b] I_{50} is expressed in µg/ml. [c] Thermostability test was made by heating samples (CTI and MTI) for 4 min. in boiling water. ND means non determined.

The adverse effect attributed to protease inhibitors can be eliminated by heating or by mixing them with sugars that are commonly present in daily food. In addition, protease inhibitors isolated from different sources may possess anticarcinogenic activity that may explain why vegetarian diets, where the protein source is mainly seeds, lead to a healthy style of life. Thus, some of the advantages that protease inhibitors offer support the interest in using them in plant genetic engineering.

C. elegans were used to determine the nematicidal effect of isolated inhibitors under laboratory conditions instead of *Meloidogyne incognita*. *M. incognita* is an obligate parasite and does not feed outside of the host. Consequently, it is not possible to test the effects of exogenous proteins *ex plant*. *M. incognita* could be used when expressing trypsin inhibitors in transgenic plants. In contrast, *C. elegans* is a free-living organism that normally feeds on bacteria *Escherichia coli*. *C. elegans* can be maintained on defined media containing added proteins such as the isolated trypsin inhibitors. Thus, *C. elegans* was used in these studies to determine the nematicidal activity of isolated trypsin inhibitors.

5.4.11 *Conclusions*

- Trypsin isoinhibitors (CTIs) were isolated from *Cassia* seeds by affinity chromatography. Three isoinhibitors were further separated and purified by ion-exchange chromatography on a FPLC system.
- After ion exchange chromatography *Cassia* inhibitors were determined to be pure showing single bands on IEF, native and SDS gels. The native inhibitor showed a MW of 31 kDa and the inhibitor treated with SDS and β-ME showed a single band with a MW of 17.7 kDa on SDS gel. *Cassia* inhibitors had a MW of 21.7 kDa according to MALDI-TOF mass analysis.
- Six peptides (M1, MTI-1 - MTI-5) were isolated from *Mucuna* seeds, one (M1) without trypsin inhibitory activity and five isoinhibitors of trypsin. M1 did not show any band on SDS-PAGE gels after silver staining.
- The molecular weight of *Mucuna* inhibitors was precisely determined by MALDI-TOF mass spectrometry. Three of them had MWs of 8.2, 8.4 and 8.4 kDa. Two of them were even smaller with MWs of 5.4 and 5.5 kDa.

- *Mucuna* trypsin inhibitors (MTI-1, MTI-2, MTI-4) showed single bands on SDS gels, native gels and Tricine-SDS gels.
- The isoelectric point for inhibitors from *Cassia* were 5.0 while the pIs for inhibitors from *Mucuna* were 5.4.
- Isolated inhibitors from *Cassia* and from *Mucuna* showed nematicidal activity against *C. elegans*. Two CTIs caused 18% and 29% nematode mortality while MTI-2 and MTI-3 caused 25 and 29% nematode mortality at a final concentration of 13 µg/ml. However, M1 which does not inhibit trypsin exhibited higher nematicidal activity with 60 % mortality at the same concentration.

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Appendix I : Research Protocols

Agglutination Assay (Lectin Assay)

Reagents: Human, rabbit, and porcine red blood cells (RBC).

1.5% RBC diluted in PBS.

PBS Buffer : 1.5 M NaCl

0.5 M Na_2HPO_4

pH 7.4 (equilibrated with 0.5 M NaH_2PO_4)

Mix 450 µl of each RBC with 2550 µl of PBS Buffer.

Protocol:

1. Add 50 µl PBS per well
2. Add 50µl of each sample
3. Add 100 µl diluted RBC to each well
4. Incubate at room temperature for 30 minutes

Agglutination of RBCs are observed using a light box.

Chymotrypsin Assay

(Method developed by Ruzhu Chen, unpublished data)

1. Prepare BTNA solution as follows:

   Dissolve in 5 ml acetone 5 mg of BTNA

   Add 5 ml 0.2 M Tris-Cl pH 8.0

2. To 0.5 ml of 0.1 M Tris-Cl, pH 8.0, add 20 µl of sample
3. Add 0.5 ml BTNA solution, mix and incubate for 5 min.
4. Measure reaction continuously at 410 nm

IEF Protocol

Gel Preparation:

dH_2O	2.5 ml
Monomer (25%T / 3%C)	1 ml
Glycerol (25%)	1 ml
Bio-lyte (ampholyte)	0.5 ml
De-gas, then add:	50 µl 10% APS
	50 µl 0.1% FMN
	5 µl TEMED

IEF Run:

100 V	15'
200 V	15'
450 V	60'

After running the gel, stain for two hours. Then, destain with 2-3 time changes.

IEF Stain:

27 % isopropanol

0.04% Coomassie Brilliant Blue R-250 dissolved in isopropanol

10 % Acetic acid

0.5% $CuSO_4.5H_2O$

0.05% crocein scarlet 4B

IEF Destain Solution:

12 % Isopropanol

7 % Acetic acid

0.5 % $CuSO_4$

Trypsin Assay

1. Add to each cuvette 1 ml of 0.1 M Tris, pH 8.0
2. Add 5 µl trypsin solution [5 mg/ml]
3. Add 20 µl of each sample and incubate for 5 min. at room temperature.
4. Add 40 µl of BAPNA [benzoyl-DL-arginine-p-nitroanilide; 40 mg/ml in dimethyl sulfoxide] to initiate reaction.
5. After incubating the reaction mixture for 5 min. at room temperature measure absorbance at 410 nm

Trypsin Affinity Column

1. Hydrate CNBr-Sepharose 4B powder in 1mM HCl for 15' (1g powder / 3.5 ml gel). Weigh 4.3 g of CNBr-Sepharose 4B for 15 ml gel.
2. Transfer swollen gel to buchner funnel (with filter paper Whatmann # 1) and wash with 1 mM HCl (>200 ml/g powder).
3. Wash briefly with coupling buffer (0.1 M NaHCO3 + 0.5 M NaCl, pH 8.3) approx. 5 ml/g powder.
4. Transfer gel to solution containing 100 mg trypsin dissolved in 30 ml coupling buffer (Gel:Buffer ratio 1:2).
5. Mix end-over end in the glass test tube for 2 hrs. at room temp. or overnight in the cold (do not use stirring bar).
6. Dry gel in the Buchner funnel with Whatman # 1 and wash with 400 ml of 1M ethanolamine pH 8.0. Transfer gel to solution containing 30 ml of 1M ethanolamine pH 8 to block remaining active groups and mix for 2 hrs at room temp.
7. Transfer gel to Buchner funnel containing Whatman #1 paper and wash under vacuum with the following buffers alternating 7 times with approx. 200 ml each time:
    a) Coupling buffer
    b) 0.1 M NaOAc + 0.5 M NaCl, pH 4.0
8. The gel was transferred to a column and washed with 100 ml of buffer 10 mM Na phosphate pH 7.5. The column was kept in cold ready to use.

SDS-PAGE

15% SDS-PAGE: separation gel

11.5 ml	30:0.8 Acrylamide : Bisacrylamide
2.8 ml	3 M Tris-Cl pH 8.9
8.0 ml	H_2O
225 µ	10% SDS

De-gass for 5 min.

Then add:

225 µl	10% APS
5 µl	TEMED

Transfer this gel solution between plates and add two or three ml of 2-butanol on top of the gel to prevent oxygen to reaching gel and let polymerize for 40 min. After polymerization discard the buthanol and rinse the gel with distilled water. The add the stacking gel solution.

3% Stacking Gel:

1.3 ml	30 : 1.03 Acrylamide : Bis
2.5 ml	0.5 M Tris-Cl pH 6.8
6.1 ml	H_20
100 µl	10% SDS

Finally add:

100 µl	10% APS
5 µl	TEMED

When ready add the stacking gel solution and place the comb carefully. Let it polymerize for 40 minutes.

Running Buffer:

1 g SDS

6 g Tris, free base

28.8g Glycine

Add deionized water to 1000 ml. pH should be 8.3 without adjusting.

Sample Dye Mixture: 1x

1.5 g sucrose

0.2 g SDS

1 mg Bromophenol blue

9.5 ml Running Buffer

Store at -20 °C in 900 µl aliquots

Gel Staining:

1) Commassie Brilliant Blue R-250

0.375 g Commassie Brilliant Blue

67 ml Methanol. Mix before adding the glacial acetic acid.

15 ml Glacial Acetic acid

Add deionized water to the final volume of 150 ml

Filter with Whatman No 1 by gravity

Destain Solution: (1 L)

100 ml acetic acid Glacial

300 ml Methanol

600 ml H_2O

After destaining the gel is stabilized in 10% acetic acid containing 4 % glycerol.

2) Silver Stain

Fix solution: 100 ml Methanol

24 ml Acetic acid

0.1 ml Formaldehyde (37%)

Bring to 200 ml with dH_2O

Pretreatment solution:

40 mg Sodium thiosulfate (NaTS) in 200 ml deionized H_2O

Silver Stain solution:

400 mg AgNO3

0.15 ml 37% Formaldehyde in 200 ml H_2O

Developer: 24 g Na_2CO_3

0.2 ml Formaldehyde

8 ml of pretreatment solution

dH_2O to a final volume of 200 ml

Stop Solution: 10 % acetic acid

Procedure of Silver Stain:

1. Fix the gel in the fix solution for two hours. Skip this step if the gel has been stained with CBB.

2. Place the gel in 50 % ethanol, twice for 30 min each. Shake

3. Replace the solution with pretreatment solution for one minute.

4. Wash the gel with deionized water three times, 20 seconds each time

5. Place the gel into silver stain solution for 20 min.
6. Wash the gel with deionized water three times.

7. Add the developer solution and develop as long as the protein bands show up. Discard the developer and stop adding the stop solution.

8. Place the gel in 10% acetic acid and 4% glycerol before drying.

In-gel Activity Assay

1. Put the gel after PAGE-electrophoresis into a flat container and wash with deionized water.
2. Immerse the gel into the trypsin solution prepared as follow:
   10 ml of 1M KH_2PO_4

10 ml of 1M K_2HPO_4

Add deionized water to a final volume of 200 ml.

Dissolve 50 mg trypsin in 150 ml 0.1 M K-phosphate buffer pH 7.0 and incubate the gel in this solution for about 20 min.

3. Carefully pipette out the trypsin solution and add the substrate solution which is made in the following procedure.

   A) 20 mg acetyl-dl-phenylalanine-b-naphtyl ester in 10 ml of dimethyl formamide firstly and

   B) 40 mg of tetrazotized orth-dianiside in 100 ml 0.05 M K-phosphate buffer (50 ml of 0.1 M Kphosphate buffer + 50 ml H_2O)

   Mix A in B and use fresh.

4. After 1h or overnight, the gel is transferred to 2% acetic acid to destain.

5. Dry the gel.

Appendix II: Solutions and Media

Acrylamide: bisacrylamide (30% : 0.8%):

Acrylamide	30 g
Bisacrylamide	0.8 g

Add deionized water to a final volume of 100 ml

Filter through a 0.45 µ filter.

Store at 4 °C in the dark.

Discard after 30 days.

BAPNA substrate:

Resuspend 40 mg of BAPNA in 1 ml of Dimethyl sulfoxide.

Store at room temperature.

BTNA substrate:

Resuspend 5 mg of BTNA (FW=405.4) in 5 ml of Acetone

Add 5 ml of 0.2 M Tris-Cl PH 8.0

Store at room temperature.

Liebermann Spray

Ethanol (95%)	800 ml
H_2SO_4	100 ml

Acetic Anhydride 100 ml

Note: Place the ethanol into an ice bath with stirring and add H_2SO_4 very carefully drop by drop and under the hood. The preparation of this reagent is extremely reactive.

Potassium Phosphate Buffer

1 M KH_2PO_4 10 ml

1 M KH_2PO_4 10 ml

Add deionized water to a final volume of 200 ml to get a 0.1 M K-phosphate buffer solution.

Sample Buffer:

4% SDS

12% Glycerol

50 mM Tris

2% ß-ME

0.01% Serva blue G

Adjust pH to 6.8 with HCl.

Sodium Phosphate Buffer pH 7.5

To prepare a 10 mM buffer mix:

1 M Na H_2PO_4.H20 (monobasic) 16 ml

1 M Na_2HPO_4 (dibasic) 84 ml

Dilute 100 times.

PBS Phosphate-buffered saline

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.2 g

Adjust the pH to 7.4 and add deionized water to 1 liter. Autoclave

Separation Buffer. 3 M Tris-Cl pH 8.9

Dissolve 39 g of Tris base in 70 ml of deionized water

Adjust to pH 8.9 with 1 N HCl

Add H_20 to 100 ml total volume

Filter or autoclave and store at 4 °C.

Stacking Buffer. 0.5 M Tris-Cl / SDS pH 6.8

Dissolve 6.5 g of Tris base in 40 ml of deionized water.

Adjust to pH 6.8 with 1 N HCl

Add H_20 to 100 ml total volume

Filter the solution through a 0.45 µ filter

Add 0.4 g of SDS and store at 4 °C.

Reagents for tricine-SDS gel electrophoresis

Anode Buffer: 0.2 M tris pH 8.9

Cathode Buffer: 0.1 M Tris pH 8.25

0.1 M Tricine

0.1 % SDS

Gel Buffer:	3 M Tris pH 8.45	
	0.2 % SDS	
Sample Buffer:	4 % SDS	
	12% glycerol	
	50 mM Tris	
	2 % β-ME	
	0.01 % Serva blue G	
49.5% T, 3% C	48 g Acrylamide	Vol = 100 ml
	1.5 g bisacrylamide	
49.5% T, 6% C	46.5 g Acrylamide	
	3 g bisacrylamide	

Tricine-SDS Gel (Vol = 30 ml) was made mixing following solutions:

	Separating Gel 16.5 % T, 6 % C	**Stacking Gel** 4 % T, 3 % C
49.5 % T, 3 % C	-	1 ml
49.5 % T, 6 % C	10 ml	-
Gel buffer	10 ml	3.1 ml
Glycerol	4 ml	-
H_2O	6 ml	8.4 ml
10 % APS	150 µl	100 µl
TEMED	15 µl	10 µl

Insect Stoneville Diet

Ingredients:

Soy Flour (ICN)	39 g
Sucrose	39 g
Wheat Germ (Pioneer)	34 g
Wesson Salt Mix	9.5 g
Vitamins	9 g
Methyl Paraben	1 g
Sorbic Acid	0.9 g
Agarose	20 g
Aureomycin	0.9 g
Streptomycin	100 mg

Diet Preparation:

1. Put 153.4 g dry ingredients in a blender
2. Add 900 ml boiling distilled water (per parts)
3. Add 2.5 ml Mold Inhibitor
4. Pour in a diet tray