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THE EFFICACY AND MODE OF ACTION OF A *PIPER* (PIPERACEAE) BOTANICAL INSECTICIDE FOR CONTROL OF INSECT PESTS OF THE HOME AND GARDEN

IAN M. SCOTT

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To Maria, Aidan and Maya.

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ABSTRACT

The present study highlights the practical application of a *Piper*-based botanical insecticide for controlling insect pests of the home and garden in urban areas in eastern Canada and northeastern North America. Biopesticides, including botanicals, can offer a safe and effective alternative to conventional insecticides for controlling major insect pests within an IPM program. Secondary compounds from the Piperaceae family, specifically the abundant isobutyl amides and lignans, have shown promise for insecticidal applications.

A method for extraction and HPLC-MS analysis of *Piper* spp. was developed in order to allow quick and accurate measure of piperamide levels in *P. nigrum*, *P. tuberculatum*, West African Guinea pepper, *P. guineense* Schum and Thonn, and in less recognized species from Central America. Extraction of leaf and peppercorn material with 50:50 ethyl acetate and water provided a greater than 80% recovery of spiked piperine. HPLC analysis using a binary gradient of acetonitrile and water provided a clean separation of the major amide peaks between 5 and 12 min. The use of APCI-MS improved the detection limit 10 fold below the 2-ng limit of the HPLC-DAD method.

Extracts from *P. nigrum*, *P. guineense* and *P. tuberculatum* were tested for efficacy against insects from five orders. Among the insect pests tested, the most sensitive species were, in order of increasing lethal concentration: Eastern tent caterpillar, *Malacosoma americanum* F. < European pine sawfly larvae, *Neodiprion sertifer* Geoffroy < spindle ermine moth larvae, *Yponomeuta cagnagella* Hübner < Viburnum leaf beetle larvae, *Pyrrhalta viburni* Paykull < stripped cucumber beetle adult, *Acalymma vittatum* Fabrcius < Colorado potato beetle adult, *Leptinotarsa decemlineata* (Say) < Japanese beetle adult, *Popillia japonica* Newman < hairy chinch bug, *Blissus leucopterus hirtus* Montandon. Greenhouse trials revealed that the pepper formulations also had a repellent activity thus protecting plant leaves from: 1) herbivory by lily leaf beetles, *Lilioceris lilii* (Scopoli) adults and larvae, and striped cucumber beetle, *Acalymma vittatum* F. adults and 2) oviposition by European corn borer, *Ostrina nubilalis* (Hübner).

When an insecticide resistant strain of potato beetle larvae was tested with the *P. tuberculatum* extract, there was less than a two fold tolerance ratio compared to the 22-fold tolerance ratio to cypermethrin, a pyrethroid. An *in vitro* polysubstrate monoxygenase (PSMO) enzyme assay, using the substrate methoxyresorufin *O*-demethylation (MROD), determined that piperine, is responsible for inhibition of that specific enzyme. A subsequent toxicokinetic study determined that piperine is quickly eliminated from the exoskeleton ($t_{1/2}$ = 16.5 h) and hemolymph ($t_{1/2}$ = 12 h) of the adult American cockroach *Periplaneta americana* L. after a topical application. However, piperine uptake in the soft tissues continued for 48 h post-dosing and the rate of piperine depuration was directly affected by the dose of piperamides. This effect was believed to be caused by the observed *in vivo* inhibition of both cytochrome P450 and b5 enzyme activity in the treated cockroaches.

Successful treatment in the mid-season was accomplished with the application of a 2% *P. nigrum* formulation to turfgrass infested with European chafer *Rhizotrogus majalis* Razoumowsky 2nd and 3rd instar larvae. The 4% pepper extracts significantly reduced the earthworm populations in treated plots compared with both controls and diazinon treated plots. Non-target toxicity to other beneficial invertebrates is a possibility, since the *P. nigrum* LC_{50} for beneficial lady beetles was 0.2%.

Piper extracts were found to have short persistence under field conditions: the residual repellent activity of the *P. nigrum* extracts on potato plants was limited to 3 h. The analysis of soil residues for piperamides determined a half-life of one day in mid-season, and 3 days in the late season. This confirmed the expectation that under field conditions the residual activity would be relatively short, thus reducing the environmental risk associated with pesticide use.

The results confirm that *Piper* extracts could be used effectively as contact botanical insect-control agents to protect vegetable and ornamental plants from developing Lepidopteran and Coleopteran larvae at concentrations less than 0.1%. There is also potential for *Piper* extracts to control insecticide-resistant populations in conjunction with other integrated pest management (IPM) strategies used in conventional and organic agriculture. The use of a botanical with a long history of human use is now recognized by regulatory agencies as an indicator of low risk to human health.

RESUME

La présente étude fait ressortir l'application d'un insecticide d'origine végétale extrait de *Piper* pour le contrôle des insectes de maison et de jardin dans les régions urbaines du Canada et de l'est et du nord-est de l'Amérique du Nord. Les biopesticides, y compris les produits d'origine végétale, peuvent offrir une alternative efficace et sans risque pour le contrôle des principaux insectes dans un programme de lutte intégrée. Les composés secondaires de la famille des Pipéracées, spécifiquement les amides et les lignanes abondants, sont très prometteurs pour des applications insecticides.

Nous avons développé une méthode pour l'extraction et l'analyse à l'HPLC-MS des espèces *Piper* afin de procéder rapidement et de façon précise pour établir la concentration des pipéramides dans *P. nigrum*, *P. tuberculatum*, le poivre de Guinée provenant de l'Afrique d'Ouest, *P. guineense* Schum et Thonn, et les espèces moins connues de l'Amérique Centrale. L'extraction des substances dans les feuilles et le poivre avec l'acétate d'éthyle et l'eau (50:50) a permis de récupérer plus de 80% de la pipérine. L'analyse par HPLC utilisant un gradient binaire avec de l'acétonitrile et de l'eau a donné une bonne séparation des pricipaux pics d'amides entre 5 et 12 minutes. L'utilisation d'un APCI-MS a amélioré la limite de détection dix fois au-dessous de la limite de 2 ng de la méthode HPLC-DAD.

Les extraits de *P. nigrum*, *P. guineense* et *P. tuberculatum* ont été examinés pour leur efficacité envers cinq ordres d' insectes. Parmi les insectes examinés, les espèces les plus sensibles ont été, par ordre croissant de sensibilité toxique: *Malacosoma americanum* F. < *Neodiprion sertifer* Geoffroy < *Yponomeuta cagnagella* Hübner < *Pyrrhalta viburni* Paykull < *Acalymma vittatum* F. < *Leptinotarsa decemlineata* Say < *Popillia japonica* Newman < *Blissus leucopterus* Montandon. Les essais dans les serres ont révélé que la formulation du poivre a aussi une activité répulsive qui protège ainsi les feuilles des plantes contre: 1) les dommages causés par les coléoptères, *Lilioceris lilii* (Scopoli) et *Acalymma vittatum* F., adultes et larves et 2) l'oviposition par la pyrale du maïs, *Ostrina nubilalis* (Hübner).

Quand une population de doryphores résistants aux insecticides a été traitée avec l'extrait de *P. tuberculatum*, il y a eu un niveau de tolérance deux fois moindre comparativement à un ratio de tolérance de 22 fois avec la cyperméthrine, un pyréthroïde. Un essai *in vitro* pour mesurer les enzymes

polysubstrats monoxygénases (PSMO), et utilisant le substrat méthoxyrésorufine par *O*-déméthylation (activité MROD), a déterminé que la pipérine est responsable de l'inhibition spécifique de l'enzyme. Une étude toxicocinétique subséquente a déterminé que la pipérine est rapidement éliminée de l'exosquelette ($t_{1/2}$=16,5 h) et de l'hémolymphe ($t_{1/2}$=12 h) des blattes adultes *Periplaneta americana* L. Cependant, l'assimilation de la pipérine dans les tissus mous a continué pendant 48 h après le traitement et le taux d'élimination de la pipérine a été directement affecté par la pipéramide totale. Cet effet est sans doute causé par l'inhibition *in vivo* de l'activité des enzymes cytochrome P450 et b5 dans les blattes.

On a pu obtenir un traitement positif à la mi-saison (en août) grâce à une application d'une formulation de 2% de *P. nigrum* sur un gazon infesté par des larves de *Rhizotrogus majalis* Razoumowsky. L'extrait de poivre de 4% a réduit de façon significative la population des vers dans les terrains traités en comparaison avec les deux contrôles et le traitement au Diazinon. La toxicité envers les invertébrés non-ciblés est une possibilité, puisque la CL_{50} de *P. nigrum* pour les insectes bénéfiques est de 0,2%.

Nous avons determiné que les extraits de *Piper* ont une faible persistance sur le terrain: l'activité répulsive résiduelle du *P. nigrum* sur les plantes de pomme de terre est limitée à 3 h. L'analyse du résidu dans le sol, pour les pipéramides, révèle une demi-vie du un jour en mi-saison, et de 3 jours en septembre. Ceci a confirmé la prédiction qu'en conditions de terrain, l'activité résiduelle serait relativement courte, réduisant ainsi les risques environnementaux associés à l'utilisation d'un pesticide.

Les résultats confirment que les extraits de *Piper* peuvent être utilisés de façon efficace comme insecticide botanique pour protéger les plantes vivrières et ornementales de l'action de lépidoptères et coléoptères à des concentrations de moins de 0,1%. Les extraits de *Piper* pourraient potentiellement contrôler les populations résistant aux insecticides en conjonction avec d'autres stratégies de lutte intégrée utilisées en agriculture conventionnelle et organique. L'utilisation de produits botaniques ayant une longue période d'utilisation est maintenant reconnue par les agences de réglementation comme un indicateur ayant peu de risques pour la santé humaine.

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

The beginning of the third millennium has seen a definite shift in public opinion regarding the use of pesticides in Canadian municipal areas. Because of the desire to do away with chemical control strategies for weeds, herbivorous insect pests and plant pathogens, the requirement for new solutions to control pests and disease in agriculture and urban areas is necessary. In Canada, concerns for human and environmental health has led to the banning or severely restricting of synthetic insecticide use in municipal areas (Globe and Mail 2003; Pest Control Canada 2003). Such legislation has been upheld by a federal judicial decision (Supreme Court decision 2001). In the United States, regulatory changes have led to streamlining of pesticide registration processes to favour low-risk products (E.P.A. 2003), which include products that are Generally Regarded As Safe (G.R.A.S.) and allow biopesticides and botanicals as a category different from conventional pesticides. Furthermore, these products are recognized for use in agricultural produce grown under certified organic practices where synthetic pesticides are not allowed. For these reasons, the present project was undertaken in order to document the potential of the plant family Piperaceae in the development of a botanical formulation considered safer for the public, less threatening for the environment, but providing active secondary plant compounds with several modes of action including contact toxicity, repellent and antifeedant properties against insects.

1.2 Literature review

1.2.1 The Piperaceae

The Piperaceae family is considered to be among the most archaic of pan-tropical flowering plants (Burger 1971). The genus *Piper* contains approximately 1000 species of herbs, shrubs, small trees and hanging vines. Several *Piper* within India, southeast Asia and Africa are of economic importance since

they are used as spices (Simpson and Ogorzaly1995). Many plant families have a global distribution, but few have the rich ethnobotanical and ethnopharmaceutical history of Piperaceae, and since the latter has been used for centuries both medicinally and as a food additive, the associated health risk to humans is generally regarded as low (Tripathi *et al.* 1996; Parmar *et al.* 1997).

Most of the documented use in health care comes from south Asia, where many species are part of the Ayurvedic system of medicine. The *Piper* genus derived its name from the Sanskrit word *Pippali* which means hot tasting and aromatic, based on the taste of the fruit of *Piper nigrum* L., black pepper (Atal *et al.* 1975). As a spice, black pepper has been traded world-wide for many centuries and represents a highly important cash crop for many tropical countries including India, Indonesia, Vietnam, Malaysia and Brazil (Simpson and Ogorzaly 1995). The unripe fruit is the source of black pepper, while the ripened fruit is the source of white pepper (Parmar *et al.* 1997). Trade between India and Europe was occurring from at least as early as the 3rd century B.C. when Greek texts mention pepper. Demand for pepper and other spices eventually led to European expansion into south and southeast Asia after the 15th century A.D.

Although the use of Piperaceae in pharmacological functions has been documented in indigenous cultures in south and central America, Africa, China and the south Pacific Islands, a great deal of knowledge about historical uses and current uses is found in Indian scientific literature. Up until 20 to 25 years ago, very little work on *Piper* species had been done outside India, except with Kava, *Piper methysticum* (Atal *et al.* 1975). Therefore, the central focus of this literature review will be the traditional uses of *Piper* species within south Asia and the current understanding of the pharmacological and insecticidal activity and associated health concerns of this group.

1.2.2 *Piper* spp. in Ayurvedic and traditional medicine

The most common and widely used of 24 *Piper* species found in India are *P. longum, P. nigrum, P. cubeba* and *P. betle* (Dasgupta and Datta 1980). *Piper nigrum* is known as *maricha* in Sanskrit. It was

used as a cerebral sedative, as an irritant and a counter-irritant and also as an antihelminthic (Chakraberty 1923). The fruit of *P. nigrum* has wide ranging activity as a stimulant in cholera, as a stomachic in dyspepsia and flatulence, antiperiodic in malarial fever and arthritic diseases, localized treatment of sore throat, piles and skin diseases (Dasgupta and Datta 1980). A recent interest in Ayurvedic medicine has led to Western medicine exploring the possibilities for new drug leads. Tripathi *et al.* (1996) provide a list of 149 Sanskrit remedies which include the use of *Piper* species. The pharmacopoeia listed by Gupta (1906) detail the specific ingredients for many of those remedies. Interestingly, two different cures for eye diseases include *P. nigrum* but the other ingredients are completely different even though the remedies are both recommended to cure most eye diseases.

Piper species are often taken as part of herbal remedies that combine other plants with different activities. One case in point is the Ayurvedic prescription of *P. nigrum*, *P. longum* and ginger, *Zingiber officinalis*, collectively known as trikatu, which in Sanskrit means three acrids (Atal *et al.* 1981). Tripathi *et al.* (1996) describe this combination as enhancing bioavailability and early references cited describe this combination as non-essential but that it helped to maintain a spiritual balance (Atal *et al.* 1981). However, in a few cases the addition of the mixture was explained as a way of increasing the efficacy of the primary plant's activity. For example, two alkaloids, vasicine and sparteine, isolated from extracts of the Ayurvedic herbs *Adhatoda vasica* and *Spartium junceum* respectively and prescribed for their anti-asthmatic properties, were tested in the presence and absence of *P. longum* fruit powder and piperine. In both cases the constituents of *Piper* significantly increased the bioavailability of the drugs in the plasma of female rats. Atal *et al.* (1981) summarized the mode of action of the trikatu group as important because it reduced the amount of active drug required and it could be given orally instead of by the parenteral route, thus decreasing the risk of infection, hypersensitivity and anaphylactic shock.

The species of Piperaceae from tropical America have the same aromaticity as those of Indian origin and have also found their way into folk-medicine. Three genera including *Piper* are represented. *Lepianthes* (Rafinesque) and *Peperomia* (Ruiz et Pavón) have several species each which have

documented medicinal use (Shultes and Raffauf 1990). Various classifications of use include abortifactions, antibiotic, arrow or fish poisons, diuretic, insect repellant, toothache remedy, tobacco snuff substitute and for witchcraft practices. Records also list uses as a carminative, for general pain relief and to cure conjunctivitis and eye diseases, febrifuge, digestive ailments and cough from tuberculosis.

The most familiar medicinal Piperaceae in Africa is the Ashanti or Guinea pepper, *P. guineense* (Iwu 1993). The leaves and fruits are used as a cough remedy and the seeds for treating stomach-aches. The roots are used for gonorrhea, syphilis, bronchitis and colds. The leaves, fruit and roots are combined to prepare formulations to fight infectious diseases. The whole fruit can be applied externally as a stimulating ointment or as a counter-irritant (Iwu 1993). Kava, *P. methysticum*, is the most recognized medicinal plant from Fiji, Samoa and other south Pacific islands (Blumenthal 2000). Kava is taken as a beverage, usually for ceremonial purposes, as it has relaxing qualities. It is also used to treat urinary tract infections, asthma, as a topical anaesthetic. Chinese traditional medicine lists *P. kadsura* as a treatment of inflammation and rheumatic conditions (Ma *et al.* 1993) and *P. macropodum* for rheumatism, toothache, epilepsy and stomach-ache (Hou *et al.* 1989). *P. wallichi*, *P. hancei* (Han *et al.* 1989), *P. futokudzura* (Chen *et al.* 1993) and *P. polysyphorum* (Ma *et al.* 1991) are also being investigated for anti-inflammatory properties.

In the recent Piperaceae literature dealing with medicinal research, there are disproportionately more studies devoted to *P. betle* and *P. methysticum* use compared to all other *Piper* species. This is probably due to the extensive use of pan or betel-nut in Asia, kava in other parts of the world and the resulting health problems which are now being identified with the use of both. In comparison, *P. nigrum* is probably consumed as much or more in the global diet yet there does not appear to be as great an interest in its pharmacological and toxicological effects.

1.2.3 Phytochemistry and *Piper* natural product diversity

The phytochemistry of most of the *Piper* spice plants is well documented, as many of these same species have been used for medicinal purposes as well (Table 1.1). The most recognized compound from *P. nigrum*, is the amide, piperine. It is present in the highest concentration of all secondary compounds in the seed of the plant; electron microscopy can even differentiate crystals of piperine in cross-section as seen in Figure 1.1 (Arnott 1999). The structure of five amides commonly present in *P. nigrum* extract: dihydropiperlonguminine; piperlonguminine; dihydropiperine; piperine and pipercide is shown in Figure 1.2A to E respectively.

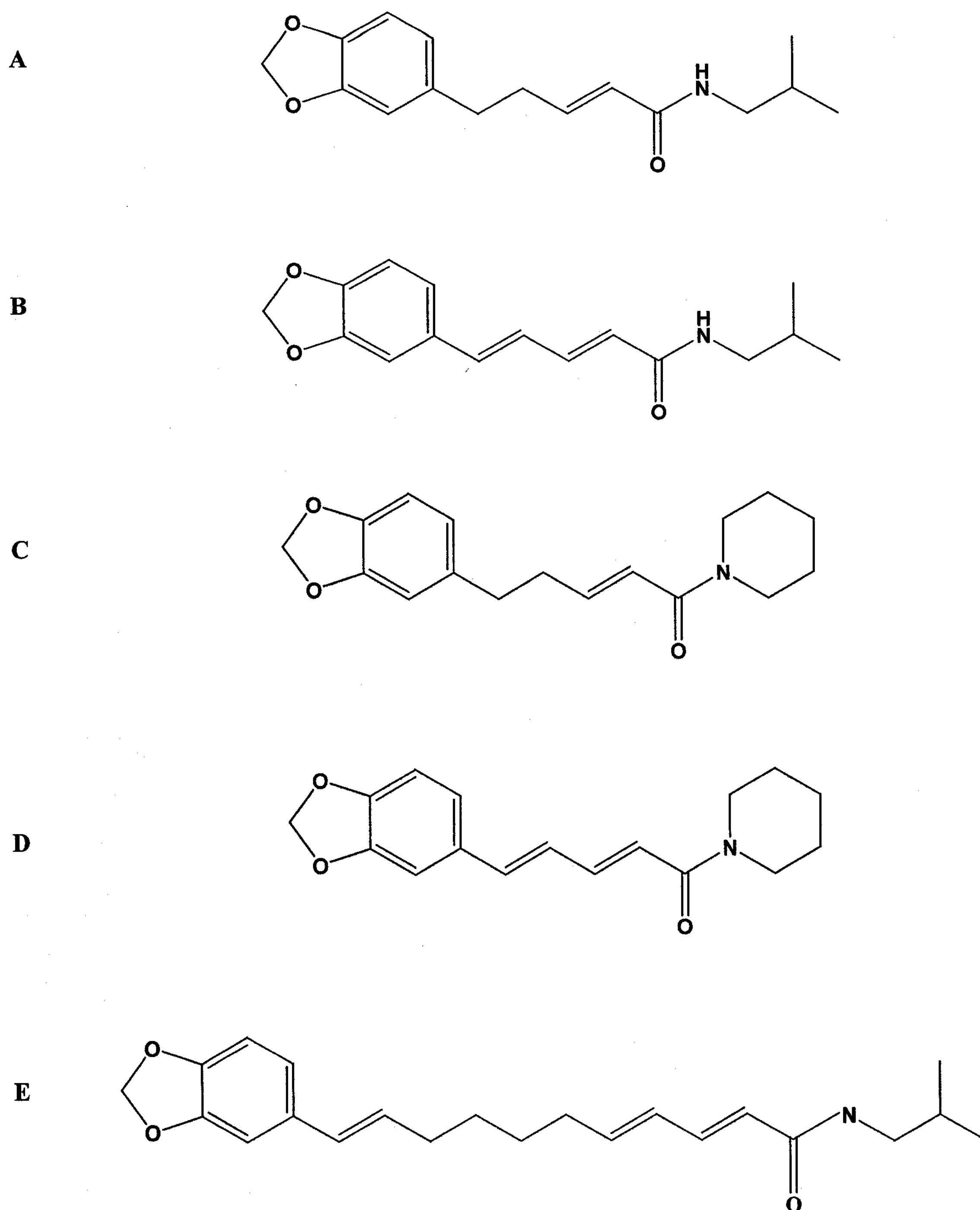
The anti-bacterial and fever-reducing activities of *Piper* extracts are well known from ancient Asian medicinal practises in South Asia as well as in other parts of the world. The compounds providing the medicinal factor are important defences for the plants in order to repel both insects and fungal pathogens (Parmar *et al.* 1997; Lee *et al.* 2001). The wide variety of secondary plant compounds found in *Piper* have already been suggested as potential leads for novel insecticides (Miyakado 1989), while many varieties are used in traditional control of insects that are vectors of disease and damage stored crops. Since these plants contain such seemingly potent insecticidal chemicals, acutely toxic amides and growth-retarding lignans, it would appear quite reasonable to assume that *Piper* would have few natural herbivores to those outside the field of chemical ecology. In fact numerous commercial insect pests and wild pepper specialist insects are reported in the literature: pests on *Piper* plants included insects found feeding on *P. nigrum* and *P. betle* in India (Ranjith *et al.* 1991; Devasahayam and Abdullah Koya 1994, Santhosh-Babu 1994; Raut and Bhattacharya 1999). A question which arose from this information was whether pest species of spice peppers were increasing due to loss of genetic diversity in the plants from selective breeding and over cultivation or because of increasing apparency to the insects. Unfortunately information on this subject specific to peppers was not available.

Table 1. 1 List of phytochemicals found in common Asian *Piper* spice and medicinal species.

Piper species	Secondary compounds	Reference
P. nigrum	N-trans-ferul-oyltyramine; N-5-(4-hydroxy-phenyl) 2E, 4E-pentadienoyl-piperidine; guineensine; N-isobutyl-2E, 4E, 8Z-eicosatrienamide; pellitorine; N-trans-feruloyl-piperidene; feruperine; dihydroferuperine; pipericide; phenolic amide; (E,E)-N-2-methylpropyl) 2,4 deca dienamide; (E,E,E)-13-(1,3 benzodioxol-5-yl)-N-(2-methylpropyl) 2,4 tridecatrienamide; piperonal; piperine; piperoleine B	Tripathi *et al.* 1996
P. longum	aromatic hydrocarbon pipataline; cinnamic acids; sitosterol; pipercide; pipernonaline; asarinine; piperundecalidine; sesamin; sesamine; pluviatilol; methyl pluviatilol;	Tripathi *et al.* 1996
	(+) - asarinin; guineensine; retrofractimide A	Parmar *et al.* 1998
P. betle	allyl pyrocatechol; allyl pyrocatechol; diacetate; carvicol; caryophyllene; charibetol; chavibetolacetate; chavicol; eugenol; hentricontane; methyl-eugenol; p-cymene; pentatricontane; steric acid; α-terpene; tricontanol; tripinylacetate;	Tripathi *et al.* 1996
	β-sitosterol; 14-benzo[1,3] dioxol-5-yl-tetradecan-2-ol; kadsurin A and B; (+) - crotepoxide; dotriacontanoic acid; tritriacontane; cepharadione A; piperine; piperlonguminine;	Parmar *et al.* 1998

Figure 1. 1. Crystal structure of piperine in *Piper nigrum* seeds. Scanning electron image by Dr. Howard J. Arnott with permission, The Center for Electron Microscopy, University of Texas at Arlington.

Figure 1. 2. Structure of piperamides: dihydropiperlonguminine (A); piperlonguminine (B); dihydropiperine (C); piperine (D) and pipercide (E).



Other information on *Piper*-insect relationships came from ecological studies conducted on Central American *Piper* species (Marquis 1984, 1990, 1991). Many neotropical *Piper* species have insect specialists associated with them. Detailed observations among species revealed significant differences in the abundance and diversity of insects that fed on them, (Marquis 1991) and among genotypes of individual species there were detectable differences in herbivore damage (Marquis 1984, 1990). A large number of environmental conditions were included as variables, such as habitat preference, soil type, plant height and abundance of each species in the study area.

In the majority of cases, the genotype or species of *Piper* explained most of the relationship between herbivore preference and resistance against the herbivores. Unfortunately, no detailed information pertaining to the chemistry of many of the same *Piper* plants examined in the insect interaction studies is available. Several recent studies (Hodge 1998, Letourneau 1998, Dyer *et al.* 2001) examined selected species in the Piperaceae and found two defence strategies that may have evolved based on the presence of piperamides. The moth *C. scriptaria* and plant *Macropiper excelsum* system is an example of co-evolved tolerance where the plant can tolerate consistent damage while the insect can tolerate the defence compounds (Hodge 1998). In the second case the ant-plant *Piper cenocladum* has a level of amides present that can provide an additional line of defence against herbivores and pathogens (Dyer *et al.* 2001).

The source of *Piper* species examined in this literature review is mainly cultivated, but several medicinal varieties collected are considered critically threatened. Although the Piperaceae are not considered among the top Ayurvedic medicinal plants in terms of representation (Shankar 1996), the many species collected by ethnic communities are just as threatened. In India as in other parts of the world, biodiversity conservation is an immediate concern to the communities that are dependent on the local plants for human and veterinary medicine. The indigenous people of India use over 7500 medicinal plants, and a subset of 1800 is recognized by the Ayurvedic system. Thus, supporting the tribal people in the protection and conservation of their lands would also benefit the other ethnic communities, since much of

the remaining plants are in locations of high regional biodiversity (Shankar 1996). This may have particular value in South and Central America, where *P. tuberculatum* and other *Piper* species are located and natural resources are similarly under pressure.

1.2.4 Biological activity of *Piper* constituents and mammalian toxicity

The importance of pepper as a spice and food additive has been demonstrated, therefore its regular use in the diet has required careful considerations of any health risks. Piperine is the major constituent in black pepper, so most of the human medicinal and toxicity studies have been conducted using the pure compound (Daware *et al.* 2000; Bhardwaj *et al.* 2002; Khajuria *et al.* 2002), with comparisons to *P. nigrum* extract or other amides. Studies have considered the acute toxicity, reproductive toxicity, effects on metabolizing enzymes, antioxidative, antimicrobial, mutagenic, antimutagenic, melanocyte-stimulating activity and allergen response of piperine. In most cases it was shown to be beneficial if applied at predetermined doses and could actually ameliorate certain conditions. Application of this information to the risks posed by the use of a *Piper* botanical are directly related, although the route and amount of exposure likely encountered under application conditions will be different.

1.2.4.1 Acute and subacute toxicity

In terms of its use in medicine, the route of piperine exposure significantly determines the acute and subacute toxicity dose in mammals (Piyachaturawat *et al.* 1983). The LD_{50}s for male adult mice were 15.1, 43, 400, 330 and 200 mg/kg for single intravenous (i.v.), intraperitoneal (i.p.), intramuscular (i.m.), intragastric (i.g.) or subcutaneous (s.c.) injections respectively. Mice that were treated either i.v., i.p. or i.g. died within 14 minutes after receiving the dose while those receiving the dose by i.m. and s.c. died after three to four hours. In comparison, *P. longum* had an $LD_{50} > 1$ g/kg and subacute toxicity tests, between 15 to 30 days after administration, failed to detect deleterious changes (Rege *et al.* 1999).

In Thailand a dose of traditional medicine contains 7.5 g of *Piper* fruit, which translates to a dose of 7 to 13 mg piperine/kg body weight, assuming an average piperine composition of 6 to 9% and an average human weight of 50 kg (Piyachaturawat *et al.* 1983). Therefore toxic concentrations can only be reached if the administration of the drug is abused within a short period of time. Death in the mice treated by the i.p. acute dosage was apparently by respiratory paralysis within four minutes. The mice treated with the i.g. acute dosage survived for up to four hours. Those mice that did not die had hemorrhaging or hyperemia in the gastrointestinal tract but no significant histopathological changes in other tissue. In subacute studies, piperine at 100 mg/kg body weight /day for seven days was not toxic. However, increasing the dose to 250 mg/kg caused weight loss in treated rats; none died, but 37% had hemorrhaging in the stomach, while 350 and 500 mg/kg killed 25% and 63% respectively within the first three days (Piyachaturawat *et al.* 1983).

1.2.4.2 Effect on metabolizing enzymes

The earlier illustration of how trikatu, a combination of *P. nigrum* and *P. longum*, affected the availability of other herbal drugs, was confirmed when piperine was shown to be a synergist for the effects of other herbals through an inhibition of the body's metabolizing enzymes (Reen and Singh 1991; Singh and Rao 1993; Singh and Reen 1994; Reen *et al.* 1993; Bhardwaj *et al.* 2002) and to cause antioxidative effects (Nakatani *et al.* 1986; Koul and Kapil 1993). Piperine inhibited the activities of arylhydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin deethylase (7ECDE) activities in rat lung microsomes (Reen and Singh 1991) and inhibited 7-methoxycoumarin demethylase (MOCD) activity in hepatoma cells with and without phenobarbitol, a substrate that induces monooxygenase activity (Singh and Reen 1994), and the main drug-metabolizing enzyme, CYP3A4, along with P-glycoprotein transport in human liver cells (Bhardwaj *et al.* 2002).

Piperine was reported to have a monophasic inhibitory effect on polysubstrate monooxygenases (PSMOs), in comparison to the biphasic effect of piperonyl butoxide, where the initial phase of inhibition

of PSMOs is followed by induction within 24 hours after treatment (Dalvi and Dalvi 1991). Piperonyl butoxide and piperine are both methylenedioxyphenyl (MDP) compounds. However the different action toward the PSMOs was thought to be the result of the marked difference in the polarity and biotransformation of their side chains. In contrast however, it was later shown that piperine can cause induction of both phase I and II P450 enzymes of the detoxification system (Singh and Rao 1993). There was a dose-dependent increase in the levels of glutathione S-transferase (GST), cytochrome b_5, cytochrome P-450, acid-soluble sulfhydryl (-SH) and malondialdehyde (MDA) suggesting that piperine may also have biphasic effect.

Piperine reduced the production of MDA in a dose-dependent manner in tert-butyl hydroperoxide (τBHP) or carbon tetrrachloride (CCl_4) treated hepatocytes (Koul and Kapil 1993). Lipid peroxidation induced by *in vivo* treatments of τBHP was reversed 27% and 36% by a 100 and 200 mg/kg piperine dose respectively. Piperine was also found to elevate glutathione (GSH) and other thiols which had been reduced by both τBHP and CCl_4; the increased levels of glutamate pyruvate transaminase (GPT) and alkaline phosphatase (AP) which had been released by hepatotoxicity were reversed. Koul and Kapil (1993) speculate that piperine inhibits the NADPH-dependent cytochrome P450-catalysed reaction which converts τBHP and CCl_4 to active radicals.

The antihepatotoxicity of piperine compares well to that of the more recognized silymarin, a flavonolignan from the milk thistle, *Silybum marianum,* but silymarin has a greater capacity to stabilize membranes and scavenge free radicals. Another flavonolignan, silibinin, has been demonstrated to: 1) stimulate the synthesis of rRNA; 2) increase the activity of DNA-dependent RNA-polymerase I, the enzyme stimulating rRNA transcription and 3) increase the rate of protein biosynthesis by 25-30% compared to controls (Sonnenbichler *et al.* 1998). Thus the flavonolignans, in comparison to the alkaloid piperine, can actually act to regenerate diseased liver. This does not take away from the fact that the presence of piperine could prevent hepatotoxicity from occurring in the first place and eliminate the requirements of therapy.

1.2.4.3 Antioxidative and antimutagenic effect

Since the constituents of *P. nigrum* and other *Piper* species are widely used as spices, one practical application of their presence with food could be the previously mentioned antioxidative activity. This idea was explored by Nakatani *et al.* (1986). Several compounds isolated from *P. nigrum* including piperine and five amides were measured for antioxidative activity and compared with the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and the natural antioxidant α-tocopherol. The results indicate that piperine has no antioxidative activity but the other phenolic amides were as active as α-tocopherol and one was as high as the synthetic BHA and BHT. Similar results were seen by Naidu and Thippeswamy (2002): piperine was the least effective in reducing oxidation of low density lipoprotein compared to other spice components such as curcumin, quercetin and capsaicin.

Thai spices including *P. nigrum* were considered to have antimutagenic activity since plant phenolic acids can reduce the mutagenicity of known carcinogens by inactivation (Ungsurbungsie *et al.*1982). A similar effect was observed with the Somatic Mutation And Recombination Test (SMART): *P. nigrum* and bell pepper, *Capsicum annum*, were found to modulate the genotoxicity of the alkylating agent methyl methanesulfonate (MMS) and promutagen agent ethyl carbamate (EC) (El Hamss *et al.* 2003). The conclusion was that piperine and capsaicin caused inactivation of cytochrome P450 enzymes in the cells of the *Drosophila melanogaster* wing responsible for the activation of the parent compounds to mutagenic metabolites, a physiological effect already observed with liver cells (Koul and Kapil 1993).

In contrast, high capsaicin content in the diet is implicated with elevated incidence of stomach cancer cases within specific ethnic populations (Archer and Jones 2002). There was less epidemiological evidence for association between "Safrole pepper" or black pepper consumption and cancer incidence. Although it is a mutagen, safrole is present at a much lower concentration in black pepper (0.2%) than capsaicin is in red, cayenne or chilli pepper (up to 1%). Interestingly, colon cancer rates were less when

capsaicin consumption was higher in the population. This was thought to relate to lowered fermentation rates in the gut by capsaicin's effect on bacterial carcinogens, whereas safrole pepper was suggested as slightly elevating the rate in populations that consume it more (Archer and Jones 2002). Several spices, including capsaicin and piperine, were observed to decrease the food transit time in rats fed for six weeks due to effects on the digestive enzymes or bile excretion (Platel and Srinivasan 2001). This action is believed to reduce the incidence of colon cancer by decreasing the colonic transit time, similar to a high fibre diet. At the same time, piperine also was found to induce alterations in intestinal membrane dynamics, permeation characteristics and protein synthesis leading to an increased absorptive surface (Khajuria *et al.* 2002). This trade-off between decreased transit time and increased absorptive capacity probably explains why no weight loss was observed during the piperine six-week feeding trial compared to controls.

1.2.4.4 Mutagenicity and carcinogenicity

Mutagenic effects have been described for black pepper and piperine when they were reacted with excess nitrite at pH 3.5, forming 6-nitropiperonal (NPA) (Wakabayahi *et al.* 1989). When metabolic activitation was included, *N*-nitrosopiperidine (NPIP) was formed, and both were determined to be major mutagens with Salmonella strains TA100 and TA98. This is not surprising since *N*-nitroso compounds such as *N*-methyl-*N*'-nitro-*N*-nitro-soguanidine and *N*-methyl-*N*-nitrosourea are known to induce stomach cancer. The leading derivatives were from phenol and indole compounds provided in certain foods such as fermented soy sauce containing tyramine, various smoked meat products and fresh vegetables including Chinese cabbage, radish root and spinach (Wakabayahi *et al.* 1989).

Further study with piperine-nitrosoation reaction detailed other products that might account for the level of mutagenicity observed (Shenoy and Choughuley 1992). Along with the *N*-nitrosation products, NPA and NPIP, three new compounds were formed from C-nitrosation: 1-5[(1,3-benzodioxol-6-nitro-5-yl)-1-oxo-2,4,E,E-pentadienyl] piperidine (MNAP); 1-5[(1,3-benzodioxol-5-yl)-1-oxo-2-nitro-

2,4,E,E-pentadienyl]piperidine (MNOP); 1-5[(1,3-benzodioxol-6-nitro-5-yl)-1-oxo-2-nitro-2,4,E,E-pentadienyl] piperidine (DNP). Along with Wakabayashi *et al.* (1989), Shenoy and Choughuley (1992), concluded that the *in vitro* conditions required for the piperine-nitrite derived mutagen formation exceeded the amount normally found in the diet and did not take into account other protective factors in the body, but that the prevalence of stomach cancer in many parts of the world could never-the-less be explained by nitrosable aromatic compounds present in the diet.

1.2.4.5 Effect on the nervous system

P. nigrum and *P. longum* are recommended for pain relief, which according to Fodor and Colosanti (1985) is due to the effect piperine has on the activity of the central nervous system (CNS). Piperine effects resemble the action of classical CNS depressants although higher doses do not produce anesthesia (Fodor and Colosanti 1985). The mechanism of action involves central serotonergic pathways, and the activity of other depressants is intensified by piperine exposure. It was determined that N-alkylamides act at site 2 of the sodium channel (Ottea *et al.* 1990), which in the mouse brain is the alkaloid activator recognition site. The isobutyl amides found in other plants such as *Echinecea* spp. are noted to have a similar anaesthetic effect: pharmacologically a voltage-gated sodium channel partial agonism, creating a tingling sensation, versus the numbing sensation caused by antagonism of the voltage-dependent increase in sodium conductance (McFerren *et al.* 2002). Other mechanisms associated with piperine interaction with nerve cell functioning will be discussed in the insecticidal activity section.

1.2.4.6 Stimulation of melanin, allergies and contraceptive effects

A further claim for *P. nigrum* in Ayurvedic medicine is as a cure for skin conditions. Earlier confirmation of this showed that piperine has antifungal activity, but Lin *et al.* (1999) were the first to show that it could also stimulate melanocyte proliferation. A *P. nigrum* fruit extract dissolved in a growth medium was observed to stimulate the mouse melan-a cell line at 0.01 and 0.1 mg/mL compared to

control, but the extracts at 1 mg/mL were cytotoxic. The actual cellular melanin content had increased between 50 and 60% indicating it was the number of cells increasing rather than the actual size of cells. When 1 μM piperine was tested it was shown to have an even greater activity than the extracts, coming close to the positive control, 20 nM tetradecanoyl phorbol acetate (TPA). It was also found that the effect of both piperine and the extract was inhibited by the presence of RO-31-8220, a selective inhibitor of protein kinase C (PKC). This suggested to Lin *et al.* (1999) that piperine is the most active principle in *P. nigrum* extract with respect to melanocyte stimulation and that the mode of action is the activation of PKC signalling.

One aspect of environmental exposure to *Piper* extracts is the potential allergic response. Piperaceae and other spice families were characterized by Ebner *et al.* (1998) by IgE-immunoblotting and by raising monoclonal antibodies against cross-reactive pollen allergens. Green and black peppercorn extracts had cross-reactive allergens with birch and mugwort pollen and celery tuber extracts. Therefore concern over the use of the pepper extracts by people already sensitive to other environmental allergies should be addressed.

A similar concern over the possible estrogenic nature of these compounds should be addressed since there has been a traditional use of black pepper extracts for contraception. In fact, piperine was shown to interfere with the estrous cycle, mating behaviour, fertilization and implantation in mice (Daware *et al.* 2000). Doses of 10 mg/kg body weight piperine reduced implantation but did not affect either

post-implant survival or sex ratio of surviving fetuses. In male rats, piperine doses of 5 and 10 mg/kg decreased the weights of testis, cauda epidymal and vas deferens significantly, along with intratesticular testosterone levels (Malini *et al.* 1999b), likely due to decreases in the testicular lipogenesis and possibly steroidogenesis (Malini *et al.* 1999a). Piperine at doses up to 75 mg/kg did not induce significant abnormalities in sperm shape, an indication of mutagenesis in male germ cells (Daware *et al.* 2000). It was thought that the reduction in fertility by 40% with 10 mg/kg may be involved with reduced prostaglandin

levels, but that these doses are well above human consumption or exposure levels and thus are unlikely to exert a harmful effect on human reproductive function. Confirmation that prostaglandin inhibition may be part of the effect *Piper* species have in treatments of inflammatory diseases such as rheumatism was provided by Stöhr *et al.* (2001). They surveyed 19 *Piper* species and found that several including *P. nigrum* were responsible for inhibiting the enzyme, cyclooxygenase-1 or COX-1, responsible for prostaglandin formation, as well as 5-lipoxygenase or 5-LOX, responsible for proinflammatory leukatriene formation.

1.2.5 Insecticidal activity

There is a current and future requirement for alternatives for pest control (National Research Council 2000) and surveys of several plant families (Lydon and Duke 1989; Isman 1994; MacKinnon *et al.* 1997) have found sources for new botanical insecticides that could possibly meet some of this demand. Piperaceae is one family that has many promising phytochemicals with insecticidal activity, the most recognized members being *P. nigrum*, *P. guineense* and *P. tuberculatum*. These species all contain high concentrations of fast-acting piperamides which provide a knock-down effect and also a recognized repellent and antifeedant activity, along with a built-in synergistic effect.

1.2.5.1 Efficacy

Early investigations with *P. nigrum* indicated that piperamides were responsible for the toxicity of the extracts to the adzuki bean weevil *Callosobruchus chinensis* L. (Miyakado *et al.* 1979 and 1980). *P. nigrum* oil formulations were found to effectively protect stored wheat from both stored grain pests, *Sitophilus oryzae* (L.) and *Rhyzopertha dominica* (Fabricius), at concentrations above 100 mg/L for up to 30 days (Sighamony *et al.* 1986). Stored beans were protected from the bruchid *Acanthoscelides obtectus* Say by ground black pepper for up to 18 weeks (Baier and Webster 1992). Three of the piperamides isolated from *P. nigrum* pipercide, pellitorine and piperine ranged in toxicity from 0.15, 2 and 20 µg/

male *C. chinensis* respectively (Dev and Koul 1997). Guineensine, isolated from *P. guineense*, had relatively the same activity as pipercide when tested topically on the cow pea weevil *Callosobruchus maculatus* (Fabricius), 0.25 versus 0.84 µg/ male (48 hour LD_{50}). *Piper guineense* treated kaolin powder at 150 µL/g reduced the average adult emergence of *C. maculatus* by 100% after 30 days treatment (Kéïta *et al.* 2000). Dust and ether-extract formulations of *P. guineense* were also effective at controlling *C. maculatus* at concentrations between 0.5 and 0.75 g/20g cow pea seed within 36 hours after treatment (Mbata *et al.* 1995). Emergence of adults from treated eggs was prevented successfully with dust and extracted oil treatments at 0.25 g/seed. Aqueous mixtures of oven-dried powdered *P. guineense* at 10 mg/L were found to be effective at controlling 4th instar mosquito *Aedes aegypti*, but were not toxic to other aquatic organisms (Okorie and Ogunro 1992).

1.2.5.2 Mode of action

Piperamides are known to act as neurotoxins in the insect. Lipid amides were initially observed to modify axonal excitability by an effect upon sodium currents, at first considered to be "pyrethroid-like" (Lees and Burt 1988). However, later it was determined to be a mechanism distinct from that of the pyrethroids when a CNS preparation from American cockroaches, *Periplaneta americana*, resistant to pyrethroids were affected by the same doses of pipercide that affected susceptible cockroaches (Miyakado *et al.* 1989) and when isobutyl amides were shown to be more potent than pyrethroids against a resistant (*super-kdr*) strain of house fly (Elliott *et al.* 1986).

Isobutyl amides from black pepper and their synthetic derivatives (de Paula *et al.* 2000) and from guinea pepper (Gbewonyo *et al.* 1993) were characterized by structure-activity relationships. Synthetic piperamides, derived from natural piperine and piperiline by substitutions at the N– position, showed no clear correlation with structure and activity, although N,N-disubstituted amides were most active against the lepidopteran *Ascia monuste orseis* (Pieridae) (de Paula *et al.* 2000). The results of this study were in contrast to the previous work of Miyakado *et al.* (1985, in de Paulo *et al.* 2000) which showed that N-

isobutyl substitution had the highest activity. Gbewonyo *et al.* (1993) found that compounds with the methylenedioxyphenyl (MDP) group were highly toxic but did not have the knockdown toxicity of the piperamides without the MDP group.

Insecticide manufacturers routinely combine nontoxic MDP containing compounds such as piperonyl butoxide (a derivative of safrole) with formulations to enhance the toxicity (Hodgson and Levi 1998). Further work with piperovatine, an isobutyl amide from *P. piscatorum*, found that concentrations greater than 10 μM increased neuronal intracellular calcium concentrations in cultured *P. americana* neuronal cells (McFerren *et al.* 2002). It was also observed that the increased Ca^{2+} concentration was not affected by a muscarine acetylcholine receptor (mAchR) agonist, atropine, indicating that isobutyl amides do not interact with that neurotransmitter system. In contrast, the piperovatine Ca^{2+} effect was eliminated by the application of a voltage-gated sodium channel blocker, tetrodotoxin (TTX), indicating the importance of the voltage-gated Na^{+} channel directly or indirectly controlling the release of intracellular Ca^{2+} stores (McFerren *et al.* 2002) and supporting the earlier findings of Ottea *et al.* (1990).

Piperamides singly, or more importantly in combination, could have a role to play in replacing contact insecticides, specifically neurotoxic compounds such as carbamates, organophosphates and pyrethroids, for which resistance has developed. A combination of these amides within a botanical formulation would thus provide the advantage of all of the previously mentioned attributes: novel target site, enzyme inhibition, low mammalian toxicity and low mutagenic properties. The important next step is to conduct efficacy trials with insect species of importance to a North American market.

1.2.6 Efficacy trial insects

The target insect species chosen for efficacy trials with the *Piper* botanicals are those currently of concern to gardeners and horticulturalists in eastern Canada and the northeastern United States and were also selected for use based on their abundance and availability. The insects tested were selected from the following orders:

Coleoptera

1) Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Chysomelidae), is recognized as one of the most damaging insect pests to potato crops in Canada. Larvae and adults will feed on several horticultural plants of importance in the Solanaceae family, including sweet pepper, eggplant, tomato and potato. Adults overwinter in the soil, appearing in early June to lay eggs. Larvae have four instars and pupate in the soil, with normally only one generation per year in Canada but two in southern areas (Boiteau and LeBlanc 1992). Colorado potato beetle larvae and adults can be controlled both through the use of cultural practices and selected insecticides, but this species has extensive insecticide resistance.

2) European chafer, *Rhizotrogus majalis* Razoumowsky (Scarabaeidae), a major pest of turfgrass. Accidentally introduced into the northeastern U.S. in the 1940s, it spread to Canada in the 1960s. Larvae consume roots of cool-season grasses, especially Kentucky bluegrass, leading to large patches of dead turf once grass dries out. Adults emerge in mid- to late June in Ontario, when huge swarms aggregate in trees, mating occurs and females lay eggs in the ground. Adults do not typically feed, but certainly cause some damage to leaves. Larvae are larger than Japanese beetle larvae and cause more damage to turf, and will also feed later in the autumn and earlier in the spring. Larvae can be controlled with carbamate, organophosphate and pyrethroid insecticides but with limited success. Bacterial pathogens and nematodes are similarly not as effective as with Japanese beetles (Potter 1998).

3) Japanese beetle, *Popillia japonica* Newman (Scarabaeidae), is a significant pest of fruits and ornamental plants in the adult stage and larvae also feed on roots of ornamentals, fruit trees and turfgrass. The adult beetles feed on a wide variety of deciduous trees and shrubs including roses, grapes, crape myrtle, flowering crab and cherry, Japanese and Norway maple, elm, Virginia creeper and many others. Accidentally introduced to U.S. in 1916, they eventually spread to Canada in the 1940s. Adults in Ontario

emerge in early July and can live for 30 to 45 days. They lay eggs in the ground which hatch over two weeks, larvae then feed until cold weather forces them to burrow deeper for the winter. Control includes the use of larvicides, biocontrol agents including pathogenic bacteria and nematodes, and selected insecticides for adults and larvae (Johnson and Lyon 1991).

4) Lily leaf beetle, *Lilioceris lilii* Scopolie (Chysomelidae), is an imported pest, first found in Montreal, Quebec, in the 1940s, which has an expanding range in Canada and the U.S. (LeSage and Elliott 2003). The adult and larvae feed principally on Asiatic lily (Lilliaceae), but will also choose lily of the valley, Solomon's seal and Fritillaria species. The adults overwinter in the ground and emerge in the early spring. Females can lay up to 300 eggs and some can live over two seasons. The larvae require two to three weeks for development and pupate for approximately three weeks in the soil. There are no registered insecticides for control of this insect although those that are recommended for general leaf beetle control may be used (Smith 2003).

5) Striped cucumber beetle, *Acalymma vittatum* Fabricius (Chysomelidae), causes damage through feeding on leaves and fruit of Cucurbitaceae plants: cucumber, squash, pumpkin and melons. Larvae also feed on the roots. Most importantly, it is a vector for bacterial wilt and squash mosaic virus, which is even more deleterious to the plant than the feeding of the beetle itself. Adults overwinter along edges of fields under plant debris and emerge in early spring and will feed on cucurbit seedlings. Eggs are deposited in the soil close to base of plants, and developing larvae feed on roots and stems for first two weeks. Several generations per season are possible but the first generation has the greatest impact on cucurbit crops (Lyon and Smith 2000). Control is through continuous monitoring of fields in order to apply insecticides such as carbamates and Imidachloprid, since certain cucurbits are very susceptible to bacterial wilt and little feeding damage can be tolerated (Foster and Brust 2000).

6) Viburnum leaf beetle, *Pyrrhalta viburni* Paykull (Chrysomelidae), is another exotic insect pest of horticultural plants, the European highbush cranberry, *Viburnum opulus*, in particular; it can also cause moderate damage to *V. lantana* and *V. rafinesquianum*. It was first reported in North America in the 1940s but not reported as established until the 1970s. Feeding larvae will strip leaves, and will kill the plants after two to three seasons of this severe defoliation. The beetle overwinters as eggs in holes in the twigs, emerging in May and feeding through June. Pupae are found in the ground and adults emerge in July. Female adults will feed until first frost, during which time eggs are deposited for the next year's generation. Control can be through removal of beetles in late spring by shaking branches onto a sheet placed underneath the shrub and drowning the insects in soapy water. Carbamate insecticides can also be used (Ventresca and Kessel 2000).

Dermaptera

The earwig, *Forficula auricularia* L. (Forficulidae), is usually just a recurring nuisance insect. Its forcep-like tail is used for defence but can give a sharp pinch (Potter 1998), and anecdotal evidence suggests that earwigs can cause damage to horticultural crops such as lettuce that can lead to added labour costs such as pre-market cleaning of produce. Eggs are laid in brood chambers in the ground, and will hatch in a few weeks or overwinter. The nymphs leave the ground a few days after hatching and then require six to seven weeks to mature. Females may lay several clutches of eggs per year. Cultural practices such as habitat removal are usually enough to keep insects away from the home, but insecticides can be applied along the foundation when infestations occur (Potter 1998).

Hemiptera

Hairy chinch bug, *Blissus leucopterus hirtus* Montandon (Lygaeidae), is a major pest of turfgrass, especially lawns. Adults and nymphs feed on grass stems and crowns by inserting sucking mouthparts to

extract juices, but at the same time introducing salivary juices into the plant. The combination of fluid loss, damage to plant tissue and drought conditions leads to dead and damaged turf in mid to late summer. The chinch bug prefers cool-season grasses like Kentucky bluegrass and perennial ryegrass and fescues. In southern parts of its range there can be two generations but usually in Ontario one generation per year is the norm with adults overwintering in the thatch. Females lay eggs in mid-to-late April, which require a month to develop. The nymphs immediately pierce grass stems and begin feeding for a four to six weeks until adults appear. Control with management practices such as thatch removal and frequent irrigation along with adequate fertilization helps to encourage grass better able to tolerate feeding damage (Potter 1998).

Hymenoptera

European pine sawfly, *Neodiprion sertifer* Geoffroy (Diprionidae), feeds from 1st to 5th larval instar on ornamental conifers such as red, Scots, Japanese red, jack, Swiss and Mugo pine. Eggs overwinter in needles and hatch in early spring. The pupae form a cocoon, from which adults emerge to deposit eggs in the needles of the host plant using the saw-like apparatus on the tip of the female abdomen. Outbreaks can strip mature needles and cause severe injury to landscape ornamentals (Johnson and Lyon 1991). Soapy water and mineral oil sprays are recommended for control.

Lepidoptera

1) European corn borer, *Ostrinia nubilalis* Hübner (Pyralidae), is a major insect pest of corn, but will feed on other horticultural crops such as sweet pepper, beans, potatoes, tomatoes, apples and other crops. It was introduced in the early 1900s, and has spread throughout most U.S. states east of the Rockies and southern Canada. In Canada, one and two generation (uni-and bivoltine) populations occur (Van Dyk 1996). Fully grown larvae over-winter in stalks of corn or other host plants. In the spring they pupate at the over-wintering site and emerge as adult in late May to mid-June. Eggs are laid on leaves and hatch in three or

more days. Larvae burrow into stalk of plants and developing kernels. In peppers, corn borers enter the fruit underneath the cap and feed extensively on the fruit, and damage may not be detected until harvest. Control can be offered with *Heliothis* traps to monitor adult activity followed by a regular spray schedule with selected insecticides (Hagerman 1997).

2) Ermine or spindle moth, *Yponomeuta cagnagella* Hübner (Yponomeutidae), introduced from Europe to Ontario in the 1960s, is a pest of ornamental *Euonymus* spp. (*E. europaea*, *E. kiautschovius* and *E. alatus*). Larvae are the damaging stage: eggs hatch in August, and overwinter as larvae, emerging early in spring to feed and make small webs which can eventually cover large branches. Larvae form cocoons in late June, and emerge as adult moths in July to lay eggs (Johnson and Lyon 1991). No control treatments are recommended; physical removal of webs would work best, although it would be labour intensive during large outbreaks.

3) Eastern tent caterpillar, *Malacosoma americanum* Fabricius (Lasiocampidae), a native defoliator, with outbreaks occurring every 10 years, causes damage to deciduous forests and ornamental shrubs and trees along with unsightly nests. It favours wild cherry, apple and crabtree, but will also feed on ash, birch, willow, witch-hazel, maple, oak and poplar. Over-wintering as eggs, larvae emerge as leaves unfold and build a web which can eventually cover several branches. When larvae reach full size they leave the tree and search for a location to spin a cocoon. The moth emerges in early summer to locate a host tree on which to oviposit egg masses (Johnson and Lyon 1991). Larvae can be controlled through removal of nests, oil sprays or selected insecticides.

Nontargets

Non-target toxicity was also evaluated using an insect species that would likely encounter the effects of the botanical treatment. Applications in the garden could affect the lady beetle *Hippodamia*

convergens Guérin-Méneville (Coccinellidae) as beneficials or predators of garden insect pests and turf treatments could affect the earthworm as exemplified by the compost worm *Eisenia fetida* Savigny (Oligochaeta: Lumbricidae).

1.3 Rationale and Objectives

A market survey by FAAR biotechnology determined that there was a requirement for alternative insect pest control products especially for the home and garden market in North America. Therefore the goal of this research was to investigate the use of *Piper* spp. as the basis of an alternative botanical insecticide. India has already begun to develop biodegradable insecticidal compounds and antitumour drugs from *Piper* species (Parmar *et al.* 1997). Publications by this group and others have described extensively the chemistry, biological activity and medicinal uses of the Piperaceae (Tripathi *et al.* 1996, Parmar *et al.* 1998). The application of these species in insect pest control appears plausible since a low degree of processing is required, which in turn significantly decreases the cost of the botanical preparation and creates a competitive product. Further, mixtures of analogous compounds can provide a successful strategy, as exemplified by neem, *Azadirachta indica.* Insect detoxification enzymes have greater difficulty metabolizing mixtures of analogs (Berenbaum and Zangerl 1996), and there is less chance of resistance development over many generations with multiple actives (Feng and Isman 1995).

Objective 1. Development of an analytical method for measuring active components in *Piper* extracts.

The validation of an HPLC and LCMS technique was undertaken in order to separate, identify and quantify the key active phytochemicals in *Piper* extracts.The processing and extraction steps were designed to reduce the amount of waste and use of toxic solvents. Sources of germplasm were assessed in terms of the isobutyl amide concentration in *P. nigrum*, *P. guineense* and *P. tuberculatum.*

Objective 2. Determine the efficacy of *Piper* botanical with selected insect pests of home and garden.

In order to assess efficacy to a variety of appropriate target insect pests and assess non-target toxicity to other invertebrates a number of toxicity trials and endpoints were used. Establishment of an effective dose for knockdown and acute toxicity (LD_{50}) and repellent effect tested in controlled treatment area was undertaken. Experiments consisted of different application techniques: sprays and dips for use around the home and garden, including a formulation for spot treatments of white grubs, *R. majalis* and *P. japonica*, for use on lawns. By the completion of the project, the final formulations were documented in terms of the chemical composition, efficacy and non-target toxicity. Recommendations for their use were also provided with the objective of integrating the use within an IPM approach for a specific insect control scenario.

Objective 3. A further examination of the inhibition of metabolizing enzymes specific to insects in order to better understand the mode of action in insects.

The literature review surveyed the biological activity of *Piper* extracts and piperamides and documented that many of the mammalian pharmacological properties claimed for *Piper* spp. are related to effects on PSMO enzyme systems. Therefore the importance of this effect in insect models was assessed for the first time. Using techniques to measure PSMO activity (cytochrome P450 and b5, MROD), the housefly (*Musca domestica*) and American cockroach (*Periplaneta americana*) were used as a model insects in order to test whether microsomes treated with piperine would show reduced activity (inhibition) or are increased (induced). Toxicity bioassays comparing insecticide-susceptible and resistant houseflies and Colorado potato beetle were used to determine whether there is a different target site for piperamides compared to other neurotoxic compounds such as pyrethroids.

Objective 4. Determine the fate of piperamides in the insect and the persistence in the environment.

This insect study was undertaken to determine distribution and residence time in target insects. A radiolabelled piperamide (piperine) synthesized in the laboratory of Dr. T. Durst, Chemistry Dept., U. of Ottawa, was applied topically to the American cockroach at sublethal levels and then sampled in the insect and frass for a period up to 96 hours. The uptake and depuration of the parent compound and metabolites were assessed in relation to the dose of piperine applied. In the environmental fate study, sunlight / UV light and shelf half- life and degradation rates in the soil after white grub treatment were investigated using standard industry protocols. Methods for soil extraction were developed and recoveries compared between different field sites.

1.4 Summary

Both extracts and single active *Piper* compounds have shown to be relatively toxic to a range of insects, including houseflies, mosquito larvae, stored grain and bean weevils (Miyakado *et al.* 1979 and 1980, Sighamony *et al.* 1986, Dev and Koul 1997). Combined with evidence that the extracts have low mammalian toxicity (Piyachaturawat *et al.* 1983) and low mutagenic potential (Wakabayahi *et al.* 1989; Shenoy and Choughuley 1992; Archer and Jones 2002), these findings suggest that these extracts will provide a safe alternative for controlling household insect pests. Several possible routes of extract application are under consideration, including aerosols for flying insects and phytophagous garden pests and through surface applications or dusts. This project has an obvious practical application, yet the understanding of the mode of action is important from the ecological plant-insect interaction perspective and also for registration. Testing both the hypotheses that the phytochemical components in *Piper* spp. interact through analogue synergism, and inhibit the insect detoxification system at the same time helps to understand the strategy applied by plants within this tropical family. Equally important is the improvement in the methods to analyze and characterize the phytochemicals both from the plant material and in other media such as soil and insect tissue. The botanical formulation is intended to function as a component

within an integrated pest management approach rather than constituting the sole solution to insect pest control.

CHAPTER 2

ANALYSIS OF PIPERACEAE GERMPLASM BY HPLC/LCMS AND BIOASSAY: ISOLATING AND IDENTIFYING PIPERAMIDE FRACTIONS FROM EXTRACTS

2.1 Introduction

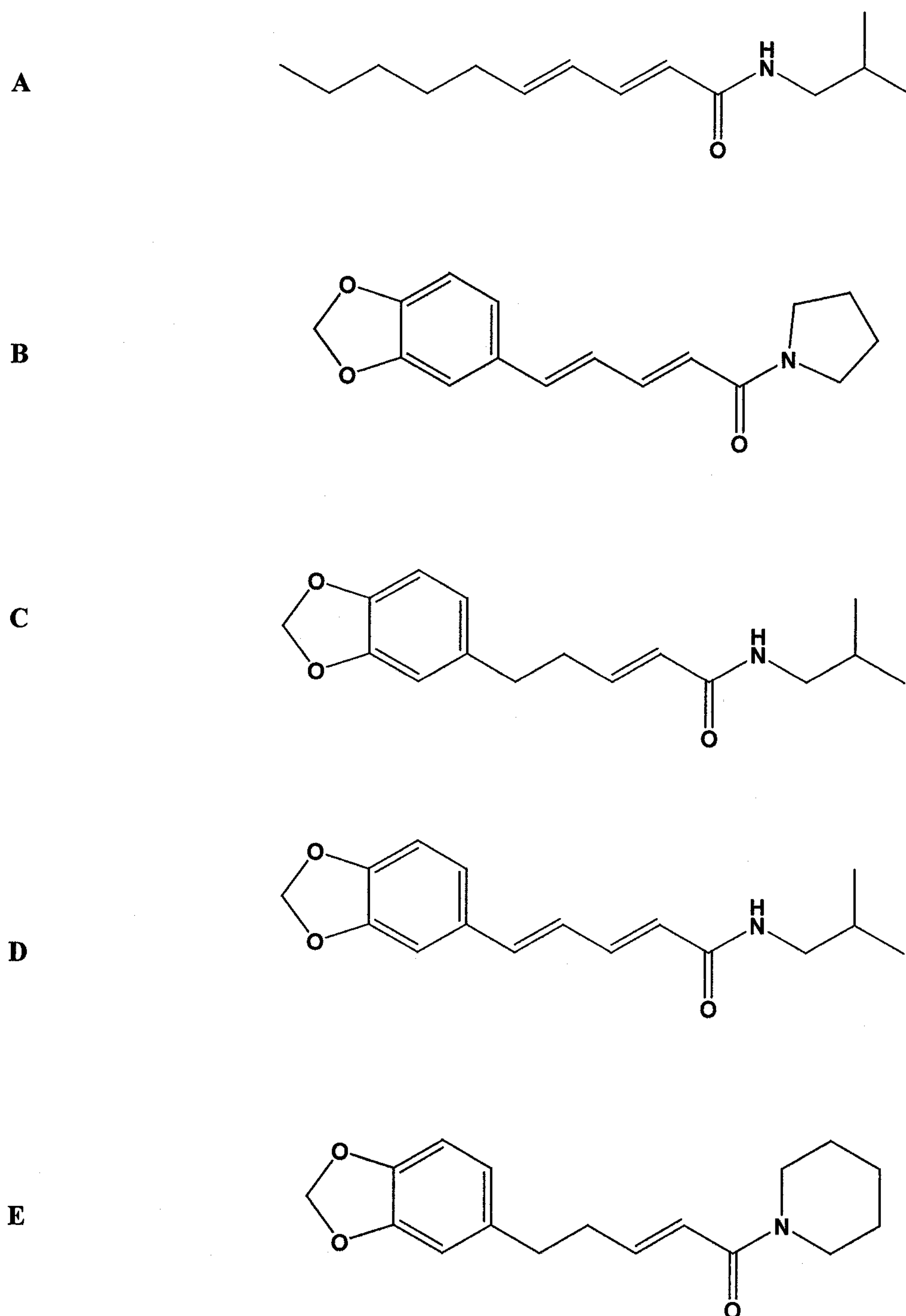
The plant family Piperaceae is a source of many biologically active phytochemicals (Parmar *et al.* 1997 and 1998) with great potential for medicinal (Tripathi *et al.* 1996) and agricultural use (Miyakado *et al.* 1989). Species in the genus *Piper* have a wide array of secondary compounds, principally alkaloids and amides (Parmar *et al.* 1997). The most widely recognized species is black pepper, *Piper nigrum* L., a spice traded around the world for hundreds, if not thousands, of years. In India, the majority (90%) of *P. nigrum* is grown along with cardamom and ginger in the southwestern state of Kerala (Narayana *et al.* 2000), where many of the world spices are believed to have originated. Other tropical countries including Indonesia, Malaysia, Brazil and Vietnam account for the remainder of the world pepper production. Use as a spice has been extended from simple culinary application in the home to industrial scale processing of essential oils and oleoresins as both food additives and pharmaceuticals (Narayana *et al.* 2000). As such there is an increasing requirement for improved extraction and analytical methods to ensure quality and consistency in the final product.

Three *Piper* species were chosen for use in the development of a separation and identification method that would be practical and applicable to many end users, especially those wishing to commercialize *Piper* species as a botanical insecticide. Those chosen, *P. nigrum* along with the West African Guinea pepper, *P. guineense* Schum and Thonn, and the Central American *P. tuberculatum* Jacq., have recognized insecticidal activity (Bernard *et al.* 1995; MacKinnon *et al.* 1997; Scott *et al.* 2002). Several extraction methods for *P. nigrum* and *P. guineense* have been described previously (Dwuma-Badu *et al.* 1976; Adaae-Mensah *et al.* 1977a and 1977b; Kiuchi *et al.* 1988; Semler and Gross 1988), the

purpose of which was to isolate and identify active compounds from the leaves, seeds and roots of those plants. High performance thin layer chromatography (HPTLC) was used to determine the main *P. nigrum* piperamide, piperine, in peppercorns for drug application in India (Kulkarni *et al.* 2001). HPLC techniques were used to fractionate *P. guineense* into peaks, bioassays indicated which were active and then compounds were identified by ^{1}H NMR (Gbewonyo and Candy 1992). Similarly *P. tuberculatum* fractions were separated by column chromatography and preparative TLC and identified by electrospray mass spectrum (ES-MS) and ^{14}C NMR (Navickiene *et al.* 2000) or by GC/MS with identification by ^{1}H and ^{14}C NMR (da-Cunha and Chaves 2001). HPLC methods have also previously been developed in order to measure the plasma and tissue levels of piperine in animals (Sunkara *et al.* 2001; Bajad *et al.* 2002).

One of the first active compounds isolated from *P. nigrum* was piperine (see Figure 2.1F), an amide that provides the pungent taste associated with black pepper. Many other amides have since been identified in *Piper* species from all tropical regions of the world (Parmar *et al.* 1997), but it was Kiuchi *et al.* (1988) who proposed the systematic nomenclature for the Piperaceous plants. The term piperamide was chosen to describe all compounds carrying an aromatic group and an amide group. The mixture of piperamides present in many *Piper* species demonstrates the diversity and apparent redundancy of phytochemicals within species (Romeo *et al.* 1996). For example, the combination of four principal amides in *P. tuberculatum* was explained previously by this group (Scott *et al.* 2002) as an example of analogue synergism and revealed phytochemical diversity at a variety of levels: different tissues of the same plant may exhibit different chemical profiles and individuals may differ in their phytochemistry within a population.

Figure 2. 1. Structure of piperamides: pellitorine (A); piperylin (B); dihydropiperlonguminine (C); piperlonguminine (D); dihydropiperine (E); piperine (F); pipercide (G).



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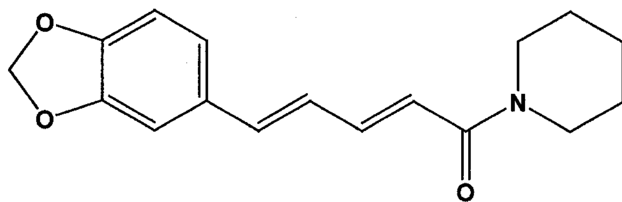

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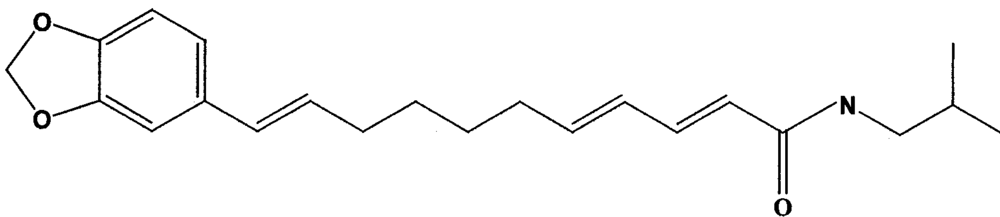


Piperine was shown to be distributed heterogeneously within parts of *P. nigrum* plants, with the fruit having the highest amount, although 2 to 10-fold differences between samples have been observed (Semler and Gross 1988). At a larger scale, geographically distinct populations of plants may differ in phytochemical concentration, potentially as a result of selective pressures imposed by the different herbivores that attack the plant throughout its range (Berenbaum and Zangerl 1996) or selection by humans. In assessing a source of germplasm as a potential supply of material for botanical use, it is necessary to compare different geographical and ecological regions. In this study, sources of germplasm were assessed in terms of the piperamide concentration in both *P. nigrum*, available commercially from a large number of wholesale suppliers of spice, and *P. tuberculatum*, collected at two sites in Costa Rica. A previous study has indicated that the efficacy of *P. nigrum* extracts from three sources in Southeast Asia is similar (Scott *et al.* 2002). *P. tuberculatum* leaves collected from locations in Costa Rica also showed similar piperamide profiles. This species is of particular interest because of the presence of piperamides in the leaf material, which has a greater potential than seed for large-scale production.

The objectives in the present study were 1) to develop methods in order to speed up the extraction of *Piper* materials, 2) to optimize the recovery and 3) to provide repeatable analysis and identification of the principal active components. This is particularly important for analyzing whole extracts since the goal is to be able to measure the quantity and quality of *Piper* germplasm and extract batches where the end use is a botanical insecticide composed of the major active compounds concentrated from the leaves and seeds.

2.2 Methods and Materials

2.2.1 *Piper* germplasm sources

Kernels of *P. nigrum* were ordered through spice distributors in Canada, the United States and Singapore. Peppercorns originated from four commercial sources in India (Malabar) and Indonesia. *P. guineense* was obtained from only one source in Togo, West Africa, and was compared with the previous

analyses by Scott *et al.* (2002). Two sites in Costa Rica were chosen as *P. tuberculatum* sources, Puntarenas and La Pacifica. Previous analyses (Scott *et al.* 2002) indicated that there was a difference in the levels of piperamides in leaves collected from these locations, La Pacifica having greater levels of the principal piperamide, 4,5-dihydropiperlonguminine. Other *Piper* species collected in Costa Rica included *P. nudifolium* C. DC., *P. cordulatum* C. DC., *P. aquale* Vahl, *P. biseriatum* C. DC., *P. pseudo-lindenii* C. DC. and a previously unknown species referred to as *Piper* species A. Leaf and peppercorn voucher specimens were deposited in the University of Ottawa Herbarium and Universidad National, Heredia, Costa Rica.

2.2.2 *P. nigrum*, *P. guineense*, *P. tuberculatum* and other Costa Rican *Piper* spp. extraction

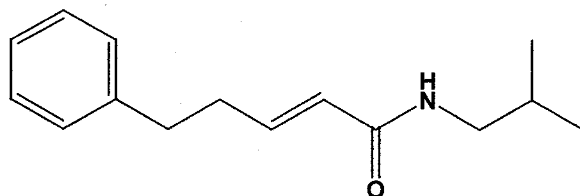
Two extraction processes for both *P. nigrum* and *P. guineense* peppercorns were chosen in order to compare extraction efficiency. The first was previously described by Scott *et al.* (2002) and the second, a modification of that technique, is as follows: *P. nigrum* or *P. guineense* peppercorns (50 g) were ground finely with a coffee grinder then covered with ethyl acetate (125 mL) and refluxed, using a water-cooled condenser, at a boil for 20 minutes. The grounds and ethyl acetate slurry were shaken for 24 h followed by suction through a Buchner funnel with Whatman No. 1 filter paper to remove insoluble material. The filter cake was rinsed four times with approximately 30 mL of ethyl acetate. The filtrate was transferred to a separateory funnel and washed twice with distilled water, approximately 1/3 of the volume of ethyl acetate (75 mL). The ethyl acetate fraction was separated and dried with anhydrous $MgSO_4$ and was re-filtered as above. The filtrate was evaporated to dryness with a rotary evaporator and vacuum pump and the extract weighed. The filter cake was dried at 60 °C overnight in the drying oven and weighed.

Piper tuberculatum leaves (50 g) were finely ground with 125 mL of distilled water in a food blender. The aqueous slurry was sonicated for 15 min and transferred to a 500 mL flask containing 125 mL of ethyl acetate and shaken for 12 h. The leaf slurry was filtered by suction in a Buchner funnel to remove insoluble material and the filter cake was rinsed 3 X with 30 mL of ethyl acetate. The filtrate was

transferred to a separatory funnel to remove the aqueous phase, and the remaining ethyl acetate fraction was washed twice with 50 mL of distilled water (or approximately half the volume of ethyl acetate). The extraction was repeated with the filter cake a second time and the ethyl acetate was separated and washed with the same procedure and then combined with the first extraction. The ethyl acetate phase was evaporated to dryness and the extract weighed. The filter cake was dried at 60 °C overnight in the drying oven and weighed.

Figure 2. 2. Structure of piperamide derivatives: EP7 (A); EP8 (B); EP6 (C); EP9 (D).

A



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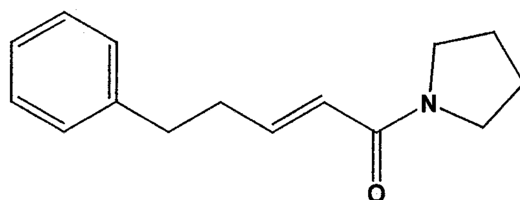

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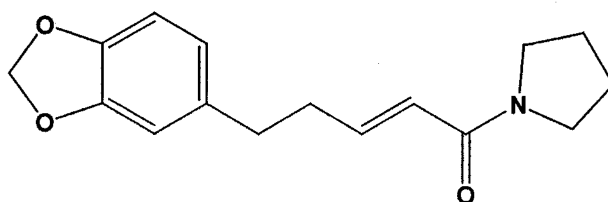

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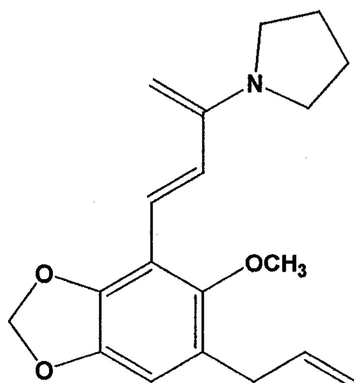


2.2.3 Piperamide recovery

Ground *P. nigrum* peppercorns were spiked with piperine (25 mg/500 mg ground pepper) at two times the observed concentration in the seed material. *P. tuberculatum* leaves were processed with 2, 3, 4 and 5 times the observed piperine content by spiking the leaves with 3, 6, 9 and 12 mg of piperine respectively at the beginning of the extraction process. The extraction of both *P. nigrum* and *P. tuberculatum* followed the methods described above.

2.2.4 HPLC method development

An existing HPLC method for separation of *Echinacea* isobutyl amides (Bergeron *et al.* 2000) was revised for the purpose of separating the piperamides in *Piper nigrum*, *P. guineense* and *P. tuberculatum* and further refined from the technique described by Scott *et al.* (2002). Four piperamide standards: 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-piperine and piperine, (Figure 2.1C, D, E and F) were synthesized as described in Scott *et al.* (2002). Pipercide (see Figure 2.1G) was obtained from G. M. Strunz, University of New Brunswick. Four piperamide derivatives were synthesized by E. Puniani, Chemistry Department, University of Ottawa (Figure 2.2A - D). A ten-point calibration curve was developed between 1 and 250 μg/mL for each amide. The optimized method used a binary gradient of acetonitrile (A) and water, beginning with 30% A, increasing to 70% in 10 minutes, 90% by 12 min and back to 30% A at 15 min. The instrument was a Varian Prostar model pump, model 330 UV/Vis photodiode array detector and model 410 autosampler (Varian Chromatography Systems, Walnut Creek, CA). The column was a Varian reverse-phase C18 (3 μm, 100 Å, 4.6 mm x 100 mm). The method was further optimized in order to improve resolution of *P. guineense* amides, analyze synthesized amide derivatives and identify unknown peaks in the *P. tuberculatum* extract.

2.2.5 LCMS method development

HPLC-MS analysis was conducted using an Agilent Technologies 1100 Series LCMS attached to a G1315B DAD, G1322A degasser, G1311A Quatpump, G1313A ALS and G1316A Colcom. Separation was achieved using a Waters YMC™ ODS-AM reverse phase column (53 μm, 120 Å, 2.0 x 100 mm). The MS detector was equipped with APCI source and operated in positive ionization mode. The MS was set on Scan-mode with positive polarity and the following parameter settings: mass range = 100-370 ms; fragmentor = 100; gain = 1.0; threshold = 150; step size = 0.1. The N_2 gas flow rate = 6.0 L/min; temperature = 300-350 °C; Nebulizer pressure = 60 psig; vaporizer temperature = 400-500 °C; capillary voltage = 3000 V positive / 3000 V negative; corona current = 4 μA positive / 15 μA negative.

2.2.6 Lipid separation procedure

In order to further concentrate piperamides, a method was devised to saponify the *Piper* extracts (see Figure 2.3). Crude extract of *Piper* species (0.5 g) was dissolved in 5 mL of methanol. A 10% methanolic KOH solution was prepared by adding 500 mg KOH to the 5 mL of methanol. The mixture was refluxed for 30 min with a water-cooled condenser. The solution was cooled and 5 mL removed and approximately 80% of the solvent of this fraction (1) was evaporated while refluxing was continued with the other fraction (2) for another 30 min. Diethyl ether (20 mL) and distilled water (20 mL) were added to fraction 1 and transferred to a separatory funnel and shaken gently in order to reduce foaming. After the aqueous layer was withdrawn, 20 mL of 10% aqeuous NaOH was added to the ether layer still in funnel and then shaken. The aqueous extracts were combined and retained. The ether fraction was dried with anhydrous $MgSO_4$ and then filtered by suction. The ether layer was evaporated to dryness by rotary evaporator and the extract weighed. Concentrated (10 M) HCl was added drop-wise to the aqueous layer until the solution was acidic by litmus paper. Ether (20 mL) was added to the aqueous solution and then the two phases were separated. The ether layer containing fatty acids was dried with anhydrous $MgSO_4$ and filtered by suction until dry and weighed. This process was carried out by A. Rao and L. Aumond in

the Chemistry Department, University of Ottawa. The ether and aqueous fractions were then analyzed for piperamide content by the previously described HPLC method.

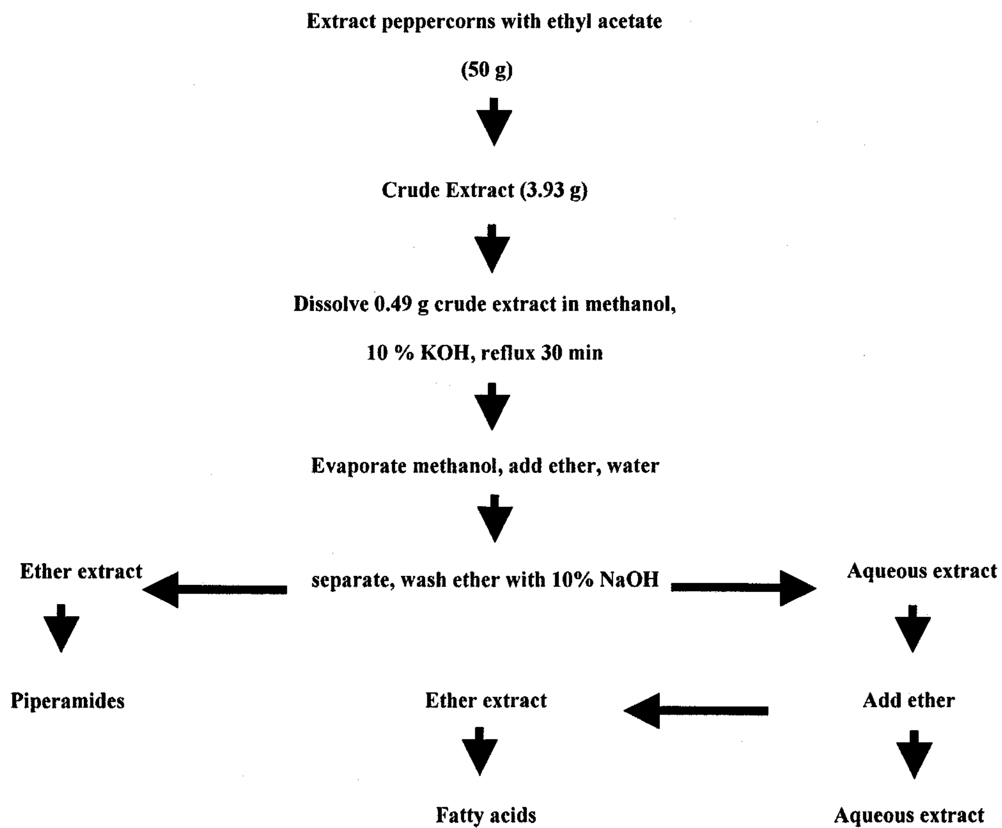
2.2.7 Assessment of biological activity

The Costa Rican *Piper* species were screened for activity by H. Jensen, Biology Deaprtment, University of Ottawa, using the previously described mosquito larval bioassay (Scott *et al.* 2002). All leaf extracts were prepared in 99% ethanol and dissolved in de-chlorinated tap water at 0.1, 1 and 10 mg / 100 mL (1, 10 and 100 ppm respectively). Twenty 2nd instar *Aedes atropalpus* larvae were added to one replicate at each concentration level. The number of surviving larvae after 24 h was assessed by probing each in order to elicit the characteristic twisting movement. *Piper* extracts producing 100 % mortality at 10 mg / 100 mL or less were retested in a narrower concentration range in order to determine the LC_{50} for that species. Three replicates of 20 2nd instar larvae were tested and the 24-h survival was assessed.

2.2.8 Statistical analyses

Comparison between extraction techniques and germplasm sources was analysed using one way ANOVA with Tukey's multiple range means test (SYSTAT 1999). Anaylses of pre- and post-lipid extraction results were compared with a one sample T-test and Bonferroni's comparison of means test and between aqueous and ether extraction by one way ANOVA (SYSTAT 1999).

Figure 2. 3. Method for separation of isobutyl amides (piperamides) from *Piper* extracts.



2.3 Results

2.3.1 HPLC separation and piperamide identification, extraction and recovery

Separation of the principal piperamides 4,5-dihydropiperlongumine, piperlonguminine, 4,5-dihydropiperine and piperine found in *P. nigrum*, *P. guineense* and *P. tuberculatum* was accomplished using the described HPLC method. The chromatogram for the four piperamide standards at 250 μg/mL demonstrates the separation and resolution obtained with the binary method (Figure 2.4). The 4,5-dihydo-piperamides have a peak absorbance at 205 nm, while piperine and piperlonguminine have a peak absorbance at 340 nm. The HPLC and LCMS detection limits were 2 and 0.2 ng respectively and the coefficient of variance was < 10.5.

Piperine was recovered with an average efficiency of 80% when added to ground peppercorns at twice the concentration determined for *P. nigrum* from the same source (Table 2.1). *P. tuberculatum* piperine recovery by the previous extraction method was 83% (Figure 2.5).The reflux extraction method was more efficient for separating active compounds from *P. nigrum* than the sonication method as shown by the greater recovery of piperine from one of two *P. nigrum* sources ($F=6.48$; $df=3,8$; $P=0.016$) (Table 2.2).

Figure 2. 4. HPLC chromatographs with piperamide standards at 205 (A) and 340 nm (B): 4,5-dihydro-piperlonguminine (1); piperlonguminine (2); 4,5-dihydropiperine (3); piperine (4) and pipercide (5).

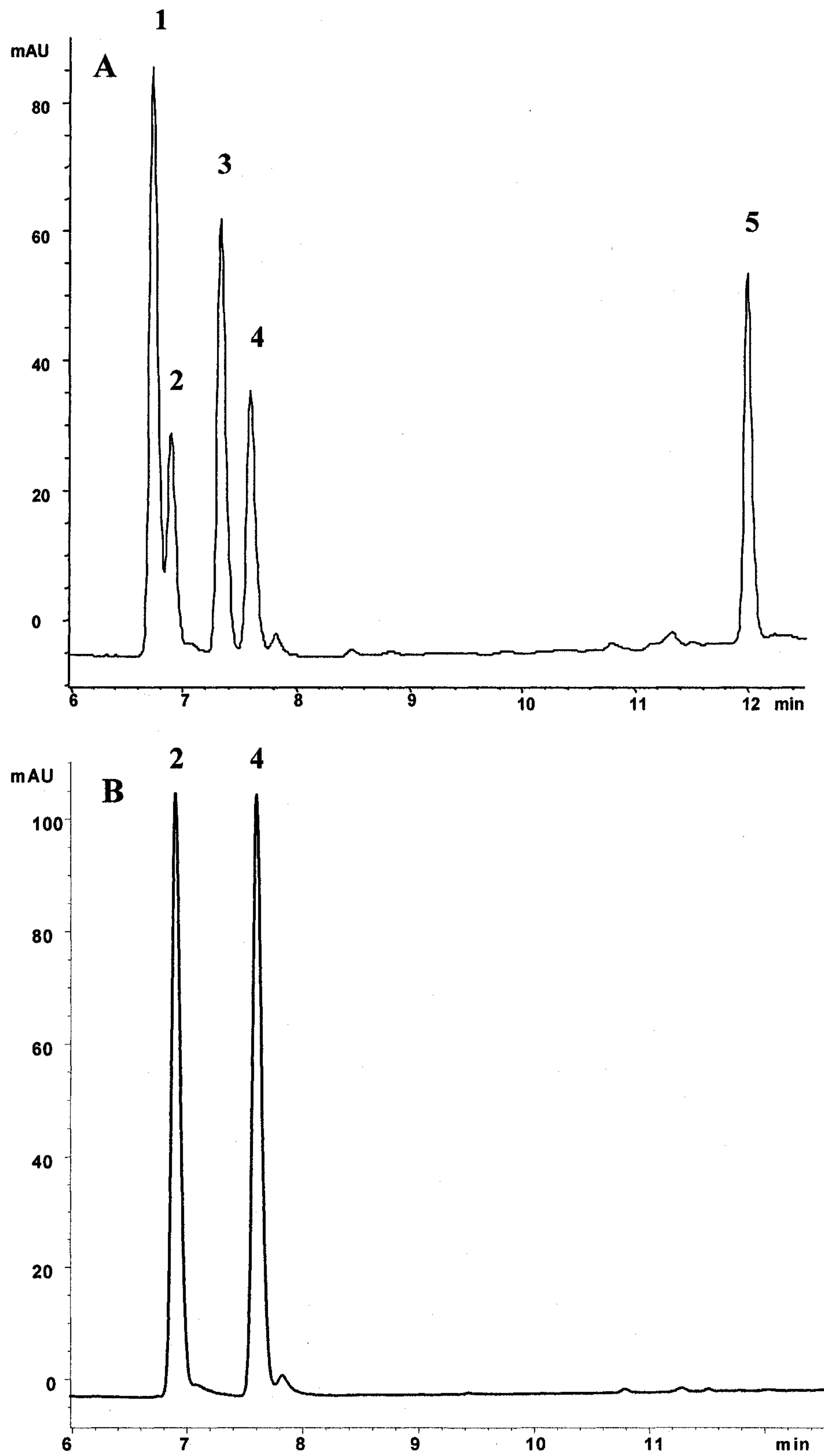


Table 2. 1. Average concentration (μg/mL) and standard error (S.E.) of four piperamides in *P. nigrum* extract analysed using HPLC to determine recovery of piperine-spiked samples (n=3).

Piperamide	Extract conc. (mg/mL)	Average concentration: (μg/mL) Piperine-spiked (S.E.)	Average concentration: (μg/mL) no piperine added (S.E.)
PLG	1	0	6 (0.4)
DHPLG	1	18 (6)	39 (4)
DHP	1	272 (20)	246 (11)
Piperine	0.1	680 (20)	548 (33)
Piperine Recovery		80 %	

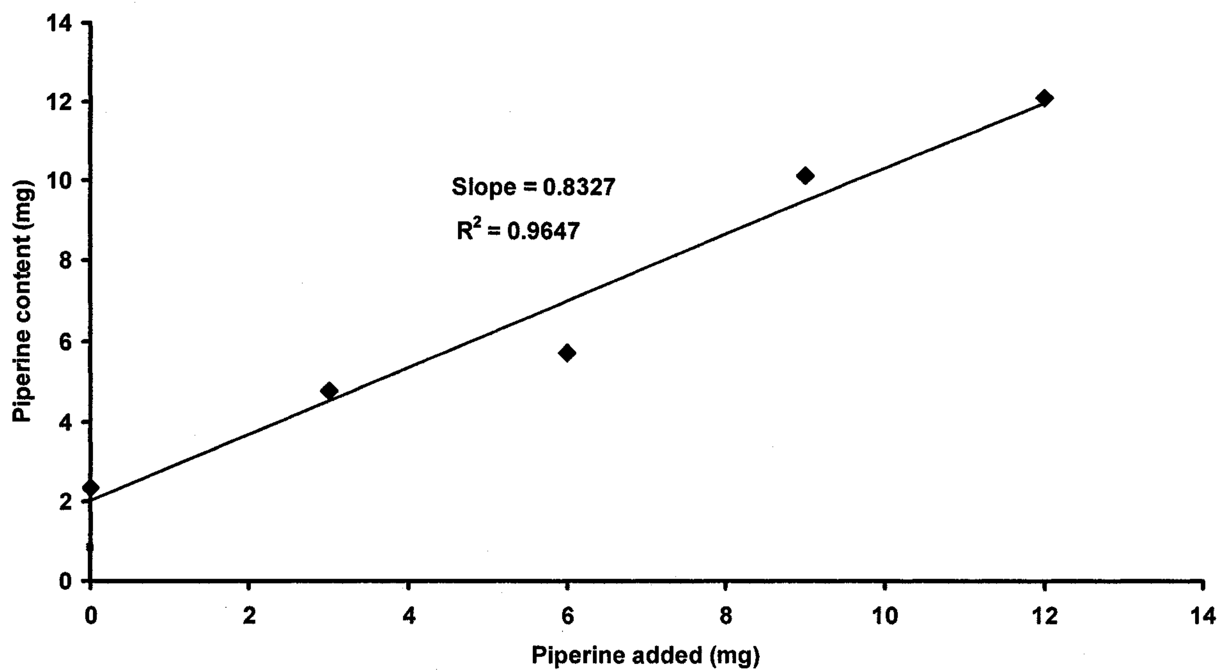
PLG = piperlonguminine, DHPLG = 4,5-dihydropiperlonguminine, DHP = 4,5-dihydropiperine

Table 2. 2. Average piperine concentration (μg/mg) and standard error (S.E.) in extracts from two sources of *P. nigrum* peppercorn and two extraction methods (n=3).

Piper nigrum peppercorn source	Average concentration of piperine in extract (μg/mL) (S.E.)	
	Sonication	Reflux
Trout Lake WA (India)	340 (10)[a]	540 (70)[b]
Gallaghers BC (Indonesia)	390 (30)[ab]	510 (40)[b]

[a] Piperine concentrations with the same letter are not significantly different (Tukey's P>0.05)

Figure 2. 5. Recovery of piperine from *P. tuberculatum* leaves extracted and analysed with HPLC-DAD method.



P. nigrum (Figure 2.6) and *P. tuberculatum* (Figure 2.7) were analysed using the same method with a 15 min run time while *P. guineense* was separated using a longer run time of 17 min (Figure 2.8). The synthetic piperamide derivatives (Figure 2.2 A, B, C and D) were found to have optimal absorption spectra at 205 nm and were conveniently separated as shown (Figure 2.9). Further identification and verification of individual piperamides was accomplished by MS analyses (see chromatograms in Appendix V).

2.3.2 Germplasm analyses

HPLC analyses of four available *P. nigrum* germplasm sources showed that three commercial sources had similar levels of piperine per dried seed material, there was only a significant difference between the highest, Gallagher's and the lowest, Spice Picks ($F=5.640$; $df=3,8$; $P=0.023$)(Table 2.3) When the total of all amides was considered, Gallagher's again had greater amounts than Spice Picks ($F=5.438$; $df=3,8$; $P=0.025$) but not Trout Lake or Country Bulk (Tukey's multiple range test, $P>0.486$). *Piper tuberculatum* leaves collected at La Pacifica had greater levels of dihydropiperlonguminine ($F=19.906$; $df=1,4$; $P=0.011$)and piperlonguminine ($F=11.110$; $df=1,4$; $P=0.029$) than at Puntarenas, whereas dihydropiperine ($F=31.840$; $df=1,4$; $P=0.005$) and piperine ($F=17.469$; $df=1,4$; $P=0.014$) were significantly lower in La Pacifica leaves. However, when the total of all amides was determined there was no significant difference between the two sites ($F=0.073$; $df=1,4$; $P=0.800$).

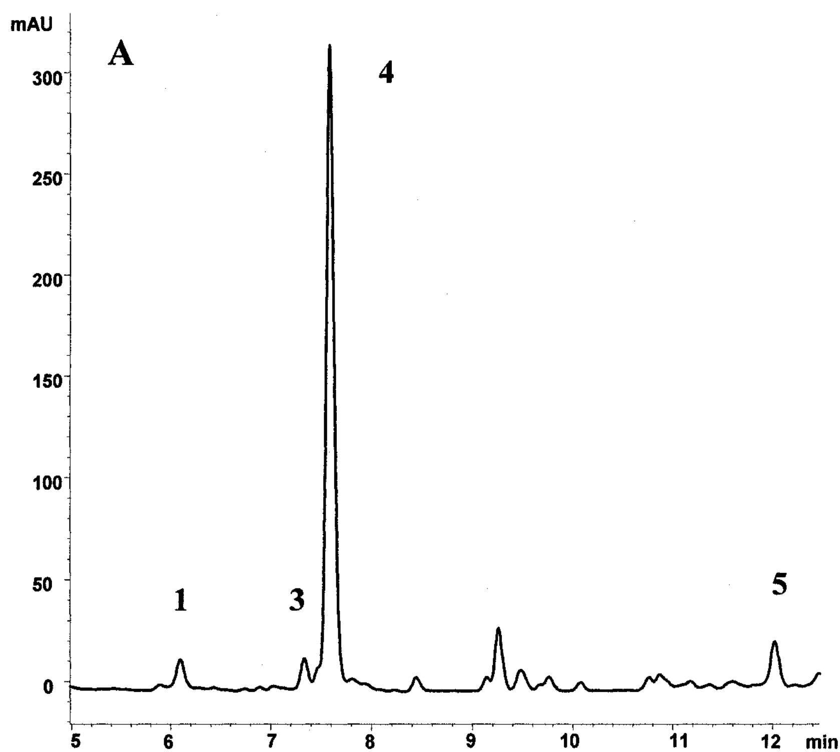
2.3.3 Lipid separation

Lipid separation of *P. guineense* and *P. tuberculatum* extracts produced higher amounts of dihydropiperlonguminine ($F=138.449$; $df=3,8$; $P<0.001$) and piperine ($F=247.497$; $df=3,8$; $P<0.001$) in the ether compared to the aqueous fraction (Table 2.4). The P. tuberculatum ether fraction contained significantly greater piperlonguminine ($F=1430.051$; $df=3,8$; $P<0.001$) than the aqueous fraction, as was the case for dihydropiperine in P. guineense ($F=288.064$; $df=3,8$; $P<0.001$). The most significant

concentrations of piperamides were in the saponified *P. tuberculatum* extract where 4,5-dihydropiperlonguminine levels were increased six fold (One sample T-test, $P=0.048$) whereas it was three fold in *P. guineense* (One sample T-test, $P<0.001$). Similary, piperlonguminine levels were five fold greater after saponification in *P. tuberculatum* (One sample T-test, $P=0.005$), piperine concentration was doubled for both *P. tuberculatum* and *P. guineense* (One sample T-test, $P<0.04$) and 4,5-dihydropiperine concentrations were 50% greater for *P. guineense* (One sample T-test, $P=0.022$).

In comparison, there was no significant difference between the ether fraction and the *P. nigrum* extract (data not shown). After lipid separation fraction 1 was a yellow, sticky solid, and weighed 0.16 g. The aqueous fraction had brown clumps when separated by ether and the precipitate clumped with $MgSO_4$. The fraction 1 aqueous phase was a white-brown solid and the ether extract weighed 0.04 g. The fraction 2 ether extract was also a yellow, sticky solid and weighed 0.2 g. The fraction 2 aqueous separation was an amber colored solid and the ether phase weighed 0.03-0.04 g. Ether and aqueous fractions were analysed by HPLC.

Figure 2. 6. HPLC chromatographs at 205 (A) and 340 nm (B) for *P. nigrum*. Peaks identified are: piperylin (1); piperlonguminine (2); dihydropiperine (3); piperine (4) and pipercide (5).



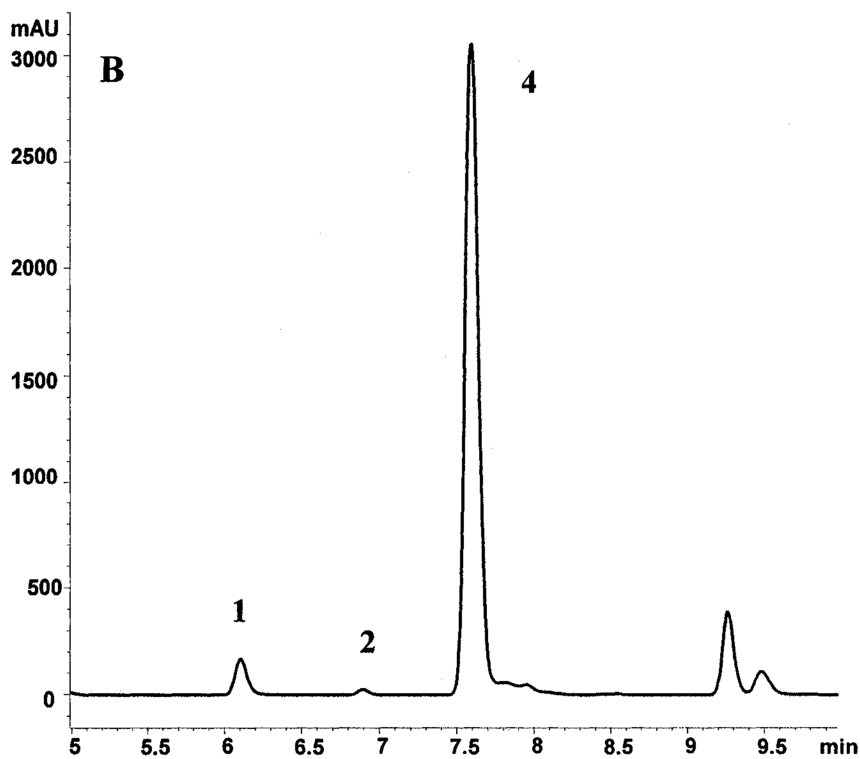
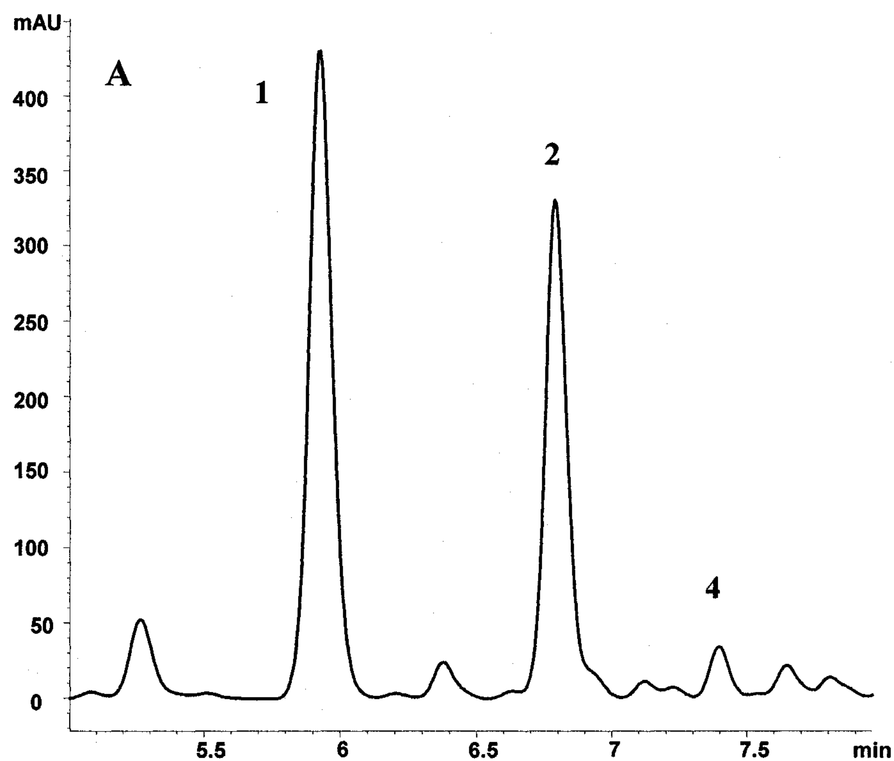


Figure 2. 7. HPLC chromatograph at 205 (A) and 340 nm (B) for *P. tuberculatum*. Peaks identified are: pellitorine (1); dihydropiperlonguminine (2); piperlonguminine (3); dihydropiperine (4) and piperine (5).



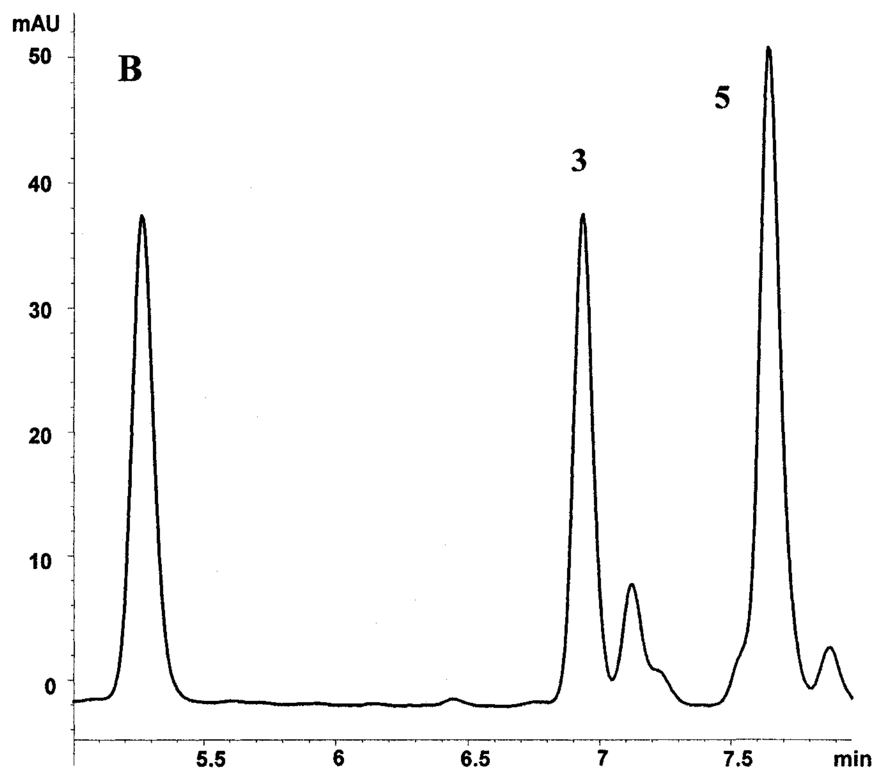
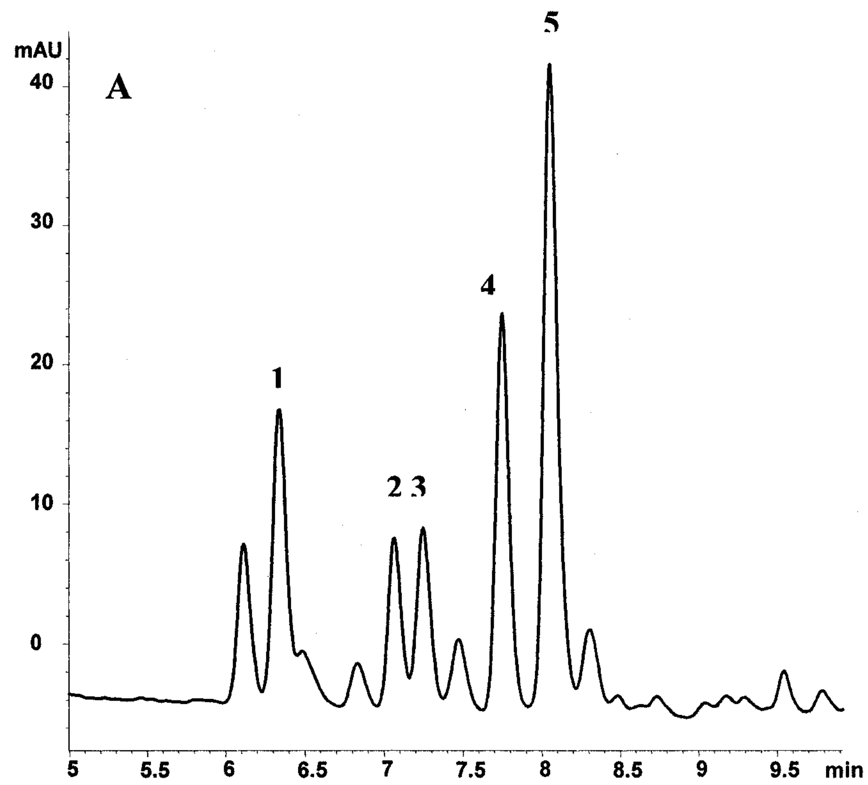


Figure 2. 8. HPLC chromatographs for *P. guineense*: piperamide peaks identified are piperylin (1); 4,5-dihydropiperlonguminine (2); piperlonguminine (3); 4,5-dihydropiperine (4) and piperine (5).



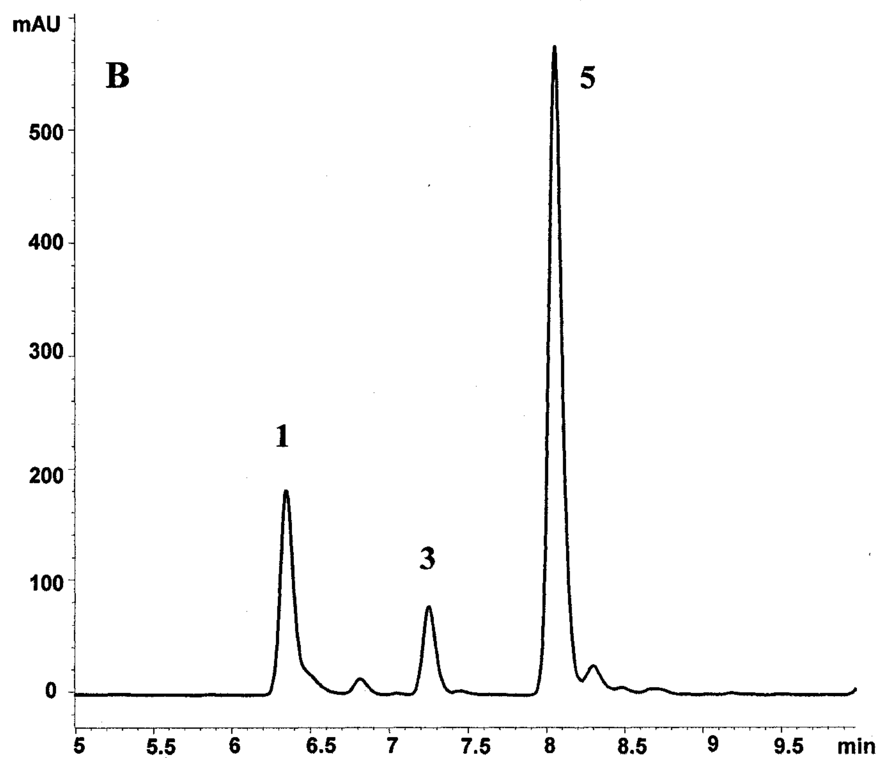


Figure 2. 9. Chromatogram of synthesized piperamide derivatives at 205 nm : EP6 (1); EP8 (2); EP7 (3) and EP9 (4).

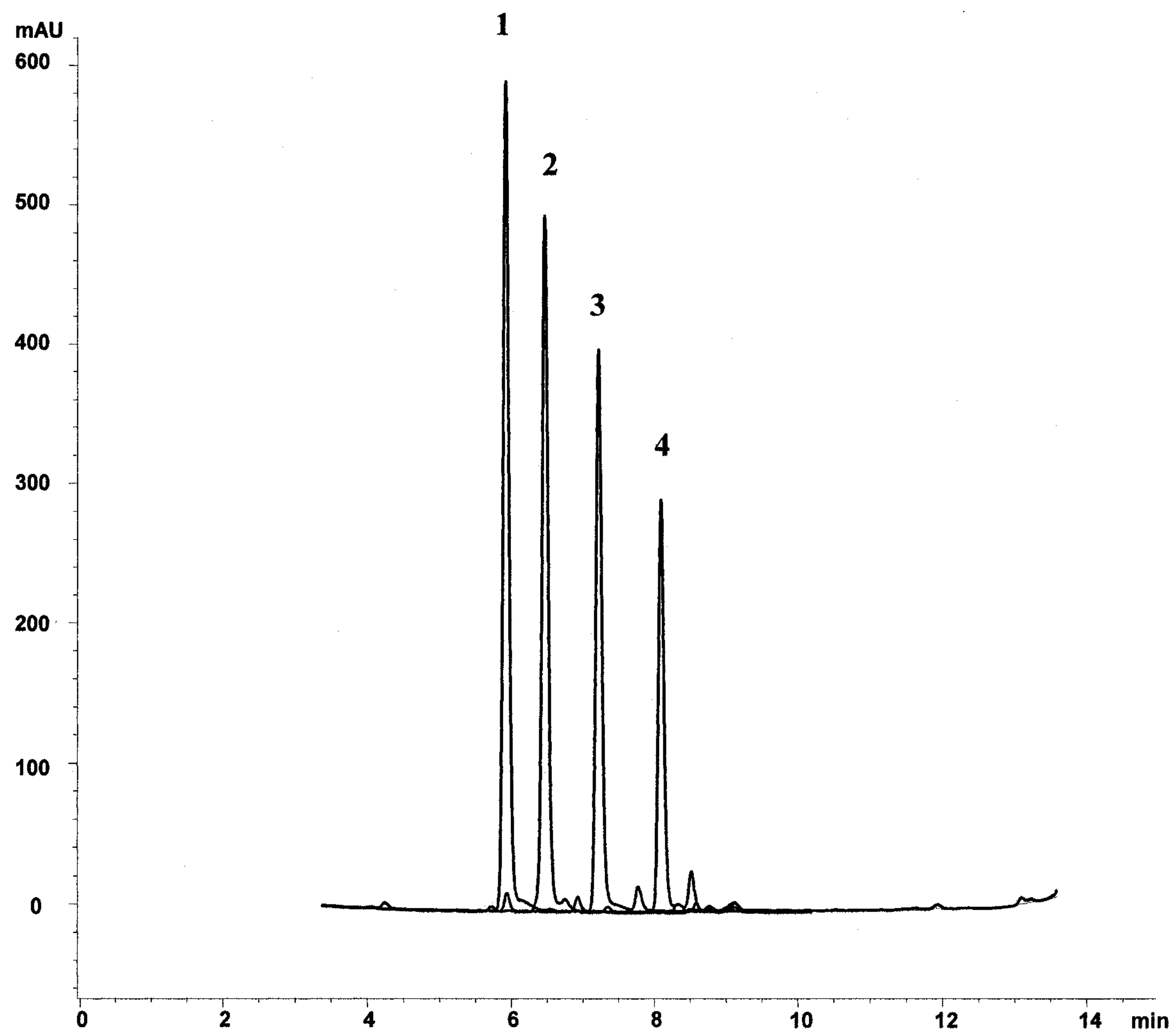


Table 2. 3. Average concentration of piperamides and standard error (S.E.) of n = 3 different samples in *P. nigrum* (µg or mg/g dried peppercorn) from four commercial suppliers and *P. tuberculatum* (µg or mg/g dried leaves) from two distinct ecological areas in Costa Rica.

Pepper supplier	DHPLG µg/g (S.E.)	PLG µg/g (S.E.)	DHPip mg/g (S.E.)	Pip mg/g (S.E.)	Total mg/g
Trout Lake	203 (51) [a]	222 (29) [c]	2 (0.3) [e]	51 (2) [ghi]	53 (3) [jk]
Country Bulk	0 [b]	81 (2) [d]	2 (0.2) [e]	43 (6) [ghi]	45 (7) [jk]
Gallagher's	223 (124) [a]	112 (31) [d]	3 (0.1) [f]	55 (3) [h]	58 (2) [j]
Spice Picks	250 (16) [a]	95 (13) [d]	3 (0.3) [ef]	34 (3) [i]	37 (3) [k]
Costa Rica pepper site	**DHPLG mg/g (S.E.)**	**PLG µg/g (S.E.)**	**DHPip µg/g (S.E.)**	**Pip µg/g (S.E.)**	**Total mg/g dry leaf**
La Pacifica	2 (0.1) [a]	351 (20) [c]	209 (16) [e]	168 (9.0) [g]	2.7 (0.3) [i]
Puntarenas	1 (0.1) [b]	224 (33) [d]	743 (93) [f]	433 (63) [h]	2.7 (0.1) [i]

DHPLG = 4,5-dihydropiperlonguminine, PLG = piperlonguminine, DHPip = 4,5-dihydropiperine, Pip = piperine. [a] Individual piperamide concentrations with the same letter are not significantly different (Tukey's P>0.05).

Table 2. 4. Concentration of piperamides (μg/g) and standard error (S.E.) in *Piper tuberculatum* and *P. guineense* extracts before and after lipid extraction procedure.

***Piper* Species/ Extraction Type**		**DHPLG μg/g (S.E.)**	**DHP μg/g (S.E.)**	**PLG μg/g (S.E.)**	**Piperine μg/g (S.E.)**
P. tuberculatum	Pre	38[a]	15	6[d]	3[g]
Aqueous	Post	6.6 (0.8)[b]	N.A.	1.5 (0.2)[e]	0.6 (0.1)[h]
Ether	Post	237 (45)[c]	N.A.	32 (1.9)[f]	10 (1.0)[i]
Ratio: Ether/Extract		**6.2**	**N.A.**	**5.3**	**3.2**
P. guineense	Pre	8.4[a]	38[d]	0	45[g]
Aqueous	Post	17 (0.3)[b]	28 (0.3)[e]	N.A.	25 (4.8)[g]
Ether	Post	32 (0.5)[c]	56 (0.5)[f]	N.A.	98 (11)[h]
Ratio: Ether/Extract		**3.8**	**1.5**	**N.A.**	**2.2**

DHPLG = 4,5-dihydropiperlonguminine, DHP = 4,5-dihydropiperine, PLG = piperlonguminine.

[a] Individual piperamide concentrations with the same letter between pre and post extracts are not significantly different (Bonferroni's, P>0.05) and piperamide concentrations with the same letter between aqueous and ether extractions are not significantly different (Tukey's P>0.05).

2.3.4 Assessment of biological activity in a convenient mosquito larvae bioassay

In addition to *P. nigrum* and *P. guineense* four of seven Costa Rican *Piper* species tested were acutely toxic to *Aedes atropalpus* larvae at 100 μg/mL (Table 2.5). When *P. aequale*, *P. cordulatum*, *P. tuberculatum* and *Piper* species A were retested, the most active was *P. tuberculatum* with an EC_{50} between 2.5 and 10 ppm (Figure 2.10). The EC_{50} for *Piper* species A was between 12.5 and 25 ppm, while *P. cordulatum* and *P. aequale* were > 50 and 100 ppm respectively. The HPLC analysis of *Piper* species A and *P. cordulatum* (Figure 2.11) indicate that there is a lower concentration of piperamides in those species compared to *P. tuberculatum* but 4,5-dihydropiperlonguminine was common to all three.

Table 2. 5. Survival of 20 *Aedes atropalpus* 2nd instar larvae after 24-h exposure to Costa Rican *Piper* species at 1, 10 and 100 μg/mL.

	Number of survivors		
***Piper* species**	**1 (μg/mL)**	**10 (μg/mL)**	**100 (μg/mL)**
P. aequale	20	20	0
P. biseriatum	20	20	18
P. cordulatum	20	20	0
P. nudifolium	20	20	19
P. pseudo-lindenii	19	20	19
Piper species A	19	16	0
P. tuberculatum	19	0	0
P. nigrum	20	0	0
P. guineense	20	0	0

Figure 2. 10. Survival of *Aedes atropalpus* 2nd instar larvae after 24-h exposure to *Piper tuberculatum* between 1 and 10 µg/mL, *Piper* species A, *P. cordulatum* and *P. aequale* between 10 and 100 µg/mL (n=20/treatment level).

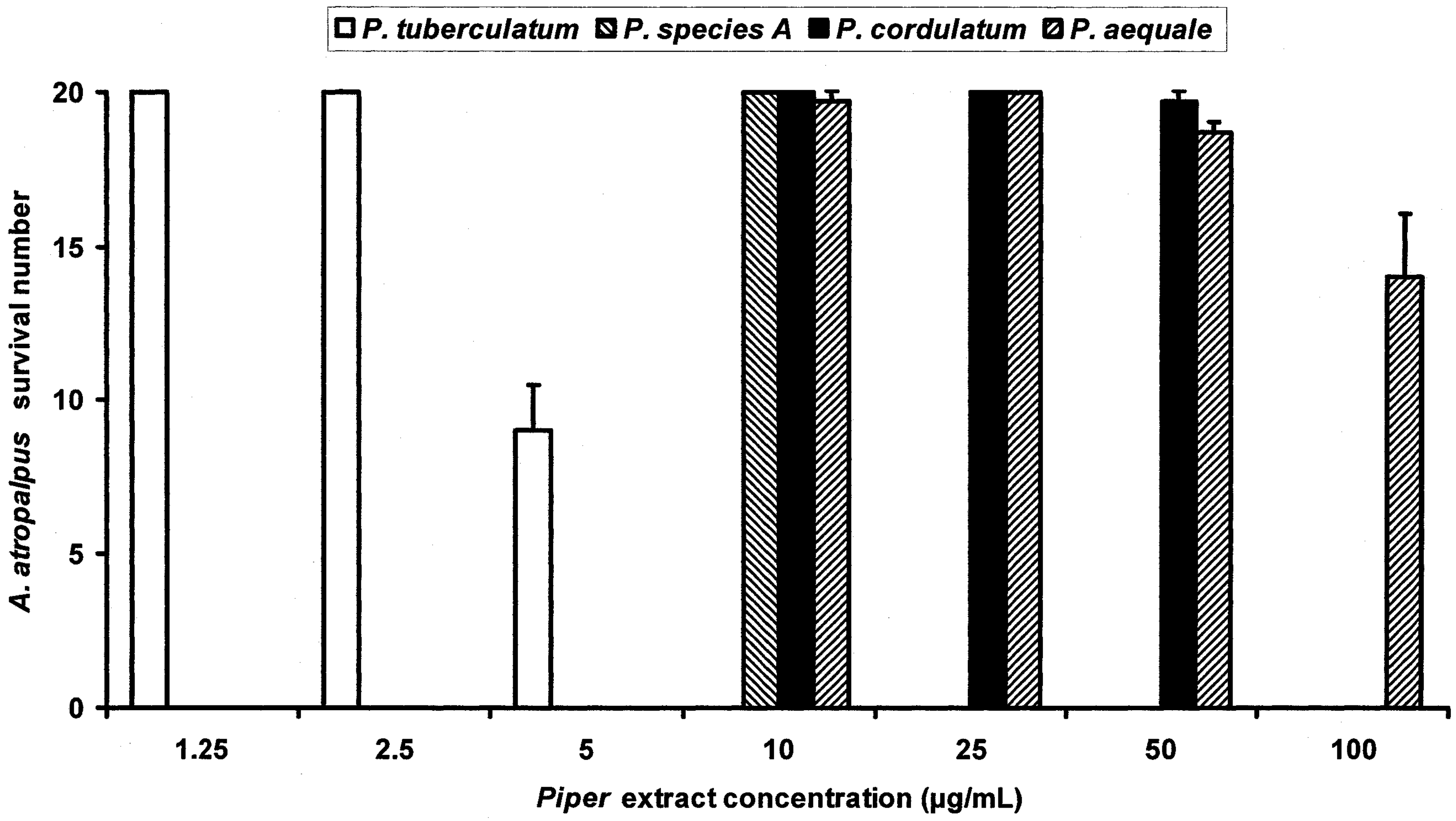
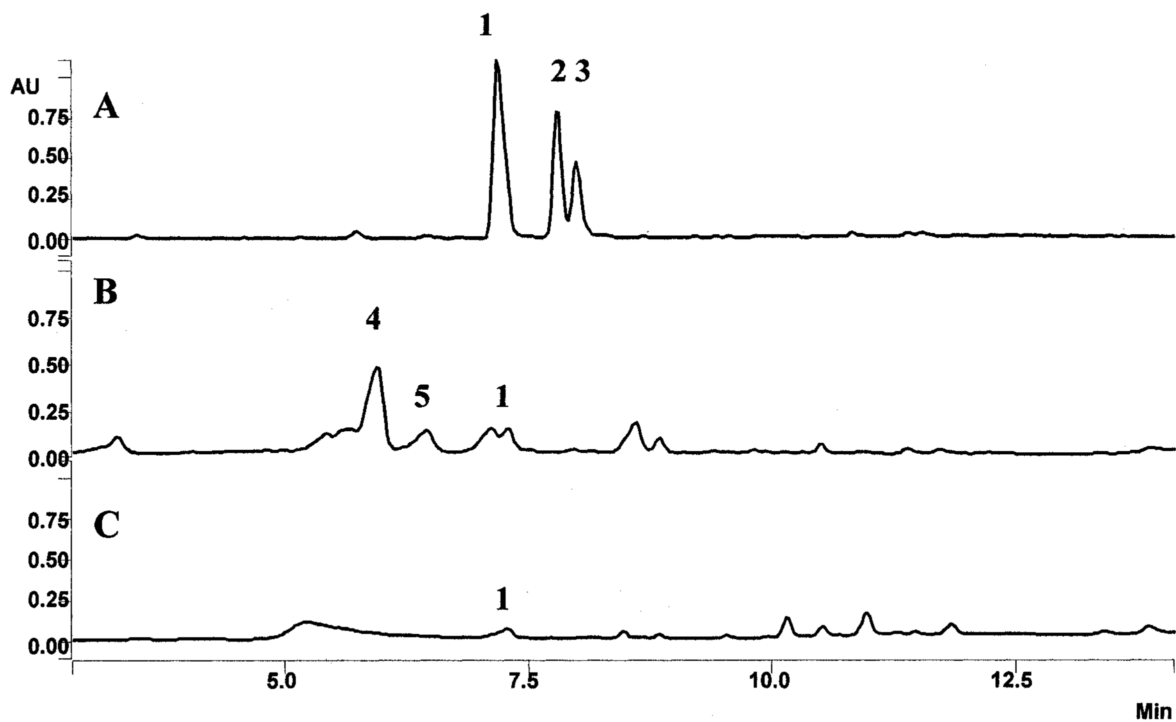


Figure 2. 11. HPLC chromatogram at 205 nm showing A) piperamide standards: dihydropiperlonguminine (1), dihydropiperine (2) and piperine (3); B) *Piper* species A with new compound (4), pellitorine (5) and dihydropiperlonguminine; and C) *Piper cordulatum* with only 1 peak identified; dihydropiperlonguminine (1).



2.4 Discussion

The extraction and HPLC methods described provided rapid and accurate analysis for assessing the level of the active components found in the three species, *P. nigrum*, *P. guineense* and *P. tuberculatum*. The addition of the LCMS improved on resolution and sensitivity from the method previously used (Scott *et al.* 2002). Although several reports of HPLC separation exist (Gbewonyo and Candy 1992; Sunkara *et al.* 2001; Bajad *et al.* 2002), this is the first report that provides a validated method including recoveries and detection limits. The present method uses small diameter columns and the present run time was half that used by Stöhr *et al.* (2001).

The reflux extraction technique, previously used with peppercorns (Kiuchi *et al.* (1988), was determined to increase piperamide yields. Extraction of peppercorns was improved through the use of refluxing rather than simple grinding and extraction in solvent alone. The refluxing step likely benefits the extraction of dried peppercorn more so than alcohol-preserved leaf material but this step should be tested with the *P. tuberculatum* leaves as well. Despite the lower recoveries the amount of piperine per peppercorn compares favourably to that determined by Kulkarni *et al.* (2001), 50.78 mg/g peppercorn, analysed using a high performance thin layer chromatography method. However, the HPLC method has better resolving power, and can separate co-eluting unknowns.

Lipid extraction was shown to improve the further separation and concentration of piperamides from the original extract (Table 2.4). The physical appearance of the three extracts was as follows: *P. nigrum* was the driest, followed by *P. tuberculatum*, while *P. guineense* was the most fluid. This might help to explain why removal of lipid material greatly increases the amide profile of the latter two versus black pepper. This suggests that the efficacy of a botanical insecticide based on *P. tuberculatum* material will further benefit from a saponification step in the production process.

The HPLC method provided clear separation of closely related piperamide derivatives, helping to explain how amide molecular structure influences the chromatography. In the case of the derivatized amides, lipophilicity and steric effects influenced separation. The MDP group present on EP6 (MW = 277, Figure 2.2A) creates a more polar compound than either EP 7 (MW = 231, Figure 2.2B) or EP 8 (MW =

230, Figure 2.2C) where it is absent (Figure 2.9). Similarly the presence of a pyridine ring moiety on the opposite end of the amide chain creates a more polar molecule. Also, the presence of a conjugated diene chain will decrease the polarity of the molecule, hence the lower retention time for 4,5-dihydropiperine (MW = 287, Figure 2.1E) versus piperine (MW = 285, Figure 2.1F), as observed in Figure 2.4A.

At extract concentrations between 0.1 and 1 mg/mL ethanol, all of the *Piper* species could be analysed and the principal active compounds identified by LCMS from five piperamide standards (MS chromatographs in Appendix V) and literature reports [pellitorine MW = 221.2 (Kiuchi *et al.* 1988); piperylin MW = 273.1 (Kiuchi *et al.* 1988; Strunz unpublished]. The lack of identifiable peaks in chromatographs for one of the Costa Rican species (Figure 2.11C) correlated well with the lack of biological activity in the insect bioassay (Figure 2.10). This is not to say that these species do not contain biologically active secondary compounds, but in comparison to the recognized *Piper* species, they do not provide a useful material for a fast-acting botanical insecticide. The one *Piper* species with comparable activity was not identified but the major peak (Figure 2.11) was analyzed by LCMS and determined to have a MW of 205.2. Based upon the MS data, the molecular formula is suspected to be $C_{13}N_2H_{19}$, a compound not recognized from the *Piper* literature we examined. Unfortunately no further extract material was available to isolate the compound by column chromatography.

In confirmation of our previous study (Scott *et al.* 2002), separate *P. tuberculatum* populations in Costa Rica have significantly different piperamide profiles. In the present study, leaves collected from plants at the La Pacifica site had levels of 4,5-dihydropiperlonguminine almost twice (2.02 versus 1.26 mg/g dried leaves) those found in leaves from Puntarenas (Table 2.3); however when the total amount of piperamides is considered, the difference between leaves from the two sites was no different (2.7 mg/g). This suggests the amount of protection afforded by the secondary compound profile is similar between populations, as observed previously (Scott *et al.* 2002), regardless of ecological differences between the two sites.

In the case of *P. nigrum*, the source of germplasm did affect the level of piperamides present: Gallagher's acquired black peppercorns from Indonesia, while Spice Picks obtained their material from

India (Table 2.3). Although it is not certain whether the region where the peppercorns where produced or other variables such as storage, handling etc are a factor, it is evident that the distributor of the pepper must be assessed in order to select the pepper with the highest piperamide content. Two hypotheses to test in the future are as follows: 1) organically produced peppercorns, such as those available through Trout Lake, experience higher pest damage in the crop that in turn induces greater amide production as a defense by the plant and 2) different environments promote a higher level of amide production. If this were true *P. nigrum* would best be obtained from Indonesian sources since the piperamide content is on the whole higher and it has been observed that the Lampong region of Indonesia produces more pungent peppercorns than the Kerala region of India.

However, since only one source of peppercorn from Indonesia was examined in the present study it maybe premature to suggest that this one source is representative of an entire region. Intra-region differences in pepper characteristics were significant when explored by cluster analysis (Mathew *et al.* 2001). Of the 51 cultivated black pepper cultivars found in Kerala, India, 10 separate and distinct genetic groupings can be made based upon 27 quantitative and qualitative morphological characters This observation may indicate that there is as much difference in the amide profiles within the cultivars, and could explain why there are differences amongst the Indian peppercorns observed in the present study. A similar use of cluster analysis produced three distinct groupings of essential oil profiles in populations of *P. lanceaefolium* from Costa Rica (Mundina *et al.* 2001). The clustering of the main phenylpropanoids, parsley apiol, dill apiol and elemicin, into three groups of two each was confirmed by principal component analysis. Since geographical origin was not considered as the prime factor, genetic control was suggested as the source of the chemical pattern composition. This in turn may explain why the *P. tuberculatum* populations were similar in piperamide profile: a greater genetic similarity than is observed between *P. lanceaefolium* populations.

The continued culinary and medicine application of *P. nigrum*, and the potential for insect-control usage in the future, will require increasing sophistication in analytical assurance. LCMS analysis will ensure that levels of piperamides in mixtures can be reported so that commercial sources of either

oleoresin or formulated extracts can have an assured range of active components. The industry of botanical natural products has ensured standardization of many popular phytomedicines, a process which is more accepted because of improved plant breeding and quality assurance. The acceptance of botanical insecticides will have to similarly reach this level of quality control before increased registration of these products will occur. The improved methods for analysis and assurance of high standards in germplasm will help to foster this movement.

CHAPTER 3

EFFICACY OF PIPER EXTRACTS FOR CONTROL OF HOME AND GARDEN INSECT PESTS

3.1 Introduction

Biopesticides of plant origin have recently been reviewed (Regnault-Roger *et al.* 2002) and it was concluded botanicals have considerable market potential as reduced-risk control agents. In addition the National Research Council (2000) in the United States recommended a number of uses where botanicals meet current and future requirements for alternative pest control. Lydon and Duke (1989), Isman (1994) and MacKinnon *et al.* (1997) have surveyed several plant families that show promise as sources of new botanical insecticides. Members of the pepper family Piperaceae produce phytochemicals with insecticidal activity. The most widely recognized species are black pepper *Piper nigrum* L. and African Guinea pepper *P. guineense* Schum and Thonn, but many other species in the family are also insecticidal (Bernard *et al.* 1995).

Early investigations with *P. nigrum* extracts indicated that isobutyl amides were responsible for the toxicity of the extracts to the adzuki bean weevil *Callosobruchus chinensis* L. (Miyakado *et al.* 1979 and 1980). Three of the isobutyl amides isolated from *P. nigrum*, pipercide, pellitorine and piperine had 48 h LD_{50}s of 0.15, 2 and 20 µg/ male *C. chinensis*, respectively (Dev and Koul 1997). Guineensine, isolated from *P. guineense*, had similar activity to pipercide when tested topically on *Callosobruchus maculatus* (Fabricius) (0.25 versus 0.84 µg/ male 48 hour LD_{50}s respectively). Pepper extracts containing mixtures of piperamides are also highly effective (Scott *et al.* 2002).

Essential oils of *P. nigrum* were found to effectively protect stored wheat from the stored-grain pests, *Sitophilus oryzae* L. and *Rhyzopertha dominica* F., at concentrations above 100 mg/L for up to 30 days (Sighamony *et al.* 1986). Stored beans were protected from the bruchid *Acanthoscelides obtectus* Say, by ground black pepper for up to 18 weeks (Baier and Webster 1992). *P. guineense*-treated kaolin

powder at 150 μL /g reduced the average adult emergence of the cowpea weevil *C. maculatus* by 100% after 30 days treatment (Kéïta *et al.* 2000). Dust and ether-extract formulations of *P. guineense* were also effective at controlling *C. maculatus*, at concentrations between 0.5 and 0.75 g/20g cow pea seed within 36 hours after treatment (Mbata *et al.* 1995). Emergence of adults from treated eggs was prevented successfully with dust and oil treatments at 0.25 g/seed.

Tests of the efficacy of *Piper* extracts against a few other insect pests has also been undertaken. The termite, *Macrotermes nigeriensis* Sjostedti, was controlled with a 5% aqueous solution of *Piper guineense* applied topically (Ivbijaro *et al.* 1993). Javier and Morallo-Rejesus (1986) determined that semi-purified organic solvent extracts of *P. nigrum* were more toxic than crude extracts against the housefly *Musca domestica* L., common cutworm *Spodoptera litura* F., black armyworm *Spodoptera exempta* Walker and diamondback moth *Plutella xylostella* L. Ewete *et al.* (1996) showed that *P. guineense* incorporated into the diet of the European corn borer, *Ostrinia nubilalis* (Hübner), at 300 mg/L, reduced larval growth by 27%, increased the time to adult emergence and reduced egg production at concentrations above 10 mg/L. In terms of non-target effects, aqueous mixtures of oven-dried powdered *P. guineense* at 10 mg/L were found to be effective at controlling 4th instar mosquito *Aedes aegypti* L. but were not toxic to other aquatic organisms (Okorie and Ogunro 1992).

Other species of *Piper* that also contain piperamides include Long pepper *P. longum* L., from south Asia, and *P. tuberculatum* Jacq., from South and Central America. The latter is of particular interest because of high concentrations of piperamides in the leaves (Scott *et al.* 2002). Along with *P. nigrum* and *P. guineense*, *P. tuberculatum* may offer promising and active pepper extracts for the development of a commercial insect control product due to the long traditional use, low associated health risk and the relative abundance of material.

Most previous evaluations have focused on major crop and nuisance pests and little research has been undertaken to date on insects of the home and garden, where a botanical product might gain ready acceptance. We developed a practical formulation that was evaluated against target insect species that concern gardeners and horticulturalists in eastern Canada and the northeastern United States and were

selected for use in efficacy trials based on their abundance and availability. These include insects from the orders: 1) Coleoptera; Colorado potato beetle, *Leptinotarsa decemlineata* Say (Chysomelidae), Japanese beetle, *Popillia japonica* Newman (Scarabaeidae), lily leaf beetles, *Lilioceris lilii* Scopoli (Chysomelidae), striped cucumber beetle, *Acalymma vittatum* Fabricius (Chysomelidae) and Viburnum leaf beetle, *Pyrrhalta viburni* Paykull (Chrysomelidae); 2) Dermaptera; earwig, *Forficula auricularia* L. (Forficulidae); 3) Hemiptera; hairy chinch bug, *Blissus leucopterus hirtus* Montandon (Lygaeidae); 4) Hymenoptera; European pine sawfly, *Neodiprion sertifer* Geoffroy (Diprionidae) and 5) Lepidoptera; European corn borer, *Ostrinia nubilalis* Hübner (Pyralidae), ermine or spindle moth, *Yponomeuta cagnagella* Hübner (Yponomeutidae) and eastern tent caterpillar, *Malacosoma americanum* Fabricius (Lasiocampidae).

Non-target toxicity was also evaluated using an insect species that would likely encounter the effects of the botanical treatment. Applications in the garden could affect the lady beetle *Hippodamia convergens* Guérin-Méneville (Coccinellidae), as a beneficial or predator of garden insect pests.

The target insect pests and non-target invertebrate were treated with *Piper* extract formulations in order to achieve the following objectives: 1) to establish concentration levels for the target insects and toxicity values to protect the non-target invertebrate, 2) to evaluate knockdown and repellent action under controlled treatment conditions, and 3) to determine residue levels for *Piper* active compounds on contact surfaces. The overall objective was to provide an in-depth evaluation of the effectiveness, toxicity and environmental fate of pepper extracts.

3.2 Materials and Methods

3.2.1 *Piper* extracts and commercial botanical formulations

Seed material for both *P. nigrum* and *P. guineense* was purchased from commercial suppliers in Canada, the United States and Togo, West Africa. Leaves of *P. tuberculatum* were collected in Costa Rica by P. Sanchez and L. Poveda near San Carlos. Voucher specimens have been placed in the University of Ottawa and the Universidad Nacional Herbarium, Costa Rica. *Piper nigrum* and *P. guineense*

peppercorns, and *P. tuberculatum* leaf material were ground and the active constituents extracted following the methods described in Scott *et al.* (2002). Natural solvents and emulsifiers were incorporated into the formulation in order to reduce the risk of toxicity to the applicator and the environment. The *Piper* extracts were formulated by R. Bradbury, Eco-Smart, as follows: 20% extract, 70% tetrahydrofurfuryl alcohol (THFA, Penn Specialty Chemicals, Memphis TN) and 10% emulsifier (Alkamuls EL-719 ethoxylated castor oil, a gift of Rhodia, Cranbury NJ). The piperamide concentration in the extracts and formulations was analysed based on the methods of Scott *et al.* (2002). HPLC analysis was conducted using a Varian Prostar model pump, model 330 UV/Vis photodiode array detector and model 410 autosampler, Varian chromatography systems (Walnut Creek, CA).

Other botanical extracts were obtained in order to act as synergists or to compare with the insecticidal and repellent activity of the *Piper* extracts. These included: Neem oil *Azadiracta indica* Juss (Meliaceae) (Ahimsa Alternative Inc., Oklahoma City, OK); concentrated garlic *Allium sativum* L. (Alliaceae) (Garlic Barrier™, a gift of Garlic Barrier Labs, Glendale, CA) and lemon grass oil *Cymbopogon citratus* [DC] Stapf. (Poaceae) (Ropel®, Burlington Scientific, NY). All commercial products were applied at rates recommended by the manufacturer.

3.2.2 Effect of sunlight and ultraviolet radiation exposure to *Piper* extracts

The effect of sunlight and ultraviolet (UV) radiation on exposed piperamides either alone or in *Piper* extracts was assessed following the methods described in Scott *et al.* 2003. Degradation of piperamides exposed to full sunlight conditions was determined by placing 50 μL aliquots of 20% extract on glass microscope slides and allowing them to dry overnight on the bench top. The slides were then exposed to full sunlight for 6 h during peak daylight hours. Readings of solar radiation were taken at 3 h intervals. The glass slides were then rinsed with 5 mL of 99% ethanol to wash off the extract residue. Treated control slides not exposed to full sunlight were rinsed using the same method. The ethanol solutions (1 mL) were then filtered through a 0.2-μm polypropylene filter and then placed in a 1.5 mL

HPLC vial in preparation for analysis. Analysis was conducted according to Scott *et al.* (2002), however a Varian Prostar model pump, model 330 UV/Vis photodiode array detector and model 410 autosampler, and a Varian reverse-phase C18 column (Varian Chromatography Systems, Walnut Creek CA) were used. The compounds were eluted with a binary gradient of acetonitrile and water, where acetonitrile was increased from 30 to 90% in 12 min, as described by Scott *et al.* (2002). Solar radiation readings were taken at each time period with a Li-Cor Quantum model LI-192SB sensor. The UV light was an Industrial F20T12/BLB blacklight blue lamp that emits in the near UV, 315 to 400 nm. The lamp has a deep blue filter to absorb visible light and transmit near UV at a 3.7 W output.

3.2.3 Insect species collected or cultured

Coleoptera

Popillia japonica adults were collected at one site in Ottawa, Ontario, in August 2001 and 2002. Adults were kept at 10° C, photoperiod 16:8 L:D, and fed crab apple *Malus sylvestris* L. Miller (Rosaceae) or mountain ash *Sorbus americana* Marsh (Rosaceae) leaves until bioassays were initiated. *Lilioceris lilii* eggs and adults were collected from several gardens in May and June 2001 and 2002 and at the Central Experimental Farm in Ottawa, Ontario, during June and July 2001 and May 2002. Adults were kept at 10° C, photoperiod 16:8 L:D, until bioassays were initiated or kept on Asiatic lily plants *Lilium* spp. (Liliaceae) in the greenhouse to produce eggs. Larvae were reared on plants until bioassays were initiated. *Pyrrhalta viburni* larvae were collected from the Kemptville Agricultural College Campus, Kemptville, Ontario, and The Log Farm, Ottawa, Ontario, in May 2002. Larvae were kept in containers with fresh European highbush cranberry *Viburnum opulus* L. (Caprifoliaceae) leaves at 10° C, photoperiod 16:8 L:D, until bioassays were initiated. *Acalymma vittatum* adults were collected from the Gloucester allotment gardens in August 2002, Ottawa, Ontario, and kept at 10°C, photoperiod 16:8 L:D, and fed cucumber slices until bioassays were initiated. Wild-type adult *L. decemlineata* were collected from organic potato fields, Hatley, Québec, and urban allotment gardens having pesticide use restrictions, Ottawa, Ontario, in July 2002. *Leptinotarsa decemlineata* eggs, insecticide-susceptible were supplied by the Southern Crop

Protection and Food Research Centre (SCPFRC), Agriculture and Agri-food Canada, London ON. Egg masses containing 25 to 30 eggs were couriered to the University of Ottawa, where they were placed on the leaves of four-week old greenhouse-grown potato plants (one to two egg masses per plant), variety Superior Gold and Russett Burbank.

Dermaptera

Forficula auricularia were collected from a garden site in Ottawa, Ontario, in July 2001. The earwigs were fed carrot and apple slices and were at held at 10° C, photoperiod 16:8 L:D, until the test was initiated.

Hemiptera

Blissus leucopterus hirtus nymphs and adults were collected in residential turf in Gatineau, Québec, in August 2002. The chinch bugs were kept in a mixture of soil and grass mulch at 10° C until tests were initiated.

Hymenoptera

Neodiprion sertifer larvae were collected from mugo pine trees *Pinus mugo* variety Mugo (Pinaceae) near Almonte and Pakenham, Ontario, in May 2002. The larvae were fed freshly cut mugo pine boughs and kept at 10°C, photoperiod 16:8 L:D, until bioassays were initiated.

Lepidoptera

Malacosoma americanum larvae were collected near Almonte and Pakenham, Ontario, in May 2002. Larvae were kept at 10°C and fed *M. sylvestris* leaves until trials began. Larvae were separated into instars and bioassays were conducted with the different age classes where numbers permitted.
Yponomeuta cagnagella were collected from the Kemptville Agricultural College Campus, Kemptville, Ontario, once in June 2001 and twice in May 2002. The individuals collected in June 2001 at the pre-

pupal, non-feeding larval stage while the earlier larval stages were fed burning bush *Euonymus alatus* Thunb. Siebold variety Compactus (Celastraceae). The larvae were kept at 10°C, photoperiod 16:8 L:D, until trials began. *Ostrinia nubilalis* larvae and adults were obtained from the University of Ottawa laboratory culture. Second instar larvae were selected 6 days after eggs hatched and the adult moths within 24 hours of emerging from the pupal stage.

Voucher specimens for all insects collected and used in efficacy bioassays were placed in the Insect Biosystematics collection, ECORC, Agriculture and Agri-food Canada, Ottawa, Ontario.

3.2.4 Bioassays for assessing insecticidal and repellent effects

3.2.4.1 24 hour LC_{50} determination

With the exception of *L. decemlineata* larvae, *B. leucopterus hirtus* adults, *F. auricularia* adults, *N. sertifer* larvae and *Y. cagnagella* larvae (due to a lack of sufficient numbers to allow for full toxicity bioassays or requirement for different test conditions) all toxicity trials were conducted by spray to drip of individual host plant leaves and then allowing them to dry at room temperature for 30 minutes. Test insect larvae or adults were then treated by spray to drip, patted dry, and placed on the leaves with the same treatment inside a glass Petri plate or 500 mL Mason® jar. Each species was treated with six *P. nigrum* concentration levels, including a formulation blank or emulsifiable concentrate (EC), based upon the results of a range-finding test. Ten larvae were used per two-three leaves and each treatment was replicated at least two times where numbers permitted. Test containers were kept at room temperature. Mortality after 24 hours was determined by touching the larvae with a probe to elicit a response.

Coleoptera

Seven days after hatching, *L. decemlineata* larvae obtained from SCPFRC were collected from plants and then dipped into treatments of either water (control), formulation blank (emulsifiable concentrate or EC), or 0.1, 0.5, 1 and 2% formulated *P. nigrum*. All treated larvae were placed into Petri

plates lined with fresh potato leaves and kept in an incubator at 27 °C, 70 RH and 16:8 L:D. Mortality after 24 h was determined by probing the larvae with tweezers to elicit a response.

Dermaptera

Forficula auricularia adults were sprayed to runoff with solutions of *P. nigrum* and *P. guineense* extracts at concentrations of 0.1, 0.5 and 1%.and then placed individually in plastic cups, 10 replicates per treatment. Controls consisted of water only and a formulation blank consisted of a 5% formulation mixture in water. Mortality was observed after 24 hours.

Hemiptera

Blissus leucopterus hirtus adults were treated with the spray to drip method but were then placed in Petri plates with Whatman filter paper. Ten chinch bugs were used per treatment with two and three replicates for the first and second trials respectively. Six *P. nigrum* concentration levels were tested, including a formulation blank or emulsifiable concentrate (EC), based upon the results of a range-finding test. After 24 hours the number of dead adults on the filter paper was determined by touching the chinch bug with a probe to elicit a response.

Hymenoptera

Neodiprion sertifer larvae were separated into instars and bioassays were conducted with the 4th-5th instar larvae where numbers permitted. *Neodiprion sertifer* larvae, 10 per treatment replicate, were placed onto the tips of needles of *P. mugo* boughs freshly cut and then were sprayed to runoff. Six *P. nigrum* concentration levels were tested, including a formulation blank or emulsifiable concentrate (EC), based upon the results of a range-finding test. The boughs were then placed inside a 500 mL Mason® jar to allow them to remain upright and covered with a mesh lid. After 24 hours the number of dead or moribund larvae at the bottom of the jars was determined.

Lepidoptera

Prepupal *Yponomeuta cagnagella* larvae collected in 2001 were treated with control (water only), 1% formulation blank and *P. nigrum* at 0.01, 0.05 and 0.1%. Larvae were dipped into the solutions, and then placed in a covered Petri plate. Ten larvae per replicate were used, with three replicates per treatment. Survival of larvae 48 hours after treatment and the number of adults that emerged successfully within the following two week period was noted.

3.2.4.2 Further acute toxicity trials

Combinations of *Piper* extracts with two promising botanical oils, Tansey (*Tanasetum vulgare* var. Crispum) and dillapiol, from dill oil *Anethum sawa* Roxb. (Umbelliferae) were applied to *P. japonica* adults, *L. lilii* adults, house flies *Musca domestica* L. (Muscidae: Diptera) adults and European chafer *Rhizotrogus majalis* Razoumowsky (Scarabaeidae) larvae. These experiments were conducted to test whether the combination would improve the contact toxicity of the *Piper* formulation. These results are reported in Appendix III

3.2.4.3 Repellent effects

All *Piper* spp. repellent trials were conducted in the greenhouse at Carleton University.

Coleoptera

Twenty *Popillia japonica* adults were caged on Explorer rose plants (Rosaceae) variety Louis Jolliet to determine the repellent effect of *P. nigrum* alone and in combination with other botanical products currently available by comparing damage to leaves caused by feeding. Treated plants were sprayed either with 100 mL of water only, or water combined with 2.5% formulation blank or 0.5% *P. nigrum*.

Pre-pupal *Lilioceris lilii* larvae and adults were tested using potted Asiatic lily plants treated with *P. nigrum* extracts. Asiatic *Lilium* (Liliaceae) were purchased in the spring as bulbs, Orange Pixie, Butter

Pixie and Latoya varieties, or as plants, Cancun, Orange Pixie, Butter Pixie and Lennox varieties, and were treated with formulated *P. nigrum* extract in the range of 0.125, 0.25, 0.5 and 1%. At each concentration level, three replicate plants were treated along with three replicate controls and a formulation blank of equal EC concentration. Ten pre-pupal *L. lilii* larvae were placed on each plant, which was checked after 24 hours to assess larval mortality, movement from the treated leaves and feeding damage to leaves.

A choice test using cucumber plants *Cucumis* spp. (Cucurbitaceae), variety Bush Pickle, was conducted where plants were sprayed to runoff with either 0.1 or 0.5% *P. nigrum* extract, and placed in a cage with both a water and formulation blank control. Sixty *Acalymma vittatum* adults were released in each of three replicate cages. Each plant was surrounded with a plastic collar which still allowed the beetles to disperse from the plant, which was capped at the end of 96 hours in order to remove each plant but not lose the insects feeding on it. The plants were then cooled to 10°C so that the adult beetles on each plant could be collected and counted and the number of damaged leaves counted.

To compare the toxic, repellent and antifeedant effect of *P. nigrum* in combination with other botanical products currently available, a trial was conducted by spraying potato plants with 100 mL of 0.5% *P. nigrum* alone, or 0.5% *P. nigrum* mixed with either the recommended dose of Garlic Barrier $^{AG+}$ Insect Repellent (Garlic Research Labs, Glendale CA) or Ropel® Plant Protect•™ (Burlington Scientific, Farmingdale NY), based on garlic *Allium sativum* Linn. and lemon grass oil *Cymbopogon citrates* [DC] Stapf. extracts, respectively. After application, 20 adult *L. decemlineata* were placed on each plant, three replicates per treatment, and covered with the mesh cage. The number of surviving adults and damaged leaves per plant was assessed after 24 h.

Lepidoptera

Green pepper plants *Capsicum annum* L. (Solanaceae) were grown to the mature fruiting stage and then chosen on the basis of damage-free fruits. *Ostrinia nubilalis* adults were collected within 24 hours of emerging from the pupal stage. Five female and five male adults were aspirated into a 500 mL

flask. Green pepper plants were treated with either water, formulation blank, 0.5% *P. guineense* extract, Ropel® or Garlic Barrier™, both at the recommended application rates. Each plant was sprayed until runoff (approximately 100 mL), with four replicates for the control, EC blank and *P. guineense* treated plants and three for the remaining treatments. Plants were left to dry and then caged by enclosing the plants with a metal frame and mesh net. The adult *O. nubilalis* were added to the cages and left in the greenhouse for 96 hours. The cages were reopened and the surviving adults, the number of leaves with egg masses and the total number of egg masses were counted.

3.2.5 Colorado potato beetle field trials

Field-grown potato plants, variety Yukon Gold, each four to five-weeks old, were sprayed with 100 mL of 0.5% *P. nigrum* to runoff. Control plants were treated with equal amounts of formulation blank. Immediately after application of control solution to three replicate plants and 0.5% *P. nigrum* to nine replicate plants, 20 field-collected *L. decemlineata* adults (10 females and 10 males) were placed on each of the control plants and three of the 0.5% *P. nigrum* plants. The plants were then covered with a fine mesh net supported by a wire cage to prevent the insects from escaping. After both 1.5 and 3 h, 20 adults were added to the remaining six replicate 0.5% *P. nigrum*-treated plants and then covered. Solar radiation readings were taken at each time period with a Li-Cor Quantum model LI-192SB sensor. After a 24-h, plants were sampled and assessed for leaf damage, survival of adults and egg mass production.

A second trial to assess contact toxicity consisted of 20 adult *L. decemlineata* (15 females and 5 males) placed on individual potato plants prior to treatment with either formulation blank or 0.5% *P. nigrum*. Plants were then sprayed with 100 mL of each treatment, three replicate controls and four replicate 0.5% *P. nigrum* treatments, to runoff. Each plant was covered with a mesh cage and left for 24 h. Plants were then sampled and assessed for survival of the adults.

3.2.6 Non-target invertebrate

Lady beetles

Adult lady beetles, *Hippodamia convergens* were purchased from Biobest, Leamington, Ontario, through Plant Products, Brampton, Ontario. The adults were kept at 4°C, and were not fed prior to or during the test. All *H. convergens* tests were initiated within two weeks of obtaining the adults. The toxicity test procedure followed those previously described for the 24 hour LC_{50} determination. Adults were dipped into *P. nigrum* solutions ranging from 0.01 to 1%, patted dry on filter paper and then placed into a plastic Petri plate with Whatman No. 1 filter paper. Ten *H. convergens* were treated per plate with three replicate plates per concentration.

3.2.7 Statistics

Probit analysis (Hubert and Carter 1990a) was used to determine the LC_{50} values for *P. nigrum*. Comparison of the LC_{50} values between trials for each species tested was conducted using a Chi Square or Z-test (Hubert and Carter 1990b). Results of all other bioassays were tested for normality and the data were transformed if necessary. A one-way analysis of variance (ANOVA) was performed with a post-hoc Tukey's multiple comparison of means test (SYSTAT 1999).

3.3 Results

3.3.1 Pepper formulation development

Black pepper extracts contained large amounts of piperine and smaller amounts of piperlonguminine, dihydro-piperine and dihydro-piperlonguminine (Table 3.1). The four piperamides from each batch were totalled to provide an estimate of the total amide content although a few minor compounds were not quantified. The total ranged from 299 to 588 mg/g in *P. nigrum*, from 49 to 197 mg/g in *P. guineense* and from 72 to 102 in *P. tuberculatum*. Piperamide levels from *P. nigrum* batches prepared for the efficacy trials in both 2001 and 2002 were found to have an overlapping range of

concentrations whereas the *P. guineense* extracts used in 2002 had higher piperamide levels than in 2001 likely due to a difference in the source of the peppercorns.

Tests with different formulation components indicated that: 1/ the three pepper extracts were soluble in ethanol and THFA and 2/ the two emulsifiers selected, Jeneil Co. biosurfactant JBR-325® and -425®, and Rhone-Poulenc castor oil Alkamus EL-719®, rated consistently high in terms of emulsion bloom in water, emulsion suspensibility and spreading area.

Table 3. 1. Range of piperamide concentrations (mg/g) in *P. nigrum*, *P. guineense* and *P. tuberculatum* extracts used in efficacy trials 2001 and 2002.

Extract	Year	Dihydropiper-longuminine	Piperlon-guminine	Dihydropiperine	Piperine	Total
P. nigrum	'02	0.4	1.9 – 2.9	17.7 – 22	370 - 427	391 - 451
	'01	0.5 – 1.8	0.9 – 5.1	10.4 – 23.7	288 - 514	299 - 538
P. guin.	'02	31.2	28.1	84.4	53.5	197
	'01	3.6 – 8	2.7 – 7.7	9.8 – 29.9	33.4 – 68.8	49 – 114
P. tuber.	'02	59 – 65.5	2.7 – 7.5	6 – 15.5	4.7 – 13.5	72 - 102

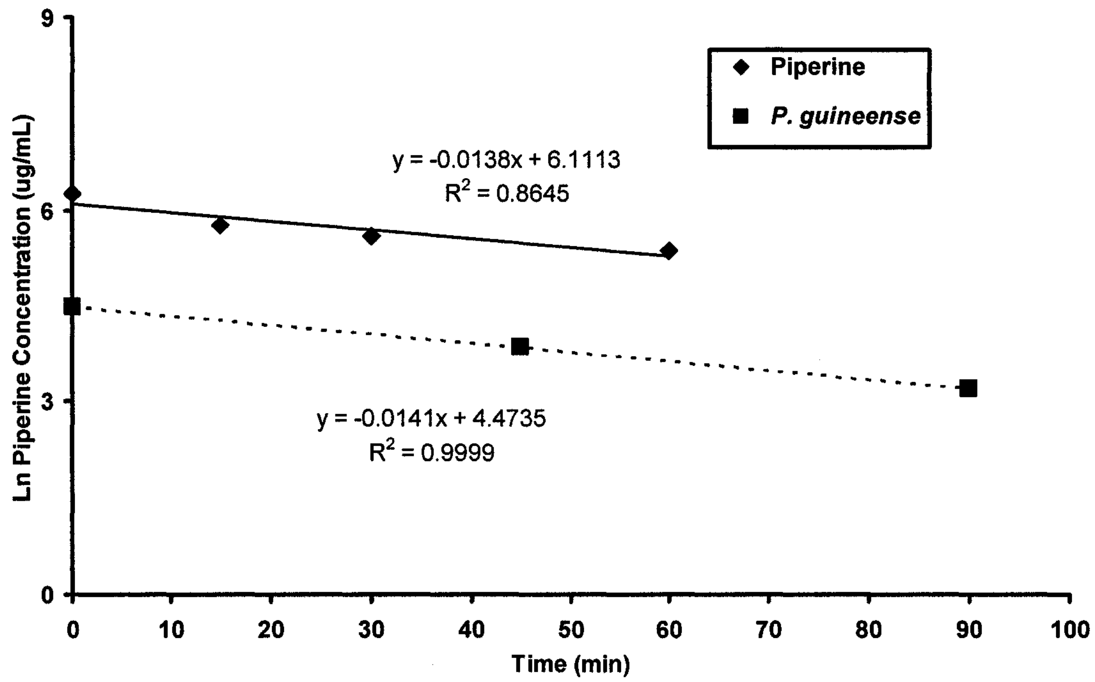


Figure 3. 1. Piperine concentration after exposure of *P. guineense* extract (dashed line) to full sunlight and piperine to UV radiation (solid line) over 1.5 hours.

THFA was chosen over ethanol based on the lower flashpoint and potential for registration and Alkamus EL-719, a synthetic ethoxylated castor oil, was preferred over JBR-425, a natural rhamnolipid, since it was less toxic in housefly toxicity trials (unpublished data). The final composition of the pepper formulation for testing was: pepper extract 20%, THFA 70% and emulsifier 10%. The formulation was diluted in water at the time of spraying to the appropriate concentration.

3.3.2 Effect of sunlight and ultraviolet radiation on *Piper* extracts

The piperamide content of extracts was found to be stable over several months in the laboratory at room temperature, but the formulations are susceptible to photodegradation. Piperine in the extracts degraded quickly after exposure to sunlight (Fig. 3.1). Pure piperine also degraded quickly under UV lamp exposure with a half-life of approximately 39 min, suggesting that the degradation was a direct photolysis, not a photosensitized reaction from some pepper pigment. After six hours exposure to sunlight, all amides in the *P. guineense* extract, including piperine ($t_{1/2}$ = 49 min), were below detection (data not shown). Light peak light levels were measured between 1230 and 1410 μEinsteins m^{-2} s^{-1}. Combinations of formulated *P. nigrum* with several antioxidants and sunscreens determined that only a sunblock such as titanium oxide would protect piperamides from photodegradation (Appendix II).

3.3.3 Insecticidal activity

Accurate LC_{50} values for the *P. nigrum* formulation were obtained with eight selected urban insect pest species and one beneficial insect (Table 3.2 and 3.3). In general the extracts were more toxic to larval insects where LC_{50} values ranged from 0.018-0.103 % (Table 3.2) than adult insects, where LC_{50} values ranged from 0.103-0.746% (Table 3.3). Lepidoptera and Hymenoptera species were more susceptible (0.018 - 0.075%) than Coleoptera or Hemiptera (0.103 – 0.746%) to the *P. nigrum* extracts.

Table 3. 2. *P. nigrum* LC_{50} (%), 95% confidence intervals and slope of probit lines for selected insect larvae.

Common Name	Order: Family	Genus Species	N	*P. nigrum* LC_{50} (%)	95% C.I. (%)	Slope
Eastern Tent Caterpillar	Lepidoptera: Lasiocampidae	*Malacosoma americanum* (Fabricius)	358	0.018	0.015, 0.022	2.84
European Pine Sawfly Larvae	Hymenoptera: Diprionidae	*Neodiprion sertifer* (Geoffroy)	354	0.046	0.04, 0.054	2.72
Spindle Ermine Moth Larvae	Lepidoptera: Yponomeutidae	*Yponomeuta cagnagella* (Hübner)	357	0.075	0.054, 0.124	1.15
Viburnum Leaf Beetle Larvae	Coleoptera: Chrysomelidae	*Pyrrhalta viburni* (Paykull)	160	0.103	0.071, 0.137	2.03

Table 3. 3. *P. nigrum* LC_{50} (%), 95% confidence intervals and slope of probit lines for selected insect adults.

Common Name	Order: Family	Genus Species	N	*P. nigrum* LC_{50} (%)	95% C.I. (%)	Slope
Striped Cucumber Beetle Adult	Coleoptera: Chysomelidae	*Acalymma vittatum* (Fabricius)	240	0.103	0.087, 0.13	3.16
Convergent Lady Beetle Adult	Coleoptera: Coccinellidae	*Hippodamia convergens* (Guérin-Méneville)	540	0.213	0.173, 0.276	2.03
Colorado Potato Beetle Adult	Coleoptera: Chrysomelidae	*Leptinotarsa decemlineata* (Say)	300	0.498	0.363, 0.652	1.45
Japanese Beetle Adult	Coleoptera: Scarabidae	*Popillia japonica* (Newman)	539	0.532	0.446, 0.616	2.11
Hairy Chinch Bug Adult	Hemiptera: Lygaeidae	*Blissus leucopterus hirtus* Montandon	214	0.746	0.518, 1.361	1.49

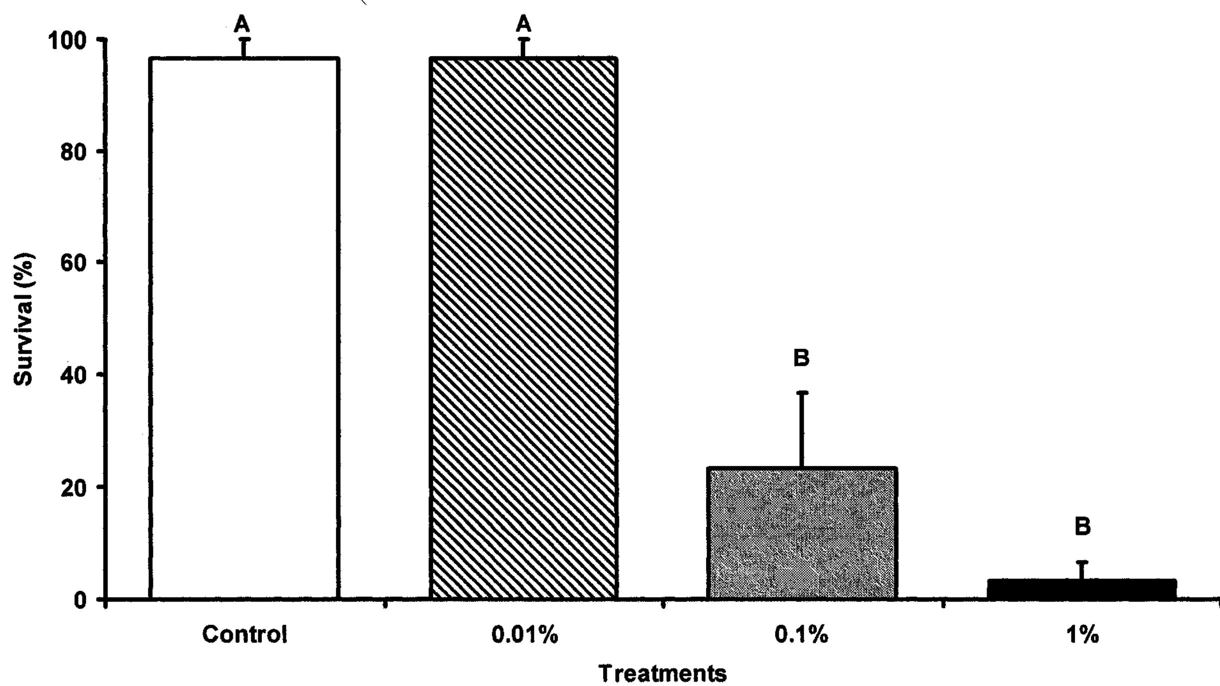


Figure 3. 2. Percent survival ± S.E. of mid stage *L. decemlineata* larvae treated with three concentrations of *P. nigrum* extract compared to the formulation control (Tukey's, P<0.05).

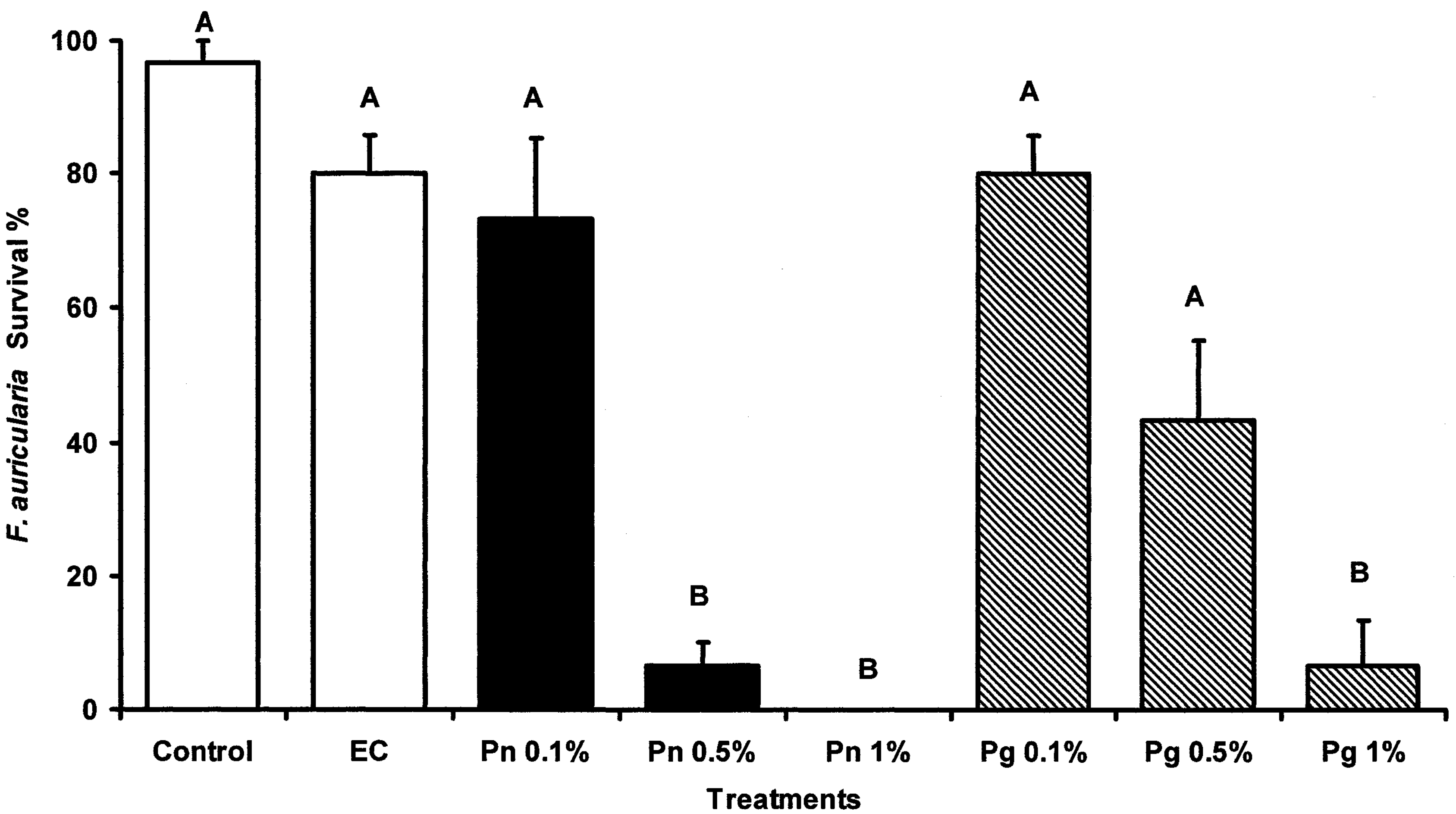


Figure 3. 3. Adult *F. auricularia* percent survival ± S.E. 24 h after water control, formulation control (EC), 0.01, 0.1 and 1% *P. nigrum* (Pn) and *P. guineense* (Pg) extract treatment. The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

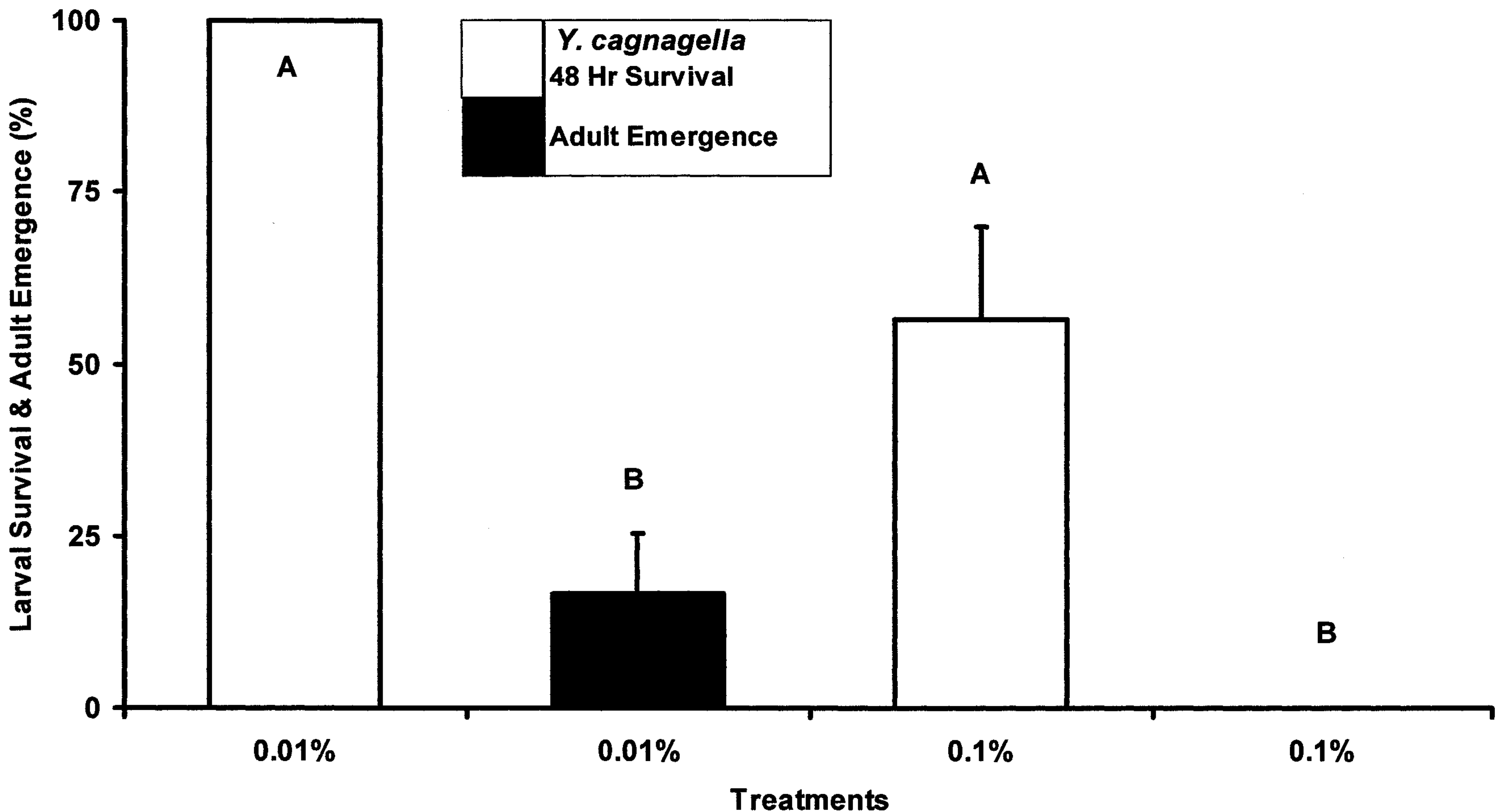


Figure 3. 4. Prepupal *Y. cagnagella* larvae percent survival and adult emergence ± S.E. after 0.01 and 0.1% *P. nigrum* treatment. The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

Although LC_{50} data were not available, toxicity assessments at several concentrations were made for other insects. The mortality of seven day-old *L. decemlineata* larvae was 75% when exposed to a 0.1% *P. nigrum* treatment while the 1% treatment caused 95% mortality (F=43.421; df=3,8; P=0.001) (Fig. 3.2). A 0.5% treatment knocked down approximately 50% of the pre-pupal larvae (data not shown). The adults appear to be equally sensitive, as the LD_{50} was determined to be 0.5% (95% confidence limits = 0.36, 0.65) *P. nigrum* (Table 3.3).

Survival of adult earwigs, *F. auricularia* decreased significantly at 0.5% and 1% *P. nigrum* (F= 14.7; df = 7,16; P<0.008) and 1% *P. guineense* (Tukey's multiple range test, P=0.001) after 24 hours (Fig. 3.3). These latter results also show comparable insecticidal activity for both the African and black pepper. *P. nigrum* extracts up to 0.1% did not reduce late instar, pre-pupal *Y. cagnagella* survival significantly after 48 hours (F=13.712; df = 3,8; P=0.855), but effects were manifested later in the life cycle where 0.01 and 0.1% *P. nigrum* reduced survival to adult emergence significantly (Tukey's multiple range test, P = 0.058 and 0.004 respectively) (Fig. 3.4).

3.3.4 Repellent effects, antifeedant and oviposition deterrent

Coleoptera

Behavior modification effects (repellent, antifeedant and oviposition effects) of pepper formulations were clearly evident with some situations, but not others. In a no-choice experimental design with *L. lilii* larvae, fewer larvae (expressed as mobile insects) remained on treated lily plants and fewer damaged leaves were observed (F=11.189; df = 15, 32; P<0.001) compared to controls as the *P. nigrum* dose was increased from 0.125 to 0.5% (Fig. 3.5). *Piper nigrum* extract at 1% burned lily plant leaf tips thus the results were not incorporated into Fig. 3.5 despite the fact that at that concentration all treated *L. lilii* larvae died within 24 h. These two concentration-dependent relations were significant (P=0.05) with r^2 = 0.86 for leaves damaged and r^2 = 0.94 for insect mobility. The pepper treatment reduced the survival of all exposed larval insects (F = 13.223; df = 3, 8; P=0.002) but not the number of dead or moribund larvae after 24 hours (F = 18.073; df = 3,8; P=0.131).

Rose plants sprayed with 0.5% *P. nigrum* had fewer adult *P. japonica* present after a 96 h choice test compared to water treated plants (F=7.57; df = 5, 12; P=0.001) but there was no significant difference compared to formulation-treated plants (Tukey's multiple range test, P=0.243) (Fig. 3.6). More importantly there was no difference in the number of leaves damaged by the beetles (Tukey's multiple range test, P>0.953).

Both 0.5 and 0.1% *P. nigrum* extracts reduced the number of *A. vittatum* adults found on cucumber plants after a 96 h choice test (F=8.563; df = 7,16; P<0.007) compared to both water and formulation controls (Fig. 3.7A and 3.7B respectively). The *A. vittatum* adults not found on plants were considered mobile and factored into the analyses but were not shown in the figures. In this situation no phytotoxicity to cucumber leaves was observed with 0.1% *P. nigrum* formulation

Mixtures of *P. nigrum* and either garlic or lemon grass oil showed no difference in terms of combination effect. No significant difference for either a toxic (F=2.769; df=2,6; P=0.141) and repellent effect (F=1.143; df=2,6; P=0.380) between *P. nigrum* extract alone or in combination with the two other botanicals was detected (Fig. 3.8A & B). Although the joint action of *P. nigrum* with lemon grass oil (Ropel®) did not reduce the damage to leaves from *L. decemlineata* feeding, there were on average higher numbers of moribund adults after 24 h. It is important to note that the 0.5% *P. nigrum* application did not cause any damage to the treated potato plants while a 1% formulated extract was observed to be phytotoxic.

Lepidoptera

Piper guineense at 0.5% reduced *O. nubilalis* oviposition compared to the control (F=3.182; df = 4,13; P=0.043) but not the formulation blank, Ropel® or Garlic Barrier™ (Tukey's multiple range test, P>0.113)(Fig. 3.9).

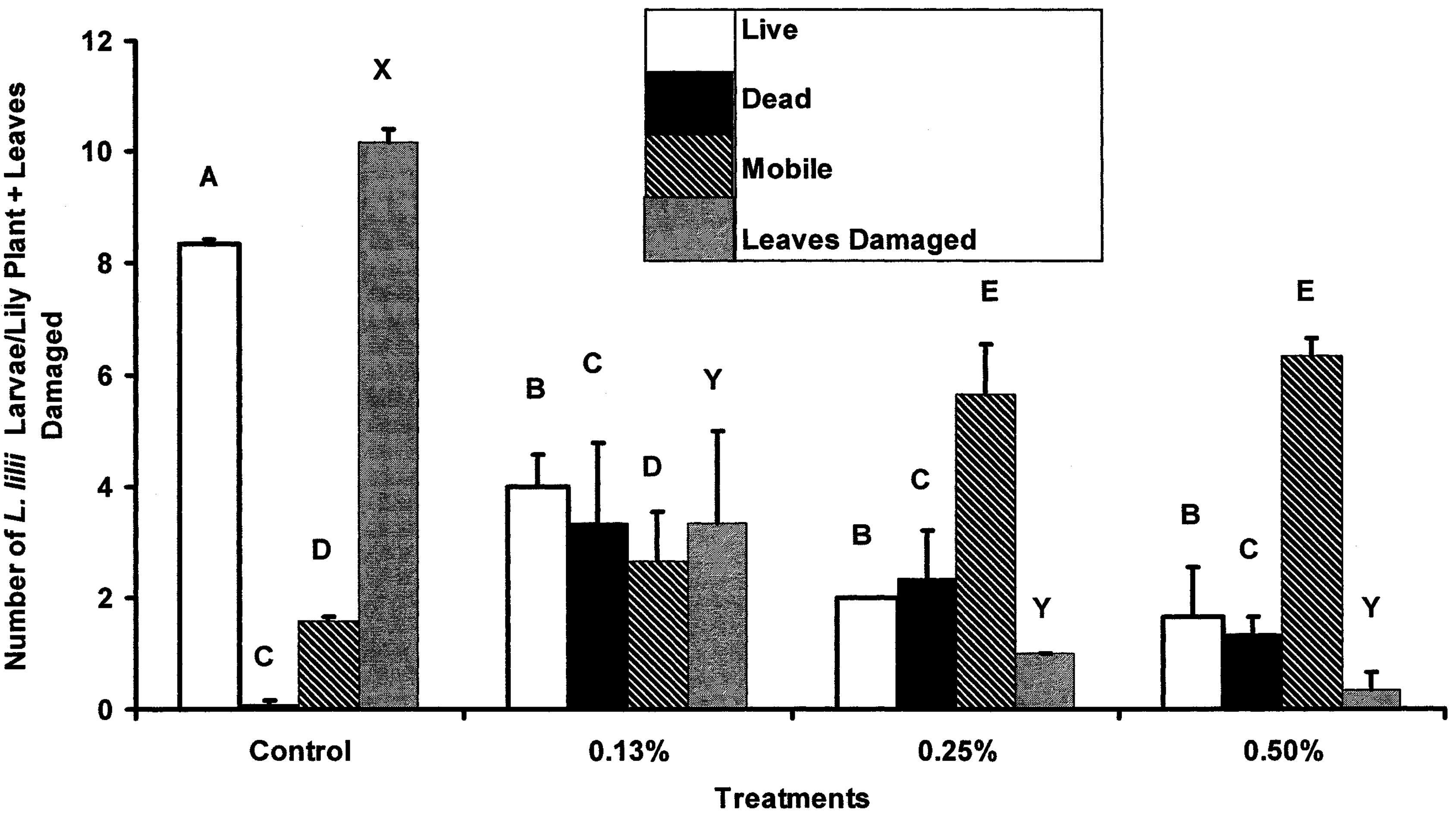


Figure 3. 5. Mean number of live, dead and mobile *L. lilii* larvae ± S.E. on *P. nigrum*-treated Asiatic *Lilium* plants along with the mean number of damaged leaves ± S.E. per plant. The treatment means for survival (clear bar), mortality (dark bar), mobile *L. lilii* larvae (hatched bar) and number of damaged leaves (shaded bar) with the same letter are not significantly different (Tukey's, $P>0.05$).

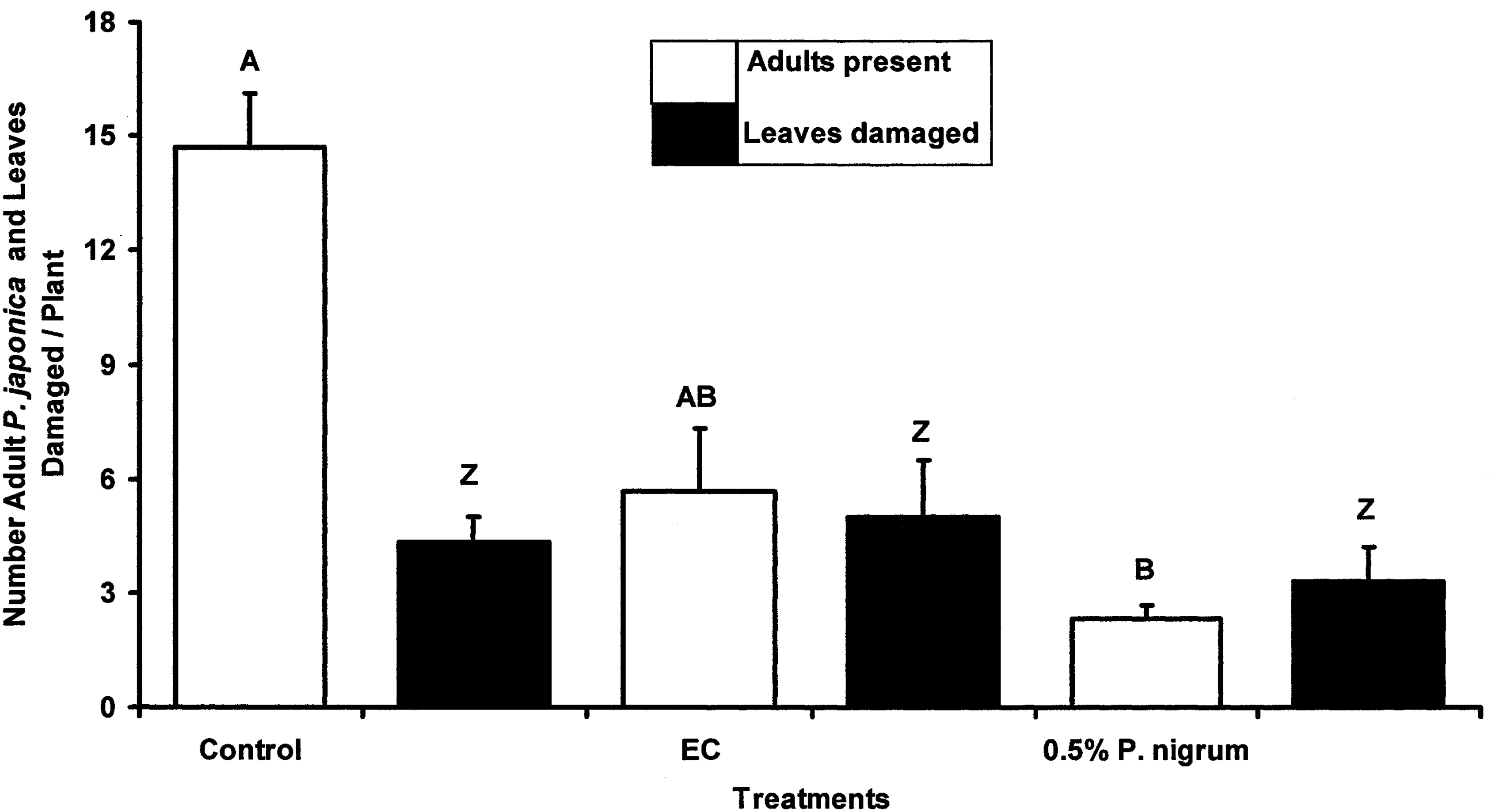


Figure 3. 6. Mean number of *P. japonica* adults and leaves damaged ± S.E. on rose plants treated with water (control), formulation control (EC) and 0.5% *P. nigrum*. The treatment means for adults/plant (clear bar) and leaves damaged (dark bar) were compared separately where the same letter indicates no significant difference (Tukey's, $P > 0.05$).

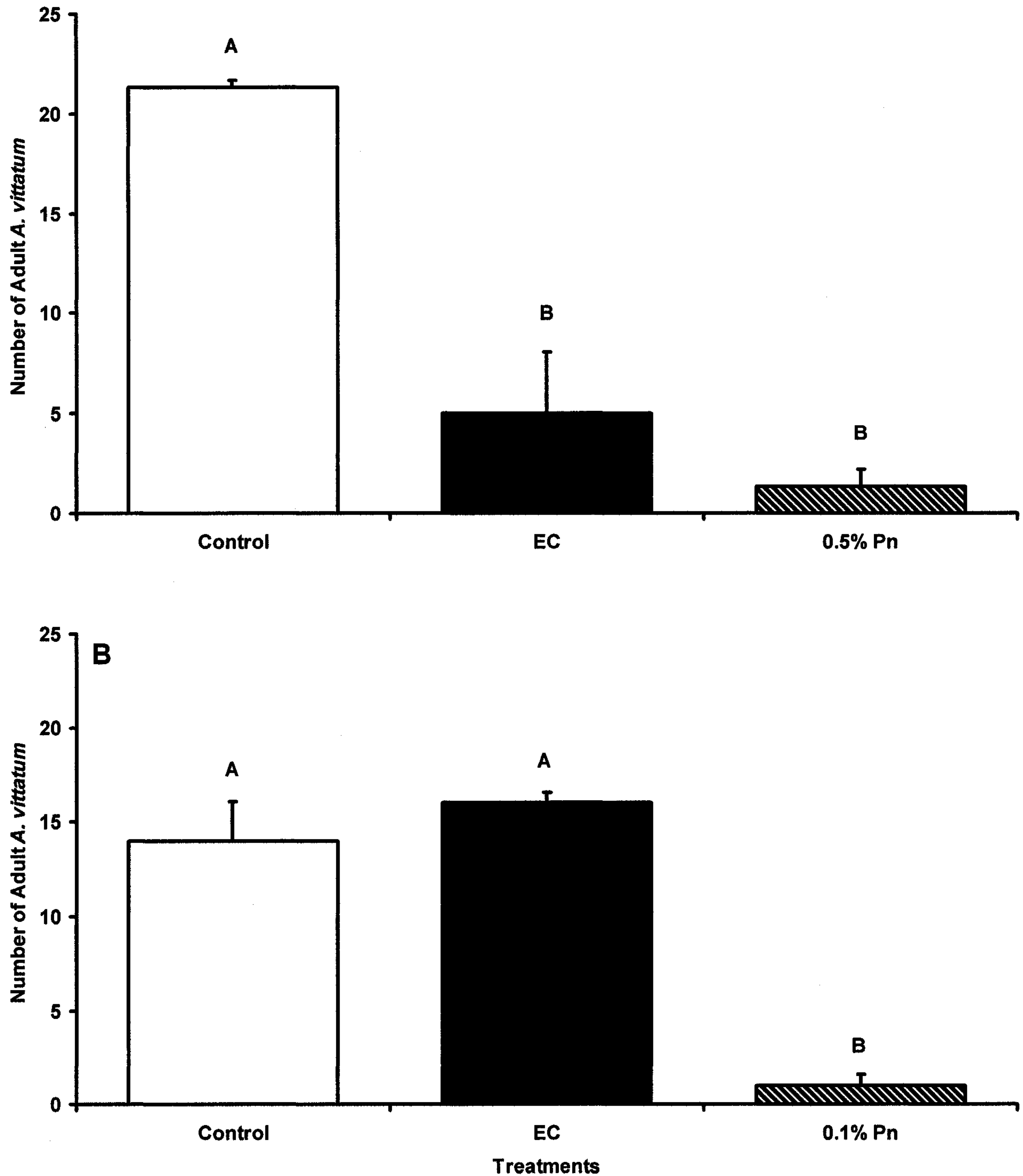


Figure 3. 7. Mean number of *A. vittatum* adults + S.E. found on cucumber plants after two 96 h choice test: (A) plants treated with water (Control), formulation control (EC) and 0.5% P. nigrum; (B) plants treated with water (Control), formulation blank (EC) and 0.1% P. nigrum. The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

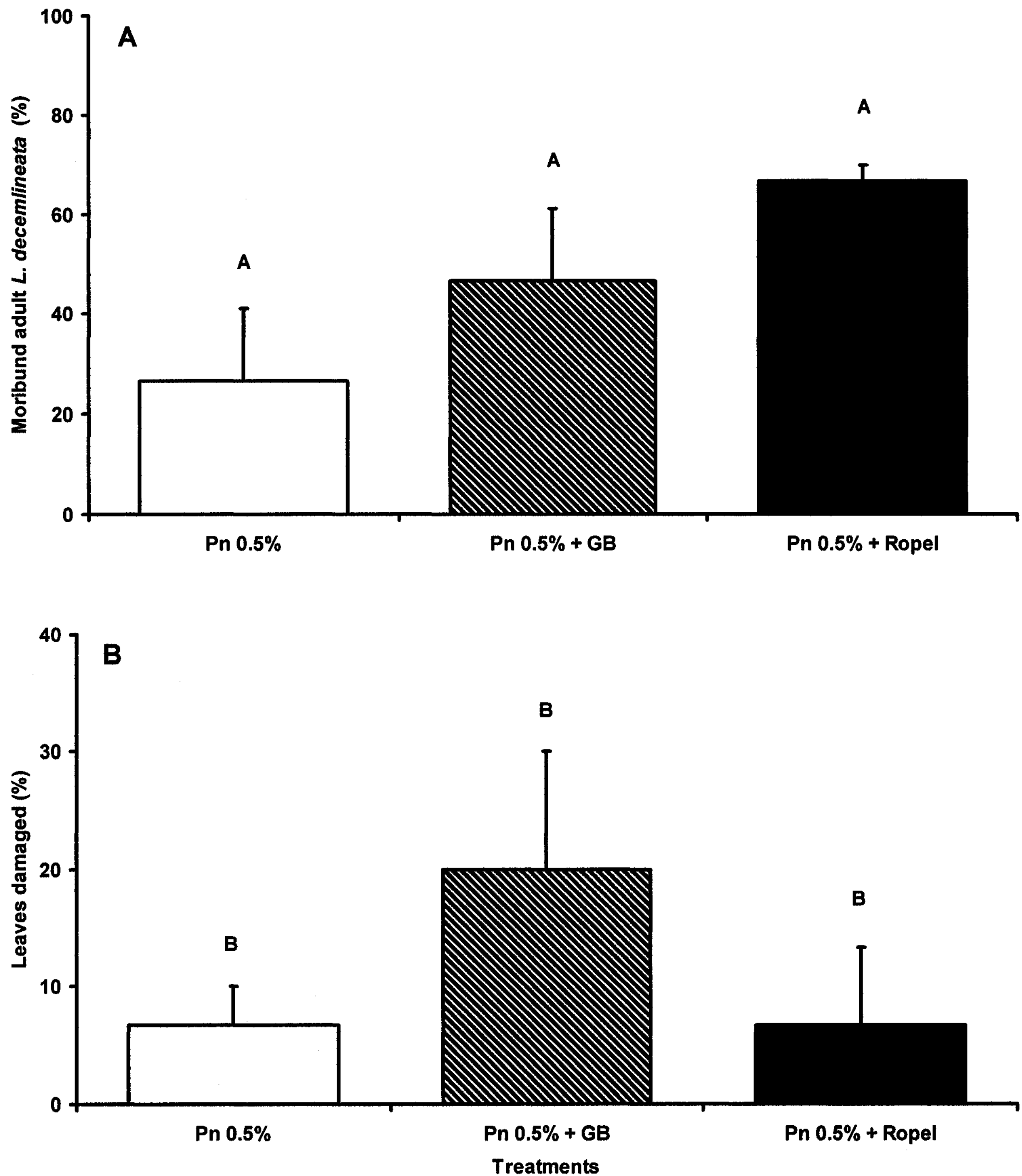


Figure 3. 8. Percent adult *L. decemlineata* morbidity ± S.E. (A) and percent damage to leaves ± S.E. (B) 24 h after potato plants were treated with 0.5% *P. nigrum* only, or in combination with either Garlic Barrier® (GB) or Ropel® commercial insect repellents. The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

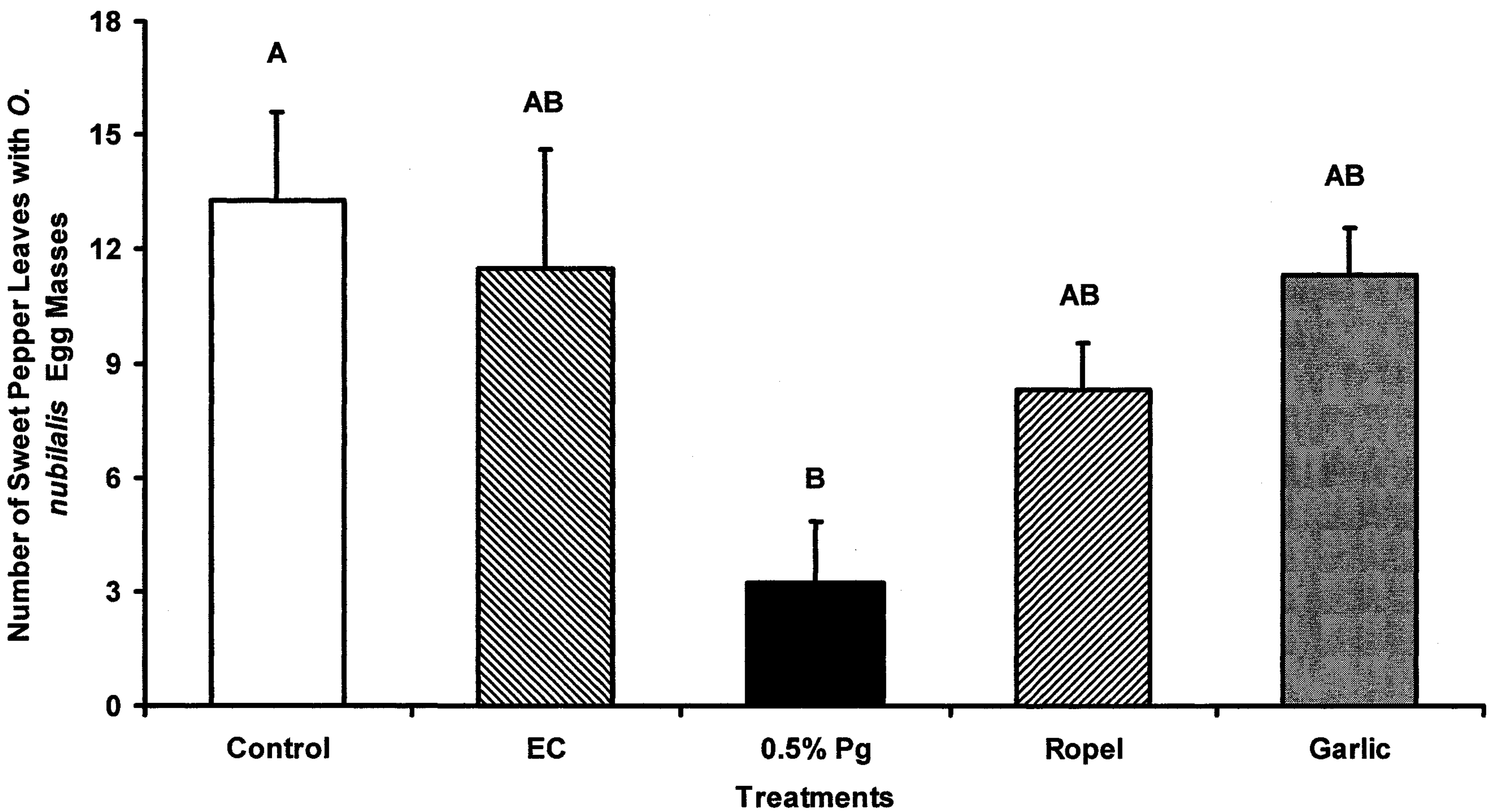


Figure 3. 9. Mean number of *O. nubilalis* egg masses found on bell pepper plants 96 h after treatment with either water (control), formulation blank (EC), 0.5% *P. guineense* (0.5% Pg), *C. citratus* (Ropel®) or *A. sativum* (Garlic Barrier™). The treatment means with the same letter are not significantly different (Tukey's, $P > 0.05$).

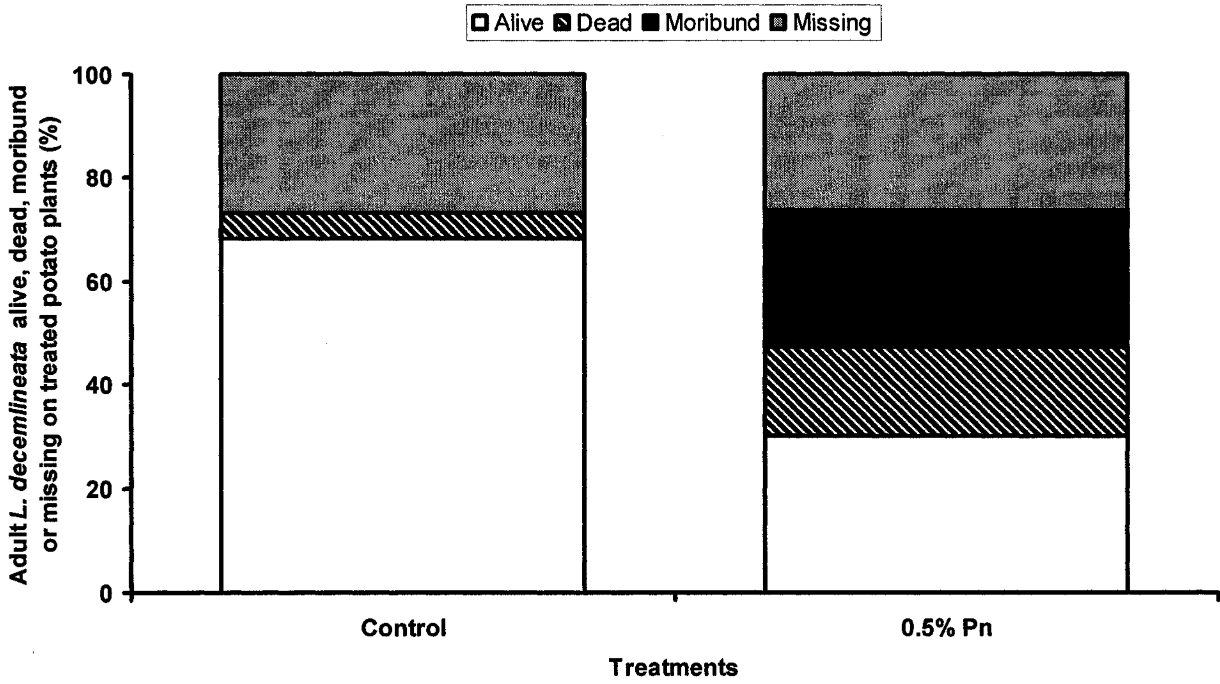


Figure 3. 10. Mean percent adult *L. decemlineata* survival and morbidity, 24 h after treatment with 0.5% *P. nigrum* extract, while feeding on field grown potato plants exposed to full sunlight.

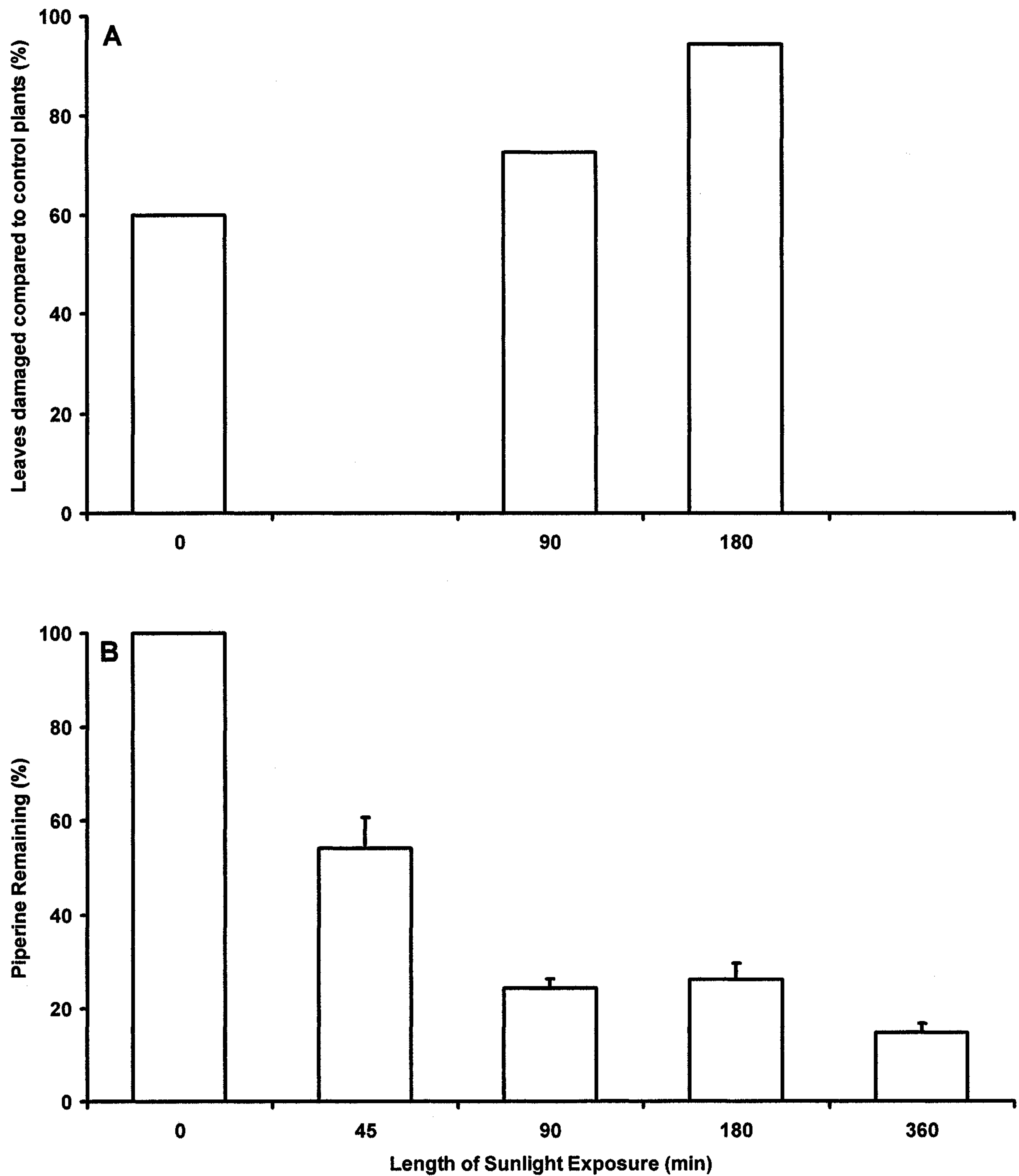


Figure 3. 11. Percent damage to potato plant leaves on plants previously treated with 0.5% *P. nigrum* and then exposed to full sunlight for either 0, 90 or 180 min before the addition of adult *L. decemlineata* (A) and the mean percent piperine remaining in *P. nigrum* extract samples exposed to full sunlight on glass slides for 0, 45, 90, 180 and 360 min (B).

3.3.5 Colorado potato beetle field trials

Knockdown of adult *L. decemlineata* was observed 24 h after a spray application of *P. nigrum* to potato plants under field conditions. The effect of a 0.5% *P. nigrum* extract treatment on adult *L. decemlineata* feeding on field-grown potato plants was comparable to that observed with the LD_{50} dose (Fig. 3.10). Less than 40% of adults survived, compared to 70% in controls (F=75.571; df=1,4; P=0.001), and the number of dead (F=13.5; df=1,4; P=0.021) and moribund (F=169.0; df=1,4; P=0.001) adults found was significantly greater than for the controls after 24 h. On both control and pepper-treated plants, 25% of the adults were not found. Since they could not escape from the cages, it is assumed that they were hiding either in the foliage or the soil. A 1% extract application was not tested due to the phytotoxicity of the formulation.

The protective effect of a 0.5% *P. nigrum* treatment was observed to decrease within three hours as the number of leaves damaged by adult *L. decemlineata* increased with the length of time that the extract was exposed to full sunlight (Fig. 3.11A). This decrease in bioactivity is likely related to the almost 80% degradation of piperine that was observed to occur over a similar length of time exposed to sunlight (R^2 =0.919) (Fig. 3.11B). Light levels were measured between 1830 and 2250 μEinsteins m^{-2} s^{-1}.

No difference in the survival of adults or the number of egg masses was found between treated potato plants exposed to sunlight for the three time periods. The survival of adults placed on the plants after the 0.5% *P. nigrum* treatment was noted to be much higher than when they are exposed directly to the spray as in Figure 3.10.

3.3.6 Non-target toxicity

The 24-h LC_{50} for *H. convergens* adults was 0.21 % (Table 3.3). Within one hour, adults treated with concentrations > 0.1% were knocked down but some had recovered by 24 h in the 0.1% treatment.

3.4 Discussion

Botanical insecticides have been used for centuries for crop protection. Only with the development of synthetic insecticides in the mid 1900s did their usage drop, as more effective products took their place. Within a relatively short time problems arose with the synthetic products: environmental contamination, poisonings of non-target species and resistance. This led many to reconsider botanical formulations as natural alternatives because they are less toxic. However, these have always had varying degrees of success and recently even their continued safe use has been questioned. Rotenone and pyrethrum, two of the most commonly applied by home gardeners and organic farmers, are being re-evaluated by the U.S. E.P.A. based on concerns regarding health effects from long-term exposure (Khera *et al.* 1982; Betarbet *et al.* 2000).

Plants with an established record for culinary or medicinal use, that therefore offer a safer starting material, have been evaluated in terms of their potential application as insecticides. For the present study these plant compounds were considered not as leads for synthetic insecticides, but for extract-based formulations that combine all of the co-occurring secondary plant compounds. The advantage of whole extracts over single active ingredients is that resistance by insects is delayed. For example resistance development occurred with pure azadiractin alone but not neem oil *A. indica* containing numerous compounds besides azadirachtin (Feng and Isman 1995). Whole extracts complicate the chemistry, but simplifies the processing and allows for a unique mixture of actives. The results of the present study correspond to many of the previous ones (Miyakado *et al.* 1979 and 1980; Sighamony *et al.* 1986; Baier and Webster 1992; Ivbijaro *et al.* 1993; Mbata *et al.* 1995; Kéïta *et al.* 2000) in terms of the promising potential efficacy found in *P. nigrum* and *P. guineense* extracts.

Piper nigrum extracts were found to provide excellent control of the lepidopteran species and European pine sawfly tested. The larvae were sensitive to *P. nigrum* and *P. guineense* treatments below 0.1% if applied as a contact insecticide although some consumption of treated plant material cannot be discounted. Both *M. americanum* and *N. sertifer* are pests of ornamental trees and shrubs (Johnson and Lyon 1991), and thus could be controlled quickly by the homeowner when damage by larvae becomes

apparent. A spray mixed between 0.05 to 0.1% *P. nigrum* would initially knock the larvae off leaves and branches, and the neurotoxic activity would prevent them from returning to the plant. Similarly, gardeners could repel *L. lilii* larvae and *Y. cagnagella* larvae from plants with 0.1% extract formulations. However, larger, hard-bodied coleopterans, like *L. decemlineata* and *P. japonica* adults, require higher doses likely due to their relatively greater body mass (Table 3.4) and the difficulty of penetrating the cuticle. Although concentrations of 0.5% *P. nigrum* were required to cause 50% mortality with both *P. japonica* and *L. decemlineata* adults, this is still a practical concentration for botanical insecticides.

A post-hoc hypothesis worthy of future examination is larger larval insects are more sensitive to pepper treatments than smaller ones regardless of insect order. This is based on the observation that the size of the larval insects (Table 3.4) was inversely correlated with the toxicity (R^2=0.913; Y=-0.002X + 0.091): the smaller *P. viburni* were less sensitive than the much larger *M. americanum* and *N. sertifer*.

Table 3. 4. Relationship between *P. nigrum* toxicity and insect mean dry weight.

Insect	Order	LC_{50} (%)	Mean Weight (mg)
M. americanum larvae	Lepidoptera	0.018	35.9
N. sertifer larvae	Hymenoptera	0.046	17.8
Y. cagnagella larvae	Lepidoptera	0.075	2.3
P. viburni larvae	Coleoptera	0.103	0.4
A. vittatum adult	Coleoptera	0.103	4.1
L. decemlineata adult	Coleoptera	0.498	20.1
P. japonica adult	Coleoptera	0.532	25.7

A more suitable explanation of the greater sensitivity of lepidopterans and the European pine sawfly larvae may be greater absorption through the cuticle compared to the coleopteran larvae. Based upon visual observations, the coleopteran, *P. viburni*, had a thicker, tougher cuticle than the representative

lepidopterans and one sawfly larvae. Structural difference in insect cuticle between different species has been documented as the reason for different rates of penetration (Smagghe *et al.* 1997; Teal *et al.* 1999). This was observed with cuticle preparations from the adult tobacco budworm moth *Heliothis virescens* Fabricius and the adult American cockroach *Periplaneta americana* L. The obviously thicker cockroach cuticle had a slower penetration rate compared to the moth (Teal *et al.* 1999). When the toxicity between the adult coleopterans are compared, cuticle thickness may not play as great a factor as size (R^2=0.967; Y=0.021X + 0.03) since the larger *P. japonica* and *L. decemlineata* required a five-times higher dose compared to the proportionally smaller *A. vittatum* (Table 3.4).

The use of *Piper* extracts for both antifeedant and oviposition deterrent activity has been well documented for stored-grain insects (Ivbijaro 1990; Ekesi 2000; Lale and Alaga 2001). In the present study, repellent activity by *Piper* extracts alone or in combination with other botanicals was observed against some common garden insect pests. The most promising results were seen when 0.125% *P. nigrum* treatments protected lily plants. The majority of *L. lilii* larvae moved or dropped off the plants, and their feeding was reduced (Fig. 3.5). This treatment level is considered safe for repeated daily applications, since no phytotoxicity was noted at concentrations below 0.5%.

In the present study, *A. vittatum* adults were deterred from cucumber plants with a 0.1% *P. nigrum* spray for a four-day period (Fig. 3.7a and b). In practical application this may prevent or reduce the infestation of the Cucurbitaceae roots and fruit by the larvae as well as the spread of the cucumber mosaic virus. Antifeedant activity was also noted for both *P. nigrum* and *P. guineense* as low as 0.01% in a *F. auricularia* feeding trial but this was attributed as much to the formulation as to the *Piper* constituents (unpublished data). The repellent effect was confirmed when 50 mg of *P. guineense* per 30 cm^2 repelled the red flour beetle *Tribolium castaneum* Herbst from treated paper discs (Lale and Alaga 2001).

In the present study oviposition by *O. nubilalis* on green pepper plants was reduced compared to controls by a 0.5% *P. guineense* spray for four days under greenhouse conditions (Fig. 3.9). Similarly, studies with *P. guineense* and *A. sativum* were equally effective at reducing the egg hatch of the legume pod borer *Maruca vitrata* Fabricius (Ekesi 2000) and oviposition of *C. maculatus* was controlled by 2 and

3 mL *P. guineense* extract / kg cowpea seeds compared to control and neem seed oil (Ivbijaro 1990). This suggests that not only would *P. guineense* extracts reduce lepidopteran pest oviposition but also the hatching success of any eggs that were placed on treated leaves.

The repellent effect of several non-host volatiles was tested (Held *et al.* 2003) in order to determine whether rose plants could be protected from *P. japonica.* None of the treatments designed to mask the host plant volatiles (that included red cedar *Juniperus virginiana* L., osage orange *Maclura pomifera* Raf. Schneid, ginko *Ginkgo biloba* L., red pepper *Capsicum frutescens* L., fennel seeds *Foeniculum vulgare* Miller and spearmint *Mentha spicata* L.) were effective at repelling *P. japonica.* Similarly the present study determined that *P. japonica,* given a choice between *P. nigrum* treated roses and controls, would still feed on the treated plants (Fig. 3.6) and, when not given a choice, would also feed on rose plants sprayed with a combination of *P. nigrum* and either *A. indica* oil or Surround®WP kaolin (Appendix III), both considered to have strong antifeedant properties. Based upon these results *P. japonica* does not appear to be a pest insect that *Piper* extracts would affect greatly.

Keeping larvae off the plants is an environmental and practical benefit of the *Piper* extracts especially since the larvicidal effects of these compounds on Lepidopteran larvae feeding inside the plant are low. According to Torto *et al.* (1992) the most potent amide in *P. nigrum* tested against Sorghum stem borer *Chilo partellus* Swinhoe is piperine. It was suggested that the methylenedioxybenzene (MDP) group common to the piperamide molecules is an important factor in antifeedant activity. In both *P. nigrum* and *P. guineense* extracts piperine is the major amide present (Scott *et al.* 2002) although several other active amides are present with the MDP group.

Both the disadvantage and advantage of using a *Piper* extract is the short residual activity, especially under full sunlight. This was further documented after exposure to UV radiation as evidenced in Figure 3.1. It was shown that under field conditions when *P. nigrum* was applied to repel *L. decemlineata* adults feeding on potato plants there was a rapid loss in repellent activity within three hours after application corresponding to an increase in leaf damage (Fig. 3.11A and B). Thus *Piper* formulations, unless prepared with a sunscreen or sunblock to extend the life of the active ingredients (see Appendix II),

will not provide an adequate repellent unless the plants are in shaded areas. Activity may be prolonged on shaded surfaces such as the underside of the leaf and applications could be timed for later in the day to work as repellents for night flying or feeding insects or possibly slugs (Mollusca: Gastropoda).

The lack of residual activity may explain why *Piper* extracts have been tested mainly against stored-products insects since sunlight is not a factor in the degradation, and where activity has been observed for up to one month (Sighamony *et al.* 1986; Baier and Webster 1992; Kéïta *et al.* 2000; Ashamo and Odeyemi 2001). Therefore under field conditions repeated sprays may be required to reduce damage to plants by larval and adult *L. decemlineata*. However the use of *Piper* extracts, primarily for their knockdown and acute toxicity, does not require the actives to remain on the plant surface for longer than the time exposed insects need to absorb them. Piperaceae extracts in combination with other management tools, such as perimeter trenches, mulching, crop rotation and intercropping, could provide better control of *L. decemlineata* and related agricultural pests for organic and small-scale farmers or gardeners in a healthier and environmentally sound manner.

Another potential disadvantage is the effect upon non-target insect species. Lady beetles *H. convergens* were affected by *P. nigrum* concentrations in the same range as those required to control other phytophagous insect species (Tables 3.2 and 3.3). Thus lady beetle populations, considered a beneficial and popular biocontrol species, would be affected when pests are being targeted. Certainly if *H. convergens* are sprayed directly with *P. nigrum* concentration between 0.1 and 0.5% they will be susceptible. However, the risk to these predators of homopterans is probably lessened considering that the extracts degrade quickly and lady beetles do not feed on treated foliage. Therefore, in an IPM context, gardeners could apply the *P. nigrum* extracts to knock down the pest insect population, wait several hours for the actives to degrade under full sunlight, then release *H. convergens*.

Combining *P. nigrum* extract with other botanicals to improve the contact toxicity provided, at best, an additive effect. A synergistic affect was suspected when 0.1 % dillapiol and 0.1% *P. guineense* were mixed together and applied to the house fly *Musca domestica* L., however the experiment has yet to be replicated (Appendix III). These encouraging results suggest further studies should be conducted to

examine the potential combination of *Piper* extracts with other botanicals such as dillapiol and essential oils.

3.4.1 Conclusions and Recommendations

In terms of a beneficial insect control alternative for organic growers and home gardeners, this study found that lepidoptera and European pine sawfly larvae could be controlled with *P. nigrum* extracts at less than 0.1%. Other important garden vegetable and ornamental pests, such as *L. lilii* and *P. viburni* larvae, and *A. vittatum* adults, were controlled with a concentration range between 0.1 and 0.2%. The activity of our *P. nigrum*-based botanical predicts that contact toxicity is effective when early instar *L. decemlineata* larvae are targeted. Late instar larvae can be knocked down with concentrations above 0.1% and 50% of the adults can be killed with a 0.5% application. There is an apparent repellent or feeding deterrent action at 0.5% as well, however oviposition is not deterred and there is little residual activity under full sunlight conditions. Gardeners would need to scout the field and spot spray plants when the larvae and adults become active.

Since the half-life of piperine and other piperamides is less than one hour under full sunlight, residual activity on plant surfaces is very short. Repellent effects were observed, but due to the short half life of active components, daily applications would be necessary for plants exposed to full sunlight. The formulations prepared and tested indicate that knockdown of insects requires only a short residual effect, but care should be taken to apply them during periods of less intense sunlight. Repeated sprays at concentrations below 0.1% would not harm the plant, but would not be practical for large scale agriculture. Similarly, concentrations of 1% or greater have been found to be phytotoxic under greenhouse conditions; however turf grass was not harmed if 4% *P. nigrum* was well watered after application (Chapter 5). Treatments to protect shaded plants, or when used indoors or in greenhouses, would likely extend the residual activity and thus the antifeedant and oviposition deterrent effect observed.

Non-target and beneficial species such as ladybird beetles (Coleoptera: Coccinellidae) and the stink bug *Perillus bioculatus* Fabricius (Hemiptera: Pentatomidae), both common predators in potato

fields, were not tested under field conditions. The *H. convergens* LC_{50} was 0.2% *P. nigrum* indicating these ladybird beetles may be protected when lower concentrations, 0.05 to 0.1% *P. nigrum* are applied for Colorado beetle larval control, but we will assume that other insect species could be susceptible to the contact action of the *Piper* extracts.

The potential benefit, as demonstrated in this study, for extracts of *P. nigrum* and other *Piper* species is the control of *L. decemlineata* larvae. The *Piper* species extracts offer not only novel activity of piperamides, but also their relative safety for use and storage and the universal availability of the seed and leaf material. In the case of black pepper, it is one of the world's most traded spices and is grown in several equatorial regions of the globe (Simpson and Ogorzaly 1995). Extracts of black pepper contain high proportions of piperine and other active piperamides, which already have been tested and applied for use in several developing regions (Arnason *et al.* 2002). Black pepper is considered a food-grade spice and categorized by the U.S. F.D.A. as "Generally Regarded as Safe" or G.R.A.S. (CITE : 21CFR182.10). In terms of the health risks, piperine is the only piperamide thus far listed on the E.P.A.s Toxic Substances Control Act (T.S.C.A.) inventory. It is a category R22, harmful if swallowed, with precautions S22-25, not to be inhaled as dust, fumes or vapours, as well as to be avoided contact with skin and eyes (Sigma-Aldrich 2002). The relatively low mammalian toxicity (Piyachaturawat *et al.* 1983) of these food-grade spices should preclude many of the necessary tests required for registration of conventional insecticides. Lowered health risks imply safer use of these extracts by applicators, however the "hot taste" associated with pepper suggests that the precautions taken when applying other chemicals should be equally observed with these extracts. The greatest risk is due to irritation of the skin and eyes, therefore applicators should take steps to avoid such exposure when spraying.

CHAPTER 4

PIPERAMIDE MODE OF ACTION IN THE HOUSE FLY AND TOXICOKINETICS IN THE AMERICAN COCKROACH: IMPLICATIONS FOR CONTROL OF THE COLORADO POTATO BEETLE

4.1 Introduction

The search for new solutions to control insect pests in agriculture and urban areas is currently influenced by four issues: 1) the banning of synthetic insecticide use in municipal areas as recently seen in Canada, 2) public perception that natural compounds are better, 3) products that are Generally Regarded As Safe (G.R.A.S.) and 4) the reliance on extracts that contain a mixture of compounds versus pure compounds. Research is again focusing on the plant kingdom for solutions since the interaction between plants and herbivores has led to the evolution of a myriad of secondary compounds that can have toxic, growth reducing and antifeedant properties against insects (Berenbaum and Zangerl 1996). The diversity of insect defences is examplified by the family Piperaceae, which includes the common spice black pepper, *Piper nigrum* L., as well as approximately 1000 other tropical species. Research on the Piperaceae over the past two decades has revealed that *Piper* species contain greater than 200 secondary compounds (Arnason *et al.* 2002).

In a previous work, 16 neotropical *Piper* species were surveyed for insecticidal activity (Mackinnon *et al.* 1997). The most active species were: 1) *Piper decurrens*, found to contain several co-occurring lignans including conocarpan, eupomatenoid-5 and -6, and decurrenal; 2) *Piper guanacastense*, with several larvicidal prenylated benzoic acid derivatives, such as methyl 3(3-methyl-2-butenyl)-4-hydroxybenzoate; and 3) *Piper aduncum*, found to have large amounts of the phenylpropanoid dillapiol, a potent polysubstrate monoxygenase (PSMO) inhibitor. However, the highest insecticidal activity observed was from *Piper tuberculatum* extracts, from which dihydropiperlonguminine (Fig. 4.1A), an isobutyl amide or piperamide, was identified (Mackinnon *et al.* 1997).

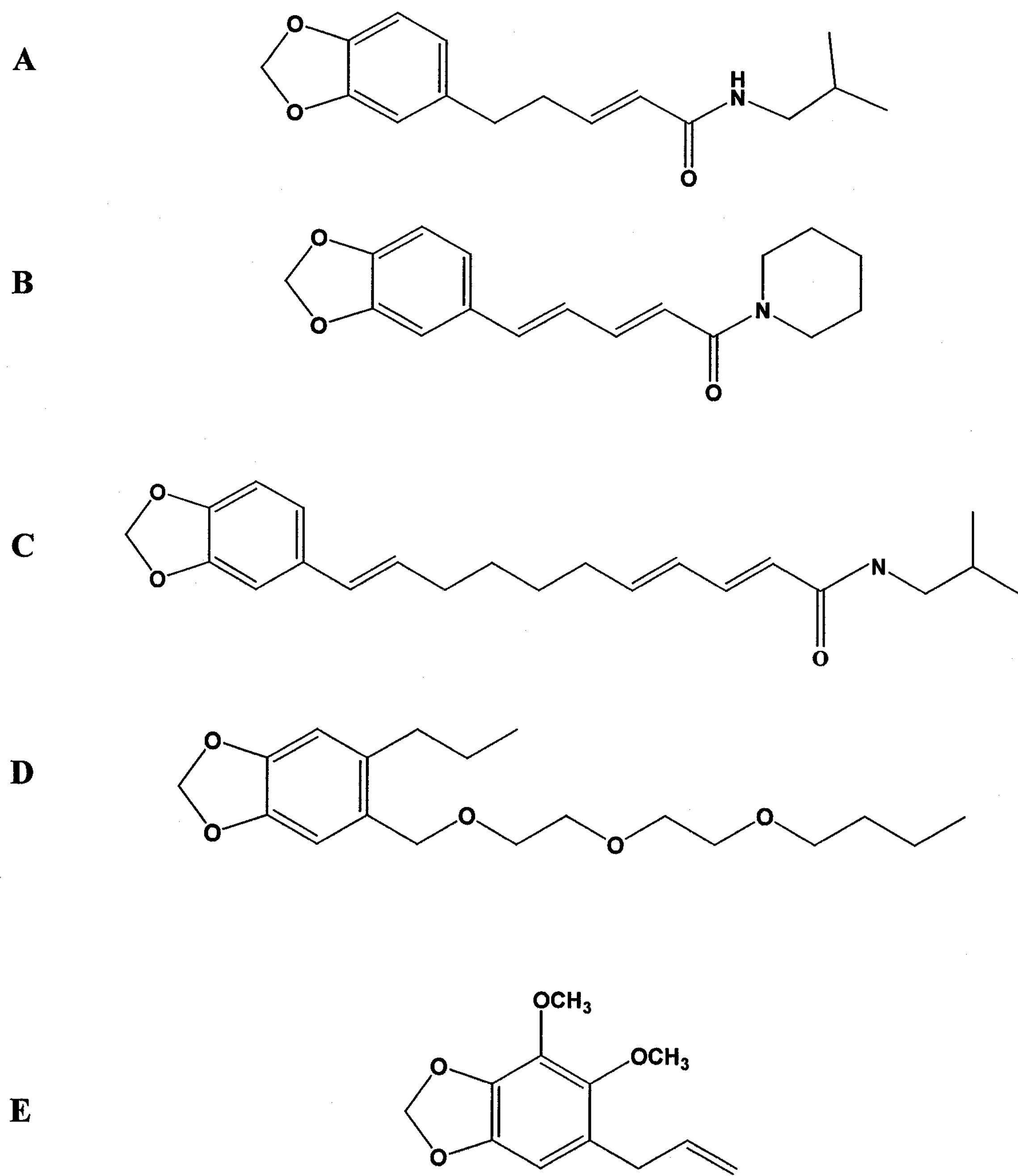


Figure 4. 1. Structure of 4,5-dihydropiperlonguminine (A), piperine (B), pipercide (C), piperonyl butoxide (D) and dillapiol (E).

The mixture of similar amides found in most *Piper* species, including the West African *P. guineense*, suggests these plants have also employed a defence strategy termed analogue synergism (McKey 1979 in Berenbaum and Zangerl 1996) whereby many similar compounds will increase the toxicity to herbivores, and render it difficult for a herbivore to adapt and become resistant (Feng and Isman 1995). In a previous study (Scott *et al.* 2002), we demonstrated that combinations of piperamides in binary, tertiary and quarternary mixtures had successively higher toxicity at equimolar concentrations. This combination of useful traits suggests that *Piper* extracts may be good candidates for use in crop protection.

The piperamides commonly found in the genus *Piper* are unique since they are bifunctional: an isobutyl amide functionality is combined with a methylenedioxyphenyl (MDP) moeity as seen in piperine (Fig. 4.1B) found in *P. nigrum* L. fruit. The most active piperamide discovered to date is pipercide (Fig. 4.1C), approximately 100-fold more active than piperine (Miyakado *et al.* 1979, 1980; Dev and Koul 1997). The piperamides are also unusual because of their dual biological activities: the amide functionality is neurotoxic and the MDP group is an inhibitor of cytochrome P450 enzymes. PSMO inhibition is demonstrated by similar MDP-containing molecules: the insecticide synergist piperonyl butoxide (PBO) (Farnham 1998) and dillapiol (Fig. 4.1D and E respectively) found in Indian dill *Anethum sowa* and *Piper aduncum* (Arnason *et al.* 2002).

There are specific cytochrome monooxygenases responsible for detoxifying different classes of insecticides (Scott 1996a). Among these, CYP6D1 is the most important isoform in the housefly, attributed with the monoxygenase-mediated pyrethroid resistance in specific strains. Known inhibitors of CYP6D1 include the plant-derived furanocoumarins, xanthotoxin, 5-methoxypsoralen and piperonyl butoxide (Scott *et al.* 1998).

In the current work we focus on piperine, the main alkaloid found in black pepper, but also found in other *Piper* spp. (Parmar *et al.* 1998; Stöhr *et al.* 2001; Scott *et al.* 2002). To assess the effect of piperine on insect monooxygense activity, we isolated microsomes from the house fly *Musca domestica* L., and used them in a recognized *in vitro* spectrofluorometric assay for measuring enzyme inhibition (Lee

and Scott 1989). Secondly, to determine the fate of piperamides in the insect we conducted a toxicokinetic study with a tritium-labelled piperine molecule. The American cockroach *Periplaneta americana* L. was chosen as a model insect due to its size, relative ease for dissection of tissues and past use in detoxification enzyme (Valles *et al.* 1999) and toxicokinetic assays (Teal *et al.* 1999). As well, based on the findings in other studies, a comparison can be made with toxicokinetics in insects of other plant compounds, for example α-tertienyl (Iyengar *et al.* 1987) and DIMBOA (Campos *et al.* 1989); or environmental contaminants, such as PCBs (Saghir *et al.* 1993 and 1994; Saghir and Hansen 1999) and benzo-(a)-pyrene (He *et al.* 1998). The pharmacokinetics of piperine has been studied in the rat (Bajad *et al.* 2002) mainly due to the interest in peppers myriad uses in culinary and traditional medicine; however nothing is known of its fate in insects. Of particular interest were potential metabolites produced by the insect, providing an idea of the type of enzyme activity occurring in the insect and one that might potentially lead to piperamide resistance after exposure for several generations or to allow insect pests to specialize on cultivated (Santhosh-babu 1994) or native pepper plants (Marquis 1991) in the tropics.

One insect pest that constantly frustrates farmers is the Colorado potato beetle *Leptinotarsa decemlineata* (Say). A North American native that has been spread to many parts of the world, this species has had a long history of development of resistance to pesticides (Georghiou 1990). *Leptinotarsa decemlineata*, like several other insect pests of economic importance, shows multiple-resistance to several insecticide classes, including carbamates, organophophates, organochlorines and pyrethoids and has led to increased efforts to develop new control strategies. As a target insect, the Colorado potato beetle provides us with the opportunity to test the hypothesis that *Piper* extracts can be used to control an insecticide-resistant pest due to the novel structure and activity of the piperamide molecule and its dual functionality.

4.2 Materials and Methods

4.2.1 *Piper* extracts and piperamide analysis

Piper nigrum peppercorns from Kerala, India, were obtained from Country Bulk Inc., a commercial spice supplier in London, Ontario. *P. tuberculatum* leaves were collected along the west coast of Costa Rica in May 2001. Leaves and peppercorns were ground and the active constituents extracted following the methods described in Scott *et al.* (2002). The extracts were formulated as follows: 20% extract, 70% tetrahydrofurfuryl alcohol (THFA, Penn Specialty Chemicals, Memphis TN) and 10% emulsifier (Alkamuls EL-719 ethoxylated castor oil, Rhodia, Cranbury NJ). Piperamide concentrations in the extracts and formulations were determined according to the methods of Scott *et al.* (2002).

4.2.2 Insects

Susceptible housefly strains and strains resistant to pyrethroids were obtained as pupae, courtesy of Benzon Research Inc., Carlisle PA, and Dr. J.G. Scott, Entomology Department, Cornell University, Ithaca NY respectively. Flies were kept at room temperature, 16:8 L:D regime and supplied with sugar water *ad libitum*. Adult and nymph *Periplaneta americana* L. were obtained from Ward's ™ Natural Science Ltd., St. Catherines Ont. They were kept in a darkened glass aquarium at room temperature and provided with crushed dog biscuits, water and apple slices *ad libitum*. Experiments were conducted after insects had acclimated at least four to five days after shipment. Insects were anaesthetized with CO_2 or ice, and kept anaesthetized on ice until dosed with *P. nigrum* extract or piperine (maximum 1 h). *Leptinotarsa decemlineata* eggs, chlorinated hydrocarbon, organophosphate and pyrethroid resistant (R strain) and susceptible (S strain), were supplied by the Southern Crop Protection and Food Research Centre (SCPFRC), Agriculture and Agri-Food Canada, London ON. Egg masses containing 25 to 30 eggs (S strain) and 10-15 eggs (R strain) were couriered to the University of Ottawa, where they were placed on the leaves of four-week old greenhouse-grown potato plants (one to two egg masses per plant), variety Superior Gold and Russett Burbank.

4.2.3 House fly PSMO study

To test whether piperamides affect PSMO enzymes directly, an established protocol for measuring microsomal activity in insects was adopted. The insect used was the housefly *Musca domestica,* rather than *L. decemlineata*, since an accepted method existed. Microsomes from the housefly were used to test whether microsomal enzymes treated with piperine would show reduced activity or inhibition using the methoxyresorufin O-demethylation (MROD) *in vitro* technique (Scott *et al.* 1998). Pyrethroid-resistant houseflies (LPR strain) (Scott and Georghiou 1985) were used since this strain had increased levels of microsomal activity (Wheelock and Scott 1992). The adults emerged from pupae over two days at room temperature and 16:8 L:D regime and fed sugar water *ad libitum*. Flies were then fed a 15% ethanol/15% sugar water solution for 72 hours. Preparation of microsomes from houseflies followed the techniques of Lee and Scott (1989) allowing for storage of microsomes for at least two months at -80 °C and repeated use in microsome assays. The MROD assay has also been described by Lee and Scott (1989). Prepared microsomes were combined with phosphate buffer in microplate wells along with methoxyresorufin substrate and challenged with doses of piperine (0, 0.125, 0.25, 0.5, 1, 2, 5 and 10 mM) dissolved in dimethyl sulfoxide (DMSO). A standard curve of 0, 0.5, 1, 2 and 4 µM resorufin was run on each microplate. Piperonyl butoxide and dillapiol at 4.3×10^{-4} mM were used as standards in the assay. The fluorescence of standards and samples was analysed with a SpectraMax Gemini XS microplate fluorometer, and SoftMax Pro Version 3.1 software (Molecular Devices Inc., Sunnyvale CA).

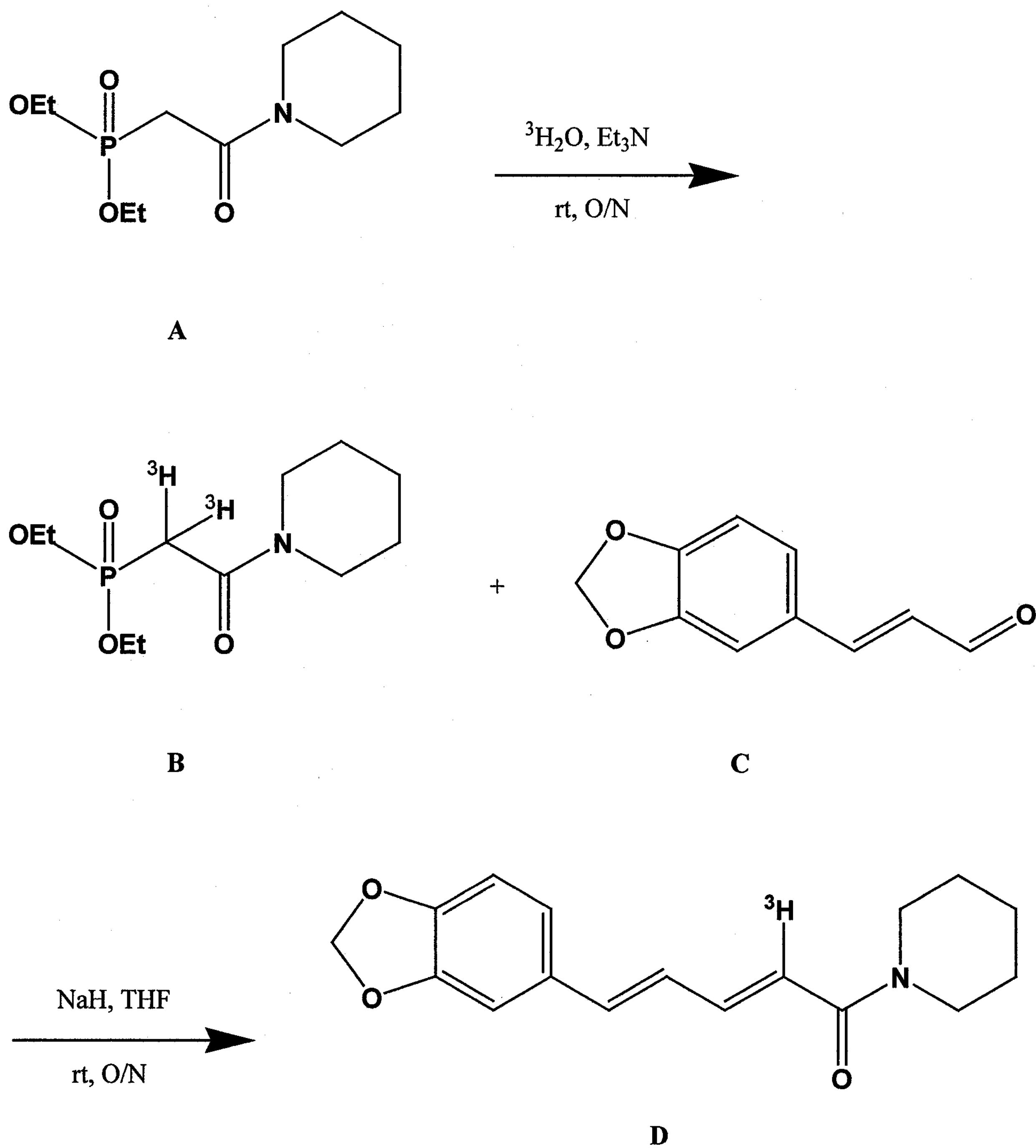


Figure 4. 2. Illustration of the steps of synthesis of ^{3}H-piperine: piperonyl phosphonate (A); radiolabelled piperonyl phosphonate (B), 3-Benzo [1,3] dioxol-5-yl-propenal (C); radiolabelled piperine (D).

4.2.4 Insecticide-resistant vs. susceptible house flies: comparison of *Piper* botanical toxicity

Pyrethroid-resistant (LPR) and insecticide-susceptible housefly strains were tested using *P. tuberculatum* extract formulated at 10%. Flies were anaesthetized with carbon dioxide and kept on ice until treated. A concentration range of 0.1, 0.2, 0.4, 0.8 and 1% *P. tuberculatum* diluted in distilled water was prepared, along with a formulation blank control. Thirty flies of each type were individually dipped into each 10-mL solution, patted dry on a Whatman No. 1 filter paper and then placed into a metal cage to recover. Flies were supplied with sugar water *ad libitum*. Mortality was observed after one and 24 hours by counting the number of flies unable to stand or fly in each cage. Each trial was repeated at least three times for both housefly strains.

4.2.5 Toxicokinetic trials

4.2.5.1 Radiolabelled piperine synthesis

Preparation of tritium-labelled piperine by Dr. T. Durst and E. Puniani, Chemistry Department, University of Ottawa, followed a two-step process (Fig. 4.2). First, a solution of phosphoric acid–(2-oxo-piperidin–1-yl-ethyl diester (1.29 g, 4.91 mmol) (A) in triethyl amine (3.4 mL) was stirred at room temperature (20 °C) for 1 hour before adding tritiated water (0.5 mL) and stirring continually overnight. Ethyl acetate (30 mL) was added to the reaction mixture before it was washed successively with water (10 mL), 10% HCl (3 x 10 mL), 5% Na_2CO_3, water (10 mL), then dried ($MgSO_4$) and concentrated *in vacuo* to yield the radiolabelled ^3H-phosphonic acid-(2-oxo-piperidin-1-yl-ethyl)-diethyl ester (620 mg, 2.31 mmol) (B). Secondly, THF (20 mL) was added to compound B along with excess NaH and then stirred for 15 minutes at room temperature, followed by the addition of 3-Benzo [1,3] dioxol-5-yl-propenal (540 mg, 3.07 mmol) (C) previously dissolved in THF (10 mL), which was then stirred continuously overnight. Lastly, the reaction mixture was quenched with water (30 mL), the THF removed *in vacuo* and the reaction mixture re-dissolved in CH_2Cl_2 (50 mL).

The aqueous layer was extracted with CH_2Cl_2 (3 x 50 mL) and the combined organic layers were dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford crude "piperine" (D) (680 mg). After silica gel

column chromatography purification, a 62%-pure radioactive ^{3}H-piperine (411 mg, 1.0 mCi total activity) was obtained. Purity of the new compound was verified by running a TLC plate with both pure piperine and ^{3}H-piperine in 50:50 ethyl acetate:hexane. A UV absorbent spot was visible 1.5 cm above the origin comparable to the piperine. When 0.5-cm squares were scraped and the activity measured by scintillation counter, the majority of activity (88%) was associated with the 1.5-cm area.

4.2.5.2 American cockroach toxicity trial

A stock concentration of *P. nigrum* extract dissolved in acetone (250 mg / mL) was prepared. Mixed (female and male) adult *P. americana* were dosed with a Hamilton syringe on the abdominal sternites using 10 μL / g body weight. A range-finding test determined that 24-h acute toxicity occurred between 1 and 10 mg *P. nigrum* extract / g body weight. Five concentrations of *P. nigrum*: (0, 0.625, 1.25, 2.5 and 5 mg/g) were then applied to five replicate *P. americana* adults. Based on the mortality from this trial, the 24-h LD_{50} was determined by probit analysis.

4.2.5.3 Experimental conditions

After topical treatment with *P. nigrum* extract, and/or ^{3}H-piperine, adult *P. americana* females were placed individually in 100-mm diameter plastic Petri dishes containing a 90-mm Whatman No. 42 ashless filter paper. The cockroaches were placed in an incubator, Conviron ™, Model E7 plant growth chamber (Controlled Environments Ltd., Winnipeg, MB) and held for pre-determined times at 27 °C, 70% RH and shaded from the fluorescent lighting (16:8 L:D) by a black plastic covering. Preliminary trials determined that depuration after 24 h was no different if the insects were supplied with or without water; therefore, during the trials the cockroaches were not fed or given water. *Perplaneta americana* toxicokinetic trials including topical treatments and sampling were carried out by R. Leduc, Biology Department, University of Ottawa

4.2.5.4 Experiment 1: ^{3}H-piperine distribution analysis

Cockroaches were weighed and then dosed from a stock solution of 25 mg *P. nigrum* (approximately 40% piperine by weight) / mL acetone and ^{3}H-piperine. Each insect was given a sublethal dose: 10% of the LD_{50} dose or 10 μL of stock combining 250 μg *P. nigrum* (100 μg piperine + other piperamides) with 6.1 μg ^{3}H-piperine, the equivalent of 24,000 dpm activity per g body weight. The dose was applied to the abdominal sternites and the cockroach was placed dorsal side down in a Petri dish to recover. Each group of four to six cockroaches was selected for a sampling period of 1, 2, 4, 6, 12, 24, 48 or 96 hours.

4.2.5.5 Sampling of American cockroach adults

Whole body extraction (separation of cuticle from muscle and other soft tissues) and feces analysis of ^{3}H piperine equivalents (both parent and ^{3}H-piperine metabolites) at each sampling period was conducted as follows: 1) *P. americana* adult females were removed from the incubator and then chilled for five to ten minutes at -20 °C to anaesthetize them; 2) after removal from the Petri dish, each individual was dissected on a new 90 mm Whatman No. 42 filter paper to collect hemolymph that escaped from the dissected body; 3) the soft tissue was separated from the exoskeleton and underlying epidermal layers with forceps and dissecting scissors and then both tissue types were individually dissolved in 10 mL of Solvable™ tissue solubilizer (Packard Bioscience, Meriden CT) overnight and 4) the two ashless filter papers / insect (collection of depurated and hemolymph samples) and 1-mL samples from each solubilized tissue solution were added to separate plastic scintillation vials containing 5-mL of Hionic- Fluor (Packard Bioscience). The amount of ^{3}H piperine equivalents in the two tissue types and the depurated and hemolymph materials was measured by a Beckman Coulter LS6500 scintillation counter (Beckman Coulter Ltd., Fullerton CA), Biology Department, University of Ottawa.

4.2.5.6 Experiment 2: ^{3}H-piperine depuration analysis

A stock solution of 0.52 mg ^{3}H-piperine / mL acetone was used to dose five cockroaches on the abdominal sternites with 10 µL / g b.w. or approximately 5.2 µg ^{3}H-piperine / insect. Each cockroach was kept in the previously described Petri dish, however the ashless filter paper was replaced every two hours for a period of 12 hours. The ashless filter paper discs removed from all five insects were combined in a 500-mL glass Mason™ jar with 25 mL of ethyl acetate and 25 mL of distilled water. The filter papers were shaken overnight and then the water fraction was removed from the ethyl acetate fraction by a separatory funnel. A 1-mL sample was collected from each of the two hour samples, both ethyl acetate and water phases and added to individual plastic scintillation vials containing 5 mL Hionic-Flour and the ^{3}H equivalents were measured as previously described.

4.2.5.7 Experiment 3: Isolation and identification of metabolites of piperine in feces

A sublethal topical application of 100 µg piperine / insect (10 µL from a stock of 10 mg piperine / mL acetone) was given to 20 cockroaches on the abdominal sternites. A second group of 20 was given a 10-µL dose of acetone only. Each cockroach was placed in an individual Petri dish and kept in an incubator under the previously described conditions. Over a 12-h elimination period, total feces was collected every two hours by the removal of the ashless filter paper and replacement with a new one. The filter papers from each of the two treatments were combined in separate 1-litre Mason™ jars with 200 mL of ethyl acetate and 200 mL of distilled water. At the end of the 12-h period, the contents of the jars were shaken overnight, and then the resultant slurry was filtered through a Buchner filter with Whatman No. 1 filter paper. The filter paper used to collect feces was then washed with 100 mL of both ethyl acetate and water. The combined ethyl acetate and water fractions were then separated by a separatory funnel.

The ethyl acetate phase was then evaporated under N_2 gas while replacing half the volume with 99% ethanol successively until a 10-mL volume remained, 6.25% ethyl acetate, for both the acetone and piperine treated insects. HPLC-MS was used to determine whether the ethyl acetate fraction contained parent compounds and/or metabolites from the piperine treatment compared to the acetone treatment.

HPLC-MS analysis was conducted using an Agilent Technologies 110 Series LCMS. Separation was achieved using a Waters YMC™ ODS-AM reverse phase column (53 μm, 120 Å, 2.0 x 100 mm) following the methods described in Chapter 2. The MS was set on Scan-mode with positive polarity and the following parameter settings: mass range = 100-370 ms; fragmentor = 100; gain = 1.0; threshold = 150; step size = 0.1. Extraction chromatographs were obtained for a pseudomolecular ion (M+1) for piperic acid (MW=219.1), the main piperine metabolite determined by Bajad *et al.* (2002).

4.2.5.8 Statistical analyses of toxicokinetic parameters

A rate-constant model was used to estimate the elimination rate of ^{3}H-piperine equivalents from the exoskeleton, soft tissue and hemolymph of the cockroach. Linear regression and corresponding rate-constants were calculated with SYSTAT (1999). The values for area under the curve (AUC) were generated by trapezoidal estimation (Newman and Unger 2000).

4.2.6 American cockroach PSMO study

To assess the effect of piperamides on detoxification enzymes and insect enzymes in specific, several techniques were employed to measure PSMO activity. A measure of the effect of piperamides on the main human drug metabolizing enzyme, CYP3A4, was made using an *in vitro* fluorometric microtiter plate assay as described by Budzinski *et al.* (2000). The four piperamides tested were 4,5-dihydropiperlonguminine, piperlonguminine, 4,5-dihydropiperine and piperine in a range producing between 0 and 100 % inhibition. The median inhibition concentration (IC_{50}) for the four compounds was determined by probit analysis.The CYP3A4 assay was conducted by J. Budzinski, Biology Department, University of Ottawa. In a second experiment, microsomes from twenty *P. americana* females treated with 10 μL of acetone and twenty treated with 10 μL of 10 mg/mL piperine for 12 h were extracted in order to test whether *in vitro* treatment with piperine reduced activity (inhibition) or increased (induced) activity. Preparation of microsomes from *P. americana* follow the techniques of Lee and Scott (1989), Valles and Yu (1996a) and Valles *et al.* (1999). Soft tissue from the abdomen of all cockroaches was processed,

except the mid gut because of possible contamination from gut contents that was shown to inhibit cytochrome P450 monoxygenases (Valles and Yu 1996b). Cytochrome P450 and b5 levels were measured according to methods adapted from Scott (1996b).

4.2.7 Colorado potato beetle study

4.2.7.1 Laboratory trials

Leptinotarsa decemlineata eggs hatched within two to three days on potato plants. Four days after hatching larvae from both the susceptible- (S) and resistant-strains (R) were collected for use in acute toxicity bioassays. The R strain *L. decemlineata* tolerance ratios for the conventional insecticides, cypermethrin, azinphosmethyl and endosulfan were documented as 11 to 22, 18 and 80, respectively (Agriculture and Agri-food Canada unpublished data). The bioassays to determine the previous tolerance ratios were conducted by the SCPFRC laboratory researchers with adult *L. decemlineata* collected from the field in 2000, and tested in the F1 to F3 generation of the strain. The 24-h toxicity trials were conducted as described previously by Hilton *et al.* (1998). S strain adults were sprayed using a Potter spray tower with a series of five concentrations for each insecticide, the range selected to kill between 0 and 100%. The insecticides (> 95 % purity technical grade) were diluted in acetone and olive oil (19:1) and 5 mL was sprayed per concentration level. Twenty adults were treated per concentration with three replicates per level. Based upon LC_{95} value determined for the S strain, the tests were then repeated with the R strain. The established LC_{50} for each insecticide with the R strain was then compared to that of the S strain to determine the tolerance ratio.

Two dose ranges with a stock solution of 10% *P. tuberculatum* formulated extract were prepared: 0.02, 0.04, 0.06, 0.08 and 0.1 μg/mL for the S strain and 0.04, 0.08, 0.12, 0.16 and 0.2 μg/mL for the R strain. The control consisted of a formulation blank equal to the highest *P. tuberculatum* concentration. Acute toxicity trials were conducted by H. Jensen, Biology Department, University of Ottawa. Individual potato leaves were dipped into the solutions then dried on the bench top for 30 minutes. Larvae were then dipped into the solutions and placed on the leaves with the same treatment inside a glass Petri plate. Ten

larvae were used per three leaves, and each treatment was replicated at least three times. Petri plates were placed in an incubator at 27 °C, 70 RH and 16:8 L:D. Mortality after 24 h was determined by probing the larvae with tweezers to elicit a response.

4.2.7.2 Greenhouse trials

Single *L. decemlineata* egg masses with 25 to 30 eggs/S-strain and 10 to 15 eggs/R-strain were pinned to leaves on four to six-week old potato plants (variety Yukon Gold). Plants were sprayed with a one-litre hand pump sprayer at concentrations of 0.01 and 0.05% *P. nigrum*. The control consisted of a 0.2% EC blank equivalent to the formulation content in the 0.05% *P. nigrum* application. Each plant was sprayed with 100 mL of solution and three replicate plants were used for each treatment. Each plant was placed inside a wooden cage covered by a fine mesh so that each cage contained one replicate of each treatment. Trays of water in the bottom of each cage kept the plants hydrated during the exposure period. Plants were sampled after eight days when number of surviving larvae, developmental stage of larvae and number of damaged leaves were assessed.

4.3 Results

4.3.1 Piperamide analysis

HPLC analyses determined that piperine concentration in *P. nigrum* stock solutions ranged from 37.0 to 42.7% by weight. *P. tuberculatum* 4,5-dihydropiperlonguminine concentration ranged from 4.1 to 4.7 % by weight in the different stock preparations (Table 4.1).

4.3.2 *In vitro* and *in vivo* PSMO inhibition studies

The PSMO inhibitory activity of piperine was demonstrated to be almost as effective as piperonyl butoxide (PBO) against multi-resistant house flies. The average IC_{50} for two MROD enzyme assays was determined at 1.2 µM piperine (1 mM dose) (Fig. 4.3) compared to less than 0.43 µM PBO, the internal standard. Dillapiol at 0.43 µM did not affect MROD activity with the LPR housefly microsomes (data not

shown). Among the four piperamides tested, the most effective CYP3A4 inhibitor was dihydropiperine, as indicated by the lowest median inhibitory concentration (Table 4.2). Although significantly less than piperine and the other two piperamides tested, all were found effective within a 0.05 mM range of each other. The cytochrome P450 activity after 12 h in cockroaches treated with 100 μg of piperine in 10 μL of acetone was not detectable compared to those treated with a 10-μL acetone dose but was not significantly different from the control (Two tailed T-test, P=0.413) (Fig. 4.4). However, the cytochrome b5 level in the control cockroaches was significantly higher than in those treated with piperine (Two tailed T-test, P=0.05).

4.3.3 Uptake, elimination and depuration of piperine from the American cockroach

The *P. nigrum* 24-h LD_{50} for adult *P. americana* was 2.5 mg/g (95% C.I. = 1, 80). As a consequence, a sublethal dose of 10% of the LD_{50} was used for the toxicokinetic study. At this selected dose, no mortality was observed during the incubation period. Recovery of ^{3}H-piperine from the filter paper and Solvable™ tissue solubilizer was > 95%.

After one-hour post-dosing, the mean amount of ^{3}H equivalents in the cockroach cuticle was approximately 80% of the total dose (Fig. 4.5A). Between two and six hours post dosing, 40 to 50% of the applied radiation was eliminated from the cuticle, followed by a slower elimination period up to 24 h. The linear relationship determined for the decrease in log ^{3}H equivalents between one and 24 hrs (Fig. 4.5B) provided an elimination rate constant of 0.04 h^{-1}, a biological half-life of 16.5 h and a mean residence time of 23.7 h (Table 4.3).

Table 4. 1. Range of concentrations (mg/mL) for piperamides determined by HPLC analysis of *P. nigrum* and *P. tuberculatum* extracts.

Extract	DHPLG	PLG	DHP	Piperine
P. nigrum	1 – 2.2	3.6 – 5.5	35.2 – 41.7	370 - 427
P. tuberculatum	41.5 – 46.6	1.6 – 2.9	5.8 – 11.3	4.9 – 9.1

DHPLG = 4,5-dihydropiperlonguminine; PLG = piperlonguminine; DHP = 4,5-dihydropiperlonguminine.

Table 4. 2. Median inhibitory concentration (IC_{50}) values for four piperamides against cytochrome P450 3A4 (CYP3A4).

	DHPLG	PLG	DHP	Piperine
IC_{50} Concentration (mM)	0.158	0.159	0.109	0.122
95% C.I. (mM)	0.153, 0.163	0.147, 0.180	0.108, 0.110	0.119, 0.127

DHPLG = 4,5-dihydropiperlonguminine; PLG = piperlonguminine; DHP = 4,5-dihydropiperine.

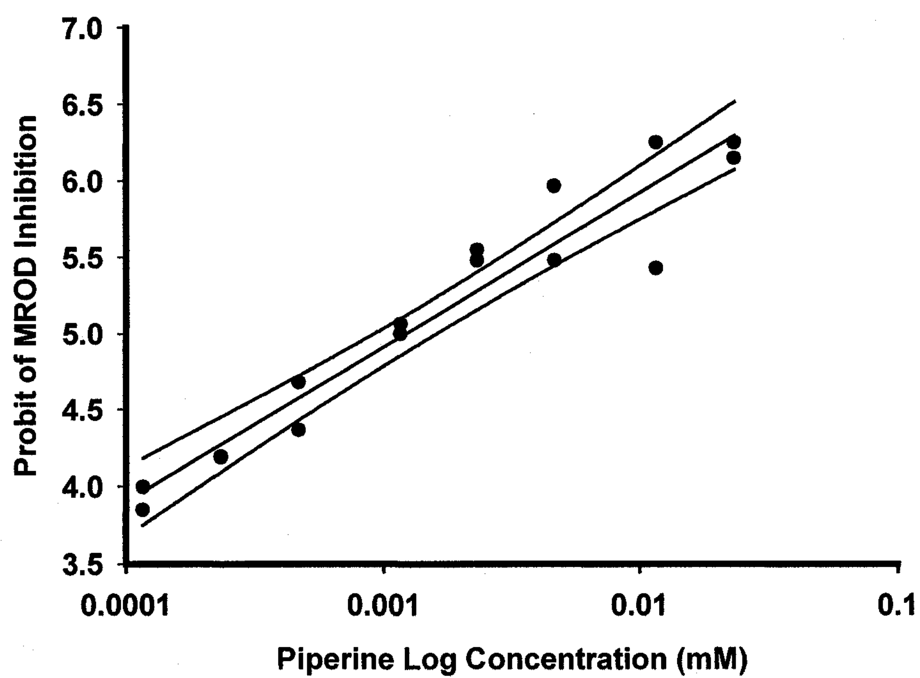


Figure 4. 3. The inhibitory effect of piperine on methoxyresorufin O-demethylation (MROD) activity as measured *in vitro* with insecticide-resistant (LPR) housefly microsomes.

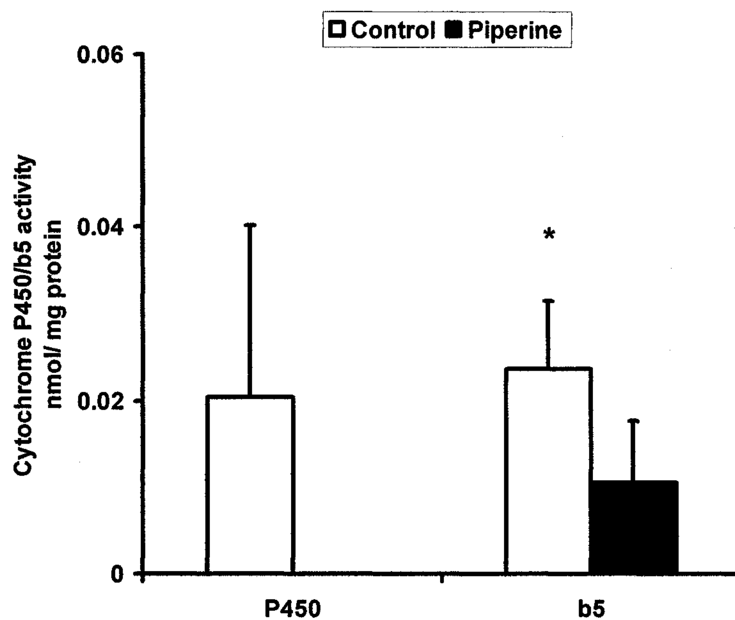


Figure 4. 4. Cytochrome P450 and b5 (nmol/mg protein) for acetone-(control) and piperine-treated female American cockroach adults (n = 40), 12 h post-dosing. Asterix above control treatment bar indicates significant difference between acetone- and piperine treatments (2-tailed T-test; P=0.05).

Table 4. 3. Elimination rates and toxicokinetic parameters for ^{3}H-piperine equivalents in the exoskeleton, hemolymph and soft tissues of female *P. americana* dosed with 6.1 μg ^{3}H-piperine/insect.

Parameter	Exoskeleton	Hemolymph	Soft Tissue
C_{max}[a]	4.92	0.2	1.24
T_{max}[b]	1	2	48
R^2[c]	0.78	0.76	
K[d]	-0.04	-0.06	
$t_{1/2}$[e]	16.5	11.9	
MRT[f]	23.7	17.1	
$AUC_{0\text{-}96}$[g]		6.1	94
AUC_{inf}[h]		0	34.5

[a] Maximum concentration (μg ^{3}H-piperine/insect), [b] time C_{max} measured, [c] regression coefficient, [d]elimination rate constant, [e]biological half-life, [f]mean residence time, [g]area under the curve for 0 - 96 h, [h]area under the curve to infinity.

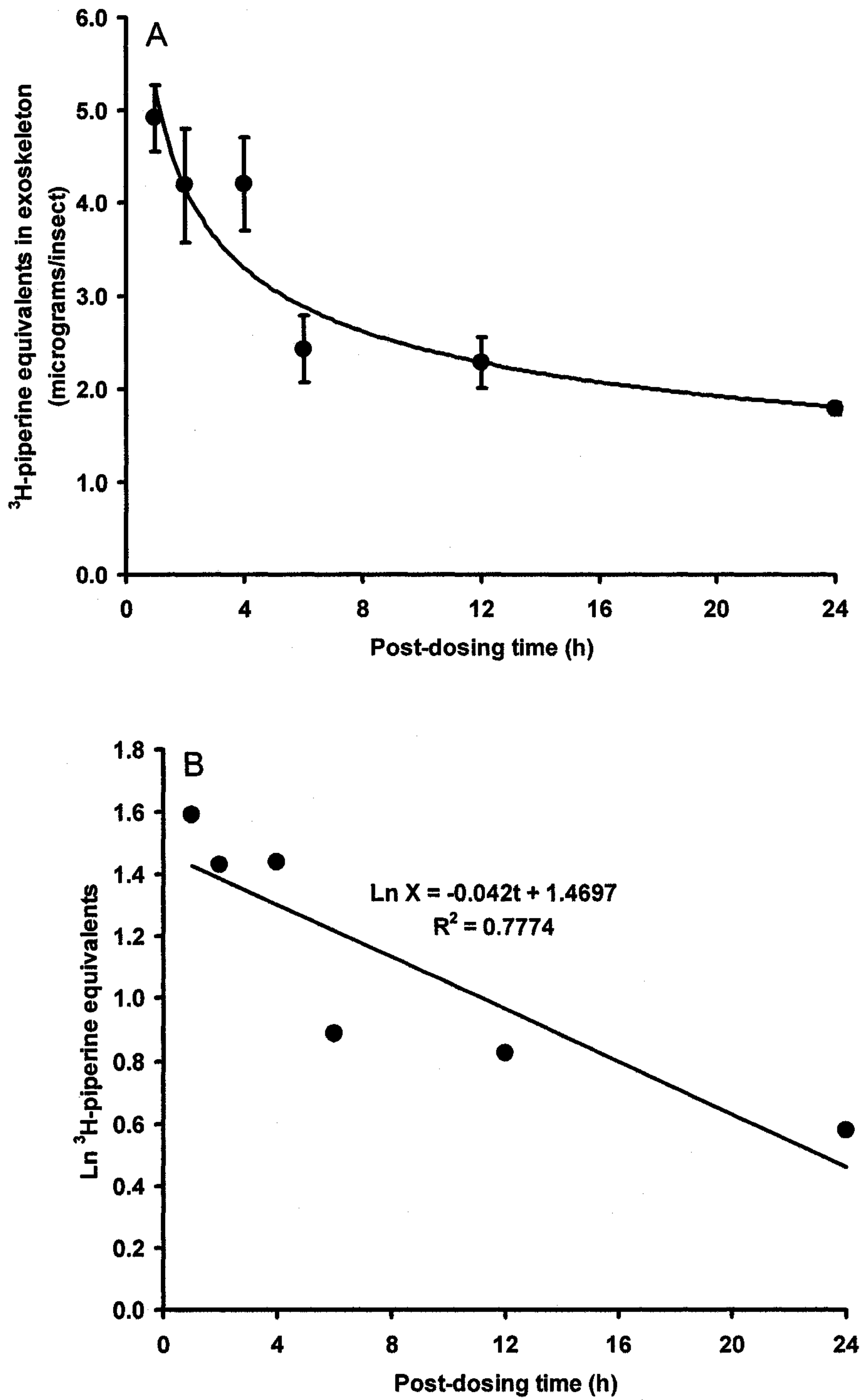


Figure 4. 5. Elimination of ^{3}H-piperine equivalents from exoskeleton of adult female *P. americana* 1, 2, 4, 6, 12 and 24 h post-dosing with 6.1 µg ^{3}H-piperine and 250 µg *P. nigrum* extract (A). The linear regression of the natural logarithm of the mean amount of ^{3}H-piperine measured in the exoskeleton versus the post-dosing time over a period of 1 to 24 h (B).

Measurement of hemolymph levels of tritiated material found that the majority of uptake occurred before one hour but peaked at two hours (T_{max} = 2 h, C_{max} = 0.2 μg ^{3}H-piperine equivalents) followed by elimination up to 96 h (Fig. 4.6). The linear relationship determined for the decrease in hemolymph ^{3}H equivalents between 2 and 24 h ($\ln X = -0.0584t - 1.5313$, $R^2 = 0.7638$) provided an elimination rate constant of 0.06 h^{-1}, a biological half-life of 11.9 h and a mean residence time of 17.1 h (Table 4.3). When the mean ^{3}H-piperine equivalents in soft tissue of the roaches was measured up to 96 h, the peak was found to be at 48 h (C_{max} = 1.24 μg ^{3}H-piperine equivalents) (Table 4.3) accounting for approximately 20% of the total dose accumulated before elimination began (Fig. 4.7).

The depuration of piperine in the cockroach *P. americana* indicates that at sublethal levels, the compounds, including metabolites, are only partially eliminated from the cuticle by 96 h post-dosing. Depuration from the female American cockroaches was relatively low in the first 24 h (0.05 to 0.3 μg ^{3}H-piperine equivalents / insect) with < 5% on average of the total dose excreted (Fig. 4.8). After 24 h the depuration rate increased and by 96 h approximately 10% of the initial dose was excreted.

The accumulated ^{3}H-piperine equivalents depurated from those cockroaches dosed with 136 μg ^{3}H-piperine / insect over a 12 h period was found to be approximately 12% of the total applied dose (Fig. 4.9). The majority of the material was soluble in ethyl acetate while a small but increasing percentage was water-soluble, indicating that few polar metabolites associated with the tritium molecule are being excreted during this period. In contrast, the amount of piperine depurated 12 h after the cockroaches were given a 100 μg dose of cold piperine was 33.7% (data not shown).

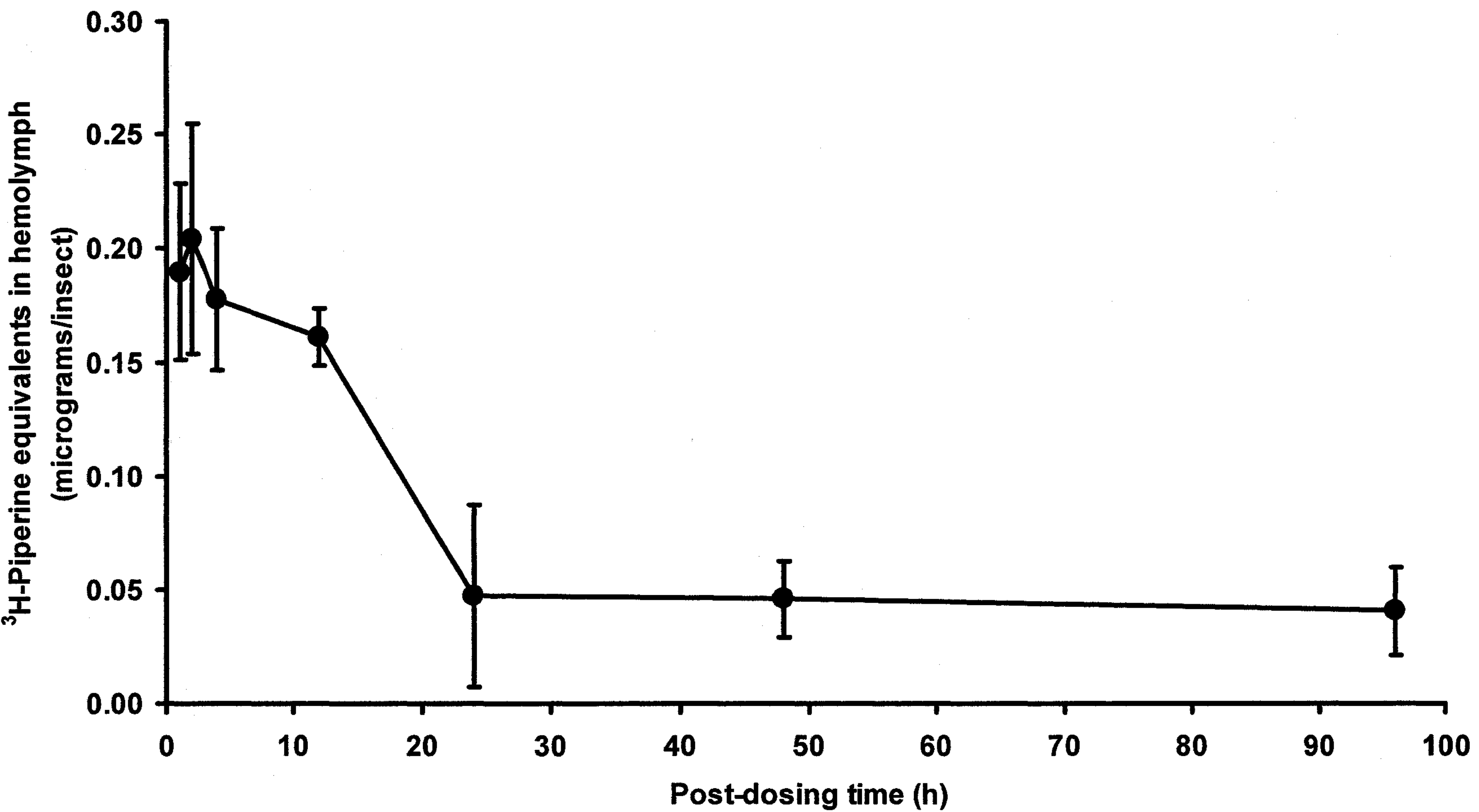


Figure 4. 6. Uptake and elimination of ^{3}H-piperine equivalents from the hemolymph in adult female *P. americana* 1, 2, 4, 12, 24, 48 and 96 h post-dosing with 6.1 µg ^{3}H-piperine and 250 µg *P. nigrum*.

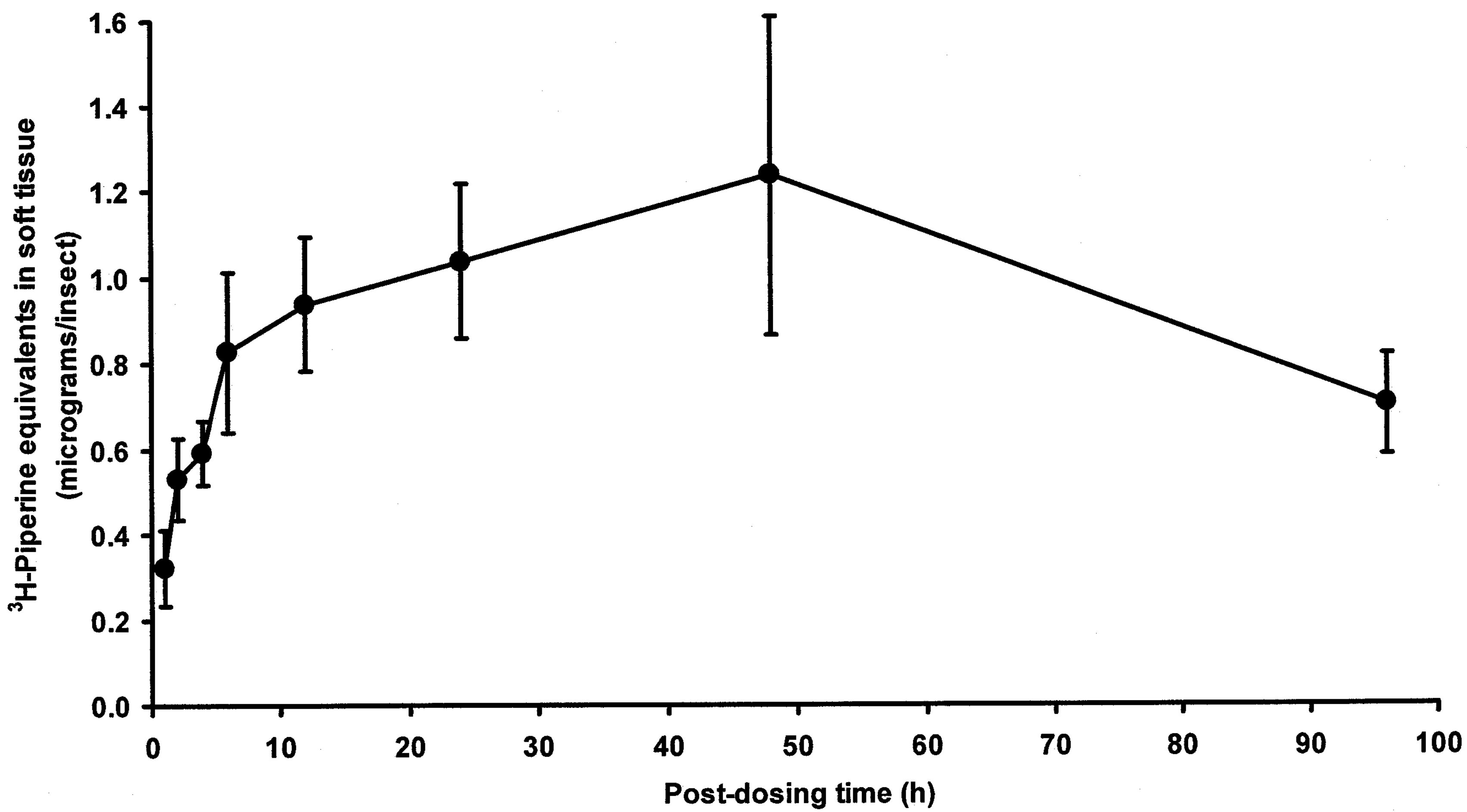


Figure 4. 7. Uptake and elimination of ^{3}H-piperine equivalents from soft tissue of adult female *P. americana* 1, 2, 4, 6, 12, 24, 48 and 96 h post-dosing with 6.1 µg ^{3}H-piperine and 250 µg *P. nigrum*.

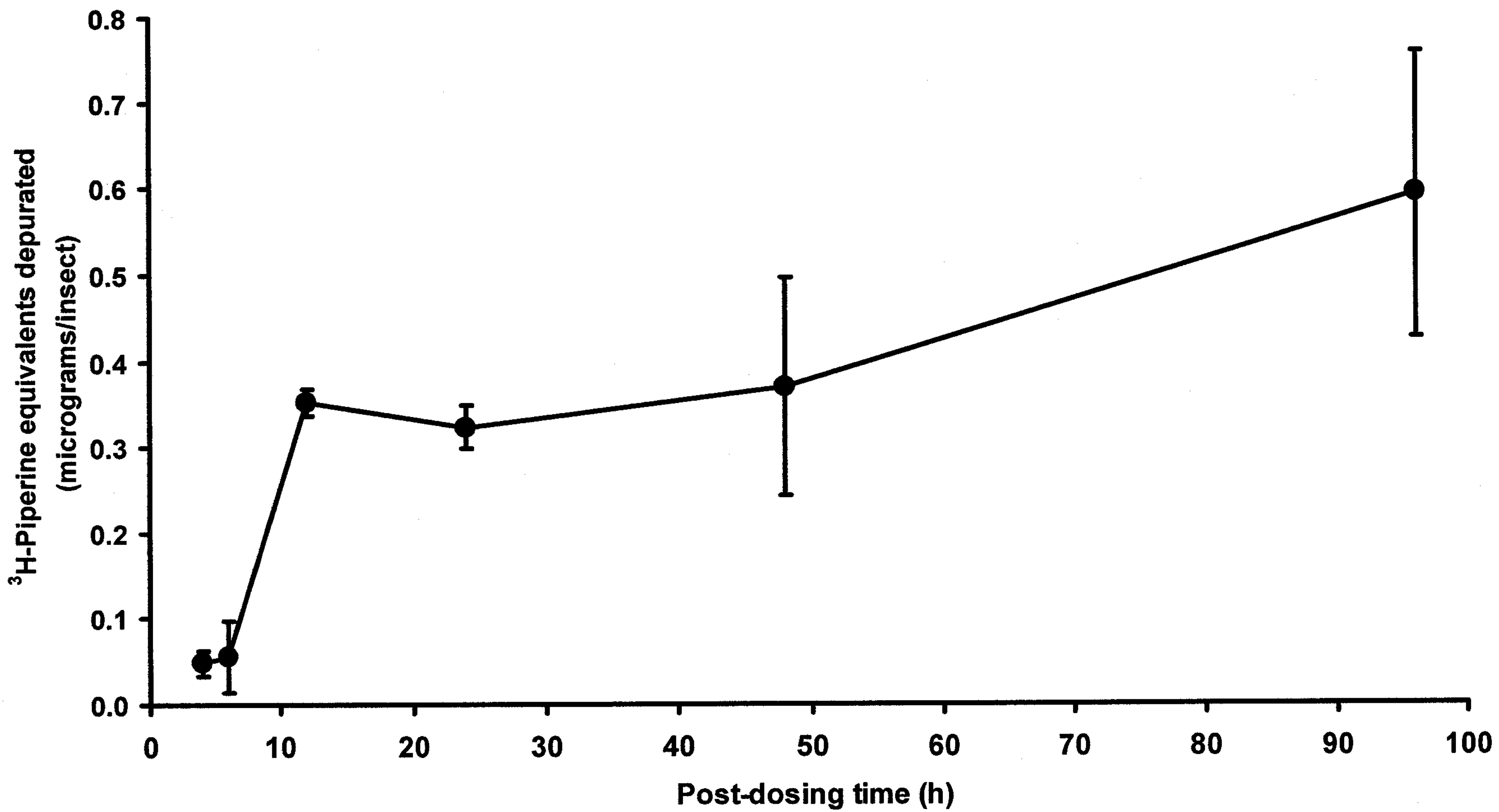


Figure 4. 8. Depuration of ^{3}H-piperine equivalents through feces from body of adult female *P. americana* 4, 6, 12, 24, 48 and 96 h post-dosing with 6.1 μg ^{3}H-piperine and 250 μg *P. nigrum*.

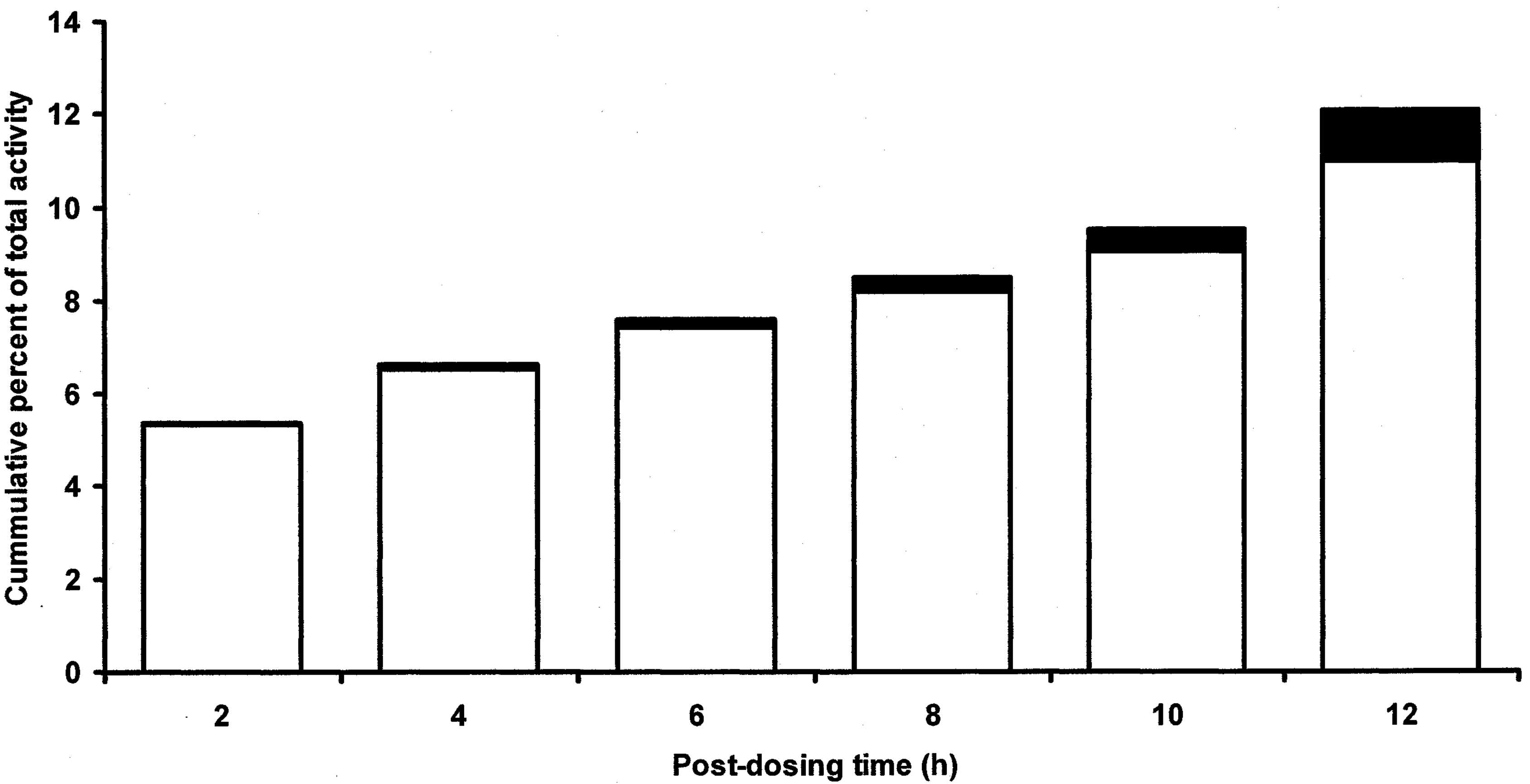


Figure 4. 9. Total accumulated depuration of ^{3}H-piperine equivalents through feces from body of adult female *P. americana* 2, 4, 6, 8, 10 and 12 h post-dosing with 136 µg ^{3}H-piperine and separated into ethyl acetate and water fractions.

4.3.4 Isolation and identification of piperine metabolites from the American cockroach

Over the entire 96-h depuration period, only a small percentage of tritiated material was depurated from the American cockroaches (Fig. 4.8 and 4.9) compared to the amount which remained in the body (Fig. 4.5A and 4.7). LCMS analysis determined two minor peaks (< 1%) present from depurated material in the piperine treated cockroaches compared to the acetone-treated ones.

The major compound depurated was identified as piperine (MW = 287.1). A second compound, also with the same molecular weight is assumed to be an isomer of piperine. Based upon the sum of the initial dose given to 20 female cockroaches, the dilution volume of ethyl acetate (350 mL), and the final piperine concentration (28.9 µg/mL), the total amount of piperine depurated was determined to be 33.74% of the initial dose. The LCMS analyses for piperic acid found none in the depurated material based upon the pseudomolecular ions selected.

4.3.5 House fly and Colorado potato beetle toxicity study

The pyrethroid-resistant housefly strain was more sensitive to the *P. tuberculatum* extract, LC_{50} = 0.49% (95% confidence limits = 0.40, 0.63) than was the susceptible strain, LC_{50} = 0.67% (95% confidence limits = 0.59, 0.78) ('Z' test, z = 2.43, z crit. = 1.96). In addition, the insecticide-resistant strain of *L. decemlineata* larvae tested with the *P. tuberculatum* extract showed a less than two-fold tolerance ratio compared to the susceptible strain. The LC_{50} values calculated for the susceptible and resistant *L. decemlineata* strains were 0.066% (95% confidence limits = 0.058, 0.076) and 0.109% (95% confidence limits = 0.088, 0.149) *P. tuberculatum* respectively. The acute bioassays indicated that the R strain was significantly less sensitive to the *P. tuberculatum* extract ('Z' test, z = 3.43, z crit. = 1.96). The relative LC_{50}s between resistant and susceptible strains, termed the tolerance ratio, was ten-fold less than for the pyrethroid cypermethrin when tested with the same R strain (Fig. 4.10).

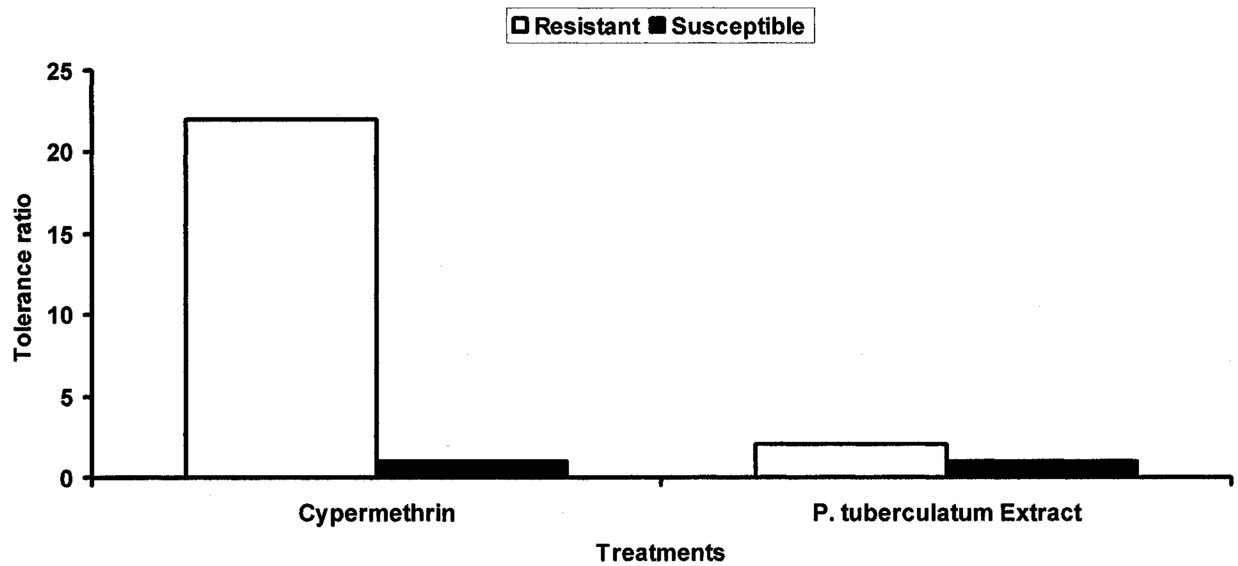


Figure 4. 10. Comparison of relative LC_{50}s (tolerance ratio) between insecticide (pyrethroid) resistant and susceptible *L. decemlineata* larvae treated with a pyrethroid and *P. tuberculatum* extract. The LC_{50}s in susceptible insects were normalized to 1.

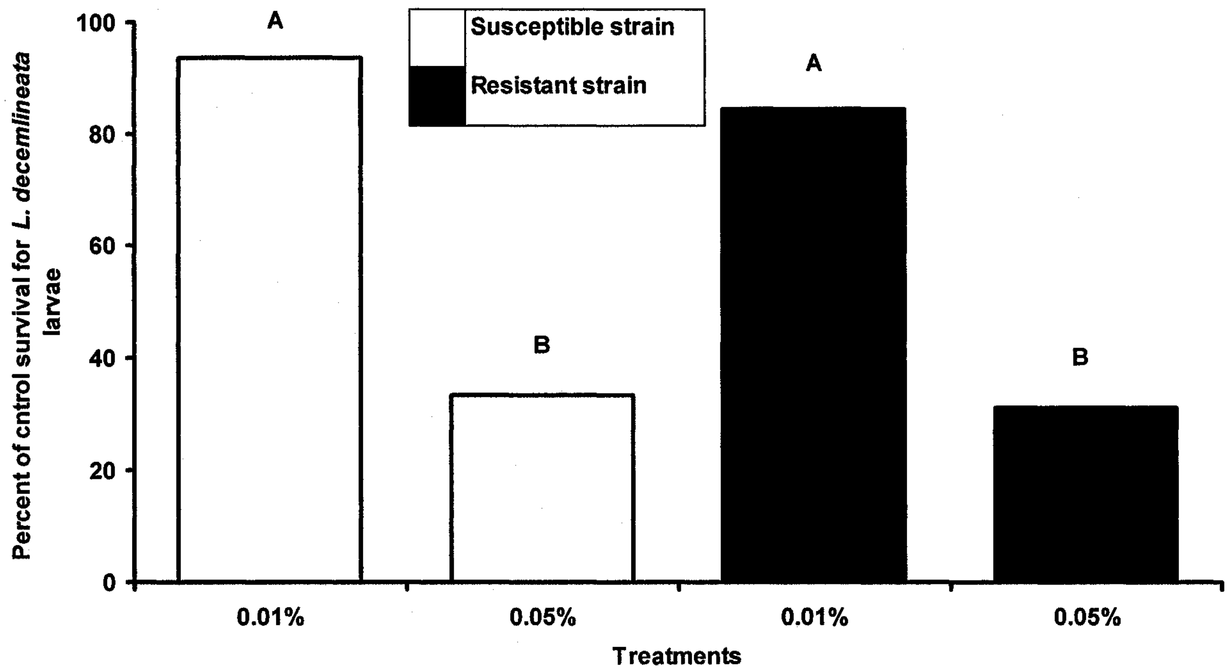


Figure 4. 11. Survival as expressed as percentage of control for susceptible (S) and pesticide-resistant (R) strain *L. decemlineata* larvae eight days after treatment of hatchlings on potato plants with two concentrations of *P. nigrum* extract (Two tailed T-test, $P<0.05$).

4.3.6 Colorado potato beetle greenhouse trials

Under more realistic conditions the *P. nigrum* extract at 0.05% had a similar effect on the R strain *L. decemlineata* larvae compared to the S strain on treated potato plants (Two tailed T-test, P=0.037 and 0.047, respectively) (Fig. 4.11). In both cases, there was a 65 to 70 % decrease in the number of larvae that survived from the hatchling stage to the eighth day compared to controls for both larval strains. *Piper nigrum* extract at 0.01% had little effect on the developing larvae (Two tailed T-test, P>0.215).

4.4 Discussion

4.4.1 Significance of insect PSMO inhibition by piperamides

The present experiments clearly show that *Piper* extracts can knock down larvae of *L. decemlineata* feeding on potato plants and will have a similar effect when applied to either multi-insecticide-resistant or susceptible populations. The initial hypothesis regarding the importance of the bifunctional nature of piperamides and the dual activity was supported by the ability of piperine to inhibit PSMO activity. Equally important from a practical aspect is the relatively low tolerance ratio between resistant and susceptible strain *L. decemlineata* larvae. This demonstrates that piperamides must have a distinct site of action compared to the other insecticides, a PSMO inhibiting effect that eliminates specific induction of enzymes in the resistant insects or both.

It has been well documented that piperine has an inhibitory effect on mammalian PSMO enzymes (Reen and Singh 1991; Singh and Reen 1994) and the specific CYP3A4 isoform (Bhardwaj *et al.* 2002). For this reason it has been patented for use as a bioavailability enhancer in drug preparations (Majeed and Badmaev 1998). What is particularly interesting is that all four piperamides tested against CYP3A4 had a similar range of inhibitory activity. A similar range of activity was found for the same compounds using the *Aedes atropalpus* mosquito larvae acute toxicity bioassay (Scott *et al.* 2002) indicating that the PSMO-inhibiting effect may be closely tied to the overall toxicity. The IC_{50} values determined in the current studyare slightly greater than those determined by Bhardwaj *et al.* (2002) (the IC_{50} values were in the range of 0.054 to 0.064 mM piperine), but this difference might be explained by their use of human liver

microsomes, with the CYP3A4 levels determined prior to measuring the inhibition of drug metabolite formation. It is also recognized that piperine will induce both phase I and II detoxification enzymes in mammals over longer time periods (Singh and Rao 1993), and many natural products can induce or inhibit insect enzyme systems (Scott *et al.* 1998). But now it is established that piperine has an *in vitro* inhibitory effect on specific enzymes which are induced in insecticide-resistant houseflies, and the *in vivo* trials with the American cockroach have documented the same activity with more general PSMO enzymes. The demonstration of an inhibitory effect with insect microsomes suggests that piperine is its own insecticidal synergist.

4.4.2 Toxicokinetics of piperamides in the insect

Elimination of piperine from the cuticle appears to be a linear relationship with first order kinetics (Figure 4.5A). Similar results were noted (Saghir *et al.* 1993 and 1994) when adult female houseflies were topically treated with tri- and tetra-chlorinated biphenyls (PCBs). They observed an initial rapid phase followed by a slow phase where it was assumed that the PCBs had penetrated and bound with lipophilic components amongst the cuticle layers and were only slowly released. In the present study the level of ^{3}H-piperine equivalents in the cuticle remains relatively constant after 24 h post-dosing. As suggested by Saghir and Hansen (1999), the matrix between cuticle layers may be slowly releasing the piperine.

The American cockroach cuticle is perhaps not representative of the type of elimination rates that would be observed in other insects treated topically by piperine. When cuticle penetration by insect pyrokinin neuropeptides (PK) analogues was compared between *P. americana* and the moth, *Heliothis virescens*, there was significantly less in the cockroach cuticle (Teal *et al.* 1999). Within 24 h after topical application there was a logarithmic elimination rate from both insect cuticles but the lower penetration seen with the cockroach could be due to the greater density and thickness of the cuticle.

T_{max} occurs quickly in the hemolymph because of the initial flush of material passing through the cuticle due in part to the relative lipophilic nature of piperine. The T_{max} was similar though to what has been observed for other toxicokinetic studies with insects (Table 4.4) despite the difference in the log P

values for the compounds compared. In the case of the cockroach, the uptake, elimination and stabilization of ^{3}H-piperine equivalents in the hemolymph appears to follow the same period of elimination and stabilization observed in the cuticle. The reason ^{3}H equivalents were removed faster from the hemolymph compared to soft tissue is likely because hemolymph is predominately water in content (Romosser and Stoffolano 1998) and lipophilic compounds are more rapidly absorbed by lipid-rich organs (Iyengar *et al.* 1987): for example the fat body, digestive tract, reproductive organs or other soft tissues. Corroborating evidence comes from the AUC values shown for both hemolymph and soft tissue where the higher value for soft tissues indicates more solubility for ^{3}H-piperine (Table 4.3).

The steady uptake of ^{3}H-piperine equivalents in the soft tissues of the cockroach between one and 48 h (Figure 4.7) suggests that, despite the stabilizing of the cuticle and hemolymph levels by 24 h, there remains a capacity for that compartment to absorb and retain the parent compound and metabolites. In comparison, the T_{max} for piperine is later than the value calculated by other insect studies (Table 4.4).

The reason higher log P compounds such as tri- and tetraCBs (Saghir *et al.* 1993; 1994) and α-terthienyl (Iyengar *et al.* 1987) have the lower T_{max} may be due to the higher penetration rate through the cuticle and passage to the lipid-rich fat body in the abdomen compared to piperine. In the case of the penetration of triCB through house fly cuticle, 90% was absorbed within the first 12 h (Saghir *et al.* 1993), and between 80 and 90% for α-terthienyl absorption into the cuticle of *O. nubilalis* and *M. sexta* respectively within 12 h (Iyengar *et al.* 1987). Another explanation was provided by Belzile *et al.* (2000) when it was observed that in the presence of the synergist, dillapiol, α-terthienyl retention in the tissues of mosquito larvae *Aedes atropalpus* increased in a dose-dependent manner and was directly correlated with the toxicity of the mixture. Therefore, in the presence of dillapiol, a PSMO inhibitor, the insect's ability to eliminate α-terthienyl was also affected. The depuration study shows that increasing the sublethal piperine dose affects the rate at which ^{3}H-piperine equivalent material is depurated. Furthermore it has been observed that piperine and other piperamide analogues inhibit PSMO activity in the same concentration range (Table 4.2).

Table 4. 4. Comparison of time of maximum concentration (T_{max}) in hemolymph and soft tissue and biological half-life ($t_{1/2}$) in hemolymph between piperine and four other compounds in six insects.

	P. americana	***O. nubilalis***[1]	***M. domestica***[2]	***A. domesticus***[3]	***H. virescens***[4]	***M. sexta***[4]
Comp.	Piperine	DIMBOA	2,2',5-triCB	B-a-P	A-Terthienyl	α-T
Appl.	topical	topical	topical	injection	topical	topical
Log P	2.78	0.65	5.16	5.9	5.39	5.39
Hemo. $t_{1/2}$	11.9			0.8		
Hemo. T_{max}	2	2			3	12
Soft tissue T_{max}	48		12		6	6

[1] Campos *et al.* 1989; [2] Saghir *et al.* 1993; [3] He *et al.* 1998; [4] Iyengar *et al.* 1987.

4.4.3 Piperine depuration and metabolites

The inhibitory effect of piperine on American cockroach PSMO enzymes *in vivo* had a negative effect on the metabolism and rate of elimination of piperine and its metabolites as the piperamide dose increased. However, the cockroach appears to have an ability to partially rid the body of piperine, apparently by passing it out unmetabolized. In the rat, piperine at a lower dose per body weight was metabolized into piperic acid (Bajad *et al.* 2002); however this was not observed in the cockroach, either because of a different metabolism or the higher inhibitory dose present in the insect. Transport of xenobiotics out of cells is often accomplished with P-glycoprotein. (Bhardwaj *et al.* 2002) Despite the fact that piperine has an inhibitory effect on mammalian P-glycoprotein at approximately the same concentration which inhibits CYP3A4 (Bhardwaj *et al.* 2002), the P-glycoprotein level in the insect may remain partially functional at piperine doses which inhibit P450 activity. This hypothesis has yet to be

confirmed but could explain how the cockroach and other insects can tolerate piperamides through quickly passing out unmetabolized compounds.

4.4.4 Mode of action combines neurotoxicity, PSMO inhibition and analogue synergism

Previous efficacy trials (Chapter 3) indicated that *P. nigrum* extracts function well, primarily due the ability to paralyze the insect through a neurotoxic effect. Amides act at site 2 of the sodium channel (Ottea *et al.* 1990), causing a modification of axonal excitability through an effect on sodium currents much like the effect of pyrethroids (Lees and Burt 1988). This effect is distinct from that of the pyrethroids since pyrethroid-resistant American cockroaches were affected by the same doses of pipercide which affected susceptible cockroaches (Miyakado *et al.* 1989). The piperamides do not have as quick a knock-down effect as pyrethroids, but the combination of analogues can be just as toxic (Miyakado *et al.* 1983). The results of the present study support these findings and further suggest that piperamides singly, or more importantly in combination, have a role to play in replacing contact insecticides for which resistance has developed. It is now apparent that this activity is enhanced by the ability of the attached MDP moiety to interfere with the detoxification enzymes of the insect, thus increasing their persistence and possibly the insecticidal activity in the insect.

The combination of novel structure of the piperamides, their mode of action and the mixture of analogues could be useful to mitigate the development of insecticide resistance, unlike many conventional products such as the single entity synthetic pyrethroids. In this case, insecticide-resistant *L. decemlineata* was found to have a tolerance ratio of less than two for *P. tuberculatum*, while the tolerance ratios for cypermethrin, azinphosmethyl and endosulfan were 11 to 22, 18 and 80 respectively (Agriculture and Agri-food Canada unpublished data). One reason for the lower tolerance ratio exhibited by *P. tuberculatum*-treated *L. decemlineata* could be that insect detoxification enzymes have greater difficulty metabolizing mixtures of analogues (Berenbaum and Zangerl 1996). In relation to Piperaceae chemical defence, it was demonstrated in *Aedes atropalpus* larval acute toxicity bioassays that tertiary and quarternary combinations of piperamides found in *P. tuberculatum* displayed synergistic activity (Scott *et*

al. 2002). The additional significance, as demonstrated by Feng and Isman (1995), is that there is less evidence of resistance development over many generations with multiple actives compared to a single-entity insecticide. Therefore, the positive implications of this study promote the use of *Piper* extracts for control of multiple-insecticide-resistant populations of insect pests best exemplified by the Colorado potato beetle.

CHAPTER 5

EFFICACY OF A *PIPER* BOTANICAL FOR CONTROL OF EUROPEAN CHAFER *RHIZOTROGUS MAJALIS* RAZOUMOWSKY (COLEOPTERA: SCARABAEIDAE)

5.1 Introduction

Many urban areas in northeastern North America have large areas of residential and public recreational land damaged by infestations of scarab beetles (Coleoptera: Scarabaeidae), the larvae of which are commonly referred to as white grubs. These larvae feed on the roots of grasses, the most preferred being Kentucky Blue grass *Poa pratensis* L. and other cool-season and transition zone turf grasses (Shetlar 2000). The damage caused by large numbers of the grubs in the late summer/early fall leads to die back in the grass, causing unsightly brown patches in lawns, parks and other turf areas (Potter 1998). A second problem occurs when predators such as skunks and crows dig up the dead turf to eat the grubs.

The most damaging turf insects in the northeast are Japanese beetle *Popillia japonica* Newman and European chafer *Rhizotrogus majalis* Razoumowsky (Brandenburg and Villani 1998) (European chafer is also referred to by the name *Amphimallon majalis* Razoumosky). The history of both Japanese beetles and European chafers in Ontario began when they entered along the Niagara Peninsula in the 1940s and 1960s, respectively, and subsequently spread east and north (Agriculture Canada 1992; Philogène 2000). As the common names suggest, both species are exotic to North America. Both populations of European chafer and Japanese beetle in Ottawa, Ontario, are near the northern extent of their range. This fact presents a novel aspect of insect life history as evidence suggests that the European chafer can take a second year to develop rather than its normal one year life-cycle (Brandenburg and Villani 1998). In the case of the Japanese beetles, urban areas provide artificial sources of heat to allow them to over-winter at this latitude. However evidence of their presence in natural areas north of Ottawa

indicates a lessening of this requirement. Japanese beetles remain a quarantined species in Canada (C.F.I.A. 1999) but European chafers have been removed from the quarantine list (C.F.I.A. 1995).

Many municipalities in Canada can no longer rely on synthetic chemical pesticides for the long-term control of white grubs. Alternative control measures in the form of biopesticides have been attempted: for example, the bacteria, *Bacillus popilliae*, and entomopathogenic nematodes, *Steinernema carpocapsae* (Weiser), *S. glaseri* (Steiner) and *Heterorhabditis heliothidis* (Khan, Brooke and Hirschmann), pathogens that live in the soil and can infest the grub and kill them (Brandenburg and Villani 1998). Experience with the commercially available nematodes suggests that the control of grubs varies from moderate to low, perhaps because the strains of nematodes available are not suited for European chafer (Shetlar 2000). A great deal of the reported success with nematodes is credited to the vigilance of the person who carries out the control exercise in terms of maintaining the correct environmental conditions to maximise the nematode population. Combining the insecticide imidacloprid, from the chloronicotinyl chemical group, with nematodes (*H. bacteriophora* Poinar) has a synergistic effect on mortality of the scarab beetle *Cyclocephala hirta* LeConte by definition of a syergist (Koppenhöffer and Kaya 1998). It is suspected that the insecticide either reduces the larval movement or other behavioural changes that facilitate increased infection rates.

Since reduction of synthetic pesticide use is desired, other forms of biopesticides are being considered, among them botanical formulations. Botanicals provide a mix of biologically active compounds, low environmental persistence and wide safety margin for non-target mammals including humans and domestic animals. One promising example is the use of neem, *Azadirachta indica* Juss, which was proven to disrupt the development of Japanese beetle larvae (Ladd *et al.* 1984). Currently registered commercial products in the U.S. for grub control include chloronicotinoids and pyrethroids, both synthetics derived from secondary plant compound leads (*eg.* pyrethroids from pyrethrins).

The pepper family, Piperaceae, was chosen for this investigation because it has shown to be promising in all of the previously mentioned categories. Being commonly associated with spice and medicinal use, many species within this family have been used around the globe for centuries without

apparent health risks (Parmar *et al.* 1997). The pepper family should not be confused with the more toxic Capsicum pepper used as an irritant spray. Extracts of *Piper* species also have insecticidal activity documented from many parts of the world. Recent studies have investigated the properties of these extracts and have identified and isolated phytochemicals that are active in a number of ways (Bernard *et al.* 1995; MacKinnon *et al.* 1997). The most promising species for botanical preparations are *Piper nigrum* L. (black pepper), *P. guineense* Schum and Thon (Guinea pepper) and *P. tuberculatum* Jacq., with isobutyl amides and lignans as some of the identified active principles. These compounds are better known as piperamides within these species, and have a high knock-down effect yet have low mammalian toxicity. The piperamide is a bifunctional molecule that combines neurotoxicity with monooxygenase inhibition, due to the activity of the amide and methelenedioxyphenyl moieties respectively. The activity is further enhanced because the extracts are a mixture of many analogues (Scott *et al.* 2002).

The objectives of the current investigation were to test the efficacy of a *Piper*-based botanical preparation to assess whether it provides an environmentally responsible alternative control method for white grubs. The main components of the research are 1) larvicidal activity; 2) non-target effects; and 3) fate of active components in the environment. Efficacy trials with field-collected European chafer larvae were conducted in the greenhouse. Non-target toxicity was evaluated using an invertebrate species that would likely encounter the effects of the botanical treatment to turf treatments: the earthworm as exemplified by the compost worm *Eisenia fetida* Savigny (Oligochaeta: Lumbricidae). Fate of pepper active compounds was evaluated over the course of the field trials by sampling the top layer of soil where the insects are located.

5.2 Materials and Methods

5.2.1 *Piper* extracts

P. nigrum peppercorns were obtained from several sources: for the earthworm bioassay and mid-season grub field trial *P. nigrum* were obtained from suppliers in Sarawak (Malayasia), Lombok and Borneo (Indonesia); Guinea pepper, *P. guineense* Schum and Thon, from Guinea, West Africa and *P.*

tuberculatum Jacq. leaves collected in Costa Rica. Voucher specimens were placed in the University of Ottawa for all species and the Herbarium, Universidad Nacional, Costa Rica for *P. tuberculatum*. The *Piper* extracts were prepared in Dr. T. Durst's laboratory, Chemistry, University of Ottawa. Seeds and leaves were ground in a blender with ethyl acetate:water (50:50) and, then the seed material allowed to sit for 24 hours before being filtered through a Buchner funnel. The seed material was mixed with ethyl acetate: water for a second 24 h soak followed by a repeat filtering. The water fraction was decanted off both sets of solvent mixes and the two ethyl acetate fractions mixed and dried by roto-evaporation. The extract material was mixed thoroughly from separate batches and added either at 25 or 50 g weights to prepare 10 and 20% extract emulsifiable concentrates with 10 % surfactant (Jeneil Co.) and 70-80% alcohol (THFA, Penn Specialty Chemicals).

For the late season field trials, peppercorns originally imported from Malabar, India, were obtained from Country Bulk Spices, London, Ontario. The peppercorns were finely ground in a mill, then mixed with ethyl acetate (100 g + 200 mL) and refluxed for 20 minutes. The mixture was shaken overnight and then filtered through Whatman No. 1 filter paper in a Buchner funnel. The ground peppercorns were washed twice with 50 mL of ethyl acetate. The filtrate was then washed once with 100 mL of distilled water in a separatory funnel. The ethyl acetate fraction was then dried by the addition of anhydrous sodium sulphate, filtered, and concentrated by rotary evaporation. The dried extract remaining was formulated by weight as: 20% extract, 70% THFA and 10% EL-719 emulsifier. Analysis of active components was conducted by sampling each 1 L stock of formulated extract prepared and analysing following the HPLC technique devised by Scott *et al.* (2002).

5.2.2 Bioassays with European chafer

Rhizotrogus majalis 3rd instar larvae were collected from Arnprior ON, in September and October 2001 and May 2002 and from Ottawa, ON, in September and October 2002. They were kept in containers with soil at 4° C until trials were initiated. *Rhizotrogus majalis* larvae bioassays were conducted in plastic seedling trays with an artificial soil /grass medium. The grass was grown in plastic seedling trays, 58 x 28

cm, divided into 6 equal sized compartments. Soil consisted of 1 part potting soil: 1 part sand, and within each compartment provided a depth of approximately 5 cm. The grass seedling mix consisted of Kentucky Blue 65%, Annual Rye Grass 20% and Fescue 15% and was distributed at 1.5 g seed per compartment, two weeks prior to the addition of the insects. Ten 3rd instar *R. majalis* were placed into each compartment so that there would be 60 larvae per tray with three replicate trays. Treatments of *P. nigrum* were based upon the average weight of soil mixture in an individual compartment. The final concentration in the soil was based upon the amount added to each compartment of soil. One to two days after the larvae were added to the trays the treatment was initiated. Each of the *P. nigrum* solutions was prepared in 100 mL of water. This included a formulation blank and five geometric extract concentrations: 0.188, 0.375, 0.75, 1.5 and 3%. The trays were watered once per day for a seven-day period after treatment. After one week, each compartment was sampled to determine the survival of the larvae. The bioassay was repeated three times. A second set of bioassays was then conducted after the soil pH was increased by the addition of 8.7 g $CaCO_3$ per compartment. Voucher specimens for *R. majalis* larvae were deposited with the Biosystematics group, Central Experimental Farm, Agriculture and Agrifood Canada, Ottawa, Ontario.

5.2.3 Soil pH and organic matter determination

Samples of the soil were taken from each compartment after both trials and analysed for both pH (A.S.T.M. 1989) and organic matter content (A.S.T.M. 1987). Oven dried soil (10 g) was mixed with deionized water (10 mL) and allowed to sit for 30 minutes. The pH meter was calibrated between pH 4.00 and 7.00. The pH was measured by immersing the electrode in the soil–water slurry. The oven dried soil was weighed into ceramic crucibles between 3 and 5 g and then placed in a muffle furnace for 3 hours at 500 °C. The difference between the oven dried weight and the ash weight relates to the soil organic content.

5.2.4 Non-target invertebrates

Earthworms

The compost earthworms, *Eisenia fetida* (Savigny) were obtained from The Worm Factory, Perth, Ontario, on two separate dates in July and August 2000. The tests with *P. nigrum* and *P. guineense* were done with the first batch and the test with *P. tuberculatum* was done with the second batch. Three toxicity tests were performed in general accordance with the ASTM Standard guide for conducting laboratory toxicity or bioaccumulation tests with the lumbricid earthworm *E. fetida* (A.S.T.M. 2000). One test using artificial soil as the test matrix was done with each *Piper* species extract. The artificial soil dry ingredients were by weight, 10% Canadian Sphagnum peat moss (that portion passing through a 2 mm screen), 20% kaolin clay, and 70% silica sand (Grade 70, that portion passing through a 0.3 mm mesh). Calcium carbonate ($CaCO_3$), 0.6%, was added to adjust the pH to near neutral. All earthworm trials were conducted by N Gagnon, Biology Department, University of Ottawa.

The earthworms were held in soil-filled metal containers with perforated black garbage bags as lids. The soil consisted of 50% peat, 11% kaolin clay and 39% silica sand by weight as previously described (A.S.T.M. 2000). The pH was adjusted with calcium carbonate. The earthworms were fed with alfalfa cakes that had been hydrated with test water and allowed to sit for at least 48 hours before being sprinkled over the surface of the soil.

For each extract, five treatment groups consisted of 75, 125, 250, 500, and 1000 μg/g extract by dry soil weight; two control groups included a natural emulsifier and a water control. The emulsifier was added at 4500 μg/g in all groups except the water control and consisted of 8 parts THFA and 1 part JBR-425 (Jeneil Biosurfactant Co., Saukville, WI) w/w. Potassium chloride (KCl) at 6030 μg/g (LC_{50}) was the reference chemical (Yeardley *et al.* 1995). Each treatment was replicated three times in 500-mL glass Mason® jars with 200 g of dry soil per jar. The soil required for three replicates was prepared as a batch. Extract and/or emulsifier were added to an amount of deionized hydration water such that when added to the artificial soil the final moisture was 35% by dry weight. After mixing, the hydrated soil was

divided evenly into three replicate jars and the jars were covered with perforated plastic wrap and allowed to equilibrate for 24 hours in the test chamber.

Total biomass of earthworms per replicate jar was measured at the beginning and end of each test. Before weighing, earthworms were rinsed and then purged of their gut contents by replacing them in Petri plates (5 per plate) with wet filter paper for 24 hours. On day 0, 10 earthworms, a minimum of 0.3 g each, were added to each replicate jar for a total of 30 earthworms per treatment group and allowed to burrow. Earthworms, 0.4 g each or more, were distributed evenly between test jars. The treatment jars were kept under continuous light in an incubator at 19°C. Burrowing was observed after 24 hours. Mortality was recorded after six and 14 days. Any dead earthworms were removed after six days. Probit analysis was used to determine LC_{50} values with 95% confidence intervals. General observations on the health of the earthworms were made at the end of the tests. After purging as previously described, relative weight loss (RWL) per worm was determined for each replicate jar and the mean $\pm$ standard error was calculated for each treatment group. RWL was only determined for treatment groups where mortality was 20% or less per jar. Soil moisture and pH were also determined for the water control and the KCl treatment group.

5.2.5 Field trials

5.2.5.1 Guelph 2000

The Guelph Turfgrass Institute (GTI), Guelph, Ontario, was selected as a field site in August 2000, based on the assessment of adequate grub numbers. Because conditions had been unseasonably wet during summer 2000 leading to healthier grass, no physical damage was noted at this site. The site was a north-facing lawn, sloping to the north-east of the main GTI building. The site, about 17 m x 8 m, contained healthy grass with little to no thatch, and consisted of mainly annual rye grass, with some fescue and Kentucky blue, and watered by sprinkler system. The grub treatment plots were randomized throughout the site. A plot was selected if the sampling instrument, a golf cup changer with 10 cm diameter, found one grub with minimal effort in each core of 10-15 cm depth, then a plastic marking stake was used to mark the site. In most cases only one grub was found per core. Plastic lawn edging material forming a

circle with a 50-cm diameter was placed around the plastic marker. Spray paint was used to outline the outside of the edging in order to define the boundary of the treatment. Numbers were written on each of the plastic markers beginning on the east side of the plot moving up and down the hill until 51 sites were marked.

In the field, 6 L of water mixed with 60 mL of both the 10 and 20% *Piper* extract formulations was applied to each plot at 0.1% and 0.2% *Piper* material respectively. *Piper* extract treatment at 0.4% was prepared by adding 120 mL to 6 L of water. The solution was poured over the 0.2 m^2 area uniformly with a watering can and post-application watering occurred daily in order to saturate the soil in the experimental area. There was sufficient material for six replications of each of the *P. nigrum* concentrations and five replications of the *P. guineense* concentrations. Controls consisted of both water applied to six sites at the same volume used in the treatments, 6 L, and EC formulation (11% surfactant and 89% alcohol) without *Piper* extract material both at 60 mL and 120 mL per 6 L of water. Diazinon at the recommended application rate was the positive control. The 51 sites were randomly assigned a treatment.

The first application took place on 30 August, 2000. One week post-application sampling of each plot was conducted using a golf-cup changer to an approximate depth of 10 to 15 cm where possible. Five cores were taken in each plot, grubs were removed from soil cores and numbers counted. All larvae were preserved in 70% ethanol for later identification. Air temperature and precipitation data were obtained from the Guelph Turfgrass Institute monitoring station for the period during the field trial.

5.2.5.2 Ottawa 2002

In late August 2002, an assessment, using 10 golf-cup changer cores, of grub populations was made in several parks of the City of Ottawa. Based upon the number of late 2nd and early 3rd instar larvae found in Grenville Glen recreational park and baseball diamond, it was chosen as the site for the treatment. Because of dry summer conditions, some damage to the outfield of the ball diamond was already noticeable. The ball diamond turf had been managed intensively while the park grass had been

top-dressed every few years. The site was split between the ball diamond outfield and the park turf area, divided by a fence delineating the edge of the playing surface.

The first application took place on 23 September, 2002 where 0.5-m^2 circular plots were chosen based upon damage to turf and the presence of grubs in the under-lying soil layer. A 10-cm wooden stake was then used to mark the centre of 42 plots, half located within the outfield of the ball diamond and half located in the adjacent park. Each of the plots was then randomly selected for one of seven treatments. Peppercorns, 15 kg, were obtained from Country Bulk, London, Ontario, and processed as previously described. A 1%-extract application was prepared by adding 80 mL of the 20% stock extract with 2 L of water, while 160 and 320 mL of *P. nigrum* formulation were used for the 2 and 4 % *P. nigrum* formulation respectively. Each treatment was prepared in 2 L of city water supplied from either a 2000-L tanker truck or adjacent water main. Water controls were 2 L water per 0.5 m^2, formulation blank controls were 80 mL and 320 mL of EC in 2 L of water and positive control (diazinon) at the recommended application rate. All treatments were replicated six times and after each application the area was watered until the ground was saturated. During the following week, the treatment area was soaked daily with water unless there was adequate rainfall.

Sampling of the plots occurred on 30 September and 1 October due to a rain delay. Within each 0.5-m^2 plot, four golf cup cores of soil, 15 cm in depth, were removed. The cores were then sorted immediately and the numbers of live, dead and moribund grubs recorded. The number of earthworms and other soil invertebrates was also noted. Air temperature and precipitation data were obtained from the Central Experimental Farm, Agriculture and Agri-Food Canada, Ottawa, for the period during the field trial.

5.2.6 Residue analyses

Soil cores in 20-mL, 2-cm diameter glass vials were obtained from the bioassay soil for residue analysis of *P. nigrum* extracts. One soil core was collected from each of the 3 % *P. nigrum*-treated replicates. The soil cores from each replicate were placed in a freezer storage bag and stored at -20° C

until analysis. Field soil residue levels of *P. nigrum* extracts were analysed through the collection of 5 cm soil cores using a 2 cm diameter copper pipe. Three soil cores were collected from each of the 4% *P. nigrum* treated plots. The soil cores from each replicate plot were placed in a freezer storage bag and stored at -20° C until analysis. Five grams of soil were extracted with acetone using the Accelerated Solvent Extractor (ASE 200, Dionex Canada Ltd., Oakville, Ontario). Conditions for extraction were as follows: 1-min preheat time; 5-min heat time at 100 °C; 10-min static at 1000 psi; 75% flush cell volume; purge for 30 sec with nitrogen at the end of 1 cycle. The acetone soil extract was then transferred to a polypropylene centrifuge tube, the volume adjusted to 25 mL and then centrifuged for 5 min at 10,000 rpm. The fraction was separated from the pellet and rotary evaporated in a 100-mL round bottom flask until dry. The residue was then dissolved in 10 mL of 99% ethanol. A 1-mL sample was passes through a 2.4 μm filter into an HPLC vial. The piperamide concentration for each sample was analysed using the method described by Scott *et al.* (2002). Final soil concentrations were based on the dry weight of each individual soil sample analysed.

Recovery of piperamides from the greenhouse soil used in bioassays and from field collected soil (Guelph Turfgrass Institute and Grenfell Glen, Ottawa) was determined by spiking 25 g of each soil type, oven dried at 60 °C, with 12.5 g of a 1% *P. nigrum* formulated extract (20 % *P. nigrum* by weight) in water, to afford a 0.5% *P. nigrum*/soil mixture ($^{w}/_{w}$). An 11-mL ASE cell was then packed with the soil mixture and run according to the above methods. Each soil type was replicated three times for the piperamide recovery analysis.

5.2.7 Statistics

Probit analysis (Hubert and Carter 1990a) was used to determine the 7-day *P. nigrum* LC_{50} values for *R. majalis* larvae. Comparison of the LC_{50} values between trials was conducted using a Chi Square or Z-test (Hubert and Carter 1990b). All field data were analysed by one-way analysis of variance (ANOVA) with SYSTAT (1999). Prior to analysis the normality and homogeneity of data were tested and, if

necessary, data were log transformed prior to ANOVA. A multiple comparison of means was accomplished using Tukey's HSD test.

5.3 Results

5.3.1 Target and non-target bioassays

The median lethal toxicity trials with *P. nigrum* extract determined that the LC_{50} for pre-winter 3rd instar *R. majalis* larvae was 2.5% (Table 5.1), which was not significantly different from the LC_{50} for post-wintering 3rd instar *R. majalis* larvae at 1.5% . The comparison of LC_{50}s in two soil with pHs of, 5.3 and 6.1 (Table 5.1), also suggested that soil pH was not a significant factor ($Z = 1.79$, Crit. $Z = 1.96$) influencing the efficacy of *P. nigrum*.

Piper extracts were toxic to earthworms exposed in closed containers, where compound avoidance was not possible. Of the three *Piper* species tested, *P. tuberculatum* resulted in the highest *E. fetida* mortality, followed by *P. nigrum* (Table 5.2). Mortality was less after six days. *P. tuberculatum* was the most toxic to *E. fetida* with a 14-day LC_{50} of 189 μg/g, less than half that of *P. guineense* (14-day LC_{50} = 540 μg/g) which was the least toxic. Earthworms were dead after a few days for all species at 1000 μg/g. At concentrations showing high mortality, some earthworms did burrow after 24 hours indicating an avoidance response to the extracts. Of the emulsifier controls from the three tests, only one out of 90 earthworms died. All live *E. fetida* appeared relatively healthy at the end of the tests. In the test with *P. nigrum*, some worms from all treatments except the water control appeared slightly spasmodic. In the test with *P. guineense*, earthworms were most active in the water controls, but they became progressively less active beginning with the emulsifier controls and with increasing concentrations of the extract.

Eisenia fetida biomass decreased for all treatment groups and all extracts as expected since the earthworms were not fed for the duration of the test (data not shown). Only *P. tuberculatum* showed a trend of increasing weight loss with increasing extract concentration.

5.3.2 Persistence of piperamides

The persistence in soil of the five isobutyl amides found in the *P. nigrum* extract was high over the seven day exposure in most of the trials and with most of the compounds (Table 5.3). There was a significant difference between the pH of the first two trials (pH 5.3-5.4) and the second two trials (pH 6.0-6.1) (F=19.430; df=3,20; P=0.001), but no difference among either pair of trials (Tukey's multiple range test, P>0.663). The difference in soil pH did not affect the level of any of the piperamides over the seven day trial (one way ANOVA, P>0.05). At the start of the second trial (pH 5.3 soil), the concentration of piperine was approximately 630 μg/g in the soil. After seven days, it had decreased to 85% or to 520 μg/g. A similar change was observed in the first pH 6.1 soil, where piperine declined from 500 to 390 μg/g or to 80% of its initial value. The soil levels of the total piperamides was also no diiferent between trials (F=1.433; df=3,8; P=0.303).

There was no evidence of large differences in the stability of the compounds and the recovery of piperamides was greater than 85% from greenhouse artificial soil and soil collected from the two field sites and spiked with *P. nigrum* at 0.5% ($^{w}/_{w}$) (Table 5.4). The pH of the greenhouse soil was significantly lower compared to that from the two field sites (F=1535.347; df=2,55; P=0.001) and the organic matter content was greater than that from the GTI site (F=5.517; df=2,67; P=0.006) but not different from the Ottawa site (Tukey's multiple range test, P=0.996). The differences in soil pH and organic matter did not affect the recovery of piperamides from the spiked soil from the three sources (one way ANOVA, P>0.05).

Table 5. 1. *P. nigrum* LC_{50} values, 95% confidence intervals and slope of probit lines for 3rd instar *R. majalis* larvae.

Test	N	LC_{50} (% extract)	95% C.I. (% extract)	Slope
Post-winter larvae pH 5.3 soil	2	1.44	0.94, 3.01	0.88
Pre-winter larvae pH 5.3 soil	3	2.49	1.90, 3.64	1.72
Pre-winter larvae pH 6.1 soil	2	4.06	2.85, 7.23	1.88

Table 5. 2. LC_{50} values for *E. fetida* worms exposed to *Piper* extracts in controlled environment. Values obtained using probit analysis and recorded in µg/g (95% confidence intervals).

Treatment	LC_{50} (6 days)[a] (µg/g)	LC_{50} (14 days) (µg/g)
P. guineense[b]	554 (478-641)	540 (462-628)
P. nigrum[c]	300 (230-416)	262 (203-351)
P. tuberculatum	223 (190-282)	189 (161-237)

[a] Value at 7 days for *P. nigrum*; [b] mortality at 1000 µg/g taken as 29/30 test organisms; [c] 250 µg/g treatment omitted as an outlier.

Table 5. 3. Mean percent (± S.E.) of piperamides in *P. nigrum* extract remaining at end of *R. majalis* 3rd instar larvae 7-day greenhouse toxicity trials (N=24).

		Percent of piperamide remaining in soil seven days post-application (S.E.)					
Trial	**Soil pH**	**DHPLG**	**PLG**	**DHP**	**Piperine**	**Pipercide**	**Total**
1	**5.42[a]**	113[c]	80.7[d]	74.5[e]	77.3[f]	80.2[g]	77.3[h]
	(0.04)	(26)	(14)	(8.6)	(11)	(14)	(11)
2	**5.27[a]**	63.9[c]	81.3[d]	73.9[e]	85.9[f]	78.1[g]	84.3[h]
	(0.06)	(2.4)	(16)	(19)	(15)	(14)	(13)
3	**6.08[b]**	90.5[c]	103[d]	71.9[e]	81.1[f]	105[g]	80.8[h]
	(0.10)	(7.5)	(17)	(10)	(20)	(8.3)	(17)
4	**5.98[b]**	74.5[c]	55.0[d]	43.3[e]	50.5[f]	77.9[g]	51.0[h]
	(0.14)	(12)	(5.1)	(7.9)	(7.4)	(11)	(7.5)

DHPLG = 4,5-dihydropiperlonguminine, PLG = piperlonguminine, DHP = 4,5-dihydropiperine

[a] pH values and percent piperamide remaining with the same letter are not significantly different between trials (Tukey's P>0.05).

Table 5. 4. Mean pH, organic matter (O.M.) content and piperamide recovery (%) ± S.E. from *P. nigrum* spiked artificial soil and ield site collected soil samples.

Parameter	Soil sample sites		
	Greenhouse	GTI Aug./Sept. 2000	Grenfell Glen Sept. 2002
pH (S.E.)	5.3 (0.05) [a]	7.4 (0.02) [b]	7.0 (0.03) [b]
N	12	34	12
% O.M. (S.E.)	9.3 (0.33) [c]	7.6 (0.42) [d]	9.3 (0.25) [cd]
N	24	34	12
Piperamide[1]	**Percent recovery (S.E.)**	**Percent recovery (S.E.)**	**Percent recovery (S.E.)**
PLG	101 [e]	97.5 [e]	101 [e]
	(2.4)	(14)	(4.3)
DHP	94.8 [f]	91.7 [f]	97.1 [f]
	(3.2)	(16)	(3.1)
Piperine	88.8 [g]	85.5 [g]	90.8 [g]
	(1.9)	(14)	(2.9)

[1] N = 3 replicates / soil type; PLG = piperlonguminine; DHP = 4,5-dihydropiperine.

[a] pH, percent organic matter and percent piperamide recovered with the same letter are not significantly different between sites (Tukey's P>0.05).

5.3.3 Field trials

5.3.3.1 Guelph 2000

The *P. nigrum* and *P. guineense* treatments reduced the grub populations observed in the treated plots by seven days post-application in a dose-dependent fashion. During the seven-day field trial period between 30 August and 6 September 2000, a one-way ANOVA showed a significant effect of treatments on grub numbers ($F=3.768$; $df=9,46$; $P=0.001$) (Figure 5.1). A post-hoc Tukey's test showed a significant difference between the average grub numbers in the 2 and 4% *P. nigrum*, 4% *P. guineense* and diazinon-treated plots compared to those in the water control plots (Tukey's multiple range test, $P<0.04$) and grub populations were well below the 5 grubs / 0.1 m^2 threshold.

The lower concentrations, 1% *P. nigrum* and 1 and 2% *P. guineense*, did not have significantly lower numbers of grubs compared to water or both formulation blank treatments (Tukey's multiple range test, $P>0.184$) and did not reduce the average number of grubs below the damage threshold of 5 grubs / 0.1 m^2. The number of grubs in the positive control plots was comparable to both formulation control treatments and both pepper treatments at all levels (Tukey's multiple range test, $P>0.166$). The number of dead larvae was not assessed, nor was the number of earthworms or other invertebrates, since the populations at this site were not sufficiently high to warrant assessment.

5.3.3.2 Ottawa 2002

One week post-application, the average number of live *R. majalis* white grubs found in the 4% *P. nigrum* and diazinon positive control treated plots was less than 5 / 0.1 m^2 (Figure 5.2). In comparison the water control and two formulation controls had between 8 and 12 / 0.1 m^2 and the 1 and 2% *P. nigrum* treatments averaged 10 / 0.1 m^2. There was a significant difference between the number of live *R. majalis* larvae found between treatments ($F=2.647$; $df=6,35$; $P=0.032$); however only the diazinon-treated plot had significantly lower numbers of live grubs compared to the water control (Tukey's multiple range test, $p=0.096$) (Figure 5.2). Since there were no significant differences between the water control and the two formulation controls (Tukey's multiple range test, $P>0.970$) the live grub counts for all

controls were combined and the results showed that there was a significant effect between treatments (F=3.839; df=4,37; P=0.01). However, only the positive control treated plots had significantly lower numbers of live grubs (Tukey's multiple range test, 0.028), while the 4% P. nigrum treated plots were no different compared to the combined controls (Tukey's multiple range test, P=0.061).

There was no significant difference (F=1.433; df=6,35; P=0.230) between the number of dead and moribund grubs found between treatments or when controls were combined (F=1.804; df=4,37; P=0.149). When the number of dead grubs alone was examined, there was no overall significant treatment effect (F=2.352; df=4,37; P=0.072): neither the 4% *P. nigrum* nor the positive control (Tukey's multiple range test, P=0.812 and 0.165 respectively) were significantly higher compared to the combined controls.

Pepper treatments were attempted later in the season but were less effective (see Appendix IV).

5.3.4 Environmental conditions during field trials

During the seven-day field trial period between 30 August and 6 September 2000, the air temperature ranged between 20 and 25 °C on average (Figure 5.3), precipitation amounted to 1.8 mm and daylight averaged 13 hours. Between 23 September and 1 October 2002 the average air temperature in Ottawa was between 15 and 20 °C (Figure 5.3), the rainfall was 34 mm and daylight averaged 12 hours. Differences in soil pH and organic matter content were negligible between sites (Table 5.4), thus these factors did not influence the efficacy results.

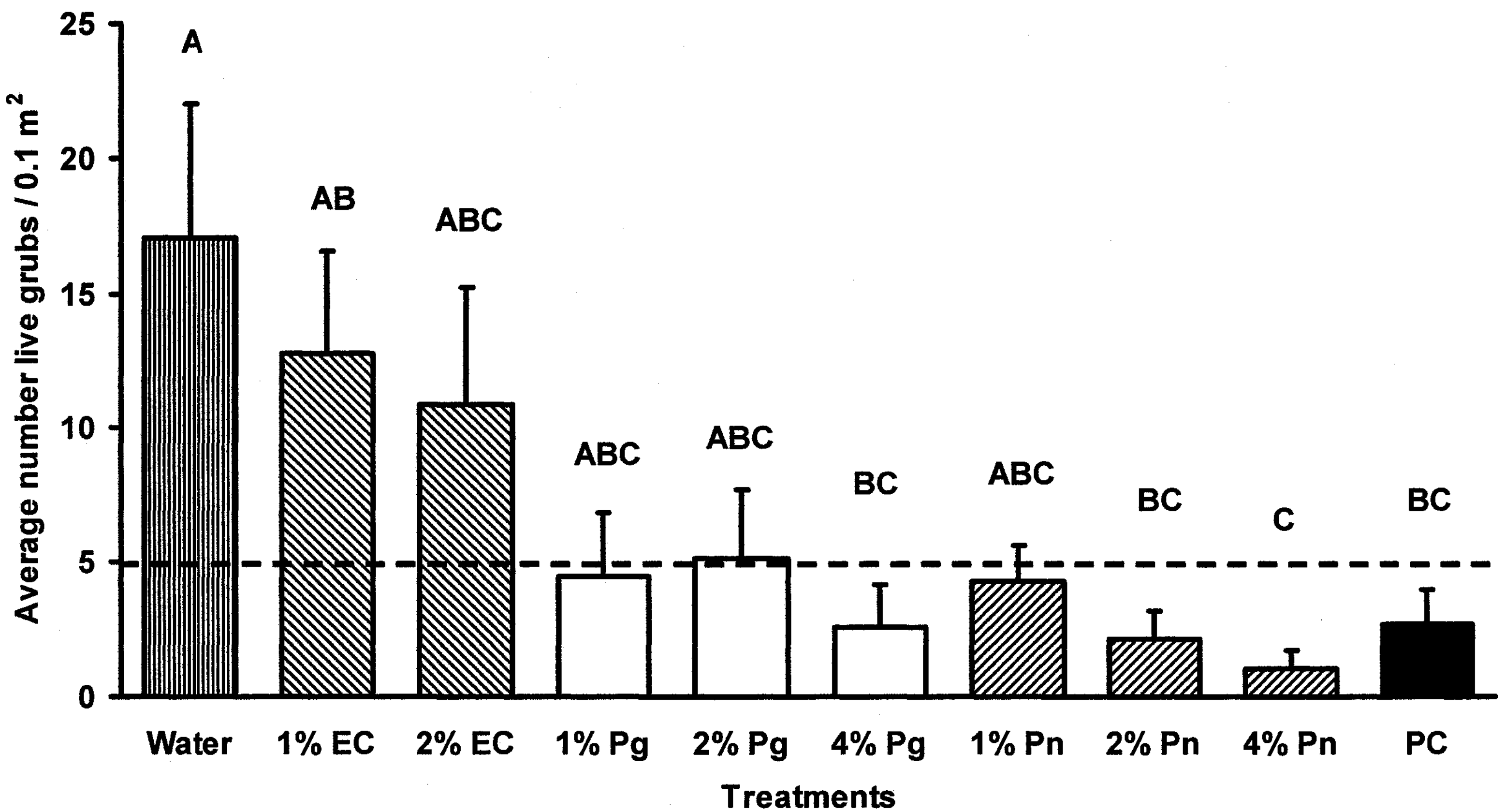


Figure 5. 1. Mean number of *R. majalis* larvae ± S.E. sampled in water control, formulation control (EC), *P. guineense* (Pg), *P. nigrum* (Pn) and positive control diazinon (PC) treated plots at Guelph Turfgrass Institute on 6 September 2000. The dashed line indicates threshold for damage in non-irigated turf (5 grubs / 0.1 m^2). Statistical differences between pepper treatments and water control were evaluated by Tukey's multiple range test (P=0.05).

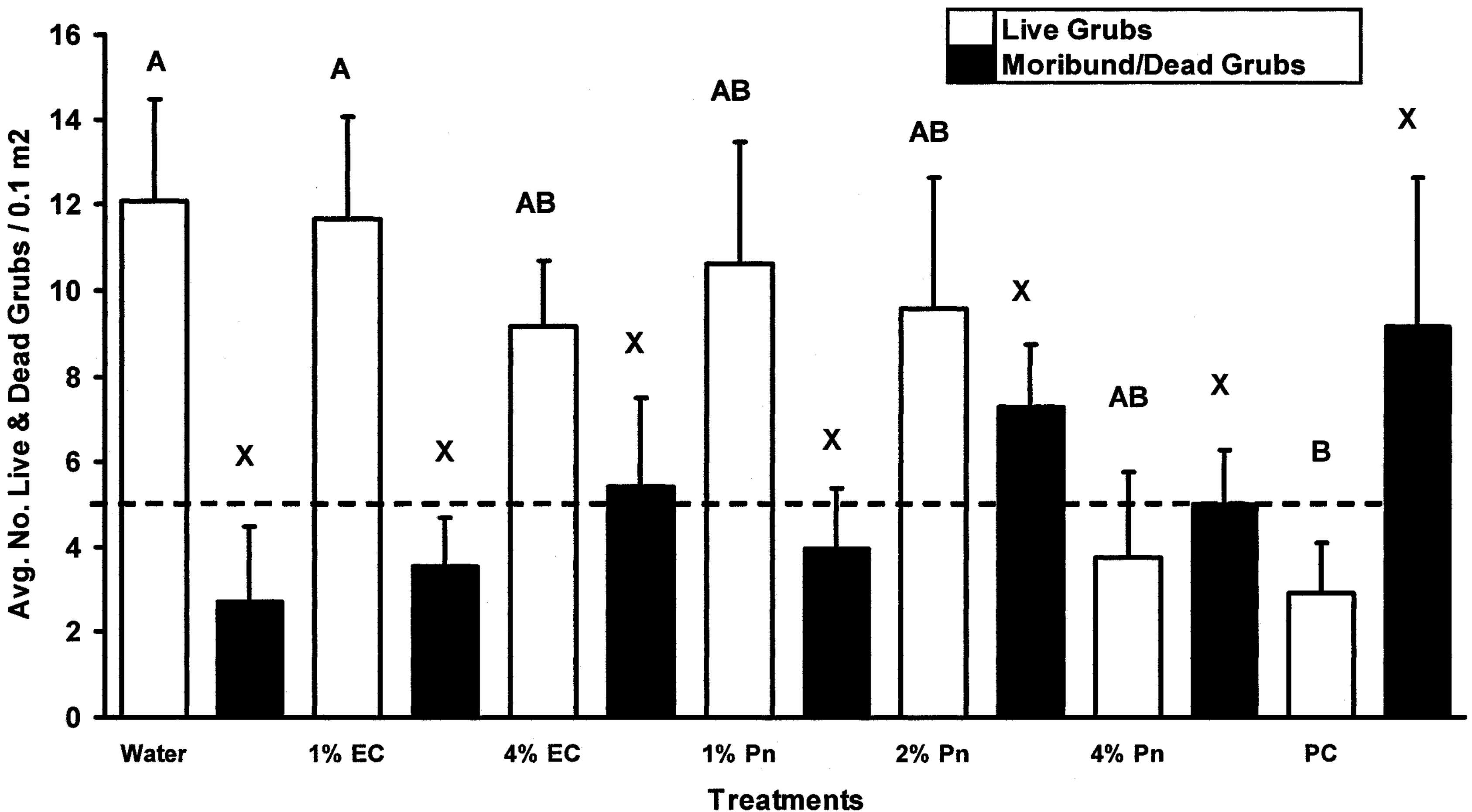


Figure 5. 2. Mean number of *R. majalis* larvae ± S.E. sampled in water control (W), formulation controls (EC1 and EC2), *P. nigrum* (Pn) and positive control (PC) treated plots at Grenfell Glen, Ottawa, on 30 September 2002. The dashed line indicates threshold for damage in non-irigated turf (5 grubs / 0.1 m^2). Statistical differences between treatments and water control were evaluated by Tukey's multiple range test (P=0.05).

5.3.5 Non-target invertebrates

5.3.5.1 Earthworms

The number of earthworms (Annelida: Lumbricidae) found between treated plots was significantly different (F=3.103; df=6,35; P=0.015) seven days after the application in September (Figure 5.4). There were significantly fewer numbers in the 4% *P. nigrum* treatment compared to the water control (Tukey's multiple range test, P=0.031) but no there was no difference between the control and positive control (Tukey's multiple range test, P=0.084). No other statistical differences were determined between treatments.

5.3.5.2 Other non-target invertebrates

In the Ottawa 2002 field trial, several other species of Coleoptera larvae, including the Scarab *Phyllophaga* spp., were identified from plot samples. Other soil invertebrates included several species of ants (Hymenoptera: Formicidae), ground beetles (Coleoptera: Carabidae), fly larvae (Diptera) and Millipedes (Diplopoda). The distribution of these invertebrates in treatment plots was not correlated with treatments (F=0.740; df=6,35; P=0.621), although in both trials, none were observed in the 4% *P. nigrum* plots (Figure 5.5). The numbers of the invertebrates sampled were low relative to the earthworms and thus do not provide as accurate an assessment of non-target effects.

5.3.6 Analysis of piperamide levels in formulated extract and soil cores

Based on the total piperamide concentration measured in the 20% *P. nigrum* formulated extracts prepared for the Ottawa field trial, there was approximately twice as much active material compared to the batch used in Guelph (F=90.636; df=2,11; P=0.001) (Table 5.5). The low recovery of piperine from seed material used in the August trial may have been responsible for the lower level measured in those batches.

The half-life of piperine in soils from the two trials was calculated from the natural log concentration lines for the first week after application (Figure 5.6). The piperine $t_{1/2}$ for the August and September treatments was 1 and 2.6 days respectively. The concentration of piperamides in the top 5 cm

of soil in the 4% *P. nigrum* treated plots two weeks after application in the September field trial was 1.3% of the initial concentration (Table 5.6).

On average the level of piperine was higher initially in the soil of the 4% *P. nigrum* plots in the September compared to August field trial (466.3 $\pm$ 36.3 versus 345.5 $\pm$ 3.3 µg/g respectively), reflecting the concentrations determined in the formulation batches applied in each case. Recovery of piperamides from the field-collected soil spiked with *P. nigrum* at 0.5% ($^w/_w$) was greater than 83% and comparable to the recovery from the artificial greenhouse soil (Table 5.4).

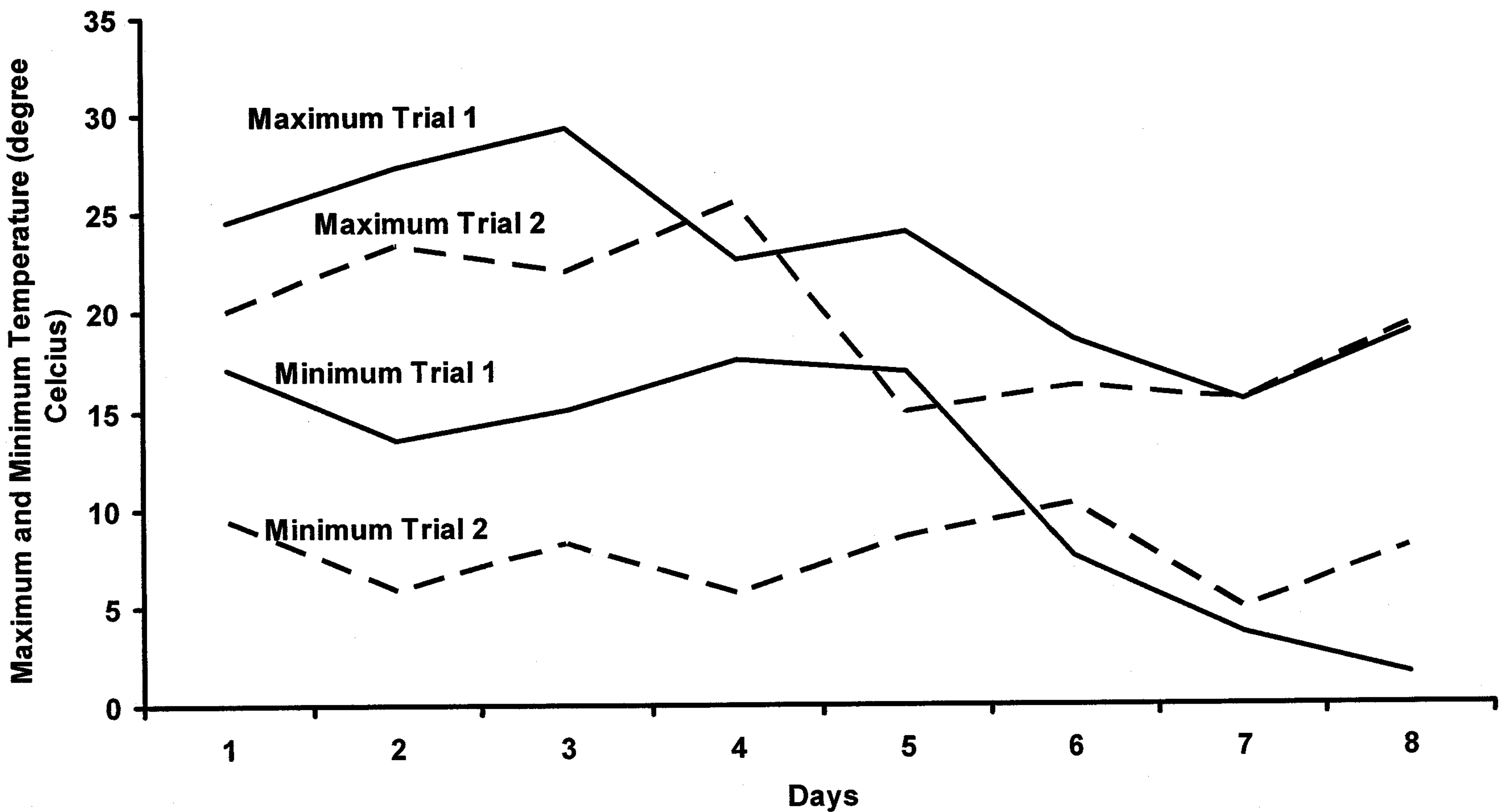


Figure 5. 3. Minimum and maximum air temperatures at Guelph Turfgrass Institute during August / September 2000 field trials and at Grenfell Glen park, Ottawa, during October 2002 field trial.

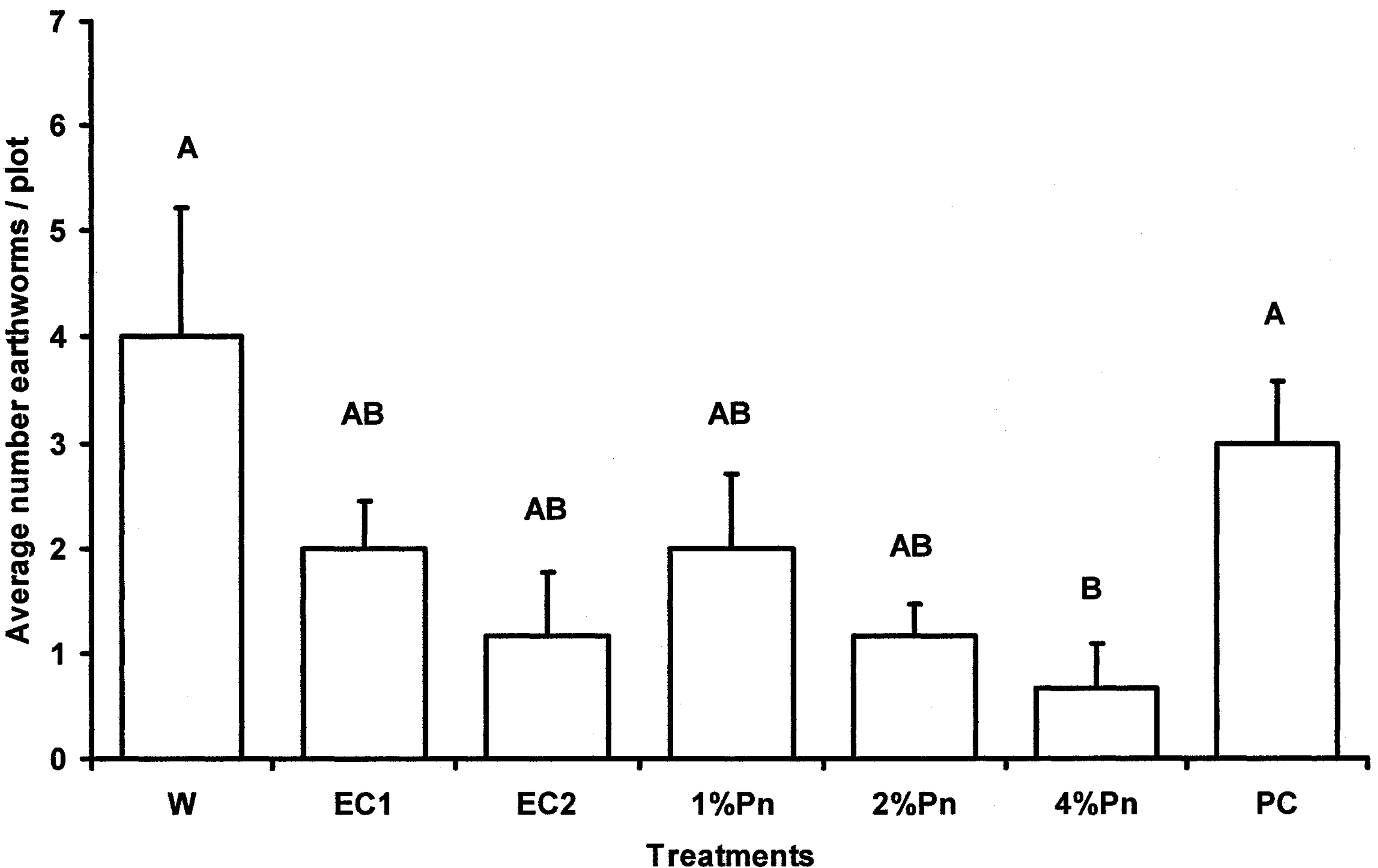


Figure 5. 4. Mean number of earthworms ± S.E. sampled in water control (W), formulation control (EC1 and EC2), *P. nigrum* (Pn) and positive control (PC) treated plots at Grenfell Glen park, Ottawa, ON, on 30 September / 1 October 2002. Statistical differences between treatments and water control were were evaluated by Tukey's multiple range test (P=0.05).

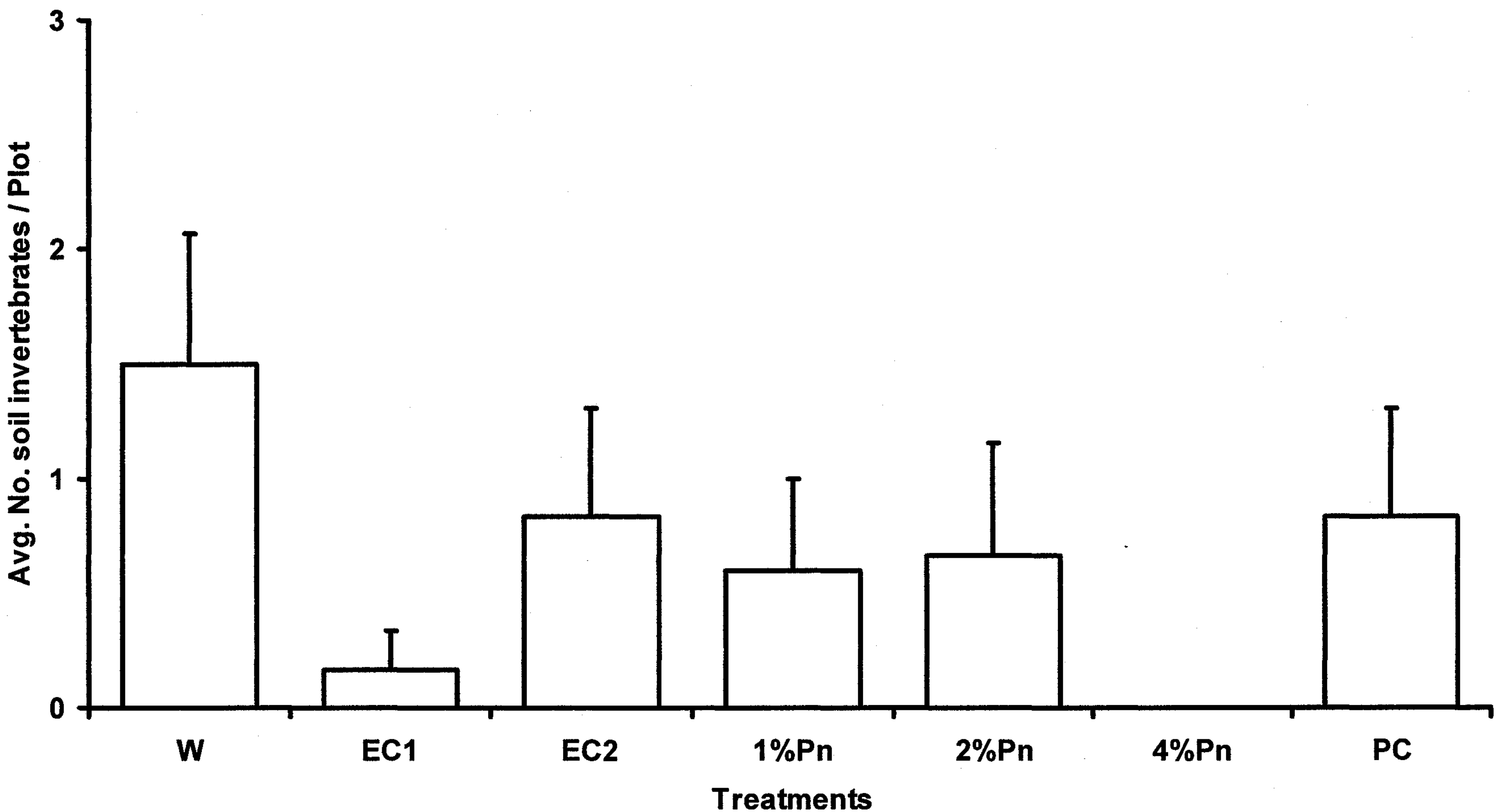


Figure 5. 5. Mean number of invertebrates ± S.E. sampled in water control (W), formulation control (EC1 and EC2), *P. nigrum* (Pn), and positive control (PC) treated plots at Grenfell Glen park, Ottawa, ON, on 30 September / 1 October 2002. No significant differences in invertebrate numbers were determined between treatments (one way ANOVA, $P>0.05$).

Table 5. 5. Mean (± S.E.) and total piperamide concentrations in 20% *P. nigrum* and *P. guineense* formulations and percent piperine (± S.E.) recovered from seed extract.

20% *P. nigrum* Formulation Batch	**Piperamide Concentration (mg/mL)**					**Piperine Recovery**
	DHPLG (S.E.)	**PLG (S.E.)**	**DHP (S.E.)**	**Piperine (S.E.)**	**Total (S.E.)**	**% (S.E.)**
Aug. 2000	0[a]	0.69[c]	0.07[f]	34.3[i]	35.0[l]	17.1[o]
P. nigrum		(0.03)	(0)	(0.18)	(0.19)	(0.09)
Aug. 2000	2.50[b]	2.29[d]	5.74[g]	15.7[j]	26.2[m]	7.80[p]
P. guineense	(1.06)	(0.02)	(0.35)	(0.18)	(1.10)	(0.09)
Sept. 2002	0[a]	0.56[e]	2.84[h]	56.6[k]	60.0[n]	28.3[q]
P. nigrum		(0.02)	(0.13)	(3.47)	(3.37)	(1.73)

DHPLG = 4,5-dihydropiperlonguminine; PLG = piperlonguminine; DHP = 4,5-dihydropiperine.

[a] Piperamide concentrations with the same letter are not significantly different between formulation batches (Tukey's P>0.05).

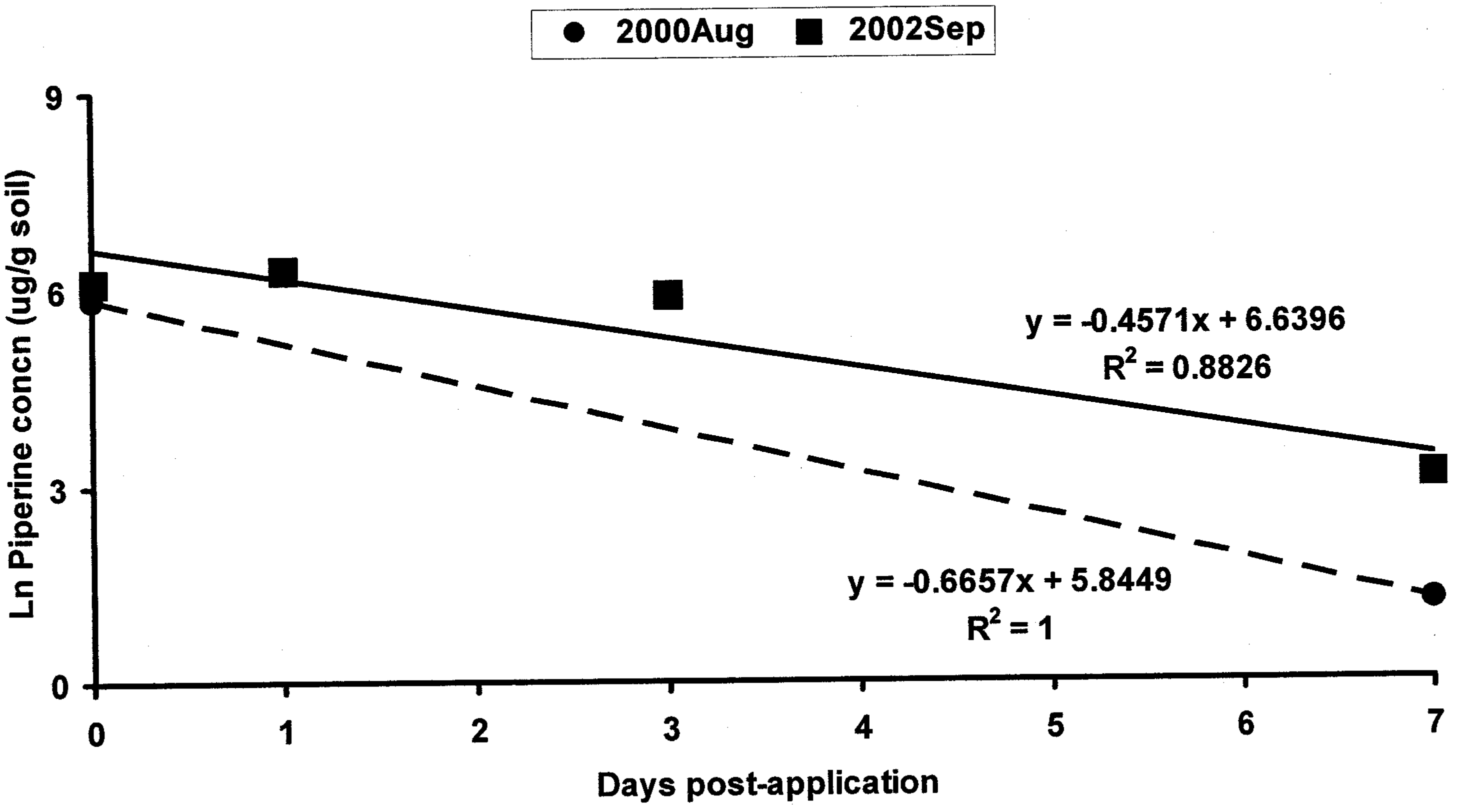


Figure 5. 6. Soil concentrations of piperine sampled during two field trials, August 2000 (dashed line) and September 2002 (solid line), 1, 2, 4 and 7 days post-application with 4% *P. nigrum* extract.

Table 5. 6. Average piperamide concentrations (± S.E.) and percent of initial residue remaining in soil samples collected from 4% *P. nigrum* treated plots at Grenfell Glen Park at start of September field trial and 2 weeks post-application.

Sampling date	**Piperamide Concentration (μg/g soil)**			
	DHPLG	**PLG**	**DHP**	**Piperine**
Sept. 23	0.9 (0.05)	4.1 (0.21)	24.1 (1.47)	466.3 (36.34)
Oct. 07	0 (0)	0 (0)	0.9 (0.5)	6.9 (1.37)
Percent remaining	**0 (0)**	**0 (0)**	**4.3 (2.5)**	**1.4 (0.3)**

DHPLG = 4,5-dihydropiperlonguminine; PLG = piperlonguminine; DHP = 4,5-dihydropiperine.

5.4 Discussion

Mid-season (late August-early September) treatments with 2% *P. nigrum* were shown to effectively reduce the number of *R. majalis* 2nd and 3rd instar larvae (Figure 5.1), with initial soil residue levels of approximately 170 μg/g soil, half the concentration measured in the 4% *P. nigrum* plots (Figure 5.6). Later in the season, applications of 2% extract, with soil concentrations between 230 and 300 μg/g, were not effective since grub numbers were no less than in control plots (Figure 5.2). This reduction in efficacy was predicted based upon the trials with other insecticides (Cowles and Villani 1996) where older grubs were more difficult to control than those found mid-season. Similarly, the efficacy of all products is reduced because the larval feeding slows as ambient temperatures drop. However, the field treatment results were within the confidence limits for *P. nigrum* extract median lethal toxicity: greenhouse trials determined that the LC_{50} for 3rd instar *R. majalis* larvae was 2.5% *P. nigrum* extract (Table 5.1). The environmental conditions at the site and during the field trial are a more realistic test of the extract's activity; therefore it is encouraging to note the similar level of mortality within the 2-4% extract range. A second positive observation was that no phytoxicity to turfgrass was associated with this treatment.

During the late season reactive treatment, there were no significant reduction in earthworm numbers in the 2% treated plots (Figure 5.4). Therefore timing of an application of pepper material in the mid-season is important to reduce the non-target effects. At the higher soil concentrations required to control the older *R. majalis*, earthworm numbers were reduced since the residue levels were between 400 and 500 μg piperine /g soil initially. This was predicted by the seven-day *P. nigrum* median lethal toxicity value for *E. fetida* of 300 μg/g (Table 5.2). Of course, a bioassay setup where the earthworms could not escape the test containers would increase the exposure and the toxicity compared to a field setting. In fact it was observed that earthworms in pepper plots were actively moving along the surface, attempting to escape from the treated soil. But because earthworm numbers in the 2% treatments after seven days were no different from those of the controls, it indicates that residue levels are either below the toxic threshold or decrease quickly below this threshold since the soil half-life is four to six days. Grub movement could

also be expected; however this occurred less than with earthworms since the number of dead and moribund grubs sampled in the 4% plots indicated that the treatment was causing mortality.

If spot applications are used for field treatments for *R. majalis* or other Scarab larvae, earthworms may well be able to escape or avoid the treated area because of the repellent effect observed in other organisms (Scott *et al.* 2004). Permanent loss of earthworms would be detrimental to the turf ecosystem since they are important to soil mixing and reduction of thatch, but treated spots may be rapidly re-colonized (Potter *et al.* 1990). Most of the insecticides currently registered showed some negative effect to earthworms, Collembola, ants and cryptostigmatid mites. Diazinon, an organophosphate, is still one of the most widely used turf insecticide, but is being phased out in Canada. It was one of the better insecticides with respect to grub control (Villani *et al.* 1988), but non-target effects still reduced earthworm populations 40-60% at ¾ of test sites (Potter *et al.* 1990). Imidacloprid was found to have a low impact on beneficial invertebrates such as mesostigmatid mites and Collembola compared to the carbamate, bendiocarb. However, a short-lived suppression of the earthworm population after imidacloprid application has been noted (Kunkel *et al.* 1999), and there may be a sublethal effect on earthworms, as imidacloprid was demonstrated to increase the percentage of sperm deformity at concentrations above 0.2 mg/kg in the soil (Luo *et al.* 1999).

Although the mid-September application of 4% *P. nigrum* did not have a significant effect on the number of live white grubs present in the treated plots (P=0.061), the average number of grubs found was less than the damage threshold for non-irrigated turf (Figure 5.2) In contrast, a repeat treatment in mid-October did not reduce the number of live white grubs in the 4% *P. nigrum* treatment below the damage threshold (Appendix IV). Two possible reasons may explain this difference: 1) the average daily temperatures were lower in October compared to September so larvae may have spent less time near the surface and were not as exposed to the treatment; and 2) the larvae had grown larger and older and were thus less susceptible to the treatments. The latter case is suspected since it has been shown that European chafer grubs are relatively insensitive to fluctuating temperatures (Villani and Wright in Villani and Nyrop 1991)

The soil residue analysis from Grenfell Glen indicated that the *P. nigrum* extract detected is less than 5% of the initial concentration or no longer detectable in the top 5 cm of soil two weeks after application with the highest treatment (Table 5.6). The difference in recovery and soil half-life between the August and September trials is likely due to the combination of lower average temperatures and shorter light period that occurred during the 2nd trial period. In aqueous medium and soil, pesticides will hydrolyze, oxidize or form isomers and when in the upper soil layer degradation will be accelerated through photolysis (Wamhoff and Schneider 1999). Therefore, based on previous exposure of piperamides to sunlight (Chapter 3), photodegradation is a major factor in the degradation of residues at or near the soil surface.

In the soil, the lipophilic nature of piperamide components will lead them to bind with organic material, and chromatographic analysis suggests that water alone does not leach the material to a greater depth in the soil. In the case of imidacloprid, sorption of parent compound and metabolites was found to increase with soil organic carbon (Cox *et al.* 1997). Similarly, the high rate of recovery of piperamides one week post-application in the low UV, greenhouse controlled environment (Table 5.3) suggests these compounds are not being washed from the soil through watering. However, field conditions, even with lower average soil temperatures, appear to degrade the materials at a faster rate (between 3 and 5 days); thus it is likely that the active components have been degraded through either photolysis or microbiological activity.

The positive aspect of these findings is that the residue of *P. nigrum* extracts will not remain in the soil much longer than is necessary to control the white grub larvae. This is important from the standpoint of reducing the exposure and thus the risk of biopesticide use. In comparison, the half life of the positive control, diazinon, is two to four weeks in the soil (EXTOXNET 1996a), while imidachloprid is 48 to 190 days (EXTOXNET 1996b), which extends the exposure time significantly longer than the piperamides. Further persistence in the environment has been documented in the case of diazinon where residue levels in surface and groundwater were a concern in Ottawa, Ontario (EXTOXNET 1996a).

Diazinon was more effective than the 1 and 2% *P. nigrum* formulations for controlling the white grubs and also did not reduce the number of earthworms in the treated plots compared to the 4% treatment. However, diazinon does pose a risk to other non-target species such as birds (LD_{50} = 2 to 40 mg/kg), fish (LC_{50} = 3 mg/L) and honeybees (EXTOXNET 1996a). The U.S. Environmental Protection Agency (EPA) and Health Canada Pest Management Regulatory Agency (PMRA) are currently reviewing diazinon due to the risks it may pose not only to wildlife but human health (E.P.A. 2000; P.M.R.A. 2001). Imidacloprid has similar mammalian toxicity to diazinon (EXTOXNET 1996a), but because of its greater systemic properties, it can be applied at lower application rates. The disadvantage is the lack of effect it has on the third instar grub, therefore it is best applied during or before the 1st instar, a potentially expensive proposition since it is difficult to predict the areas necessary for treatment (Koppenhöffer and Kaya 1998). There is no present evidence to suggest that piperine or other piperamides have the same level of avian toxicity as diazinon. The repellent activity associated with black pepper will likely preclude any large ingestion by birds or mammals. However, since it has not previously been considered for turf insect control or use in the environment, more studies are required.

Based on medicinal and culinary uses, both past and present, and since they have been shown to possess low mammalian toxicity, Piperaceae extracts were selected for the white grub efficacy trials. Extracts of black pepper contain high proportions of piperine and other active piperamides that have been applied for use in several developing regions (Arnason *et al.* 2002). Black pepper is considered a food grade spice and categorized by the United States Food and Drug Agency (FDA) as "Generally Regarded as Safe" or G.R.A.S. However, piperine is listed on the EPA's Toxic Substances Control Act (T.S.C.A.) inventory, based upon concerns of ingestion, inhalation and contact with skin and eyes (Sigma-Aldrich 2002). Thus the greatest risk will be from irritation of skin and eyes and an applicator should take steps to avoid such contact when spraying.

The application of low-risk, no-residual-effect botanical products for insect control requires that they be part of an established integrated pest management (IPM) program. This includes developing a proper identification of the pest along with a diagnostic procedure, and monitoring injury and damage

levels. Monitoring of populations using light traps and volunteers can predict where adult activity is high, but not necessarily the site where larval populations will be a problem; therefore treatment of the early larval stage is difficult due to unpredictability of adult activities. As well, it is difficult to monitor the early stages of the *R. majalis* lifecycle since the larvae are small, delicate and often move down in the soil column for moisture. Early instar larvae less than a month old may not feed on grass roots but rather will choose any organic matter (Potter 1998).

Currently, efforts are directed at monitoring damage late in the season after grubs have matured and caused turf damage. Early and mid-season monitoring would allow time to implement reactive treatments while grubs are still susceptible. Watering strategies will have to be scheduled in order to ensure that any applications are followed by adequate moisture to enable the treatments to be effective. Moisture in the soil before, during, and following these applications is necessary to ensure that: 1) contact with extracts and grubs is maximized and 2) grubs remain close to the surface and do not move away from extracts. Because of the impact upon non-target invertebrates, it is suggested that monitoring for grubs take place before damage is evident, for example in mid to late August, and then spot treatments be applied to the highest density grub areas. This will ensure that the more susceptible stage of the grub lifecycle is targeted, and that lower concentrations will be effective.

The use of cultural practices and entomopathogenic nematodes may reduce the damage associated with moderate grub populations. Again these steps require adequate moisture to ensure both a vigorous root growth and a healthy nematode population. Perhaps a multifunctional approach between nematodes and a *Piper* botanical could prove equally effective as previously demonstrated with imidacloprid (Koppenhöffer and Kaya 1998). A further long-term investment in each field or park is necessary through top-dressing, fertilizing, aerating, watering and other turfgrass management steps (GTI 2003; OMAFRA 2003). In summary, our results indicate that *P. nigrum* botanical extracts can offer an alternative to synthetic chemical insecticides when high pest densities have overcome the management practices and the use of biological controls.

CHAPTER 6

GENERAL DISCUSSION

6.1 Pepper botanicals as insecticides

The present work determined that selected species in the pepper (Piperaceae) family have great potential to provide botanical extracts as an alternative method for home gardeners and organic farmers to control outbreaks of common insect pests. From the start, the basic premise was that food-grade plant material offers a safe but effective botanical insecticide. In the case of Piperaceae, there is a long association with culinary and medicinal use as well as recognized insecticidal properties. The unwavering focus of the project was to work with the whole plant extract, thus ensuring a starting material which was already acceptable as a food-grade product generally regarded as safe (G.R.A.S.). The intent was also to facilitate the registration process that has become more streamlined due to recent changes in the regulatory process in the United States and with similar changes on the horizon in Canada.

The first step was the development of a convenient method to extract and analyze by a validated HPLC-MS method the active components found in pepper species (Chapter 2). This method was used to compare different populations of the Central American *P. tuberculatum* (Scott *et al.* 2002), where the recovery of active ingredients compared well to other analytical methods (Kulkarni *et al.* 2001). A novel element of the project was the use of less toxic solvents in the extraction process (ethyl acetate instead of hexane or methylene chloride) and the concentrated extract material was formulated with constituents considered safer for the applicator and the environment. Since only one piperamide is available commercially, it was necessary to synthesize several amides found in three active species: *P. nigrum*, *P. guineense* and *P. tuberculatum*, to provide standards for the HPLC method used in Scott *et al.* (2002 and 2003). An HPLC method was determined using a binary gradient of acetonitrile and water that allowed for a clean separation of peaks and a standard curve between 1 and 250 µg/mL in ethanol for each amide had a detection limit of 2 ng. The detection limit of *Piper* compounds was reduced to 0.2 ng with the use of the LCMS.

Applying the previous extraction and analytical method, a survey of commercially available pepper determined no differences in total levels of piperamide compounds among germplasm sources, however individual compounds differed significantly (Chapter 2). This suggested that peppercorn from black pepper or leaf material from *Piper tuberculatum* may be obtained from several growing regions or suppliers without major differences in total active components. More importantly, it was determined that the extraction technique is the step which can optimize the level of piperine content in the final extract to comparable levels found by another group (Kulkarni *et al.* 2001).

The analytical method was used to verify that piperamides are associated with the activity in the *Aedes atropalpus* mosquito bioassay. The results confirmed previous surveys which found that, of the neotropical *Piper* species, *P. tuberculatum* was the most active, in comparison to *P. nudifolium*, *P. cordulatum*, *P. aquale*, *P. biseriatum, P. pseudo-lindenii* and one unidentified Costa Rican species (Chapter 2). Only those species with piperamides present in the extracts were found to be acutely toxic in the range of 1 to 10 ppm, while few to no piperamides were identified in the inactive species. The benefit of this finding is a source of plant material which can provide biological active compounds for botanical insecticide development similar in activity to West African *P. guineense* and Asian *P. nigrum*.

Based on efficacy trials with common insect pests of northeastern North America, recommendations were made regarding the type of insect, species and life-stage which would be a suitable target for these extracts (Chapter 3). The conclusion was that, of the insect species tested, larvae of selected Lepidoptera and the European pine sawfly were susceptible target species. It was established that they could be controlled with extract concentrations that were lower than were required to control larvae and adult Coleoptera. As well, it was determined *P. nigrum* extract concentrations below 0.1% would be less likely to harm the lady beetle, a beneficial predator and biocontrol insect.

Cuticle penetration is considered the likely factor influencing the overall susceptibility of each insect because the mortality rate was greater when both ingestion and contact exposure occurred rather than ingestion alone (Chapter 3). It was important to document that when the pepper botanicals were applied at sublethal concentrations, they exhibited a repellent and antifeedant effect, even though under

normal summer sunlight conditions, this effect would not last longer than two to three hours on the plant and one to three days in the soil, as the active ingredients were degraded by UV radiation exposure (Chapter 3). It can be concluded that, with the current formulation, the pepper extracts would not provide much residual activity, but because of the rapid degradation under those conditions, there would be less concern regarding long-term toxicity to humans or the environment, compared to many conventional synthetic insecticides.

It was determined through soil bioassays conducted with European chafer larvae in a prepared soil medium that it would be practical to test the same concentration range under field conditions (Chapter 5). Results obtained from three field trials concluded that pepper botanical applications in the range of two to four percent would reduce the population of grubs below the damage threshold. This information encourages the application of the pepper botanical for control of the most destructive turfgrass insect pest in the Ottawa area. The timing of this work appears equally favorable because of the current intense political pressure to re-evaluate the use of lawn and garden pesticides and provide alternatives.

A further important finding was that piperamides in the soil would degrade quickly, ensuring that non-target invertebrates such as earthworms could re-colonize the treated areas within one week after application (Chapter 5). However, non-target invertebrates which were exposed to a direct application with the four percent extract would be repelled from the treatment area or would also suffer the fate of the target insect. The findings indicate that the pepper extracts, although possessing a much shorter half-life in the soil compared to the conventional synthetic insecticides, diazinon and imidacloprid, have relatively greater toxicity to non-target invertebrates. It is recommended that these formulations should only be applied as spot treatments, and not broadcast as other conventional treatments are used. These treatments would be practical when applied as part of an integrated pest management approach to controlling European chafer.

The findings of this project also shaped a better understanding of the mode of action of piperamides and how this relates specifically to the insect. It was previously known from studies with mammalian cell cultures that the most recognized piperamide, piperine, found in the three *Piper* species

studied, was responsible for both inhibition and induction of monoxygenase enzymes (Reen and Singh 1991; Singh and Rao 1993). In pharmacological terms, this means that ingestion of pepper can produce both enhanced or reduced detoxification and metabolism, a critical concern when one considers interactions with drugs in the body. This information was of interest to this project as it would suggest that similar activity in insect detoxification enzymes could enhance the toxic effect of the extract material. Based on the structure of the piperamide molecule, it had already been suggested that the attached methelenedioxyphenyl (MDP) moiety produces a bifunctional compound: both a neurotoxin and PSMO enzyme inhibitor (Scott *et al.* 2002). Previous studies with insects indicated piperamides with and without the MDP had different levels of activity: the neurotoxic effect is sustained when the MDP group was present (Gbewonyo *et al.* 1993). Therefore, it was hypothesized that the piperamide was directly inhibiting the insect polysubstrate monoxygenase (PSMO) enzymes, allowing for greater activity of the amide neurotoxin. This was tested by applying both extracts and piperine to the cuticle of the insect and measuring the dose-dependent rate of metabolite depuration and PSMO activity. It was determined from the toxicokinetic study with the American cockroach that the amount of piperine and piperine metabolites depurated over a 12-h period post-dosing was inversely correlated with the piperine dose the insect received (Chapter 4). At the same time, microsomal enzyme activity measured from piperine-treated insects had lower cytochrome P450 and b5 levels than control insects. This confirms that the inhibition of PSMO activity plays an important role in the mode of action within the time required for acute toxicity to occur.

Further study of PSMO inhibition by piperine within insects investigated the *in vitro* effect of microsomes prepared from the pyrethroid-resistant housefly (Chapter 4). The median inhibitory concentration (IC_{50}) of piperine for *O*-demethylase activity, induced in the pyrethroid-resistant LPR strain housefly, was within the same range as the IC_{50} determined for piperonyl butoxide, the insecticide synergist. These encouraging results not only further established the importance of PSMO inhibitory activity of piperine in the insect, but also provided evidence that these extracts could be effective controls against insect strains that had resistance to other insecticides through physiological tolerance. A laboratory

and greenhouse investigation with a strain of multi-insecticide-resistant Colorado potato beetles confirmed, with both *P. nigrum* and *P. tuberculatum*, that larvae could be controlled at the concentrations used for insecticide-susceptible strains (Chapter 4). This activity will be of great value not only in solving current resistance problems but also in increasing the length of time that is required for insect populations exposed to *Piper* extracts to develop resistance to piperamides. Partially responsible is the mixture of piperamide analogues that were determined in equi-molar concentrations of tertiary and quarternary combinations to produce greater-than-additive effects or potentiation in the *A. atropalpus* bioassay (Scott *et al.* 2002). The fact that one of the piperamides used in the combination was non-toxic in the dose range tested implies a certain level of synergism between these compounds, referred to as analogue synergism.

In summary, the three species of pepper investigated have sufficient advantages and should be considered further for development into botanical insecticides. Efficacy trials (Chapter 3) compared the pepper botanicals to several other commercially available botanical products (neem oil, lemongrass oil and garlic) and established that pepper was as effective or better in certain cases. Further comparisons are warranted however; based on current knowledge, pepper extracts can be judged to have certain advantages over other commercial botanicals not tested in the present work and those which are still at the experimental stage (Table 6.1). The advantage pepper has over pyrethrum is that it does not require piperonyl butoxide to synergise the activity due to the PSMO inhibition of piperamides. It is faster acting than neem botanicals and lasts longer than essential oils.

Table 6. 1. Comparison of pepper botanicals with other registered and experimental botanical insecticides.

Botanical: Registered	Actives	Mode of Action	Mammalian Toxicity	Non-target Toxicity
Pyrethrum Chrysanthemum cinerariaefolium Asteraceae	Pyrethrin esters: Pyrethrin I and II	Neurotoxic: affects Na channels, causes knock-down	[1]Low: $LD_{50} > 1$ g/kg Allergic response [ie] Contact dermatitis	[1]Fish are sensitive: 96 h $LC_{50} = 9 - 50$ ppb; [2]not selective
Derris *Lonchocarpus* Leguminosae	Rotenone	Respiratory inhibition: Site I, mitochondrial electron transport	[3]Possible link with Parkinson's	Used traditionally as piscicide: very toxic to fish
Neem *Azadirachta indica* Meliaceae	Azadirachtin	[4]Growth reducer and antifeedant; acts on neuroendocrine regulation of hormones	[5]Low toxicity, used medicinally	[2]Selective to herbivores, low toxicity to bio-control agent
Essential oils *Eugenia caryophyllata* Myrtaceae	Monoterpene Eugenol	[6]Neurotoxic: blocks octopamine receptors	[6]Low toxicity since octopamine specific to arthropods; not suitable with odor sensitivity	[6]Not specific, recommended as fumigant

Table 6.1 (Cont'd)

Botanicals: Experimental	**Actives**	**Mode of Action**	**Mammalian Toxicity**	**Non-target Toxicity**
Piper botanicals Piperaceae	Piperamides	Neurotoxic: affects Na channel, acts as antifeedant, PSMO inhibitor	G.R.A.S., potential for dermal and eye irritation	Not specific, unlikely to affect domestic animals or wildlife
Photoactivated thiophenes Marigolds Asteraceae	α-Terthienyl	Photooxidants through production of singlet oxygen under near-UV light	[7]Rat LD_{50} = 110 mg/kg; dermal irritation	[8]Mosquito larvicide use does cause invertebrate drift
Asarones *Acorus calamus*	(Z)- and (E)-asarones from essential oil	[9]Feeding and growth inhibiting effects, antigonadal activity, chemosterilant	[9]*In vivo* carcinogenic effect, *in vitro* mutagenic activity, chromatid induction	[9]Recommended as a fumigant, not specific to pest insect
Asimicin Annonaceae	Acetogenins: Adjacent *bis*-tetrahydrofuran (THF) rings	[10]Inhibitors of mitochondrial NADH in complex I of electron transport	[10]Low toxicity, no mutagenic effects, weak skin sensitizer	[10]Not specific, but recommended for bait use

[1] TOXNET(2003), [2]Simmonds *et al.* (2002), [3]Betarbet *et al.* (2000), [4]Rembold (1989), [5]Saxena (1989) [6]Enan (2001), [7]Marles *et al.* (1995), [8]Kumar *et al.* (2000), [9]Park *et al.* (2003), [10]Alali *et al.* (1998).

Pepper extracts are also considered to have low mammalian toxicity and lack the health concerns associated with Rotenone. In comparison to other experimental botanical compounds currently under development, *Piper* extracts are not effective at the same concentrations as α-terthienyl, asarones or asimisin. However, the *Piper* materials do have lower mammalian health risks than some of asarones, but share similar issues regarding non-specificity as do α-terthienyl, and asimicin. These differences suggest that each will have an advantage in specific applications: asarones for fumigant use, asimicin in insect baits and *Piper* botanicals on fruits, vegetables and ornamentals.

6.2 Piperamides as natural defences: role in plant herbivore interactions

These findings raise the question of the function of piperamides in the plant defense strategy. In the case of *P. tuberculatum*, are the levels of piperamides present in leaf tissue great enough to afford a protection from herbivores and/or disease? The findings of this study and a previous one (Bernard *et a*l. 1995) determined that piperamides are present at levels that are physiologically important to the insect. The concentrations of piperamides found in *P. tuberculatum* leaf material were greater than 0.2% of the leaf dry weight (Chapter 2) and feeding bioassays determined that at 0.4% in the diet *P. tuberculatum* reduced growth and survival of a generalist herbivore (Bernard *et al.* 1995). Since there must be a cost to the plant to produce them, these results suggest that protection against herbivores may offset this cost.

Based upon the dual biological activity of piperamides, these compounds present an efficient use of resources by the plant: multiple analogues of a toxic compound with PSMO enzyme inhibting activity. The importance of PSMO enzymes in insects relates not only to metabolism of xenobiotics but catabolism of hormones and other precursors in critical biochemical pathways during development stages (Scott *et al.* 1998). Disruption of enzymes at these stages could lead to a number of different outcomes for the insect, the least disruptive of which could be delayed development. In addition the antifeedant activity attributed to these extracts suggests it is also a factor in the *Piper* defense strategy. The studies in this thesis are directed toward house and garden insect pests and further work is needed in the field with real herbivores of *Piper* spp..

A number of separate ecological studies have identified a cross-section of strategies employed by *Piper*–herbivore systems which also relate to the species examined in the current research. Early work by Miyakado *et al.* (1979, 1980, 1983), Su and Horvat (1981) and others documented the insecticidal activity of the extracts of black pepper and Guinea pepper and their usefulness in tropical regions of the world. Similarly, ecological studies by Marquis (1991, 1992), Hodge *et al.* (1998), Letourneau (1998) has documented the unique relationship of *Piper* species to specialized insect fauna. It would be interesting to study the specific interactions of *P. tuberculatum* in Costa Rica with its unique insect fauna, as well as related species of Piper and their fauna. This might shed some light on whether the plant-insect relationships are examples of co-evolutionary theory (Ehrlich and Raven 1964) such as parallel cladogensis or perhaps sequential evolution through host switching (Jermy 1976). Any case study of insect specialists is a practical question to ask in this forum since the choice of any *Piper* species for use as an insecticide would benefit from some foresight into types of specialist insects feed on pepper plants and their strategies for adaptation.

Chemical redundancy (Jones and Firn 1991) and analogue synergism (Berenbaum and Zangerl 1996) were both put forward as occurring in *P. tuberculatum* (Scott *et al.* 2002). The mixture of similar amide compounds with comparable biological activity fit well into the analogue synergism theory that plants produce many like compounds in order to confound the herbivore by synergist action, and by reduction of the ability of the insect to become resistant (Feng and Isman 1995). This theory has not been tested elsewhere in the Piperaceae, and has not been tested with an adapted insect hervivore on *P. tuberculatum.*

In Costa Rica, a positive correlation between larger and more abundant *Piper* species and greater herbivore richness has been observed (Marquis 1991). When two *Piper* species of same size and equal abundance were compared, there was no difference in damage to leaves produced by the respective herbivore fauna even though one of the plants had 10 times as many leaf-chewing herbivore species. The pepper with a low number of herbivores may have toxic defenses to which only a few specialists are adapted. The species with more herbivores may have less toxic defenses allowing oligo and polyphagous

herbivores to feed. In another case which examined the effect of concentrated folivory on localized seed production in *P. arieianum* (Marquis 1992), it was concluded that the induction of secondary metabolites in injured parts of the plant caused herbivores to move, thus dispersing damage and changing the pattern to minimize the detrimental reproductive effects. Both of these examples suggest phytochemical defenses are relevant in the field.

The hypotheses that *Piper* species can induce chemical defenses was tested using the New Zealand pepper tree *Macropiper excelsum* and its main insect herbivore *Cleora scriptaria* (Walker) (Lepidoptera: Geometridae), previous leaf damage did not appear to induce chemical defences in order to limit feeding (Hodge *et al.* 1998). A regular distribution of damage between and within leaves was noted however, suggesting that the larvae feed for short periods and move on. This could be due to a number of factors including: 1) interruption in feeding for digestion of toxins; 2) avoiding predators; 3) uniform distribution; 4) a legacy of moving to cope with the defences of earlier plant hosts or 5) avoiding competition. Further work with this system showed that previous leaf damage in *M. excelsum* did not affect the feeding of either the pre-adapted *C. scriptaria* or another polyphagous larvae, the lightbrown apple moth, *Epiphyas postvittana* (Walker) (Hodge *et al.* 2000a), and *M. excelsum* did not compensate for leaf damage by increased photosynthetic capacity through increased chlorophyll content (Hodge *et al.* 2000b). The conclusions were that the *C. scriptaria* / *M. excelsum* system is one of co-evolution since the plant can function with consistent and regular distribution of damage (Nishida *et al.* 1983). Similarly, the insect can survive by feeding on the plant tissue despite the presence of insecticidal and antifeedant compounds, one of which has been identified as a juvenile hormone mimic with a methylenedioxyphenyl ring. Therefore, if chemicals are induced in the plant, the effects on the herbivore are limited to a behavioural response rather than effects on population dynamics

A second well studied insect-plant relationship in the Piperaceae is the ant-plant species. Small ants live and feed on several species of *Piper*, but the mechanisms by which the ants afford a net fitness to these plants were at first not as obvious as the advantages provided to the ants. Letourneau (1998) assumed that the relationship was mutualistic because of the obligatory nature of the interaction for the

forel ants, *Pheidole bicornis* (Hymenoptera: Formicidae: Myrmicinae), and the special morphological features of the plants. These features include hollow stems, and the adaxial margins of the sheathing leaf-base are closely contiguous, thus producing a petiolar chamber which can be inhabited by the ants (Burger 1972). The ants can get into the hollow stems by removing soft tissue thus opening an aperture at the base of the leaf-axil. The ants are attracted to the plants because, in the presence of *P. bicornis*, opalescent food bodies rich in lipids and proteins are formed on the adaxial surface of the leaf-base sheath. These are consumed by the ants. The ants then hollow out the stem pith tissue to occupy the entire plant. Of the *Piper* ant-plants studied, *P. sagittifolium*, *P. fimbriulatum* and *P.obliquum* showed > 90% average occupancy rates (Letourneau 1998).

Studies showed that when the ant *P. bicornis* was excluded, the plants exhibited lower vigor and potential fitness relative to control plants with intact ant colonies (Letourneau 1998). This was thought to occur because the ants were responsible for decreased folivory, disruption of stem-boring weevils, the removal of spores and other minute particles from the leaves thus reducing disease on the inflorescences. In a further set of experiments, Dyer and Letourneau (1999) showed that the presence of the ants also had an indirect effect on plant size in field experiments and a direct positive effect on stem and petiole biomass in shadehouse experiments with the ant-plant *P. cenocladum.* When ants were excluded, this same plant increased the production of several dihydropyridone alkaloids, including piplartine, 4'-desmethylpiplartine and cenocladamide (Dodson *et al.* 2000). The three-fold increase in the amide content in the leaves of *P. cenocladum* was thought to show an induction of chemical defences in the absence of mechanical defences. However, the fact that the plants without ant colonies were less fit and failed to grow as well as those with the colonies was evidence that the amide production came at a higher cost than the production of food bodies. Dyer *et al.* (2001) felt that the level of amides present in the ant-defended plants was still sufficient to provide an additional line of defence against herbivores and pathogens, and further illustrates defensive redundancy.

Piper tuberculatum has a comparable defense strategy to that of *M. excelsum* relative to that observed in *P. cenocladum.* It is not known whether *P. tuberculatum* accepts the level of foliar damage

exhibited by the former *Piper* species but it does not encourage a mutualistic relationship with beneficial insects or have inducible defences like the latter *Piper* species. Based upon the findings of previous surveys (Bernard *et al.* 1995) and the current work, *P. tuberculatum* is considered to contain some of the more active insecticidal compounds found in the genus. It is assumed that these are not inducible defences since the levels of total amides were consistent between populations, although the extent to which herbivory plays a part in this is unknown. Nonetheless, *P. tuberculatum* has made a large investment in maintaining these defences at high concentrations presumably in order to decrease both herbivores and pathogens. Because these plants are shrubs and do not require abundant resources for large-scale growth, there may well be more investment made in defence compounds, even though this will be lost as leaves drop off. By producing bifunctional amides with monoxygenase inhibition capabilities, the plant has become more efficient with its resources by ensuring the compounds are retained longer by the insect herbivore, thus improving the overall biological activity. As attested by ethnobotanical use and pharmacological studies, piperamides also have an antimicrobial activity consistent with a defence strategy against pathogens and secondary infection. There is also some evidence to suggest that the methelenedioxyphenyl group confers a repellent property (Harmatha and Nawrot 2002).

The specific occurance of secondary plant metabolites varies with the species of *Piper*, although phytochemical analyses to date have shown that similar types of amides are commonly found throughout the genus (Parmar *et al.* 1997). The development of resistance to these types of compounds or adaptations in order to become a specialist on plant species which have these chemicals in them may require alterations to physiological mechanisms in the insect. Physiological mechanisms leading to resistance can 1) alter the target site, 2) increase excretion, or 3) increase metabolic detoxification and degradation by P450 monooxygenases. In the case of the first mechanism, it is most relevant in terms of the insect sodium channels, documented by the knockdown resistance (KDR) phenomenon which occurs because of a structural change at the receptor in the insect sodium channel (ISC) (Zlotkin 1999). Recent evidence suggests that KDR to pyrethroids is through mutations in the ISC gene as was observed in both the housefly and German cockroach.

Detoxification enzymes are considered "a crucial line of defence for insects coming into contact with natural plant chemicals and is often the key to adaptation by herbivores to their host plants" (Feyereisen 1999). The most important mechanism for insecticide resistance development by insects was increased detoxification mediated by the monooxygenases (Scott *et al.* 1998). In the case of furanocoumarin detoxification, P450 activities were induced approximately five fold in the black swallowtail, *Papilio polyxenes*. *P. polyxenes* also evolved PSMO enzymes with reduced sensitivity to MDP-containing inhibitors (Berenbaum and Neal 1985), and certain herbivores can detoxify bioactive secondary compounds even in the presence of PSMO inhibitors (Lindroth 1991), a fact which directly relates to potential for piperamide resistance.

Increased production and breeding of *P. nigrum* for spice export may have selected for varieties with lower leaf defence compounds. The literature reveals that insect species previously unassociated have recently become pests of black pepper (Ranjith *et al.* 1991, Devasahayam *et al.* 1994, Santhosh-Babu 1994). A monoculture of pepper increases its apparency and the overall effect is to increase the incidence of pests on these plants within a much shorter time period than would have occurred in nature. However, in the case of the beetle, *Longitarsus nigripennis* Mots., the larvae feed directly on the fruit, containing the highest amount of amide compounds, and develop normally (Santhosh-Babu 1994). In the case of *P. tuberculatum*, no breeding has occurred and yet the levels of secondary compounds appear to be influenced more by genetics rather than site variables (Scott *et al.* 2002). Therefore, this is an ideal species for investigating the plant-insect relationship between host and herbivore and the ability of the insect to adapt to the inherent chemical defences.

6.3 Recommendations for future work of ecological and insecticidal significance

Based upon the ecological studies completed to date with *Piper* species, it appears that an increased ability to deal with PSMO-inhibiting MDP compounds allows associated herbivores to consume more leaf material than related insect species without this trait. An experimental approach to prove this possibility would involve making observations of the insect assemblage associated with *P. tuberculatum* in its native

range, Costa Rica, and collecting common herbivore species to determine the level of PSMO induction in the presence of leaf material. Further observations will include the time spent by the herbivore on an individual leaf, the percent damage, the preference for leaves of different ages or species. A feeding study will be used to determine the choice preference for different leaves.

A further evaluation of the mode of action for piperamides could be pursued by studying the possible target sites. Applying what is known of pyrethroid target sites is useful since previous studies compared pyrethroid-resistant and susceptible insect strains and showed that they were equally affected by piperamides. However, as was demonstrated by Miyakado *et al.* (1989), the effect of pipercide was different on the axon of pyrethroid-resistant American cockroach compared to the pyrethroid effect. This suggests a different site of action and could be further examined by using an assay for voltage-sensitive sodium, calcium and potassium channel at rat brain presynaptic nerve terminals. This approach could provide a better understanding of how these compounds affect the insect nervous system, provide a structure-activity relationship and determine the activity of piperamides compared to pyrethroids.

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APPENDIX I

GLOSSARY

Antiperiodic	Preventing the regular reoccurrence of a disease
APCI-MS	Atmospheric pressure chemical ionization–mass spectrometry
Carminative	Antiflatulence, expulsion of gas from stomach and intestines
^{13}C-NMR	A supplement for NMR based on ^{13}C signal
Counter irritant	An irritation to relieve an irritant in another part of the body (see irritant)
CYP3A4	*Homo sapiens* cytochrome P450, subfamily IIIA, polypeptide 4
DMSO	Dimethyl sulfoxide
Febrifuge	Antipyretic, fever reducing
GSH	Glutathione, a tripeptide, source of cellular sulfhydril groups
GST	Enzymes which detoxify compounds by covalent bonding with glutathione
HPLC-DAD	High performance liquid chromatography-diode array detector
HPLC-MS	High performance liquid chromatography–mass spectrometry
IPM	Integrated pest management
Irritant	A chemical which causes a reversible inflammatory effect on living tissue at contact site
LC_{50}	Median lethal concentration
LCMS	Liquid chromatography mass spectrometry
MDA	Malondialdehyde, a product of lipid peroxidation
MROD	Methoxy resorufin O-deethylase
NMR	Nuclear magnetic resonance
PKC	Protein kinase C, calcium / phospho-lipid dependent enzymes, important in cell signaling
PSMO	Polysubstrate monooxygenase
Stomachic	Agent which strenghtens or stimulates the stomach

APPENDIX II

ASSESSMENT OF ANTIOXIDANTS AND SUNSCREENS FOR *PIPER* BOTANICAL FORMULATION

P. nigrum extract was exposed to sunlight by placing a 50 μL solution (20% extract) on a glass slide and exposing it to sunlight for 6 h at mid-day (10 am to 4 pm). The extract was combined with antioxidants (hemp oil, Chinese flavanoids) or sunscreens (titanium oxide, octyl 4-methoxycinnamate, 2,4-dihydroxybenzophenone) and similarly exposed to sunlight to assess the protective effect.

Materials and Methods

Trial 1: All antioxidants and sunscreens, at 40% (lower concentrations tested but not shown) (Figure 1).

Trial 2: Increased concentration of hemp oil and Chinese licorice flavanoids at 50 and 90% (Figure 2).

Trial 3: Tested octyl 4-methoxycinnamate (O4M) and Titanium oxide (TO) at 100 mM concentration (Figure 3).

Trial 4: Tested 2,4-dihydroxybenzophenone at 0.01, 0.1 and 1 M concentrations (Figure 4).

Results and Discussion

The flavanoids tested did not improve stability under sunlight conditions (Figure 1 and 2). The sunscreens, TO (Figure 3) and 2,4DHBP (Figure 4), at concentrations greater than 100 mM were more effective at reducing degradation by 10-15%. A combination with kaolin clay (Surround®WP) also was effective (data not shown).

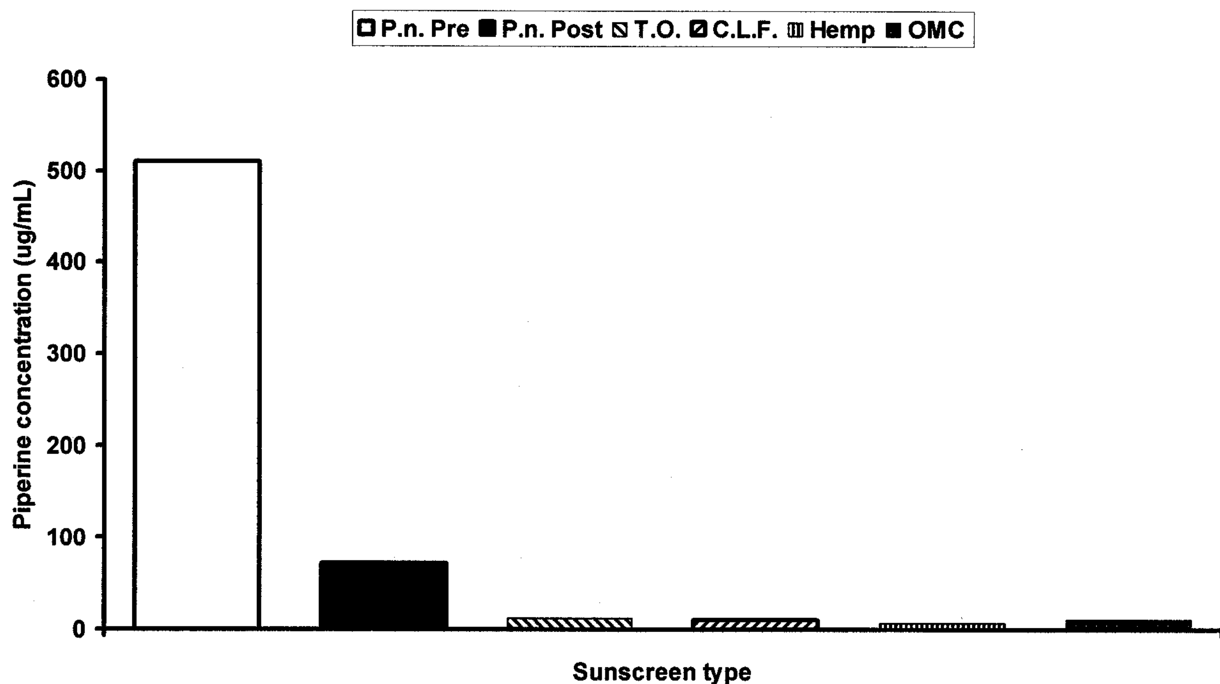


Figure1. Comparison of pre- and post-sunlight exposed *P. nigrum* extract, to *P. nigrum* combined with several antioxidants and sunscreens at 40%.

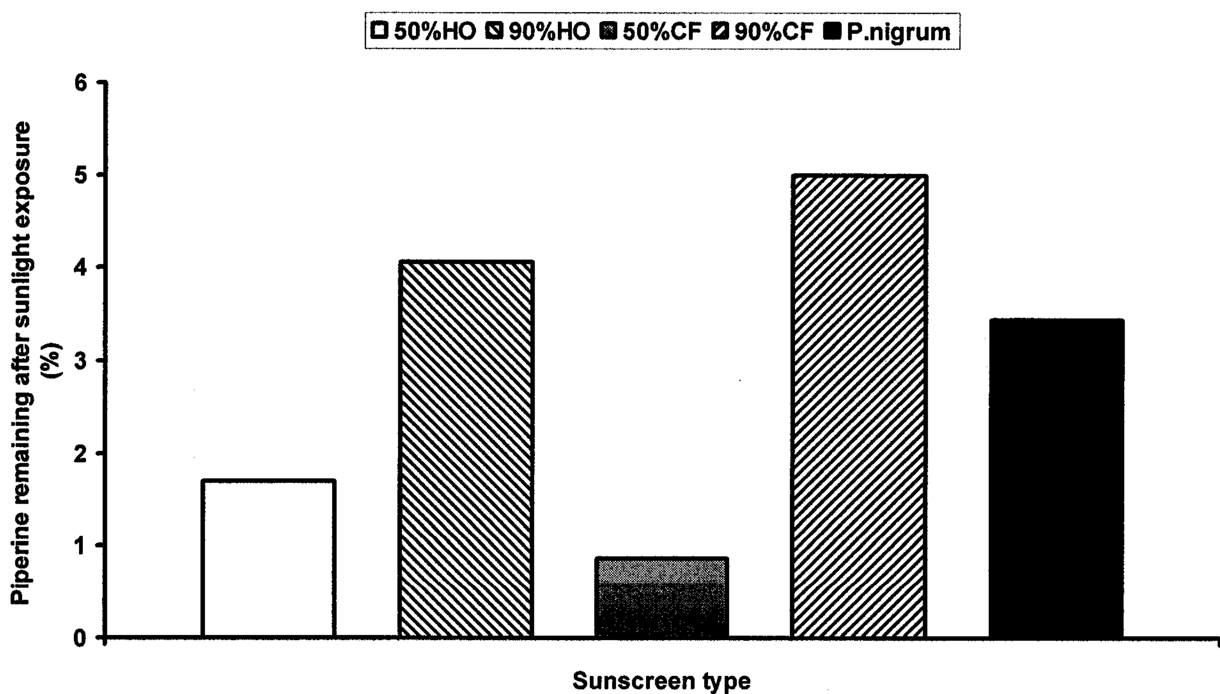


Figure 2. Comparison of *P. nigrum* combined with hemp oil and licorice flavanoids oil at 50 and 90% with untreated *P. nigrum* after exposure to sunlight.

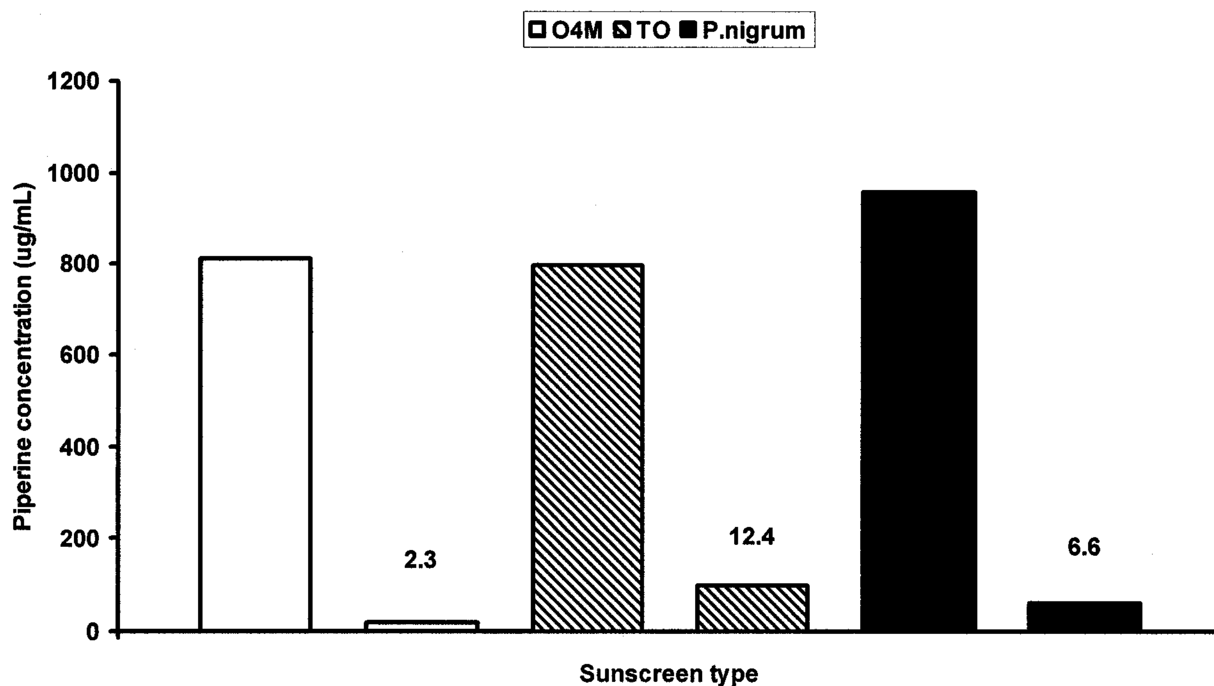


Figure 3. Comparison of *P. nigrum* extract combined with O4M and TO at 100 mM or alone, pre- and post-sunlight exposure.

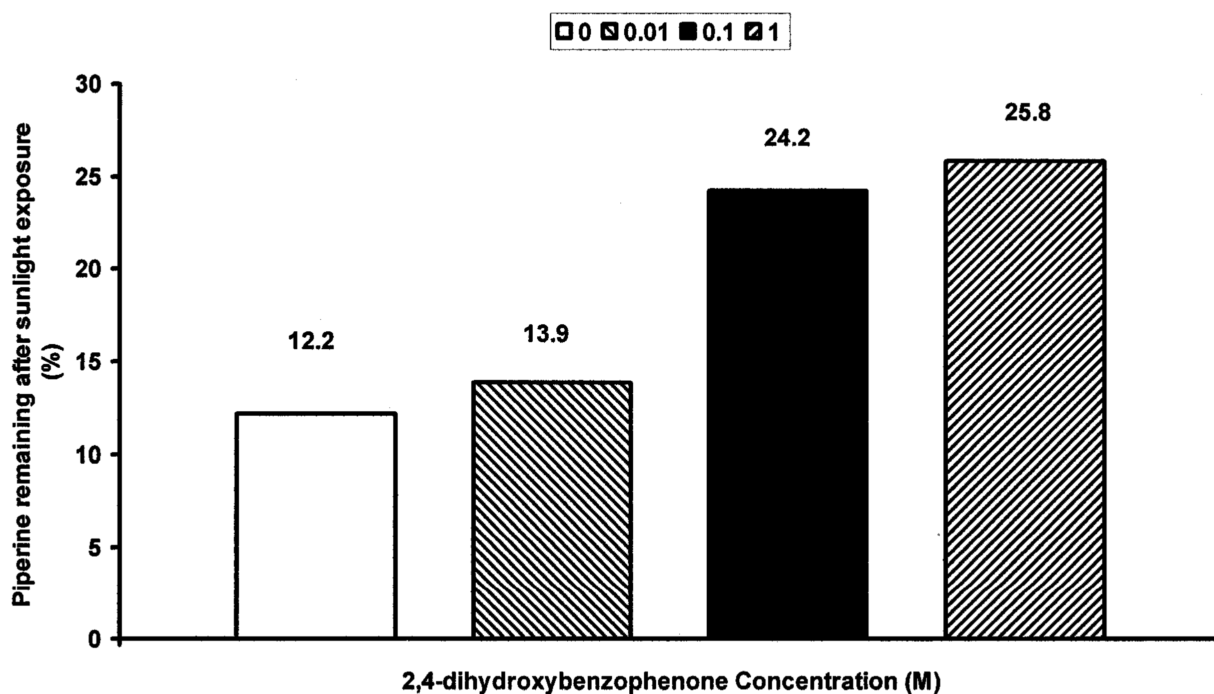


Figure 4. Comparison of 2,4-dihydrobenzophenone at 0, 0.01, 0.1 and 1 M concentrations combined with *P. nigrum* and exposed to sunlight.

APPENDIX III

COMBINATIONS OF PLANT EXTRACTS FOR INCREASING *PIPER* BOTANICAL FORMULATION ACTIVITY

Three commercial botanical extracts and one obtained from Costa Rica tested as synergists or to compare with the insecticidal and repellent activity of the *Piper* extracts. These included: Central American *Cedrela odorata* (Meliaceae) extract; Neem oil *Azadiracta indica* Juss (Meliaceae) (Ahimsa Alternative Inc., Oklahoma City, OK); lemon grass oil *Cymbopogon citratus* [DC] Stapf. (Poaceae) (Ropel®, Burlington Scientific, NY) and formulated kaolin clay (Surround®WP a gift of Engelhard, Iselin, NJ). All commercial products were applied at rates recommended by the manufacturer.

Modification of toxicity through extract combinations was assessed by mixing *P. nigrum* or *P. guineense* with either Tansey oil, *Tanasetum vulgare* var. Crispum (Gaspesia Pharma, QC), or dillapiol from Dill oil from *Anethum sawa* Roxb. (Umbelliferae). Acute toxicity trials, 24 to 48 h, were used to assess whether the mixtures were more toxic than the individual extracts against Japanese beetle *P. japonica* adults; European chafer *R. majalis* larvae; lily leaf beetle *L. lilii* adults; house fly *M. domestica* adults and ermine moth *Y. cagnagella* larvae.

Materials and Methods

Trial 1 Adult *Lilioceris lilii* were also placed in three large cages, where three lily plants per cage were treated either with 1% P. nigrum, Neem oil or Ropel®. The choice test was checked after 96 hours to assess damage to leaves from feeding.

Trial 2 Classic and Orange Sunblaze roses variety Miepinjid and Meijikatar respectively plants were sprayed with 1% *P.nigrum* alone, or 1% *P. nigrum* combined with either the recommended dose of Neem oil or Surround®WP in 100 mL of water. Each treatment was replicated three times with 20 *P. japonica*

per plant. Each plant was individually caged with a wire frame and mesh covering. The plants were checked for damage to leaves due to feeding after 96 (first trial) and 48 hours (second trial) respectively.

Trial 3 Green pepper plants *Capsicum annum* L. (Solanaceae) were grown to the early fruiting stage and then chosen on the basis of damage-free fruits. Ten plants per treatment were sprayed with either *Cedrela odorata* extract plus dillapiol, 95% dill oil (1:1 for the highest concentration and 0.6:1 for the lowest, due to availability of extract) at 0.1 and 0.03 % extract or *P. tuberculatum* extract at 0.1 and 0.05 %. The control plants were sprayed with 4:1 95% ethanol: distilled water. The following day the plants were hand infested with three 2nd instar *O. nubilalis* larvae plus one egg mass. On the third day the plants were re-sprayed. After 11 days the fruits and plants were tallied for surviving larvae. This trial was conducted by R. Nichol, Biology Department, University of Ottawa.

Trial 4. *Popillia japonica* adults were treated with the LC_{50} dose of *P. nigrum* (0.5%) in combination with *T. vulgare* oil at the same concentration and compared with 0.5% *T. vulgare* alone and a formulation blank control. Three replicates of 10 adults per treatment were used. Mortality was assessed after 24 h.

Trial 5. *Rhizotrogus majalis* 3rd instar larvae were placed in soil compartments described in Chapter 5. The soil was treated with either 1 or 2% *P. nigrum*, or a combination of 1% *P. nigrum* and 1% dillapiol. The control was formulation blank. Three replicates of 10 adults per treatment were used. Seven days later the mortality of larvae in treated and control plots was assessed.

Trial 6. *Lilioceris lilii* adults were treated with either 0.5% *P. guineense* or 0.5% *P. guineense* and 0.1% dillapiol combined and compared to a formulation blank control. Three replicates of 10 adults per treatment were used. Mortality of adults was assessed after 24 h.

Trial 7. Adult *Musca domestica* L. (Diptera: Muscidae) (Carolina Biological Supply Company, Burlington NC) anaesthetised with CO_2 and then kept immobilized on ice. *P.guineense* at 0.5% alone or in combination with 0.1% dillapiol , and 0.1% dillapiol alone as the control was used to dip flies. Each treatment used 25 to 30 flies and post-treatment flies were transferred to metal cages for recovery where they were provided with a dissolved sugar solution. Mortality was assessed after 24 h by tapping the cage and observing whether the flies on the bottom of the cage would respond by flying.

Results and Discussion

In a choice experiment with *L. lilii* adults, *P. nigrum* at 1% significantly reduced adult feeding damage after 96 h compared to the formulation blank ($F = 3.542$; $df = 3, 11$; $P=0.074$) (Fig. 1). *P. nigrum* at 1% was not significantly more effective than either Ropel® or neem oil (Tukey's multiple range test, $P>0.108$) and the 1% pepper spray caused phytotoxicity: burning of lily leaf tips, which was not observed at lower concentrations that effectively repelled larval *L. lilii.*

Rose plants sprayed with 1% *P. nigrum* and combined with neem oil showed significantly less leaf damage compared to untreated rose leaves (1-tailed T-test; $P = 0.041$) in a 48-h choice test (Fig. 2). There was no significant difference when *P. nigrum* was combined with Surround®WP (1-tailed T-test; $P = 0.11$).

The mixture of *C. odorata* and dillapiol at 0.1% had a significant impact on *O. nubilalis* larvae survival ($P<0.01$) but *P. tuberculatum* at 0.05 and 0.1% had no significant effect (Fig. 3). When a higher range of *P. nigrum* and *P. guineense* concentrations were tested there was also no effect on *O. nubilalis* larvae (unpublished data).

The *P. nigrum* LC_{50} for *P. japonica* adults was 0.5%, similar to that produced by *T. vulgare* (Fig. 4). When both extracts were mixed at equal parts (0.5%) there was no significant increase in mortality (one-way-ANOVA, $F=4.568$; $df = 2,6$; $P=0.312$), although the mixture mortality was significantly higher than for the controls (Tukeys test $P=0.053$).

The toxicity of 1% *P. nigrum* to *R. majalis* larvae was not improved significantly when combined with 1% dillapiol (one-way-ANOVA, F=87.669; df=3,14; P=0.228) (Fig. 5) but it was comparable to 2% *P. nigrum* treatment (Tukeys test P=0.63) and all treatments had significantly higher mortality than controls (P<0.001).

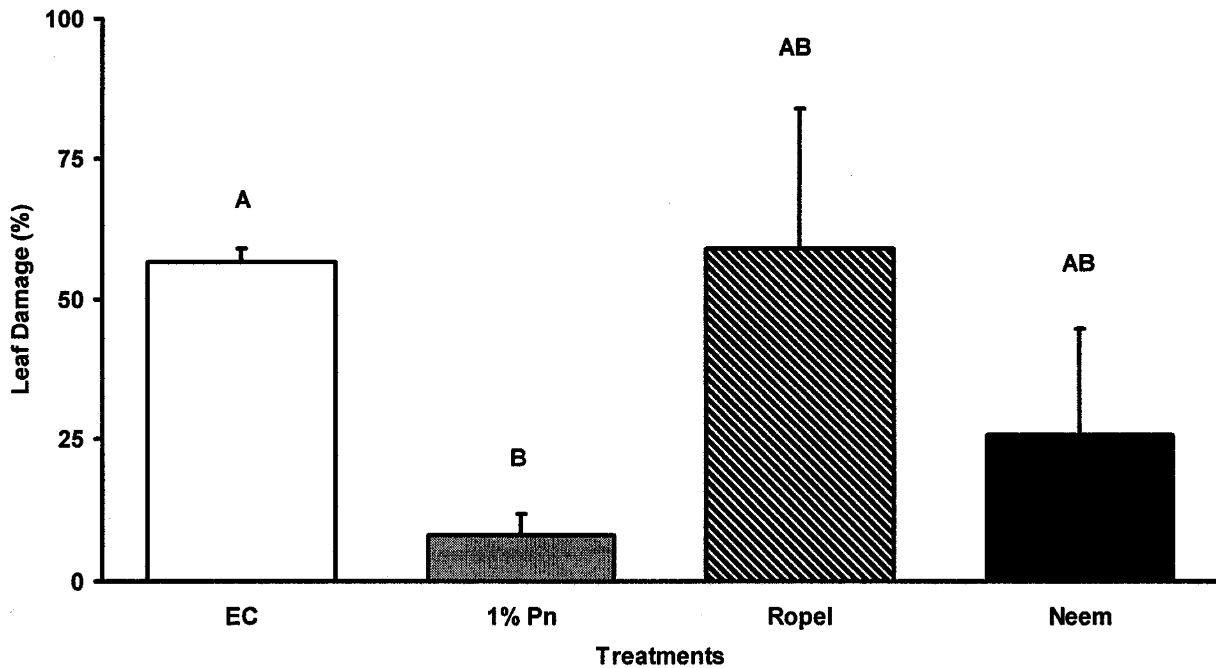


Figure 1. Percent leaf damage ± S.E. on Asiatic *Lilium* plants by *L. lilii* adults during 96-h choice test: plants treated with either formulation control (EC), 1% *P. nigrum* (1% Pn), *C. citratus* oil (Ropel®) or *Azadirachta indica* oil (Neem). The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

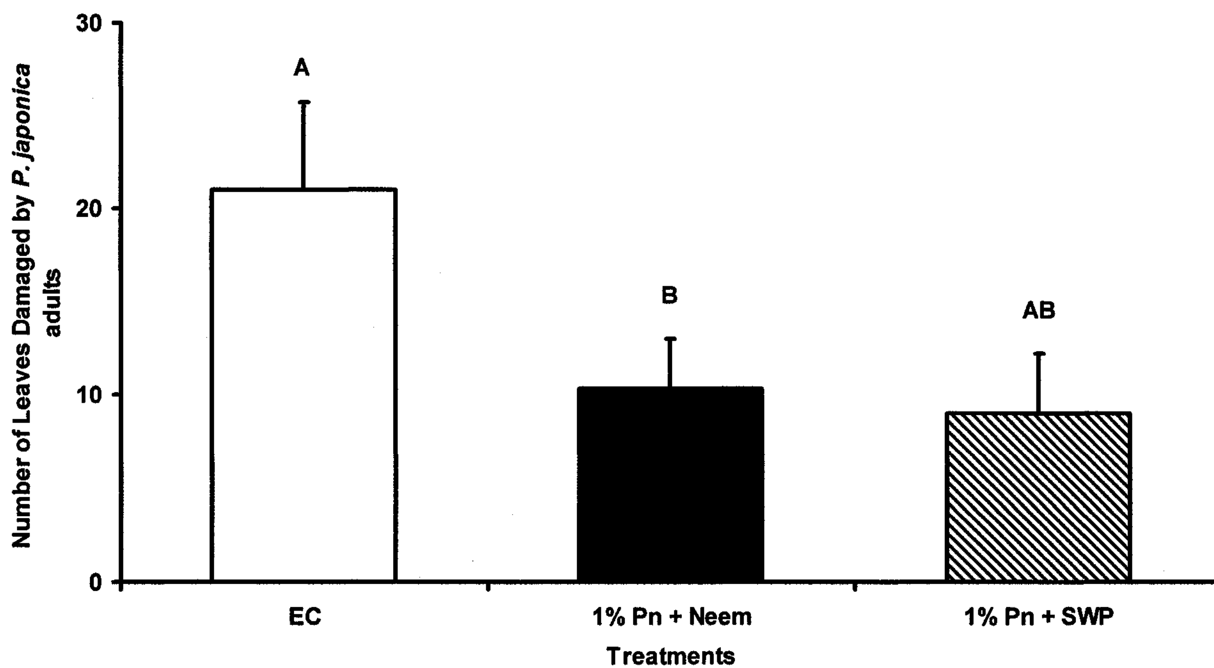


Figure 2. Mean number of leaves damaged ± S.E. by *P. japonica* adults on rose plants treated with either formulation blank (EC), 1% *P. nigrum* combined with neem oil or 1% *P. nigrum* combined with Surround®WP. The treatment means with the same letter are not significantly different (T-test, P >0.05).

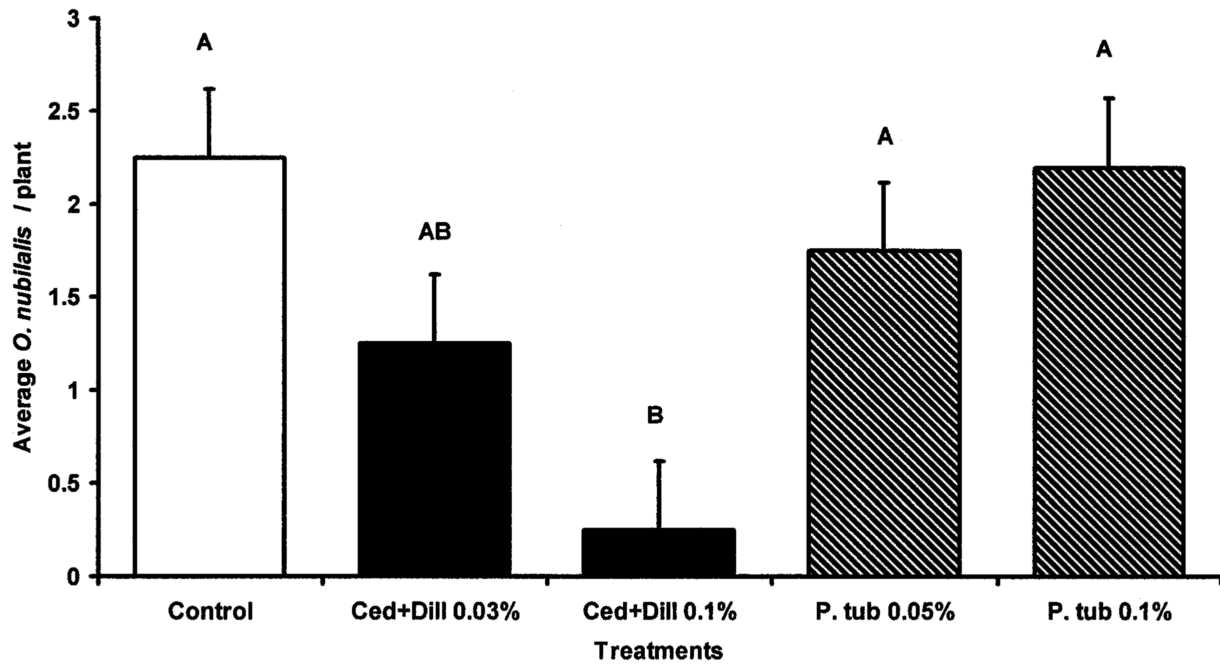


Figure 3. Mean number of *O. nubilalis* larvae ± S.E. found on bell pepper plants 11 days after treatment with water (control), combined *C. odorata* and 0.03% or 0.1% dillapiol (Ced+Dill), and 0.1% or 0.05% *P. tuberculatum* (P.tub). The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

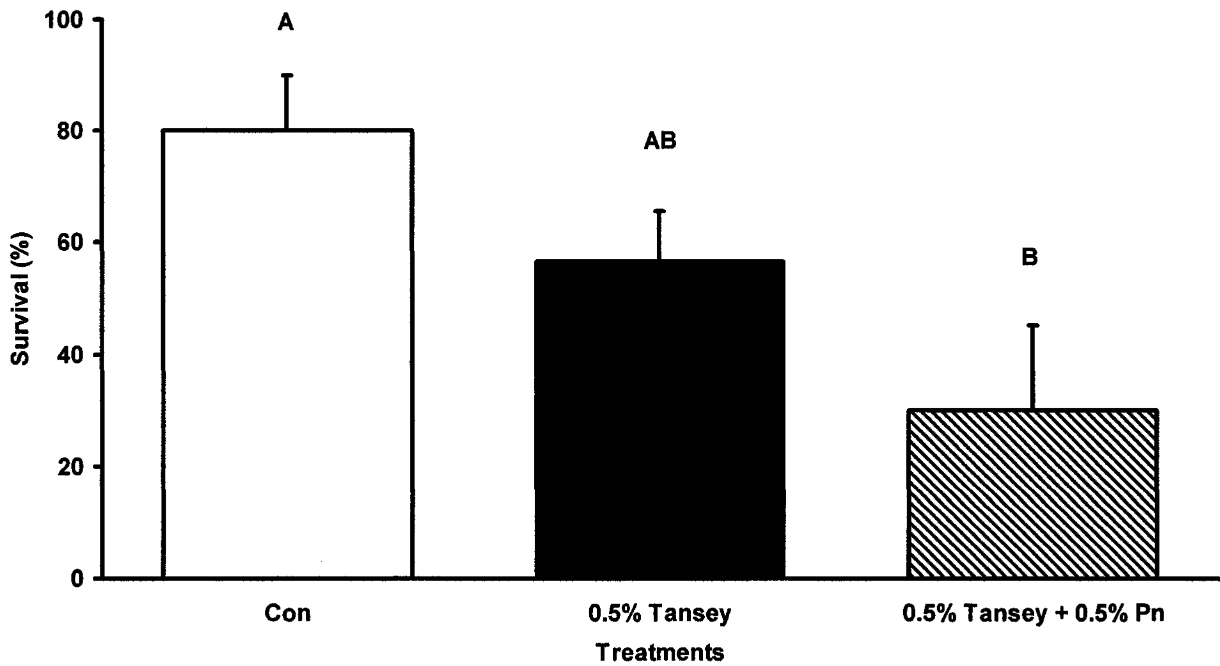


Figure 4. Adult *P. japonica* percent survival ± S.E. 24 h after treatment with 0.5% *T. vulgare* extract alone or in combination with 0.5% *P. nigrum*. A or B, The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

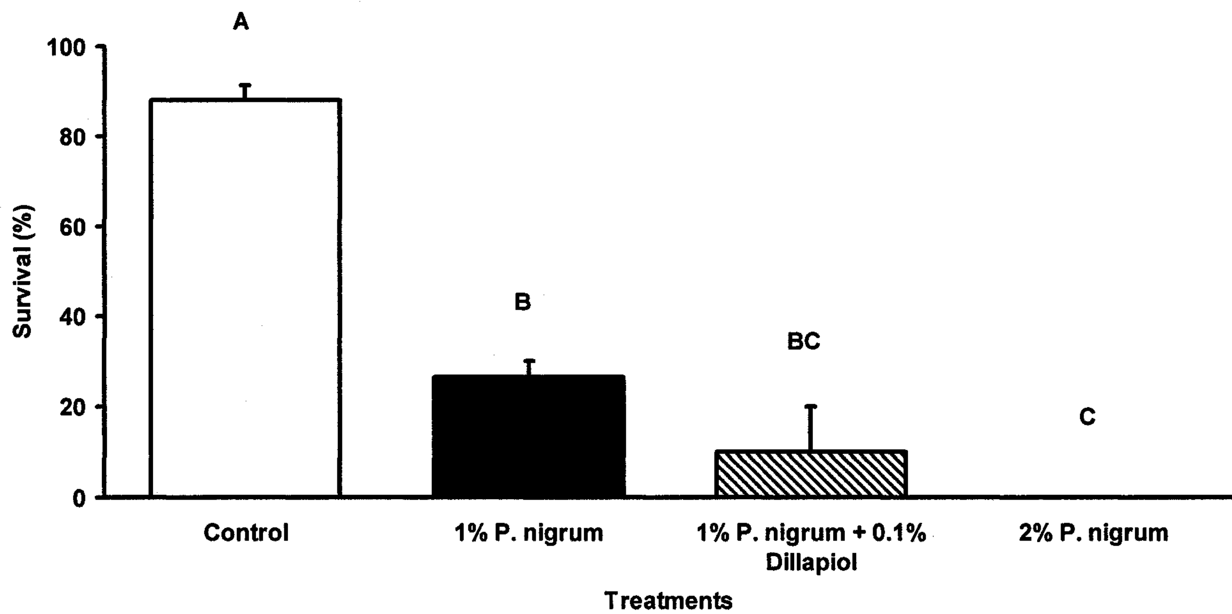


Figure 5. *R. majalis* larvae percent survival ± S.E. 7 days after soil treatments with 1% *P. nigrum* alone or in combination with 1% dillapiol compared to 2% *P. nigrum* alone. A, B or C, The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

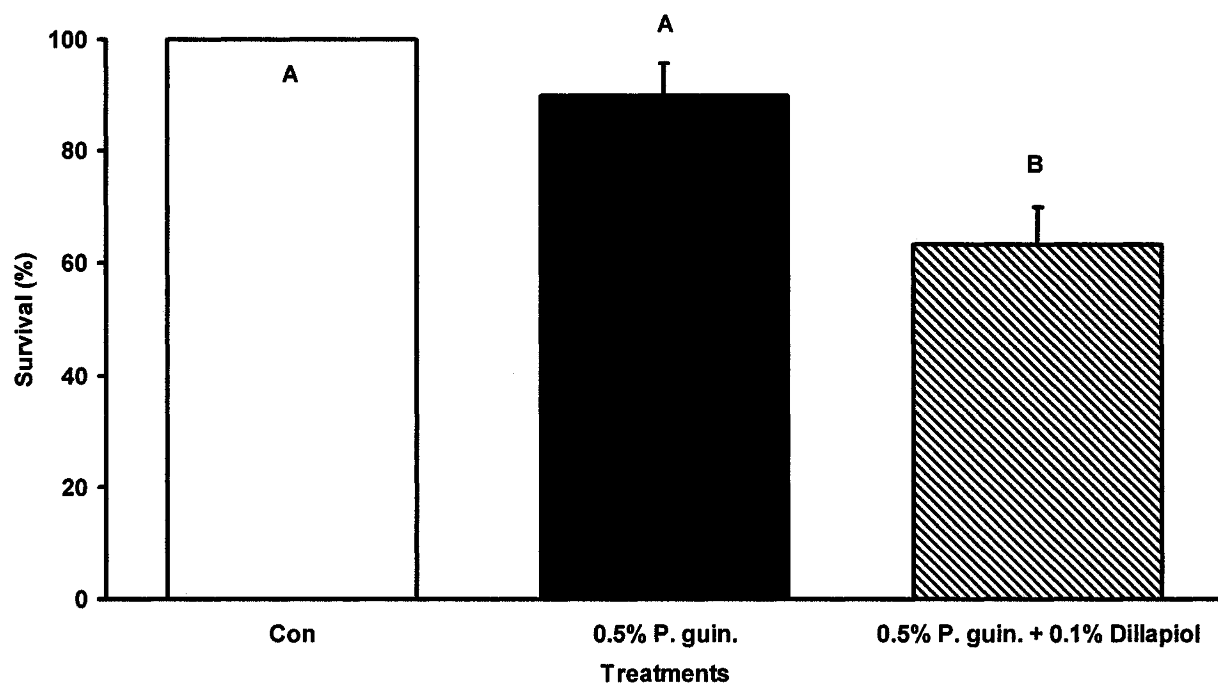


Figure 6. Adult *L. lilli* percent survival ± S.E. 24 h after *P. guineense* treatment alone and in combination with 0.1% dillapiol. A or B, The treatment means with the same letter are not significantly different (Tukey's, P >0.05).

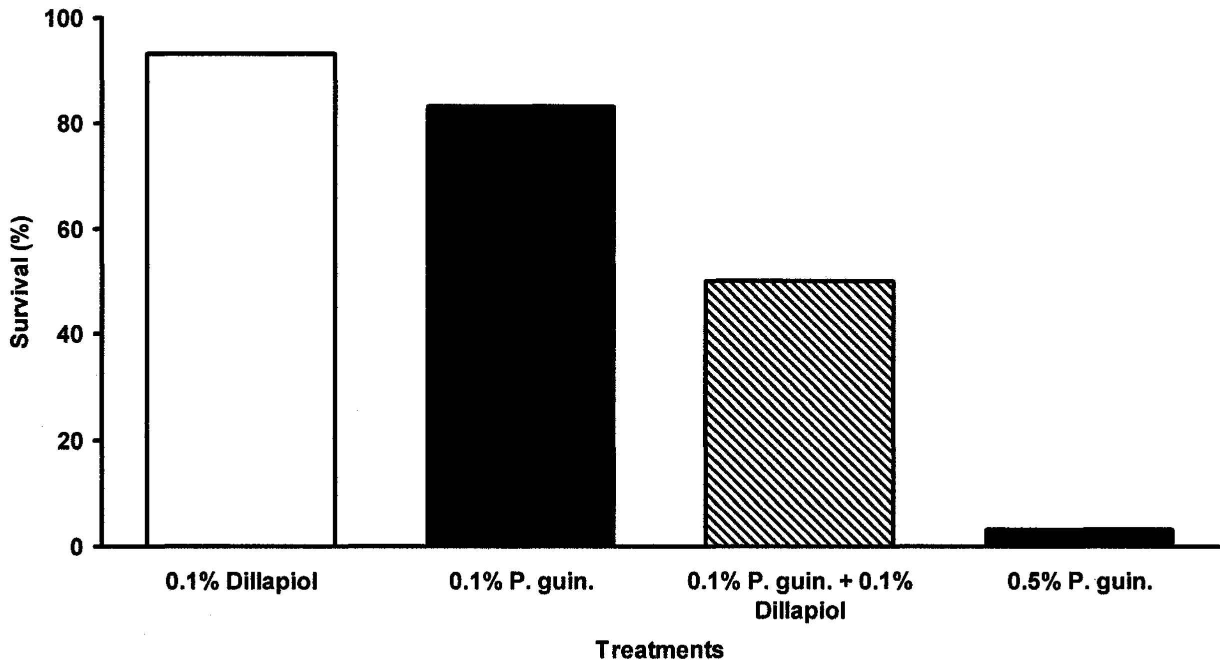


Figure 7. *M. domestica* adult percent survival 24 h after 0.1% dillapiol and 0.1% *P. guineense* alone or combined and compared to 0.5% *P. guineense* alone.

Effective control of *L. lilli* adults was observed with *P. guineense* alone only when concentrations were > 0.5% (one-way-ANOVA, F=13.857; df = 2,6; P=0.404), but beetles treated with 0.5% *P. guineense* plus 0.1% dillapiol had significantly higher mortality compared to controls and 0.5% *P. guineense* alone (Tukeys test P<0.023) (Fig. 6).

Synergism between *P. guineense* and dillapiol was observed in *M. domestica* trials where dillapiol alone was not toxic at 0.1% but when 0.1% *P. guineense* was combined with 0.1% dillapiol the mortality was greater than 0.1% *P. guineense* alone but not as active as 0.5% *P. guineense* (Fig. 7).

As was observed in this study, *P. tuberculatum* at 0.1% did not repel or effect *O. nubilalis* larvae placed on treated green peppers one day after spraying (Fig. 3). However *C. odorata* was more effective as a result of the systemic activity recognized for extracts containing liminoids like azadirachtin from *A. indica* (Gagnon 1992). When the same trial was repeated with double the concentration of *P. nigrum* and *P. guineense* no significant effect on *O. nubilalis* survival was noted (unpublished data).

The activity of the *Piper* extracts was improved in some cases when they were combined with other plant extracts: dillapiol and *T. vulgare*. In the case of *M. domestica* and *L. lilli*, dillapiol did synergise the activity of *P. guineense*. *T. vulgare* essential oil was found to be as toxic as *P. nigrum* but in 1:1 combination only additive toxicity was noted against *P. japonica* adults (Figure 1) and *R. majalis* larvae (unpublished data). *T. vulgare* essential oil contains limonene, camphene, camphore and α- and β-thujone (personal communication Gaspesia Pharma) which have a mode of action different from piperamides. *P. nigrum* essential oils were the most toxic of six tested against the rice weevil *Sitophilus zeamais* (Motsch.) (Ngamo *et al.* 2001) and *P. guineense* was as effective as *A. indica* and *Occimum gratissimum* L. (Lauraceae) in reducing survival of *S. zeamais* in maize (Banjo *et al.* 2001). However *O. gratissimum*, containing similar essential oils to *T. vulgare*, was more effective than *P. nigrum* when ingested by *S. zeamais* compared to a topical application (Ngamo *et al.* 2001). This suggests that *T. vulgare* may be more toxic in an extended feeding trial than *Piper* extracts which have a faster insecticidal action through contact application.

Dillapiol is recognized as an inhibitor of P450 enzymes in insects (Bernard *et al.* 1995) and humans (Budzinski *et al.* 2000) along with the main constituent of *P. nigrum*, piperine (Bhardwaj *et al.* 2002). It was hypothesized that the combination of both would be synergistic since there would be double the inhibition of insect detoxification enzymes thus increasing the *P. nigrum* activity. However, it appears that piperine with dillapiol may not produce any greater toxicity since both compounds may be targeting the same P450 enzymes. This has not yet been proven with insect P450 systems as of yet although piperine inhibits O-demethylase activity in houseflies (Chapter 4). The additive effect observed with *M. domestica* may occur since the dillapiol improves the uptake of the active components of the formulation through physical means: for example improved adjuvant properties. Dillapiol and *T. vulgare* essential oil may prove to be a better combination since piperine and dillapiol act similarly. Both dillapiol and *P. nigrum*, when combined with pyrethrum, did exhibit a synergistic effect with houseflies (unpublished data) similar to what was observed by Gersdorff and Piquett (1957).

A 1:1 combination with 0.1% dillapiol created a synergistic mixture against housefly adults, however this effect was not observed with other insects. Generally the combination of *P. nigrum* with dillapiol or with other botanical extracts created only additive toxicity and repellent effect. This is not a problem since combinations of actives could reduce the development of resistance in the same way as mixtures of analogues were thought to improve the efficacy of piperamides (Scott *et al.* 2002). Another improvement in terms of integrated pest management is selecting for resistant cultivars in combination with botanical essential oil products. For example *P. guineense* was applied more successfully to decrease *C. maculates* first instar larval survival and egg hatch in combination with increasingly resistant cultivars of groundnut (Ajayi and Lale 2001).

APPENDIX IV

THIRD EUROPEAN CHAFER FIELD TRIAL

A third field trial was conducted between 8 and 15 October 2002 following the same procedure outlined for the September 2002 trial in Ottawa (Chapter 5).

Results and Discussion

Ottawa, October 2002

The average number of live *R. majalis* larvae found on 15 October in all treatment plots one week after application was not significantly different (F = 1.353, error d.f. = 35, p=0.261) (Figure 1). All of the *P. nigrum* and positive control treatment plots had greater than five live larvae / 0.1 m^2 while the control treatment plots had on average 12-13 grubs / 0.1 m^2. However, there was a significant difference in the number of dead grubs between treatments (F = 2.249, error d.f. = 35, p=0.061) as well as when the dead and moribund grub numbers were combined (F = 2.136, error d.f. = 35, p=0.074).

The average number of dead grub larvae in the 4% *P. nigrum* treated plots was approximately 7 / 0.1 m^2 while it was between 1 and 4 / 0.1 m^2 on average in the control plots but the treatment numbers were not significantly different (F = 2.249, error d.f. = 35, p > 0.305). However, when the water control and two formulation control values were combined, there were significantly more dead grubs in the 4% *P. nigrum* treated plots compared to controls (F = 2.47, error d.f. = 37, p=0.073), but not for the positive control treated plots versus the controls (Tukeys test p=0.852). When compared to the combined controls, 4% *P. nigrum* treated plots had significantly higher numbers of dead and moribund (F = 2.688, error d.f. = 37, p=0.085) in contrast to the positive control (Tukeys test p=0.596).

After the October treatment the number of earthworms found in the 4% *P. nigrum* plots was significantly less than the water control (F= 4.202, error d.f. = 35, p=0.023) and high EC control (Tukeys test p= 0.001), but not compared to the number in the positive control plots (Tukeys test p=0.189) (Figure

2). The number of earthworms sampled in the 2% *P. nigrum* plots was also significantly lower than in the high EC plots (Tukeys test $p=0.077$), but not in comparison to the water or low EC controls (Tukeys test $p > 0.575$).

During the seven-day period between 8 and 15 October the average air temperature in Ottawa was between 5 and 10 °C (Figure 3), the total rainfall was 11 mm and daylight averaged 11 h. The soil pH and organic matter (Table 1) was identical to the values determined for the September trial at the same site (Chapter 5) and the concentration of piperamides in the formulation was slightly greater than the batch used for the September trial (Table 2).

The piperine $t_{1/2}$ for the October treatment was 5.7 days. This was longer than the half-life determined for piperine in both the previous Guelph and Ottawa trials (Chapter 5). This is likely due to the combination of lower light conditions and colder temperatures during the October trial. The concentration of piperamides in the top 5 cm of soil in the 4% *P. nigrum* treated plots two weeks after application was 5% of the initial concentration (Table 3).

The positive control, diazinon, had no effect on the grub population in treated plots, indicating that a reactive treatment this late in the season with a conventional synthetic insecticide was not worthwhile. The combination of colder temperatures and decreased grub activity close to the surface lessons the effectiveness of any contact insecticide treatment. Therefore it is not recommended to apply a pepper-based this late in the season, rather late summer, when monitoring of an infestation level warrants a treatment.

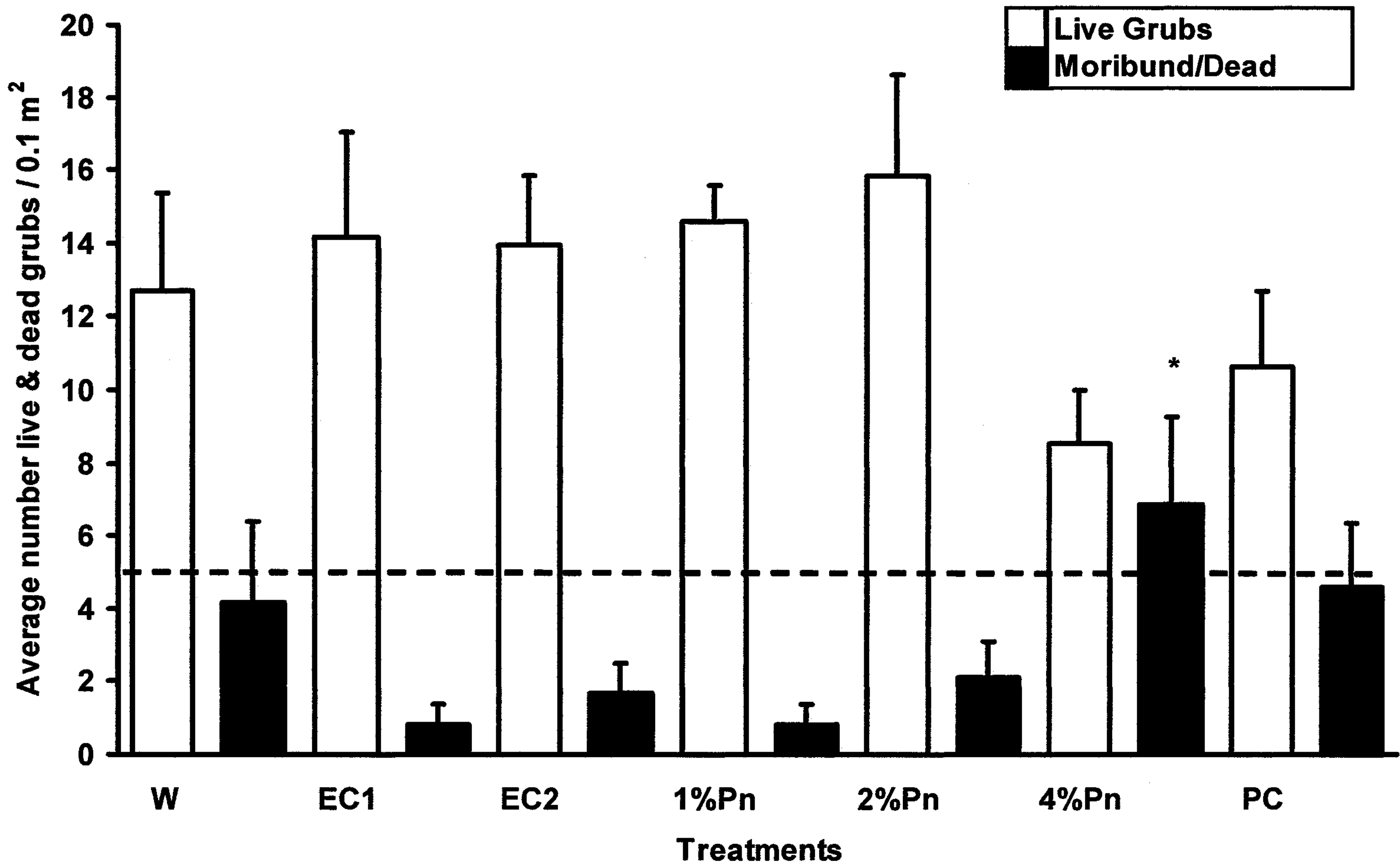


Figure 1. Mean number of *R. majalis* larvae ± S.E. sampled in control and *P. nigrum* treated plots at Grenfell Glen on October 15th, 2002. Statistical differences between treatments and water control are indicated by * ($p < 0.1$).

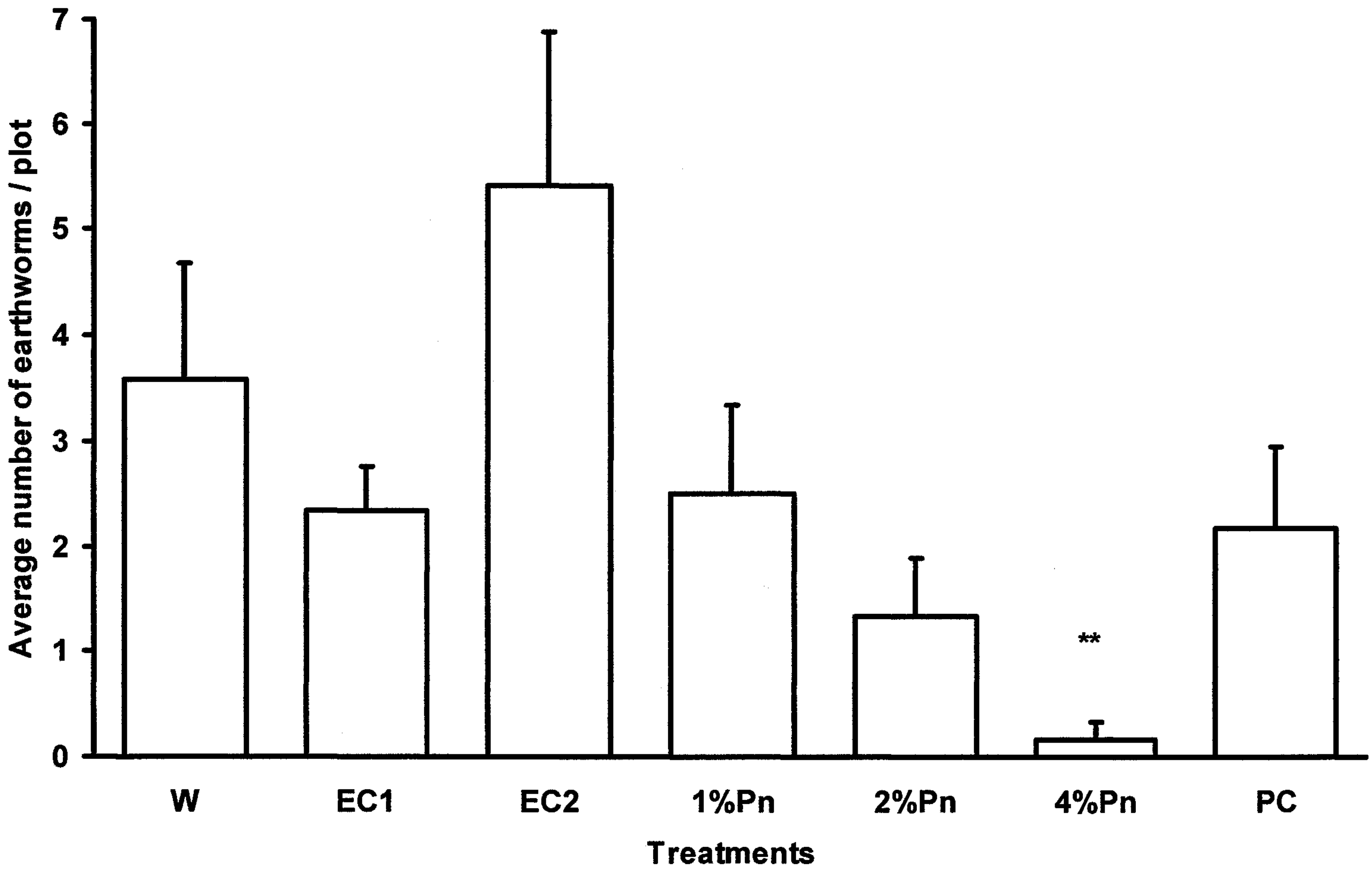


Figure 2. Mean number of earthworms ± S.E. sampled in control and *P. nigrum* treated plots at Grenfell Glen park, Ottawa, ON, on October 15th, 2002. Statistical differences between treatments and water control are indicated by ** ($p < 0.05$).

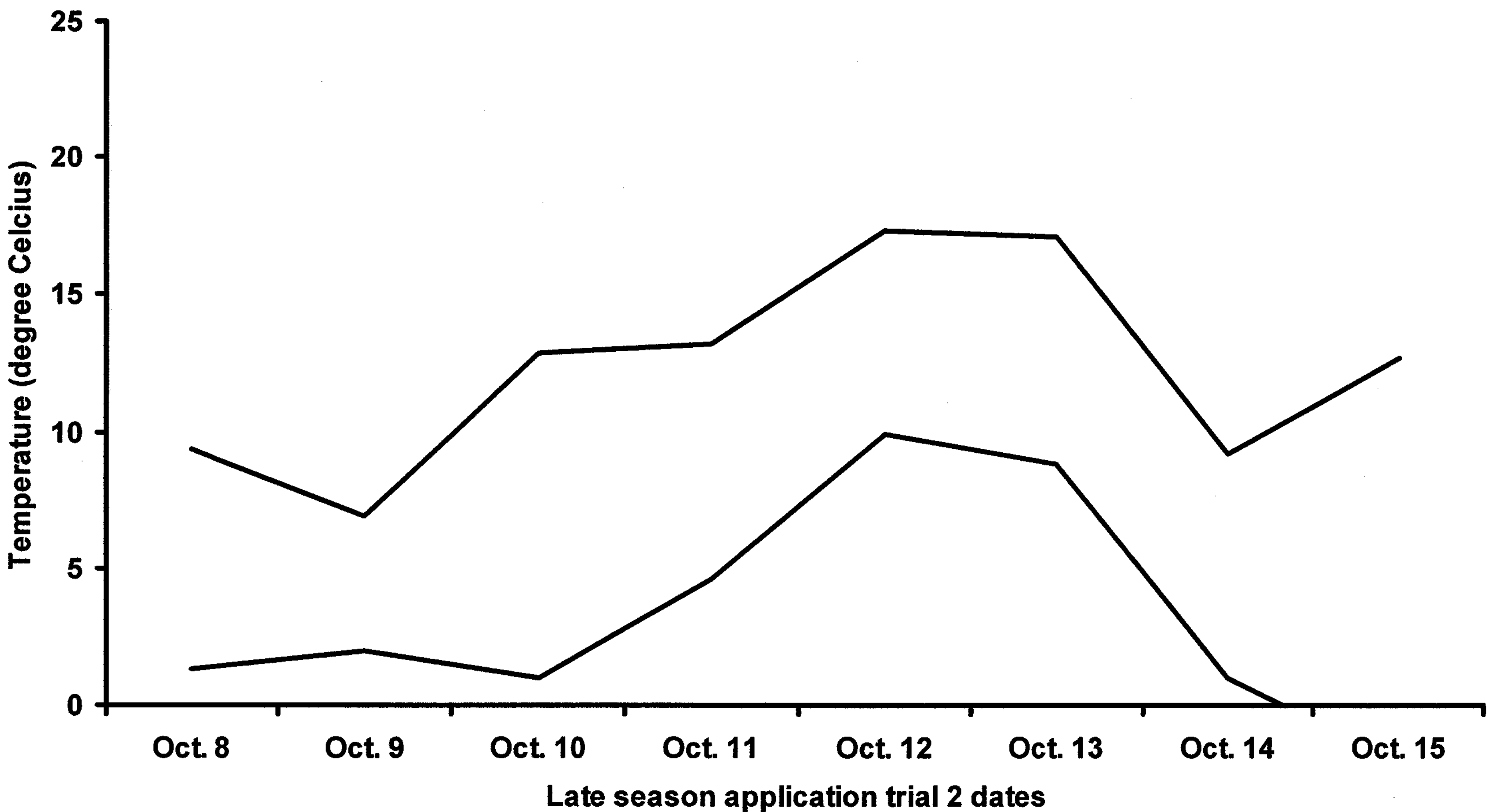


Figure 3. Minimum and maximum air temperatures at Grenfell Glen park, Ottawa, during the October 2002 field trial.

Table 1. Mean pH and organic matter content (± S.E.) in grub treatment field site collected soil samples one week post-application.

Parameter	Soil sample sites
	Glenfell Glen
	Oct. 2002
pH (S.E.)	6.9 (0.05)
N	12
% O.M. (S.E.)	9.9 (0.23)
N	12

Table 2. Mean (± S.E.) and total piperamide concentrations in 20% *P. nigrum* formulations and percent piperine (± S.E.) recovered from seed extract.

20% *P. nigrum* Formulation Batch	Piperamide Concentration (mg/mL)					Percent Piperine Recovery
	DHPLG	PLG	DHP	Piperine	Total	
Oct. 2002 *P. nigrum*	0.04 (0.04)	0.56 (0.014)	2.81 (0.11)	63.9 (5.76)	67.3	31.9 (2.9)

DHPLG = 4,5-dihydropiperlonguminine; PLG = piperlonguminine; DHP = 4,5-dihydropiperine.

Table 3. Average piperamide concentrations (± S.E.) and percent of initial residue remaining in soil samples collected from 4% *P. nigrum* treated plots at Grenfell Glen Park at start of October field trial and 2 weeks post-application.

Sampling Date	**Piperamide Concentration (µg/g soil) (S.E.)**			
	4,5-dihydro piperlong.	**Piperlonguminine**	**4,5-dihydropiperine**	**Piperine**
Oct. 08	1.0 (0.1)	4.3 (0.33)	28.0 (2.9)	601.6 (49.84)
Oct. 22	0	0.4 (0.23)	1.6 (0.93)	27.3 (1.81)
Percent Remaining	**0**	**10.0 (6.4)**	**7.6 (4.6)**	**4.3 (0.4)**

APPENDIX V

PIPERAMIDE MASS SPECTRA

The HPLC-MS method developed in Chapter 2 included the verification of individual piperamides through the mass of the standards. If standards were not available, data on the mass of identified compounds was obtained in the literature. The following HPLC and MS chromatograms provide the details for the identification of the peaks quantified in *P. nigrum*, *P. guineense* and *P. tuberculatum*.

Figure 1. MS chromatogram of dihydropiperlonguminine standard (A) and mass spectra (B).

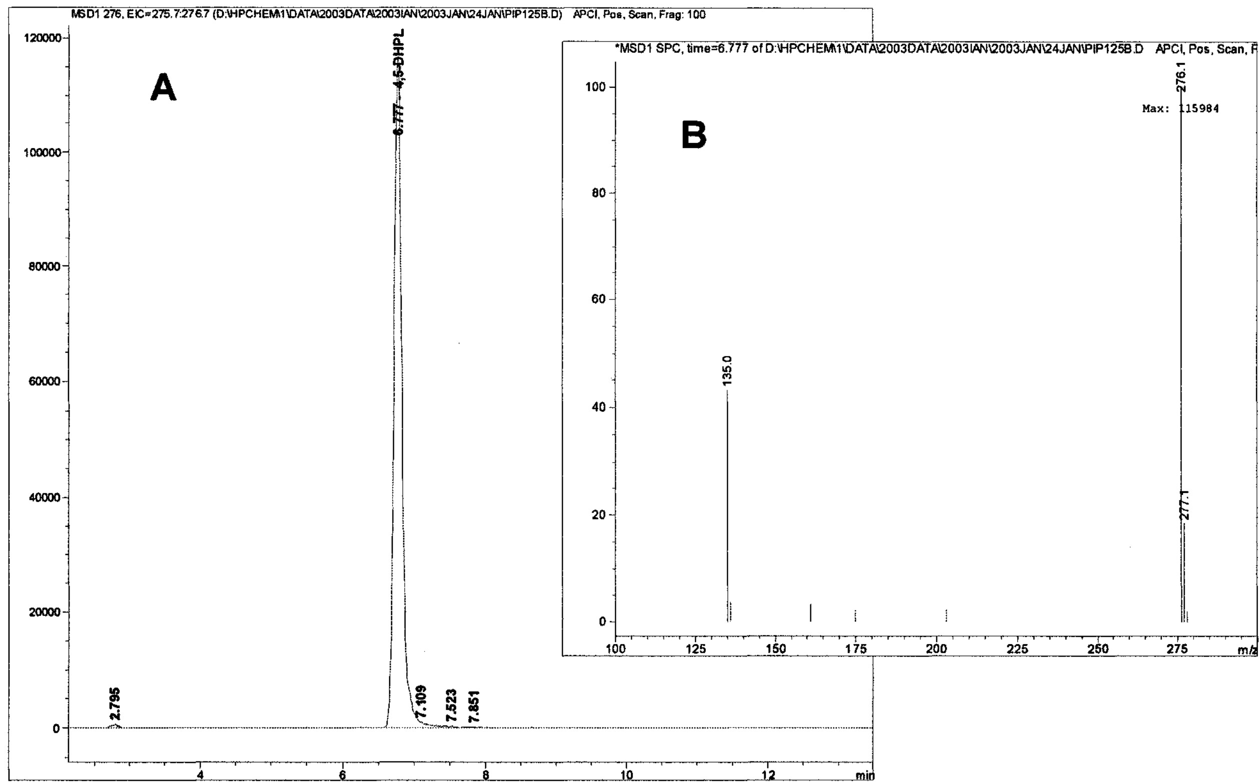


Figure 2. MS chromatogram of piperlonguminine standard (A) and mass spectra (B).

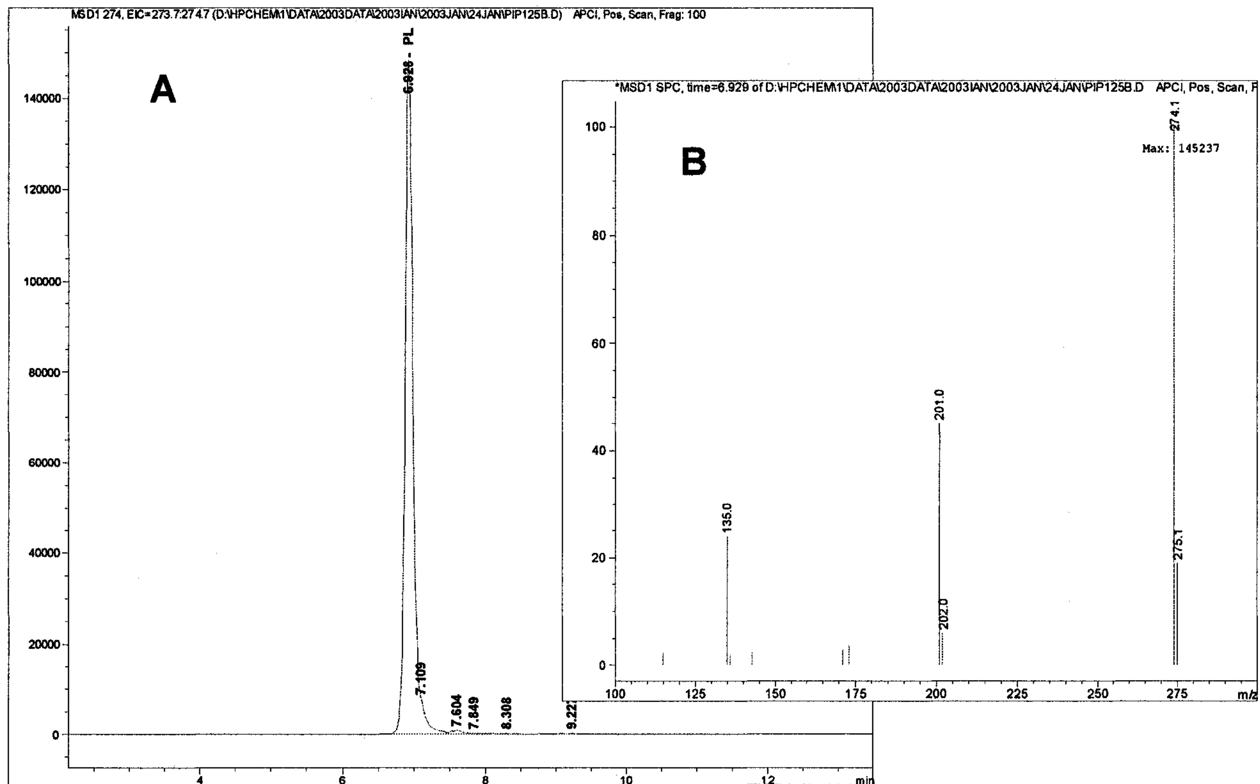


Figure 3. MS chromatogram of dihydropiperine standard (A) and mass spectra (B).

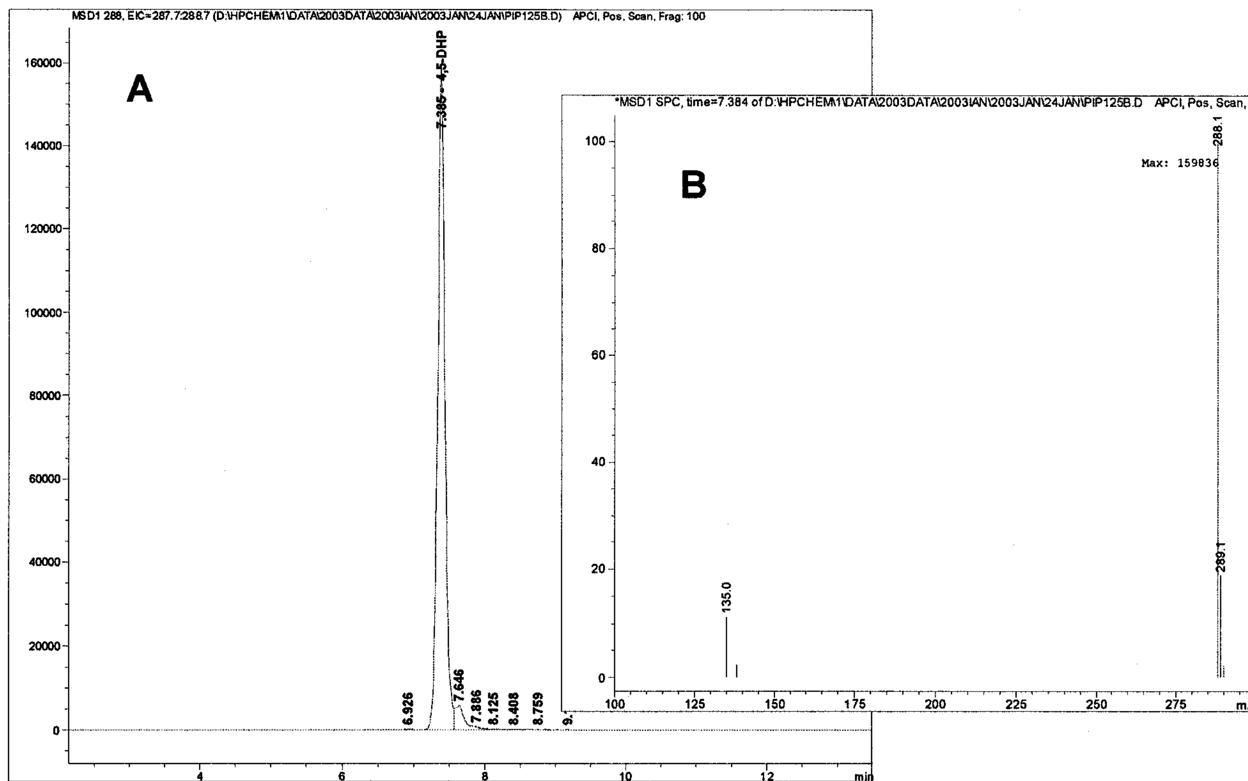


Figure 4. MS chromatogram of piperine standard (A) and mass spectra (B).

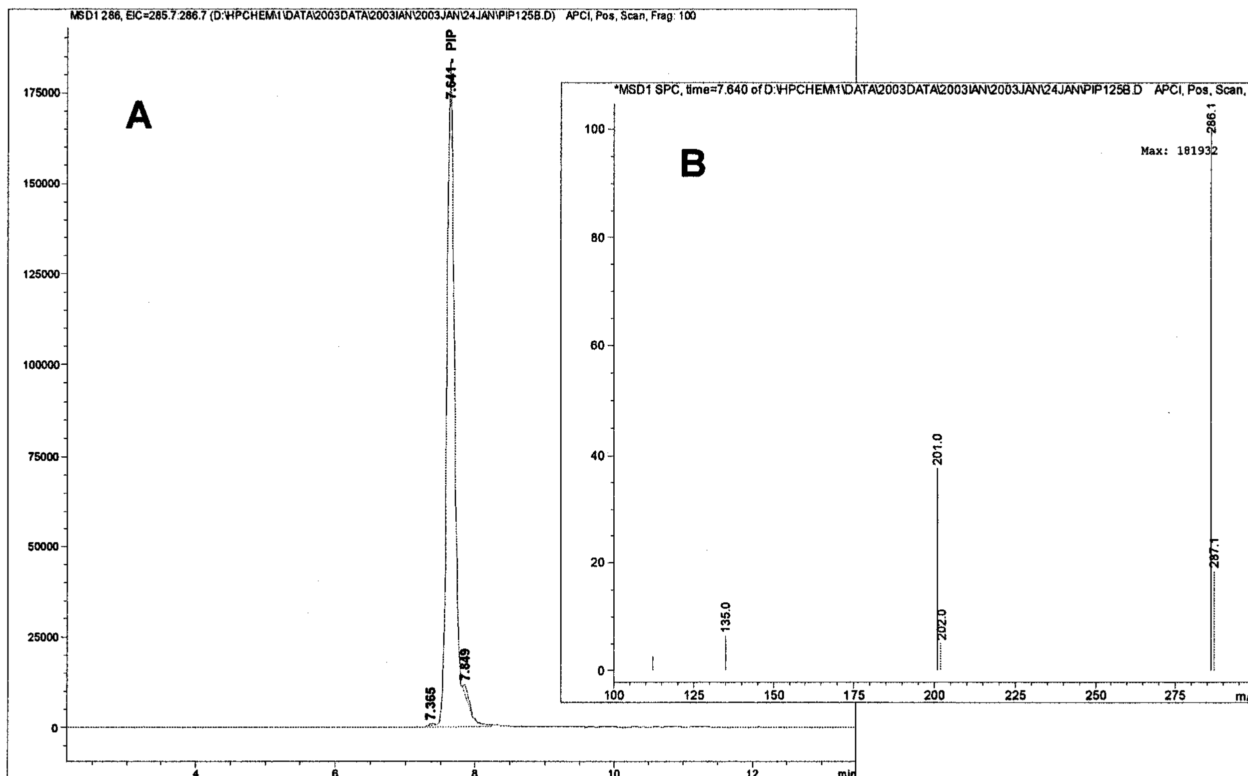


Figure 5. MS chromatogram of pipercide standard (A) and mass spectra (B).

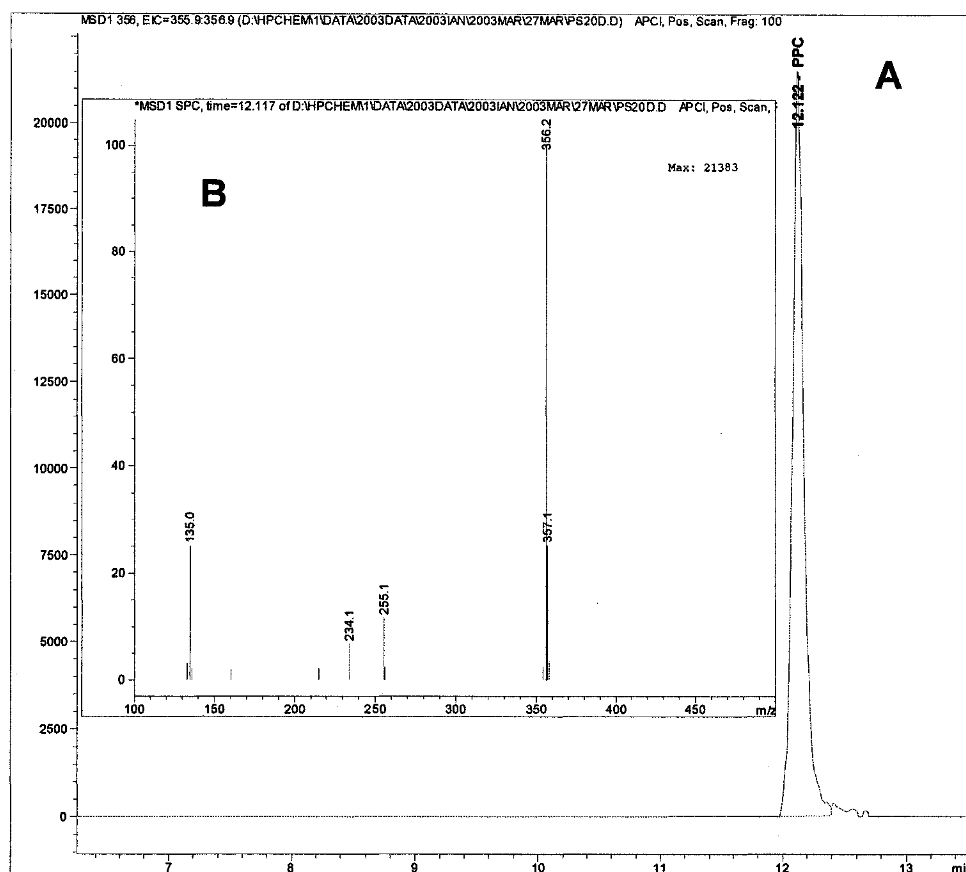


Figure 6. MS chromatogram of *P. nigrum* extract with dihydropiperlonguminine (A) and mass spectra (B), piperine (C) and mass spectra (D).

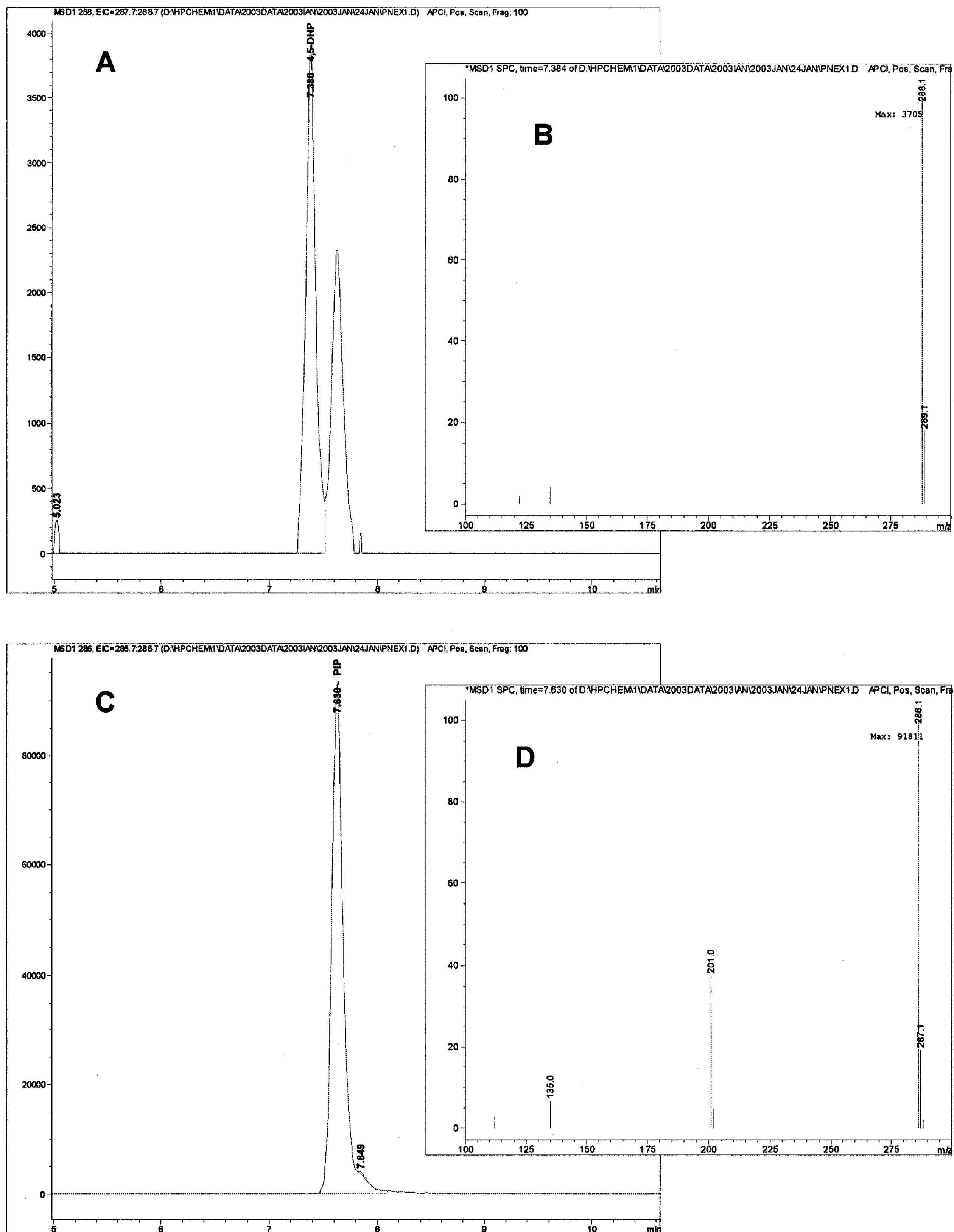


Figure 7. MS chromatogram of *P. nigrum* extract with piperlonguminine (A) and mass spectra (B), pipercide (C) and mass spectra (D).

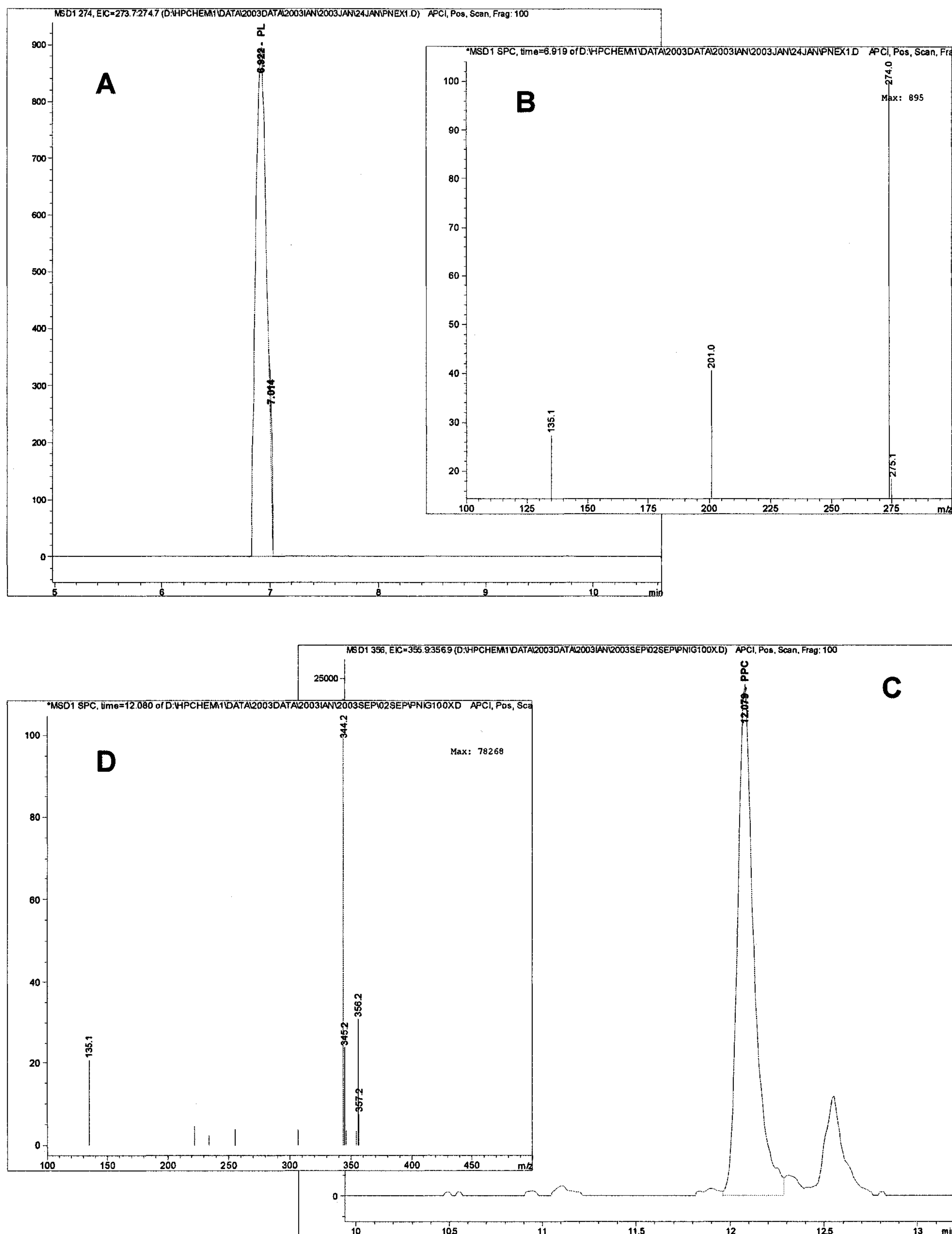


Figure 8. MS chromatogram of *P. guineense* extract with dihydropiperine (A) and mass spectra (B), piperine (C) and mass spectra (D).

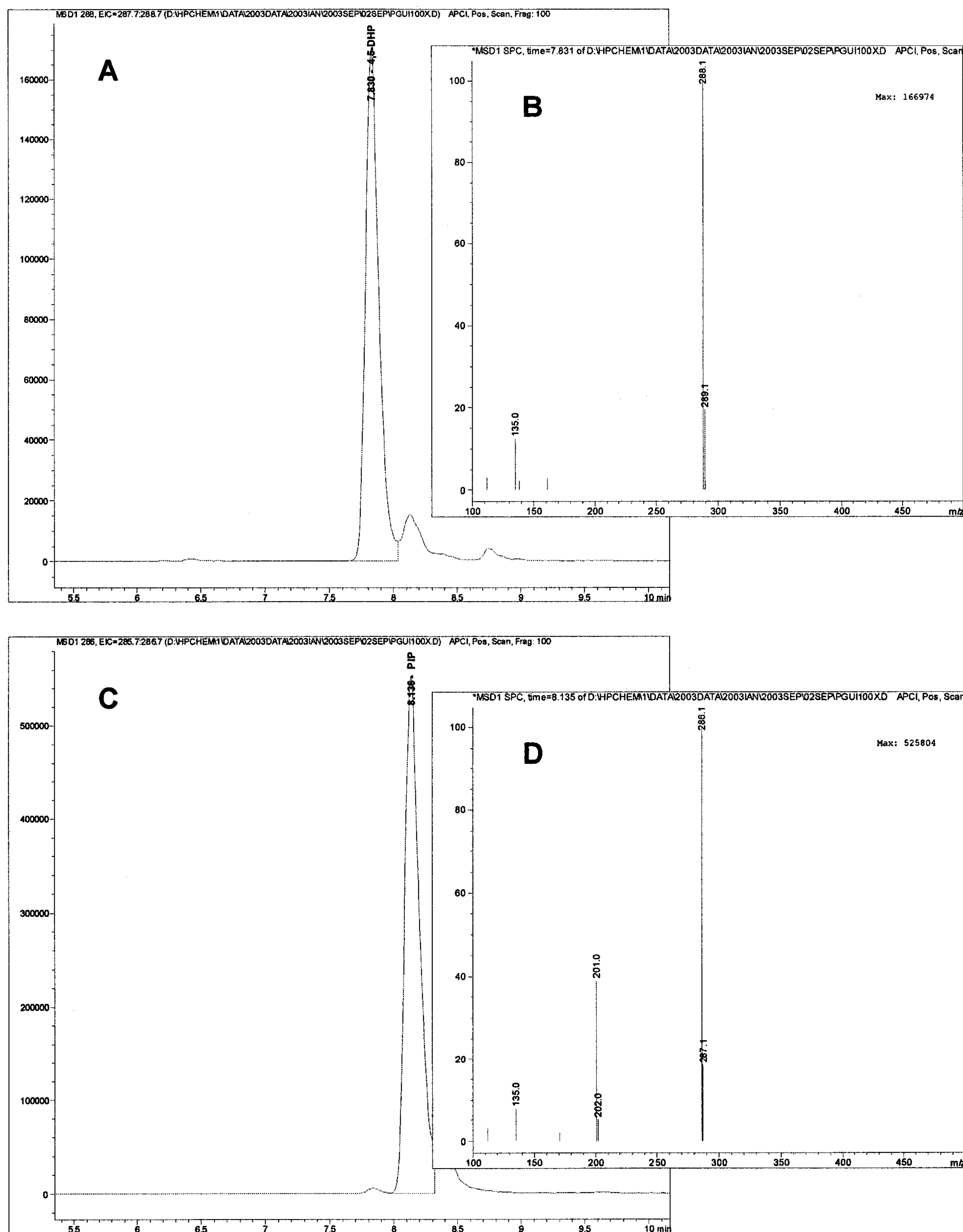


Figure 9. MS chromatogram of *P. guineense* extract with piperlonguminine (A) and mass spectra (B), piperlyn (C) and mass spectra (D).

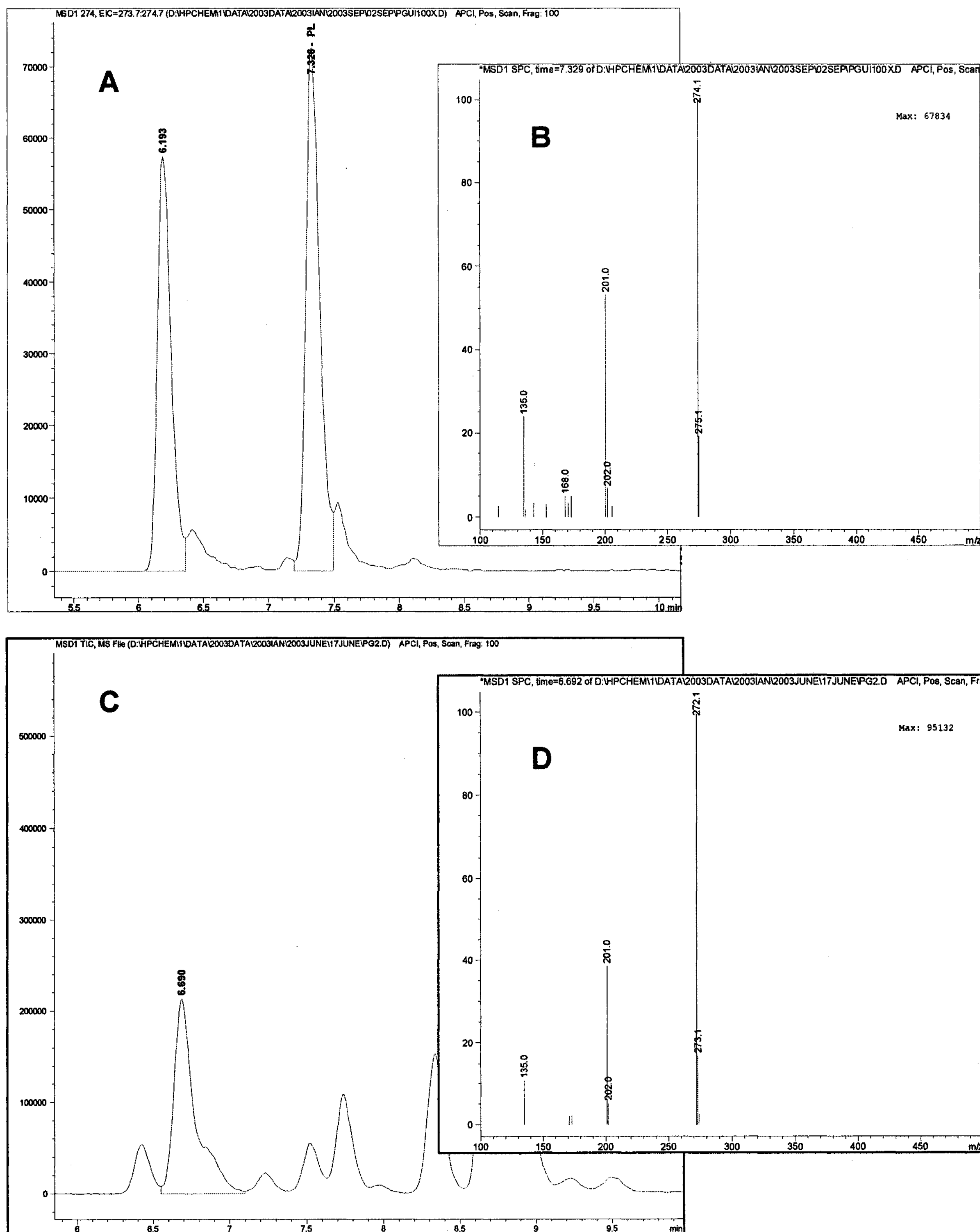


Figure 10. MS chromatogram of *P. tuberculatum* extract with dihydropiperlonguminine (A) and mass spectra (B), dihydropiperine (C) and mass spectra (D).

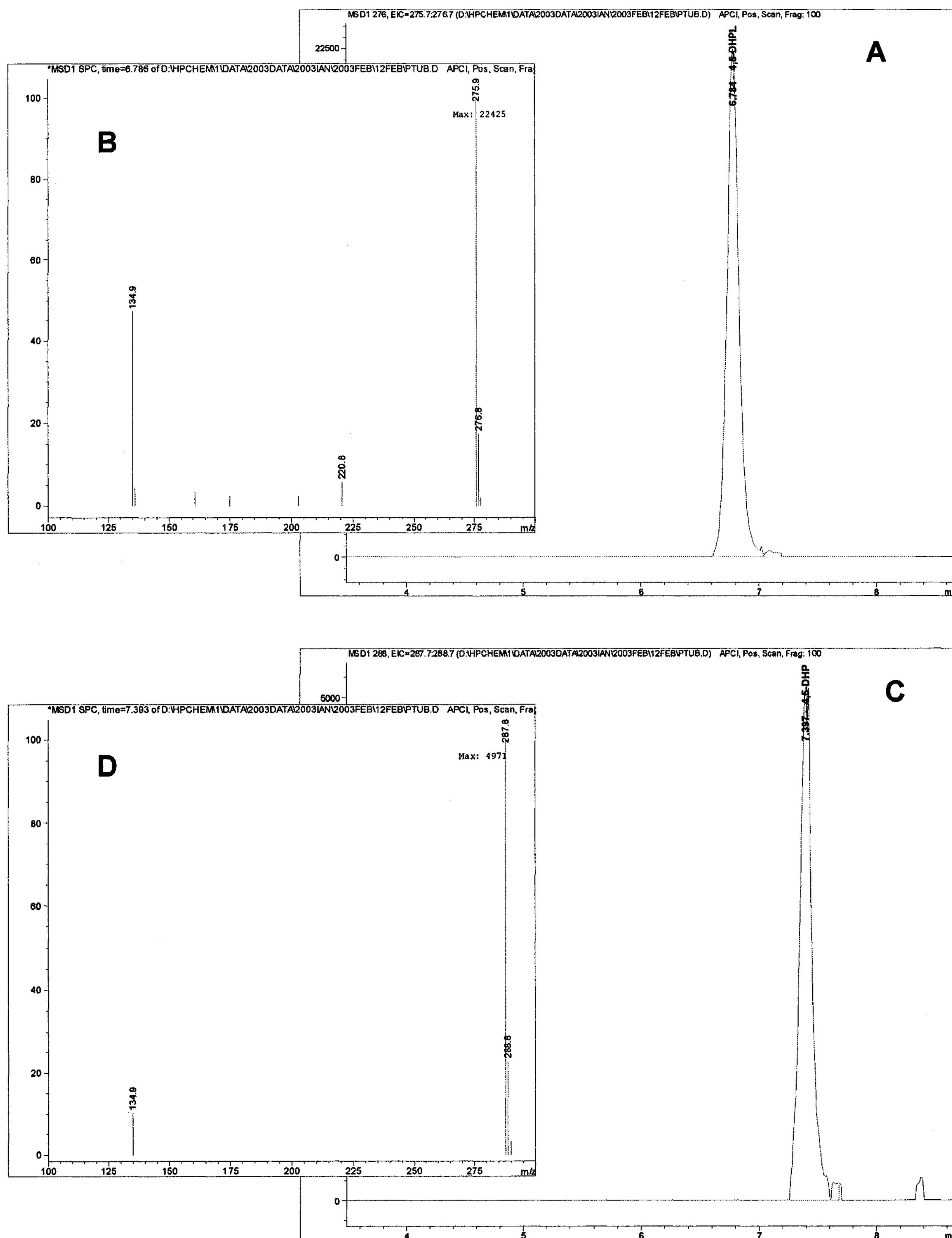
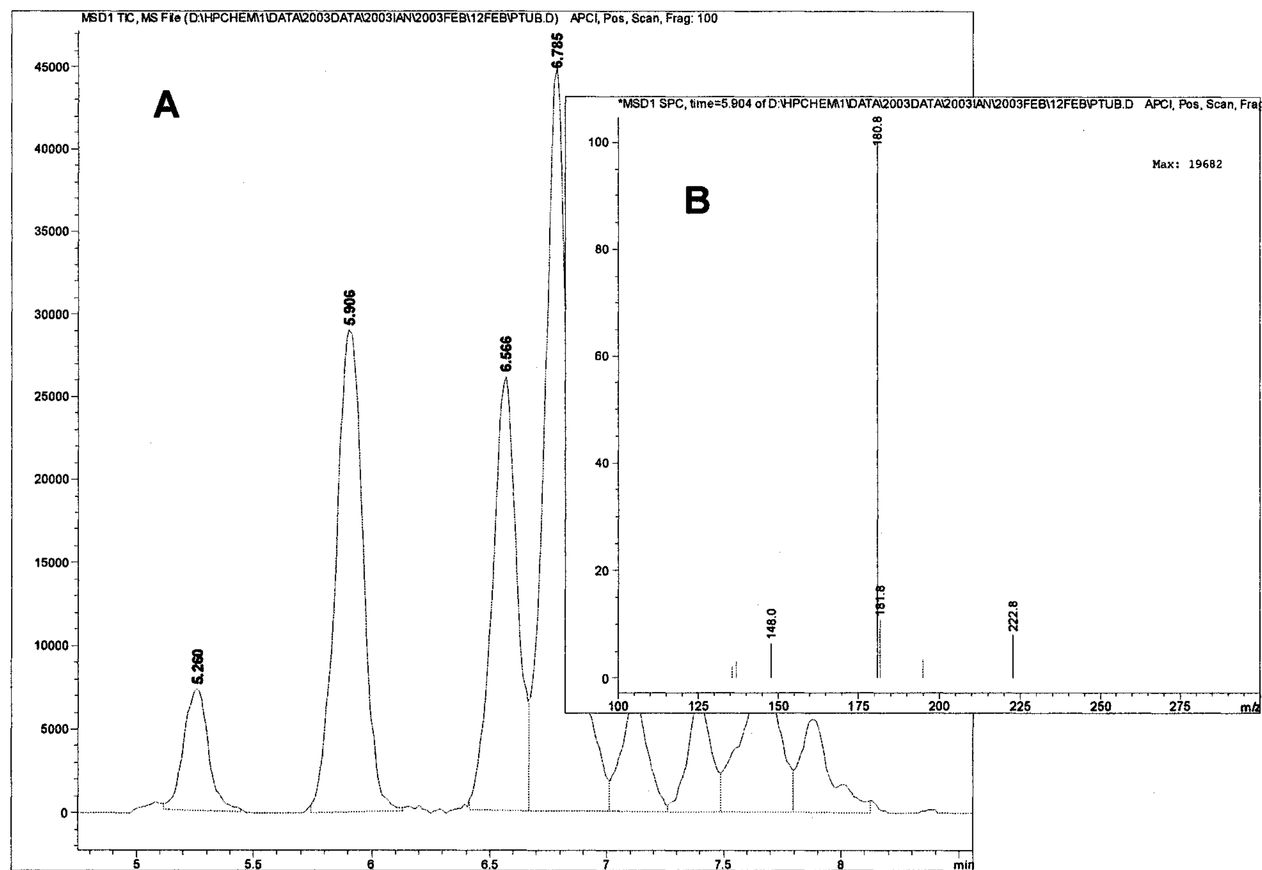


Figure 11. MS chromatogram of *P. tuberculatum* extract with pellitorine at 5.9 min (A) and mass spectra (B).



APPENDIX VI

ESTIMATED COST OF PRODUCING A *PIPER NIGRUM*-BASED BOTANICAL

Formulation price

- Pepper price (bulk) = $15 Cdn/kg
- 1 L 20% formulation = $300
- To treat 1 ha @ 1% = $472.50
- If peppercorn "2nds" used (20% cost) = $94.50/ha

Pyrethrum comparison

- $210 US/gallon (PyGanic 1.4% ai)
- Recommend 1 gallon/acre = $26.25/acre
- 1% Pepper = $37.80 Cdn or $28.35 US/acre
- Reduce price if 0.1% extract used