INSECTICIDAL ACTIONS OF *CITRUS AURANTIUM*

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To my Giota

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SUMMARY

This thesis was aimed at the extraction, isolation and structure elucidation of insecticidally active secondary metabolites of *Citrus aurantium* plant parts for potential use either as commercial insecticides or as lead compounds.

Fruits, leaves and shoots of *C. aurantium* were extracted in methanol and the chemicals recovered were fractionated by liquid-liquid partitioning using organic solvents of increasing polarity (petroleum ether, dichloromethane, ethyl acetate). Petri dish exposure bioassays revealed that only the petroleum ether fraction of the fruit methanol extract was toxic against *Bactrocera oleae* adults. The bioactive chemicals present in the fruits were produced and/or accumulated in the peels.

Petri dish exposure and topical application bioassays revealed that a petroleum ether peel extract was toxic against *B. oleae* and *Ceratitis capitata* adults. In several cases, differences in susceptibility were revealed between the two species and between the two sexes of the same species.

First gravity column fractionation of the peel extract revealed that only the F_2 fraction was active, while fraction F_1 has a synergistic effect. Further purification of the F_2 fraction on the second gravity column, followed by HPLC, resulted in the isolation of three major compounds.

The chemical structure of these components was elucidated by spectroscopic methods (UV, FTIR, GC-MS and ^{1}H NMR) and was assigned as osthol, bergapten and 6′,7′-epoxybergamottin. Of the three isolated compounds, only the 6′,7′-epoxybergamottin was active and its toxicity was the same to the synthesised 6′,7′-epoxybergamottin. However, the synergism revealed between isolated bergapten and 6′,7′-epoxybergamottin was not confirmed when isolated bergapten was replaced by synthetic bergapten, indicating that minor components present in the isolated bergapten were responsible for the synergistic effect.

Crude or semi-purified peel extracts of *C. aurantium* may have potential for insect control. Moreover, 6′,7′-epoxybergamottin has apparently never been reported to have deleterious effects on insects. At this early stage, the potential of 6′,7′-epoxybergamottin as a lead compound for a new class of insecticides remains to be determined.

CONTENTS

ABBREVIATIONS

ANOVA:	Analysis of variance
BO:	*Bactrocera oleae*
b.p.:	Boiling point
br:	Broad
°C:	Degrees Celsius
ca.:	Approximately
CA:	*Citrus aurantium*
CC:	*Ceratitis capitata*
$CDCl_3$:	Deuteriochloroform
$CHCl_3$:	Chloroform
cm:	Centimeter
Conv. S-L:	Conventional solid-liquid extraction method
d:	Day or doublet, depending on context
DCI:	Direct Contact Inlet
DCM:	Dichloromethane
DEPT:	Distortionless enhancement through polarisation transfer
df:	Degrees of freedom
EI:	Electron impact (ionisation)
EtOAc:	Ethyl acetate
eV:	Electron volt
FID:	Flame Ionisation Detector
FTIR:	Fourier Transform Infrared Spectroscopy
g:	Gram
GC:	Gas Chromatography
h:	Hour
Hex:	n-Hexane
H_2O:	Water
HPLC:	High Performance Liquid Chromatography
Hz:	Hertz
i.d.:	Internal diameter
IPM:	Integrated Pest Management

IR:	Infrared
l:	Litre
LC_{50}:	Median lethal concentration: Statistically derived concentration expected to kill 50 % of test organisms in a given population
LD_{50}:	Median lethal dose: Statistically derived dose expected to kill 50 % of test organisms in a given population
μA:	Microamper
μg:	Microgram
μl:	Microlitre
μm:	Micrometer
m:	Multiplet
mA:	Milliamper
MCPBA:	3-Chloroperbenzoic acid
MeOH:	Methanol
mg:	Milligram
MHz:	Megahertz
min:	Minute
ml:	Millilitre
mm:	Millimeter
MS:	Mass Spectrometry
m z:	Mass-to-charge ratio
n:	Number of sampling units in a sample
Na_2SO_4:	Sodium sulphate
nm:	Nanometer
NMR:	Nuclear Magnetic Resonance
P:	Probability
PE:	Petroleum ether (b.p. 40-60 ^{o}C)
PTFE:	Polytetrafluoroethylene (Teflon)
R_f:	Retention factor
s:	Singlet
SE:	Standard error
sec:	Second
SIT:	Sterile Insect Technique
t:	Triplet

TLC:	Thin Layer Chromatography
UV:	Ultraviolet
v:	Volume
χ^2:	Chi-squared

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INSECTS AS PESTS

From a purely anthropocentric standpoint, insects are pests when they harm man, his crops, animals or property. More specifically, in agronomy an insect is classified as a pest if the damage it causes to a crop or to livestock is sufficient to reduce the yield and/or quality of the harvested product by an amount that is unacceptable to the farmer (Dent, 1991). The estimated number of insect species varies from less than 5 million to as many as 80 million (Gullan and Cranston, 1994). However, only around 1 million insect species have been described up to now. Of these, fewer than 10,000 can be considered as pests, some 3,500 requiring regular attention and as few as 600 requiring control measures (Pedigo, 1996).

Insects may become crop pests for many reasons:

- The accidental or intentional introduction of insects into a favourable new area without the controlling influence of their natural enemies (Gullan and Cranston, 1994).
- The adoption of introduced crop species by native insects (van Driesche and Bellows, 1996).
- The simplification of agroecosystems by large-scale culture of a single crop, with uniform planting and harvesting schedules (van Driesche and Bellows, 1996).

- The use of broad spectrum insecticides which cause non-pest species to become major pests by eliminating their natural enemies (Johansen, 1962).
- The use of less resistant, but more high-yielding cultivars compared to wild relatives (Dent, 1991).
- The adoption of cultural practices, such as the continued cultivation of a single crop, without crop rotation, sanitation and a fallow period, which allows the build-up of insect pest numbers (Gullan and Cranston, 1994).

Since the development of agriculture, man has employed a variety of control measures to protect his crops from pest insects. These are: control through plant resistance (Dent, 1991), cultural control (Coaker, 1987), biological control (van Driesche and Bellows, 1996), chemical control (Matsumura, 1975) and control techniques that interfere with the normal physiological function or behaviour of insect pests (Dent, 1995) such as the use of pheromones (Jones, 1998) and the sterile insect technique (Boller, 1987). However, since the development of DDT the control of insect pests has been accomplished largely by the use of synthetic insecticides.

Over the past 30 years the concept of Integrated Pest Management (IPM) has completely changed the philosophy of pest control. Instead of using a single control technique (mainly the application of pesticides), to control a specific pest problem, various control measures are combined to control the whole range of pest species in a cropping system. IPM is a pest management strategy that, in the socioeconomic context of farming systems, the associated environment and the population dynamics of pest species, utilises all suitable techniques and methods in as compatible a manner

as possible, and maintains the pest population levels below those causing economic injury (Dent, 1991).

Plant-derived chemicals can play a critical role in IPM systems, as their properties – rapidly biodegradable, species-specific, environment-friendly – are compatible with the demands of IPM. However, it is generally acknowledged that synthetic insecticides are more effective, efficient and stable than natural insecticides (Assabgui *et al.*, 1997).

1.2 PLANT-DERIVED CHEMICALS AS A SOURCE OF INSECTICIDES

Plant-derived chemicals have played a crucial role in the history of humankind, as people had recognised and used plant-derived substances to cure illness and to protect their crops from pests (Hedin and Hollingworth, 1997). The estimated number of plant species in the world is 0.4-0.5 million (Hostettmann and Wolfender, 1997). However, only a tiny fraction of these species (around 10 %) has been investigated phytochemically and an even smaller fraction has been screened for activity against insect pests (Benner, 1993). Furthermore, even plant extracts that have been judged to be not toxic against a specific insect pest can exhibit considerable toxicity when tested against other insect pests, as secondary plant metabolites are often active against a narrow spectrum of insects (Ahn *et al.*, 1997). Thus, it can be concluded that there is a great potential for isolating chemicals from plants that can exhibit a range of biological activity against insects pests. These chemicals can cause acute toxicity, can be antifeedants, oviposition deterrents and/or repellents, and/or can interfere with growth and/or development (Coats, 1994).

Plants are known to produce a variety of secondary metabolites such as terpenoids, alkaloids, phenolics, polyacetylenes, flavonoids, unusual amino acids and sugars (Ahn *et al.*, 1997; Hostettmann and Wolfender, 1997). These substances have no apparent function to primary metabolism and their main role is to protect plants from microbial pathogens, insects, vertebrate herbivores and other plants (Coats, 1994). Other roles they have are as pollinator attractants and as chemical adaptations to environmental stresses (Balandrin *et al.*, 1985).

The renewed interest over recent years in plant-derived chemicals as insecticides has been fuelled by:

- The increasing political and consumer pressures to reduce the usage of synthetic insecticides due to their adverse environmental effects, residues on agricultural products and uncertain long-term ecological and biological effects (Isman *et al.*, 1997).
- The decreasing efficacy of synthetic insecticides due to the development of resistance (Ahn *et al.*, 1997).
- The decreasing developmental rate of new synthetic insecticides, due to stricter requirements on efficacy, selectivity, toxicology and general environmental impact (McLaren, 1986).
- The lower costs of the discovery and development of natural insecticides compared to synthetic insecticides (McChesney, 1994).
- The development of more sophisticated techniques and instruments for isolating, purifying and characterising the active substances from plant extracts (Hedin *et al.*, 1997).

Plant-derived insecticides appear to have several advantages over conventional broad-spectrum insecticides:

- They are likely to be more rapidly degradable, and consequently have lower environmental persistence than conventional insecticides; thus, they pose much less of a threat to the environment (Nair, 1994).
- They may also show increased specificity and so reduce the risks to beneficial organisms and consequently reduce the likelihood of outbreaks of secondary pests (Plimmer, 1993).
- In the case that new modes of action are validated, insecticide resistance problems may be reduced (Plimmer, 1993).

Chemical substances isolated from plants for their insecticidal properties can be used in three different ways to combat insect pests:

- In very exceptional cases as products *per se* (Benner, 1993).
- As lead structures for programmes of synthetic chemistry. The success story of the synthetic pyrethroids is the best example supporting the use of natural products as leads for further chemical synthesis (Miyakado *et al.*, 1997).
- As a source of new modes of action (Escoubas *et al.*, 1994), which is very important as the major compounds currently used to control insect pests are neurotoxins acting at limited targets (Copping and Hewitt, 1998).

There are numerous examples in the literature of plant-derived chemicals with insecticidal properties. The most important and significant application of a plant natural product centres on the insecticidal properties of pyrethrum, which is obtained from *Chrysanthemum cinerariaefolium* (Asteraceae). Pyrethrum is a powerful

insecticide which causes a rapid paralysis (knockdown), but it is unstable to sunlight and rapidly hydrolysed. Pyrethrum, which is a contact nerve poison, has served as the model for synthesis of the synthetic pyrethroids, several of which are of considerable current agricultural importance (Graves *et al.*, 1999).

The botanical insecticide which is attracting considerable interest nowadays is neem, a derivative of the seeds of the Indian neem tree, *Azadirachta indica* (Meliaceae). Neem is unusual among botanicals in that it is not an acute toxin. Its numerous reported effects include repellency, feeding deterrency, oviposition deterrency, interference with growth and development, and reproduction (Schmutterer, 1990).

A very well-known compound which has been used as a commercial insecticide is nicotine. Nicotine is found in many species of *Nicotiana* (Solanaceae). *Nicotiana rustica* is a much better source of this compound than the more familiar *Nicotiana tabacum* and has been cultivated specifically for nicotine extraction (Benner, 1993).

Rotenone is a botanical insecticide which still retains popularity with gardeners. Rotenone and related rotenoids are derived from the roots and tubers of South American and Southern Asian legumes in the genera *Derris*, *Lonchocarpus* and *Tephrosia* (Benner, 1993). Other botanical insecticides include sabadilla, ryania, hellebore, strychnine, nornicotine, and anabasine (Graves *et al.*, 1999).

Jacobson (1989) pointed out that the most promising botanicals as sources of novel plant-based insecticides for use in the present and in the future are species of the

families Meliaceae, Rutaceae, Asteraceae, Anoonaceae, Labiatae and Canellaceae. Among these six plant families, the Meliaceae and the Rutaceae predominate.

1.3 THE FAMILY RUTACEAE

The Rutaceae is distributed in warm and tropical areas of both hemispheres, with its centres of speciation in South Africa and Australia. There are 150 genera and some 900 species. The most important genus commercially is *Citrus*.

1.3.1 The genus *Citrus*

Citrus species are small, often spiny shrubs and trees of the tropics and subtropics native to Asia and Malaysia. A number are cultivated for their edible fruit: citron, orange, sweet orange, lemon, lime, pomelo, grapefruit, mandarin orange and tangerine.

Citrus plants have long been known to be a source of insect control agents. Back and Pemberton (1915) reported that *Citrus* peel oils were toxic to eggs and larvae of the Mediterranean fruit fly, *Ceratitis capitata*. Volatiles from *Citrus* peel oils have also been found to be toxic to eggs and larvae of the Caribbean fruit fly, *Anastrepha suspense* (Greany *et al.*, 1983). Abbassy *et al.* (1979) studied the contact activity of grapefruit, lemon, lime, mandarin, navel orange, native orange and sweet orange peel oils against two stored-product insects by the Petri dish exposure bioassay. They found that against the confused flour beetle, *Tribolium confusum*, lime oil was the most toxic, followed by navel orange, lemon, grapefruit and mandarin oils; sweet and native orange oils were the least toxic. Against granary weevil, *Sitophilus granarius*, lime oil was again the most toxic, followed by the oils of grapefruit, navel orange and

mandarin; lemon and sweet orange oils were the least toxic, while native orange oil was not toxic.

Sheppard (1984) demonstrated both the fumigant activity of grapefruit, lime, lemon and orange peel oils against foraging workers of the red imported fire ant, *Solenopsis invicta*, and the contact toxicity of orange peel oil by the topical application bioassay against house flies, stable flies, black soldier flies, paper wasps and house crickets. More recently, Ezeonu *et al.* (2001) studied the fumigant activity of the volatile extracts of sweet orange and lime against mosquito, cockroach and house fly. These authors found that volatiles of sweet orange showed greater insecticidal potency, while the cockroach was the most susceptible to the orange peels among the three insects studied.

Limonene, a major constituent of *Citrus* oils, exhibited contact and fumigant activity against several insects (Taylor and Vickery, 1974). Rani and Osmani (1980) reported that citral, a minor constituent of *Citrus* oils, was very active as a fumigant against house fly; contact toxicity was also exhibited by citral but to a lesser degree. Styer and Greany (1983) compared the insecticidal activity of citral and limonene against the Caribbean fruit fly. They found that citral was considerably more toxic than limonene to the eggs and larvae of this species. However, citral and limonene were equally effective to house fly adults by the topical application and moreover they were toxic to adults of the twospotted spider mite, *Tetranychus urticae*, by the leaf-dip bioassay (Lee *et al.*, 1997).

Su *et al.* (1972b) demonstrated that even when the volatile materials (about 30-35 %, mostly terpene compounds), were removed by lyophilisation from *Citrus* peel oils, the remaining non-volatile components exhibited activity when applied topically to stored-product insects. Terpeneless peel oils of lemon, grapefruit, lime, kumquat, tangerine, orange, tangelo and temple orange were moderately toxic to rice weevil at dosages of 25 and 50 $\mu g/cm^2$. Moreover, terpeneless peel oils of lemon, grapefruit, lime, kumquat and tangerine were highly toxic to cowpea weevil, whereas orange, tangelo and temple orange oils were inactive. Furthermore, none of the 8 peel oils studied showed any appreciable toxicity to black carpet beetle larvae, Indian meal moth larvae, cigarette beetle, red flour beetle and confused flour beetle. In addition, Su *et al.* (1972a) examined the efficacy of these 8 oils as surface protectants of black-eyed peas from cowpea weevil attack. They reported that all oils tested were effective in preventing nearly all development of an F_1 generation at 1.0 and 0.75 % by weight of the peas.

Salvatore *et al.* (2004) showed that diethyl ether, ethyl acetate and methanol lemon peel extracts were toxic against *C. capitata* larvae when these extracts were incorporated into their diet. Moreover, a crude aqueous extract of lemon peel was toxic to *Culex pipiens* larvae (Thomas and Callaghan, 1999). However, even powdered sun-dried *Citrus* peels possess insecticidal chemicals: Don-Pedro (1985) demonstrated that powdered sun-dried orange and grapefruit peels applied as food admixtures, exhibited toxic and repellent properties against *Dermestes maculatus* and *Callosobruchus maculatus*.

Limonoids, a group of chemically related bitter tetranortriterpene derivatives, are the most distinctive secondary metabolites of the plant order Rutales (Champagne *et al.*, 1992). *Citrus* limonoids, present in large quantities in the by-products of *Citrus* industry (Klocke and Kubo, 1982), may act as either feeding deterrents or toxins, depending on the insect species (Mendel *et al.*, 1991b). The three major neutral limonoids occurring in the seeds of *Citrus* species are limonin, nomilin and obacunone (Mendel *et al.*, 1991b). Limonin, the principle bitter component of *Citrus*, represents a potential pest management tool for many insect species (Klocke and Kubo, 1982). Its antifeedant activity has been reported against the Colorado potato beetle, *Leptinotarsa decemlineata* (Alford *et al.*, 1987; Mendel *et al.*, 1991a, 1991b) and the fall armyworm, *Spodoptera frugiperda* (Mendel *et al.*, 1991a, 1993). Nomilin has been found to deter feeding of *Reticulitermes speratus* (Serit *et al.*, 1992), *Ostrinia nubilalis* (Arnason *et al.*, 1987), *Leptinotarsa decemlineata* (Mendel *et al.*, 1991b) and *Spodoptera frugiperda* (Mendel *et al.*, 1993). Antifeedant activity has also been reported for obacunone towards *Reticulitermes speratus* (Serit *et al.*, 1992) and *Leptinotarsa decemlineata* (Mendel *et al.*, 1991b). Limonin, obacunone and nomilin were reported to act not only as feeding deterrents against the Colorado potato beetle, but also as toxins (Mendel *et al.*, 1991b). Moreover, these three limonoids were reported to exhibit moult inhibiting activity in mosquito *Culex quinquefasciatus* larvae (Jayaprakasha *et al.*, 1997).

Apart from the insecticidal properties of *Citrus* species, many other activities of these species have been reported. Volatile components of several *Citrus* fruit essential oils reported to exhibit antimicrobial action on *Penicillium digitatum* and *Penicillium italicum* (Caccioni *et al.*, 1998). *Citrus* peel and seed extracts have an interesting

antioxidant activity with regard to citronellal (Bocco *et al.*, 1998). Nogata *et al.* (1996) reported that *Citrus* fruit extracts inhibited the enzyme activity of rat platelet cyclooxygenase and lipoxygenase. *Citrus aurantium* fruit extracts reduced food intake and body weight gain, in rats and also produce significant pathological changes in electrical activity of myocardium; their potential as antiobesity herbal medicine was discussed (Calapai *et al.*, 1999). Moreover, the antimicrobial, antifeeding, insecticidal and antitumour activities of *Citrus paradisi* are well documented (Tirillini, 2000).

Although the insecticidal properties of *Citrus* species are well documented, bitter orange *C. aurantium*, has received little attention, like many other members of this genus. Moreover, most reports concerning studies on the insecticidal properties of *Citrus* species were focused on peel and seed extracts and not enough information is available on the insecticidal properties of extracts derived from other *Citrus* plant parts (e.g. leaves, shoots, etc.). In this study *C. aurantium* has been chosen in order to study in detail the insecticidal potency of some of its tissues.

Citrus aurantium (Linnaeus) (Rutaceae)

Citrus aurantium, also known as bitter or sour orange, is a small tree with a smooth, greyish-brown bark and branches that spread into a fairly regular hemisphere. The leaves are evergreen, 3 to 4 inches long, glossy, dark green on the upper side, paler beneath and have sometimes a spine in the axil. The bitter orange and edible orange trees closely resemble one another, but their leaf-stalks show a marked difference: that of bitter orange being broadened out in the shape of a heart. The fruit is globular, a little rougher and darker than that of the common sweet orange.

1.4 THE FAMILY TEPHRITIDAE

The Tephritidae (fruit flies) is a moderately large family with about 4500 species which belongs to the order Diptera (flies). Fruit flies are distributed throughout the temperate, subtropical, and tropical areas of the world. Individual flies vary in body length from 1 to over 20 mm. Adult flies are often brightly coloured, their wings may be banded, they feed on sugary food and they are long-lived (5-6 months). The females of the main agricultural pests lay eggs in fruits, the developing larvae feed inside the fruit and most leave when fully grown to pupate in soil. These pests are thus difficult to kill as larvae, and most control measures have to be applied against the adult flies. Pest species of Tephritidae fall mostly into the following genera: *Anastrepha*, *Ceratitis*, *Bactrocera* and *Rhagoletis* (Hill, 1997).

1.4.1 *Bactrocera oleae* (Gmelin) (Diptera: Tephritidae)

Bactrocera oleae, also known as the olive fruit fly, exists in all Mediterranean olive-growing countries and is considered to be the most serious pest of olive fruits.

The adult (Figure 1.1) measure 4 to 5 mm in length. The thorax is reddish-yellow, with a black dorsum encircled by four greyish bands. The wings are hyaline, with veins and a dark mark at the apex. The abdomen is of blond colour with segments one to four decorated with two lateral black marks of varying size. Larvae are white when living in green olives and dirty-looking purple when living in black olives, and are *ca.* 7 mm long when full-grown. The colour of the puparium varies from pale yellow to brown.

Figure 1.1 *Bactrocera oleae*, adult female.

B. oleae's oviposition and food acquisition for larval growth are restricted to fruits of the genus *Olea*, from cultivated as well as wild species. Other essential activities and resources, such as adult feeding, mating and shelter sites, may also be found on non-host plants. The *B. oleae* female is attracted to the host plant when the olives are suitable for oviposition. Each female tends to oviposit on suitable olives where no other egg had previously been laid. The annual population development of *B. oleae* is interrupted in northern Mediterranean regions mostly by the harsh climatic conditions of winter. In intermediate Mediterranean zones, it is interrupted by unfavourable conditions occurring both winter and summer. In southern Mediterranean regions, *B. oleae* population development is interrupted only by the high temperature-low relative humidity conditions of summer.

Attack by *B. oleae* may potentially account for 50-60 % of the total insect pest damage and falls into three main categories: (a) premature fruit fall, (b) decreased

yield and quality of oil and (c) spoiling of fruit for consumption as table olives (Walton, 1995).

1.4.2 *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae)

Ceratitis capitata, also known as the Mediterranean fruit fly or Med fly, is the single most important pest of the family Tephritidae. It is native to central Africa and as a result of transport by humans, it is now found in practically all the subtropical regions of the world.

The adults (Figure 1.2) are similar in size to house flies, have a yellow and black thorax and a yellow abdomen with two silver crossbands, while their wings are transparent with bands of yellow, brown, and black. Larvae are white, *ca.* 10 mm long when full-grown, and the puparium is brown.

Figure 1.2 *Ceratitis capitata*, adult female.

Adults or puparia overwinter in cool regions, but in warmer areas, the species is active all year. Female flies, which may produce up to 600 eggs, oviposit 2-10 eggs in holes under the skin of the fruit. Eggs hatch in 2-20 days, and larvae feed within fruits for about one week. After completion of development, larvae fall to the ground and pupate in soil for *ca.* 10 days. Generation time is variable (3-12 weeks), depending on climatic conditions.

The Med fly is among the most destructive pests of more than 100 species of fruit including orange, peach, grapefruit, plum, apple, and pear. In subtropical areas, larvae feed on the pulp of the fruits, causing the development of infection courts for fruit diseases. Also, holes made by female flies during oviposition scar the fruits, which lowers quality (Pedigo, 1996).

The predominant method of control of *B. oleae* and *C. capitata* during recent decades has been through the use of conventional pesticides. Two types of insecticide treatment have generally been used: (a) cover sprays with organophosphorous insecticides which kill adults (on contact) and larvae in the fruit (by a semi-systemic effect) and (b) bait sprays using protein hydrolysates and insecticide which attract and kill the adults. By applying bait sprays, only a part of the tree need to be sprayed and as a result the quantity of insecticide required, the damage to the fauna of beneficial insects as well as the insecticide residues, is greatly reduced (Katsoyannos, 1992). However, ecological, toxicological and environmental problems related to the use of pesticides have given the impetus for the development of alternative methods of controlling these pests. These are: the use of traps, the sterile insect technique (SIT), biological control (Kapatos *et al.*, 1977; Wong *et al.*, 1992), insect growth regulators

(Budia and Viñuela, 1996; Mazomenos *et al.*, 1997), microbial insecticides (Alberola *et al.*, 1999; Peck and Mcquate, 2000) and botanical insecticides (Stavroulakis *et al.*, 2001).

A variety of traps utilising one or more attractants (visual, food and sex) have been designed and evaluated and several attempts to control these flies with such traps have been made, with encouraging results (Broumas and Haniotakis, 1994; Katsoyannos and Papadopoulos, 2004). Whether these results can be considered satisfactory for control purposes, depends on the population levels of the species in the area, the density of the traps used and the effectiveness of the traps during a particular period (Kapatos, 1989).

Yellow sticky traps, baited with a food attractant (usually slow release ammonium salts) and the female sex pheromone (Baker *et al.*, 1980; Mazomenos and Haniotakis, 1981, 1985) for *B. oleae* and different parapheromones (trimedlure, methyleugenol and 'Cue-lure') (Jones, 1998) for *C. capitata* give very acceptable crop protection at low to medium pest population densities (Bueno and Jones, 2002; Katsoyannos and Papadopoulos, 2004).

One disadvantage of such trap, which is based totally or partially on attraction to colour, is that it also attracts non-target insects, and at high trap densities can cause damage to beneficial insect populations (Neuenschwander, 1982). By using grey, green or brown colours attraction of beneficial insects is reduced and thus their populations are conserved (Mazomenos *et al.*, 2002).

Sticky traps used for mass trapping suffered from their limited catching capacity (especially in areas with high populations). Sticky traps have thus been superseded by 'target' devices treated with insecticides to kill flies once they have made contact with the device. Using such 'lure and kill', problems of trap saturation are overcome because the insect, having picked up a lethal dose of insecticide from the target device, then flies or walk away from it until the toxic effects of the insecticide manifest themselves (Jones, 1998).

An alternative method to control *C. capitata* is the sterile insect technique. The SIT is amongst the most non-disruptive pest control methods. Unlike some other biologically-based methods it is species-specific, does not release exotic agents into new environments and does not even introduce new genetic material into existing populations as the released organisms are not self-replicating. However, the SIT is only effective when integrated on an area-wide basis, addressing the total population of the pest, irrespective of its distribution (Hendrichs *et al.*, 2002). In different parts of the world (e.g., North, Central and South America, Japan, Australia, etc.) the massive releases of sterile flies proved to be a technology capable of suppressing or eradicating *C. capitata* populations on an area-wide scale with negligible adverse effects to the environment (Schwarz *et al.*, 1989; Hendrichs, 1996). In recent years this control method has become even more cost-effective due to new technological breakthroughs such as better diets for mass-rearing, development of male only strains, increased precision in sterile fly releases, better sterile: fertile discrimination techniques and more sensitive monitoring networks (Hendrichs *et al.*, 1995; Hendrichs *et al.*, 2002).

SIT has been examined for use against *B. oleae* and much knowledge has been gained on rearing techniques and the quality and competitiveness of the sterile flies. However, the two small scale field trials made in Greece in 1973 and 1974 and again in 1979, 1980 and 1981, were only partially successful and there are still a number of political and techno-biological problems that have to be adequately resolved before SIT could be used for the control of *B. oleae* (Kapatos, 1989). Several differences in physiological and behavioural characteristics between mass-reared and wild flies have been reported, which may account for the failure of SIT control. These include longevity, reproductive pattern and capacity, male competitiveness, flight ability, field dispersal, eye colour and vision and pheromone production (Katsoyannos, 1992).

1.5 AIM AND OBJECTIVES

The aim of this study is to investigate whether *C. aurantium* plant parts synthesise and/or accumulate chemical compounds that can be used as alternatives of the synthetic insecticides currently used for the control of Tephritidae.

The objectives of this study are:

- To screen *C. aurantium* fractions and extracts for insecticidal activity against *B. oleae* adults.
- To investigate the insecticidal activity of the most promising *C. aurantium* extract by contact and fumigant bioassays to both *B. oleae* and *C. capitata* adults.
- To isolate the active substances of the most promising *C. aurantium* extract by using bioassay-guided procedures and *B. oleae* as the test insect.

- To elucidate the structure of the isolated substances by spectroscopic methods and to compare the activity of the isolated substances with synthetic ones.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Methods specific to individual chapters are described in those chapters.

2.1 MATERIALS

2.1.1 Insects

Artificially-reared *Bactrocera oleae* (BO) flies, were mainly used in this study, while artificially-reared *Ceratitis capitata* (CC) flies, were used only in order to compare the susceptibility of the two insects to the final petroleum ether peel extract of *Citrus aurantium* (CA). Preliminary studies showed that, in both fly species, there were no differences in susceptibility between artificially-reared and wild adults. Laboratory-reared flies were preferred, because they can be produced in high numbers under standard conditions, their development is faster, and they are available over the whole year. Wild insects, by contrast, are available only 3-4 months per year, and their rate of development depends upon environmental conditions which vary significantly.

Laboratory-reared flies

Adults of both fly species were obtained from artificially-reared colonies maintained in the insectarium of the Biology Institute NCSR Demokritos for many generations. The rearing method for BO under laboratory conditions was described by Tsitsipis (1975, 1977a, 1977b). CC was reared under laboratory conditions using the method used for BO, with slight modifications concerning caging and oviposition system. The same adult and larval diets were used for both insect species. All the insects' stages

were maintained in separate rooms under the same rearing conditions: 25 ± 2 °C, 65 ± 5 % relative humidity, 12 h:12 h light:dark regime and artificial light (3000 lux) provided by fluorescent light. BO and CC adults were maintained in wood-framed cages (100 cm long x 40 cm wide x 30 cm high) (Figure 2.1) and in Plexiglas cages (30 x 30 x 30 cm) (Figure 2.2) respectively and provided with adult solid diet and water. Adult solid diet was a mixture of yeast hydrolyzate (Nutritional Biochemical Corporation, USA), sucrose and fresh egg yolk, in a ratio of 1:4:0.7, plus 0.05 % streptomycin sulphate (Tsitsipis, 1977b). After collection, eggs were transferred with a brush onto a filter paper impregnated with 0.3 % propionic acid, in a Petri dish, for an incubation period of 24 h at 25 °C (Manoukas and Mazomenos, 1977) and subsequently placed on the larval rearing medium. Around 5 eggs were placed for each gram of larval diet (Tsiropoulos and Manoukas, 1977). The latter had the following composition (Tsitsipis, 1977b): tap water 55 ml, cellulose powder (No. 123, Schleicher and Schüll, Germany) 30 g, brewer's yeast (Schwechat, Austria) 7.5 g, soya hydrolyzate enzymatic (Nutritional Biochemical Corporation) 3 g, sucrose 2 g, olive oil (0-1 acidity) 2 ml, Tween-80 0.75 ml, potassium sorbate (Merck, Germany) 0.05 g, Nipagin (methyl-p-hydroxybenzoate) (Merck) 0.2 g, and HCl solution (2N) 3 ml. The fully developed larvae were allowed to pupariate in sawdust. The puparia were sieved to remove the sawdust 1-2 days before adult emergence and then placed in open plastic boxes which were finally introduced into the colony cages.

Figure 2.1 Wood-framed colony cage in which artificially-reared BO adult flies were maintained.

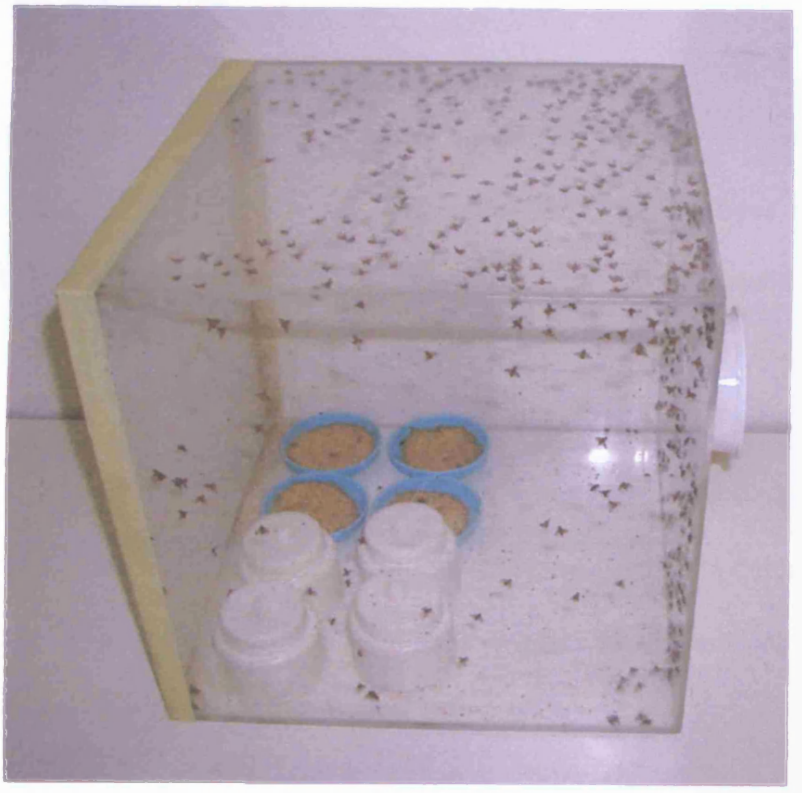

Figure 2.2 Plexiglas colony cage in which artificially-reared CC adult flies were maintained.

Handling of flies used in experiments

2-3 d-old adults were used in all bioassays. To ensure that adult flies used in this study were approximately of the same age, they were allowed to emerge for 24 h in a Plexiglas cage (30 x 30 x 30 cm) and then the remaining puparia were transferred every 24 hours to separate cages. Sufficient number of insects of the same generation (batch) for the five successive days of screening experiments was achieved, by placing half of the puparia at the optimum storage temperature (25 °C) and the other half at 18 °C. When all puparia are placed at 25 °C, eclosion of flies occurs for only 3 days. By placing half of the puparia at 25 °C and half at 18 °C, eclosion of flies occurs for 5 days, as a result of delayed eclosion of flies placed at 18 °C (Tsiropoulos, 1972).

2.1.2 Plant materials

All plant materials used in this study were collected from CA trees (Figure 2.3) located in the Zografos area of Athens, Greece. Ten trees were selected for sampling. These were approximately 10 years old, of similar size (4-5 m high) and healthy. In each collection the same numbers of fruits, leaves and shoots were obtained from each of the 10 sampling trees. The plant samples were transferred immediately to the laboratory and cleaned thoroughly in the following order: washed in detergent, rinsed with tap water and finally rinsed with distilled water. All plant materials were extracted fresh unless otherwise stated.

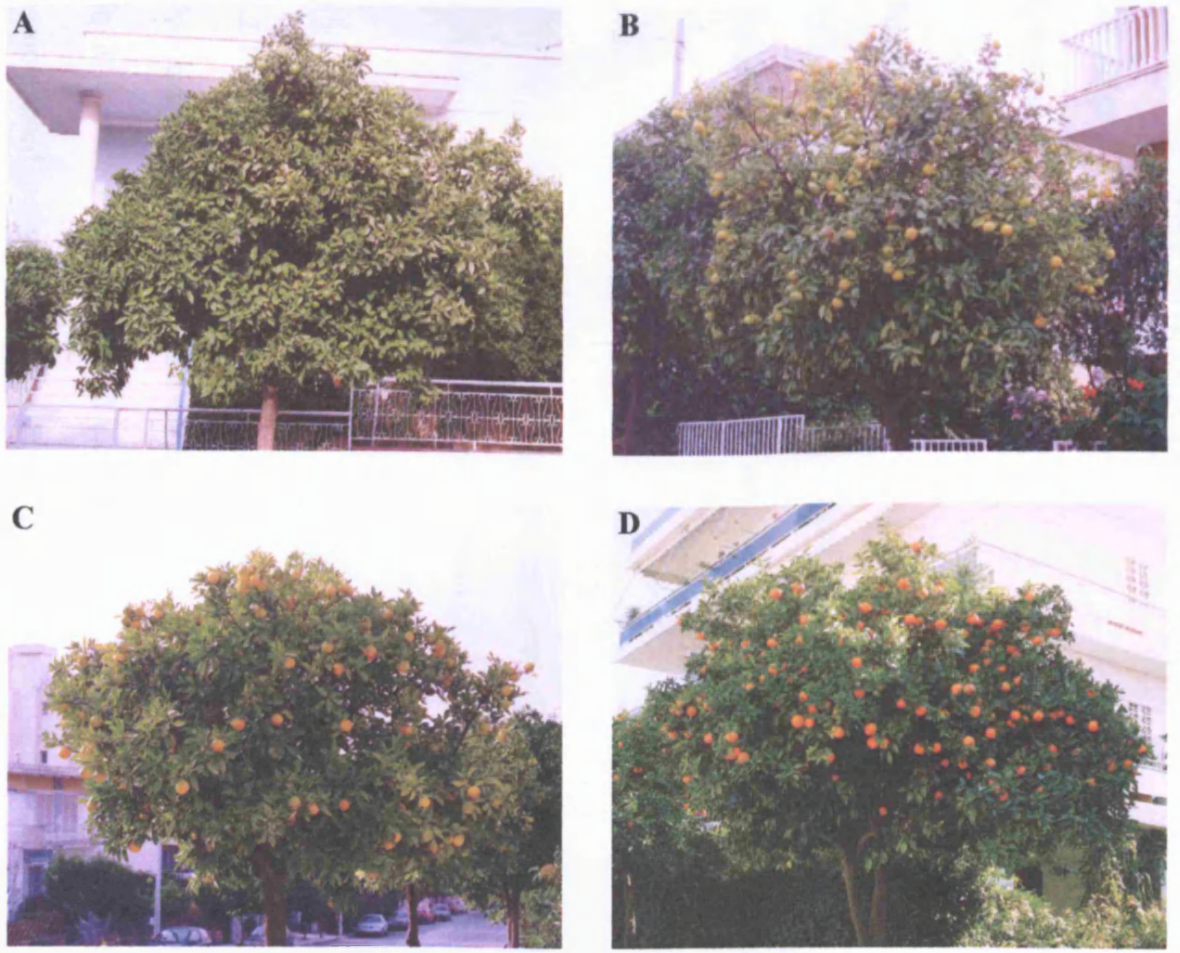

Figure 2.3 CA trees with fruits at different ripening stages.

(A) Unripe fruits (green); **(B)** Semi-ripe fruits (green-yellow); **(C)** Semi-ripe fruits (yellow-orange); **(D)** Ripe fruits (orange).

2.1.3 Chemicals

All chemicals were stored according to supplier's recommendations.

Standards

Bergamottin: Rotichrom HPLC, Carl Roth, Germany.

Bergapten: 99 %, Aldrich, USA.

Rotenone: 95-98 %, Sigma, USA.

Reagents

3-Chloroperbenzoic acid (MCPBA): ~ 70 %, Fluka, Switzerland.

Sodium sulphate (Na_2SO_4): Sodium sulphate anhydrous GR, Merck, Germany.

Solvents

Dichloromethane, ethyl acetate, n-hexane, methanol, petroleum ether (b.p. 40-60 %) and water, were HPLC grade (Lab-Scan, Ireland).

2.2 METHODS

2.2.1 Extraction methods

Two extraction methods were employed throughout this study: conventional solid-liquid extraction and ultrasound extraction. Prior to extraction, plant materials were homogenised with the appropriate extraction solvent (approximately 1 ml/g fresh plant material or dried equivalent to 1 g fresh) for 5 min in a blender.

Conventional solid-liquid extraction

Homogenised plant materials (100 g fresh or dried equivalent to 100 g fresh) were placed in a 2 l Erlenmeyer flask and cold solvent (20-22 °C) was added (final extraction solvent volume 1 l). The flask was then placed on a shaker (Orbital Shaker S01, Stuart Scientific, UK). Conventional solid-liquid extraction involved the maceration of the plant material with the extraction solvent for 24 h at room temperature (20-22 °C). Continuous shaking was applied in order to increase the efficiency of the method (Silva *et al.*, 1998). Although the solubility of compounds in a solvent increases with increasing solvent temperature, and also higher temperatures facilitate penetration of the solvent into the cellular structures of plant materials extracted, hot extraction solvents were not used due to the possibility that some compounds could be unstable at higher temperatures, giving rise to compound artifacts (Houghton and Raman, 1998).

Ultrasound extraction

Homogenised plant materials (100 g fresh) were placed in a 2 l Erlenmeyer flask and cold solvent was added (final extraction solvent volume 300 ml). The ultrasound extraction method involved the sonication of the samples in an ultrasonic bath (Bransonic Ultrasonic Cleaner B-5200 E1, Branson Ultrasonics Corporation, USA) at room temperature for a total of 30 min.

2.2.2 Bioassays

Three types of bioassay were used for the evaluation of the insecticidal activity of CA fractions and extracts: (a) Petri dish exposure bioassay, (b) Fumigation bioassay and (c) Topical application bioassay. All bioassays were conducted in the laboratory under

the same conditions as described for the rearing of insects, and commenced 2 h after the onset of photophase. Insects were considered dead if they did not move any of their appendages even when picked up with forceps. Mortalities were recorded every 24 h for 3-6 d depending on the bioassay. General descriptions of the bioassays are as follows:

Petri dish exposure bioassay

Glass Petri dishes of the following dimensions were used in this bioassay: bottom internal diameter 5 cm, sides height 1.3 cm and lid internal diameter 5.7 cm. The surface area of the bottom, sides and lid is 19.6, 20.4 and 25.5 cm^2, respectively. Test samples in 1 ml of the appropriate solvent were prepared by dilution from stock solutions. By using a 100 μl syringe (Hamilton, Switzerland) 299, 312, and 389 μl were applied to the bottom, sides and lid, respectively in order that all the treated surface area contained the same sample concentration ($\mu g/cm^2$). After sample application to the bottom and the lid, they were distributed manually under a fume hood during evaporation so that the test sample was deposited as uniformly as possible over the bottom and the lid surfaces. The bottom was then held horizontally for the application of the test sample to the sides and, after application, it was continuously rolled, manually, over a flat surface under the fume hood until all the solvent had evaporated, so that the test sample was deposited evenly over the sides. The bottom and the lid were then left under the fume hood for 1h in order to ensure complete evaporation of solvent traces.

Groups of 10 randomly selected adult flies, 2-3 d-old, were immobilised by chilling for a period of 5 min and subsequently introduced into each Petri dish, which was

then covered. Four thin cardboard strips (1 cm wide x 1.4 cm high, and less than 1 mm thick), oriented in four different directions, were placed between the sides and the lid of each Petri dish so that the dishes were not airtight (Figure 2.4). Control dishes were treated in a similar manner with solvent only, the same numbers of insects were introduced into them and run simultaneously with the treatments. After 12 h exposure to treated and control Petri dishes, flies of the same treatment were transferred to wood-framed cages (12 x 12 x 12 cm) containing solid adult diet and water (Figure 2.5). Prior to transfer, the Petri dishes were chilled briefly in order to decrease the flies' mobility, and consequently prevent insects from escaping them during transfer. After each test the Petri dishes were thoroughly cleaned and placed in the oven at 100 °C for 24 h.

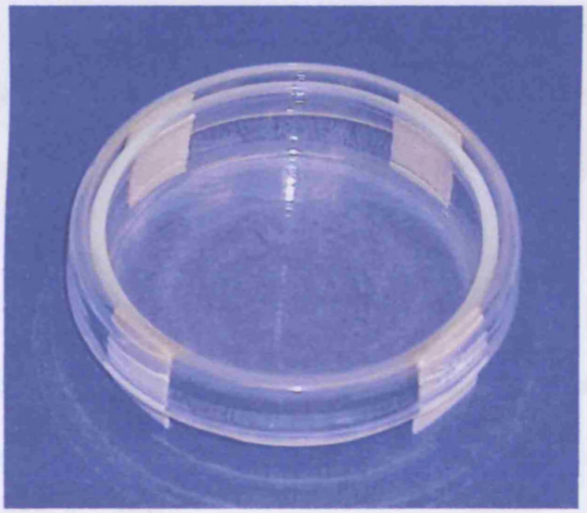

Figure 2.4 A Petri dish used in the Petri dish exposure bioassay in which the test sample was applied to the entire inner surface and thin cardboard strips were placed between the sides and the lid.

Figure 2.5 Wood-framed cages, containing solid adult diet and water, in which tested flies were transferred (10 flies/cage) after 12 h exposure to treated and control Petri dishes.

The method described above involves two modifications compared to the standard Petri dish exposure bioassay described by Abbassy *et al.* (1979): (a) the samples are applied uniformly to all of the inner surface of the Petri dishes, instead of only to the bottom, and (b) cardboard strips are placed between the sides and the lid of the Petri dishes.

There are certain advantages to be gained using these modifications:

- When a sample that possesses repellent properties is applied only to the bottom of the Petri dish, the test insects have the potential to discriminate between treated and untreated surfaces. Moreover, when a sample is applied only to the bottom, there is a possibility that an external stimulus (e.g. light) can influence the distribution of insects in the Petri dishes in a way such that the insects prefer the untreated to the treated surfaces. In both cases, insects acquire less amount of test sample and consequently their mortality will be

lower. In contrast, when the entire inner surface is lined with the test samples, insects cannot avoid acquiring residues of the test samples from the Petri dishes.

- The application of the same amount of test samples gives 3.3 times the concentration ($\mu g/cm^2$) when applied only to the bottom than when applied to the total inner surface. Consequently, in the case of crude extracts, in which activity is not pronounced and/or the texture is sticky, the possibility of the insects adhering to the extract film is reduced.
- By using the cardboard strips between the sides and the lid the possibility that mortality is due to fumigant action rather than contact is reduced, as the Petri dishes were not air-tight and consequently volatiles could escape.

Note that the time required for the preparation of the Petri dishes using the two modifications was much greater than that required for the unmodified apparatus.

Fumigation bioassay

This bioassay was designed in order to investigate the possibility that the observed mortality in the Petri dish exposure bioassay was due to fumigation rather than contact. For that purpose a sheet of gauze was fitted inside the Petri dishes to prevent direct contact between deposit and insect. Moreover, in order to determine whether the test samples have fumigant activity, a Petri dish fitted with a sheet of gauze was sealed with Parafilm.

Four Petri dishes were prepared for each concentration (mg/Petri dish) tested. In one Petri dish the test solution was applied to the total inner surface of the Petri dish as in

the Petri dish exposure bioassay, and in the other three Petri dishes only to the bottom. A sheet of gauze was fitted inside two Petri dishes in which the test sample was applied only to the bottom. Groups of 10 randomly selected adult flies, 2-3 d-old, were immobilised by chilling and subsequently introduced into each Petri dish, which was then covered. Cardboard strips were placed then between the sides and the lid of the Petri dishes as in the Petri dish exposure bioassay except one in which the test sample was applied to the bottom and a sheet of gauze was fitted. This Petri dish was sealed with Parafilm. In Figure 2.6 the 4 Petri dishes described above are shown. Control dishes were treated in a similar manner with solvent only, consisted of the same numbers of insects and run simultaneously with the treatments. Tested flies were

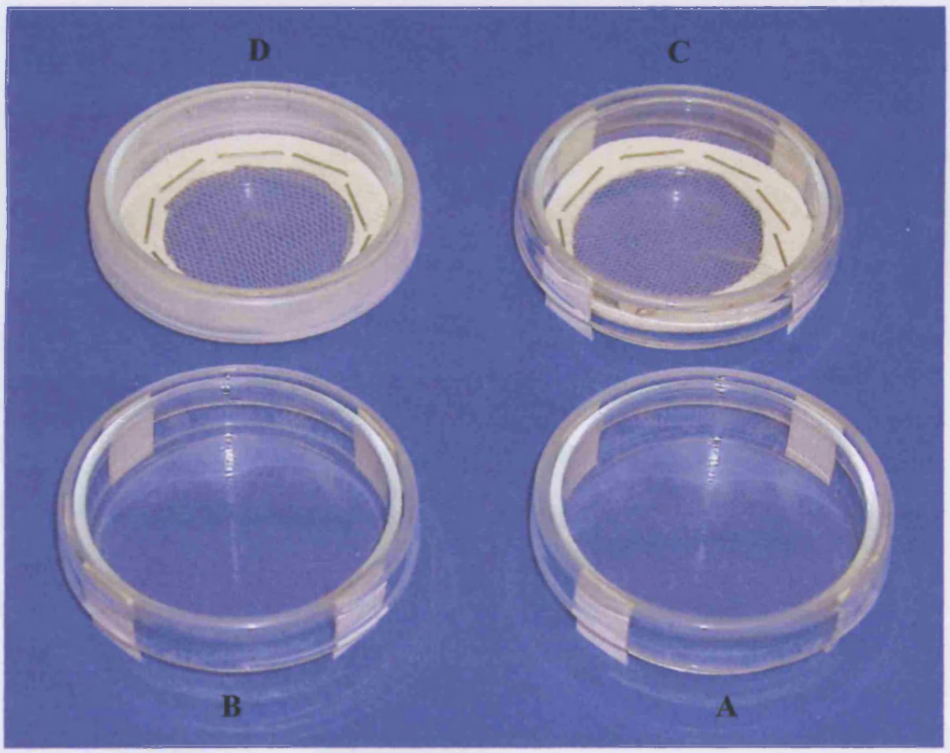

Figure 2.6 The 4 Petri dishes used in the fumigation bioassay.

(A) Test sample was applied to the total inner surface and thin cardboard strips were placed between the sides and the lid; **(B)** Test sample was applied only to the bottom and thin cardboard strips were placed between the sides and the lid; **(C)** Test sample was applied only to the bottom, a sheet of gauze was fitted inside the Petri dish and thin cardboard strips were placed between the sides and the lid; **(D)** Test sample was applied only to the bottom, a sheet of gauze was fitted inside the Petri dish and sealed with Parafilm.

transferred in individual wood-framed cages (12 x 12 x 12 cm) containing solid adult diet and water, 12 h after their introduction to the Petri dishes.

Topical application bioassay

In this study topical application bioassays were conducted according to the standard method 'number 1' described by McDonald *et al.* (1970). Groups of 10 randomly selected adult flies, 2-3 d-old, were immobilised by chilling and subsequently transferred into a Petri dish lined with filter paper. The Petri dish was then placed on ice to prevent flies from reviving before treatment. One μl of the test solution was applied to the dorsal surface of the thorax of each fly with a hand micro-applicator (Burkard, England) (Arnold, 1967) (Figure 2.7). The control run simultaneously consisted of the same numbers of insects treated with 1 μl of solvent each. After

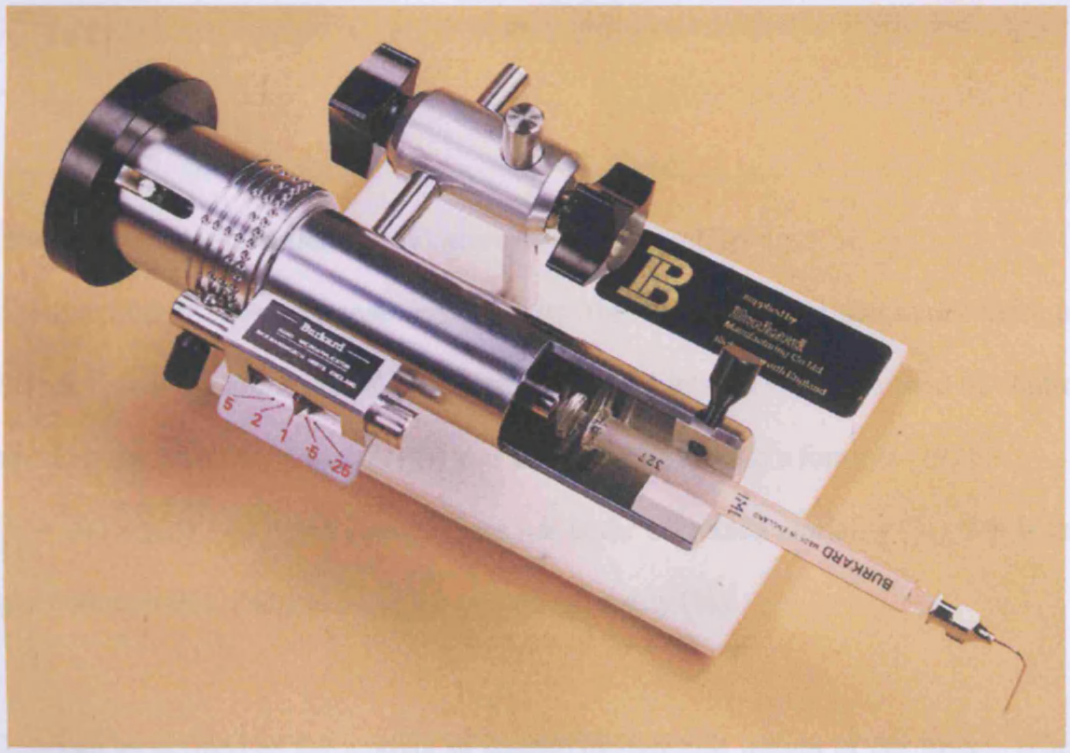

Figure 2.7 The Burkard hand micro-applicator, used to apply topically 1 μl of solvent or test solution to the dorsal surface of the thorax of each fly.

treatment, flies were transferred in individual wood-framed cages (12 x 12 x 12 cm) (10 flies/cage) containing solid adult diet and water.

2.2.3 Statistics

The program SPSS 8.0 for Windows provided the analysis environment for running statistical tests (ANOVA, Tukey's honestly significant difference test, probit analysis, Pearson's χ^2 goodness-of-fit test, etc.) and transformations (arcsine, $\log_{10}$, etc.).

Comparison of means

Mortality data were analysed by one-way analysis of variance (ANOVA) with dose/concentration as the independent variable. Mortality was transformed to arcsine$\sqrt{p}$ before ANOVA, where p is the proportion of dead insects, in order to equalise the variance among treatments. Comparisons and separations of means were performed by Tukey's honestly significant difference (Tukey's HSD) test (Tukey, 1953) at $P = 0.05$.

Estimation of median lethal dose concentration (LD_{50}/LC_{50})

In dose/concentration-response experiments the median lethal dose/concentration (LD_{50}/LC_{50}) were estimated by probit analysis (Finney, 1971). In the case of control mortality the recorded mortality (%) was corrected by Abbott's formula (1925):

$Mc = 100 \times (Mp - Mt)/(100 - Mt)$, where Mc is the corrected mortality (%), Mp is the recorded mortality (%) and Mt is the control mortality (%).

Dose can be defined as the amount of insecticide per unit weight or per insect. When the exact dose given to the insect cannot be determined, then concentration is used

which can be defined as the amount of insecticide per unit of an external media (Matsumura, 1975).

The median lethal dose/concentration (LD_{50}/LC_{50}) was in each case estimated from the regression of the cumulative corrected probit mortality versus $\log_{10}$ of the doses/concentrations. The probit regression line allowed the estimation of LD_{50}/LC_{50} and their corresponding 95 % confidence limits, and the slope and intercept with their corresponding standard errors. Moreover, Pearson's χ^2 goodness-of-fit test was performed in order to test how well the data fitted the probit model. When $P > 0.05$, data fitted the probit model adequately. In contrast, when $P < 0.05$ data did not fit adequately the probit model and a heterogeneity factor was automatically used in the calculations of confidence limits by SPSS. LD_{50}s/ LC_{50}s were considered significantly different when confidence limits did not overlap. From a biological point of view, the slope of a probit regression estimates the change in activity per unit change in dose or concentration (Robertson and Preisler, 1992). The length of 95 % confidence limits provides a measurement of the precision of an LD/LC estimate. Shorter lengths indicate greater precision (Finney, 1971) which allow differences between treatments to be revealed. Precisely estimated LD_{50}/LC_{50} are obtained when responses are evenly distributed between 25 and 75 % (Robertson *et al.*, 1984).

CHAPTER 3

SCREENING FOR INSECTICIDAL ACTIVITY IN *CITRUS AURANTIUM* EXTRACTS

3.1 INTRODUCTION

Variation in collection-site altitude, plant age, climate, and soil type can all influence the concentration levels of secondary metabolites and even the kinds of compounds biosynthesised in certain cases (Silva *et al.*, 1998). Furthermore, the time of collection, the time and temperature of extraction and the use of additional substances during extractions are factors that can affect the chemical composition of plant extracts (Houghton and Raman, 1998). Variation in the chemical profile of plant material will probably cause a difference in the activity of extracts when tested biologically (Houghton and Raman, 1998).

In the early stages of phytochemical investigations, one of the first and most obvious questions to ask is whether the bioactive chemicals are localised to certain parts of the plant (Cannell, 1998). Different organs in the plant are known to produce and/or accumulate different profiles of secondary metabolites (Silva *et al.*, 1998) and probably their extracts can exhibit different degree of activity when tested against insects. Gallo *et al.* (1996) reported that the crude hexane extract of *Mammea americana* (Guttiferae) seeds was significantly more active against larvae of *Trichoplusia ni* (Lepidoptera: Noctuidae) than the crude hexane extract of leaves when an ingestion bioassay was used. Screening experiments conducted by Kadir *et*

al. (1989) with *Spilanthes acmella* (Compositae) leaf and flower head extracts against adults of *Periplaneta americana*, using the topical application bioassay, revealed that the active chemicals occurred in greater concentrations in the flower heads than in the leaves. Weaver *et al.* (1994) examined the insecticidal activity of floral, foliar, and root extracts of *Tagetes minuta* (Asterales: Asteraceae) against adult Mexican bean weevils (Coleoptera: Bruchidae) using the Petri dish exposure bioassay. The results of this study revealed that the root extract was the most active, followed by the flower extract while the foliar extract was the least active.

Nogata *et al.* (1996) studied the inhibitory activity of different fruit tissue extracts (flavedo, albedo, pulp and juice) of unripe and ripe fruits of *Citrus lumia* and *Citrus reticulata* against cyclooxygenase and lipoxygenase, respectively. They reported that the flavedo and the albedo extracts of *Citrus lumia* inhibited cyclooxygenase to the same degree and were more inhibitory than the pulp extract, whereas the juice extract had no effect on the enzyme. The flavedo, albedo and juice extracts of *Citrus reticulata* unripe fruits inhibited lipoxygenase to the same degree and were more inhibitory than the pulp. In contrast, the flavedo and the albedo extracts of *Citrus reticulata* ripe fruits inhibited lipoxygenase to the same degree and were more inhibitory than the juice extract, whereas the pulp extract exhibited slightly activity.

Variation in the chemical composition of plant samples can also occur due to treatment after collection (Houghton and Raman, 1998). In most cases, plant material is dried prior to extraction (Silva *et al.*, 1998). Drying is the most common method of preservation commercially and is achieved by leaving the material in warm, dry air (Houghton and Raman, 1998). Temperature must not exceed 30 °C, and plant samples

must kept away from direct sunlight because ultraviolet radiation may produce chemical reactions giving rise to compound artefacts (Silva *et al.*, 1998). Oils obtained from dried *Citrus* peels were reported to be much less toxic to the house fly and red imported fire ant (Sheppard, 1984). However, Don-Pedro (1985) demonstrated that powdered sun-dried orange and grapefruit peels applied as food admixtures, exhibited toxic and repellent properties against *Dermestes maculatus* and *Callosobruchus maculatus*.

Gbewonyo (1991) pointed out that it is important in studies involving the examination of plants for insecticidal constituents, to test extracts obtained with different solvents and methods. Ideally, an extraction solvent should be easy to remove, and be inert, non-toxic, and not easily flammable (Silva *et al.*, 1998). The nature of the extraction solvent determines the type of chemicals it is likely to extract based on the "like dissolves like" general principle; non-polar solvents will extract out non-polar substances, and polar materials will be extracted out by polar solvents (Houghton and Raman, 1998). Salvatore *et al.* (2004) compared the activity of lemon peel extracts obtained with different solvents using an ultrasonic bath (20 °C for 20 min) by a larval toxicity bioassay against CC. They found that the diethyl ether extract was the most toxic, followed by the methanol extract, whereas the ethyl acetate extract was the least toxic.

Morgan and Wilson (1985) emphasised that extraction methods involving heating are inappropriate for the isolation of some insecticidal components from plants since some of these compounds may be heat-labile. This view was supported by Gbewonyo (1991) who reported that although Soxhlet extraction of the male root of *Piper*

guineense gave more extraction yield, its activity against *Musca domestica* by the topical application method, was less than the extract obtained by cold extraction.

The fact that plant secondary metabolite profiles may vary both quantitatively and qualitatively among different batches of the same plant part collected at different times has important consequences in making plant recollections for scale-up work (Silva *et al.*, 1998). Chloroform-methanol extracts of ripe and unripe fruits of *Piper guineense* did not differ significant in activity by the topical application bioassay against *Musca domestica* and *Periplaneta americana* adults although the activity of the ripe fruit extract was greater against both insects (Gbewonyo, 1991). The inhibitory activity against cyclooxygenase of *Citrus lumia* pulp extract decreased during ripening, while there was little difference in extract inhibition between unripe and ripe flavedo, albedo and juice extracts (Nogata *et al.*, 1996). Moreover, the inhibitory activities of *Citrus reticulata* pulp and juice extracts declined during ripening, while the activities of flavedo and albedo extracts remained almost constant.

Even though it is well documented that *Citrus* species contain chemicals that exhibit a wide range of insecticidal properties against a broad spectrum of insect pests, CA among other *Citrus* species had received little attention. That fact suggests that the investigation of the insecticidal actions of CA is worthwhile.

The objectives of this component of the study were:

- To screen various solvent fractions obtained from different plant parts of CA for insecticidal activity against adults of BO.

- To determine whether the active chemicals present in the fruits are localised to specific fruit tissues.
- To study the effect of different drying treatments on the stability of the bioactive chemicals present in the peels.
- To compare the effectiveness of different extraction methods and solvents on the recovery of the insecticidal chemicals.
- To study the effect of fruit maturation on the biosynthesis and/or bioaccumulation of the active chemicals.

3.2 METHODS

3.2.1 Extraction of plant materials

Plant materials from the selected CA trees were collected at different dates. In each collection date the same number of fruits, leaves and shoots were collected from each of the 10 sampling trees. The samples were transferred immediately to the laboratory, cleaned and extracted fresh unless otherwise stated. The collection data for CA samples are shown in Table 3.1.

Table 3.1 Collection data for CA samples.

Collection date	Plant part	Plant part weight (g)	Plant part description	Number of plant parts collected
01/08/1998	Fruits	30-40	Dark green	20
01/08/1998	Leaves	1.0-1.5	Annual	160
01/08/1998	Shoots	4-6	Annual	40
20/08/1998	Fruits	40-60	Dark green	50
10/09/1998	Fruits	50-80	Dark green	30
01/10/1998	Fruits	60-100	Light green	60
01/08/1999	Fruits	30-40	Dark green	30
01/09/1999	Fruits	50-70	Dark green	30
01/10/1999	Fruits	60-100	Light green	30
01/11/1999	Fruits	70-120	Green-yellow	30
01/12/1999	Fruits	80-130	Yellow-orange	30
01/01/2000	Fruits	90-140	Orange	30
01/02/2000	Fruits	90-140	Orange	30

Extraction of CA plant parts

Fruits, leaves and shoots of CA (Figure 3.1) were collected on 01/08/98 in order to investigate whether these plant parts contain chemicals with insecticidal activity. Ten

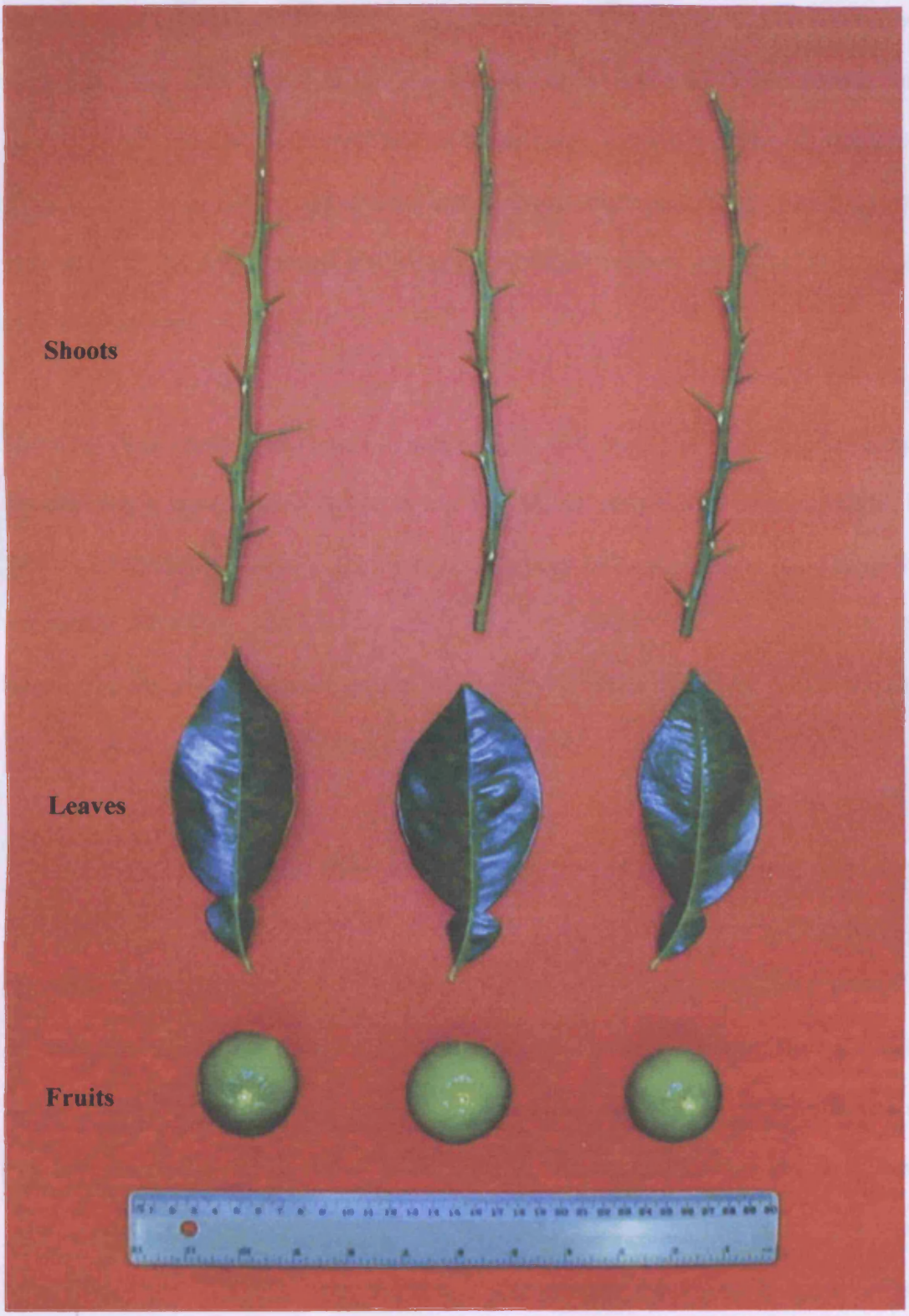

Figure 3.1 Fruits, leaves and shoots of CA.

fruits (1 from each tree), 80 leaves (8 from each tree) and 20 shoots (2 from each tree) weighing 350, 100 and 100 g, respectively were cut into small pieces and homogenised separately with methanol in the blender. A portion of the homogenised fruits equivalent to 100 g fruits and the homogenised leaves and shoots were extracted separately by the conventional solid-liquid extraction method using methanol as the extraction solvent.

Methanol was used as the initial extraction solvent as it can extract compounds representing a wide polarity range and is a so-called 'all-purpose' solvent (Cannell, 1998). Furthermore, methanol efficiently penetrates cell membranes, permitting the extraction of high amounts of endocellular components and also prevents, by denaturing the plant enzymes responsible, enzymatic processes or reactions that start after the plant material is collected (Silva *et al.*, 1998).

The resulting mixtures were filtered through a double-layer Gen-purpose filter paper (Schleicher & Schüell, Germany) on a Büchner funnel with a slight suction and the methanol extracts (filtrates) were concentrated to dryness under reduced pressure at 35 °C in a rotary evaporator (a Rotavapor R-114 fitted with a waterbath B-480, Büchi, Switzerland). The partitioning procedure of CA methanol extracts is displayed in Figure 3.2. The dried methanol extracts were suspended in 100 ml water and partitioned successively using separatory funnels, 3 times with 100 ml of petroleum ether (b.p. 40-60 °C), dichloromethane and ethyl acetate. After gravity filtration and solvent evaporation, the petroleum ether (MeOH-PE), dichloromethane (MeOH-DCM) and ethyl acetate (MeOH-EtOAc) fractions of the fruit, leaf and shoot methanol extracts were obtained. The remaining aqueous layers were concentrated in

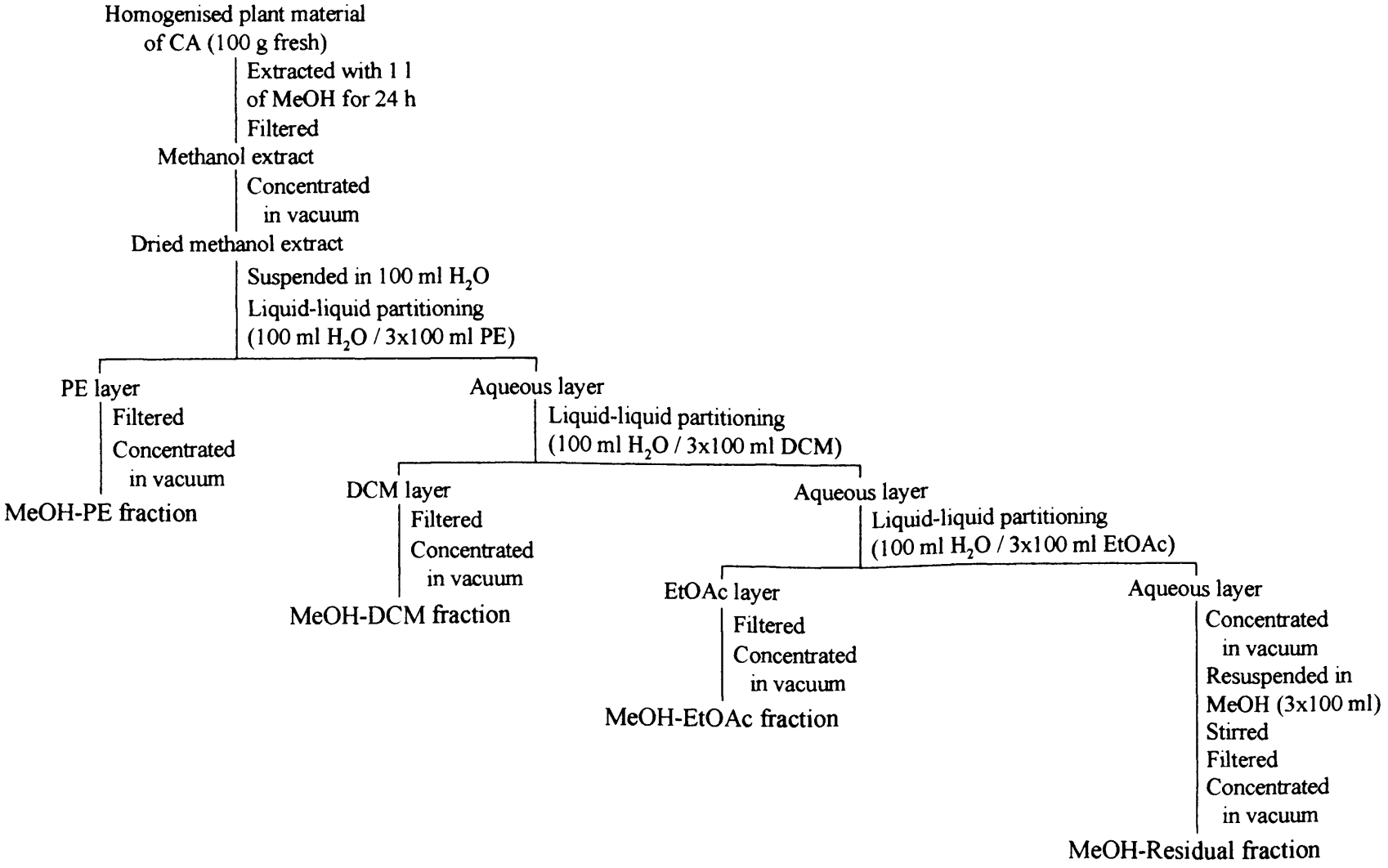

Figure 3.2 Partitioning procedure of CA methanol extracts.

the rotary evaporator, resuspended in methanol (3x100 ml), stirred each time for 5 min in the Büchi rotavapor without applying suction, gravity filtered and finally concentrated, yielding the residual fraction (MeOH-Residual) of the fruit, leaf and shoot methanol extracts.

The above extraction strategy was used in preference to a series of stepwise extractions (exhausted extraction of plant material by using successively extraction solvents of increasing polarity) as it is less time- and solvent-consuming.

The 10 remaining fruits were cut in half and, together with the remaining leaves (n = 80) and shoots (n = 20) which were cut into small pieces, were dried to constant weight under darkness at 50 °C in a well-ventilated oven (B50, Memmert, W. Germany) and their percentage dry weight was estimated. Percentage dry weight was used in order to express the extraction yields as milligram extractable material per gram of dried plant material extracted.

Extraction of CA fruit tissues

Fruits of CA were collected on 20/08/98 in order to determine whether the insecticidal active chemicals present in the MeOH-PE fraction of the fruits are localised to a specific fruit tissue. Leaves and shoots of CA were not further examined as all their fractions were inactive.

Twenty fruits (2 from each tree) were dissected into peels (flavedo), albedo and flesh (Figure 3.3). The peels, the albedo, the flesh and 10 intact (whole) fruits (1 from each tree) weighing 100, 100, 320 and 500 g, respectively were cut into small pieces and

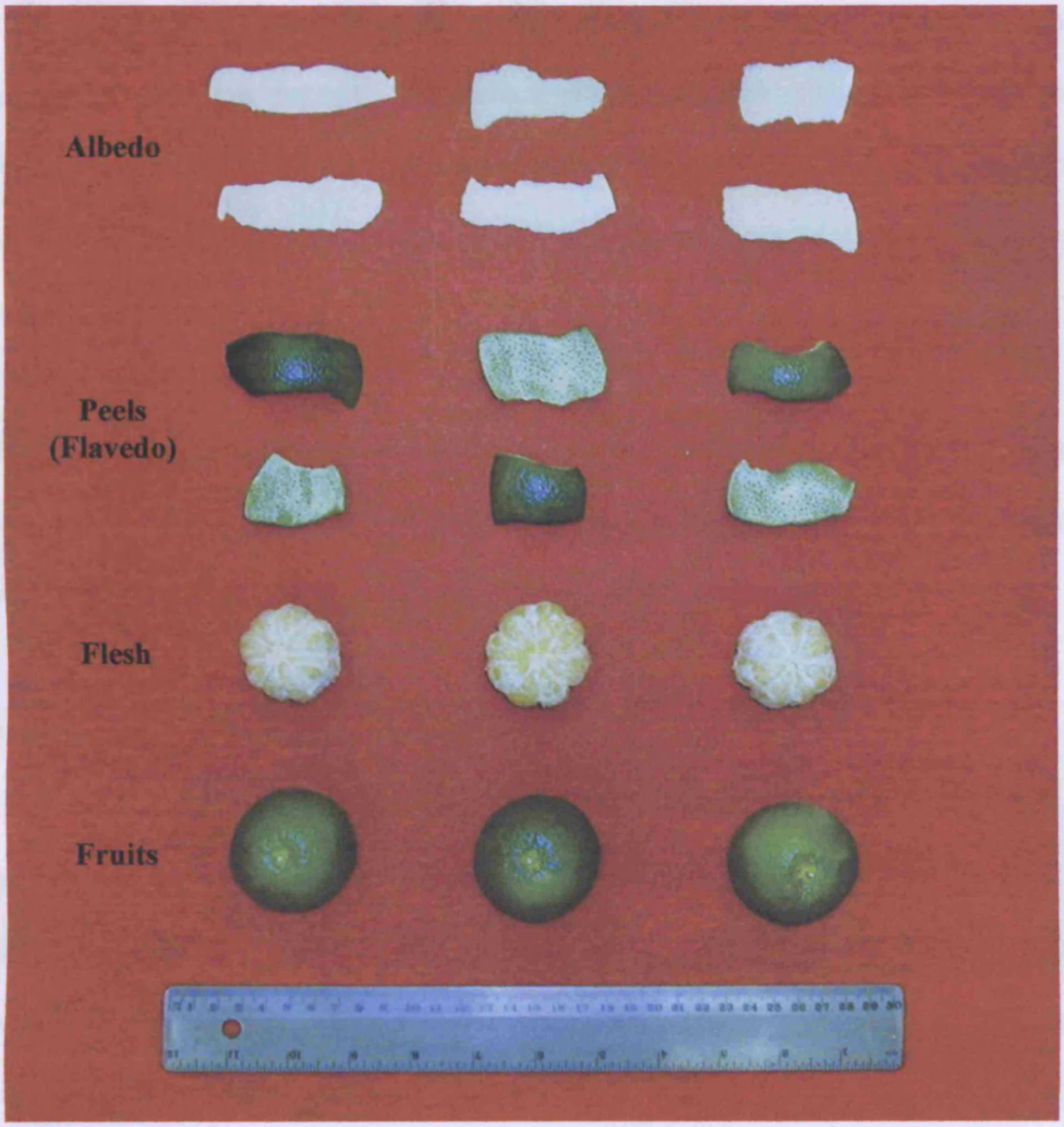

Figure 3.3 Fruit tissues of CA.

homogenised separately with methanol. A portion of the homogenised fruits and flesh equivalent to 100 g fruits and flesh, respectively and the homogenised peels and albedo were extracted separately by the conventional solid-liquid extraction method using methanol as the extraction solvent. The resulting mixtures were suction-filtered and then the methanol extracts (filtrates) were concentrated to dryness under reduced pressure at 35 °C in the rotary evaporator. The dried methanol extracts were then suspended in 100 ml water and partitioned only with petroleum ether (3 times with

100 ml each time), yielding after gravity filtration and solvent evaporation the petroleum ether fractions of the fruit, peel, albedo and flesh methanol extracts. The remaining aqueous layers after their partitioning with petroleum ether were discarded as the dichloromethane, ethyl acetate and residual fractions of the fruit methanol extract were inactive.

Ten fruits (1 from each tree) were cut in half and placed in the oven at 50 °C in order to estimate their percentage dry weight. The 10 remaining fruits were dissected into peels, albedo and flesh in order to estimate their percentage dry weight.

Extraction of fresh and dried peels of CA

Fruits of CA were collected on 10/09/98 in order to investigate whether drying of peels prior to extraction can affect the insecticidal activity of their MeOH-PE fractions. Intact fruits, albedo and flesh were not further studied as the activity of the MeOH-PE peel fraction was significant greater than that of the whole fruit, whereas the albedo and flesh fractions were inactive.

The bulk sample of peels obtained by knife-peeling of the fruits was divided into 3 sub-samples (100 g each). One sub-sample was randomly selected to be extracted fresh and the other two sub-samples were randomly assigned to the two oven-drying treatments (30 °C for 4 d and 50 °C for 2 d). Oven-drying was preferred than sun drying as ultraviolet radiation may produce chemical reactions giving rise to compound artefacts (Silva *et al.*, 1998).

The fresh and the two dried peel sub-samples were extracted separately using the conventional solid-liquid extraction method and methanol as the extraction solvent. The petroleum ether fractions of the fresh and the two dried peel methanol extracts were obtained as in the extraction of the fruit tissues.

Extraction of CA peels by different extraction methods and solvents

Fruits of CA were collected on 01/10/98 in order to investigate the effect of two different extraction methods and three different initial extraction solvents on the insecticidal activity of peel fractions and extracts obtained by the extraction of fresh peels. Dried peels were not further examined, as their MeOH-PE fractions were inactive.

The ultrasound and the conventional solid-liquid extraction methods were compared. The ultrasound extraction method was selected in order to be compared with the conventional solid-liquid extraction method, as it is more rapid and it employs less solvent than the conventional solid-liquid extraction (Poole and Poole, 1991). Furthermore, the efficacy of methanol, water and petroleum ether, as initial extraction solvents was compared with both extraction methods. Water was used in order to investigate whether it can extract the non-polar insecticidal active compounds present in the MeOH-PE fraction. Petroleum ether was used in order to investigate whether the concentration of the insecticidal active compounds was greater in the petroleum ether extracts than in the petroleum ether fractions of the methanol extracts.

The bulk sample of peels obtained by knife-peeling of the fruits was divided into six sub-samples (100 g each). Three sub-samples were randomly assigned to be extracted

separately with petroleum ether, methanol and water as initial extraction solvents using the conventional solid-liquid extraction method. The other 3 sub-samples were randomly assigned to be extracted with the three different initial extraction solvents using the ultrasound extraction method. The methanol extracts obtained from the two extraction methods were partitioned with petroleum ether as in the extraction of fruit tissues, yielding the conventional solid-liquid petroleum ether (Conv. S-L MeOH-PE) and the ultrasound petroleum ether (Ultrasound MeOH-PE) fractions of the methanol extracts. The aqueous extracts from each extraction method were concentrated to 100 ml and then partitioned with petroleum ether (3x100 ml), yielding the conventional solid-liquid petroleum ether (Conv. S-L H_2O-PE) and ultrasound petroleum ether (Ultrasound H_2O-PE) fractions of the aqueous extracts. The petroleum ether extracts from both extraction methods were suction-filtered and concentrated to dryness, yielding the conventional solid-liquid petroleum ether (Conv. S-L PE) and ultrasound petroleum ether (Ultrasound PE) extracts.

Extraction of peels obtained from CA fruits at different ripening (maturity) stages

Seven collections of fruits of CA with a month interval starting on 01/08/99 (3 months after fruit set) until full maturity (last collection on 01/02/00) were performed in order to investigate whether there is within-seasonal variation in the insecticidal activity of the petroleum ether peel extracts.

The bulk sample of peels of each collection was divided in two sub-samples (100 g each) which were randomly assigned to be extracted and dried. The peels were extracted by using the ultrasound extraction method and petroleum ether as the extraction solvent, yielding the petroleum ether peel extracts.

Although the insecticidal activity of the peel extracts obtained by both methods using petroleum ether as the extraction solvent was similar, peels were extracted by the ultrasound method as this method is less time- and solvent-consuming than the conventional solid-liquid extraction method. Methanol and water were not further used as initial extraction solvents since the H_2O-PE fractions of both methods and the ultrasound MeOH-PE fraction were inactive, whereas the petroleum ether extracts of both methods exhibited significantly greater insecticidal activity than the conventional solid-liquid MeOH-PE fraction.

The weight (mg) of all the fractions and extracts was estimated by concentrating to near-dryness, an appropriate portion or the whole fraction or extract in the rotary evaporator at 35 °C. The portion of each fraction or extract was selected in order to weigh 50-100 mg, on the basis of preliminary weighing of 5 % of the whole fraction or extract. The concentrated fraction or extract was subsequently transferred in a 15-ml vial (15-ml clear glass vial, screw top solid cap with PTFE liner, Supelco, USA) immersed in a water bath at 35 °C and concentrated to constant weight under a stream of nitrogen. All weighing was performed on an electronic four-figure balance (AB54-S, Mettler Toledo, Switzerland). All fractions and extracts were concentrated to dryness and stored in the dark at -20 °C until used.

3.2.2 Bioassay of fractions and extracts

The Petri dish exposure bioassay was used in order to evaluate the insecticidal activity of different fractions and extracts of CA against 2-3 d-old adults of BO. Five replicates of 10 BO flies each (5 males and 5 females) were employed for each fraction/extract concentration and the control. The 5 replicates were conducted on 5

successive days, utilising the same generation (batch) of flies. Different concentrations of screened fractions/extracts were not run simultaneously as screening experiments at lower concentrations included only the more active fractions/extracts. Stock solutions were prepared in DCM, as were subsequent dilutions except of the plant parts fractions, which were prepared in a mixture of MeOH:DCM (3:1, v/v). Fresh test solutions were prepared for each replicate. The syringe used for the application of samples was thoroughly cleaned after the application of each sample. Mortalities were recorded every 24 h for 4 d after which there was no further increase in mortality.

3.2.3 TLC analysis of fractions and extracts

Thin layer chromatography (TLC) is an extremely sensitive analytical method which can analyze a large number of samples simultaneously, although it does not require special and expensive equipment (Touchstone and Dobbins, 1983). TLC analyses were performed on 20 x 20 cm Sigma glass plates pre-coated with 250 μm silica gel 60 F_{254}. CA fractions and extracts were spotted (100 μg in 5 μl of the appropriate solvent) using a 10 μl Hamilton syringe onto the origin line (a line marked 2 cm from one end of the plate). All fractions and extracts were dissolved in DCM, except the MeOH-EtOAc, and MeOH-Residual fractions of fruits, leaves and shoots which were dissolved in MeOH. A glass chamber (29 cm long x 10 cm wide x 27 cm high, Sigma-Aldrich, USA) was used for plates' development. Prior to the introduction of the appropriate developing solvent system, the inner surface of the chamber was lined with filter paper to aid in saturating the atmosphere with the solvent vapours. The appropriate developing solvent system was used in each case in order to give the best possible separation based on preliminary analyses. After introducing the developing

solvent system (100 ml) to the glass chamber an interval of 30 min was allowed for saturation of the atmosphere with the solvent vapours. TLC plates were developed in the saturated chamber to a distance of 15 cm. After development, the TLC plates were air-dried for 1 h at room temperature under a fume hood. Photographs of the TLC plates were obtained by a Camedia digital camera (C-2000Z, Olympus, Japan) under UV light of 254 nm provided by a Spectroline lamp (ENF-280C/F, Spectronics Corporation Westbury, USA). Under UV light of 254 nm eluted components present in CA fractions and extracts were visible as dark spots or areas against a greenish fluorescent background.

3.3 RESULTS

3.3.1 Extraction yield, chemical profile and insecticidal activity of various fractions of CA plant parts

Petroleum ether, dichloromethane, ethyl acetate and residual fractions of the methanol extracts of CA fruits, leaves and shoots were obtained as described in section 3.2.1 in order to investigate whether these fractions contain insecticidal active chemicals.

Extraction yield

The extraction yield of all fractions and their contribution to the sum of fractions of the same plant part are shown in Table 3.2. The residual fractions accounted for 79.0-87.8 % of the sum of fractions of the same plant part, whereas the petroleum ether, dichloromethane and ethyl acetate fractions attained only 1.8-17.0, 1.6-4.7 and 2.0-6.3 %, respectively. The chemicals present in the petroleum ether fractions exhibited a wide range of contribution to the sum of fractions of the same plant part, represented 1.8, 9.2 and 17.0 % of the sum of fruit, shoot and leaf fractions, respectively.

Table 3.2 Extraction yield of various fractions of CA plant parts and % contribution of each fraction to the sum of fractions of the same plant part.

Fraction[a]	Plant part					
	Fruit	Leaf	Shoot	Fruit	Leaf	Shoot
	Extraction yield (mg/g fresh)[b]			Contribution (%)		
MeOH-PE	1.80	16.65	4.92	1.8	17.0	9.2
MeOH-DCM	4.72	1.97	0.87	4.7	2.0	1.6
MeOH-EtOAc	5.78	1.91	3.33	5.7	2.0	6.3
MeOH-Residual	88.32	77.28	44.12	87.8	79.0	82.9

[a]MeOH-PE, MeOH-DCM, MeOH-EtOAc and MeOH-Residual: Petroleum ether, dichloromethane, ethyl acetate and residual fractions, respectively. [b]Extraction yield of fractions was recorded as fractions weight (mg) per g of fresh plant material extracted.

The high contribution of the residual fractions to the sum of fractions of the same plant part was expected as the initial extraction solvent used was methanol. Methanol extracts mainly polar compounds, based on the "like dissolve like" general principle. These polar compounds cannot be extracted by the solvents used (petroleum ether, dichloromethane and ethyl acetate) during the liquid-liquid partitioning of CA methanol extracts and thus remained in the residual fractions.

The dry weight (%) of CA fruits, leaves and shoots and the extraction yield of the sum of fractions of each plant part are shown in Table 3.3. The dry weight (%) of shoots was 1.2 and 2.3 times greater than that of the leaves and fruits, respectively. The extraction yield of the sum of fractions of fruits and leaves when expressed as milligrams per gram of fresh plant material extracted was similar and at least 1.8 times greater than that of the shoots. However, when extraction yield was expressed as milligrams per gram of dried plant material extracted, the extraction yield of fruits was 2-fold and 4.4-fold greater than that of the leaves and shoots, respectively. This finding indicates that the differences among extraction yields of the different plant

Table 3.3 Dry weight (%) of CA plant parts and extraction yield of their sum of fractions.

Plant part	Fresh amount extracted (g)	Dry weight (%)	Sum of fractions	
			Extraction yield	
			mg/g fresh[a]	mg/g dried[b]
Fruit	100	24.7	100.62	407.37
Leaf	100	48.6	97.81	201.26
Shoot	100	57.2	53.24	93.08

[a]Extraction yield of the sum of fractions was recorded as sum of fractions weight (mg) per g of fresh plant material extracted. [b]Extraction yield of the sum of fractions was recorded as sum of fractions weight (mg) per g of dried plant material extracted.

parts were not due to the dry weight extracted (equivalent to 100 g fresh), but rather to the nature of the secondary metabolites present in each plant part.

Chemical profile

The various fractions of CA fruits, leaves and shoots were analysed by TLC in order to compare their chemical profiles. Different developing solvent systems were employed for the analyses of these fractions as secondary metabolites present in them represented a wide range of polarity. Preliminary analyses showed that the most appropriate developing solvent system in order to analyse simultaneously the four fractions of the three plant parts of CA was ethyl acetate (100 %). Comparison among chemical profiles of dichloromethane fractions was performed using this developing solvent system. By using Hex:EtOAc (50:50, v/v) as the developing solvent system the petroleum ether fractions were compared. Furthermore, the ethyl acetate and the residual fractions which contained mainly polar compounds were analysed by using as developing solvent system DCM:MeOH (85:15, v/v) and DCM:MeOH (80:20, v/v), respectively. The solutions used to spot the TLC plates were prepared in DCM for the petroleum ether and dichloromethane fractions, while the ethyl acetate and residual fraction were prepared in MeOH.

In Figure 3.4 the analysed fractions (the four fractions of the three plant parts of CA) using EtOAc (100 %) as the developing solvent system are shown. Great quantitative and qualitative differences were found among petroleum ether, dichloromethane, ethyl acetate and residual fractions of the same plant part. In Figures 3.5A, 3.5B and 3.5C the analysed petroleum ether, ethyl acetate and residual fractions of fruits, leaves and shoots of CA using Hex:EtOAc (50:50, v/v), DCM:MeOH (85:15, v/v) and

DCM:MeOH (80:20, v/v) as developing solvent systems, respectively are shown. Quantitative and quantitative differences were found among dichloromethane fractions (Figure 3.4), petroleum ether fractions (Figure 3.5A), ethyl acetate fractions (Figure 3.5B) and residual fractions (Figure 3.5C), of fruits, leaves and shoots of CA.

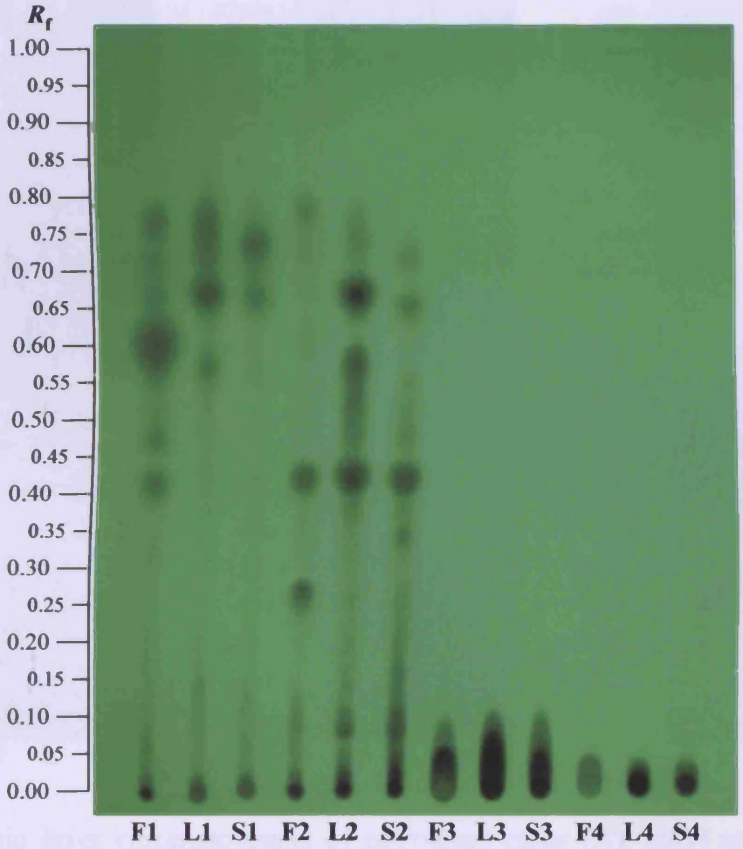

Figure 3.4 Thin layer chromatogram of various fractions of CA plant parts.

F1, **F2**, **F3** and **F4**: Petroleum ether, dichloromethane, ethyl acetate and residual fruit fractions, respectively; **L1**, **L2**, **L3** and **L4**: Petroleum ether, dichloromethane, ethyl acetate and residual leaf fractions, respectively; **S1**, **S2**, **S3** and **S4**: Petroleum ether, dichloromethane, ethyl acetate and residual shoot fractions, respectively; R_f: Retention factor. Developing solvent system: 100 % EtOAc. UV light: 254 nm. Amount of fractions per spot: 100 μg.

The differences in the range and proportions of chemical substances among the different fractions of the same plant part indicate that the fractionation procedure (liquid-liquid partitioning with solvents of increasing polarity) of the methanol extracts was effective, as different types of compounds based on their polarity were

present in each different fraction. The quantitative and qualitative differences among fruits, leaves and shoots of the same solvent fraction indicate that fruits, leaves and shoots of CA produce and/or accumulate different profiles of secondary metabolites.

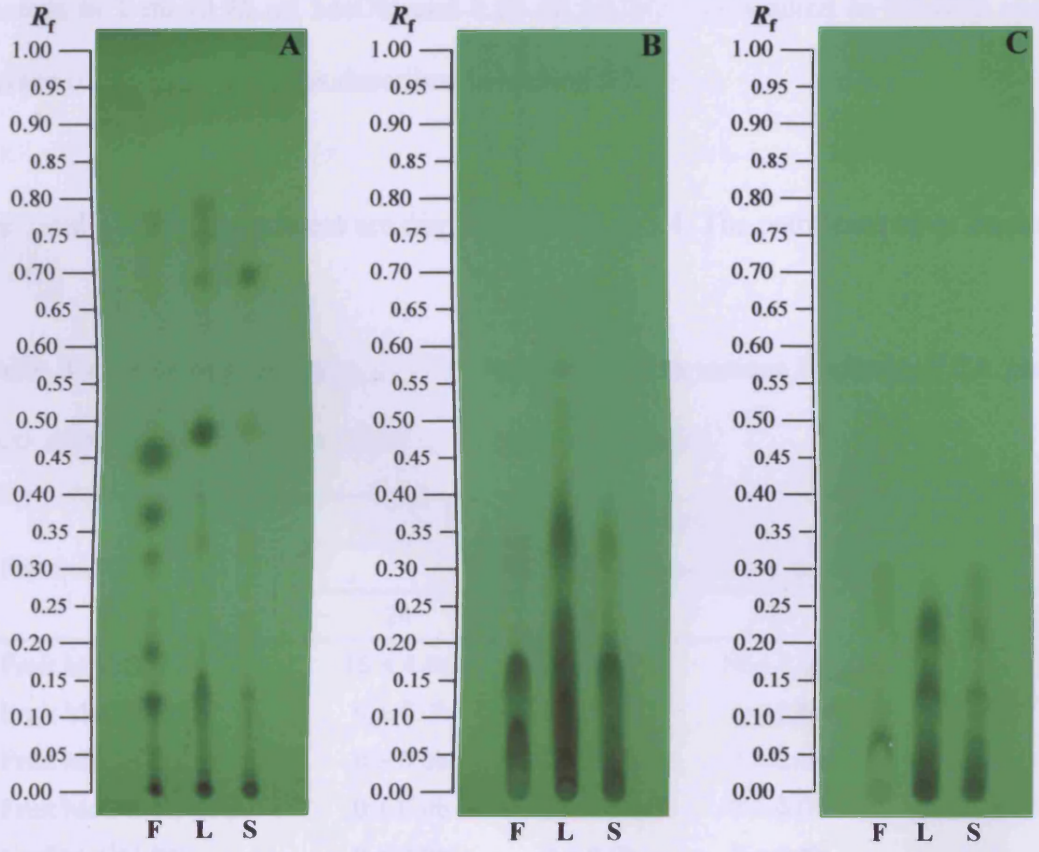

Figure 3.5 Thin layer chromatograms of petroleum ether (A), ethyl acetate (B) and residual (C) fractions of CA plant parts.

F: Fruit; **L**: Leaf; **S**: Shoot; R_f: Retention factor. Developing solvent systems: **(A)** Hex:EtOAc (50:50, v/v); **(B)** DCM:MeOH (85:15, v/v); **(C)** DCM:MeOH (80:20, v/v). UV light: 254 nm. Amount of fractions per spot: 100 μg.

Insecticidal activity

The various fractions of CA fruits, leaves and shoots were screened for insecticidal activity against BO adults by the Petri dish exposure bioassay. As these fractions contained chemicals with a wide range of polarity a solvent mixture of MeOH:DCM

(3:1, v/v), which possesses the solubility characteristics to dissolve all fractions was employed. By finding one solvent mixture which can dissolve all fractions, only one control was used which was treated with this solvent mixture only. The screening concentration was set at 200 $\mu g/cm^2$ and so 13.1 mg (200 $\mu g/cm^2$ x 65.5 cm^2) of each fraction in 1 ml (0.75 ml MeOH and 0.25 ml DCM) was applied to the total inner surface of the Petri dishes as described in section 2.2.2.

The results of this experiment are displayed in Table 3.4. The petroleum ether fraction

Table 3.4 Percentage mortality of BO adults exposed to various fractions of CA plant parts at 200 $\mu g/cm^2$ using the Petri dish exposure bioassay.

Fraction[a]	% Mortality			
	Mean[b] ± SE[c], at hours after treatment			
	24	48	72	96
Fruit MeOH-PE	16 ± 4.0a	60 ± 5.5a	74 ± 5.1a	76 ± 5.1a
Fruit MeOH-DCM	0 ± 0.0b	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
Fruit MeOH-EtOAc	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
Fruit MeOH-Residual	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
Leaf MeOH-PE	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
Leaf MeOH-DCM	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
Leaf MeOH-EtOAc	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
Leaf MeOH-Residual	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b	4 ± 2.4b
Shoot MeOH-PE	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
Shoot MeOH-DCM	0 ± 0.0b	4 ± 2.4b	4 ± 2.4b	4 ± 2.4b
Shoot MeOH-EtOAc	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
Shoot MeOH-Residual	0 ± 0.0b	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
Control[d]	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b

[a]MeOH-PE, MeOH-DCM, MeOH-EtOAc and MeOH-Residual: Petroleum ether, dichloromethane, ethyl acetate and residual fractions, respectively. [b]Mean of 5 replicates of 10 insects each (5 males and 5 females). [c]SE: Standard error of mean. [d]Treated with a solvent mixture of MeOH:DCM (3:1, v/v). Means within a column followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

of the fruit methanol extract was the only fraction which exhibited activity against BO adults (76 % mortality 96 h after treatment). Its activity was significantly greater from all other fractions and the control at all mortality recording times.

The results indicate that the MeOH-PE fraction of the fruits contained one or more chemical substances that can kill BO adults. All other fractions either did not contain insecticidally active chemicals or contained them in such proportions that are not toxic at the screening concentration used.

3.3.2 Extraction yield, chemical profile and insecticidal activity of petroleum ether fractions of CA fruit tissues

Petroleum ether fractions of the methanol extracts of CA intact fruits, peels, albedo and flesh were obtained as described in section 3.2.1 in order to determine whether a specific fruit tissue was mainly responsible for the insecticidal activity of the petroleum ether fraction of the fruit methanol extract.

Extraction yield

The dry weight (%) of CA fruit tissues and the extraction yield of their petroleum ether fractions are displayed in Table 3.5. The dry weight (%) of peels and albedo was similar and at least 1.4 and 1.9 times greater than that of the intact fruits and flesh, respectively. The extraction yield of the petroleum ether fractions of the peels and flesh when expressed as milligrams per gram of fresh fruit tissue extracted was approximately the same and at least 1.2 and 2.5 times respectively greater than that of the intact fruits and the albedo. However, when the extraction yield was expressed as milligrams per gram of dried fruit tissue extracted, the petroleum ether fraction of

intact fruit was 1.2 and 3.0 times greater than that of the peels and albedo, respectively but 1.7 times less than that of the flesh.

Table 3.5 Dry weight (%) of CA fruit tissues and extraction yield of their petroleum ether fractions.

Fruit tissue	Fresh amount extracted (g)	Dry weight (%)	Petroleum ether fractions	
			Extraction yield	
			mg/g fresh[a]	mg/g dried[b]
Intact fruit	100	24.4	2.02	8.28
Peel	100	35.0	2.47	7.06
Albedo	100	35.8	0.99	2.77
Flesh	100	18.2	2.50	13.74

[a]Extraction yield of fractions was recorded as fractions weight (mg) per g of fresh fruit tissue extracted.
[b]Extraction yield of fractions was recorded as fractions weight (mg) per g of dried fruit tissue extracted.

These findings indicate that the observed differences in the extraction yield among petroleum ether fractions of CA fruit tissues were not due to their dry weight extracted (equivalent to 100 g fresh), but rather to the chemical composition of these fruit tissues.

Chemical profile

Comparison of the chemical profiles of the petroleum ether fractions of CA fruit tissues was performed by TLC using Hex:EtOAc (50:50, v/v) as the developing solvent system. By using this single developing solvent system the petroleum ether fractions were analysed adequately as they contained chemicals of similar polarities (mainly non-polar chemicals).

In Figure 3.6 the analysed petroleum ether fractions of CA fruit tissues using Hex:EtOAc (50:50, v/v) as developing solvent system are shown. Great quantitative and qualitative differences were revealed among the three fruit tissue fractions. Among the four fractions analysed, the peel and the intact fruit chemical profile look more similar than any other pair of fractions, although that between them quantitative and qualitative differences were evident. TLC analysis indicates that different fruit tissues of CA produce and/or accumulate different profiles of secondary metabolites.

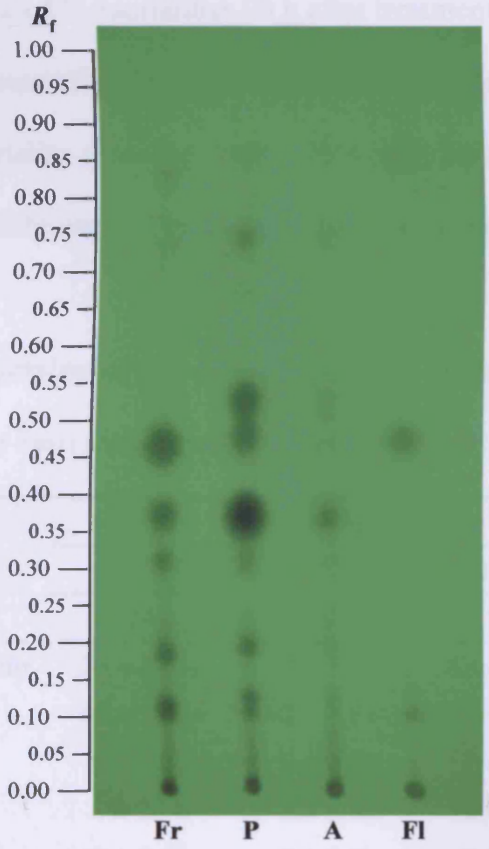

Figure 3.6 Thin layer chromatogram of petroleum ether fractions of CA fruit tissues.

Fr, **P**, **A** and **Fl**: Petroleum ether fractions of intact fruit, peel, albedo and flesh, respectively; R_f: Retention factor. Developing solvent system: Hex:EtOAc (50:50, v/v). UV light: 254 nm. Amount of fractions per spot: 100 μg.

Insecticidal activity

The petroleum ether fractions of CA fruit tissues were tested against BO adults, using the Petri dish exposure bioassay, in order to investigate in which fruit tissue fraction the insecticidal active chemicals were present in greater concentrations. The screening concentration used for the initial experiment was 200 $\mu g/cm^2$. Only fractions that gave significant activity at this concentration were further tested at 100 $\mu g/cm^2$.

The results of these experiments are shown in Table 3.6. The albedo and the flesh fractions were inactive (2 - 4 % mortalities 96 h after treatment) at 200 $\mu g/cm^2$. At the same concentration the mortality of the peel fraction was greater than that of the intact fruit fraction at all mortality recording times. However, only at 72 and 96 h after treatment were those differences significant. Furthermore, the peel fraction caused

Table 3.6 Percentage mortality of BO adults exposed to petroleum ether fractions of CA fruit tissues using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Fruit tissue	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
200	Intact fruit	26 ± 10.3a	62 ± 5.8a	68 ± 8.0b	70 ± 7.1b
	Peel	46 ± 11.7a	82 ± 8.6a	96 ± 2.4a	100 ± 0.0a
	Albedo	0 ± 0.0b	0 ± 0.0b	0 ± 0.0c	2 ± 2.0c
	Flesh	2 ± 2.0b	2 ± 2.0b	4 ± 2.4c	4 ± 2.4c
	Control[c]	0 ± 0.0b	0 ± 0.0b	0 ± 0.0c	0 ± 0.0c
100	Intact fruit	0 ± 0.0b	12 ± 4.9b	14 ± 4.0b	14 ± 4.0b
	Peel	14 ± 7.5a	66 ± 12.1a	80 ± 10.5a	84 ± 6.8a
	Control	0 ± 0.0b	0 ± 0.0b	0 ± 0.0c	0 ± 0.0c

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with DCM. The two experiments (200 and 100 $\mu g/cm^2$) were not run simultaneously. Means within a column of a single concentration followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

significantly more mortality to BO adults than the intact fruit fraction at all mortality recording times at 100 $\mu g/cm^2$.

The results indicate that the peel fraction possesses, in greater proportions, the insecticidally active chemical compounds present in the intact fruit fraction. The albedo and flesh fractions either did not contain these chemicals or contained them in such proportions that cannot exhibit any evidence of activity even at 200 $\mu g/cm^2$. It seems that the bioactive chemicals present in the intact fruit fraction are mainly produced and/or accumulated in the peel tissue of CA fruits.

3.3.3 Extraction yield, chemical profile and insecticidal activity of petroleum ether fractions obtained from fresh and dried peels of CA

Petroleum ether fractions of the methanol extracts obtained by the extraction of fresh and dried peels were prepared as described in section 3.2.1 in order to investigate whether chemical and/or enzymatic processes or reactions possibly occurring during the two drying processes of the peels can change the insecticidal activity of their petroleum ether fraction.

Extraction yield

The extraction yield of the petroleum ether peel fractions obtained from fresh, oven-dried at 30 °C for 4 d and oven-dried at 50 °C for 2 d peels, recorded as milligrams per gram fresh peel was 2.66, 1.60 and 1.76, respectively. The extraction yield of the two petroleum ether peel fractions obtained from dried peels was similar and at least 1.5 times less than that of the petroleum ether fraction obtained from fresh peels.

The reduced extraction yields of the two fractions obtained from dried peels could be attributed to the transformation, during drying treatments, of some chemicals present in the fraction obtained from fresh peels, to more polar chemicals due to enzymatic and/or chemical processes or reactions. These artefacts possibly cannot be extracted from the methanol extracts by the liquid-liquid partitioning with petroleum ether, due to their greater polarity.

Chemical profile

Comparison of the chemical profiles of the petroleum ether peel fractions obtained from fresh and oven dried peels was performed by TLC using Hex:EtOAc (50:50, v/v) as the developing solvent system. Figure 3.7 shows the analysed petroleum ether peel fractions of CA. TLC analysis revealed that there were great similarities among the chemical profiles of the three fractions. However, the two fractions obtained from dried peels were almost the same (only quantitative difference between spots with R_f: 0.30), and they differ with the fraction obtained from fresh peels only quantitatively (spots with R_f: 0.00, 0.12, 0.19, 0.30 and 0.46).

The quantitative differences revealed when the fraction obtained from fresh peels was compared with the two fractions obtained from dried peels could be attributed to the transformation of some chemicals or part of them to more polar chemicals during drying treatments. However, the greater proportions of the chemicals with R_f: 0.12, 0.19 and 0.30 in the fractions obtained from dried peels compared to the fraction obtained from fresh peels could be explained by the lower yield of these fractions rather than the greater amount of these chemicals in the fractions. Furthermore, the

small difference between the fractions obtained from dried peels must have been due to the effect of the different temperatures and exposure times of the drying treatments.

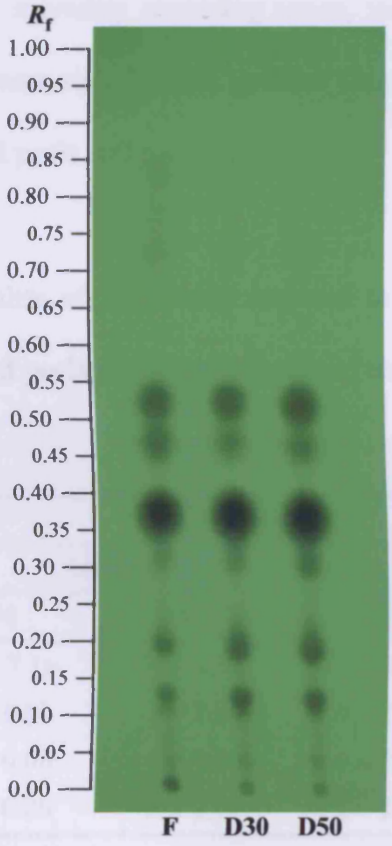

Figure 3.7 Thin layer chromatogram of petroleum ether fractions obtained from fresh and dried peels of CA.

F: Fresh peels; **D30**: Dried peels (30 °C for 4 d); **D50**: Dried peels (50 °C for 2 d); R_f: Retention factor. Developing solvent system: Hex:EtOAc (50:50, v/v). UV light: 254 nm. Amount of fractions per spot: 100 μg.

Insecticidal activity

Comparison of the insecticidal activity of the petroleum ether fractions obtained from fresh and dried peels was done against BO adults using the Petri dish exposure bioassay. The screening concentration was set at 100 $\mu g/cm^2$.

The results of this experiment are displayed in Table 3.7. The fraction obtained from fresh peels was highly active (94 % mortality 96 h after treatment), whereas the fractions obtained from dried peels were inactive (0-4 % mortalities 96 h after treatment). Moreover, at all mortality recording times, the mortality of the fraction obtained from fresh peels was significantly greater than the mortality of the two fractions obtained from dried peels and the control.

Table 3.7 Percentage mortality of BO adults exposed to petroleum ether fractions obtained from fresh and dried peels of CA at 100 $\mu g/cm^2$ using the Petri dish exposure bioassay.

Peel	% Mortality			
	Mean[a] ± SE[b], at hours after treatment			
	24	48	72	96
Fresh	20 ± 7.1a	84 ± 4.0a	92 ± 2.0a	94 ± 2.4a
Dried 30 °C	0 ± 0.0b	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b
Dried 50 °C	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
Control[c]	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with DCM. Means within a column followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

These results indicate that the insecticidal active chemicals present in the fraction obtained from fresh peels were at least partly decomposed during the drying treatments. Consequently, the proportions of the insecticidally active chemicals in the fractions obtained from the dried peels, if present, were not so great as to produce any sign of activity against BO adults.

3.3.4 Effect of different extraction methods and solvents on extraction yield, chemical profile and insecticidal activity of peel fractions and extracts of CA

Peel fractions and extracts of CA were obtained as described in section 3.2.1 in order to determine the combination of extraction method and initial extraction solvent, which recovered the insecticidal active chemicals in greater proportions.

Extraction yield

The extraction yields of peel fractions and extracts of CA are presented in Table 3.8. There were no differences between extraction yields obtained using the same initial extraction solvent, but different extraction method. Moreover, the extraction yield of the petroleum ether extracts was more than 1.4 and 6.5 times the yield of MeOH-PE and H_2O-PE fractions, respectively.

Table 3.8 Extraction yield of peel fractions and extracts obtained from 100 g fresh peels of CA using different extraction methods and initial extraction solvents.

Extraction solvents[a]	Extraction method	
	Conventional solid-liquid	Ultrasound
	Extraction yield (mg/g fresh)[b]	
PE	3.74	4.02
MeOH-PE	2.65	2.60
H_2O-PE	0.55	0.58

[a]PE: Initial extraction with petroleum ether; MeOH-PE: Initial extraction with methanol, liquid-liquid partitioning with petroleum ether; H_2O-PE: Initial extraction with water, liquid-liquid partitioning with petroleum ether. [b]Extraction yield of fractions/extracts was recorded as fractions/extracts weight (mg) per g of fresh peels extracted.

Although the ultrasound extraction method employed less solvent and was much more rapid compared to the conventional solid-liquid extraction method, similar yields were obtained by both methods, indicating that the ultrasound extraction method is

more efficient than the conventional solid-liquid extraction method. The low yield obtained by both methods using water for the initial extraction is explained by the inability of water to extract non-polar chemical compounds. Moreover, the lower yield obtained by both methods using methanol than petroleum ether for the initial extraction can be explained by the ability of petroleum ether to extract selectively non-polar compounds, whereas methanol is extracting compounds representing a wide polarity range.

Chemical profile

Comparison of the chemical profiles of peel fractions and extracts were performed by TLC using Hex:EtOAc (50:50, v/v) as the developing solvent system. In Figure 3.8 the analysed petroleum ether peel fractions and extracts of CA are shown. TLC analysis revealed small quantitative differences between the chemical profiles of fractions/extracts obtained by using the same initial extraction solvent, but different extraction methods. Moreover, the chemical profiles of the H_2O-PE fractions obtained by both extraction methods differ substantially both quantitatively and qualitatively with the four other chemical profiles. Furthermore, among the two MeOH-PE fractions and the two PE extracts there were some differences, but not such great as that stated above.

These findings indicate that the effect of extraction method in the chemical profile of the fractions/extracts was not crucial. By contrast, the choice of initial extraction solvent greatly affected the chemical profiles of these fractions/extracts. Moreover, the inability of water to extract non-polar chemical compounds is obvious. It must be noticed that although some chemicals of the H_2O-PE fractions with R_f below 0.5 were

present in greater proportions compared to the other four extracts, the amount of these chemicals in these fractions was lower as their extraction yields were at least 4.5 and 6.5 times less compared to the MeOH-PE fractions and PE extracts, respectively.

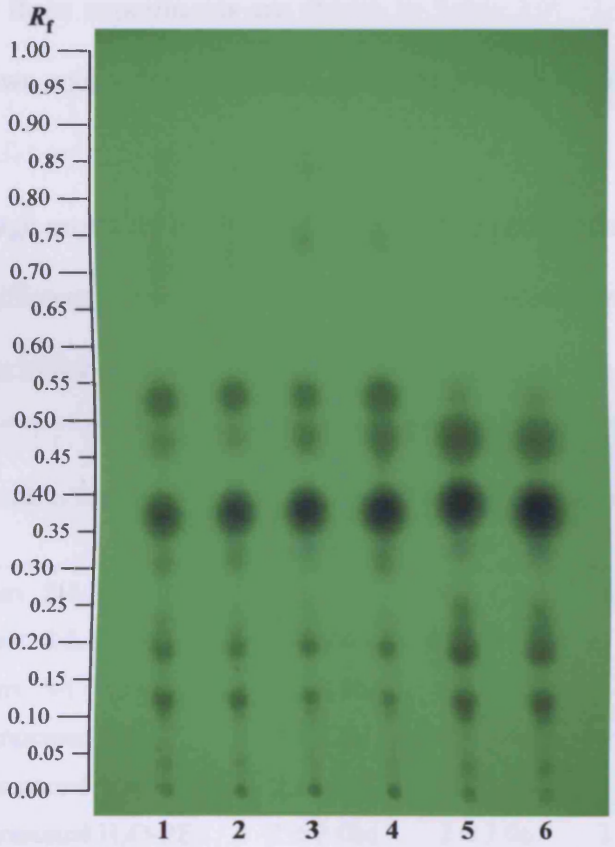

Figure 3.8 Thin layer chromatogram of peel fractions and extracts of CA obtained by different extraction methods and initial extraction solvents.

1: Conventional solid-liquid PE; **2**: Ultrasound PE; **3**: Conventional solid-liquid MeOH-PE; **4**: Ultrasound MeOH-PE; **5**: Conventional solid-liquid H_2O-PE; **6**: Ultrasound H_2O-PE; R_f: Retention factor. Developing solvent system: Hex:EtOAc (50:50, v/v). UV light: 254 nm. Amount of fractions/extracts per spot: 100 μg.

Insecticidal activity

Peel fractions and extracts of CA were evaluated for their insecticidal activity against BO adults using the Petri dish exposure bioassay. The screening concentration used

for the initial experiment was 100 $\mu g/cm^2$. Only fractions/extracts that gave significant activity at this concentration were further tested at 50 $\mu g/cm^2$ and moreover, only active fractions/extracts at 50 $\mu g/cm^2$ were tested at 25 $\mu g/cm^2$.

The results of the three experiments are shown in Table 3.9. The H_2O-PE fractions obtained by the two extraction methods and the MeOH-PE fraction obtained by the

Table 3.9 Percentage mortality of BO adults exposed to peel fractions and extracts of CA obtained by different extraction methods and initial extraction solvents using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Fraction/Extract[a]	% Mortality			
		Mean[b] ± SE[c], at hours after treatment			
		24	48	72	96
100	Conv. S-L PE	42 ± 8.0a	94 ± 4.0a	98 ± 2.0a	100 ± 0.0a
	Conv. S-L MeOH-PE	14 ± 6.8b	62 ± 5.8b	80 ± 5.5b	82 ± 6.6b
	Conv. S-L H_2O-PE	2 ± 2.0bc	4 ± 2.4c	4 ± 2.4c	4 ± 2.4c
	Ultrasound PE	52 ± 8.0a	92 ± 3.7a	96 ± 2.4a	100 ± 0.0a
	Ultrasound MeOH-PE	0 ± 0.0c	2 ± 2.0c	2 ± 2.0c	2 ± 2.0c
	Ultrasound H_2O-PE	2 ± 2.0bc	2 ± 2.0c	2 ± 2.0c	2 ± 2.0c
	Control[d]	0 ± 0.0c	2 ± 2.0c	2 ± 2.0c	2 ± 2.0c
50	Conv. S-L PE	10 ± 4.5a	48 ± 7.3a	84 ± 6.0a	86 ± 5.1a
	Conv. S-L MeOH-PE	2 ± 2.0a	20 ± 7.1b	24 ± 5.1b	24 ± 5.1b
	Ultrasound PE	6 ± 4.0a	50 ± 5.5a	76 ± 6.0a	80 ± 4.5a
	Control	0 ± 0.0a	2 ± 2.0c	2 ± 2.0c	2 ± 2.0c
25	Conv. S-L PE	4 ± 4.0a	24 ± 8.7a	34 ± 7.5a	36 ± 7.5a
	Ultrasound PE	4 ± 2.4a	18 ± 5.8a	32 ± 7.3a	38 ± 7.3a
	Control	0 ± 0.0a	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b

[a]Conv. S-L: Conventional solid-liquid; PE: petroleum ether extract; MeOH-PE: petroleum ether fraction of methanol extract; H_2O-PE: petroleum ether fraction of aqueous extract. [b]Mean of 5 replicates of 10 insects each (5 males and 5 females). [c]SE: Standard error of mean. [d]Treated with DCM. The three experiments (100, 50 and 25 $\mu g/cm^2$) were not run simultaneously. Means within a column of a single concentration followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

ultrasound extraction method were inactive (2-4 % mortalities 96 h after treatment) at 100 $\mu g/cm^2$. The mortality caused by the MeOH-PE fraction obtained by the conventional solid-liquid extraction method was significant lower compared to the PE extracts obtained by both methods at 100 $\mu g/cm^2$ at all mortality recording times and at 50 $\mu g/cm^2$ at 48, 72 and 96 h after treatment. Moreover, there were no significant statistical differences between the two PE extracts at the three screening concentrations at all mortality recording times.

The results of the three experiments indicate that the insecticidal active chemicals were present in the two PE extracts in similar proportions, but greater compared to the MeOH-PE fraction obtained by the conventional solid-liquid extraction method. The H_2O-PE fractions obtained by both extraction methods and the MeOH-PE fraction obtained by the ultrasound extraction method either did not contain these chemicals or contain them in such concentrations that can not cause mortality even at 100 $\mu g/cm^2$.

3.3.5 Effect of fruit ripeness on extraction yield, chemical profile and insecticidal activity of petroleum ether peel extracts of CA

Petroleum ether peel extracts were obtained as described in section 3.2.1 in order to investigate whether there were differences in the insecticidal activity of the petroleum ether peel extracts obtained from fruits collected at different times in the same growing season.

Extraction yield

The dry weight (%) of peels obtained from CA fruits at various ripening stages and the extraction yield of their petroleum ether extracts are displayed in Table 3.10. The

dry weight of peels varied from 30.4 to 34.1 %. There were no differences among extraction yields of the petroleum ether extracts obtained by the extraction of peels of fruits collected in August, September and October and also for peels of fruits collected in November, December, January and February. Moreover, the extraction yield obtained by the extraction of peels on August, September and October was at least 1.3 times greater than the extraction yield obtained by the extraction of peels on November, December, January and February, either expressed as milligrams per gram of fresh peel or milligrams per gram of dried peel extracted.

Table 3.10 Dry weight (%) of peels obtained from CA fruits at various ripening stages and extraction yield of their petroleum ether extracts.

Collection	Fresh amount extracted (g)	Dry weight (%)	Extraction yield	
			mg/g fresh[a]	mg/g dried[b]
August	100	34.1	4.59	13.5
September	100	33.5	4.70	14.0
October	100	32.3	4.43	13.7
November	100	31.7	3.13	9.9
December	100	30.9	3.21	10.4
January	100	31.5	3.31	10.5
February	100	30.4	3.07	10.1

[a]Extraction yield of extracts was recorded as extracts weight (mg) per g of fresh peels extracted.
[b]Extraction yield of extracts was recorded as extracts weight (mg) per g of dried peels extracted.

These findings indicate that the greater yields of extracts on August, September and October compared to November, December, January and February, were due, not to the dry weight extracted in each case, but rather to changes in the chemical composition of the peel occurring during the third and fourth collection (during the change in fruit colour from light green to green-yellow).

Chemical profile

Comparison of the chemical profiles of the petroleum ether peel extracts were performed by TLC using Hex:EtOAc (50:50, v/v) as the developing solvent system. In Figure 3.9 the analysed petroleum ether peel extracts of CA are shown. TLC analysis revealed that below R_f 0.55 the chemical profiles of the seven extracts had minor quantitative differences. However, the proportions of chemicals with R_f above 0.55 increased during the ripening process. That finding indicates that the chemical composition of the peels was affected by the ripening process of the fruit.

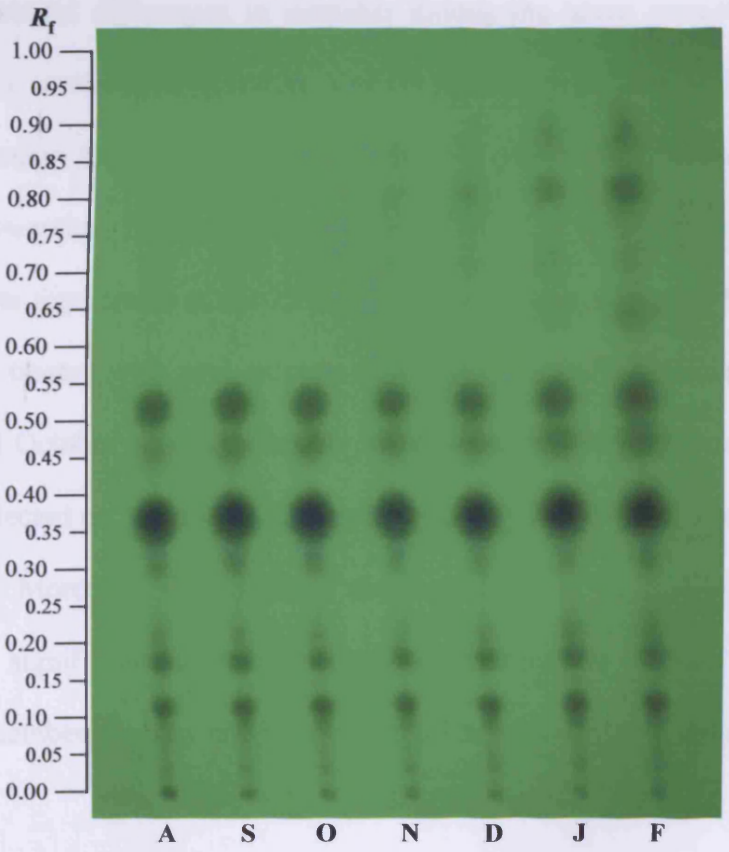

Figure 3.9 Thin layer chromatogram of petroleum ether peel extracts of CA obtained by fruits at various ripening stages.

A, **S**, **O**, **N**, **D**, **J** and **F**: Petroleum ether peel extracts obtained by fruits collected on August, September, October, November, December, January and February, respectively; R_f: Retention factor. Developing solvent system: Hex:EtOAc (50:50, v/v). UV light: 254 nm. Amount of extracts per spot: 100 μg.

Insecticidal activity

The petroleum ether peel extracts of CA obtained from fruits collected monthly from August to February were evaluated for their insecticidal activity against BO adults using the Petri dish exposure bioassay. The screening concentration used for the initial experiment was 50 $\mu g/cm^2$. As all extracts were significant active at this concentration, all extracts were subsequently tested at 25 $\mu g/cm^2$. Only active extracts at 25 $\mu g/cm^2$ were further tested at 12.5 $\mu g/cm^2$.

The results of the three experiments are shown in Table 3.11. Although there were no significant statistical differences in mortality among the seven extracts, 96 h after treatment at the screening concentration of 50 $\mu g/cm^2$, there was evidence that the extracts obtained on August, September and October were not as active as the extracts obtained on November, December, January and February. This was confirmed from the results of the experiment at the 25 $\mu g/cm^2$ concentration where the flies mortality in Petri dishes treated with peel extracts obtained from fruits collected on August, September and October was significantly lower than that of peel extracts obtained from fruits collected on November, December, January and February at 72 and 96 h after treatment. Moreover, the results of the experiment at 12.5 $\mu g/cm^2$ shown that there was no significant statistical differences among the extracts obtained on November, December, January and February at all mortality recording times.

The results indicate that the insecticidal active chemicals were present in greater proportions in the extracts obtained on November, December, January and February compared to the extracts obtained on August, September and October. It can be

concluded that changes in the chemical composition of the peels occurring during the third and fourth collection affected the insecticidal activity of the extracts.

Table 3.11 Percentage mortality of BO adults exposed to petroleum ether peel extracts of CA obtained by fruits at various ripening stages using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Extract	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
50	August	6 ± 2.4a	42 ± 5.8b	82 ± 4.9a	88 ± 4.9a
	September	6 ± 2.4a	44 ± 5.1b	88 ± 3.7a	90 ± 4.5a
	October	10 ± 3.2a	52 ± 8.6ab	90 ± 4.5a	92 ± 3.7a
	November	22 ± 8.0a	68 ± 6.6ab	92 ± 5.8a	98 ± 2.0a
	December	18 ± 4.9a	70 ± 11.0ab	100 ± 0.0a	100 ± 0.0a
	January	14 ± 7.5a	64 ± 7.5ab	94 ± 4.0a	98 ± 2.0a
	February	22 ± 5.8a	78 ± 5.8a	96 ± 2.4a	100 ± 0.0a
	Control[c]	2 ± 2.0a	4 ± 2.4c	4 ± 2.4b	4 ± 2.4b
25	August	0 ± 0.0a	18 ± 3.7b	22 ± 4.9b	24 ± 4.0b
	September	2 ± 2.0a	22 ± 5.8ab	28 ± 7.3b	28 ± 7.3b
	October	4 ± 2.4a	20 ± 4.5ab	28 ± 3.7b	30 ± 4.5b
	November	8 ± 3.7a	44 ± 8.1ab	70 ± 4.5a	72 ± 3.7a
	December	6 ± 2.4a	56 ± 10.8a	80 ± 8.9a	84 ± 8.1a
	January	8 ± 3.7a	46 ± 12.1ab	74 ± 9.3a	80 ± 7.1a
	February	10 ± 3.2a	56 ± 11.2a	82 ± 6.6a	86 ± 5.1a
	Control	0 ± 0.0a	0 ± 0.0c	0 ± 0.0c	0 ± 0.0c
12.5	November	6 ± 4.0a	32 ± 8.6a	34 ± 9.3a	36 ± 8.1a
	December	2 ± 2.0a	24 ± 8.7a	40 ± 7.1a	42 ± 8.0a
	January	4 ± 2.4a	24 ± 5.1a	28 ± 4.9a	28 ± 4.9a
	February	4 ± 2.4a	30 ± 7.1a	36 ± 6.8a	36 ± 6.8a
	Control	0 ± 0.0a	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with DCM. The three experiments (50, 25 and 12.5 $\mu g/cm^2$) were not run simultaneously. Means within a column of a single concentration followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

3.4 DISCUSSION

Screening experiments using the Petri dish exposure bioassay against adults of BO shown that the petroleum ether fraction of the fruit methanol extract was the only insecticidal active fraction among the different solvent fractions of fruits, leaves and shoots of CA. TLC analyses revealed that the proportion and range of chemical substances differ greatly among the fractions (petroleum ether, dichloromethane, ethyl acetate and residual) of the same plant part and also among the plant parts. The different insecticidal potency of CA fractions should be accounted to their differences in chemical composition. The bioactive chemicals are of low polarity since they were recovered in a non-polar solvent such as petroleum ether. Fractions that did not show any sign of activity either did not contain these chemicals or contain them in low concentrations.

Evaluation of the insecticidal activity of the different fruit tissues revealed that the bioactive chemicals present in the petroleum ether fraction of the fruits were mainly synthesised and/or accumulated in the peels. TLC analysis revealed that the chemical composition of fruit tissues differ substantially indicating that chemical substances are produced and/or accumulated specifically in certain fruit tissues. *Citrus* peel extracts obtained with non-polar solvents have been documented to be insecticidally active by different bioassay methods. Abbassy *et al.* (1979) showed that lime, navel orange and grapefruit peel oils obtained using benzene as the extraction solvent exhibited significant activity against *Tribolium confusum* and *Sitophilus granarius* by the Petri dish exposure bioassay. In addition, Su *et al.* (1972b) reported that lemon, grapefruit, lime, kumquat and tangerine peel oils obtained using hexane as the extraction solvent exhibited significant activity against *Sitophilus oryzae* and *Callosobruchus maculates*

by the topical application bioassay. It is worth-noted that Su *et al.* (1972b) had removed the volatile materials of their extracts by lyophilisation, whereas the extracts obtained by Abbassy *et al.* (1979) contained both volatile and non-volatile chemical substances. In this study, most volatiles have removed from all fractions/extractions under a stream of nitrogen.

Petroleum ether peel extracts lost their insecticidal activity when peels were oven-dried prior to the extraction, either at 30 °C for 4 d or 50 °C for 2 d. Moreover, the extraction yields of the two petroleum ether peel fractions obtained from dried peels were similar and at least 1.5 times less than that of the petroleum ether fraction obtained from fresh peels. Considering these results, it can be concluded that the bioactive chemicals present in the peels were transformed at least partly to less toxic or non-toxic chemicals due to enzymatic and/or chemical processes or reactions. TLC analysis revealed only quantitative differences when the two petroleum ether peel fractions obtained from dried peels were compared to the petroleum ether fraction obtained from fresh peels. These differences may be accountable for the inactivity of the two petroleum ether peel fractions obtained from dried peels. Sheppard (1984) reported that oils from dried peels were less active than oils obtained from fresh. In addition, Salvatore *et al.* (2004) found that toxicity against larvae of CC decreased when diethyl ether extracts of lemon peel were obtained from stored lemons (storage conditions: 25 ± 2 °C temperature and 60 - 70 % relative humidity). These authors attributed that decrease in toxicity to the decrease in the concentration of total coumarins after harvest (35 % over 2 months). Moreover, Thomas and Callaghan (1999) noted that a crude aqueous lemon peel extract had low persistence as a larvicide. Activity of this extract against *Culex pipiens* larvae was reduced even 72 h

after the extract had been added to the water. The lack of persistence of lemon peel extract was related by these authors to the volatility of the active ingredients. In contrast, Don-Pedro (1985) demonstrated that powdered sun-dried orange and grapefruit peels applied as food admixtures, exhibited toxic and repellent properties against *Dermestes maculatus* and *Callosobruchus maculatus*. However, Don-Pedro had not used fresh peels in order to compare the activity between fresh and sun-dried peels.

Bioassays conducted with peel fractions/extracts obtained using three different initial extraction solvents and two different extraction methods, revealed that the petroleum ether extracts obtained by both methods were the most active. Since the ultrasound extraction method is more rapid and employs less solvent than the conventional solid-liquid extraction and also it afforded greater extraction yield, this method was preferred in all subsequent extractions in this thesis. The reduced activity of the petroleum ether fraction obtained by the conventional solid-liquid extraction method indicate that petroleum ether extract more selectively the insecticidal chemicals than methanol. Diethyl ether, ethyl acetate and methanol lemon peel extracts were obtained by Salvatore *et al.* (2004) using an ultrasound bath at 20 °C for 20 min. Results of a larval toxicity bioassay against CC shown that the diethyl ether extract, which contain mainly non-polar chemicals, was the most toxic, supporting our results.

Fruit maturity affected petroleum ether peel extracts bioactivity. Results indicate that extracts from ripe fruits, collected on November, December, January and February, were more active than extracts obtained from unripe fruits, collected on August, September and October. TLC analysis revealed that the chemical composition of the

peels was affected by the ripening process of the fruit. It seems that changes in the chemical profile of peels during October were responsible for the increased activity of ripe peel extracts. *Citrus* fruits are resistant to attack by tephritid fruit flies prior to the occurrence of peel senescence (Greany *et al.*, 1994). The resistance of lemons to CC (Back and Pemberton, 1918; Quayle, 1914, 1929) and to *Anastrepha suspense* (Greany *et al.*, 1983) was attributed by these authors, among other reasons, to the peel oil. More specifically, da Silva Branco *et al.* (2000) reported that oxygenated monoterpene aldehydes, like citral, are responsible for the chemical resistance of lemons to attack by CC. Although unripe fruits are more tolerant to insect attack than ripe, our results show that insecticidal active chemicals were present in greater proportion to ripe fruits. That discrepancy can be explained by the presence in our petroleum ether peel extract of non-volatile compounds mainly, whereas fruit tolerance is attributed to volatile compounds.

Screening experiments revealed that CA peels possess chemical substances that are bioactive to BO flies. This finding prompted us to continue our study in two directions: (a) to evaluate the insecticidal activity of a peel extract by different bioassays against BO and CC adults, and (b) to isolate the bioactive chemicals of the active peel extract using bioactivity-guided fractionation procedures. These studies are reported in Chapters 4 and 5, respectively.

CHAPTER 4

EVALUATION OF THE INSECTICIDAL ACTIVITY OF A PETROLEUM ETHER PEEL EXTRACT OF *CITRUS AURANTIUM* TO *BACTROCERA OLEAE* AND *CERATITIS CAPITATA* ADULTS

4.1 INTRODUCTION

Insecticide bioassays are experiments done with an insecticide to estimate the probability that an insect population will respond in the desired manner and so be made innocuous (Robertson and Preisler, 1992). Initial evaluation of a candidate insecticide takes place in the laboratory and if it is promising it is further tested in a series of glasshouse and field trials. Several bioassays methods have been developed in order to test insecticides in the laboratory.

The most commonly employed method is topical application in which the insecticide is dissolved in a relatively nontoxic and volatile solvent and applied directly to a specific part of the outer surface of an insect. Usually combinations of a constant amount of solvent with varied concentrations of the insecticide are used for this purpose in order to keep the area of contact, as well as the effect of solvent, constant (Matsumura, 1975). Different solvents, solvent volumes and sites of placement [dorsal surface of the thorax (Iwuala *et al.*, 1981), ventral abdomen (Nigg *et al.*, 1994), legs and head (Scott *et al.*, 1986), left eye of moths (Forrester *et al.*, 1993)] have been used in topical application bioassays. The topical application bioassay

permits quantification of the amount of toxicant received by each individual test insect, but does not reflect the mechanism by which residual insecticides are acquired by insects (Hinkle *et al.*, 1985), as most insects physically encounter surface residues by tarsal contact, not dorsal contact (Stark *et al.*, 1995). The clearest advantage of topical application is that the doses are independent of insect activity. A disadvantage is that this method may overcome penetration barriers. It is also tedious and requires manual dexterity (Moyses and Gfeller, 2001).

The treated surface exposure method is another way of exposing insects to an insecticide. The insecticide in a solvent is applied to the container or the panel surface where insects are to walk or rest. The solvent is evaporated by rotating the container or the panel, so that the insecticide is evenly spread over a known area (Matsumura, 1975). Petri dishes (Studebaker and Kring, 2003), vials (Stark *et al.*, 2004), jars (Hu and Prokopy, 1998) and filter papers (Obeng-Ofori and Reichmuth, 1997) have been used in treated surface exposure bioassays. The treated surface exposure bioassay gives a realistic simulation of the field situation, where insects are exposed to insecticides mainly by tarsal contact. However, this method does not allow the actual amount of insecticides acquired by insects to be determined (Choo *et al.*, 2000).

Based on the various types of practical control measures and the insect's mode of life, several other bioassay methods have been developed for testing the effectiveness of insecticides toward various insect species. These are: injection (Jefferies *et al.*, 1992), dipping (Adán *et al.*, 1996), ingestion (Medina *et al.*, 2004), fumigation (Konstantopoulou *et al.*, 1992), leaf-dip (Lee *et al.*, 1997) and systemic bioassay (Siskos, 1997).

Assessing of mortality is the most immediate concern when insecticides are tested. However, other effects on insects such as knockdown (Gbewonyo *et al.*, 1993), repellency, feeding and oviposition deterrency (Talukder and Howse, 1994), reduced fertility, fecundity and offspring development (Jimenez-Peydro *et al.*, 1995) could be of great importance. The toxicity of insecticides to a particular insect is expressed as LD_{50}/LC_{50} (median lethal dose/concentration). LD_{50}/LC_{50} is the statistically derived dose of an insecticide/concentration of an insecticide in an environmental medium, expected to kill 50 % of test insects in a given population under a defined set of conditions (Duffus, 1993).

Intrinsic factors (species and stage specificity, sex, size and resistance), extrinsic factors (temperature, humidity, illumination, population density and food), experimental factors and experimental techniques are factors which affect the evaluation of an insecticide (Matthews, 1997).

In this chapter the insecticidal activity of a petroleum ether peel extract obtained using the ultrasound extraction method was evaluated against males and females of BO and CC adults by the topical application and the Petri dish exposure bioassay. In addition, a fumigation bioassay was employed in order to examine whether the activity of the extract in the Petri dish exposure bioassay is due to fumigation rather than contact and moreover to investigate the fumigant toxicity of this extract.

4.2 METHODS

4.2.1 Extraction of CA peels

Twenty-five green-yellow fruits of CA weighing 70-120 g were collected from each of the 10 sampling trees on 1-5 November 1998 in order to obtain sufficient amount of petroleum ether peel extract for the study of its insecticidal properties. Three-hundred grams of peel per tree were obtained and a series of 30 extractions were performed by using the ultrasound extraction method and petroleum ether as extraction solvent (see section 2.2.1). The extractions were performed using the same amount of peel (100 g) used in all previous extractions, as the proportions and range of substances extracted can change when scaling up a process (Houghton and Raman, 1998). The extractable materials of the 30 extractions were combined and the extraction yield of the total extract was estimated by concentrating to near-dryness 1 % of the total extract in the rotary evaporator at 35 °C. The concentrated extract was subsequently transferred to a 15-ml glass vial, immersed in a water bath at 35 °C and concentrated to constant weight under a stream of nitrogen. The remaining extract was concentrated to dryness and stored in the dark at -20 °C until used. The weighing was performed on the Mettler Toledo balance.

4.2.2 Bioassays

The insecticidal properties of the petroleum ether peel extract were evaluated using the Petri dish exposure, fumigation and topical application bioassays (see section 2.2.2) against adults of BO and CC. Stock solutions of the petroleum ether peel extract were prepared in DCM, as were subsequent dilutions. Fresh solutions were prepared for each replicate. Concentrations/doses for each replicate were applied from the lowest to the highest.

In the Petri dish exposure and topical application bioassays, males and females of BO and CC were tested separately, but simultaneously. For each sex, 5 replicates of 10 flies each were employed for the 5 concentrations/doses and the control. The 5 replicates were conducted using 5 different generations of flies. As the main aim of the experiments performed with the Petri dish exposure and the topical application bioassays was the precise estimation of LC_{50}s/LD_{50}s, respectively five concentrations/doses were chosen for each sex of the two test insects on the basis of preliminary range-finding studies performed by the two biological evaluation techniques, in order to produce mortalities evenly distributed between 25 and 75 % at the final day of recording of mortality (Robertson *et al.*, 1984).

Petri dish exposure bioassay

The concentrations tested against both sexes of BO were 10, 15, 20, 25 and 30 $\mu g/cm^2$. The concentrations employed against males of CC were 50, 60, 70, 80 and 90 $\mu g/cm^2$, while the concentrations used against females of CC were 100, 120, 140, 160 and 180 $\mu g/cm^2$. Mortality for males and females of BO was recorded every 24 h for 4 d and for males and females of CC every 24 h for 3 d, after which no further increase in mortality to both insect species was recorded.

Topical application bioassay

The doses employed against both sexes of BO and the males of CC were 20, 30, 40, 50 and 60 μg/insect, while the doses employed against females of CC were 30, 50, 70, 90 and 110 μg/insect. BO and CC mortalities of both sexes were recorded every 24 h for 6 and 4 d, respectively after which there was no further increase in mortality to both species. Ten adults of each sex of the two test insects were randomly selected at

the end of each replicate and their body weight was measured. The mean body weight for male and female flies of BO and CC was determined and so doses can be expressed, not only as μg/insect, but also as micrograms per milligram adult body weight (μg/mg).

Fumigation bioassay

Males and females of BO were equally susceptible when exposed to the petroleum ether peel extract using the Petri dish exposure bioassay. Considering that result, males and females were tested together (5 males and 5 females/Petri dish) in the fumigation bioassay. The concentration used was 4mg/Petri dish, which is approximately 2-fold greater than the highest concentration used against BO in the concentration-response Petri dish exposure bioassay.

Males were significantly more susceptible than females of CC in the Petri dish exposure bioassay. Consequently, males and females were tested in separate experiments (10 males or females/Petri dish). The concentration employed against males was 12 mg/Petri dish, while the concentration employed against females was 24 mg/Petri dish. These concentrations were approximately 2-fold greater respectively than the highest concentration used against males and females of CC in the concentration-response Petri dish exposure bioassay.

Four Petri dishes for each concentration and their corresponding controls (see section 2.2.2), were replicated 5 times using flies of different generations. Mortality of BO adults was recorded every 24 h for 4 d, whereas mortality of CC adults (both sexes) every 24 h for 3 d.

4.3 RESULTS

4.3.1 Petri dish exposure bioassays

Petri dish exposure bioassays were performed in order to compare the susceptibility of BO and CC adults to the petroleum ether peel extract of CA. Moreover, as males and females of both species were tested separately the susceptibility of sexes was also compared.

BO

The results obtained by the Petri dish exposure bioassay against males and females of BO are presented in Figure 4.1 and Figure 4.2, respectively. At 24 h after treatment, mortality of males and females of BO was not significantly different from the control even at the highest concentration of the petroleum ether peel extract tested (30 $\mu g/cm^2$). However, at 48, 72 and 96 h after treatment, mortality of males and females was significantly higher compared to the control at concentrations of 15, 20, 25 and 30 $\mu g/cm^2$ of the petroleum ether peel extract. Mortality of males and females caused by the concentration of 10 $\mu g/cm^2$ was greater than the control, but not significantly different. The mortality results reveal that mortality in males and females of BO increased with increasing concentration, indicating that mortality is concentration-dependent.

A summary of probit analyses of mortality data for males and females of BO exposed to different concentrations of the petroleum ether peel extract of CA using the Petri dish exposure bioassay is presented in Table 4.1. Regression lines obtained from probit analyses are shown in Figure 4.3. The concentrations selected to estimate the LC_{50} of males and females at the end-point mortality (96 h after treatment) cause low

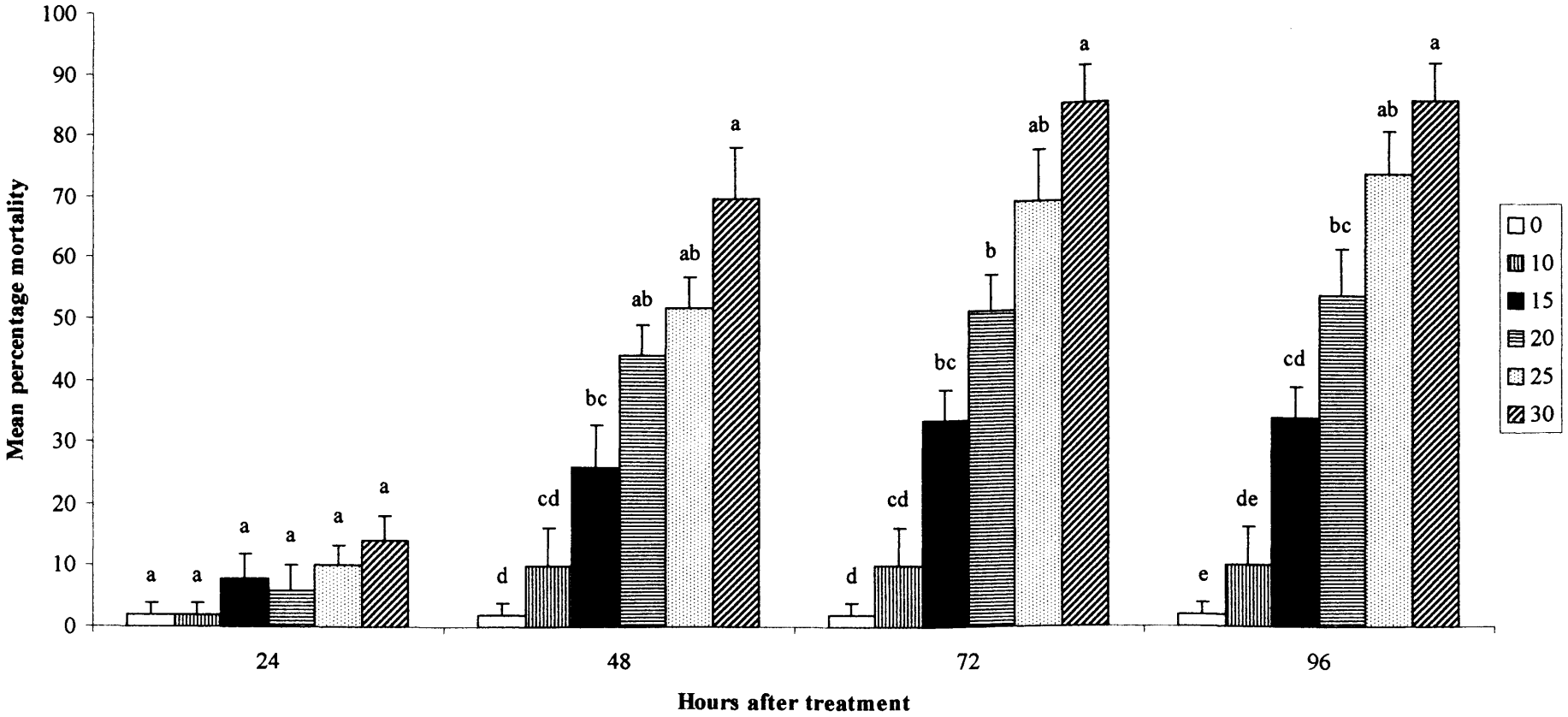

Figure 4.1 Percentage mortality of BO male adults exposed to different concentrations ($\mu g/cm^2$) of the petroleum ether peel extract of CA using the Petri dish exposure bioassay.

Columns represent mean of 5 replicates of 10 males each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

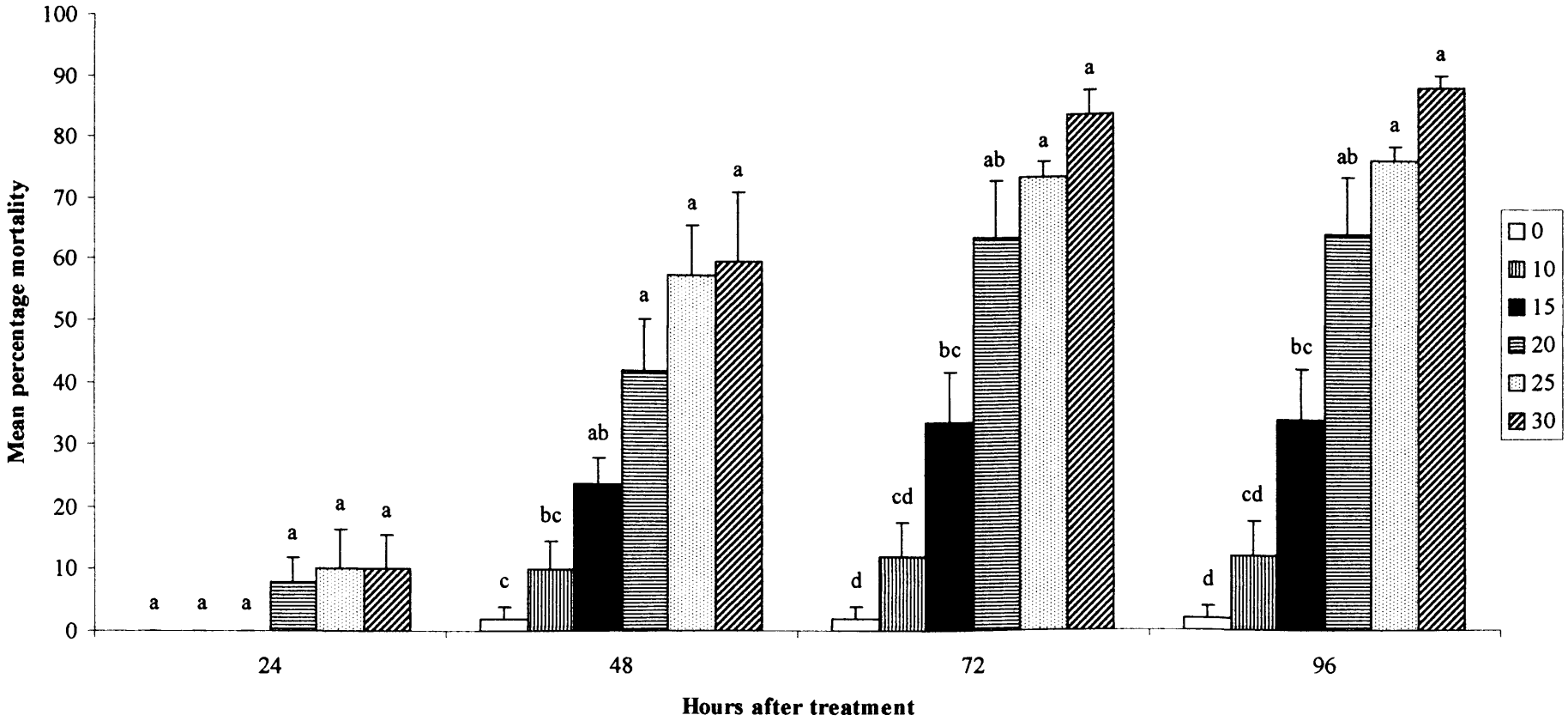

Figure 4.2 Percentage mortality of BO female adults exposed to different concentrations ($\mu g/cm^2$) of the petroleum ether peel extract of CA using the Petri dish exposure bioassay.

Columns represent mean of 5 replicates of 10 females each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

Table 4.1 Summary of probit analyses of mortality data for males and females of BO exposed to different concentrations of the petroleum ether peel extract of CA using the Petri dish exposure bioassay.

h	Sex	n	LC_{50} ($\mu g/cm^2$)	95 % CL	Slope ± SE	Intercept ± SE	χ^2	df	*P*
48	♂	250	23.0	20.8 - 26.2	3.76 ± 0.57	-0.13 ± 0.75	24.8	23	0.358
48	♀	250	23.8	21.3 - 27.5	3.60 ± 0.57	0.04 ± 0.75	27.7	23	0.225
72	♂	250	19.1	17.6 - 20.8	4.88 ± 0.60	-1.25 ± 0.78	27.0	23	0.257
72	♀	250	18.1	16.6 - 19.7	4.77 ± 0.58	-1.00 ± 0.75	20.7	23	0.599
96	♂	250	18.8	17.3 - 20.4	5.02 ± 0.60	-1.40 ± 0.78	26.5	23	0.280
96	♀	250	17.8	16.4 - 19.3	5.10 ± 0.60	-1.38 ± 0.77	19.2	23	0.691

Column headings are abbreviated as follows: h is the hours after treatment; n is the numbers of insects tested excluding the controls; 95 % CL is the 95 % confidence limits; Slope ± SE is the slope ± standard error; Intercept ± SE is the intercept ± standard error; χ^2 is the Pearson's χ^2 goodness-of-fit test; df is the degrees of freedom; *P* is the probability.

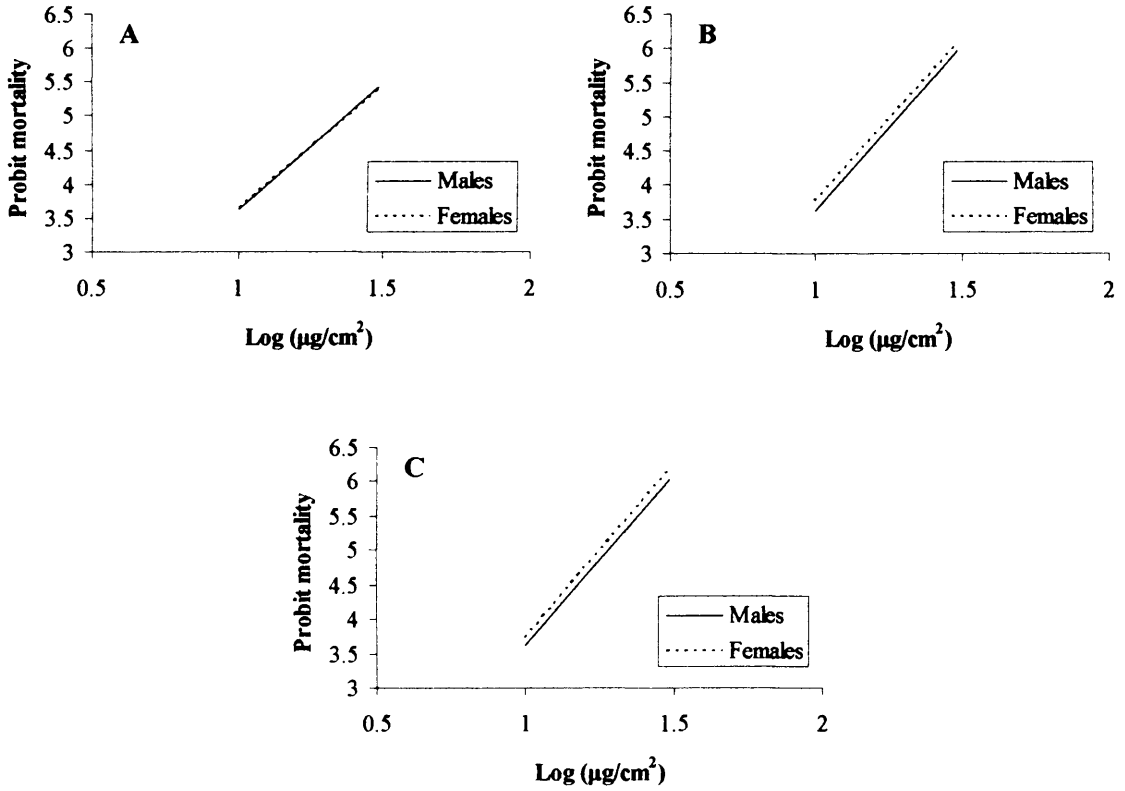

Figure 4.3 Log ($\mu g/cm^2$) - probit mortality lines calculated by probit analysis for males and females of BO exposed to the petroleum ether peel extract of CA using the Petri dish exposure bioassay at 48 (A), 72 (B) and 96 (C) h after treatment.

mortality (14 % or less) at 24 h after treatment; consequently LC_{50}s were not estimated at this time. All *P*-values of Table 4.1 were much greater than 0.05 and so Pearson's χ^2 goodness-of-fit tests were not significant, indicating that the data fit the assumptions of the probit model adequately.

The LC_{50} estimated from probit analysis for males at 48 h after treatment, was slightly lower (23 $\mu g/cm^2$) than females (23.8 $\mu g/cm^2$), whereas the LC_{50}s for males at 72 and 96 h after treatment (19.1 and 18.8 $\mu g/cm^2$, respectively) were slightly higher than for females at the same mortality recording time (18.1 and 17.8 $\mu g/cm^2$, respectively). However, the 95 % confidence limits of males and females overlapped at all mortality recording times, indicating that males and females of BO were equally susceptible to the petroleum ether peel extract of CA. Moreover, the action of the petroleum ether peel extract against males and females of BO was the same, as the slopes of the regression lines were not significantly different at all mortality recording times.

CC

The results obtained by the Petri dish exposure bioassay against male adults of CC are presented in Figure 4.4. At 24 h after treatment, only the mortality caused by the concentration of 90 $\mu g/cm^2$ was significantly greater than that of the control, although 10 % mortality was caused by the concentrations of 60, 70 and 80 $\mu g/cm^2$. At 48 and 72 h after treatment, mortalities caused by all concentration tested were significantly greater than that of the control.

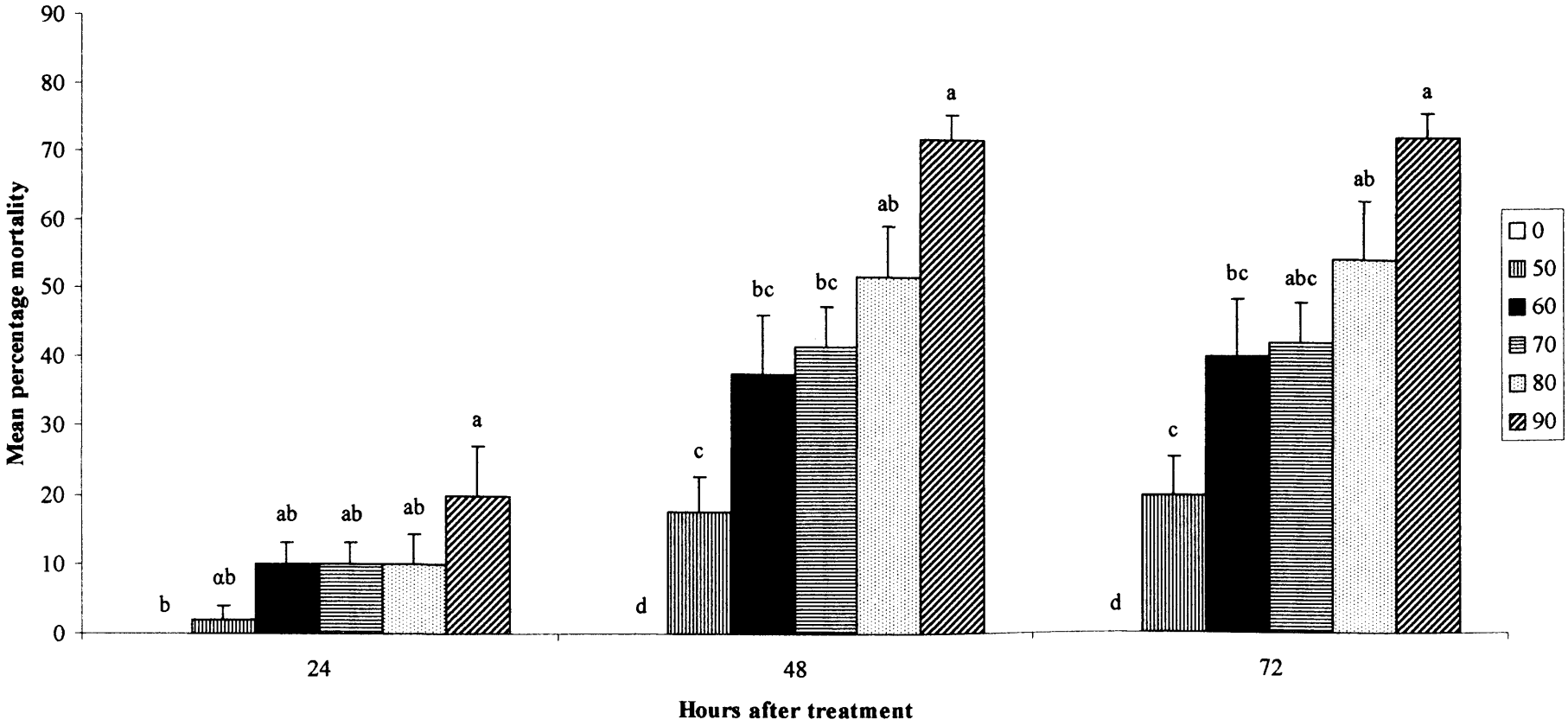

Figure 4.4 Percentage mortality of CC male adults exposed to different concentrations ($\mu g/cm^2$) of the petroleum ether peel extract of CA using the Petri dish exposure bioassay.

Columns represent mean of 5 replicates of 10 males each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

Mortality trends of female adults of CC (Figure 4.5) were quite similar to those of male adults of CC, although the concentrations tested against females were 2-fold greater than that tested against females. 24 h post-treatment, even the highest concentration tested (180 $\mu g/cm^2$) which caused 14 % mortality was not significantly different from the control. At 48 and 72 h after treatment, mortality levels caused by the concentrations of 120, 140, 160 and 180 $\mu g/cm^2$ were significantly greater than that of the control, while the mortality (10 %) caused by the lowest concentration tested (100 $\mu g/cm^2$) was not significantly different from the control. Greater concentrations caused greater male and female mortalities, showing a clear concentration-response relationship.

A summary of probit analyses of mortality data for males and females of CC exposed to different concentrations of the petroleum ether peel extract of CA using the Petri dish exposure bioassay is presented in Table 4.2. Regression lines obtained from the probit analyses are shown in Figure 4.6. All the concentrations selected to estimate the LC_{50} of males and females at the end-point mortality (72 h after treatment) did not produce significant mortality levels during the first 24 h. For that reason, LC_{50}s were not estimated at this time. All *P*-values of Table 4.2 were much greater than 0.05 and so Pearson's χ^2 goodness-of-fit tests were not significant, indicating that the data fit the assumptions of the probit model adequately.

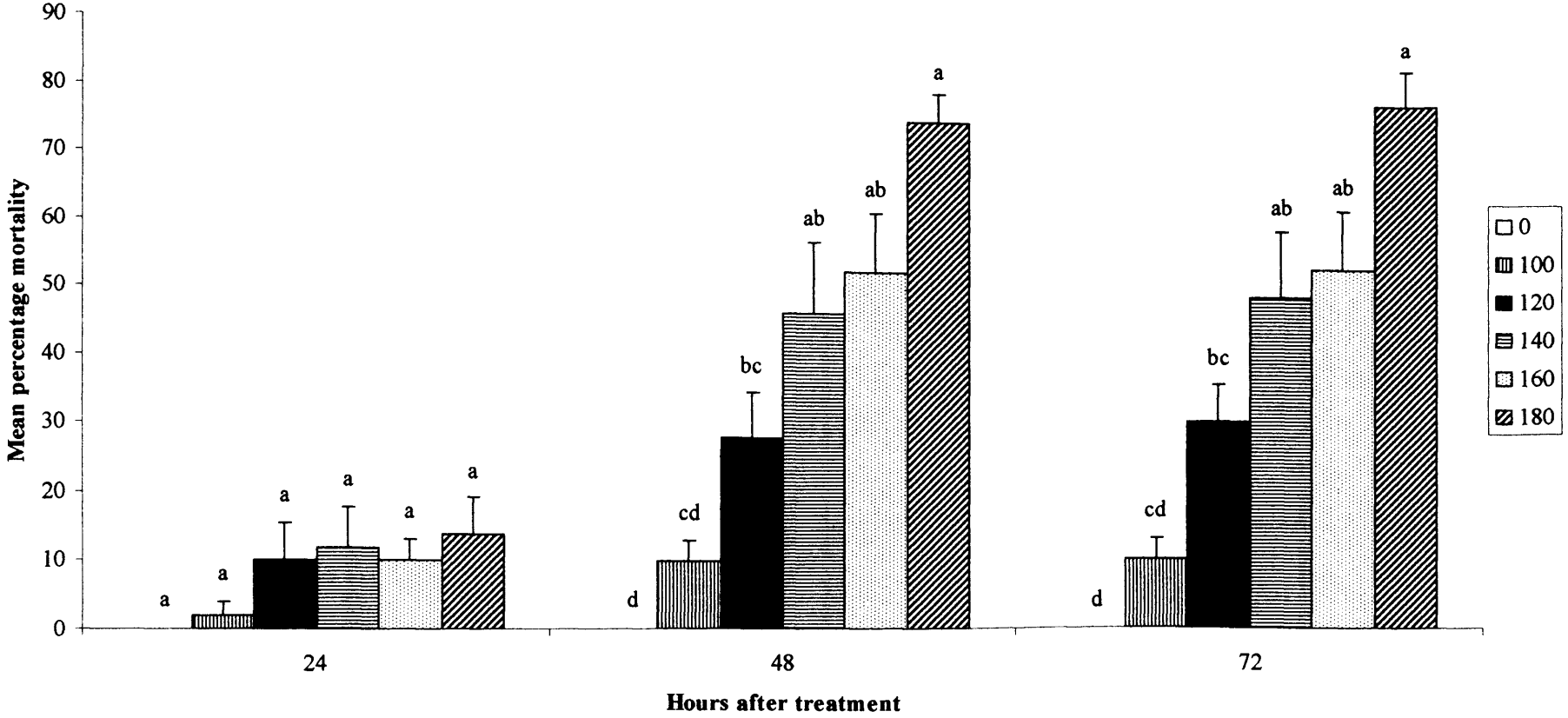

Figure 4.5 Percentage mortality of CC female adults exposed to different concentrations ($\mu g/cm^2$) of the petroleum ether peel extract of CA using the Petri dish exposure bioassay.

Columns represent mean of 5 replicates of 10 females each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

Table 4.2 Summary of probit analyses of mortality data for males and females of CC exposed to different concentrations of the petroleum ether peel extract of CA using the Petri dish exposure bioassay.

h	Sex	n	LC_{50} ($\mu g/cm^2$)	95 % CL	Slope ± SE	Intercept ± SE	χ^2	df	*P*
48	♂	250	73.6	68.4 - 80.5	5.18 ± 0.96	-4.67 ± 1.76	20.4	23	0.617
48	♀	250	149.0	140.9 - 159.1	6.93 ± 1.03	-10.07 ± 2.21	24.4	23	0.381
72	♂	250	72.6	67.3 - 79.5	4.95 ± 0.95	-4.21 ± 1.74	22.2	23	0.510
72	♀	250	147.1	139.2 - 156.8	7.00 ± 1.03	-10.17 ± 2.20	23.8	23	0.418

Column headings are abbreviated as follows: h is the hours after treatment; n is the numbers of insects tested excluding the controls; 95 % CL is the 95 % confidence limits; Slope ± SE is the slope ± standard error; Intercept ± SE is the intercept ± standard error; χ^2 is the Pearson's χ^2 goodness-of-fit test; df is the degrees of freedom; *P* is the probability.

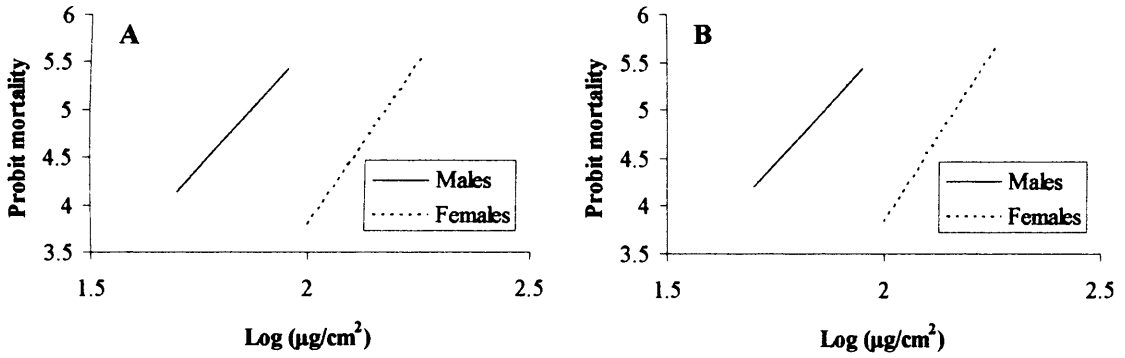

Figure 4.6 Log ($\mu g/cm^2$) - probit mortality lines calculated by probit analysis for males and females of CC exposed to the petroleum ether peel extract of CA using the Petri dish exposure bioassay at 48 (A) and 72 (B) h after treatment.

The LC_{50}s estimated from probit analysis for females of CC at 48 and 72 h after treatment (149.0 and 147.1 $\mu g/cm^2$, respectively), were 2-fold greater than that of males (73.6 and 72.6 $\mu g/cm^2$, respectively). At both mortality recording times, males of CC were significantly more susceptible to the exposure of the petroleum ether peel extract of CA than females as their 95 % confidence limits did not overlap.

At the end-point mortality of BO and CC (96 and 72 h after treatment, respectively) the LC_{50} of CC males was 3.9 times greater than that of BO males. Moreover, the LC_{50} of CC females was 8.3 times greater than that of BO females. That difference (more than 2-fold) in the degree of susceptibility between males and females accounted to the difference in susceptibility between CC males and females. These results indicate that at the end-point mortality, BO males and females were significantly more susceptible when exposed to the petroleum ether peel extract of CA using the Petri dish exposure bioassay than CC males and females, respectively. Moreover, CC adults reached the end-point mortality one day earlier than BO adults, indicating that the extract acted more rapidly against CC adults.

4.3.2 Fumigant bioassays

In the following bioassays, the possibility that mortality caused by the petroleum ether peel extract of CA, in the Petri dish exposure bioassay, was due to fumigation rather than contact and the fumigant action of the petroleum ether peel extract was investigated.

BO

The results obtained by the fumigation bioassay against BO adults (males and females were tested together) are presented in Table 4.3. At all mortality recording times only the two treatments in which adults of BO were exposed to the petroleum ether peel extract (either applied to the total inner surface or only to the bottom of the Petri dishes) by contact, caused significant mortality. This result reveals the absence of fumigant action in the Petri dish exposure bioassays and indicates that mortality observed was the result of contact of BO adults with the petroleum ether peel extract

of CA. Moreover, as no significant mortality was observed when BO adults were exposed only to the fumes of the petroleum ether peel extract in Petri dishes sealed with Parafilm, a lack of fumigant activity of the petroleum ether peel extract against BO adults even at a concentration of 157 $\mu g/cm^3$, was revealed.

Table 4.3 Percentage mortality of BO adults exposed to the petroleum ether peel extract of CA at 4mg/Petri dish using the fumigation bioassay.

Treatment[a]	% Mortality			
	Mean[b] ± SE[c], at hours after treatment			
	24	48	72	96
P1	26 ± 6.0a	74 ± 6.8a	100 ± 0.0a	100 ± 0.0a
P2	32 ± 8.6a	76 ± 7.5a	96 ± 2.4a	100 ± 0.0a
P3	0 ± 0.0b	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b
P4	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b	4 ± 2.4b
C1	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
C2	0 ± 0.0b	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
C3	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
C4	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b	4 ± 2.4b

[a]**P1**: The extract was applied to the total inner surface of the Petri dishes; **P2**: The extract was applied only to the bottom of the Petri dishes; **P3**: The extract was applied only to the bottom of the Petri dishes and a sheet of gauze was fitted inside them; **P4**: The extract was applied only to the bottom of the Petri dishes, a sheet of gauze was fitted inside them and sealed with Parafilm; **C1**, **C2**, **C3** and **C4**: Controls corresponding to P1, P2, P3 and P4, respectively which were treated with DCM only. [b]Mean of 5 replicates of 10 insects each (5 males and 5 females). [c]SE: Standard error of mean. Means within a column followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

CC

Similar results were obtained when the same bioassay was conducted against CC (males and females were tested separately) (Table 4.4). Only the two treatments, in which males and females of CC were allowed to contact the petroleum ether peel extract, produced significant mortality at all mortality recording times. This result

Table 4.4 Percentage mortality of CC male and female adults exposed to the petroleum ether peel extract of CA at 12mg/Petri dish and 24 mg/Petri dish, respectively using the fumigation bioassay.

Treatment[a]	% Mortality					
	Mean[b] ± SE[c], at hours after treatment					
	24	48	72	24	48	72
	Males			Females		
P1	40 ± 6.3a	100 ± 0.0a	100 ± 0.0a	38 ± 8.6a	96 ± 4.0a	98 ± 2.0a
P2	28 ± 6.6a	94 ± 4.0a	96 ± 2.4a	32 ± 5.8a	92 ± 3.7a	94 ± 4.0a
P3	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
P4	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
C1	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
C2	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
C3	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
C4	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]**P1**: The extract was applied to the total inner surface of the Petri dishes; **P2**: The extract was applied only to the bottom of the Petri dishes; **P3**: The extract was applied only to the bottom of the Petri dishes and a sheet of gauze was fitted inside them; **P4**: The extract was applied only to the bottom of the Petri dishes, a sheet of gauze was fitted inside them and sealed with Parafilm; **C1**, **C2**, **C3** and **C4**: Controls corresponding to P1, P2, P3 and P4, respectively which were treated with DCM only. [b]Mean of 5 replicates of 10 adults of the same sex each. [c]SE: Standard error of mean. Means within a column followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

reveals the absence of fumigant action in the Petri dish exposure bioassays and indicates that mortality of CC males and females, as in the case of BO, was due to contact. Moreover, no mortality was recorded when CC males and females were exposed only to the fumes of the petroleum ether peel extract in Petri dishes sealed with Parafilm, indicating a lack of fumigant activity of the petroleum ether peel extract against CC males and females even at a concentration of 471 and 942 $\mu g/cm^3$, respectively.

4.3.3 Topical application bioassays

Topical application bioassays were performed in order to compare the susceptibility of BO and CC adults to the petroleum ether peel extract of CA. Moreover, as males and females of both species were tested separately the susceptibility of sexes was also compared.

BO

The body weight (mean ± standard error of mean) of BO males and females was 6.0 ± 0.14 and 7.7 ± 0.17 mg, respectively. For completeness, as there was variation in weight between males and females of BO, doses tested are expressed not only as μg per insect but also as μg per mg of adult body weight. When doses were expressed as μg/insect, the same doses were employed against males and females of BO (20, 30, 40, 50 and 60). In contrast, when doses were corrected for the difference in body weight between males and females, the doses employed against males of BO were 3.34, 5.01, 6.68, 8.35 and 10.02 μg/mg, while the doses employed against females of BO were 2.60, 3.90, 5.20, 6.50 and 7.80 μg/mg. The slightly lower doses (μg/mg) employed against females was a result of their greater body weight.

The results obtained by the topical application bioassay against male adults of BO are presented in Figure 4.7. At 24 h after treatment, even mortality caused by the highest dose tested (60 μg/insect) was not significantly different from the control. 48 h post-treatment, only the two higher doses tested (50 and 60 μg/insect) caused significantly greater mortality than that of the control. Doses of 30, 40, 50 and 60 μg/insect caused significantly greater mortality than that of the control at 72, 96, 120 and 144 h after treatment, whereas the lowest dose tested (20 μg/insect) was significantly greater from the control only 72 and 144 h post-treatment.

Mortality trends of female adults of BO (Figure 4.8) were quite similar to those of male adults. At 24 and 48 h after treatment, even mortality caused by the highest dose tested (60 μg/insect) was not significantly different from the control. However, 72, 96, 120 and 144 h after treatment, doses of 30, 40, 50 and 60 μg/insect caused significantly greater mortality than the control, while the lowest dose tested (20 μg/insect) was significantly different from the control only at 120 h after treatment. The results presented in Figure 4.7 and Figure 4.8 show that mortality of BO males and females increased with increasing dose, indicating a dose-response effect.

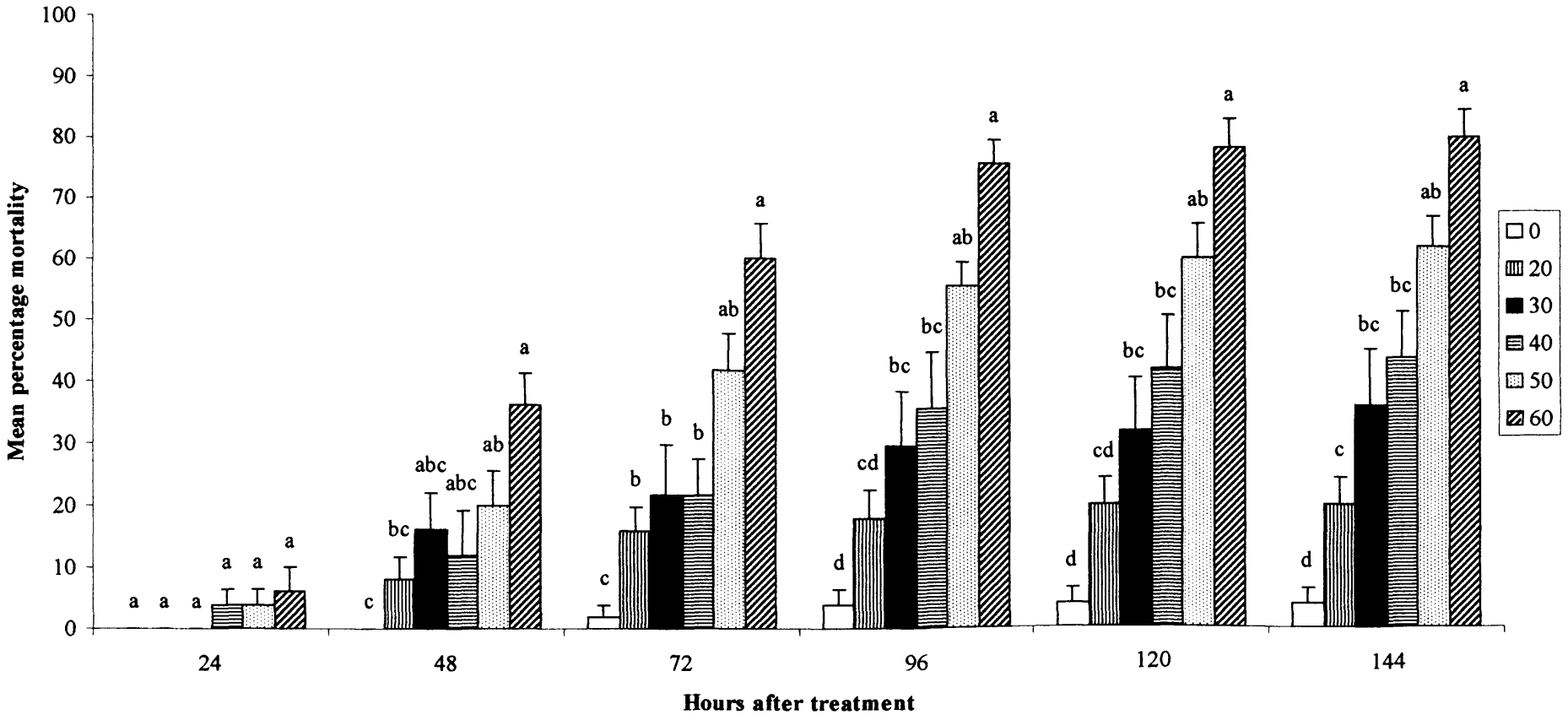

Figure 4.7 Percentage mortality of BO male adults treated with different doses (μg/insect) of the petroleum ether peel extract of CA using the topical application bioassay.

Columns represent mean of 5 replicates of 10 males each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

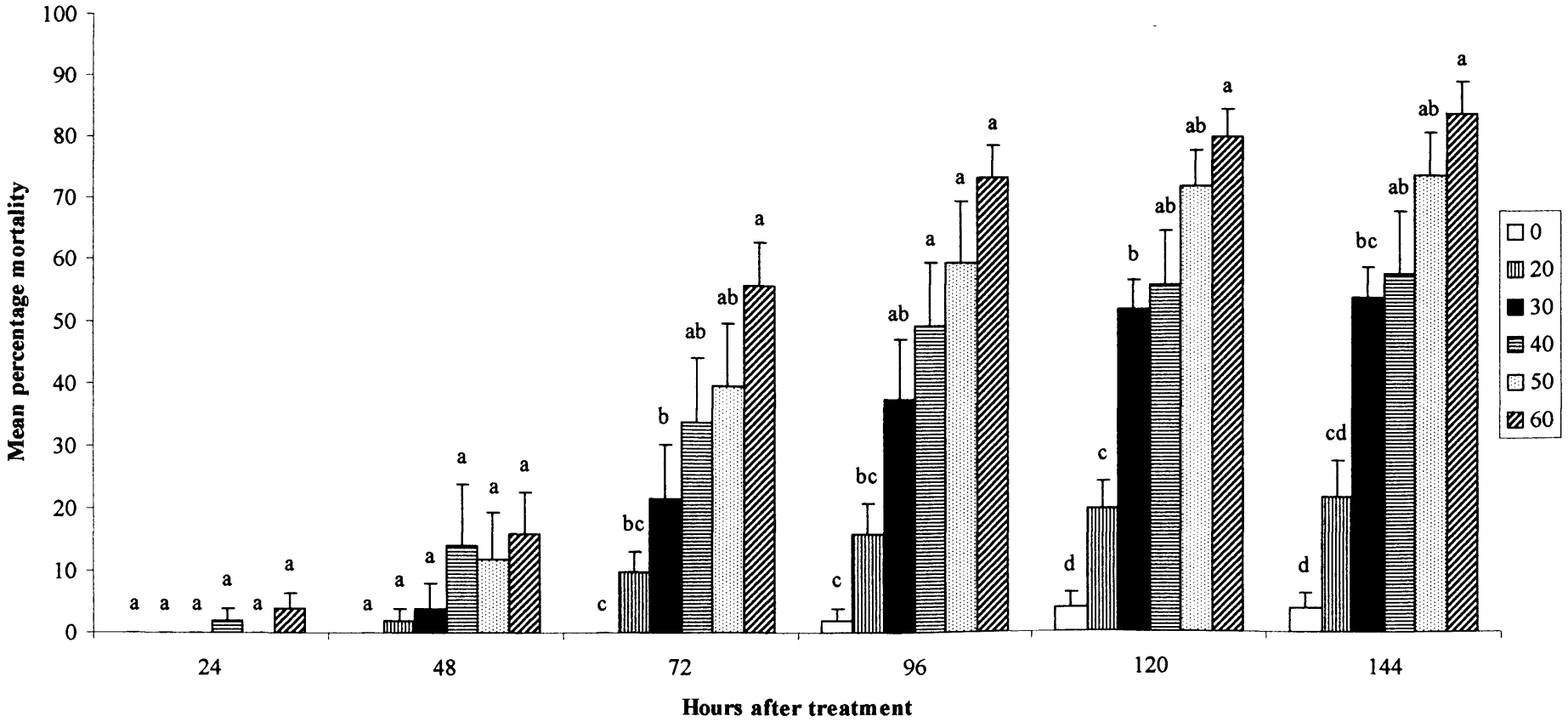

Figure 4.8 Percentage mortality of BO female adults treated with different doses (μg/insect) of the petroleum ether peel extract of CA using the topical application bioassay.

Columns represent mean of 5 replicates of 10 females each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

A summary of probit analyses of mortality data for males and females of BO treated with different doses (expressed as μg/insect and μg/mg) of the petroleum ether peel extract of CA, using the topical application bioassay, is presented in Table 4.5 and Table 4.6, respectively. Regression lines [probit mortality versus log (μg/insect) and probit mortality versus log (μg/mg)] obtained from the probit analyses are shown in Figure 4.9 and Figure 4.10, respectively. The doses selected to estimate the LD_{50} of males and females at the end-point mortality (144 h after treatment) did not cause significant mortality at 24 and 48 h after treatment and so LD_{50}s were not estimated at these times. All *P*-values of Table 4.5 and Table 4.6 were greater than 0.05 and so Pearson's χ^2 goodness-of-fit tests were not significant indicating that the data fit the assumptions of the probit model adequately.

Table 4.5 Summary of probit analyses of mortality data for males and females of BO treated with different doses (μg/insect) of the petroleum ether peel extract of CA using the topical application bioassay.

h	Sex	n	LD_{50} (μg/insect)	95 % CL	Slope ± SE	Intercept ± SE	χ^2	df	*P*
72	♂	250	59.6	50.2 - 82.4	2.67 ± 0.55	0.26 ± 0.88	21.5	23	0.551
72	♀	250	56.4	48.4 - 73.6	2.87 ± 0.56	-0.02 ± 0.90	32.8	23	0.084
96	♂	250	44.8	40.0 - 51.5	3.42 ± 0.54	-0.65 ± 0.87	22.7	23	0.479
96	♀	250	40.1	35.7 - 45.4	3.34 ± 0.53	-0.35 ± 0.85	30.7	23	0.130
120	♂	250	41.9	37.5 - 47.6	3.43 ± 0.54	-0.56 ± 0.85	21.8	23	0.532
120	♀	250	34.6	30.5 - 38.6	3.46 ± 0.53	-0.33 ± 0.83	17.3	23	0.793
144	♂	250	40.2	36.0 - 45.3	3.48 ± 0.53	-0.59 ± 0.85	19.8	23	0.652
144	♀	250	33.0	29.1 - 36.8	3.57 ± 0.53	-0.42 ± 0.83	23.8	23	0.416

Column headings are abbreviated as follows: h is the hours after treatment; n is the numbers of insects tested excluding the controls; 95 % CL is the 95 % confidence limits; Slope ± SE is the slope ± standard error; Intercept ± SE is the intercept ± standard error; χ^2 is the Pearson's χ^2 goodness-of-fit test; df is the degrees of freedom; *P* is the probability.

Table 4.6 Summary of probit analyses of mortality data for males and females of BO treated with different doses (μg/mg) of the petroleum ether peel extract of CA using the topical application bioassay.

h	Sex	n	LD_{50} (μg/mg)	95 % CL	Slope ± SE	Intercept ± SE	χ^2	df	*P*
72	♂	250	9.9	8.4 - 13.8	2.67 ± 0.55	2.33 ± 0.46	21.5	23	0.551
72	♀	250	7.3	6.3 - 9.6	2.87 ± 0.56	2.52 ± 0.41	32.8	23	0.084
96	♂	250	7.5	6.7 - 8.6	3.42 ± 0.54	2.01 ± 0.45	22.7	23	0.479
96	♀	250	5.2	4.6 - 5.9	3.34 ± 0.53	2.61 ± 0.38	30.7	23	0.130
120	♂	250	7.0	6.3 - 8.0	3.43 ± 0.54	2.10 ± 0.44	21.8	23	0.532
120	♀	250	4.5	4.0 - 5.0	3.46 ± 0.53	2.74 ± 0.37	17.3	23	0.793
144	♂	250	6.7	6.0 - 7.6	3.48 ± 0.53	2.12 ± 0.44	19.8	23	0.652
144	♀	250	4.3	3.8 - 4.8	3.57 ± 0.53	2.74 ± 0.37	23.8	23	0.416

Column headings are abbreviated as follows: h is the hours after treatment; n is the numbers of insects tested excluding the controls; 95 % CL is the 95 % confidence limits; Slope ± SE is the slope ± standard error; Intercept ± SE is the intercept ± standard error; χ^2 is the Pearson's χ^2 goodness-of-fit test; df is the degrees of freedom; *P* is the probability.

Except from the different estimates of LD_{50}s and their corresponding 95 % confidence limits when doses were expressed as μg/insect and μg/mg adult body weight, the only other difference between Table 4.5 and Table 4.6 was the intercept and its standard error. At all mortality recording times, the LD_{50}s estimated for males of BO were greater than that of females, either doses expressed as μg/insect or μg/mg of adult body weight. When doses expressed as μg/insect, the 95 % confidence limits overlapped at all mortality recording. In contrast, when doses were expressed as μg/mg of adult body weight, the 95 % confidence limits did not overlap at 96, 120 and 144 h after treatment, whereas they overlapped at 72 h after treatment. That results indicate that males and females of BO did not differ significantly in susceptibility when doses were expressed as μg/insect, whereas females were more susceptible than males when doses were expressed as μg/mg adult body weight. Moreover, the action of the petroleum ether peel extract against BO males and females is similar as the

slopes of the regression lines did not differ significantly, in all mortality recording times.

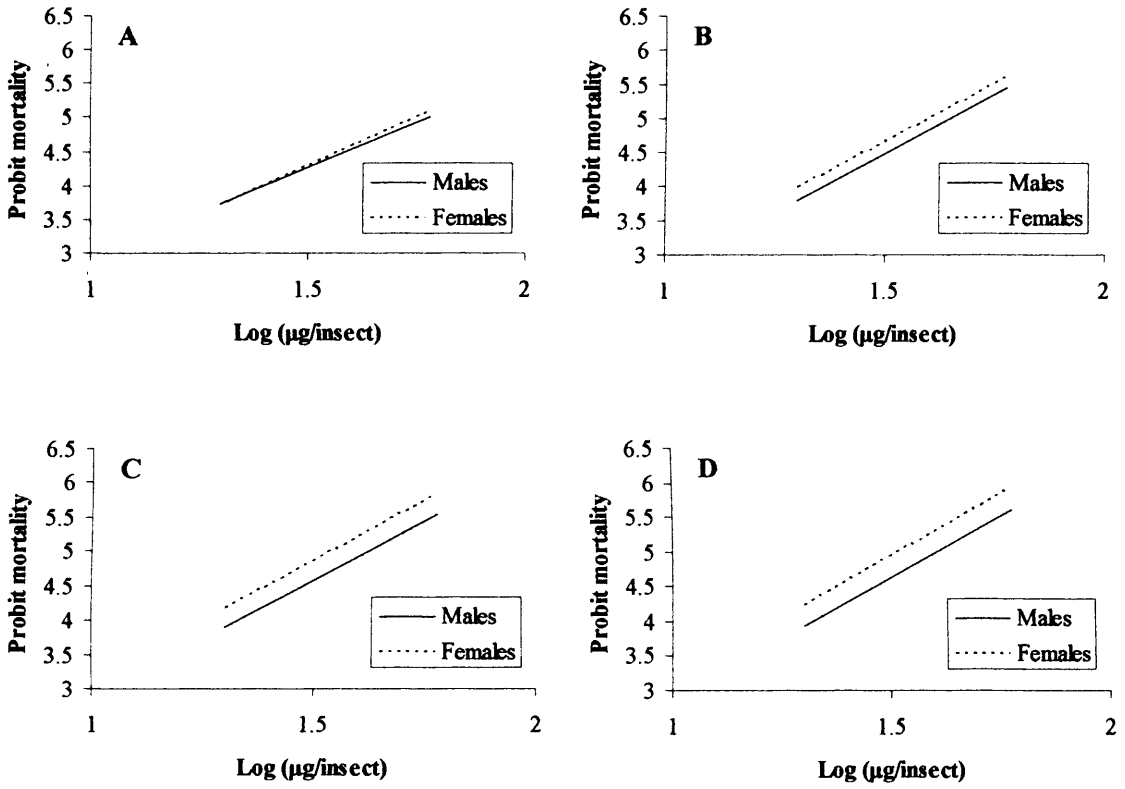

Figure 4.9 Log (μg/insect) - probit mortality lines calculated by probit analysis for males and females of BO treated with the petroleum ether peel extract of CA using the topical application bioassay at 72 (A), 96 (B), 120 (C) and 144 (D) h after treatment.

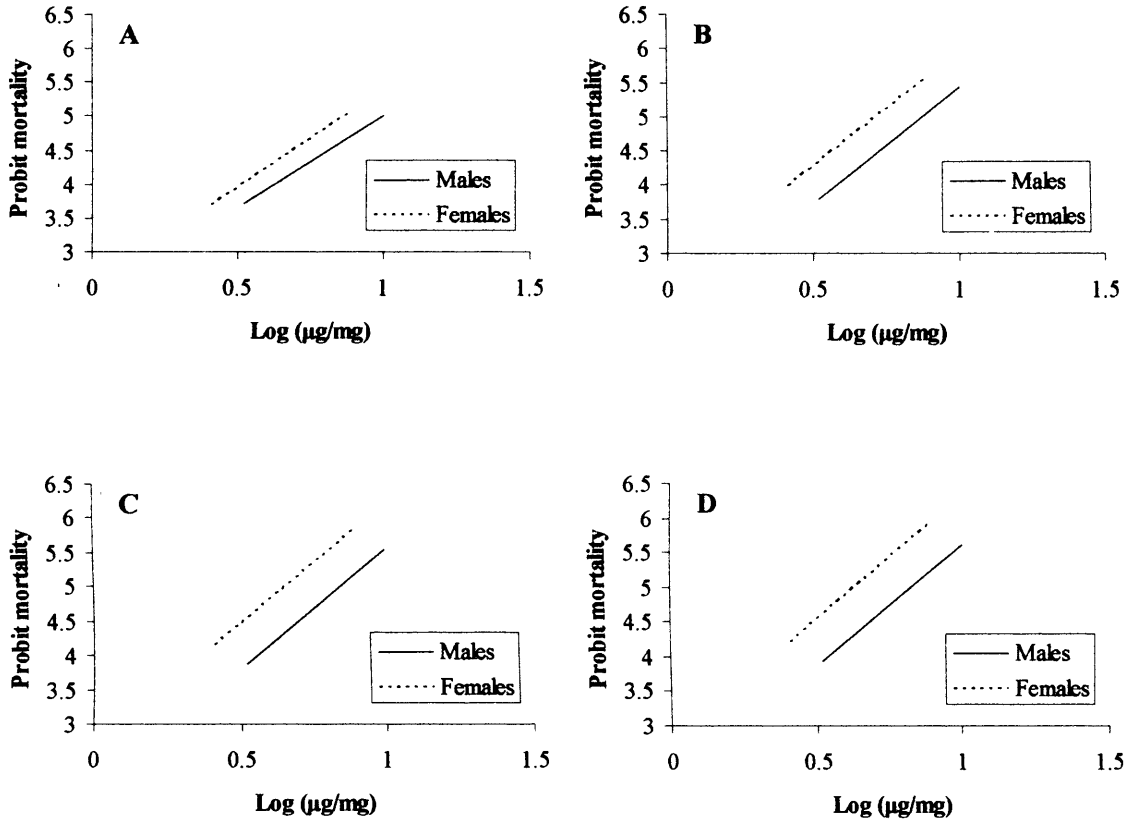

Figure 4.10 Log (μg/mg) - probit mortality lines calculated by probit analysis for males and females of BO treated with the petroleum ether peel extract of CA using the topical application bioassay at 72 (A), 96 (B), 120 (C) and 144 (D) h after treatment.

CC

The body weight (mean ± standard error of mean) of CC males and females was 6.9 ± 0.16 and 8.0 ± 0.25 mg, respectively. For completeness, as there was variation in weight between males and females of CC, doses tested are expressed not only as μg per insect but also as μg per mg of adult body weight. The doses (μg/insect) tested were 20, 30, 40, 50 and 60 against males and 30, 50, 70, 90 and 110 against females of CC. However, when doses were expressed as μg/mg, the doses tested were 2.90, 4.35, 5.80, 7.25 and 8.70 against males and 3.75 6.25, 8.75, 11.25 and 13.75 against females of CC. As a result of the greater body weight of females, the difference

between the doses tested against males and females was lower when doses were expressed as μg/mg.

The results obtained by the topical application bioassay against male and female adults of CC are presented in Figure 4.11 and Figure 4.12, respectively. At 24 and 48 h after treatment, only the male mortalities caused by the two lower doses (20 and 30 μg/insect) were not significantly different from the control. However, at 72 and 96 h after treatment only the lowest dose tested (20 μg/insect) against males did not cause significantly greater mortality than that of control. In contrast, at all mortality recording times all doses tested against females of CC caused mortalities significantly greater from the control. The results presented in Figure 4.11 and Figure 4.12 reveal the existence of a dose-dependent effect on mortality of males and females of CC.

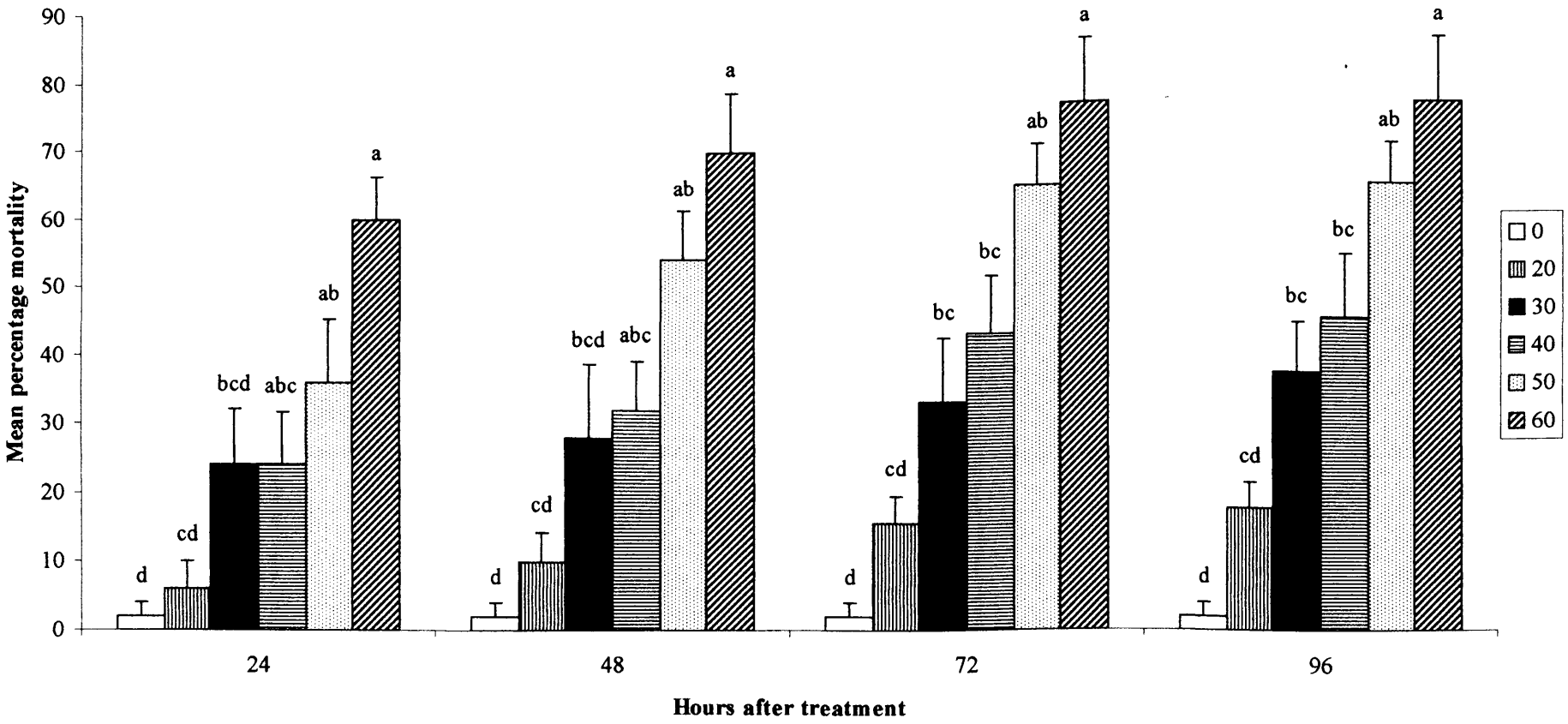

Figure 4.11 Percentage mortality of CC male adults treated with different doses (μg/insect) of the petroleum ether peel extract of CA using the topical application bioassay.

Columns represent mean of 5 replicates of 10 males each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

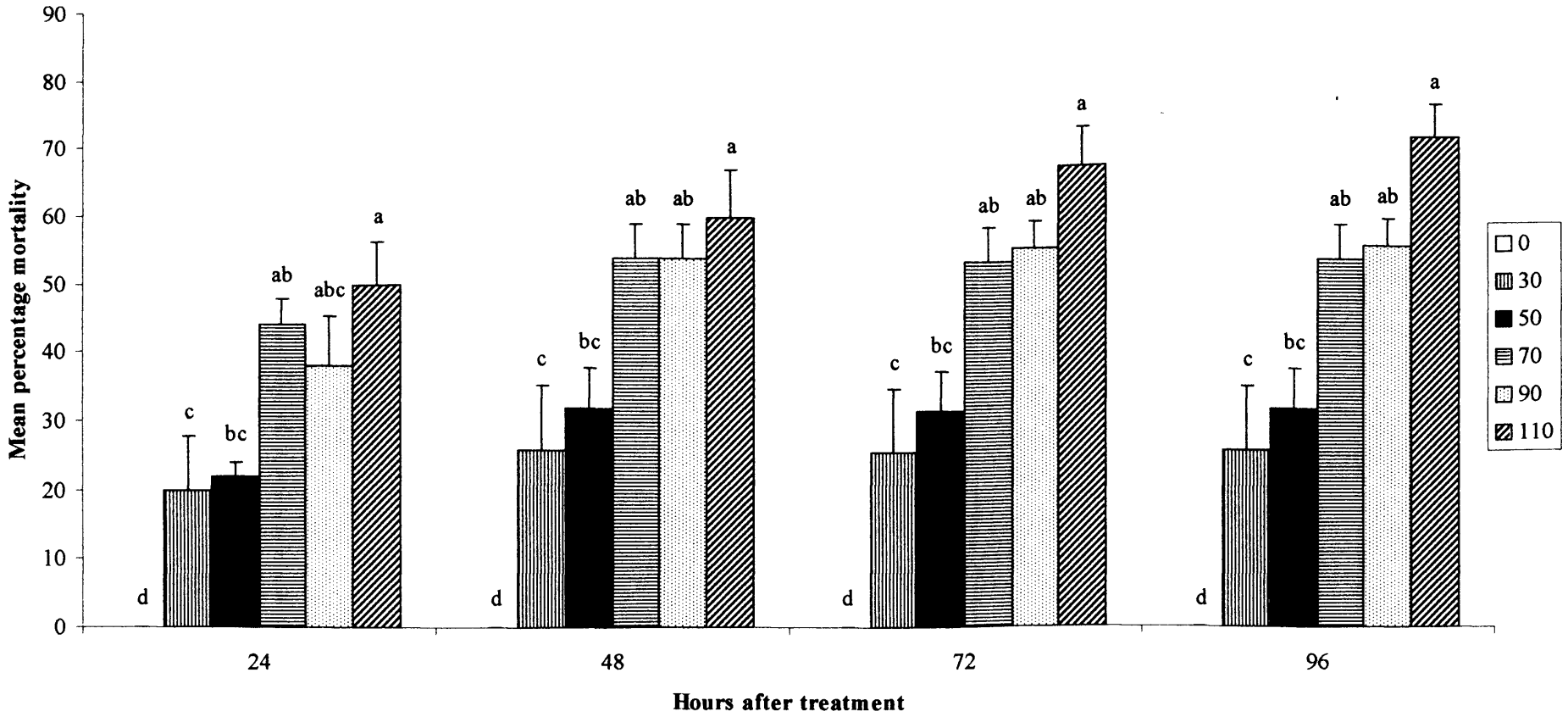

Figure 4.12 Percentage mortality of CC female adults treated with different doses (μg/insect) of the petroleum ether peel extract of CA using the topical application bioassay.

Columns represent mean of 5 replicates of 10 females each. Bars at each data point indicate the standard error. Columns of the same mortality recording time labelled by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means and standard errors of untransformed data are presented.

A summary of probit analyses of mortality data for males and females of CC treated with different doses (expressed as μg/insect and μg/mg) of the petroleum ether peel extract of CA using the topical application bioassay are presented in Table 4.7 and Table 4.8, respectively. Regression lines [probit mortality versus log (μg/insect) and probit mortality versus log (μg/mg)] obtained from the probit analyses are shown in Figure 4.13 and Figure 4.14, respectively. Except from the different estimates of LD_{50}s and their corresponding 95 % confidence limits when doses were expressed as μg/insect and μg/mg adult body weight, the only other difference between Table 4.7 and Table 4.8 was the intercept and its standard error. The *P*-value at 48 h after treatment for male adults of CC was 0.039 (significant) and so the data did not fit the probit model adequately. In this case a heterogeneity factor was automatically used in the calculations of confidence limits by SPSS. All other *P*-values of Table 4.7 and

Table 4.7 Summary of probit analyses of mortality data for males and females of CC treated with different doses (μg/insect) of the petroleum ether peel extract of CA using the topical application bioassay.

h	Sex	n	LD_{50} (μg/insect)	95 % CL	Slope ± SE	Intercept ± SE	χ^2	df	*P*
24	♂	250	57.9	50.7 - 72.3	3.52 ± 0.62	-1.20 ± 1.01	33.5	23	0.072
24	♀	250	117.6	88.2 - 258.0	1.54 ± 0.43	1.82 ± 0.79	20.4	23	0.617
48	♂	250	47.4	41.3 - 57.7	3.77 ± 0.58	-1.32 ± 0.93	36.3	23	0.039
48	♀	250	75.8	61.1 - 103.1	1.71 ± 0.42	1.78 ± 0.76	22.2	23	0.508
72	♂	250	40.0	36.0 - 44.6	3.77 ± 0.55	-1.03 ± 0.87	29.7	23	0.158
72	♀	250	69.7	57.7 - 86.7	2.01 ± 0.42	1.29 ± 0.77	20.7	23	0.598
96	♂	250	38.8	34.7 - 43.6	3.52 ± 0.53	-0.59 ± 0.85	27.4	23	0.240
96	♀	250	67.8	56.6 - 82.4	2.15 ± 0.42	1.07 ± 0.77	20.8	23	0.594

Column headings are abbreviated as follows: h is the hours after treatment; n is the numbers of insects tested excluding the controls; 95 % CL is the 95 % confidence limits; Slope ± SE is the slope ± standard error; Intercept ± SE is the intercept ± standard error; χ^2 is the Pearson's χ^2 goodness-of-fit test; df is the degrees of freedom; *P* is the probability.

Table 4.8 Summary of probit analyses of mortality data for males and females of CC treated with different doses (μg/mg) of the petroleum ether peel extract of CA using the topical application bioassay.

h	Sex	n	LD_{50} (μg/mg)	95 % CL	Slope ± SE	Intercept ± SE	x^2	df	*P*
24	♂	250	8.4	7.3 - 10.5	3.52 ± 0.62	1.75 ± 0.49	33.5	23	0.072
24	♀	250	14.7	11.0 - 32.3	1.54 ± 0.43	3.20 ± 0.41	20.4	23	0.617
48	♂	250	6.9	6.0 - 8.4	3.77 ± 0.58	1.84 ± 0.45	36.3	23	0.039
48	♀	250	9.5	7.6 - 12.9	1.71 ± 0.42	3.33 ± 0.39	22.2	23	0.508
72	♂	250	5.8	5.2 - 6.5	3.77 ± 0.55	2.13 ± 0.42	29.7	23	0.158
72	♀	250	8.7	7.2 - 10.8	2.01 ± 0.42	3.11 ± 0.39	20.7	23	0.598
96	♂	250	5.6	5.0 - 6.3	3.52 ± 0.53	2.36 ± 0.41	27.4	23	0.240
96	♀	250	8.5	7.1 - 10.3	2.15 ± 0.42	3.01 ± 0.39	20.8	23	0.594

Column headings are abbreviated as follows: h is the hours after treatment; n is the numbers of insects tested excluding the controls; 95 % CL is the 95 % confidence limits; Slope ± SE is the slope ± standard error; Intercept ± SE is the intercept ± standard error; χ^2 is the Pearson's χ^2 goodness-of-fit test; df is the degrees of freedom; *P* is the probability.

Table 4.8 were greater than 0.05 and so Pearson's χ^2 goodness-of-fit tests were not significant, indicating that the data fit the assumptions of the probit model adequately.

At all mortality recording times, the LD_{50}s estimated for female adults of CC were at least 1.6 times greater than that estimated for the males, when doses expressed as μg/insect. As at all mortality recording times the 95 % confidence limits did not overlap it can be concluded that males were significantly more susceptible than females of CC when doses were expressed as μg/insect. Moreover, the LD_{50}s estimated for female adults of CC were at least 1.4 times than those for the males, when doses expressed as μg/mg of adult body weight. At 24, 72 and 96 h after treatment, the 95 % confidence limits did not overlap, indicating that males were more susceptible than females of CC at these mortality recording times. In contrast, at 48 h

after treatment the 95 % confidence limits overlapped, indicating that males and females did not differ significantly in susceptibility at this time.

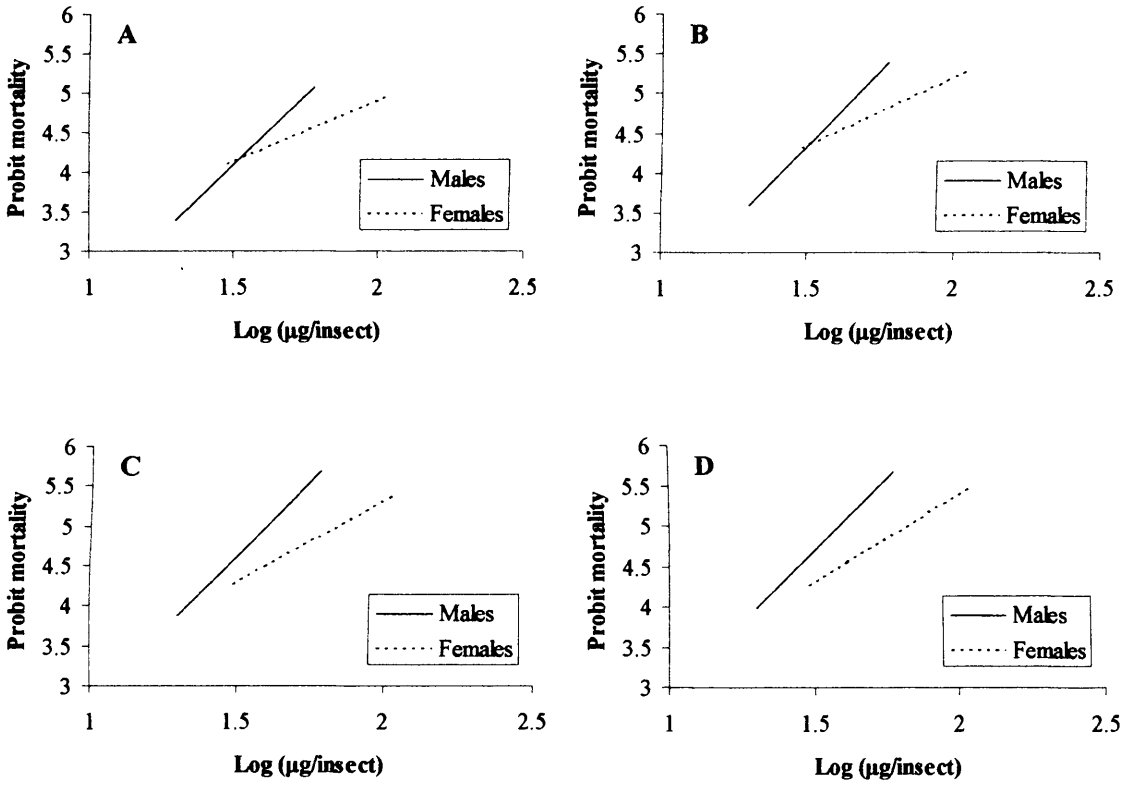

Figure 4.13 Log (μg/insect) - probit mortality lines calculated by probit analysis for males and females of CC treated with the petroleum ether peel extract of CA using the topical application bioassay at 24 (A), 48 (B), 72 (C) and 96 (D) h after treatment.

According to the results presented in Table 4.5, Table 4.6, Table 4.7 and Table 4.8, at the end-point mortality of BO and CC (144 and 96 h after treatment, respectively) males of both insect species were equally susceptible (either doses expressed as μg/insect or μg/mg adult body weight) to the petroleum ether peel extract when treated topically. In contrast, LD_{50}s estimated for females of CC were about 2-fold greater (doses expressed either as μg/insect or μg/mg adult body weight) than LD_{50}s estimated for females of BO. That results indicate that regardless of dose expression,

males of both species were equally susceptible (95 % confidence limits overlapped), whereas BO females were more susceptible than CC females (95 % confidence limits did not overlap) when treated topically with the petroleum ether peel extract of CA. Moreover, CC adults reached the end-point mortality two days earlier than BO adults, indicating that the extract acted more rapidly against CC adults.

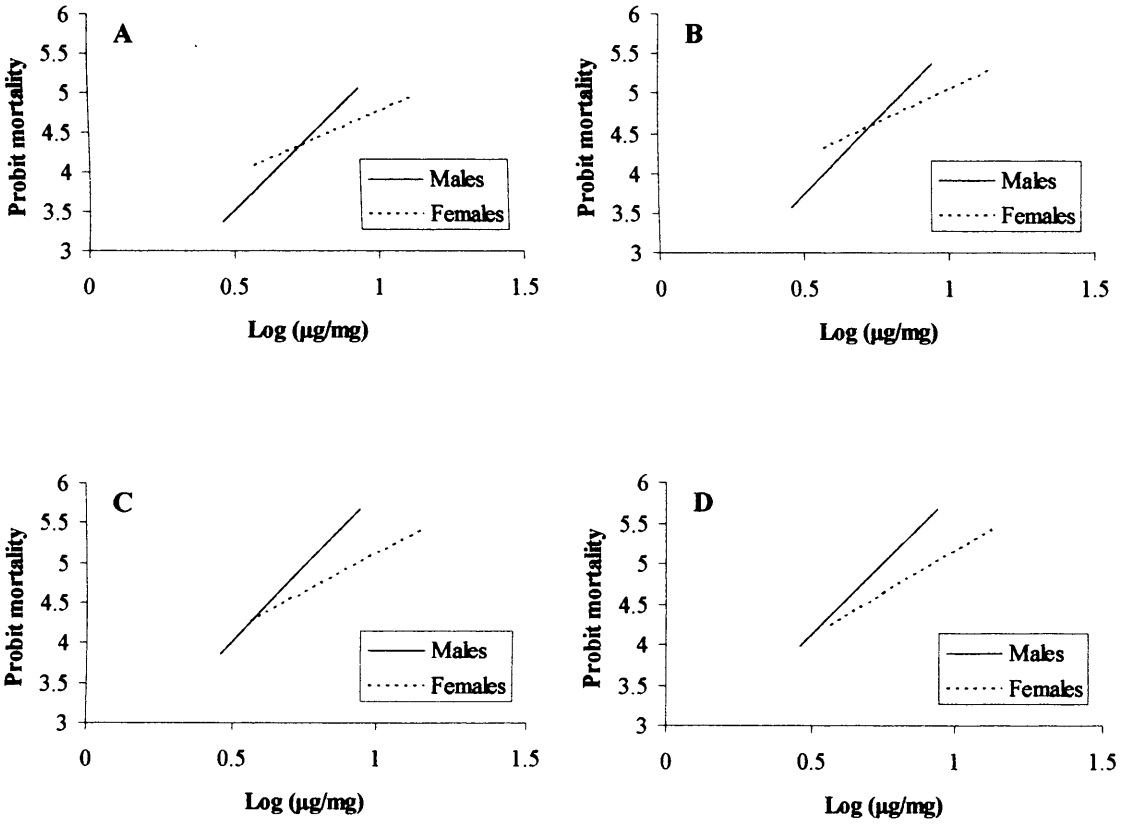

Figure 4.14 Log (μg/mg) - probit mortality lines calculated by probit analysis for males and females of CC treated with the petroleum ether peel extract of CA using the topical application bioassay at 24 (A), 48 (B), 72 (C) and 96 (D) h after treatment.

4.4 DISCUSSION

The results obtained by the two contact bioassays suggested that the petroleum ether peel extract of CA is active to both fly species tested showing a clear dose/concentration-dependent effect. However, in some cases, differences in susceptibility were detected not only between the two species, but also between the two sexes of the same species.

According to the results obtained by the Petri dish exposure bioassays males and females of BO are equally susceptible. On the contrary, females were more tolerant than males of CC. That difference in susceptibility between males and females of CC (about 2-fold) could not be fully explained by the slightly greater body weight of females. Moreover, personal observations during Petri dish exposure bioassays did not reveal any difference in behaviour between males and females of CC. The most plausible explanation for the higher tolerance of CC females is that females might possess higher quantities of enzymes or more efficient mechanisms that can detoxify the insecticidal active chemicals present in the petroleum ether peel extract. Weaver *et al.* (1994) reported that males of the Mexican bean weevils were more susceptible than females when exposed to filter papers treated with floral, foliar and root extracts of *Tagetes minuta*. However, these authors attributed that difference to the smaller size of males rather than to an innate difference in the mode of susceptibility.

Comparing the results obtained by the Petri dish exposure bioassays against BO and CC adults, it is apparent that males and females of BO are significantly more susceptible than males and females of CC, respectively. That difference in susceptibility between the two species could not be fully explained by the slightly

greater body weight of males and females of CC compared to males and females of BO, respectively. The most plausible explanation for the greater tolerance of males and females of CC compared to males and females of BO, respectively is that CC adults are less behaviourally active than BO adults and consequently they acquired smaller quantities of the insecticidal active chemicals when the flies are placed in the Petri dishes. That difference in the behavioural activity between BO and CC was confirmed by personal observations during the Petri dish bioassays. Moreover, the difference in the degree of susceptibility between the two sexes (males and females of CC was 3.9 and 8.3 more tolerant than males and females of BO, respectively) could be accounted to the higher quantities of enzymes or more efficient mechanisms of females compared to males of CC. Abbassy *et al.* (1979) reported that *Tribolium confusum* was significant more susceptible than *Sitophilus granaries* when lime, lemon, sweet orange and native orange peel oils were tested using the Petri dish exposure bioassay. However, when navel orange, grapefruit and mandarin peel oils were tested the two insects were equally susceptible.

Bioassays performed in order to investigate whether mortality of BO and CC caused in the Petri dish exposure bioassays was due to fumigation rather than contact, reveal the absence of fumigant action. Moreover, even when the Petri dishes were sealed with Parafilm, mortality of both species did not occur, indicating the relevant unimportance of fumigant compared to contact activity of the petroleum ether peel extract of CA. In contrast, Don-Pedro (1996) reported the relevant unimportance of the contact toxicity of citrus oils. That discrepancy can be explained as Don-Pedro used industrially extracted citrus peel oils, which contain mainly volatile compounds, while volatiles of our extract were removed.

The results obtained from the topical application bioassays reveal that when doses were expressed as μg/insect, males and females of BO are equally susceptible, which is in agreement with the results obtained by the Petri dish exposure bioassay in which no correction was done for the difference in body weight between males and females. However, when doses were expressed as μg/mg adult body weight (corrected for the difference in body weight between males and females), females are more susceptible than males. Generally, female insects have a greater tolerance to insecticides, when tests are corrected for any differences in body weight, which is attributable to their metabolism (Matthews, 1997). That was not the case when BO was treated topically with the petroleum ether peel extract. Since topical application bioassay is independent of insect activity, the most possible explanation for the greater tolerance of males could be that males possess higher quantities of enzymes or more efficient mechanisms that can detoxify the insecticidal active chemicals present in the petroleum ether peel extract.

Moreover, males of CC were more susceptible than females when treated topically with the petroleum ether peel extract, either dose expressed as μg/insect or μg/mg adult body weight. This finding is fully in accordance with the results obtained by the Petri dish exposure bioassay, indicating that the explanation for the greater tolerance of females compared to males of CC in both bioassays should be their ability to metabolise the insecticidal active chemicals. Albrecht and Sherman (1987) found that males of two *Bactrocera* species (*B. dorsalis* and *B. curcubitae*) were more susceptible than females, whereas male and female of CC were equally susceptible, when treated topically with avermectin B_1 (doses expressed as μg/g). It is worth mentioning that although male and female of CC were equally susceptible (their 95 %

confidence limits did not overlap), the LD_{50} estimated for males was 2-fold greater than that estimated for females. It is obvious that generalisations about sex susceptibility of insect species can not be made since different chemicals substances produce different results although the same biological evaluation technique was used.

Comparing the results obtained by the topical application bioassays against BO and CC adults it is apparent that males of both species are equally susceptible, whereas females of BO are more susceptible than females of CC either dose expressed as μg/insect or μg/mg adult body weight. The difference in the degree of susceptibility between the two sexes (2-fold when doses expressed as μg/insect and 1.7-fold when doses expressed as μg/mg adult body weight) when the two species were compared is fully in accordance with the results of the Petri dish exposure bioassay. The higher quantities of enzymes or more efficient mechanisms of females compared to males of CC could explain not only the difference between BO and CC females, but also the difference in the degree of susceptibility between the two sexes. Su *et al.* (1972b) reported that although peel oils of lemon, grapefruit, lime, kumquat, tangerine, orange, tangelo and temple orange were moderately toxic to rice weevil, none of these oils showed any appreciable toxicity to black carpet beetle larvae, Indian meal moth larvae, cigarette beetle, red flour beetle and confused flour beetle using the topical application bioassay (doses expressed as μg/insect). Moreover, Albrecht and Sherman (1987) reported great differences when the susceptibility of CC, *B. dorsalis* and *B. curcubitae* of the same sex was compared; males of CC were 13.8 and 6.9 more tolerant than *B. dorsalis* and *B. curcubitae* males, respectively while females of CC were 48.3 and 23.2 more tolerant than *B. dorsalis* and *B. curcubitae* females, respectively. Apart from the detoxification mechanisms, Stark and Sherman (1989)

suggested that significant differences in the ranking of toxicity between species can be attributed to different penetration rates and excretion (Stark and Sherman, 1989).

Petri dish exposure and topical application bioassays produced different results when the susceptibility of the same sex between the two fly species was compared. Although in the topical application bioassay males of both species were equally susceptible, in the Petri dish exposure bioassay BO males were 3.9 times more susceptible than CC males. Moreover, females of BO were more susceptible than females of CC in both bioassays, but the degree of susceptibility differed significantly (8.3 and 2.0 times for the Petri dish exposure and topical application bioassay, respectively). The greater activity of BO compared to the CC resulted in greater susceptibility of BO compared to the CC in the Petri dish exposure bioassay, since this bioassay is not independent of insect activity.

Slope values for the regression lines of both insect species were significantly greater in the Petri dish exposure than in topical application bioassay. Since higher slopes indicate a more sensitive technique (Dahm *et al.*, 1961) it can be concluded that the Petri dish exposure is more sensitive than the topical application bioassay when the petroleum ether peel extract was tested against both BO and CC adults.

Petri dish exposure bioassay was not only more sensitive than topical application in this study, but also in that bioassay the end-point mortality was reached earlier. In the Petri dish exposure bioassay, BO and CC adults reached the end-point mortality two and one days respectively earlier than in the topical application.

In both bioassays CC adults reached the end-point mortality earlier than BO adults. In the Petri dish exposure bioassay CC adults reached the end-point mortality one day earlier than BO adults, whereas in the topical application bioassay CC adults reached the end-point mortality two days earlier than BO adults.

CHAPTER 5

ISOLATION OF INSECTICIDALLY ACTIVE COMPONENTS FROM THE PETROLEUM ETHER PEEL EXTRACT OF *CITRUS AURANTIUM*

5.1 INTRODUCTION

Fractionation is the process in which the components of a mixture, such as a plant extract, can be separated into groups of compounds sharing similar physicochemical characteristics (Houghton and Raman, 1998). Thus, solubility, volatility, polarity, size, shape, electrical charge and functional groups present may influence the grouping (Harwood *et al.*, 1999). The methods used for achieving fractionation are: precipitation, liquid-liquid partitioning, distillation, dialysis, chromatographic techniques and electrophoresis. However, it should be noted that chromatography in its various forms is the most common method and is exploited in most fractionation procedures carried out nowadays (Houghton and Raman, 1998).

Chromatography depends upon the differential distribution of various components of a mixture between two phases, the mobile phase and the stationary phase. The mobile phase may be either a liquid or a gas and the stationary phase either a solid or liquid. Various combinations of these components give the main types of chromatographic techniques (Harwood *et al.*, 1999). The main chromatographic techniques are: column chromatography (gravity and flash), planar chromatography (thin layer and paper),

gas chromatography, high performance liquid chromatography (normal and reverse phase), ion exchange chromatography and gel permeation chromatography.

Different fractionation strategies can be followed during a fractionation process. Collecting large number of very small fractions means that its fraction is more likely to contain a pure compound, but it requires more work for testing every fraction. This also runs the risk of spreading the target compound over so many fractions that, if originally present in only low concentrations, it may evade detection in any one of the fractions. Collecting a few large, but relatively crude fractions resulted in more fractionation steps, but the work in each step is considerably less (Cannell, 1998).

A common phenomenon during fractionation processes was discussed by Cannell (1998) and Houghton and Raman (1998). They concluded that lack or disappearance of activity occurred after fractionation may be due to the following reasons:

- The active components may remain in the system used for fractionation.
- The active components may have decomposed during the fractionation process.
- Observed activity in the crude extract is due to synergism between components which are separated as a result of the fractionation process.
- The active components may have been lost due to a process such as co-distillation or sublimation when liquid fractions are concentrated.
- The starting sample was not prepared so as to be fully compatible with the mobile phase, so that a large proportion of the active component precipitated when loading on the top of the column.

- Most of the active component(s) spread across a wide range of fractions in a concentration too low to be detected by the assay.

Only a few efforts have been made to isolate the insecticidally active components of promising *Citrus* extracts. Su (1976) reported the isolation of a single active component from lyophilised hexane lemon peel oil. Fractionation was carried out by three sequential TLC (silica gel F_{254}) separations using three different developing solvent systems. Fractionation was guided by topical application and the test insect was adult cowpea weevil. However, although Su had achieved the isolation of a pure active compound, she did not make any attempt to elucidate its structure. More recently, Su and Horvat (1987) isolated and identified the four major components of lyophilised hexane lemon peel oil. Fractionation was carried out by TLC (silica gel F_{254}) followed by HPLC (C_{18} column) and was not guided by bioassays.

The majority of studies aiming to isolate the active ingredients from insecticidally active plant extracts involve liquid-liquid partitioning, gravity column or flash column chromatography, preparative thin layer chromatography and high performance liquid chromatography. Dadang *et al.* (1996), using bioassay-guided procedures, isolated from a methanol tuber extract of nutgrass an insecticidal sesquiterpenoid, which was identified as α-cyperone (4, 11-selinadien-3-one). Fractionation was carried out by using in sequence liquid-liquid partitioning (MeOH/n-hexane), gravity column chromatography (silica gel), preparative TLC (silica gel F_{254}) and finally HPLC. Rodriguez-Saona *et al.* (1997, 1998) using flash chromatography on silica gel separated an avocado idioblast cell oil into eight fractions. Two fractions were revealed as promising against *Spodoptera exigua*. The most active fraction was

further purified by HPLC (silica column) resulting in the isolation and identification of persin (Rodriguez-Saona *et al.*, 1997). The other active fraction was further fractionated by two sequential flash columns resulting in the isolation of several insecticidal compounds (Rodriguez-Saona *et al.*, 1998). Gbewonyo and Candy (1992a) reported that gas chromatography allowed the purification of pellitorine from a piper extract in a single step. However, they found that the activity of the extract was decreased on passage through the GC column, which was attributed to the instability of some insecticidal or synergistic compounds at the high temperatures used. They concluded that GC is unsuitable for the separation and isolation of these compounds. The same authors (1992b) reported the purification and identification of five active amides of the same piper extract using different bioassay-guided fractionation procedures. This time, fractionation was carried out by three sequential TLC separations using three different developing solvent systems followed by reversed-phase HPLC.

In this chapter the chromatographic procedure followed to isolate the bioactive chemicals present in the petroleum ether peel extracts of CA and the obtained results will be discussed.

5.2 METHODS

5.2.1 Fractionation procedure of the petroleum ether peel extract of CA

The active petroleum ether peel extract of CA obtained in section 4.2.1 was fractionated using chromatographic techniques in order to isolate the insecticidally active secondary metabolites it possessed. The fractionation procedure of the petroleum ether peel extract of CA is shown in Figure 5.1 and was guided by Petri dish exposure bioassays.

First gravity (open) column fractionation

Gravity column chromatography is the oldest form of chromatographic technique (Houghton and Raman, 1998). Although it does not require special equipment and is the most economic of the chromatographic techniques, as it requires only the most basic grades of chromatographic adsorbents, it can give sufficient separation at the initial fractionation steps (Harwood *et al.*, 1999).

A glass column (500 mm x 45 mm i.d.) was used for the fractionation of 2 g of the petroleum ether peel extract. This column was fitted at one end with a fine porosity sintered glass disk above the stopcock in order to support the stationary phase. The column was wet packed with 200 g of silica gel 60 for column chromatography (0.04-0.063 mm/230-400 mesh ASTM, Macherey-Nagel, Germany). The 1 % sample/stationary phase ratio used, offers sufficient separation while loss by irreversible adsorption to the stationary phase was minimised. Prior to packing, silica gel was suspended in DCM, which was the least polar solvent used for the fractionation. The height of silica gel into the column was 32 cm. Wet packing was used in preference to dry packing in order: (a) to achieve a narrow band of applied

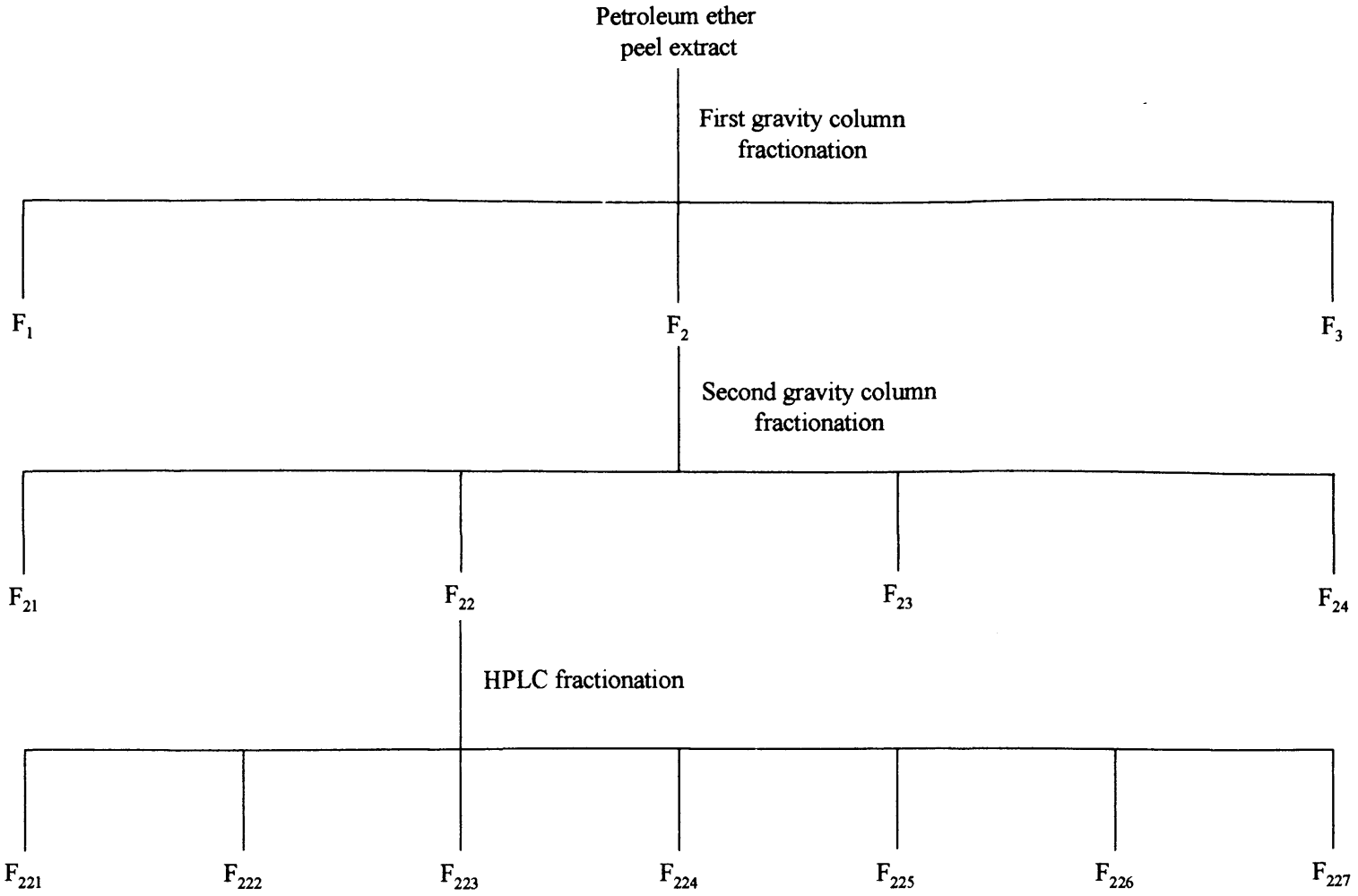

Figure 5.1 Fractionation procedure of the petroleum ether peel extract of CA.

sample, (b) to achieve uniform flow of the mobile phase, and (c) to avoid air bubbles, often trapped as the mobile phase passes through, which disrupt the flow (Houghton and Raman, 1998).

As the force of the eluting solvents tends to disturb the flat surface of the stationary phase, about 1 cm of anhydrous sodium sulphate was placed above the silica gel surface in order to protect it. A petroleum ether peel extract solution (2 g/20 ml DCM) was introduced on to the column. The stopcock was opened until the solution reached the surface of the anhydrous sodium sulphate without touching it and then the stopcock was closed. Above the sample, 1 cm of anhydrous sodium sulphate was added in order to avoid sample dilution. Silica gel and anhydrous sodium sulphate were washed with MeOH and then activated at 130 °C for 24 h prior to use. Three fractions were collected by sequentially eluting the column with 1 l each (approximately 3 dead volumes) of DCM (F_1 fraction), EtOAc (F_2 fraction) and MeOH (F_3 fraction).

Second gravity column fractionation

The same column as above was used for the fractionation of 1 g of the active F_2 fraction, obtained from the first gravity column fractionation. The column was wet packed with 100 g silica gel (suspended in 20 % Hex in EtOAc). The height of silica into the column was 16 cm. 1 g of the F_2 fraction dissolved in 50 ml of 20 % Hex in EtOAc was introduced on to the column. Four fractions were collected by sequentially eluting the column with 500 ml each (approximately 3 dead volumes) of 20 % Hex in EtOAc (F_{21} fraction), 40 % Hex in EtOAc (F_{22} fraction), 60 % Hex in EtOAc (F_{23} fraction) and 100 % MeOH (F_{24} fraction).

HPLC fractionation

High Performance Liquid Chromatography (HPLC) was used to fractionate further the active F_{22} fraction, obtained from the second gravity column fractionation. HPLC was used for the final stage of fractionation as it is a much more efficient technique than gravity column chromatography and can lead to the isolation of pure substances. There are many advantages of HPLC over conventional chromatography, and these include increased speed, higher resolution, higher sensitivity, greater reproducibility, and better sample recovery (Patel, 1997).

A binary LC pump 250 equipped with a LC-235 diode array detector (Perkin Elmer, USA) was used. The Simple peak 202 (SRI Instruments, USA) software was employed for recording the chromatograms. After testing, different columns, stationary phases, mobile phases, elution profiles, flow rates, injection volumes and UV detection wavelengths, the following chromatographic conditions were chosen for performing the fractionation. A 250 mm x 10 mm i.d. LiChrospher 100 CN column with a particle size of 10 μm (Merck, Germany) supplied with a 10 mm precolumn packed with the same stationary phase was used. Two solvents, n-hexane (A) and ethyl acetate (B), which were degassed by sonication before use, were selected. The elution profile was: 0-35 min A:B 90:10 % (isocratic), 35-40 min from A:B 90:10 % to 100 % B (linear gradient), 40-60 min 100 % B (isocratic). Prior to injection of sample the column was re-equilibrated for 10 min at the initial conditions. The flow rate used was 4 ml/min, the column temperature was ambient and the absorbance was monitored at 310 nm (optical bandwidth 15 nm). The F_{22} fraction (1mg/20 μl EtOAc per injection) was introduced onto the column via a Rheodyne 7125 injector (USA) fitted with a 20 μl sample loop. Seven fractions were collected (F_{221}, F_{222}, F_{223}, F_{224},

F_{225}, F_{226} and F_{227}) (see Figure 5.4). Totally, 100 mg (100 runs) of the F_{22} fraction were fractionated. All fractions were filtered, concentrated, weighed and stored in the dark at -20 °C until used.

5.2.2 Bioassay of fractions

To monitor the activity of the fractions obtained during the fractionation procedure of the petroleum ether peel extract of CA the Petri dish exposure bioassay was used (see section 2.2.2). Five replicates of 10 (5 males and 5 females) 2-3 d-old BO flies each, were employed for each treatment and the control. The 5 replicates were conducted on 5 successive days, using the same generation of flies. Different concentrations of treatments were not run simultaneously, as screening experiments at lower concentrations included only the more active treatments. Stock solutions were prepared in DCM, as were subsequent dilutions. Fresh test solutions were prepared for each replicate. Because the amount of the fractions obtained from the HPLC fractionation was limited, during tests on these fractions, olive oil was added to each treatment solution and control (DCM) so that each cm^2 of the total inner surface of the Petri dishes was layered with 0.1 μl of olive oil. Olive oil was used in order to reduce the amount of the HPLC fractions tested, as it served as a carrier to facilitate pick up of residues of the test materials from the Petri dish (Gbewonyo and Candy, 1992a). The syringe used for the application of samples was thoroughly cleaned after the application of each sample. Mortalities were recorded every 24 h for 3 d, when olive oil was added to the treatments, whereas when olive oil was not added, mortalities were recorded every 24 h for 4 d, after which there was no further increase in mortality.

5.2.3 TLC analysis of fractions

TLC analyses were carried out as in section 3.2.3 in order to check the component profile of the fractions obtained from the first gravity column fractionation against that of the petroleum ether peel extract and moreover to check the component profile of the fractions obtained from the second gravity column fractionation against that of the F_2 fraction. In each case fractions were checked using the same amount per spot (100 μg/spot for the first gravity column fractionation and 62.7 μg/spot for the second gravity column fractionation) or equivalent amount to the initial extract/fraction compared. The petroleum ether peel extract and all fractions were dissolved in DCM. All analyses were performed using Hex:EtOAc (50:50, v/v) as the developing solvent system. Photographs of the TLC plates were obtained by the Camedia digital camera under 254 nm UV light

5.2.4 HPLC analysis of fractions

HPLC analyses were carried out in order to investigate whether the three major peaks of the F_{22} fraction were separated adequately during the HPLC fractionation. All chromatographic conditions were the same as in section 5.2.1 except the elution profile which was Hex:EtOAc (90:10, v/v) for all the time required for the analyses of fractions F_{221}, F_{222}, F_{223}, F_{224}, F_{225} and F_{226}. However, when analysing the F_{227} fraction the same elution profile as in section 5.2.1 was set. Each fraction was analysed in triplicate. Fraction solutions analysed were equivalent to 200 μg/injection of the F_{22} fraction. All fractions were dissolved in EtOAc. Percentages of the three major peaks present in each fraction obtained from the HPLC fractionation were calculated.

5.3 RESULTS

5.3.1 First gravity column fractionation

Three fractions (F_1, F_2 and F_3) were obtained from the first gravity column fractionation of the petroleum ether peel extract of CA (see section 5.2.1) in order to detect whether the insecticidally active chemicals present in the petroleum ether peel extract were recovered during the first fractionation step in a single fraction.

Recovery

The weights of fractions and their % recovery after the first gravity column fractionation of the petroleum ether peel extract of CA, are shown in Table 5.1. The amount recovered in the F_2 fraction was 2.3 and 9.1 times greater than that of the F_1 and F_3 fractions, respectively. The low amount recovered in the F_3 fraction can be explained, by the use of a non-polar solvent such as petroleum ether for the extraction of peels. Chemicals extracted from peels of CA using petroleum ether were mainly non-polar, unionised substances, which were already eluted from the column (fractions F_1 and F_2) prior elution with MeOH. It must be noticed that 3.3 % (66 mg) of the petroleum ether peel extract were not eluted from the column. These chemicals were mainly the more polar chemicals present in the petroleum ether peel extract and

Table 5.1 Weights of fractions and their % recovery after the first gravity column fractionation of 2 g of the petroleum ether peel extract of CA.

Fraction	Elution solvent	Amount recovered (mg)	Recovery (%)
F_1	DCM	542	27.1
F_2	EtOAc	1254	62.7
F_3	MeOH	138	6.9
Sum of fractions		1934	96.7

were adsorbed irreversibly to the stationary phase.

TLC analysis

The component profile of F_1, F_2, F_3 fractions was checked against that of the petroleum ether peel extract by TLC using Hex:EtOAc (50:50, v/v) as the developing solvent system. In Figure 5.2A, 100 μg and the petroleum ether peel extract and 27.1, 62.7, 6.9 μg (equivalent to 100 μg of the petroleum ether peel extract) of the F_1, F_2, F_3 fractions, respectively were spotted on the baseline of the TLC plate. In Figure 5.2B, 100 μg of the F_1, F_2, F_3 fractions and the petroleum ether peel extract were spotted. Figures 5.2A and 5.2B indicate that the first gravity column effectively fractionated the petroleum ether peel extract as different types of compounds based on their polarity were present in each different fraction. Moreover, Figure 5.2A indicates that decomposition during fractionation had not occurred, as compounds present in the petroleum ether peel extract had not disappear, and moreover, compounds not present in the petroleum ether peel extract did not appear, after fractionation.

Insecticidal activity

The petroleum ether peel extract and its fractions obtained from the first gravity column fractionation were tested for activity using the Petri dish exposure bioassay in order to detect which fraction contain the insecticidal active chemicals. The initial screening concentration was set at 100 $\mu g/cm^2$. Only fractions that exhibited activity at this concentration were further tested at 50 $\mu g/cm^2$.

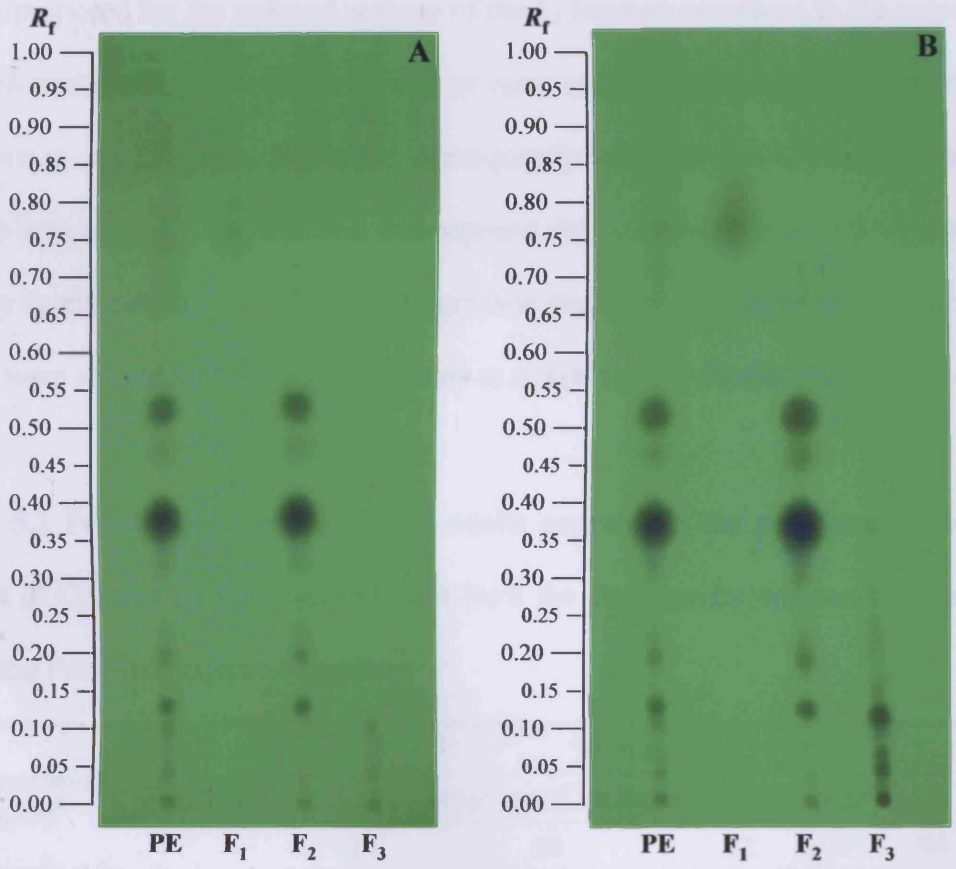

Figure 5.2 Thin layer chromatograms of the fractions obtained from the first gravity column fractionation of the petroleum ether peel extract.

PE: Petroleum ether peel extract; $\mathbf{F_1}$, $\mathbf{F_2}$ and $\mathbf{F_3}$: DCM, EtOAc and MeOH fractions, respectively; R_f: Retention factor; **(A)** PE: 100 μg/spot; F_1, F_2 and F_3: 27.1, 62.7 and 6.9 μg/spot, respectively (equivalent to 100 μg/spot of PE); **(B)** PE, F_1, F_2 and F_3: 100 μg/spot. Developing solvent system: Hex:EtOAc (50:50, v/v). UV light: 254 nm.

The results of the two experiments are presented in Table 5.2. The only active fraction at 100 $\mu g/cm^2$ was the F_2 fraction, whereas the F_1 and F_3 fractions were inactive. Moreover, the activity of the petroleum ether peel extract was significantly greater than that of the F_2 fraction at both concentrations tested at all mortality recording times.

Results indicate that the activity of the petroleum ether peel extract cannot be fully explained by the activity of the F_2 fraction. There are three possible explanations that

can be proposed for the reduced activity of the F_2 fraction compared to the activity of the petroleum ether peel extract: (a) one or more active components were irreversible adsorbed in the stationary phase and consequently were not eluted from the column, (b) one or more active components decomposed during fractionation, and (c) observed activity in the petroleum ether peel extract was due to synergism among components which were separated in different fractions as a result of the fractionation process.

Table 5.2 Percentage mortality of BO adults exposed to the petroleum ether peel extract of CA and its fractions obtained from the first gravity column fractionation using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Fraction	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
100	PE[c]	46 ± 12.1a	98 ± 2.0a	100 ± 0.0a	100 ± 0.0a
	F_1	0 ± 0.0b	0 ± 0.0c	0 ± 0.0c	0 ± 0.0c
	F_2	8 ± 5.8b	46 ± 6.8b	60 ± 8.9b	64 ± 8.7b
	F_3	2 ± 2.0b	2 ± 2.0c	2 ± 2.0c	2 ± 2.0c
	Control[d]	2 ± 2.0b	2 ± 2.0c	2 ± 2.0c	4 ± 2.4c
50	PE	30 ± 5.5a	74 ± 6.0a	96 ± 2.4a	98 ± 2.0a
	F_2	2 ± 2.0b	10 ± 3.2b	12 ± 3.7b	14 ± 5.1b
	Control	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]PE: Petroleum ether peel extract. [d]Treated with DCM. The two experiments (100 and 50 $\mu g/cm^2$) were not run simultaneously. Means within a column of a single concentration followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

In order to clarify the reason for the reduced activity of the F_2 fraction, an experiment was designed in which the activity of the petroleum ether peel extract was compared to the activity of the sum of the three fractions. The contribution of each fraction to the concentrations of the sum of fractions tested was equivalent to the screening

concentration of the petroleum ether peel extract. In detail, at the screening concentration of 50, 25 and 15 $\mu g/cm^2$ the concentration of the F_1 fraction was 13.6, 6.8 and 4.1 $\mu g/cm^2$, the concentration of the F_2 fraction was 31.4, 15.7 and 9.4 $\mu g/cm^2$ while the concentration of the F_3 fraction was 3.4, 1.7 and 1.0 $\mu g/cm^2$, respectively.

The results of the three experiments are shown in Table 5.3. There were no significant differences in activity between the petroleum ether peel extract of CA and the sum of fractions ($F_1 + F_2 + F_3$) at the three concentrations tested at all mortality recording times.

The results indicate that active chemicals were not decomposed or remain irreversibly adsorbed to the stationary phase during the fractionation process. Moreover, it was evident that synergism between chemicals of different fractions took place, which explains why the activity of the F_2 fraction was significantly lower than that of the petroleum ether peel extract.

A further experiment was designed in order to detect whether the activity of the petroleum ether peel extract could be fully explained by the activity of a binary blend of the three fractions. The petroleum ether peel extract and the three binary blends of fractions ($F_1 + F_2$, $F_1 + F_3$ and $F_2 + F_3$) were tested. The contribution of each fraction to the concentration of each binary blend was equivalent to the screening concentration of the petroleum ether peel extract (for details see previous experiment). Only binary blends that were active at the initial experiment were further tested.

Table 5.3 Percentage mortality of BO adults exposed to the petroleum ether peel extract of CA and the sum of its fractions obtained from the first gravity column fractionation using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Treatment	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
		1st Experiment			
50	PE[c]	34 ± 9.3a	76 ± 7.5a	98 ± 2.0a	98 ± 2.0a
13.6 + 31.4 + 3.4	$F_1+F_2+F_3$	32 ± 9.2a	82 ± 3.7a	100 ± 0.0a	100 ± 0.0a
0	Control[d]	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b	4 ± 2.4b
		2nd Experiment			
25	PE	6 ± 4.0a	58 ± 7.3a	74 ± 7.5a	78 ± 5.8a
6.8 + 15.7 + 1.7	$F_1+F_2+F_3$	10 ± 4.5a	50 ± 7.1a	68 ± 5.8a	70 ± 5.5a
0	Control	0 ± 0.0a	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
		3rd Experiment			
15	PE	2 ± 2.0a	16 ± 4.0a	28 ± 6.6a	32 ± 8.0a
4.1 + 9.4 + 1.0	$F_1+F_2+F_3$	4 ± 2.4a	18 ± 3.7a	36 ± 5.1a	38 ± 5.8a
0	Control	2 ± 2.0a	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]PE: Petroleum ether peel extract of CA. [d]Treated with DCM. The three experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

The results of the two experiments are displayed in Table 5.4. The $F_1 + F_3$ and $F_2 + F_3$ binary blends were inactive at 25 $\mu g/cm^2$, whereas the petroleum ether peel extract and the $F_1 + F_2$ binary blend were active and did not differ significantly at both experiments at all mortality recording times.

The results of these experiments indicate that synergism occurred between chemicals that were eluted during fractionation to the F_1 and F_2 fractions. The activity of the petroleum ether peel extract of CA can be fully ascribed to the activity of the $F_1 + F_2$

binary blend. Moreover, the chemicals present in the F_3 fraction did not have any effect to the insecticidal activity of the petroleum ether peel extract.

Table 5.4 Percentage mortality of BO adults exposed to the petroleum ether peel extract of CA and different binary blends of its fractions obtained from the first gravity column fractionation using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Treatment	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
		1st Experiment			
25	PE[c]	10 ± 5.5ab	48 ± 7.3a	62 ± 9.7a	64 ± 8.7a
6.8 + 15.7	F_1+F_2	10 ± 3.2a	38 ± 9.2a	56 ± 8.1a	60 ± 7.1a
6.8 + 1.7	F_1+F_3	2 ± 2.0ab	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
15.7 + 1.7	F_2+F_3	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
0	Control[d]	0 ± 0.0b	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
		2nd Experiment			
15	PE	6 ± 4.0a	14 ± 5.1ab	26 ± 5.1a	30 ± 3.2a
4.1 + 9.4	F_1+F_2	2 ± 2.0a	20 ± 4.5a	34 ± 5.1a	36 ± 6.0a
0	Control	2 ± 2.0a	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]PE: Petroleum ether peel extract of CA. [d]Treated with DCM. The two experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

5.3.2 Second gravity column fractionation

Bioassays conducted with the fractions obtained from the first gravity column indicate that the only fraction which exhibit activity when tested individually was the F_2 fraction. Hence, fractionation was continued with this fraction. Four fractions (F_{21}, F_{22}, F_{23} and F_{24}) were obtained from the second gravity column fractionation of the F_2

fraction (see section 5.2.1) in order to detect the fraction that contain the insecticidal active chemicals present in the F_2 fraction.

Recovery

The weights of fractions and their % recovery after the second gravity column fractionation of the F_2 fraction are shown in Table 5.5. The highest amount of chemicals of the F_2 fraction was recovered in the F_{23} fraction (633 mg). The amount of chemicals recovered in fraction F_{22} was 2.6 times less than that recovered in the F_{23} fraction, but 3.0 and 5.9 times more than that recovered in the F_{24} and F_{21} fractions, respectively. 99.7 % of the total amount of the F_2 fraction placed into the column was recovered in the four fractions (F_{21}, F_{22}, F_{23} and F_{24}) collected. The 0.3 % (3 mg) of the F_2 fraction that seems not to be present in the fractions could be attributed to uncertainties associated with weighing rather than irreversible adsorption to the stationary phase.

Table 5.5 Weights of fractions and their % recovery after the second gravity column fractionation of 1 g of the F_2 fraction.

Fraction	Elution solvents	Amount recovered (mg)	Recovery (%)
F_{21}	Hex:EtOAc (80:20, v/v)	41	4.1
F_{22}	Hex:EtOAc (60:40, v/v)	243	24.3
F_{23}	Hex:EtOAc (40:60, v/v)	633	63.3
F_{24}	MeOH	80	8.0
Sum of fractions		997	99.7

TLC analysis

The component profile of F_{21}, F_{22}, F_{23} and F_{24} fractions was checked against that of the F_2 fraction by TLC using Hex:EtOAc (50:50, v/v) as the developing solvent system. In Figure 5.3A, 62.7 μg of the F_2 and 2.6, 15.2, 39.7, 5.0 μg (equivalent to 62.7 μg of the F_2 fraction) of the F_{21}, F_{22}, F_{23} and F_{24} fractions, respectively were spotted on the baseline of the TLC plate. In Figure 5.3B, 62.7 μg of the F_2, F_{21}, F_{22}, F_{23}, F_{24} fractions were spotted. Figures 5.3A and 5.3B indicate that the second gravity

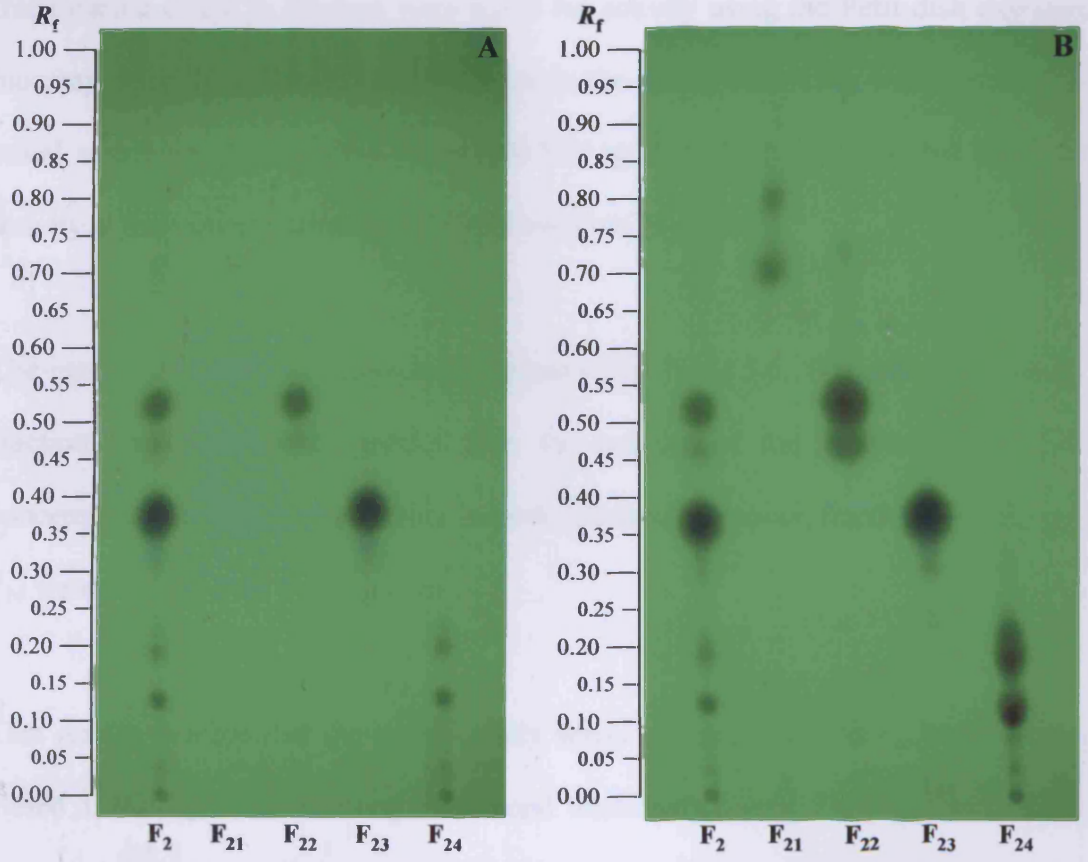

Figure 5.3 Thin layer chromatograms of the fractions obtained from the second gravity column fractionation of the F_2 fraction.

F_2: EtOAc fraction; **F_{21}**, **F_{22}**, **F_{23}** and **F_{24}**: 20 % EtOAc, 40 % EtOAc, 60 % EtOAc and MeOH fractions, respectively. ***R_f***: Retention factor. **(A)** F_2: 62.7 μg/spot; F_{21}, F_{22} F_{23} and F_{24}: 2.6, 15.2, 39.7 and 5.0 μg/spot, respectively (equivalent to 62.7 μg/spot of F_2). **(B)** F_2, F_{21}, F_{22}, F_{23} and F_{24}: 62.7 μg/spot. Developing solvent system: Hex:EtOAc (50:50, v/v). UV light: 254 nm.

column provided sufficient fractionation, since different types of compounds based on their polarity were present in each individual fraction. Moreover, Figure 5.3A indicates that decomposition during fractionation had not occurred, as compounds present in the F_2 fraction did not disappear, and moreover, compounds not present in the F_2 fraction did not appear, after fractionation.

Insecticidal activity

The F_2 fraction and its fractions obtained from the second gravity column fractionation of the F_2 fraction were tested for activity using the Petri dish exposure bioassay in order to detect in which fraction the active chemicals were eluted. The initial screening concentration was set at 100 $\mu g/cm^2$. Only fractions that exhibited activity at this concentration were further tested at 50 $\mu g/cm^2$.

The results of the two experiments are presented in Table 5.6. The activity of the F_{22} fraction was significantly greater than the activity of the F_2 fraction at both concentrations tested at all mortality recording times. Moreover, fractions F_{21}, F_{23} and F_{24} were inactive even at 100 $\mu g/cm^2$.

That results indicate that the insecticidally active chemicals of the F_2 fraction were eluted to the F_{22} fraction during the second fractionation step. However, in order to investigate whether the activity of the F_2 fraction could be fully explained by the activity of the F_{22} fraction, a further experiment was designed. In this experiment the insecticidal activity of the F_2 fraction at 100 and 75 $\mu g/cm^2$ was compared to the activity of the equivalent concentration of the F_{22} fraction (24.30 and 12.15 $\mu g/cm^2$), respectively.

Table 5.6 Percentage mortality of BO adults exposed to the F_2 fraction and its fractions obtained from the second gravity column fractionation using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Fraction	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
100	F_2	12 ± 3.7b	30 ± 4.5b	56 ± 5.1b	58 ± 6.6b
	F_{21}	2 ± 2.0b	2 ± 2.0c	4 ± 2.4c	4 ± 2.4c
	F_{22}	74 ± 8.1a	94 ± 2.4a	98 ± 2.0a	100 ± 0.0a
	F_{23}	0 ± 0.0b	0 ± 0.0c	0 ± 0.0c	0 ± 0.0c
	F_{24}	2 ± 2.0b	2 ± 2.0c	2 ± 2.0c	2 ± 2.0c
	Control[c]	0 ± 0.0b	2 ± 2.0c	2 ± 2.0c	2 ± 2.0c
50	F_2	2 ± 2.0b	4 ± 2.4b	6 ± 4.0b	8 ± 3.7b
	F_{22}	32 ± 6.6a	68 ± 5.8a	96 ± 2.4a	100 ± 0.0a
	Control	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with DCM. The two experiments (100 and 50 $\mu g/cm^2$) were not run simultaneously. Means within a column of a single concentration followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

The results of the two experiments are presented in Table 5.7. The activity of the F_{22} fraction did not differ significantly to the activity of the F_2 fraction at both experiments at all mortality recording times, indicating that only the active chemicals present in the F_{22} fraction were responsible for the observed activity of the F_2 fraction. Chemicals present in the F_{21}, F_{23} and F_{24} fractions made no contribution to the insecticidal activity of the F_2 fraction.

Table 5.7 Percentage mortality of BO adults exposed to the F_2 and F_{22} fractions using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Fraction	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
		1st Experiment			
100.00	F_2	12 ± 3.7a	44 ± 6.8a	56 ± 6.0a	58 ± 4.9a
24.30	F_{22}	10 ± 3.2a	44 ± 7.5a	64 ± 8.1a	66 ± 6.8a
0.00	Control[c]	2 ± 2.0a	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
		2nd Experiment			
50.00	F_2	4 ± 2.4a	20 ± 3.2a	30 ± 5.5a	32 ± 5.8a
12.15	F_{22}	6 ± 4.0a	26 ± 5.1a	30 ± 5.5a	30 ± 5.5a
0.00	Control	0 ± 0.0a	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with DCM. The two experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

5.3.3 HPLC fractionation

Seven fractions (F_{221}, F_{222}, F_{223} F_{224}, F_{225}, F_{226} and F_{227}) were obtained from the HPLC fractionation of the F_{22} fraction as shown in Figure 5.4 in order to isolate the compound(s) responsible for the activity of the F_{22} fraction.

Recovery

The weights of fractions and their % recovery after the HPLC fractionation of the F_{22} fraction are shown in Table 5.8. There was no fraction that accounted for less than 9.1 % and more than 22.3 % of the amount of the F_{22} fraction fractionated. The amount recovered in the F_{226} fraction (highest recovery) was 2.5 times greater than the amount recovered in the F_{222} and F_{224} fractions (lower recovery). 99.8 % of the total amount

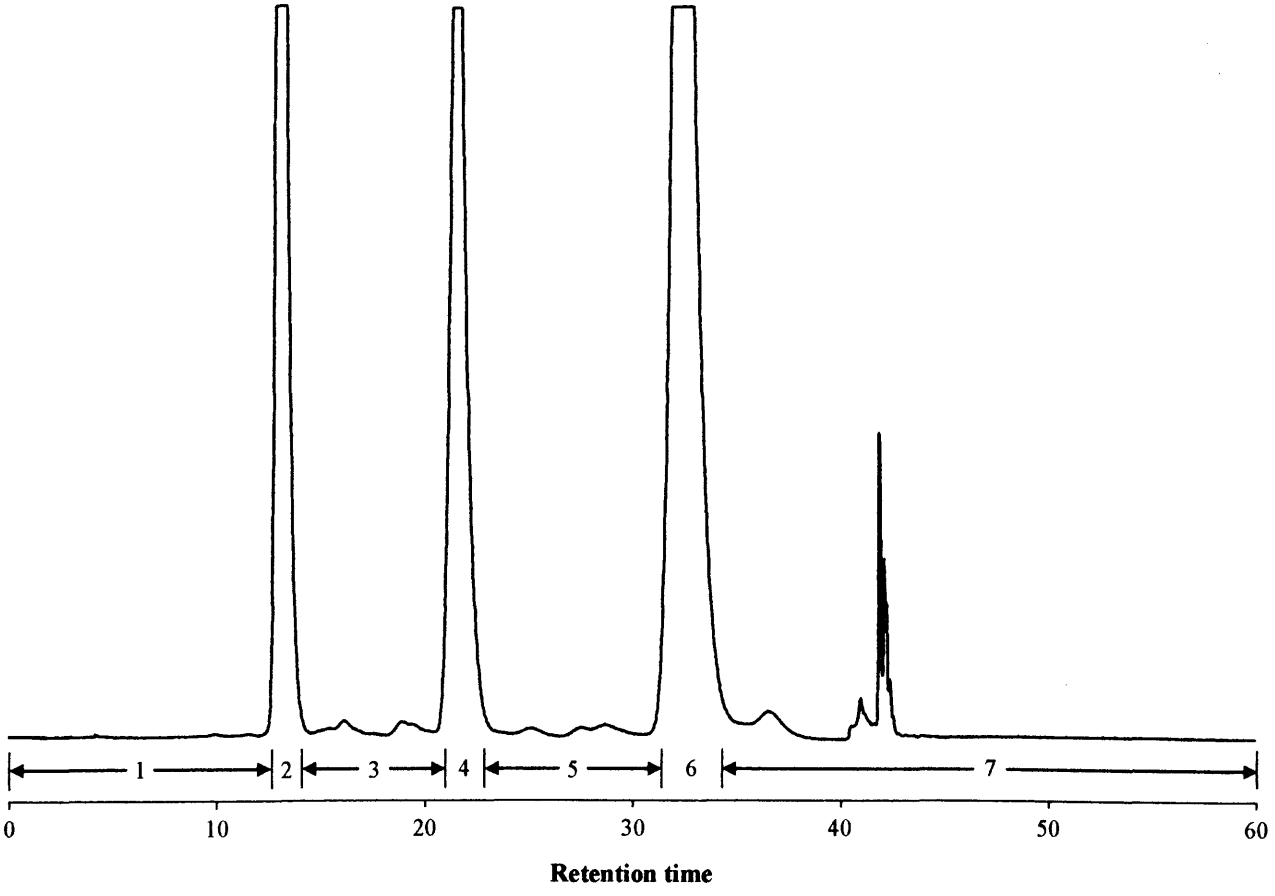

Figure 5.4 HPLC chromatogram of the F_{22} fraction.

Column: 250 x 10 mm i.d. supplied with a 10 mm precolumn; stationary phase: LiChrospher 100 CN with a particle size of 10 μm; mobile phase: n-hexane (A) and ethyl acetate (B); elution profile: 0-35 min A:B 90:10 % (isocratic), 35-40 min from A:B 90:10 % to 100 % B (linear gradient), 40-60 min 100 % B (isocratic); flow rate: 4 ml/min; column temperature: ambient; UV detection at 310 nm; amount injected: 1mg/20μl EtOAc; 1, 2, 3, 4, 5, 6 and 7: F_{221}, F_{222}, F_{223}, F_{224}, F_{225}, F_{226} and F_{227} fractions, respectively.

of the F_{22} fraction placed into the column was recovered in the HPLC fractions collected. The 0.2 % (2 mg) of the F_{22} fraction that seems not to be present in the fractions could be attributed to uncertainties associated with weighing rather than irreversible adsorption to the stationary phase.

Table 5.8 Weights of fractions and their % recovery after the HPLC fractionation of 100 mg of the F_{22} fraction.

Fraction	Retention time (min)	Amount recovered (mg)	Recovery (%)
F_{221}	0.00 - 12.7	18.9	18.9
F_{222}	12.7 - 14.0	9.1	9.1
F_{223}	14.0 - 21.0	18.0	18.0
F_{224}	21.0 - 22.8	9.1	9.1
F_{225}	22.8 -31.5	9.4	9.4
F_{226}	31.5 - 34.3	22.3	22.3
F_{227}	34.3 - 60.0	13.0	13.0
Sum of fractions		99.8	99.8

HPLC analysis

F_{221}, F_{222}, F_{223} F_{224}, F_{225}, F_{226} and F_{227} fractions were analysed by HPLC in order to investigate whether the three major peaks of the F_{22} fraction were separated adequately during the HPLC fractionation. The results obtained from the analyses (Table 5.9) reveal that the three major peaks were separated adequately and recovered in more than 94.2 % in a single fraction indicating that loss of these peaks (distribution to other fractions) during their isolation was kept to minimum.

Table 5.9 Distribution (%) of the three major peaks of fraction F_{22} to the fractions obtained from the HPLC fractionation of 100 mg of the F_{22} fraction.

Fraction	Major peaks retention time (min)		
	13.2	21.7	32.5
	% Distribution (mean[a] ± SE[b])		
F_{221}	1.7 ± 0.07	-	-
F_{222}	96.8 ± 0.21	-	-
F_{223}	1.5 ± 0.17	2.8 ± 0.15	-
F_{224}	-	94.2 ± 0.47	-
F_{225}	-	3.1 ± 0.35	2.3 ± 0.12
F_{226}	-	-	95.3 ± 0.21
F_{227}	-	-	2.4 ± 0.10

[a]Mean of 3 replicates. [b]SE: Standard error of mean.

Insecticidal activity

The F_{22} fraction and its fractions obtained from the HPLC fractionation were tested for activity using the Petri dish exposure bioassay in order to isolate the active compound(s) present in the F_{22} fraction. The initial screening concentration was set at 5 $\mu g/cm^2$. Only fractions that exhibited activity at this concentration were further tested at 2.5 $\mu g/cm^2$. At both experiments olive oil was added (0.65 % v/v) to each fraction solution and the control.

The results of the two experiments are presented in Table 5.10. The only active fractions at 5 $\mu g/cm^2$ were the initial F_{22} and the recovered from the HPLC F_{226}, whereas all the other HPLC fractions were inactive. Moreover, the activity of the F_{226} fraction was significantly greater than that of the F_{22} at the concentration of 2.5 $\mu g/cm^2$ at all mortality recording times.

Table 5.10 Percentage mortality of BO adults exposed to the F_{22} fraction and its fractions obtained from the HPLC fractionation using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Fraction	% Mortality		
		Mean[a] ± SE[b], at hours after treatment		
		24	48	72
5	F_{22}	60 ± 5.5b	94 ± 2.4a	98 ± 2.0a
	F_{221}	2 ± 2.0c	2 ± 2.0b	4 ± 2.4b
	F_{222}	0 ± 0.0c	0 ± 0.0b	0 ± 0.0b
	F_{223}	0 ± 0.0c	0 ± 0.0b	0 ± 0.0b
	F_{224}	2 ± 2.0c	2 ± 2.0b	2 ± 2.0b
	F_{225}	0 ± 0.0c	0 ± 0.0b	0 ± 0.0b
	F_{226}	82 ± 5.8a	96 ± 2.4a	100 ± 0.0a
	F_{227}	2 ± 2.0c	2 ± 2.0b	2 ± 2.0b
	Control[c]	0 ± 0.0c	2 ± 2.0b	2 ± 2.0b
2.5	F_{22}	14 ± 5.1b	20 ± 6.3b	20 ± 6.3b
	F_{226}	36 ± 6.8a	64 ± 7.5a	68 ± 5.8a
	Control	0 ± 0.0c	0 ± 0.0c	0 ± 0.0c

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with 1 ml of DCM plus olive oil (0.65 % v/v). The two experiments (5 and 2.5 $\mu g/cm^2$) were not run simultaneously. Means within a column of a single concentration followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

That results indicate that the insecticidally active chemicals of the F_{22} fraction were eluted to the F_{226} fraction during the HPLC fractionation. However, in order to investigate whether the activity of the F_{22} fraction could be fully explained by the activity of the F_{226} fraction a further experiment was designed. In that experiment (Table 5.11) the insecticidal activity of the F_{22} fraction at 5.0 $\mu g/cm^2$ was compared to the activity of the equivalent concentration of the F_{226} fraction (1.1 $\mu g/cm^2$). F_{22} fraction was significantly more active than the F_{226} fraction at all mortality recording times indicating that the activity of the F_{22} fraction cannot be fully explained by the activity of the F_{226} fraction. Two explanations can be proposed for the reduced

activity of the F_{226} fraction compared to the activity of the F_{22} fraction: (a) one or more active components were decomposed during fractionation, and (b) observed activity of the F_{22} fraction is due to synergism among components which were separated in different fractions as a result of the fractionation process.

Table 5.11 Percentage mortality of BO adults exposed to the F_{22} and F_{226} fractions using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Fraction	% Mortality		
		Mean[a] ± SE[b], at hours after treatment		
		24	48	72
5.0	F_{22}	72 ± 7.3a	94 ± 4.0a	96 ± 2.40a
1.1	F_{226}	2 ± 2.0b	4 ± 2.4b	6 ± 2.4b
0.0	Control[c]	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with 1 ml of DCM plus olive oil (0.65 % v/v). Means within a column followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

In order to clarify the reason for the reduced activity of the F_{226} fraction a set of experiment was conducted in order to compare the activity of the F_{22} fraction against that of the sum of its fractions obtained from the HPLC fractionation. The F_{22} fraction and the sum of its fractions were tested at three concentrations (5, 4 and 3 $\mu g/cm^2$). The contribution of each fraction to the concentrations of the sum of fractions tested was equivalent to the screening concentration of the F_{22} fraction. In detail, at the screening concentration of 5, 4 and 3 $\mu g/cm^2$, the concentration of the F_{221} fraction was 0.945, 0.756 and 0.567 $\mu g/cm^2$, the concentration of the F_{222} fraction was 0.455, 0.364 and 0.273 $\mu g/cm^2$, the concentration of the F_{223} fraction was 0.900, 0.720 and 0.540 $\mu g/cm^2$, the concentration of the F_{224} fraction was 0.455, 0.364 and 0.273 $\mu g/cm^2$, the concentration of the F_{225} fraction was 0.470, 0.376 and 0.282 $\mu g/cm^2$, the

concentration of the F_{226} fraction was 1.115, 0.892 and 0.669 $\mu g/cm^2$, and the concentration of the F_{227} fraction was 0.650, 0.520 and 0.390 $\mu g/cm^2$, respectively.

The results of the three experiments are shown in Table 5.12. There were no significant differences in activity between the F_{22} fraction and the sum of its fractions ($F_{221} + F_{222} + F_{223} + F_{224} + F_{225} + F_{226} + F_{227}$) at the three concentrations tested at all mortality recording times.

The results clearly show that active chemicals were not decomposed during the HPLC fractionation. Moreover, it was evident that synergism between chemicals present in different fractions took place which can fully explained why the activity of the F_{226} fraction was significant lower than that of the F_{22} fraction.

A further set of experiments was conducted in order to detect among which fractions synergism occurred. The F_{22} fraction and three different combinations of the HPLC fractions were tested. These combinations were: the active F_{226} together with the two other major peaks ($F_{222} + F_{224} + F_{226}$); the active F_{226} together with all other fractions except the two other major peaks ($F_{221} + F_{223} + F_{225} + F_{226} + F_{227}$); and all fractions except the active F_{226} ($F_{221} + F_{222} + F_{223} + F_{224} + F_{225} + F_{227}$). The contribution of each fraction to the concentration of each combination was equivalent to the screening concentration of the F_{22} fraction, 4 and 3 $\mu g/cm^2$, (for details see previous experiment). Only fractions combinations that were active in the initial experiment were tested further.

Table 5.12 Percentage mortality of BO adults exposed to the F_{22} fraction and the sum of fractions obtained from the HPLC fractionation using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Treatment	% Mortality Mean[a] ± SE[b], at hours after treatment		
		24	48	72
	1st Experiment			
5.000	F_{22}	62 ± 6.6a	88 ± 3.7a	90 ± 3.2a
0.945 + 0.455 + 0.900 + 0.455 + 0.470 + 1.115 + 0.650	$F_{221} + F_{222} + F_{223} + F_{224} + F_{225} + F_{226} + F_{227}$	64 ± 5.1a	92 ± 2.0a	96 ± 2.4a
0.000	Control[c]	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
	2nd Experiment			
4.000	F_{22}	56 ± 7.5a	80 ± 3.2a	80 ± 3.2a
0.756 + 0.364 + 0.720 + 0.364 + 0.376 + 0.892 + 0.520	$F_{221} + F_{222} + F_{223} + F_{224} + F_{225} + F_{226} + F_{227}$	50 ± 4.5a	72 ± 9.7a	76 ± 7.5a
0.000	Control	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
	3rd Experiment			
3.000	F_{22}	18 ± 5.8a	36 ± 4.0a	38 ± 4.9a
0.567 + 0.273 + 0.540 + 0.273 + 0.282 + 0.669 + 0.390	$F_{221} + F_{222} + F_{223} + F_{224} + F_{225} + F_{226} + F_{227}$	20 ± 3.7a	36 ± 6.8a	36 ± 6.8a
0.000	Control	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with 1 ml of DCM plus olive oil (0.65 % v/v). The three experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

The results of the two experiments are displayed in Table 5.13. The $F_{221} + F_{223} + F_{225} + F_{226} + F_{227}$ and $F_{221} + F_{222} + F_{223} + F_{224} + F_{225} + F_{227}$ combinations of fractions were inactive at the initial experiment, whereas the F_{22} fraction and the $F_{222} + F_{224} + F_{226}$ combination of fractions were active and did not differ significantly at both experiments at all mortality recording times.

The results of these experiments indicate that synergism occurred between chemicals that were eluted during the HPLC fractionation to the F_{222}, F_{224} and F_{226} fractions. The activity of the F_{22} fraction can be fully ascribed to the activity of the $F_{222} + F_{224} + F_{226}$ blend. Moreover, the chemicals present in the F_{221}, F_{223}, F_{225} and F_{227} fractions did not have any contribution to the insecticidal activity of the F_{22} fraction.

A further set of experiments was conducted in order to detect whether the activity of F_{22} fraction could be fully explained by the activity of a binary blend of the F_{222}, F_{224} and F_{226} fractions. The contribution of each fraction to the concentration of each binary blend was equivalent to the screening concentration, 4 and 3 $\mu g/cm^2$, of the F_{22} fraction (for details see previous experiment). Only binary blends that were active at the initial experiment were tested further.

Table 5.13 Percentage mortality of BO adults exposed to the F_{22} fraction and different combinations of the fractions obtained from the HPLC fractionation using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Treatment	% Mortality		
		Mean[a] ± SE[b], at hours after treatment		
		24	48	72
	1st Experiment			
4.000	F_{22}	46 ± 6.8a	68 ± 6.6a	68 ± 6.6a
0.364 + 0.364 + 0.892	$F_{222} + F_{224} + F_{226}$	38 ± 6.6a	52 ± 8.0a	54 ± 6.8a
0.756 + 0.720 + 0.376 + 0.892 + 0.520	$F_{221} + F_{223} + F_{225} + F_{226} + F_{227}$	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
0.756 + 0.364 + 0.720 + 0.364 + 0.376 + 0.520	$F_{221} + F_{222} + F_{223} + F_{224} + F_{225} + F_{227}$	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
0.000	Control	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
	2nd Experiment			
3.000	F_{22}	16 ± 4.0a	40 ± 6.3a	42 ± 5.8a
0.273 + 0.273 + 0.669	$F_{222} + F_{224} + F_{226}$	16 ± 5.1a	34 ± 6.8a	34 ± 6.8a
0.000	Control	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with 1 ml of DCM plus olive oil (0.65 % v/v). The two experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

The results of the two experiments are displayed in Table 5.14. The F_{222} + F_{224} and F_{222} + F_{226} binary blends were inactive at the initial experiment, whereas the F_{22} fraction and the F_{224} + F_{226} binary blend were active and did not differ significantly at both experiments at all mortality recording times.

Table 5.14 Percentage mortality of BO adults exposed to the F_{22} fraction and different binary blends of the three major peaks of the F_{22} fraction using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Treatment	% Mortality		
		Mean[a] ± SE[b], at hours after treatment		
		24	48	72
		1st Experiment		
4.000	F_{22}	40 ± 5.5a	64 ± 5.1a	66 ± 5.1a
0.364 + 0.364	F_{222} + F_{224}	34 ± 5.1a	56 ± 5.1a	56 ± 5.1a
0.364 + 0.892	F_{222} + F_{226}	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
0.364 + 0.892	F_{224} + F_{226}	0 ± 0.0b	0 ± 0.0b	2 ± 2.0b
0.000	Control[c]	2 ± 2.0b	4 ± 2.4b	4 ± 2.4b
		2nd Experiment		
3.000	F_{22}	20 ± 4.5a	34 ± 7.5a	36 ± 6.8a
0.273 + 0.669	F_{224} + F_{226}	18 ± 3.7a	26 ± 6.0a	26 ± 6.0a
0.000	Control	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]Treated with 1 ml of DCM plus olive oil (0.65 % v/v). The two experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

The results of these experiments indicate that synergism occurred between chemicals that were eluted during HPLC fractionation to the F_{224} and F_{226} fractions. The activity of the F_{22} fraction can fully be ascribed to the activity of the F_{224} + F_{226} binary blend. Moreover, the chemical(s) present in the F_{222} fraction did not made any contribution to the insecticidal activity of the F_{22} fraction.

In a final set of experiments the activity of the petroleum ether peel extract was compared to the activity of a blend consisted of the F_1, F_{224} and F_{226} fractions, in order to confirm that the activity of the petroleum ether peel extract was due to the presence of these fractions in the extract. The contribution of each fraction to the concentration of the blend was equivalent to the screening concentration of the petroleum ether peel extract. In detail, at the screening concentration of 25 and 15 $\mu g/cm^2$ the concentration of the F_1 fraction was 6.78 and 4.07 $\mu g/cm^2$, the concentration of the F_{224} fraction was 0.35 and 0.21 $\mu g/cm^2$, while the concentration of the F_{226} fraction was 0.85 and 0.51 $\mu g/cm^2$, respectively. At this experiment olive oil was not added to treatment solutions.

The results of these experiments are presented in Figure 5.15. There were no significant differences in activity between the petroleum ether peel extract and the blend of the F_1, F_{224} and F_{226} fractions at both concentrations at all mortality recording times.

The results clearly show that the activity of the petroleum ether peel extract was the result of the synergistic action among the F_1, F_{224} and F_{226} fractions. Since F_1 fraction consisted of a mixture of compounds it can be concluded that at least three compounds contribute for the overall activity of the petroleum ether peel extract.

Table 5.15 Percentage mortality of BO adults exposed to the petroleum ether peel extract of CA and a blend consisted of the F_1, F_{224} and F_{226} fractions using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Treatment	% Mortality			
		Mean[a] ± SE[b], at hours after treatment			
		24	48	72	96
		1st Experiment			
25	PE[c]	8 ± 3.7a	60 ± 8.4a	78 ± 8.6a	80 ± 7.1a
6.78 + 0.35 +0.85	$F_1 + F_{224} + F_{226}$	4 ± 2.4a	54 ± 8.7a	66 ± 10.3a	66 ± 10.3a
0	Control[d]	0 ± 0.0a	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b
		2nd Experiment			
15	PE	2 ± 2.0a	22 ± 7.3a	38 ± 5.8a	40 ± 5.5a
4.07 + 0.21 + 0.51	$F_1 + F_{224} + F_{226}$	2 ± 2.0a	24 ± 5.1a	34 ± 4.0a	36 ± 5.1a
0	Control	0 ± 0.0a	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b

[a]Mean of 5 replicates of 10 insects each (5 males and 5 females). [b]SE: Standard error of mean. [c]PE: Petroleum ether peel extract of CA. [d]Treated with DCM. The two experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different ($P = 0.05$, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

5.4 DISCUSSION

Bioassay-guided fractionation of the petroleum ether peel extract resulted in the isolation of three pure compounds (fractions F_{222}, F_{224} and F_{226}). Only the F_{226} fraction was toxic when tested individually. The F_{224} fraction was found to act synergistically to the F_{226} fraction, whereas the F_{222} fraction did not show any significant activity. The fractionation procedure of two sequential gravity silica columns followed by HPLC for the final step of purification was proven sufficient to isolate the insecticidally active chemicals present in the peel extract.

The amount of chemicals recovered in each fractionation step was considerably high (lowest recovery 96.7 % for the first gravity column whereas for the second gravity column and HPLC recovery was *ca.*100 %), indicating that only a very small portion of compounds were not eluted from the columns. TLC analysis on the fractions obtained from the two gravity columns and HPLC analysis on the HPLC fractions indicate that decomposition did not occur during the whole fractionation process and that adequate separation was achieved in each fractionation step.

Bioassays conducted with the fractions obtained from the fractionation process revealed that the activity of the petroleum ether peel extract prior to fractionation can be fully explained by the synergistic activity among F_1, F_{224} and F_{226} fractions That finding indicates that the peel extract did not loss its activity when passed through the columns.

Synergism among several compounds, usually chemically related, is in most cases accountable for the activity of plant extracts. Plant extracts have different biological

characteristics from pure compounds. Mixtures may be more effective due to synergism or joint action of active compounds with different mechanisms (Assabgui *et al.*, 1997). Berenbaum *et al.* (1991) investigated whether a mixture of 6 furanocoumarins isolated from fruits of *Pastinaca sativa* (Apiaceae) is toxicologically more effective against *Heliothis zea* larva than is an equivalent amount of xanthotoxin, which is the major furanocoumarin present in the mixture. They found that the mixture of six furanocoumarins was more effective than equivalent amount of pure xanthotoxin in reducing growth of *Heliothis zea* larva.

Moreover, the evolution of resistance to plant extracts is extremely slow in contrast to resistance to pure compounds which develops rapidly. Feng and Isman (1995) found that peach aphid lines selected with pure azadirachtin, developed a 9-fold resistance to this substance over 40 generations, but a parallel line selected with neem seed extracts containing azadirachtin and other materials did not develop significant resistance. It was suggested that the reason underlying the extremely slow evolution of resistance to plant extracts is that it is more difficult for an insect to develop several adaptations simultaneously (Brattsten *et al.*, 1986).

McLaughlin *et al.* (1997) pointed out that by the use of crude, chemically unrefined, plant extracts, containing mixtures of the bioactive plant components, rather than the use of pure individual components, insect resistance is much less likely to develop, the environmental load of individual components is lessened and the products of crude extracts are cheaper to prepare. In addition, Weaver *et al.* (1997) stated that plant extracts subjected to simple chemical fractionation procedures may have a great potential for insect control.

Considering the advantages of using plant extracts and taking into account the synergistic activity of the petroleum ether peel extract it can be concluded that crude or partially purified petroleum ether peel extracts of CA could have great potential, particularly in the developing countries, for the control of insect pests.

Having obtained reasonable quantities of fractions F_{222}, F_{224} and F_{226} (pure compounds), the next step, which is presented in Chapter 6, is to fully characterise these components using spectroscopic methods. Moreover, the activity of the characterised compounds was compared with standards or synthesised compounds.

CHAPTER 6

IDENTIFICATION OF ISOLATED INSECTICIDALLY ACTIVE COMPONENTS

6.1 INTRODUCTION

Generally, the methods employed for the determination of the structure of unknown compounds can be divided into two categories: chemical and physicochemical (spectroscopic). In essence, chemical methods involve the use of degradative techniques to produce simpler and more readily analysed compounds from an unknown. Success of these methods hinges on the ability to work with small quantities of material, and the accurate observation and interpretation of the results obtained (Harwood *et al.*, 1999). Addition reactions, reductive and oxidative processes are the main chemical methods used (Stevens, 1998). However, nowadays structure determination relies heavily on spectroscopic methods. The use of these techniques has revolutionised structure organic chemistry, and their impact cannot be overstated (Harwood *et al.*, 1999). With the advent of modern chemistry, the structures of many biologically active compounds became known, and the systematic studies of natural products that protected plants from pests became a recognised activity within the field of chemistry (Hedin and Hollingworth, 1997).

The most widely spectroscopic techniques used are: ultraviolet (UV) spectroscopy, infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and mass (MS) spectrometry (Williams and Fleming, 1995). With the exception of MS, all

other spectroscopic methods are non-destructive, thus permitting further examination of the sample; fortunately MS requires the least quantity of sample (Houghton and Raman, 1998). UV, IR and NMR come under the general heading of absorption spectrometry while MS is fundamentally different to these in that it does not involve absorption of radiation (Harwood *et al.*, 1999). The uses of these methods are stated by Williams and Fleming (1995). UV spectroscopy is used to detect conjugated systems, because the promotion of electrons from the ground state to the excited state of such systems gives rise to absorption in this region. IR is used to detect and identify the vibrations of molecules, and specifically the characteristic vibrations of the double and triple bonds present in many functional groups. NMR uses a longer wavelength of the electromagnetic spectrum to detect changes in the alignment of nuclear magnets in strong magnetic fields. MS measures the mass-to-charge ratio of organic ions. Structural information comes from the moderately predictable fragmentation organic molecules undergo; the masses of the fragment ions can often be related to likely structures. However, it cannot be overemphasized that the method of greatest importance in structure determination is NMR. As NMR increased in power over the last three decades, UV and IR have decreased in importance.

In this chapter the structure of the isolated chemicals present in the petroleum ether peel extract of CA was determined using the following spectroscopic methods: UV, IR, MS and NMR. Moreover, biological and spectroscopic studies with isolated and synthetic compounds were performed in order to confirm our results.

6.2 METHODS

6.2.1 Spectroscopic methods

The following spectroscopic methods were employed in order to elucidate the chemical structure of the three isolated compounds from the petroleum ether peel extract.

UV Spectroscopy

UV spectra of F_{222}, F_{224}, F_{226} fractions were obtained during the HPLC fractionation of the F_{22} fraction of the petroleum ether peel extract (for details see section 5.2.1). UV spectra were recorded in the region of 245-365 nm and printed (GP-100 Graphics Printer, Perkin Elmer).

Gas chromatography - Fourier Transform Infrared Spectroscopy.

GC-FTIR analysis of F_{222}, F_{224}, F_{226} fractions was carried out on a Perkin Elmer Autosystem gas chromatograph interfaced to a Perkin Elmer System 2000 (Perkin Elmer Ltd., Buckinghamshire, England) Fourier transform infrared spectrometer equipped with a liquid-nitrogen cooled narrow-band (4000 - 750 cm^{-1}) infrared detector (mercury cadmium telluride). Helium was used as the carrier gas (2 ml/min) and the effluent from the GC was mixed with the sweep gas helium (0.5 ml/min; transfer line temperature 280 °C) and passed through the IR light pipe with KBr windows. All spectra were obtained at 8 cm^{-1} resolution. All samples were chromatographed on a 30 m x 0.32 mm x 1 μm film thickness DB-5 column (J&W Scientific). The oven temperature program was 50 °C for 2 min, then 5 °C/min to 280 °C and held for 60 min. Splitless injections were made (1 μl) at an injector temperature of 250 °C and a splitless period of 90 sec.

Gas Chromatography - Mass Spectrometry.

Gas chromatography - mass spectrometry analysis of F_{222}, F_{224}, F_{226} fractions was carried out on a Hewlett Packard 5890 Series II gas chromatograph interfaced to a Fisons VG Trio 1000 (Manchester M23 9BE, UK) quadrupole mass spectrometer. Electron impact ionization was used, with an electron energy of 70 eV and a trap current of 200 μA. The interface was kept at 290 °C. All samples were chromatographed on a 60 m x 0.25 mm x 0.1 μm film thickness DB-5 column (J&W Scientific). The oven temperature was set at 50 °C for 2 min, then raised to 250 °C at 5 °C/min, held for 1 min, then 2 °C/min to 280 °C and held for 50 min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Splitless injections were made (1 μl) at an injector temperature of 250 °C and a splitless period of 90 sec. 6',7'-epoxybergamottin could not be chromatographed on the GC as it probably decomposes on the hot injector temperature. For this compound, mass spectrometric data were obtained by the use of the Direct Contact Inlet (DCI) probe of the VG Trio 1000 mass spectrometer. The DCI was set at 0 mA for 2 min, then raised to 200 mA at 100 mA/min, held for 2 min, then 300 mA/min to 1000 mA held for 4 min and then ramped down to 0 mA at 200 mA/min where it stayed for 5 min.

NMR Spectroscopy

NMR spectra of F_{222}, F_{224}, F_{226} fractions were recorded on Bruker Advance DPX-400 spectrometer. $CDCl_3$ was used as solvent. Tetramethylsilane or residual solvent peaks (e.g. $CHCl_3$) were used as frequency standards. Coupling constants were determined using the computer program Multiplet and are quoted in hertz (Hz). Values are reported to 0.1 Hz, but have an uncertainty of ± *ca.* 0.3 Hz (at 400 MHz), due to the

digital resolution of the FID accumulation and Fourier transformation. ^{1}H NMR spectra were simulated using RACCOON.

6.2.2 Synthesis of 6′,7′-epoxybergamottin

20 mg bergamottin and 20 mg MCPBA, in 5 ml DCM, were left to stand for 3 h at room temperature (20 - 22 °C) (Dreyer and Huey, 1973; Bellevue *et al.*, 1997). The DCM solution was extracted three times with H_2O (5 ml each time) and the aqueous layer was discarded. Na_2SO_4 was added then to the DCM solution in order to absorb the water remained. The DCM solution was filtered and concentrated. Purification of the 6′,7′-epoxybergamottin was done by HPLC using the same chromatographic conditions as in section 5.2.1.

6.2.3 Bioassay of isolated and synthetic compounds

The Petri dish exposure bioassay was used in order to compare the insecticidal activity of the isolated and synthetic compounds against 2-3 d-old adults of BO. Stock solutions were prepared in DCM, as were subsequent dilutions. Olive oil was added to each treatment solution and control (DCM) so that each cm^2 of the total inner surface of the Petri dishes was layered with 0.1 μl of olive oil. Fresh test solutions were prepared for each replicate. The syringe used for the application of samples was thoroughly cleaned after the application of each sample. Mortalities were recorded every 24 h for 3 d after which no further increase in mortality was recorded.

Estimation of LC_{50}s

Five replicates of 10 BO flies each (5 males and 5 females) which were conducted using 5 different generations of flies, were employed for the 5 concentrations and the

control. The concentrations tested for each chemical were chosen on the basis of preliminary range-finding studies. The concentrations of the isolated and synthesised 6',7'-epoxybergamottin tested were 1.7, 2.0, 2.3, 2.6 and 2.9 $\mu g/cm^2$. Rotenone, a commercial plant-derived insecticide was used for comparison. The concentrations of rotenone tested were 0.015, 0.020, 0.025, 0.030 and 0.035 $\mu g/cm^2$. Concentrations for each replicate of each chemical were applied from the lowest to the highest.

Screening experiments

Five replicates of 10 BO flies each (5 males and 5 females) were employed for each treatment and the control. The 5 replicates were conducted on 5 successive days, utilising the same generation of flies. Different concentrations of screened treatments were not run simultaneously as screening experiments at lower concentrations included only the more active treatments.

6.3 RESULTS

6.3.1 Spectroscopic data of the isolated compounds

In the present work the isolated compounds were subjected to UV, IR, MS and ^1H NMR analysis. Considering the spectral data obtained and comparing them with that present in the literature we assigned fraction F_{222} as osthol, fraction F_{224} as bergapten and fraction F_{226} as 6',7'-epoxybergamottin. Their chemical structures are presented in Figure 6.1.

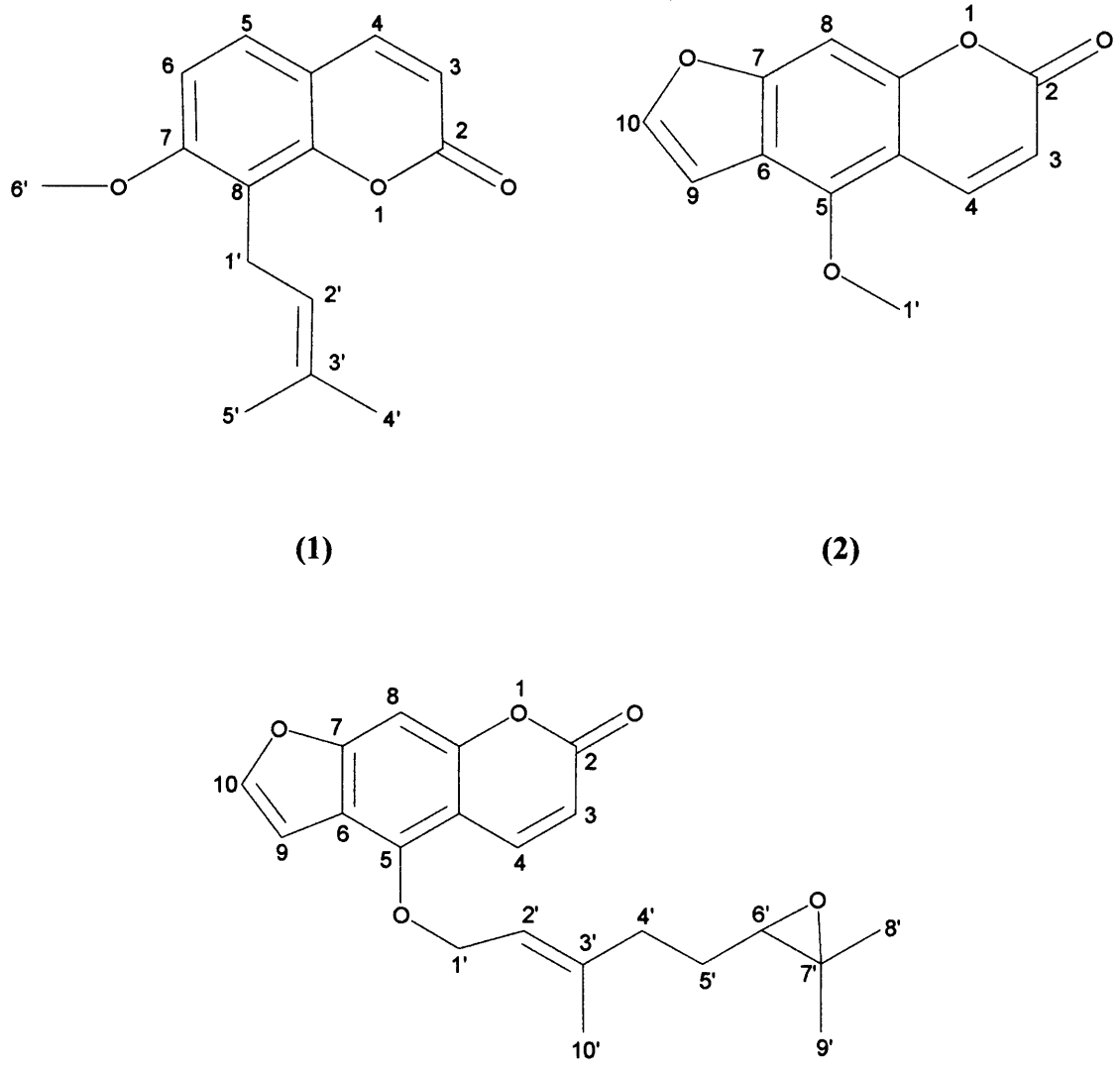

Figure 6.1 Chemical structures of osthol (1), bergapten (2) and 6',7'-epoxybergamottin (3).

Spectroscopic data of osthol (fraction F_{222})

UV Spectroscopy

The UV spectrum of osthol showed two absorption maxima (Figure 6.2). The absorbance at 310 nm was considerably higher than that at 250 nm. This spectrum is consistent to that reported by Hadaček *et al.* (1994) for osthol isolated from *Peucedanum* (Apiaceae: Apioideae) species.

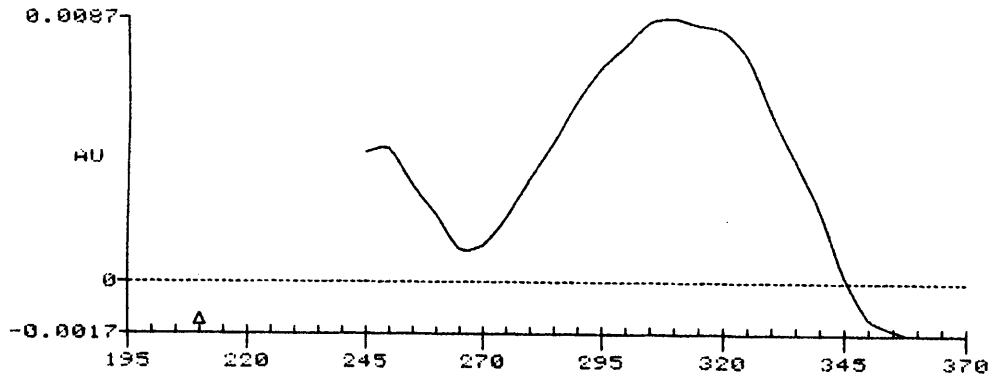

Figure 6.2 UV spectrum of osthol.

IR Spectroscopy

The most intense peak in the IR spectra of osthol (Figure 6.3) is the peak at 1770 cm^{-1} that originates from the carbonyl absorption of the lactone moiety. Peaks at 1600 cm^{-1} to 1450 cm^{-1} are characteristic of the conjugated aromatic system. The C-O stretching of the lactone moiety and the absorptions of the =C-O-C group occur in the region of 1300 cm^{-1} to 1050 cm^{-1}. The absorption at *ca.* 830 cm^{-1} is characteristic of the C-H out of plane vibration of the aromatic ring the frequency of which is determined by the number of adjacent hydrogen atoms of the ring. Finally the peaks in the region of 3000 cm^{-1} to 2800 cm^{-1} are due to the absorption frequencies of the CH, CH_2 and CH_3 groups of the compounds.

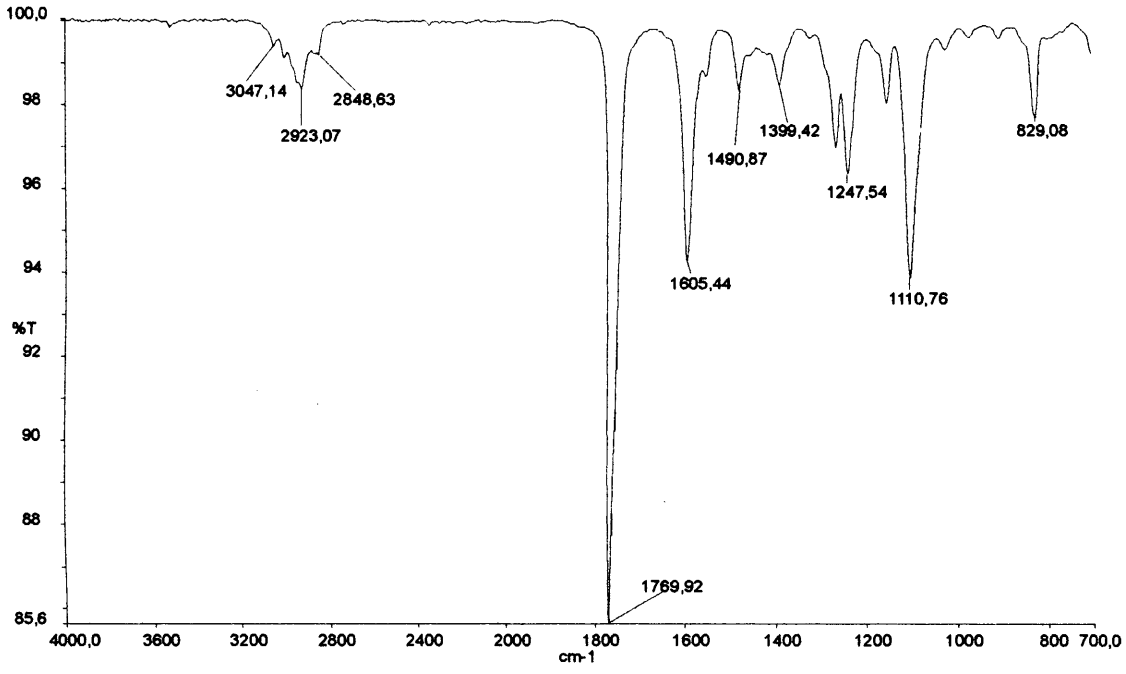

Figure 6.3 IR spectrum of osthol.

Mass Spectroscopy

For osthol, the parent ion *m/z* 244 (100 %) is the base peak of the spectrum (Figure 6.3). Loss of the CH_3 group probably occurs from the side chain and gives the peak *m/z* 229 (77 %), and then the loss of CO gives the peak *m/z* 201 (72 %). The peak *m/z* 213 (48 %) is due to loss of the CH_3O group from the molecular peak. Fission of the *β*-chain to the ring to remove a C_4H_7 radical gives the ion *m/z* 189 (81 %), which, after hydrogen rearrangement, subsequently loses a CH_2O group to give *m/z* 159 (40 %). Finally, the expulsion of CO from the pyran ring gives the ion *m/z* 131 (62 %). This spectrum is very similar to that reported by Zobel *et al.* (1991) for osthol isolated from *Apium graveolens* and by Buiarelli *et al.* (1996) for osthol isolated from the non-volatile fraction of grapefruit essential oil.

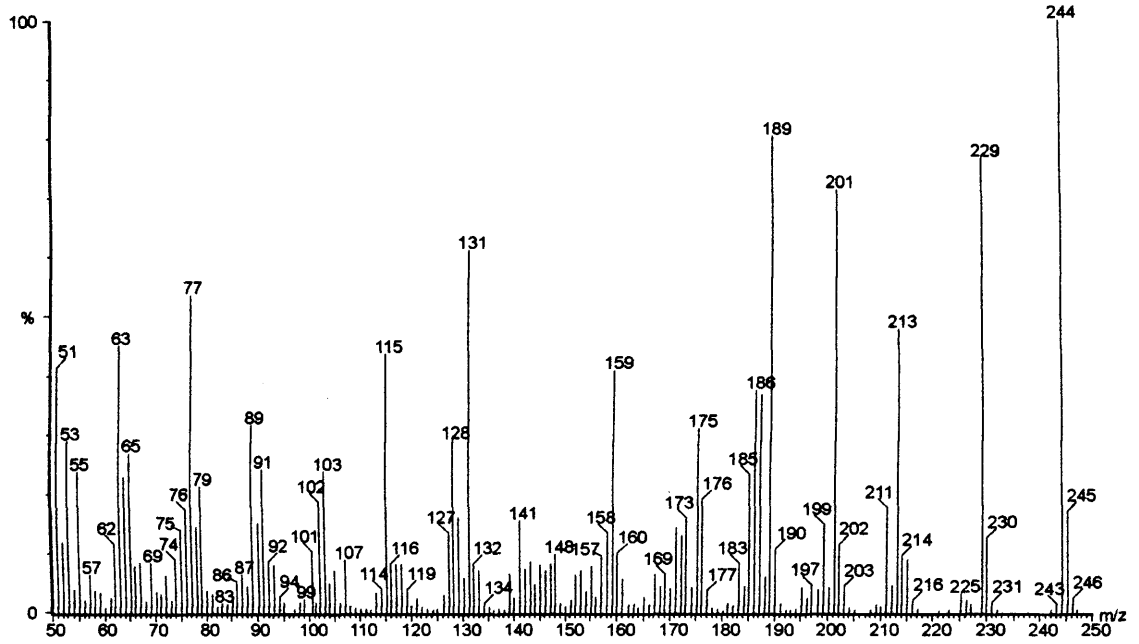

Figure 6.4 MS spectrum of osthol.

NMR Spectroscopy

δ_H ($CDCl_3$, 400 MHz) 7.55 (d, 1H, *J* 9.4 Hz, 4-H), 7.20 (d, 1H, *J* 8.6 Hz, 5-H), 6.77 (d, 1H, *J* 8.6 Hz, 6-H), 6.17 (d, 1H, *J* 9.4 Hz, 3-H), 5.16 (t, 1H, *J* 7.2 Hz, 2′-H), 3.85 (s, 3H, 6′-H_3), 3.45 (d, 2H, *J* 7.2 Hz, 1′-H_2), 1.78 (s, 3H, 4′-H_3), 1.59 (s, 3H, 5′-H_3). The ^1H NMR spectrum of osthol is presented in Figure 6.5.

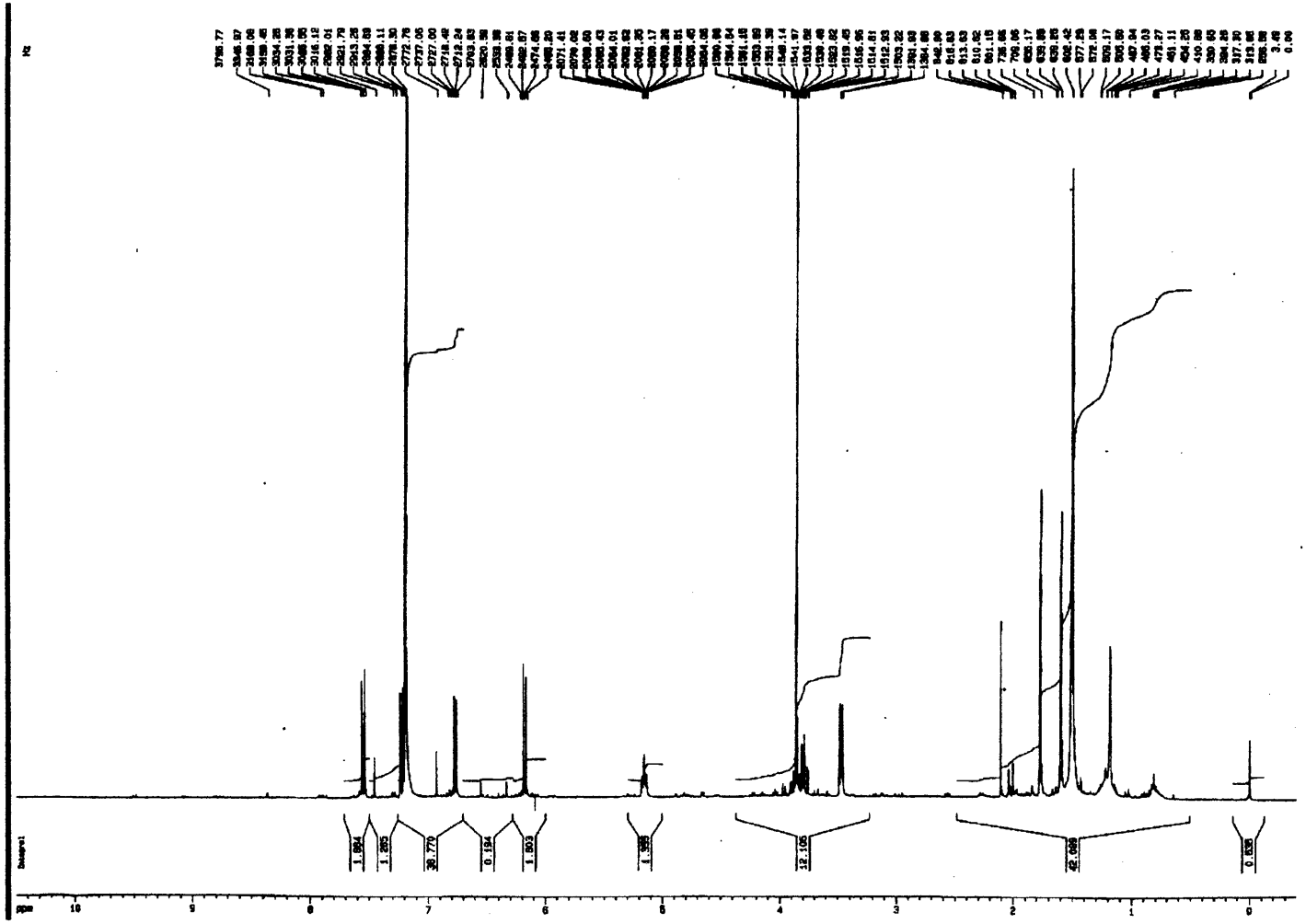

Figure 6.5 ^{1}H NMR spectrum of osthol.

Spectroscopic data of bergapten (fraction F_{224})

UV Spectroscopy

The UV spectrum of bergapten showed two absorption maxima (Figure 6.6). The absorbance at 250 nm was considerably higher than that at 305 nm. This spectrum is consistent to that reported by Hadaček *et al.* (1994) for bergapten isolated from *Peucedanum* (Apiaceae: Apioideae) species.

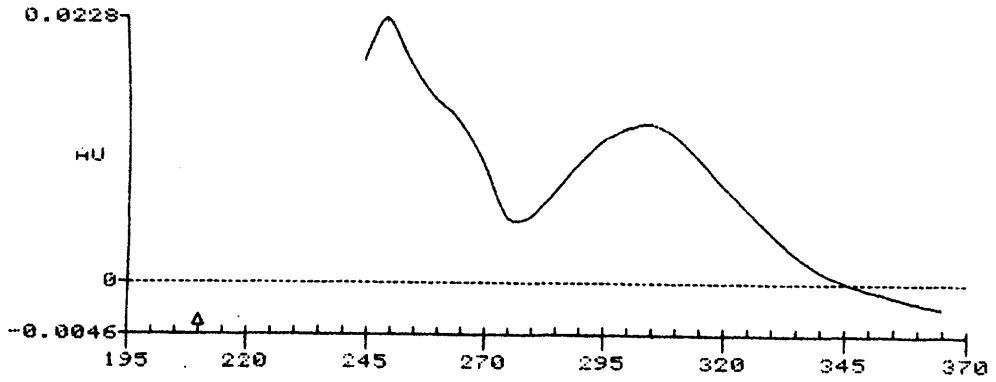

Figure 6.6 UV spectrum of bergapten.

IR Spectroscopy

The gas phase IR spectra of bergapten (Figure 6.7) is very similar to that of osthol since these two compounds possess common functional groups, such as the conjugated aromatic system, the lactone moiety and the ether group, and consequently they both exhibit the characteristic absorptions of these groups.

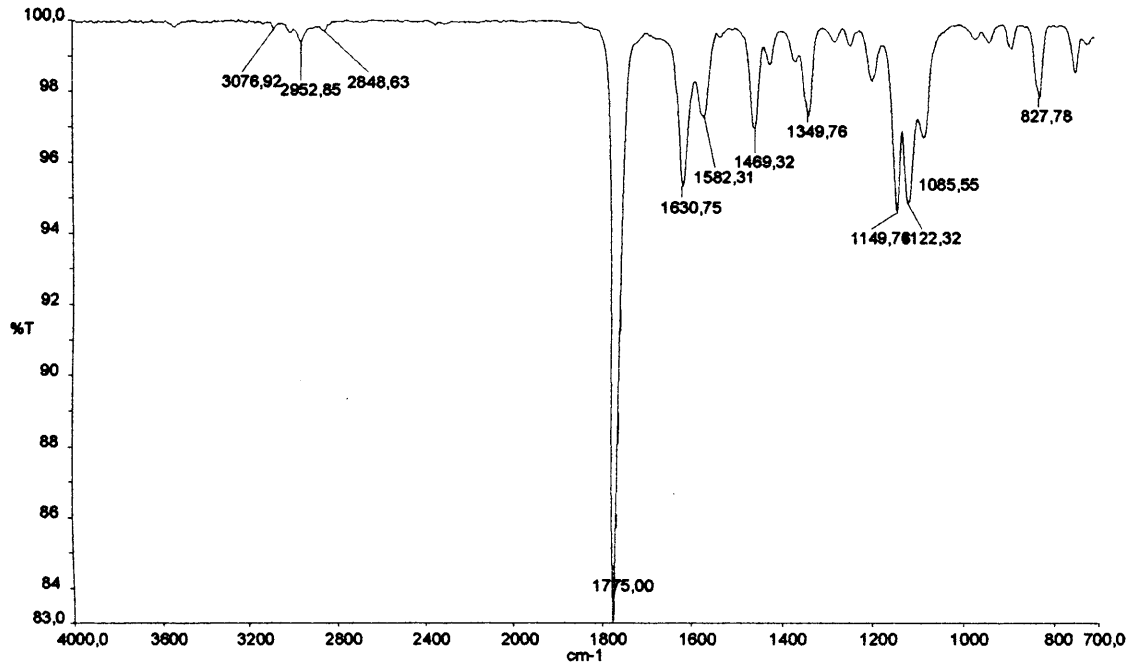

Figure 6.7 IR spectrum of bergapten.

Mass Spectroscopy

The parent ion *m/z* 216 (100 %) is the base peak of the mass spectrum of bergapten (Figure 6.8). Loss of the CH_3 radical from the parent ion gives the peak *m/z* 201 (35 %) whilst loss of the CO group leads to the formation of the peak *m/z* 188 (15 %). The formed ion *m/z* 201 undergoes sequential losses of a CO group to give the peaks at *m/z* 173 (64 %) and *m/z* 145 (33 %) respectively. This spectrum is consistent to that reported by Valenciennes *et al.* (1999) for bergapten isolated from *Euodia borbonica* (Rutaceae).

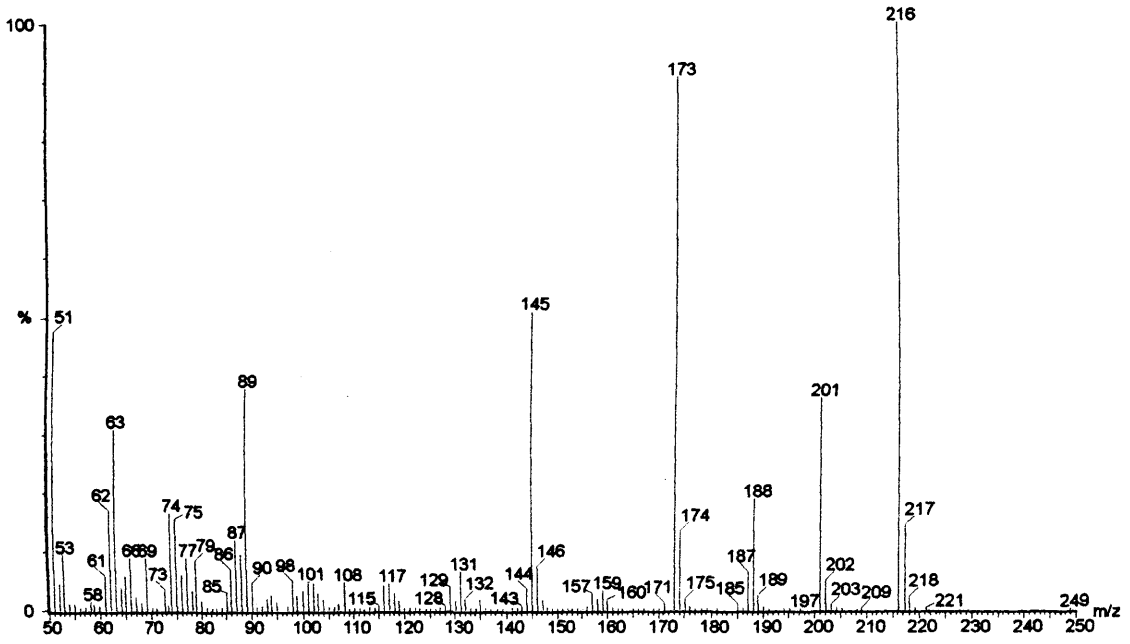

Figure 6.8 MS spectrum of bergapten.

NMR Spectroscopy

δ_H ($CDCl_3$, 400 MHz) 8.14 (d, 1H, *J* 9.9 Hz, 4-H), 7.57 (d, 1H, *J* 2.4 Hz, 10-H), 7.11 (s, br, 1H, 8-H), 7.01 (dd, 1H, *J* 2.4 and 0.8 Hz, 9-H), 6.27 (d, 1H, *J* 9.9 Hz, 3-H), 4.27 (s, 3H, 1'-H_3). The ^{1}H NMR spectrum of bergapten is presented in Figure 6.9. This spectrum is consistent to that reported by Sasaki *et al.* (1980) for bergapten isolated from *Glehnia littoralis* (Umbelliferae).

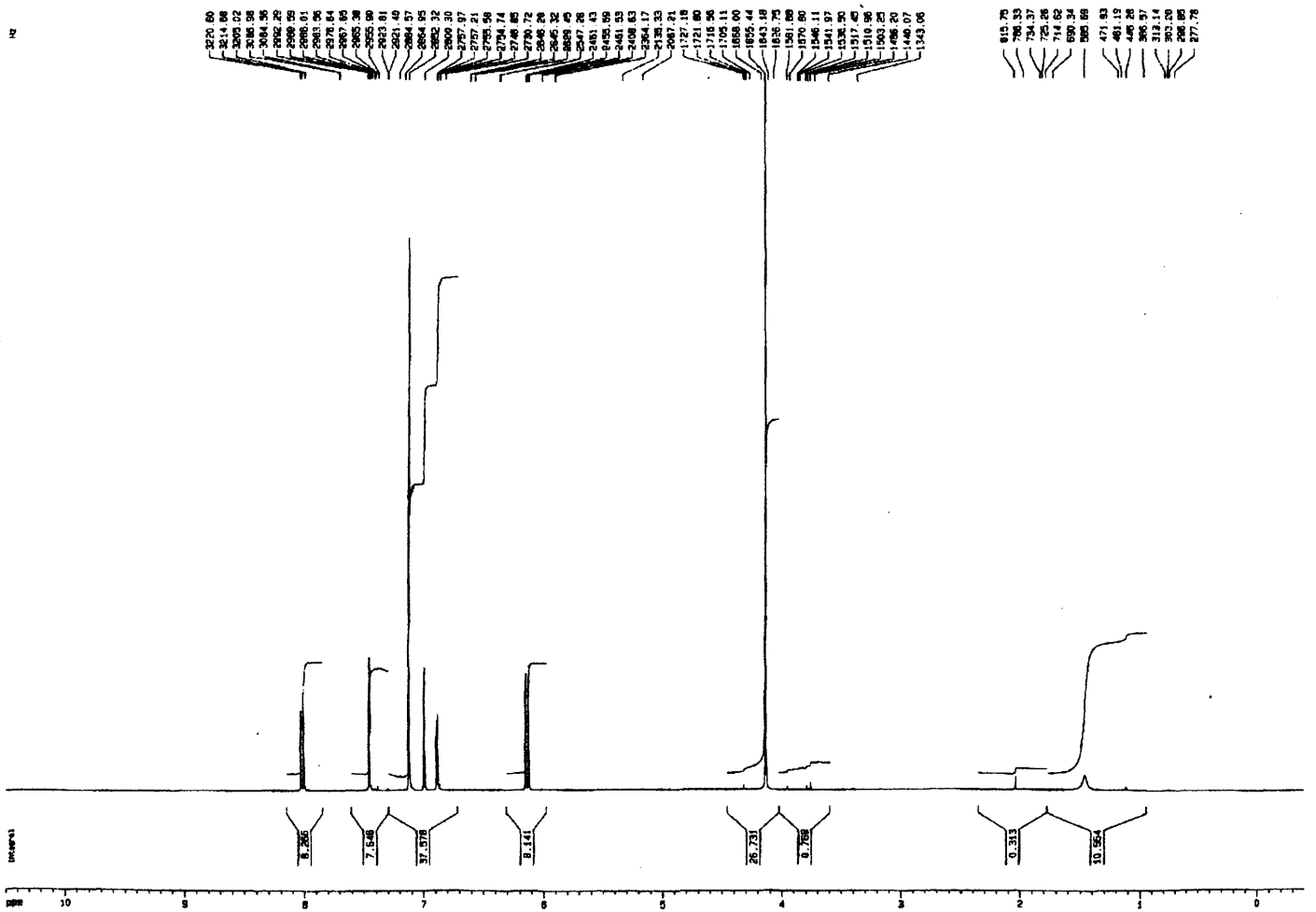

Figure 6.9 ^{1}H NMR spectrum of bergapten.

Spectroscopic data of 6',7'-epoxybergamottin (fraction F_{226})

UV Spectroscopy

The UV spectrum of 6',7'-epoxybergamottin was very similar to bergapten's spectrum (Figure 6.10). The absorbance at 250 nm was considerably higher than that at 305 nm. This spectrum is consistent to that reported by Dreyer and Huey (1973) for 6',7'-epoxybergamottin isolated from *Citrus macroptera*.

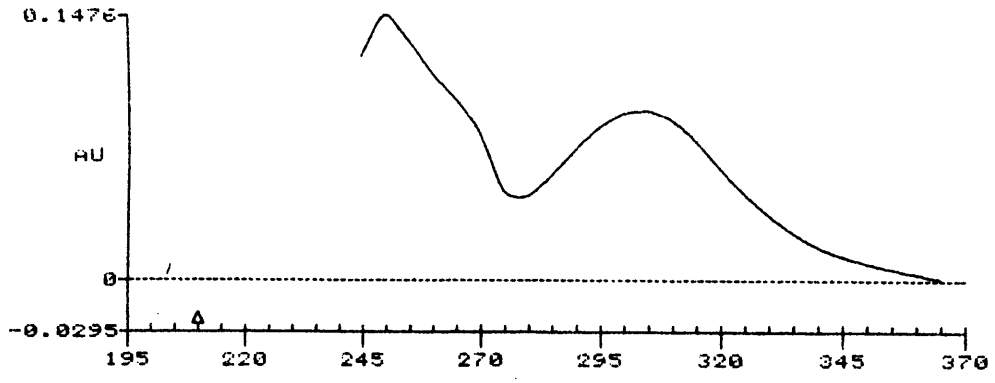

Figure 6.10 UV spectrum of 6',7'-epoxybergamottin.

Mass Spectroscopy

The EI mass spectrum of 6',7'-epoxybergamottin does not exhibit the molecular ion at *m/z* 354 as it rapidly fragments following the typical fragmentation pattern of the epoxides with preferential cleavage *alpha* to the epoxy group. This cleavage gives rise to the base peak of the spectrum at *m/z* 71 (100 %) corresponding to the C_4H_7O fragment (Figure 6.11). The peak *m/z* 202 (69 %) originates probably from *alpha* cleavage to the ether group after hydrogen rearrangement and charged retained at the furanocoumarin moiety (loss of the $C_{10}H_{16}O$ group from the molecule of the 6',7'-epoxybergamottin). The same cleavage, but this time with the charged retained at the aliphatic moiety gives the peak at *m/z* 153 (30 %) corresponding to the $C_{10}H_{17}O$

fragment. Loss of the CO group from the *m/z* 202 ion gives the peak at *m/z* 174 (42 %). The peak *m/z* 145 (27 %) probably originates from the *m/z* 174 fragment with loss of the CHO group.

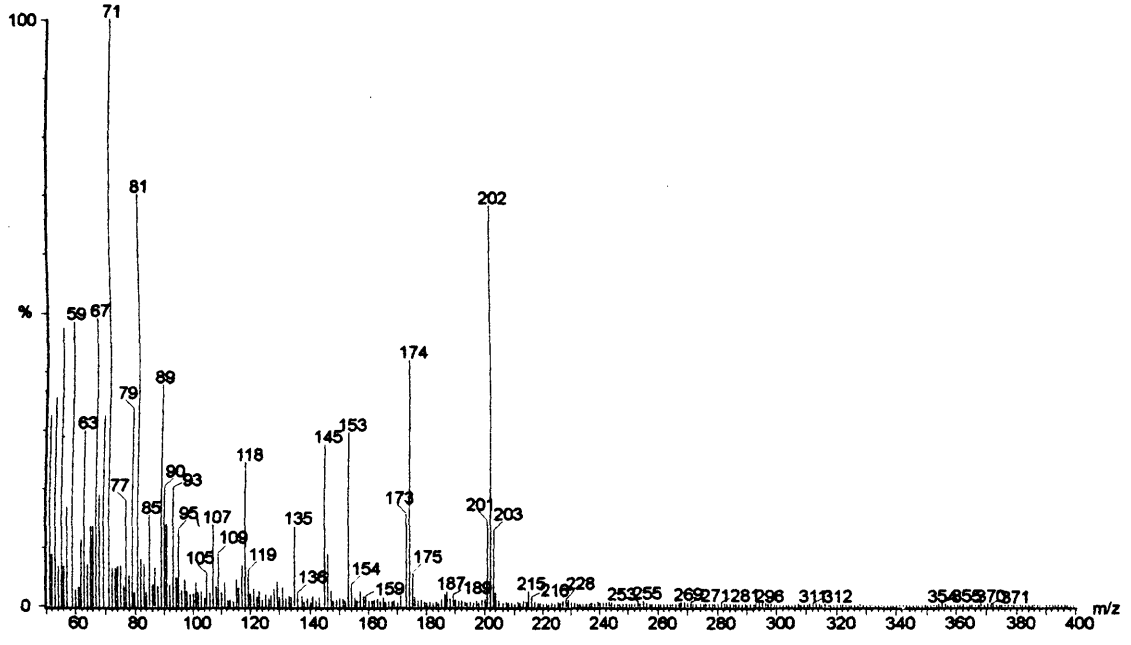

Figure 6.11 MS spectrum of 6′,7′-epoxybergamottin.

NMR Spectroscopy

δ_H ($CDCl_3$, 400 MHz) 8.09 (d, 1H, *J* 9.9 Hz, 4-H), 7.53 (d, 1H, *J* 2.5 Hz, 10-H), 7.09 (s, 1H, 8-H), 6.88 (m, 1H, *J* 2.5 and 0.9 Hz, 9-H), 6.21 (d, 1H, *J* 9.9 Hz, 3-H), 5.52 (m, 1H, 2′-H), 4.89 (d, 2H, *J* 6.9 Hz, 1′-H_2), 2.64 (dd, 1H, *J* 7.0 and 5.4 Hz, 6′-H), 2.15 (m, 2H, 4′-H_2), 1.67 (s, 3H, 10′-H_3), 1.55 (m, 2H, 5′-H_2), 1.25 (s, 3H, 9′-H_3), 1.23 (s, 3H, 8′-H_3). The ^{1}H NMR spectrum of 6′,7′-epoxybergamottin is presented in Figure 6.12. This spectrum is consistent to that reported by Dreyer and Huey (1973) for 6′,7′-epoxybergamottin isolated from *Citrus macroptera* and by Ohta *et al.* (2002) for 6′,7′-epoxybergamottin isolated from grapefruit juice.

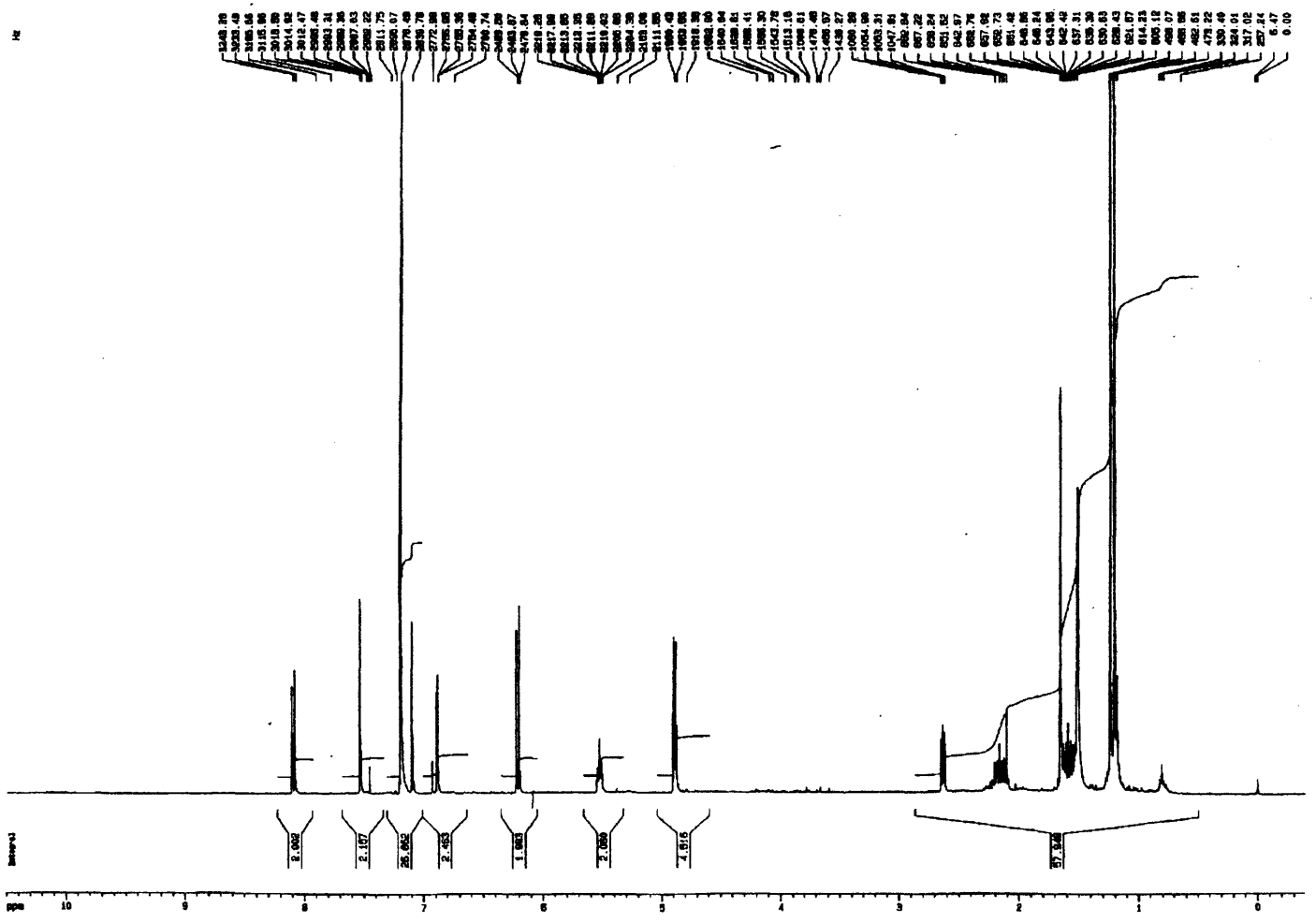

Figure 6.12 ^{1}H NMR spectrum of 6′,7′-epoxybergamottin.

6.3.2 Synthesised 6′,7′-epoxybergamottin

Selective epoxidation of the terminal bond of bergamottin gave the 6′,7′-epoxybergamottin in 69 % yield. Bellevue *et al.* (1997) obtained 6′,7′-epoxybergamottin in similar yield (65 %).

6.3.3 Spectroscopic data of synthetic osthol, bergapten and 6′,7′-epoxybergamottin

All the spectroscopic data of the synthesised 6′,7′-epoxybergamottin, synthetic bergapten purchased from Sigma and synthetic osthol kindly provided by May Berenbaum, were identical to the isolated 6′,7′-epoxybergamottin, bergapten and osthol, respectively.

6.3.4 Bioassays

Petri dish exposure bioassays were conducted in order to investigate whether there were any difference in activity between isolated and synthetic 6′,7′-epoxybergamottin, bergapten and osthol.

Estimation of LC_{50}s

LC_{50}s were estimated for the isolated and the synthesised in our laboratory 6′,7′-epoxybergamottin in order to confirm that the observed activity of the isolated 6′,7′-epoxybergamottin was not due to minor compounds possibly present in it. Moreover, LC_{50}s of the botanical insecticide rotenone were estimated in order to compare its activity to that of the 6′,7′-epoxybergamottin.

A summary of probit analyses of mortality data for BO adults exposed to different concentrations of the isolated and the synthetic 6′,7′-epoxybergamottin and the botanical insecticide rotenone using the Petri dish exposure bioassay is presented in Table 6.1. LC_{50}s were not estimated at 24 h after treatment as mortality at this time was not significant high to estimate precisely the lethal concentrations. All *P*-values of Table 6.1 were much greater than 0.05 and so Pearson's χ^2 goodness-of-fit tests were not significant, indicating that the data fit the assumptions of the probit model adequately.

Table 6.1 Summary of probit analyses of mortality data of BO exposed to different concentrations of the isolated and the synthetic 6′,7′-epoxybergamottin and the botanical insecticide rotenone using the Petri dish exposure bioassay.

h	n	LC_{50} ($\mu g/cm^2$)	95 % CL	Slope ± SE	Intercept ± SE	χ^2	df	*P*
				Isolated 6′,7′-epoxybergamottin				
48	250	2.23	2.12 - 2.35	7.75 ± 1.09	2.29 ± 0.40	20.4	23	0.615
72	250	2.19	2.07 - 2.31	7.53 ± 1.09	2.43 ± 0.39	20.4	23	0.617
				Synthetic 6′,7′-epoxybergamottin				
48	250	2.38	2.27 - 2.53	7.33 ± 1.10	2.23 ± 0.41	18.5	23	0.728
72	250	2.35	2.23 - 2.48	7.62 ± 1.10	2.17 ± 0.41	22.3	23	0.503
				Rotenone				
48	250	0.025	0.023 - 0.027	5.88 ± 0.75	14.42 ± 1.21	29.0	23	0.180
72	250	0.024	0.023 - 0.026	5.71 ± 0.74	14.21 ± 1.19	27.7	23	0.228

Column headings are abbreviated as follows: h is the hours after treatment; n is the numbers of insects tested (mixed sexes) excluding the controls; 95 % CL is the 95 % confidence limits; Slope ± SE is the slope ± standard error; Intercept ± SE is the intercept ± standard error; χ^2 is the Pearson's χ^2 goodness-of-fit test; df is the degrees of freedom; *P* is the probability.

Isolated and synthetic 6′,7′-epoxybergamottin did not differ significantly as the 95 % confidence limits overlap at both mortality recording times. Moreover, the insecticidal activity of rotenone was at least 95 times greater than the activity of either isolated or

synthetic 6′,7′-epoxybergamottin. That results confirm the insecticidal activity of the 6′,7′-epoxybergamottin. However, its toxicity was substantially lower when compared to the toxicity of the botanical insecticide rotenone. Moreover, it must be noticed that either the isolated or the synthetic bergapten and osthol did not show in sign of toxicity against BO adults even at 10 $\mu g/cm^2$.

A very interesting finding was revealed when bergamottin was tested against BO adults. Even at 10 $\mu g/cm^2$ bergamottin did not exhibit any sign of activity. Since the only difference between bergamottin (Figure 6.13) and 6′,7′-epoxybergamottin (see Figure 6.1) is the epoxide in the 6′,7′ position it can be concluded that the epoxide has a critical role for the activity produced by the 6′,7′-epoxybergamottin.

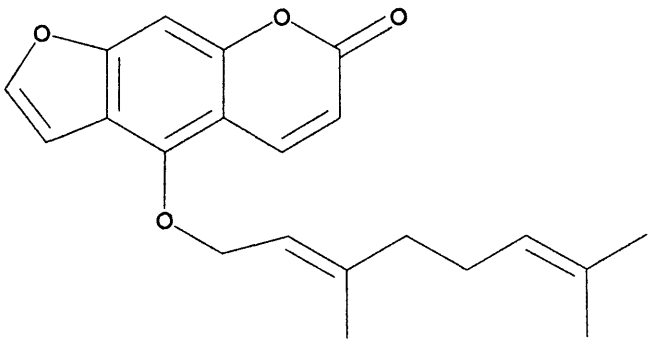

Figure 6.13 The chemical structure of bergamottin.

Screening experiments

In order to confirm the synergistic action between 6′,7′-epoxybergamottin and bergapten a set of experiments with the following treatments was conducted: isolated 6′,7′-epoxybergamottin plus isolated bergapten, isolated 6′,7′-epoxybergamottin plus synthetic bergapten, synthetic 6′,7′-epoxybergamottin plus isolated bergapten and

synthetic 6′,7′-epoxybergamottin plus synthetic bergapten. The contribution of each compound to each blend was equivalent to 4 and 3 $\mu g/cm^2$ of the F_{22} fraction. In detail, the concentration of either isolated or synthetic bergapten tested was 0.364 and 0.273 while the concentration of either isolated or synthetic 6′,7′-epoxybergamottin tested was 0.892 and 0.669 $\mu g/cm^2$, in the initial and the subsequent experiment respectively.

The results of these experiments (Table 6.2) reveal that synergism between 6′,7′-epoxybergamottin and bergapten occur only when the isolated bergapten was tested. When the isolated bergapten was substituted by the synthetic bergapten synergism was not evident indicating that other minor compounds present in the isolated bergapten must be responsible for the synergistic effects. Moreover, there was no difference in activity when the isolated 6′,7′-epoxybergamottin was substituted by the synthetic 6′,7′-epoxybergamottin confirming that the 6′,7′-epoxybergamottin was responsible for the observed synergism.

Table 6.2 Percentage mortality of BO adults exposed to different combinations of isolated and synthetic 6′,7′-epoxybergamottin and bergapten using the Petri dish exposure bioassay.

Concentration $\mu g/cm^2$	Treatment[a]	% Mortality		
		Mean[b] ± SE[c], at hours after treatment		
		24	48	72
		1st Experiment		
0.892 + 0.364	IE + IB	40 ± 4.5a	64 ± 5.1a	66 ± 6.0a
0.892 + 0.364	SE + IB	44 ± 4.0a	62 ± 5.8a	62 ± 5.8a
0.892 + 0.364	IE + SB	0 ± 0.0b	2 ± 2.0b	2 ± 2.0b
0.892 + 0.364	SE + SB	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
0.000	Control	0 ± 0.0b	0 ± 0.0b	0 ± 0.0b
		2nd Experiment		
0.669 + 0.273	IE + IB	22 ± 5.8a	38 ± 8.6a	40 ± 7.1a
0.669 + 0.273	SE + IB	18 ± 3.7a	28 ± 5.8a	30 ± 5.5a
0.000	Control	2 ± 2.0b	2 ± 2.0b	2 ± 2.0b

[a]IE: Isolated 6′,7′-epoxybergamottin; SE: Synthetic 6′,7′-epoxybergamottin; IB: Isolated bergapten; SB: Synthetic bergapten; Control: Treated with 1 ml of DCM plus olive oil (0.65 % v/v). [b]Mean of 5 replicates of 10 insects each (5 males and 5 females). [c]SE: Standard error of mean. The two experiments were not run simultaneously. Means within a column of a single experiment followed by the same letter are not significantly different (P = 0.05, Tukey's HSD test). Mortalities were transformed before ANOVA to arcsine $\sqrt{p}$, where p is the proportion of dead insects. Means ± SE of untransformed data are reported.

6.4 DISCUSSION

Considering the data obtained from the UV, FTIR, MS and ^{1}H NMR studies the isolated pure compounds, fractions F_{222}, F_{224} and F_{226} were assigned as osthol, bergapten and 6',7'-epoxybergamottin, respectively. Comparison of our data to that reported in the literature and authentic components revealed that the spectra were identical, confirming our results.

The coumarin, osthol and the linear furanocoumarins or psoralens, bergapten and 6',7'-epoxybergamottin among other coumarins and furanocoumarins have been recently isolated from the oxygen heterocyclic fraction of bitter orange essential oil by normal-phase HPLC (Dugo *et al.*, 1996). Coumarin and psoralen compounds are common to plants of the Rutaceae family (Gray and Waterman, 1978), to which *Citrus* species belong. The coumarin and psoralen compounds present in the *Citrus* peel oils are essentially non-volatile and, consequently, are not found in the distilled oils (Stanley and Jurd, 1971). They exhibit strong absorption in the ultraviolet region (Stanley and Jurd, 1971). Berenbaum (1991) reported that high performance liquid chromatography has provided a fast, efficient, and sensitive method for separating and quantifying coumarins and psoralens at the nanogram level by both normal- and reverse-phase. In this study, these advantages during HPLC purification of osthol, bergapten and 6',7'-epoxybergamottin were proven.

Since it is always possible that minor components can give rise to all or part of the biological activity (Cannell, 1998) the activity of the isolated 6',7'-epoxybergamottin was compared to the activity of 6',7'-epoxybergamottin synthesised in our laboratory. It was revealed that the activity of the isolated and synthetic 6',7'-epoxybergamottin

did not differ significantly indicating that the 6′,7′-epoxybergamottin was responsible for the mortality of BO adults. This is the first report of the insecticidal activity of 6′,7′-epoxybergamottin. However, another activity of 6′,7′-epoxybergamottin has been reported recently. Wangensteen *et al.* (2003) were found that 6′,7′-epoxybergamottin can inhibit the activity of the enzyme cytochrome P450 3A4 (CYP3A4).

Comparison of the insecticidal activity of 6′,7′-epoxybergamottin and bergamottin showed that the epoxide is essential for the observed activity of 6′,7′-epoxybergamottin, since bergamottin was inactive even at considerably higher concentrations than that used in the concentration-response experiments with 6′,7′-epoxybergamottin. Although bergamottin did not show any significant activity against BO in the Petri dish exposure bioassay, it has been reported that species of Lepidoptera were deterred from feeding when bergamottin was incorporated into their diets (Stevenson *et al.*, 2003).

Comparison of 6′,7′-epoxybergamottin with the commercially available botanical insecticide rotenone revealed that 6′,7′-epoxybergamottin was considerably less toxic than rotenone. However, it should be noted that any work dealing with natural compounds as sources of new insecticides, should consider positive results as an indication of potential in a chemical series, rather than trying to discover compounds usable *per se*. Few plant-derived chemicals can be as effective as the modern synthetic insecticides. Bioactive natural compounds should therefore be considered models which can serve for the development of series of more efficient, biodegradable, safer insecticides (Escoubas *et al.*, 1994). Moreover, since plant-

derived chemicals are known to exhibit increased specificity, it is possible that 6',7'-epoxybergamottin can exhibit greater activity against other insect pests.

Bioassays conducted with isolated and synthetic bergapten and 6',7'-epoxybergamottin revealed that minor compounds present in the isolated bergapten should be accounted for the synergistic action between bergapten and 6',7'-epoxybergamottin. Moreover, it should be noted that isolated and synthetic bergapten did not produce any sign of toxicity against BO by the Petri dish exposure bioassay even at considerably higher concentrations than that used in the concentration-response experiments with 6',7'-epoxybergamottin. However, the antifeedant activity of bergapten is well documented (Escoubas *et al.*, 1992; Berdegue *et al.*, 1997; Calcagno *et al.*, 2002; Stevenson *et al.*, 2003).

Petri dish exposure bioassays conducted with isolated and synthetic osthol did not exhibit any activity against BO even at considerably higher concentrations than that used in the concentration-response experiments with 6',7'-epoxybergamottin. However, osthol is known that posses antibacterial (Stanley and Jurd, 1971), antimalarial (Harborne and Baxter, 1993), antimutagenic (Harborne and Baxter, 1993), cytostatic (Gawron and Glowniak, 1987) and cytotoxic (Yang *et al.*, 2003) activity.

CHAPTER 7

GENERAL DISCUSSION

Plants in general have the ability to synthesise compounds that, while having no apparent function in primary metabolism, are physiologically active in insects and other organisms, so providing plants with one of their most important defense mechanisms. During recent years, increasing political and consumer pressures to reduce the use of synthetic insecticides, and the decreasing efficacy of them due to the development of resistance, have fuelled the search among plants for potentially useful insecticides. Plant-derived insecticides are likely to be more rapidly degradable and consequently more environmentally and toxicologically safe and more selective, which accords with the requirements of IPM.

Citrus species are known to contain chemicals that exhibit different properties (toxicity, deterrence, feeding deterrence) against insects. Studies undertaken so far on the insecticidal potency of *Citrus* species have mainly concentrated on *Citrus limon*, while no attention was given to *Citrus aurantium*.

The results of my research reveal that peels of *C. aurantium* contain chemicals with insecticidal properties. Petri dish and topical application bioassays shown that a petroleum ether peel extract obtained using the ultrasound extraction method was toxic against *Bactrocera oleae* and *Ceratitis capitata* adults. Bioassay-guided fractionation of this extract resulted in the isolation of three compounds which were

elucidated by spectroscopic methods and were assigned as osthol (coumarin), bergapten and 6′,7′-epoxybergamottin (furanocoumarins). However, only the 6′,7′-epoxybergamottin was toxic against *B. oleae* and its toxicity was the same to the synthesised 6′,7′-epoxybergamottin. It should be noticed that the insecticidal activity of the petroleum ether peel extract can only be fully explained by the synergistic action between the 6′,7′-epoxybergamottin and two or more compounds, which were not isolated and identified in this project.

Coumarins are secondary plant metabolites which can be divided into three major classes: simple hydroxycoumarins, furanocoumarins and pyranocoumarins. Simple hydroxycoumarins and their glucosides are found regularly in higher plants, having been recorded in *ca.* 100 families (Murray, 1991). The furano- and pyranocoumarins, which usually occur in the free state in fruits and roots, are mainly restricted to two large families, the Umbelliferae (Apiaceae) and the Rutaceae, where they occur widely. They have been recorded occasionally in six other families: the Asteraceae, Fabaceae, Moraceae, Pittosporaceae, Solanaceae and Thymeleaceae (Harborne and Baxter, 1993). The site of storage or accumulation of furanocoumarins in plants is also the site of synthesis, and especially for the rutaceous genus *Citrus*, furanocoumarins are localised in oil glands in the peel (Berenbaum, 1991), as revealed in this study.

Furanocoumarins are more biologically active than simple coumarins (Harborne and Baxter, 1993). Their biological roles in their host plants have not been well understood, but a likely function is to protect, through toxicity, plants from a wide array of natural enemies (Guo and Yamazoe, 2004). This is supported by the

correlation of bacterial toxicity and insect resistance with higher concentrations of furanocoumarins in celery and other furanocoumarin-containing plants (Trumble *et al.*, 1990; McCloud *et al.*, 1992; Cianfrogna *et al.*, 2002). Moreover, UV light induces the biosynthesis of furanocoumarins, which are often produced in tissue exposed to the sun. Their accumulation in high light environments may reflect their ability to function as UV-absorbent screens, protecting plant ptotosynthetic apparatus from damaging radiation (Berenbaum, 1991).

Furanocoumarins are unusual among plant secondary compounds because their toxic properties are greatly enhanced in the presence of UV light. Photoactivity of furanocoumarins is due to the formation of an excited triplet state on absorption of a proton. The excited triplet state furanocoumarin can react directly with molecules such as pyrimidine bases. Alternatively, the excited triplet state furanocoumarin can react with ground state oxygen. By energy transfer, singlet oxygen can be generated, or, by either electron or charge transfer, toxic oxyradicals such as hydroxy radical or superoxide anion radicals can be formed. These molecules can then proceed to react with molecules, including DNA, to cause toxicity (Berenbaum, 1991, 1995; Sastry *et al.*, 1997).

Furanocoumarins elicit behavioural responses and physiological effects in herbivorous insects. They are repellents and feeding deterrents (Klocke *et al.*, 1989; Berdegué *et al.*, 1997) as well as toxins (Berenbaum, 1978). In addition to their inherent toxicity, they may act synergistically (Brewer *et al.*, 1995) or antagonistically (Diawara *et al.*, 1993) when mixed with other furanocoumarins. Moreover, synergism has also been observed in mixtures of the furanocoumarin xanthotoxin with the

nonfuranocoumarins myristicin, safrole, and fagaramide (Berenbaum and Neal, 1985; Neal, 1989). Toxicity in most instances is greatly enhanced in the presence of UV light, suggesting that photoactivation, possibly involving DNA, is a major mechanism of toxicity (Berenbaum, 1991). However, there are also toxic effects of furanocoumarins that are independent of UV light (Berenbaum, 1978). Insects feeding on plants with high contents of furanocoumarins possess adaptations for reducing the toxicity of furanocoumarins. Many associates of furanocoumarin-containing plants avoid light and thereby avoid the photoactivating effects of UV on furanocoumarins (Berenbaum, 1990). Moreover, in some insects a biochemical resistance to furanocoumarins has been evolved (Hung *et al.*, 1995).

Furanocoumarins are toxic against a wide variety of organisms, including bacteria, fungi, plants, nematodes, molluscs, fish, birds and mammals (Berenbaum, 1991). Moreover, some linear furanocoumarins disrupt the detoxification capability of organisms by acting as suicide substrates for cytochrome P-450 monooxygenases, an enzyme system involved in detoxification processes in mammals (Koenigs and Trager, 1998) and insects (Neal and Wu, 1994).

Phytophotodermatitis, an epidermal reaction to furanocoumarins in the presence of UV light, is the most widely recognised manifestation of furanocoumarin activity. In all organisms afflicted, phytophotodermatitis manifests itself in the form of erythema, bullous eruptions, and pigmentation at the point of contact with furanocoumarin-rich plant tissue (Fleming, 1990). These symptoms are well known from celery workers and the causal agents are the linear furanocoumarins psoralen, 5-methoxypsoralen (bergapten), and 8-methoxypsoralen (xanthotoxin) (Austad and Kavli, 1983;

Ashwood-Smith *et al.*, 1985). Human food plants known to cause photodermatitis include carrots, celery, parsnip, dill, fennel, caraway, parsley, lovage, anise, and chervil in the Apiaceae; figs in the Moraceae; and lemon, orange, and grapefruit in the Rutaceae (Murray *et al.*, 1982).

Furanocoumarins have been used since ancient times to treat human skin disorders such as skin depigmentation (vitiligo), psoriasis, mycosis fungoides, polymorphous photodermatitis and eczema (Musajo and Rodighiero, 1962; Scott *et al.*, 1976). However, the use of these compounds in medicine has been associated with higher incidence of skin cancer (Musajo and Rodighiero, 1962; Stern *et al.*, 1979; Grekin and Epstein, 1981). Moreover, furanocoumarins have been reported to be carcinogenic and mutagenic (Roelandts, 1984). Therefore, benefit and risk should be carefully balanced against each other when furanocoumarins are used in medicine (Daniel *et al.*, 1999).

Citrus oils are used in the formulation of perfumes and cosmetics, and coumarins are used for their flavour quality and are added to sunscreen formulations to enhance tanning induced by ultraviolet radiation (Mabry and Ulubelen, 1980). Moreover, several plants containing furanocoumarins, e.g. lemon, celery, parsnip, parsley and carrots, are part of the human diet (Daniel *et al.*, 1999). Based on the average consumption and furanocoumarin contents of different food items, the uptake of furanocoumarins in the United States was calculated to be 1.3 mg per person per day (Wagstaff, 1991). It can be concluded that the carcinogenic potential of furanocoumarins is low, although their combination with exposure to UV radiation could pose a hither to unquantified risk.

Further studies will need to be done in order that the full insecticidal potential usefulness of *C. aurantium* can be explored. Different stages of insects from a broad range of families should be tested using different bioassay methods. Since botanical insecticides are generally species-specific and also different bioassays produce different results, it is highly likely that a more pronounced activity will be shown. Even fractions, extracts and isolated compounds that were inactive by the bioassays and insect species used in this study, can be revealed to be very promising as pest control chemicals. Moreover, further work is needed in order to determine the chemical structure of the synergistic chemicals present in the peel extract. Besides toxicity studies, it is also important to determine other factors such as persistence and effects on man and other non-target organisms.

Roark (1942) suggested that the reasons for seeking new natural insecticides included the continuing need to combat insects which had not yielded to effective control, to eliminate chemicals poisonous to man, to find substitutes for existing chemicals, and to provide a supply to meet insecticidal shortages. These reasons appear as valid today as they did then, despite the dominance of synthetic insecticides, many of which are now environmental hazards.

CHAPTER 8

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