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Repellent, Antifeedant & Molluscicidal Effects of *Commiphora* spp. Oleoresins, and their extracts, on *Deroceras reticulatum* and *Helix aspersa*

A thesis presented for the degree

of Doctor of Philosophy

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Abstract

The oleoresin exudates from two species of *Commiphora* trees, and their extracts, were evaluated as novel methods of controlling terrestrial molluscs. Various test methods were employed including terraria trials, leaf disc assays, caged field trials and spray trials.

Laboratory terraria trials with *C. molmol* (myrrh) and *C. guidotti* (opoponax) oleoresins, showed them to be effective repellent barriers against the terrestrial molluscs *Deroceras reticulatum*, *Arion hortensis* and *Helix aspersa*. Solid repellent barriers comprised of reduced amounts of myrrh oleoresin, mixed with inert materials (sawdust, corncob and sharp sand) and sawdust coated with extracts of myrrh and opoponax, were also very effective in repelling terrestrial molluscs.

The botanical origin of myrrh and opoponax oleoresins were confirmed using gas chromatography-mass spectrometry (GC-MS) analytical techniques. The chemical compounds identified for myrrh were consistent with those reported for *C. molmol*, comprising mainly of sesquiterpenes and furano-sesquiterpenes, whilst the chemical compounds identified for opoponax were consistent with those reported for *C. guidotti,* comprising mainly of monoterpenes and sesquiterpenes.

Comparison of the chemicals identified for commercial myrrh (Yemeni) and Somali myrrh (Guban) showed them both to contain the same bouquet of chemical compounds. Differences were observed in the gas chromatographic profile of Somali and Yemeni myrrh. Somali myrrh contained high levels of β-elemene, whilst Yemeni myrrh was dominated by the furano-sequiterpenes, curzerene and Furanoeudesma -1,3-diene. α-Santalene was the major sesquiterpene identified for the liquid extracts of opoponax, whilst *trans*-β-ocimene was the dominant chemical identified for the volatile odour associated with the opoponax oleoresin and its extracts.

Leaf disc assays, with *D. reticulatum* slugs, confirmed the extracts of myrrh and opoponax, to be strong antifeedants at concentrations of 0.5% and 1% respectively. Both extracts significantly reduced the feeding behaviour of the slugs. A number of terpenoid chemicals were also evaluated, using the leaf disc assay, and showed significant antifeedant properties. The most potent of these chemicals was found to be *trans*-β-ocimene, a major component of opoponax, and was found to possess both antifeedant and molluscicidal properties towards slugs. The molluscicidal nature of this monoterpene depended upon the polarity of the medium used to prepare it.

Leaf discs assays with *H. apersa* snails, showed that higher concentrations of myrrh and opoponax extracts (3%) was required to deter the snails from feeding on the lettuce leaf discs. In addition higher concentration levels of *trans*-β-ocimene (5%) was required to cause a similar antifeedant effect. In contrast to the slugs, no snail mortality was observed with these strong antifeedant extracts, however 100% snail mortality was observed after treating lettuce leaf discs with pure *trans*-β-ocimene oil.

Emulsion stability was found to be dependent upon nature of the non-ionic surfactant incorporated into the formulation. Oil in water emulsions based on myrrh, opoponax and *trans*–β-ocimene oils, containing 3 to 5% surfactant, were stable for time

periods ranging from two weeks to more than 10 months. Emulsions based on Synperonic 91/8 were stable for two weeks, whilst those containing Tween 80 and Tween 20 were stable for approximately 10 months to one year.

Caged field trials with repellent physical barriers comprised of 100% myrrh and opoponax oleoresins, reduced myrrh oleoresin mixed with inert substrates, and sawdust treated with ethanol and essential oil extracts of myrrh, all showed significant repellency properties towards *D. reticulatum* slugs for 14 days.

Spray trials with myrrh, opoponax and *trans*-β-ocimene, under controlled temperature conditions, showed them to be very effective in deterring slugs and snails from consuming lettuce plants. Myrrh essential oil and *trans*-β-ocimene were also molluscicidal against the small field slug. Little slug mortality was observed when ethanol extracts of myrrh were employed, whilst still maintaining its strong repellent properties. No incidences of snail mortalities were observed throughout the spray trials.

Myrrh and opoponax oleoresins were found to have no toxic effects on earthworms and their 3% extracts showed very little phytotoxic effects against lettuce plants. *Trans*–β-ocimene (5%) extracts were well tolerated but marginally affected one variety of curly lettuce.

This study has shown the novel application of myrrh and opoponax oleoresins, their extracts, and their chemical components in affecting the feeding activity of terrestrial molluscs.

Publications

Peer Reviewed

1. Ali, A. Y., Müller, C. T., Randerson, P. and Bowen, I. D. (2003) Screening African plants for mollusc repellency. In: *Slugs & Snails Agricultural, Vetinary & Enviromental Perspectives*, BCPC Symposium Proceedings, **80**, 319-324.

2. Ali, A. Y., Müller, C. T., Randerson, P. and Bowen, I. D. (2003) Molluscicidal and repellent properties of African plants. In: *Slugs & Snails Agricultural, Vetinary & Enviromental Perspectives*, BCPC Symposium Proceedings, **80**, 135-141.

3. Ali, A. Y. and Bowen, I. D. (2005) Antifeedant and molluscicidal activity of scented myrrh applied as a spray. In: *OILB/IOBC Working Meeting On Integrated Control of Soil Pests*, Stuttgart, Germany. In Press.

Patents

1. Bowen, I. D. and Ali, A. (2003) The use of plant materials as a terrestrial molluscicidal and/or mollusc repellent agent. International Patent WO 03/092385 A1.

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CHAPTER 1:

General Introduction

1. INTRODUCTION

1.1 **Deroceras reticulatum** Müller (1774)

1.1.1 Description, Classification and Life History

The field slug, *D. reticulatum* (Müller), is considered to be the most common slug species found in the British Isles. This slug, although indigenous to Europe (Quick, 1960), has been introduced to most of North and South America, South Africa and Australia (Chichester and Getz, 1996), through trade and commerce. It is approximately 2 to 5 cm long and has an appearance which varies from pale cream to brown, often blotched with dark grey pigmentation, see figure 1.1. The pigment often accumulates in grooves forming a reticulum, hence the origin of its name. The mantle extends over half the body length, at the end of which is the respiratory pore. The slug has a pointed tail, a short truncated keel and a light brown sole. The mucous is thin and colourless until irritated when it becomes milky and sticky.

In Britain *D. reticulatum* was formally known as *Agriolimax reticulatum* until it was re-classified by Waldén (1976).

The taxonomical classification of *D. reticulatum*, as defined by Godan (1983), is shown below:

Phylum:	Mollusca
Class:	Gastropoda
Sub-Class:	Pulmonata
Order:	Stylommatophora
Family:	Limacidae
Genus:	Deroceras
Species:	reticulatum

Figure 1.1 The field slug, *D. reticulatum.* Photograph reproduced from Godan (1983).

Godan (1983) described five stages in the reproductive cycle of terrestrial gastropods: courtship, copulation, nest building and embryonic development followed by hatching.

Slugs lay eggs, about ten days after mating (Quick, 1960), onto soils which must be 40% to 80% water saturated before oviposition and development can begin. According to Carrick (1942) oviposition occurs at temperatures between 3°C and 20°C and times of development can vary from 105 days (at 5°C to 18°C) to 18 days at 20°C. On average *D. reticulatum* slugs lay between 20 and 30 eggs at a time which corresponds to over 300 during their life time (Godan, 1983). At the time of emergence eggs are approximately 4mm in diameter. The average slug has a life span of between 9 to 13 months.

1.1.2 Feeding behaviour

Slugs are nocturnal animals, coming out to feed at night, thereby avoiding coming into contact with many predatory animals. *D. reticulatum* slugs spend most of their time feeding on green plant material found near or on the surface of the soil (Brooks *et al.*, 2003). Most gastropods normally eat only small amount of grasses, however Pallant (1972) found the slug *D. reticulatum* to be an exception. He found large proportions of the grass *Holcus lanatus* in the diet of grassland populations of this slug. The crops at most risk, from damage, are cereals (such as winter wheat, soya bean and corn), and a number of horticultural crops (Watkins *et al.*, 1996; Hammond, 1996).

The ability of slugs, including *D. reticulatum*, to discriminate between the leaves of different plants has been verified by Cook *et al.* (1996). They showed that the presence of highly palatable weeds (such as Dandelion, Shepherd's Purse and Wild Clover), as alternative food sources, reduced the consumption of winter wheat seeds. Brooks *et al.*, (2003) also investigated the use of alternate food sources, in the form of legumes, to reduce crop damage by *D. reticulatum*. They demonstrated a hierarchy of slug acceptability, with red clover, lucerne, lupin and white clover being consumed more than six other species of legumes. In addition they found that, with choice tests, red clover reduced the consumption of winter wheat seeds by 50%.

Dobson and Bailey (1987) were the first to demonstrate the negative correlation between the duration of feeding and crop fullness in *D. reticulation.* They found that the crop part of the slug's gut was not always full when it stopped feeding and was not always empty when feeding was resumed. They suggested that external factors, such as chemoreceptors, might be initiating feeding. Pickett and Stephenson (1980) also reported the behaviour of slugs, towards food plants, to be mediated by chemicals. They suggested that the volatile cues were detected at a distance by olfaction, thereby acting as attractants, whilst non volatile compounds with a low volatility would be more likely to influence feeding behaviour by gustatory mechanisms, such as taste, once the plant had been located.

1.2 **Helix aspersa** Müller (1774)

1.2.1 Description, Classification and Life History

The common garden snail, *H. aspersa*, although widely distributed throughout the British Isles originates from the warmer climates of the Mediterranean (Godan, 1983). According to *H. aspersa* snails are recognised by the distinct colour pattern on their shell, which is brown with irregular broken bands of pale yellow, crossed by zig-zag streaks. Their shell size can grow up to 38 mm breadth and 35 mm height, and have a bluntly rounded spire (Godan, 983). They have a moist grey skin as shown in figure 1.2.

The recent adoption of the name *Cantareus aspersus,* for this snail, is somewhat controversial after being long referred to as *H. aspersa* (Barker, 2002). The taxonomic arguments for not adopting the former name are described in detail by Thorsson (2005). To avoid the confusion of taxonomic nomenclature the familiar name, *H. aspersa*, will be used throughout this thesis.

According to Godan (1983) the taxonomical classification of *H. aspersa* is described as:

Phylum:	Mollusca
Class:	Gastropoda
Sub-Class:	Pulmonata
Order:	Stylommatophora
Family:	Helicinae
Genus:	Helix
Species:	aspersa

Figure 1.2 Photograph of the common garden snail, *Helix aspersa*

Moisture combined with a certain depth of soil is a prerequisite for egg laying by terrestrial snails such as *H. aspersa* (Godan, 1983). Adult females lay approximately 80 eggs at one time into crevices in the top soil and can lay up to six batches of eggs per year (Bezemer and Knight, 2001). The eggs are approximately 3 mm in diameter, spherical and white in appearance. Temperature, humidity and light can influence the rate of oviposition for snails. According to Stephens and Stephens (1966), exposure of *H. aspersa* to 15 hours illumination, followed by nine hours of darkness, resulted in vigorous egg laying. It takes two years from hatching for a juvenile snail to fully mature. During cold weather snails hibernate in the top soil, while during hot or dry periods they aestivate by sealing themselves off with a tough membrane (Bezemer and Knight, 2001).

1.2.2 Feeding behaviour

Land snails such as *H. aspersa* are generalist herbivores, feeding on a wide variety of plants (Chevalier *et al.*, 2001). They feed at night when there is less chance of being troubled by enemy predators.

Although slugs and snails generally avoid eating grasses, due to the high silica and fibre contents of the leaves, high levels of grasses was found to be eaten by *H. aspersa (* Chevalier *et al.*, 2001). Chevalier also showed the two plants *Urtica dioica* and *Picris echioides* to be the main diet of *H. aspersa*, irrespective of seasonal variations. Iglesias and Castillejo (1999) suggested the high calcium content of *U. dioica* and *P. echioides* to be the main reason for them being the snails preferred choice of food.

Mineral intake is the primary factor governing the food preferences of snails (Williamson and Cameron, 1976). Minerals such as calcium are essential for the biological development of snails (Godan, 1983). Recent studies have shown that food consumption by juvenile snails could also be increased by external factors such as temperature (Bezemer and Knight, 2001).

1.3 Terrestrial Molluscs As Pests

Slugs and snails are the major pests of agricultural and horticultural crops in many parts of the world. In the UK the field slug, *D. reticulatum,* is widely regarded as being the most destructive mollusc (Watkins *et al.*, 1996; Choi, *et al.*, 2003). The cold, moist climate is particularly favourable to this pest which causes extensive damage to field crops such as winter wheat, potatoes, and oil seed rape (Howlett and Port, 2003).

In the UK slug damage is estimated to cost the farming industry approximately £4 million per annum, for wheat crops alone (Shirley *et al.*, 2001). The most serious damage done to winter wheat occurs soon after sowing. The slugs hollow out the seed germ and endosperm, or eat through the base of the seedlings stem (Watkins *et al.*, 1996) resulting in the seed failing to germinate or resulting in the death of the seedling shortly after emergence. The two main mollusc pests, in the UK's horticultural nurseries, have been reported to be the slug *Deroceras panormitanum* and the snail *Oxyloma pfeifferi* (Schüder *et al.*, 2003).

Of the snail species the family Helicinae, including *H. aspersa and Cepaea* spp., constitutes the greatest pest, particularly in southern Europe, the Mediterranean region,

and the warmer parts of the USA (Godan, 1983). The snail is an important pest in citrus orchards, however the degree of damage they cause is weather dependent. During hot dry periods snails are minor pests, however, during the rainy seasons their status can elevate to number one pest (Sakovich, 1996) with crop losses sometimes being as high as 40%.

In the UK, an estimated 4,800 tonnes of molluscicidal products are applied, annually, to agricultural and horticultural crops, at a cost of approximately £10 million (Garthwaite and Thomas, 1996). In France, Europe's largest molluscicidal market, their usage is approximately three times greater (Meredith, 2003).

1.4 Methods of Controlling Terrestrial Molluscs

1.4.1 Chemical Control

Three major chemical compounds are used in the control of terrestrial molluscs, namely: metaldehyde, methiocarb and thiodicarb (Barker and Watts, 2002). In the UK, over 800,000 hectares of agricultural and horticultural crops are treated with chemical molluscicides each year (Garthwaite & Thomas, 1996). Out of the total area treated metaldehyde accounts for 55%, methiocarb 40% and thiodicarb 5%. The remainder (less than 1%) being made up by compounds such as aluminium sulphate, sodium tetraborate and iron chelates/iron phosphate. The two major chemicals used to control molluscs, metaldehyde and methiocarb, are described in more detail in sections 1.4.1.1 and 1.4.1.2.

1.4.1.1 Metaldehyde

The first major advance in the chemical control of molluscs was in 1934, with the discovery of the molluscicidal properties of metaldehyde in South Africa (Kelly and Martin, 1989). At the time metaldehyde, a polymer of acetaldehyde was sold commercially as a solid fuel. Today, in the UK, metaldehyde is sold as a 5% bait pellet formulation and is marketed under the trade name Meta®. The chemical structure of metaldehyde is illustrated in figure 1.3.

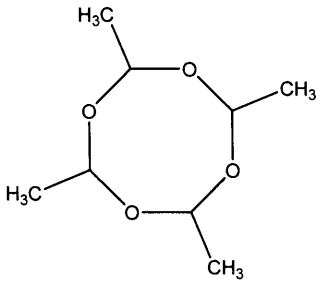

Figure 1.3 Chemical structure of Metaldehyde.

According to Godan (1983) the molluscicidal properties of metaldehyde is affected through ingestion and dermal contact. Triebskorn and Ebert (1989) described, in detail, the impact of metaldehyde on *D. reticulatum* slugs. They showed metaldehyde induced an immediate secretion of mucus immediately after application, followed by severe ultrastructural cell damage, which prevented further production of mucous. According to Meredith (2003), the mode of action of metaldehyde is dehydration, through excessive production of mucous, eventually leading to death. At temperatures around 20°C the activity of metaldehyde is optimised but at low temperatures its toxic effect is reduced (Triesbskorn *et al.*, 1998). Metaldehyde has a secondary neurotoxic effect, contributing to loss of motor activity (Coloso *et al.*, 1998).

1.4.1.2 Methiocarb

Methiocarb, common name mercaptodimethur, was initially developed as an insecticide and became available as a molluscicidal pellet around 1967 (Martin and Forrest, 1968), many decades after the discovery of metaldehyde. The chemical structure of methiocarb is illustrated in figure 1.4. In the UK methiocarb is commercially available as a 4% bait pellet formulation and is marketed under the trade name Draza ®. The pellets are applied at an application rate of 5.5kg/ha.

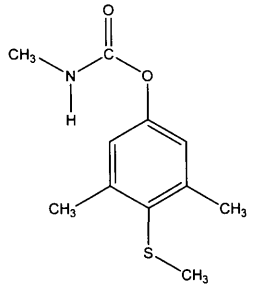

Figure 1.4 Chemical structure of Methiocarb

According to Meredith (2003), methiocarb has a different mode of action to metaldehyde, acting on nerve tissue through the inhibition of the neurotransmitter acetylcholine esterase. Poisoned slugs initially become hyperactive, lose muscle tone and eventually die (Godan, 1983). In contrast to metaldehyde, methiocarb works well at lower temperatures (2°C), which is advantageous for controlling *D. reticulatum*.

Both metaldehyde and methiocarb pellets show very high activity against slugs, nevertheless, both have their drawbacks. Slugs which have been immobilised after eating metaldehyde pellets have been observed to re-hydrate after encountering a moist environment, often leading to full recovery (Barker, 2002; Crawford-Sidebotham,

1970). Methiocarb has a more serious drawback than metaldehyde, related to its high mammalian toxicity profile. The oral toxicity of methiocarb (LD_{50}), against mice, is 52-58 mg/kg compared to metaldehyde which is 425 mg/kg (Simms and Wilson, 2003). Methiocarb has been shown to be toxic to earthworms, carabid beetles, spider mites, and many other non target organisms (Biery *et al.*, 1989; Purvis, 1996; Tomlin, 2000). It is environmental concerns, such as these, which make alternative chemical molluscicides more attractive to the public. Two examples of chemical molluscicides, regarded as less toxic, are iron chelate and iron phosphate pellets although high application rates (up to 50 kg/ha) may be required for them to effectively control molluscs (Meredith, 2003).

1.5 Biological Control

There has been much interest, recently, in the biological approach to mollusc control as an alternative to the chemical bait pellets. Molluscs have a large number of natural enemies, including pathogenic bacteria, viruses, predatory molluscs, flatworms, predatory arthropods, reptiles, birds and mammals (Barker and Watts, 2002). Recently the two most researched areas for biological control of molluscs has involved predator carabid beetles (*Abax* spp. and *Pterostichus* spp.) and the rhabiditid nematode *Phasmarhabditis hermaphrodita.*

1.5.1 Carabid Beetles

Using one metre square plots, Symondson (1993) demonstrated the use of the generalist predator carabid beetle, *Abax parallelepipedus*, to reduce *D. reticulatum* slug

populations. In support of these findings, Asteraki (1993) showed that two species of carabid beetles (*Pterostichus madidus* and *Abax parallelepipedus*) were as effective as methiocarb pellets in controlling slugs, in grass and clover swards. More recently Armsworth *et al.* (2005) showed that *D. reticulatum* slugs avoided areas which had previously been in contact with the carabid beetle *Pterostichus melanarius*. They concluded that the chemical cues from the beetle were responsible for the slug's antipredator behavioural response. Oberholzer and Frank (2003) studied the predation, by carabid beetles (*P. melanarius* and *Poecilus cupreus*), on the terrestrial slugs *Arion lusitanicus* and *D. reticulatum* and their eggs. Their study showed *P. melanarius* beetles consumed *D. reticulatum* eggs in preference to *Arion lusitanicus* eggs and was not distracted from feeding by alternative food choice, whereas the smaller *P.cupreus* beetle only consumed the smaller eggs laid by *D. reticulatum*. In addition they found only *P. melanarius* beetles killed and consumed the smaller *D. reticulatum* slugs in the presence of alternative prey.

Although carabid beetles have been shown to reduce slug populations, the major problem with their application is their lack of specificity. The carabid beetles are generalist predators, hence may feed on alternative food, such as crickets or aphids, in preference to slugs if they are available (Oberholzer and Frank, 2003).

1.5.2 Nematodes

The use of nematodes, as a biological control agent, has been very successful in reducing mollusc populations and has resulted in the commercialisation of the rhabtitid nematode *P. hermaphrodita,* in 1994, in the form of Nemaslug® (MicroBio Ltd, UK). *P. hermaphrodita* is a bacterial-feeding nematode and is a lethal parasite of slugs of the families Arionidae, Limacidae and Milacidae (Wilson *et al.*, 1993). Many studies have been published showing the high mollucicidal activity of nematodes. Glen *et al.* (2000) performed laboratory trials with *P. hermaphrodita* nematodes and showed that the feeding behaviour of *D. reticulatum* slugs was significantly reduced, after exposure to two different concentrations of juvenile nematodes. The high concentration (300,000 juvenile nematodes per arena), resulted in high slug mortality in a short space of time, whereas the lower concentration (7,000 juvenile nematodes per arena) showed low slug mortality but strongly inhibited feeding. Grewal *et al.* (20001) used green house trials to show that applying *P. hermaphrodita* nematodes to slug shelters was an effective method of slug control and was more economical than broad application to the soil. They concluded that the use of nematodes was equally or more effective than metaldehyde pellets, in terms of leaf damage to *Impatiens* and *Hosta* plants.

Many other researchers have validated the effectiveness of the rhabtitid nematode, *P. hermaphrodita*, as an effective form of mollusc control (de Werd *et al.*, 2001; Glen *et al.*, 2001; Castillejo *et al.*, 2001 and Ester and Van Rozen, 2001). Unlike chemical molluscicides, this form of mollusc control poses no threat to non-target organisms (Wilson *et al.*, 2001). Application on an agricultural scale, however, raises

the problem of availability and shelf-life of the product. There have been some problems with maintaining nematode/bacterial virulence (Bowen, I. D., Personal communication). The major drawback of using nematodes as molluscicides is the high price. Chemical molluscicides cost approximately €20 per hectare compared to commercial nematodes retailed at €200 per hectare. These high prices are expected to be reduced, with increasing demand for this biological form of mollusc control (Verdun and Linton, 2004).

1.6 Botanical Control

Currently, there is renewed interest in plant molluscicides as a cheaper and safer means of mollusc control. Historically, the development of plant molluscicides was directed at controlling aquatic molluscs which are vectors of tropical diseases such as schistomiasis. According to Marston and Hostettman (1985) over 1000 plant species had been evaluated as sources of natural chemicals to control aquatic molluscs, resulting in the isolation of 70 natural products with strong molluscicidal activity. One of the most well known plants used to control aquatic molluscs is the Ethiopian *endod* plant, *Phytolacca dodecandra.* The berries of this plant were demonstrated to have strong molluscicidal activity against the aquatic mollusc, *Biomphalaria pfeifferi*, the intermediate snail host of *Schistosoma mansoni* (Lemma *et al.*, 1972). In our laboratories the leaves and bark of the Nigerian plants, *Detarium microcarpum* and *Ximenia americana*, were shown to have potent molluscicidal activity against the golden apple snail *Pomacea canaliculata* (Arthur *et al.*, 1996), as well as terrestrial molluscs, such as *D. reticulatum*, (Ali *et al.*, 2003a).

The use of plants as a source of controlling terrestrial molluscs has recently become more popular in Europe, mainly due to the environmental problems associated with chemical control products such as metaldehyde and methiocarb pellets. Current trends, however, seem to be to steer away from killing molluscs and instead to modify their feeding behaviour using chemicals of low or zero toxicity as part of an integrated pest management system (Clark *et al.*, 1997). Since many plants synthesise chemicals for protection against herbivores, a reservoir of potential antifeedant compounds already exists, for testing against terrestrial molluscs. Such an approach also helps protect biodiversity, including that of target species.

Table 1.1 shows a selected number of plants, and their chemical components, that have been used to control the activity of terrestrial molluscs.

Table 1.1 Examples Of Plants Used To Control The Feeding Behaviour Of Terrestrial Molluscs

Plant Name	Active Component	Mollusc Tested	Method of Application/ Mode of Action	Reference
Ghalqa (*Pergularia tomentosa*)	Cardenolide	***Snails**: Monacha obstructa*	Spray /Molluscicide	Hussein *et al.* (1999)
Potato (*Solanum tuberosum*)	α-Solanine, α-Chaconine	***Snails**: H. aspersa*	Paper discs/ Antifeedant	Smith *et al.* (2001)
Lichen (*Letharia vulpina*)	Vulpinic acid	***Slugs:** D. reticulatum*	Spray/ Antifeedant	Clark *et al.* (1998)
Garlic (*Allium sativum*)	Allicin	***Slugs**: D. panormitanum,* ***Snails**: Oxyloma pfeifferi* snails	Spray / physical barrier	Schüder *et al.* (2003)
Yucca schidigera	Saponins	***Slugs:** Arion hortensis, Arion circumscriptus,* ***Snails**: H. aspersa*	Spray /Antifeedant	Mason *et al.* (1994)
Hedera helix	Saponins	***Slugs:** Arion hortensis, Arion circumscriptus,* ***Snails**: H. aspersa*	Spray /Antifeedant	Mason *et al.* (1994)
Cinnamon (*Cinnamomum* spp.)	Cinnamamide	***Slugs**: D. reticulatum, D. panormitanum,* ***Snails**: Oxyloma pfeifferi*	Seed dressing /Antifeedant	Watkins *et al.* (1996)
Fennel (*Feoniculum vulgare*)	(+) - Fenchone	***Slugs**: D. reticulatum*	Seed dressing/Antifeedant	Garrway *et al* (1991)
Hemlock (*Conium maculatum*)	γ-Coniceine,	***Slugs**: D. reticulatum*	Repellent /Antifeedant	Birkett *et al.* (2004)
Hemlock (*Conium maculatum*) *Petroselinum crispum*	Not tested	***Slugs**: D. reticulatum*	Repellent /Antifeedant	Dodds *et al.* (1999)
Coriandrum sativum	Not tested	***Slugs**: D. reticulatum*	Bait Pellets /Antifeedant	Dodds *et al.* (1999)

1.7 Burseraceae

The Burseracea family of trees are widespread in all tropic, and some subtropic, regions and comprise of 17 genera and 500-600 species (Vollesen, 1989; Thulin, 1999). They are often the dominant vegetation type in dry lowland areas. In Somalia and Ethiopia there are 2 genera (***Commiphora*** and ***Boswellia***) and over 58 species. Both genera have ducts present in the bark. When the bark is wounded, the tissues between the ducts break down to form large cavities which are filled with granular secretions (Grieve, 1992). These secretions flow freely and on exposure to air solidify to form solid oleoresins such as **myrrh** (*C. molmol*), **opoponax** (*C. guidotti*) and **frankincense** (*B. carteri*).

1.8 **Boswellia** Roxb. (1807)

These trees have an outer bark which often peels, leaves which are often clustered at the tips of the branches and flowers which are bisexual in panicles or racemes (Vollesen, 1989; Thulin, 1999). There are about 20 species in the dry regions ranging from western Africa to southern Arabia and south to Tanzania and India. The genus *Boswellia* is centered in north east Africa, where approximately 75% are endemic (Vollesen, 1989). The most important members of the *Boswellia* species are the frankincense producing trees such as: *B. frereana* Birdw. (1870), which only grows in northern Somalia, *B. carteri* Birdw. (1870) found in northern Somalia and southern Arabia and *B. sacra* Flück (1867), which is indigenous to southern Arabia and is synonymous with the Somali species *B. carteri* (Thulin, 1999).

1.9 **Commiphora** Jacq. (1797)

The genus *Commiphora* is comprised of 150-200 species and is found widespread throughout the drier parts of tropical Africa. The genus extends as far as India, via the Arabian peninsular, and Iran (Thulin, 1999). A few species are also found in Mexico and Brazil. The most economically important members of this genus are the myrrh producing trees (*C. molmol* and *C. myrrha*) common to the horn of Africa and southern Arabia, and the opoponax bearing trees (*C. guidotti*) found throughout Somalia and some regions of eastern Ethiopia (Bale).

1.9.1 ***Myrrh:*** *C. molmol* (Engl.) Engl. (1931) / *C. myrrha* (Nees) Engl. (1883)

Myrrh (Somali name: **Malmal**; Arabic name: **Murr**) is the name used for the solid oleoresin obtained from the stem of *C. molmol* and *C. myrrha*. The trees are found growing wild in north-eastern Africa and southern Arabia (BP Codex, 1968).

The myrrh trees grow up to 5 metres tall and have branches which often terminate in spines and an outer bark often peeling away from the green underbark. Many *Commiphora* spp. trees are leafless for most of the year and have flowers, which are unisexual and dioecious (Vollesen, 1989; Thulin, 1999). The flowers are usually produced before the leaves appear.

True myrrh oleoresins are only obtained from *C. myrrha* (Nees) Engl. (1883) indigenous to southern Arabia and *C. molmol* (Engl.) Engl. (1931) found growing widely in Somalia, eastern Ethiopia and northern Kenya (see figure 1.5). The two

species are considered to be synonymous (Hedberg and Edwards, 1989). Some researchers have argued for *C. molmol* to be reserved for the African myrrh tree and *C. myrrha* for the Arabian myrrh tree (Cufodontis, 1956). There are many other *Commiphora* spp. trees producing oleoresins in the "horn of Africa", such as *C. abyssinica*, *C. holtziana*, *C. sphaerocarpa* and *C. hildebrandtii*. Some times "true" myrrh oleoresins are found adulterated with similar looking oleoresins collected from these other *Commiphora* spp trees. Myrrh oleoresins are found in the form of irregular or rounded tears, 1.5 cm to 10 cm in diameter, and are reddish-brown or reddish yellow in colour (BP Codex, 1968). South Arabian myrrh (*C. myrrha*) has been described as being paler, less bitter and less fragrant than African myrrh (*C.molmol*).

The Somalis recognise two qualities of myrrh: ***guban malmal*** collected from trees on the coastal plain and ***ogo malmal*** gathered from the inland plateau (Drake-Brockman, 1912). There are clear differences between the appearances of the two myrrh oleoresins. Ogo malmal has a reddish yellow colouration and has been described as being more friable, powdery and bitter in taste than guban malmal (Groom, 1981). Guban malmal has a blood red coloration and has higher oil content than Ogo malmal. The appearance of Ogo myrrh correlates well with that described for Yemeni myrrh (BP Codex, 1968) and is illustrated in figure 1.6.

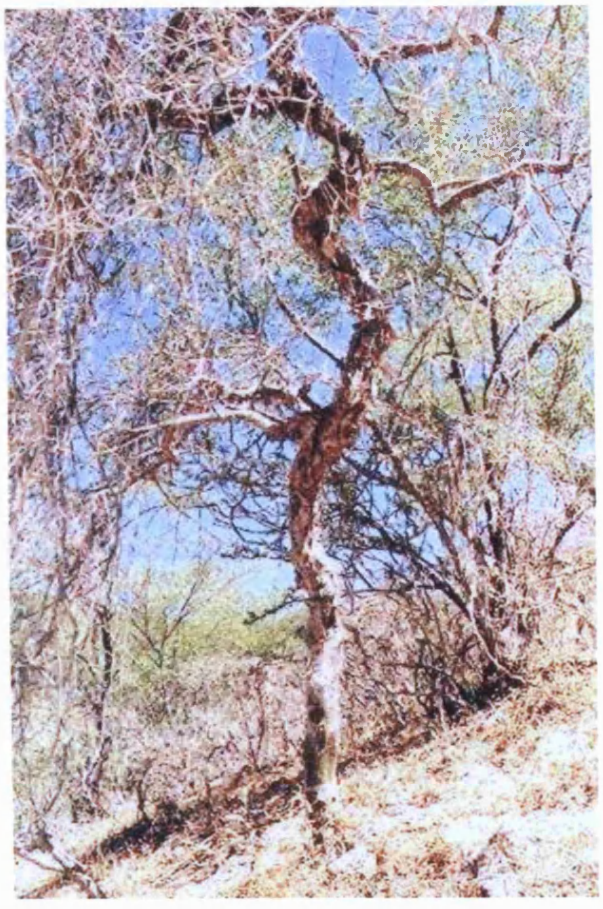

Figure 1.5 Myrrh Tree (*C. myrrha*) growing in southern Arabia (Yemen). Reproduced from Crystal (2005).

Figure 1.6 **Myrrh** (*C. molmol*) oleoresin collected from: **A)** Yemen (Baldwin) and **B)** Somalia. Notice the small dry oleoresin granules of the Yemen myrrh compared to the large oily lumps of Somali myrrh oleoresins.

1.9.2 **Opoponax:** *C. guidotti* Chiov. (1932)

Until recently the origin of this oleoresin was thought to be *C. erythraea* var. *glabrescens* Engl. (1883), which is found to grow only in southern Somalia and northern Kenya. In 1991 Thulin and Claeson confirmed the botanical origin of opoponax to be *C. guidottii* which is indigenous to most parts of Somalia and eastern Ethiopia. *C. guidotti* is found to grow in open *Acacia-Commiphora* bushland on stony slopes and are always found on gypsum soils (Vollesen, 1989; Thulin, 1999), see figure 1.7. The tree grows up to 5 metres tall and has a smooth bark which usually peels off as yellow or white flakes. The leaves are described as 1 to 5 foliates with petioles 0.5 to 10 cm long. This tree is said to be synonymous with another *Commiphora* spp called *C. sessiliflora* Vollesen (1985).

Opoponax oleoresins are commonly known as ***scented myrrh*** (in Europe and USA) ***bissabol myrrh*** in India and as ***habak haddi*** in Somalia. The oleoresin is red in colour and has a high oil content (see figure 1.8). Drake-Brockman (1912) described opoponax oleoresins as being large irregular lumps of much the same colour as myrrh with the additional occurrence of white areas which resemble toffee. Unique to opoponax oleoresins is the infrequent presence of black odourless granules, which comprise older pieces of opoponax oleoresin. The strong sweet smell associated with opoponax oleoresins is quite unlike that of myrrh (balsamic smell); hence the two oleoresins are rarely mixed together by accident.

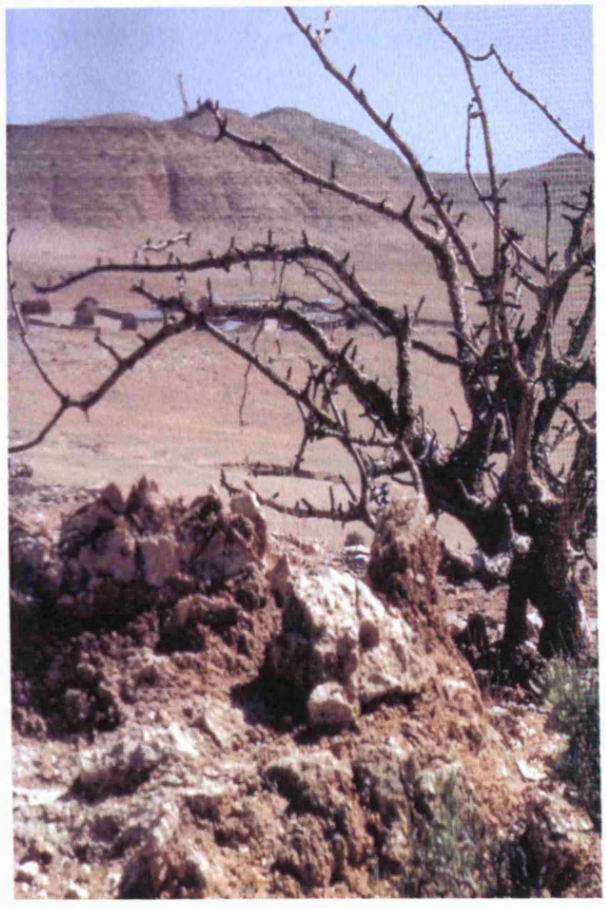

Figure 1.7 Opoponax Tree (*C. guidotti*) growing in Somalia (Laascaanood region) Reproduced from Thulin (1999).

Figure 1.8 Opoponax oleoresins collected from Somalia. Notice the characteristic white bone-like streaks present on some of the lumps.

Figure 1.9 The Painting on the Wall of Queens Hatshepsut's Temple at Dier al-bahri shows two "Punite" men depicted as carrying *Commiphora* spp trees, as gifts to the Ancient Egyptians (Source: http:// www.ancient-egypt.org/glossary /punt.html.).

Figure 1.10 The above map shows the ancient trade routes used for Myrrh (*Commiphora* spp) and Frankincense (*Boswellia* spp.). The map also illustrates the geographical distribution of these oleoresin producing trees. Place names are in written in the Greek language. The map is reproduced from Porter (2005).

1.11 Historical, Traditional And Current Uses Of Myrrh and Opoponax

Myrrh has been regarded as one of the finest treasures of the Far East for thousands of years, starting from the Egyptian period to the Arabian spice trade era.

According to Groom (1981) pure myrrh was the main ingredient of the holy anointing oil of the Jews described in the Bible (Exodus) and was commonly added to wine to "diminish drunkenness". In the 5th century BC, the Greek historian Herodotus described, in detail, how the Egyptians used myrrh for embalming bodies (Waterfield, 1998). In the fourth century AD, a Greek physician gave a series of lectures on medicine which were translated to the Syrian language. These lectures were known as the "Syriac Book of Medicine" (Groom, 1981). This book contains many references to the medicinal applications of myrrh, such as its use for toothache, stomach pains, pleurisy, constipation, jaundice and insect bites. At the beginning of the twentieth century the medicinal use of myrrh became official, in both Britain and the USA. Its medicinal applications were described in detail in the British pharmaceutical codex (1911) and the 1926 edition of the National Formulary (now known as the United States Pharmacopoeia).

Traditionally, the Somalis apply emulsions of myrrh to the skin to heal cuts and wounds. They dissolve a few grains of myrrh in warm water to cure back pain, whilst a warm bath containing myrrh is used by women after giving birth. The smokes produced, by burning myrrh, frankincense and opoponax, are used by women to repel snakes and flies from their houses. Opoponax has traditionally been used in Somalia to

treat ailments such as stomach pain, cholera, wounds and for facilitating the expulsion of the placenta after child birth (Thulin, 1999).

Due to its astringent properties, myrrh is used in western countries, to sooth inflamed tissues in the mouth and throat. Both myrrh and opoponax are regularly used in the flavour and fragrance industry. Myrrh is still used today as a component in western perfumes. Myrrh has excellent fixative properties which enables it to retain the volatile "top notes" in perfumes for long periods. Myrrh is employed in modern perfumes as an absolute, essential oil or resinoid (Watt and Sellar, 1996). Examples of perfumes which contain myrrh are *Le Jardin* (Max factor), *Opium* (St Laurent), *Le Sport* (Coty) and *Givenchy III* (Givenchy).

A number of academic publications have validated the traditional medicinal and pesticidal uses of myrrh and opoponax. Tariq *et al.* (1985) highlighted the significant anti-inflammatory properties of myrrh (*C. molmol*), while Al-Harbi *et al.* (1994) highlighted the cytoxic and anti-carcinogenic potential of *C. molmol,* after testing it against mouse solid tumours, induced by Ehrlich carcinoma cells. An aqueous suspension of myrrh (*C. molmol*) has been shown to protect against stomach ulcers (Al-Harbi *et al.*, 1996). The significant antioxidant and antibacterial properties of myrrh has also been demonstrated (Assimopoulou *et al.*, 2004; Hammer *et al.*, 1999).

Myrrh oleoresin has, recently, been cited as a successful treatment for schistosomiasis, a widely occurring helmintic disease mainly affecting developing countries (Massoud *et al.*, 2003; Massoud, *et al.*, 2004; Sheir *et al.*, 2001). This exciting discovery is, however, controversial as many researchers have not been able to

reproduce the very high schistosomicidial activities reported for myrrh (Fenwick *et al.*, 2003; Botros *et al.*, 2004; Botros *et al.*, 2005). Massoud *et al.* (2001) also demonstrated myrrh's high therapeutic efficacy against fascioliasis, a zoonotic disease caused by Fasciola (liver fluke), which normally affects sheep, goats, cattle, and increasingly humans.

A hexane extract of opoponax, incorrectly classified as *C. erythrae,* was shown by Carrol *et al.*, (1989) to have a repellent and toxic effect against three species of animal ticks. Wilson *et al.*, (1993) demonstrated one of the major components of opoponax, α-bisabolene and its analogues, to be strong repellents against the house fly (*Musca domestica*) and mosquito's (*Aedes aegypti*). Massoud *et al.* (2001) found the resin extract of myrrh to have strong larvicidal activity against mosquito larvae (*Culex pipiens*).

Allam *et al.* (2001) showed myrrh oil (*C. molmol*) extracts to have significant molluscicidal activity against aquatic molluscs (*Biomphalaria alexandria*, *Bulinus truncatus* and *Lymnaea cailliaudi*) and their eggs. According to Abel-Hay *et al.* (2002) the oil fraction was responsible for the molluscicidal activity of myrrh, as opposed to the resin (alcoholic) fraction.

1.12 Aims and Objectives

Both myrrh (*C. molmol*) and opoponax (*C. guidotti*) oleoresins contain a wide bouquet of chemicals which contribute to their highly valued pleasant aromas. Myrrh oleoresins have a balsamic, medicinal smell whilst opoponax oleoresins have a perfume-like, sweet smell associated with it. These volatile chemicals may play a major part in the many medicinal and agrochemical applications that have been reported for these oleoresins, both anecdotal and scientific.

The main aim of this thesis is to evaluate myrrh and opoponax oleoresins and their extracts as potential mollusc control agents. The chemical profile of these oleoresins will be investigated and identified chemical components assessed for their mollusc control properties.

Their repellent, antifeedant and molluscicidal effects upon terrestrial molluscs (*D. reticulatum*, *A. hortensis* slugs and *H. aspersa* snails) will be assessed using a number of screening methods both under laboratory and field conditions.

In summary the main objectives of this PhD thesis are:

1. To evaluate the repellent and molluscicidal properties of solid barriers comprised of 100% myrrh and opoponax oleoresins and their mixtures with inert substrates (sawdust, sharp sand and corncob), using laboratory terraria methods.

2. To evaluate the repellent and molluscicidal properties of solid barriers comprised of sawdust, treated with ethanol and essential oil extracts of myrrh and opoponax, using laboratory terraria methods.

3. To determine any toxicity effects of *Commiphora* spp. oleoresins against non-target organisms (earthworms) and phytotoxicity effects towards winter wheat seeds.

4. To identify and characterise the chemical profiles of the liquid extracts of myrrh and opoponax extracts using gas chromatography-mass spectrometry (GC-MS) analytical techniques.

5. To identify and characterise the chemical profiles of the volatile cues associated with myrrh and opoponax oleoresins and their extracts, using GC-MS and solid phase microextraction (SPME) analytical techniques.

6. To investigate the antifeedant properties of liquid extracts of myrrh and opoponax oleoresins, and their chemical components, towards the terrestrial molluscs, *D. reticulatum.*, using the leaf disc assay method.

7. To develop a stable spray emulsion formulation based on myrrh, opoponax and their chemical components.

8. To ascertain the antifeedant activity of spray emulsion formulations towards *H. aspersa*, using the leaf disc assay method.

9. To assess the efficacy of emulsion spray formulations, based upon *Commiphora* spp. extracts and their components, against *D. reticulatum* and *H. aspersa*, under spray trials conditions.

10. To evaluate any phytotoxicity effects of emulsion spray formulations, based upon *Commiphora* spp. extracts and their components, on lettuce plants, under spray trial conditions.

11. To ascertain the efficacy of physical barriers comprised of myrrh and opoponax, their mixtures with sawdust and their plant extracts coated onto sawdust, under caged-field conditions.

CHAPTER 2:

TERRARIA TRIALS

2.1 INTRODUCTION

Many types of barriers are used to deter slugs and snails from damaging garden and horticultural plant beddings. Copper is commonly used as a barrier against molluscs. Copper barriers, usually about six inches tall, can be erected around planting beds and buried several inches below the soil to prevent slugs crawling beneath the barrier. Commercially, copper foils such as Snail-Bar™ are available to wrap around planting boxes. According to Flint (2003), copper barriers are thought to react with the slime produced by the slug or snail, resulting in the generation of a flow of electricity that deters it from any further contact.

Hata, *et al.* (1997) evaluated the barrier properties of copper, aluminium, fibreglass and paper against the terrestrial slugs *Vaginula plebeian* and *Veronicella cubensis*. They found the copper barriers to be the most effective in preventing slugs from feeding on moistened slug baits.

The barrier efficacies of other types of materials, including copper, were evaluated against *Deroceras panormitanum* slugs and *Oxyloma pfeifferi* snails (Schüder *et al.*, 2003). They evaluated the vertical and horizontal efficacy of various barriers comprising of copper foil, its solution as copper ammonium carbonate as well as many other treatments including aluminum foil, garlic solution (2.5% and 5%), Snail Ban ™ (a kaolin mineral commercial barrier), a 1% solution of cinnamamide and a 6% solution of ureaformaldehyde. On testing the various barriers they concluded that garlic, ureaformaldehyde and cinnamamide were the three best products for controlling mollusks, resulting in mortality rates between 20% and 94% over a seven day period

and reduced feeding by 41% to 100%. Their findings also validated the use of copper foil as a horizontal barrier resulting in barrier efficacies of 20% and 80% against *D. panormitanum* slugs and *O. pfeifferi* snails respectively.

The barrier trial with garlic and cinnamamide is only one of many reports investigating the use of plant extracts and their chemical components to repel terrestrial molluscs. Mason, *et al.* (1994) showed that compositions containing saponins from the plants *Yucca schidigera* and *Hedera helix* prevented terrestrial molluscs from feeding upon plants. Increasing the saponin contents from 0.05% to 0.15% changed the mechanism of action from an antifeedant effect to a molluscicidal one.

Grubisic *et al.* (2003) investigated the repellent and molluscicidal effect of some botanical treatments (lavender and rosemary), physical barriers (sawdust), a product based on bicarbonate of soda as well as the chemicals methiocarb, metaldehyde and iron phosphate pellets on protecting beans, lettuce and Swiss chard crop against the slug pests *Arion rufus* and *D. reticulatum*. In trials carried out in lettuce and beans, they noticed the phytotoxic effects of the lavender mixture and the product based on bicarbonate of soda. In the field experiments, all the treatments failed to have a positive effect on slug control after the sixth day of assessment.

Recently, we have shown in our laboratories that certain Nigerian plants and their extracts exhibit both repellent and molluscicidal properties against the field slug *D. reticulatum* (Ali *et al.*, 2003). Barriers comprised of Nigerian plant material *Detarium microcarpum* bark, the leaves and bark of *Ximena americana* and *Polygonum limbatum* shoots as well as their mixtures with sawdust (50:50) and sawdust coated

with their ethanol extracts (20%) were evaluated. *D. microcarpum* raw material (100%) was found to be a potent molluscicide with 60% slug mortality whilst the alcoholic extracts of the barks of *X. americana* and *D. microcarpum* were highly repellent to slugs but caused low mortality. Although the Nigerian plants were highly effective mollusc barriers against slugs in laboratory and in field conditions, their registration as a commercial product could prove a lengthy process, since the safety profile of these plants are not well documented.

In this chapter, the barrier properties of two odoriferous Somali oleoresins called myrrh (*C. molmol*) and opoponax (*C.guidotti*) will be evaluated against the field slug *D. reticulatum* using terraria methods similar to those used for evaluating the Nigerian plants. These Somali oleoresins are initially liquid exudates, produced on wounding the tree bark, and solidify on exposure to air. These oleoresins are highly odoriferous and would be good candidates for testing as mollusc repellents.

Both *Commiphora* spp. oleoresins are known through local folklore to be repellent to insects especially flies and mosquitoes, when the oleoresins are applied as fumigants. Opoponax extracts have been reported to be active against ticks (Maradufu, 1982; Carrol *et al.*, 1989). Myrrh has been shown to possess larvicidal activity against *Culex pipiens* and *Aedes caspius* mosquito larvae. In addition to these repellent activities, both oleoresins are taken orally in Somalia and Ethiopia as herbal medicines and are acknowledged to be relatively non-toxic to humans.

Both of these oleoresins have wide commercial uses in Europe and the USA mainly in the flavour and fragrance industries. In the western hemisphere, myrrh is

employed in oral health care products such as mouthwashes and toothpaste. Both oleoresins are registered as GRAS (generally regarded as safe) hence are expected to have a better safety profile against non target organisms than Nigerian plants which are potent contact molluscicides and have no traditional history of being used as oral medicines.

2.2 Aims and Objectives

The main aim of this chapter is to develop a mollusc repellent barrier, of botanical origin, which can be used in the home and garden. Various barriers will be tested against terrestrial molluscs (*D. reticulatum, A. hortensis and H. aspersa*), using laboratory terraria trials, with the aim of determining whether:

i) When applied as barriers, myrrh and opoponax solid oleoresins have repellent or molluscicidal properties towards terrestrial molluscs.

ii) When applied as barriers various inert materials, such as sawdust (Spruce and Beech), kaolin clay, corn cob and sand are suitable inert substrates for mixing with the solid oleoresins.

iii) When applied as barriers, sawdust treated with myrrh and opoponax extracts have repellent or molluscicidal properties.

iv) When applied as barriers, sawdust treated with *trans*-β-ocimene, a major chemical component of opoponax, has repellent or molluscicidal properties.

v) Myrrh oleoresin barriers have any toxic effects on non-target organisms (earthworms).

vi) Myrrh and opoponax extracts have any phytotoxic effects on winter wheat seeds.

In this chapter inert" is defined as materials which when applied as barriers alone demonstrates little repellence or molluscicidal activity towards terrestrial molluscs. The purpose of screening inert substrates is to determine a suitable substrate or carrier that can be used to mix with the more expensive *Commiphora* spp. oleoresins.

2.3 MATERIAL AND METHODS

Myrrh (*C. molmol*) oleoresin, originally sourced from Yemen, was purchased from Baldwin and Co., (Middlesex, London) and opoponax (*C. guidotti*) oleoresin from Hargeisa (Somalia). Myrrh (*C. molmol*) and opoponax (*C. guidotti*) essential oils and dimethyl sulfoxide (D.M.S.O) (analar grade) were all purchased from Sigma-Aldrich (Poole, Dorset). Alcoholic tincture of myrrh (20%) was obtained from Thornton and Ross (Huddersfield, Yorkshire). Spruce sawdust, Beech sawdust (Lignocel^{TM}) and corn cob were kindly provided by RS Biotech Limited (Finedon, Northants). Sharp sand (lime free agricultural grade) was purchased from Wickes Limited (Cardiff, South Glamorgan). Absolute ethanol and methanol (HPLC grade) were purchased from Fisher Scientific (Loughborough). Peat soil and a commercial mollusc barrier called Slug Stop® (Growing Success), comprising of amorphous silica flakes (Diatomite), were purchased from B & Q (Cardiff, South Glamorgan). Blotting paper (A5 size) was purchased from Office Depot (Andover, Hampshire).

2.3.1 *Preparation Of Alcoholic Extracts Of Myrrh And Opoponax Oleoresins (20%)*

Alcoholic oleoresin extracts (20%) were prepared by transferring 40g of myrrh and opoponax granules to separate containers, diluting to 200ml with absolute ethanol and leaving overnight. Both oleoresin extracts were separately filtered, under vacuum, using a Büchner flask.

2.3.2 Preparation Of Myrrh Essential Oil Extract in Ethanol (1%)

Myrrh essential oil extract was prepared as a 1% essential oil solution in ethanol by weighing 2g of essential oil into a 200ml volumetric flask and diluting to volume with absolute ethanol.

2.3.3 Preparation Of Myrrh, Opoponax And Trans-β-Ocimene Oil Extracts (0.5 And 1%) In Aqueous DMSO

Myrrh and *trans*-β-ocimene oil extracts (0.5%) were separately prepared by adding 0.5g of the oil to a 100ml volumetric flask containing 2.5g of DMSO. This was homogenously mixed together by shaking and then diluted to volume with de-ionised water. Opoponax oil extract (1%) was prepared by adding 1g of opoponax oil to a 100 ml volumetric flask, containing 2.5g of DMSO, shaken to mix and diluted to volume with de-ionised water. A control was prepared similarly comprising of DMSO (2.5g) in 100ml water.

2.3.4 Preparation Of Myrrh, Opoponax and Trans-β-Ocimene Oil extracts (0.5 and 1%) in Aqueous Tween 80

Myrrh and *trans*-β-ocimene oil extracts (0.5%) were separately prepared by adding 0.5g of myrrh oil to a 100ml volumetric flask containing 0.2g of Tween 80. This was homogenously mixed together by shaking and then diluted to volume with deionised water. Opoponax essential oil (1%) was prepared similarly, with the variation of adding 1g of opoponax essential oil to a 100 ml volumetric flask containing

0.2g of Tween 80. A control was prepared comprising of 0.2g of Tween 80 in 100ml water only.

2.3.5 Procedure For Coating Sawdust With Plant Extracts

Sawdust (100g) was covered with 100ml of myrrh, opoponax or ocimene extract and mixed homogenously with a spatula. This was transferred to a polythene bag and further shaken for two minutes. The treated sawdust was emptied on to a clean dry surface and the solvent allowed to evaporate to air.

2.3.6 Evaluation of Inert Substrates As Barriers

Initial pre-screening of inert materials showed cellulose powder and silica powder to be unsuitable as a repellent barrier. Both powders dissolved on contact with water. Cellulose paper granules (Grantex) were also found to be unsuitable since its light weight would have a propensity to be blown around in a field. The materials initially tested for their inertness as repellent barriers were Kaolin, Spruce sawdust, and Sharp Sand. Later in the terraria tests, Beech sawdust (Lignocell^{TM}) and corn cob became available for further testing as inert materials (see section 2.3.8).

2.3.7 Evaluation of Myrrh And Opoponax As Barriers

Myrrh (Baldwin) oleoresin was purchased as grain sized granules, hence its physical nature did not need to be altered for the tests. Somali myrrh (*C. molmol*) and opoponax (*C. guidotti*) oleoresin, however were purchased as large lumps. These large lumps were broken down into smaller granules, in a mortar and pestle, before use. Both these materials were placed around winter wheat seeds and evaluated as repellent barriers.

2.3.8 Evaluation of Mixtures Of Myrrh Oleoresin and Inert Substrates As Barriers

Myrrh (Baldwin) oleoresin was mixed with various inert substrates to determine their effectiveness as repellent barriers. The inert substrates that were separately mixed with myrrh comprised of beech sawdust (Lignocell ^{TM}), corn cob and sharp sand. The former two substrates were prepared as 50:50 mixtures (i.e. 50% myrrh oleoresin / 50% inert substrate). The myrrh content of the myrrh / sharp sand barrier, however, was increased to 60%, due to difference in density of the two materials in order to maintain a consistent volume of repellent barrier i.e 2.4g of myrrh was mixed with 1.6g of sand (total weight of barrier was 4g). Myrrh / substrate mixtures were placed around the winter wheat seeds as repellent barriers. Four controls were also prepared comprising unprotected winter wheat seeds, Spruce sawdust, corncob and sharp sand barriers. Typical dimensions of the barriers evaluated are illustrated in figure 2.3.

2.3.9 Evaluation of Mixtures Of Myrrh Oleoresin and Inert Substrates As Barriers (Peat Soil)

Baldwin myrrh (100%) oleoresin granules and mixtures with sawdust and corncob inert materials were evaluated as described in 2.3.8 with the variation of replacing the blotting paper substrate with peat soil.

2.3.10 Evaluation of Mixtures Of Myrrh Oleoresin and Corn Cob Substrate As Barriers (Myrrh Dose-Activity Relationship)

The relationship between Baldwin myrrh dose concentration and barrier efficacy was evaluated by preparing barriers containing myrrh and corncob, in the ratio 0.8g/3.2g, 1.2g/2.8g and 1.6g/2.4g. These weights corresponded to a myrrh content of 20%, 30% and 40% w/w respectively.

2.3.11 Evaluation of Alcoholic Extracts of Myrrh and Opoponax Oleoresins as Barriers

Sawdust samples previously coated separately with either alcoholic myrrh (Baldwin) or opoponax oleoresin extracts (20%) were placed around three groups of eight winter wheat seeds as repellent barriers as shown in figure 2.3. A barrier comprised of sawdust coated with a commercial ethanolic myrrh (Thornton & Ross) extract was similarly prepared. Three controls were also tested comprising unprotected winter seeds, sawdust, and sawdust treated with absolute ethanol as repellent barriers.

2.3.9 Evaluation of Mixtures Of Myrrh Oleoresin and Inert Substrates As Barriers (Peat Soil)

Baldwin myrrh (100%) oleoresin granules and mixtures with sawdust and corncob inert materials were evaluated as described in 2.3.8 with the variation of replacing the blotting paper substrate with peat soil.

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2.3.11 Evaluation of Alcoholic Extracts of Myrrh and Opoponax Oleoresins as Barriers

Sawdust samples previously coated separately with either alcoholic myrrh (Baldwin) or opoponax oleoresin extracts (20%) were placed around three groups of eight winter wheat seeds as repellent barriers as shown in figure 2.3. A barrier comprised of sawdust coated with a commercial ethanolic myrrh (Thornton & Ross) extract was similarly prepared. Three controls were also tested comprising unprotected winter seeds, sawdust, and sawdust treated with absolute ethanol as repellent barriers.

2.3.12 Evaluation Of Myrrh, Opoponax And trans-β-Ocimene Oils, Coated Onto Sawdust, As Barriers

Winter wheat seeds were surrounded with barriers of sawdust coated with 0.5% myrrh essential oil, 1% opoponax essential oil and 0.5% *trans*-β-ocimene oil. These oils were solubilised in ethanol, aqueous DMSO and aqueous Tween 80. Controls were prepared by coating sawdust with the various solvents used. Any residual solvent was allowed to evaporate before use.

2.3.13 Evaluation Of Toxicity Effects Of Myrrh And Opoponax Barriers On Non-Target Organisms: Earthworm (*Lumbricus terrestris*)

The test method used for assessing non target effects of myrrh barriers, against earthworms was a modification of the method published by Bieri *et al.* (1989). The lower end of a glass funnel (diameter 12cm) was connected to a 20cm length of clear plastic tubing and sealed with a cork stopper, as shown in figure 2.1. Peat soil was added to the glass funnel filling it to a three quarter depth (for peat soil this corresponds to approximately 18g and for garden soil approximately 40g). A hole was drilled in the middle of the soil, using a pencil, to produce a burrow for the earthworm. One earthworm (*L. terrestris*) was introduced into each glass funnel, approximately 2 g of plant material added evenly to the soil surface and a little water to moisten the soil before covering the funnel with para-film. Experiments were conducted in the dark, at a temperature of 18°C, and monitored for mortality over a seven day period. Earthworms were recorded as dead when failing to respond to a 9-volt stimulus.

Observations were recorded on day 3 and day 7. A control was prepared similarly with the omission of the plant material. Twenty replicates were prepared per condition.

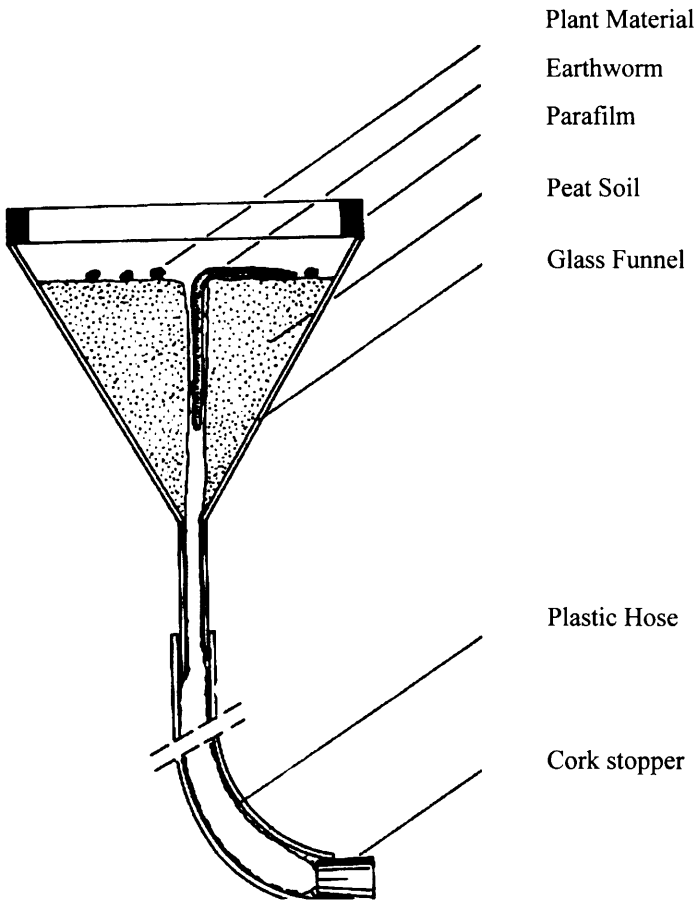


Figure 2.1 Section Through A Funnel For Earthworm Observations. Reproduced From M. Bieri *et al.* (1989).

2.3.14 Evaluation Of Phytotoxic Effects Of Myrrh Oleoresin Barriers on Winter Wheat Seeds

Plastic trays (32cm x 22cm x 5cm, 0.07m^2) were lined with peat soil and three groups of eight winter wheat seeds added to the surface. These seeds were surrounded by three 4 g barriers of myrrh oleoresin granules (approximately 2 cm width) and moistened with water. The trays were kept in a controlled temperature unit with constant environmental conditions (12 hour light: dark regime 15°C, 90% humidity). The coleoptile (shoot) length of the germinated wheat seeds and the percentage seed germination were recorded daily.

The winter wheat seeds were considered to have germinated when the coleoptile had emerged from caryopsis (kernel) at stage 07 of the decimal code for the growth stages of cereals (Tottman and Broad, 1987), see figure 2.2.

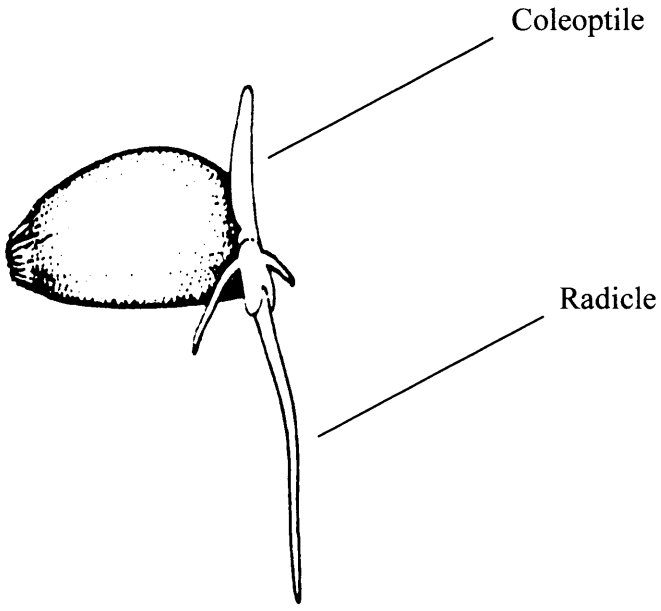


Figure 2.2 Wheat Seed Showing Coleoptile Emerging From Caryopsis. Reproduced From Tottman and Broad (1987).

2.3.15 Evaluation Of Phytotoxic Effects of Plant Extracts on Winter Wheat Seeds

Twenty four winter wheat seeds were placed in a stoppered glass vial (30 ml) and one ml of plant extract added. Seeds were left to soak overnight, removed and placed in plastic containers (17 x 11 cm), lined with unbleached blotting paper, which had been saturated with water. Seeds were monitored for germination and the coleoptile length measured, on days 3, 5, 7 and 9. Experiments were performed in duplicate.

The various treatments evaluated consisted of:

i) 0.5% Myrrh essential oil in aqueous Tween 80 (0.2%).

ii) 1% Opoponax essential oil in aqueous Tween 80 (0.2%).

iii) 0.5% *Trans*-β-ocimene in aqueous Tween 80 (0.2%).

iv) Aqueous Tween 80 (0.2%).

v) Water.

2.3.16 Test animals

Adult *D. reticulatum* and *A. hortensis* slugs were collected from nearby fields and maintained in plastic trays lined with moist, unbleached, absorbent paper. They were housed in the dark at a constant temperature of 10° ± 1°C and were regularly fed on lettuce. Prior to testing, slugs were starved for 24 hours and acclimatised to 15°C.

Adult *H. aspersa* snails were purchased from Blades Biological (Kent, UK). They were housed in plastic aquariums, regularly fed on lettuce plants and placed in an environmentally controlled chamber unit (12 hour light, 15°; 12 hour dark, 15°C).

Earthworms (*Lumbricus terrestris*) were collected from nearby fields and maintained in plastic trays lined with moist soil, obtained from their respective surrounding habitats. These were kept in the dark, at ambient temperatures (16 to 20°C) and fed on vegetation obtained from their surrounding habitats, as well as iceberg lettuce.

2.3.17 Terraria Trials (D. reticulatum And A. hortensis)

Experiments were performed as described by Bowen and Antoine (1995) and involved either four or five replicates using 0.07 m^2 plastic trays (32 x 22 cm) lined with moist, unbleached, absorbent paper. Tests were performed under environmentally controlled conditions (12 hour light 15°C: 12 hour dark 15°C). Three groups of eight winter wheat seeds were placed into each tray and each group surrounded with a continuous barrier of test material (4 g). As indicated in figure 2.3, the 4g oleoresin barriers were arranged to have a barrier width dimensions between 1.5 and 2.0 cm with a barrier aperture between 3.0 and 3.5cm. Four or five pre-starved slugs were introduced to the middle region of each tray and a glass lid placed on the top to confine the slugs to this arena. The terraria trials involving inert materials, 100% *Commiphora* spp. oleoresins and their ethanolic extracts were performed over a period of seven days, after which all slugs were removed, replaced with naive ones and the trials continued for a further seven days. The remaining terraria experiments were evaluated over an uninterrupted test period of either seven or eleven days. The level of crop protection and slug mortality was recorded on a daily basis. The level of crop protection was obtained by recording the number of hollowed or damaged seeds. Slug mortality was monitored by observing their responses to a short nine volt DC electrical stimulation. Healthy slugs responded by secreting a white mucous and moved away, paralysed slugs reacted similarly but failed to move, while dead slugs showed no response at all. Both % seed hollowing and % mortality data were calculated cumulatively over the test period investigated.

2.3.18 Terraria Trials (H. aspersa)

H. aspersa snails would not eat winter wheat seed, hence the terraria method described in 2.3.14 was modified by replacing the 24 wheat seeds, per tray, with 24 lettuce leaf discs (1.4 cm^2). Five pre-starved *H. aspersa* snails were added to each tray.

2.3.19 Summary of Materials Tested As Repellent Barriers Using The Terraria Method

Blotting Paper Substrate: D. reticulatum

i) 100% Inert materials comprising Spruce sawdust, kaolin clay, and sand.

ii) 100% Myrrh (Baldwin) and opoponax oleoresin granules.

iii) Myrrh (Baldwin) oleoresin granules mixed separately with sand, corncob and sawdust (Lignocel ^{TM}) inert materials. The Myrrh / corn cob barrier was applied in the ratio of 50:50 whilst myrrh/ sawdust and myrrh / sand were mixed in the ratio of 60:40.

iv) Reduced amounts of myrrh (Baldwin) oleoresin granules mixed with inert corn cob material. The amount of myrrh incorporated into the repellent barrier was 20, 30 and 40% in order to determine the relationship between myrrh dose and its activity against *D. reticulatum* slugs.

v) Spruce sawdust coated separately, with 20% ethanolic extracts of myrrh oleoresin (Baldwin), 20% ethanolic extracts of opoponax and a commercial source of ethanolic myrrh extract (Thornton & Ross).

vi) Spruce sawdust coated separately with 0.5% myrrh essential oil solubilised in ethanol, aqueous Tween 80 (0.2%) and aqueous DMSO (2.5%).

vii) Spruce sawdust coated separately, with 1% opoponax essential oil solubilised in aqueous Tween 80 (0.2%) and aqueous DMSO (2.5%).

viii) Spruce sawdust coated separately with 0.5% *trans*-β-Ocimene (a major component of opoponax essential oil), separately solubilised in aqueous Tween 80 (0.2%) and aqueous DMSO (2.5%).

Blotting Paper Substrate: A. hortensis

i) 100% Myrrh (Baldwin) oleoresin granules (*C. molmol*).

Peat Soil Substrate: D. reticulatum

i) Myrrh (Baldwin) oleoresin granules mixed separately with sand (60:40), corncob (50: 50) and Beech sawdust (Lignocel®) (60:40) and a commercial mollusc repellent barrier called Slug Stop®, comprised of amorphous silica granules (Diatomite), placed on a peat soil substrate.

Blotting Paper Substrate: H. aspersa

i) 100% Myrrh (Somali) and opoponax oleoresin granules (8 g).

ii) Myrrh (Somali) oleoresin granules mixed Beech sawdust (Lignocel®) inert material in the ratio of 60:40 (4.8g / 3.2g).

iii) Opoponax oleoresin granules mixed Beech sawdust (Lignocel®) inert material in the ratio of 60:40 (4.8g / 3.2g).

iv) Control comprising of 100% Beech sawdust (Lignocel®) (8g)

Because of the higher density of Beech sawdust (Lignocel®) compared to Spruce sawdust 8g was applied, instead of 4g, in order to generate a barrier with comparable dimensions (barrier thickness between 1.5 and 2.0 cm and barrier aperture between 3.0 and 3.5 cm), as illustrated in figure 2.3.

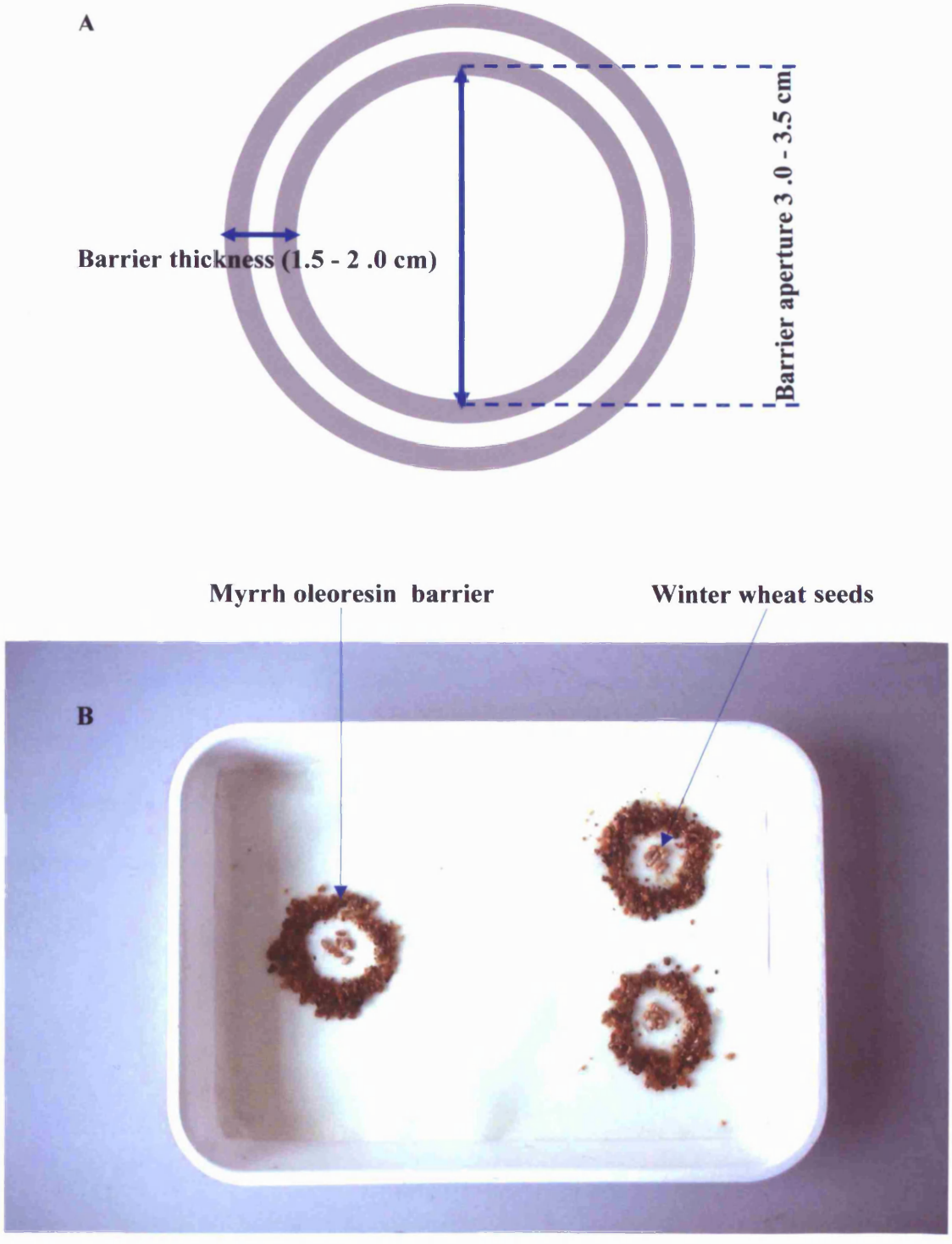


Figure 2.3. **(a)** Dimensions of a typical barrier used in terraria trials experiments. **(b)** Terraria tray with three groups of winter wheat seeds surrounded by repellent barriers comprised of myrrh oleoresin granules.

2.3.20 Statistical Methods

Analysis of variance (Anova) was used to compare group means in cases where parametric criteria were appropriate i.e. between group variances that were homogenous according to Bartlett's or Levene's test. Parametric criteria were also fulfilled if the residuals were normally distributed using the Anderson-Darling test (Fry, 1993). Fisher's *a priori* test for least significant difference of the means (LSD) was used to compare the means of multiple treatments to a control. The Tukey-Kramer *a posteriori* test for minimum significant differences (MSD) was used for pairwise comparisons of the means within multiple treatment groups.

In cases where the parametric criteria were not met, the non parametric Kruskal-Wallis test was used to determine the significant differences between group medians. The Mann-Whitney test was used to compare group medians of multiple treatments with the control. For all four statistical tests the level of significance was chosen as $P = 0.05$.

2.4 RESULTS

2.4.1 Evaluation of Inert Materials (100%) As Repellent Barriers

Seed Hollowing

During the first three days, sawdust was found to be the only inert material showing barrier properties against slugs, with only 27% seed hollowing compared to 58% for the unprotected seeds (control), as indicated in figure 2.4. This was confirmed using Fisher's *a priori* test to estimate the least significance difference (LSD) between the means. After seven days, however, all of the inert barriers, including sawdust, exhibited insignificant repellent barrier properties against *D.reticulatum* slugs in comparison to the unprotected winter wheat seeds (Anova, $P = 0.144$). Kaolin clay was found to have very poor mechanical properties as it turned into a liquid paste within the first few days.

After the second week of terraria testing, significant differences in barrier efficacies were observed for the various treatments (Anova, $P < 0.001$), as indicated in figure 2.5. Fisher's LSD test, however, showed Kaolin clay with 56% seed hollowing to be the only inert material with barrier properties significantly better than the control (96% seed hollowing).

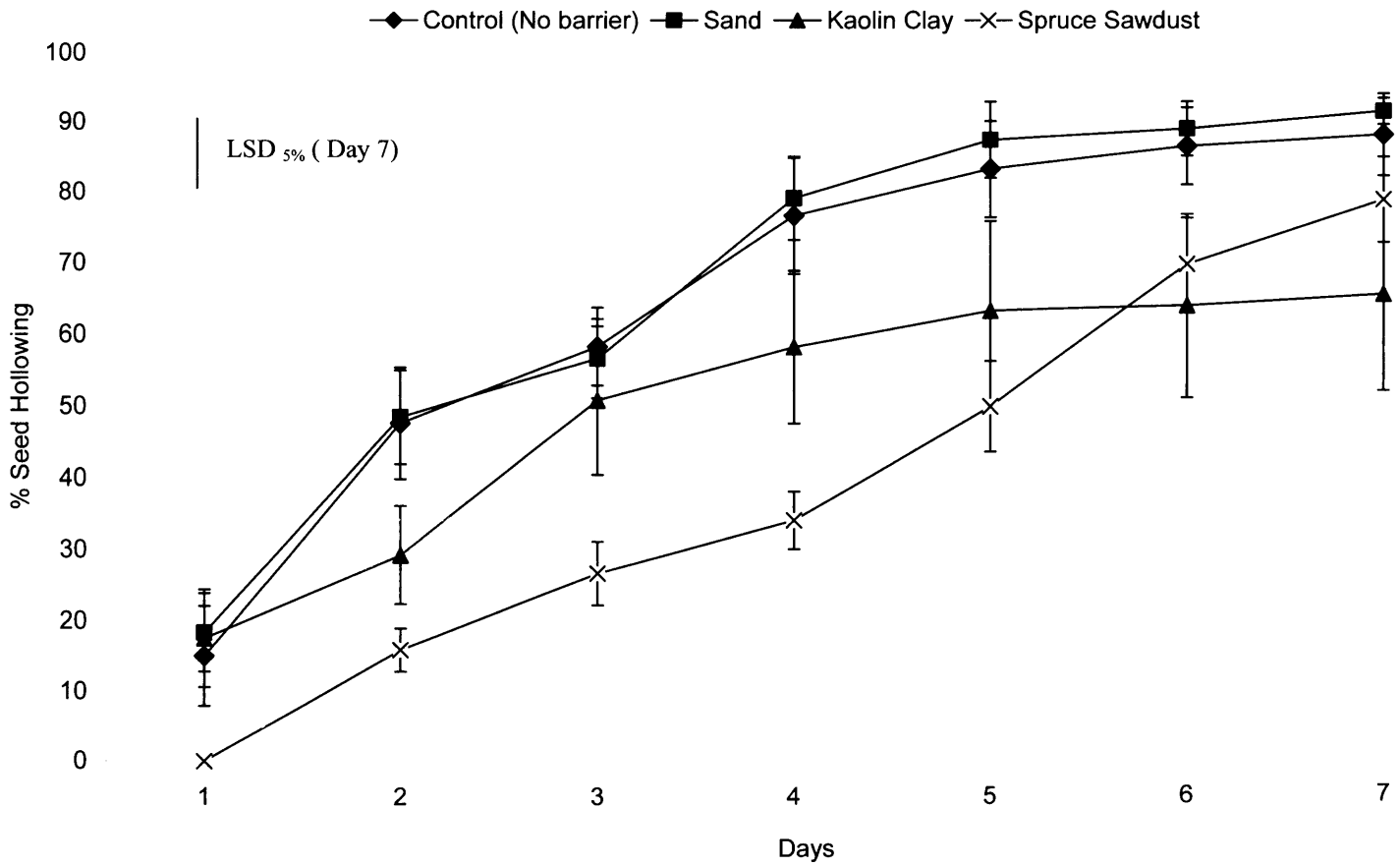


Figure 2.4 Evaluation Of The Barrier Properties Of Inert Materials (100%). Assessment Of The Amount Of Seed Hollowing (7 Days).

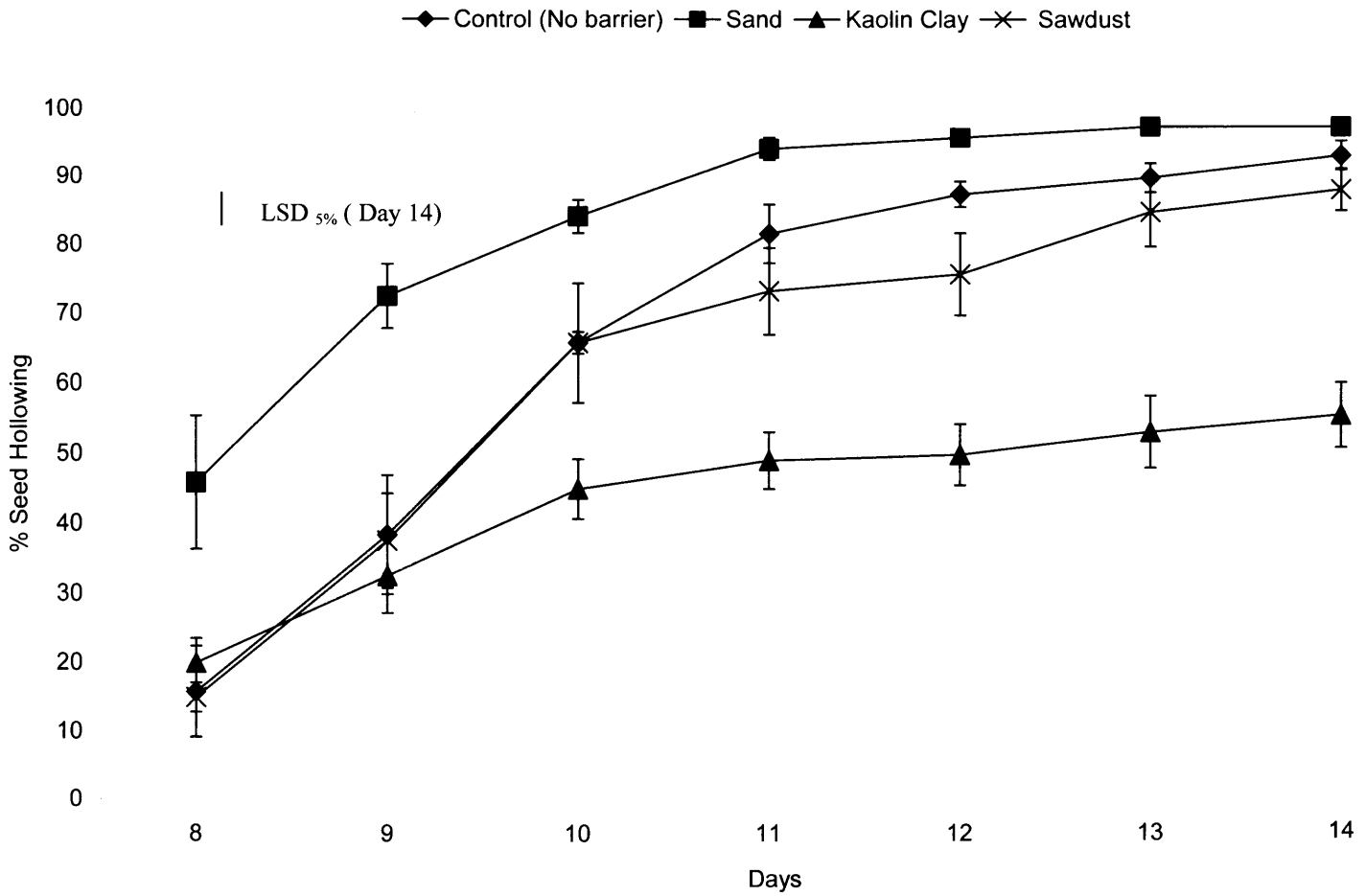


Figure 2.5 Evaluation Of The Barrier Properties of Inert Materials (100%). Assessment Of The Amount of Seed Hollowing (14 Days).

Slug Mortality

None of the inert materials tested showed significant incidences of slug mortality after seven days (Anova, $P = 0.762$). Slug mortality rates were relatively low, the maximum being 25% for the Kaolin and sand barriers compared to 20% for the control. The second week of the terraria trials showed no significant increase in slug mortalities for any of the inert materials tested (Anova, $P = 0.461$). Maximum slug mortality was 10% for both the Kaolin clay and sand barriers compared to 15% for the control.

2.4.2 Repellent Barriers Using Myrrh and Opoponax Oleoresins (100%)

Seed Hollowing

The use of myrrh and opoponax oleoresins as repellent barriers significantly reduced the amount of seed hollowing caused by the *D. reticulatum* slugs, during the first seven days (Kruskal-Wallis test, $P = 0.02$). As shown in figure 2.6 there were very few seeds damaged by the slugs during the first week with 0% median number of seeds hollowed for oleoresin barriers, compared to 92% for the control. Pair-wise comparisons of the medians showed no significant differences in the repellent properties of the myrrh and opoponax oleoresins (Mann-Whitney test, $P = 0.423$).

In the second week the myrrh and opoponax barriers continued to significantly repel the slugs from feeding on the winter wheat seeds (Kruskal Wallis test, $P = 0.002$). Pairwise comparisons of the group medians for myrrh and opoponax barriers, with the

control, showed significant barrier protection for both *Commiphora* spp. oleoresins against *D. reticulatum* (Mann-Whitney test, $P < 0.05$).

Comparing the barrier properties of the two oleoresins, in terms of the amount of seed damage showed opoponax to be significantly more effective in deterring slugs than myrrh (Mann-Whitney test, P=0.007). The median number of seed damage observed with the myrrh and opoponax barriers were 8% and 0% respectively, compared to 100% for the control, see figure 2.7.

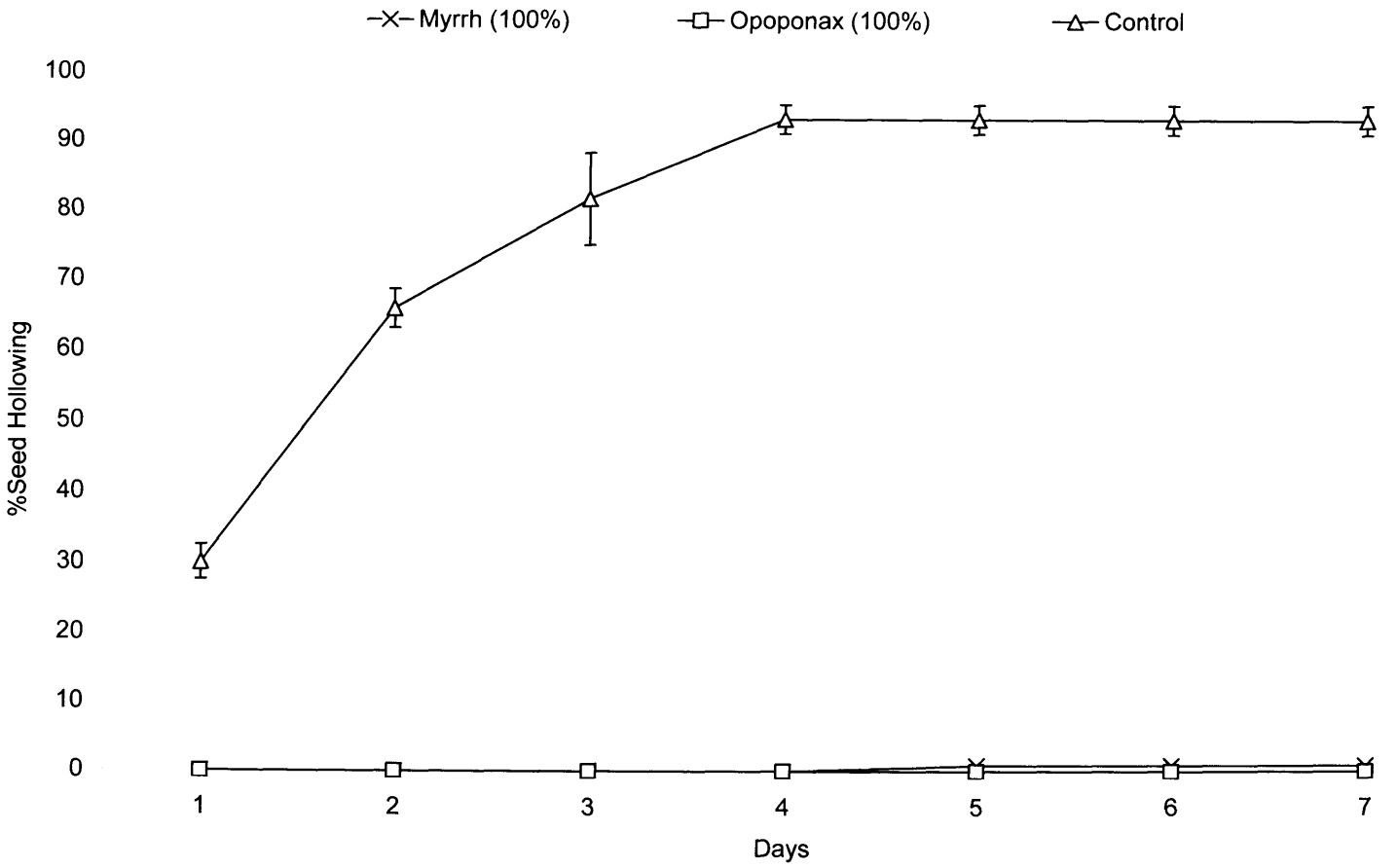


Figure 2.6 Evaluation Of The Barrier Properties Of Myrrh And Opoponax Oleoresins (100%).

Assessment Of The Amount Of Seed Hollowing (7 Days)

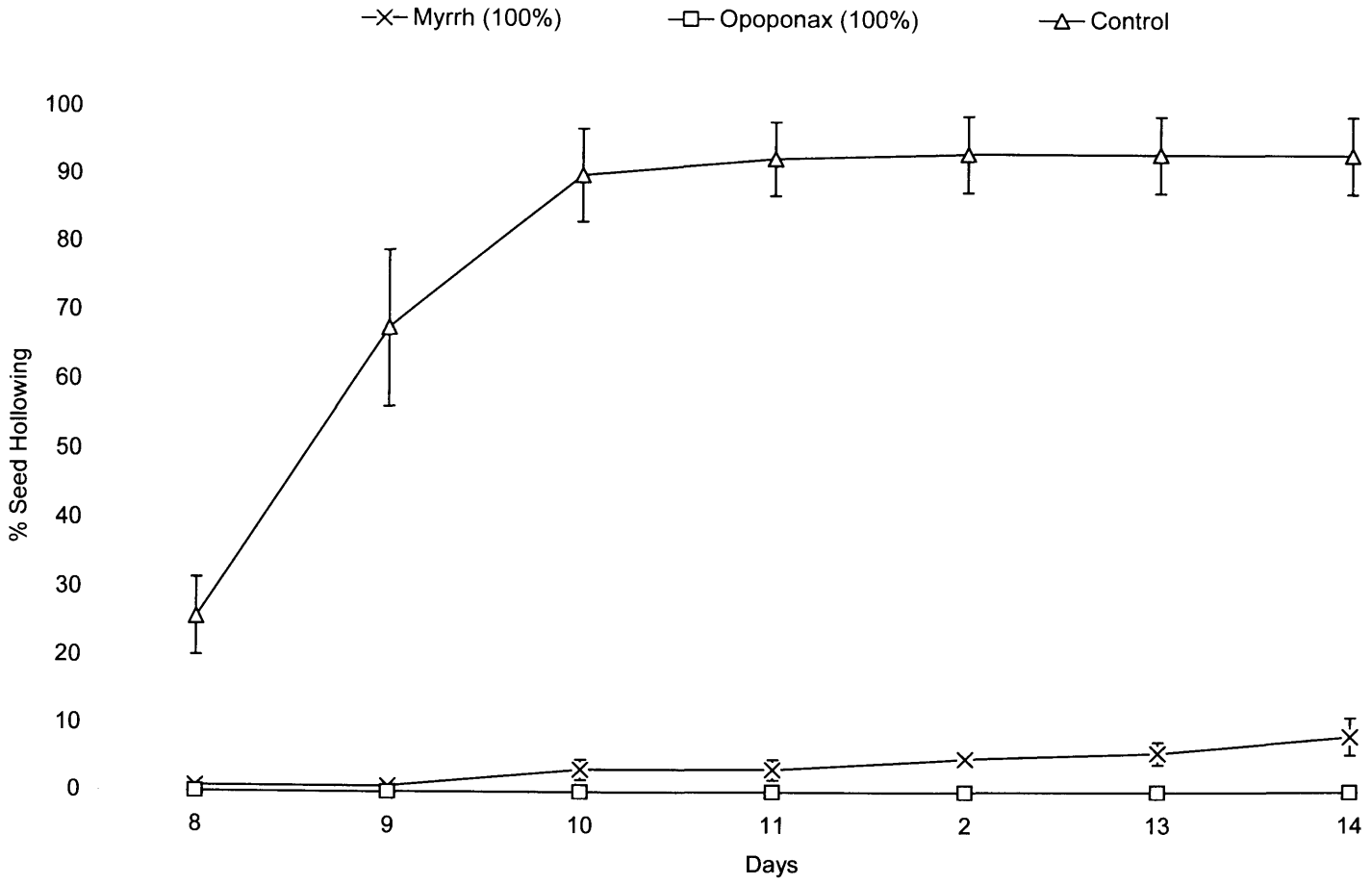


Figure 2.7 Evaluation Of The Barrier Properties Of Myrrh And Opoponax Oleoresins (100%). Assessment Of The Amount Of Seed Hollowing (14 Days).

Slug Mortality

After the seven day test period significantly high incidences of slug mortality were recorded, when myrrh and opoponax oleoresins were applied as repellent barriers, (Anova, $P = 0.011$). The mean percentage slug mortality for myrrh and opoponax barriers were 70% and 85% respectively compared to 15% for the control, as shown in figure 2.8. Pairwise comparisons of the mean percentage mortality showed no significant difference between the two oleoresins, using the parametric Tukey-Kramer test. This test showed that the difference between the pair of means was less than the minimum significant difference (MSD, 53%)

Testing the myrrh and opoponax barriers for a further seven days (8 to 14 days), with naïve slugs, showed no significant increase in mortality for either of the oleoresins (Anova, $P = 0.242$). Myrrh and opoponax barriers showed only 10% and 20% slug mortality respectively, compared to 0% for the control.

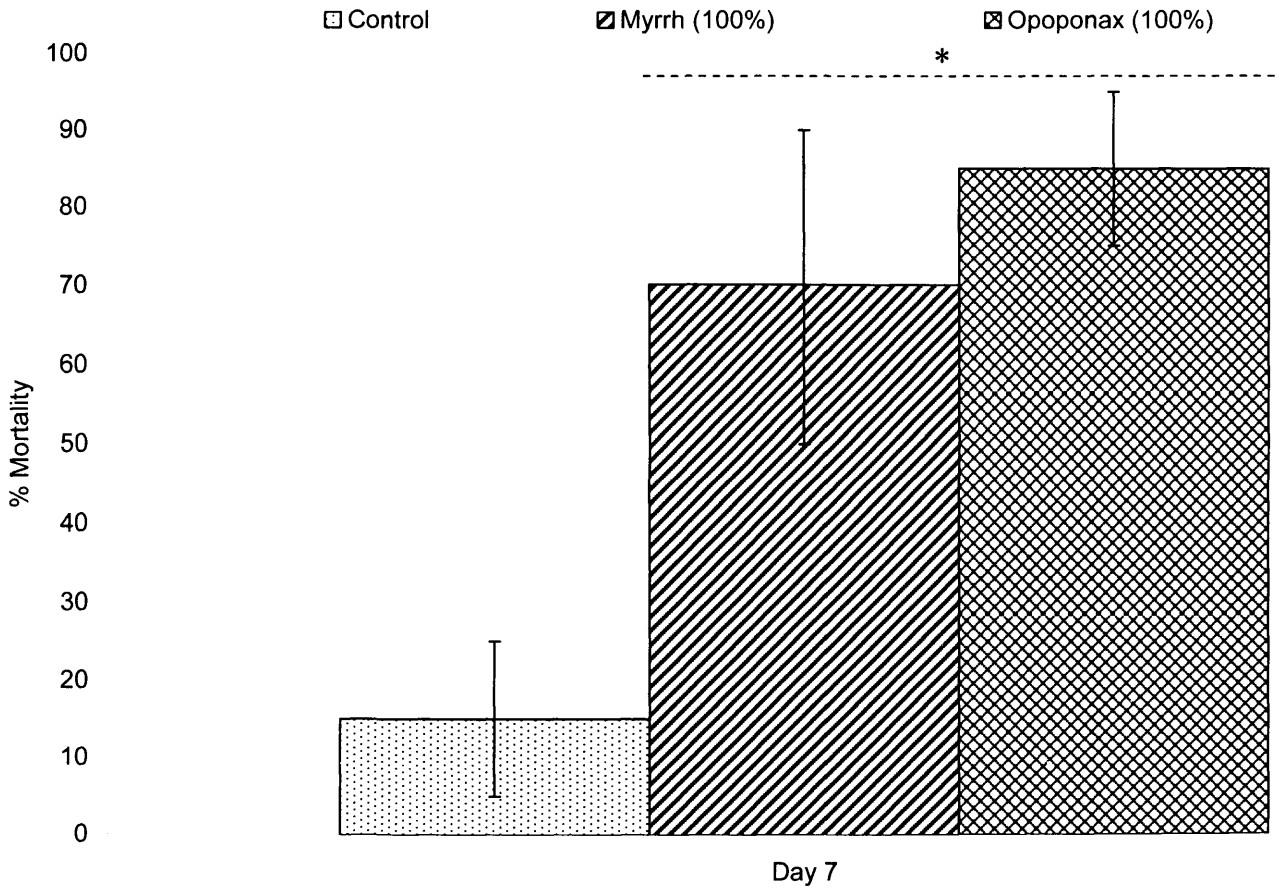


Figure 2.8 Evaluation Of The Barrier Properties Of Myrrh And Opoponax Oleoresins. Assessment Of Slug Mortality (7 Days). * = Significant difference ($p< 0.05$).

2.4.3 Repellent Barriers Using New Inert Materials (100%) And Mixtures Of Myrrh Oleoresins With Inert Materials

Seed Hollowing

As expected, none of the inert materials, tested as barriers, deterred the slugs from feeding on the winter wheat seeds, over the eleven day test period. Kruskal-Wallis analysis showed that in terms of barrier properties, there was no significant differences between the protected seeds (inert barriers) and the unprotected seeds (control) (Kruskal-Wallis test, $P = 0.749$).

In contrast, all of the barriers which comprised mixes of myrrh and inert material were remarkably effective as repellent barriers (figure 2.9). All of the myrrh barriers significantly reduced feeding on the winter wheat seeds, regardless of which inert material was incorporated into the barrier (Kruskal-Wallis test, $P = 0.002$). Pair-wise comparisons of group medians, using the Mann-Whitney test, showed that there was no significant difference ($P > 0.05$) in the barrier efficacies of the three myrrh barriers tested (myrrh granules mixed with sharp sand, sawdust and corncob).

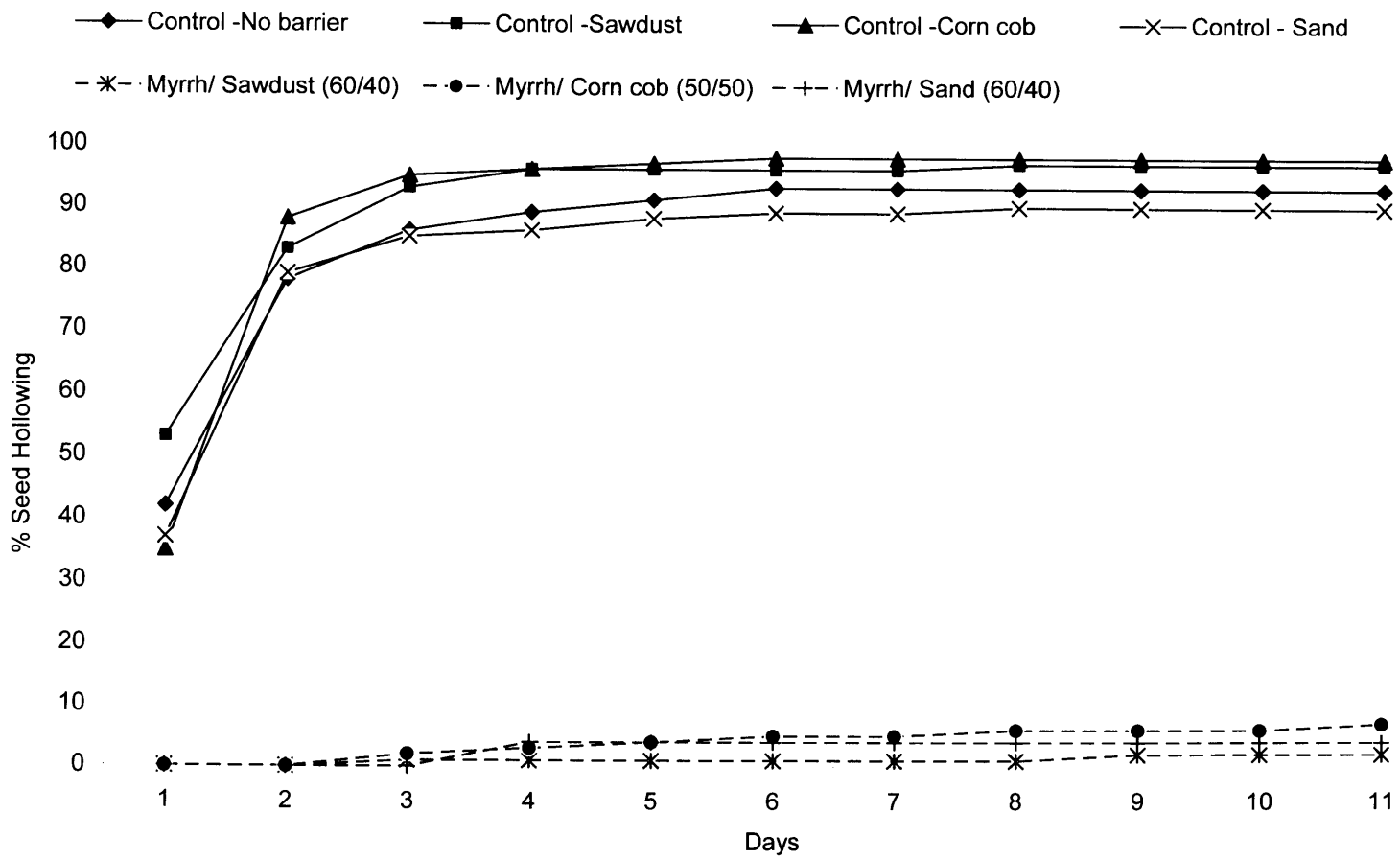


Figure 2.9 Evaluation Of The Barrier Efficacy Of Myrrh Oleoresin Mixed With Different Inert Materials. Assessment Of Slug Mortality (11 Days).

Slug Mortality

There was a significant effect on slug mortality when the myrrh oleoresins were evaluated as repellent barriers over eleven day (Anova, $P = 0.008$). Figure 2.10 shows that myrrh's combination with sand was the main contribution to the increase in slug death. This was confirmed using Fisher's test for least significance differences (LSD) to compare the mean slug mortality for all myrrh treatments. Only the repellent barrier comprised of myrrh and sand (60%: 40%) exceeded the LSD level for slug mortality with a mean slug mortality of 70%.

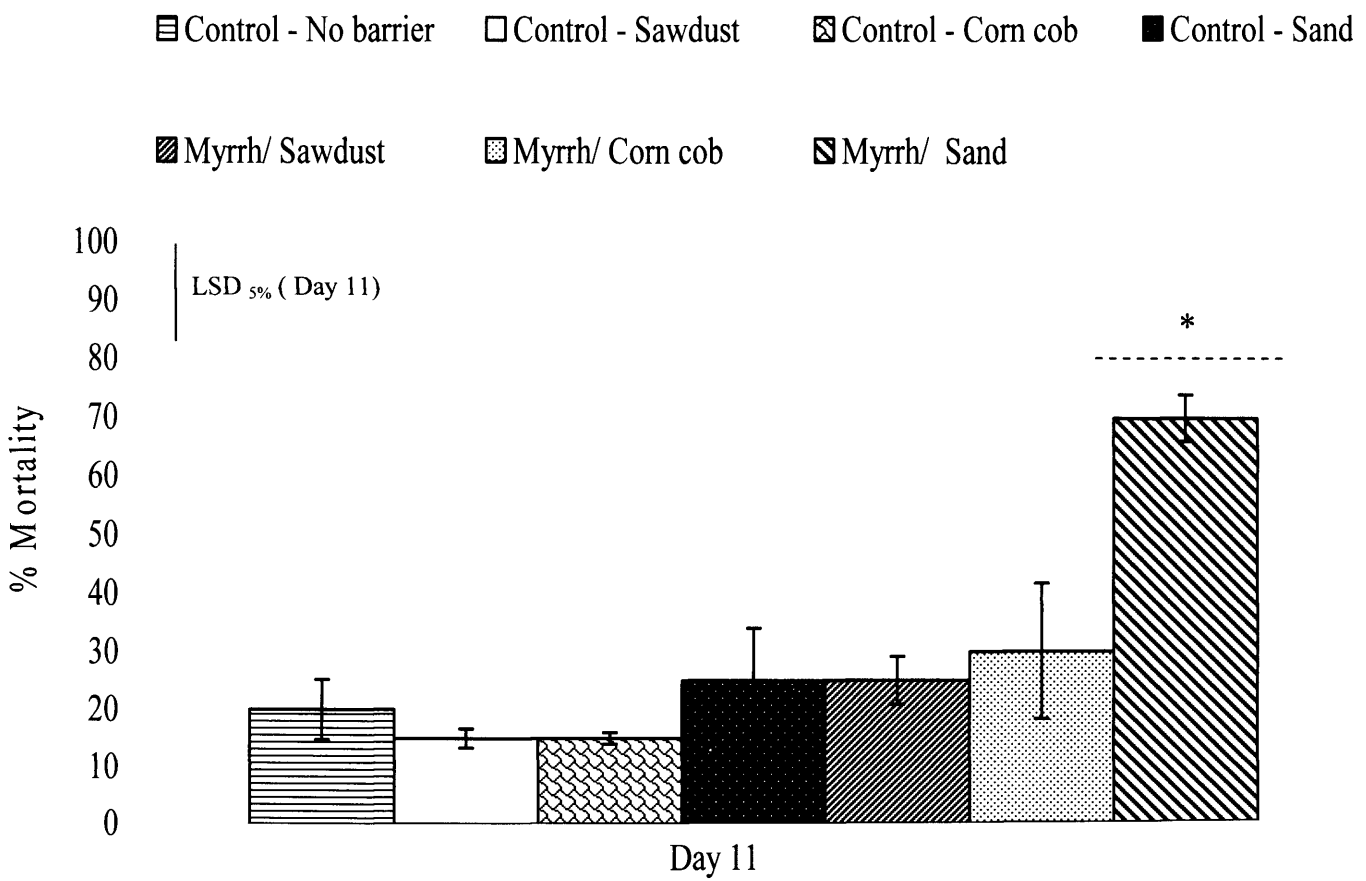


Figure 2.10 Evaluation Of The Barrier Efficacy Of Myrrh And Opoponax Oleoresins Mixed With Different Inert Materials. Assessment Of Slug Mortality (11 Days). * = Significant Difference ($P < 0.05$).

2.4.4 Repellent Barriers Using Lower Amounts Of Myrrh Granules Mixed With Corncob (Myrrh Dose-Activity Relationship)

Seed Hollowing

Reducing the amount of myrrh oleoresin incorporated into the myrrh / corncob barriers, from 50% to 20%, had no detrimental effect on their repellence properties against *D. reticulatum* over the eleven day test period (Anova, $P < 0.001$). As shown in figure 2.11, myrrh oleoresin granules mixed with inert corncob substrates in the ratios 40:60, 30:70 and 20:80 resulted in only 7%, 6% and 10% mean percentage seed hollowing. As expected a high occurrence of seed damage occurred with the controls resulting in 98% mean percentage seed hollowing when the seeds were surrounded with corncob and 93% when they were left unprotected. Pairwise comparisons of the means for minimum significant differences (MSD), using the Tukey-Kramer, test showed no significant differences in the mean percentage seed damage between the three myrrh repellent barriers

Slug Mortality

Comparing the mean percentage slug mortality for the various reduced myrrh barriers, over the eleven day test period, showed no significant increases in mortality (Anova, $P = 0.092$). Further statistical analysis, however, using Fisher's LSD test showed the 30/70% myrrh/corncob barrier to have a significantly higher contribution to slug mortality (63%) compared to control (25%). The 40/60 and 20/ 80 myrrh/corncob barriers both gave 50% slug mortality, as shown in figure 2.12.

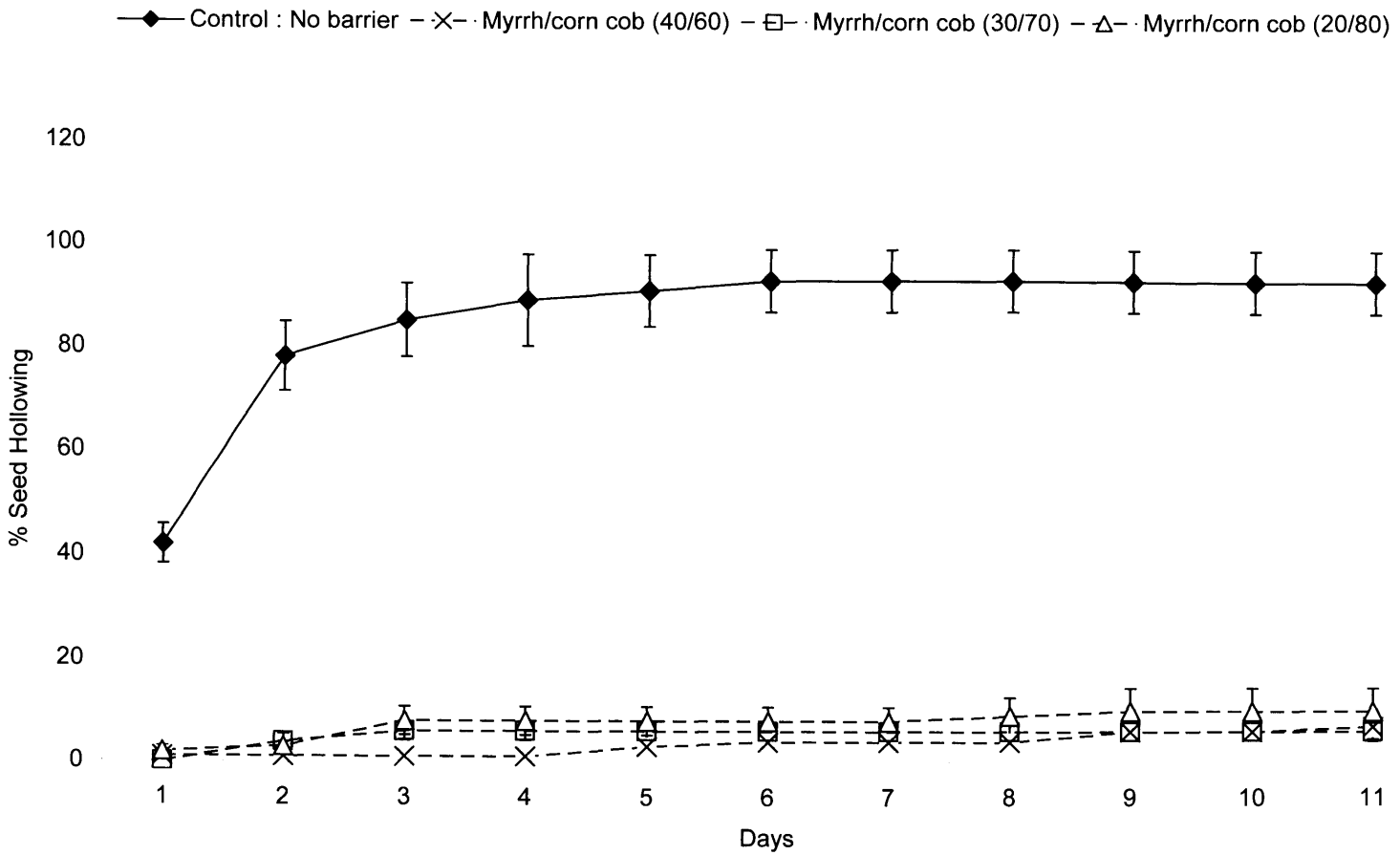


Figure 2.11 Effect Of Myrrh Concentration On Barrier Repellency (Dose activity Relationship).

Assessment Of The Amount Of Seed Hollowing (11 Days).

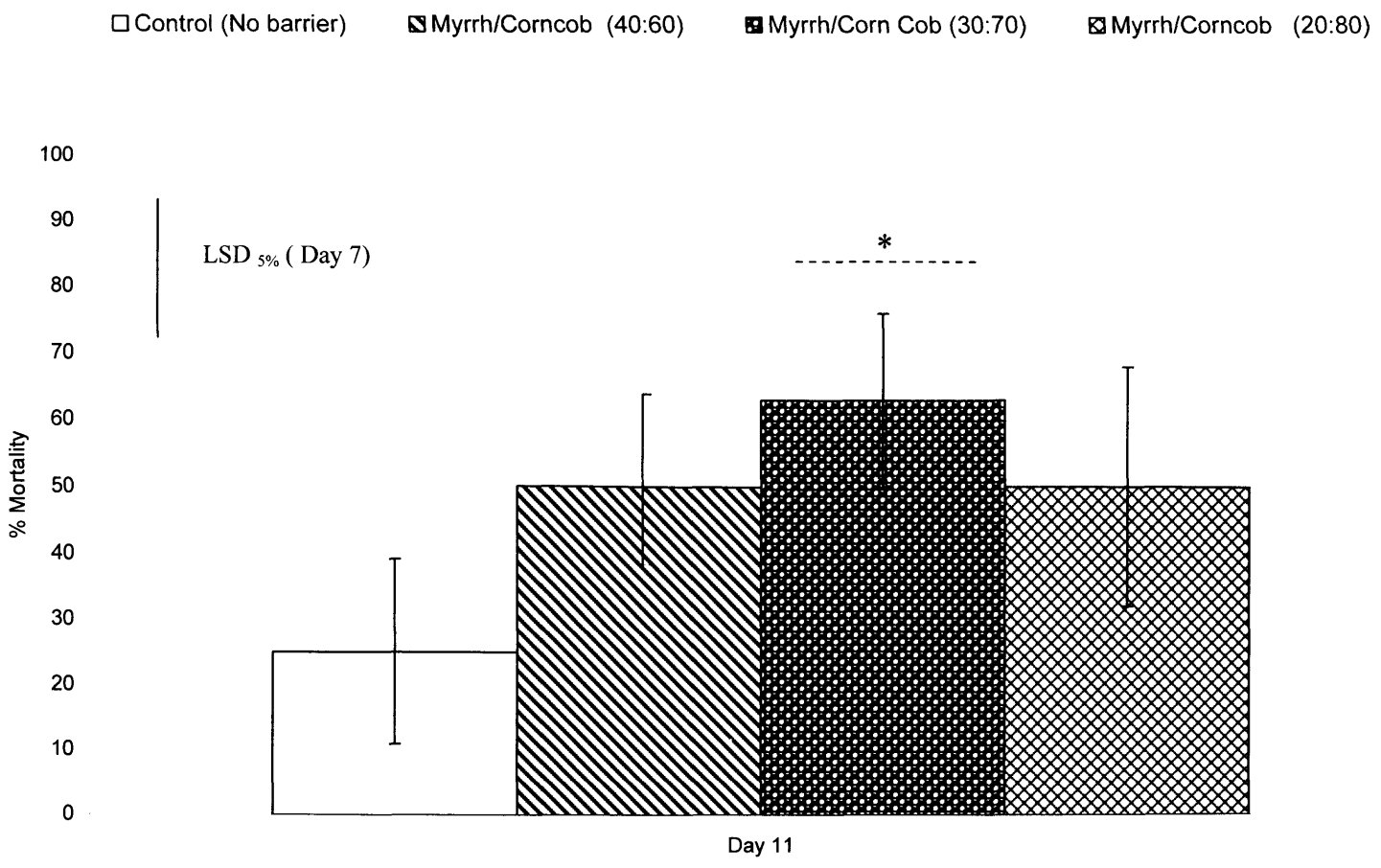


Figure 2.12 Effect Of Myrrh Concentration On Barrier Repellency (Dose-Activity Relationship).

Assessment Of Slug Mortality (11 Days).

2.4.5 Repellent Barriers using Myrrh Oleoresins mixed with inert materials on a Peat Soil substrate

Seed Hollowing

Kruskal-Wallis analysis showed a significant difference in the mean percentage seed hollowing between the control and the various treatments over the seven days ($P = 0.002$), see figure 2.13. Further statistical analysis using the non parametric Mann-Whitney test showed only the three myrrh barriers (myrrh only, myrrh/sawdust and myrrh corncob) to have significantly reduced the amount of seed hollowing on comparison to the control (no barrier) ($P < 0.05$).

Pairwise comparisons of the group medians showed no significant differences in the barrier efficacies of the 100% myrrh oleoresins and the myrrh/sawdust mixtures (Mann-Whitney, $P = 0.563$). There was, however, significant differences in the amount of seed damage caused by the slugs when 100% myrrh oleoresin and the myrrh / corncob barriers were statistically compared as barriers (Mann-Whitney, $P = 0.040$). Surprisingly the commercial physical barrier, Slug Stop®, showed very little barrier properties (median 100% seed hollowing), hence, did not have any effect on the feeding behaviour of the slugs (Mann-Whitney, $P = 1.000$).

Slug Mortality

Incidences of slug death on peat soil were significantly low for all treatments, after the seven day test period (Anova, $P = 0.276$). No mortality was observed for the 100% myrrh and myrrh/ sawdust barriers and very little slug mortality (5%) recorded for the myrrh/corncob barriers. The controls (no barrier and sawdust only) showed 15% and 10% slug mortalities respectively.

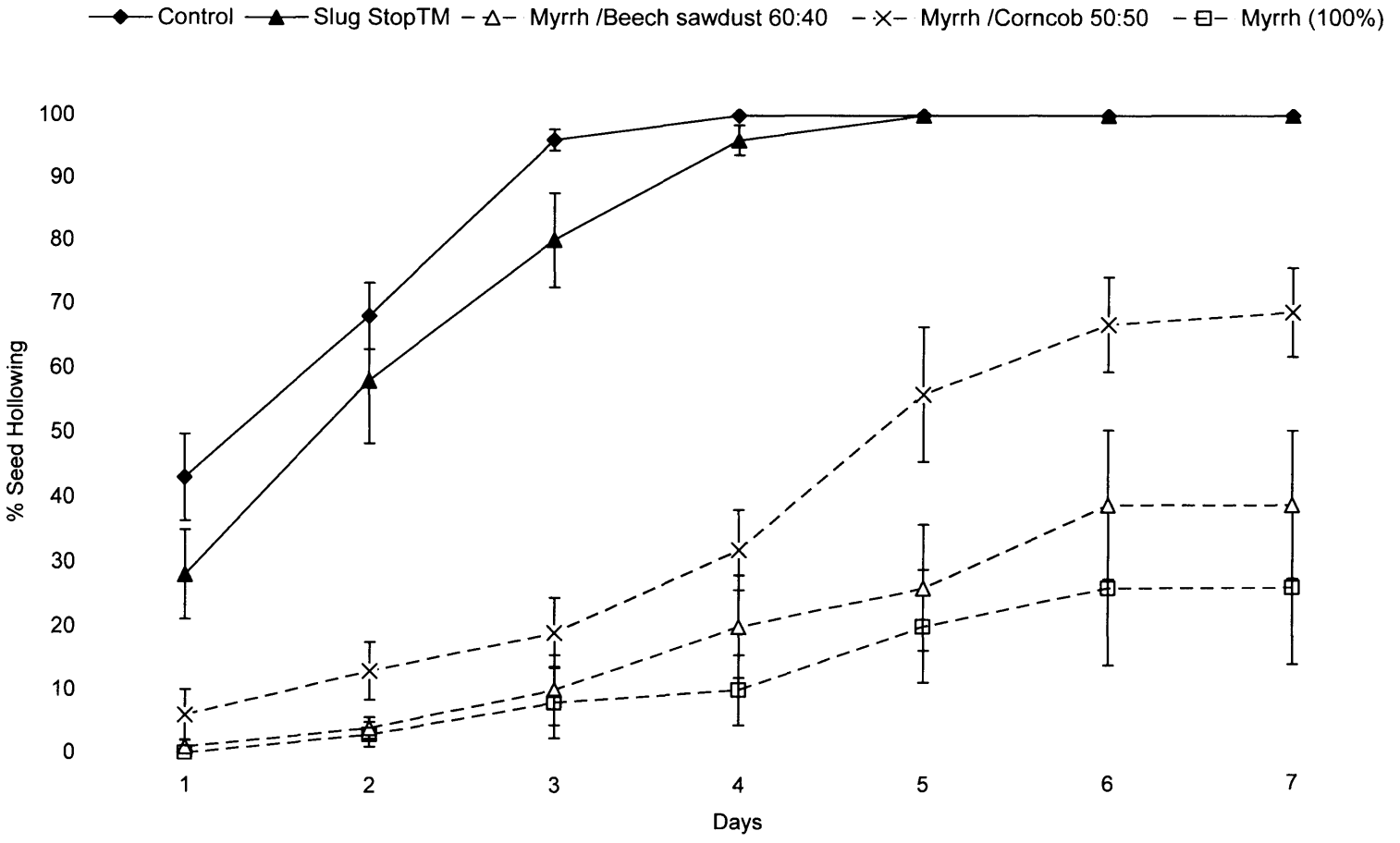


Figure 2.13 Effect Of Myrrh-Inert Substrate Mixes On Barrier Repellency (Peat Soil).

Assessment Of Seed Hollowing (7 Days).

2.4.6 Repellent Barriers Using Sawdust Coated With Ethanolic Extracts Of Myrrh And Opoponax Oleoresins

Seed Hollowing

Over the seven day test period barriers comprised of sawdust coated with ethanolic extracts of myrrh and opoponax oleoresins showed a highly significant reduction in the feeding behaviour of the slugs (Kruskal-Wallis, $P<0.001$), see figure 2.14. Mann-Whitney pairwise comparisons of group medians showed that there was no significant differences between the barrier efficacies of the ethanolic myrrh (Baldwin), commercial ethanolic myrrh (Thornton & Ross) and ethanolic opoponax (Mann-Whitney, $P>0.05$) resulting in 33%, 17% and 13% median seed hollowing respectively.

The non-parametric Mann-Whitney test also showed no significant differences between the median percentage seed hollowing for the two inert barriers, sawdust and sawdust coated with ethanol, compared to the control (no barrier) over the seven day test period ($P>0.05$).

The ethanolic extracts of myrrh and opoponax continued to be significantly effective repellent barriers, against *D. reticulatum*, for a further seven days (8 to 14 days) (Anova, $P< 0.01$).

Tukey-Kramers test for minimum significant differences (MSD) showed there were no significant differences in the mean percentage seed hollowing when sawdust coated with ethanolic myrrh (Baldwin), commercial ethanolic myrrh (Thornton & Ross)

and ethanolic opoponax extracts were applied as repellent barriers, resulting in 15%,15% and 11% seed hollowing as indicated in figure 2.15. This test also confirmed that there was no significant differences between the mean percentage seed hollowing for the three control barriers (no barrier, sawdust and sawdust coated with ethanol) resulting in 79%, 77% and 88% seed hollowing respectively.

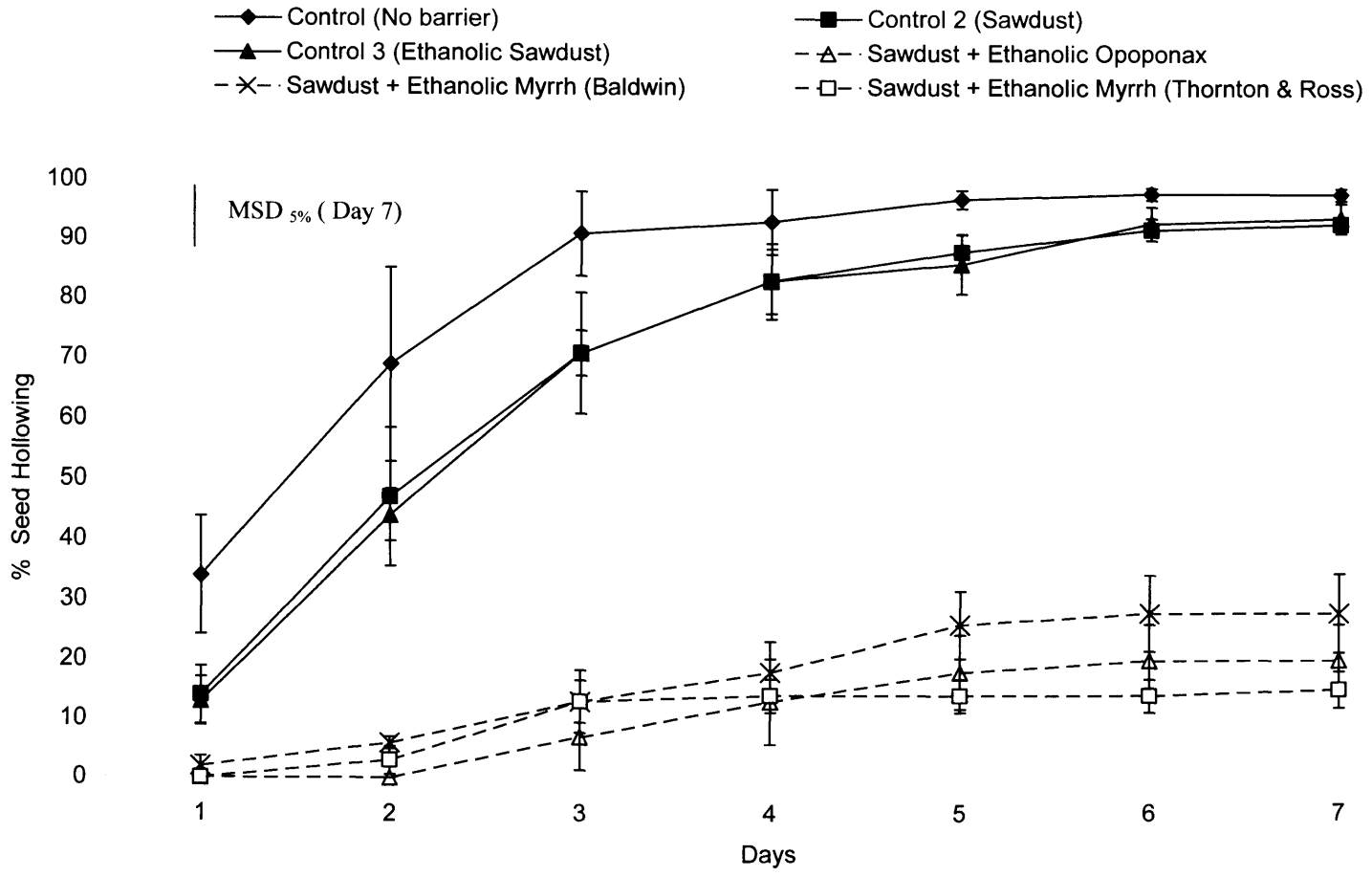


Figure 2.14 Evaluation Of Barrier Properties Of Ethanol Extracts Of Myrrh And Opoponax Oleoresins. Assessment Of Seed Hollowing (7 Days).

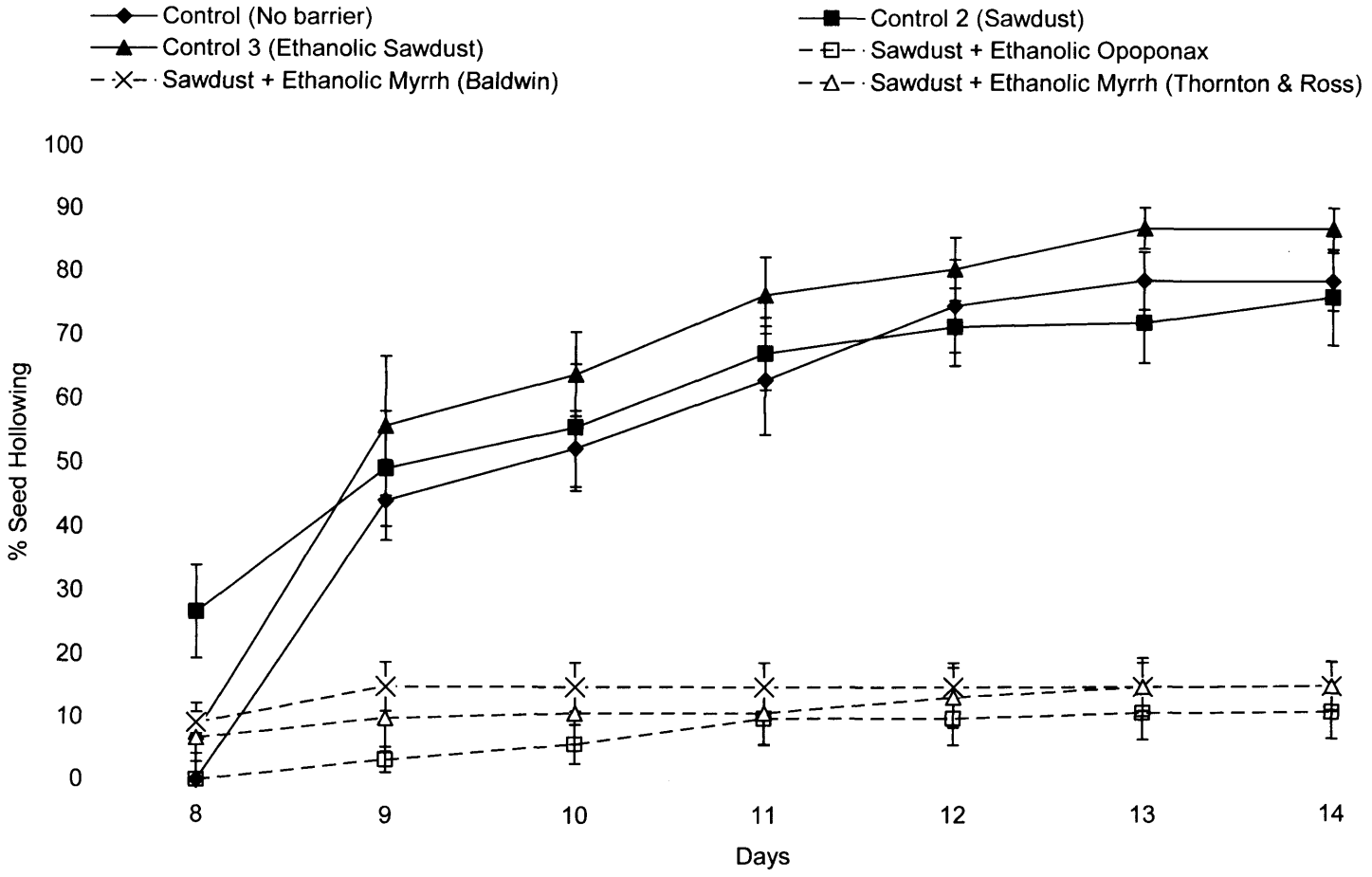


Figure 2.15 Evaluation Of Barrier Properties Of Ethanol Extracts Of Myrrh And Opoponax Oleoresins. Assessment Of Seed Hollowing (14 Days).

Slug Mortality

Log transformation $\log_{10}$ (N + 1) of the seven day mortality data showed a significant increase in slug mortalities (Anova, p = 0.009). Using Fisher's *a priori* test, for least significant differences (LSD), to compare the efficacies of the various barriers showed only sawdust coated with the commercial grade of ethanolic myrrh (Thornton & Ross) made a significant contribution to slug mortality (40%). Mortalities for all the other treatments were not significantly different from the unprotected winter wheat seeds (Fisher's test, P>0.05), as shown in figure 2.16.

After the second week the pattern of slug mortality changed with sawdust barriers coated with ethanolic opoponax extracts giving significantly high mortalities (60%). Mortalities for all the other repellent barriers were not significantly different from the control (Fisher's test, P>0.05), as shown in figure 2.17.

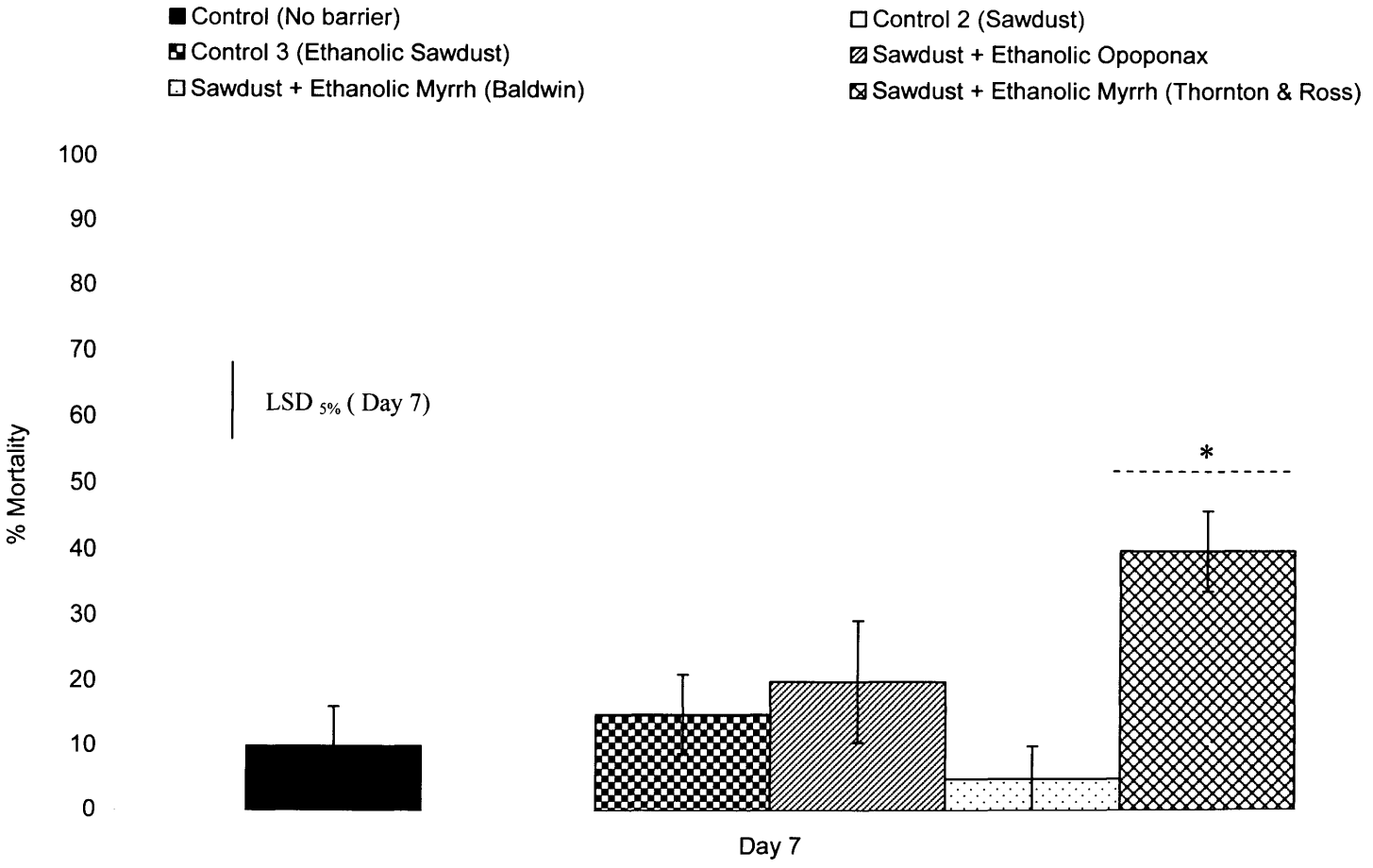


Figure 2.16 Barrier Properties Of Ethanol Extracts Of Myrrh And Opoponax Oleoresins. Assessment Of Slug Mortality (7 Days). * = Significant Difference ($P < 0.05$).

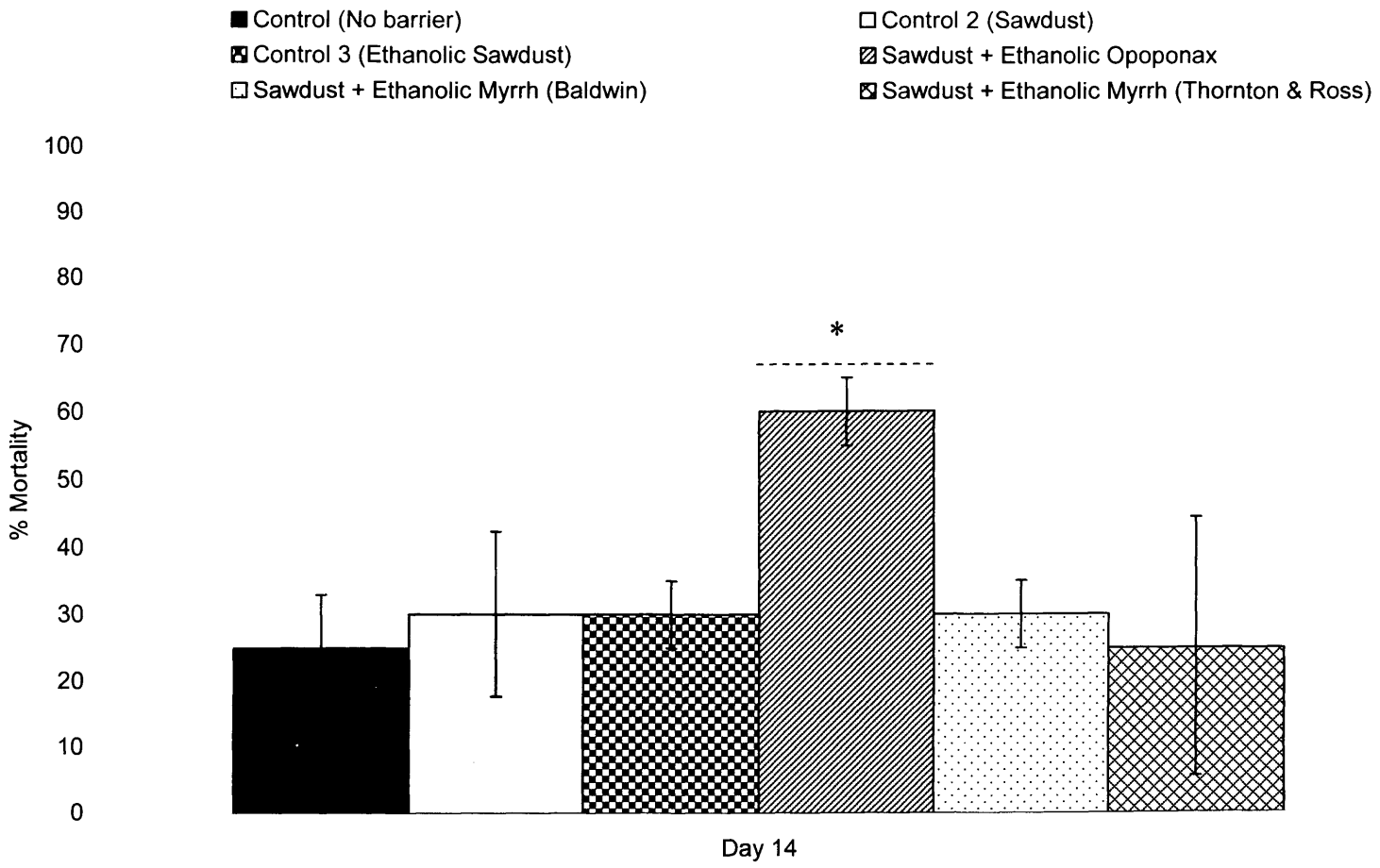


Figure 2.17 Evaluation Of Barrier Properties Of Ethanol Extracts Of Myrrh And Opoponax Oleoresins. Assessment Of Slug Mortality (14 Days).

2.4.7 Repellent Barriers Using Sawdust Coated With Myrrh And Opoponax Essential Oils

Seed Hollowing

Statistical analysis indicated that all of the sawdust barriers coated with myrrh essential oil significantly reduced the amount of seed hollowing, on comparison to the control (sawdust only), regardless of which solvent was used as a medium for the myrrh essential oil (Mann-Whitney test $P < 0.05$). The median percentage seed hollowing for the sawdust barriers coated with myrrh essential oil (0.5%) when solubilised in ethanol, aqueous Tween 80 and aqueous DMSO were 25%, 31% and 0% respectively.

As shown in figure 2.18 the barrier properties of the sawdust barriers coated with the opoponax essential oil was much more dependent upon the medium used to solubilise the oil than for myrrh essential oil. This was confirmed using Mann-Whitney for pairwise comparisons of the group medians with the sawdust only control. This nonparametric statistical analysis showed a significant effect in reducing the amount of seed hollowing ($P = 0.026$) for sawdust coated with 1% opoponax essential oil solubilised in 2.5% aqueous DMSO to have with a median seed damage of 50%, compared to the control (100%). The sawdust barriers coated with 1% opoponax essential oil solubilised in 0.2% aqueous Tween 80, however, showed very little protective barrier properties against *D. reticulatum* (Mann-Whitney, $P = 0.163$) resulting in a median seed hollowing of 94%.

The control barriers, as expected, showed very high occurrences of seed damage. The median percentage seed hollowing for the barriers comprised of sawdust, sawdust coated with ethanol and sawdust coated with aqueous Tween 80 was 100% compared to 90% when the sawdust was coated with aqueous DMSO. Statistical analysis of the latter control, showed sawdust coated with aqueous DMSO, to be significantly different to the other control barriers (Mann-Whitney test $P = 0.025$).

Slug Mortality

Insignificant levels of slug mortality was observed over the seven day test period (Kruskal-Wallis, $P = 0.199$). This was confirmed using Mann-Whitney pairwise comparisons of the median mortality for all of the barrier treatments with the sawdust control. Median mortalities for the sawdust barriers coated with 0.5% myrrh essential oil solubilised in ethanol, aqueous Tween 80 and aqueous DMSO was 25%, 40% and 37% respectively compared to the sawdust barriers coated with the same solvents (median mortalities of 0, 10 and 25%). Similarly, the sawdust barriers coated with 1% opoponax essential oil showed no increase in slug mortality when solubilised in aqueous Tween 80 or aqueous DMSO, when compared to the sawdust control (Mann-Whitney , $P > 0.05$), yielding median mortalities of 12.5% and 20% respectively.

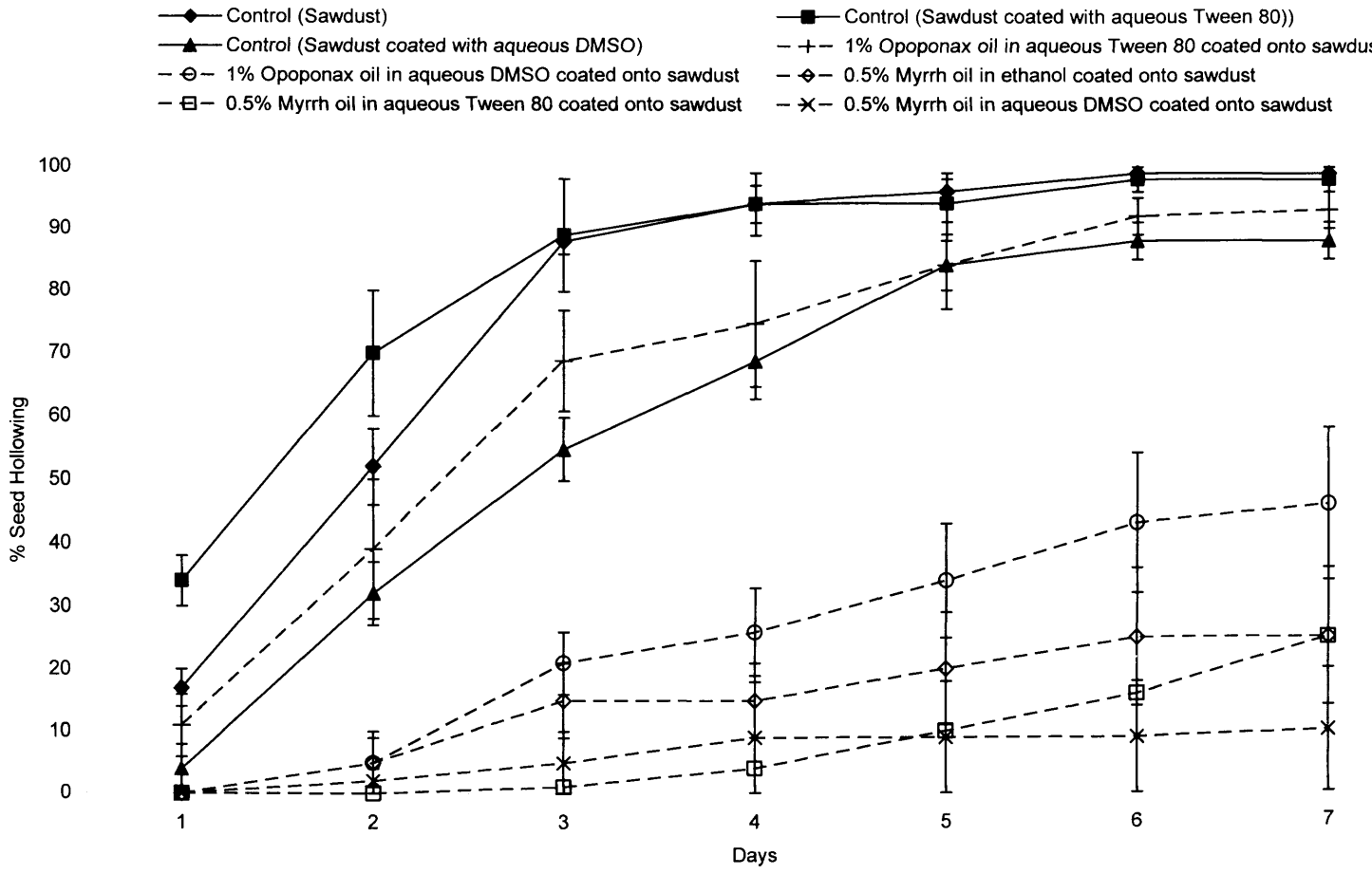


Figure 2.18 Evaluation Of Barrier Efficacy Of Sawdust Coated With Essential Oil Extracts Of Myrrh And Opoponax. Assessment Of Seed Hollowing (7 Days).

2.4.8 Repellent Barriers Using Sawdust Coated With trans-β-Ocimene Solubilised In Aqueous Tween 80 And Aqueous DMSO

Seed Hollowing

Sawdust coated with the chemical *trans*-β-ocimene, solubilised in aqueous Tween 80 (0.2%), was not very effective in deterring *D. reticulatum* slugs from feeding on the wheat seeds (Mann-Whitney, P =0.443) as shown in figure 2.19. The median number of damaged seeds for the barriers treated with *trans*-β-ocimene was 81% compared to the 94% for the unprotected seeds. Solubilising *trans*-β-ocimene in aqueous DMSO (2.5%), however, had a marked effect on increasing its barrier properties with the median number of seeds hollowed being 27%. Sawdust coated with 0.5% ocimene/aqueous DMSO significantly reduced the amount of seed damage over the seven day test period compared to sawdust only (Mann -Whitney, P = 0.015).

Slug Mortality

Statistical analyses showed no significant increases in slug mortality, for the various barriers tested over the seven day test period (Kruskal-Wallis, P=0.182). This was confirmed using the non parametric Mann-Whitney test to compare median mortalities of the two *trans*-β-ocimene treated barriers with the sawdust only barriers (P>0.05).

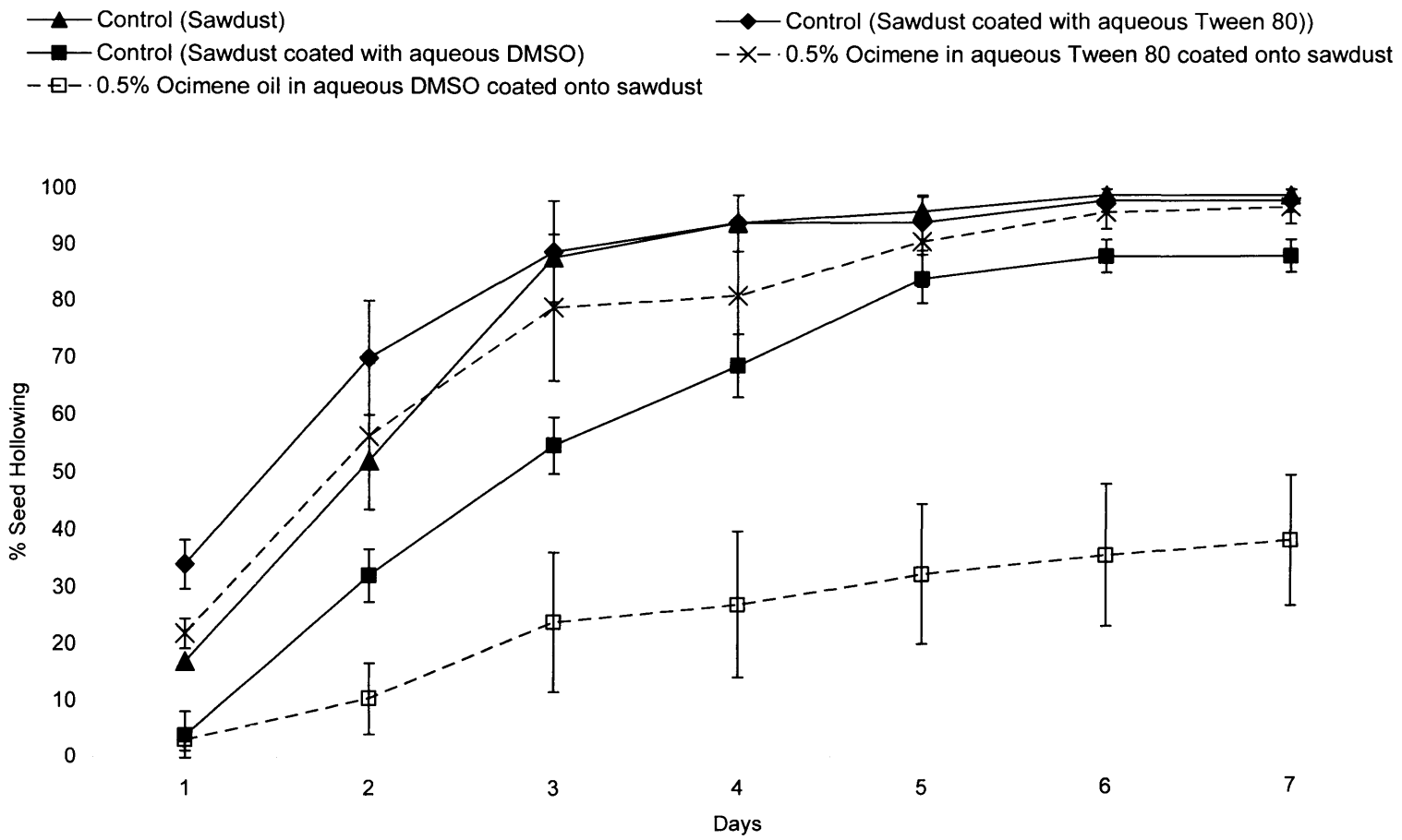


Figure 2.19 Evaluation Of Barrier Efficacy Of Sawdust Coated With Aqueous Extracts Of Ocimene.

Assessment Of Seed Hollowing (7 Days).

2.4.9 Repellent Barriers Using Myrrh Oleoresins (100%) Evaluated Against Arion hortensis Slugs

Seed Hollowing

The use of myrrh oleoresins as repellent barriers significantly reduced the amount of seed hollowing caused by the *A. hortensis* slugs. No occurrences of seed hollowing were observed over the entire seven day test period.

Slug Mortality

No incidences of slug mortality were observed over the seven day test period. Excess secretions of white gel-like mucous were, however, produced by the slugs on the slugs contact with the myrrh barriers, see figure 2.20.

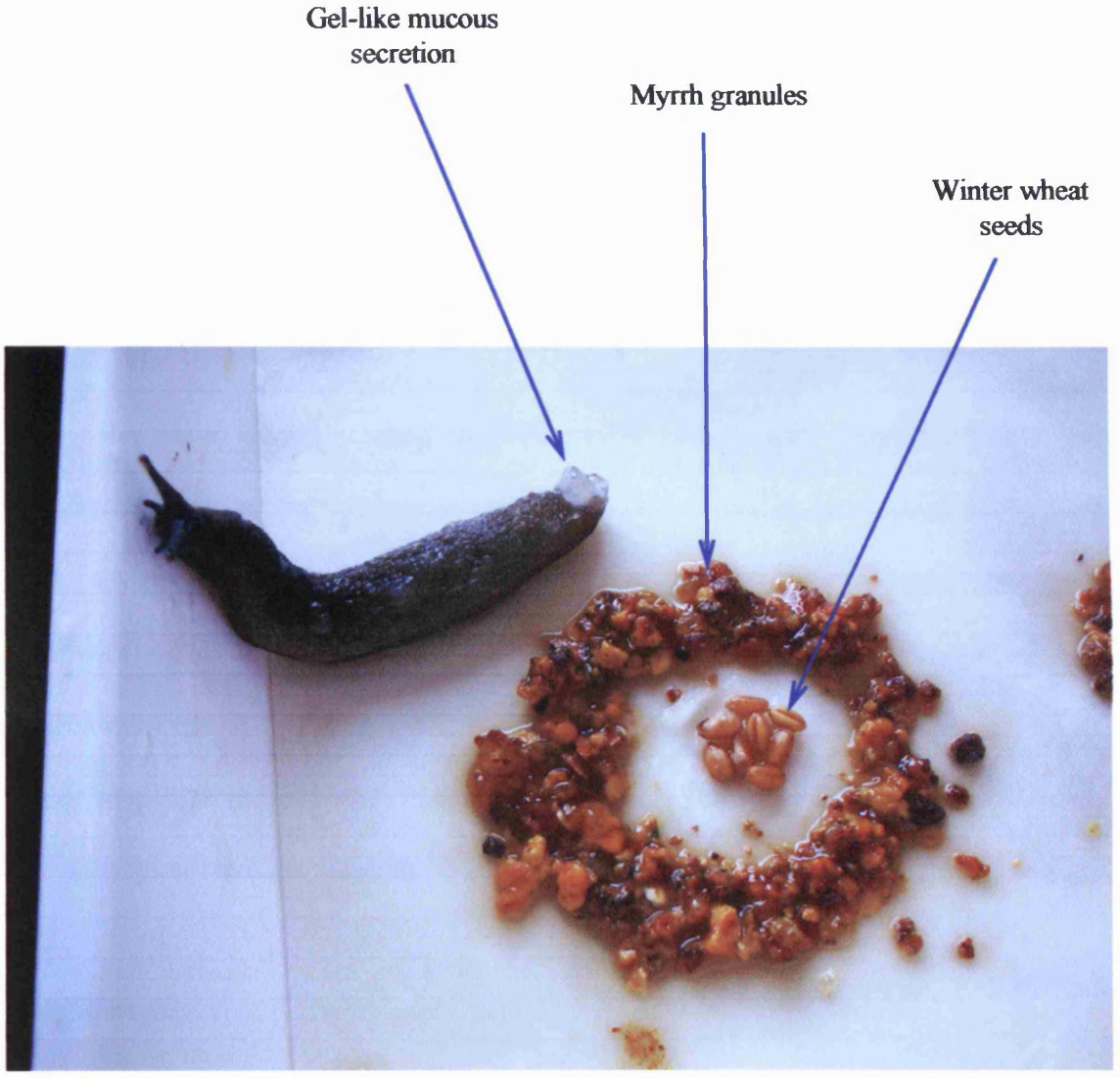


Figure 2.20 Large slug (*Arion* spp.) avoiding contact with the repellent barrier comprised of myrrh oleoresin granules. Although the slug was pre-starved for 24 hours, the winter wheat seeds remained undamaged after seven days. Notice the gel-like mucous secretions on the tail end of the slug indicating signs of irritation, on contact with the myrrh granules.

2.4.10 Repellent Barriers Using Myrrh and Opoponax Oleoresins (100%) and their mixtures with sawdust, Evaluated Against H. aspersa Snails

Seed Hollowing

Repellent barriers comprised of 100% myrrh and opoponax oleoresins significantly reduced the number of lettuce leaf discs eaten by *H. aspersa* snails for 7 days, compared to the sawdust control (Mann-Whitney test, P=0.017 for both oleoresins). Pair-wise comparisons of the medians showed that the strong repellency properties of the two oleoresins was still maintained, even after reducing the amount of myrrh oleoresins to 60% (P=0.021) and opoponax oleoresins to 60% (P =0.020) when incorporated as repellent barriers. This is illustrated in figure 2.21.

Snail Mortality

Very little incidences of snail mortality were observed after the seven days. Maximum median snail mortality was found to be 10% for both of 100% myrrh and opoponax oleoresins. Mann-Whitney pair-wise comparison of the medians to the control showed incidences of mortality to be not significant (P> 0.05).

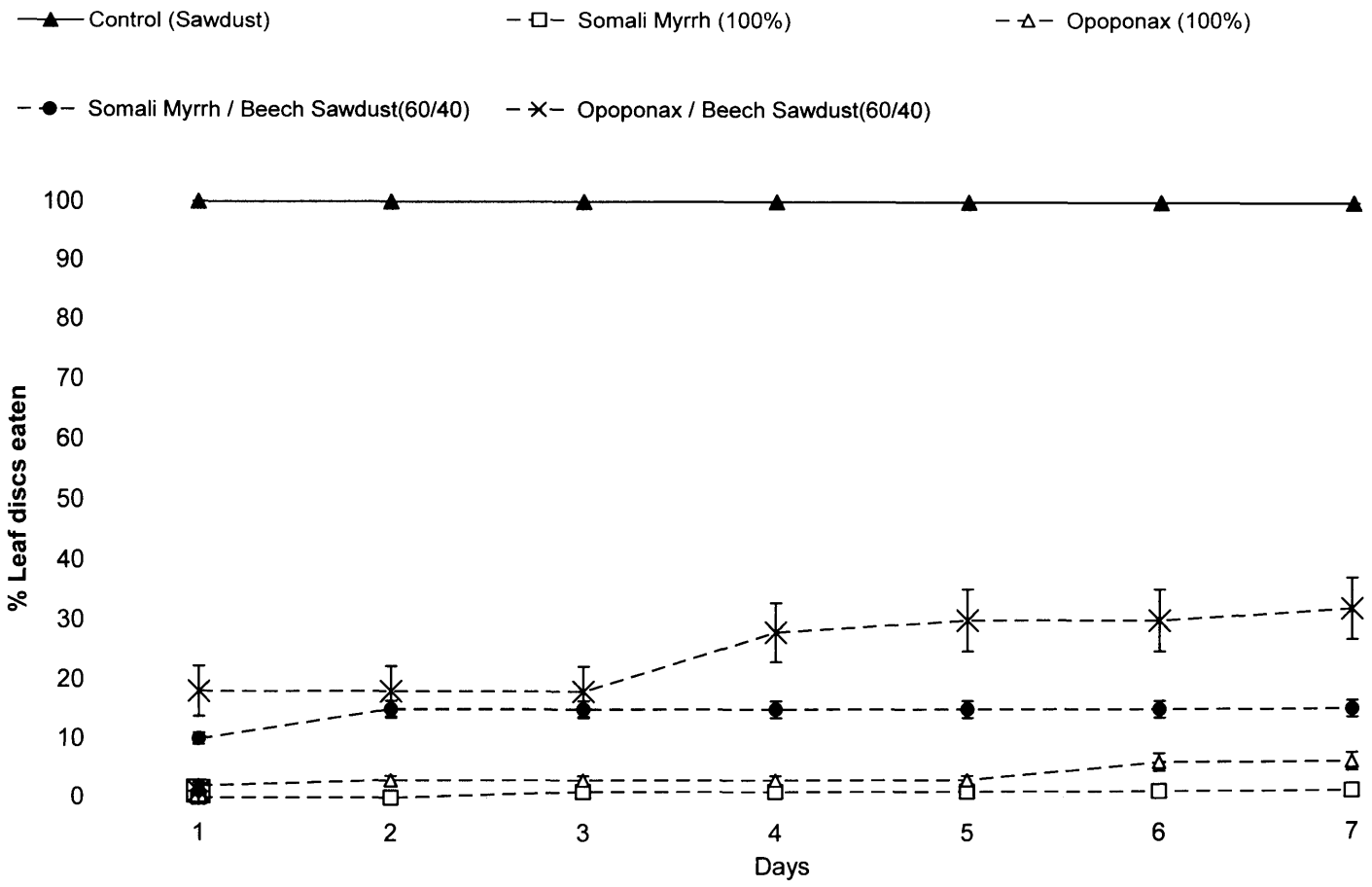


Figure 2.21 Evaluation of Barrier Properties of 100% Myrrh and Opoponax Oleoresins, Myrrh/Sawdust Mixes and Opoponax/Sawdust Mixes, On Barrier Repellency Against *H. aspersa* Snails. Assessment Of Seed Hollowing (7 Days).

2.4.11 Evaluation of Toxicity Effects of Myrrh Barriers: Non-Target Organisms(Earthworms)

As shown in table 2.1 very low incidences of earthworm mortality (median 0%) were observed for the sawdust (control) and opoponax barriers and represented only one dead earthworm out of 20. Comparison of the group medians using the non parametric Mann-Whitney test, showed these incidences of mortality to be not significant ($P < 0.05$). No earthworm mortality was observed for barriers of myrrh oleoresin after the seven day test period

Table 2.1 Effect of Myrrh and Opoponax Barriers on Earthworms On Day 7

	Control (Sawdust)		Myrrh Oleoresin		Opoponax Oleoresin	
	Mean ± S.E.M	Median	Mean ± S.E.M	Median	Mean ± S.E.M	Median
% Mortality	5 ± 5	0	0 ± 0 NS	0	5 ± 5 NS	0
	M-W test		P =1.000		P =0.342	

N = 20 earthworms. NS = Not significant (P >0.05). M-W = Mann-Whitney.

2.4.12 Evaluation of Phytotoxic Effects of Myrrh Barriers on Winter Wheat Seeds

Effect on Germination

Surrounding winter wheat seeds with barriers of myrrh had no significant effect on seed germination at the end of the seven day test period (Anova, $P = 0.115$). The wheat seeds surrounded with myrrh barriers gave similar germination rates to the wheat seeds surrounded with the sawdust (control) barriers resulting in 95% and 87% seed germination respectively, see figure 2.22.

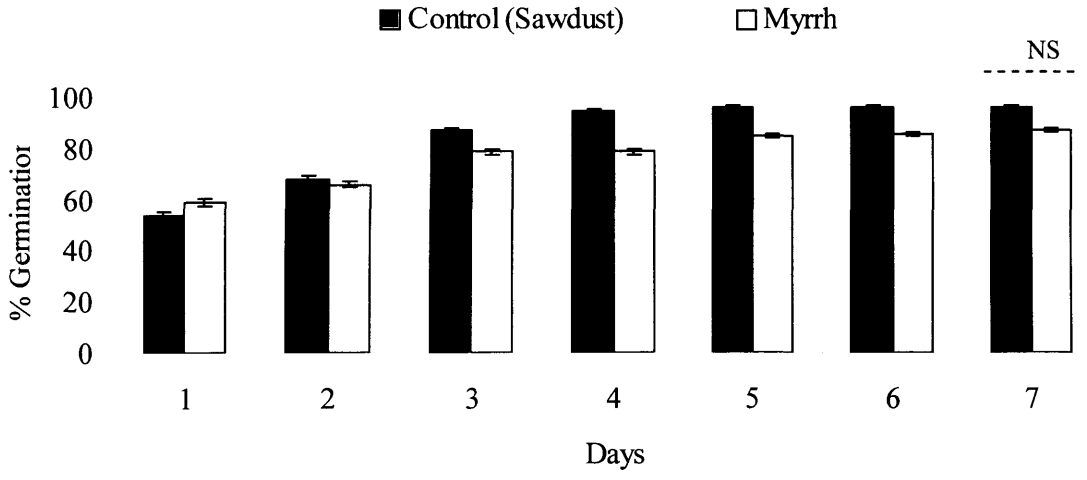


Figure 2.22 Effects of myrrh barriers on the germination rates of winter wheat seeds at day 7. NS = No significant difference ($P > 0.05$).

Effect on Coleoptile Length

After seven days the coleoptile lengths of the winter wheat seeds bordered by barriers of myrrh showed no significant differences to those bordered by barriers of sawdust (Anova, $P = 0.182$), see figure 2.23.

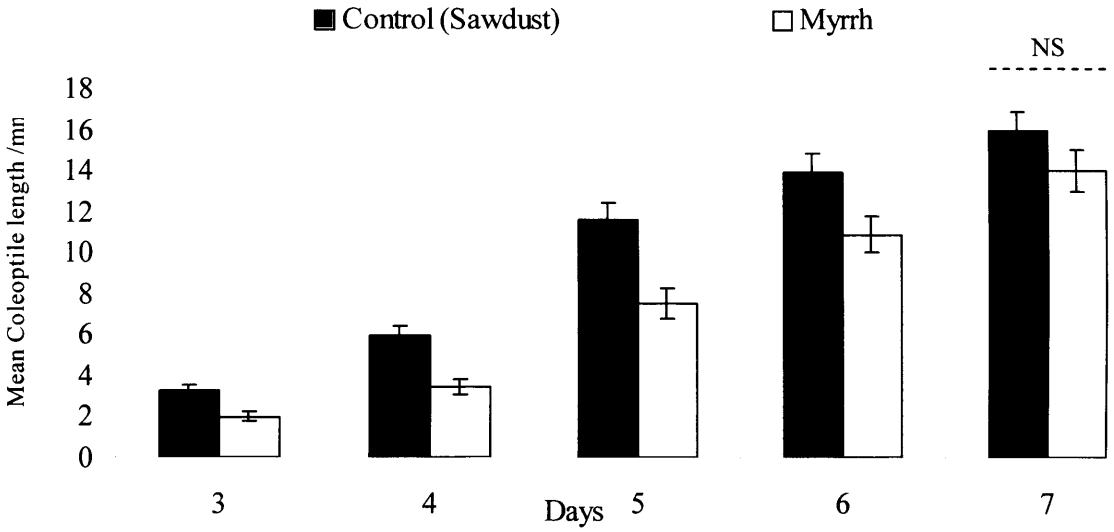


Figure 2.23 Evaluation of phytotoxic effects of myrrh barriers on the coleoptile lengths of winter wheat seeds at day 7. NS = No significant difference ($P > 0.05$).

See appendix A1, for photograph of fully developed winter wheat seed coleoptiles surrounded by barriers of myrrh oleoresin.

2.4.13 Evaluation of Phytotoxic Effects, of Myrrh, Opoponax and Ocimene Extracts On Winter Wheat Seeds

Effect on Germination

As indicated in figure 2.24, soaking the winter wheat seeds in the plant extracts had no significant effect on seed germination (Anova, $P = 0.741$).

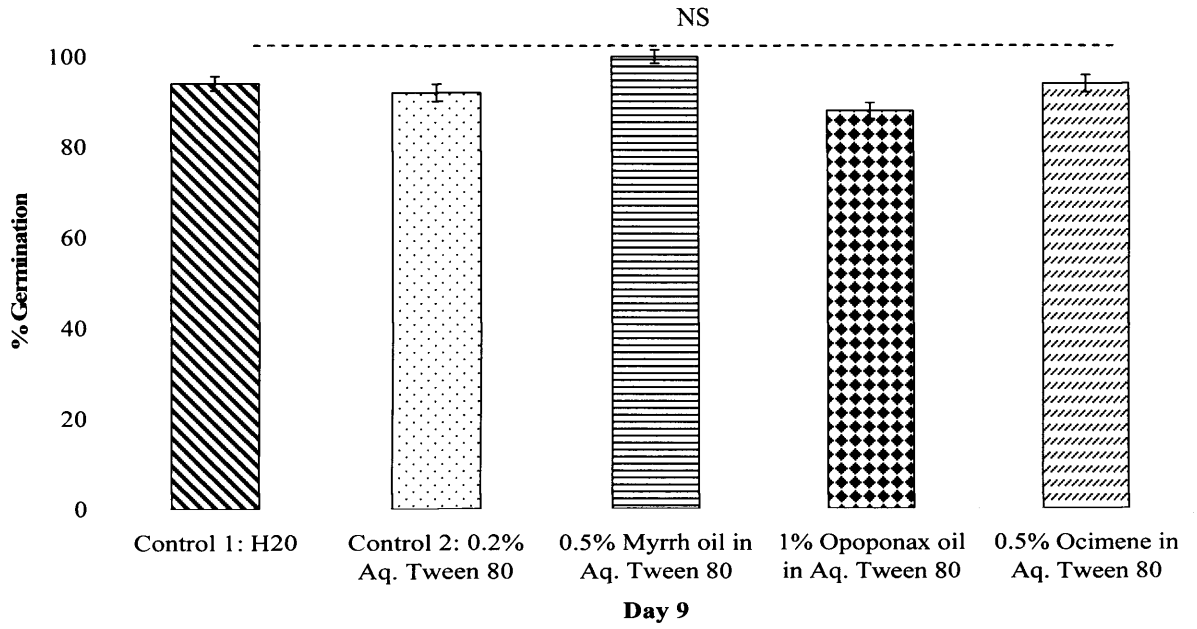


Figure 2.24 Evaluation Of Phytotoxic Effects of Plant Extracts on the Germination Rates of Winter Wheat Seeds. NS = No significant differences ($P > 0.05$).

Effect on Coleoptile Length

During the first five days of the experiment the myrrh and opoponax essential oils caused the winter wheat seed coleoptiles to develop significantly slower than the controls (Anova, $P = 0.002$), as indicated in figure 2.25. The mean coleoptile lengths on day five, for aqueous Tween 80, Myrrh oil, Opoponax oil and ocimene were 16, 5, 7 and 13 mm in length respectively, compared to 21mm for the control. This retardant effect was short lived as the aqueous Tween 80 extracts of myrrh, opoponax and ocimene had only marginal effects on coleoptile length over the nine day test period (Anova, $P = 0.052$), compared to the control (water).

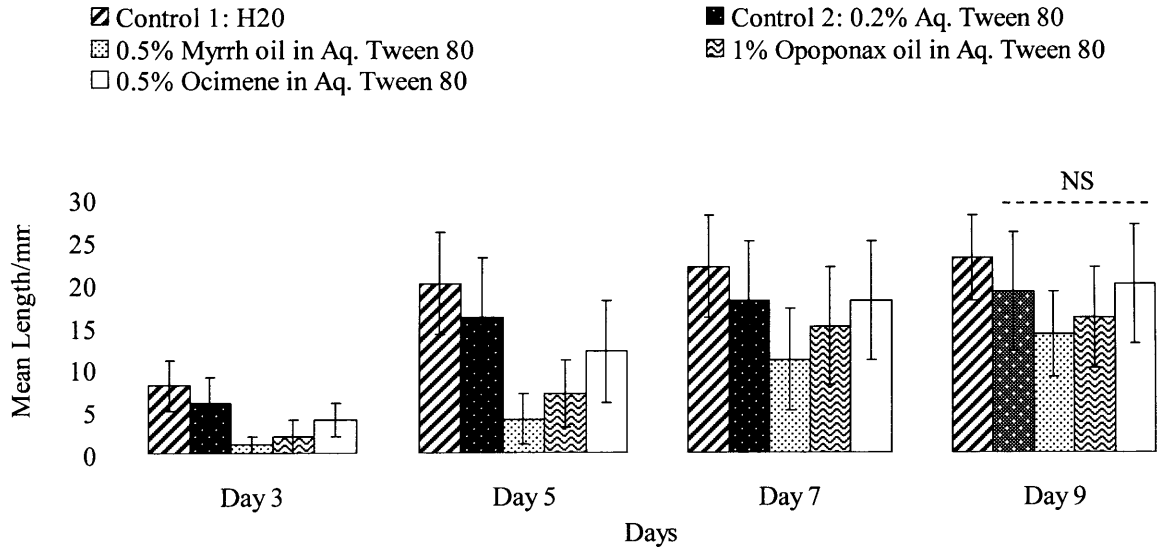


Figure 2.25 Evaluation of Phytotoxic Effects of Plant Extracts on the Coleoptile Lengths of Winter Wheat Seeds. NS = No significant difference ($P > 0.05$)

Multiple statistical comparisons, using the parametric Tukey-Kramer test, showed only the myrrh extracts to have a very minor effect on the coleoptile length on day nine (mean length 15 mm), compared to the control (mean length 23 mm). This coleoptile length bordered on the limits of significance, with the difference between group means being 9.0 compared to the critical value of 8.9. All the other seed treatments had no significant effect on the length of the seed coleoptiles, with aqueous Tween 80, opoponax and ocimene having mean coleoptile lengths of 21, 18 and 22 mm respectively with differences in group means being less than the critical value. See appendix A2 for photograph of fully developed winter wheat seed coleoptiles surrounded by sawdust coated with aqueous Tween 80 extracts of myrrh essential oil.

2.4.14 Summary

The various materials evaluated as mollusc repellent barriers, in the terraria trials, are summarised in table 2.2.

Table 2.2 Summary Of Terraria Trials.

Test Barrier	Test Period	Slug Species	Substrate	Results
100% Inert Materials (Sawdust, Sand, Kaolin Clay).	7 days	*D. reticulatum*	Blotting Paper	Sawdust was an effective barrier for 3 days only. After 7 days all the materials tested were not significantly more effective, as barriers, than the unprotected seeds (control). Kaolin clay changed from a powder to a liquid paste after a few days. No significant incidences of mortality were observed.
	14 days	"	"	After 14 days the sawdust and sand barriers were not significantly more effective, as barriers, than the unprotected seeds (control). No significant incidences of mortality were observed.
100% Myrrh and opoponax oleoresins.	7 days	"	"	Both oleoresins showed potent barrier properties. Very high incidences of slug mortality.
	14 days	"	"	Both oleoresins showed potent barrier properties. Low incidences of slug mortality.
Myrrh / Inert substrate mixes. (Myrrh/sawdust 60:40 and myrrh/corncob, myrrh/ sand both 50:50).	11 days	"	"	All the myrrh/ inert substrate barriers showed potent barrier properties. Only the myrrh/ sand barrier showed significant slug mortalities.
Myrrh / corncob mixes Dose-activity relationship. (40:60, 30:70 and 20:80).	11 days	"	"	All the myrrh/corncob barriers were effective barriers. Only the 30:70 myrrh/corncob barriers gave significant slug mortalities.
Myrrh / Inert substrate Mixes. (Myrrh/sawdust 60:40 and myrrh/corncob 50:50, myrrh (100%) and Slug Stop™.	7 days	*D. reticulatum*	Peat Soil	All the myrrh/inert substrate barriers showed significant barrier efficacy. No significant incidences of slug mortality observed.
Ethanolic *Comiphora* extracts. Sawdust, Sawdust coated with ethanol. Ethanolic myrrh (Baldwin), ethanolic myrrh (Thorton & Ross) and ethanolic opoponax extracts.	7 days	"	Blotting Paper	Sawdust and sawdust barriers coated with ethanol were not effective barriers against slugs All the ethanolic myrrh barriers exhibited strong barrier properties. Only sawdust barriers coated with ethanolic myrrh (Thornton &Ross) showed significant slug mortalities.
	14 days	"	Blotting Paper	Sawdust and sawdust barriers coated with ethanol were not effective barriers against slugs All the ethanolic myrrh barriers exhibited strong barrier properties. Only sawdust barriers coated with ethanolic opoponax showed significant slug mortalities.

Table 1. Continued

Test Barrier	Test Period	Slug Species	Substrate	Result
Aqueous Essential Oil extracts Sawdust coated with ethanol, aqueous DMSO and aqueous Tween 80, Sawdust coated with 0.5% myrrh oil (in aqueous DMSO & aqueous Tween 80), Sawdust coated with 1% opoponax oil (in aqueous DMSO & aqueous Tween 80)	7 days	*D. reticulatum*	Blotting Paper	All the sawdust barriers coated with only solvents showed poor barrier properties. All sawdust barriers coated with myrrh oil were strong barrier repellents against slugs. Sawdust coated with opoponax oil, solubilised in aqueous Tween 80, exhibited very poor barrier properties. Sawdust coated with opoponax oil, solubilised in aqueous DMSO, was significantly effective as repellent barriers.
***Trans*-β-ocimene extracts** Sawdust coated with aqueous DMSO and aqueous Tween 80, Sawdust coated with 0.5% ***Trans*-β-ocimene** (in aqueous DMSO & aqueous Tween 80)	7 days	"	Blotting Paper	All the sawdust barriers coated with only solvents showed poor barrier properties. Sawdust coated with *trans*-β-ocimene, solubilised in aqueous Tween 80, exhibited very poor barrier properties. Sawdust coated with *trans*-β-ocimene, solubilised in aqueous DMSO, was significantly effective as repellent barriers.
100% Myrrh oleoresins.	7 days	*A. hortensis*	"	Myrrh oleoresins were highly effective repellent barriers. No occurrences of seed damage. No incidences of slug mortality.
100% Myrrh oleoresin, **60/40 Myrrh /sawdust mixes** **100% Opoponax oleoresin** **60/40 Opoponax /sawdust mixes**	7 days	*H. aspersa*	"	100% Myrrh and opoponax oleoresins were highly effective repellent barriers against snails. Myrrh and opoponax mixed with sawdust still maintained their strong barrier repellency properties. Low incidences of snail mortality observed.

2.5 DISCUSSION

Evaluation of the inert materials as barriers showed only kaolin clay to have a significant effect on reducing seed hollowing over fourteen days. This was mainly due to the change in physical state from a powder to a thick liquid paste over a very short period of time. Kaolin clay was discarded from further testing as a suitable inert substrate should maintain its solid physical state for it to be useful as a diluent with the bio-active *Commiphora* spp. oleoresins. Although unsuitable as a physical barrier against terrestrial molluscs, Kaolin may be more suited formulated as a mollusc repellent spray/coating. Kaolin is registered in the USA as a pesticide for many crops, in particular apples, and has proven to be as effective as organo-phosphates in controlling mites, moths maggots and thrips (Anon., 2000).

The other "inert" substrates evaluated as barriers (sharp sand, corncob and sawdust) had no significant effect on the feeding behaviour of the slugs and in addition continued to hold their original physical formation over the period under test. None of the inert substrates, evaluated against *D. reticulatum* slugs, were found to be molluscicidal in nature. Grubisic *et al.* (2003) also evaluated sawdust, as a physical barrier, against *Arion rufus* slugs in field trials with Swiss chard, beans and lettuce plants. Although they reported sawdust barriers to have reduced the amount of leaf damage over six days, compared to the control, they found it's barrier properties was not statistically significant. In this study, laboratory terraria trials with barriers comprised of Spruce sawdust was some times found to significantly deter the slugs from feeding for two to three days but was found to loose its protective properties after that initial period. This initial barrier effect in addition to its inert properties gives an

added benefit for mixing sawdust with myrrh oleoresin as a repellent barrier. Although sawdust, sand and corncob were not very effective repellent barriers in these trials, they are useful for diluting expensive plant materials, such as myrrh and opoponax oleoresins, which themselves have proven to be highly active mollusc repellents.

In terraria trials both myrrh and opoponax oleoresins were found to be very effective slug repellents, when applied as barriers, with very little seed damage occurring over two weeks. Although myrrh has been shown to be not toxic to mice (Rao *et al.*, 2001), high incidences of slug mortality were recorded for both oleoresins during the first week. This was reduced to insignificant levels during the second week. Most dead slugs were found either on top of the myrrh / opoponax oleoresins barriers or a very short distance away. Both oleoresins are very sticky in nature and on contact the slugs become retarded or trapped. They try to move away by secreting copious amounts of mucous, eventually exhausting their supply of mucous. The mechanism of slug death, therefore, appears to be one of dehydration. This is in some ways a similar mechanism to that incurred when slugs come into contact with the molluscicide metaldehyde.

Replacing the blotting paper lining of the tray arena with peat soil resulted in negligible slug mortalities whilst maintaining significant repellence properties for the 100% myrrh and myrrh/sawdust barriers. This situation reflects more closely the scenario found in the field and clearly reduces incidences of slug mortality.

The myrrh oleoresin granules were also very effective barriers against the larger species of slugs and snails (*A. hortensis and H. aspersa*) completely retarding

their feeding behaviour with little or no mortality effects. Because of the small physical size of *D. reticulatum,* many of them cannot recover from the excessive mucous production. However, this phenomenon is not observed with the larger *Arion* spp. and *H. aspersa*, which have a much higher survival rate. Other researchers have also observed *D. reticulatum* to be more sensitive to physical contact with chemicals than *H. aspersa* (Parella *et al.*, 1985; Davis *et al.*, 1996).

Myrrh mixtures including sawdust, corncob and sharp sand were found to be exceptional repellent barriers preventing the slugs from feeding on the winter wheat seeds. In general slug mortality levels appeared much lower than with barriers comprised only of myrrh oleoresin, the exception being the sand mixture where mortality was significantly higher. The sand granules may be enhancing slug mortality by severely irritating or cutting the surface skin of the slug as it moves over it. Mixtures with sawdust and corncob may therefore, be preferable repellent barriers in terms of maintaining biodiversity and conservation by avoiding mortalities. All the myrrh / inert substrate mixes were much superior repellent barriers than the commercial product (Slug Stop ^{TM}) which was not significantly different from the unprotected winter wheat seeds.

Coating the ethanolic plant extracts onto sawdust also had significant effects in reducing seed hollowing over fourteen days for both species of *Commiphora* spp. oleoresins. Slug mortality was highly variable, with ethanolic myrrh and opoponax being significantly molluscicidal on alternative weeks over a two week test period.

Barriers of sawdust treated with essential oil showed only the myrrh extracts to give effective crop protection, against the slugs, particularly the aqueous DMSO extract. Opoponax essential oil was not as effective, as myrrh oil, in deterring the slugs from feeding on the winter wheat seeds. In all the barrier treatments with essential oil, incidences of slug mortality were very low.

The repellence properties of myrrh and opoponax oleoresin granules and their extracts, however, are enhanced by moist conditions, possibly by increasing the adhesive properties of the barrier, making it very difficult for molluscs to move over it. In contrast, a metal based barrier such as copper tends to lose its protective property in wet conditions due to the formation of a thin film of moisture, enabling the slug to crawl over it (Godan, 1983).

Barriers comprising of myrrh and opoponax oleoresins had no toxic effects against the beneficial non-target earthworms, using the funnel test. These barriers also had no phytotoxic effects against winter wheat seeds in terms of germination rate and coleoptile development, when tested in a peat soil terrarium. In addition, no phytotoxic effects against winter wheat seeds were observed with myrrh and opoponax barriers in terraria trials, when blotting paper was used as a substrate. This is encouraging as in wet conditions leachate from barriers of *Commiphora* spp. oleoresins would come into contact with the winter wheat seeds.

Winter wheat seeds soaked in 0.5-1% o/w emulsions of myrrh, opoponax and ocimene showed, in terms of coleoptile development, a slight retardant effect up to day five. This was short lived, as, by day nine, only myrrh oil extracts were marginally

affected. No effect on the germination rate was observed for any of the aqueous oil treatments. These results are very encouraging as other investigators have found chemicals of plant origin, including monoterpenes, to be phytotoxic to winter wheat seed when applied as seed dressings (Powell *et al.*, 1996). They found the chemicals of plant origin, in particular cinnamyl alcohol, salicyladehyde, carvone and menthone completely inhibited seed germination, whereas the chemicals vanillin, thymol and a-terpineol significantly affected coleoptile development.

The harmful effects of plants on one another through the production of secondary chemicals is called *allelopathy* and the substances which inhibit growth and germination termed *allelochemicals*. Members of the *Brassicaceae* have frequently been cited as allelopathic crops (Turk and Tawaha, 1973). Components such as allyl-isothiocyanates from black mustard (*Brassica niagra*) are implicated as inhibiting the establishment of grass species in natural grassland communities. Rothamstead laboratories investigated the antifeedant properties of more than sixty plant species, with the most active being both a root from the horseradish plant *Amoracia rusticana* and also a scented geranium (*Pelargonium graveolens*) (Warrel, 1991). The chemical responsible for the antifeedant property of the horseradish plants was found to be 2-phenylethyl isothiocyanate. Unfortunately this proved to be phytotoxic to winter wheat seedlings.

In summary, these laboratory terraria trials have confirmed the potent repellence, and in some cases molluscicidal, properties of myrrh (*C. molmol*) and opoponax (*C. guidottii*) oleoresins against terrestrial molluscs, *D. reticulatum, A. hortensis*, and *H. aspersa*, when applied as barriers. In contrast to chemical means of

mollusc control, no non-target effects against earthworms were observed for both *Commiphora* spp. oleoresins when applied as 100% barriers. In addition, neither myrrh or opoponax exhibited any phytotoxic effects towards winter wheat seeds.

CHAPTER 3:

Chemistry of Myrrh and Opoponax

3.1 INTRODUCTION

3.1.1 The Chemistry Of Myrrh (C. molmol)

Chemical studies, involving *Commiphora* spp. oleoresins are often complicated by the lack of quality control procedures when first obtaining the plant samples. Authentication of the *Commiphora* species, from which the oleoresin has originated, is fundamental for accurate reporting of the chemical profile of these samples. Dekebo *et al.* (2002) highlighted additional problems associated with studying the chemistry of *Commiphora* spp. oleoresins which arise as a result of deliberate adulteration with oleoresins collected from related *Commiphora* species such as *C. sphaerocarpa* (Bale region, Ethiopia), *C. holtziana* (Isiolo, Kenya) and *C. kataf* (Samburu district, Kenya).

"True" myrrh trees (*C. molmol and C. myrrha*) grow in the arid regions of Somalia, eastern Ethiopia (Ogaden) and Yemen. Although these two myrrh species are synonymous (Hedberg and Edwards, 1989), there are ostensible differences in their appearance, which can probably be attributed to differences in the oleoresins volatile oil content. The appearance of myrrh oleoresins from north Somalia (Somaliland) is bright blood-red in colour, with a very high oil content, whereas the Ethiopian and Yemeni myrrh varieties have a dull yellow-brown colouration with a brittle appearance and are associated with a very low volatile oil content. As mentioned in chapter 1, Cufodontis (1956) argued for *C. molmol* to be reserved for the African myrrh tree and *C. myrrha* for the Arabian myrrh tree.

Myrrh oleoresins obtained from *C. molmol* and *C. myrrha* trees, are reported to be comprised of 7% to 17% volatile oil, 25% to 40% resin and up to 57% to 61% gum (polysaccharide). The resin is thought to contain three free commiphoric acids (α, β and γ isomers), an ester of a resin acid called commiphorinic acid as well as two phenolic resins α- and β-heerabomyrrhol (BP Codex 1968).

In the early twentieth century scientists researching the chemistry of myrrh concentrated mostly on the identification of the monoterpenes present in the volatile oil and the chemistry of the organic acids present in the resin. Today the chemistry of myrrh oleoresin and its extracts is generally agreed to be comprised of several terpenoid compounds, predominantly sesquiterpenes and furano-sesquiterpenes as illustrated in figure 3.1. Ma *et al.* (1991) used supercritical fluid extraction to isolate the volatile oil from myrrh oleoresin and used gas chromatography-mass spectrometry (GC-MS) to identify a number of oxygenated furano-sequiterpenes, including curzerenone and furanodienone.

Dekebo *et al.* (2002) also used GC-MS to analyse the essential oil extract of myrrh oleoresin, collected from southern Ethiopia (Gode), but failed to detect any oxygenated sesquiterpenes. They concluded that previous reports of their presence, in myrrh oleoresins, were due to adulteration from related *Commiphora* spp. oleoresins such as *C. sphaerocarpa* (Bale region, Ethiopia), *C. holtziana* (Isiolo, Kenya) and *C. kataf* (Samburu district, Kenya).

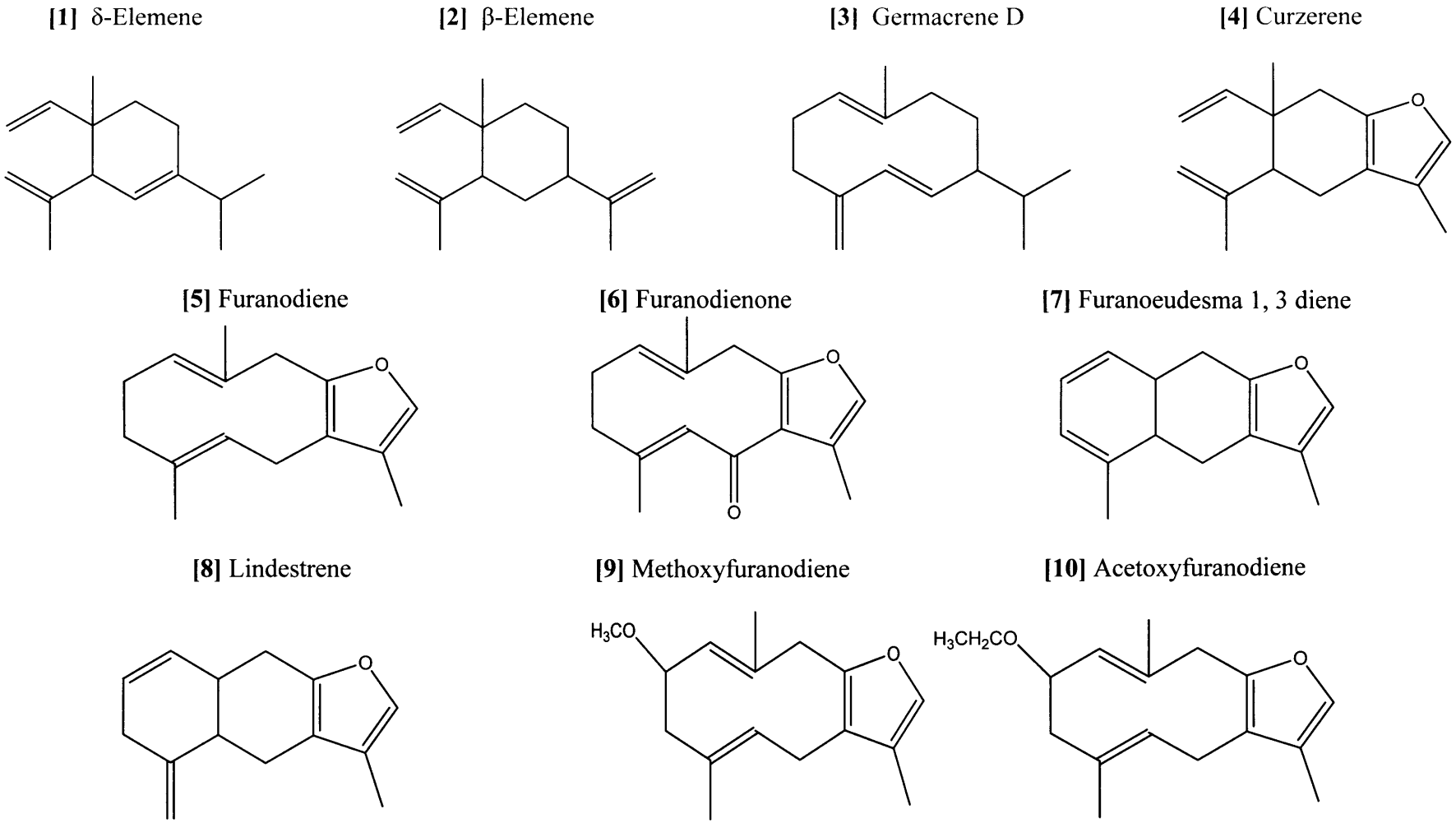


Figure 3.1 Chemical Structures Of Sesquiterpenes And Furano-Sesquiterpenes Reported To Be Present In Myrrh (*C. molmol and C. myrrha*).

Another difficulty that might be incurred when performing chemical analysis of *Commiphora* spp. oleoresins using gas chromatography (GC), can be attributed to the high temperatures associated with the injector port resulting in the formation of chemical artefacts such as those illustrated in figure 3.2. Hikino *et al.* (1968) were the first researchers to propose that pyrolysis of the furano-sesquiterpene, furanodiene, would result in a Cope rearrangement resulting in the formation of curzerene. This phenomenon was also observed by Maradufu (1982) who found that the two furano-sesquiterpenes, methoxyfuranodiene and acetoxyfuranodiene, formed their respected furano-elemanes after pyrolysis. The occurrence of thermal rearrangement products, in gas chromatography, is relatively easy to detect as an increase in the baseline noise usually occurs, resulting in a hump-like peak, suggesting that not only the injector but the oven temperature is a major contributing factor to this phenomena (Baldovini *et al.*, 2001).

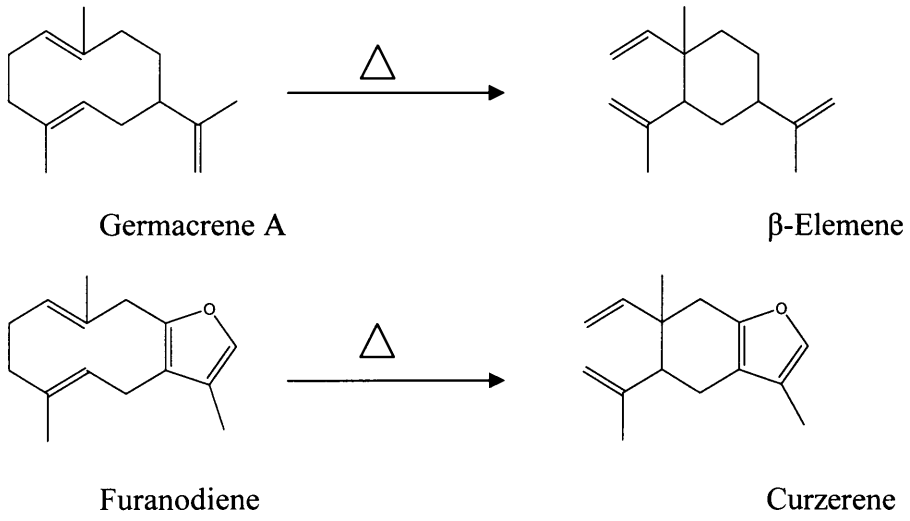


Figure 3.2 Example Of Thermal Rearrangement Products Associated With Germacrene A and Furanodiene.

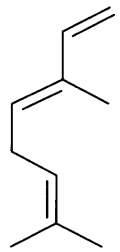
3.1.2 The Chemistry Of Opoponax (C. guidotti)

The appearance of opoponax oleoresins are quite distinct from myrrh oleoresins, exhibiting white bone-like streaks on the surface and are recognised by the sweet aroma associated with them, compared to the balsamic aroma arising from myrrh oleoresins.

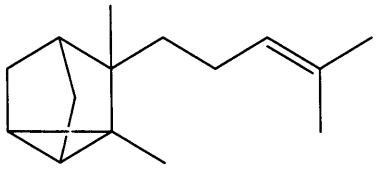
In contrast to myrrh, the volatile oil of opoponax, contains very few furano-sesquiterpenes, only furanodiene, and is defined predominately by the ubiquitous sesquiterpenes α-santalene, bisabolene (α, β and γ isomers), β-bergamotene, β-farnesene and the monoterpenes trans-β-ocimene and 3-carene.

Craveiro *et al.* (1983) analysed opoponax essential oil and found the major components to be the sesquiterpenes α-santalene and α–bisabolene and the furano-sesquiterpene furanodiene. Other researchers (Baser *et al.*, 2003; Jingai and Shangmei 1996; Moyler and Clery, 1997), however, found the monoterpene *trans*-β-ocimene in addition to the sesquiterpenes α-santalene and α–bisabolene to be the major components of opoponax essential oil. Baser (2003) also confirmed furanodiene, to be only a minor component of opoponax essential oil, occurring only at a level of 0.1%.

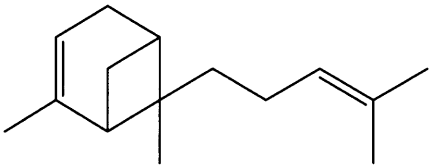
[1] *trans*-β-Ocimene



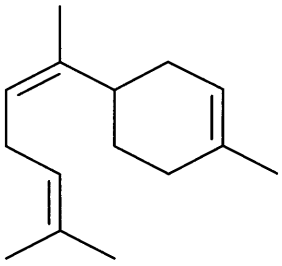
[2] α-Santalene



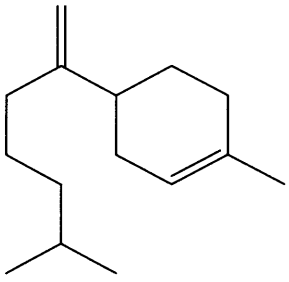
[3] *trans* α- bergamotene



[4] α-Bisabolene



[5] β-Bisabolene



[6] γ-Bisabolene

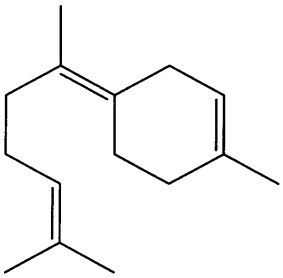


Figure 3.3 Chemical Structures Of Various Terpenoid Compounds Reported To Be Present In Opoponax (*C. guidotti*) Essential Oil.

In chapter 2 Laboratory terraria trials showed barriers of *Commiphora* spp. oleoresins to induce significant changes in the feeding behaviour of three terrestrial mollusc, *D. reticulatum*, *A. hortensis* and *H. aspersa*. Some behavioural changes were elicited a distance away from the barriers (i.e. repellency) whilst other changes in behaviour, could be attributed to avoidance of close physical contact with the barriers. On contact with the barriers, the slugs were severely irritated by the sticky nature of the oleoresins.

There are many *Commiphora* spp. oleoresins, from the "horn of Africa", which are odoriferous and have similar appearances to myrrh and opoponax; hence the need to authenticate the botanical origin of these two oleoresin samples. The most reliable and accurate way to do this is via the use of gas-chromatography, for the separation of volatile and semi-volatile, chemical components and the use of mass spectrometry to identify the chemicals present. In this chapter the two analytical techniques coupled together will be used, and is called gas chromatography-mass spectrometry (GC-MS). The major chemical components of the oleoresins will be identified and if commercially available will be evaluated for their repellence/ antifeedant properties against terrestrial molluscs.

In summary the main aim of this chapter is to use GC-MS techniques to:

i) Identify the chemical compounds present in various liquid extracts (hexane and ethanol) of commercial Yemeni myrrh (G. Baldwin).

ii) Identify the chemical compounds present in two commercial sources of myrrh essential oil (Aldrich and Tisserand).

iii) Determine the major chemicals associated with the volatile odours of commercial myrrh oleoresin and myrrh essential oil.

ii) Identify the chemical components present in the liquid extract (hexane) and volatile aroma of Somali myrrh oleoresins.

iv) Compare the chromatographic profile of commercial myrrh liquid extracts with those from Somali myrrh oleoresins.

iii) Identify the chemical components present in various liquid extracts of opoponax oleoresin.

iv) Ascertain the major chemicals associated with the volatile odours of opoponax oleoresin.

3.2 MATERIALS AND METHODS

Myrrh (*C. molmol*) oleoresin was purchased from G. Baldwin and Co., (London) and according to the supplier originated from Yemen. Opoponax (*C. guidottii*) oleoresins and a second source of myrrh were purchased from Hargeisa (Somaliland). The essential oils of myrrh and opoponax were purchased from Sigma-Aldrich limited (Poole, Dorset). Cis-β-ocimene (purity 99.6%) was also purchased from Sigma-Aldrich limited (Poole, Dorset). Myrrh essential oil and sandalwood essential oil (*Santalum album*), both manufactured by the Tisserand Aromatherapy Company, were also purchased from Neal's Yard Remedies (Cardiff). The following chemicals were kindly provided by R C Treat limited (Suffolk): *trans*-β-ocimene (purity 90%), γ-bisabolene (purity 70 %), germacrene D (purity 40%). Absolute ethanol (HPLC grade) was obtained from Fisher Scientific (UK). The plant *Smyrnium olusatrum* was purchased from the Derry Watkins Company (Chippenham).

3.2.1 Gas Chromatography Mass Spectrometry (GC - MS): Instrument Conditions (Liquid Injection)

Samples were analysed using a Finnegan GC 8000 gas chromatograph equipped with a MD 800 mass selective detector and an AS 800 Finnegan autosampler. A DB-5 fused silica capillary column (J&W Scientific) was used with the following dimensions: 30m x 0.32mm id. and 0.25μm film thickness. The oven temperature was programmed from 50°C to 240°C at a rate of 3°C min^{-1} and maintained at this final temperature for two minutes. The helium carrier gas was set at a flow-rate set of 1ml min^{-1}, maintained under constant pressure. The injector and source temperatures were both set at 260°C.

The mass detector was used in the positive electron impact ionisation mode (EI+) using an ionisation voltage of 70 eV. A scan range of 35 to 450 mass units in 0.45 seconds was used for acquiring mass spectra with an interscan time of 0.08 seconds. Data acquisition was performed using the MassLab (ver 1.4) computer software.

Injection volumes were 1μL for all test samples and were injected in the splitless mode with the purge switched off for one minute. A solvent delay of three minutes and a desorption time of one minute was programmed for all liquid injections. An ethanol solvent wash was incorporated between sample injections.

Identification of individual peaks was made by:

i) Comparing retention times and sample mass spectra to those of authentic standards,

ii) Comparing sample mass spectra to those stored in the NIST library database and

iii) Comparing sample mass spectra to published literature values.

The NIST libraries contain over 54,000 spectra. A reverse fit method was used for identification throughout. This method normalises data to 1000. Compounds with library fits greater than 900 have a high likelihood of being correctly assigned. The common name as well as the chemical name will be reported in this chapter.

3.2.2 *Gas Chromatography Mass Spectrometry (GC - MS)*: Instrument Conditions (Solid Phase Micro Extraction - SPME)

The instrument conditions were the same as described in 3.2.1 with the following variations:

i) Solvent delay time was reduced to 0.01 minutes (no solvents present),

ii) Desorption time was increased to 2 minutes.

3.2.3 Sample Preparation For GC-MSAnalysis: Liquid Injection

A known amount of plant extract was accurately weighed into individual glass hplc vials (see table 3.1) and 1ml of absolute ethanol added. The mixture was vortex mixed for one minute and 1 µl samples injected onto the GC-MS using the chromatographic conditions described earlier in section 3.2.1.

Table 3.1 Preparation Of Myrrh And Opoponax Liquid Extracts For GC-MS Analysis

	File Names	Description	Type of Extraction[1]	Weight of Plant Extract (mg)	Volume of Ethanol (ml)
Myrrh	Molmol 76, 77, 78, 79	Oleoresin (Baldwin)	Hexane	2	1
	Molmol 187, 188, 189	Oleoresin (Somalia)	Hexane	2	1
	Molmol 71, 72, 73, 74	Oleoresin (Baldwin)	Ethanol	2	1
	Molmol 94, 95, 96, 97	Essential oil (Aldrich)	Steam distillation	2	1
	Molmol 83, 84, 85	Essential oil (Tisserand)	Steam distillation	2	1
Opoponax	Molmol 49, 52, 55, 63	Oleoresin (Somalia)	Hexane	2	1
	Molmol 50, 53, 56, 64	Oleoresin (Somalia)	Ethanol	2	1
	Molmol 51, 54, 57, 65	Essential oil (Aldrich)	Essential oil	2	1

Note:

[1] See chapter 4 for the preparation of the hexane and ethanolic extract of myrrh and opoponax oleoresins.

3.2.4 *Preparation of Authentic Standards (Liquid Injections)*

The root part of the *S. olusatrum* plant was cleaned with water to remove soil debris, cut into small pieces and 10g placed in a glass vessel containing 100 ml of hexane. This was covered and left for seven days in a dark cupboard and stirred occasionally. After this time period the extract was filtered under vacuum through a Büchner flask using a Whatman filter and dried using a rotatory evaporator. A bright yellow extract was obtained. This extract was used as a standard for the confirmation of the presence of the furano-sesquiterpenes curzerene and furanodiene. The *S. olusatrum* plant extract and the commercially available chemicals were weighed (2 mg) into glass HPLC vials, 2ml of ethanol added and then mixed at high speed using a Whirly ™ vortex mixer.

Table 3.2 Preparation Of Authentic Standards For GC-MS Analysis

Standard Chemicals	Solvent	Weight of Chemical (mg)	Volume of Ethanol (ml)
cis β-Ocimene	Ethanol	2	1
trans- β-Ocimene	Ethanol	2	1
Germacrene D	Ethanol	2	1
γ-Bisabolene	Ethanol	2	1
Sandalwood oil (*S. album)*[1]	Ethanol	2	1
Alexanders Oil (*S. olusatrum*)[2]	Ethanol	2	1

Note: [1]Sandalwood oil was used to confirm the presence of α-Santalene, in opoponax.

[2] *S. olusatrum* was used to confirm the presence of curzerene and furanodiene in myrrh.

3.2.5 Solid Phase Micro Extraction (SPME) of Myrrh and Opoponax

A known weight of plant extract or solid oleoresin was placed in a clear glass headspace vial (15mm x 45mm, Supelco) and allowed to equilibrate at a constant temperature using a water bath. The fiber needle was penetrated through the septum and the headspace sampled for a fixed time, as indicated in the tables 3.3 and 3.4. The fiber holder was placed in the injection port, the fiber exposed and allowed to desorb for two minutes.

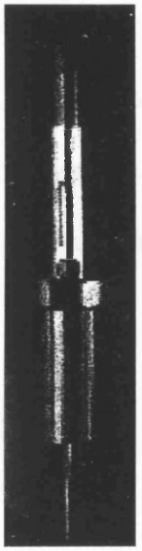


Figure 3.4 Solid Phase Microextraction (SPME) Fiber Holder For Manual Sampling. Reproduced from Anon. 2002.

3.2.6 *Optimising SPME Sampling Conditions for Myrrh and Opoponax*

SPME is a relatively new technique for sampling both liquids and volatile compounds. Sampling, once optimised, is very quick compared to liquid/liquid extractions and has the distinct advantage of not requiring any solvents for injecting onto the gas chromatograph. SPME fibers (Supeleco limited) can be used to extract and concentrate non-volatile and volatile analytes from liquids or odorous solids. They can also be reused many times. In this thesis a polyacrylate fiber (85 μm) was used throughout for the spme analysis of myrrh and opoponax.

Due to differences in the volatility of the *Commiphora* spp. extracts, a number of different parameters had to be varied, to prevent overloading the G.C capillary column. The parameters investigated were the temperature of the water bath, equilibration time and the amount of time the fiber was exposed to the sample (sampling time).

Table 3.3 Optimisation Of The Sampling Conditions For SPME Analysis Of Myrrh (*C. molmol*)

File name	Description	Sample Weight (g)	Water bath Temperature (°C)	Equilibration Time (mins)	Fiber Sampling Time (mins)
Molmol 15	Oleoresin	2.6	47	120	30
Molmol 24	Oleoresin	3.5	25	120	1
Molmol 31	Hexane extract	0.13	25	120	1
Molmol 33	Hexane extract	0.13	47	120	1
Molmol 34	Ethanol extract	0.27	47	120	1
Molmol 35	Ethanol extract	0.27	47	180	120
Molmol 40	Essential oil	0.49	25	10	1

Table 3.4 Optimisation Of The Sampling Conditions For SPME Analysis Of Opoponax (*C. guidotti*)

File name	Description	Sample Weight (g)	Water Bath Temperature (°C)	Equilibration Time (min)	Fiber Sampling Time (min)
Molmol 18	Solid oleoresin	3.40	47	120	30
Molmol 20	Solid oleoresin	3.51	25	60	30
Molmol 21	Solid oleoresin	3.12	25	60	5
Molmol 22	Solid oleoresin	3.01	25	60	1
Molmol 36	Hexane extract	3.21	25	10	30
Molmol 37	Hexane extract	3.06	25	10	1
Molmol 38	Ethanol extract	3.10	47	120	1
Molmol 39	Essential oil	0.73	25	10	1

3.3 RESULTS

3.3.1 Chemical Analysis of Myrrh (Liquid injection): Identification of Compounds

Many of the chemicals detected in the liquid extracts of myrrh oleoresin (hexane, and ethanol) could not be identified using the computer library database, as they are relatively uncommon compounds. Instead identification was performed by comparing the mass spectra with previously published literature. Fortunately many researchers have analysed various extracts of myrrh using GC-MS, hence making some chemical identification possible. Although only 39-56% of the compounds present in the liquid oleoresin extracts of myrrh could be analysed this way, the major chemical compounds were still identified, see table 3.5 and figure 3.3.

Quantification of the essential oils (Aldrich and Tisserand) was much easier because of the reduced number of compounds detected, see table 3.7

Table 3.5 Identification Of Compounds Present In Myrrh Oil Hexane Extract: Liquid Injection (File Name: Molmol 76)

Peak No.	Retention Time (Minutes)	Common Name	Chemical Name or Mass Spectra of Unknown Chemical
[1]	19.21	δ-Elemene [b, c]	[1S-(1α,2β,4β)]-1-ethenyl-1-methyl-2,4 -bis (1-methylethenyl)-cyclohexane
[2]	21.15	β-bourbonene [b]	1,2,3,3a,3bβ,4,5,6,6aβ,6bα-decahydro-1α-isopropyl-3aα-methyl-6-methylene cyclobuta [1,2:3,4] dicyclopentene
[3]	21.55	β-Elemene [c]	(3R-trans)-4-ethenyl-4-methyl-3-(1-methyl ethenyl)-1-(1-methylethyl)-cyclohexene
[4]	25.23	Germacrene D [a, b, c]	(S-(*E,E*)) -1- methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene
[5]	25.98	Curzerene [a, c]	4,5,6,7-tetrahydro-3,6-dimethyl-5-(prop-1-en-2-yl)-6-vinylbenzofuran
[6]	30.34	Furanodienone [c]	(5E,9Z)-7,8-dihydro-3,6,10-trimethyl cyclodeca[b]furan-4(11*H*)-one
[7]	31.18	Furanoeudesma -1,3-diene [c]	4,4a,8a,9-tetrahydro-3,5,8a-trimethylnaptho [2,3-*b*]furan
[8]	31.38	Lindestrene [c]	4,4a,5,6-hexahydro-3methyl-5methylene naptho [2,3-*b*]furan
[9]	34.68	2-Methoxyfuranodiene [c]	(5*E,*9*Z*) -8-methoxy -4,7,8,11-tetrahydro 3,6,10–trimethylcyclodeca [*b*] furan
[10]	35.39	Epicurzerenone [c]	(E)-6,7,10,11-tetrahydro-3,6-dimethyl-10-methylenecyclodeca[b]furan-4(5H)-one
[11]	40.84	2-Acetoxyfuranodiene [c]	(5*E,*9*Z*)-8-ethoxy-4,7,8,11-tetrahydro-3,6,10 -trimethylcyclodeca[*b*]furan

Compound identification was confirmed by: [a] Comparing RT and MS to authentic standard, [b] Comparing mass spectra to NIST library and [c] Comparing mass spectra to published literature. Note: RT= retention time and MS = mass spectra.

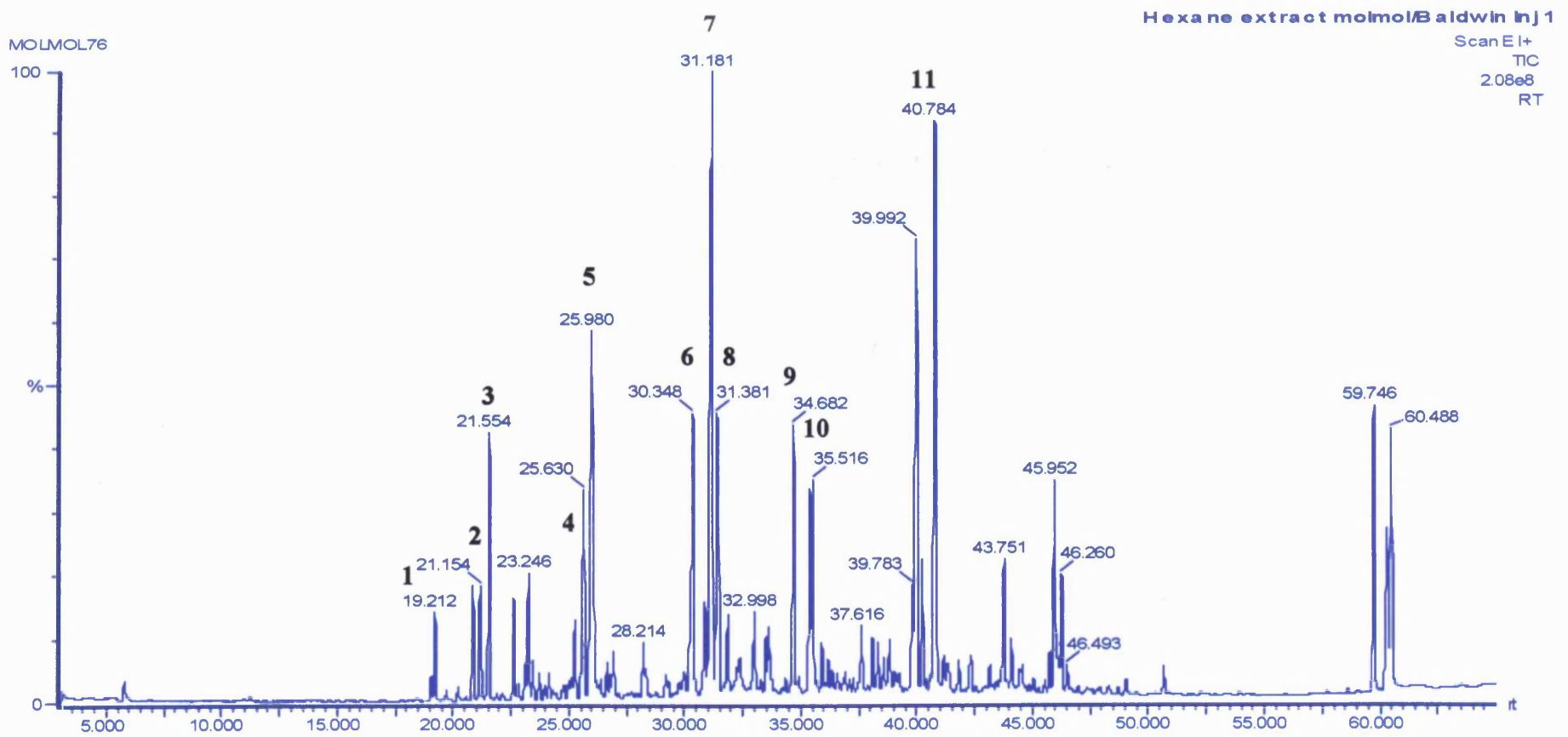


Figure 3.5 Total Ion Current (TIC) Generated Chromatograph of the Hexane Extract of Myrrh (C. *molmol*) Oleoresin (Baldwin): Liquid Injection. Peak Numbers Correspond To The Chemical Compounds Identified In Table 3.5.

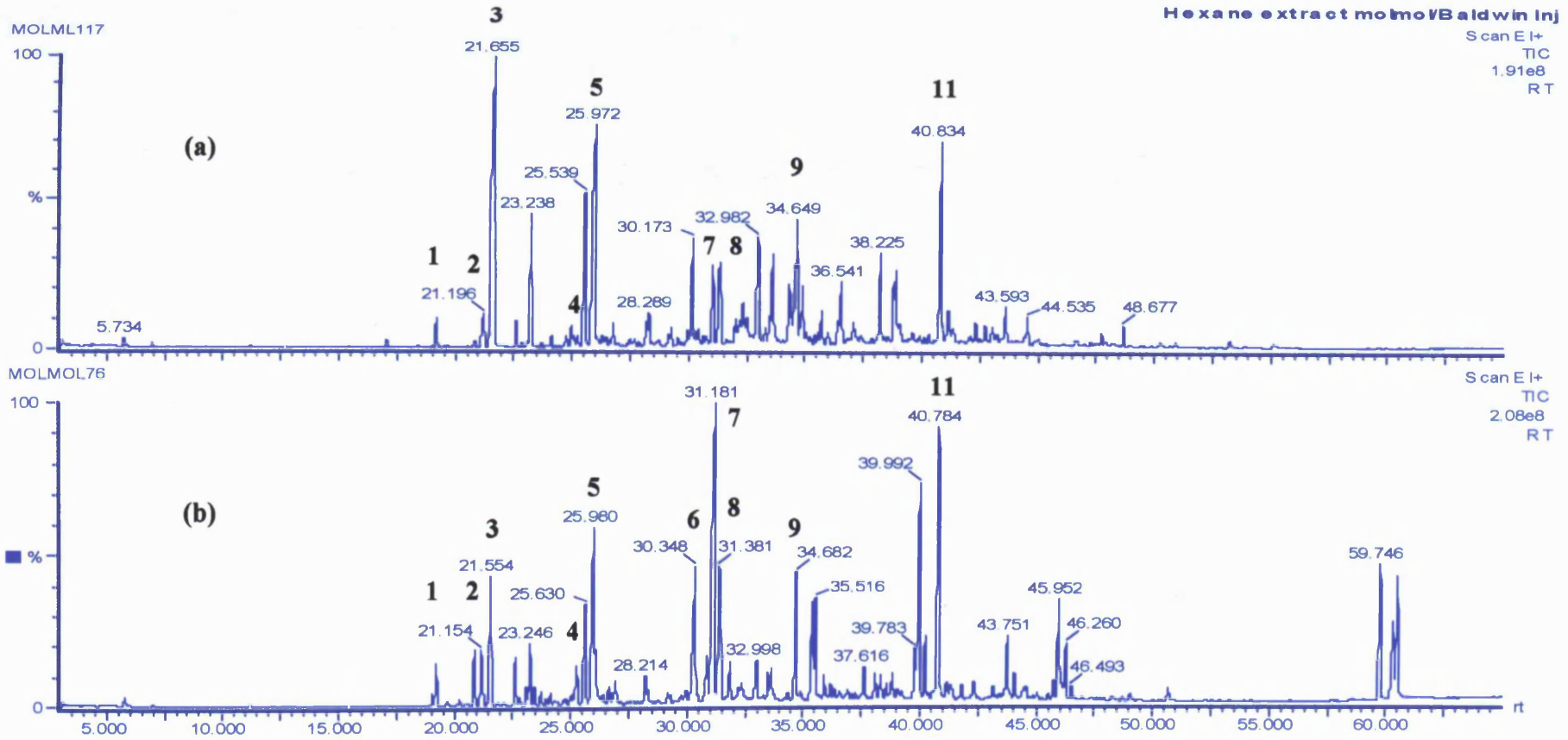


Figure 3.6 Comparison Of TIC Generated Gas Chromatograms Obtained From The Hexane Extracts Of Two Sources Of Myrrh:

a) Somaliland Myrrh (*C. molmol*) b) Yemen Myrrh (*C. molmol*) (Baldwin & Co.).

Peak Numbers Correspond To Compounds Listed in Table 3.5.

Table 3.5 Quantification Of Chemicals Present In Myrrh Oleoresin Extracts (Liquid Injection)

Retention Time (Minutes)	Compounds	Mean % Area ± S.E.M		
		Oleoresin Ethanolic Extract (Baldwin)	Oleoresin Hexane Extract (Baldwin)	Oleoresin Hexane Extract (Somaliland)
19.2	**δ-Elemene**	**0.2 ± 0.0**	**0.8 ± 0.0**	**0.6 ± 0.0**
20.81	Unknown Sesquiterpene	0.6 ± 0.0	1.1 ± 0.0	trace
21.15	**β-Bourbonene**	**0.7 ± 0.0**	**1.4 ± 0.0**	**1.3 ± 0.0**
21.58	**β-Elemene**	**2.0 ± 0.0**	**2.5 ± 0.0**	**13.9 ± 0.2**
23.24	Unknown Sesquiterpene	N D	2.7 ± 0.0	3.4 ± 0.0
25.23	**Germacrene D**	**0.5 ± 0.0**	**0.9 ± 0.0**	**0.2 ± 0.0**
25.63	Unknown Sesquiterpene	2.0 ± 0.0	2.9 ± 0.0	4.6 ± 0.0
26.14	**Curzerene**	**4.1 ± 0.0**	**4.8 ± 0.0**	**9.0 ± 0.0**
30.17	Unknown Sesquiterpene	N D	N D	2.9 ± 0.0
30.37	Unknown Sesquiterpene	N D	N D	N D
30.41	**Furanodienone**	**3.1 ± 0.0**	**4.4 ± 0.4**	**N D**
31.34	**Furanoeudesma -1,3 diene**	**8.0 ± 0.1**	**10.6± 0.1**	**2.1 ± 0.0**
31.38	**Lindestrene**	**2.9 ± 0.0**	**4.3 ± 0.0**	**3.2 ± 0.0**
32.31	Unknown Sesquiterpene	N D	N D	N D
32.99	Unknown Sesquiterpene	N D	N D	3.4 ± 0.0
33.62	Unknown Sesquiterpene	N D	N D	2.7 ± 0.0
34.68	**2-Methoxyfuranodiene**	**2.4 ± 0.0**	**2.7 ± 0.0**	**3.0 ± 0.0**
34.89	Unknown Sesquiterpene	N D	N D	1.7 ± 0.0
35.39	**Epicurzerenone**	**3.4 ± 0.0**	**2.4 ± 0.0**	**ND**
35.51	Unknown furano-sesquiterpene	1.9 ± 0.0	2.0 ± 0.0	N D
36.55	Unknown Sesquiterpene	N D	N D	1.5 ± 0.0
38.22	Unknown Sesquiterpene	N D	N D	2.0 ± 0.0
38.80	Furano-sesquiterpene	N D	N D	1.2 ± 0.0
38.90	Unknown Sesquiterpene	N D	N D	1.7 ± 0.0
39.99	Unknown Sesquiterpene	9.7 ± 0.1	5.7± 0.0	N D
40.24	Unknown Sesquiterpene	1.4 ± 0.0	1.1 ± 0.0	N D
40.84	**2-Acetoxyfuranodiene**	**9.7 ± 0.2**	**7.5 ± 0.0**	**6.1 ± 0.0**
41.13	Unknown Sesquiterpene	N D	N D	1.3 ± 0.0
43.60	Furano-sesquiterpene	N D	N D	1.2 ± 0.0
43.75	Unknown furano-sesquiterpene (furanoazulene type)	1.9 ± 0.1	1.6 ± 0.0	N D
45.95	Unknown furano-sesquiterpene	4.7 ± 0.0	2.8 ± 0.0	N D
46.26	Unknown furano-sesquiterpene	2.2 ± 0.0	1.1 ± 0.0	N D
59.75	Unknown Furano-sesquiterpene	ND	3.2 ± 0.0	N D
60.32	Unknown Furano-sesquiterpene	ND	2.1 ± 0.0	N D
60.49	Unknown Furano-sesquiterpene	ND	2.9 ± 0.0	N D
	Total %	61.4	71.5	67
	% Identified	56.7	42.3	39.4

Note: N = 4 replicates per extract. N D = Not detected. Identified compounds are highlighted in bold.

Table 3.6 Quantification Of Chemicals Present In Myrrh Essential Oil Extracts (Liquid Injection)

		Mean % Area ± S.E.M	
Retention Time (Minutes)	**Compounds**	**Essential Oil Extract (Aldrich) [1]**	**Essential Oil Extract (Tisserand) [2]**
19.2	**δ-Elemene**	**0.7 ± 0.0**	**1.3. ± 0.1**
20.81	Unknown Sesquiterpene	0.1 ± 0.0	0.2 ± 0.0
21.15	**β-Bourbonene**	**0.4 ± 0.0**	**0.6 ± 0.0**
21.58	**β-Elemene**	**2.9 ± 0.0**	**6.3 ± 0.2**
23.24	Unknown Sesquiterpene	5.4 ± 0.0	4.1 ± 0.2
25.23	**Germacrene D**	**1.0 ± 0.0**	**1.1 ± 0.0**
25.63	Unknown Sesquiterpene	1.2 ± 0.0	N D
26.14	**Curzerene**	**16.0± 0.1**	**23.4 ± 0.5**
30.17	Unknown Sesquiterpene	N D	N D
30.37	Unknown Sesquiterpene	6.9 ± 0.0	N D
31.34	**Furanoeudesma -1,3 diene**	**19.7 ± 0.0**	**27.8 ± 0.6**
31.38	**Lindestrene**	**7.0 ± 0.0**	**7.9 ± 0.1**
32.21	Furano-sesquiterpene	ND	1.8 ± 0.1
32.31	Unknown Sesquiterpene	4.0 ± 0.0	ND
34.68	**2-Methoxyfuranodiene**	**5.7 ± 0.0**	**5.2 ± 0.1**
35.39	**Epicurzerenone**	**N D**	**N D**
40.84	**2-Acetoxyfuranodiene**	**3.3 ± 0.0**	**0.9 ± 0.1**
	Total %	74.2	81.3
	% Identified	56.7	74.5

Note: [1] N = 4 replicates per extract. [2] N = 3 replicates per extract.
N D = Not detected. Identified compounds are highlighted in bold.

A number of peaks (25) were not identified in the various myrrh extracts. From these peaks, a large proportion (8) were found to containing the prominent m/z 108 peak (furan moiety) confirming them to be furano-sesquiterpenes. The remaining peaks showed mass fragment patterns similer to sesquiterpenes. In appendix B1 a list of unidentified compounds for myrrh extracts, with their retention times and eight prominents peaks is tabulated.

3.3.2 Chemical Analysis of Myrrh (SPME)

Initial experiments demonstrated that SPME sampling at 25°C for 1 minute delivered optimal recovery of volatiles from myrrh oleoresin, myrrh essential oil and hexane extracts of myrrh oleoresin (table 3.6). The essential oil was the most volatile of the myrrh samples tested hence less equilibration time was required. In contrast the ethanol extract of myrrh oleoresin was quite viscous and had no odours associated with it. A temperature of 45° was required to increase the volatility of the ethanol myrrh extracts.

Quantification of the volatile compounds associated with the SPME extracts of myrrh was much easier due to the reduced number of compounds present, compared to the liquid extracts, see table 3.7 and figure 3.6.

Table 3.6 Optimising Sampling Conditions For SPME Analysis Of Myrrh Oleoresin And It's Extracts

File name	Description	Water Bath Temperature (°C)	Equilibration Time (mins)	Fiber Sampling Time (mins)	TIC (10^7)	Column Loading
Molmol 15	Oleoresin	47	120	30	29.2	High
Molmol 24	**Oleoresin**	**25**	**120**	**1**	**4.2**	**Optimum**
Molmol 31	**Hexane extract**	**25**	**120**	**1**	**4.4**	**Optimum**
Molmol 33	Hexane extract	47	120	1	10.2	High
Molmol 34	Ethanol extract	47	120	1	0.79	Low
Molmol 35	**Ethanol extract**	**47**	**180**	**120**	**10.9**	**Optimum**
Molmol 40	**Essential oil**	**25**	**10**	**1**	**0.97**	**Optimum**

Note: The optimum conditions for sampling myrrh oleoresin and extracts are highlighted in bold.

Table 3.7 Quantification Of The Chemical Components Present In *C. molmol* Solid Phase Micro Extracts (SPME Injections)

Retention Times (Minutes)	Compounds	Mean % Area ± S.E.M	
		Myrrh Oleoresin	Myrrh Essential Oil (Tisserand)
		Molmol 24	Molmol 40
20.04	**δ-Elemene**	**2.0**	**3.7**
21.53	Unknown sesquiterpene	2.7	trace
22.04	**β-Bourbonene**	**3.6**	**1.8**
22.42	**β-Elemene**	**8.0**	**19.0**
23.32	Unknown Sesquiterpene	4.8	ND
24.11	Unknown Sesquiterpene	5.8	4.9
25.98	**Germacrene D**	**4.4**	**0.7**
26.47	**Unknown**	**9.7**	**trace**
26.87	**Curzerene**	**8.7**	**31.2**
26.89	Unknown Sesquiterpene	3.7	ND
32.09	**Furanoeudesma-1,3- diene**	**18.2**	**15.7**
32.33	**Lindestrene**	**5.7**	**6.0**
33.68	Unknown Sesquiterpene	0.9	ND
35.35	**2-Methoxyfuranodiene**	**0.6**	**trace**
36.06	Unknown Sesquiterpene	0.4	ND
36.18	Unknown Sesquiterpene	0.2	ND
36.60	Unknown Sesquiterpene	0.3	ND
	Total %	82.7	83
	% Identified	54.2	78.1

Note: ND = Not detected. Identified compounds are highlighted in bold.

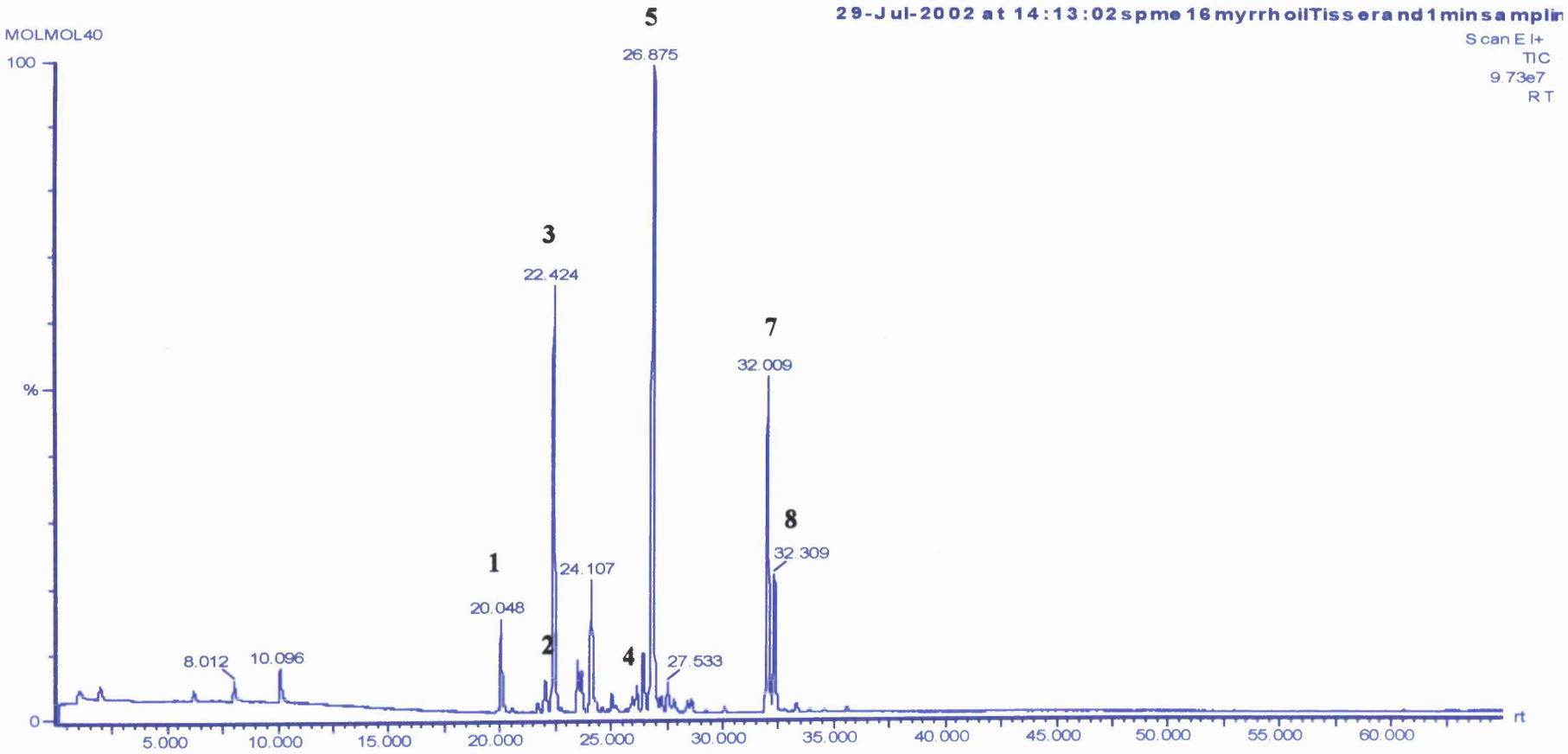


Figure 3.7 Total Ion Chromatogram Obtained Using SPME Analysis Of Myrrh (*C. molmol*) Essential Oil (Tisserand)

3.3.3 *Chemical Analysis of Opoponax (Liquid injection)*

The major peaks identified for opoponax extracts are tabulated in table 3.8.

Table 3.8 Identification of Compounds Present in Opoponax Essential oil (File name: Molmol 51)

Peak Numbers	Retention Time (min)	Compound	Chemical Name	Library Fit (Reverse)
	7.2	*cis*-β-Ocimene [a,b,c]	3,7-dimethyl-1,3,6-octatriene	977
[1]	7.7	*trans*-β-Ocimene [a,b,c]	3,7-dimethyl-1,3,6-octatriene	923
[2]	22.5	*cis*-α-Bergamotene [a,b,c]	bicycle (3.1.1) hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-, (1S- (1.alpha., 5.alpha., 6 beta.))-	957
[3]	22.9	α-Santalene [a,b,c]	tricycle (2.2.1.02,6), 1,7-dimethyl-7-(4-methyl-3-pentenyl)-,	955
[4]	23.5	*trans*-α-Bergamotene [a,b,c]	bicycle (3.1.1) hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-, (1S- (1.alpha., 5.alpha., 6 alpha.))-	934
[5]	25.5	cis-β-Farnesene [b,c]	1,6,10-dodecatriene, 7,11-dimethyl-3-methylene, (Z)-	980
[6]	26.4	α-Bisabolene [a,b,c]	cyclohexene, 1-methyl-4-(6-methyl hepta-1,5-dien-2-yl)	961
[7]	26.6	β-Bisabolene [a,b,c]	cyclohexene, 1-methyl-4-(5-methyl-1-methylene -4-hexenyl)-, (s)-	956
[8]	27.3	γ-Bisabolene [a,b,c]	cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-.	925

Compound identification was confirmed by:

[a] Comparing RT and MS to authentic standard,

[b] Comparing mass spectra to NIST library and

[c] Comparing mass spectra to published literature.

Note: RT= retention time and MS = mass spectra.

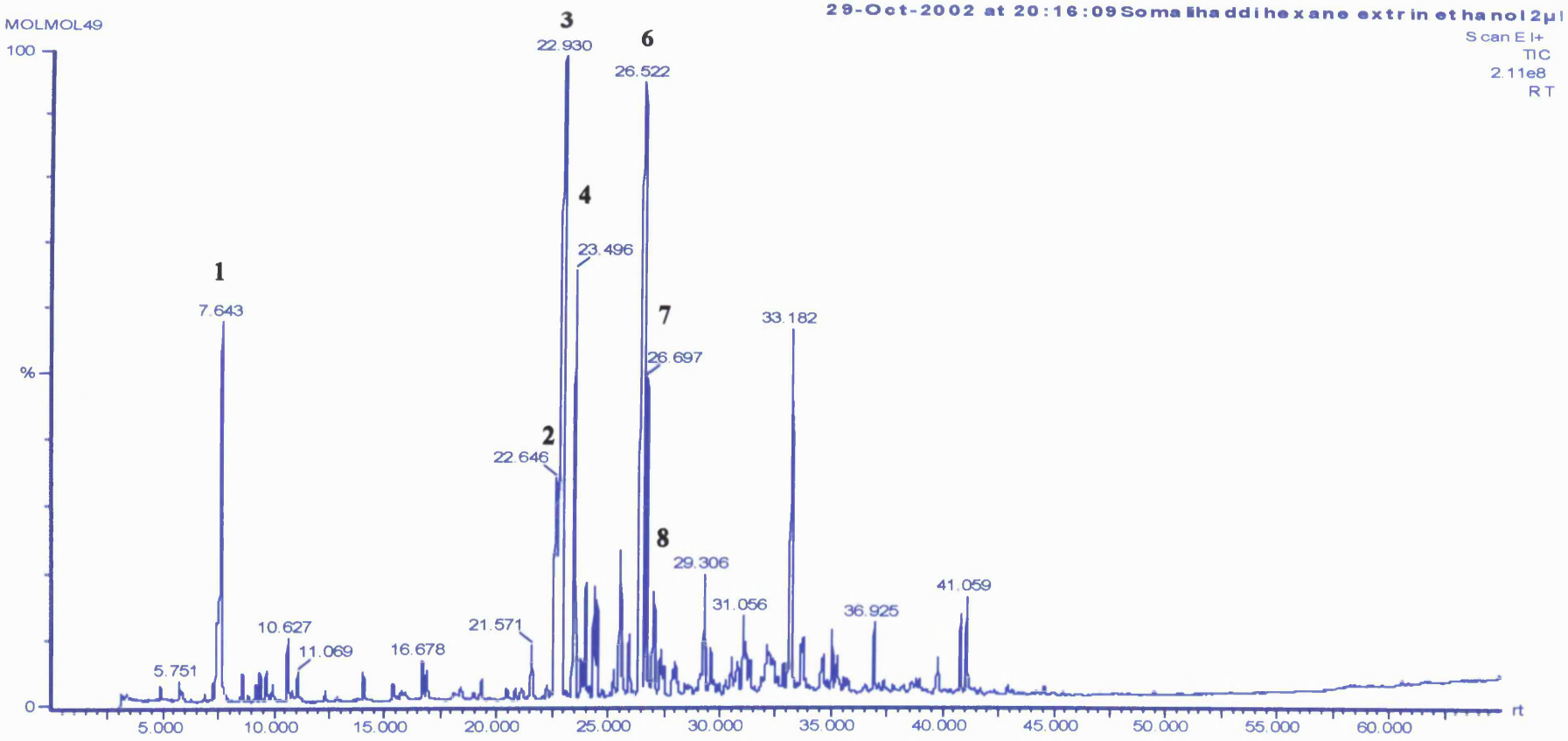


Figure 3.8 TIC Generated Chromatograph Of Opoponax (C. *guidotti*) Oleoresin Hexane Extract: Liquid Injection.

Peak Numbers Correspond To The Chemical Compounds Identified In Table 3.8.

The mass spectral fragmentation pattern of *trans*-β-ocimene (figure 3.9) showed a base peak at m/e 93. This peak corresponds to the loss of $C_3H_7^+$ ion and was the most stable organic species, which is reflected in its 100% relative abundance. The relative abundance of the molecular ion, at m/e 136, was much lower (20%).

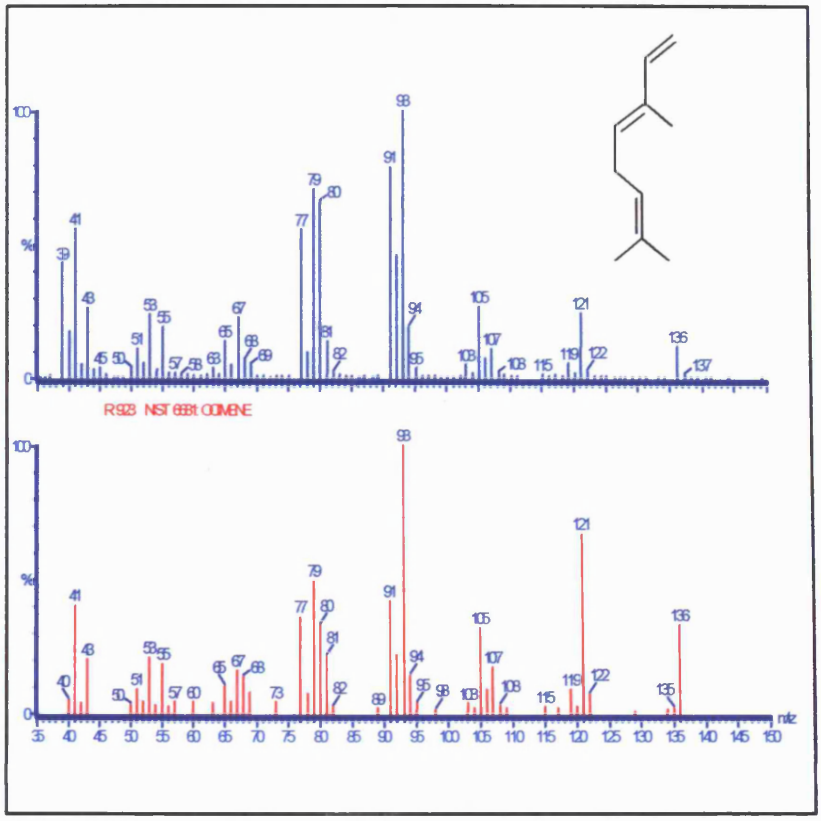


Figure 3.9 Mass spectra of *trans*-β-ocimene identified in opoponax extracts. Below the sample spectra (Blue) is the identified mass spectra from the NIST library database (Red).

α-Santalene is chemically classed as a sesquiterpene and its chemical skeleton comprises of three isoprene units, containing fifteen carbons and twenty-four hydrogens ($C_{15}H_{24}$) per molecule. The mass fragmentation pattern of α-Santalene showed a base peak at m/e 94, closely followed by a peak at m/e 93. These peaks are the most stable organic species for α-Santalene and correspond to the loss of a $\mathbf{C_3H_8^{\cdot}}$ and $\mathbf{C_3H_7^{\cdot}}$ ions respectively. The molecular ion peak was clearly visible at m/e 204 (see appendix B2). α-Santalene was found to be the most abundant compound present in the opoponax extracts (hexane, ethanol and essential oil liquid) occurring at levels between 20%-22% and were found to be present at similar levels (20%-34%) in the volatile headspace (spme) above the opoponax hexane extracts and oleoresin , see tables 3.9 and 3.11.

Another sesquiterpene *trans*-α-bergamotene, was found to be present in the hexane, ethanol and essential oil opoponax extracts at levels of 7 to 10 %. This is one of two isomers of *alpha*-bergamotene present in opoponax, the other isomer being *cis*-α-bergamotene, which was found to be present in the hexane, ethanol and essential oil opoponax extracts at levels of 5 to 6%, see table 3.9. The mass spectra for both isomers were found to be identical.

Three isomers of bisabolene (α, β and γ) were identified in opoponax, and all were found to have a base peak at m/e 93. At the retention time of 26.6 the NIST library incorrectly identified the β-bisabolene peak as γ-bisabolene. Since the discriminating power of the computer depends upon the available stored data, it will not always assign the correct isomer. The sample mass spectra for β-bisabolene, however, entirely agreed with published literature values (Robert Adams, 2001).

α-Bisabolene, was found to be a major chemical component of opoponax liquid extracts and was present in the hexane, ethanol and essential oil extracts at levels between 13% to 19 %, see table 3.9. Due to the lack of commercial availability of the α- and β-bisabolene compounds, only the γ-bisabolene isomer was evaluated in this thesis as possible repellent/antifeedants against terrestrial molluscs.

β-farnesene was present in both the liquid and spme extracts of opoponax at levels between 0.9-2%. Curzerene was detected at similar levels to farnesene (1-2%) in the liquid extracts (hexane and essential oil) but not detected in the ethanol extract. Curzerene, however, was not detected in the volatile aroma above the headspace of opoponax oleoresin and its extracts.

The chemical compound which eluted at a retention time of 33.1 minutes was found to be present in all the opoponax liquid extracts and could not be identified either from the NIST library or by reviewing published literature. This compound produced the mass fragmentation spectra pattern as shown in figure 3.10.

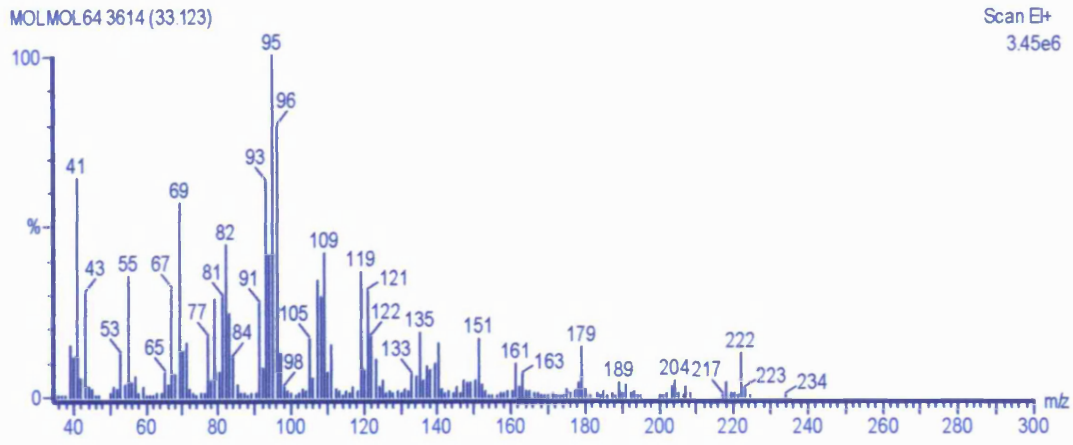


Figure 3.10 Mass spectra of unknown compound present in opoponax essential oil.

The eight most abundant peaks for this unknown compound were found:

m/e (%): 95(100), 96(82), 41(72), 69(61), 93(60), 82(47), 94(46), 119(42).

The molecular ion peak might be the m/e 222 ion which was found to be abundant at 22%.

Table 3.9 Quantification of the components present in the liquid extracts of Opoponax (*C. guidotti*) Using TIC generated gas chromatograms.

Retention Time (mins)	Compounds	Liquid Injection Mean % Area ± S.E.M Hexane Extract (molmol 49,52,55)	Ethanol Extract (molmol 50,53,56)	Essential Oil Extract (molmol 48,51,54)
7.3	cis-β-Ocimene	0.2 ± 0.0	ND	0.3 ± 0.0
7.6	trans-β-Ocimene	9.3 ± 0.1	1.3 ± 0.1	12.6 ± 0.2
22.6	Cis- α-Bergamotene	6.2 ± 0.1	4.6 ± 0.1	26.0 ± 0.5
22.9	α -Santalene	20.2 ± 0.2	22.1 ± 0.7	(co-elution)
23.5	trans α-Bergamotene	8.7 ± 0.1	9.7 ± 0.2	7.2 ± 0.1
24.0	Santalene isomer	1.9 ± 0.0	1.3 ± 0.0	2.1 ± 0.0
24.5	Santalene	1.4 ± 0.0	1.1 ± 0.0	1.4 ± 0.0
25.2	Germacrene D	0.4 ± 0.0	ND	ND
25.5	*trans*-β-Farnesene	2.1 ± 0.0	1.9 ± 0.0	2.1 ± 0.0
25.9	Curzerene	1.1 ± 0.0	N D	2.0 ± 0.0
26.5	α -Bisabolene	18.9 ± 0.0	13.0 ± 0.4	16.1 ± 0.3
27.3	γ-Bisabolene	5.2 ± 0.1	4.6 ± 0.1	4.8 ± 0.1
33.1	Unknown compound	5.3 ± 0.1	4.3± 0.0	5.1 ± 0.0
34.6	2-Methoxyfuranodiene	trace	ND	1.7 ± 0.0
	Total (%)	78.8	62	79.3

n = 3 injections, SEM = standard deviation of the mean

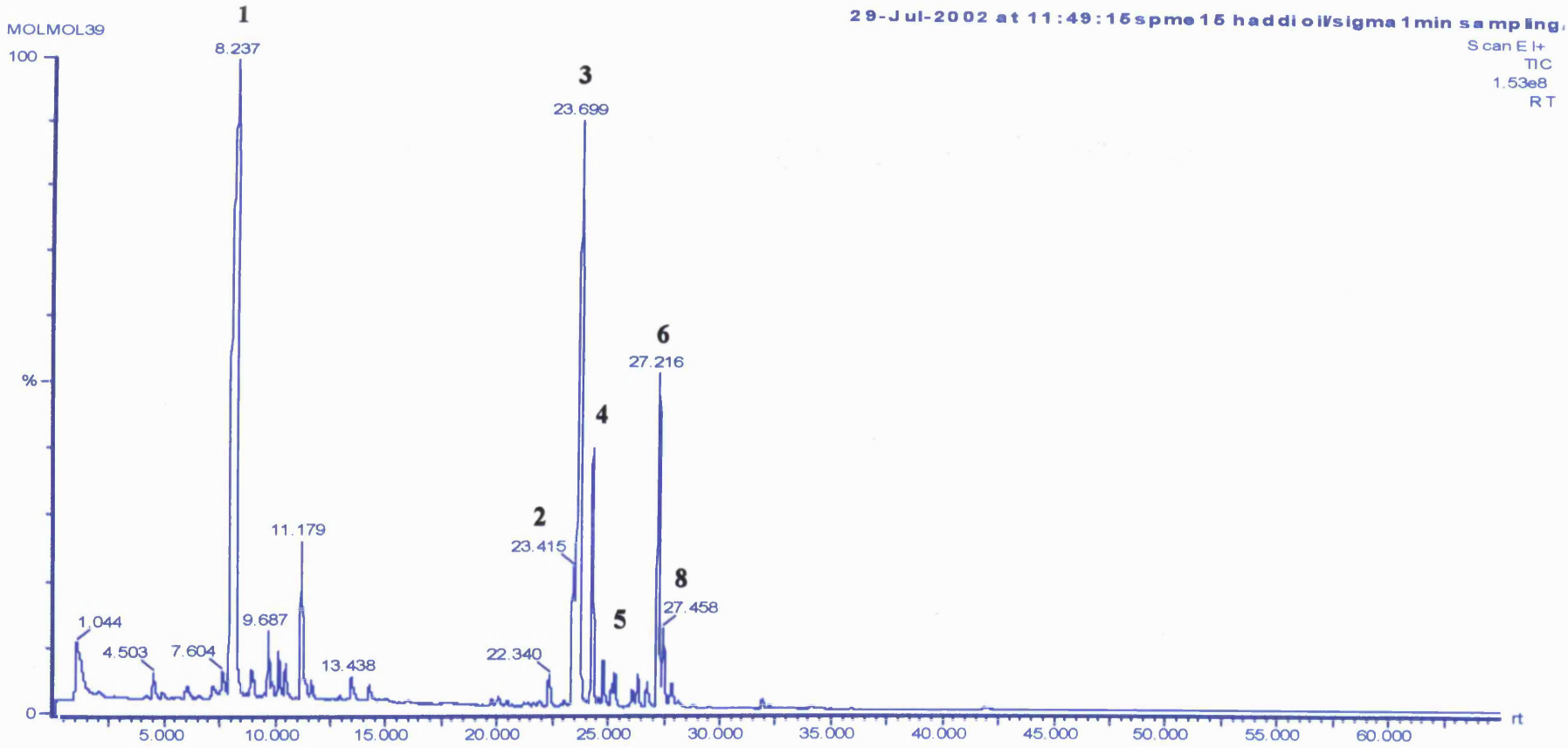


Figure 3.11 Total Ion Chromatogram Obtained Using SPME Analysis Of Opoponax (*C. guidotti*) Essential Oil (Aldrich).

Peak Numbers Correspond To The Chemical Compounds Listed In Table 3.8.

		Peak Area (%)			
	Filename:	Molmol 38	Molmol 39	Molmol 37	Molmol 22
Retention Time (Minutes)	Compound	Ethanol	Essential Oil	Hexane	Oleoresin
7.6	cis-β-Ocimene	trace	0.5	1.0	1.3
8.2	trans-β-Ocimene	trace	37.7	32.7	32.7
23.4	cis-α-Bergamotene	6.5	4.5	5.2	5.0
23.7	α-Santalene	34.2	22.2	25.6	20.4
24.3	trans α-Bergamotene	17.4	7.1	9.5	8.9
24.8	Santalene isomer	2.3	1.1	1.1	1.2
26.3	β-farnesene	2.3	0.85	1.0	1.2
27.3	α-Bisabolene	26	9.3	11.3	14.8
27.5	γ-Bisabolene	4.5	1.5	2.0	3.3
	Total (%)	93.2	84.7	89.4	88.8

Table 3.12 Retention Times of Commercially Available Standards

Retention Time (Minutes)	Chemical Name
7.3	*cis*-β-Ocimene
7.6	*trans*-β-Ocimene
25.2	Germacrene D
23.7	α-Santalene [1]
25.9	Curzerene [2]
34.1	Furanodiene [2]
27.5	γ-Bisabolene

Note:

[1] Confirmed by injecting sandalwood oil (*S. album*). This oil contains 2% α-Santalene.

[2] Confirmed by injecting ethanolic root extract of Alexanders plant (*S.olustatrum*).

This oil contains 7% curzerene and 13% furanodiene.

3.4 DISCUSSION

3.4.1 Chemistry of Myrrh

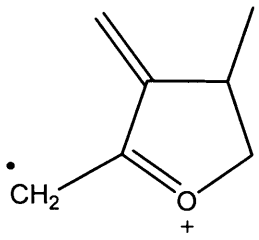
GC-MS analysis of the liquid extracts of commercial myrrh oleoresins demonstrated a similar bouquet of chemicals for all three extracts (hexane, ethanol and essential oil). All liquid extracts of myrrh contained a mixture of sesquiterpenes (δ-elemene, β-elemene, germacrene D, and β-bourbonene) and furano-sesquiterpenes (curzerene, furanoeudesma-1, 3-diene, lindestrene, methoxyfuranodiene and acetoxy-furanodiene).

The chromatographic profiles of the two sources of myrrh essential oil (Aldrich and Tisserand) were found to be very similar. The major components of these oils were found to be the furano-sequiterpenes furanoeudesma-1, 3-diene (19.7% and 27.8% respectively) and furanodiene (16.0% and 23.4% respectively). These levels of furano-sequiterpenes for myrrh essential oil agreed closely with those reported by Moyler and Clery (1997).

The major components of the hexane extract were identified to be furanoeudesma-1, 3-diene (10.6%) and 2-acetoxyfuranodiene (7.5%), whilst the major components of the ethanol extract were furanoeudesma-1, 3-diene (8.0%), 2-acetoxy-furanodiene (9.7%) and an unknown compound (9.7%).

The hexane extract of myrrh was the only extract to contain the three chemical compounds eluting between 59 and 61 minutes. These unknown compounds, present at

levels between 2.1 to 2.9%, are likely to be furano-sesquiterpenes from observation of their mass spectral patterns. The mass spectra of all three unknown compounds exhibit a base peak at the mass/charge ratio (m/z) 108 attributable to the cleavage of bonds β to the furan ring system resulting in the formation of the ion $\mathbf{C_7H_8O^+}$ (Craveiro *et al.*, 1983). The structure of the furan moiety which gives rise to the m/z 108 base peak is shown below:



Chemical analysis of the volatile odours associated with commercial myrrh oleoresin, using solid phase micro extraction (SPME), showed a different chromatographic profile to the liquid extracts of commercial myrrh oleoresins. Elemene (β) was found to contribute much more to the aroma of the myrrh oleoresin (12.7%) and the myrrh essential oil (19.0%) than that observed for the liquid extracts of myrrh oleoresin (1.9 - 2.9%). Furanoeudesma-1, 3-diene also contributed significantly to the volatile head space region above the myrrh oleoresin (18.2%) and myrrh essential oil (15.7%) in quantities similar to those observed for the liquid extract. The SPME chromatographic profile obtained for myrrh oleoresin granules agreed well with published literature (Ham *et al.*, 2004).

Curzerene (synonym: isofuranogermacrene) was found to be present in all the myrrh liquid extracts analysed, with the highest levels occurring in the two essential oil extracts (16 and 23%) and the hexane extract of Somali myrrh oleoresin (9%). This

furano-sesquiterpene was present at similar levels in the volatile headspace (SPME) above the commercial myrrh oleoresin (Baldwin) and in even higher levels above the headspace of myrrh essential oil (31.2%).

As mentioned earlier in this chapter, the major problem with analysing essential oil extracts, such as myrrh, by gas chromatography is the problem of thermal rearrangement of terpenes in the injector port. If the terpenes are subjected to high temperatures (>200°C) some sesquiterpenes can rearrange themselves to more thermally stable chemical. If this occurs the chromatographic baseline usually increases just before the compound under going thermal rearrangement (Baldovini *et al.*, 2001). This was not observed in any of the gc injections performed with myrrh and opoponax but did occur with those injections involving the root extracts of *Smyrnium olustatrum.* The change in baseline occurred just before the furanodiene hence confirming the process of thermal rearrangement to curzerene. This analysis confirmed the retention times of the furano-sesquiterpenes curzerene and furanodiene.

The ethanolic extract of *S. olustatrum* root was found to contain curzerene and furanodiene at 7% and 13% levels respectively, lower than that reported by Mölleken *et al.* (1998) who reported 20.5 and 17% respectively. Hikino *et al.* (1968) also detected both furanodiene and curzerene in the rhizome of *Curcuma zedoria (Zingiberacaea.)*

Elemene (β) has been found to occur at levels greater than 15% in the leaves of the *Pittosporum* species of plants (*P. anomalum*), which are native to New Zealand, (Western, 2004) and at similar levels in the volatile oil of *Salvia* plant species (*S. aethiopis*) which grow wild in Iran (Rustaiyan *et al.*, 1999). Harada *et al.* (1996)

reported β-Elemene to be responsible for the significant attractive properties of the fish bait all spice (*Pimenta officinalis*) towards the black abalone fish (*Haliotis discus*). This attractant property of β-Elemene was also observed when bark beetles (*Hylurgopinus rufipes*) were found colonising American elm trees that had been infected with Dutch elm disease (Millar *et al.*, 1986). They analysed the volatiles from the infected elm tree and found it to contain fourteen sesquiterpenes of which only cadinene (δ and γ isomers), α-cubebene, γ-muurolene and β-elemene elicited significant responses from *Scolytus multistriatus* bark beetles. Elemene (β) has been also been shown to be present in soldier ants defence secretions when analysed by gas chromatography-mass spectrometry (gc-ms) (Nelson *et al.*, 2001). They suggested that its presence may have been detected as a result of thermal rearrangement from its precursor compound Germacrene A.

Furanoeudesma-1, 3-diene, however, has only been previously reported to be present in *Commiphora molmol* and *C. myrrh* oil (Maradufu, 1982). Maradufu was the first researcher to isolate 2-Methoxy furanodiene and 2-acetoxy furanodiene from the hexane extract of myrrh oleoresin resulting in yields of 2% and 4% respectively. He also discovered that furanodienone, 2-Methoxy furanodiene and 2-acetoxy furanodiene possessed moderate ixodicidal activity against *Rhipicephalus appendiculatus* tick larvae. The occurrence of furanodienone and other oxygenated furano-sesquiterpenes in myrrh (*C. molmol*) has been questioned recently by Dekebo *et al.* (2002). They suggested that the presence of the oxygenated furano-sesquiterpenes was due to adulteration by oleoresins from trees related to myrrh, *C. sphaerocarpa,* which are located in the southern Ethiopian region of Bale.

Lindestrene has been previously characterised as a component of the pleasant smelling root oil of *Lindera strychnifolia* (*Lauraceae*) (Günther, 1994).

Significant differences in the TIC generated chromatographic profiles of different myrrh oleoresins were observed on analysing the hexane extracts of Somali myrrh and commercial myrrh obtained from Yemen (Baldwin & Co). The most obvious difference between the two myrrh extracts was the large peak eluting relatively early (approximately 21minutes), for the Somali myrrh (13.9%) compared to 2.5% for commercial myrrh (Yemen). This peak was identified to be the sesquiterpene β-elemene. Another major difference observed between the two sources of myrrh oleoresin was the very low amount of furano-eudesma-1, 3-diene detected in the Somali myrrh hexane extracts (2.1%) compared to 10.6% for the hexane extract of the commercial variety from Yemen (Baldwin & Co). The curzerene content of the Somali myrrh hexane extract (9.0%) was found to be double the level detected in the hexane extract of the commercial myrrh variety (4.8%).

3.4.2 Chemistry of Opoponax

The GC-MS analytical method for the liquid injections did not require any modification as the major factor for avoiding column overloading was the weight of oil extract transferred into the vial, 2mg being the optimum

Development and optimisation of the SPME analytical method for sampling opoponax was, however, essential as this is a sensitive and relatively new technique for analysing aromatic samples. A number of parameters had to be varied to prevent the gc

capillary column from being overloaded with the test samples. In general, one minute was found to be a good time period for exposing the fiber to the samples. The major parameters that needed controlling were the temperature of the water bath and the amount of time the sample was allowed to attain thermal equilibrium, in the water bath (equilibration time). All the samples (oleoresin, hexane extract and essential oil) with the exception of the ethanol extract had a sweet volatile aroma associated with them. The ethanol extract was found to be more viscous, than the other samples hence the higher temperature and longer equilibration times required for achieving optimum chromatography. Optimum column loading was attained when good chromatographic peak shape and satisfactory resolution was observed for each injection. This was reflected in the good peak shape and resolution of the chemical compounds.

Unlike the myrrh extracts the majority of the chemical compounds present in opoponax were identified using the NIST computer library. Both the liquid and SPME analysis of the opoponax extracts (hexane and essential oil) displayed the same bouquet of terpenoid compounds. GC-MS analysis, however, also highlighted the absence of the monoterpene *trans*-β-ocimene, in the ethanol extract of opoponax oleoresin. Although high levels of *trans*-β-ocimene were detected in the hexane and essential oil liquid extracts (9% and 13 % respectively) much higher levels were found to be present in the volatile aroma of opoponax oleoresin (32.7%), the hexane extract (32.7%) and the essential oil (37.7%), when sampled with the SPME fibers. The relative molecular weight of *trans*-β-ocimene is relatively small (136) and is more volatile than sesquiterpenes, hence, would be expected to contribute more to the volatile odours produced by the opoponax samples.

Both the monoterpenes and sesquiterpenes found to be present in opoponax have been have been investigated by various researchers in other plant species.

Urzua (2002) found *trans*-β-ocimene to be a major constituent of the headspace, in extracts, of four *Pseudognaphalium* plant species, accounting for 41% to 87% of emitted volatiles. *Trans*-β-ocimene identified, in the floral volatile of alfalfa, was found to behave neither as an attractant or repellent to the honey bee *Apis mellifera.*

Kessler and Baldwin (2001) found that when the leaves of the plant *Nicotiana attenuata* was attacked by different pests, the volatiles *trans*-α-ocimene, β-farnesene and *trans*-α-bergamotene were emitted from the leaves after 24 hours. The alcohol 3-hexenol was produced in greatest amounts for all the pests. When attacked by the hornworm (*M. quinquemaculata*) the leaves produced significant amounts of *trans*-α-ocimene, *trans*-α-bergamotene and β-farnesene volatiles, compared to the control. When the plant was attacked by leaf bug (*Dicyphus minimus*) and the flea beetle (*Epitrix hirtipennis*) significant amount of volatiles, involving trans-β-ocimene and bergamotene, were produced.

Singh *et al.* (2003) found *trans*-β-ocimene to be the major compound in the essential oil of *Tagetes erecta*, accounting for 42% of the total oil and possessed significant antifungal (*Aspergillius terreus*, *Colletotrichum falcatum*) and insecticidal activity against the white termite (*Odontotermes obesus*). They found that Bergamotene increased the amount of eggs eaten by predators. They concluded that the defensive behaviour of *Tagetes erecta* works by attracting predators to the plant.

Trans-β-ocimene and α-bergamotene may have a semio-chemical role, by attracting parasitoids, when being attacked by herbivores (Rayko *et al.,* 2000).

Both α-farnesene and β-farnesene have been implicated as semio-chemicals which modify the behaviour of mice. The male mice tend to avoid areas where they come into contact with these particular terpenes. In contrast the same chemicals were found to be attractive to female mice (Ma *et al.*, 1999).

α-Santalene is one of the more common components found in the essential oil of Sandalwood oil (*Santalum album)* at moderately high levels (24%) which, along with opoponax essential oil (*C. guidotti),* is one of the highest levels of this sesquiterpene found in nature. The presence of α-santalene and α-bergamotene may be responsible for the resistance of the wild tomato (*Lysopersicon hirsutum*) against the spider mite, Colorado potato beetle and the beet armyworm (Rutger *et al.*, 2000). They inferred that the bioactivity of the wild tomato could be related to the production of α-santalene and *trans*-α-bergamotene since these compounds were not found in the cultivated tomato (*L. esculentum*). In contrast the sesquiterpenes β-carophyllene and α-humulene were identified to be the major chemicals present in the cultivated tomato.

Mixtures containing α-Bisabolene was found to repel both the housefly (*Musca domestica*) and mosquitos (*Aedes aegypti*) (Wilson *et al.*, 1993). α-Bisabolene is found in the resin exudates produced by the Grand fir tree (*Abies grandis*) when it is attacked by bark beetles.

α-Bisabolene was found in a large abundance in the opoponax extracts (9%-26%) and has recently been reported to be present in similar quantities (23%) in the essential oil of *Helichrysum rugulosum* (Bougatsos *et al.*, 2003). They found that this essential oil possessed antibacterial activity.

This study has shown the highest chemical contribution to the liquid extracts of opoponax to be α-santalene followed by α-bisabolene and then β-ocimene, whilst for the volatile SPME injections the largest contributors were found to be β-ocimene, α-santalene and α-bisabolene. The level of α-santalene, detected in both the spme and liquid extracts of opoponax, stayed relatively constant. In contrast the levels of *trans*-β-ocimene and α-bisabolene detected, in opoponax, were highly variable depending on the type of analysis employed (liquid or spme injection). *Trans*-β-ocimene was the largest contributor to the volatile aroma associated with opoponax whilst α-bisabolene contributed more to its liquid extracts.

In summary, it is recommended that before one commences chemical analysis of myrrh oleoresins one should spend some time authenticating its country of origin although this is difficult as suppliers often do not divulge this information correctly. Since a few *Commiphora* oleoresins look and smell similarly (balsamic) it is easy for adulteration to take place. In contrast to myrrh, the authentication of opoponax oleoresins is much easier, because of the unique sweet smell associated with them, making it more difficult for deliberate adulteration to take place.

This study is the first scientific comparison of the chromatographic profile of myrrh oleoresins, indigenous to north Somalia (Somaliland), with commercial varieties,

obtained from Yemen. Both of these "true" myrrh varieties are classified either as *C. molmol* or *C. myrrha.* Cufodontis (1956) stated the two species to be clearly synonymous and suggest that *C. myrrha* be reserved for the Arabian species and *C molmol* for the African species. This study also confirms the two myrrh species to contain the same bouquet of chemicals, the major difference being their abundance. These slight variations may be may be due to the differences in their geographical location and the environmental conditions that the myrrh tree is growing under.

This is also the first chemical quantification of the volatile aroma of opopanax (*C. guidotti*) oleoresins using the relatively new solid phase micro-extraction (spme) technique. This technique has been used previously to study the volatiles associated with frankincense (*Boswellia* spp.) and myrrh (*C. myrrha*) oleoresins (Hamm *et al.*, 2003; Hamm *et al.*, 2004).

Gas chromatography-mass spectrometry (gc-ms) has shown to be a powerful method for identifying chemical compounds as well as characterising the chromatographic profile of *Commiphora* spp. oleoresins. GC-MS is an essential tool for authenticating the botanical origin of *Commphora* spp. oleoresins, such as myrrh and opoponax, and can be used as a routine method for the quality control of these highly valued natural products.

CHAPTER 4:

Antifeedant Properties of Myrrh and Opoponax Extracts, and their Components, against *D. reticulatum* Slugs

4.1 INTRODUCTION

Every year slugs and snails cost millions of pounds of damage to horticulture and agricultural crops. The costs of slug control in UK cereal crops have been estimated to be over £8 million a year alone (Schüder *et al.*, 2003).

Current methods of slug control rely mostly on chemical baits such as metaldehyde and methiocarb pellets. These chemical methods although having a reasonable track record of killing molluscs are being continuously reviewed for their impact on the environment and in particular non-target organisms. Synthetic chemicals, such as metaldehyde and methiocarb, have been implicated as being toxic to animals and birds (Godan, 1983). As the use of synthetic molluscicides in the agricultural and horticultural industry is increasingly becoming unpopular new means of deterring or repelling slugs and snails are actively being sought.

An alternative form of mollusc control is the use of non-toxic plant extracts and their components as antifeedants or repellents. There have been many reports, over the years, evaluating the repellent and antifeedant properties of natural compounds but none so far have been exploited commercially (Barker, 2002). Airey *et al.* (1989) identified a number of chemicals of plant origin which prevented slugs from feeding (2-phenylethyl isothiocyanate, and fenchone), however, the former was found to be phytotoxic and the latter too volatile to be persistent in the field. More recently Schüder *et al.* (2003) found garlic to be an effective barrier against the terrestrial mollusc, *Deroceras panormitanum* and *Oxyloma pfeiffer,* resulting in 95% and 30% mortality respectively. Dodds *et al.* (1999) used electrophysiological and feeding bioassays to identify *Petroselinum*

crispum, Conium maculatum, and *Coriandrum sativum* plant extracts as being both neuroactive and antifeedant against slugs. This work was studied further by Birkett *et al.* (2004). They identified antifeedant compounds from three species of apiaceae to be active against the field slug, the most potent being the alkaloid γ-coniceine from *C. maculatum* plant extracts. Hollingsworth *et al.* (2002) found caffeine, a natural product present in coffee, to be both repellent and toxic to slugs and snails. In field tests aqueous caffeine extracts (1% and 2%) applied to potted orchids and infested with mature orchid snails *(Zonitoides arboreus*) resulted in 60% and 95% snail mortality respectively. Similar tests using slugs (*Veronicella cubensis*) showed 92% slug mortality in 48 hours. Simms and Wilson (2003), however, questioned the environmental usefulness of caffeine as a molluscicide stating it to be more toxic to mammals than metaldehyde, the most commonly used commercial molluscicide.

Leaf discs experiments are relatively simple bioassays and are usually the principal test employed for screening the antifeedant properties of plant extracts and newly discovered chemical entities. Lopez-Olguin *et al.* (1998) used potato leaf discs to demonstrate the potent antifeedant activity of natural and synthetic neoclerodane diterpenes from *Teucrium* spp. plant extracts, against the Colorado potato beetle larvae. The leaf disc assay confirmed some of the neoclerodane chemicals to have potent antifeedant activities at concentrations as low as 50 ppm.

Paper leaf discs were used by Smith *et al.* (2001) to validate the chemicals α-solanine and α-charconine, both glycoalkaloids extracted from the potato (*Solanum* spp.), to be potent antifeedant compounds towards the snail *Helix aspersa* appreciably inhibiting feeding at concentrations of 1mM. Wood *et al.* (2000) used the lettuce leaf

disc assay to demonstrate the antifeedant property of 1-octen-3-ol, towards the banana slug *Ariolimax columbianus*. This alcohol which was extracted from the mushroom *Clitopilus prunulus* and was shown to be a potent slug antifeedant, deterring feeding at concentrations as low as 8.3μg per lettuce disc.

Govindachari *et al.* (1999) investigated the antifeedant activity of six diterpenes towards fourth instar nymphs and adults of the potato beetle *Henosepilachna vigntioctopunctata* using potato leaf discs. They found the diterpene caryoptin to be the most effective antifeedant which deterred feeding at concentrations as low as $0.05\mu g/cm^2$. The antifeedant activity of sesquiterpene lactones has been demonstrated against fifth instar larvae of the Asian armyworm *Spodoptera litura* using cabbage leaf discs (Passreiter and Isman, 1997). Faini *et al.* (1997) used lettuce leaf disc assays to investigate the antifeedant activity of the resinous shrub *Flourensia thurifera* towards *Spodoptera littoralis* larvae. They found that the resin extracts from the stem and leaves showed high levels of antifeedant activity and isolated a number of potentially active compounds which included coumarins, chromenes, a benzofuran, a flavanoid, and *p*-coumaric.

In this study the resinous exudates from the stems of *C. molmol* and *C. guidotti* and their components will be evaluated, as possible antifeedants/repellents towards the field slug *Deroceras reticulatum*, using the lettuce leaf disc method as a rapid chemical screening bioassay.

In chapter three chemical analysis of opoponax essential oil revealed the presence of a number of mono-and sesquiterpenes. From that selection of chemicals

only *trans*-β-ocimene, γ-bisabolene and germacrene D were commercially available and procured for testing as novel slug repellents/ antifeedants. An isomer of β-farnesene, called α-farnesene, and its alcoholic analogue (farnesol) were also found to be commercially available. An alcoholic analogue of santalene called santalol (in the form of sandalwood oil) was also evaluated using no-choice lettuce leaf disc assays.

The main aim of this chapter is to evaluate the antifeedant activity of extracts of myrrh, opoponax and selected chemicals towards the field slug *D. reticulatum* using the leaf disc screening method. The following treatments were evaluated:

i) Hexane and ethanol extracts of myrrh oleoresin extracts (1, 5 and 10% w/v).

ii) DMSO extracts of myrrh oleoresin extracts (10%w/v).

iii) Myrrh essential oil in ethanol (0.5, 1, 5 and 10% w/v).

iv) Myrrh essential oil in 10% aqueous DMSO (0.5, 1, 5 and 10%w/v).

v) Myrrh essential oil in 0.2% aqueous Tween 80 (0.5%w/v).

vi) Myrrh essential oil in water (0.5% and 1%w/v).

vii) Opoponax essential oil in ethanol (1, 5 and 10% w/v).

viii) Opoponax essential oil in 10% aqueous DMSO (1 and 5%w/v).

ix) Opoponax essential oil in 0.2% aqueous Tween 80(0.5 and 1%w/v).

x) Opoponax essential oil in water (0.5 and 1 %w/v).

xi) 1% α-Bisabolene, α-farnesene, *cis*,*trans*-farnesol and germacrene D in ethanol.

xii) *Trans*-β-ocimene solubilised in ethanol (0.1, 0.5 and 1% w/v).

xiii) *Trans*-β-ocimene solubilised in 10% aqueous DMSO (0.25, 0.5, 1 and 3%w/v).

xiv) *Trans*-β-ocimene solubilised in 0.2% aqueous Tween 80 (0.5%w/v).

xv) *Trans*-β-ocimene solubilised in water (0.5%w/v).

xvi) 1% Santalol (α, β) in 10% aqueous DMSO.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

Absolute ethanol and hexane (HPLC grade) were obtained from Fischer Scientific (UK). The following chemicals were purchased from Sigma-Aldrich limited (Dorset): Dimethyl sulfoxide (DMSO) (analar grade), Tween 80 (Polyoxyethlene sorbitan monooleate), *cis*-β-ocimene (purity 70%), *cis, trans*-farnesol (purity 95%), myrrh essential oil and opoponax essential oil.

The following chemicals were kindly provided by R C Treat limited (London): *Trans*-β-*o*cimene (purity 90%), γ-bisabolene (purity 70%), germacrene D (purity 40%), and α-Farnesene (purity 70%).

Myrrh oleoresin (*C. molmol*) was purchased from G. Baldwin & Sons Limited (London), whilst opoponax oleoresin (*C. guidotti*) was collected from Hargeisa, Somalia.

4.2.2 Test animals

Adult *D. reticulatum* slugs were collected from nearby fields and maintained in plastic trays lined with moist, unbleached, absorbent paper. They were housed in the dark and at a constant temperature of 10° ± 1°C. Slugs were regularly fed on a mixture of iceberg lettuce and carrots. Slugs, with a weight 300-600mg, were pre-starved for 24 hours and maintained constant temperature of 15° ± 1°C prior to testing.

4.2.3 Preparation of Myrrh Oleoresin Extracts (Ethanol & Hexane)

Various amounts of myrrh oleoresin (1, 5 and 10 g) were weighed into separate 250 ml beakers and 100ml of hexane or ethanol added to them, corresponding to myrrh concentrations of 1, 5 and 10%w/v. The mixture was stirred for two minutes, the beakers covered with aluminium foil and then left in a dark cupboard for one week. The extracts were filtered under vacuum through a Büchner flask. The filtered hexane extracts were a clear golden colour in appearance which after drying in a rotatory evaporator, gave a golden-yellow oil (approximately 2% yield). The dried ethanol extracts, in contrast, were amber brown in appearance with an extract yield of approximately 22%. The evaporated ethanol extracts were very sticky with a tar-like texture and very little odour associated with them. The ethanol extracts were also very viscous compared to the free flowing hexane extracts.

4.2.4 Myrrh Oleoresin Extracts (10% w/v) in DMSO

Myrrh oleoresin (10g) was weighed into a 250ml glass beaker and 100 ml of DMSO added. The mixture was stirred for two minutes, the beaker covered with aluminium foil and then left in a dark cupboard left for one week. The extracts were filtered under vacuum through a Büchner flask, containing a Whatman™ filter paper producing a dark brown solution.

4.2.5 Preparation of Myrrh, Opoponax and trans-β-Ocimene Extracts

The oils of myrrh, opoponax and *trans*-β-ocimene were prepared in different solvents by aliquoting various volumes of oil extracts (10, 25, 50, 100, 500 and 1000µl) into a 10 ml volumetric flask and diluting to volume with solvent yielding final concentrations of 0.1, 0.25, 0.5, 1, 5 and 10 % v/v. Solutions were shaken well to allow homogenous mixing.

4.2.6 Leaf Disc Assay Method

A known volume of plant extract (50µl) was accurately pipetted onto individual lettuce leaf discs (1.4cm^2) and any residual solvent left to evaporate for a minimum time of 30 minutes.

Slugs, previously starved for 24 hours, were introduced to the Petri dishes and placed in an environmentally controlled chamber (15°C: 12 hours day, 15°C: 12 hours night) for 24 hours.

The amount of leaf disc damaged (consumed) was quantified by comparing digital photographs of treated leaf discs with untreated (control) leaf discs, using the computer software products "*Adobe Photoshop*™" and "*Sigma Scan*™". The experiment was repeated 20 times for each treatment.

Figure. 4.1 Leaf disc assay showing slug avoiding lettuce leaf disc treated with 1% opoponax essential oil solubilised in ethanol.

4.2.7 Statistical Analysis

As the between-treatment variances were not homogenous (Bartlett's/Levene's tests) and the residuals not normally distributed (Anderson-Darling test) the data were analysed using the non-parametric Kruskal-Wallis test to show significance of differences between group medians or the Mann-Whitney test for pairwise comparisons of the medians. In situations where zero percent slug mortality was observed for the control treatments, the non-parametric Wilcoxon signed rank test was used. This one-tailed test enables a standard median integer, in this case zero, to be compared with the median data for test samples. In all cases, the level of significance was established at P=0.05.

4.3 RESULTS

4.3.1 Evaluation of The Antifeedant Properties Of Myrrh Oleoresin Extract

Leaf damage

Coating lettuce leaf discs with myrrh oleoresin extracts significantly modified the feeding behaviour of *D. reticulatum* slugs. As indicated in table 4.1 all myrrh oleoresin extracts (hexane, ethanol and aqueous DMSO) showed highly significant reductions in leaf damage, by *D. reticulatum* slugs (Kruskal-Wallis, $P< 0.001$).

Increasing the concentration of myrrh (hexane extract) added to the lettuce leaf discs, from 1% to 5 and 10%, significantly reduced the quantity of leaf discs consumed by the slugs from a median leaf damage of 66% to 0% and 0% respectively. Mann-Whitney pair wise comparisons of the medians for the 1% and 5% hexane extracts of myrrh oleoresin showed a significant difference in their antifeedant properties ($P = 0.021$). Significant differences were also found between the medians of the 1% and 10% myrrh oleoresin hexane extracts ($P <0.001$) and also between the 5% and 10% myrrh oleoresin hexane extracts with ($P =0.039$).

Similarly all concentrations of ethanol extracted myrrh (1%, 5% and 10%), when added to the lettuce leaf discs, significantly reduced lettuce leaf disc consumption by the slugs (Kruskal-Wallis, $P < 0.001$), resulting in a median leaf damage of 0% for all ethanol extracts of myrrh compared to 100% for the control. Pair wise comparison of median leaf damage for the 1% ethanol extracts of myrrh oleoresin with the increased

concentrations (5% and 10%) also showed significant differences in the antifeedant properties towards *D. reticulatum* (Mann-Whitney, $P < 0.05$). In contrast there was no significant differences between the 5% and 10% myrrh (ethanol) extract concentrations ($P = 0.615$), both myrrh concentrations being equally repellent to the slugs.

The non parametric Mann-Whitney test showed the 1% ethanol extract of myrrh oleoresin to be a significantly better antifeedant, against slugs, than the 1% hexane extract resulting in median % leaf damage of 0% and 66% respectively. Similarly the 5% ethanolic myrrh extract was found to be a more effective antifeedant than the corresponding hexane extract, see table 4.1.

Both of these solvent extracts (hexane and ethanol) showed better antifeedant activity than the aqueous DMSO extract, the latter only being active at 10% myrrh concentration levels.

Slug Mortality

Incidences of slug mortality were only observed for the 5% and 10% hexane extracts of myrrh oleoresin and the 10% aqueous DMSO of myrrh oleoresin. These extracts caused very low mortalities (mean 5%, median 0%) which were not deemed to be significant (Wilcoxon, $P = 0.500$), compared to the control.

Table 4.1 Antifeedant and Molluscicidal Properties Of Myrrh Oleoresin Extracts

Treatment	%Leaf Eaten			% Mortality		
(N = 20 slugs were used per treatment)	Mean ± SEM	Median	Mann-Whitney	Mean ± SEM	Median	Wilcoxon
Myrrh Oleoresin						
a) Hexane extracts						
1% myrrh oleoresin	58 ± 9	66*	$P < 0.001$	0 ± 0	0	
5% myrrh oleoresin	27 ± 9	0*	$P < 0.001$	5 ± 5	0 NS	$P = 0.500$
10% myrrh oleoresin	4 ± 3	0*	$P < 0.001$	5 ± 5	0 NS	$P = 0.500$
Control (Ethanol)	95 ± 5	100		0 ± 0	0	
K-W test		$P < 0.001$*			$P = 0.567$ NS	
b) Ethanol extracts						
1% myrrh oleoresin	24 ± 9	0*	$P < 0.001$	0 ± 0	0	
5% myrrh oleoresin	3 ± 3	0*	$P < 0.001$	0 ± 0	0	
10% myrrh oleoresin	2 ± 2	0*	$P < 0.001$	0 ± 0	0	
Control (Ethanol)	95 ± 5	100		0 ± 0	0	
K-W test		P < 0.001*				
c) Aqueous DMSO extract						
10% myrrh oleoresin	28 ±10	0*	$P < 0.001$	5 ± 5	0 NS	$P = 0.500$
Control (aq. DMSO (10%))	97 ± 3	100		0 ± 0	0	

K-W = Kruskal–Wallis test (P =0.05). Mann-Whitney = Mann-Whitney test (P =0.05). SEM = Standard error of the mean. Wilcoxon = Wilcoxon signed rank test (P=0.05). * = Significant difference (P <0.05) between the medians of the test sample and the control. NS = No significant difference (P >0.05) between the medians of the test sample and the control.

4.3.2 Evaluation Of The Antifeedant Properties Of Myrrh Essential Oil

Leaf damage

Myrrh essential oil extracts were highly effective in reducing the feeding activity of *D. reticulatum* slugs on lettuce leaf discs, as indicated in table 4.2 (Kruskal-Wallis, P <0.001). Myrrh essential oil was found to be a potent slug antifeedant, regardless of which solvent medium was employed for its solubilisation (ethanol, aqueous Tween 80 or aqueous DMSO).

Between treatment effects for myrrh oil solubilised in ethanol showed moderate significant differences between the amount of leaf damage observed with lettuce leaf discs treated with 0.5% and 1% myrrh oil (Mann-Whitney, P = 0.040). The mean and median leaf eaten for leaf discs treated with 0.5% myrrh oil was 14% and 0% respectively compared to a mean and median of 0% for leaf disc treated with 1% myrrh oil. Pair wise comparisons of the median leaf eaten for lettuce leaf discs treated with higher concentrations of myrrh oil (1, 5 and 10%) showed no significant differences in their strong antifeedant properties towards *D. reticulatum* slugs (median leaf damage 0% for each extract).

The strong antifeedant property of myrrh oil was increased further by using DMSO as a solubilising solvent. This solvent enabled the concentration of myrrh oil to be reduced to concentrations as low as 0.1% and 0.25% whilst still maintaining significant antifeedant effects (50% and 0% Median leaf eaten respectively) compared to 97% median leaf eaten observed for the control (Mann-Whitney, P < 0.001). The lowest amount of myrrh oil, when solubilised in 10% aqueous DMSO, which elicited negligible levels of leaf eaten was 0.5% (median leaf eaten 0%, mean leaf eaten 0.5%). This concentration of myrrh oil (0.5%) showed highly significant

antifeedant properties compared to the control (Mann-Whitney, $p<0.001$) when solubilised in 10% aqueous DMSO.

The strong antifeedant property of myrrh oil, at a concentration of 0.5%, was confirmed by solubilising in 0.2% aqueous Tween 80 resulting in no lettuce leaf discs being consumed by the slugs. As expected treating lettuce leaf discs with 0.5% myrrh, solubilised in water only, resulted in poor protection against the slugs (100% median leaf eaten) although being significantly different to the control (Mann-Whitney, $P = 0.029$).

Pair wise comparisons of the median leaf eaten for 0.5% myrrh essential oil solubilised in various media (ethanol, 10% aqueous DMSO and 0.25 Tween 80) indicated no significant differences in the amount of lettuce leaf discs consumed by *D. reticulatum* slugs (Mann-Whitney, $P < 0.001$).

Slug Mortality

General occurrences of slug mortality were very low for various concentrations of myrrh essential oil solubilised in different media. Low incidences of slug mortality were observed for 1% myrrh oil solubilised in 10% aqueous DMSO (mean 20%, median 0%) but was found to be moderately significant compared to the control (Wilcoxon, $P = 0.050$).

Table 4.2 Antifeedant And Molluscicidal Properties Of Myrrh Essential Oil Solubilised In Different Media

Treatment	%Leaf Eaten			Mortality		
(N = 20 slugs were used per treatment)	Mean ± SEM	Median	Man-Whitney	Mean ± SEM	Median	Wilcoxon
Myrrh Essential Oil						
a) Ethanol						
0.5% myrrh oil	14 ± 7	0*	$P < 0.001$	0 ± 0	0	
1% myrrh oil	0 ± 0	0*	$P < 0.001$	5 ± 5	0 NS	$P = 0.500$
5% myrrh oil	0 ± 0	0*	$P < 0.001$	0 ± 0	0	
10% myrrh oil	0 ± 0	0*	$P < 0.001$	0 ± 0	0	
Control (Ethanol)	95 ± 5	100		0 ± 0	0	
K-W test		$P < 0.001$*			$P = 0.406$ NS	
b) Aqueous DMSO (10%)						
0.1% myrrh oil	43 ± 9	50*	$P < 0.001$	0 ± 0	0 NS	
0.25% myrrh oil	29 ± 10	0*	$P < 0.001$	0 ± 0	0 NS	
0.5% myrrh oil	0.5 ± 0.5	0*	$P < 0.001$	0 ± 0	0 NS	
1% myrrh oil	0 ± 0	0*	$P < 0.001$	20 ± 0	0*	$P = 0.050$
Control (10% aq. DMSO)	97 ± 3	100		0 ± 0		
K-W test		$P < 0.001$*			$P = 0.002$*	
c) Aqueous Tween 80 (0.2%)						
0.5% myrrh oil	0 ± 0	0*	$P < 0.001$	0 ± 0	0	
Control (aq. Tween 80 (0.2%))	94 ± 4	100		0 ± 0	0	
d) Water						
0.5% myrrh oil	83 ± 7	100*	$P = 0.029$	0 ± 0	0	
1% myrrh oil	42 ± 10	45*	$P < 0.001$	5 ± 5	0 NS	$P = 0.500$
Control (water)	95 ± 5	100		0 ± 0	0	
K-W test		$P < 0.001$*			$P = 0.368$ NS	

K-W = Kruskal – Wallis test (P =0.05). Mann-Whitney = Mann-Whitney test (p=0.05). SEM = Standard error of the mean. Wilcoxon = Wilcoxon signed rank test (P=0.05). * = Significant difference (P <0.05) between the medians of the test sample and the control. NS = No significant difference (P >0.05) between the medians of the test sample and the control

4.3.3 Evaluation Of The Antifeedant Properties Of Opoponax Essential Oil

Leaf damage

All opoponax essential oil treatments were found to be effective antifeedants as shown in table 4.3. Solubilising opoponax essential oil (1 to 10%) in different media (ethanol, aqueous Tween 80, aqueous DMSO and in water) gave significant reductions in the amount of leaf damage caused by *D. reticulatum* slugs (Kruskal-Wallis, $P < 0.001$).

In general the optimum opoponax oil concentration required to reduce lettuce leaf disc consumption was found to be at the 1% concentration level. Solubilising opoponax oil (1%) in different media (water, ethanol, aqueous Tween 80 and aqueous DMSO) had significant antifeedant effects towards slugs (Kruskal-Wallis, $P = 0.171$). Solubilising opoponax essential oil in different media had no significant effects on it strong antifeedant properties (Kruskal-Wallis, $P = 0.171$).

Surprisingly opoponax oil, at concentrations as low as 0.5%, was found to be a potent antifeedant when solubilised in water, compared to the control (Mann-Whitney, $P < 0.001$).

Slug Mortality

Incidences of slug mortality were not deemed to be significant compared to the controls (Wilcoxon, $P > 0.05$).

Table 4.3 Antifeedant And Molluscicidal Properties Of Opoponax Essential Oil Solubilised In Different Media

Treatment (N = 20 slugs were used per treatment)	%Leaf Eaten Mean ± SEM	Median	Mann-Whitney	Mortality Mean ± SEM	Median	Wilcoxon
a) Opoponax Ess. Oil in Ethanol						
1% opoponax oil	35 ± 10	0*	$P = 0.002$	0 ± 0	0	
5% opoponax oil	0 ± 0	0*	$P < 0.001$	5 ± 5	0 NS	$P = 0.500$
10% opoponax oil	0 ± 0	0*	$P < 0.001$	10 ± 7	0 NS	$P = 0.186$
Control (Ethanol)	95 ± 5	100		0 ± 0	0	
K-W test		P <0.001*			$P = 0.689$ NS	
b) Opoponax Ess. Oil in aq. DMSO (10%)						
1% opoponax oil	12 ± 6	0*	P <0.001	0 ± 0	0	
5% opoponax oil	0 ± 0	0*	P <0.001	10 ± 7	0 NS	$P = 0.186$
Control (10% aq. DMSO)	97 ± 3	100		0 ± 0		
K-W test		P <0.001*			$P = 0.343$ NS	
c) Opoponax Ess. Oil in aq. Tween 80 (0.2%)						
0.5% opoponax oil	33 ± 10	0*	P <0.001	0 ± 0	0	
1% opoponax oil	20 ± 90	0*	P <0.001	0 ± 0	0	
Control (0.2%aq. Tween 80)	94 ± 4	100		0 ± 0	0	
K-W test		P <0.001*				
d) Opoponax Ess. Oil in water						
0.5% opoponax oil	11 ± 6	0*	P <0.001	5 ± 5	0 NS	$P = 0.500$
1% opoponax oil	10 ± 6	0*	P <0.001	0 ± 0	0 NS	
Control (water)	95 ± 5	100		0 ± 0	0	
K-W test		P <0.001*			$P = 0.146$ NS	

K-W = Kruskal – Wallis test (P =0.05). Mann-Whitney = Mann Whitney test (P =0.05). SEM = Standard error of the mean. Wilcoxon = Wilcoxon signed rank test (P=0.05). * = Significant difference (P <0.05) between the medians of the test sample and the control. NS = No significant difference (P >0.05) between the medians of the test sample and the control.

4.3.4 Evaluation of the Antifeedant Properties Of Selected Chemical Compounds Solubilised In Ethanol

Leaf damage

All the chemicals evaluated at the 1% concentration level, were found to significantly deter the slugs from feeding on lettuce leaf discs on comparison to the control (Mann-Whitney, $P< 0.001$), see table 4.4. Pairwise comparisons of the medians of all the chemicals, at the 1% concentration level, showed only *trans*-β-ocimene treated leaf discs to have significantly different amounts of leaf eaten ($P< 0.05$), see table 4.5.

Out of the selection of chemicals tested, *trans*-β-ocimene was found to be the most potent chemical antifeedant, towards *D. reticulatum* slugs, resulting in only 5% mean leaf eaten compared to 21-54% leaf damage for the other chemicals evaluated using the leaf disc assay method.

Reducing the *trans*-β-ocimene concentration from 1% to 0.5% and 0.1% caused a sharp increase in the consumption of the lettuce leaf discs, by the slugs, resulting in 62% mean leaf eaten (median 100%) and 90% mean leaf eaten (median 100%), hence significantly reducing the chemicals antifeedant properties.

Table 4.4 Antifeedant And Molluscicidal Properties Of Terpenoid Chemicals Solubilised In Ethanol

Treatment	%Leaf Eaten			Mortality		
	Mean ± SEM	Median	Mann-Whitney	Mean ± SEM	Median	Wilcoxon
0.1% β-Ocimene (*trans*)	90 ± 70	100 NS	$P = 0.573$	0 ± 0	0	
0.5% β-Ocimene (*trans*)	62 ± 10	100*	$P = 0.005$	0 ± 0	0	
1% β-Ocimene (*trans*)	5 ± 5	0*	$P < 0.001$	5 ± 5	0 NS	$P = 0.500$
1% γ-Bisabolene	27 ± 8	0*	$P < 0.001$	0 ± 0	0	
1% α-Farnesene	36 ± 10	0*	$P < 0.001$	0 ± 0	0	
1% Farnesol (*cis*, *trans*)	21 ± 8	0*	$P < 0.001$	0 ± 0	0	
1% Germacrene D	21 ± 7	0*	$P < 0.001$	0 ± 0	0	
1 % β-Carophyllene	54 ± 11	92*	$P < 0.001$	15 ± 8	0 NS	$P = 0.091$
Control (Ethanol)	95 ± 5	100		0 ± 0	0	
K-W test		$P < 0.001$*			$P = 0.987$ NS	

K-W = Kruskal – Wallis test (P=0.05). Mann-Whitney = Mann Whitney test (P =0.05). Wilcoxon = Wilcoxon signed rank test (P=0.05). SEM = Standard error of the mean. * = Significant difference (P<0.05) between the medians of the test and control samples. NS = No significant difference (P>0.05) between the medians of the test and control samples. N = 20 slugs were used per treatment.

Table 4.5 Mann-Whitney Pair-wise Comparisons of % leaf eaten *of trans*-β-Ocimene with selected chemicals related to those present in opoponax essential oil

	Mann-Whitney				
Chemicals	1% *trans*-β-Ocimene	1% γ-Bisabolene	1% α-Farnesene	1% Farnesol (*cis*, *trans*)	1% Germacrene D
1% *trans*-β-Ocimene	-	$P = 0.007$*	$P = 0.010$*	$P = 0.028$*	$P = 0.014$*
1% γ-Bisabolene	$P = 0.007$*	-	$P = 0.661$ NS	$P = 0.550$ NS	$P = 0.695$ NS
1% α-Farnesene	$P = 0.010$*	$P = 0.661$ NS	-	$P = 0.390$ NS	$P = 0.480$ NS
1% Farnesol (*cis*, *trans*)	$P = 0.028$*	$P = 0.550$ NS	$P = 0.390$ NS	-	$P = 0.839$ NS
1% Germacrene D	$P = 0.014$*	$P = 0.695$ NS	$P = 0.480$ NS	$P = 0.839$ NS	-

* = Significant difference ($P < 0.05$) between the medians of test samples. NS = No significant difference ($P > 0.05$) between the medians of test samples. Mann-Whitney = Mann-Whitney test ($P = 0.05$).

4.3.5 *Evaluation of the Antifeedant Properties Of Selected Chemical Compounds Solubilised In Aqueous DMSO* (*trans*-β-Ocimene and Santalol)

Leaf damage

As indicated in table 4.6, coating lettuce leaf discs with a wide concentration range of the chemical *trans*-β-ocimene (0.25% to 3%), solubilised in aqueous DMSO indicated strong antifeedant properties towards *D. reticulatum* slugs (Kruskal-Wallis, $P< 0.001$). Pair wise comparisons of the medians, using the non parametric Mann-Whitney test, indicated that the lowest *trans*-β-ocimene concentration (0.25%) did not significantly deter the slugs from feeding on the leaf discs ($P = 0.336$). No significant differences were observed between the strong antifeedant properties of the lettuce leaf discs treated with the higher *trans*-β-ocimene concentrations (0.5% to 3%), towards the slugs (Mann-Whitney, $P > 0.05$).

Treating lettuce leaf discs with 1% santalol, solubilised in aqueous DMSO also showed highly significant antifeedant properties towards *D. reticulatum* slugs, when compared to the control (Mann-Whitney, $P > 0.001$).

Slug Mortality

All the higher concentrations of *trans*-β-ocimene (0.5% to 3%), solubilised in aqueous DMSO, were found to exhibit potent molluscicidal properties with median mortalities of 100%. No incidences of slug mortality were observed on treating leaf discs with *trans*-β-ocimene (0.25%) and 1% santalol solubilised in aqueous DMSO.

Table 4.6 Antifeedant And Molluscicidal Properties Of Terpenoid Chemicals Solubilised In Aq. DMSO

Treatment	%Leaf Eaten			Mortality		
	Mean ± SEM	Median	Mann-Whitney	Mean ± SEM	Median	Wilcoxon
0.25% *trans*-β-Ocimene	95 ± 50	100 NS	$P = 0.336$	0 ± 0	0	
0.5% *trans*-β-Ocimene	10 ± 7	0*	$P < 0.001$	70 ± 0	100*	$P = 0.001$
1% *trans*-β-Ocimene	8 ± 6	0*	$P < 0.001$	75 ± 10	100*	$P < 0.001$
3% *trans*-β-Ocimene	0 ± 0	0*	$P < 0.001$	95 ± 5	100*	$P < 0.001$
Control (aq. DMSO 10%)	92 ± 6	100		0 ± 0	0	
K-W test		$P < 0.001$*			$P < 0.001$*	
Santalol solubilised in aq. DMSO (10%)						
1% Santalol (α & β isomers) in aq. DMSO (10%)	9 ± 5	0*	$P < 0.001$	0 ± 0	0	
Control (aq. DMSO 10%)	92 ± 6	100		0 ± 0	0	

K-W = Kruskal – Wallis test (P =0.05). Mann-Whitney = Mann Whitney test (P =0.05). Wilcoxon = Wilcoxon signed rank test (P=0.05). SEM = Standard error of the mean. * = Significant difference (P <0.05) between the medians of the test sample and the control. NS = No significant difference (P >0.05) between the medians of the test sample and the control. Santalol was used in the form of Sandalwood oil (containing >90% Santalol mixed α & β isomers. N = 20 slugs were used per treatment.

4.3.6 *Evaluation of the Antifeedant Properties Of Selected Chemical Compounds Solubilised In Aqueous Tween 80* (*trans*-β-Ocimene and *cis*-β-Ocimene)

Leaf damage

Both geometric isomers of β-ocimene (*cis* and *trans*) solubilised in 0.2% aqueous Tween 80 were found to be potent antifeedants towards *D. reticulatum* slugs, when coated onto lettuce leaf discs (Kruskal-Wallis, $P < 0.001$), see table 4.7. Pair wise comparisons of the median % leaf eaten, using the non parametric Mann-Whitney test, showed no significant differences between the antifeedant properties of these two isomers of β-ocimene ($P = 0.713$), both yielding 0% median leaf damage.

Slug Mortality

Both isomers of β-ocimene (*cis* and *trans*) gave low incidences of slug mortalities (median 0%). Maximum slug mortality was observed with lettuce leaf disc treated with *trans*-β-ocimene (mean 20%, median 0%) but was statistically on the margins of significance, compared to the control (Wilcoxon, $P = 0.050$).

4.3.7 Evaluation of the Antifeedant Properties Of Selected Chemical Compounds Solubilised In Water (trans-β-ocimene)

Leaf damage

Treating lettuce leaf discs with 0.5% *trans*-β-ocimene, solubilsed in water, surprisingly resulted in none of the leaf disc being consumed by slugs. By contrast, most of the control leaf discs (water treated) were eaten (median 100%, mean 88%), see table 4.7.

Slug Mortality

Highly significant slug mortalities (median 100%, mean 90%) were observed when lettuce leaf discs were treated with 0.5% *trans*-β-ocimene solubilised in water in comparison to the control (Wilcoxon, $P < 0.001$).

Table 4.7 Antifeedant and Molluscicidal Properties Of Ocimene solubilised in aq. Tween 80 (0.2%) and Water

Treatment	%Leaf eaten			% Mortality		
	Mean ± SEM	Median Mann-Whitney		Mean ± SEM	Median Wilcoxon	
0.5% Ocimene oil in aq. Tween 80 (0.2%)						
0.5% *cis*-β-Ocimene	14 ± 7	0*	$P < 0.001$	15 ± 8	0 NS	$P = 0.091$
0.5% *trans*-β-Ocimene	10 ± 6	0*	$P < 0.001$	20 ± 9	0	$P = 0.050$
Control (aq. Tween 80 (0.2%))	88 ± 8	100		0 ± 0	0	
K-W test		$P < 0.001$			$P = 0.127$ NS	
0.5% Ocimene oil in water						
0.5% *trans*-β-Ocimene	0 ± 0	0*	$P < 0.001$	90 ± 7	100 *	$P < 0.001$
Control (water)	95 ± 5	100		0 ± 0	0	

K-W = Kruskal – Wallis test (P =0.05). Mann Whitney test (P =0.05). SEM = Standard error of the mean. Wilcoxon = Wilcoxon signed rank test (P =0.05). * = Significant difference (P <0.05) between the medians of the test sample and the control. NS = No significant difference (P >0.05) between the medians of the test sample and the control.

4.3.8 A Graphical Summary Of The Antifeedant And Molluscicidal Properties Of Myrrh, Opoponax And Selected Chemicals

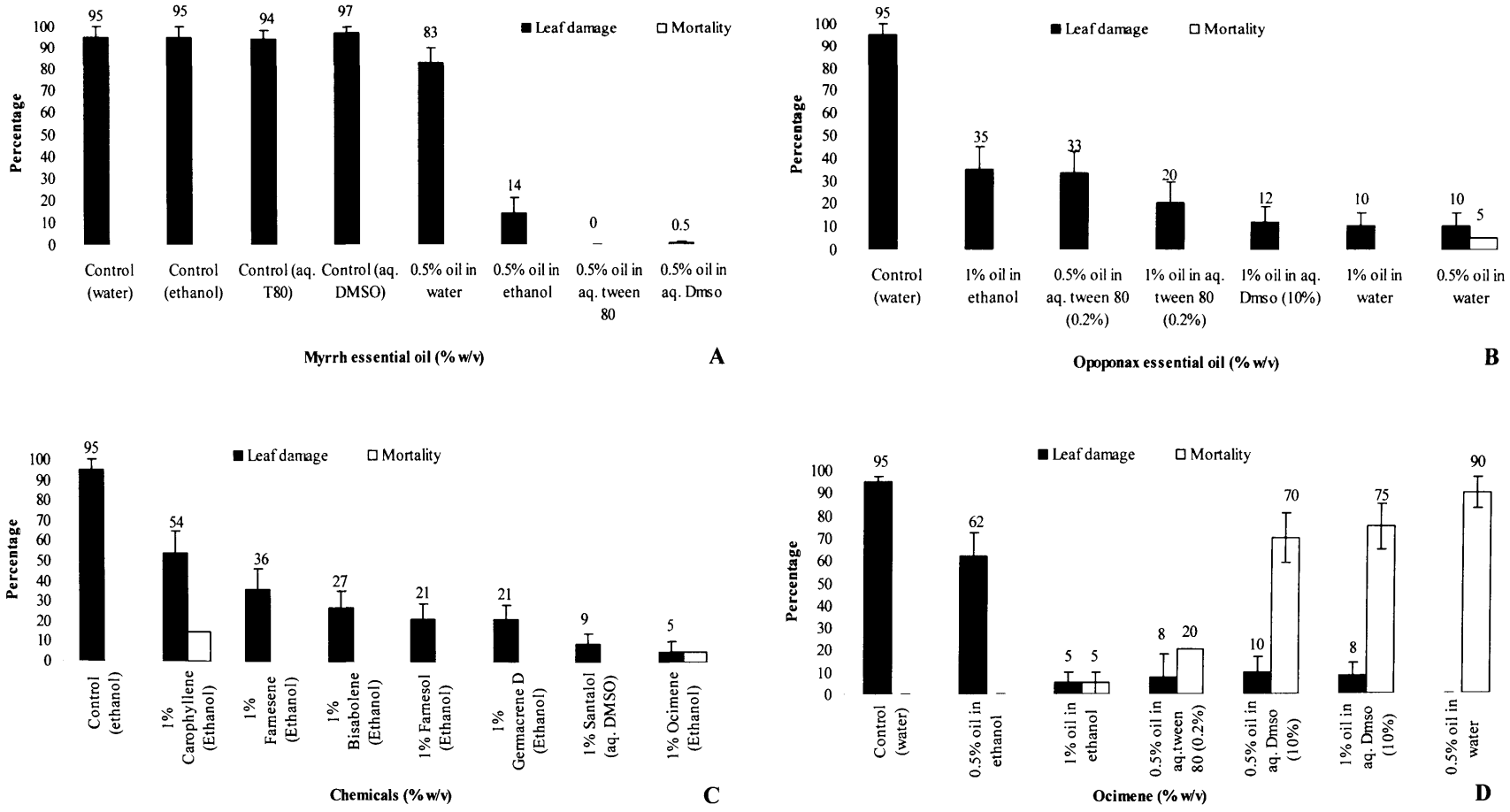


Figure 4.8 Comparison of the antifeedant and molluscicidal properties of *Commiphora* spp. extracts and related chemicals components.

The various graphs correspond to: A) Myrrh essential oil, B) Opoponax essential oil, C) Selected chemicals and D) *trans*-β-Ocimene.

4.4 DISCUSSION

In this chapter, none of the solvents tested were found to have any antifeedant effects on *D. reticulatum* slugs when evaluated using the leaf disc assay. Ethanol, being very volatile, did not remain very long on the leaf discs, while DMSO is widely regarded as being a relatively non toxic solvent, hence, both of these solvents were not expected to have any antifeedant effects. The aqueous solvents containing the nonionic surfactant Tween 80 also showed no antifeedant effects towards the field slugs. This latter solvent effect confirmed the findings of Dawson *et al.* (1996) who found Tween 80 to have no repellent properties against *D. reticulatum* slugs when tested on Chinese cabbage leaf discs.

In this study , lettuce leaf discs treated with hexane and ethanol extracts of myrrh (*C. molmol*) oleoresin, showed both extracts to be effective antifeedants at high concentration levels (10% and 5% respectively) with negligible incidences of slug mortality.

Replacing myrrh oleoresin extracts with myrrh essential oil demonstrated stronger antifeedant activity towards the slugs. The optimum concentration of myrrh essential oil, eliciting effective antifeedant activity towards *D. reticulatum* slugs was found to be 0.5% w/v regardless of whether the oil was solubilised in ethanol, aqueous DMSO or aqueous Tween 80. The antifeedant properties of myrrh essential oil, as expected, was very poor when mixed with water only. This was expected as myrrh essential oil being nonpolar does not disperse well in polar media such as water.

Differences in the bio-activity of myrrh essential oil compared to myrrh oleoresin extracts have been recently reported in other biological studies. Abel-Hay *et al.* (2002) claimed the oil fraction to be more active in killing aquatic snails (*Biomphalaria alexandria*) than the alcoholic

resin. Assimopoulou *et al.* (2004) found the essential oil fraction of myrrh to possess much stronger antioxidant activity, in sunflower oil and lard substrates, than the resin extract. Massoud *et al.* (2001), however, found the oleoresin extract of myrrh to have stronger larvicidal activity against mosquito larvae (*Culex pipiens*) than the oil extract.

It may be possible to correlate the difference in the antifeedant properties between the oleoresin and essential oil extracts with the chemical components present in the extracts. As shown in chapter 3, the GC-MS profile of these three extract were markedly different. The hexane and ethanol extracts showed an increase in the presence of non polar components (i.e. peaks with long retention times). The hexane extract of myrrh had the greatest occurrence of these long eluting chemicals followed by the ethanol extract of myrrh oleoresin and finally the myrrh essential oil. These long eluting chemicals may serve to reduce the antifeedant properties of myrrh extracts, thereby behaving in a synergistic manner.

Opoponax essential oil extracts also possessed very strong antifeedant properties against *D. reticulatum* slugs. The antifeedant activity was improved when opoponax essential oil was solubilised in water or aqueous DMSO than when it was solubilised in ethanol and aqueous Tween 80, indicating that its antifeedant property was enhanced when solubilised in polar media. Dose-activity experiments showed 1% to be the optimum level of opoponax essential required to elicit a significant antifeedant effect against slugs, when solubilised in aqueous DMSO and water alone. This is a very unusual phenomenon as essentials oils are not usually known for their solubility in water.

On contact with the lettuce leaf discs coated with *trans*-β-ocimene the slugs reacted violently by wriggling vigorously left to right while continuously secreting a white layer of

mucous. This observation was noted regardless of the nature of the solvent medium used to solubilise it. Dose-activity experiments showed 0.5% to be the optimum concentration of ocimene required to elicit an antifeedant effect against *D. reticulatum* slugs, when solubilised in aqueous media. As observed for the opoponax essential oil extracts, *trans*-β-ocimene's antifeedant activity was strongly enhanced when solubilised in polar aqueous media. *Trans*-β-ocimene being a nonpolar monoterpene was not expected to have any antifeedant activity in aqueous media. It is proposed that *trans*-β-ocimene may be the major compound contributing to the antifeedant properties of the nonpolar opoponax essential oil when formulated in aqueous media.

In addition to its strong antifeedant activities *trans*-β-ocimene also possessed potent molluscicidal properties. This molluscicidal activity was enhanced with increasing solvent polarity. Maximum mortality was observed when 0.5% ocimene was solubilised in water only, resulting in 90% slug death. By contrast, when *trans*-β-ocimene was solubilised in aqueous Tween 80 media only 20% mortality was observed. *Trans*-β-ocimene, being a relatively small molecule (molecular weight 136), may be partially soluble in water although most chemical citations suggest the opposite (Susan Budavari, 1996). This partial solubility may have been reduced when formulated with non-ionic surfactants such as Tween 80. The nonpolar end of the surfactant molecule adheres to the nonpolar *trans*-β-ocimene molecule hence changing its chemical and solubility characteristics. When 0.5% *trans*-β-ocimene was solubilised in the polar solvent aqueous DMSO, its solubility properties were enhanced resulting in high slug mortalities (70%).

Out of all the selected chemicals evaluated as mollusc antifeedants only farnesene has been implicated in mollusc control. Khallouki *et al.* (2000) demonstrated the antibacterial and

molluscicidal activity of the volatile fraction of *Chrysanthemum viscidehirtum*. The oil, which contained 24% β-farnesene in addition to many oxygenated sesquiterpenes, showed molluscicidal activity against the aquatic snails, *Bulinus truncatus* and antibacterial activity against many bacterial strains in particular *Salmonella typhi* and *Proteus mirabilis*.

In summary all the myrrh and opoponax extracts tested showed significant antifeedant properties against *D. reticulatum* slugs using the leaf disc method. In contrast to myrrh, both opoponax and *trans*-β-ocimene were also highly effective antifeedants when solubilised in water alone. All the other terpenoid chemicals evaluated were also significant in deterring the feeding behaviour of the slugs but preliminary tests have showed them not to be effective when mixed with water (data not published). It is proposed that the enhanced activity of opoponax, in water, is mainly due to the presence of *trans*-β-ocimene, which is not only a potent antifeedant, but is also strongly molluscicidal when solubilsed in water.

Chapter 5:

Antifeedant Properties of Myrrh and Opoponax Essential Oil Extracts, and their Components, Against *H. aspersa* Snails

5.1 INTRODUCTION

Slugs and snails are in general polyphagous and have distinct feeding preferences. A number of researchers have tested the palatability of various plants to terrestrial molluscs. Dirzo (1980) and Grime *et al.* (1968) both concluded that the plant *Plantago lanceolata* was not eaten by the terrestrial molluscs *Deroceras caruanae* and *Cepaea nemoralis* whilst Wardle *et al.* (1998) reported that this plant was only slightly eaten by *D. reticulatum.* This plant was, however, eaten extensively by the snail *Helix aspersa* (Grime *et al.* 1996).

According to Grime *et al.* (1996) there are three major factors which affect the palatability of certain plants to herbivores. These are chemical defence (bitter taste or toxic compound), physical defence (hardness of plant tissue) and substances which promote feeding such as sweet tasting compounds.

Hussein *et al.* (1996) found that a cardenolide extract, from the African plant *Calotropis procera*, showed strong antifeedant properties against the land snail *Theba pisana* at a low concentration of 1.3μg/cm^2. Likewise Hussein *et al.* (1999) found another cardenolide extract, this time from the Saudi Arabian plant *Pergularia tomentosa*, to be both molluscicidal and a feeding deterrent when tested against the terrestrail snail *Monacha obstructa*. The plant extracts were applied onto the surface of the snail body and the effective lethal dose (LD_{50}) determined over 72 hours. The LD_{50} for the plant extract was found to be 61μg/snail compared to 12 and 27 μg/snail for the commercial molluscicides Methomyl and Methiocarb.

activity of petasin, at a lower level of 0.05% and found it to significantly affect food choice behaviour. The bitterness of petasin, they concluded, could be responsible for the avoidance behaviour of the snails, whereas the increased sensitivity towards the sesquiterpene could be as a result of associated learning.

The aim of this research study is to demonstrate the use of myrrh (*Commiphora molmol*) and opoponax (*C. guidotti*) and their components in deterring the common garden snail *H. aspersa* from feeding upon lettuce leaf discs. A number of oil in water emulsions, containing non-ionic surfactants, were tested in order to reduce the palatability of the lettuce leaf discs. Surfactants are necessary as they help disperse these nonpolar oils into water as well as increasing the shelf life of the emulsion formulations.

All the *Commiphora* spp. oils have pungent aromas which might have the additional advantage of behaving as potential mollusc repellents/antifeedants. This research study will investigate whether their extracts have any antifeedant effects against *H. aspersa* snails.

The main aims of this chapter are:

i) To assess the antifeedent properties of the essential oils of myrrh against the garden snail *H. aspersa.*

ii) To assess the antifeedent properties of the essential oils of opoponax, against the garden snail *H. aspersa.*

iii) To assess the antifeedant properties of *trans*-β-ocimene, a major component of opoponax essential oil, against the garden snail *H. aspersa.*

5.2 MATERIALS AND METHODS

5.2.1 Reagents

The non-ionic surfactants Tween 80 and Tween 20 were purchased from Sigma-Aldrich (Dorset). The non-ionic surfactant Synperonic 91/8, was kindly supplied by Grotech Productions limited (Goole, Yorkshire). The essentials oils of myrrh (*C. molmol*) and opoponax (*C. guidotti*) were obtained from Sigma-Aldrich (Dorset). The chemical *trans*-β-ocimene was kindly supplied by R C Treatt & Co. Limited (Suffolk).

5.2.2 Test Animals

Adult *Helix aspersa* snails, obtained from Blades Biological Limited (Kent) were kept in an aquarium, layered with peat, soil and maintained at room temperature (18 to 22°C). Snails were regularly fed on lettuce. The snails were starved twenty four hours prior to testing and acclimatised to a temperature of 15°C, in an environmentally controlled chamber.

5.2.3 Test Formulations Prepared For The Leaf Disc Assay

The oils of myrrh, opoponax, and *trans*-β-ocimene were prepared as oil in water emulsions by employing nonionic surfactants as emulsifying agents. The oils were weighed into a stoppered glass vial. To this was also added the selected surfactant and the mixture vortex mixed for approximately one minute, using a Whirli^{TM} mixer

(Fisons). The formulation was diluted to volume (10ml) with deionised water and vigorously vortex mixed for 2 minutes.

It was essential that the water was added to the mixture only after the oil and surfactant had been blended together for at least 1minute. We found that if this order of addition was not followed, the stability of the emulsion produced was adversely affected. The following spray formulations (10ml) were prepared for the leaf disc assay for testing against the garden snail, *H. aspersa*:

5.2.4 Myrrh Oil

i) 3% Aqueous myrrh oil spray formulations containing Synperonic 91/8, and Tween 20 at 0.025% w/v surfactant levels were prepared separately.

iv) 3% Aqueous myrrh oil spray formulations containing Tween 80 (0.2% and 3% w/v) were prepared separately

vi) 3% Aqueous myrrh oil spray formulations containing Synperonic and Tween 20 at 3% w/v surfactant levels were prepared separately.

vii) Pure myrrh essential oil (100%)

5.2.5 Opoponax Oil

i) 3% Aqueous opoponax oil spray formulations containing 0.025% w/v Synperonic 91/8and Tween 20 surfactant levels were prepared separately.

ii) 3% Aqueous opoponax oil spray formulations containing 3% w/v Synperonic 91/8, and Tween 20 surfactant levels were prepared separately.

iii) Pure opoponax essential oil (100%)

The following spray formulations (10ml) were prepared for the leaf disc assay for testing against the garden snail, *H. aspersa*:

5.2.6 Trans-β-Ocimene

i) 5% w/v Aqueous ocimene oil spray formulations containing 0.025% w/v Synperonic 91/8, and Tween 20 were prepared separately.

ii) 5% w/v Aqueous opoponax oil spray formulations containing 5% w/v Synperonic 91/8, Tween 80 and Tween 20 were prepared separately.

iii) 5% and 10% w/v ocimene in water.

iv) Pure ocimene oil (100%).

5.3 Statistical Treatment

Between-treatments effects were determined using the non-parametric Kruskal-Wallis (K-W) test to evaluate significance differences between group medians. In all cases, between-group variances were not homogenous (Bartlett's or Levenes's test) and residuals were not normally distributed (Anderson-Darling test), hence parametric comparisons were not fulfilled. The non parametric Mann-Whitney test was used for pair-wise comparisons of the medians. In situations where 100% leaf damage was observed for the control treatments, i.e no variability, the non-parametric Wilcoxon signed rank test was used. This one-tailed test enables a standard median integer, in this case 100, to be compared with the median data for test samples. In all statistical tests, the level of significance was established at P=0.05.

5.4 RESULTS

5.4.1 Effect Of Aqueous Surfactants On The Feeding Behaviour Of H. aspersa

All of the lettuce leaf discs, treated with aqueous surfactants, were consumed within 24 hours. This feeding behaviour was identical to that obtained for the leaf discs treated with water only, see table 5.1.

Table 5.1 Antifeedant Properties Of Various Surfactants

Sample Description	% Leaf Disc Eaten Mean ± SEM
Control (Water)	100 ± 0
Aqueous Tween 80 (5%)	100 ± 0
Aqueous Tween 80 (3%)	100 ± 0
Aqueous Tween 80 (0.2%)	100 ± 0
Aqueous Tween 20 (5%)	100 ± 0
Aqueous Tween 20 (3%)	100 ± 0
Aqueous Synperonic (5%)	100 ± 0
Aqueous Synperonic (3%)	100 ± 0
Aqueous Synperonic (0.025%)	100 ± 0

N = 20 snails per test condition. S.E.M = Standard error of the mean.

No incidences of snail mortality were observed for any of the treatments.

5.4.2 *Evaluation of 3% Myrrh Essential Oil Emulsion Formulations*

As shown in table 5.2, all the 3% myrrh oil emulsions were highly significant in reducing the amount of lettuce leaf disc consumed by the slugs (Kruskal-Wallis, $P < 0.001$). At the 3% concentration level for myrrh oil, the amount of surfactant added had no effect on the antifeedant nature of the formulation, but had a large bearing on the stability of the emulsion formed, as described in chapter 6. The only treatment that showed signs of leaf damage was 3% Myrrh / aq. Tween 80 (0.2%) resulting in a mean of 17% lettuce leaf discs being eaten (median 0%) which was not statistically significant, compared to the control (Wilcoxon, $P < 0.01$).

Table 5.2 Antifeedant Properties Of 3% Myrrh Emulsion Formulations

	% Leaf Eaten		
Sample Description	Mean ± SEM	Median	Wilcoxon
3% Myrrh / aq. Synperonic (0.025%)	0 ± 0	0	
3% Myrrh / aq. Synperonic (3%)	0 ± 0	0	
3% Myrrh / aq. Tween 80 (0.2%)	17 ± 8	0*	$P < 0.01$
3% Myrrh / aq. Tween 80 (3%)	0 ± 0	0	
3% Myrrh / aq. Tween 20 (0.025%)	0 ± 0	0	
3% Myrrh / aq. Tween 20 (3%)	0 ± 0	0	
Control (Water)	100 ± 0	100	
K-W test		$P < 0.001$	

N = 20 snails per test condition, * Significant difference ($p<0.05$),
aq. = aqueous, SEM = Standard error of the mean. Wilcoxon = Wilcoxon signed rank test ($P=0.05$).

No incidences of snail mortality were observed for any of the treatments.

5.4.3 *Evaluation of 3% Opoponax Essential Oil Emulsion Formulations*

As indicated in table 5.3, all the emulsion formulations tested were significantly effective in deterring the garden snail, *H. aspersa*, from consuming treated lettuce leaf discs.

Table 5.3 Antifeedant Properties Of 3% Opoponax Emulsion Formulations

	% Leaf Disc Eaten		
Sample Description	Mean ± SEM	Median	Wilcoxon
3% Opoponax oil / aq. Synperonic (0.025%)	10 ± 7	0*	$P < 0.001$
3% Opoponax oil / aq. Synperonic (3%)	0 ± 0	0	
3% Opoponax oil / aq. Tween 20 (0.025%)	5 ± 5	0*	$P < 0.001$
3% Opoponax oil / aq. Tween 20 (3%)	0 ± 0	0	
3% Opoponax oil / aq. Tween 80 (3%)	0 ± 0	0	
Control (Water)	100 ± 0	100	-
K-W test		$P< 0.001$	

N = 20 snails per test condition, * Significant difference (P <0.05),
aq. = aqueous, SEM = Standard error of the mean. Wilcoxon = Wilcoxon signed rank test (P=0.05).

No incidences of snail mortality were observed for any of the treatments.

5.4.4 Evaluation of 5% and 10% Trans-β-Ocimene Emulsion Formulations

All the *trans*-β-ocimene/surfactant formulations tested showed strong antifeedant properties against the snails, when applied to the lettuce leaf discs (Kruskal-Wallis, $P<0.001$). Testing 5% aqueous ocimene in the absence of a surfactant, although significant compared to the control (Wilcoxon, $P=0.030$), was ineffective in reducing the feeding behaviour of the snails (100% median leaf disc consumed) as indicated in table 5.4.

Table 5.4 Mollusc Repellency Properties Of *trans*–β-Ocimene Formulations (5 and 10%)

	% Leaf Disc Eaten		
Sample description	Mean ± SEM	Median	Wilcoxon
5% *trans*–β-Ocimene oil / aq. Synperonic (0.025%)[$]	2 ± 2	0 *	$P < 0.001$
5% *trans*–β-Ocimene / aq. Synperonic (5%)	0 ± 0	0	
5% *trans*–β-Ocimene oil /aq. Tween 20 (5%)	0 ± 0	0	
5% *trans*–β-Ocimene oil / Water	79 ± 9	100*	$P=0.030$
10% *trans*–β-Ocimene oil / Water	0 ± 0	0	
Control (Water)	100 ± 0	100	-
K-W test		$P < 0.001$	

N = 20 snails per treatment, except where indicated. [$]N = 30 snails, Wilcoxon = Wilcoxon signed rank test ($P=0.05$). * = Significant difference ($P<0.05$), aq. = aqueous, SEM = Standard error of the mean.

Increasing *trans*-β-ocimene from 5% to 10%, in water only, deterred the snails from eating lettuce leaf discs (0% leaf disc eaten). The only incidence of snail mortality was observed for the emulsion containing 0.025% synperonic (mean 17%, median 0%) but was not significant (Wilcoxon, $P=0.030$).

5.4.5 Evaluation Of 3% Myrrh Aqueous Ethanol Extracts

As shown in table 5.5, lettuce leaf discs treated with 3% ethanolic myrrh extracts showed significant antifeedant properties towards *H. aspersa* snails resulting in 0% of lettuce leaf discs being eaten.

Table 5.5 Mollusc Repellency Properties Of Myrrh Formulations (3%)

Sample Description	% Leaf Disc Eaten	
	Mean ± SEM	Median
3% Myrrh Aq. Ethanol	0 ± 0	0
Control (Ethanol)	100± 0	100

N = 20 molluscs per test condition, aq. = aqueous, SEM = Standard error of the mean.

No incidences of snail mortality were observed in these tests.

5.4.6 *Evaluation Of The Pure Oils Of Myrrh, Opoponax And trans-β-Ocimene*

Treating lettuce leaf discs with the 100% pure oils of myrrh, opoponax and *trans*-β-ocimene completely inhibited feeding by the snails - see figure 5.1.

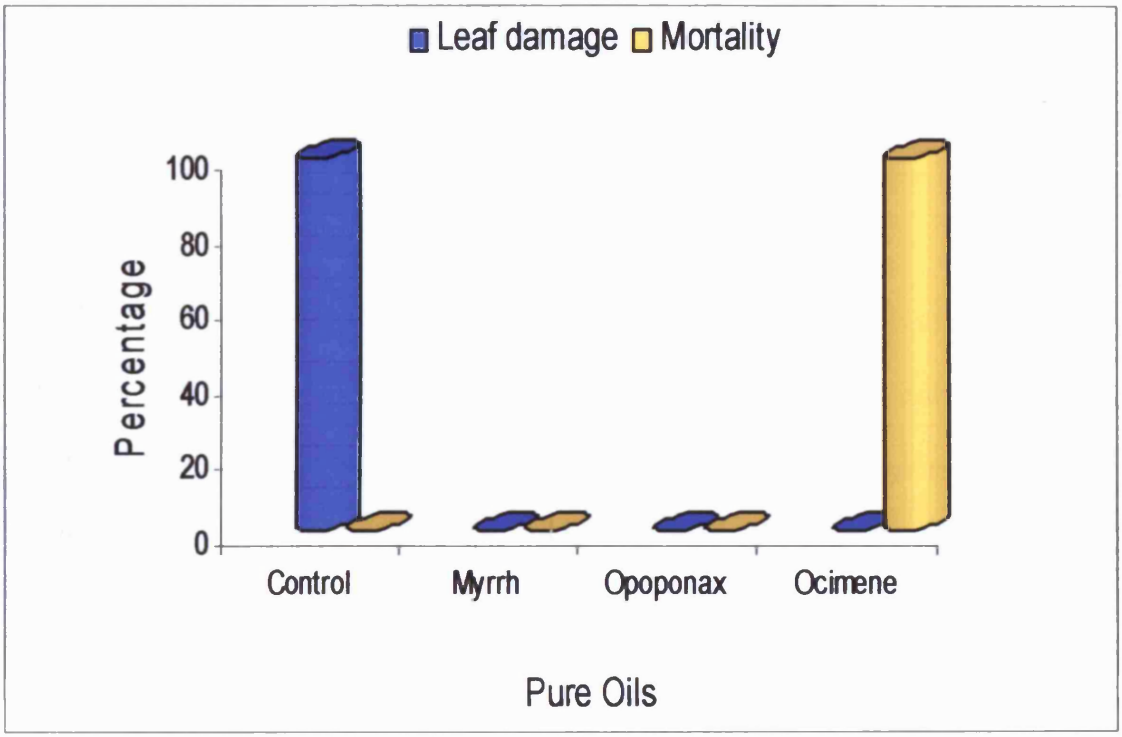


Figure 5.1 Mollusc antifeedant and molluscicidal properties of Pure (100%) Myrrh, Opoponax and *trans*-β-Ocimene oils.

When the snails came into contact with 10% aqueous *trans*-β-ocimene formulations, a bright yellow secretion was produced by some of the snails. The yellow mucous secretions was observed to occur to even greater extent when the snails came into contact with lettuce leaf discs that had been treated with pure *trans*-β-ocimene (100%). In the latter case the yellow secretions were produced by all the snails prior to their death, resulting in 100% mortality.

This phenomenon was not observed when the snails came into contact with the pure essential oils of myrrh and opoponax as they did not appear to have any toxic or irritant effects. Chemical controls containing leaf discs treated with pure ocimene oil, covered with perforated plastic top had no fumigant effect on the snails.

A physical control was employed by observing their responses to a short 9 V DC electrical stimulation. This resulted in accumulation of thick yellow mucous only on the contact points of the stimulation device. In contrast to *trans*-β-ocimene, continuous electrical stimulation resulted in the production of a clear liquid containing many bubbles.

A chemical control, comprising of 4% aqueous formaldehyde, was applied to the foot of *H. aspersa* resulting in the production of many bubbles of yellow mucous secretions, prior to death. The snail, therefore, responded similarly for both chemicals; aqueous formaldehyde (4%) and pure *trans*-β-ocimene.

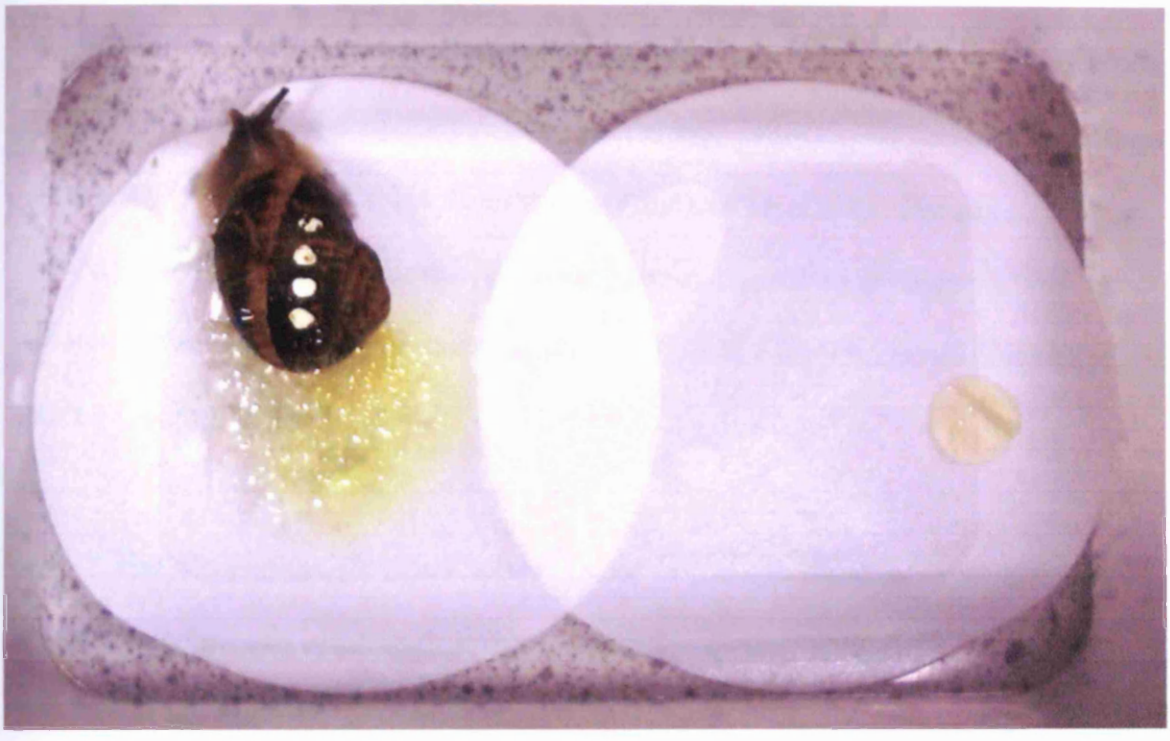


Figure 5.2 *H. aspersa* snails producing yellow mucous after being in contact with lettuce leaf disc coated with 100% *trans*-β-ocimene. The treated leaf disc, on the right hand side of the photograph, remained undamaged.

5.5 DISCUSSION

None of the nonionic surfactants tested were found to have any repellent or antifeedent properties, when tested alone, against the common garden snail *H. aspersa*. This is in good agreement with the findings of Dawson *et al.* (1996) who found that Chinese cabbage leaf discs treated with 50μg/cm^2 of the nonionic surfactant, Tween 80 had little effect on the feeding behaviour of the field slug, *D. reticulatum*. The cationic surfactants, however, did have antifeedant properties. In particular those formulations containing ammonia were found to be the best mollusc repellents. For example cetyl dimethylethyl ammonium chloride, possessed strong repellency properties. The anionic surfactant dioctylsulfosuccinate (DSS) also had a significant repellent effect on *D. reticulatum*.

The garden snail (*H. aspersa*) was found to be more resistant to the oils of myrrh, opoponax and ocimene than that previously reported for the field slug *D. reticulatum (*Chapter 4). In those leaf disc experiments 0.5% myrrh, *trans*-β-ocimene and 1% opoponax oils were very effective in reducing the palatability of the lettuce leaf discs at these low oil concentrations. In this study myrrh, opoponax, and *trans*-β-ocimene oils were found to be effective mollusc repellents at concentrations of 3% and 5% respectively. This is not totally surprising, as the epithelial foot of the garden snails, *H. aspersa*, is much thicker than that of the field slug *D. reticulatum*, and hence, it can absorb much higher levels of chemical contaminants. The garden snail is much hardier than the field slug (*D. reticulatum*) and can live between two and five years, depending on the climate and it can take between 10 months and two years for the snail to reach sexual maturity. In contrast *D. reticulatum* takes 3 to 4 months to reach maturity and

has a much shorter life cycle, approximately 530 days (South, 1982).

Although all the oils possessed strong mollusc repellency properties when formulated with low surfactant levels (0.025%), it was found that the stability of the emulsion was compromised at these low levels. To achieve a stable emulsion formulation surfactant levels approaching the same level as the oil was required. For a 3% myrrh and opoponax emulsion formulation 3% surfactant level was required, whilst 5% ocimene formulations required a surfactant level of 5%.

Recently Schuder *et al.* (2003) reported the use of a 5% garlic solution as a barrier against the horizontal movement of the snail *Oxloma pfeiffer* and the slug *Deroceras panormitanum.* The 5% garlic solution had a barrier efficacy of 65% with no mortality. This is similar to that reported by Ali *et al.* (2003b) who used inert sawdust (Spruce) coated with 0.5% myrrh essential oil, as a physical barrier, against the horizontal movements of *D. reticulatum.* Using 0.5% myrrh oil they obtained a 70% barrier efficacy, in laboratory terraria trials, and 30% slug mortality.

The interaction between land snails and essential oil containing Oregano plants (*Origanum vulgare* subspecies *hirtum* and *O. vulgare* subsp. *Vulgare*) was studied by Vokou *et.al.* (1998). They offered flour pellets containing 10% origano essential oil, to three species of snails (*Helix aspersa*, *H. lucorum* and *Eobania vermiculata*). All the snails rejected food containing high concentrations of subsp. *Hirtum* essential oil. In particular the snail *H. lucorum,* which is not native to the same environment as the subsp. *Hirtum* plants, was the least tolerant to its essential oil.

Chapter 4 demonstrated the molluscicidal properties of 0.5% aqueous *trans*-β-ocimene, against the field slug *D. reticulatum.* In this study, however, the garden snails (*H. aspersa*) showed no mortality when the lettuce leaf disc was treated with 3% and 5% aqueous *trans*-β-ocimene. This is in agreement with Davis *et al.* (1996) as they found copper spray to induce significant mortality with *D. reticulatum* but not with *H .aspersa.* Although low mortality was observed for *H. aspersa* they recorded clear repellency. Parella *et al.* 1985 also found *D. reticulatum* slugs to be more sensitive to chemical contact than *H. aspersa* snails.

At the higher *trans*-β-ocimene concentration (10%), in water, the formulation was found to be 100% effective in preventing the garden snails from consuming the lettuce leaf discs, with negligible snail mortality (5%).

When the pure oils of myrrh, opoponax and ocimene were tested using the leaf disc assay, only *trans*-β-ocimene resulted in snail death (100%), thus confirming the molluscicidal nature of this monoterpene. The other two oils (myrrh and opoponax) were not toxic to the garden snails but were just as effective in repelling them (100% snail repellency). Normal mucous secretions from *H. aspersa* are generally colourless and are produced in order to keep their bodies moist as well as assist with locomotion. Mucous is also thought to protect the snails against parasites and irritants. The formation of the yellow mucous, therefore, was clearly a result of a severe irritation by the chemical *trans*-β-ocimene. All of the snails produced yellow mucous secretions when they came into contact with lettuce leaf discs coated with pure *trans*-β-ocimene oil (100%). In the latter case the production of yellow secretions was an indication of lethal injury as it was directly followed by mortality.

Treatment of snails with 4% formaldehyde also resulted in the immediate production of yellow mucous followed by rapid mortality. The mechanism of action for formaldehyde is well known (Hayat, 1981). Formaldehyde penetrates animal tissues quite rapidly, reacting with amino acids, proteins and lipids. It forms addition products with free aminogroups in amino acids and proteins, causing irreversible damage to mucus cells.

H. aspersa was found to have a higher sensitivity threshold, when irritated with a 9 V DC electrical stimulation, having tolerated it for 3 minutes. Visible side effects were observed, with only little yellow mucous being secreted at the points of contact. This indicates that the secretion of the yellow mucous was as a direct response to the applied chemicals *trans*-β-ocimene and formaldehyde.

The production of yellow mucous by terrestrial molluscs has been previously reported for the slug species *Arion subfuscus* (Cameron, 1983), *Prophysaon andersoni* (Kozloff, 1976), *Limax flavus*, *Limax tenellus, Milax sowerbyi, Arion intermedius* and *Arion hortensis* (Godan, 1983). The marine snail *Calliostoma canaliculatum* is, apparently, the only snail that has been reported to produce yellow mucous secretions (Kelley *et al.*, 2003).

The significance of the yellow mucous secretions produced by the snails is not yet known. However it is not unreasonable to hypothesise that this behaviour was developed as a reaction to noxious chemicals entering its body. The excretory cells of *H. aspersa* contain large yellow excretory granules which contain the elements sulphur, phosphorous and calcium (Kohler *et al.*, 2001). *Trans*-β-ocimene could possibly be

causing irreversible damage to these cells. Further research is required to confirm the significance of these yellow mucous secretions in the life cycle of *H. aspersa* snails.

In summary, the leaf disc assay confirmed the antifeedant nature of the essential oils of myrrh, opoponax and their chemical components against the terrestrial mollusc *H. aspersa*. The feeding behaviour of the snails was clearly modified, when offered lettuce leaf discs treated with 50µl of 3%to 5% myrrh, opoponax and ocimene oils.

This is the first report of myrrh, opoponax and *trans*-β-ocimene oils being used to modify the feeding behaviour of terrestrial snails.

CHAPTER 6:

Formulation Development

& Stability Studies

of a Mollusc

Antifeedant Spray

6.1 INTRODUCTION

Previous terraria and leaf disc experiments (chapters 2 and 4) have demonstrated the effective use of myrrh (*C. molmol*) and opoponax (*C. guidiotti*) oleoresins and their extracts in repelling the terrestrial mollusc *D. reticulatum.* As an extension of this work a mollusc repellent/molluscicidal spray emulsion formulation will be developed based upon the essential oils of myrrh, opoponax and *trans*-β-ocimene (a major chemical component of opoponax oil).

Because of the poor solubility of these oils in water "wetting agents" or "emulsifying agents" called surfactants will be used to disperse the oils into water. Once the emulsion is formed these surfactants will also serve to prolong their stability.

6.1.1 Emulsions

Emulsions are used to a great extent in industry as carriers for active ingredients, such as pesticides, cosmetics and pharmaceuticals; hence their physical stability is of great importance (Taylor, 1998).

An emulsion can be defined as a two phase system, where one phase is finely dispersed in another with which it is immiscible (Shi *et al.*, 1999). In order to produce an emulsion mechanical energy (agitation) has to be put into the system, thus making the emulsion thermodynamically unstable (McClements and Dungan, 1995). To make the

emulsion stable, one has to make the system kinetically stable with the use of emulsifiers (surfactants). Emulsifiers (surfactants) readily absorb at interfaces and encourage emulsion formation by lowering the interfacial (surface) tension (Guzey *et al.*, 2004).

The type of emulsion formed generally depends upon the chemical characteristics of the surfactant added. Figure 6.1 illustrates the formation of two different types of emulsions. These are categorised as oil in water (o/w) or water in oil (w/o) depending upon the environment surrounding the disperse phase.

In **oil in water** (o/w) emulsions the oil is generally classed as the disperse phase and water as the continuous phase (figure 6.1c.). When water is the disperse phase and the oil is the continuous phase the emulsion is classed as **water in oil** (w/o) (figure 6.1b).

In an emulsion, surfactant molecules can exist in a number of different environments (McClements and Dungan, 1995): at the oil/water interface, as individual molecules in the aqueous phase or as micelles (aggregates) which can solubilise lipophilic materials, which otherwise would not be soluble in water.

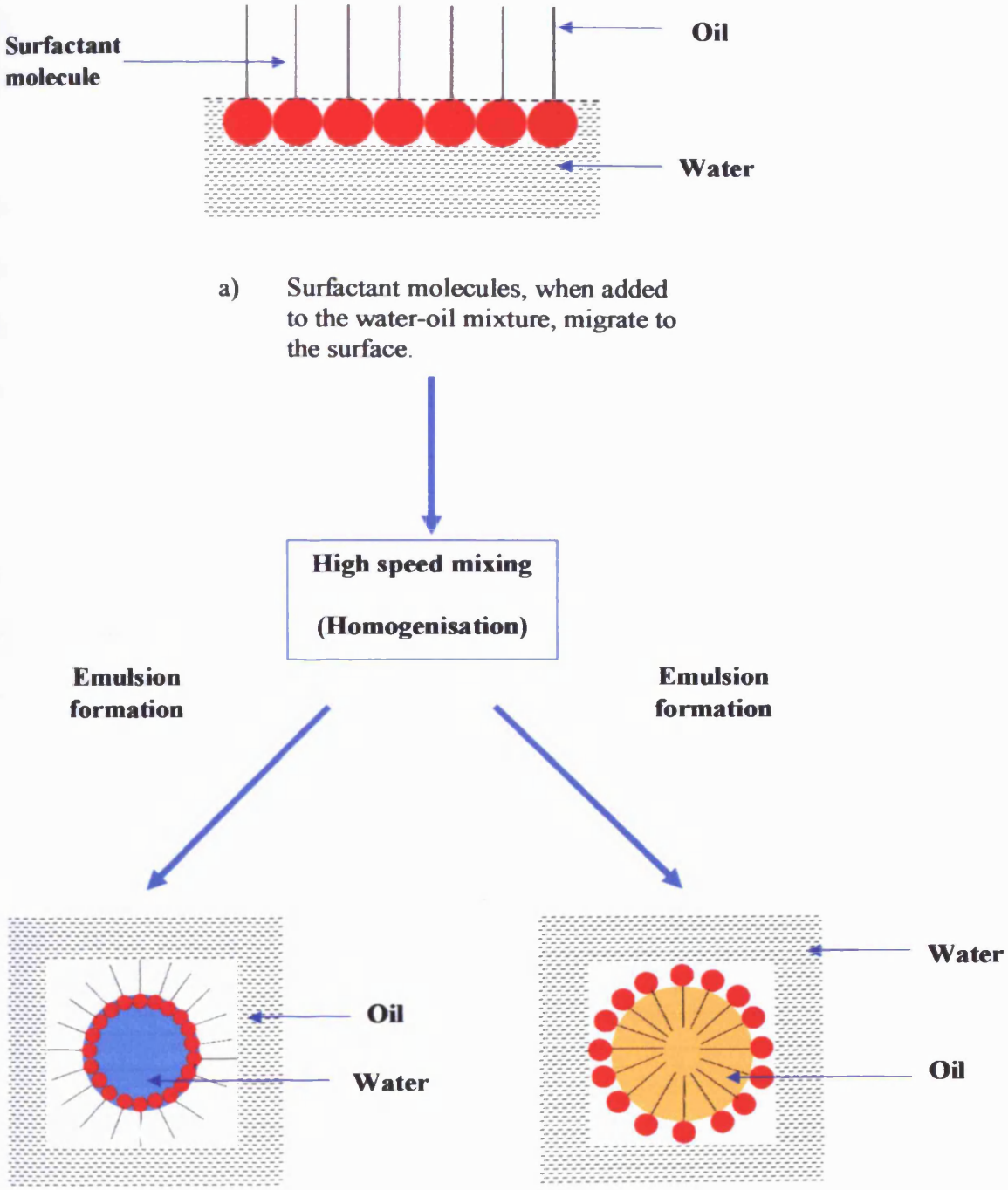


Figure 6.1 Behaviour Of Surfactants In Different Environments.

6.1.2 Emulsion stability

Some physical factors which may affect emulsion stability are: temperature, surfactant type, surfactant concentration, oil type, oil/surfactant ratio, homogeniser speed and homogenisation time. The destabilisation of emulsions may occur through a number of different processes including creaming, sedimentation, flocculation and coalescence. These processes are described in more detail below:

Creaming and **sedimentation** occur due to the density differences between the disperse and continuous phases. Creaming results when the disperse phase (oil droplets) move upwards due to having a lower density than the surrounding liquid (continuous phase), whilst sedimentation results when the disperse phase has a high density (Basaran *et al.*, 1998).

Flocculation can be described as the aggregation of particles due to weak attractive forces between colloids (Rousseau, 2000). Aggregation of the dispersed droplets form loose clusters within the emulsion and may occur before or after creaming.

Coalescence is a process where after the collision of oil droplets, the interfacial film between flocculated droplets is disrupted, resulting in them merging together to form larger droplets. Both creaming and flocculation allow the oil droplets, in the emulsion, to get closer to each other and facilitate the occurrence of coalescence. These emulsion destabilisation processes are illustrated in figure 6.2.

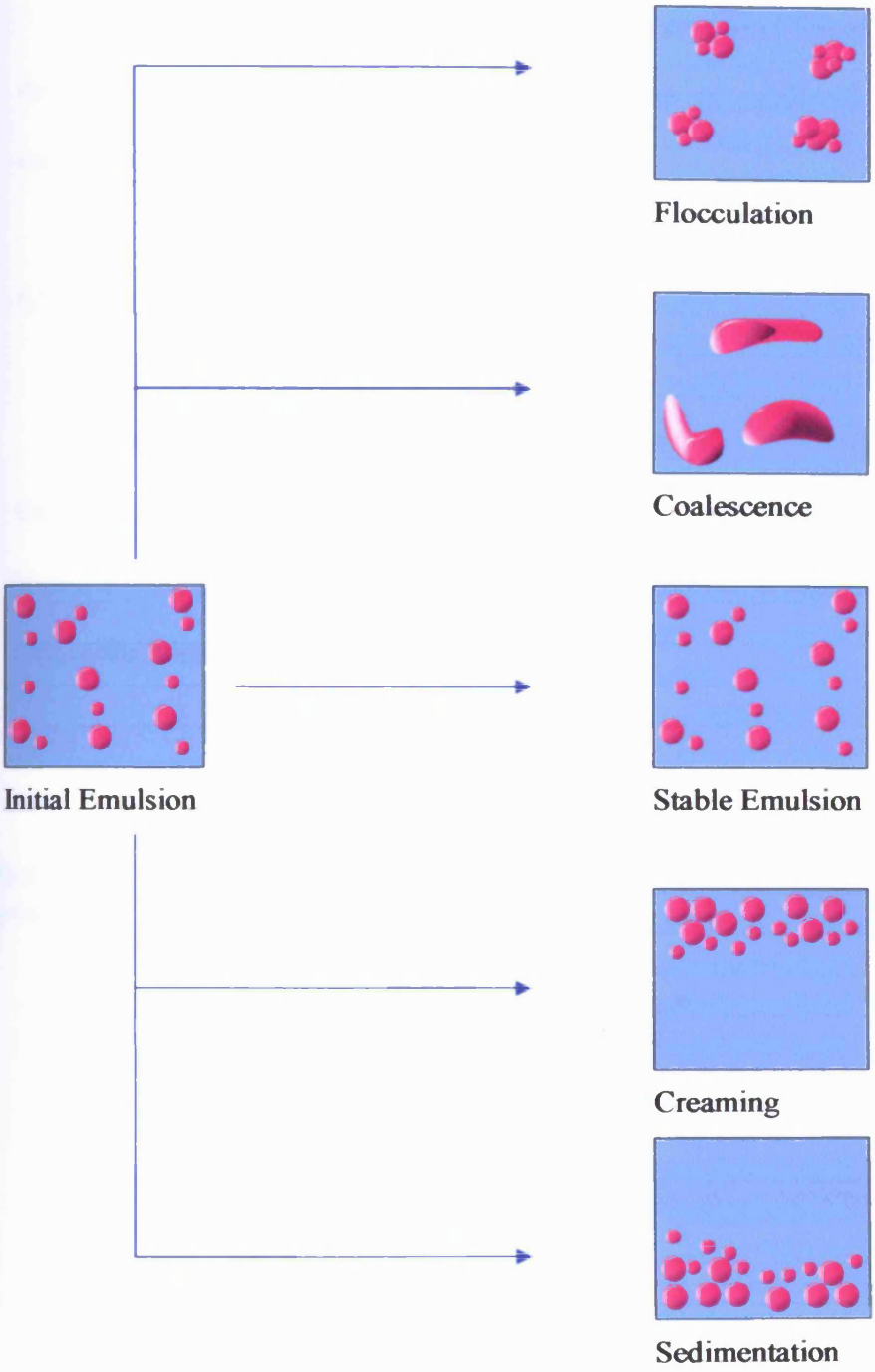


Figure 6.2 Different Types of Emulsion Instability

A stable emulsion will be formed if the repulsive forces between colliding oil droplets are predominant, as they will rebound of each other, thereby preventing coalescence (Ivanov and Kralchevsky, 1997).

6.1.3 Surfactants

Surfactants are molecules which when added to a liquid changes the properties of that liquid at the surface or interface. Surfactants are composed of two parts, a water-loving or hydrophilic group at one end and an oil-loving or lipophylic group at the other end, as illustrated in figure 6.3.

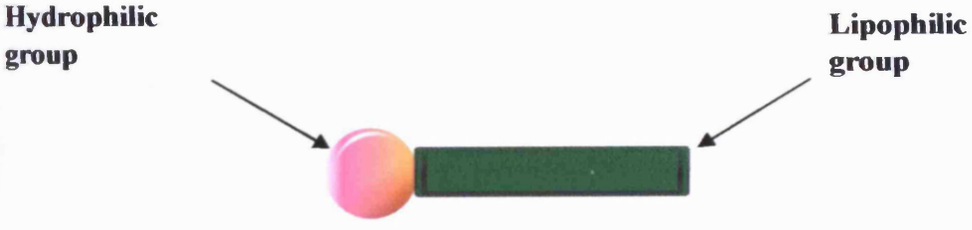


Figure 6.3 A Typical Surfactant Molecule.

The hydrophilic end is usually a polar or ionic group, whereas the lipophilic end is usually a long hydrocarbon chain. This dual functionality provides the basis for their useful properties in developing emulsion formulations. When surfactants are added to an oil/water mixture the lipophilic part, usually a hydrocarbon, disperses in the oil phase,

whilst the hydrophilic (water-loving) portion of the molecule orientates themselves into clusters (micelles) with the hydrophilic portion facing towards the water molecules.

This effect reduces the surface tension of the oil/water interface layer facilitating the formation of an emulsion. The surfactant prevents the coalescing of the oil droplets, thereby preventing the two phases from separating.

Surfactants are classified by the nature of the ionisable hydrophilic portion of the molecule and are categorised into three types; anionic, cationic or non-ionic.

Nonionic surfactants achieve stabilisation by surrounding the oil particles with a hydrated layer of surfactant which prevents molecules coalescing together by using steric hindrance. Nonionic surfactants have large hydrophilic groups which extend away from the droplet surface a significant distance imposing a steric barrier to encounter.

Anionic and cationic surfactants have ionisable hydrophilic groups, which when adsorbed onto the interface of the emulsion droplets increases the surface charge. Electrostatic forces are then developed that oppose the rate at which oil droplets encounter each other, thereby preventing coalescence and flocculation from occurring.

Guidelines for choosing the optimum surfactant are aided by many formulation rules two of which are:

i) Bancroft rule (Bancroft, 1913) and

ii) Griffins Hydrophilic-lipophylic balance (HLB) scale (Griffin, 1949).

Bancroft rule states that "in order to have a stable emulsion the surfactant must be soluble in the continuous phase". This suggests that a surfactant which has an affinity for water favours the formation of an oil in water (O/W) emulsion and that a surfactant which has an affinity for oil favours the formation of water in oil (W/O) emulsion.

Griffin's HLB scale is essentially a balance of the size and strength of the hydrophilic and lipophilic properties of a surfactant molecule. He created the "**HLB number**" which is a scale ranging from 0 to 20 to describe the solubility properties of non-ionic surfactants. In general a low HLB number indicates an oil loving surfactant (liophilic) and a high HLB number indicates a water loving surfactant (hydrophilic). Griffin's HLB scale is still regularly used today, however temperature and interactions with the aqueous and oil phases, which he overlooked, have to be incorporated into the HLB number if it is to be a useful tool for predicting emulsion stability (Davis, 1994). Stable emulsions are best formulated with emulsifiers having HLB values close to that required of the oil phase (Aulton, 1995).

The hydrophilic-lipopilic balance (HLB) system has proven to be a useful rule for preparing oil/water emulsions (Xu *et al.*, 2001). It is generally accepted that surfactants

with a low HLB value (3-6) stabilise water in oil emulsions whereas those with a high HLB value (8-18) stabilise oil in water emulsions.

In this thesis three surfactants are evaluated for their ability to form stable emulsions with myrrh, opoponax and ocimene oils. The main advantages of using non-ionic surfactants in emulsion formulations, compared to ionic surfactants, are:

i) They are not destabilised by the presence of electrolytes (Palla and Shah, 2002).

ii) They display relatively low viscosities.

iii) They are equally effective in both aqueous and nonaqueous media.

iv) They have superior antibacterial properties (Wilkinson, 1982).

6.1.4 *Analytical Techniques For Measuring Emulsion Stability*

The stability of an emulsion can be assessed by using the following methods:

i) Monitoring physical changes in their appearance with time.

ii) Using a spectrophotomer to measure turbidity by recording the amount of light transmittance (%) through the emulsion over time.

iii) Applying a centrifugal force to the emulsion sample.

iv) Measuring the zeta potential of an emulsion.

v) Measuring the conductivity of an emulsion.

vi) Measuring changes in particle size (droplet size) using microscopy.

vii) Measuring changes in particle size (droplet size) using light scattering techniques.

According to Kim *et al.* (2000) the most common method is droplet size analysis and microscopy, which are very time consuming techniques. In this thesis the first method (physical appearance) will be used to assess emulsion stability. Changes in emulsion turbidity will be assessed over a finite time period. Emulsion formulations will be considered stable if no phase separation is detected for a defined period of time.

The main aims of this chapter are:

i) To screen three non-ionic surfactants for their ability to emulsify/stabilise non polar essential oils in water.

ii) To determine whether 3% and 5% are optimum levels of surfactant to produce stable o/w emulsion formulations.

iii) To monitor the stability of emulsion formulations by recording any changes in their physical appearances with time.

The term "**stability**" is difficult to define in regards to emulsion formation, as it may be understood differently in different applications (Wiacek *et al.*, 2002). In this chapter the appearance of an emulsion is defined as stable in reference to its turbidity. The criteria for defining a stable emulsion is the formation of one homogenous phase mixture comprising a turbid appearance. If the emulsion is stable for twenty four hours it is classed as a *short term stable emulsion* whilst if no changes in appearance occurs over a time period greater than one week, it is classed as a *long term stable emulsion.* The turbidity of an emulsion generally decreases with time because of the emulsion breaking processes which take place (Kim *et al.*, 2000).

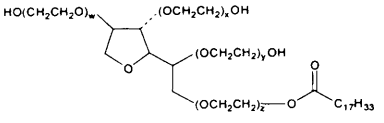
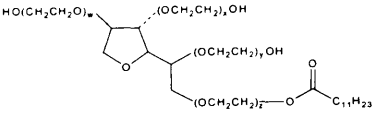
Preliminary evaluations of emulsion stability with 3% myrrh and opoponax oils containing low levels of surfactants (0.025 – 1%) showed poor stability profiles (1 to 24 hours) hence this study will focus on higher levels of surfactants (3% and 5%).

6.2 MATERIALS AND METHODS

6.2.1 Reagents

The essentials oils of myrrh and opoponax were obtained from Sigma-Aldrich (USA). *Trans*-β-ocimene, a major component of opoponax oil, was kindly supplied by RC Treatt & Co. Limited (Suffolk).

Table 6.1 Properties of Selected Surfactants Used For Preparing Emulsions

Name	Chemical Structure	HLB No.	Supplier
Synperonic 91/8	Polyoxyethylene (8) synthetic primary C9/C11 alcohol	14.1*	Grotech Production, Goole, Yorkshire.
Tween 80	 Polyoxyethylene (20) sorbitan monooleate	15.0 †§	Sigma-Aldrich, Poole, Dorset.
Tween 20	 Polyoxyethylene (20) sorbitan monoolaurate	16.7 †	Sigma-Aldrich, Poole, Dorset.

† Al-Sabagh (2002), * Personnel communication ICI (2004), § Bogman *et al.* (2003).

6.2.2 Blending Protocol

The oils of myrrh, opoponax, and *trans*-β-ocimene were prepared as oil in water emulsions by adding various surfactants. The oils were weighed into a stoppered glass vial, containing the selected surfactant and the mixture vortex mixed for approximately 2 minutes, using a Whirli^{TM} mixer (Fisons) at full speed. The formulation was diluted to volume (10ml) with deionised water and vortex mixed for another 2 minutes. It is important that the water is not added to the mixture until the oil and surfactant have been blended together for at least two minutes. We found the stability of the resulting emulsion to be adversely affected if this order of addition was not followed.

6.2.3 Preparation of Oil in Water (O/W) Emulsion Spray Formulations For Stability Studies

Oil in water emulsion spray formulations were all prepared using the blending protocol described in section 6.2.2. Myrrh, opoponax, and *trans*-β-ocimene oils were all added at concentration levels of 3% and 5%, whilst the surfactants were added to them at 0.025%, 3% and 5% levels. These were made up to volume with de-ionised water. Samples were prepared in duplicate. The nonionic surfactants used in this study were:
i) Synperonic 91/8 ii) Tween 80 and iii) Tween 20.

The total amount of formulation prepared was approximately 10ml, as shown in table 6.2. The glass vials were stored at room temperature (16–25°C). The appearances of the prepared formulations were recorded after one hour and any subsequent changes in their physical appearances monitored for a time period between one and 56 weeks.

Table 6.2 Preparation Of Oil In Water Emulsions Containing Various Amounts Of Nonionic Surfactant (3% to 5%).

Components	Amount Added	%
3% Oil + 3% Surfactant		
Oil	0.3 g	3
Surfactant	0.3 g	3
Water	9.4 ml	94
5% Oil + 5% Surfactant		
Oil	0.5 g	5
Surfactant	0.5 g	5
Water	9.0 ml	90

The surfactants evaluated were Synperonic 91/8, Tween 80 and Tween 20.

6.3 RESULTS

6.3.1 Stability Of 3% Myrrh Oil In Water Emulsions Containing 3% Surfactant

Synperonic 91/8 stabilized the emulsion formulation for a period of two weeks, after which the emulsion separated into two phases. A yellow oil phase was formed as a top layer and a beige emulsion bottom layer, see figure 6.4. The appearance of the other two myrrh emulsions, containing 3% Tween 20 and Tween 80, were unaffected for a period of 43 weeks (approx. 10 months) compared to the initial appearance of the emulsion formulations, see table 6.3. After a period of 44 weeks the myrrh emulsion containing aqueous Tween 20 became physically unstable and separated into two distinct phases. The myrrh emulsion containing Tween 80 remained stable after a period of 56 weeks.

Table 6.3 Stability Evaluation Of 3% Myrrh Oil Emulsion Formulations Containing 3% Surfactant Levels

	Changes in Emulsion Stability with Time						
Formulation	1 week	2 weeks	3 weeks	13 weeks	43 weeks	44 weeks	56 weeks
3% Myrrh / Synperonic	**Stable**	**Stable**	Unstable	Unstable	Unstable	Unstable	Unstable
3% Myrrh / Tween 80	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**
3% Myrrh / Tween 20	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	Unstable	Unstable

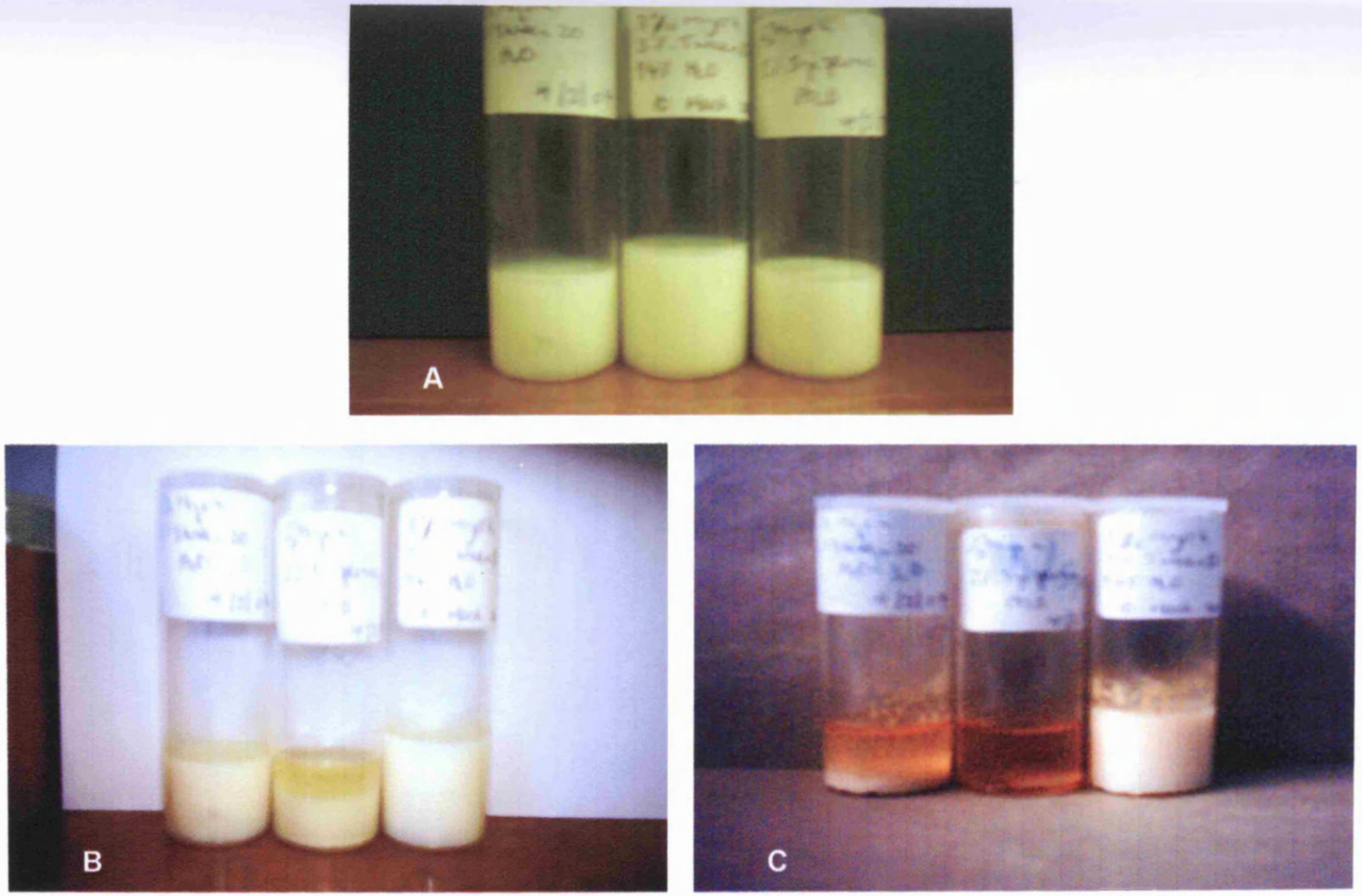


Figure 6.4 Appearance of 3% aqueous myrrh emulsions after a period of: **A) 0 weeks (Initial)**, **B) 3 weeks** and **C) 43 weeks**. After 3 weeks the emulsion formulation containing Synperonic 91/8 had become unstable, separating to form two distinct phases. After 44 weeks the emulsion formulation containing the surfactant Tween 20 had also become unstable. The emulsion formulation containing the surfactant Tween 80 remained stable for a time period greater than 56 weeks.

6.3.2 *Stability Of 3% Opoponax Oil In Water Emulsions Containing 3% Surfactant*

The stability assessment of the aqueous extracts of opoponax oil essentially mirrored that of myrrh, with the slight variation of producing bright white coloured emulsions with all the surfactants. The synperonic based emulsion was stable for two weeks when formulated with a surfactant level of 3%. Tween 20 and Tween 80 surfactants stabilised the oil in water emulsions for approximately 56 weeks. The stability profile is summarized in table 6.4.

Table 6.4 Stability Evaluation Of 3% Opoponax Oil Emulsion Formulations Containing 3% Surfactant Levels

	Changes in Emulsion Stability with Time						
Formulation	1 week	2 weeks	3 weeks	13 weeks	43 weeks	44 weeks	56 weeks
3%Opoponax /Synperonic	**Stable**	**Stable**	Unstable	Unstable	Unstable	Unstable	Unstable
3% Opoponax /Tween 80	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**
3% Opoponax /Tween 20	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**

6.3.3 *Stability Of 5% Trans-b-Ocimene Emulsions Containing 5% Surfactant*

The aqueous emulsions of ocimene gave bright white emulsions with all the surfactants evaluated. Synperonic 91/8 stabilised the emulsion formulations for only 1 week, whereas emulsion formulations containing aqueous Tween 80 were stable for 43 weeks after which they became physically unstable. Tween 20 based emulsions were stable for over 56 weeks, see table 6.5.

Table 6.5 Stability of 5% *Trans*-β-Ocimene Oil Emulsion Formulations

	Changes in Emulsion Stability with Time						
Formulation	1 week	2 weeks	3 weeks	13 weeks	43 weeks	44 weeks	56 weeks
5% Ocimene/ Synperonic	**Stable**	Unstable	Unstable	Unstable	Unstable	Unstable	Unstable
5% Ocimene/ Tween 80	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	Unstable	Unstable
5% Ocimene/ 5% Tween 20	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**	**Stable**

6.4 DISCUSSION

Many agrochemicals are formulated as emulsifiable concentrates which, when added to water, produce oil-in-water emulsions (Piscureanu *et al.*, 2001). These are usually prepared on the day of use and are generally stable for four to seven hours, long enough for the end user (farmer or horticulturist) to spray his crops.

In this study the turbidity of the o/w spray formulations was found to give a good indication of emulsion stability. Long periods of emulsion stability was achieved for the 3% myrrh, 3% opoponax and 5% ocimene oils containing equal levels of surfactants (3% and 5%). Formulations containing 3% and 5% Synperonic 91/8 were stable for 1 to 2 weeks, after which they had become clear in appearance whereas formulations containing Tween 20 and Tween 80 were found to be stable for approximately 10 months (43 weeks). All of the oil emulsions containing 3% surfactant could be classed as *long term stable emulsions*. After 56 weeks emulsions containing 3% myrrh/Tween 80, 3% opoponax / Tween 20, 3% opoponax / Tween 80 and 5% ocimene / Tween 20 were the only emulsion formulations that could be classed as stable.

The long periods of emulsion stability obtained for Tween 20 and Tween 80, compared to Synperonic 91/8, could be attributed to their high HLB numbers (Hydrophilic-lipophilic balance) which are 16.7, 15 and 14.1 respectively. Surfactants with a high HLB number are generally thought to favour the stabilisation of o/w emulsions due to their high solubility in water.

In addition to concentration and surfactant type another major factor in emulsion stability is the process or manufacturing conditions used to prepare the emulsion. These can be divided into mixing speed and the order of addition of the various excipients. In this study, the oil and surfactant components were first mixed together at full speed using a vortex mixer followed by the addition of water. This mixture was, once again, homogenised at high speed. Preliminary formulation studies, with myrrh oil, showed that if this order of addition was not followed, the stability of the emulsion was compromised. Gullapalli and Sheth (1999) also found that adding the surfactant to the oil phase first, formed more stable emulsions (no creaming) than when the surfactant was added first to the water phase (creaming).

The stability of these spray formulations might also be increased when formulated on a larger manufacturing scale size, since much faster mixing speeds can be achieved. Lashmar *et al.* (1995) recognized mixing speed to be the single most important factor for emulsion stability.

The one year plus stability profiles achieved for these 'ready to use' spray formulations are encouraging considering the small batch size formulated (10ml) and the basic nature of the mixing apparatus used.

CHAPTER 7:

Caged-field and Spray Trials

7.1 INTRODUCTION

Laboratory experiments such as terraria studies and leaf discs assays give a good indication of the effectiveness of new products for mollusc control. However, to obtain a more realistic picture of their effectiveness, larger scale testing such as field trials has to be conducted. There have been many reports using these types of trials mainly with commercial molluscicides such as metaldehyde and methiocarb and occasionally with alternative mollusc controls agents. Hata *et al.* (1997) conducted greenhouse trials with thirteen molluscicides containing metaldehyde, three molluscicides containing metaldehyde plus methiocarb and one molluscicide containing only methiocarb for efficacy against the slugs *Vaginula plebeian* and *Veronicella cubensis*. They found that without exception, all the molluscicides were effective against both slug species. They also reported that physical barriers composed of copper and fibreglass screens were effective repellents towards both slug species.

The use of chemical molluscicides to control molluscs is, however, increasingly becoming unpopular in agriculture and horticulture. In organic farming the use of chemical molluscicides is not allowed and instead the use of alternative methods of mollusc control such as physical barriers/fences, beer traps and hand collecting are encouraged (Speiser, 1996). European Union regulations still allow the use of metaldehyde in organic farming as long as it is used in traps and the bait pellet formulation contains an animal repellent (Gardner, 2003). Today, iron pellets are the only chemical form of mollusc control accepted for use in organic farming and are regarded as GRAS (generally regarded as safe) in the USA.

The alternative use of iron pellets as molluscicides has also been investigated by Young (1996) in the form of iron chelate pellets. He confirmed the efficacy of two bait formulations incorporating iron chelate in the form of iron EDTA (Ethylenediamine acetic acid) against three species of snails (*Helix aspersa, Theba pisana* and *Cernuella virgata*) and two species of slugs (*Deroceras reticulatum* and *Limax maximus*). The two formulations, which differed only in the bran content (25% and 50%), showed high kill rates against all the molluscs tested under laboratory conditions.

More recently Speiser and Kistler (2002) evaluated the use of iron phosphate pellets as an alternative means of reducing slug damage to lettuce and rape seedlings, under miniplot trial conditions. The plots were either treated with pellets containing 1% iron phosphate (Ferramol ®), 5% metaldehyde (Metarex R.G®) or left untreated. They found that metaldehyde and iron phosphate pellets not only reduced the number of *Arion lusitanicus* slugs but also the amount of lettuce leaf loss and the percentage of damaged rape seedlings.

Commercially, there is plenty of scope for developing new molluscicides in the form of sprays, since there have not been many advances in this particular area. Aqueous sprays containing copper sulphate, despite being toxic, was included in UK recommendations for controlling gastropods in arable crops as recently as 1984 (Anon, 1984). In practice, however, copper sulphate has been largely superseded by commercial baits, whereas in some parts of Europe, such as the Netherlands, the use of copper sprays has been banned (Gardner, 2003), hence the impetus for developing alternative more acceptable spray products for controlling molluscs.

Metaldehyde has been applied in emulsified form as a spray for controlling molluscs but its application has been limited (Barker, 2002).

The antifeedant properties of lichen plant extracts, applied as sprays, were evaluated by Clark *et al.* (1998). They tested 15 species of lichen against the field slug and all but 3 showed some antifeedant activity, the most effective extract being from *Letharia vulpine*. They found vulpinic acid to be the major active responsible for the extract's antifeedant behaviour and was tested as a foliar spray to turnip plants. A 3% suspension of vulpinic acid was found to be a potent antifeedant against *D. reticulatum* slugs but unfortunately possessed some phytotoxic properties.

The main aims of this chapter are to evaluate the:

i) Efficacy of solid myrrh (*C.molmol*) and opoponax (*C. guidotti*) oleoresins, and their mixtures with inert substrate, as repellent barriers towards slugs, under caged field trials conditions.

ii) Effectiveness of myrrh extracts coated onto sawdust, as repellent barriers towards slugs, under caged field trials conditions.

iii) Antifeedant properties of myrrh and opoponax oleoresin extracts, towards slugs and snails, under spray trial conditions.

iv) Antifeedant properties of *trans*-β-ocimene, a major component of opoponax oil, towards slugs and snails, under spray trial conditions.

7.2 MATERIALS AND METHODS

7.2.1 Reagents

Opoponax (*C. guidotti*) oleoresin was obtained from Hargeisa (Somaliland) whilst myrrh (*C. molmol*) oleoresin was purchased from G. Baldwin & Co. (London). Spruce sawdust was purchased from RS Biotech Limited (Fidon,). Slug Stop™ a commercial mollusc barrier comprised of amorphous silica (diatomite) was purchased from B&Q (Cardiff). Myrrh and opoponax essentials oils and the non-ionic surfactants Tween 20 and Tween 80 were all purchased from Sigma-Aldrich (Pole, Dorset). Ethanolic myrrh extract (20% w/v) was purchased from Flavex limited (Hereford). *Trans*-β-ocimene, a major component of opoponax essential oil, was kindly supplied by RC Treatt & Co. Limited (Bury St Edmonds, Suffolk). The nonionic surfactant Synperonic 91/8 was kindly supplied by Grotech Production limited (Goole, Yorkshire). The following lettuce seedlings were supplied by Gerway Nurseries (Exeter): 'Iceberg' crisp head, 'Cultivar' all year round, 'Charles' butterhead, 'Emerald' butterhead, ' Radja' curly variety and 'Little Gem' cos.

7.2.2 Preparation of Myrrh Treated Sawdust Barriers

See chapter 2 (sections 2.3.1 and 2.3.2) for the preparation of sawdust barriers treated with ethanolic myrrh (20%) and sawdust treated with 1% myrrh essential oil.

7.2.3 *Blending Protocol for Spray Trials*

7.2.3.1 Myrrh and Opoponax Emulsion Sprays (3% Essential Oil)

The essential oil (6g) was weighed into a 500ml duran bottle and 6g of surfactant (Tween 80 or Tween 20) added. The two ingredients were mixed together for two minutes by resting the edge of the bottle on a Whirly mixer TM and vortex mixed for two minutes at maximum speed. The mixture was diluted to a volume of 200ml and shaken together, vigorously, for a further two minutes until the surfactant/oil mixture had completely gone into solution forming a homogenous turbid emulsion.

7.2.3.2 *trans*-β-Ocimene (5%) Emulsion Sprays

The protocol described in 7.2.3.1 was followed with the variation of adding of *trans*-β-ocimene (10g) and 10g of surfactant (Tween 80 or Tween 20), both corresponding to 5%w/v.

7.2.3.3 Myrrh Oleoresin Emulsion Sprays (3% and 5%Aqueous Ethanol Extracts)

Aqueous ethanol extracts of myrrh oleoresin (3% and 5%) were prepared by adding 30ml and 50ml, respectively, of a commercially available myrrh aqueous alcohol extract (containing 20% myrrh and 95% ethanol) and diluting to 200ml with deionised water.

7.2.4 Test animals

Adult *H. aspersa* snails were obtained from Blades Biological (Kent) and kept in an aquarium maintained at room temperature (16 to 25°C) and fed regularly on lettuce. Snails were acclimatised to a temperature of 15°C in an environmentally controlled chamber and pre-starved for 24 hour prior to testing.

Adult *D. reticulatum* slugs were collected from nearby fields and maintained in plastic trays lined with moist, unbleached, absorbent paper. They were housed in the dark and at a constant temperature of 10° ± 1°C. Slugs were regularly fed on a mixture of lettuce and carrots. Twenty four hour prior to testing slugs were starved for 24 hour and acclimatised to a temperature of 15°C, in an environmentally controlled chamber.

7.2.5 Caged-Field Trials

Caged field trials were conducted in October 2001 and April 2002, in 1m^2 arenas with rigid pvc side panels (200mm high). Fluon® (polytetrafluoroethylene) was applied to the sides of the cages to contain the slugs within the testing arena. The enclosures were filled with medium loam soil to a depth of approximately 5-6 cm and four young lettuce seedlings (Iceberg) with 4-8 true leaves were planted in each. Each lettuce was surrounded with a barrier consisting of 15g of test material. Ten slugs were introduced into each arena representing a slug population equivalent to 100,000 slugs per hectare. The amount of leaf damage per lettuce was visually assessed daily for fourteen days. % leaf damage was measured by visual assessing the % leaf eaten for each leaf and the

mean value for all the leaves calculated. The field trials were conducted using four replicates.

The materials tested, in October 2001, as repellent barriers comprised:

i) Myrrh oleoresin (*C. molmol*).

ii) Opoponax oleoresin (*C. guidotti*).

iii) Myrrh essential oil extract (1% in ethanol) coated onto sawdust.

iv) Myrrh ethanolic extract (20% in ethanol) coated onto spruce sawdust.

v) No barrier as a control.

The materials tested in April 2002, as a repellent barrier, comprised:

i) Sawdust and corncob raw materials.

ii) Myrrh oleoresin mixed with spruce sawdust (50%).

iii) Myrrh oleoresin mixed with corncob (50%).

iv) Slug Stop ® flakes (commercial barrier) and

v) No barrier as control.

Figure 7.1 Caged field trials with lettuce seedlings surrounded by repellent solid barriers comprised of myrrh oleoresins (*C. molmol*).

7.2.6 *Laboratory Spray Trials against D. reticulatum slugs (24 hours)*

Lettuce leaves ('Little Gem', Cos) were sprayed with 12 ml of a 3% aqueous oil emulsion containing 0.2% Tween 80 as the surfactant. Once sprayed the leaves were placed in small plastic trays containing peat soil and left for 30 minutes to remove the excess liquid drops. After this time period two adult slugs (*D. reticulatum*), previously starved for 24 hour, were added to each plastic tray and left for 24 hour in a controlled temperature environment (15°C, 12 hour light; 15°C , 12 hour dark). A control (water) was treated similarly. Four replicates were prepared for each treatment. The amount of leaf damage (consumed) was calculated using digital photographs before and after exposure to the slugs. The following emulsion formulations (200ml) were evaluated:

i) 3% Myrrh oil in 0.2% aqueous Tween 80.

ii) 3% Opoponax oil in 0.2% aqueous Tween 80.

iii) 3% *trans*-β-Ocimene oil in 0.2% aqueous Tween 80.

iv) Control (water only)

7.2.7 *Controlled Temperature Unit Spray Trials Against H. aspersa Snails (7 Days)*

Plastic bowls (approximately 30 x 30 cm) were painted with Fluon to prevent snails from leaving the arena. The bowls were then layered with approximately 2cm depth of peat soil. Three young lettuces ('Charles', butterhead), at the 6 to 8 leaf stage, were planted per bowl and each plant sprayed with about 5 ml of spray formulation. In addition to this, the adjacent soil area to each lettuce plant was sprayed with 5 ml of

spray formulation. Eight *H. aspersa* snails were added per arena. The trials were performed in a controlled environment with temperatures maintained at 15°C: 12 hour day, 15°C: 12 hour night. The amount of leaf damage (consumed) was assessed daily and recorded over a three day period. Any incidental snail mortality was also noted. Each leaf was estimated visually for damage and the mean leaf damage calculated per plant. The amount of leaf damage was recorded daily for each lettuce plant. Experiments were performed in triplicate. On day two the lettuce plants were sprayed with water to prevent them from drying. The following 200ml emulsion formulations were evaluated:

i) 3% Myrrh essential oil in 3% aqueous Tween 20.

ii) 3% Opoponax essential oil in 3% aqueous Tween 20.

iii) 5% *trans*–β-Ocimene oil in 5% aqueous Tween 20.

iv) 3% Myrrh ethanol extract (Flavex) in water (containing 14% ethanol).

v) Control (water)

7.2.8 Controlled Temperature Unit Spray Trials Against D. reticulatum slugs (7 Days): May 2004

Method was the same as described for 7.2.7 with variation of adding five previously starved slugs (*D. reticulatum*), for 24 hour, per bowl. Three young lettuce seedlings of the 'Radja' curly variety were placed in each bowl.

The following 200ml spray formulations were evaluated:

i) 3% Myrrh essential in aqueous Tween 20 (3%).

ii) 3% Opoponax essential oil in aqueous Tween 20 (3%).

iii) 5% *trans*-β-Ocimene in aqueous Tween 20 (5%).

iv) 3% Myrrh aqueous ethanol extract, (containing 14.5% ethanol).

v) Control (water).

7.2.9 Controlled Temperature Unit Spray Trials Against D. reticulatum slugs (7 Days): December 2004

The aim of this experiment was to compare the efficacy of the different concentrations of aqueous ethanol extracts of myrrh and to confirm the inertness of aqueous Tween extracts. The method was conducted as described in section 7.2.8, with the variation of employing the 'Emerald' butterhead type of lettuce and spraying each plant and adjacent soil area with 10ml of spray formulation.

The following 200ml emulsion formulations were evaluated:

i) 3% Myrrh ethanol extract in water (contains 14% ethanol).

ii) 5% Myrrh ethanol extract in water (contains 24% ethanol).

iii) 3% Aqueous Tween 80.

iv) 3% Aqueous Tween 20 and

v) Control (Water).

7.2.10 Phytotoxic Effects of Plant Extracts on Lettuce Plants

Spray trials 1 to 4 (April - December2004)

During the spray trials, described in sections 7.2.6 to 7.2.9, the condition of the lettuce leaves were monitored for signs of phytotoxicity. % Phytotoxicity was measured by visual assessing the % discolouration (chlorosis) for each leaf and the mean value for all the leaves calculated. The assessments were recorded daily for the length of the spray trials. The spray trials assessed for phytotoxicity were:

ii) *Spray trial 1: Controlled Temperature Unit (May 2004).*

iv) *Spray trial 2: Controlled Temperature Unit (May 2004).*

v) *Spray trial 3: Controlled Temperature Unit (December 2004).*

7.3 Statistical Methods

Between-treatment effects were determined using the non-parametric Kruskal-Wallis (K-W) test to show the significance of differences between group medians. In the majority of cases, between-group variances were not homogenous (Bartlett's or Levene's test) and residuals were not normally distributed (Anderson-Darling test), hence conditions for non-parametric comparisons were fulfilled. The non-parametric Mann-Whitney test was used for pair wise comparisons of group medians. Where the criteria for parametric analysis were met, data were analysed using analysis of variance (ANOVA) to compare group means. The parametric Tukey-Kramer test, for maximum significance differences, was used for pairwise comparisons within treatments. In all cases the level of significance was established at P=0.05.

7.4 RESULTS

7.4.1 Caged Field Trials

In the first caged field trial, conducted in October 2001, both *Commiphora* spp. oleoresins and their extracts were significantly effective in reducing crop damage over the seven day test period (Kruskal-Wallis, P=0.014). The median percentage leaf damage observed when young lettuce seedlings were surrounded by barriers of myrrh (100%), opoponax (100%), myrrh oil extract (1%) and myrrh ethanol extract were 2%, 0%, 4% and 0% respectively, compared to 37.5% for the control (no barrier). Pairwise comparisons of the medians, using the nonparametric Mann-Whitney test, showed each test barrier to have significantly better protective properties than the control (P <0.05).

The repellency properties of the barriers continued until day fourteen. The median percentage leaf damage observed, after fourteen days, for barriers comprised of 100% myrrh oleoresin, 100%opoponax oleoresin, 1% myrrh essential oil extract and 20% myrrh ethanol extract were 9%, 13.5%,18 % and 13.5% respectively, compared to 47% the control (no barrier). Pairwise comparisons of the medians showed each test barrier to have significantly better protective properties than the control (Mann-Whitney, P <0.05), see figure 7.3

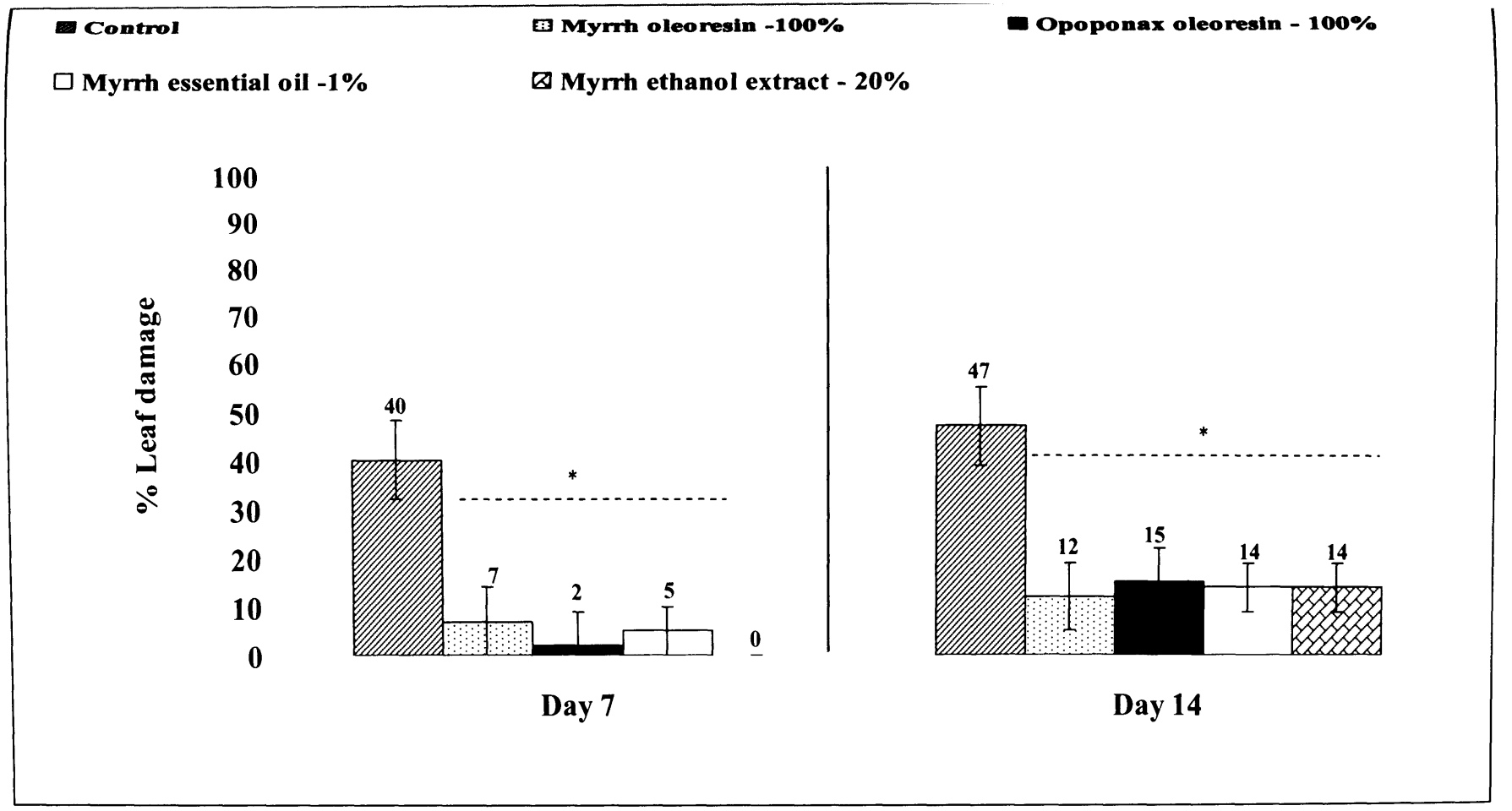


Figure 7.3 Caged Field Trials (October 2001): Evaluation Of *Commiphora* Spp. Oleoresins, And Their Extracts, As Repellent Barriers Against *D. reticulatum* Slugs. * = Significant Differences ($P<0.05$).

7.4.2 Caged Field Trials

In the second caged field trial, conducted in April 2002, the myrrh / sawdust repellent barrier was significantly effective in reducing crop damage over the seven day test period (Mann-Whitney, $P = 0.029$) resulting in 8% percentage leaf damage on comparing median percentage leaf damage to a median of 44.5% for the control (no barrier). Although the second myrrh barrier, mixed with corncob, was very effective in reducing crop damage the large variation in the data showed it to be not significant when analysed by the Mann-Whitney test ($P = 0.060$). The barriers comprised of the inert materials (sawdust and corncob) and the commercial barriers (Slug Stop ^{TM}) were not very effective in protecting the lettuce seedlings from being eaten by the slugs (Mann-Whitney, $P > 0.05$) over seven days.

Extending the trial over fourteen days showed both myrrh barriers to be effective in preventing the slugs from consuming the lettuce seedlings (Mann-Whitney, $P < 0.05$) resulting in 17% median leaf damage for the myrrh/sawdust barrier and 20% median leaf damage when myrrh was mixed with corncob, see figure 7.4.

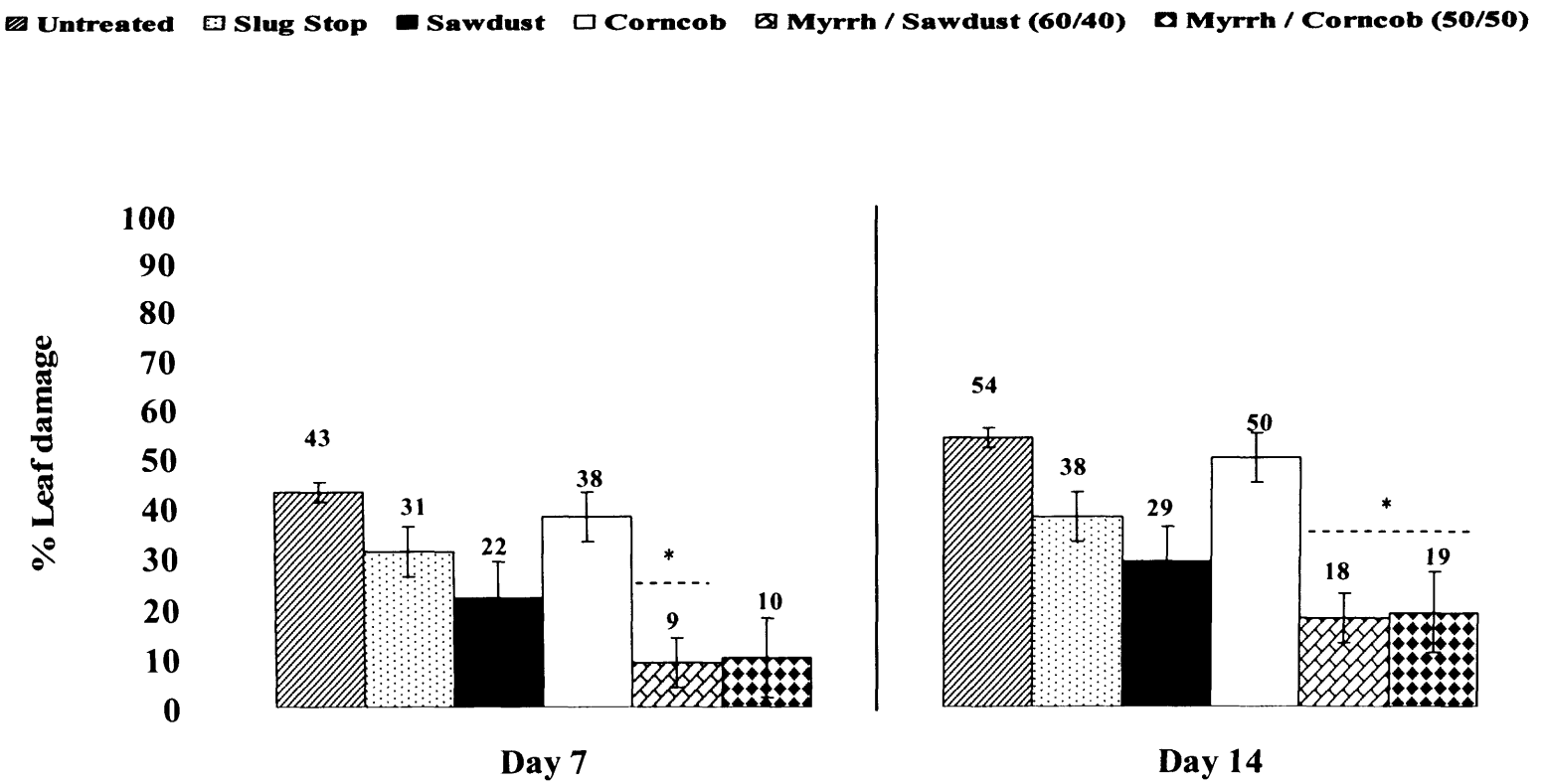


Figure 7.4 Caged Field Trials (April 2002): Evaluation Of *Commiphora* Spp. Oleoresins/ Inert substrate Mixes As Repellent Barriers against *D. reticulatum* slugs. * = Significant differences ($P < 0.05$).

7.4.3 Laboratory Spray Trials Against D. reticulatum Slugs

Kruskal-Wallis analysis of the median percentage leaf damage showed significant differences between group medians when the lettuce leaves were sprayed with the various plant extracts (P =0.010). No incidences of leaf damage were observed after spraying with myrrh and opoponax,, see table 7.1. Although visual observations indicated a strong antifeedant effect on slugs when the lettuces were sprayed with 3% *trans*-β-ocimene, statistically it was not considered significantly effective because of the large variation in the data (Mann-Whitney, P = 0.112).

No slug mortalities were observed for any of the treatments over the 24 hour test period.

Table 7.1 Evaluation of the Antifeedant Activity of Aqueous Emulsions of Myrrh, Opoponax and *trans*–β-Ocimene Oils, against the Field Slug *D. reticulatum.*

	Leaf damage (%)	
Treatment	Mean ± SEM	Median
3% Myrrh oil in 0.2% aqueous Tween 80	0 ± 0	0
3% Opoponax oil in 0.2% aqueous Tween 80	0 ± 0	0
3% Ocimene in 0.2% aqueous Tween 80	7 ± 4	5 NS
Control (Water)	31 ± 10	25
K-W test		P = 0.010

N = 4 lettuce leaves per treatment. NS = No significant difference (P >0.05).

7.4.4 *Controlled Temperature Unit Spray Trials against H. aspersa Snails, (7 days)*

As indicated in table 7.3, pair wise comparison of the medians, showed the 3% myrrh formulations (both essential oil and ethanolic) to significantly deter the snails from feeding on the lettuce plants (Mann-Whitney, P <0.001). On contact with the myrrh treatments the snails were observed to immediately retract into their shells. Spraying the 3% opoponax formulation onto the lettuces also had a significant effect in reducing the feeding behaviour of the snails over the seven day test period (Mann-Whitney, P = 0.033), although its antifeedant effect was highly variable i.e. three plants were untouched while the remaining nine plants suffered various degrees of damage. The spray formulations containing 5% *trans*-β-ocimene were highly effective mollusc repellents for only three days (Mann-Whitney, P <0.001) with only 9% median leaf damage. This repellent effect was found to be on the margins of significance on day four (Mann-Whitney, p=0.058) and not significant after seven days (Mann-Whitney, P = 0.374).

No snail mortality was observed over the seven day test period

Table. 7.3 **Spray Trials: Assessment Of Lettuce Leaf Damage Caused By *H. aspersa* Snails,**

After Spraying With Various Aqueous Emulsions

Treatment	% Leaf Damage							
	Mean ± SEM							Median
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 7**
3% Opoponax oil	0	0	1 ± 1	11 ± 11	22 ± 22	38 ± 23	56 ± 30	100*
3% Myrrh oil	0	0	0	0.5 ± 0.5	1.3 ± 1.3	1.3 ± 1.3	3.5 ± 2	3*
3% Ethanolic Myrrh	0	0	0	0	0.5 ± 0.5	0.5 ± 0.5	2.9 ± 2	0*
5% *trans*-β-Ocimene	0	5 ± 2	7 ± 5	38 ± 18	87 ± 7	94 ± 6	94 ± 6	100 NS
Control (Water)	16 ± 7	31 ± 7	68 ± 11	81 ± 6	100 ± 0	100 ± 0	100 ± 0	100 NS
K-W test								P <0.001

N = 12 lettuce plants per treatment. * = Significant difference ($P < 0.05$). NS = Not significant ($P > 0.05$)

7.4.5 Controlled Temperature Unit Spray Trials against Slugs (May 2004)

All spray formulations significantly deterred the slugs from feeding on the lettuce plants for the duration of the seven day testing period (Kruskal-Wallis, P <0.001), as indicated in table 7.4. Pairwise comparisons of the median % lettuce leaf damage with the control confirmed 3% opoponax esential oil and 3% ethanolic myrrh spray treatments to significantly reduce the feeding behaviour of the slugs compared to the control (Mann-Whitney, $P< 0.05$). Myrrh essential oil (3%) and 5% ocimene treatments also proved to be potent antifeedants, both treatments resulting in 0% leaf damage.

Highly significant incidences of slug mortality were observed over the seven day test period (Kruskall-Wallis, P <0.001). In particular the sprays containing myrrh essential oil and ocimene were highly molluscicidal in nature (Mann-Whitney, $P< 0.01$), as indicated in table 7.5. The opoponax spray formulations also gave significant mortalities, when applied as a spray (Mann-Whitney, P=0.02). The slugs secreted excess amounts of white mucous on contact with all the oil emulsion spray formulations, see figure 7.5. This effect was not observed with the ethanolic myrrh extracts.

Table. 7.4 Spray Trials: Assessment Of Lettuce Leaf Damage, Caused By *D .reticulatum* Slugs, After Spraying With Various Aqueous Emulsions

Treatment	% Leaf Damage							
	Mean ± SEM							Median
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 7**
3% Opoponax Essential Oil [a]	0	0	1±1	1±1	3±2	5±3	7±4	0*
3% Myrrh Essential Oil [b]	0	0	0	0	0	0	0	0
3% Ethanolic Myrrh [a]	2±1	3±1	10±7	10±7	16±11	17±12	19±14	10*
5% Ocimene Oil [b]	0	0	0	0	0	0	0	0
Control (Water)	40± 4	50±9	67±8	69±10	69±10	73±12	79±13	100
K-W test								P <0.001

N = 12 lettuce plants per treatment. [a] Mann-Whitney test used (P=0.05). * = Significant difference (P< 0.05).

Table. 7.5 Spray Trials: Assessment Of Slug Mortality After Spraying With Various Aqueous Emulsion

Treatment	% Mortality							
	Mean ± SEM							Median
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 7
3%Opoponax Oil	0±0	40±20	40±20	40±20	40±20	40±20	47±18	40*
3%Myrrh Oil	53±24	53±24	67±18	67±18	80±12	80±12	87±7	100*
3% Ethanolic Myrrh	7±7	13±7	13±7	13±7	27±13	27±13	27±13	0
5% Ocimene Oil	67±	80±12	87±7	87±7	93±7	93±7	93±7	100*
Control (water)	0± 0	0±0	0±0	0±0	7±7	7±7	7±7	0
K-W test								$P<0.01$

N = 20 slugs per treatment. * = Significant difference ($P < 0.05$).

Figure 7.5 Spray Trials: Lettuce plants and soil treated with 3% *trans*-β-ocimene in aqueous Tween 80 (3%). This formulation was a potent contact molluscicide. Notice the bright white mucous secretions produced on contact with the formulation, indicating its strong irritation properties. This effect was also seen for spray formulations containing 3% myrrh and 3% opoponax essential oils solubilised in 3% aqueous Tween 20.

7.4.6 *Controlled Temperature Unit Spray Trials against D. reticulatum Slugs, Over 7 Days (December 2004)*

Both aqueous ethanol myrrh spray formulations (3% and 5%) were found to significantly deter the *D. reticulatum* slugs from feeding on the lettuce plants (Mann-Whitney, $P < 0.05$), with median leaf damages of 1 and 2% respectively. The aqueous Tween 20 and Tween 80 spray formulations (3%), however, gave quite contrasting results. Aqueous Tween 80 had no significant antifeedant effect against the slugs over the seven day test period (Mann-Whitney, P=0.185), whereas aqueous Tween 20 surprisingly did have a significant on the feeding behaviour of the slugs (Mann-Whitney, P =0.021), see table 7.6.

Over the seven day test period both ethanolic myrrh treatments (3% and 5%), resulted in high incidences of slug mortality, when applied as sprays. Because of the large variability of the data obtained for the 5% myrrh extracts, i.e. 3 terraria trays gave 80-100% mortalities and one gave 0% mortality, the Mann-Whitney test was not able to detect significant differences compared to the control (P=0.180). This has to be interpreted cautiously as one can see clearly from table 7.7 that sprays containing 5% myrrh had relatively high incidences of slug mortality. Sprays containing 3% myrrh extracts gave less variable results, showing significant incidences of slug mortality (Mann-Whitney, P=0.020). Both aqueous Tween extracts (20 and 80) were not molluscicidal in nature compared to the control (Mann-Whitney, P = 1.000).

Table. 7.6 Monitoring Amount Of Lettuce Leaf Damage Caused By *D. reticulatum* Slugs, After Spraying With Aqueous Emulsions Of Myrrh Oleoresin and Nonionic Surfactants

Treatment	% Leaf Damage							
	Mean ± SEM							Median
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 7**
3% Aq.Tween 80	24 ± 1	47 ± 9	61 ± 5	77 ± 4	80 ± 4	80 ± 4	97 ± 2	100 NS
3% Aq.Tween 20	13 ± 7	27 ± 5	35 ± 4	45 ± 6	48 ± 8	48 ± 8	48 ± 8	44 *
3% Ethanolic Myrrh	0 ± 0	0 ± 0	3 ± 2	3 ± 2	3 ± 2	3 ± 2	3 ± 2	0 *
5% Ethanolic Myrrh	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 2	0 *
Control (water)	44 ± 8	71 ± 5	91 ± 5	95 ± 3	100 ± 0	100 ± 0	100 ± 0	100
K-W test								$P< 0.01$

N = 12 lettuce plants per treatment. * = Significant difference ($p< 0.05$). NS = Not significant ($p > 0.05$)

Table. 7.7 Assessment Of Slug Mortality After Spraying With Aqueous Emulsions Of

Myrrh Oleoresin and Nonionic Surfactants

Treatment	% Mortality							
	Mean ± SEM							Median
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 7
Control (water)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	10 ± 10	20 ± 10	20 ± 10	10 NS
3% Aq.Tween 80	0 ± 0	0 ± 0	5 ± 5	5 ± 5	5 ± 5	15 ± 10	20 ± 11	20 NS
3% Aq.Tween 20	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	5 ± 5	20 ± 14	10 NS
3% Ethanolic Myrrh	10 ± 6	10 ± 6	60 ± 14	80 ± 0	80 ± 0	80 ± 0	80 ± 0	80*
5% Ethanolic Myrrh	5 ± 5	30 ± 17	35 ± 15	60 ± 20	65 ± 22	65 ± 22	65 ± 22	80 NS
K-W test								$P = 0.039$

N = 20 slugs per treatment. * = Significant difference ($p < 0.05$). NS = Not significant ($p > 0.05$)

7.4.7 Phytotoxic Effects of Plant Extracts on Lettuce Plants

Spray trial 1: Controlled Temperature Trials (May 2004)

No incidences of phytotoxicity were observed for the lettuce leaves that had been sprayed with the various plant extracts. The young lettuce seedlings sprayed with the aqueous Tween 20 extracts of myrrh essential oil (3%) spray showed slight brown staining which was confined to one or two midriff veins of the lettuce leaves per plant.

Spray trial 2: Controlled Temperature Trials (May 2004)

Only the lettuce leaves that had been sprayed with aqueous tween 20 extracts of *trans*-β-ocimene showed signs of phytotoxicity, see appendix. The middle of the lettuce leaves showed large spots of yellow-brown pigmentation with occurrences of yellow brown discolouration on the leaf edges. Overall these incidences of phytotoxicity accounted for approximately 20% of the whole plant. A few pin hole sized dark grey spots was also observed with the aqueous Tween 80 extracts of myrrh essential oil which accounted for less than 1% of the whole plant. No signs of phytotoxicity were observed for the 3% aqueous Tween 80 extracts of opoponax and the 3% ethanolic extracts of myrrh.

Spray trial 3: Controlled Temperature Unit (December 2004)

As shown in table 7.8, significant incidences of phytotoxicity, over seven days, was only observed for the 5% ethanolic extract of myrrh (Mann-Whitney, $p = 0.037$). Negligible phytotoxic effects were observed for the 3% ethanolic myrrh extracts and the 3% aqueous Tween 80 extracts of myrrh oil. Some tiny grey pinhole spots were, however, observed for the latter spray extracts (myrrh oil) with only one or two tiny light grey spot being observed per leaf for three terraria trays, whilst no grey pin sized spots were observed for the fourth terraria. No systemic phytotoxic effects were observed on applying the 3% ethanolic myrrh extracts to the soil. Although no signs of phytotoxicity were observed for both of the aqueous Tween spray formulations containing 3% Tween 20, slight changes in the texture of the lettuce leaves were observed. The waxy cuticle of the lettuce leaves sprayed with 3% aqueous Tween 20, were not as smooth as those sprayed with water (control). This effect was not observed with spray formulations containing 3% aqueous Tween 80.

	Median % Phytotoxicity						
	Control (Water)	3% Myrrh Aqueous Ethanol (Leaves)	3% Myrrh Aqueous Ethanol (Soil)	5% Myrrh Aqueous Ethanol (Leaves)	3% Myrrh oil in Aqueous Tween 80 (Leaves)	3% Aqueous Tween 20	3% Aqueous Tween 80
	0 (1)	0 (2)	0 (0)	18 (22)	0 (0)	0 (0)	0 (0)
Mann-Whitney test		$P = 1.000$ N.S	$P = 1.000$ N.S	$P = 0.037$ *	$P = 1.000$ N.S	$P = 1.000$ N.S	$P = 1.000$ N.S

N= 9 lettuce seedlings per treatment. Figures in brackets are mean % phytotoxicity.

* = Significant differences ($p < 0.05$). N.S = Not significant ($p > 0.05$).

7.5 DISCUSSION

Both caged-field trials conducted in October 2001 and April 2002 yielded encouraging results. In the first caged-field trial all the *Commiphora* spp. plant materials, and their extracts, were significantly effective in reducing lettuce leaf damage over the seven day test period. The protective properties of the barriers continued until day fourteen. The second cage-field trial showed that diluting the myrrh raw material with sawdust or corncob inert materials had moderately significant crop protection properties, over the fourteen day test period. The myrrh raw materials mixed with inert diluents were much superior in reducing crop damage than the commercial physical barrier (Slug Stop ^{TM}).

Laboratory spray trials, conducted with *D. reticulatum* slugs over 24 hours, showed no lettuce leaf damage for sprays containing 3% myrrh and opoponax oils and very little leaf damage (median 5%) for the spray containing 3% *trans*-β-ocimene compared to the untreated lettuce leaves (median 25%). Due to the large variability in the data, however, the non-parametric Mann-Whitney test identified the formulations containing *trans*-β-ocimene as not having significant antifeedant effects. The large variability in data could be attributed to the non-standardization of the nozzles fitted onto the commercial garden spray bottles resulting in some sprays having better delivery and leaf coverage than others.

Controlled temperature unit spray trails, conducted with *H. aspersa* snails, resulted in little leaf damage occurring when the lettuces were sprayed with myrrh extracts. Both the 3% myrrh oil and the 3% myrrh aqueous ethanol extracts deterred the

snails from eating the lettuce plants for seven days, whereas opoponax oil was effective in deterring the snails for up to five days. High variations in the antifeedant behaviour of the snails were observed when lettuce leaves were sprayed with aqueous emulsions of 3% opoponax essential oil. This could be attributed to the basic spraying techniques employed. The use of electrostatic or air assisted spray apparatus would be a more efficient method of application when spraying crops than the hand held spray bottles used. The snail repellency properties of 5% trans-β-ocimene were not very long lasting and wore off after a short period of three days. *Trans*- β-ocimene being a relatively low molecular weight monoterpene is quite volatile in nature, hence formulations based on it would be highly affected by surrounding environmental conditions. Frank *et al.* (2002) found this to be the case with trials with lettuce treated with carvone, a natural compound isolated from caraway seeds. This compound significantly reduced the feeding behaviour of *Arion lusitanicus* in laboratory based no-choice experiments. In field trials, however, lettuces treated with carvone were not effective deterrents against slugs when sprayed at an application rate of 0.75ml litre^{-1}. They attributed this to carvone's high volatility and recommended it to be incorporated into mulches at higher concentrations. In this study, no snail mortalities were observed in any of the spray trials conducted with the *H. aspersa* snails.

Controlled temperature unit spray trails, conducted with *D. reticulatum* slugs, were found to be encouraging for all of the oils tested, resulting in little leaf damage, over the 7 day test period. Both 3% myrrh and 5% ocimene prevented leaf damage over this test period whilst plants treated with 3% opoponax and 3% ethanolic myrrh suffered low occurrences of leaf damage.

In contrast to the spray trials conducted with *H. aspersa* snails, high mortalities were observed for the spray trials with *D. reticulatum* slugs. Myrrh oil and ocimene oil sprays, in particular, gave high slug mortalities as early as day one. Low slug mortality rates were observed for the 3% ethanolic myrrh extracts when low application rates (5ml) were employed. This observation is in agreement with the controlled temperature spray trials where no snail mortalities were observed when lettuce leaves and adjacent soil were treated with the 3% ethanolic extracts of myrrh, using similar spray application rates.

Controlled temperature unit spray trails, found 3% and 5% ethanolic extracts of myrrh oleoresin to be potent antifeedant/ repellents against the field slug. Low incidences of leaf damages occurred over seven days. High molluscicidal activities were observed for these extracts, after employing higher application rates (10ml), yielding mean slug mortalities of 65% and 80% respectively.

In summarising the performances of all the aqueous spray formulations, it appears that the 3% myrrh aqueous emulsion formulations (essential oil and ethanolic) provided the best protection for the lettuce plants with both formulations being effective in reducing the feeding behaviour of *H. aspersa* and *D. reticulatum*.

In general the spray formulations behaved as both antifeedants and contact molluscicides depending on which type of terrestrial mollusc came into contact with the treated areas. An explanation for the different in tolerance levels between the two terrestrial molluscs has been proposed by Davis *et al.* (1996). They suggested that the different behaviour of the slugs and snail upon contact with chemicals could be due to

the smaller size and the greater amount of exposed body surface area of the slug. They confirmed this by showing that juvenile snails and slugs were more susceptible to copper sprays than adult snails. Morphological differences in the epithelial cells of the foot, could also explain why the sole foot of *H. aspersa* snails might be able to absorb much higher levels of chemical contaminants than smaller molluscs like, *D. reticulatum.* Bradley *et al.* (1996) confirmed the mechanism of cell death to be necrosis when *H. aspersa* snails come into contact with high levels of heavy metal contaminants. They showed the occurrence of sloughing of the foot epithelial cells on contact with solutions containing copper creating open lesions, which on contact with bacteria results in the production of granulomas and extensive tissue necrosis.

Opoponax and ocimene oil emulsion sprays at concentrations of 3% and 5% were potent antifeedants and molluscicides against field slugs but not as effective against snails. Their repellency/antifeedant properties against snails lasted for approximately 5 and 3 days respectively. The advantage of using opoponax and ocimene as mollusc repellents/antifeedants is apparent. However, their application could be improved by implementing new formulation technologies, such as the incorporation of slow release synthetic polymer resins. The use of adjuvants, such as silicone oil, could also be a useful way of prolonging their repellent/antifeedant activities. These adjuvants would increase both the retention and the spray deposition onto foliages. This has been demonstrated by many investigators (Holloway *et al.*, 2000; Gaskin *et al.*, 2000; Hollingsworth et al., 2003). In the case of trans-β-ocimene it's high volatility may be reduced by adding a non volatile middle chain length alcohol, like undecenol to the formulation. On contact with the spray emulsions the slugs were observed to sway vigorously from side to side, simultaneously secreting copious

amounts of white mucous. Once the mucous secretion was exhausted the slug dehydrated, resulting in a severe reduction of body mass. This is a similar mechanism of death as that induced by the commonly used molluscicide, metaldehyde. This is a potent contact molluscicide, which causes *D. reticulatum* slugs to secrete excessive amounts of mucous on contact.

Spray trials are often performed using lettuce plants as they are one of the most sensitive crops to incidences of phytotoxicity. In this study no signs of phytotoxicity were observed for any of the three species of lettuce seedlings sprayed with the emulsion formulations containing 3% opoponax essential oil and 3% ethanolic myrrh. Some occurrences of brown staining as well small pinhole grey spots were observed on some lettuce leaves, when sprayed with 3% myrrh essential oil emulsion. Sprays containing 5% ocimene, however, were found to be strongly phytotoxic to one of three species of the lettuce plants ('Curly' variety). The curly variety of lettuce retained the spray emulsions more than the other varieties of lettuce. The effect of the high retention of the spray emulsion was the same as one of over application, resulting in some phytotoxicity at localised sites.

It is interesting that no signs of phytotoxicity was observed with the 3% ethanolic extract of myrrh oleoresin applied as an emulsion spray formulation whilst 3% myrrh essential oil showed some phytotoxic effects (slight brown staining and dark pin hole type spots). One explanation for this is the different chemistries of the two extracts. Preliminary GC-MS studies on these two extracts showed the major compounds for the essential oil to elute between 21 and 34 minutes whilst the GC-MS profile for the ethanolic extract of myrrh oleoresin mirrored that of the essential oil with

the presence of late eluting compounds. The major compounds for the ethanolic extract eluted between 21 and 46 minutes. The late eluting compounds observed for the ethanolic myrrh extracts may be responsible for reducing the phytotoxic effects of ethanolic extracts myrrh oleoresin.

Grubisic *et al.* (2003) performed similar spray trials, with beans and lettuce plants using spray formulations containing 0.5% lavender essential oil and an aqueous extract of dry rosemary leaves. They reported the aqueous extract of lavender essential oil to be too phytotoxic to lettuce and beans crops but not against Swiss chard. No phytotoxicity effects were reported for the aqueous rosemary leaf extracts. This is in good agreement with our observations of lettuce leaves sprayed with myrrh essential oil and the ethanolic extract of myrrh oleoresins.

Sprays based upon 5% *trans*-β-ocimene were found to be marginally phytotoxic only against the 'Radja' curly variety of lettuce seedlings. This disparity in plant sensitivities to spray formulations, based on natural products, were also observed by Hollingsworth *et al.* (2002). They found 2% caffeine to be phytotoxic to ferns, bromeliads and lettuce causing the yellowing of foliage but not phytotoxic to the foliage of Dracaena, Anthurium, palms or orchids. They found the addition of a corn flour / sugar adjuvant (4%) reduced phytotoxicity against cabbage leaves (*Brassica rapa* var. *pekinensis*) as well as increasing the efficacy of the caffeine sprays (Hollingsworth *et al.*, 2003) by increasing the uniform deposition of caffeine on the leaf surface. This could be a way forward for reducing phytotoxicity for ocimene based sprays, as build up of extracts on lettuce leaves was observed at local sites, suggesting not only uneven distribution of extracts but over-application on certain leaf areas.

CHAPTER 8:

General Discussion

8.1 General Discussion

Slugs and snails are major pests in the UK costing millions of pounds a year to both horticulturists and farmers alike (Schüder and Port, 2003). Chemical methods for mollusc control are, currently, becoming less popular in Europe and are restricted in organic farming; hence alternative methods of mollusc control are very much needed.

As an alternative to toxic chemical methods of mollusc control the odiferous myrrh and opoponax oleoresins, collected from the trees *Commiphora molmol* and *Commiphora guidotti,* were evaluated as mollusc repellent barriers against terrestrial slugs, *D. reticulatum* and *Arion hortensis,* as described in Chapter 2. Terraria studies with *D. reticulatum* slugs showed very little amount of winter wheat seed damage, when encountering repellent barriers comprised of myrrh and opoponax oleoresins, oleoresin mixtures with inert substrates and sawdust treated with ethanolic and essential oil extracts of myrrh and opoponax. Incidences of slug mortality with 100% oleoresins were high when blotting paper was used to line the tray arenas, but was much reduced when the oleoresins were either mixed with sawdust and corncob or if the extracts were coated onto sawdust. Evaluating repellent barriers comprised only of 100% myrrh granules, using a peat soil substrate, showed no incidences of slug mortality whilst maintaining its strong slug repellent properties.

The use of *Commiphora* spp. oleoresins as repellent barriers is not only a novel application, of these highly valued plant materials, but has also been shown to be a highly effective means of deterring molluscs from feeding on plant materials.

This new repellent barrier method of mollusc control compares favourably with reported mollusc control methods, including those comprised of copper, fibre glass, garlic extracts, yucca plant extracts and short chain fatty acids, (Hata *et al.*, 1997; Schüder et al., 2003, Mason *et al.*, 1994; Puritich *et al.*, 1993).

The barrier properties of sawdust treated with 0.5% *trans*-β-ocimene was found to be dependent upon the medium used to solubilise the chemical. This monoterpene seemed to be much more polar than expected, showing more repellent activity when solubilised in polar media (aqueous DMSO) than in aqueous Tween 80 which is a non-ionic surfactant. These terraria studies also demonstrated the poor repellent barrier properties of commercial mollusc repellent barriers (Slug Stop®) comprised of amorphous silica flakes (diatomite).

The chemistry of myrrh and opoponax extracts has been studied extensively (Dekebo *et al.*, 2002; Baser *et al.*, 2003; Jingai and Shangmei, 1996; Moyler and Clery, 1997; Craveiro *et al.*, 1983). This thesis (Chapter 3) authenticated the identity of the myrrh and opoponax oleoresins used in the terraria trials, using GC-MS analytical methods. The chemical profile of the liquid extracts of commercial myrrh (Baldwin) and opoponax agreed well with published literature values (Moyler and Clery, 1997). However the liquid extracts of Somaliland myrrh showed uniquely high levels of the sesquiterpene β-elemene. To my knowledge, this chemical profile, has never been reported before, which may suggest that most of the commercial myrrh used in Europe is either collected from Yemen (*C. myrrha*) or perhaps from Ogo malmal (*C. molmol*) from myrrh trees in eastern Ethiopia (Ogaden region). The high levels of β-elemene observed in Somaliland myrrh may be specific to myrrh samples

from the Guban region of Somaliland, which are known to have high oil contents. Both myrrh trees (*C. myrrha* and *C. molmol*), however, contained the same bouquet of chemicals (mainly furano-sesquiterpenes) and differed only in the relative amounts present in the oleoresins.

The SPME analysis of myrrh agreed well with that reported by Hamm *et al.* (2004) and comprised mainly of furano-sesquiterpenes. This is the first SPME report of the volatile compounds associated with opoponax oleoresins and its extracts. In contrast to the liquid extracts of opoponax, the major chemical component detected for the spme anlysis of the solid opoponax oleoresin was found to be *trans*-β-ocimene. This would suggest that on wounding the stem bark of *C. guidotti* the monoterpene *trans*–β-ocimene would be the first volatile chemical emitted by the tree, followed somewhat later by the sesquiterpenes. This behaviour was also observed by Steel *et al.* (1998) in ground fir trees. They reported that the synthesis of monoterpenes was fast reaching maximum levels after 2 to 4 days, whereas the synthesis of the sesquiterpene α-bisabolene was slow, reaching maximum levels after 12 days after wounding.

The *Commiphora* spp. oleoresins contain a complex mixture of terpenes, comprising monoterpenes, sesquiterpenes and furanosesquiterpenes. All terpenes are derived from isopentyl diphosphate, IPP, (Trapp and Croteau, 2001). The location of this compound in the plant cell dictates which biochemical pathway is undertaken. The presence of IPP in the plastids of higher plants results in the synthesis of monoterpenes (C_{10}), diterpenes, (C_{20}) and tetraterpenes (C_{40}), via the

pyruvate-glceraldehyde-3-phosphate pathway (Bruick and Mayfield, 1999), as illustrated in figure 8.1.

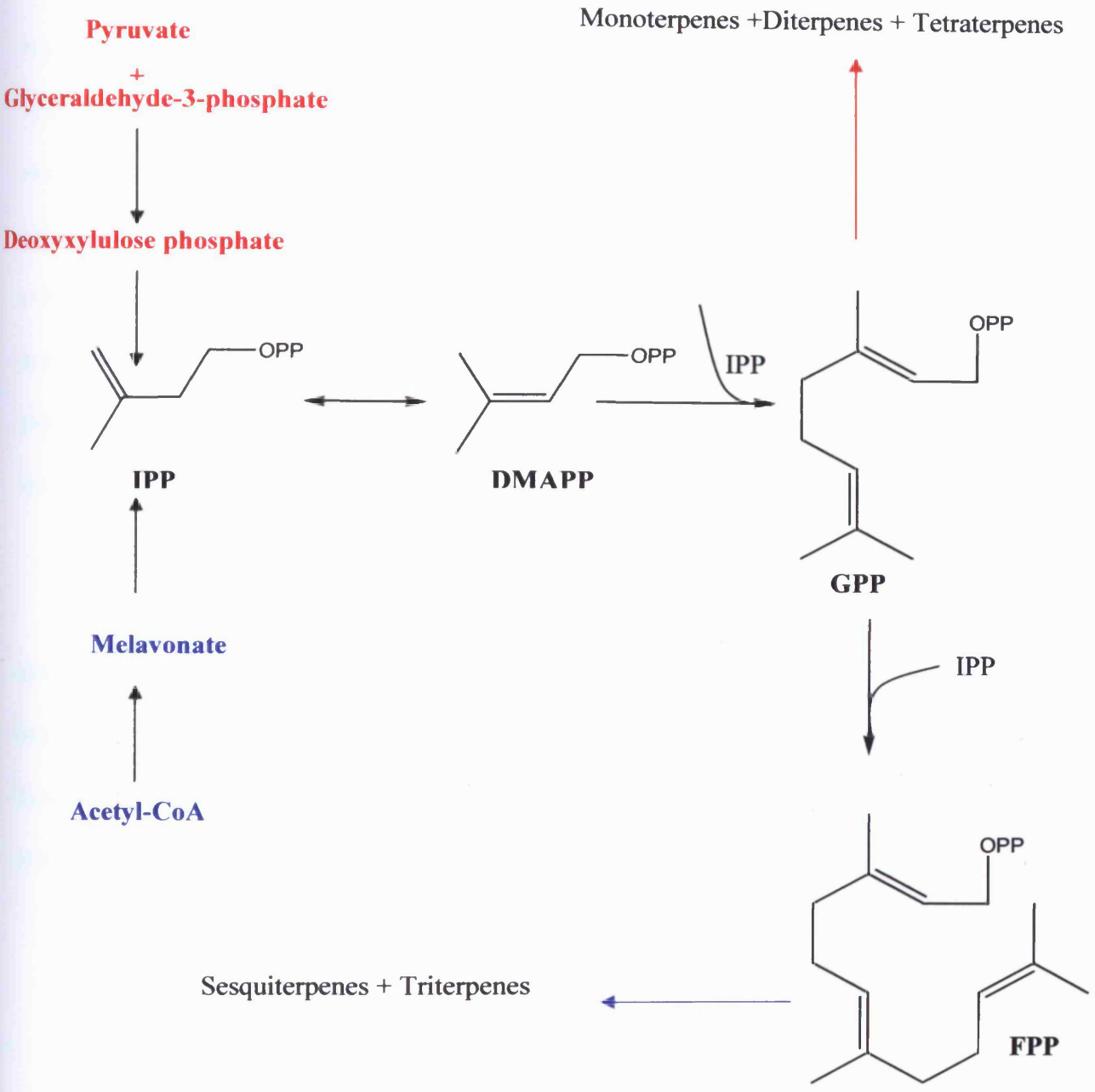


Figure 8.1 Terpene biosynthesis of monoterpenes and sesquiterpenes in higher plants, showing two pathways for the production of isopentenyl diphosphate (**IPP**) and dimethylallyl diphosphate (**DMAPP**). The **blue** pathway indicates the acetate-mevalonate pathway which synthesises geranyl diphosphate (GPP), the precursor of monoterpenes. The **red** pathway, involving pyruvate-glyceraldehyde-3-phosphate, produces farnesyl diphosphate (**FPP**) the precursor of sesquiterpenes. The cytosolic pool of isopentenyl diphosphate (IPP), derived from mevalonic acid, is the precursor of farnesyl diphoshate (FPP) and ultimately the sesquiterpenes and triterpenes (Trapp and Croteau, 2001).

The relationship between the terpenoid chemicals present in oleoresins and the surrounding ecology i.e. nearby fauna, such as insects, is a complicated one and has recently been highlighted by Assad *et al.* (1997). He showed that the essential oil extracted from the twigs and leaves of *Commiphora quadricincta* had a maturation effect on the desert locust (*Schistocerca gregaria*). Carlisle *et al.* (1965) found a similar effect using monoterpenes found in the essential oil of myrrh (*C. myrrha*), namely eugenol, α and β-pinene and limonene. Both Assad (1997) and Carlisle (1965) acknowledge the symbiotic role of *Commiphora* spp., volatile terpenoid emissions, with the sexual maturation of locusts in a desert environment. Oleoresin producing trees have evolved elaborate terpene based defence mechanisms to deter insect pests and their symbiotic fungal pathogens (Phillips and Croteau, 1999).

Phillips and Croteau (1999) showed that the enzyme (E)-α-bisabolene synthase, to be responsible for the synthesis of α-bisabolene on wounding grand fir conifer trees (*Abies grandis*). They considered this sesquiterpene, derived via the farnesyl diphosphate pathway, to be the precursor of the insect juvenile hormone mimics, todomatuic acid and juvabione see figure 8.2.

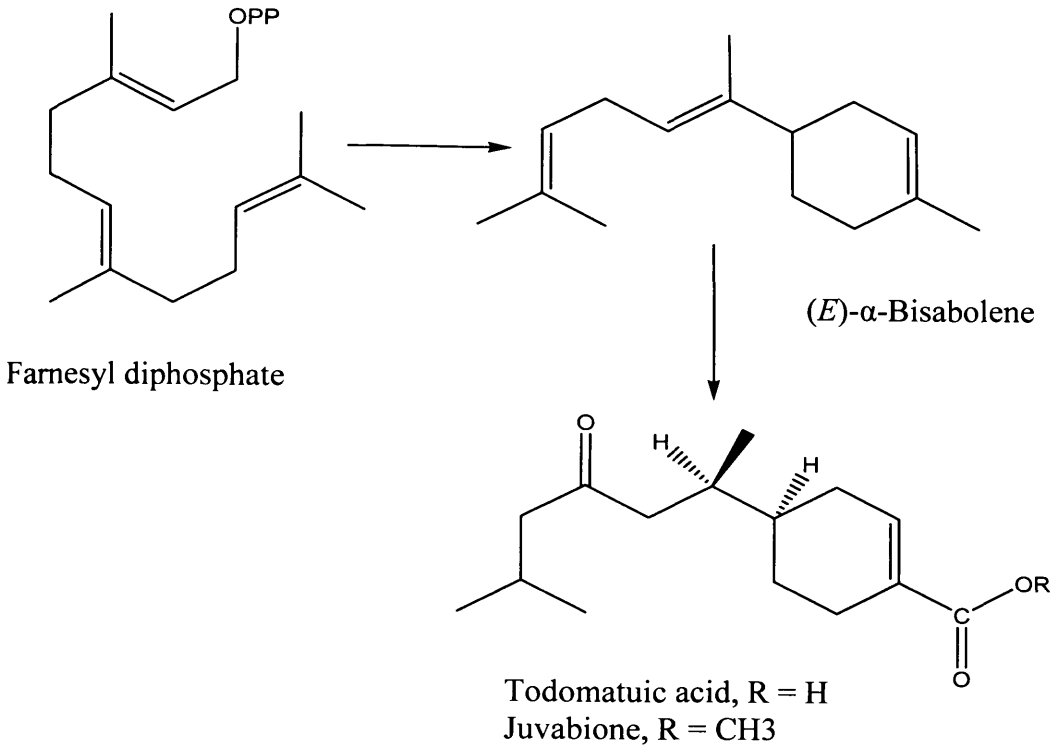


Figure 8.2 (*E*)-α-Bisabolene is synthesized from farnesyl diphosphate, by the enzyme (E)-a-bisabolene synthase, and is further transformed to the products which stimulate the action of juvenile hormone III, an insect molting hormone. Reproduced from Phillips and Croteau (1999).

Bowers (1976) also argued that the sesquiterpenes, produced by the grand fir oleoresins, functioned as hormones, interfering with the insect reproduction and development. Juvabione has been shown to be responsible for the failure of certain insects to molt into adults (Slama and Williams, 1966). Accumulation of todomatuic acid, the precursor of juvabione, in grand fir after being wounded by insect feeding, suggests that biosynthesis of the juvenile hormone analogue is induced as a response to insect attack.

The role of sesquiterpenes, produced by trees, could be to form a secondary line of defence against insect herbivores and fungal pathogens, which is distinct from the primary line of defence provided by monoterpenes (Bohlmann *et al.* ,1998). According to Assad (1997) the faster production of the toxic monoterpenes by the wounded trees might have antifeedant and antifungal functions, whereas the slower production of the sesquiterpenes (such as α-santalene and α-bisabolene) might play a role in reducing development and sexual reproduction of the pests, especially if there is a large infestation.

The production of oleoresins by *Commiphora* spp. trees may be a line of defence from the wide diversity of pests co-habiting its semi-arid environment, such as insect, bacterial and fungal pathogens, as well as stem borer beetles. Assad (1996) suggested that the sexual maturation of the adult desert locust coincided with the bud burst of certain desert shrubs (*B. neglecta* and *C. myrrha*) at the beginning of the rainy season.

The monoterpene *trans*-β-ocimene is one of the most widespread volatile compounds emitted by plants (Parre and Tumilson, 1997). According to Birckett *et al.* (2000) *trans*-β-ocimene can affect the plant defence system by stimulating the activity of parasitoid and predatory insects. For example the black-currant plant, *Ribes nigrum*, was very attractive to aphids as well as lady-birds (an aphid predator) when *trans*-β-ocimene production was stimulated by *cis*-jasmone. Many predatory insects are attracted to the volatile monoterpene *trans*-β-ocimene, such as mites *Amblyseius potentillae*, *Phytoseiulus persimilis* (Krips *et al.*, 2001). Plants such as *Gerbera jamesonii* produce this volatile monoterpene, when the leaves of ornamental

crops are damaged by the feeding behaviour of insects such as the spider mite, signalling their presence to enemy predator mites.

Chapter 4 described the first reporting of the defensive nature of the monoterpene, *trans*-β-ocimene. It was demonstrated to be a potent mollusc antifeedant and molluscicide towards the field slug *D. reticulatum* at concentrations as low as 0.5%, when coated onto lettuce leaf discs. As mentioned earlier (chapter 2) the repellency properties of *trans*-β-ocimene was dependent on the medium that it was solubilised in. Greatest molluscicidal activity was found when *trans*-β-ocimene was solubiled in polar media, water alone or aqueous DMSO. Solubilising *trans*-β-ocimene in aqueous Tween 80 or ethanol reduced the molluscicidal nature of this monoterpene to negligible levels, whilst maintaining its antifeedant nature. The antifeedant activity of myrrh and opoponax essential oil was also demonstrated towards *D. reticulatum* slugs using the leaf disc assay.

Although low oil concentrations (0.5 and 1%) were adequate to control the feeding behaviour of *D. reticulatum* slugs (Chapter 2), the spray emulsions developed in chapter 6, were formulated at higher oil levels (3% and 5%) for repelling the larger terrestrial mollusc *H. aspersa*. Chapter 5, highlighted how these snails can tolerate higher levels of chemical contaminants than the smaller terrestrial slugs, *D. reticulatum*. This is in good agreement with observations made by Davis *et al.* (1996). They found, during spray trials that copper spray formulations were highly repellent to the terrestrial molluscs, *D. reticulatum* and *H.aspersa*, but only induced significant mortality with *D. reticulatum*.

Most oil in water pesticide formulations are prepared on the day of use, hence avoiding problems associated with the poor emulsion stability of oil in water emulsions. In chapter 6 the use of non-ionic surfactants to stabilise aqueous oil emulsion formulations was investigated. The non-ionic surfactants, Tween 20, Tween 80 and Synperonic 91/8, were incorporated at the same level as the oils (3 – 5%) resulting in the formation of emulsions with long term stability (1week to one year). In general Synperonic 91/8 based emulsions were stable only for 2 to 3 weeks, whereas as Tween 20 and Tween 80 stabilised emulsions for 43 weeks or longer. The two non-ionic surfactants, Tween 20 and Tween 80, have higher HLB numbers (15.0 and 16.7) than Synperonic 91/8 (14.1) indicating their higher affinity for water. The higher surfactant HLB numbers enable the *Commiphora* spp oils, and their components, to be emulsified in water for longer periods.

There is a great demand for new molluscicides, especially those which are effective when applied as sprays (Davis *et al.*, 1996). Apart from the chemical molluscicides (metaldehyde and methiocarb) the only other chemical frequently used to control mollusc are those based upon copper. Copper salts are known to possess strong molluscicidal properties (Godan, 1983) and copper sulphate in particular has been extensively used to control pest molluscs. Ryder and Bowen (1977) reported copper sulphate to be a contact molluscicide, being absorbed through the foot epithelium of the slug *Agriolimax reticulatus* (now known as *D. reticulatum*).

Spray trials based on emulsion myrrh and opoponax essential oil and their chemical component *trans*-β-ocimene, were found to be potent antifeedants as well as

strong contact molluscicides, towards the smaller *D. reticulatum* slugs (Chapter 7). Little molluscicidal activity was observed on spraying the lettuce plants with ethanolic myrrh emulsions whilst still maintaining strong mollusc repellency. Replacing the slugs with the larger land molluscs (*H. aspersa*) demonstrated the myrrh based emulsions (essential oil and aqueous ethanol) to be the most repellent spray formulations. Opoponax essential oil showed some protection against the snails however the repellency properties of the chemical *trans*-β-ocimene was short lasting. Little indications of phytotoxicity was observed throughout the spray trials.

In summary the myrrh based emulsion formulations (essential oil and aqueous ethanol) were the most effective mollusc antifeedants during the spray trials conducted in control temperature units. Applying these emulsion formulations, using modern spraying techniques such as electrostatic spraying, would provide a viable alternative to toxic chemical based spray formulations.

The mode of action of the *Commiphora* spp. oleoresins, when applied as physical barriers, can be said to be one of repellency and possibly contact dehydration. Video recordings of myrrh and opoponax barriers, surrounding lettuce plants, being approached by *H. aspersa* snails in semi-field conditions (data not published) showed the snails to initially move towards the lettuce plants. When they were in close proximity to the barriers they turned back and went in the opposite direction. A repellent is defined as eliciting an effect from a distance; hence these barriers in the field can be defined as such. In smaller test conditions such as terraria trials pre-starved molluscs will eventually come into contact with the barriers. Once in contact with the barriers the smaller molluscs, *D. reticulatum*, become stuck to the

sticky oleoresins and release copious amounts of mucous, resulting in dehydration and eventually death.

The mode of action of the *Commiphora* spp. extracts towards the slugs was observed to be mostly antifeedant in laboratory terraria experiments, as little lettuce leaf disc were consumed. However visual observations indicated that the slugs some times took up to 5 hours before feeding, which also indicates an initial repellent effect. A similar antifeedant / repellent effect was observed for *H. aspersa* snails.

In addition to *trans*-β-ocimene a number of terpenoids compounds were shown to have significant antifeedant properties towards *D. reticulatum* slugs at the 1% concentration level including *cis*-β-ocimene, γ-bisabolene, germacrene D, *cis* and *trans* farnesol and *trans, trans*-α-farnesene (*E,E*) when solubilised in ethanol. *Trans*-β-ocimene, however, is the only terpene whose molluscicidal activity was enhanced when solubilised in water. The mode of action of *trans*-β-ocimene, a major compound present in opoponax extracts, was found to be both antifeedant and a contact molluscicide towards *D. reticulatum* slugs, depending on the media chosen for solubilising the chemical. Lettuce leaf discs studies on *H. aspersa* snails, when treated with pure oils of myrrh, opoponax and trans-β-ocimene (Chapter 5) resulted in only *trans*-β-ocimene possessing potent molluscicidal activity. On contact with *trans*-β-ocimene, the snails became highly irritated and secreted copious amount of a yellow coloured mucous. To my knowledge this is the first report of terrestrial snails producing this type of coloured secretion, when irritated. Chemical literature publications, such as the Merck Index (Susan Budavari, 1996) declare *trans*-β-ocimene to be practically insoluble in water but soluble in ethanol. This thesis is the

first report of a non polar monoterpene being more bioactive in water or polar media than in solvent/ surfactant based media. Possible explanations for this new phenomenon could be due to:

i) Increased solubility of *trans*-β-ocimene in water, when vortex mixed at high speeds, resulting in the formation of bioactive stable emulsions.

ii) Formation of polar artefacts, such as diols, when reacting with water. The acyclic monoterpene, (Z)-3,7-dimethyl, 2, 6-octadiene, in the presence of the micro organism *Diplodia gossypina*, can result in the formation of the corresponding diol (Abraham *et al.*, 1985), see figure 8.3.

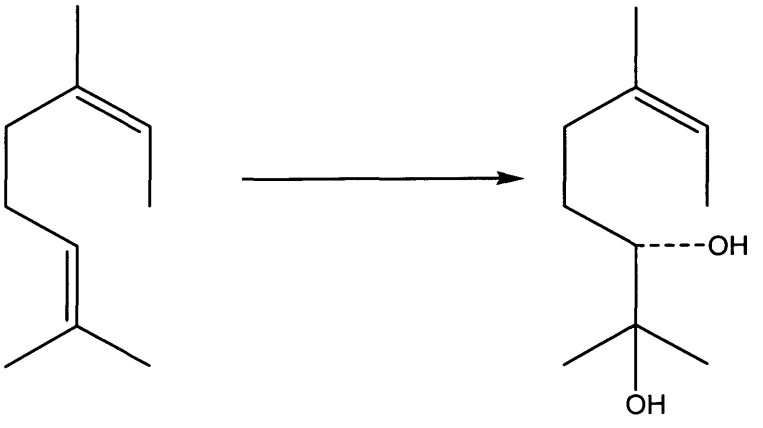


Figure 8.3 Microbial oxidation of the acyclic monoterpene, (Z) 3, 7-dimethyl-2,6-octadiene, to the corresponding 2,3 dihydro-2,3-diol.

Recently Birkett *et al.* (2004) tested sixteen chemical compounds, identified from three species of Apiaceae, for their antifeedant activity against the field slug, *D. reticulatum*. Interestingly the major compounds identified in the volatile cues from

Conium maculatum were found to be γ-coniceine, ocimene and myrcene. They passed the hexane extract of these chemicals over the slug's posterior tentacle to record the electrophysiological response and treated winter wheat seeds to ascertain antifeedant activity. Both tests showed the alkaloid γ-coniceine to be the most effective antifeedant and ocimene to be the least! There may be two possible explanations as to why they achieved contrasting results to this thesis:

i) The hexane extracts were passed over the slug's posterior tentacle. According to Ungless (1998) the slug's anterior tentacle and not the posterior tentacles are required for proximal olfaction.

ii) The chemical concentrations they used to treat the winter wheat seeds (0.1%), was lower than the concentration used in this thesis for treating sawdust barriers (Chapter 2, 0.5%).

All the plant extracts, evaluated in this thesis, have good safety profiles in respect to mammalian toxicity. Hence they should not have the same environmental problems encountered when using metaldehyde and methiocarb. Myrrh, opoponax and *trans*-β-ocimene are regarded in the USA as G.R.A.S (generally regarded as safe) and are regularly used materials in the food and flavour industry. Both non-ionic surfactants Tween 80 and Tween 20 are both approved pharmaceutical excipients (British Pharmacopoeia, 2003) and belong to a class of food additives known as the polysorbates. In addition these plant extracts causes minimal phytotoxicity to plants during spray trials.

8.2 Conclusion

This research study has successfully demonstrated the novel application of myrrh and opoponax oleoresins, their extracts and chemical components as repellent barriers towards the terrestrial molluscs *D. reticulatum*, *A. hortensis* and *H. aspersa*.

In laboratory terraria studies, repellent barriers containing reduced amounts of myrrh, mixed with inert substrates (sawdust, corncob) maintained the strong repellency properties of myrrh oleoresins while reducing slug mortality. Mixing myrrh with sharp sand increased incidences of slug mortality. Lining the tray arena with peat soil instead of blotting paper also reduced incidences of slug mortality. Extending these laboratory terraria experiments to caged field conditions confirmed the good slug repellent properties of myrrh and opoponax oleoresin barriers. Barriers of sawdust treated with myrrh extracts (essential oil and ethanolic) showed equivalent repellence properties towards slugs. Unlike current methods of mollusc control, these oleoresins became more effective under wet conditions due to the increased glue-like "stickiness" of the oleoresin. Myrrh and opoponax oleoresins showed no toxic effects against non-target organisms such as earthworms

The botanical origin of the myrrh and opoponax oleoresins was confirmed by GC-MS analytical techniques. The major chemical components identified in myrrh were not commercially available due to the complexity of their chemistries (furano-sesquiterpenes). Opoponax contained a simpler bouquet of chemical compounds, out of which only *trans*-β-ocimene, germacrene D and γ-bisabolene were commercially available. The former monoterpene was found to be a major component of opoponax.

The gas chromatographic profile of Somali myrrh was found to be dominated by elevated amounts of β-elemene, unlike commercial myrrh samples (Yemeni) whose chemistry was dominated by the furano-sesquiterpenes curzerene and furanoeudesma 1, 3 diene. This is the first report demonstrating the chromatographic differences between Somali (Guban) myrrh and commercial myrrh obtained from Yemen.

Spray emulsion formulations based on myrrh, opoponax and *trans*-β-ocimene were developed which were found to be stable between 43 and 56 weeks. Most agricultural spray formulations based on oil in water emulsions (o/w) are stable only for one day. Spray trials with these emulsion formulations protected lettuce plants from both slugs and snails. *D. reticulatum* slugs were not only significantly deterred from feeding but also suffered high incidences of mortality on contact with emulsion spray formulations based on the essential oils of myrrh, opoponax and the chemical *trans*-β-ocimene. Little slug mortality was observed when plants were sprayed with ethanolic myrrh, whilst still significantly reducing the slugs feeding behaviour. Spray trials with *H. aspersa* snails also showed significant antifeedant behaviour when lettuce plants were treated with these emulsion formulations. No incidences of snail mortality were observed throughout the spray trials. These spray formulations were well tolerated by the lettuce plants, showing little evidence of phytotoxicity.

This is the first report of *trans*-β-ocimene and *Commiphora* spp. extracts being employed as a spray to control terrestrial mollusc pests. Most reports concerning the volatile monoterpene *trans*-β-ocimene involve its role in attracting insects to plants; however this is the first to report its strong contact pesticide properties.

8.3 Future Work

These emulsion spray formulations should be evaluated for possible phytotoxicity effects against a wider range of crops such a peas, Brussels sprouts as well as horticultural plants. The residual effects of these sprays on the leaves of plants should also be evaluated, if these sprays are going to be applied to food crops. The optimum application rate should be determined, for these sprays, using modern spray application techniques.

A commercial slug/snail barrier product has been launched recently called ***Slugs.biz*** based on 50; 50 mixture of myrrh and sawdust. The antibacterial and antifungal properties of *Commiphora* spp. extracts could be utilised by combining them with wood/bark chips to protect and enhance domestic and municipal gardens and landscapes. This could be investigated further in the near future.

The repellent properties of myrrh, opoponax and *trans*-β-ocimene have been extended to aquatic organisms which commonly adhere to the sides of ships. Preliminary laboratory results showed *Commiphora.* spp extracts and selected chemical components prevented barnacle cyprids from adhering to treated plastic surfaces. Future work should involve the incorporation of these extracts and terpenoid chemicals into antifouling paints and tested under marine conditions.

CHAPTER 9:

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9. REFERENCES

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APPENDIX

Appendix A1

Figure A1. Terraria Studies: Evaluation Of Phytotoxicity Effects of 100% Myrrh Barriers on Winter Wheat Seeds (Day 7). Notice the fully developed coleoptiles.

Appendix A2

Figure A2. Terraria Studies: Evaluation of phytotoxicity effects of myrrh extracts on winter wheat seeds (Day 7), when coated onto sawdust. No detrimental effects on the coleoptile lengths, of the wheat seeds, were observed.

Table B1: Mass Spectra of Unknown Compounds Present In Myrrh Extracts: Liquid Injection

Retention Time (Minutes)	Origin Of Unknown Compound	% Peak Area	Eight Most Prominent m/z Peaks (in decreasing order of intensity)
20.81	All myrrh extracts	0.1-1.1	161 (100), 119 (93), 105 (88), 93 (69), 91 (52), 92 (40), 81(37), 120 (36)
23.24	All myrrh extracts except Baldwin ethanol extract	2.7-5.4	121 (100), 93 (91), 107 (88), 105 (84), 91 (81), 119 (80), 161 (76)
25.63	All myrrh extracts except Tisserand essential oil	1.2-4.6	105 (100), 107 (94), 93 (91), 79 (75), 81 (67), 91 (65), 121 (64), 108 (58)
30.17	Only in Somali Oleoresin (Hexane extract)	2.9	135 (100), 121 (98), 107 (98), 93 (84), 122 (75), 91 (75), 41 (66)
30.37	Only in Aldrich Essential Oil	6.9	150 (100), 151 (94), 95 (93), 135 (78), 107 (76), 81 (74), 43 (74), 109 (72)
32.21	Only in Tisserand Essential Oil	1.8	**108** (100), 216 (85), 93 (34), 91 (23), 79 (21), 95 (19), 109 (19), 77 (17)
32.30	Only in Aldrich Essential Oil	4.0	161(100), 149 (88), 135 (85), 204 (85), 59 (83), 105 (77), 107 (77), 119 (72)
32.99	Somali and Baldwin Oleoresins (Hexane extract)	3.4	43 (100), 93 (89), 161 (81), 121 (60), 107 (58), 81 (42), 105 (42), 119 (40)
33.62	Only in Somali Oleoresin (Hexane extract)	2.7	107 (100), 135 (99), 136 (96), 67 (74), **108** (67), 121 (66), 91 (65), 105 (52)
34.89	Only in Somali Oleoresin (Hexane extract)	1.7	93 (100), 81 (90), 107 (84), 95 (71), 71 (67), 121 (65), 91 (64)
35.51	Baldwin myrrh oleoresin (Hexane and ethanol extract)	1.9– 2.0	175 (100), 159 (98), 232 (74), 161 (67), 91 (58), 122 (53), 162 (49), 147 (44)
36.55	Only in Somali Oleoresin (Hexane extract)	1.5	177 (100), 159 (82), 220 (80), 123 (70), 107 (70), 93 (57), 91 (54), 135 (53)
38.22	Only in Somali Oleoresin (Hexane extract)	2.0	149 (100), 107 (99), 121 (79), 135 (77), 41 (63), 67 (61), 91 (56), 93 (46)
38.80	Only in Somali Oleoresin (Hexane extract)	1.2	**108** (100), 109 (90), 43 (58), 148 (55), 106 (51), 91 (32), 95 (29), 147 (26)
38.90	Only in Somali Oleoresin (Hexane extract)	1.7	167 (100), 68 (97), 67 (86), 121 (85), 41 (63), 107 (53), 105 (37)
39.99	Baldwin myrrh oleoresin (Hexane and ethanol extract)	5.7-9.7	159 (100), 146 (86), 185 (82), 186 (79), 160 (77), 145 (75), 175 (74), 228 (74)
40.24	Baldwin myrrh oleoresin (Hexane and ethanol extract)	1.1-1.4	215 (100), 145 (88), 230 (81), 216 (47), 159 (39), 115 (37), 91 (28), 173 (26)
41.13	Somali myrrh oleoresin (Hexane extract)	1.3	232 (100), **108** (91), 109 (58), 199 (38), 213 (38), 43 (30), 91 (29), 123 (28)
43.60	Somali myrrh oleoresin (Hexane extract)	1.2	**108** (100), 197 (96), 212 (64), 43 (43), 169 (35), 183 (31), 105 (30), 155 (27)
43.75	Baldwin myrrh oleoresin (Hexane and ethanol extract)	1.6– 1.9	175 (100), 176 (69), 260 (59), 174 (39), 213 (34), 91 (28), 115 (24), 71 (21)
45.95	Baldwin myrrh oleoresin (Hexane and ethanol extract)	2.8-4.7	175 (100), 43 (89), 162 (80), 85 (59), 111 (53), 188 (50), 145 (46), 213 (46)
46.26	Baldwin myrrh oleoresin (Hexane and ethanol extract)	1.1-2.2	162 (100), 213 (97), 188 (63), 43 (61), 85 (47), 186 (44), 161 (37), 199 (35)
59.75	Baldwin myrrh oleoresin (Hexane extract)	3.2	94 (100), **108** (98), 95 (93), 93 (87), 79 (84), 107 (84), 205 (84), 81 (81)
60.32	Baldwin myrrh oleoresin (Hexane extract)	2.1	**108** (100), 93 (85), 94 (84), 107 (79), 79 (68), 95 (61), 81 (53), 91 (51)
60.49	Baldwin myrrh oleoresin (Hexane extract)	2.9	**108** (100), 94 (99), 95 (87), 93 (86), 207 (86), 107 (81), 79 (79), 135 (77)

Note: [1]Figures in bracket are the relative abundance of each fragment peak.
[2] Peaks highlighted in blue indicates the presence of a furan moiety (m/z 108).

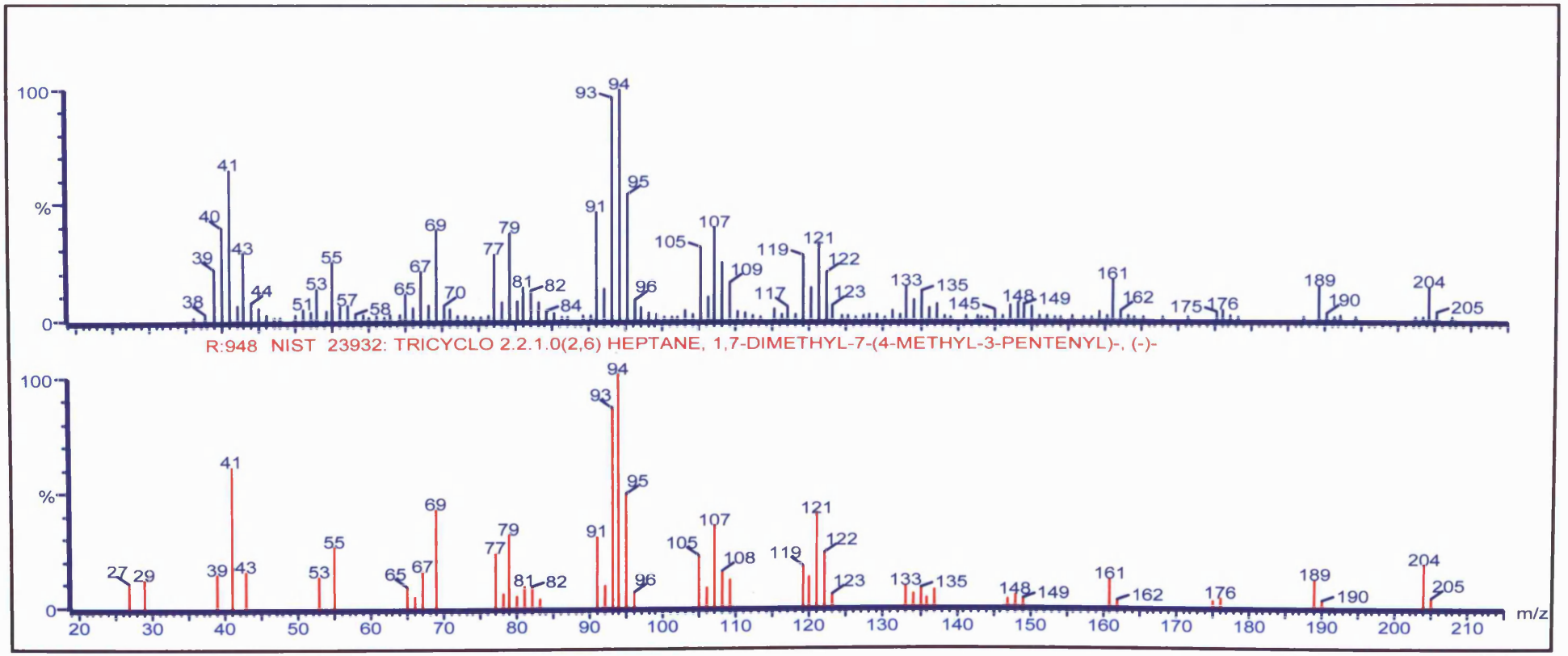

Figure B1: EI (+) mass spectra of α-Santalene. A major chemical compound identified in opoponax extracts. Below the sample spectra (Blue) is the identified mass spectra from the NIST computer library database (Red).

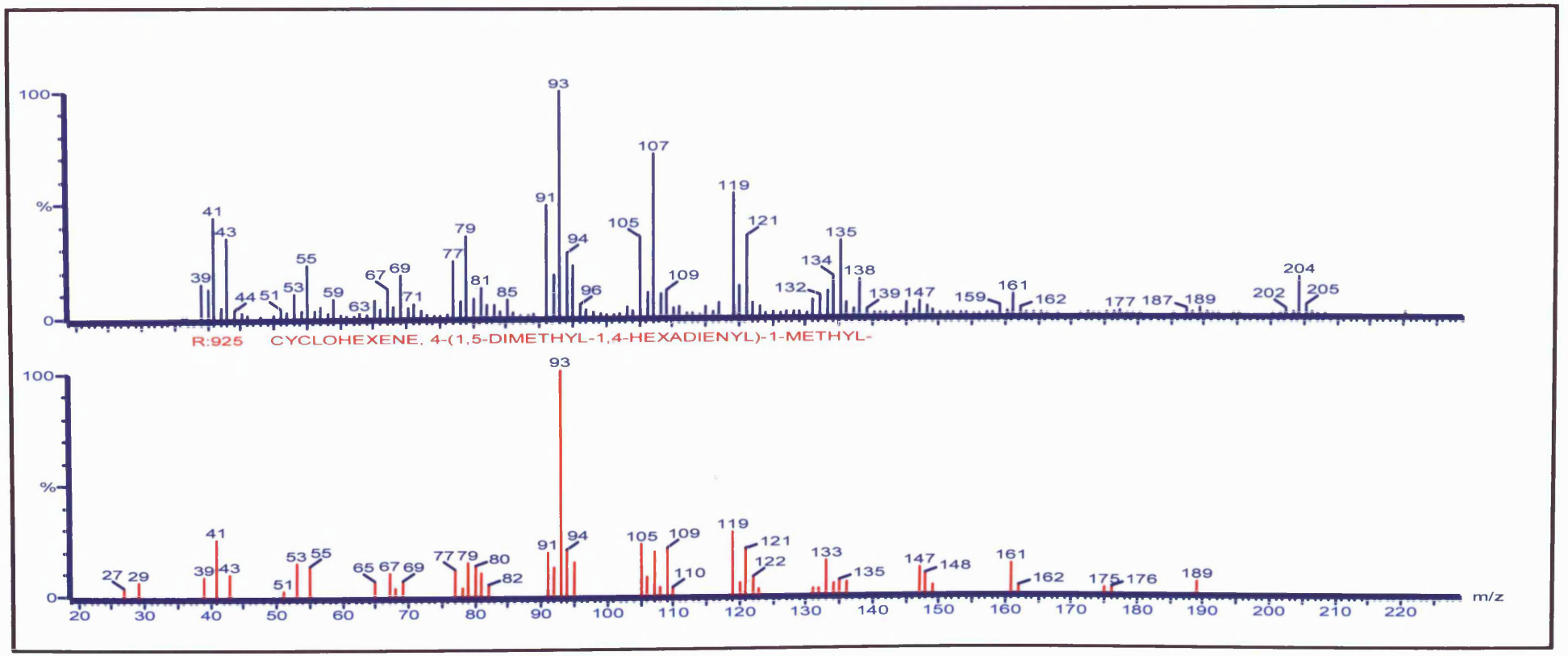

Figure B2. EI (+) mass spectra of g-Bisabolene. A major chemical compound identified in opoponax extracts. Below the sample spectra (Blue) is the identified mass spectra from the NIST computer library database (Red).

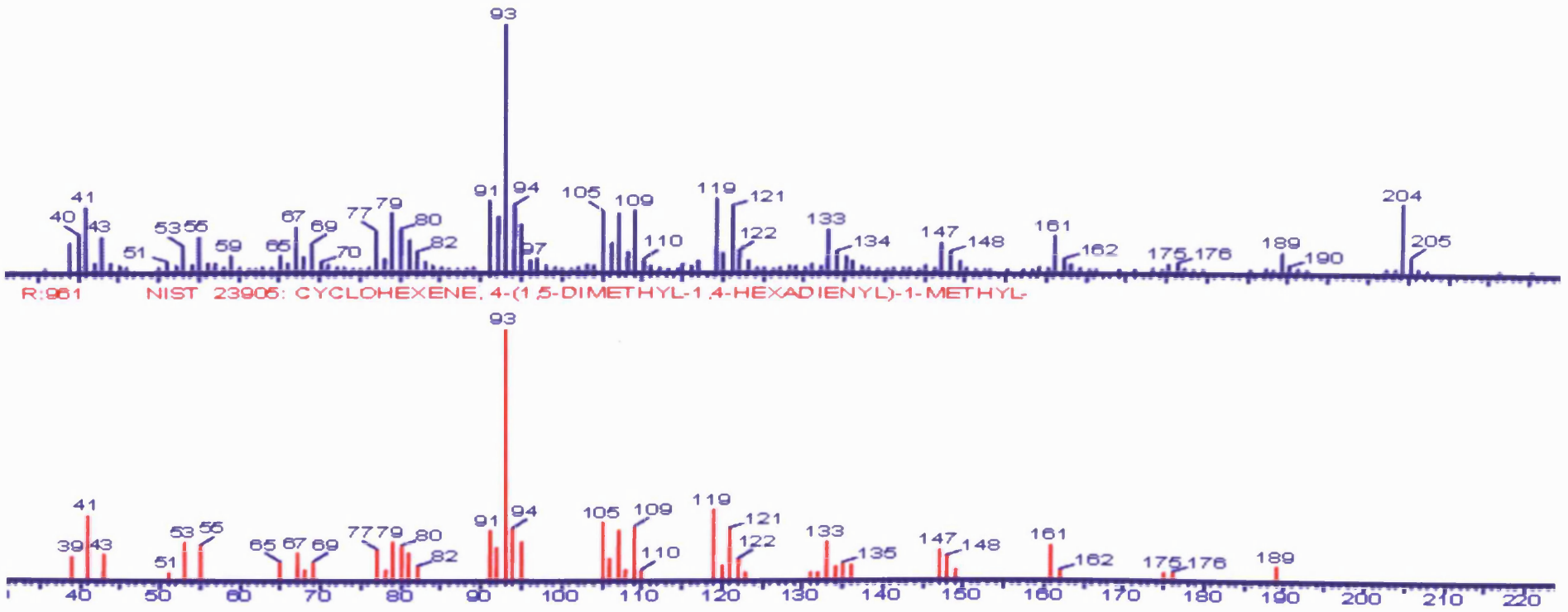

Figure B3. EI (+) mass spectra of β-Bisabolene. A chemical compound identified in opoponax extracts. Below the sample spectra (Blue) is the identified mass spectra from the NIST computer library database (Red).

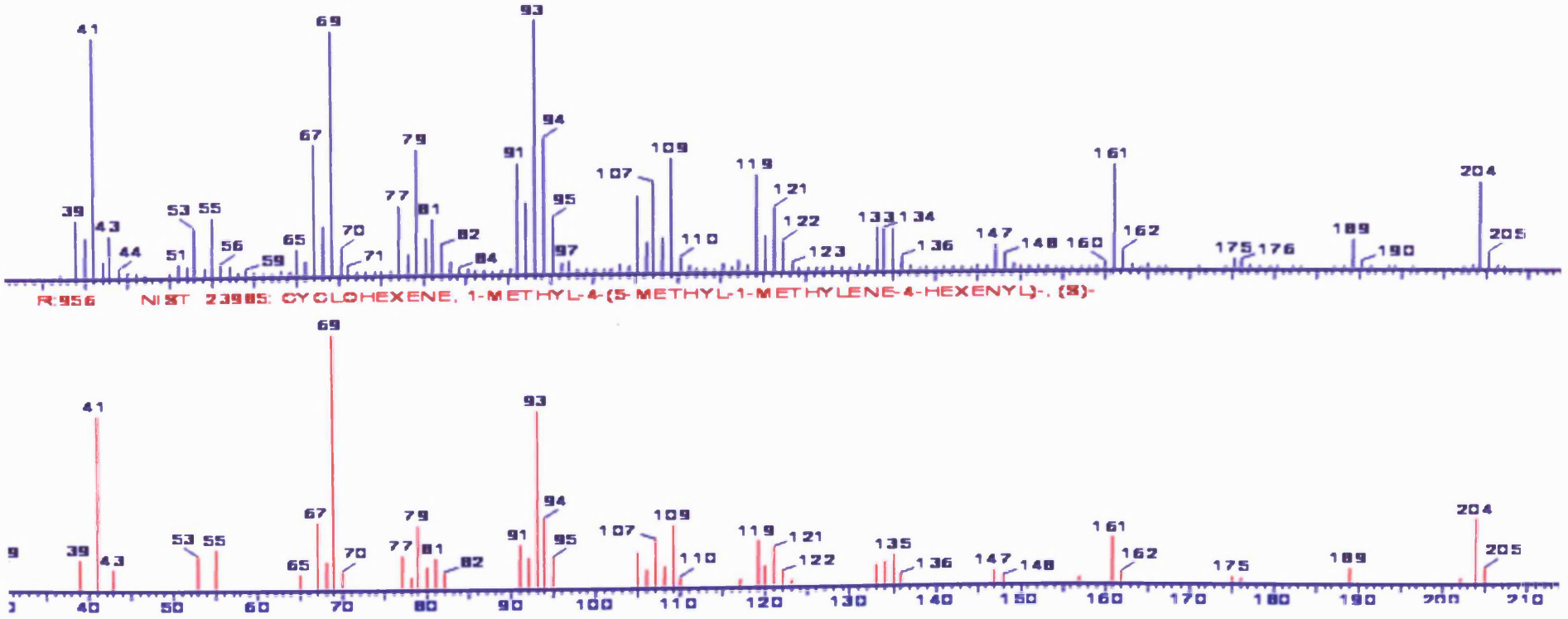

Figure B4. EI (+) mass spectra of α-Bisabolene. A chemical compound identified in opoponax extracts. Below the sample spectra (Blue) is the identified mass spectra from the NIST computer library database (Red).

Appendix C

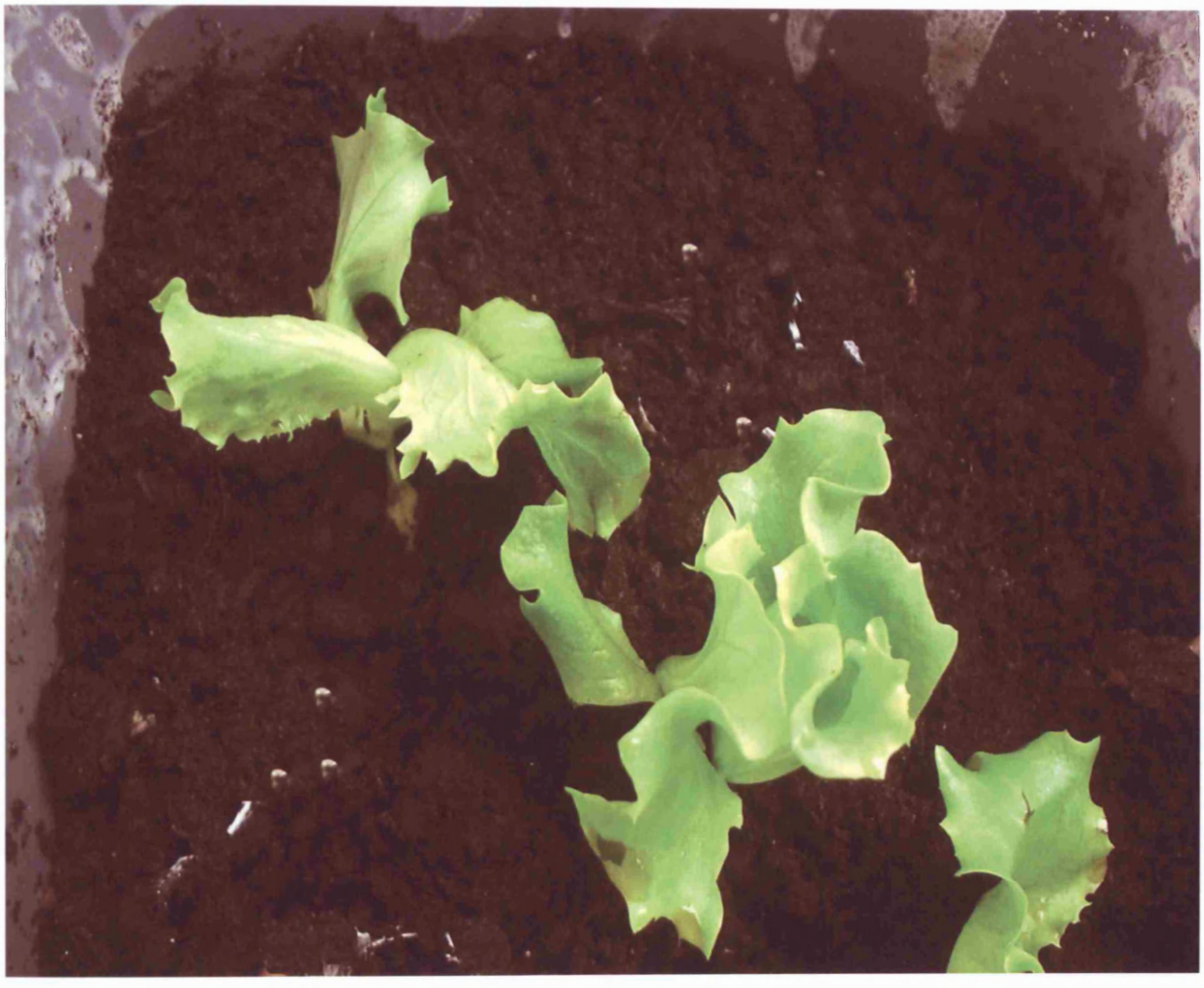

Figure C. Spray Trials: Evaluation For Phytotoxicity Effects of 5% Aqueous Tween 20 Extracts of *trans*-β-Ocimene. Notice the large yellow-brown spots on the leaf surface. (Controlled Temperature Unit Spray Trial No: 2, May 2004)

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2003 BCPC SYMPOSIUM PROCEEDINGS NO. 80:
Slugs & Snails: Agricultural, Veterinary & Environmental Perspectives

Molluscicidal and repellent properties of African plants

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ABSTRACT

Screening of Nigerian plants, for aquatic molluscicides has been previously reported, however their use as terrestrial mollusc repellents is examined here for the first time.

The plants evaluated were ***Detarium microcarpum*** (bark), ***Ximenia americana*** (bark and leaves) and ***Polygonum limbatum*** (shoot). The plant raw materials as well as their aqueous and alcoholic extracts were tested, against the field slug ***Deroceras reticulatum,*** using a variety of assays including; terraria trials, split substrate, contact toxicity, and caged field trials. Split substrate studies demonstrated the repellent nature of all the plant extracts. Laboratory terraria trials showed that all the plant raw materials exhibited significant mollusc repellency properties, when applied as a barrier. Metaldehyde pellets (4%) and ***Detarium microcarpum*** demonstrated potent molluscicidal properties in the first week. Mixing the plant materials with sawdust (50:50) as well as coating the alcoholic extracts on to sawdust provided an alternative option for application as a barrier. The barks from ***Detarium microcarpum*** and ***Ximenia americana*** showed significant mollusc repellency properties. Contact toxicity tests for all of the plants showed high slug mortality over a 24 hour period. Promising results obtained, from caged field trials, validate the use of these indigenous Nigerian plants as a natural barrier for slug control.

INTRODUCTION

Many laboratories are today screening plants as a potential source of cheaper, safer and more effective molluscides. African plants, in particular, have been widely reported as possessing molluscicidal activity (Adewunmi, 1991). The molluscicidal activity of Nigerian plants was reported to be very effective against 12 week old ***Lymnaea natalensis*** (Kela ***et al.,*** 1989) the aquatic intermediate host of ***Fasciola gigantica,*** the parasite responsible for transferring the disease schistosomiasis. Previous reports have shown the molluscicidal activity of Nigerian plants, ***(Detarium microcarpum, Ximenia americana*** and ***Polygonum limbatum)*** against ***Pomacea canaliculata*** (Lamarck), in an aquatic environment (Arthur ***et al.,*** 1996) and tested their toxicity to non-target species.

The use of raw plant material, raw plant material/sawdust mixes and plant extracts coated on to sawdust was evaluated against terrestrial molluscs in laboratory terraria experiments. The main aim of the study was to develop an environmentally friendly barrier against terrestrial molluscs, which has little or no toxic effects against non-target organisms. In this form the material could find beneficial use as a home and garden product.

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MATERIALS AND METHODS

All plants were kindly provided and identified by Professor S L Kela from the Bauchi area of Nigeria. Spruce sawdust was obtained from RS Biotech, Northants, UK. Sand (lime free agricultural grade) was obtained from Wickes, Cardiff, UK. Absolute ethanol and methanol (hplc grade) was purchased from Fisher Scien tific, Loughborough, UK.

Test animals

Adult Deroceras reticulatum were collected from nearby fields and maintained in plastic trays lined with moist, unbleached, absorbent paper. They were housed in the dark and at a constant temperature of 10° t 1°C. Slugs were regularly fed on a mixture of iceberg lettuce and carrots. Slugs were pre-starved for 24 hours prior to testing. The earthworm Lumbricus terrestris were also collected from the field for non-target testing.

Preparation of test materials

Plant raw materials were ground to a powder using a Multiquick hand blender (Braun).
Plant material/sawdust mixtures (50%) were prepared by adding 1008 of powdered plant material to 100g of sawdust. Alcoholic plant extracts (30%) were prepared by transferring 60g of the powdered barks of*D.* microcarpum and X. americana to separate containers, diluting to 200ml with either absolute ethanol or methanol and left overnight. The plant extracts were filtered, under vacuum, using a Buchner flask. Sawdust (30g) was covered with 100ml of alcoholic plant extract, mixed homogenously with a spatula and the solvent allowed to evaporate to air.

Terraria trials

Experiments were performed as described by Ali & Bowen (2003) and involved five replicates using 0.07 m2 plastic trays lined with moist, unbleached, absorbent paper.
Four pre-starved slugs were placed in each tray, equivalent to 570,000 slugs/ha, which is regarded as representing a heavy infestation. The following test materials (1-2g) were tested as barriers, against *D.* reticulatum: 100% plant material, 50:50 plant material/sawdust, and sawdust coated with alcoholic plant extracts. Controls were employed consisting of untreated trays, sawdust and alcoholic sawdust. Experiments were conducted over fourteen days.

Split substrate test

Experiments were performed as described by Bowen & Antoine (1995) in which treated filter paper sectors are tested in petri dishes to measure slug trail coverage. Once dry the filter papers were photographed to measure slug trail coverage, with a digital camera and manipulated using photographic software (Adobe Photoshopm). The area of the slug slime trail was calculated using an image analyser (Sigma Scan*). The repellency/attractancy properties of the following aqueous extracts (10%) were tested against the field slug D. refculatum; Detarium microcarpum bark, Ximenia americana bark, Ximenia americana leaf, Polygonum limbatum shoot.

The repellency index (R.I) was determined using the following equation:

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$$R.I\ (\%) = 100 \times \frac{\%\ \text{slug trail area (control)} - \%\ \text{slug trail area (test)}}{\text{slug trail area (control + test)}}$$

Contact toxicity test

A glass tube (75mm x 25mm) was filled with one third plant raw material, i.e. approximately 3.5g, and moistened with about 4ml of de-ionised water. One slug was added to each tube followed by a piece of moistened cotton wool, thus forcing the slug to be in contact with the moistened plant material. The tube was stoppered with a cork and the test replicated a total of ten times. The test was performed under environmentally controlled conditions ((12 hour light; 15°C: 12 hour dark; 15°C) over a 24 hour period. Slugs were recorded as dead if they did not respond to a 9 volt electrical stimulus.

The 100% raw plant materials were tested against D. reticulatum using sand filled tubes as controls. The method of Bieri et al., (1989) was used to test the impact of the plant materials on the non-target species Lumbricus terrestris. The earthworm was allowed to move about within a soil filled glass filter funnel and did not have enforced contact with the test materials. Mortalities were recorded over seven days.

Caged field trials

Caged field trials were conducted in October 2001, in 1 m2 hardwood arenas, with rigid pvc foam core side panels (200mm high) using the method described by Ali & Bowen (2003). A known population of slugs (10) was introduced into each arena to represent a population equivalent to 100,000 slugs per hectare. The plant raw materials (100%) were tested in the field against *D.* reticulatum using untreated plots as a control. The percentage area of leaf damage was assessed for each plant over a fourteen day period.

Statistical methods

Between treatment effects were determined using the non-parametric Kruskal-Wallis (K-W) test to show the significance of differences between group medians. In the majority of cases, between group variances were not homogeneous and residuals were not normally distributed, hence parametric comparisons of group means were not appropriate.

RESULTS

Terraria trials

Table 1 shows the crop protection obtained after a fourteen day exposure of winter wheat seeds to *D.* reticulatum, when Nigerian plant raw materials, their sawdust mixtures and their alcoholic plant extracts are applied as barriers. Over the first seven day period all the plant materials gave significant crop protection against the molluscs, compared to the control. *D.* microcarpum was equally as effective as the commercial molluscicide metaldehyde both showing negligible seed hollowing. On comparison of X. americana bark and leaf, the former showed good barrier properties resulting in low seed damage. X. americana leaf and P. limbatum shoot gave only moderate crop protection over the seven day period. Continuation of the experiment for a further seven days showed *D.* microcarpum and X.

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americana to have good barrier properties, whereas the total protection with metaldehyde resulted from slug mortality. The leaves and shoot from X. americana and P. limbatum gave relatively poor crop protection. In terms of mortalities, metaldehyde gave the highest slug death over the first seven day period followed by D. microcarpum. The other plant materials gave relatively low slug mortalities. After a fiuther seven days metaldehyde gave 100% mortality and of the plant materials only X. americana bark showed moderate molluscicidal properties.

Mixing the raw plant materials 50% with sawdust reduced the efficacy of D. microcarpum bark, yielding very poor crop protection properties over the seven day period, whilst mixing X. americana with the same amount of sawdust maintained the same high level of crop protection, as before. The level of crop protection afforded by the two plants over the next seven days was only moderate. The highest slug mortality for X. americana was obtained in the first seven days. In terms of molluscicidal properties X. americana was more effective than D. microcarpum over the fourteen day period.

Table i Mean (±SEM) and median percentage seed protection and percentage slug mortality with barriers of plant origin

Treatment	lay 7				- Day 14			
	Hollowed Seeds		Mortality		Hollowed Seeds		Mortality	
(n= 20 slugs)	Mean	Median	Mean	Median	Mean	Median	Mean	Median
100 % Plant raw material								
Control (No barrier)	98 t 1	100	5 t 5	0	87 f 11	100	15 f 10	0
Detarium microcarpum bark	1 t 1	0	60 t 13	50	6 t 2	8	10 t 10	0
Mmenta americana bark	8 f 1	8	10 f 6	0	12 t 4	8	30 t 15	25
xmenia americena leaf	19 f 7	13	10 t 6	0	4815	46	5 t 5	0
Polygonan limbatwn shoot	27 t 6	29	25 t 8	25	50 t 4	48	15 t 6	25
Metaldehyde (40/6)	1 f 1	0	90 f 10	100	0 ± 0	0	100 t 0	100
K-W test		P<0.001		P<0.001		P<0.001		P=0.007
50'/oPlant raw mat./sawdust								
Control (No barrier)	93 f 2	92	15 f 10	0	93 f 6	100	Of 0	0
50%D. microcarpum/sawdust	51 t 3	13	5 t 5	0	38 t 10	42	5±5	0
50% X. americana/sawdust	10 t 4	50	40 f 6	50	28 t 9	21	15 t 10	0
K-W test		P=0.002		P=0.028		P=0.009		P=0.291
Sawdust + alcoholic extracts								
Control (No barrier)	98 t 1	100	10 t 6	0	79 f 5	75	25 f 8	25
Sawdust	93 t 2	92	0 t 0	0	77 f 8	83	30 t 12	25
Ethanolic sawdust	94 f 3	92	15 f 6	25	88 f 3	92	30 t 9	25
Methanolic sawdust	99 t 1	100	5 t 5	0	88 t 2	92	30 f 5	25
Amicrocerpum (Ethanolic)	36 t 9	38	5 t 5	0	72 f 6	63	15 t 6	25
Rmicrocarpum (Methanolic)	49 t 14	46	10 f 6	0	39 t 8	38	4016	50
X americane (Ethanolic)	12 t 6	8	15 t 6	25	32 t 11	17	45 f 15	50
X americana (Methanolic)	3 t 3	0	15 f 15	0	52 f 6	50	15 t 15	0
K-W test		P<0.001		P--0.504		P<0.001		P=0.285

Sawdust treated with plant extracts of D. microcarpum showed relatively modest levels of crop protection and mollu scicidal activity. However the ethanolic and methanolic extracts of X. amer1cana showed superior crop protection, with little seed damage occurring over the first seven days. Crop protection decreased in the second week but the molluscicidal activity of ethanolic X. americana increased. The repellent nature of all the plant extracts was confirmed by the high repellency indices obtained in the split substrate test (Table 2).

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Table 2 Mean LSEM) slug trail area and the repellency index calculated from the split substrate test evaluating aqueous Nigerian Plant extracts

Treatment (n = 20 replicates)	Percent slug trail in control sector	Percent slug trail in plant extract sector	Repellency Index (%)
Detarium microcarpum bark	3 f 1	30 t 4	89 f 4
Ximenia americana leaf	10 t 2	44 t 2	65 t 4
Ximenia americana bark	9 f 1	43 f 3	66 f 5
Polygonum limbatum shoot	8 t 2	50 t 4	78 t 4

Enforced contact with all the raw plant materials, over 24 hrs, induced high slug mortalities (80-100%) compared to the control (20%). D. microcarpum induced relatively low mortalities (10%) against L. terrestris as the animal was allowed to withdraw away from the test source. Even so, X. americana leaf and bark were found to be more toxic to earthworms, 40% and 30% respectively, over the seven days. i

Table 3. Mean (± SEM) and median percentage leaf damage to lettuce with barriers of plant caged field trials

Treatment (n = 16 lettuces)	Leaf damage (%) Day? Mean	Day? Median	Day 14 Mean	Day 14 Median
Control o barrier	40 t 3 38		47 f 4 47	
Detarium microcar um bark	5 t 1 7		6 f 1 7	
xmenia americana bark	11 t 3 9		19 f 2 17	
K-W test	P=0.022		P--0.013	

As shown in Table 3, both X. americana and *D.* microcarpum conferred good crop protection over seven days, when tested in the field. Thus protection was extended over fourteen days.

DISCUSSION

All plant materials and their extracts showed some measure of mollusc repellency. Using *D.* microcarpum plant raw material on its own was particularly effective in both laboratory and caged field trials, matching the performance of metaldehyde in crop protection.

D. microcarpum in addition shows very strong molluscicidal activity especially during the first week but declining in the second week, whilst X. americana takes longer to exert an effect as a molluscicide. When the raw material is mixed 50:50 with sawdust the best performance is achieved by samples containing X. americana, whilst the activity of *D.* microcarpum appears to decline with time. Only the alcoholic extract ofX. americana bark, applied to sawdust, demonstrated good crop protection properties for the first seven days. The repellent nature of the Nigerian plant extracts were confirmed by the split substrate assay resulting in high repellency indices for all the aqueous plant extracts. In aquatic studies, Arthur et al., (1996) also concluded that X. americana was more potent than D. microcarpum. In this respect X. americana extracts compare well with a range of naturally occurring compounds tested as seed treatments by Powell & Bowen (1996).

Bourne et al., (1988) showed that methiocarb demonstrated better seed protection than metaldehyde and subsequently demonstrated the relative efficacies of the two commercial molluscicides. The efficacies achieved by metaldehyde in terms of crop protection are

Appendix D1

Bourne *et al.*, (1988) showed that methiocarb demonstrated better seed protection than metaldehyde and subsequently demonstrated the relative efficacies of the two commercial molluscicides. The efficacies achieved by metaldehyde in terms of crop protection are matched here by *D. microcarpum* (presented as 100% raw material) and approach the same level of molluscicidal activity over a seven day period. The molluscicidal activity of *D. microcarpum,* however, is more labile and declines rapidly over fourteen days. Enforced contact between *D. reticulatum* and the plant raw materials resulted in slug death, confirming that in a terrestrial context all the plants tested are molluscicidal. In terms of impact on non-target species enforced contact is not normally experienced. Earthworms will normally move away from molluscicidal slug pellets (Bieri *et al.*, 1989). In this study where the earthworms *L. terrestris* have some contact with the test materials some mortalities were observed, although these were minimal with *D. microcarpum* over a seven day period. Caged field trials represent a more natural application and resulted in very low slug mortalities (3%) for both plant materials as well as good crop protection especially in the case of *D. microcarpum* where there was negligible leaf damage over fourteen days. Overall the data presented here support the use of particular Nigerian plant materials as successful slug repellents and would have minimal impact on ecological biodiversity.

ACKNOWLEDGMENTS

We wish to thank Professor S K Kela for identifying and supplying the Nigerian plants and the Compton Group for sponsoring this PhD research project and their continued support.

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Appendix D2

2003 BCPC SYMPOSIUM PROCEEDINGS NO. 80:
Slugs & Snails: Agricultural, Veterinary & Environmental Perspectives

Screening African plants for mollusc repellency

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ABSTRACT

Myrrh (Commiphora molmol) and opoponax (C. guidotti) produce resinous plant materials when wounded These raw plant materials and their extracts were tested in terms of their slug repellency and mortality using a variety of assays including; laboratory terraria trials, split substrate, and caged field trials. Parallel experiments were carried out using various mixes of myrrh resin with sawdust, sand and corncob granules. Sawdust was also used as a base for a range of extracts including aqueous/surfactant, aqueous/dimethyl sulfoxide (DMSO) and ethanol. All the tests confirmed the repellent nature of the plant materials and their extracts. The raw materials successfully repelled Deroceras reticulatum (Mailer) over fourteen days resulting in less seed hollowing and high slug mortality. When mixed 50:50 with either sawdust, sand or corncob, repellency was broadly maintained, but with reduced slug mortality. Sawdust treated with extracts was also an efficient slug repellent, in particular, beech sawdust treated with aqueous /DMSO extract resulted in only 10% seed hollowing and low slug mortality. In caged field trials, these plants outperformed a leading commercial product as a natural physical barrier against D. reticulatum.

INTRODUCTION

In this study we used the oleoresins derived from myrrh (Commiphora molmol) and opoponax (Commiphora guidotti). These materials have been prized for their medicinal and fragrant uses, in north-eastern Africa and southern Arabia, since ancient times. They are formed from exudates that the trees produce on being wounded, which form resinous gums upon exposure to air. The hexane extract of both oleoresins have been reported to have repellent activities against ticks (Maradufu, 1982). Commiphora oleoresins contain secondary metabolites such as monoterpenes and sesquiterpenes which play a vital role in protecting the tree against insects and pathogenic micro organisms. It is anticipated that these terpenes may be responsible for the repellent properties of these resinous exudates.

This paper is the first report of Commiphora sp. oleoresins being used as barriers against terrestrial molluscs, in this case Deroceras reticulatum (Mailer). The aim of this work was to develop a simple repellent or molluscicidal barrier, compromising Commiphora sp. oleoresins, which could be developed for home and garden use.

MATERIAL AND METHODS

Myrrh gum (C. molmol) oleoresin was purchased from Baldwin, London, whilst opoponax

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(C guidotti) was obtained from Hargeisa, Somalia. Sawdust (spruce and beech) and corncob were kindly provided by RS Biotech, Northants. Sharp sand was purchased from B&Q Cardiff. Absolute ethanol was obtained from Fischer Scientific (UK) and dimethyl sulfoxide (DMSO) was obtained from Aldrich Chemical Company (UK). Charcoal was obtained from BDH limited (UK). The essentials oils of C. molmol and C guidottt were purchased from the Aldrich Chemical Company, USA.

Test animals

Adult *D.* reticulatum were collected from nearby fields and maintained in plastic trays lined with moist, unbleached, absorbent paper. They were housed in the dark and at a constant temperature of 10° t 1°C. Slugs were regularly fed on a mixture of iceberg lettuce and carrots. Slugs, with a weight of 300-600mg, were pre-starved for 24 hours prior to testing.

Laboratory terraria trials

Experiments were performed as described by Bowen and Antoine (1995) and involved either four or five replicates using 0.07 m2 plastic trays lined with moist, unbleached, absorbent paper. Tests were performed under environmentally controlled conditions (12 hour light 15°C: 12 hour dark 15°C). Three groups of eight winter wheat seeds were placed into each tray and each group surrounded with a continuous band of test material (4 g). Four or five pre-starved slugs were placed in each tray. The terraria trials of the 100% plant raw material and ethanolic extracts were performed over a period of seven days, after which all slugs were removed, replaced with naive ones and the trials continued for a further seven days. For the plant material/substrate and essential oil/substrate terraria trials, the experiments were monitored over a seven day period. The level of crop protection and slug mortality was recorded on a daily basis. The following materials (4 g) were tested as barriers, against *D.* reticulatum:

i) Oleoresin raw material (grain size) of C. molmol or C. guidotti.
ii) 50-60% w/w Commiphora sp. raw materials mixed with sawdust (beech), sharp sand and corncob.
ii) 20% w/v Commiphora sp. ethanolic extracts, coated onto sawdust (beech), which had been air-dried.

Split substrate test

Experiments were performed as described by Bowen and Antoine (1995) in which treated filter paper sectors were tested in Petri dishes to measure slug trail coverage. The arena was split into two, one half containing the treated filter paper and the other, control, half treated with water. The arena was exposed to one slug for 24 hours after which both filter paper sectors were sprinkled with charcoal. Once washed and dried, the filter papers were photographed with a digital camera and the image manipulated using photographic software (Adobe Photoshop®). The area of the slug slime trail was calculated using an image analyser (Sigma Scan®) to estimate the trail coverage on each segment of filter paper. The essential oils of C. molmol and C. guidoti at 0.5% and 1% levels respectively were initially solubilised in surfactant (0.2%) and diluted to volume with deionised water. A control was employed comprising 0.2% aqueous surfactant. Essentials oil were similarly prepared using DMSO (2.5%) as an extraction medium instead of surfactant and a corresponding control prepared containing 2.5% aqueous DMSO. The repellency index (R.I) was determined using the following equation:

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$$R.\ 1(\%) = 100 \times \frac{(\%\ \text{trail area on control} - \%\ \text{trail area on sample})}{(\%\ \text{trail area on control} + \%\ \text{trail area sample})}$$

Caged field trials

Caged field trials were conducted in October 2041 and April 2002, in lm2 arenas with rigid PVC side panels (200mm high). Fluon® (polytetrafluoroethylene) was applied to the sides, of the cages, to contain the slugs within the testing arena. The enclosures were filled, with medium loam soil, to a depth of approximately 5-6 cm and four young lettuce seedlings (Iceberg) with 4-8 true leaves were planted in each. Each lettuce was surrounded with a barrier consisting of 15 g of test material. Ten slugs were introduced into each arena to represent a population equivalent to 100,000 slugs per hectare. The trial was run over a fourteen day period. The amount of leaf damage per lettuce was visually assessed over fourteen days. The field trials were conducted using four replicates. The materials tested in October 2001 were: Control (no barrier), C, molmol oleoresin raw material (100%), C. guidotti oleoresin raw material (100%), C molmol essential oil (1%) in ethanol, coated onto sawdust (spruce) and C. molmol oleoresin raw material (20%) in ethanol, coated onto sawdust (spruce). The materials tested in April 2002 were: Control (no barrier), sawdust (spruce), corncob, C. molmol oleoresin raw material mixed 50;50 with sawdust (spruce), C. molmol oleoresin raw material mixed 50:50 with corncob and a commercial barrier (Slug Stop ~ comprising coconut soap.

Statistical methods

Between-treatment effects were determined using the non-parametric Kruskal-Wallis (K-W) test to show the significance of differences between group medians. In the majority of cases, between-group variances were not homogeneous (Bartlett's or Levene's test) and residuals were not normally distributed (Anderson-Darling test), hence parametric comparisons of group means were not appropriate (Fry 1993). In cases where these parametric criteria were fulfilled, analysis of variance (Anova) was used to compare group means.

RESULTS

The presence of oleoresin barriers from either Commiphora sp. significantly reduced seed damage to negligible amounts over the fourteen day period, as shown in Table Ia. Significantly high slug mortalities were obtained for both barriers in the first week, but not in the second week). Coating the ethanolic plant extracts onto sawdust also had significant effects on reducing seed hollowing over fourteen days, as shown in Table lb, for both species of Commiphora. Mortality was highly variable and differences between treatments were not significant.

Mixing the plant material with inert substrates (sawdust and corncob) did not apparently reduce the crop protective properties of the oleoresins, as shown in Table 2a. However, mortality levels appear much lower than with raw plant materials alone (Table 1), with the exception of the sand mixture, where mortality was significantly higher.

Coating C. molmol essential oil extracts onto sawdust also had a significant *effect in* reducing seed hollowing, as shown in Table 2b-d. The best result for the Commiphora sp.

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essential oils was obtained using 2.5% aqueous DMSO, as a solubilising medium, resulting in significant crop protection over seven days (Table 2d). Slug mortalities, were in general, low for both Commiphora sp. essential oils in all cases (Table 2b-d).

Table 1. Mean (± SEM) and median percentage seed protection and percentage slug mortality with barriers of Commiphora oleoresin and sawdust coated with ethanolic Commiphora sp. extracts (20%)

Treatment	Day 7				Day 14			
	Hollowed Seeds		Mortality		Hollowed Seeds		Mortality	
(n = 20 slugs)-	Mean	Median	Mean	Median	Mean	Median	Mean	Median
a) 100% Plant raw material								
Control (No barrier)	93 f 2	92	15 t 10	0	93 f 6	100	0 t 0	0
C. mohmol	1 ± 1	0	70 ± 20	100	8 ± 2	8	10 ± 6	0
C. guld20	0 f 0	0	85 t 10	100	0 f 0	0	20 t 12	0
K-W test		P=0.002		P=0.028		P=0.001		P=0.270
b) Sawdust + ethanolic extracts								
Control (No barrier)	98 t 1	100	10 ± 6	0	79 f 5	75	25 t 8	25
Spruce sawdust	93 ± 2	92	0 f 0	0	77 ± 8	83	30 f 12	25
Ethanolic sawdust	94 f 3	92	15 f 6	25	88 ± 3	92	30 f 9	25
C.guidottict	20 ± 6	13	20 ± 9	25	11 ± 4	13	60 f 5	75
CmohWI	28 t 7	33	5 ± 5	0	15± 4	17	30 t 5	25
K-W test		P=0.001		P=0.208		P=0.001		P=O. 152

Table 2. Mean `SEM) and median percentage seed protection and percentage slug mortality over 7 days with barriers of Commiplmra oleoresin mixed with inert materials and sawdust coated with essential oil plant extracts

Treatment	Hollowed Seeds		Mortality -	
[Except where indicated n=20 slugs (` n = 16)]	Mean	Median	Mean	Median
_ 0 ComMphora sp./substrate mixes				
Control (No barrier)	93 ± 6	98	20 t 14	10
Sawdust	96 f 3	98	15 ± 10	10
Comcob	99 ± 1	100	0 t 0	0
Sharp sand	89 t 9	98	20 t 8	20
C mobnol/sawdust (60%/40%)	2 f 1	2	20 ± 8	20
C. mohnol/sand (60%140%)	1 t 1	0	60 t 8	60
C. mohnol/comcob (50%/50%)	8 f 3	9	5 t 5	0
C. molmol/comcob (40%/60%)'	4 t 2	4	38 ± 13	50
K-W test		P-0.001		P=0.028
b) Essential oil extracts in ethanol				
Control 1 (no barrier)	99 ± 1	100	0 ± 0	0
C. molmol essential oil (0.5%)	30 ± 6	25	25 ± 11	25
K-W test		P-0.007		P=0.053
c) Essential oils + 0.2 % aqueous surfactant				
Control I (sawdust only)	99 f 1	100	0	0
Control 2 (sawdust + 0.2% aq. surfactant)	98 f 2	100	15 f 10	10
C. guideni (1%)	93 ± 4	94	15 t 5	20
C. molmol (0.5%)	25 ± 9	32	30 f 10	40
K-W test		P=0.013	P=O. 115	
d) Essential oil extracts + 2, 5% aqueous DMSO				
Control (sawdust + 2.5% aqueous DMSO)'	88 t 3	90	25 t 10	25
C. guidotf (1 %)'	46 t 12	50	13 t 7	13
e moJmol (0.5%)'	10110	0	38 t 16	38
K-W test		P=0.012		P=0.396

The repellent nature of the essential oil extracts was confirmed using the split substrate test as shown in Table 3, with both plants giving significantly high repellency indices compared

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to the control. The slugs spent less time on the plant extract sectors, with only 5 and 12% of the filter paper sector being covered with trails of slug slime, compared to 54% for the control.

Table 3. Mean (± SEA) and median percent slug trail area and the repellency index calculated from the split substrate test evaluating Commiphora sp. essential oil extracts in 0.2% aqueous surfactant

Treatment (n = 20 replicates)	Percent slug trail in control sector (0.2% aqueous surfactant)	Percent slug trail in plant extract sector	Repellency Index (%) mean	median
Control (0.2% aqueous surfactant)	55 f3	54 f4	2 f3	1
C molmol (0.5%)	65 f9	5 f1	84 t4	94
C guidow (1%)	52 f3	IZt 2	63 f5	66
K-W test				P<0.001

Caged field trials conducted in October 2001 and April 2002 yielded encouraging results, as shown in Table 4. In the first field trial, all the plant raw materials and their extracts were significantly effective in reducing crop damage over the seven days (Table 4a). The protective properties of the barriers continued until day fourteen. The second field trial showed that diluting the C molmol raw material with sawdust or corncob inert materials had moderately significant crop protection properties over the seven day test period (Table 4b). The Commiphora sp. raw materials mixed with inert diluents were superior in reducing crop damage compared to the commercial physical barrier.

Table 4 Mean (± SEM) percentage leaf damage to lettuce with barriers of Commiphora oleoresin and extracts mixed with inert materials in caged field trials

Treatment (n = 16 lettuces)	Day 7	Day 14
a) October 2001		
Control (No barrier)	40 t 7	47 f8
C.molmol oleoresin (100%)	7 t 6	12 t 7
C.mohnol essential oil (1%)	5 t 3	14 t 6
C.mohmol ethanolic extract (20%)	0 f0	14 f5
Cguidold oleoresin (100%)	2 t 2	15 f7
Anova test	P<0.o01	P=0.006
b) April 2002		
Control (No barrier)	43 t 7	54 t 2
Control (corncob)	38 t 13	50 t 17
Control (sawdust)	22 +10	29 f10
Commercial product	31 t 9	38 f11
Cmolmol / sawdust (60%: 40%)9 f1		18 t 5
C. molmol / corncob (50'/6:50%)	10 f6	19 t 8
Anova test	P=0.043	P–.0.089

DISCUSSION

Myrrh showed strong slug repellency in split substrate tests, in terraria and in caged field trials.

Although myrrh has been shown to be non-toxic to mice (Rao, et al., 2001), high slug mortalities were recorded in laboratory terraria trials over fourteen days. On wetting, the resin takes on a glutinous sticky texture and the movement of slugs may be physically

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retarded or they may be trapped. In cage trials, repellency was maintained but there was little evidence of mortality. When raw materials were mixed with other substrates repellency was maintained, but with low mortalities, with the exception of sand where higher mortalities were observed over seven days. Mixes with corncob or beech sawdust produced similar levels of crop protection with little seed hollowing, but much reduced mortality over seven days. This mix may thus be preferable in terms of maintaining biodiversity and conservation. In terms of substrates treated with essential oil extracts, only the aqueous/DMSO gave effective crop protection. Split substrate experiments show that aqueous/surfactant extracts are an effective repellent to slugs.

Some effective barriers used to protect plants include the use of copper and fibreglass (Rata, *et al.*, 1997). These were found to be successful in reducing the feeding behaviour of *Vaginula plebeian* and *Veronicella cubensis*. Mason, *et al.*, (1994) showed that compositions containing saponins from the plants *Yucca schidigera* and *Hedera helix* prevented terrestrial molluscs from feeding upon plants. Metal based barriers, such as copper lose their protective properties in wet weather due to the formation of a thin film of moisture, enabling the slug to crawl over it (Godan, 1983). *Commiphora* sp. oleoresins performed even better under these conditions, possibly due to the increased adhesive properties and the high essential oil content of the resin. Some plant based barriers e.g. Yucca plants containing saponins can be toxic if ingested, whereas *Commiphora sp.* essential oils are approved for use in food (Watt & Sellar,1996).

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