THE ISOLATION AND CHARACTERIZATION OF PHYTOALEXIN AND CONSTITUTIVE AGENTS FROM PLANTS FOR MYCOTOXIN CONTROL

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ABSTRACT

Plant medicine is an important area of commercial activity in South Africa. This is a rapidly expanding market, thus we are evaluating natural and stress-induced compounds (phytoalexins) from plants as agents that may be able to control mycotoxins. Natural compounds from *Bridelia micrantha*, *Warburgia salutaris*, *Lippia javanica* and *Scenecio serratuloides* and stress-induced compounds (phytoalexins) from *Citrus sinensis* cv Valencia were screened for antifungal and antimycotoxic activity by bioautography against a test organism (*Cladosporium cladosporoides*) and mycotoxin producing fungi (*Fusarium moniliforme* and *Aspergillus flavus*). The active compound isolated from stress-induced fruit showed antifungal and antimycotoxic activity and was chemically identified by Ultraviolet and Infrared spectrophotometry and Near Magnetic Resonance (^{1}H NMR) spectroscopy.

Quantitation of this compound was accomplished by High Performance Liquid chromatography (UV detection - 350nm). HPLC results showed that Valencia oranges contained a trace amount (0.36 μg/g) of scoparone in untreated fruit but levels increased in UV illuminated fruit (15.2 μg/g). However, the best yield of 35,51μg/g was obtained in fruit infected with *Penicillium digitatum* (natural spoilage mould of citrus fruits). UV absorption wavelengths for the two main functional groups of scoparone were identical for the standard (aromatic CH_2 backbone: 1450cm^{-1}; $(CH_2)_2$ -substituent group: 800–1000cm^{-1}) and extracted compound (aromatic CH_2 backbone: 1446 cm^{-1}; $(CH_2)_2$ - substituent group: 800-1000cm^{-1}). Further verification by IR

spectrophotometry and ^{1}H NMR spectroscopy (360 MHz, $CDCl_3$) resulted in the chemical identification of the isolated compound as 6, 7 dimethoxycoumarin. *Warburgia salutaris* contained four antifungal compounds which showed the following identical spectral data: IR ν^{max} 3460cm^{-1} (OH), 2850cm^{-1} (CHO), 1723cm^{-1} CHO), 1687cm^{-1} (C=C-CHO). The above spectral data were in good agreement with those of the sesquiterpenoid dialdehydes (warburganal, ugansidial, polygonal and isopolygoidal) isolated from *W. ugandensis* (Meinwald *et al.*, 1978). Mass spectra further verified the compounds as drimane-type sesquiterpenoids. This is the first report showing the stress-induced compound, 6,7 dimethoxycoumarin isolated from *P. digitatum* infected Valencia fruit and the constitutive compound isolated from *W. salutaris* conferring resistance against the mycotoxigenic fungi, *A. flavus* and *F. moniliforme*.

PREFACE

This study represents original work by the author and has not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Biological Sciences, Faculty of Science, M.L. Sultan Technikon, under the supervision of Dr. B. Odhav and Professor M. F Dutton.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDICES

CHAPTER ONE

1. INTRODUCTION

Mycotoxins are a structurally diverse group of mostly small molecular weight compounds, produced by the secondary metabolism of fungi, are ubiquitous in a broad range of commodities and feeds, and are toxic to mammals, poultry and fish. In structural complexity, mycotoxins vary from simple C_4 compounds, e.g., moniliformin, to complex substances such as the tremorgenic mycotoxins (Steyn and Vleggar, 1985). Mycotoxins induce powerful and dissimilar biological effects. Some are carcinogenic (aflatoxins and fumonisins), mutagenic (aflatoxins and sterigmatocystin), teratogenic (ochratoxins), oestrogenic (zearalenone), heamorrhagic (trichothecenes), immunotoxic (aflatoxins and ochratoxins), nephrotoxic (ochratoxins) and neurotoxic (ergotoxins and penitrems), whereas others display antitumour, cytotoxic and antimicrobial properties. The human ingestion of mycotoxins is due to the consumption of the mycotoxins in plant-based foods and their residues and metabolites in animal-derived foods, e.g., aflatoxin M_1 in milk.

The global nature of the mycotoxin problem is based on well-documented human mycotoxicoses such as the ergotism in Europe, alimentary toxic aleukia (ATA) in Russia, acute aflatoxicoses in South and East Asia and human primary liver cancer (PLC) in Africa and South East Asia (Steyn. 1995).

Measures have to be implemented to control mycotoxin-producing fungi. Current control measures include physical destruction and chemical detoxification. Biological control of mycotoxins is currently an uncharted field. Little or no support has been given to elucidate the nature of disease resistance in plants. Thus the aim of this study was the isolation and identification of constitutive and stress induced compounds for mycotoxin control.

Plant organs are used in traditional systems of medicine, folklore and ethnomedical products, and include a broad category of compounds. In the first two categories of plants there is a record of long clinical use that is helpful in choosing appropriate biological test systems. In case of plants having no documented therapeutic use the problem is rather more difficult and very often a broad-based screening is required. A broad-based programme of biological screening of medicinal plants was initiated at the Central Drug Research Institute, Lucknow, in 1963 and the first results were reported in 1968 (Dhar *et al.*, 1968). The programme continues, and results on over 3000 (Feng *et al.*, 1962, Aswal *et al.*, 1984) plant materials have been published. In addition results of testing 967 plants for antiviral activity (Babbar *et al.*, 1982) 1600 plants spermicidal and sernen coagulant activity, (Setty *et al.*, 1977; Khamboj and Dhawan. 1977), and 644 plants for leather tanning and insecticidal activity have also been reported (Fernandes and Costa Pereira, 1977) The programme is expanded to cover the marine flora and fauna (Naqvi *et al.*, 1980; Kamat *et al.*, 1981). Fifty-percent ethanolic extracts from about 3000 plant materials have been run through the programme. The various activities observed and the plants showing a particular

activity are summarized in Table 1.1. The analysis covers results from approximately 2700 materials. About 21% of the plants tested have significant activity and about 33% of the plants had a low (though significant) level of activity.

Table 1.1 Summary of primary biological screening results of 2700 plant extracts at CDRI

Sample number	Pharmacological activity	Number of active extracts
1.	Abortifacient	12
2.	Antiamoebic	1
3.	Anticancer	132
4.	Antihelminthic	2
5.	Anti-implantation	10
6.	Anti-inflammatory	31
7.	Antimicrobial (antibacterial + antifungal)	13
8.	Antiviral	13
9.	Cardiovascular effects (hypotensive, hypertensive, cardiotonic)	71
10.	CNS depressant	77
11.	Diuretic	39
12.	Hypoglycaemic	12
13.	Hypolipaedemic	5
14.	Insecticidal	21
15.	Oxytocic	14
16.	Semen coagulant	49
17.	Spasmolytic	52
Total		554

CHAPTER TWO

2.1 PHYTOALEXINS

2.1.1 General Overview

Muller and Borgers work in 1940 showed that *Phytophthora* resistance of potatoes led to decisive experimental progress in the elucidation of plant defense mechanisms. Plants defend themselves against the parasite by sacrificing the tissue layers infected with fungus. During this reaction which is termed "defense necrosis", a defense substance is formed which is called "phytoalexin". Phytoalexins are defined as defense substances with antimicrobial properties that are produced post-infectionally by the host (Kuc, 1972).

Phytoalexins play an important role in resistance against pathogens (Darvill and Albersheim, 1984; Hahlbrock and Scheel, 1987; Ingham, 1973). The presence of phytoalexins in citrus tissue has been reported (Afek and Sztejnberg, 1988; Afek and Sztejnberg, 1988a; Arimoto and Homma, 1988; Arimoto *et al.*, 1986; Arimoto *et al.*, 1986a). Most of antifungal compounds in citrus tissues are observed following inoculation with *Phytophthora* sp. (Musumeci and Oliveria, 1975). Several coumarins, including xanthyletin (Khan *et al.*, 1985) and seselin (Vernenghi *et al.*, 1987) accumulated in trunk and root tissue of citrus infected by *Phytophthora* sp.

Ismail *et al.* (1978) reported that the synthesis of umbelliferone (7-hydroxycoumarin) was greatly enhanced during healing of injured grapefruit. Afek and Sztejnberg, 1988 isolated another coumarin, scoparone, (6, 7 dimethoxycoumarin) from the bark

of citrus following inoculation with *P. citrophora.* This compound was induced in fruit, leaves and twigs of Satsumi mandarin infected with *Diaporthe citri* (Faw.) and was not detected in the healthy tissue of the citrus (Arimoto and Homma, 1988; Arimoto *et al.*, 1986; Arimoto *et al.*, 1986a).

Scoparone was isolated also from orange peel infected with *Guignardia citricarpa* Kiely, the cause of black spot (De Lange *et al.*, 1976), and from peel of gamma-irradiated grapefruit (Riov, 1971), but it was not found in the non-irradiated fruit or in the fruit inoculated with the pathogen. Ben-Yehoshua *et al.* (1988) isolated several antifungal substances from pomelo fruit, some of which are coumarin derivatives. However, Baudoin and Eckert (1985) did not find any significant accumulation of antifungal compounds in wounded tissues of lemons inoculated with *Geotrichum candidum* Link ex Pers.

2.1.2 Sources of Phytoalexins

Phytoalexins have been found from Leguminosae and Solanaceae plant families. Most phytoalexin research has been conducted with plants in the Leguminosae and Solanaceae families due to the ease of extraction and chemical characterization of phytoalexins from these families, but relatively few reports on the accumulation of phytoalexins in plants of the Gramineae family (e.g. momilactones of rice) are available. The major phytoalexins from these flowering plant families are summarized in Table 2.1

Table 2.1 Phytoalexins from different plant families.

Family	Species	Phytoalexin	Reference
Leguminosae	*Glycine max*	Maackiain	Cartwright *et al.*, 1977
	Pisum sativum	Pisatin	Keen and Kennedy 1974
Solanaceae	*Monilinia fructicola*	Capsidiol	Stoessel et al., 1972
Amaryllidaceae	*Narcissus pseudonarcissus*	7-hydroxyflavan 7.4` -dihydroxyflavan 7,4 dihyroxy- 8- methylflavan	Coxon *et al.*, 1980
Compositae	*Carthamus tinctorius*	Safynol	Allen and Thomas, 1971a
		Dehydrosafynol	Allen and Thomas, 1971b
Convolvulaceae	*Ipomoea batatas*	Ipomeamarone	Kubota and Matsuura,
		Dehydroipomeamarone	1953
		Ipomeamaronol	Oguni and Uritani, 1974a
			Kato *et al.*, 1971
Gramineae	*Oryza sativa*	Momilactone A	Cartwright *et al.*, 1977
		Momilactone B	
Malvaceae	*Gossypium barbadense*	Hemigossypol	Zaki *et al.*, 1972
	Gossypium hirsutum	Isohemigossypol	Sadykov *et al.*, 1974
		Gossyvertin	Karimdzhanov *et al.*, 1976
		6-methoxyhemigossylpol	Bell *et al.*, 1975
		6-deoxyhemigossypol	Bell. 1967
		Gossypol	Stipanovic *et al.*, 1975b
		6-methoxygossypol	
		6,6`-dimethoxygossypol	
Moraceae	*Morus alba*	Moracin A	Takasugi *et al.*, 1978
		Moracin B	
		Oxyresveratol	
	Broussonetia papyrifera	Broussonin A	Takasugi *et al.*, 1978a
		Broussonin B	Takasugi *et al.*, 1980
Orchidaceae	*Orchis militaris*	Orchinol	Hardegger *et al.*, 1963
	Loroglossum hircinum	Loroglossol	Ward *et al.*, 1975
		Hircinol	Urech *et al.*, 1963
Rutaceae	*Citrus limon*	Xantoxylin	Hartman and Nienhaus,
			1974
Umbrelliferae	*Daucus carota*	6 –methoxymellein	Condon and Kuc, 1962
		Falcariniol	Harding and Heale, 1980
		Xanthotoxin	Johnson *et al.*, 1973
Vitaceae	*Vitis vinifera*	ε-viniferin	Langcake and Price,
		α-viniferin	1977a
		Pterostilbene	Pryce and Langcake,
			1977
			Langcake *et al.*, 1979

2.1.3 Mechanisms of Phytoalexin Accumulation

Phytoalexins are produced by plants in response to interactions with fungi, bacteria, viruses, nematodes, other living organism, by treatment with many chemicals and by irradiation with ultra-violet light. They are diverse compounds contributing to many of the major groups of secondary metabolites, including simple phenolics, flavonoids, isoflavonoids, stilbenes, terpenes and polyacetylenes.

2.1.3.1 The Role of fungi in phytoalexin accumulation

Early isolation of phytoalexins was based on experiments with fungi, which are very efficient phytoalexin inducing agents (VanEtten and Bateman, 1971). The structures of phytoalexins are determined solely by the plant producing them (Cruickshank and Perrin, 1963) and tissues infected by different fungi generally contain a similar range of phytoalexins. For example, phaseollin, phaseollidin and phaseollin isoflavan were produced by French bean in response to infection by *Botrytis cinerea* (Fraile *et al.*, 1980; Van den Heuvel and VanEtten, 1973*), C. lindemuthianum* (Bailey, 1974), *Fusarium solani* (VanEtten and Bateman, 1971), *Rhizoctonia solani* (Smith and Bull, 1978) or *Thielaviopsis basicola* (VanEtten and Bateman, 1971). Similarly, medicarpin was isolated from *Medicago sativa* infected with *C. phomoides, Stemphylium loti, S. botryosum, Phoma herbarum or Leptosphaeria briossiana* (Higgins, 1972) and glyceollins from soybean infected with *Phytophthora megasperma* var. *sojae* or *P. eactorum.*

Other less frequent reports have indicated some interesting exceptions. Kievitone was the predominant phytoalexin in French bean tissues infected with *Rhizoctonia solani* (Smith and Bull, 1978) and also occurred in tissues infected with *Colletotrichum lindemuthianum* (Bailey, 1974) or *Botrytis cinerea* (Fraile *et al.,* 1980). It was not, however, detected in lesions caused by *Fusarium solani or Thielaviopsis basicola* (VanEtten and Bateman, 1971). Analysis of sesquiterpenes produced by several cultivars of potato infected with various isolates of *Phytophthora infestans, Fusarium avenaceum* or *Phoma exigua* also revealed differences in the phytoalexins produced. Solavetivone was produced most consistently, but rishitin, although present in tissue infected with most isolates of *P. infestans* was rarely present in tissues infected with *F. avenaceum or Phoma exigua (Price et al.,* 1976).

The quantities of phytoalexins in infected tissue also change during the expression of resistant reactions. Normally this is evident as a gradual or a rapid increase in concentration to a degree, which is often maintained or only slightly reduced. Other patterns have, however, been reported. Smith and Bull (1978) found that kievitone accumulated very rapidly in young lesions of *Rhizoctonia solani* on French bean stems. Within a few days the amounts of kievitone present were very much lower, whereas the concentrations of phaseollin had increased during this period. A similar reduction in the concentration of rishitin was observed in potato tubers following the hypersensitive reaction (Kuc, 1972).

2.1.3.2 Bacterial Elicitation

Bacteria cause less phytoalexin accumulation than fungi. Several bacteria failed to cause pisatin to form in pea pods (Cruickshank and Perrin, 1963) and only small amounts of phaseollin were produced by pods of French bean (Cruickshank and Perrin, 1971). These results, based on diffusate techniques, may have discouraged further work, but in 1971 Stholasuta *et al.* reported large concentrations of phaseollin (22.6 μg/g tissue) in French bean leaves which had reacted hypersensitively to infiltration with an incompatible strain of *Pseudomonas phaseolicola*. It has been subsequently found that phaseollidin, phaseollinisoflavan, kievitone and coumestrol are also produced (Gnanamanickan and Patil, 1977). The non-pathogenic bacterium *P. morsprunorum* was also effective but phytoalexins were not obtained from leaves inoculated with the saprophyte *P. fluorescens* (Lyon and Wood, 1975). An earlier failure to obtain phytoalexins from leaves infiltrated with *P. morsprunorum* (Stholasuta *et al.,* 1971) may have been due to very rapid dehydration of entire inoculated leaves. *Pseudomonas* sp. causes phytoalexins to accumulate in other plant species. Glyceollin and coumestrol, along with daidzein and sojagol, were produced by soybean infected with *P. glycinea* (Keen and Kennedy, 1974).

Studies with soybean leaves and cotyledons illustrate the potential complexities of responses to bacteria. Leaves inoculated with *P. pisi* produced three glyceollin isomers, isoformonenetin, glyceofuran, 9-0-methylglyceofuran and glyceocarpin (Ingham *et al.,* 1981). Cotyledons inoculated with *Erwinia carotovora* also produced

the glyceollin isomers, but the major product was a trihydroxypterocarpan, glycinol (Weinstein *et al.,* 1981). Soybean leaves did not produce glycinol.

Sesquiterpene phytoalexins are produced in bacteria-infected tissues of solanaceous plants. In 1972, Lyon reported that rishitin and phytuberin occurred at high concentrations (100 to 1000 μg/g tissue) in potato tubers colonized by *E. atroseptica.* These compounds were also present, with several other sesquiterpenes, in potatoes infected with *E. carotovora.* Rishitinol and lubimin were not detected, although they had been obtained from fungus infected tissues (Coxon *et al.,* 1974). *E. carotovora* also caused capsidiol to form in pepper fruits (Ward *et al.,* 1975) and phytuberin was isolated from leaves of *Nicotiana tabacum* inoculated with *P. lachrymans* (Hammerschmidt and Kuc, 1979)

2.1.3.3 Viruses

The formation of virus-induced local lesions is associated with many biochemical changes, including production of phenolic compounds and pigments (Farkas and Kiraly, 1962). Antifungal activity was first reported using extracts of soybean leaves infected with tobacco necrosis virus (TNV) (Hammerschlag and Klarman, 1969). Subsequently phaseollin, phaseollidin, phaseollinisoflavan and kievitone were obtained from TNV-infected French beans (Burden *et al.,* 1972) and glyceollin from virus-infected soybean (Klarman and Hammerschlag, 1972). Since this work, virus induced local lesions in several plants have been shown to be an excellent source of phytoalexins. Phascollin, phaseollidin, kievitone and 2-0-methylphaseollidin-isoflavan were obtained from infected *Vigna* spp. (Bailey, 1973; Preston, 1975),

pisatin from pea (Bailey, 1973) and medicarpin from *Canavalia ensiformis* (Lampard, 1974). Similarly, several phytoalexins were produced by virus-infected tobaccos. Capsidiol, solavetivone, 3-hydroxy- solavetivone, solascone, phytuberin and phytuberol were obtained from TNV- infected *Nicotiana tabacum* (Bailey *et al.,* 1976) and glutinosone from *N. glutinosa* infected with tobacco mosaic virus. Glutinosone was not obtained from *N. glutinosa* infected systemically with TNV (Bailey *et al.*, 1976). The amounts of phytoalexins obtained from these sources can be large (10 to 500 μg/g tissue), particularly if the integrity of the infected tissues can be maintained for several days after symptoms appear. Thus virus-infected tissues have been useful not only for the isolation of some new phytoalexins, but also for the provision of quantities sufficient for studies on their metabolism and toxicity (Bailey and Skipp, 1978).

2.1.3.4 Animals

Infestation of plant tissues by weevils or nematodes leads to the production of phytoalexins. Ipomeamarone, ipomeamarol and dehydroipomeamarone were obtained from sweet potatoes infected with *Cylas formicarius or Euseepes postfasciatus* (Uritani and Oshima, 1965). Various coumestans, phaseollin or glyceollin occurred in lima bean (Rich *et al.,* 1977), French bean (Abawi *et al.,* 1971) or soybean (Kaplan *et al.,* 1980) infected with *Pratylenchus Beer, P. penetrans* or *Meloidogyne incognita* respectively. *M. incognita* also caused several terpene aldehydes, including gossypol, to accumulate in cotton (Veech, 1978).

2.1.3.5 Chemicals (abiotic elicitors)

Many chemicals cause phytoalexins to accumulate; the same chemical causing production of phytoalexins by different plant species. Salts of heavy metals, e.g. mercury and copper, induce accumulation of many phytoalexins. Isoflavonoids were produced by pea (Perrin and Cruickshank, 1965). French bean (Hargreaves, 1981) and soybean (Yoshikawa, 1978); wyerone and other acetylenes by *Vicia faba* (Mansfield, 1980); 6- methoxymellein by carrot (Coxon *et al.,* 1973); furanoterpenes by sweet potato (Uritani and Oshima, 1965) and sesquiterpenes by potato (Tomiyama and Fukaya, 1975). The amounts of phytoalexins produced were sometimes small and in some investigations could not be obtained from chemically treated tissues (Lisker and Kuc, 1977). Respiratory inhibitors, e.g. sodium iodoacetate, sodium fluoride, potassium cyanide and 2,4-dinitrophenol are also effective in producing isoflavonoids (Perrin and Cruickshank, 1965), sesquiterpenes (Cheema and Haard, 1978) and furanoterpenes (Uritani and Oshima, 1965). Several surfactants can also act as elicitors. Glyceollin was produced by soybeans treated with Triton X-100 or Nonidet P40 (Yoshikawa, 1978); furanoterpenes by sweet potato treated with sodium dodecyl sulphate (Oba *et al.,* 1976) and phaseollin, kievitone and licoisoflavone A by French bean treated with several phytotoxic Triton surfactants (Hargreaves, 1981).

2.1.3.6 Physical agents

2.1.3.6.1 Wounds.

Wounding tissue, e.g. by cutting, bruising or pricking rarely leads to the accumulation of phytoalexins. Phytoalexins could not be detected in water placed on pea pod tissue or on tobacco leaves which had been pricked with a sterile needle (Cruickshank and Perrin, 1963). However, extract of leaves of *Vicia faba,* which had been bruised, became fungi toxic, although the identity of the active components, possibly acetylenic phytoalexins, was not established (Deverall and Vessey, 1969).

2.1.3.6.2 Partial freezing.

It is a general observation that phytoalexins are not present in extracts of uninfected or untreated tissues which have been obtained either before or after storage at -20 °C and phytoalexins do not accumulate when completely frozen tissues are thawed and incubated (Rahe and Arnold, 1975). However, these workers found that phaseollin was produced in French bean hypocotyls which had been frozen by touching with solid carbon dioxide at separate sites along their length. Phaseollin was generally restricted to the wounded areas. This discovery was extended by the finding that several isoflavonoids were produced by hypocotyls which had been thawed and incubated after being partially frozen by placing at - 20°C for 10 to 20 minutes or by contact with liquid nitrogen.

2.1.3.6.3 Irradiation

Short wavelength (254 nm) UV light is cytocidal or, at sub-lethal doses, mutagenic. It is another important method of producing phytoalexins in significant quantities. In 1971, Hadwiger and Schwochau obtained pisatin from irradiated pea pods and more recently several other isoflavonoids have been isolated from various legumes (Ingham and Dewick, 1980; Martin and Derwick, 1979; Munn and Drysdale, 1975; Reilly and Klarman, 1980; Weinstein *et al.,* 1981). The effect of UV light is not restricted to legumes. Irradiation (260 to 270 nm) was the major treatment for the production of pterostilbene and resveratrol from *Vitis vinifera* (Langcake and Pryce, 1977) and also caused rishitin and lubimin to form in potato tuber tissues (Cheema and Haard, 1978). Gamma irradiation caused pisatin to form in pea leaves (Hadwiger *et al.,* 1976).

2.1.4 Toxicity of Phytoalexins

Compounds recognized as phytoalexins come from a wide variety of chemical classes. They possess different modes of action and also exhibit differential toxicity across much of the biological spectrum (prokaryotic and eukaryotic) and exhibit inhibitory effects, for example, by inactivating extracellular enzymes (Bull and Smith, 1981). They are fungitoxic, antibacterial, phytotoxic and also exhibit animal toxicity.

2.1.4.1 Fungitoxicity

The mode of action of any toxic agent is usually derived by determining the earliest physiological effect on the target organism. Two principal features of activity that phytoalexins probably represent as multi-site toxicants and the dysfunction of membrane systems, particularly the plasmalemma, is instrumental in their toxicity. Examples of evidence supporting multi-site activity are: (a) many phytoalexins are broad-spectrum toxicants; (b) a variety of biochemical and physiological repercussions are apparent soon after fungi are treated, such that many "secondary" effects apparently exist; (c) comparatively high concentrations (10^{-4}M) are often required to achieve inhibition; (d) indiscriminate binding probably occurs; and (e) where tolerance is found, detoxification is often involved (Bull, 1981; Fuchs *et al.*, 1980; VanEtten and Bateman, 1971). Such multi-site activity may be of selective advantage to the plant. Site-selective compounds are probably more easily countered by fungi as alterations in only one fungal gene may be sufficient to induce a change at the site of action and so confer resistance (Delp, 1980).

The preceding considerations of common cytological and physiological effects of phytoalexins underline the likelihood that membrane dysfunction is an important part of the mode of action. Substantial loss of dry weight, leakage, swelling and bursting of affected hyphae, all testify to membrane damage, particularly damage to the plasmalemma (Fig 2.1B). The apparent importance of a lipophilic nature for many of the compounds would also be consistent with such an activity. Multi-site toxicants would be capable of affecting other reactions, resulting in interference with

respiration (VanEtten and Bateman, 1971), inhibition of enzymes (Bull and Smith, 1981; Ravise and Kirkiacharian, 1976), and possible prevention of cell wall biosynthesis.

For many phytoalexins, there may be no one site of action, but a range of targets. Selective inhibition of one particular intracellular process, such as protein synthesis, respiration, or nucleic acid transcription seems unlikely, since a considerable time would probably have to elapse before secondary effects would be apparent, and ultrastructural damage would not be evident after only a few minutes treatment, as is the case for several terpenoids and isoflavonoids (Bull, 1981; Harris and Dennis, 1977; Smith and Bull, 1978). A disruption of preformed components necessary for cellular maintenance (e.g. of the cytoskeleton) would also be consistent with some of the immediate effects observed (Smith and Bull, 1978).

2.1.4.2 Antibacterial activity

It is difficult to generalize from results obtained with individual phytoalexins in various assays against different species or isolates of bacteria. Even the effect of a single phytoalexin on the same bacterium is greatly affected by the test conditions (Lyon and Bayliss, 1975). As with the fungi, estimates *in vitro* of phytoalexin toxicity towards bacteria are affected by many procedural variables, including the type of assay employed, the composition of the medium, inoculum density and incubation period.

Rishitin (360 μg/ml) caused a decrease in the viable count of *Erwinia atroseptica* from 10^9 cells/ml to < 3 cells/ml in 4 hours at 25 °C when incorporated in 0.1% peptone water medium widely used to maintain bacterial viability without allowing a significant increase in viable counts. The effectiveness of related compounds, however, varies considerably. Whereas rishitin was bactericidal, phytuberin and spirovetiva-1, 1-1-dien-2-one were not, even when much higher concentrations were employed (up to 800 μg/ml).

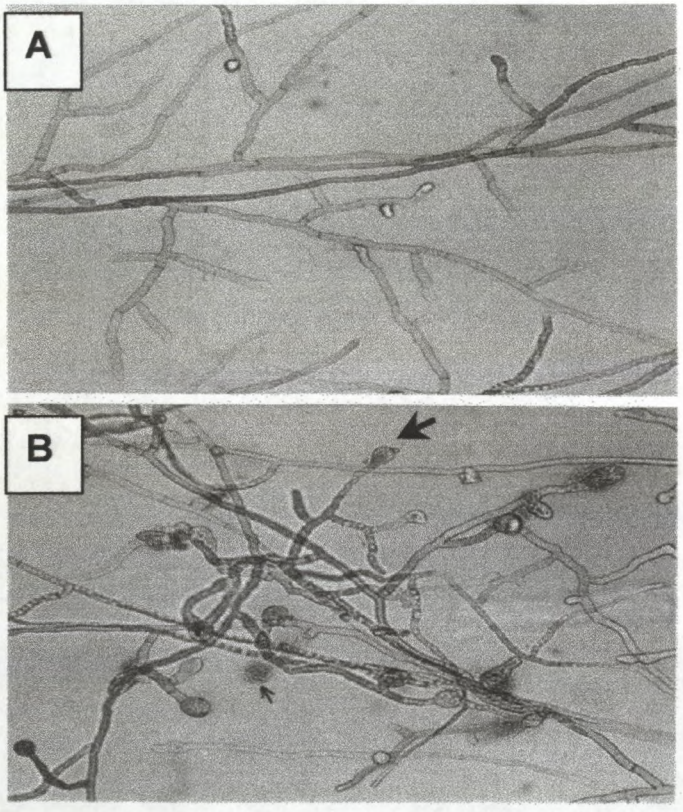


Figure 2.1 Observation of phytoalexin fungitoxicity by light microscopy. (A) Typical hyphal growth of *Rhizoctonia solani* on agar, 24 h after inoculation. (B) Typical hyphal growth of *R. solani* on agar incorporating 25 μg kievitone/ml in 0.5 % ethanol, 24 h after inoculation. (x 175). Note in (B) the disorganized pattern of growth and frequency of swollen hyphal tips, some of which have lysed (arrowed).(Smith and Bull, 1978).

2.1.4.3 Animal toxicity

Two quite separate considerations arise with respect to animal toxicity. Firstly, there is the possibility that phytoalexin accumulation is an effective defensive component useful in protecting plants against animal pathogens, particularly insects (Jacobson, 1977; Russell *et al.,* 1978; Sutherland *et al.,* 1980), and nematodes (Kaplan *et al.,* 1980). Secondly, there may be toxic consequences to animals, including humans, consuming plant tissues containing phytoalexins (Kuc and Currier, 1976). Instances of acute poisoning occurring after ingestion of diseased plant tissues are of historical significance, though the toxic chemicals are usually of microbial, not plant origin (Uraguchi and Yamazaki, 1978). Nevertheless, potentially serious problems might arise if new resistant crop varieties are selected on the basis of the ability to accumulate toxic chemicals when stressed.

One of the most striking concerns the observations that several isoflavonoid phytoalexins- including glyceollin, kievitone, medicarpin, pisatin and phaseollin (Bull, 1981; Oku *et al.,* 1976; VanEtten, 1972; VanEtten and Bateman, 1971) will lyse red blood cells. The bright, refractive appearance of human erythrocytes (Figure 2.2A) changed considerably after a ten minute exposure to phaseollin (45ug/ml) (Figure 2.2B); haemoglobin was lost and the individual cells exhibited something of the echinocytic change noted using pisatin (Oku *et al.,* 1976)

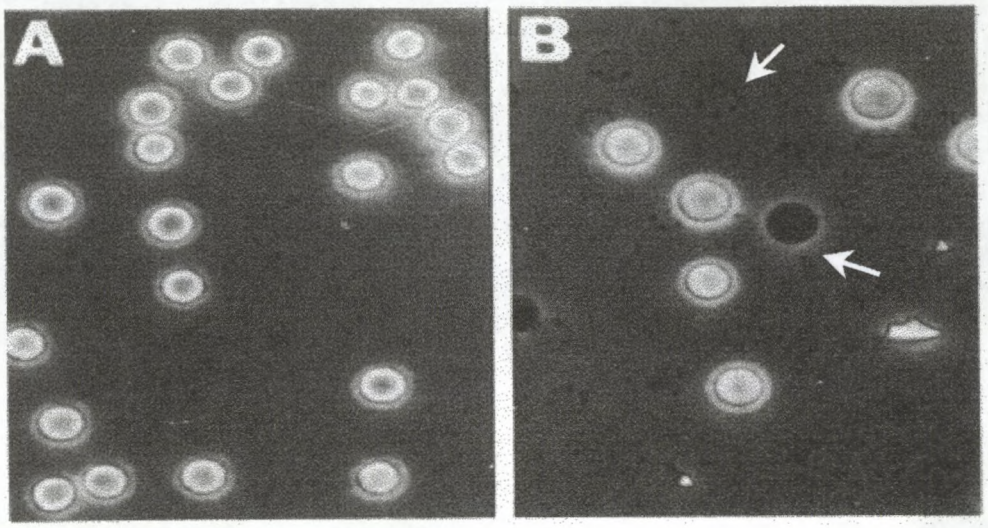


Figure 2.2 Observation of phytoalexin toxicity to animal cells by phase contrast microscopy. (A) Phase contrast photomicrograph of human red blood cells exposed to ethanol (0.5 %) for twenty minutes. Note the bright, refractive appearance. (X 630). (B) Phase contrast photomicrograph of human red blood cells ten minutes after treatment with phaseollin (45ug/ml) in 0.5% ethanol). Note that many of the cells are no longer bright and refractive, and now appear as "ghosts" with small dark inclusions (x 630).

Haemolysis is often more rapid, being almost complete in ovine erythrocytes five minutes after treatment with 30 ug phaseollin/ml (VanEtten and Bateman, 1971). Apart from lysis of red blood cells, reports exist citing numerous and, apparently, miscellaneous, toxic effects of phytoalexins. Ipomeamarone (Uritani and Oshima, 1965) and pisatin (Oku *et al.,* 1976) repressed respiration in isolated rat liver mitochondria; ipomeamarone inhibited electron transport and oxidative phosphorylation, whereas pisatin served as an uncoupler.

It is not only isolated systems that are affected; whole animals are also vulnerable. Ipomeamarone derivatives, 4-ipomeanol, 1-ipomeanol, ipomeamine and 1,4-ipomcaidol, produced pulmonary oedema, congestion, and even death, in mice

(Boyd *et al.,* 1973; Kuc and Currier, 1976). Liver and kidney damage may also arise. Concern is justified by the occurrence of these compounds, which are not destroyed by heat, in slightly blemished roots of sweet potato available for human consumption. The most serious danger, both in terms of the amounts occurring in plant tissues and its toxicity, seems to be associated with 4- ipomeanol.

2.1.4.4 Phytotoxicity

Many phytoalexins, including some furanoacetylenes, isoflavonoids and terpenoids, have been shown to be phytotoxic. Cell death and darkening of potato tuber slices has been observed on rishitin infected potatoes (Ishiguri *et al.,* 1978) and rishitin-incited lysis of potato and tomato protoplasts (Lyon and Mayo, 1978) has also been observed. Lyon (1980) investigated the effect of rishitin on potato leaf tissues and concluded that its phytotoxicity reflects a primary site of action on membranes. Lyon (1980) postulates that rishitin may increase membrane fluidity and so contribute to cell dysfunction. Glazener and VanEtten (1978) noted cell death and initial reduced growth of suspension cultures after phaseollin treatment of *Phaseolus aureus and P. vulgaris.* Although both species seemed able to metabolize the phytoalexin, enhanced tolerance of *P. vulgaris* was not evident upon treatment with low doses prior to higher concentrations; this is unlike the situation with some fungi (Van den Heuvel and VanEtten, 1973).

2.2 CONSTITUTIVE COMPOUNDS

2.2.1 General Overview

A fungal spore landing on the surface of the plant has to combat a complex series of defense barriers set up by the plant before it can germinate, grow into the plant tissues and survive. The arsenal of weapons against the fungus includes physical barriers and chemical ones. The chemical compounds can be preformed in the plant, the so-called "constitutive antifungal substances". Many antifungal compounds from plants have been reported (Grayer and Harborne., 1994). These are mainly flavanoids and isoflavonoids. The flavonones of *Humulus lupulus* (hops) have shown high antifungal activity against *Trichophyton mentagrophytes* and *T. rubrum.*

Tanaguchi *et al.* (1993) screened East African plants for antimicrobial activity. The plant specimens were collected mainly on the basis of information gathered from 'Bwana Mganga" (Swahili medicine man) on medicinal plants. Seventy-nine extracts representing seventy-two species were subjected to microbial tests. Of the seventy-nine extracts, forty gave initial positive results, with sixteen being shown to be antifungal against *Saccharomyces cerevisiae* and *Penicillium crustosum.*

Alves *et al.* (2000) screened sixty medicinal plant species from Brazilian savanna that could contain useful compounds for the control of tropical disease. Plant extracts were screened for molluscicidal activity, brine shrimp toxicity, antifungal acitvity and antibacterial activity. Forty-two species afforded extracts that showed

some degree of activity in one or more of these bioassays. Kott *et al.* (1999) screened five species of Argentine medicinal plants for antiviral activity. Aqueous extracts of the medicinal plants were assayed *in vitro* to detect antiviral activity against herpes simplex virus type 1 (HSV-1), respiratory syncytial virus (RSV) and adenovirus serotype 7 (ADV-7). *Polygonum punctatum*, *Lithraea molleoides*, *Sebastiania brasiliensis, Sebastiania klotzschiana* and *Myrcianthes cisplatensis* showed *in vitro* antiherpetic activity with 50% effective dose (ED50) ranging from 39 to 169 μg/ml. None of the extracts had antiviral activity against RSV and ADV-7.

Plants need to protect themselves against predators. This is often done by producing repellents, antifeedants and toxins. An antifeedant has been defined (Munakata, 1975) as a compound that inhibits feeding but does not kill the predator directly, although it may die by starvation. For instance, the African neem tree *Melia azedarach* is never attacked by desert locusts, because it contains the powerful antifeedant terpene, azadirachtin (Fig 2.3a) (Nakanishi, 1975).

The sesquiterpene, warburganal (Fig 2.3b), which has been produced by the East African tree *Warburgia stuhlmannii*, is a specific antifeedant against larvae of the african army worm. However, it does not have any repellent effect on locusts. Warburganal interferes with the stimulus transduction process in the chemoreceptor cells of the army worm (Ma, 1977).

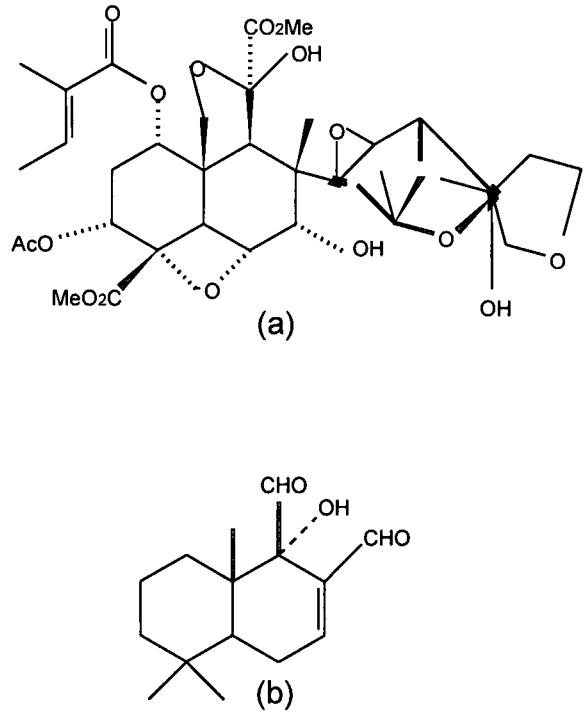


Figure 2.3 Some insect attractants/repellents produced by plants. (a) azadirachtin and (b) warburganal (Ma, 1977).

Warburganal contains an α,β-unsaturated 1,4 –dialdehyde moiety (Fig 2.4) in conformity with several other repellent or antifeedant terpenoids. Their repellency implies a role in the natural defense systems of the host organisms (Cimino *et al.*, 1983). In some cases, the predator does not even have to chew the plant. For instance, aphids have been shown to detect polygodial (Fig 2.6), another plant metabolite containing unsaturated dialdehyde functionality, with sensilla located on the antennal tips (Powwell *et al.*, 1995). Until now, 81 terpenoid unsaturated

dialdehydes have been isolated from most kingdoms in nature. Most of them are sesqui-, di-, or sesterterpenes, while a few are monoterpenes.

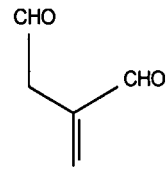


Figure 2.4 The α,β-unsaturated 1,4-dialdehyde moiety.

Several unsaturated dialdehydes with a drimane skeleton have been isolated from Cannellaceae, a small family of plants consisting of nine species, grouped into four genera. Of these, *Winterana* and *Cinnamodendron* are endemic to South America, *Warburgia* to East Africa and *Cinnamosma* to Madagascar. Five unsaturated dialdehydes have been isolated (Kubo *et al.*, 1977) from the stem bark of *Canella winterana,* a tree that grows in the subtropical areas of the Florida Keys and throughout the Caribbean. The East African genus *Warburgia* consists of two species, *W. stuhlmannii* and *W. ugandensis*. The barks of these are employed widely in folk medicine and as spices in food. The bark extract, containing some unsaturated dialdehydes, possesses antifeedant activity against African armyworms *Spodoptera littoralis* and *S. exempta*, widely occuring *African* crop pests.

Another important field of research involving *Warburgia* spp. is its application as plant molluscicides. In rural communities the cost of synthetic molluscicides or chemotherapy prohibits their use thus plant molluscicides provide a cheap, effective and enviromentally acceptable alternative. Studies carried out by Clarke *et al.* (1997)

show that *W. salutaris* had the greatest potential for cultivation and also showed the highest molluscicidal activity.

Although a lot of work has been conducted on the attractant/repellent properties and molluscicidal activity of medicinal plants, very few studies have focused on the effect of medicinal plants or plant extracts on mycotoxigenic fungi. This type of research has only recently come into focus thus this study concentrates on the effect of aromatic compounds, sesquiterpenoid dialdehydes (isoprene compounds) on mycotoxigenic fungi.

2.2.2 Plants used in this study

2.2.2.1 *Crocosmia aurea* (Pappe ex. Hook.) Planch

These plants are deciduous herbs with globose corms with membranous or fibrous tunics. Extracts of bulbs of *C. crocosmiiflora* (Nicholson.) showed strong antitumour activity against *Erlich carcinoma* (Nagamoto *et al.*, 1988). The active principles are saponins with medicagenic and polygalacic acid as the major sapogenins and glucose, xylose, arabinose, rhamnose and fucose as the sugar moieties. Various anthocyanin flavonoids have been reported in this genus (Gibbs, 1974). Zulu medicinal usage include the treatment of dysentery, or diarrhoea using corn decoctions and also for the treatment of infertility (Gerstner, 1941). Watt and Breyer-Brandwijk (1962) also suggested that the Sotho used *C. aurea* for human and bovine diarrhoea. Plant decoctions are reported to act as sedative diuretics (Bryant, 1966).

2.2.2.2 *Scenecio serratuloides* DC. var. *serratuloides*

Tea made from leaves is taken against infections and leaves are directly applied to purulent sores as a drawing agent. Leaf decoctions are taken as blood purifiers for skin eruptions (syphilis), and powdered leaves or powder from charred roots is applied to burns and sores (Watt and Breyer-Brandwijk, 1962). Mixtures of unspecified parts of the plant are taken in small doses for swollen gums and chest pains and has been suspected of causing the death of a woman who had taken a large dose of the mixture of unspecified parts of the plant as a purgative (Watt and Breyer-Brandwijk, 1962). The symptoms indicated a slow irritant poisoning. Extracts tested on cats produced weakness, drowsiness and slowing of respiration, followed by unsteadiness on the legs, slight transitory salivation and purgation.

2.2.2.3 *Chrysanthemoides monilifera* (L.) var. *canescens*

Infusions from the leaves are administered as enemas for feverish conditions (Palmer and Pitman, 1972). Small frequent doses of juice from the fruit are reported to be administered by the Zulu, Xhosa and Sotho as blood strengtheners and purifiers to men suffering from impotence or weakened by intestinal ailment (Roberts, 1990). They are also used by adolescents to clear up pimples. In Lesotho, leafy branches are burned as a cure in the huts of madmen (Jacot-Guillarmod, 1971). The ash of this plant contain alkali from which soap has been made (Palmer and Pitman,1972).

2.2.2.4 *Bulbine frutescens* (L.) Willd.

Application of leaf sap is used to treat eczema and similar itchy conditions (Watt and Breyer-Brandwijk, 1962). Leaf and tuber decoctions are also taken for these ailments. Tubers are sometimes rubbed on the limbs of children who suffer from undeveloped muscles in the belief that this will strengthen their muscles. Unspecified plant parts are used as ingredients in infusions taken by young men to harm the rivals of their unfaithful girlfriends (Bryant, 1966). Decoctions are taken by groups of men and adolescent boys in purification rites aimed at the prevention of antisocial behavior (Hutchings, 1989). Tuber preparations are used for urinary complaints, dysentery and diarrhoea, and also for convulsions in children (Pujol, 1990). Smith (1895) cites reports of use of the tubers for lumbago by the Xhosa and Dutch settlers, who also used tubers for rheumatism and as blood purifiers.

Plant preparations are reported to act as sedative diuretics (Bryant, 1966). Tubers are reported to have blood-purifying properties and also to produce depression, acidity and flatulence (Smith, 1895). Small doses tend to produce constipation but large doses produce diarrhoea.

2.2.2.5 *Bridelia micrantha* (Hochst.) Baill.

Bark infusions are taken as emetics. Decoctions made from roots with a pinch of roasted, powdered roots of a *Dichrostachys* species are taken or rubbed into incisions for lung pain (Palmer and Pitman, 1972). Leaves and stembark are used

for coughs and diabetes and also as laxatives in West Africa (Dalziel, 1937). Leaf sap is used by the Haya for sore eyes and in decoctions for conjunctivitis in West Africa (Watt and Breyer-Brandwijk, 1962). Roots are used for severe epigastric pain and as purgatives or powdered and rubbed into the scalp for headaches in various parts in Africa. The Vavenda use barks for burns, wounds, venereal disease and toothache, and leaves for painful eyes, fevers and headaches (Mabogo, 1990). In Zimbabwe, roots are used for infant's coughs while leaves are used as abortifacients and also as charms to liberate prisoners (Gelfand *et al.* 1985). In East Africa, bark decoctions are taken for stomach ache, tapeworm and diarrhoea and are also administered as tonics to children (Kokwaro, 1976). Root decoctions are taken for aching joints and pounded bark infusions are used to combat snoring in cattle.

The death of a patient four hours after taking cough medicine made from the plant has been reported (Verdcourt and Trump, 1969). Chemical constituents from *Bridelia micrantha* include friedelin, taraxerone, epifriedelinol, taraxerol, gallic acid and ellagic acid. The presence of the anthocyanidin, delphinidin and caffeic acid was indicated in the leaves (Verdcourt and Trump, 1969).

2.2.2.6 *Lippia javanica* (Burm. f.) Spreng.

Hot leaf infusions are widely used for coughs and colds, most frequently as inhalants but also taken orally. Leaves are also used to treat febrile rashes and are sometimes smeared on the body as a protection against dogs and crocodiles (Roberts, 1990). They are also used in washes and poultices for chest ailments

(Roberts, 1990). Poultices from leaves are also applied to warm up the lower limbs. Cold leaf infusions are taken for a condition referred to as gangrenous rectitis (Bryant, 1966).

Weak leaf and stem infusions are taken for coughs, colds and bronchial ailments and, with the addition of *Artemisia afra* Jacq. ex Willd., are also used for fevers and measles by the Xhosa (Smith, 1895; Watt and Breyer-Brandwijk, 1962). Plants are also used to disinfect suspected anthrax-infested meat. Leaves are used for a variety of ailments including asthma, headaches, febrile and respiratory complaints, convulsions, weak joints, cataracts and sore eyes in Zimbabwe (Gelfand *et al.* 1985). Roots are used for headaches, scabies and backache, and infusions are used to bathe marasmic infants. The Vavenda use leaf infusions as anthelmintics, for respiratory and febrile ailments and as prophylactics against dysentery, diarrhoea and malaria (Mabogo, 1990). Powder from burnt roots is applied to scarifications made around sprained joints. Roots are used as antidotes for suspected food poisoning and for bronchitis and sore eyes in Botswana (Hedberg and Staugard, 1989). In West Africa, leaves and roots are used for fevers, headaches and skin diseases (Dalziel, 1937; Irvine, 1961).

Leaves are strongly aromatic. Photosensitivity has been produced in sheep (Kellerman *et al.* 1988). The icterogenic principles, pentacyclic triterpenoids known as lantadene A and lantadene B have been isolated (Louw, 1943; Louw, 1948; Louw, 1949). Lantadene A is responsible for the photosensitisation in sheep and

cattle. Leaves yield stearic, palmitic, myristic, oleic, arachidic, behenic and lignoceric acids and triacontane alkanes (Neidlein and Staehle, 1973). The main acid components of a waxy fraction were found to be lauric (26,5%), myristic (11,6%) and palmitic (47,2%) acids and the neutral fraction included hexacosanol (58,2%), triacontanol (19,8%) and docosanol (12,9%) alcohols (Neidlein and Staehle, 1973). Alanine, asparagine, arginine and proline were the main amino acids while the main essential oil components were caryophyllene, linalool and p-cymene (Neidlein and Staehle, 1974). Glucose was the only sugar component found and choline was also present. Preliminary screening tests indicate the presence of platelet activating factor (PAF) antagonists in extracts from leaves (Munjeri and Chagwedera, 1988). The extracts inhibited histamine- and (PAF)-induced contractions in guinea-pig trachea.

2.2.2.7 *Warburgia salutaris* (Bertol. f.) Chioy.

A pinch of powdered bark is taken in a spoonful of cold water or smoked, mixed with *Cannabis sativa* L. leaves, for a dry cough (Bryant, 1966). Bark is used in emetics or purgatives for febrile complaints and for rheumatism or the ailments known as *isibhobo or amanxeba,* which are traditionally thought to be caused by sorcery. These ailments appear to be mainly intercostal neuralgia but may also be rheumatism or a symptom of liver disease. Powdered bark mixed with fat is used as ointment for penile irritation. Lotions made from pounded leaves with stalks of *Hibiscus surattensis* L., are applied to the penis in cases of inflammation of the urethra, sores and other irritation. Ointments from pounded leaves and stalks, mixed

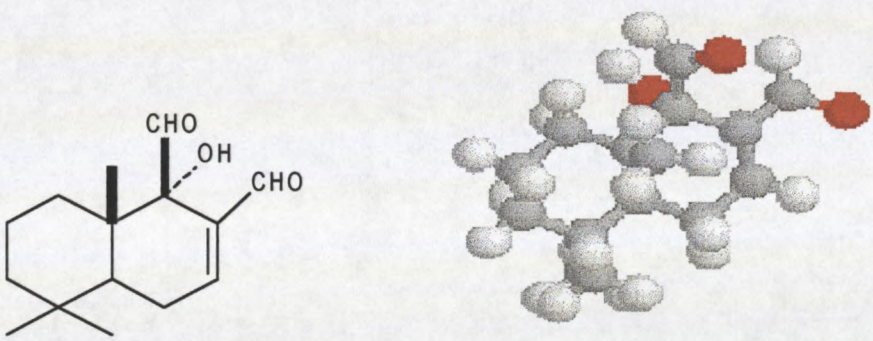
with bark and any kind of fat, may also be used. Cooked roots are taken for coughs (Gerstner, 1939).

Root and stembark are widely used in southern Africa as expectorants or smoked for colds and coughs (Watt and Breyer-Brandwijk, 1962). Bark is used in Tanzania for toothache. The Vavenda use the bark to make dogs more alert and ferocious and to make bees aggressive and thus protected. In Kenya, bark is used for constipation and stomach ache (Johns *et al.* 1990). Bark and roots are used in West Africa for influenza, fevers, pains, stomach ache and other gastro-intestinal disorders (Dalziel, 1937).

Preliminary screening of extracts from inner bark show highly toxic effects in experiments currently being undertaken at the University of Stellenbosch. These may be related to the haemolytic and cytotoxic properties which are known in warburganal (Nakanishi, 1982a). Inner bark, rootbark and leaves have a pungent ginger or pepper-like taste (Watt and Breyer-Brandwijk, 1962). Crushed leaves emit a strong odour of eugenol and taste peppery. Bark smells of cinnamon.

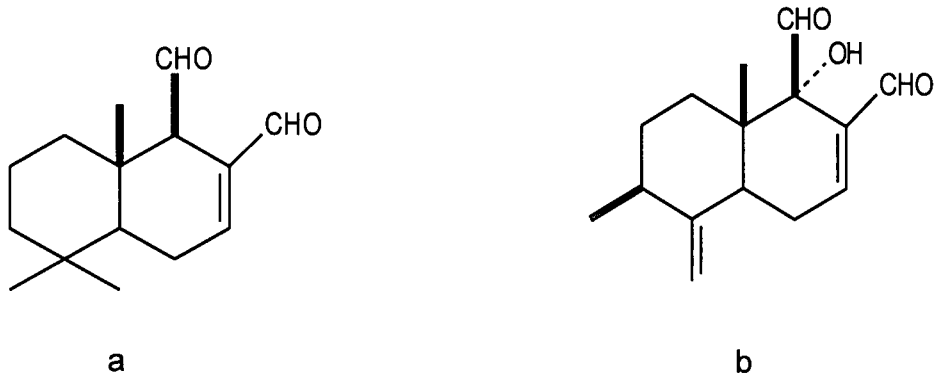
Bark is reported to contain tannin and about 3% mannitol (Watt and Breyer Brandwijk, 1962). Sesquiterpenoid dialdehydes isolated from bark include warburganal, muzugadial, polygodial, mukaadial and ugandensidial (Nakanishi, 1980; Warthen *et al.* 1983; Taniguchi and Kubo, 1993). Drimenin from the bark has insect antifeedant properties. Warburganal (Fig 2.5a and 2.5b), a potent antifeedant

against *Spodoptera eempta* (the African armyworm), is also strongly cytotoxic and has haemolytic properties (Nakanishi, 1982). Warburganal, muzugadial (Fig 2.6a) and polygodial (Fig 2.6b) showed broad antimicrobial activity against all of various yeasts and moulds tested and were highly active against *Saccharomyces cerevisae, Candida utilis and Sclerotinia libertiana* (Taniguchi and Kubo, 1993). Polygodial showed the most potent activity against these microorganisms and displayed activity comparable to that of amphotericin B. It has been found to significantly enhance the antifungal activities of actinomycin D and rifampicin and also to synergise the antifungal activity of maesanin against *Candida utilis* (Yano *et al.* 1989). Stem and rootbark have been reported ineffective in experimental malaria (Watt and Breyer- Brandwijk, 1962)



a b

Figure 2.5 A drimane-type sesquiterpene dialdehyde, warburganal (a) and ball and stick configuration (b) (Nakanishi, 1982).



a b

Figure 2.6 Polygodial (a) and Muzigadial (b) are related drimane-type sesquiterpenoids which showed intense antifeedant and plant growth inhibitory activities (Taniguchi and Kubo, 1993).

2.2.3 Biochemical Pathways for the Production of Isoprene Compounds

The basic biochemical pathways for isoprene production are similar in both plants and animals. It is to the secondary plant products, that the majority of plant drugs owe their therapeutic activity. The production of secondary metabolites is dependent on the fundamental metabolic cycles of the living tissue (Fig 2.7). Photosynthesis is the initial pathway involved in the production of isoprene compounds (triterpenoids, sesquiterpenoids, steroids and other basic terpenes). It is a simple process that involves the conversion of CO_2 of the atmosphere into sugars as illustrated in this reaction:

$$CO_2 + H_2O \longrightarrow (CH_2O) + O_2$$

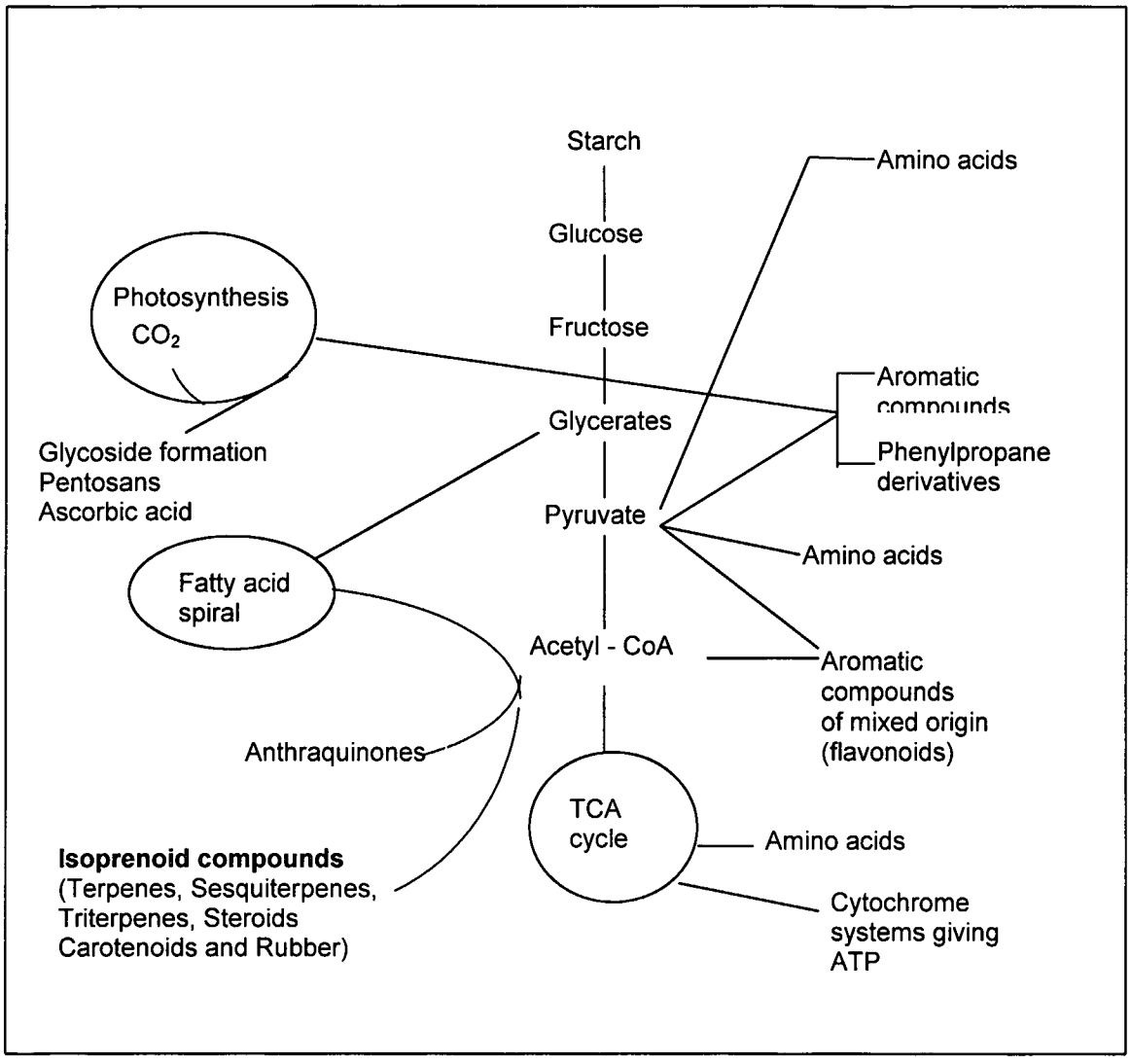


Figure 2.7 Origins of some secondary metabolites in relation to basic metabolic pathways (Cunningham, 1991).

These carbohydrates are then made available for energy production by a process which involves conversion to pyruvate and then Acetyl coenzyme A. A number of pathways for the initial metabolism of glucose are known for various living tissues. For the production of isopreniod compounds, the glucose is metabolized via the Embden – Meyerhof scheme of glycolysis. (Fig 2.8)

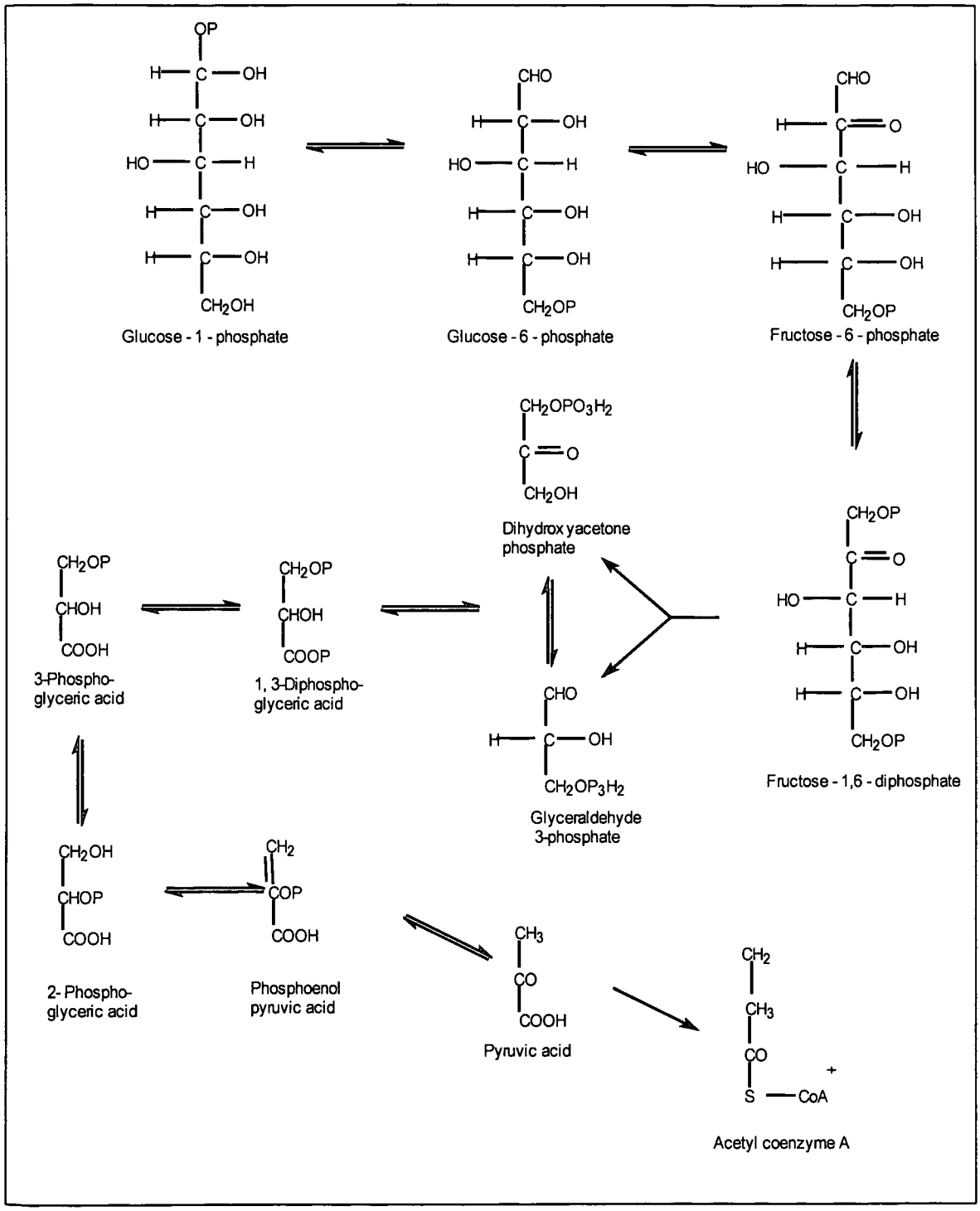


Figure 2.8 Outline of the Embden-Meyerhof scheme of glycolysis (Cunningham, 1991).

Studies on the pyrogenic decomposition of rubber led workers in the latter half of the last century to believe that isoprene could be regarded as the fundamental building block for the production of rubber, carotenoids, triterpenes, sesquiterpenes and steroids. As a result, Ruzicka published in 1953 his "biogenetic isoprene rule". The value of this rule lay in its broad unifying concept, which allowed postulation of a rational sequence of events that might occur in the biogenesis of unrelated compounds. Examples of various compounds to which this rule can be applied are indicated in Figure 2.9.

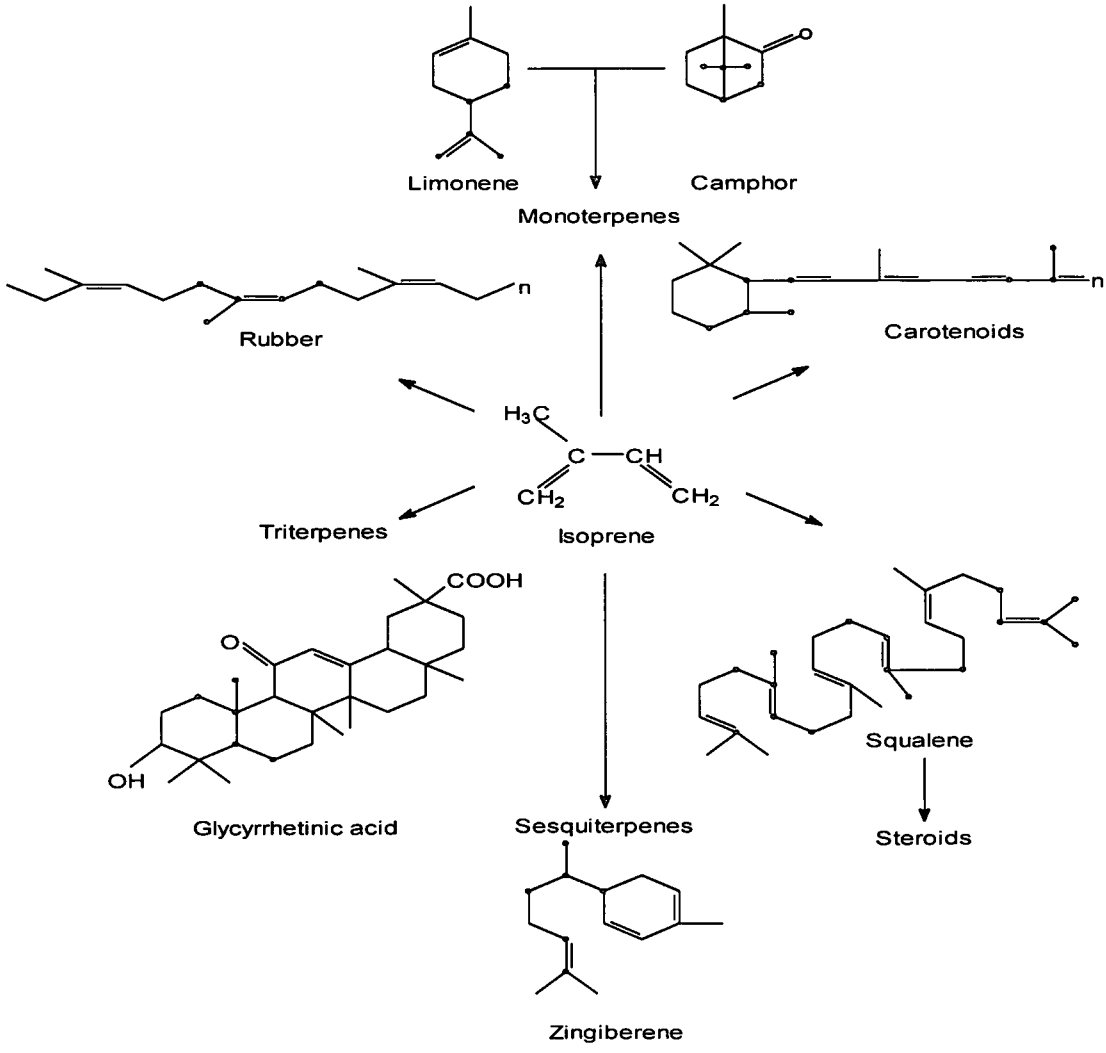


Figure 2.9 Application of the isoprene rule (Cunningham, 1991).

The discovery of acetyl CoA in 1950 gave support to the role of acetate in the biosynthetic processes. The next major advance was the discovery, in 1956, of mevalonic acid. Mevalonic acid (3, 5 –dihydroxy 3-methylvaleric acid) is a 6-carbon acid and is not the active isoprene unit, which forms the basic building block of isoprenoid compounds. During the next four years of research, it was established that the 6-carbon compound for which the biochemists had been seeking so long, was isopentylpyrophosphate, derived from mevalonic acid pyrophosphate by decarboxylation and dehydration. Isopreniod synthesis then proceeds by the condensation of isopentylpyrophosphate with the isomeric dimethy allyl-pyrophosphate to yield geranylpyrophosphate. Further 5-carbon units are added by the addition of more isopentenylpyrophosphate. Preliminary stages of the biosynthesis of isoprenoid compounds are shown in Figure 2.10.

From geranyl and farnesyl pyrophosphate various structures can be built up including sesquiterpenoids (Fig 2.11).

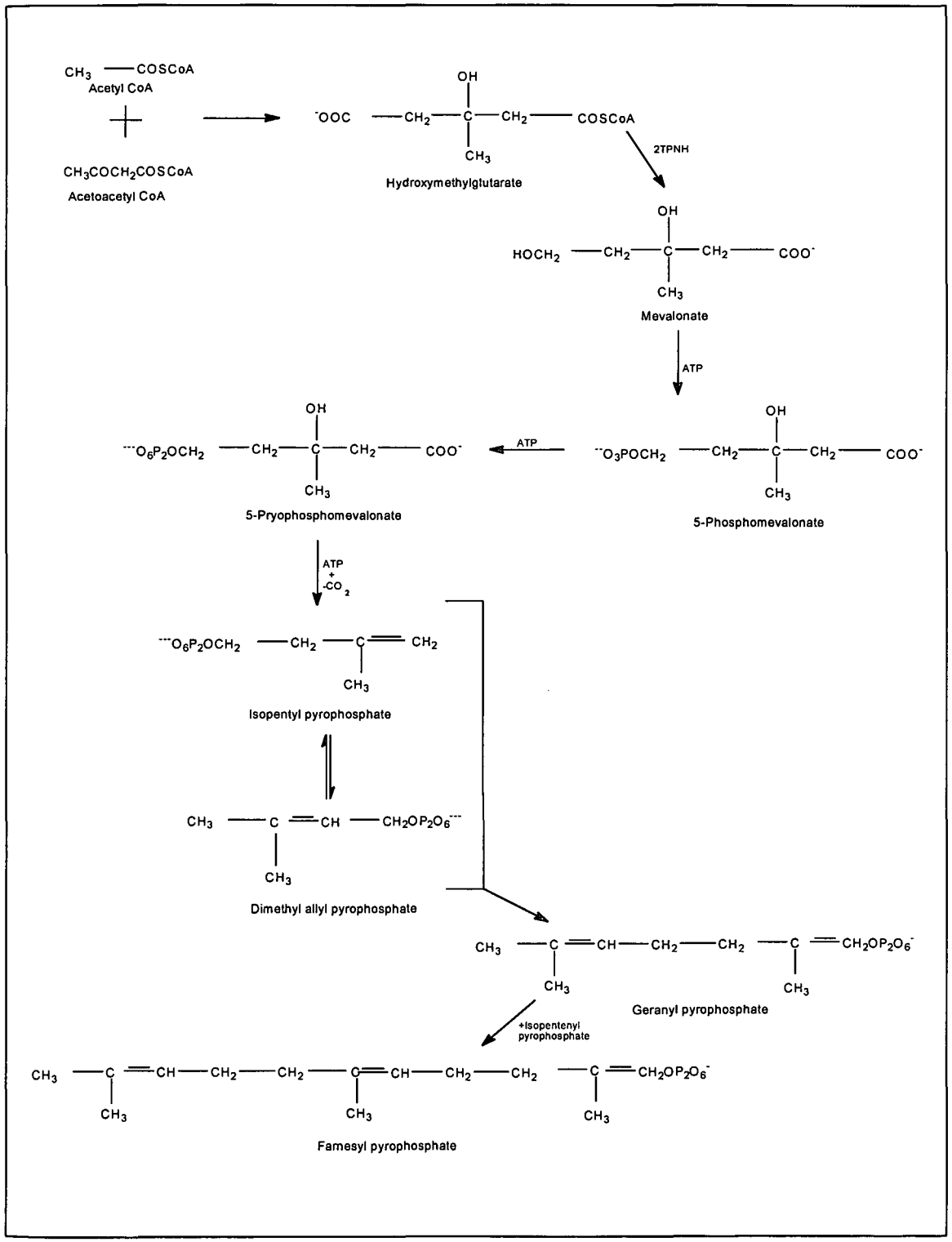


Figure 2.10 Preliminary stages in the biosynthesis of isoprenoid compounds (Cunningham, 1991).

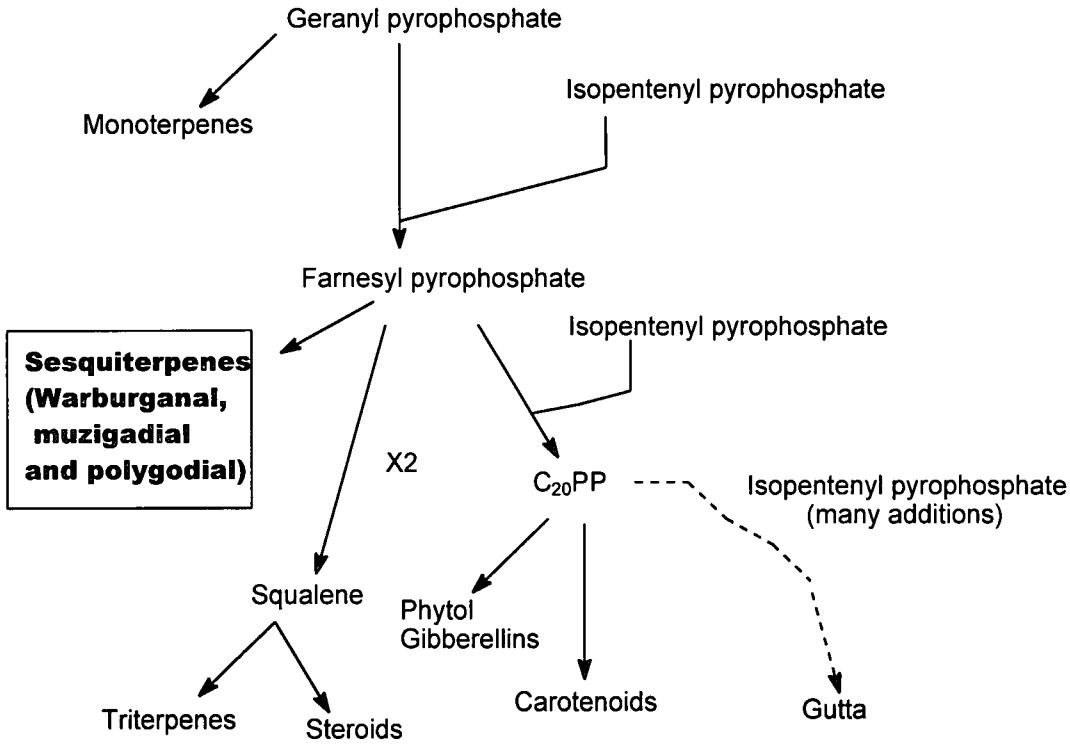


Figure 2.11 Biosynthesis of isoprenoid compounds (Cunningham, 1991).

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 METHODOLOGY: PHYTOALEXINS

3.1.1 Introduction

Lemons (*Citrus limon* cv. Eureka) and oranges (*Citrus sinesis* cv. Valencia) are small fruited relatives of the citrus genus which has not been investigated for its natural antifungal activity. The composition of flavonoids and terpenoids (groups to which many defense-related compounds belong) in lemons and oranges, differ from that of other species (Horowitz and Gentilli, 1977). This work involved the induction of phytoalexin in citrus fruit by UV illumination and *Penicillium digitatum* infection. Isolation of fractions was achieved by Thin Layer Chromatography. Purification and concentration of the isolated fraction was achieved using preparative TLC. The antifungal activity of this fraction against *Aspergillus flavus* and *Fusaruim moniliforme* was verified by direct bioautography and the chemical characterization of the active fraction were determined by UV, IR and ^{1}H NMR spectroscopy. Since 6,7 dimethoxycoumarin is the only known phytoalexin isolated from Valencia fruit, commercially avialable 6, 7 dimethoxycoumarin was used as a reference standard for identification of the induced compound.

3.1.2 Fungal Strains and Growth Media

Aspergillus flavus (ATCC 15546), *Cladosporium cladosporiodes* (CRCC 30812) and *Fusarium moniliforme* (PPRI 1059) were purchased from the American Type Culture Collection (ATCC) and the National Collection of Fungi (PPRI). The cultures were maintained and subcultured fortnightly on Czapek-Dox sucrose agar medium.

Penicillium digitatum (ATCC 10030) was maintained and subcultured monthly as recommended by the American Type Culture Collection on a potato dextrose agar (PDA) (diced potatoes, 300g; glucose, 20g; agar, 15g; distilled water, 1000 ml). This culture was then transferred to Sabouraud Dextrose Agar (SDA) and incubated for 10 days at 26 °C. Spores were collected and concentration was determined with a haemacytometer. The spore suspension was then diluted to a final concentration of 4.2×10^6 ml^{-1} just before inoculation.

All cultures were stored in distilled water with 30% glycerol as the cryo-protective agent in 2 ml cryovials (Dow-Corning) in a –70 °C bio-freezer and were plated out on SDA or PDA plates when required.

3.1.3 Phytoalexin Induction in Citrus Fruits.

Mature fruits of lemons (*Citrus limon* cv. Eureka) and oranges (*Citrus sinesis* cv. Valencia) were obtained directly from Sunrise orchards (Ceres). Fruits of uniform size and appearance, originating from the same orchard, were subjected to fungal infection and UV treatments at random. The citrus fruits were washed with 70% ethanol and divided into four groups for phytoalexin induction, by fungal infection using *P. digitatum* and UV illumination. Fungal treatment of citrus fruit was according to the method used by Kim *et al.* (1991). Spore concentrations of *P. digitatum* were adjusted to 4.2×10^6 spores/ml using a Neubar Counting Chamber. These were inoculated into the fruits by piercing four of each fruit with 100µl spore suspension. Each experiment was carried out in triplicate.

Fruits were also subjected to UV illumination in a chamber with two UV lamps (220 volts UV output) (CAMAG). The fruits were placed 15 cm away from the UV source for 15 -20 minutes and then incubated in darkness after treatment and stored at 30 °C for 30 days. *P. digitatum* infection treatment was also studied over a 30 day time period with duplicate samples being removed at 5 day intervals for analysis. Non-treated fruits were subjected to the same storage conditions and were used as control.

3.1.4 Extraction and Cleanup

3.1.4.1 Preparation of Crude Extract

Extraction procedure used for citrus fruit phytoalexins is a modified method of Kim *et al.* (1991). Citrus fruit tissue (20g) was excised with a scalpel and extracted using an organic solvent, CH_2Cl_2 1:4(w/v) for 48 hours. The tissue was homogenized in an industrial blender (Waring) and the homogenate was filtered *in vacuo* through a Whatman number 1 filter paper. The residue was re-homogenized with the same volume of dichloromethane and filtered *in vacuo*. The combined filtrate was dried over $MgSO_4.7H_2O$ and concentrated *in vacuo* on a Buchi Rotavapor RE 120. The extract was collected, dried under nitrogen gas and kept in a freezer (- 20 ^{0}C) until use.

3.1.4.2 Thin Layer Chromatography of the Crude Extract

Thin Layer Chromatography of the crude extract was carried out by the method described by Kim *et al.* (1991) using 6,7 dimethoxycoumarin as the standard. Crude

extracts (10 mg) were dissolved in dichloromethane (1ml) and 15µl of each crude extract and 6, 7dimethoxycoumarin as well as the phytoalexin standard solution (100 µg/ml) (i.e. reconstituted in dichloromethane) were spotted 15mm away from the base of silica gel 60 F_{254} plates (0.22 mm thickness). Toluene : Ethyl acetate (4:1 ; v/v) was used as the developing solvent and the plates were developed for 45 minutes. The developed plates were dried and exposed to UV light (366 nm) for the detection of the fluorescent compounds. Migration rates were calculated using the following equation:

$$Rf = \frac{\text{Distance of sample from baseline}}{\text{Distance of solvent front from baseline}}$$

3.1.5 Isolation of Antifungal Substances Induced by Irradiation and Infection Treatments

The antifungal activity of isolated plant compounds against *Aspergillus flavus* and *Fusarium moniliforme* was analyzed by the bioautography technique described by Homans *et al.* (1970). In this method the compounds are resolved by thin layer chromatography on two plates and one plate is sprayed and used as a template, the second plate is sprayed with fungal spores in a minimal medium. Zone of inhibition at site of compound indicates presence of an antifungal compound.

3.1.5.1 Sample Collection and Preparative Thin Layer Chromatography

Crude extracts from the irradiated and infected plants were dissolved in dichloromethane and spotted on TLC plates. The TLC plates were developed with toluene: ethyl acetate (4:1; v/v). The developed plates were exposed to UV light (366 nm) to identify the bands showing similar migration rates as the standard (6,7

dimethoxycoumarin). Individual bands were collected and extracted with dichloromethane. The extract was concentrated *in vacuo*, collected in dichloromethane, and rechromatographed with a more polar solvent, toluene : ethyl acetate (1:1). Bands were recollected, extracted and concentrated *in vacuo*. The extract was dried under nitrogen and stored for use in the antifungal assay and for quantitative analysis of the compound.

3.1.5.2 Detection of Antifungal activity on TLC plates

The isolated individual bands were bioassayed for antifungal activity directly on TLC plates with *Cladosporium cladosporoides* as the control organism and *Fusaruim moniliforme* and *Aspergillus flavus* as the mycotoxigenic fungi. Samples (20 μl) of the purified compound were placed onto TLC plates and were developed with toluene : ethyl acetate (1:1; v/v). These plates were air-dried overnight at room temperature and sprayed with *Cladosporium cladosporoides, F. moniliforme* and *A. flavus* (10^5 spores/ml) respectively in a mineral salts stock solution and 30 % aqueous glucose solution. The sprayed plates were placed in a humid plastic tray and incubated at 24 ^{0}C for 5 to 7 days under aseptic conditions. Antifungal activity was indicated by the absence of mycelium around the spot of the compound on the plate.

3.1.6 Chemical Characterization of the Active Fraction

3.1.6.1 High Performance Liquid Chromatography

The quantitation of the antifungal fraction was achieved using the method by Afek and Sztejhberg (1988) conducted on a Merck "La Chrom" liquid chromatograph system equipped with a Merck L7400 UV lamp. Merck Reverse Phase 18 (3.4 X 250 mm, 5 μm particles) column was used for HPLC separations. A guard column (1cm) was also

used. The mobile phase was methanol : water (4:1, v/v) and the flow rate was constant at 0.5 $ml.min^{-1}$ for 12 minutes. Peaks were detected at a fixed wavelength of 335nm. The phytoalexin content in the citrus fruit were calculated from the peak areas using the following equation:

$$A(ng) = G/H \times S$$

where A = ng phytoalexin present in test extract injected into HPLC

G = phytoalexin peak area in test sample

H = phytoalexin peak area in standard

S = amount of phytoalexin injected into HPLC (10µl)

The concentration (C) of phytoalexin present in the test extract in ng/g was calculated using the following equation:

$$C\ (ng/g) = \frac{A \times T \times D}{I \times W}$$

Where A is calculated above,

T = total volume of sample

D = dilution volume used

I = injection volume used

W = test portion equivalent weight (Thiel *et al.*, 1993).

3.1.6.2 UV spectroscopy

The λ_{max} for the compound was obtained by UV spectrophotometry using the Varian DMS 100 double-beam spectrophotometer with 1 cm path length quartz cuvettes using dichloromethane as the reference solvent (λ^{DICH}_{max} nm:229, 292 and 341). The experimental λ_{max} for the unknown compound was compared to the standard (6, 7 dimethoxycoumarin). The corresponding absorbitivity constant (a) was calculated using the Beers Law equation:

$$A = abc$$

Where (A)= absorbance value

(b)= path length

(c) = concentration of the compound ($\mu.mol^{-1}$).

The wavelength ranged from 0 to 350nm.

3.1.6.3 Infrared spectroscopy

The structure of the compound was confirmed with its IR spectrum (γ CH_2Cl_2 $_{max}$ cm^{-1}: 3400, 2970, 2800, 2200, 1450 and 1000) in a Nicolet – OMNIC impact 410 system with a PIKE IR cell. Dichloromethane (MP -97°C; BP 40°C) was used as running solvent. Infrared beam was 9mm in diameter and 9 cm from baseplate. Helium/Neon (He/Ne) laser was used with λ set at 632.8 nm.

3.1.6.4 ^{1}H NMR spectroscopy

Electromagnets with a field of 14000 gauss were used between two pole pieces and the performance specifications for this magnet was stringent because of the high resolution work. The magnetic field sweep was used to maintain field homogeneity. Sweep range was set at 1175 milligauss for the 300 MHz instrument. The radio frequency source (transmitter) had a power output less than 1 watt and the signal detector amplified the signal generated by 10^{5}, which allowed for recording of signal. NMR sample cell: 5mm (outer diameter) glass tube, which contained about 0.4 ml sample. The sample probe (supplied with a driver turbine) rotated the sample tube along its longitudinal axis at 700 rpm^{-1}. This rotation was used to obtain sharper lines and better resolution and it also canceled out effect of inhomogenities in the magnetic field. Deuterized chlorine ($CDCl_3$) was used as the reference solvent.

3.2 Constitutive Compounds

3.2.1 Plant Materials

The plant materials were collected from the University of Zululand herbarium, Kwa-Zulu Natal, South Africa in September 1998. These plants include *Bridelia micarantha*, (Hochst.) Bail., *Warburgia salutaris*, (Bertol.f.) Chiov., *Bulbine fructesce*ns (L.) Willd., *Crysanthermoides monilifera* (L.) T. Norl., *Crocosmia aurea* (Pappe ex Hook.) Planch., *Erythrina latissima* E. Mey., *Scenecio serratuloides* DC. var. *serratuloides*, *Lippia javanica* (Burm. f.) Spreng. Table 3.1 indicates plant name and variety, collector, primary collector and collector number and botanical institutions where herbarium specimens are deposited. Plant species were further divided into leaves, heartwood and bark and the active fractions were extracted by soxhlet extraction and an extraction method outlined by Kardono *et al.* (1990) with a few modifications. The extracted fractions were then tested for antibacterial and antifungal activity and then chemically characterized by UV and IR spectrophotometry, ^{1}H NMR spectroscopy and Gas chromatography – Mass spectroscopy (GC-MS).

3.2.2 Preparation of Extract

3.2.2.1 Soxhlet Extraction

The plant compounds were extracted by soxhlet extraction according to a method by Katsui *et al.*, 1973.

The apparatus for this technique is outlined in Figure 3.1. The material (B) being extracted is placed in the soxhlet basket (C), which is a vessel with perforated sides and bottom so that liquid can fall through it. When gentle heat is applied to the main collection vessel (D), the solvent begins to evaporate and the solvent vapours reach the cold condenser (A) at the top of the flask and begin to liquefy on the sides of the condenser. The re-condensed solvent on the sides of the condenser begin flowing down the sides of the condenser and begin dripping off of drip points on the end of the condenser. This solvent drips into the top of the soxhlet basket where it saturates the material being extracted. The solvent flows through the basket and out the holes in the bottom of the basket carrying the extract with it into the bottom collection vessel. The extract laden solvent falling from the soxhlet basket is dark in colour and as it becomes clearer the process nears completion.

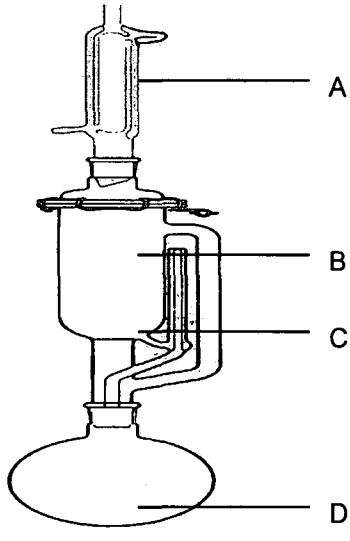


Figure 3.1 Soxhlet apparatus used for the distillation and extraction of plant material. A – condensation chamber, B - plant material, C – soxhlet basket and D – solvent collection vessel.

The leaves, bark and heartwood were air dried and blended. 100 g of powdered plant material was mixed with 95% (v/v) ethanol and was subjected to soxhlet extraction for 48h. The total soxhlet extract was then mixed with 5% HCl/chloroform (v/v) to release the chloroform extractives, acids and neutrals (acid/base separation). 5% sodium hydroxide (v/v) was added to the chloroform extract releasing the neutral components. 90% methanol/hexane (v/v) was added to the neutral components causing the release of the methanol extractives, terpenes, sterols and polar lipids.

Table 3.1 Plant name and variety, collector, primary collector and collector number and botanical institutions where herbarium specimens are deposited.

Plant Name	**Collector**	**Primary collector and number**	**Botanical Institutes**	**Reference**
Bridelia micarantha, (Hochst.) Bail.	A. Hutchings	E. J Moll (1558)	National herbarium, Pretoria	Hutchings *et al.*, 1996
Warburgia salutaris, (Bertol.f.) Chiov.	A. Hutchings	Cunningham (2515)	University of Natal herbarium, Pietermaritzburg	Hutchings *et al.*, 1996
Bulbine fructescens (L.) Willd.	A. Hutchings	Bantu Cancer Registry Collection (308)	University of Fort Hare herbarium	Hutchings *et al.*, 1996
Crysanthermoides monilifera (L.) T. Norl.	A. Hutchings	Cunningham (2104)	University of Natal herbarium, Pietermaritzburg	Hutchings *et al.*, 1996
Crocosmia aurea (Pappe ex Hook.) Planch.	A. Hutchings	Cunningham (2336)	University of Natal herbarium, Pietermaritzburg	Hutchings *et al.*, 1996
Erythrina latissima E. Mey.	A. Hutchings	Gerstner (4656)	National herbarium, Pretoria	Hutchings *et al.*, 1996
Scenecio serratuloides DC. var. *serratuloides*	A. Hutchings	Klusener (5131)	University of Zululand herbarium	Hutchings *et al.*, 1996
Lippia javanica (Burm. f.) Spreng.	A. Hutchings	Gelfand (1895)	National herbarium, Grahamstown	Hutchings *et al.*, 1996

3.2.2.2 Modified Extraction Method (Kardono *et al.*, 1990)

The second extraction method using a protocol outlined by Kardono *et al.* (1990) used solvents of differing boiling points. The plant material (50g) was initially mixed with 50ml petroleum ether and boiled at 45°C to release the ether extractives. The marcerate from the petroleum ether extraction was re-extracted with methanol at 70°C to release methanol extractives. The macerate was then discarded. The methanol extracts were then dried and reconstituted in dichloromethane and hexane. These four extracts were filtered using Whatman No. 4 filter paper and concentrated to dryness using nitrogen. This process is outlined in Figure 3.2.

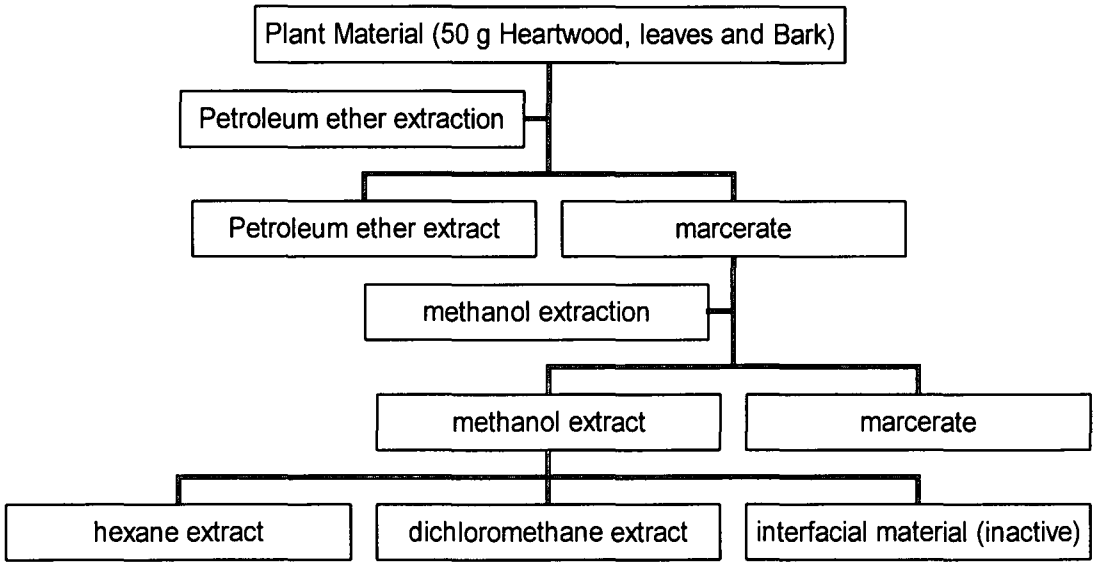


Figure 3.2 Extraction process used by Kardono *et al.* (1990) for the release of the petroleum ether, hexane and dichloromethane extracts.

3.2.3 Preparation of Bacterial Cultures

Six bacterial cultures, *Esherishia coli.*, (ATCC 8739), *Micrococcus luteus* (NCTC 8340), *Bacillus subtilus* (NCTC 3610), *Serratia marcescens* (ATCC 8100), *Klebsiella pneumonia* (NCTC 7427), and *Pseudomonas aeroginosa* (ATCC 25619), were maintained on nutrient agar slants (Oxoid) and was recovered for testing by growth in nutrient broth for 24 hours. *Staphylococcus aureus* (NCTC 6511) was maintained on Blood Agar (Oxoid).

3.2.3.1 Antibacterial Test

The plates were filled with two layers of nutrient agar, each of approximately 15ml (sufficient volume to give a final agar depth of 3 – 4 mm as recommended by British Pharmacopoeia). The lower base layer was poured and allowed to attain an even surface. The plate was allowed to cool and set. Once the base layer was set, 1 ml of the seven bacterial cultures was added aseptically to the molten agar (temperature $\geq$ 45 °C). This was mixed by 'swirling' and poured evenly over the base layer. The poured plate was then allowed to set. The settled plate was placed over a 6 X 6 latin square template and wells were cut at appropriate locations by means of a sterile well cutter. The agar plugs were carefully removed from the wells with a sterile needle, taking care not to damage the surface of the plate or the sides of the wells. 100 µl of the plant extracts was then added to these wells. One blank plate containing nutrient agar and solvents without the extracts served as a negative control. Tetracycline (30 iu) was used

as a positive control for the gram positive bacteria and Penicillin G (5 iu) for gram negative bacteria. Each test was carried out in triplicate.

3.2.3.3 Antifungal Tests

The mycotoxigenic fungi *F. moniliforme* (PPRI 1059) and *A. flavus* (ATCC 15546) were cultivated on Sabourand Dextrose Agar slants (SDA), at 25 °C for 14 and 21 days respectively. The spores were collected in 10ml sterile distilled water and the concentration was adjusted to approximately 10^6 spores/ml. SAB plates were poured and allowed to settle, to form a base layer. Fungal spores were added to molten SAB agar (50 °C) and poured to form a top layer. Wells were cut out using a sterile well cutter, test extracts (100 µl), positive control, (amphotericin B, 5 µg/ml), and negative controls (solvents) only were added to the wells in triplicate. The results were evaluated by measuring the zones of clearing around the wells.

3.2.4 Chromatography of Crude Extracts

3.2.4.1 Sample preparation:

Extracted plant samples were reconstituted in 6 ml of methanol and heated at 60°C for 5 minutes. The sample was filtered with Whatman No. 4 filter paper, and only the clear filtrate was used for TLC analysis.

3.2.4.1 Thin Layer Chromatography and Detection

Method 1

The plant material (5µl) was spotted on silica gel F_{254} precoated TLC plates (Merck). The solvent system used was ethyl acetate: methanol: water (100:13.5:10 v/v/v). Detection was achieved using UV light at 365nm.

Method 2

The plant material (5µl) was spotted on adsorbent silica gel 60 plates. The solvent system used for the first dimension was toluene: ethyl acetate (93:7, v/v), and benzene: acetone (2:1, v/v) was used for the second dimension. Three different spray reagents was used for detection:

Spray reagent 1: Vanillin/sulphuric acid reagent (5% ethanolic sulphuric acid – solution 1, 1% ethanolic vanillin – solution 2. Plate was sprayed vigorously with 10 ml of solution 1 followed by 5 – 10 ml of solution 2. Plates were heated at 110°C for 5 – 10 min).

Spray reagent 2: 30 % sulphuric acid spray reagent (Plates were heated at 100°C for 3 – 5 min and evaluated in visible or UV - 360 nm light)

Spray reagent 3: Dinitrophenylhydrazine reagent (DNPH): (0.1g of 2.4 dinitrophenylhydrazine was dissolved in 100 ml methanol, followed by addition of 1 ml of 36% HCL. After spraying with 10 ml, plate was evaluated under visible light. Spray reagent used for detection of ketones and aldehydes).

3.2.4.2 Circular Thin Layer Chromatography

The solvent migrates circularly from the point of application to form arcs. Silica Gel F_{254}, precoated plates (20 x 20 cm) (Merck, Germany) were used. Toluene/ethyl acetate (93:7) was used as the developing solvent. Two diagonal pencil lines are drawn from the corner of the TLC plate. The centre point of the plate is marked and the circle is drawn around it with a diameter of approximately 2cm. The circle is divided into four segments, the perimeter of each segment was used for the application of 5 μl of plant extract.

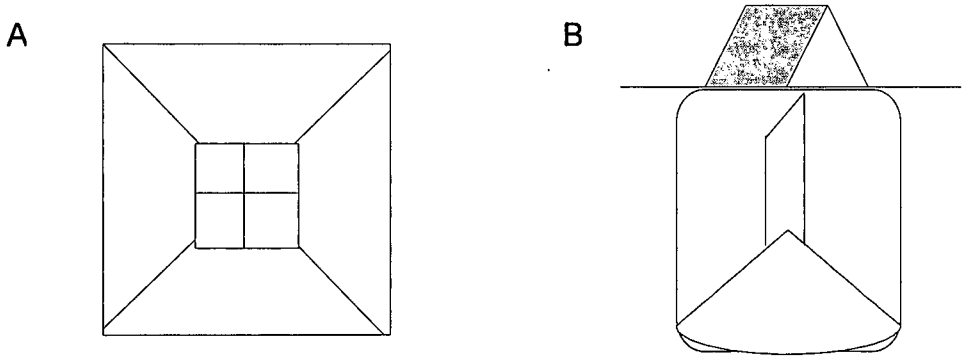


Figure 3.3 Circular Thin Layer Chromatography: A – the perimeter of each segment serves as a point for the application of plant extracts. B – basic apparatus for circular thin layer chromatography.

Development: 100 ml of solvent was placed in 1000 ml beaker (ca. 20 cm diameter), a glass funnel was loosely packed with cotton wool which extends as a wick through the tube of the funnel. The funnel was placed on the solvent system, so that the solvent soaks into the cotton wool. The loaded side of the plate faces downwards, and the TLC plate was placed over the top of the beaker, so that the wick from the funnel makes contact exactly at the marked center. The

solvent spreads as a circle on the TLC plate, and the zones of the plant extract form arcs, which increases in length from the starting point to the periphery of the spreading solvents.

3.2.5 Detection of Antifungal Activity on TLC plates (Bioautography)

Antifungal activity of the active components of the plant extracts was bioassayed directly onto thin layer chromatography plates with *A. flavus* and *F. moniliforme*. 15–20μl of the plant extract was spotted on the TLC plates which was developed with toluene:ethyl acetate (93:7). The plate was air-dried and sprayed with the *A. flavus* and *F. moniliforme* spore suspension (10^6 spores/ml) in a mineral salt: 30% glucose media (Appendix 1). The sprayed plate was placed in a closed humid tray and incubated at 25°C for 72h. Antifungal activity of the plant components was indicated by the absence of mycelia around the migrated compound on the plate.

3.2.5.1 Isolation of active components from plants showing antifungal activity (Preparative Thin Layer Chromatography)

Crude extract of active plant material was dissolved in chloroform and spotted on TLC plates (toluene : ethyl acetate, 93:7 v/v). Plates were run in duplicate and the template was used to identify the active components by developing one plate. Individual active bands were extracted and collected with chloroform. The extract was then filtered to remove the silica using Whatman number 4 filter paper and concentrated by drying under nitrogen. The isolated individual bands were then

reconstituted and spotted on TLC plates and rechromatographed with a more polar solvent, toluene : ethyl acetate (50:50 v/v). These bands were collected and rechromatographed with toluene : ethyl acetate (7:93 v/v). The bands were scraped off the TLC plate and extracted with chloroform, filtered and concentrated by drying under nitrogen. These extracts were used for the structural determination of the individual components of the plant by Infrared, UV, ^{1}H NMR spectroscopy and Gas Chromatography - Mass spectroscopy (GC-MS).

3.2.6 Structural Identification of Active Compounds from Medicinal Plants

3.2.6.1 UV spectroscopy

The λ_{max} for the compound was obtained by UV spectrophotometry using the Varian DMS 100 double-beam spectrophotometer with 1 cm path length quartz cuvettes using dichloromethane as the reference solvent (λ^{CHCl3}_{max} nm:229, 292 and 341). The experimental λ_{max} for the unknown compound was used to determine the absorbance in the UV range.

3.2.6.2 Infrared spectroscopy

Method discussed in section 3.1.6.3 except that λ = 350 nm.

3.2.6.3 ^{1}H NMR spectroscopy

Method discussed in section 3.1.6.4.

3.2.6.4 Gas Chromatography - Mass spectroscopy (GC-MS)

3.2.6.4.1 Sample preparation

Extracted and purified plant sample was reconstituted by thoroughly shaking in dichloromethane (1ml).

3.2.6.4.2 Instrumentation

One microlitre of the above solution was injected into the Hewlett-Packard 6890 series Gas Chromatograph interfaced to a Hewlett-Packard 5793 mass selective detector (MSD)(Fig 3.4) with a Hewlett-Packard Chemstation software (Version b.02.05, 1989 – 1991). The chromatographic separation was achieved using the Hewlett-Packard Mass Spectroscopy Capillary column (30.0m X 250μm X 0.25μm). The column stationery phase was a (5%-phenyl) – methylpolysiloxane. The gas chromatograph parameters were as follows: Oven temperature: 50°C (held for 2.0 min) and ramped at 20°C/min to 280°C (held for 7.5 min). Injection was splitless at 250°C. Matches were obtained using the HPPest, PMW_TOXR and Wiley275 libraries.

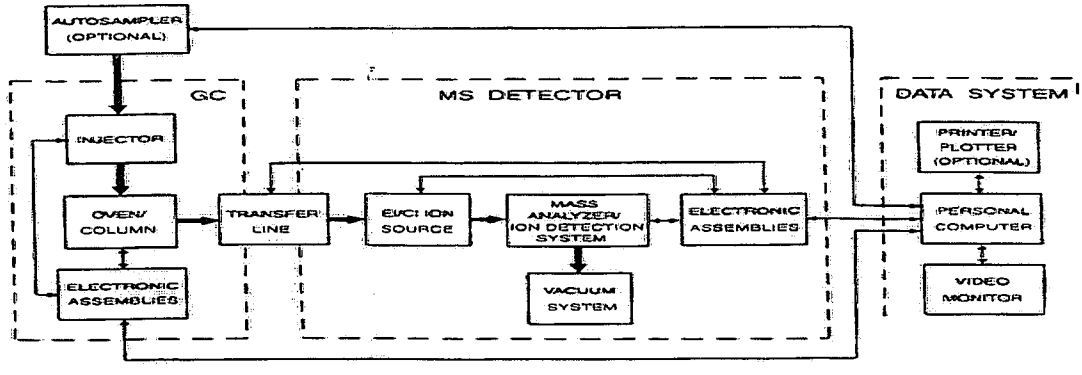


Figure 3.4 Functional block diagram of the GC-MS system. The broad, single-headed arrow represents the flow of sample molecules through the system

CHAPTER FOUR

4.1 Results: Phytoalexins

4.1.1 Isolation and Identification of active component

The induction of phytoalexins in valencia oranges and lemons using *P. digitatum* infection and UV illumination revealed the presence of several fluorescent compounds that were not present in unstressed fruits (Fig 4.1). There were more compounds present in *P. digitatum* infected Valencia fruits (Lanes 4 and 5) than in UV illuminated fruit (Lane 3) or in *P. digitatum* induced lemons (Lanes 7). The antifungal activity of these compounds using bioautography revealed a compound with an Rf value of 0.65 inhibiting both *F. moniliforme* (Fig 4.2B) and *A. flavus* (Fig 4.2C). This compound has the same Rf value as 6, 7 dimethoxycoumarin, which is the only known phytoalexin in Valencia fruit.

Individual compounds collected by preparative TLC using solvents (toleune:ethyl acetate, 4:1) and decreasing polarity of the resolving solvent to toleune:ethyl acetate, 1:1 showed a compound that correlated to 6, 7 dimethoxycoumarin having strong antifungal activity (Fig 4.3). The TLC plate with the resolved induced compound was sprayed with dinitrophenylhydrazine spray reagent to indicate the resolution factor of the compound. This was then used as as a template against which zones of clearing on the TLC plate were compared (Fig 4.2A).

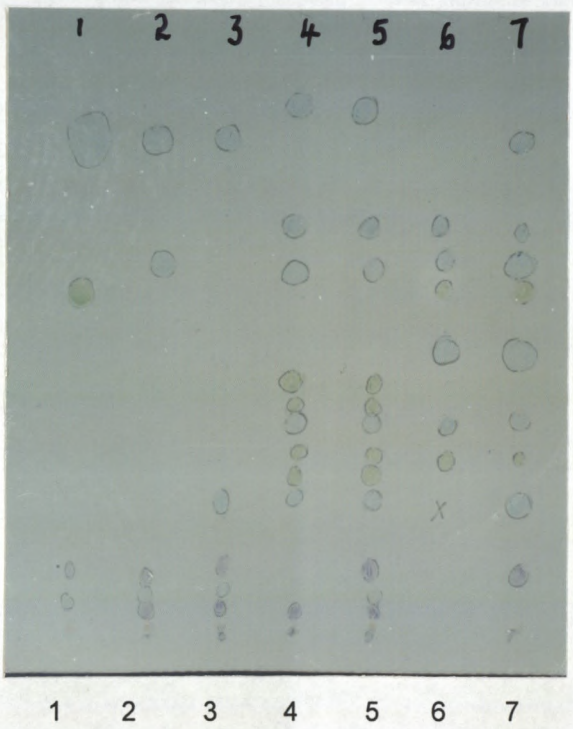


Figure 4.1 Separation of fluorescent compounds from oranges (*Citrus sinesis* cv. Valencia) and lemon (*Citrus limon* cv. Eureka). Lanes 1 and 2- Unstressed Valencia oranges (control), Lane 3- UV illuminated Valencia fruit, Lane 4- *P.digitatum* infected Valencia fruit, Lane 5- UV illuminated and infected Valencia fruit. Lane 6- Lemon control, Lane 7- Infected lemon fruit. X – no compound present in unstressed fruit.

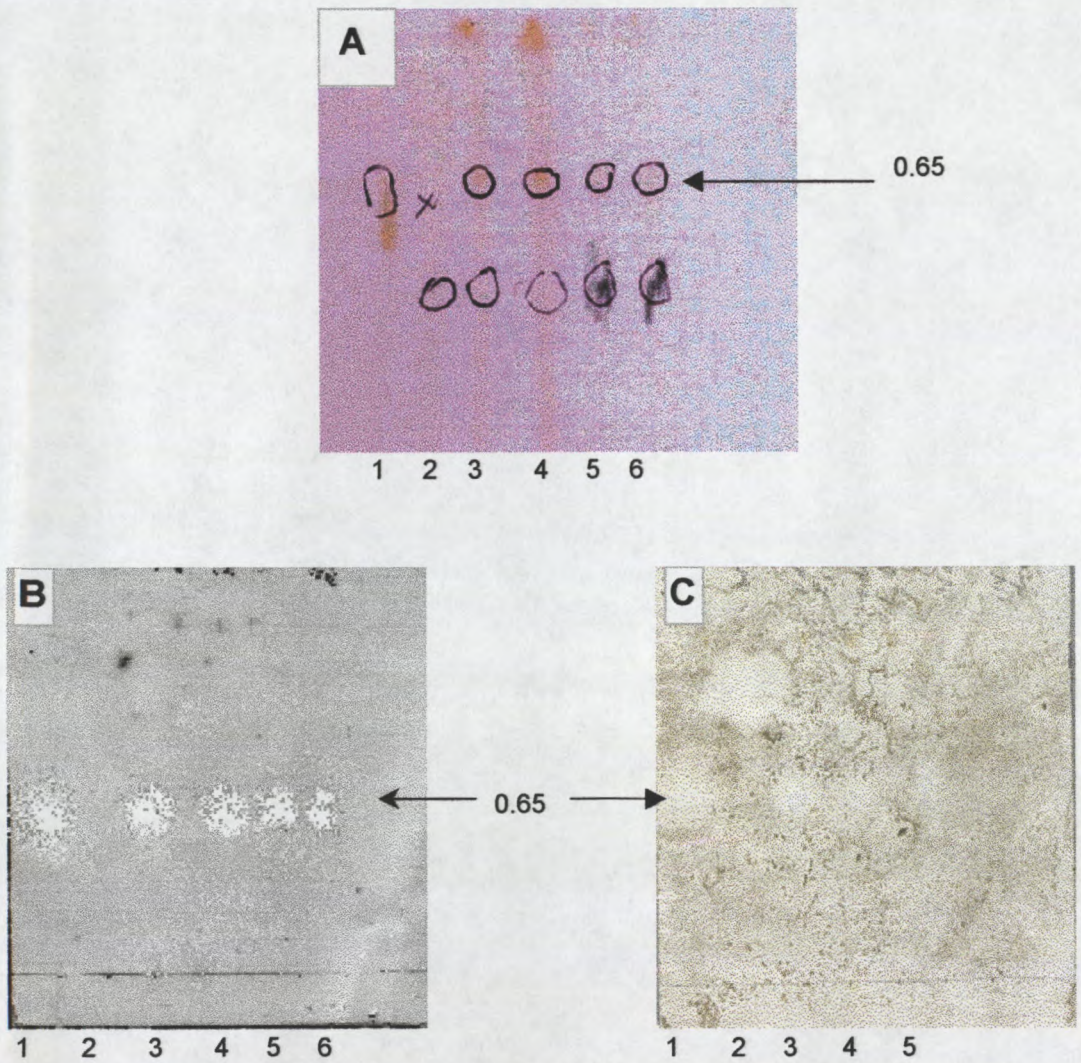


Figure 4.2 Phytoalexin bioassays on thin layer chromatograms. (A) Template - Lane 1- Standard (6,7 -dimethoxycoumarin); Lane 2- control; Lanes 3, 4, 5 and 6 -purified compound from infected Valencia oranges with identical Rf value as standard (0.65). (B) Thin layer chromatogram bioassay of extracts from *P. digitatum* inoculated Valencia orange. The fungal culture used was *F.moniliforme*. The standard, 6,7 dimethoxycoumarin (Lane 1) showed slight antifungal activity at Rf = 0.65. Lane 2 - control (uninfected fruit); Lanes 3, 4, 5 and 6 - treated fruit showed similar antifungal zones at Rf = 0.65. (C) A similar bioassay of extracts from *P.digitatum* inoculated Valencia oranges using *A. flavus* as the test fungi. The control, 6,7 dimethoxycoumarin (Lane 1) showed slight antifungal activity at Rf = 0.65. Lane 2 - control (uninfected fruit); Lanes 3,4 and 5 - purified compounds from treated Valencia oranges showed similar cleared zones at Rf = 0.65.

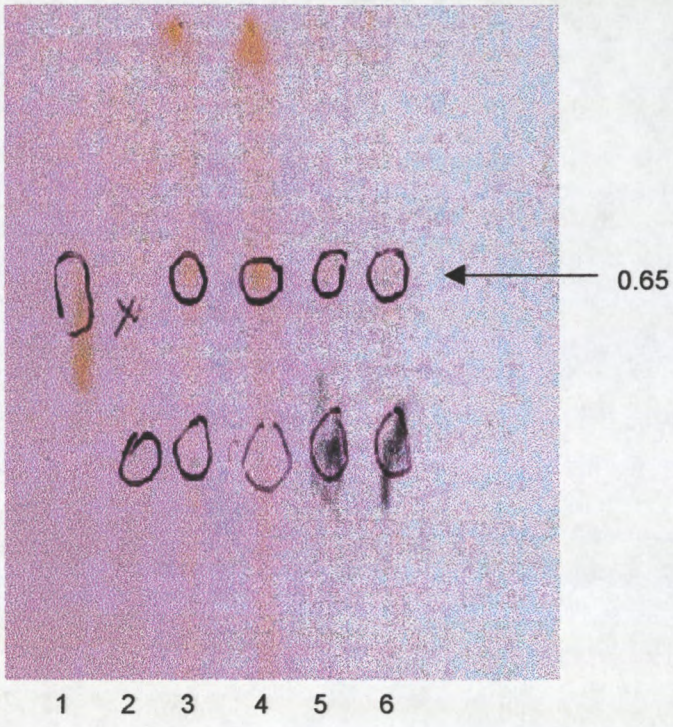


Figure 4.3 Preparative Thin Layer Chromatography of active compound which manifested high antifungal activity (Rf = 0.65). Lane 1- Standard (6,7 -dimethoxycoumarin); Lane 2- control; Lanes 3, 4, 5 and 6 -purified compound from infected valencia oranges with identical Rf value as standard (0.65).

4.1.2 High Performance Liquid Chromatography

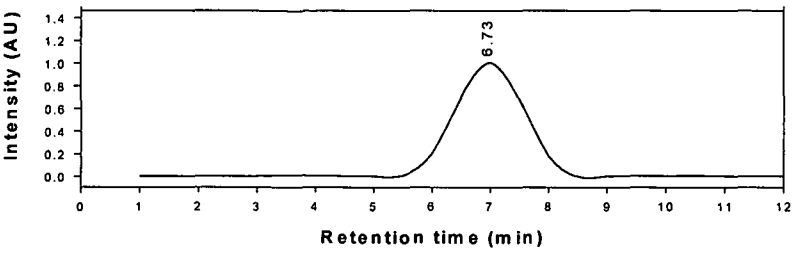
The quantification of the induced phytoalexin was accomplished by High Performance Liquid Chromatography. Verification of the induced compound with 6, 7dimethoxycoumarin was achieved by comparing the retention times of the commercially available 6, 7 dimethoxycoumarin (Fig 4.4A, Chromatogram 1 – 3) to the induced compound (Fig 4.4B, Chromatogram 4 – 8) which were both eluted at approximately 6.70 minutes. Method optimization with decreasing concentrations of 6, 7 dimethoxycoumarin indicated that the lowest detectable limit of this compound was 0.125 μg/g.

Different methods of phytoalexin induction revealed that non-illuminated oranges contained only 0.36 μg/g (Chromatogram 4), UV illuminated fruits contained 12.1 μg/g (Chromatogram 5) and this increased to 15.2μg/g (Chromatogram 6) upon storage for six days at 50°C. *P. digitatum* infected fruit produced a two fold increase i.e. 35.5 μg/g (Chromatogram 7) as compared to UV illuminated fruit. The results obtained using the different induction treatments are summarized in Table 4.1.

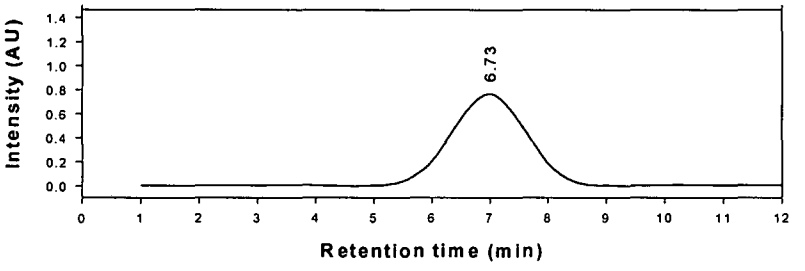
Table 4.1 Concentration of 6, 7 dimethoxycoumarin induced by different treatments.

Sample	**Concentration (μg/g)**
Standard (6,7 dimethoxycoumarin)	100 μg/g, 75μg/g and 50 μg/g (Fig 19A chromatogram 1, 2 and 3)
Control	0.36 μg/g (Fig 19B; chromatogram 4)
UV-dried	15.2 μg/g (Fig 19B; chromatogram 6)
UV- not dried	12.5 μg/g (Fig 19B; chromatogram 5)
P. digitatum infected fruit	35.51 μg/g (Fig 19B; chromatogram 7 and 8)

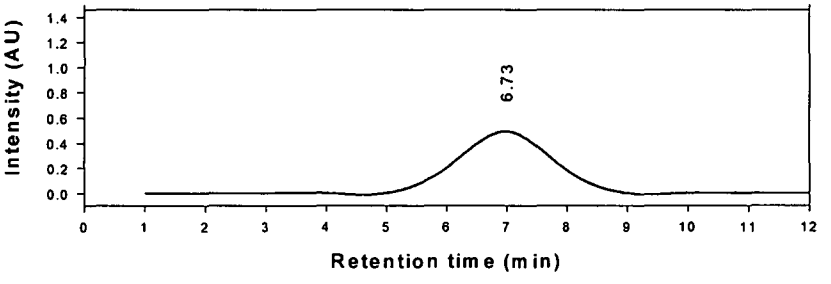
4.1.2.1 Method Optimization using 6,7 dimethoxycoumarin standards



Chromatogram 1

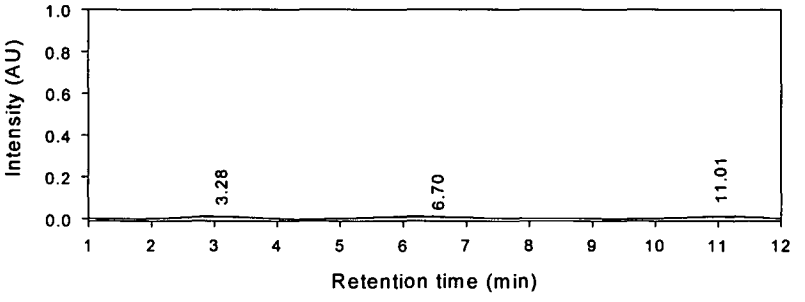


Chromatogram 2

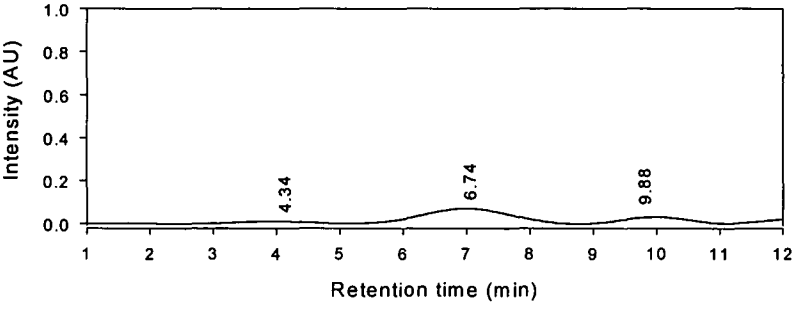


Chromatogram 3

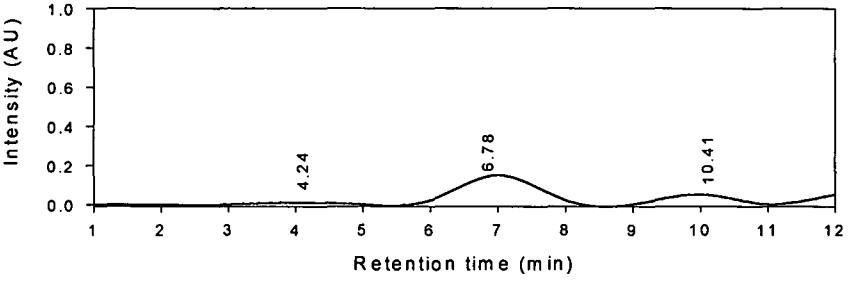
Figure 4.4 A Standard (6,7 dimethoxycoumarin) at different concentrations. Retention time: 6.70 and the concentrations are as follows; 0.1mg/ml (Chromatogram 1); 0.75 g/ml (Chromatogram 2) and 0.50 g/ml (Chromatogram 3).



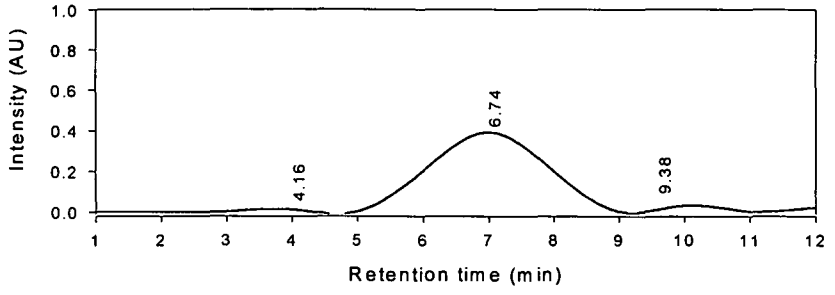
Chromatogram 4



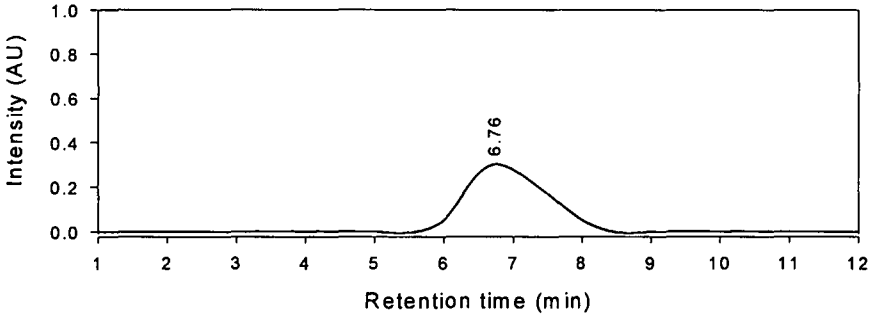
Chromatogram 5



Chromatogram 6



Chromatogram 7



Chromatogram 8

Figure 4.4 B Control: no UV treatment or infection (Chromatogram 4), UV illuminated and dried valencia tissue (Chromatogram 5), UV illuminated and not dried valencia tissue (Chromatogram 6), *P. digitatum* infected valencia fruit (Chromatogram 7) and purified compound isolated from *P. digitatum* infected valencia fruit (Chromatogram 8). Retention time 6.70 - 6.77. Peak eluting at 6.70 – 6.77 was later identified as 6, 7 dimethoxycoumarin compared to result with a commercially available standard.

The increase in induced compound content in UV and *P.digitatum* treated valencia fruit was accompanied by a rise in antifungal activity in the tissue as indicated by bioassay results (Fig. 4.2B-C)

The effect of *P. digitatum* infection over a 30 d period indicated that 6, 7 dimethoxycoumarin appeared after five days of inoculation and maximum levels (35.5 μg/g) were obtained at day fourteen. Thereafter, there was a decline and no phytoalexin was detected after 30 days (Fig 4.5).

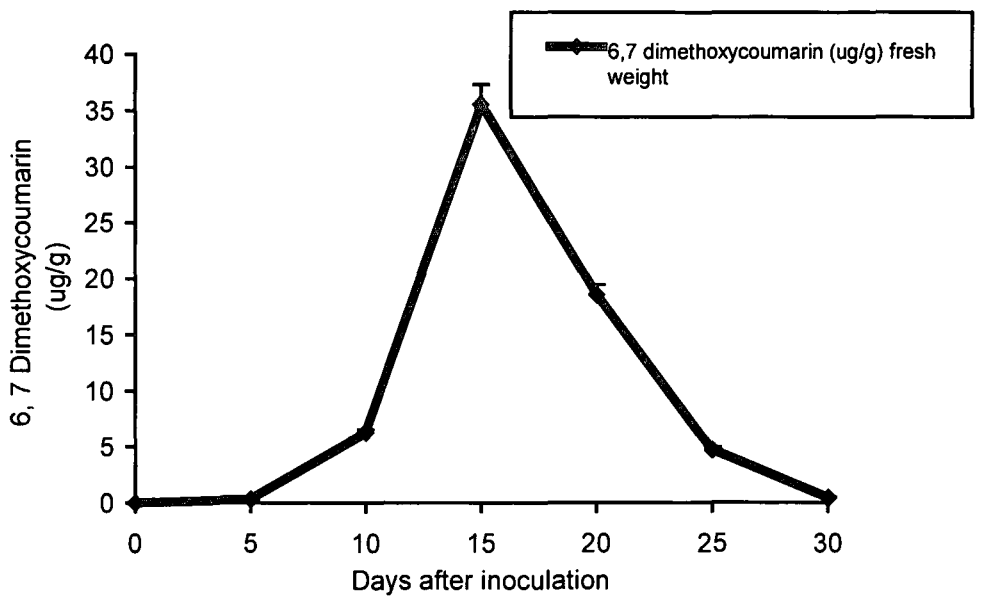
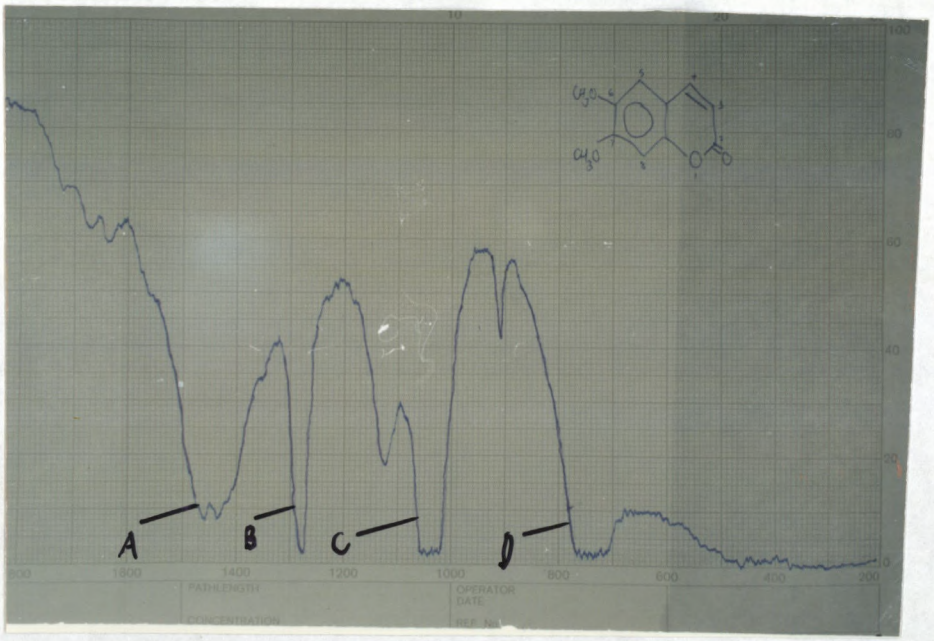


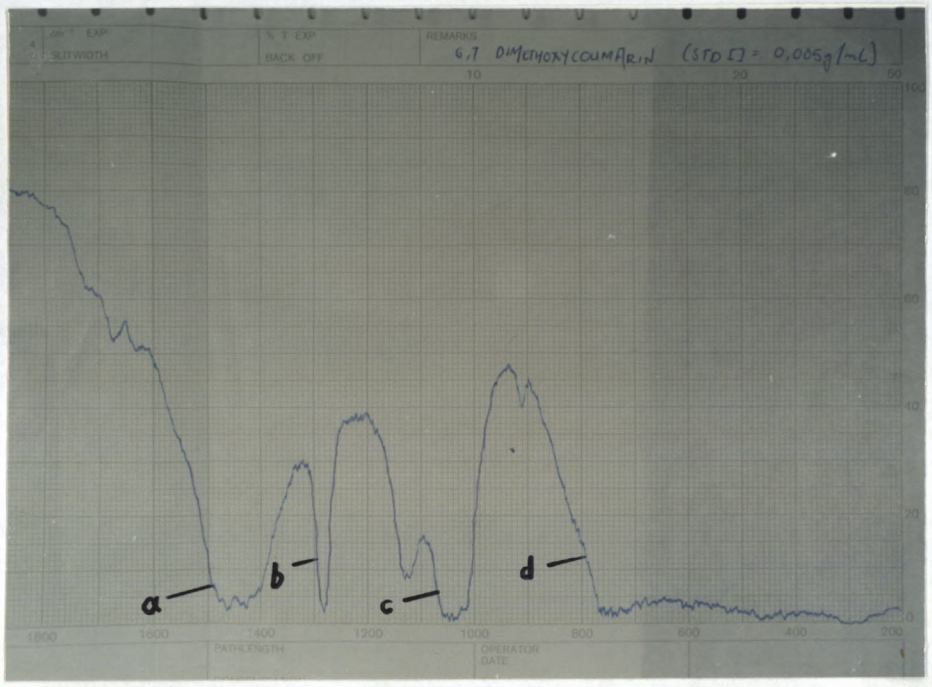
Figure 4.5 Effect of *P. digitatum* infection on the accumulation of 6, 7 dimethoxycoumarin in Valencia tissue.

4.1.3 Structural Determination of the compound

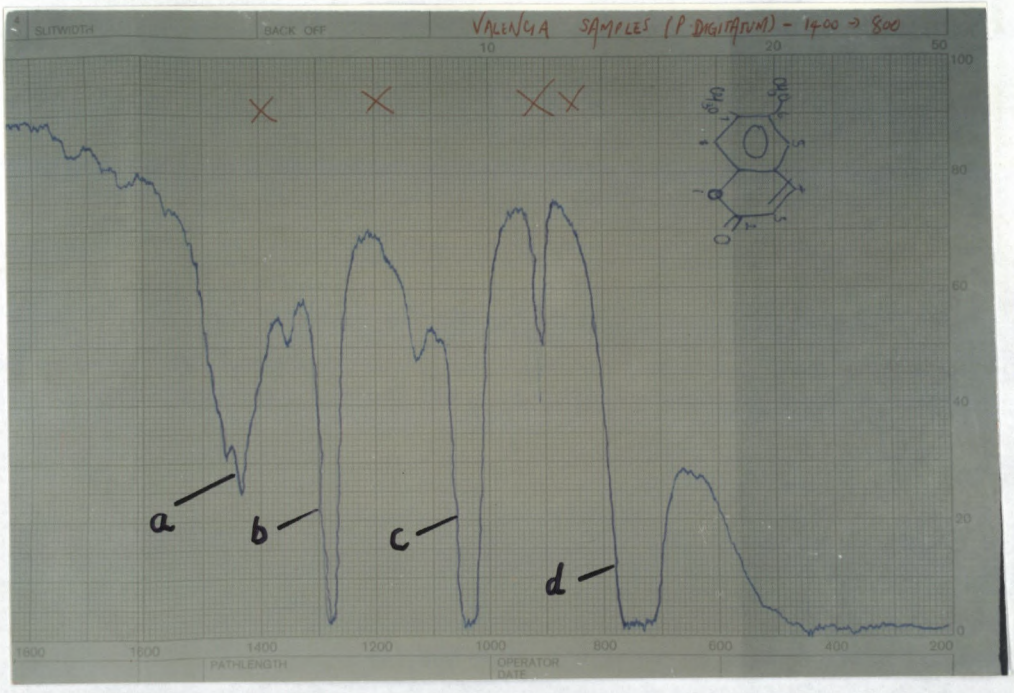
Structural determination was achieved by comparing the structural data of the induced compound to the commercially available standard, 6,7 dimethoxycoumarin by UV, IR and ^{1}H NMR. Correlation charts comparing the absorption wavelength in the UV spectral range of the aromatic backbone, alkene, conjugated alkene and dimethyl substituent groups indicated identical structures for the induced compound and the standard (Fig 4.6). The peaks a, b, c and d were at identical wavenumbers and these results are summarized in Table 4.2.



(1)



(2)



(3)

Figure 4.6 UV scan of the scoparone standards (1 and 2) and Induced compound (3) obtained from *P. digitatum* infected valencia fruit (a - aromatic backbone, b - alkene, c - conjugated alkene and d – dimethyl substituent group)

Table 4.2 Ultra-Violet absorption wavelengths for the 6, 7 dimethoxycoumarin standards and induced compound from *P. digitatum* infected fruit.

Functional Groups	Standard [0.01g/ml]	Standard [0.005 g/ml]	Induced compound
1. Aromatic backbone (a)	1450 cm	1450 cm	1446cm
2. Alkene (b)	1290 cm	1290 cm	1290cm
3. Conjugated alkene (c)	1050-1150 cm	1050-1150 cm^{-1}	1050-1140cm
4. Dimethyl substituent groups (d)	800 - 1000 cm	800 – 1000cm	800 – 1000cm

Further confirmation of structural similarities between the standard and induced compound was achieved by the determination of absorption frequencies in the Infrared spectral range. Both the standard and sample showed similar absorption scan patterns (Fig 4.7a and Fig 4.7b). The details of the absorption patterns are shown in Table 4.3.

Table 4.3 Infra-red absorption frequencies of the 6, 7 dimethoxycoumarin standard and induced compound from *P.digitatum* infected oranges.

	Scoparone standard [0.01 g/ml]	Induced compound	Structural groups
Peak 1 (a)	3294.46 cm^{-1}	3356.04 cm^{-1}	ortho-disubstituted CH_2
Peak 2 (b)	2941.70 cm^{-1}	2937.51 cm^{-1}	
Peak 3 (c)	2824.11 cm^{-1}	2824.61 cm^{-1}	
Peak 4 (d)	1436.64 cm^{-1}	1436 cm^{-1}	Aromatic hydrocarbon backbone
Peak 5 (e)	1042.63 cm^{-1}	1042.76 cm^{-1}	

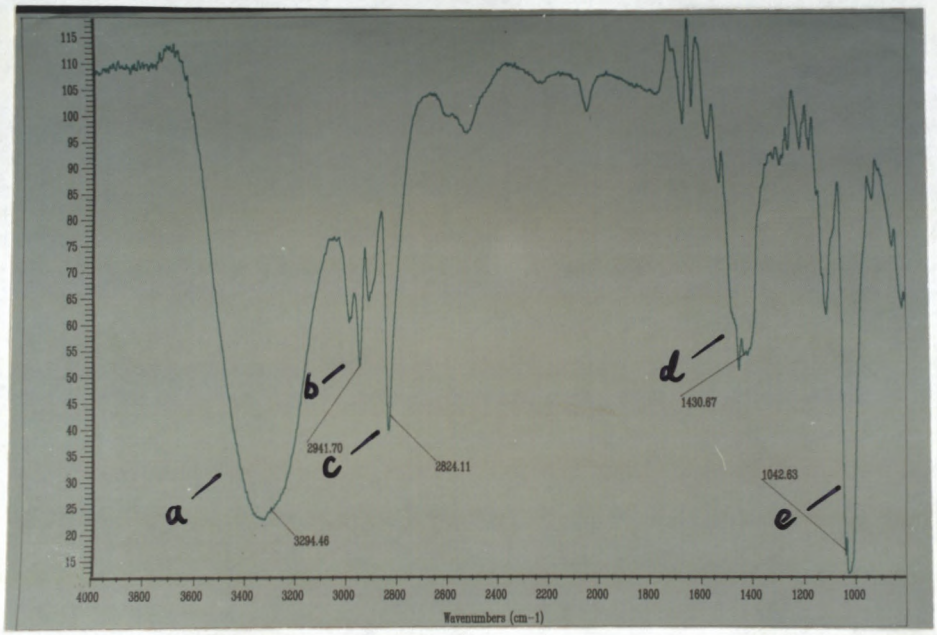


Figure 4.7a Infra red scan of authentic standard, 6,7 dimethoxycoumarin.

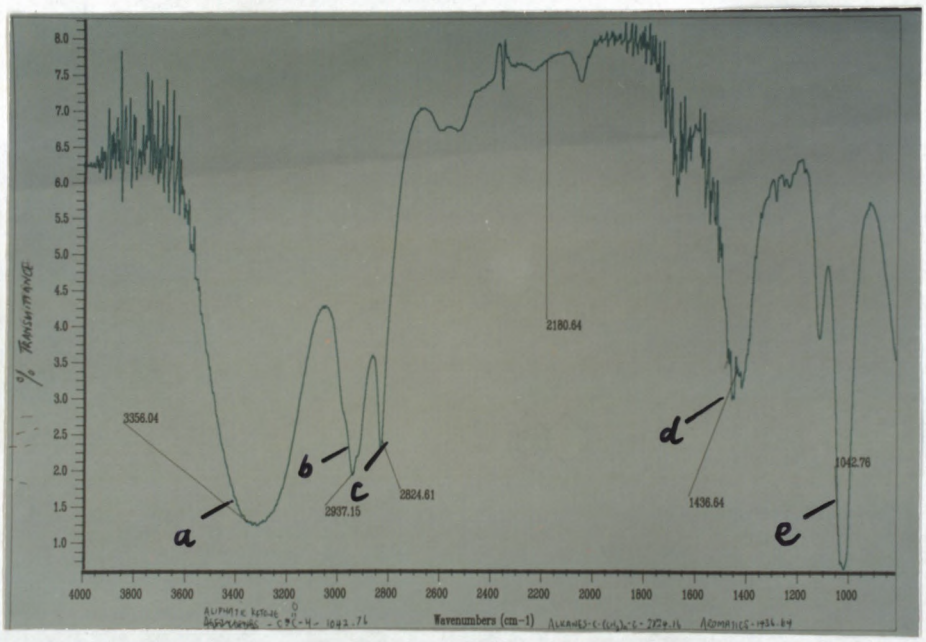
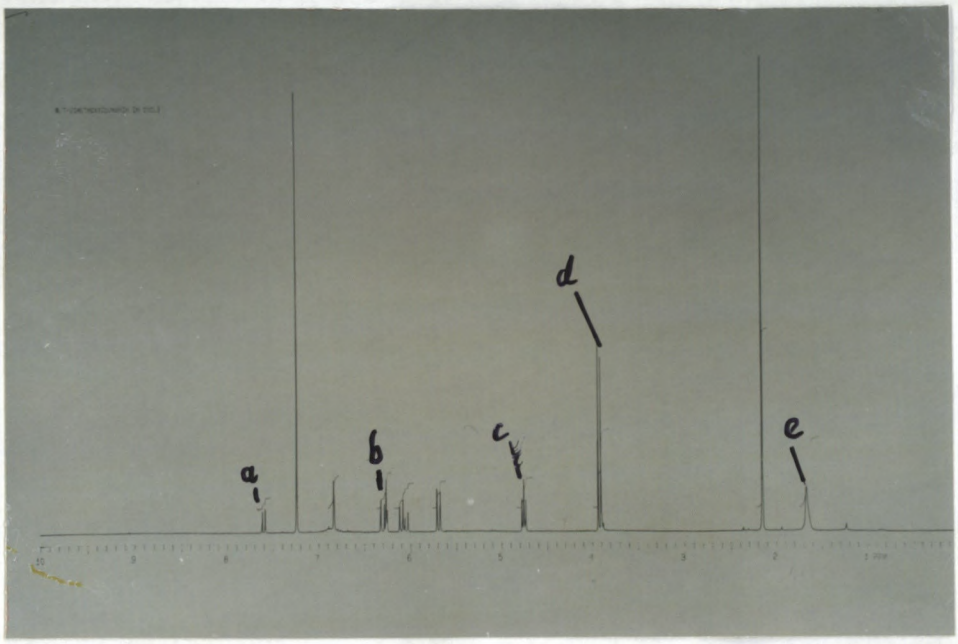
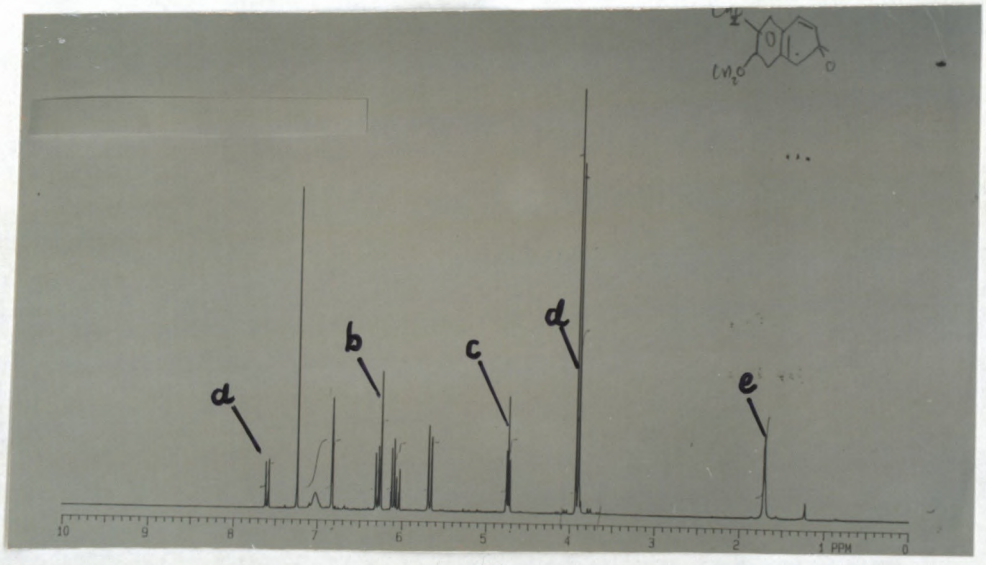


Figure 4.7b Infrared scan of the induced compound obtained from *P. digitatum* infected valencia fruit.

Final confirmation of structural similarities for the purified sample and standard was done using ^{1}H NMR spectroscopy. Figures 4.8 (1 and 2) shows the results of ^{1}H NMR spectroscopy.



(1)



(2)

Figure 4.8 Mass-spectra of scoparone. Samples: (1) commercial 6, 7 dimethoxycoumarin; (2) induced compound extracted from *P.digitatum* infected fruit.

Mass spectral data revealed that the induced compound had identical peaks at the same frequencies. ^{1}H NMR scans were compared to correlation charts. The correlating results for the standard and induced compound are listed below and all the ^{1}H NMR spectral results are shown in Table 4.4. The scoparone standard showed peaks of interest at the following frequencies:

(1) 2285.49 - 7.616 ppm - aromatic backbone group (arrowed a)
(2) 1898.13 - 6.326 ppm - conjugated alkene group (arrowed b)
(3) 1432.46 - 4.774 ppm - alkene group (arrowed c)
(4) 1179.71 - 3.931 ppm - ortho-disubstituted functional group (arrowed d)
(5) 499.51 - 1.665 ppm - alkene group (arrowed e)

The induced compound showed peaks of interest at the following frequencies:

(1) 2284.88 – 7.614 ppm – aromatic backbone group (arrowed a)
(2) 1894.53 - 6.314 ppm - conjugated alkene group (arrowed b)
(3) 1429.16 - 4.763 ppm - alkene group (arrowed c)
(4) 1178.61 - 3.928 ppm - ortho-disubstituted functional group (arrowed d)
(5) 509.40 - 1.698 ppm - alkene group (arrowed e)

The spectral data obtained from the induced compound suggests that the compound isolated from *P. digitatum* infected Valencia fruit has an identical structure to the standard (6,7 dimethoxycoumarin) thus verifying the isolation and structural elucidation of 6, 7 dimethoxycoumarin from Valencia oranges.

Table 4.4 ^{1}H NMR spectral analysis results for 6,7 dimethoxycoumarin standard and induced compound from *P. digitatum infected* Valencia oranges.

Standard (6,7 dimethoxycoumarin)				**Induced compound**			
Peak number	Frequency	ppm	Intensity	Peak number	Frequency	ppm	Intensity
1.	2285.49	7.616	9.915	1.	2284.88	7.614	23.579
2.	2276.15	7.585	11.560	2.	2275.35	7.583	23.316
3.	2172.61	7.240	192.217	3.	2172.61	7.240	150.914
4.	2050.70	6.834	23.150	4.	2108.69	7.027	7.297
5.	2048.99	6.828	20.922	5.	2107.17	7.022	7.838
6.	1898.13	6.326	10.768	6.	2050.15	6.832	53.415
7.	1896.73	6.321	13.894	7.	2048.38	6.826	46.711
8.	1886.23	6.286	12.235	8.	1894.53	6.314	27.011
9.	1881.10	6.269	18.753	9.	1893.07	6.309	24.558
10.	1879.70	6.264	22.682	10.	1885.19	6.282	30.504
11.	1876.65	6.254	10.772	11.	1877.50	6.257	51.628
12.	1835.99	6.118	11.651	12.	1875.97	6.252	66.942
13.	1825.73	6.084	14.280	13.	1838.61	6.127	30.180
14.	1808.70	6.027	8.774	14.	1828.58	6.094	34.155
15.	1712.67	5.707	18.603	15.	1828.54	6.070	16.140
16.	1701.13	5.669	17.187	16.	1811.51	6.037	19.682
17.	1432.46	4.774	13.964	17.	1706.63	5.687	40.489
18.	1426.11	4.752	24.641	18.	1697.77	5.658	33.113
19.	1419.76	4.731	13.795	19.	1696.25	5.653	35.595
20.	1179.71	3.931	84.612	20.	1429.16	4.763	29.530
21.	1170.80	3.902	78.778	21.	1422.63	4.741	55.373
22.	644.99	2.149	409.555	22.	1416.40	4.720	26.053
23.	499.51	1.665	19.228	23.	1178.61	3.928	200.000
				24.	1169.64	3.898	180.20
				25.	1166.40	3.884	15.159
				26.	514.34	1.714	40.091
				27.	509.40	1.698	17.739
				28.	368.19	1.227	7.851

4.2 CONSTITUTIVE COMPOUNDS

Extracts from *B. micrantha, B. frutescens, C. aurea, C. monilifera, E. latissima, L. javanica, S. serratuloides and W. salutaris* using soxhlet extraction, solvent extraction and Thin Layer Chromatography separation showed that all the plants had compounds at differing Rf values. These results are shown in Figure 4.9. Most of the compounds were concentrated in the bark (Lanes 1 and 2 from *Warburgia salutaris*; Lane 6 from *Bridelia micrantha*) compared to the heartwood and leaves.

4.2.1 Antimicrobial Activity

The crude extracts of the plants were investigated for antibacterial and antifungal properties. *B. micrantha* (heartwood and bark) showed antibacterial activity against *S. aureus* and *M. luteus*. *B. micrantha* (leaves) also showed activity against *M. luteus*. *W. salutaris* (bark) showed activity against *S. aureus* and *B. subtilus*. *W. salutaris* (leaves extract in dichloromethane) showed activity against *E. coli*, *S. aureus* and *B. subtilus*. *Scenecio serratuloides* (leaf extract) showed activity against *S. aureus*, *B.subtilus*, *M. luteus* and *S. marcescens*. *Lippia javanica* (leaf extract) showed activity against *E. coli*, *S. aureus*, *B. subtilus*, *M. luteus* and *S. marcescens*. *Erythrina lattisma* (leaf extract) showed excellent activity against *M. luteus*. *B. micrantha* and *W. salutaris* showed antifungal activity against *F. moniliforme.* Tables 4.5 and 4.6 shows the results of antimicrobial tests respectively.

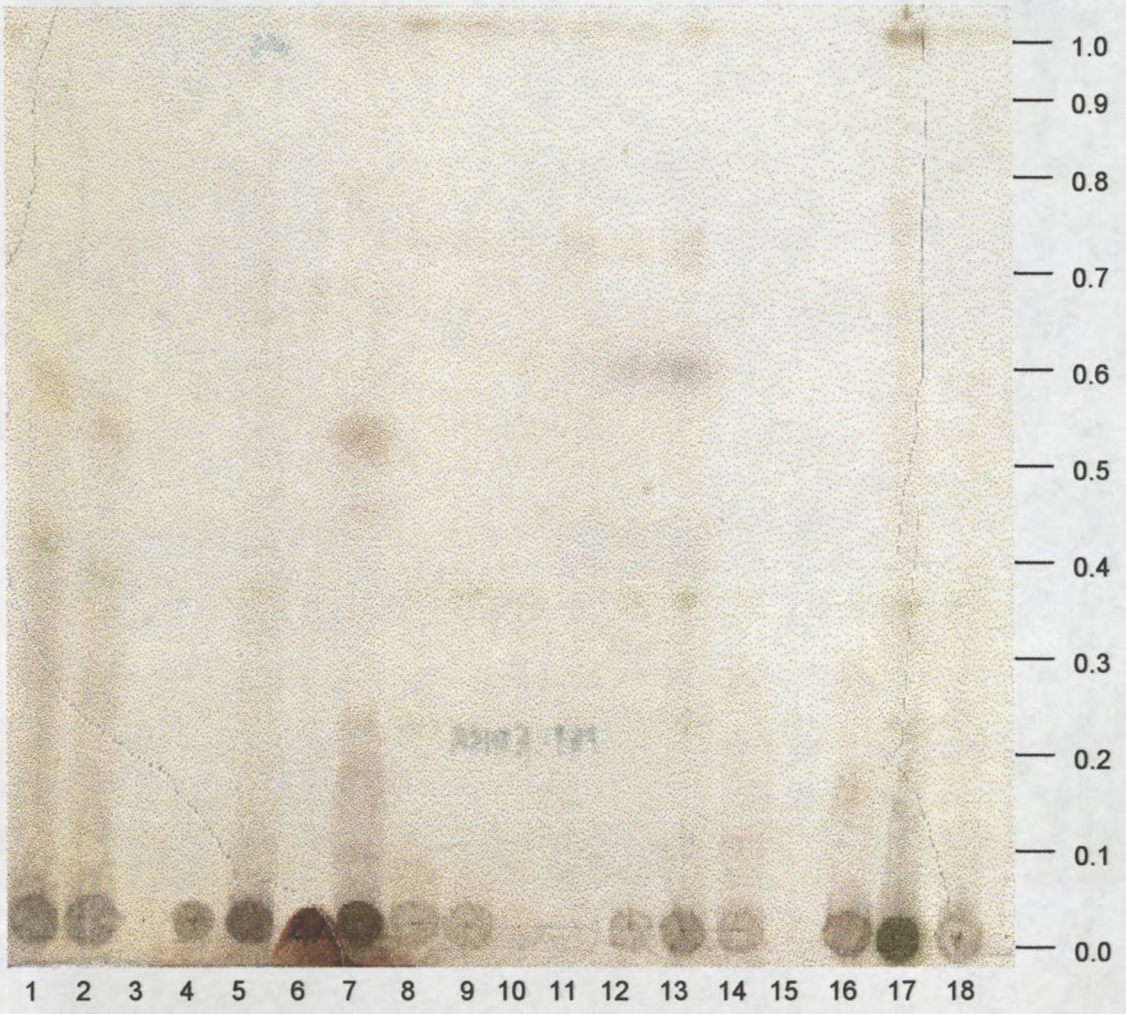


Figure 4.9 Separation of components from 20 different extracts derived from 8 plants using two different exrtraction techniques. Lane 1 – *Warburgia salutaris* (ethyl acetate, 60°C), Lane 2 – *Warburgia salutaris* (dichloromethane, 60°C), Lane 3 - *Bulbine fructescens leaves* (soxhlet, dichloromethane), Lanes 4, 5 and 6 – *Bridelia micrantha* heartwood, leaves and bark repectively (soxhlet, dichloromethane), Lane 7 – Traditional Indian herbal remedy (dichloromethane, 60°C), Lanes 8, 9, and 10 – *Chrysanthemoides monilifera* leaves (soxhlet extracted in dichloromethane, ethyl acetate and hexane respectively), Lanes 11, 12 and 13 – *Lippia javanica* leaves (hexane 60°C, dichloromethane 60°C and ethyl acetate 60°C respectively), Lanes 14, 15 and 16 – *Erythrina latissima* leaves (soxhlet extracted in dichloromethane, ethyl acetate and hexane respectively, Lane 17 - *Crocosmia aurea* bulb (soxhlet extracted in dichloromethane), Lane 18 – *Scenecio serratuloides* leaves (soxhlet dichloromethane).

Table 4.5 Results of antibacterial tests

Botanical Name	Tissue	Solvent	Zone of inhibition (mm)						
			E. coli	*S. aureus*	*B. subtilus*	*P. aeroginosa*	*M. luteus*	*S. marcescens*	*K pneumonia*
Bridelia micrantha	Heartwood	Methanol	-	5	2	-	4	2	2
	Bark	Methanol	-	5	-	-	5	-	-
	Leaf	Methanol	-	-	-	-	4	2	-
Warburgia salutaris	Heartwood	Methanol	-	2	3	-	-	-	-
	Bark	Methanol	-	5	6	-	-	-	-
	Leaf	Methanol	-	-	4	-	-	-	-
	Leaf	Hexane	-	-	-	-	-	-	-
	Leaf	Ethyl acetate	3	4	3	-	-	-	-
	Leaf	Dichloro	4	5	4	2	-	-	-
Senecio serratuloides	Leaf	Ethyl acetate	-	4	-	-	5	2	-
	Leaf	Dichloro.	-	3	2	-	-	-	2
	Leaf	Hexane	-	-	-	-	NE[1]	-	NE
Crocosmia aurea	Leaf	Ethyl acetate	-	-	-	-	-	-	-
	Leaf	Dichloro.	-	-	-	-	-	-	-
	Leaf	Hexane	-	-	-	-	-	-	-
	Leaf	Petroleum ether	-	NE	NE	-	NE	-	-
Lippia javanica	Leaf	Ethyl acetate	-	4	2	-	5	2	2
	Leaf	Dichloro.	-	2	2	-	2	2	-

	Leaf	Hexane	-	-	-	-	2	-	-
Chrysanthemoides monilifera	Leaf	Ethyl acetate	-	-	-	-	-	-	-
	Leaf	Dichloro.	-	-	-	-	-	-	-
	Leaf	Hexane	-	-	-	-	7	-	1
Erythrina lattisma	Leaf	Ethyl acetate	4	4	3	3	10	-	2
	Leaf	Dichloro.	3	4	3	-	4	-	-
	Leaf	Hexane	-	-	-	-	-	-	-
Bulbine fructescens	Leaf	Ethyl acetate	-	5	-	-	-	-	-
	Leaf	Dichloro.	NE	2	NE	-	-	-	-
	Leaf	Hexane	NE	-	-	-	-	-	-

[1] NE – not extracted

Table 4.6 Results of antifungal tests

Botanical Name	Tissue	Solvent	*Aspergillus flavus*	*Cladosporuim cladosporoides*	*Fusarium moniliforme*
Bridelia micrantha	Leaf	Ethyl acetate	-	-	8
	Heartwood	Dichloromethane	-	-	10
	Bark	Hexane	-	-	-
Warburgia salutaris	Leaf	Dichloromethane	-	-	15
	Heartwood	Ethyl acetate	-	-	15
	Bark	Hexane	-	-	12
Bulbine fructescens	Leaf	Dichloromethane	-	NE[1]	5
	Leaf	Ethyl acetate	-	NE[1]	-
	Leaf	Hexane	-	NE[1]	-
Crocosmia aurea	Leaf	Dichloromethane	-	-	9
	Leaf	Ethyl acetate	-	-	8
	Leaf	Hexane	-	-	6
Lippia javanica	Leaf	Dichloromethane	-	-	10
	Leaf	Ethyl acetate	-	-	10
	Leaf	Hexane	-	-	6
Chrysanthemoides monilifera	Leaf	Dichloromethane	-	-	11
	Leaf	Ethyl acetate	-	-	15
	Leaf	Hexane	-	-	10

Erythrina latissma	Leaf	Dichloromethane	-	-	4
	Leaf	*Ethyl acetate*	-	-	3
	Leaf	Hexane	-	-	6
Senecio serratuloides	Leaf	Dichloromethane	-	-	10
	Leaf	Ethyl acetate	-	-	9
	Leaf	Hexane	-	-	NE

[1] NE – not extracted

4.2.2 Separation of Components from *Warburgia salutaris* and *Bridelia micrantha*.

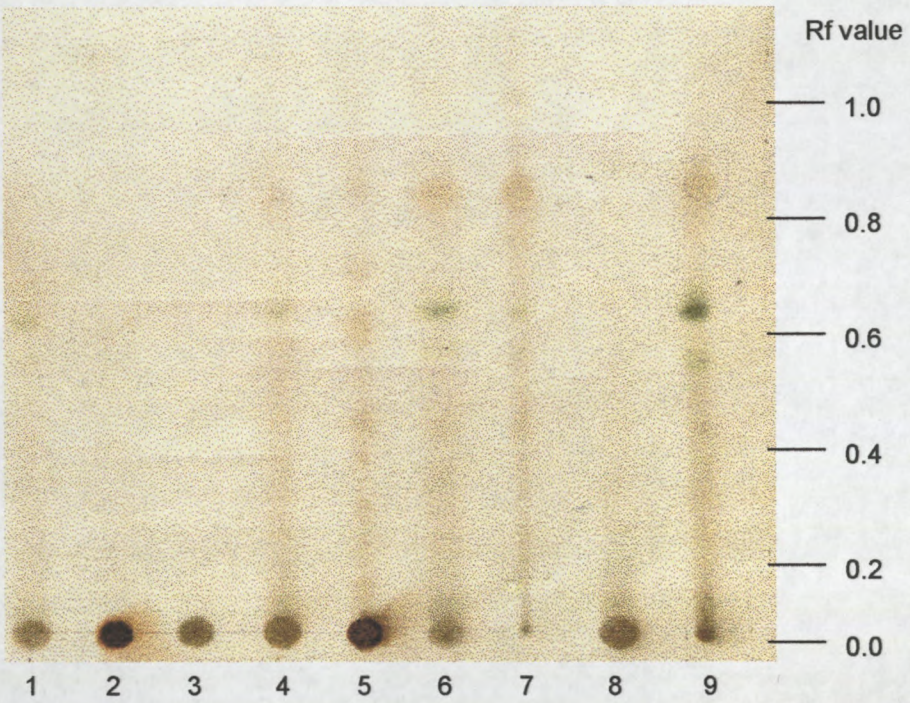


Figure 4.10 Separation of components from *W. salutaris* and *B. micrantha*. Lane 1 – *Bridelia micrantha* leaves (soxhlet extracted in methanol), Lane 2 – *Bridelia micrantha* bark (soxhlet extracted in methanol), Lane 3 – *Bridelia micrantha* heartwood (soxhlet extracted in methanol), Lane 4 – *Warburgia salutaris* leaves (soxhlet extracted in methanol), Lane 5 - *Warburgia salutaris* bark (soxhlet extracted in methanol), Lane 6 - *Warburgia salutaris* leaves (ethyl acetate, 60°C), Lane 7 - *Warburgia salutaris* leaves (hexane, 60°C), Lane 8 - *Warburgia salutaris* leaves (soxhlet extracted in dichloromethane), Lane 9 - *Warburgia salutaris* leaves (dichloromethane, 60°C).

Figure 4.10 shows the plant material extracted by heating at 60°C in a water bath for 5 minutes with 6 ml of solvent (ethyl acetate, dichloromethane or hexane) Lanes 6, 7 and 9 showed better separation compared to soxhlet extracted plant materials (Lanes 1, 2, 3, 4, 5 and 8). The heat-treated solvent extraction technique was used as the preferred extraction technique.

B. micrantha showed both antibacterial and antifungal activity however this compound is cytotoxic. Death of a patient four hours after taking cough medicine made from this plant has been reported by Verdcourt and Trump. (1969). Thus further experimentation with this plant was considered non-beneficial.

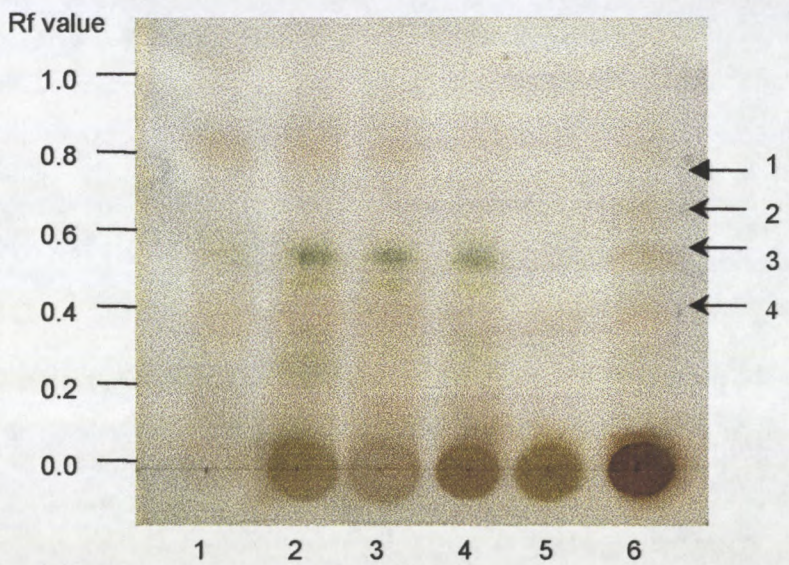


Figure 4.11 Separation of individual components from *Warburgia salutaris* leaves and bark extracts. Lane 1 - *Warburgia salutaris* leaves (soxhlet extracted in dichloromethane, Lane 2 - *Warburgia salutaris* leaves (dichloromethane, 60°C) Lane 3 - *Warburgia salutaris* leaves (ethyl acetate, 60°C), Lane 4 - *Warburgia salutaris* leaves (hexane, 60°C), Lane 5 - *Warburgia salutaris* bark (Soxhlet extracted in dichloromethane) and Lane 6 - *Warburgia salutaris* bark (dichloromethane, 60°C). Arrows 1, 2, 3 and 4 are the components of interest found in *Warburgia salutaris* bark.

Warburgia salutaris bark showed the most potent antifungal activity thus the most effort was placed on the isolation and identification of the active compounds found in *Warburgia salutaris* bark (Figure 4.11). Further separation of these

fractions using dinitrophenylhydrazine (DNPH) (Figure 4.12A) as a spray reagent and circular TLC (Figure 4.12B) confirmed the presence of four fractions.

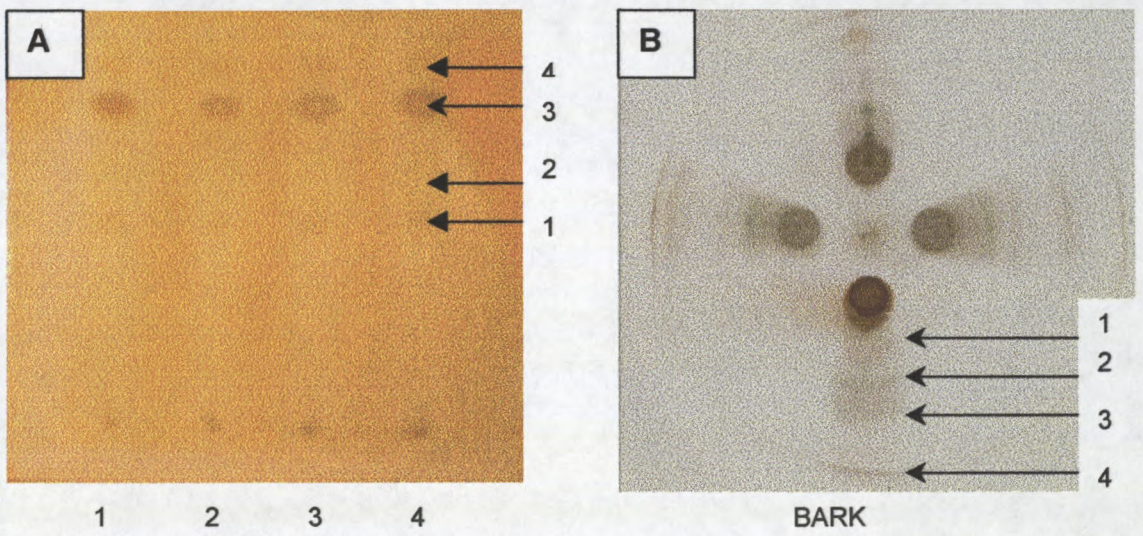


Figure 4.12 Separation of fractions from *Warburgia salutaris* bark. (A) – Longitudinal TLC showing four distinct fractions (arrowed 1-4) and identical migration rates in all four lanes. Rf value for fractions 1, 2, 3 and 4 were 0.55, 0.64, 0.75 and 0.90 respectively. Spray reagent – dinitrophenylhydrazine (DNPH) – selective for ketones and aldehydes. (B) – Circular TLC verifies the presence four fractions (arrowed 1 –4). Compound migration is shown as arcs.

4.2.3 Antifungal Activity: Bioautography

Antifungal activity of *Warburgia salutaris* bark extracts was determined by direct Bioautography on TLC plates sprayed with the mycotoxigenic fungi, *Aspergillus flavus* (Fig 4.13C) and *Fusarium moniliforme* (Fig 4.13B).

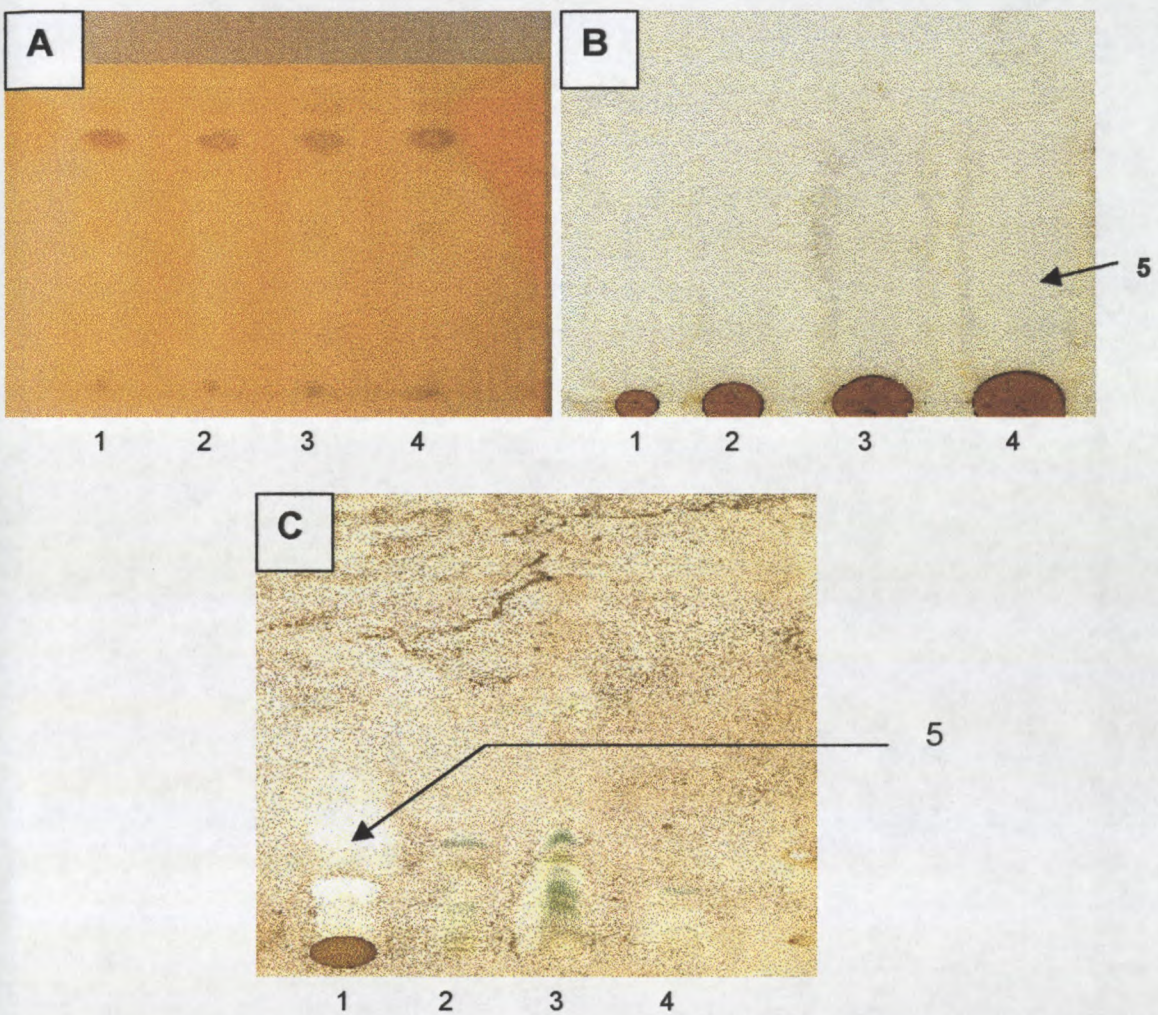
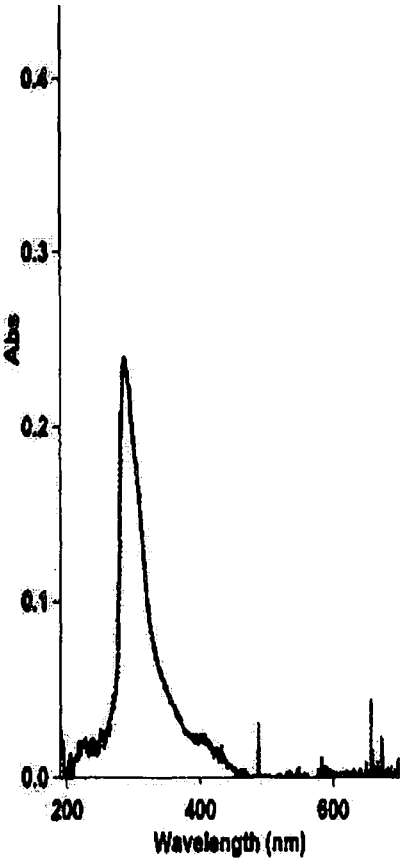


Figure 4.13 Antifungal activity of *Warburgia salutaris* bark. (A) Template (B) *Aspergillus flavus*, Lane 1 – *Warburgia salutaris* bark (lane of inhibition), Lane 2, 3 and 4 - *Warburgia salutaris* leaves (moderate zones of inhibition). (B) *Fusarium moniliforme*, Lanes 1, 2, 3 and 4 – *Warburgia salutaris* bark at different capillary volumes (5, 10, 15 and 20 μL respectively) – shows clearance of the migration front of the extract (arrowed - 5)

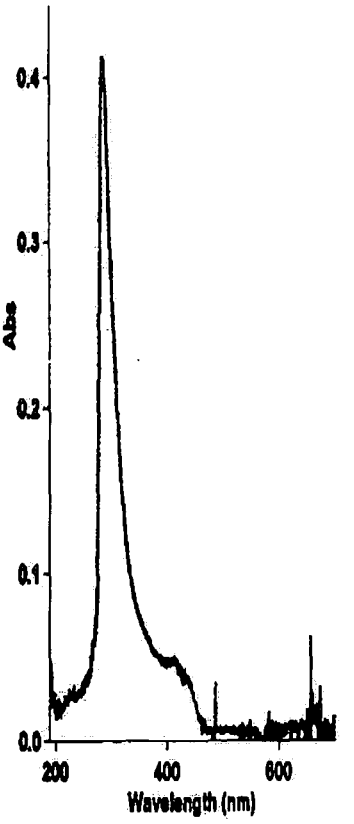
4.2.4 Structural Identification of the Four Active Fractions Isolated from *Warburgia salutaris* stem bark

Ultra-Violet absorption for the four active fractions was determined to be at the highest at a wavelength of 300 nm. Maximum absorption value was 0.42 (Fig 4.14B) and the minimum value was 0.27 (Fig 4.14A). The absorption value for fraction 3 and 4 are illustrated in Fig 4.14C and Fig 4.14D respectively. All four fractions contained compounds that absorbed UV light at wavelength 300 nm.

4.2.4.1 UV Spectroscopy



A



B

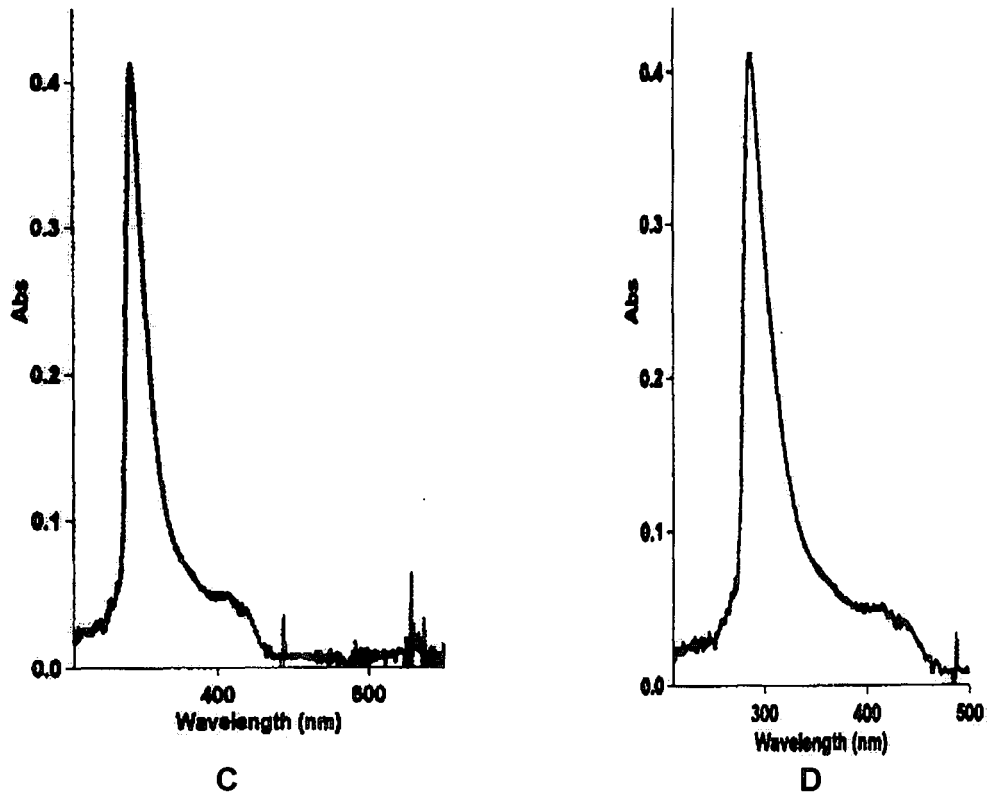
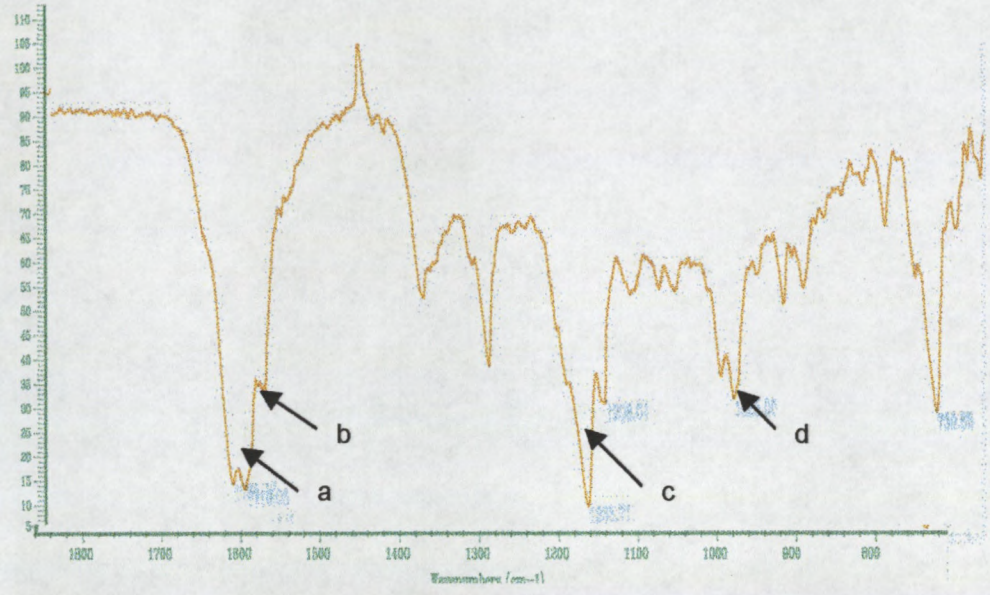


Figure 4.14 Ultra-Violet absorption for the four active fractions isolated from *W. salutaris* bark. A – fraction 1, B – fraction 2, C – fraction 3 and D – fraction 4.

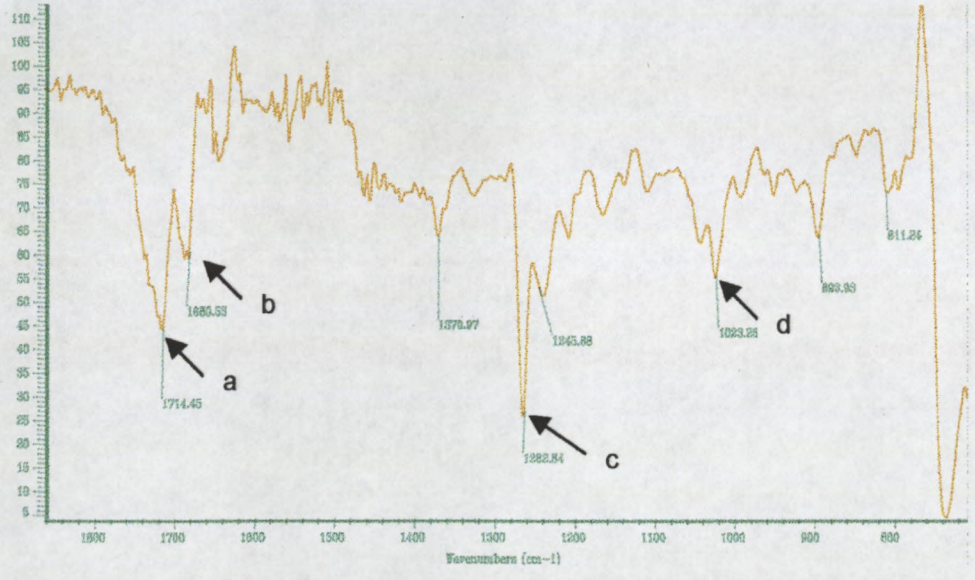
4.2.4.2 IR Spectroscopy

The four active fractions absorb light in the IR spectral range at identical wavenumbers. They were found to have imides, condensed aromatic system, ortho-disubstituted aromatic group and aromatic esters by comparing to IR spectral correlation charts. The four fractions contained the ortho-disubstituted aromatic group at a wavenumber ~ 1200 –1300 cm^{-1}, this could be a probable site for the attachment of the methyl and hydroxyl group side chains. The condensed aromatic system was also found in all four fractions at a wavenumber ~ 1670 –1680 cm^{-1}, this observation is in agreement with that of Fukuyama *et al.*(1982) in which a group of three aromatic rings, two benzene rings and one

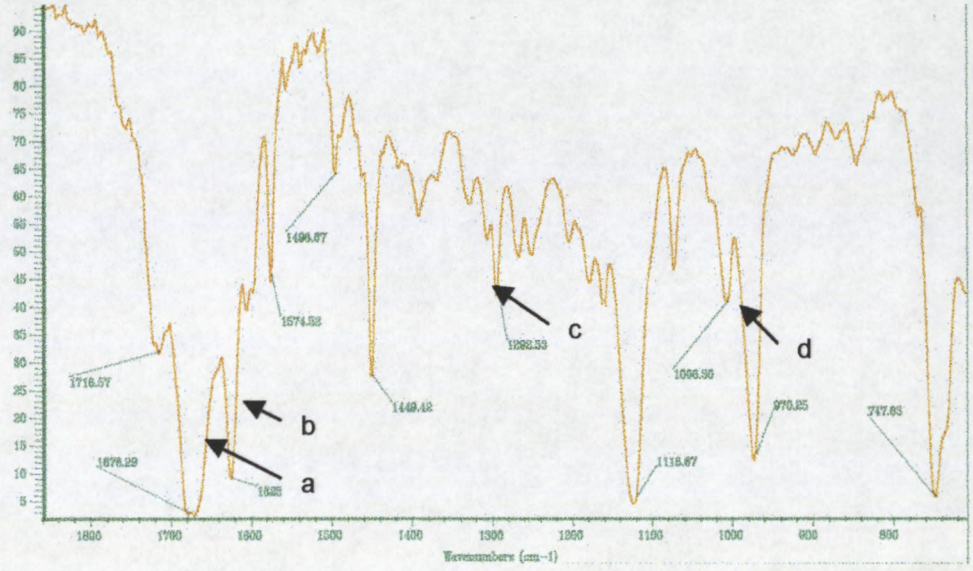
furan ring make up the structural backbone of the compounds. These results are summarized in Table 4.7.



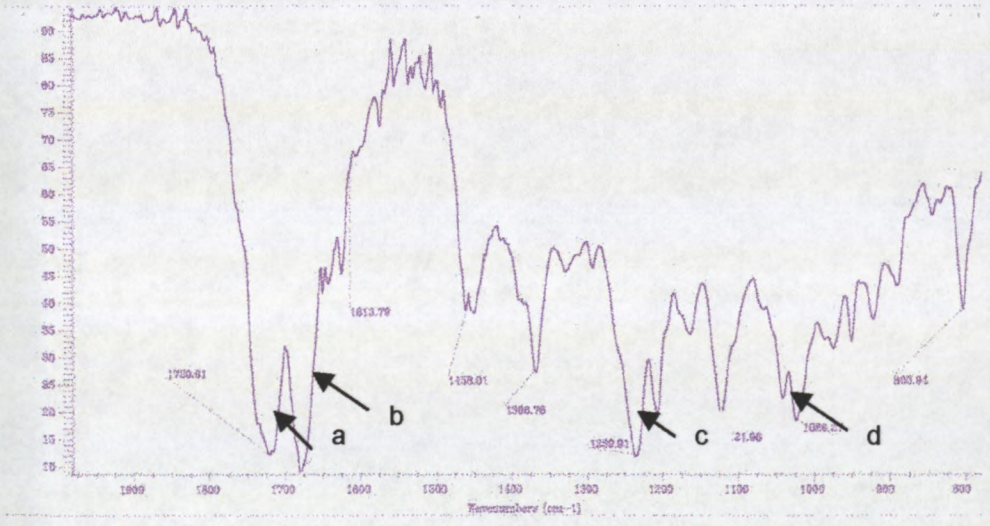
A



B



C



D

Figure 4.15 Infra-Red scans of the four active fractions isolated from *Warburgia salutaris* bark. A - Fraction 1, B – Fraction 2, C – Fraction 3 and D – Fraction 4.

Table 4.7 Similarities of IR absorption wavenumbers (cm^{-1}) for the four active fractions isolated from *Warburgia salutaris*.

	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Corresponding functional groups
Peak 1 (a)	1718.05	1714.45	1716.57	1720.61	Imides
Peak 2 (b)	1670.00	1680.53	1676.29	1676.11	Condensed aromatic system
Peak 3 (c)	1235.71	1282.84	1292.53	1239.91	Ortho-disubstituted aromatic ring
Peak 4 (d)	1025.00	1023.26	1006.30	1026.27	Aromatic esters

4.2.4.3 Gas Chromatography – Mass Spectroscopy

Fractions 1 and 2 contained 1, 2 benzenedicarboxylic acid, dibutyl ester and eicosane. 1, 2 benzenedicarboxylic acid, dibutyl ester is a phthalate derivative, which is used as plasticizers and is a common contaminant of mass spectra of compounds. The percentage quality matches for the four fractions verified these results, which is given in Table 4.8.

Table 4.8 Identification and corresponding percentage quality matches for the four active fractions from *Warburgia salutaris*.

Compound Identity	Library Match Quality (%)
Fraction 1	
1,2 benzenedicarboxylic acid, dibutyl ester	91
Eicosane	85
Fraction 2	
1,2 benzenedicarboxylic acid, dibutyl ester	91

Eicosane	82	
Fraction 3		
Eicosane	83	
Hexadecanoic acid, ethyl ester	97	
1,2 benzenedicarboxylic acid, dibutyl ester	91	
(5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-Octahydro-6, 6, 9A-trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) (arrowed A)	80	(Figure 4.18)

Fraction 4		
1,2 benzenedicarboxylic acid, dibutyl ester	90	
Eicosane	91	
Hexadecanoic acid, ethyl ester	99	
5, 10-Dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan (arrowed B)	91	(Figure 4.16, 4.19)
(5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-Octahydro-6, 6, 9A-trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) (arrowed A)	98	(Figure 4.16, 4.18)
Tetradecane	83	
1, 5, 5, 8-Tetramethyl-12-Oxabicyclo [9.1.0] dodeca-3, 7-diene	87	
9, 10 dimethylphenantrene	90	
Docosane	91	
3, 8-Dimethyl decane	9	
17-Pentatriacontene	81	

Fractionation GC-MS scans are illustrated in Appendix 2. The above identifications are based on a HPPest, PMW_TOXR and Wiley275 library search and are by no means absolute.

Fractions 1 and 2 did not contain the drimane-type sesquiterpenoid compound but contained a high level of the phthalate contaminant and fraction 3 contained a higher abundance of the contaminant (1,2 benzenedicarboxylic acid, dibutyl ester) than the compounds of interest (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-

Octahydro-6, 6, 9A- trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) (Table 4.8, Figure 4.16). Further work with these three fractions could not be carried out because of the high level of contaminant in the sample.

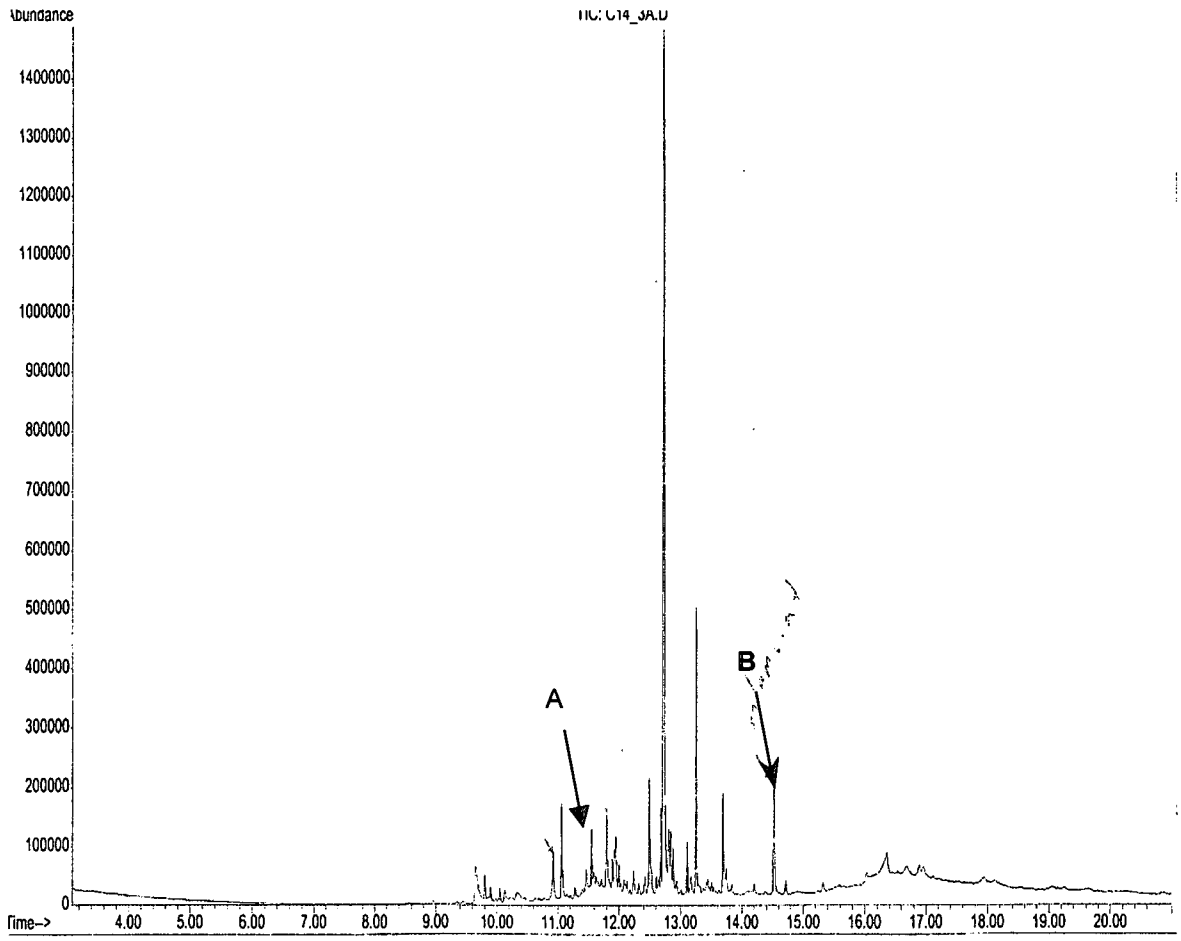


Figure 4.16 GC-MS of fraction 3 showing (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-Octahydro-6, 6, 9A- trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) and contaminant, 1,2 benzenedicarboxylic acid, dibutyl ester (arrowed B)

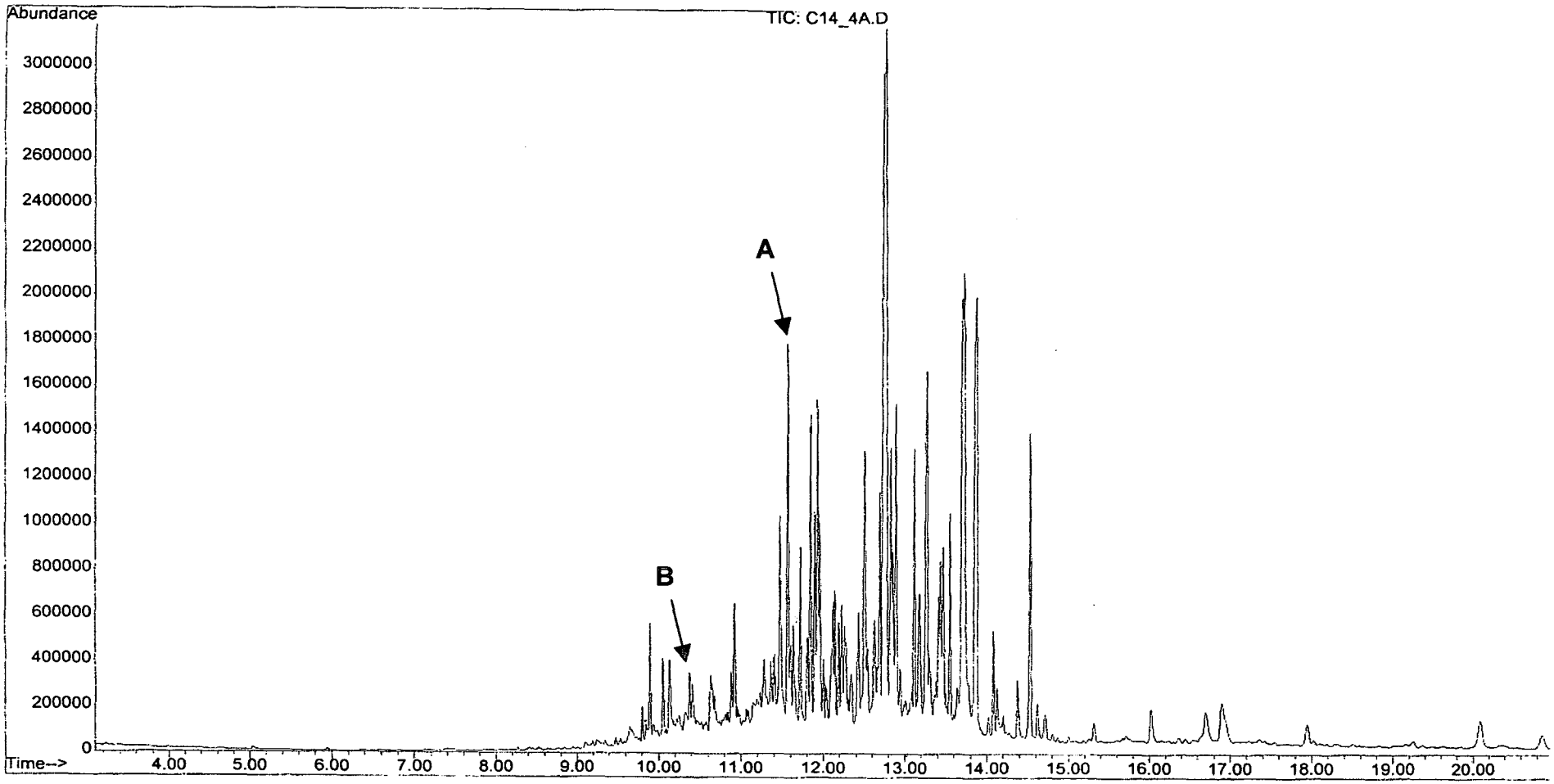


Figure 4.17 GC-MS scan of fraction 4. The peaks for the sesquiterpeniod lactones, (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-Octahydro-6, 6, 9A- trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) (arrowed A) and 5, 10-Dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan (arrowed B) are shown.

4.2.4.3.1 Fractionations of the GCMS scan

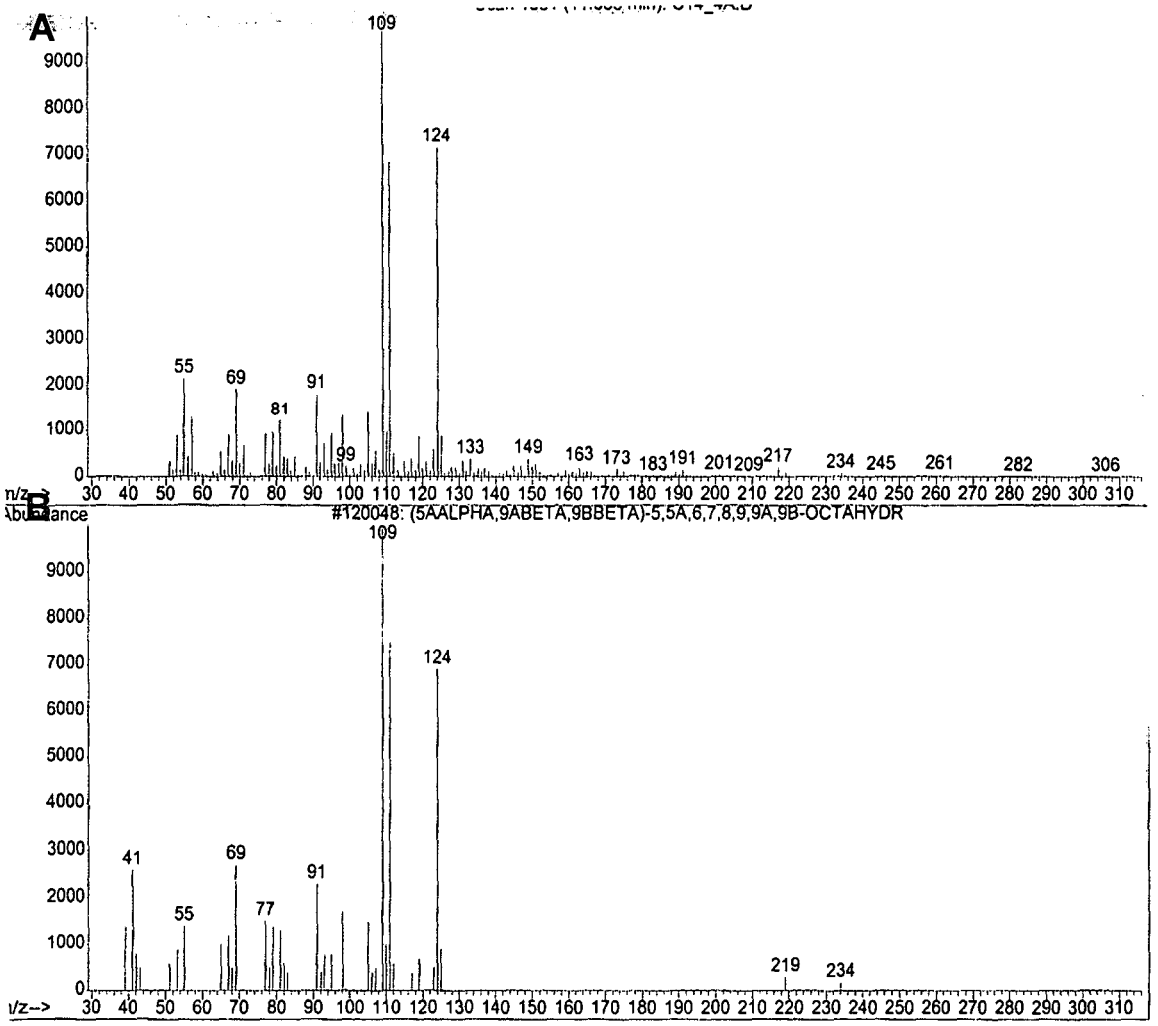


Figure 4.18 GC-MS fractionation scan comparing the purity of compound isolated from *Warburgia salutaris* (A) fraction 4 against a standard, (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-Octahydro-6, 6, 9A- trimethylnaphtho[1(B) furan-1-(3H)-one.

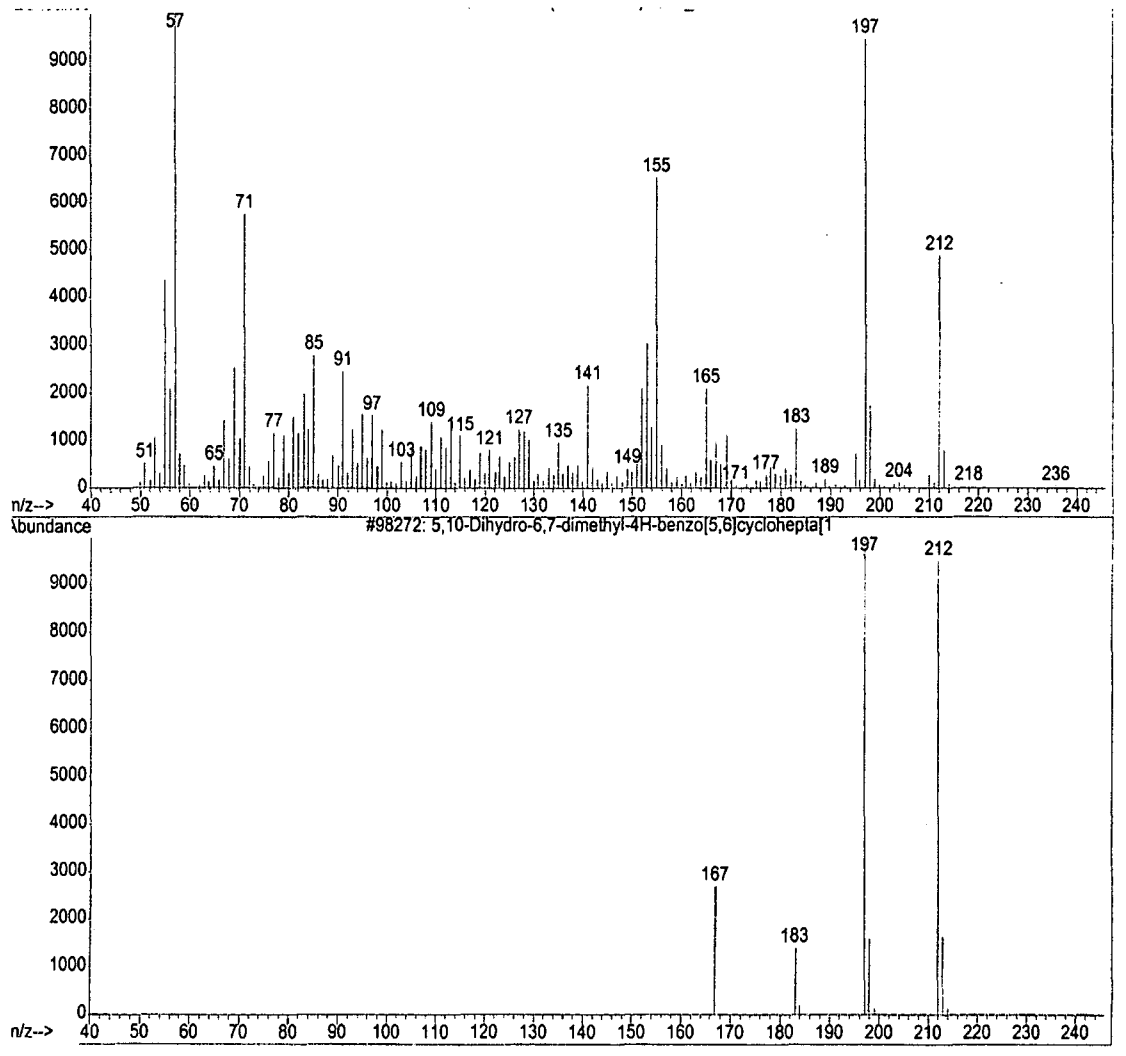


Figure 4.19 GC-MS fractionations scan comparing the purity of compound isolated from *Warburgia salutaris* (A) fraction 4 against a standard, 5, 10-Dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan.

Fractionation GC-MS scans shows that two compounds isolated from *Warburgia salutaris* fraction 4 are drimane-type sesquiterpenoids with known prolific antifungal compounds.

CHAPTER FIVE

5 DISCUSSION

5.1 Phytoalexins

UV illumination and *P. digitatum* infection were successful in the induction of several fluorescent compounds from Valencia oranges. The antifungal activity of the fluorescent compounds by bioautography revealed a compound with the Rf value of 0.65 to be antifungal against *A. flavus* and *F. moniliforme*. The compound with the most potent antifungal activity was later identified as 6,7 dimethoxycoumarin. Further purification and quantitation was achieved by HPLC.

Kim *et al.* (1991) found trace amounts of 6, 7 dimethoxycoumarin ~1 – 2 μg/g fresh weight of non-inoculated flavedo in fruit. Figure 5 shows that 6, 7 dimethoxycoumarin appeared after inoculation, but its concentration increased rapidly in the *P. digitatum* infected fruit. The concentration of the compound increased to 35.5μg/g fresh weight after 14 days inoculation. After reaching that peak, 6, 7 dimethoxycoumarin concentration declined to 5μg/g fresh weight in the fruit 30 days after inoculation. After reaching a peak of 35.51μg/g at 14 days inoculation the concentration of the compound declined. Such a pattern of accumulation and degradation is typical of phytoalexins in plant tissues (Bailey *et al.*, 1976).

It was also observed that 6, 7 dimethoxycoumarin accumulated at the inoculation sites of Valencia oranges but was not detected on the side opposite the

inoculation site or even 1 cm away from the inoculation site. This observation agrees with the report (Ingham, 1973) that phytoalexin biosynthesis is confined to the infected cells and their immediate vicinity in most plants.

UV illumination was also an effective elicitor of phytoalexin. This is in agreement with an earlier report (Hardwiger and Schwochau, 1971). It appears that unlike mechanical injury or drying treatments, UV illumination induced production of 6, 7 dimethoxycoumarin without *Penicillium* inoculation in Valencia tissue (Ben-Yehoshua, 1985). Drying of the UV illuminated tissue showed an increase in 6, 7 dimethoxycoumarin concentration (Chromatogram 5), thus showing the effect of temperature on 6, 7 dimethoxycoumarin production. Arimoto and Homma. (1988) found that the largest quantity of 6, 7 dimethoxycoumarin was produced in the melanose spots or scars at 25 °C, with progressively low levels at lower temperatures until no 6, 7 dimethoxycoumarin was detected at 5 to 10 °C. Afek and Sztejnberg (1988) reported that the concentration of 6, 7 dimethoxycoumarin in the bark of rough lemon was sevenfold higher at 28 °C than at 20 °C, in parallel with the increase of resistance to *Penicillium citrophora*. The level of 6, 7 dimethoxycoumarin accumulated by UV elicitation was sufficient to inhibit the growth of the fungus in the fruit. UV illumination elicited small concentrations of 6, 7 dimethoxycoumarin accumulation compared to pathogen inoculation, demonstrating the major role of pathogen in the elicitation of defense response. Phytoalexin accumulation in lemon fruit showed good correlation to reports by Baudion and Eckert (1985) who did not find any phytoalexin or antifungal activity in lemons, due to rapid decaying of the infected fruit, hence insufficient time for

phytoalexin accumulation. Results have thus far indicated that 6, 7 dimethoxycoumarin was absent in non-infected Valencia oranges and appeared only after inoculation with a *P. digitatum*, however it was also noted that 6, 7 dimethoxycoumarin was present at low levels in healthy fruit that was not stressed (chromatogram 4). Tatum and Berry. (1977) reported the presence of 6, 7 dimethoxycoumarin in just-harvested oranges, however the quantities of the harvested fruit were insufficient to bring out the antifungal effect of 6, 7 dimethoxycoumarin (Kim *et al.*, 1991). The concentrations of 6, 7 dimethoxycoumarin produced by UV treatment was sufficient to inhibit the development of decay.

These points support previous conclusions (Afek and Sztejnberg, 1988; Afek and Sztejnberg, 1988a; Arimoto and Homma, 1988; Arimoto *et al.*, 1986; Arimoto *et al.*, 1986a) that 6, 7 dimethoxycoumarin is a phytoalexin. The spectral data obtained from the induced compound showed the presence of the four functional groups viz., aromatic backbone, conjugated alkene, alkene and dimethyl substituent group which is in good agreement with Afek and Sztejnberg (1988), proving the structural identity of the induced compound as 6, 7 dimethoxycoumarin.

Application of phytoalexins in corn for mycotoxin prevention encompasses prevention of toxin biosynthesis and metabolism in the field or in storage. Mycotoxin detoxification refers to post-harvest treatments to remove, destroy or reduce toxic effects of the mycotoxigenic fungi. Failure to prevent mycotoxin

formation in the field or in storage will lead to an increased health risk and economic loss. Since *F. moniliforme* is a seed-borne, symptomless endophyte in corn, its elimination will be difficult. Detoxification strategies based on physical, chemical or microbiological factors can detoxify corn by destroying, modifying or absorbing the mycotoxin so as to reduce or eliminate its toxic effects. Another possible post -harvest detoxification strategy is the use of phytoalexins. These induced compounds have a wide spectrum of action against a large number of fungi and also some bacteria and viruses. According to the results obtained in this study, stimulation of decay resistance in UV illuminated and *P. digitatum* infected fruit could be related to the phytoalexin, 6, 7 dimethoxycoumarin elicited in the fruit by the treatment. Other stress-induced processes, such as lignification (Brown *et al.*, 1978) and PR-protein formation (Christ and Mosinger, 1989), may also contribute to this effect. Pathogen inhibition in this study is moderate, considering the ED_{50} of 6, 7 dimethoxycoumarin and the level of its accumulation in treated fruit.

Further optimization of fruit treatment and storage conditions could enhance positive *P. digitatum* induction and UV effects and diminish undesirable ones. Finally these phytoalexins may provide a stimulus for conceiving novel, non-polluting pesticides.

5.2 Constitutive Compounds

The antibacterial activity of the plant extracts tested was found mainly against the Gram-positive bacteria. *W. salutaris* (ethyl acetate and dichloromethane extracts) showed activity against *S. aureus, S. marcescens* and *P. aeroginosa*. The negative results obtained against the Gram negative bacteria was not surprising as, in general, these bacteria are more resistant than Gram positive ones (Rios *et al.*, 1991; Rabe and van Staden, 1997). The two different extraction techniques employed in this study resulted in the release of various volatile compounds. The heat-treated solvent extraction technique proved more effective in the release of isoprene compounds (terpenoids). *Warburgia salutaris* has been reported to release a wide variety of isoprene compounds. The drimane type sesquiterpene dialdehyde , Warburganal isolated from *Polygonum hydropiper* exhibited potent antifeedant; antibiotic and molluscicidal activity (Fukuyama *et al.*, 1982, Clarke et al., 1997). Other drimane type sesquiterpenes polygodial, isopolygodial, isodrimenol, drimenol, confertifolin and a nor-sesquiterpene monoaldehyde, polygonal has also been isolated from *Warburgia* spp.

A typical pungent odour and bitter taste of *Warburgia* spp. was also exhibited by *W. salutaris*. The bitter characteristic refers to the bitter tasting monoterpenoid lactones known as iridoids. Iridoids are components of volatile oils, which have been used to stimulate actions within the body, such as mucosal or gastric secretion. Iridoids usually occur in angiosperms, especially valerian, gentian, blue flag and orris root and can have therapeutic uses/properties such as

expectorants; anthelmintics; insecticides; antiseptics; antimicrobial; antileukemic properties (Ingham, 1973) and interferon-like activity (Smolarz and Skwarek, 1999).

Fukuyama *et al.* (1982) reported the presence of various sesquiterpenoid dialdehydes from *Warburgia* spp. They also reported the distribution of seven different drimane-type sesquiterpenoids in *Polygonum hydropiper* (Table 5.1)

Table 5.1 Distribution of dimane-type sesquiterpenoids in *Polygonum hydropiper and* two *Warburgia spp.*

		Compounds														
Species		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Polygonaceae	*P. hydropiper*	+	+	+	-	-	+	+	+	-	+	-	-	-	-	-
Canellaceae	*W. stuhlmanni*	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
	W. ugandesis	-	-	+	+	-	-	-	-	-	-	+	-	-	+	+

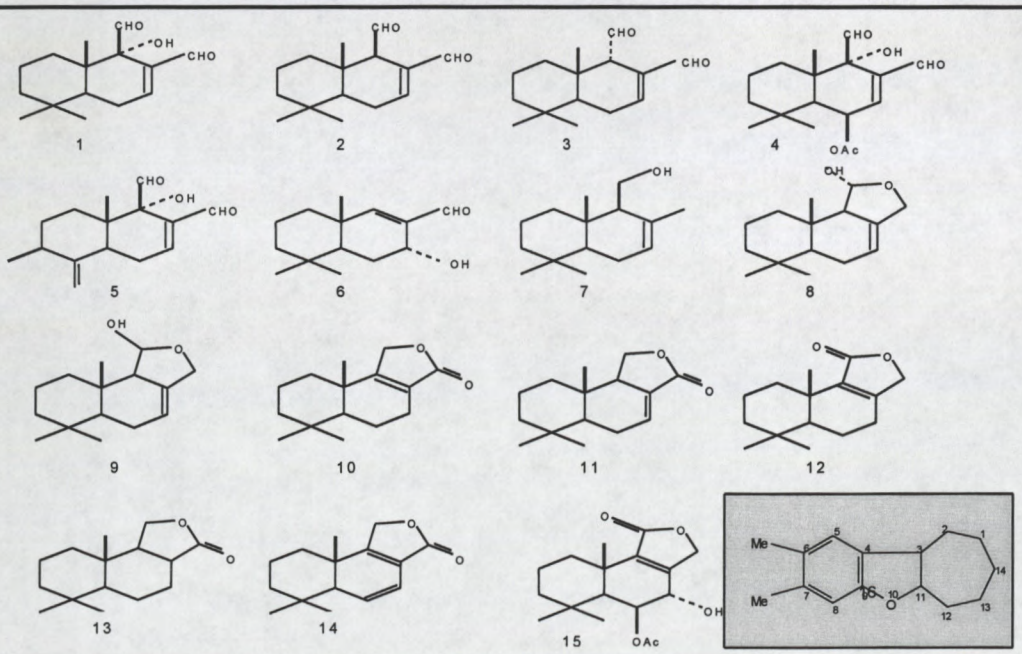


Figure 5.1 Structural configuration of drimane-type sesquiterpenoids isolated from *W. stuhlmanii, W. ugandensis* and *P. hydropiper*

The structural similarities between the sesquiterpenoids isolated from *Warburgia stuhlmanii* and *W. ugandensis* and the constitutive compound isolated from *W. salutaris* is shown in Fig 5.1. Warburganal (Fig 5.1; 1), Polygodial (Fig 5.1; 2), Ugansidial (Fig 5.1; 4), Muzigadial (Fig 5.1; 5) and precursors of drimanolides (Fig 5.1; 11, 14 and 15) isolated from *Warburgia stuhlmanii* and *W. ugandensis* were similar in structure in that they all contained condensed aromatic ring system, hydroxyl groups, an unsaturated aldehyde group and tertiary methyl groups. A compound isolated and identified from *Warburgia salutaris* showed the presence of a condensed aromatic system in which a benzene ring was attached to a furan ring system, which was coupled with a 7-carbon aromatic ring. 5, 10-dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan (Fig 5.1; 16) also contained two hydroxyl groups at positions C-5 and C-10, and a pair of primary methyl groups at positions C-5 and C-6.

Acetylation of isodrimenol (Fig 5.1; 8) yielded a labile monoacetate and a β, β-disubstituted furano compound (Fig 5.1; 16). A similar compound was isolated from *W. salutaris*, (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-octahydro-6, 6, 9A-trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin). (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-Octahydro-6, 6, 9A- trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) and 5, 10-dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan isolated from *Warburgia salutaris* shows a good similarity in structure to the drimane-type unsaturated sesquiterpenoid dialdehydes (Fig 5.1) isolated from *Warburgia stuhlmanii* and *W. ugandensis* which is responsible for the high antifungal activity, thus showing the potential of (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9,

9A, 9B-octahydro-6, 6, 9A- trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) and 5, 10-dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan as antifungal agents.

The hot-tasting sesquiterpenoids isolated from *W. salutaris* were identical to potent African armyworm antifeedants isolated from *W. ugandensis.* The (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-octahydro-6, 6, 9A- trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) and 5, 10-dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan isolated from *W. salutaris* form part of a large number of phytochemicals that have been isolated as antimicrobial agents. Their activity is usually not potent enough to be considered for practical application. Future studies to enhance their biological activities are needed. Applications could also include the treatment of systemic fungi. The difficulty involved with the treatment of systemic fungi is the similarity between fungal cells and mammalian cells, "What kills fungi also puts the host cell at risk" (Kamuhabwa *et al.*, 2000). It is important therefore, to search for compounds that can target fungal cells specifically, and be administered at low dosages. The fungicidal activity of (5Aα, 9Aα, 9Bβ)-5, 5A, 6, 7, 8, 9, 9A, 9B-octahydro-6, 6, 9A- trimethylnaphtho[1, 2c] furan-1-(3H)-one (drimenin) and 5, 10-dihydro-6, 7-dimethyl-4H-benzo [5, 6] cyclohepta [1, 2-b]-furan , with possible mechanism of synergism with other naturally occuring compounds, makes these two compounds promising antimicrobial agents.

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APPENDICES

APPENDIX 1: Materials and Method for Fungal Bioautography

Media and Reagents

Mineral Salts Stock Solution:

KH_2PO_4	7g
$Na_2HPO_4.2H_2O$	3g
KNO_3	4g
$MgSO_4$-$7H_2O$	1g
NaCl	1g
Tap water	1000 ml

30 % aqueous glucose solution

Methodology.

1. The mineral salts stock solution and the 30 % aqueous glucose solution were sterilized by filtering through a 0.22 gm-pore-diameter filter.
2. 5μL of each of the above plant extract-chloroform solutions were spotted onto two TLC plates.
3. TLC plates were developed using a Toluene: Ethyl acetate(2: 1) solvent system and then air dried.
4. TLC plate was sprayed with vanillin-sulphuric acid solution, dried in a drying oven, and used as a template
5. 10mL of the 30 % aqueous glucose solution was added per 60ml of mineral salts stock solution, to which fungal spores were added.
6. The other TLC plate was then sprayed with the spore suspension, and then incubated under warm (25°C), moist conditions for 5 - 7 days.

7. After incubation the TLC plate was compared with the template for the presence or absence of inhibition zones.

APPENDIX 2: Fractionation GC-MS results for fractions 3 and 4 isolated from *Warburgia salutaris.* Quality matches > 80%

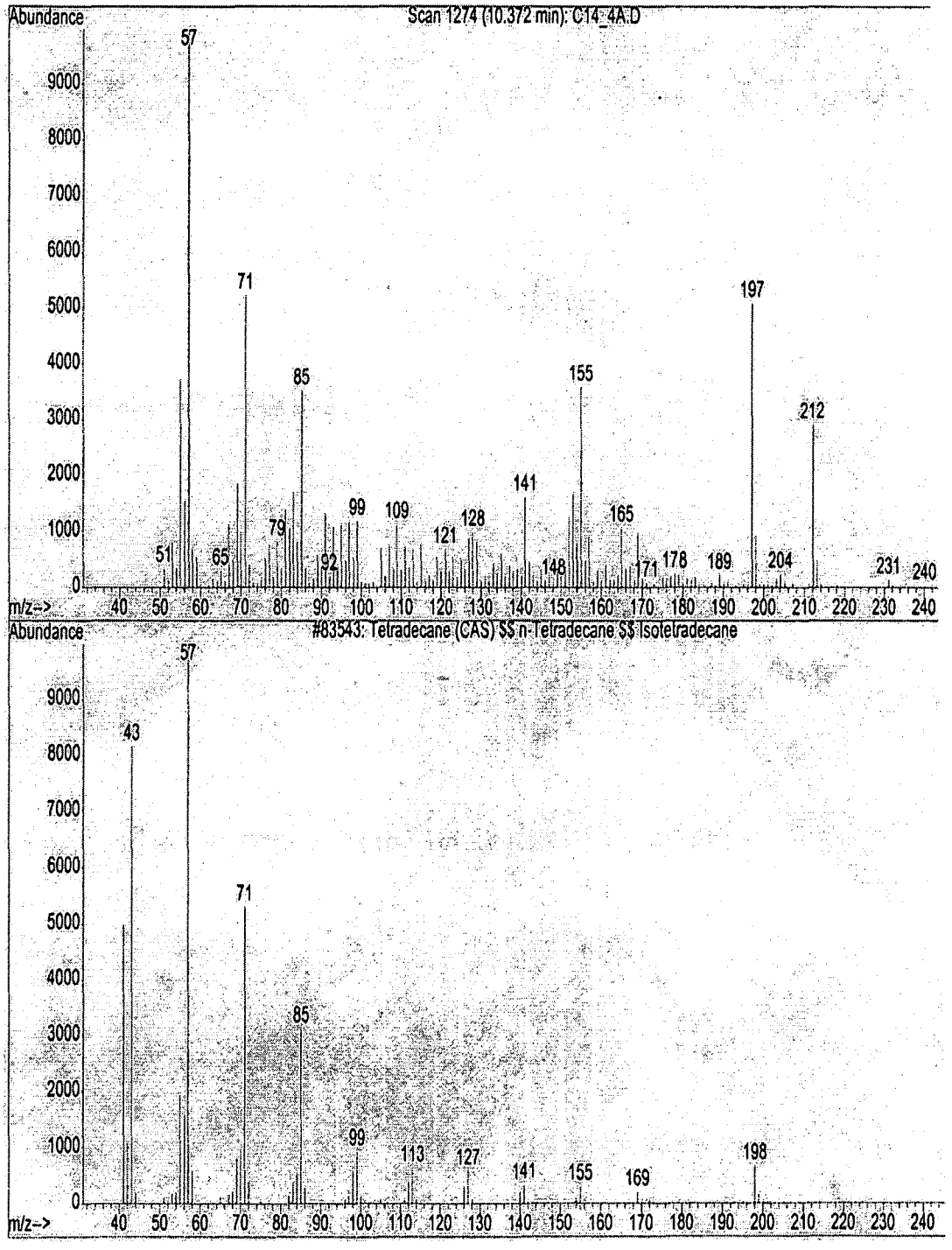

Figure 1. Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, tetradecane.

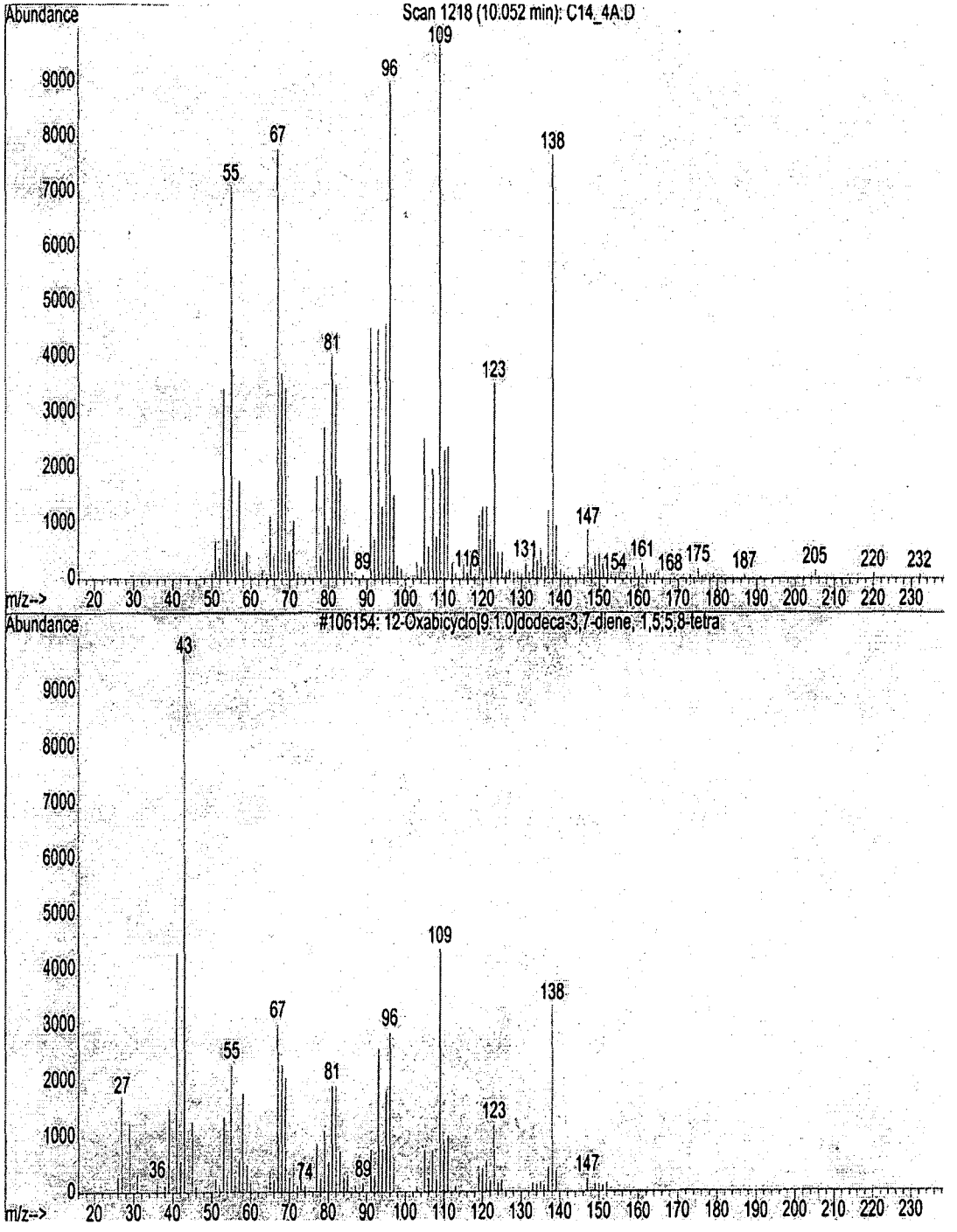

Figure 2 Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, 12-Oxabicyclododeca-3, 7-diene.

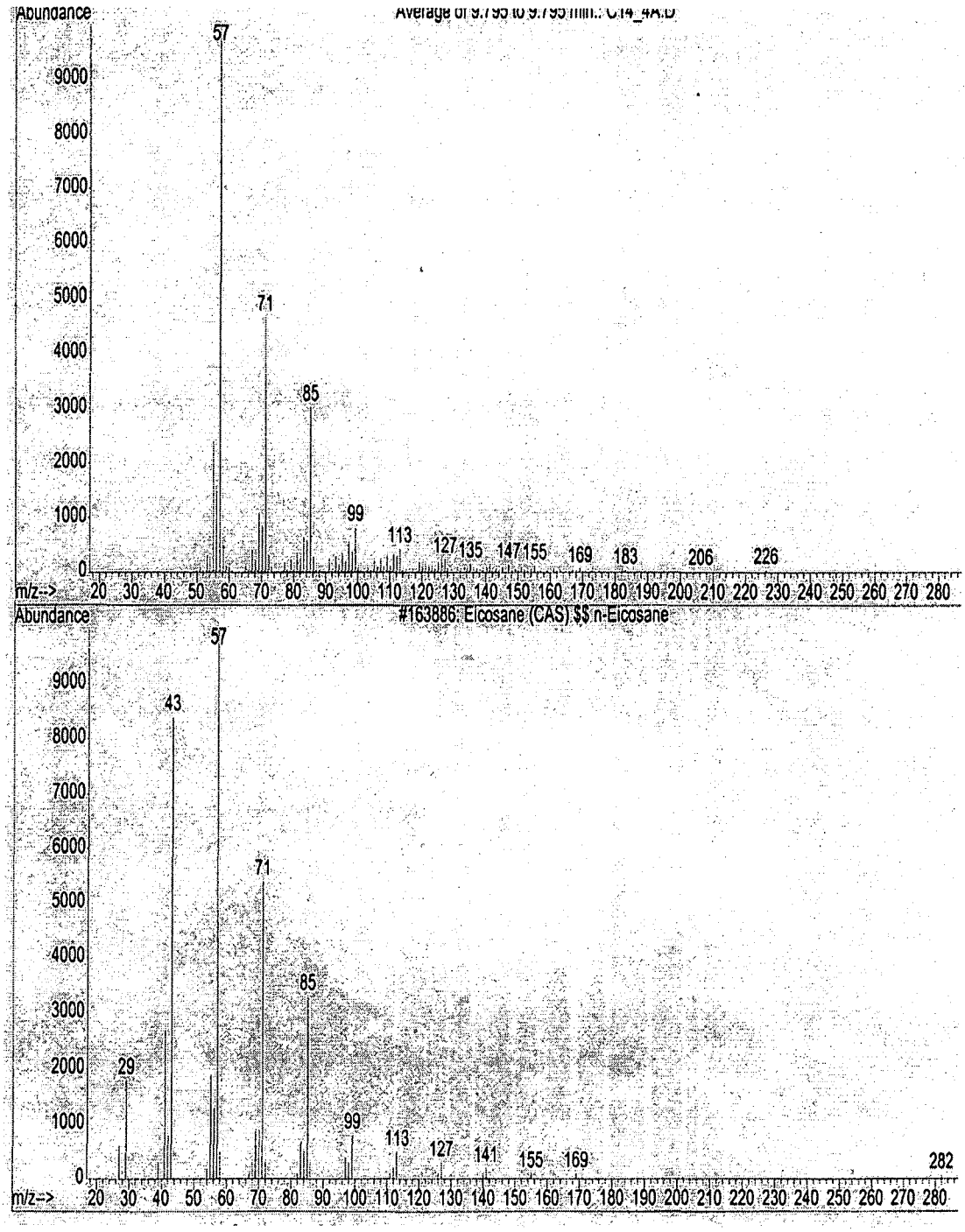

Figure 3. Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 3 and 4 against a commercial standard, n-Eicosane.

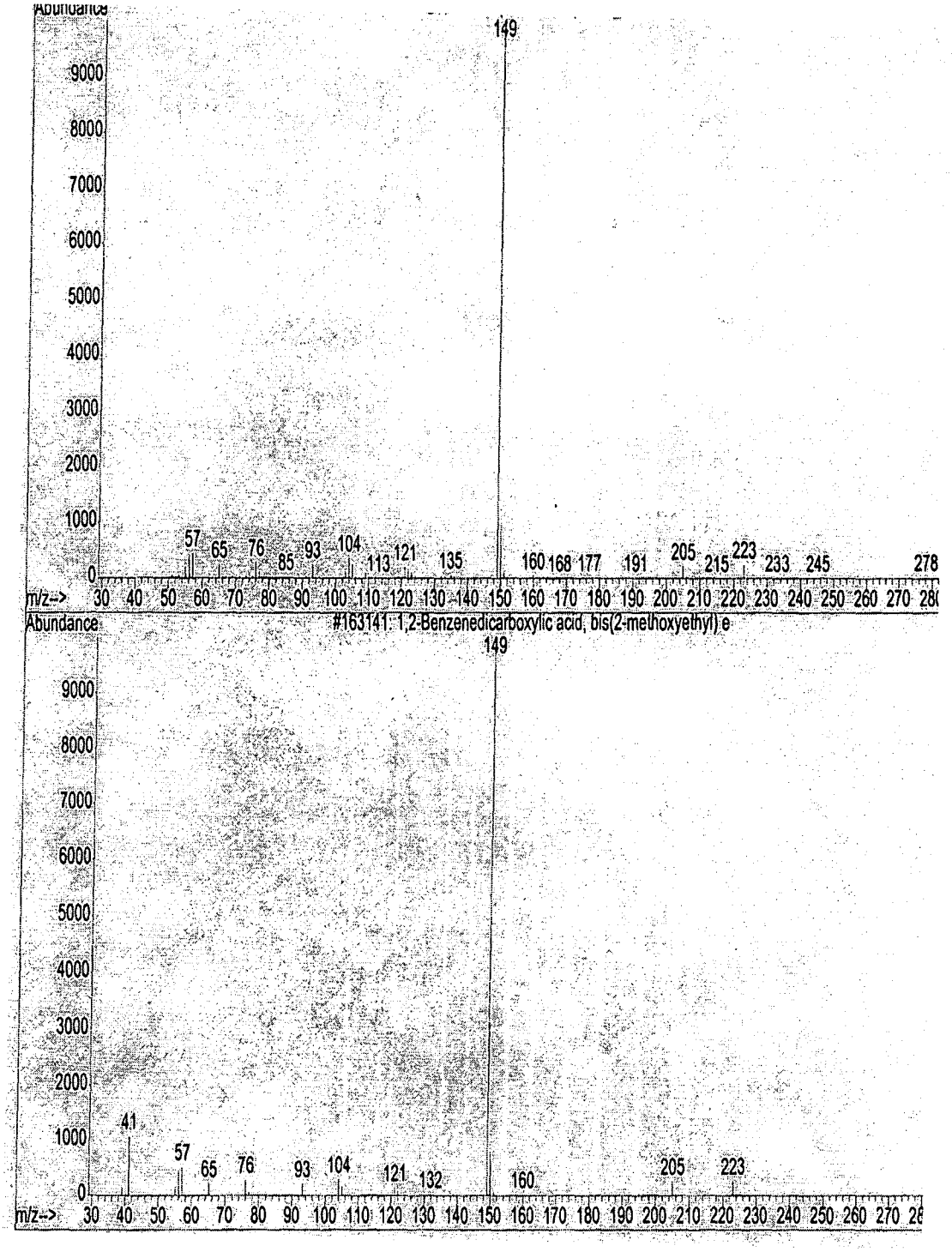

Figure 4. Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 3 and 4 against a commercial standard, 1.2 benzenedicarboxylic acid.

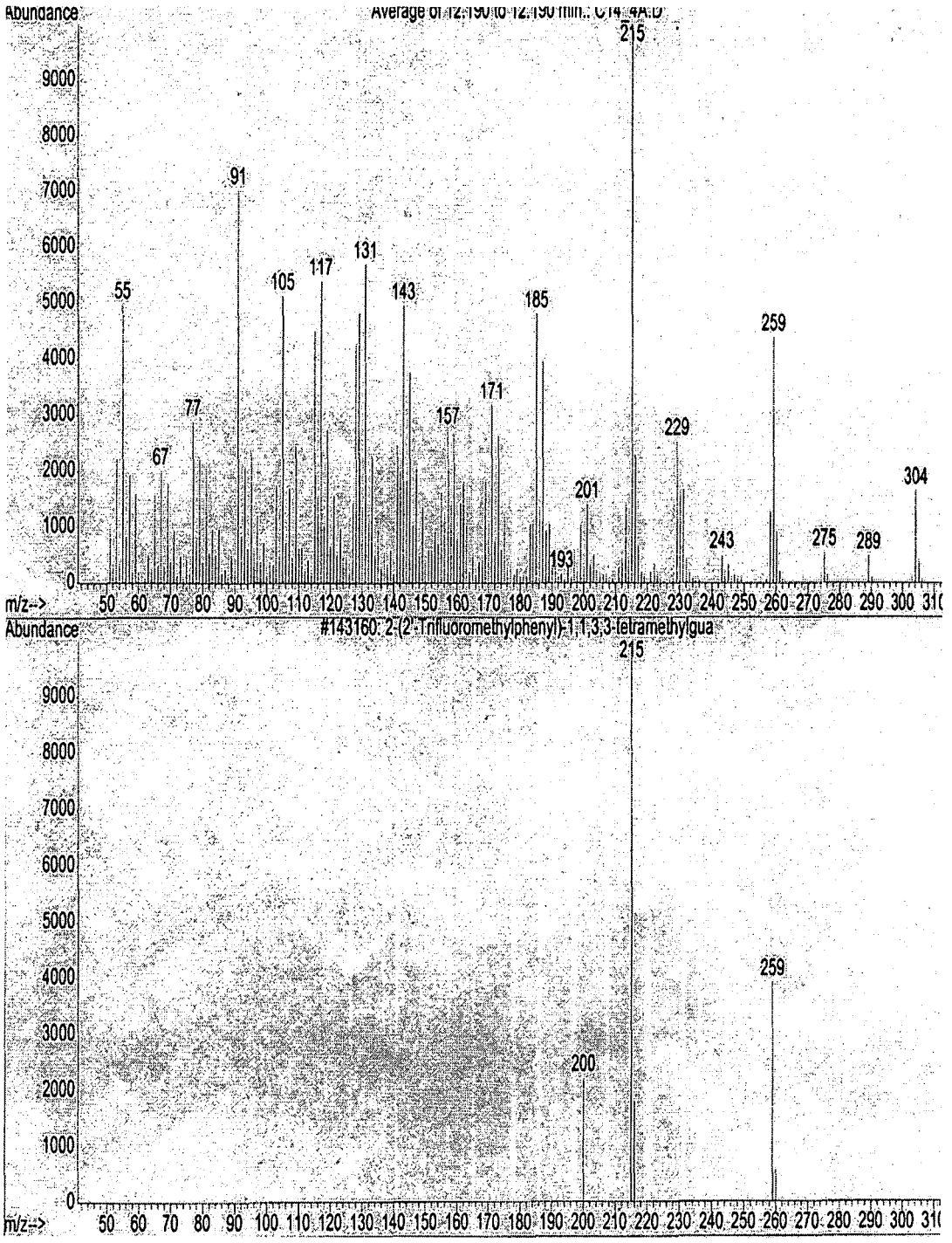

Figure 5. Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, 1, 1, 3, 3- tetramethylguanidine

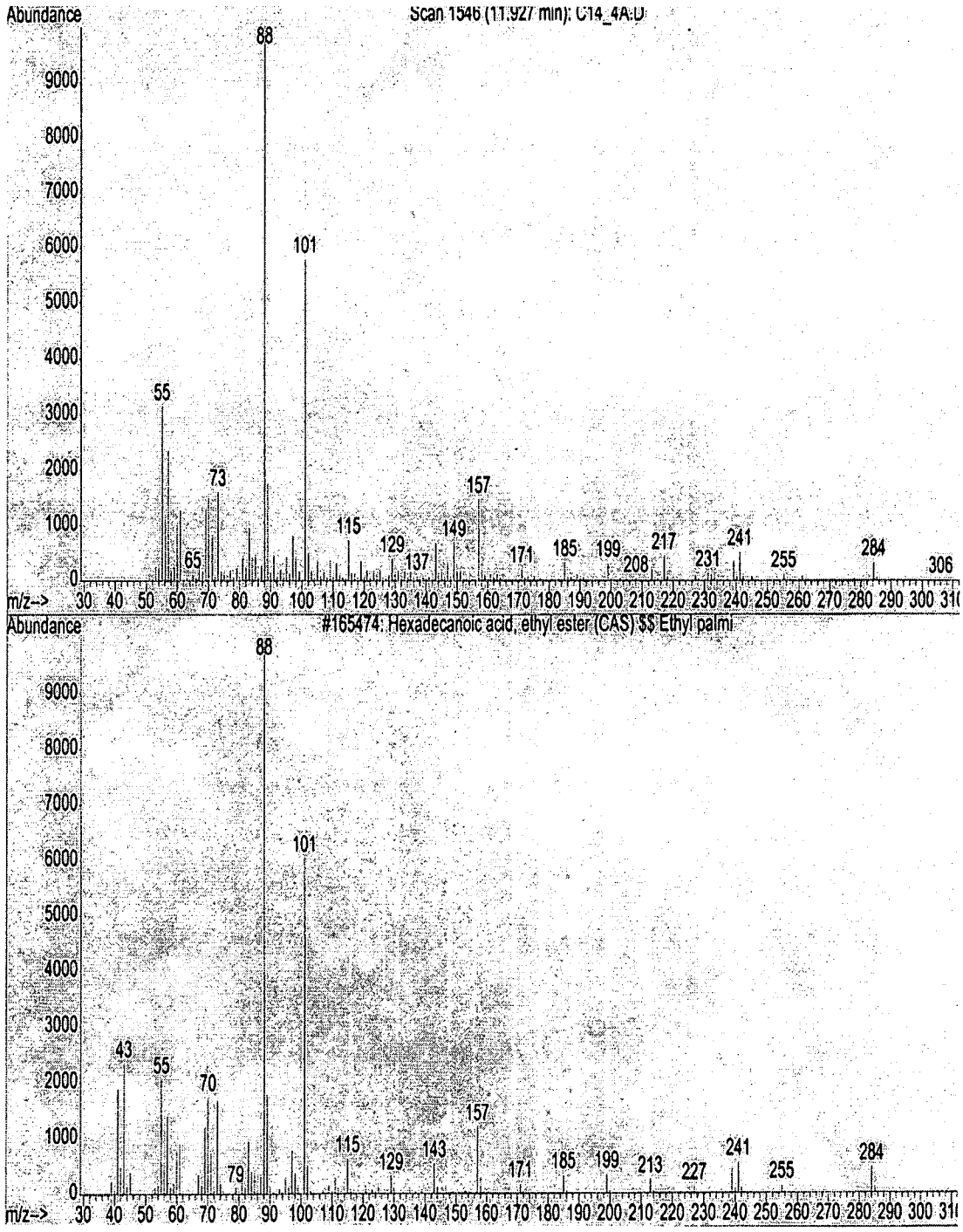

Figure 6. Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 3 and 4 against a commercial standard, Hexadecanoic acid, ethyl ester.

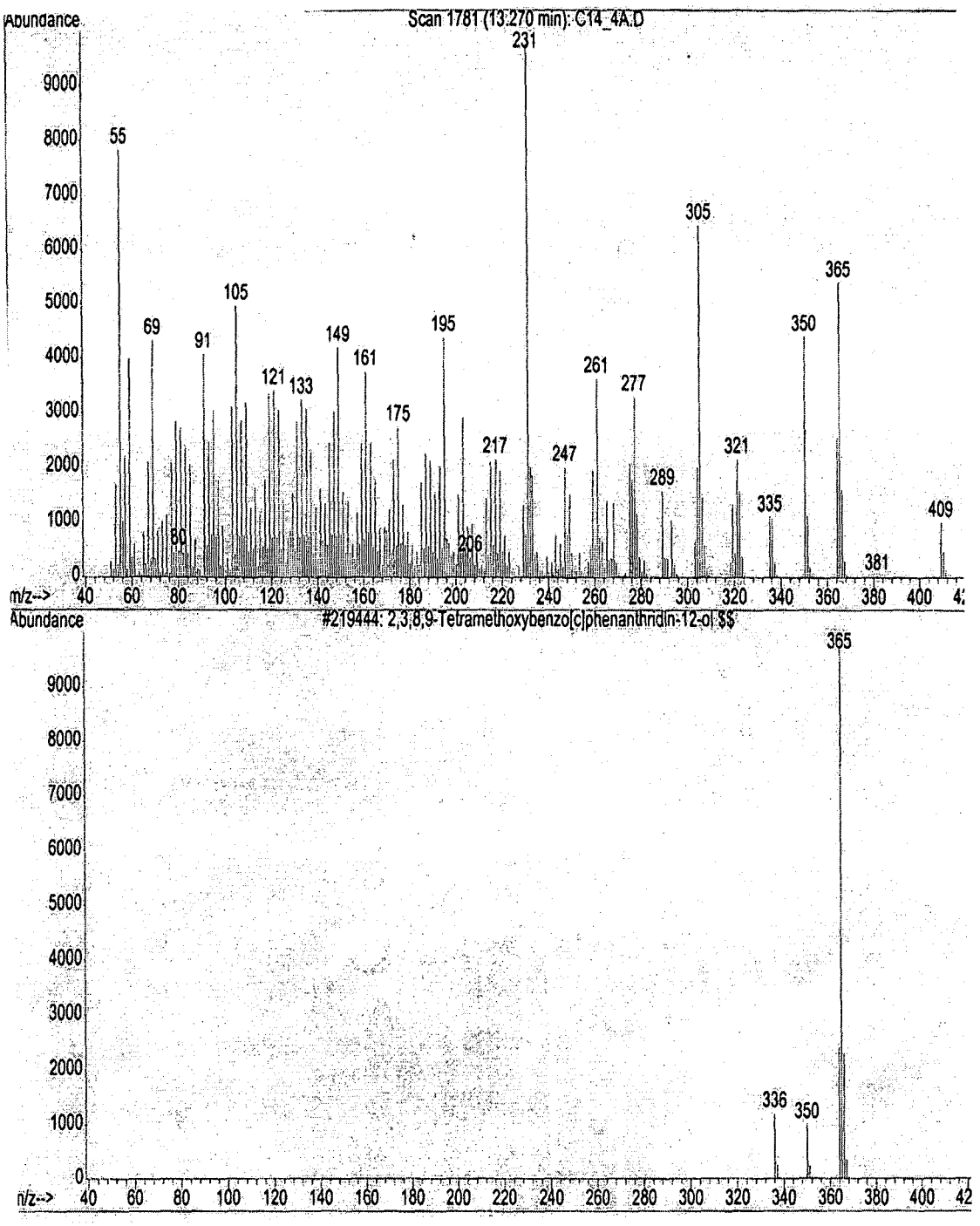

Figure 7 Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, 2, 3, 8, 9 Tetramethoxy benzophenanthridin – 12- ol.

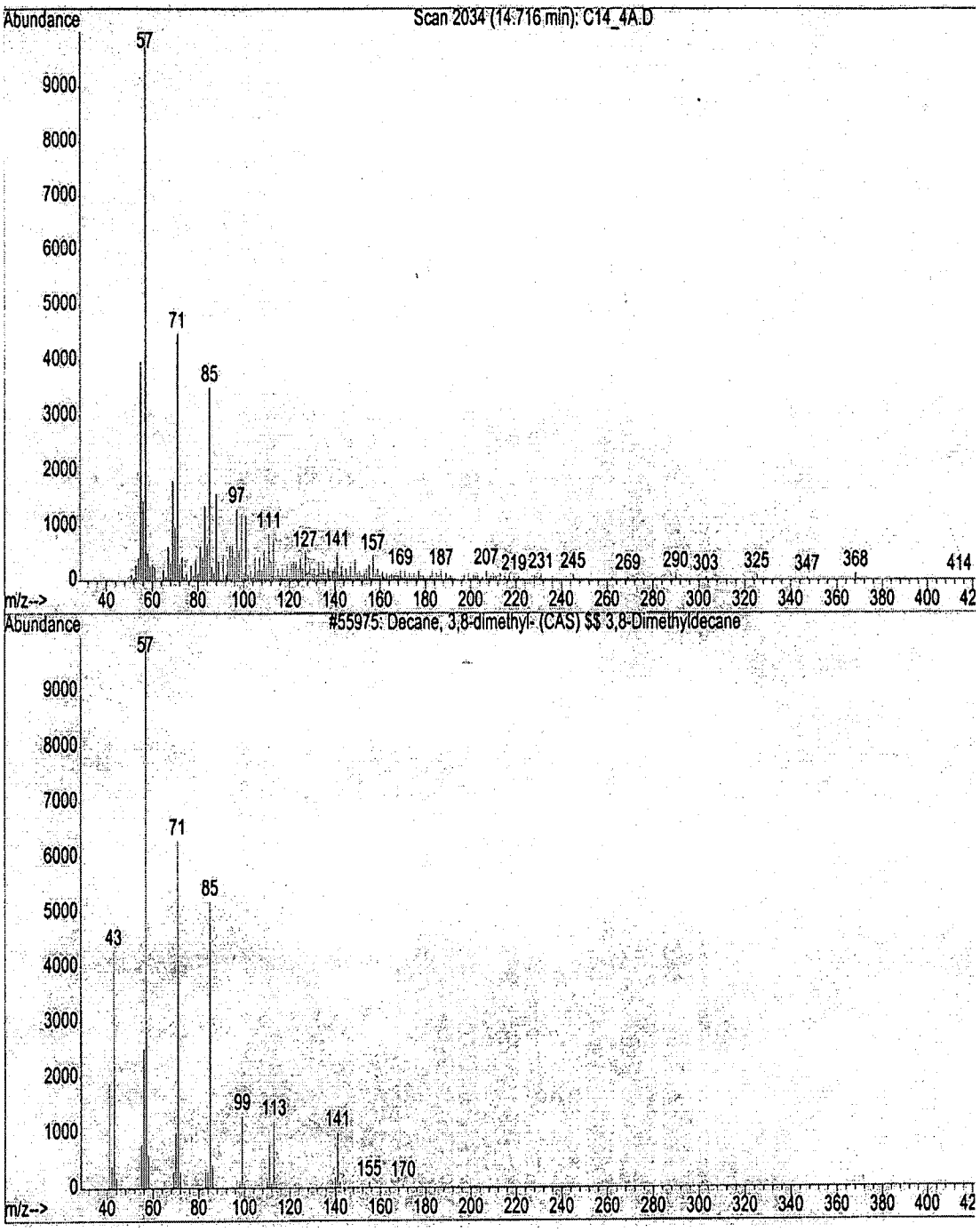

Figure 8 Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, 3, 8 dimethyl decane.

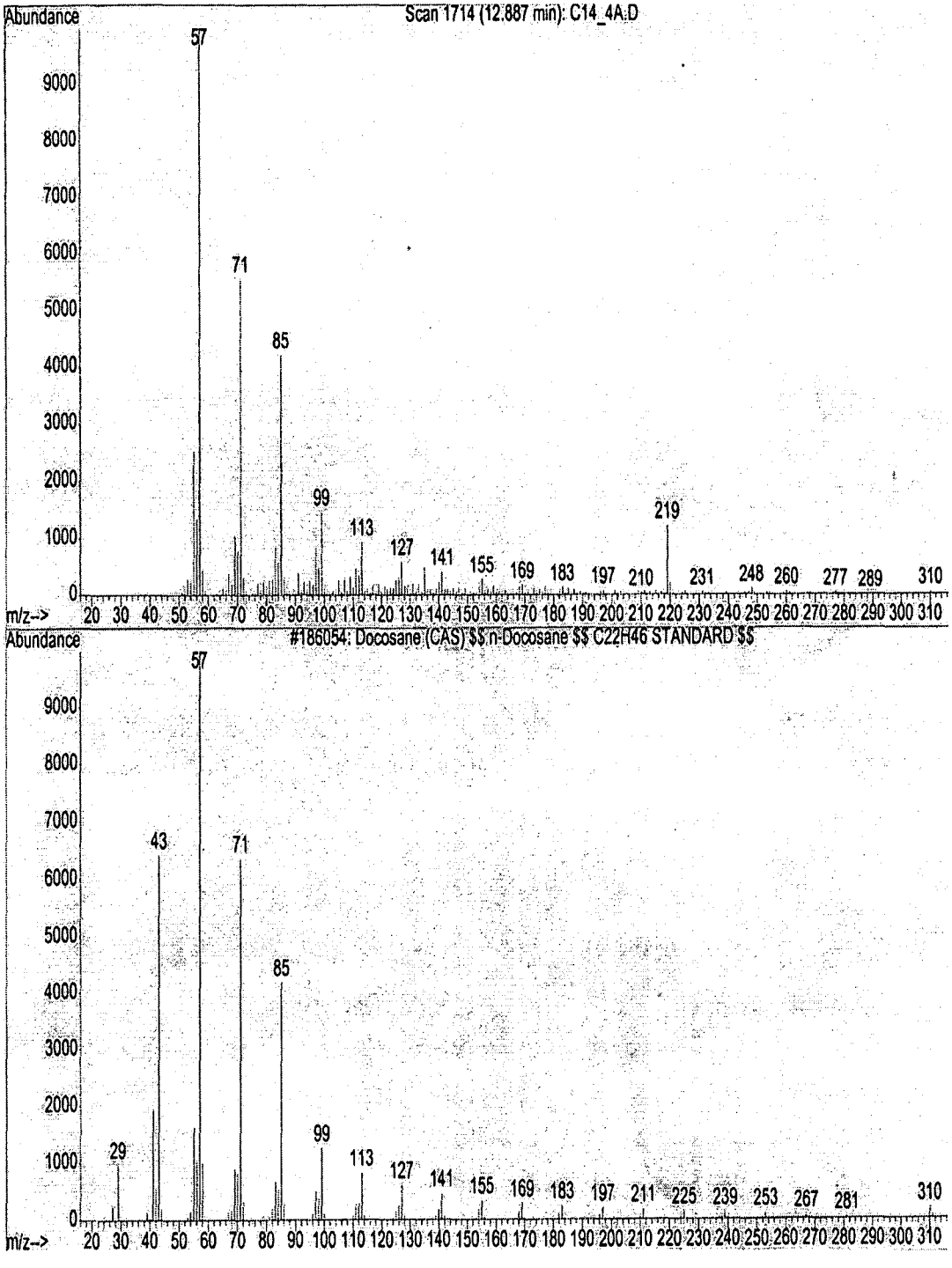

Figure 9 Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, n-Docosane.

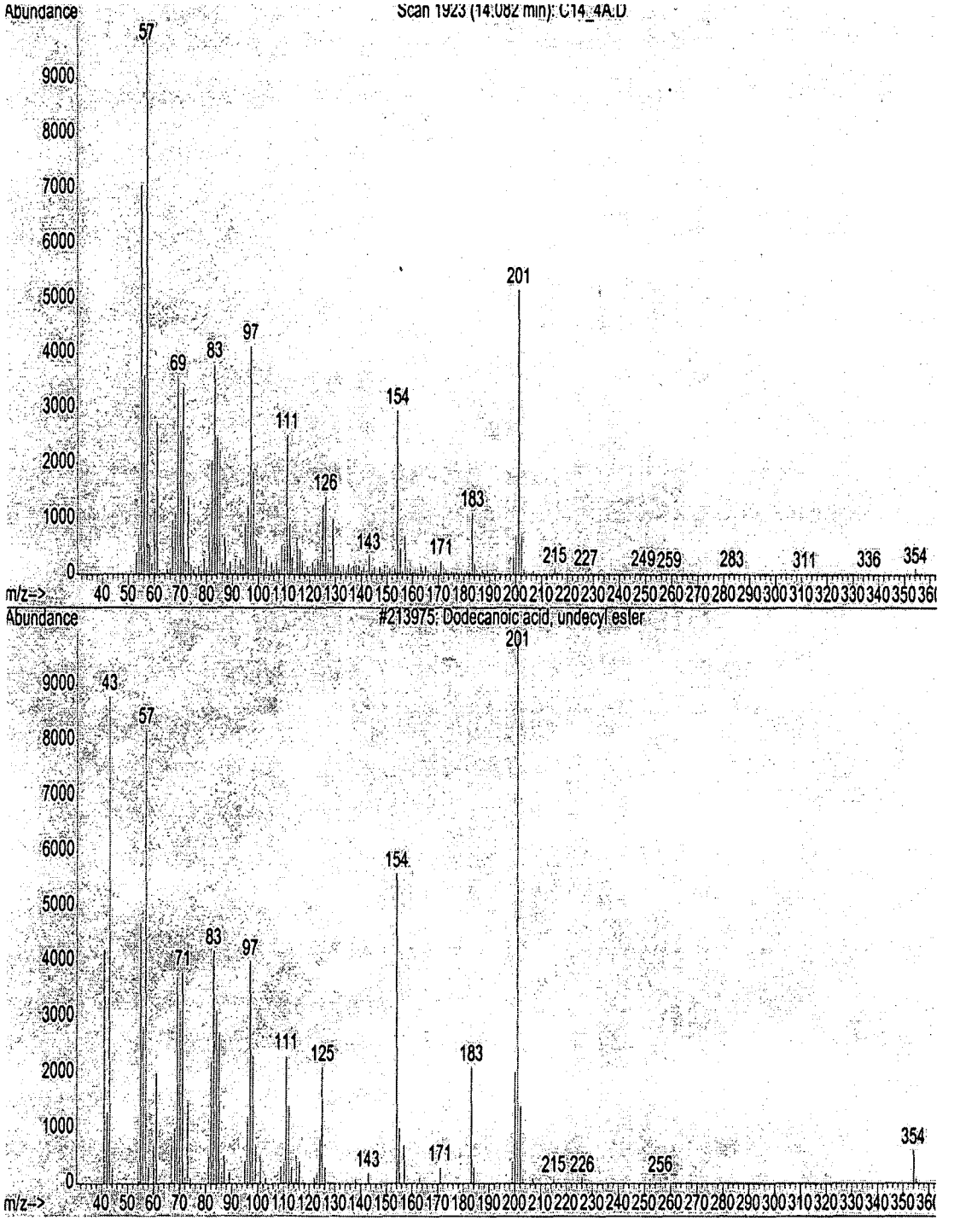

Figure 10 Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, Dodecanoic acid, undecyl ester.

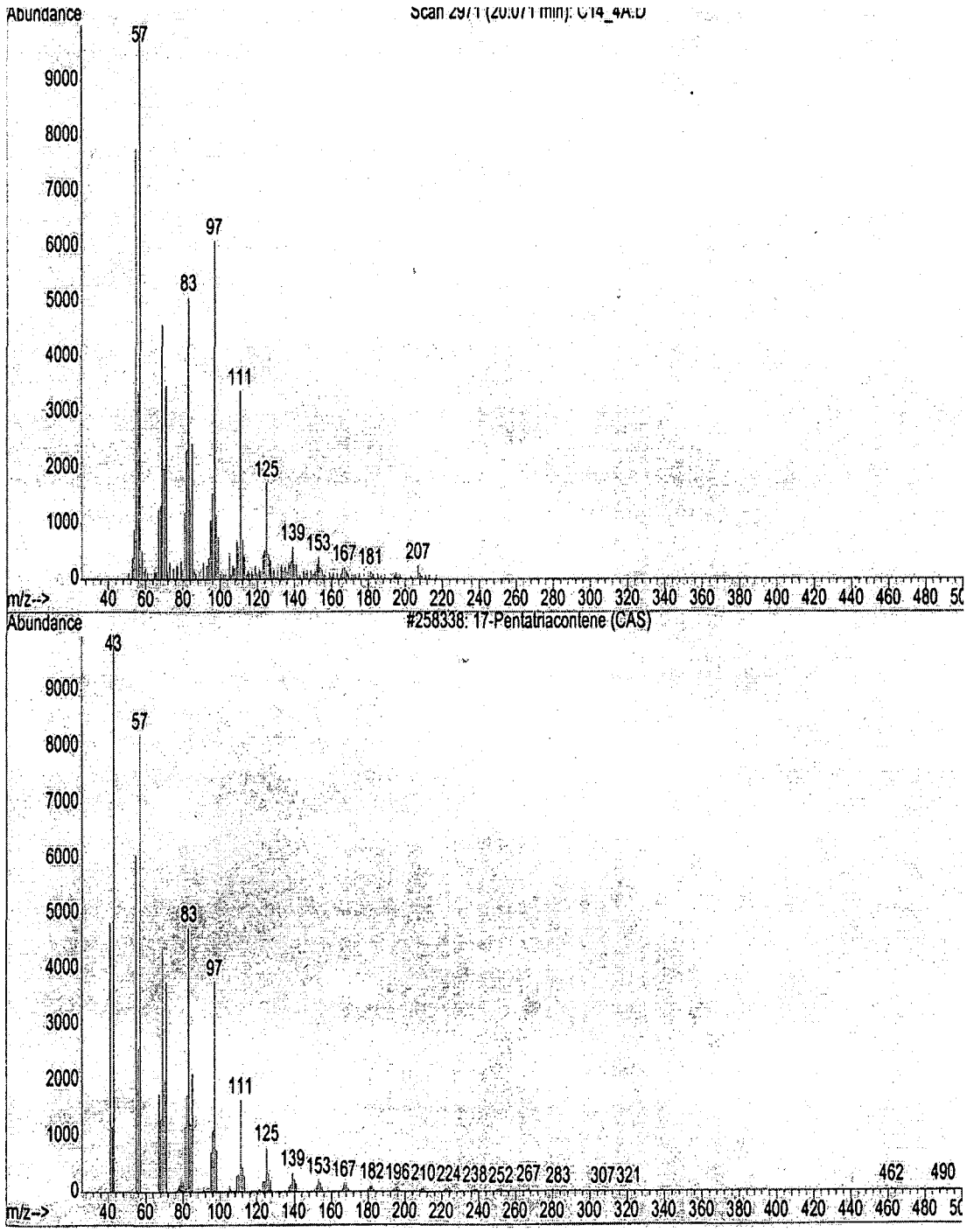

Figure 11 Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, 17-Pentatriacontene.

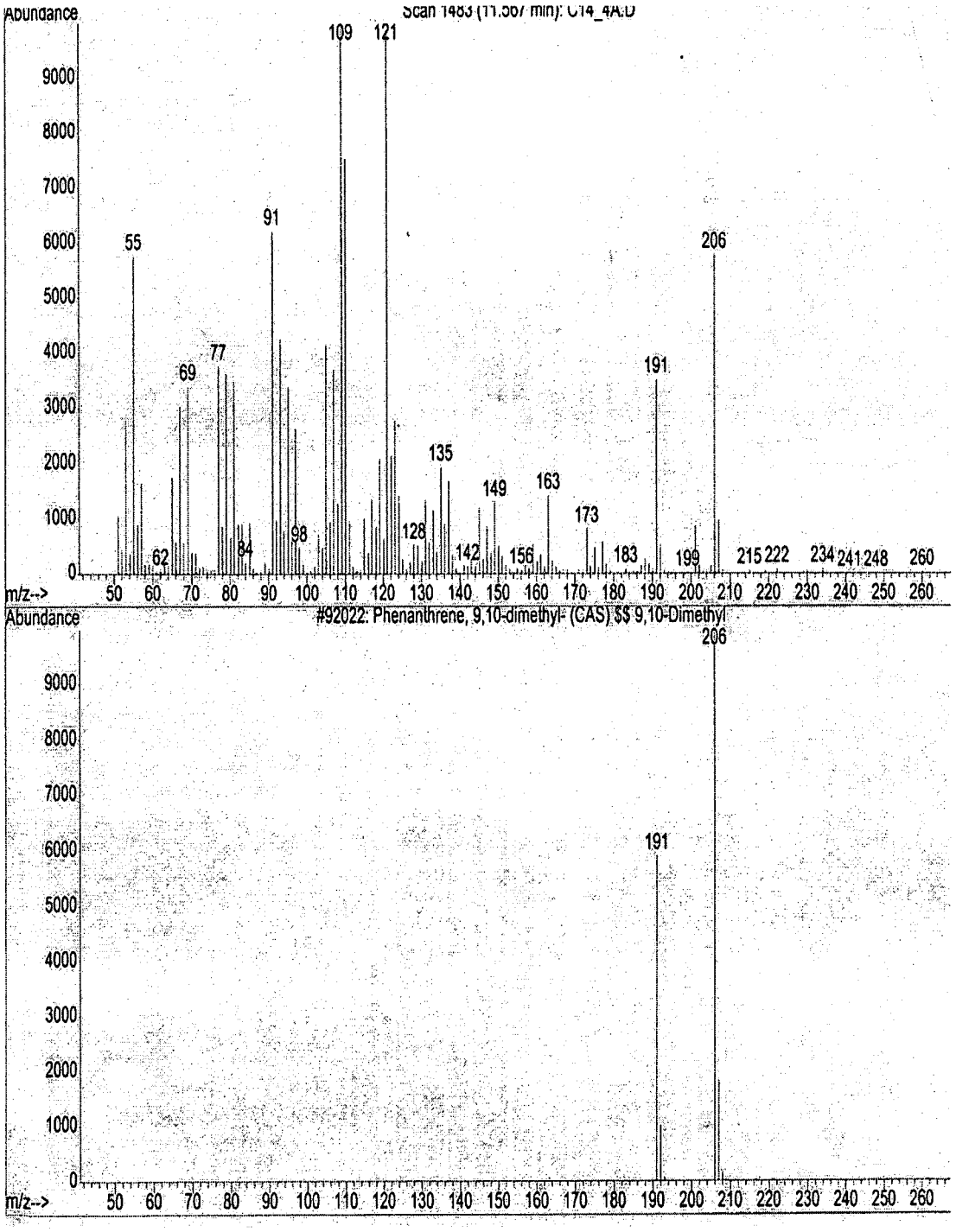

Figure 12 Fractionation GC-MS scan of a compound isolated from *W. salutaris* fraction 4 against a commercial standard, Phenanthrene.