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DIPLOMARBEIT

Phytochemical and Pharmacological Aspects of Australian *Plectranthus* sp.

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I, Elena Pašoski, declare that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

Abstract

The chemical composition of the essential oils of five different *Plectranthus* species has been determined. The major components in *Plectranthus graveolens* and *Plectranthus nitidus* are diterpenes. *Plectranthus suaveolens* essential oil contains mostly mono-, and sesquiterpenes and the major components are β-cedrene, 1,10-di-epi-cubenol and camphor with 51.49%, 18.22% and 20.08% of relative distribution, respectively. The oil of *Plectranthus sp.* 'Hahn Tableland' contains mostly monoterpenes with δ-3-carene and limonene as major components with 39.08% and 15.79% of relative distribution. Ornamental *Plectranthus* oil contains mono- and sesquiterpenes with carvacrol and δ-3-carene as the major components with 23.47% and 20.68% relative distribution, respectively.

The oils were screened for antioxidant and cytotoxic activity. The antioxidant activity was determined by the ORAC assay and the highest antioxidant activity was observed from ornamental *Plectranthus* (2215 ± 377 μmol TE/g), which was attributed to its high carvacrol content. Cytotoxic activity was investigated against P388 mammalian cell line with curcumin and chlorambucil as positive controls. Highest activity was observed from *P. nitidus* with IC_{50} value of 32 μg/mL, followed by *P. graveolens* and *P. suaveolens* with IC_{50} values of 60 μg/mL and 61 μg/mL, respectively. Cytotoxic activity of *P.nitidus* was attributed to its diterpene content, but because of time and material restraints no closer investigation has been done.

Fractionation of the *Plectranthus sp.* 'Hahn Tableland' was carried out using SPE. Fractions were screened for antioxidant and cytotoxic activity and fraction P4B-D showed the highest antioxidant activity of 2368 μmol TE/g, which was attributed to its carvacrol content. Fraction P4B-F was the most cytotoxic against P388 cell line and had IC_{50} of 29 μg/mL, which was attributed to its intermedeol (11-selinen-4-ol; (4β,5β,7β,10α)-form) content. Further fractionation was done by preparative HPLC and 6 components were isolated: carvone; carvacrol; 3,6,6-trimethyl-2,4-cycloheptadien-1-one; 3-caren-2-one; *m*-cymen-8-ol; and *m*-mentha-4,6-dien-8-ol. The compounds 3,6,6-trimethyl-2,4-cycloheptadien-1-one, *m*-cymen-8-ol and *m*-mentha-4,6-dien-8-ol have not been reported in the genus before. 3-Caren-2-one was not originally found in the oil but is possibly an oxidation product of δ-3-carene, the major compound of the oil.

Fractionation of the red oil of *P. nitidus* has been carried out by preparative HPLC and the fractions were subjected to LCMS analysis and two compounds were isolated: a red crystal and a yellow oil. The red crystal was identified as 12-hydroxy-6,8,12-abietatriene-

11,14-dione ($[M+1]^+$=315.2) and it is also the major component in the oil. The yellow oil was tentatively identified as 7,9(11)-abietadiene-13-ol.

Zusammenfassung

Diese Arbeit befasst sich mit der Untersuchung der chemischen Zusammensetzung von den ätherischen Ölen aus fünf *Plectranthus* species. Die Hauptkomponenten in den ätherischen Ölen von *Plectranthus graveolens* und *Plectranthus nitidus* waren Diterpene. Das Öl vom *Plectranthus suaveolens* enthielt meistens Mono- und Sesquiterpene mit den Hauptinhaltsstoffen β-Cedren, 1,10-di-epi-Cubenol und Campher mit 51.49%, 18.22% bzw. 20.08% relativer Verteilung. In *Plectranthus sp.* 'Hahn Tableland' waren hauptsächlich Monoterpene vorhanden mit δ-3-Caren und Limonen als Hauptkomponenten mit 39.08% bzw. 15.79% relativer Verteilung. Im ätherischen Öl des dekorativen *Plectranthus* waren Mono- und Sesquiterpene mit Carvacrol und δ-3-Caren die Hauptinhaltstoffe mit 23.47% bzw. 20.68% relativer Verteilung.

Die Öle wurden auf zytotoxische und antioxidative Aktivität geprüft. Die antioxidative Aktivität wurde mittels ORAC-Assay ermittelt und die höchsten Werte wurden mit dem Öl des dekorativem *Plectranthus* beobachtet (2215 ± 377 μmol TE/g). Diese hohe Aktivität wurde dem hohen Gehalt an Carvacrol zugeschrieben. Die zytotoxische Aktivität wurde gegen P388 Zellen geprüft. Als Kontrollsubstanzen wurden Curcumin und Chlorambucil verwendet. Ebenfalls eine sehr starke Aktivität wurde mit dem Öl von *P. nitidus* beobachtet, mit einem IC_{50} Wert von 32 μg/mL. *P. graveolens* und *P. suaveolens* erwiesen sich mit IC_{50} Werten von 60 μg/mL bzw. 61 μg/mL weniger aktiv. Die zytotoxische Aktivität von *P. nitidus* wurde seinem Diterpen-Gehalt zugeordnet. Auf Grund der Zeit- und Materialbeschränkung konnte aber eine detaillierte Analyse nicht mehr durchgeführt werden.

Die Fraktionierung des ätherischen Öls von *Plectranthus sp.* 'Hahn Tableland' wurde mittels SPE durchgeführt. Die Fraktionen wurden auf antioxidative und zytotoxische Aktivität geprüft und die Fraktion P4B-D zeigte den höchsten Wert von 2368 μmol TE/g. Das wurde dem hohen Gehalt dieser Fraktion an Carvacrol zugeordnet. Auch die Fraktion P4B-F erwies sich sehr stark zytotoxisch aktiv gegen den P388 Zellen (IC_{50} lag bei 29 μg/mL), was ihrem Gehalt an Intermedeol (11-Selinen-4-ol; (4β,5β,7β,10α)-Form) zugeordnet wurde. Die weitere Fraktionierung wurde mittels HPLC durchgeführt und so die folgenden 6 Komponenten isoliert: Carvon; Carvacrol; 3,6,6-Trimethyl-2,4-cycloheptadien-1-on; 3-Caren-2-on; *m*-Cymen-8-ol; und *m*-Mentha-4,6-dien-8-ol. Die Komponenten 3,6,6-Trimethyl-2,4-cycloheptadien-1-on, *m*-Cymen-8-ol und *m*-Mentha-4,6-dien-8-ol wurden bis jetzt in dieser Gattung nicht beschrieben. 3-Caren-2-on war ursprünglich nicht im Öl zu finden aber es ist wahrscheinlich ein oxidatives Produkt der Hauptkomponente δ-3-Caren.

Die Fraktionierung des roten Öls aus *P. nitidus* wurde mittels HPLC durchgeführt, dann wurden die Fraktionen mittels LCMS untersucht und zwei Komponenten isoliert: der rote Kristall und das gelbe Öl. Der rote Kristall - die Hauptkomponente des betreffenden Öls - wurde als 12-Hydroxy-6,8,12-abietatrien-11,14-dion ($[M+1]^+$=315.2) identifiziert. Dem gelben Öl kommt vorläufig die Strukturformel von 7,9(11)-Abietadien-13-ol zu.

Acknowledgments

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Publications Arising from this Research

Elena Pasoski, Leema Marangattil, Myrna A.Deseo, David N. Leach and Paul Forster, Preliminary investigation into the phytochemical and pharmacological aspects of *Plectranthus* sp., Royal Australian Chemical Institute (RACI) Natural Products Chemistry Group Annual One Day Symposium, University of New Castle, New Castle, NSW, Australia, 2nd October 2009.

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1. Introduction

1.1. Aims and overview of the thesis

Plectranthus is one of the genera of Lamiaceae family, subfamily Nepetoideae. It comprises about 300 species worldwide [1]. They usually inhabit the southern hemisphere and are typical in sub-Saharan Africa, India, the Indonesian archipelago, Australia and some Pacific Islands. In Australia, 28 species can be found, 22 are endemic, and 6 are introduced [2]. Although *Plectranthus* species have been used in traditional medicine for a long time, some of them are yet to be described. The lack of present information on Australian *Plectranthus* species renders this genus interesting for further investigation in botanical, phytochemical and pharmacological aspects. *Plectranthus sp.* 'Hahn Tableland' is a species that has not been described yet, and it is typical for Hahn Tableland region of north-east Queensland. *Plectranthus nitidus* occurs in south-eastern Queensland and north-eastern New South Wales, and although it has been described, little is known about this species.

The aims of this thesis were:

1. To determine chemical composition of the essential oils of five different *Plectranthus* species
2. To screen the volatile oils for antioxidant and cytotoxic activity
3. To investigate the active compounds of the *Plectranthus sp.* 'Hahn Tableland' and *Plectranthus nitidus* oil using bioactivity-guided fractionation.

1.2. An overview of family Lamiaceae and genus *Plectranthus*

Lamiaceae, also known as mint-family, is a large plant family containing 236 genera with 7173 species worldwide. They occur almost throughout the world, except of the coldest regions of high latitude and altitude. In Australia 44 genera were described containing 750 species in all states. They usually appear as shrubs and herbs with tetragonal stems and opposite leaves. The original name of the family was Labiateae (labia is the Latin for "lip"), which was given to this family because of the shape of its flowers. They are zygomorphic, and the corolla usually has an upper and lower lip. The upper lip possesses two lobes and forms a hood over the lower lip, which has three lobes. Flowers occur mostly in heads, spikes, cymes or in panicles.

Figure 1. Typical flowers of *Plectranthus* [3].

They are usually aromatic plants and some of the popular kitchen herbs like rosemary or oregano belong to this family. Lots of them are used in traditional medicine because of their antiseptic, antimicrobial and antioxidant properties. Their volatile oils are commonly used in aromatherapy. *Plectranthus* species occur as herbs, subshrubs or shrubs. They are often succulent and with opposite leaves. Inflorescence is terminal or in the upper leaf axils and flowers are in compact cymose clusters.

Figure 2. Typical leaves of *Plectranthus* species.

In Australia they are typically found in the east coast of NSW and Qld, mostly on rocky outcrops and creek-beds. In this study five different oils from *Plectranthus* species that grow along east Australian coast have been profiled.

Plectranthus graveolens (Latin = "strong smelling") is a small spreading shrub with strongly aromatic toothed leaves and violet flowers. Branches and leaves are densely covered with hairs with orange-red sessile glands (Figure 3).

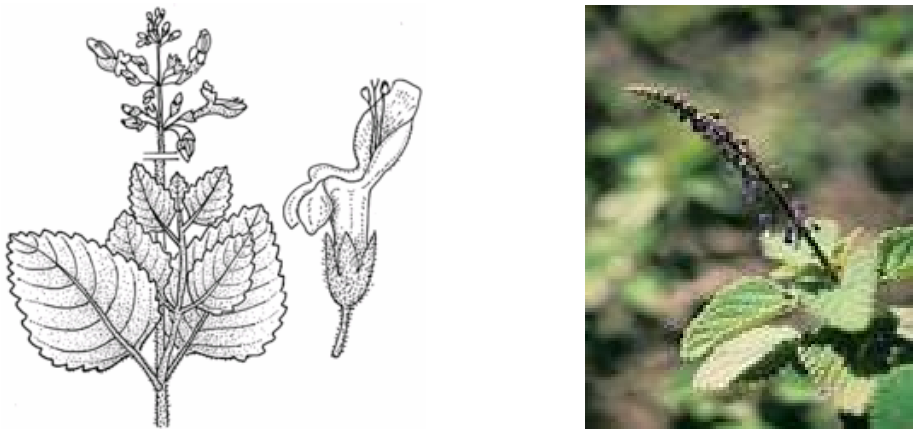


Figure 3. *Plectranthus graveolens* [2].

The volatile oil is intense red coloured with strong aromatic smell. It is distributed widely from south-east NSW to north-east Qld.

Plectranthus suaveolens is a sweetly aromatic shrub to 80 centimeters in height**.** The branches and leaves are densely covered with short white, retorse hairs and many shorter gland-tipped hairs. Corolla is blue to violet and hairy as well (Figure 4). The volatile oil is orange with pleasant aromatic smell.

They usually inhabit rocky and shallow soil sites and are found in the north coast and northern Tablelands districts of NSW, but also occur in the Darling Downs and Moreton Districts of Queensland.

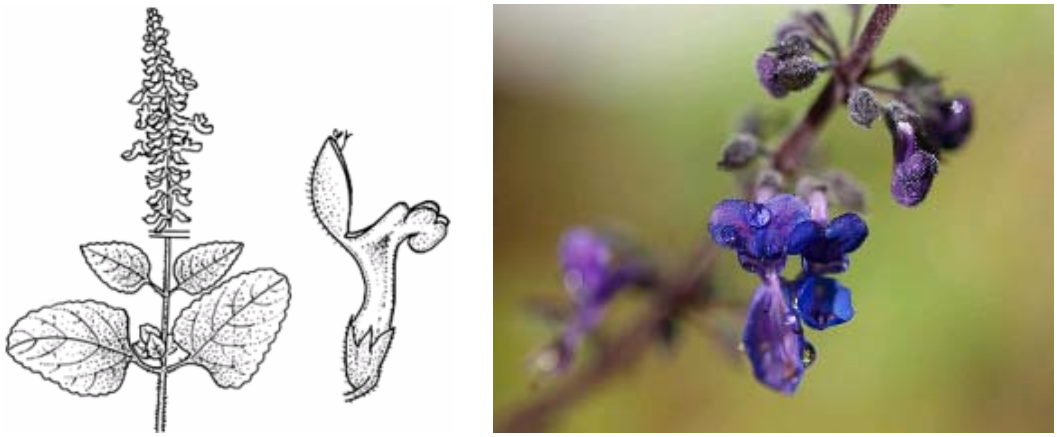


Figure 4. *Plectranthus suaveolens* [2].

Plectranthus nitidus (Nightcap *Plectranthus*) is a small shrub that grows 30–150 cm tall. The leaves are fleshy and rounded with a green upper and a purple lower surface. The branches are erect and sparsely covered with short hairs. The flowers are lilac to blue and have a long lower lip (Figure 5). *Plectranthus nitidus* occurs on rocky cliff faces or amongst rocky outcrops and is restricted to south-east Queensland and north-east NSW where it occurs from the Nightcap Range north to the McPherson Ranges.

Figure 5. *Plectranthus nitidus* (Picture by Peter Richards).

Plectranthus* sp. *'Hahn Tableland'

P. sp 'Hahn Tableland' was first found in the Hahn Tableland region of north Queensland [4].It is and aromatic plant with a pleasant scent. The leaves are succulent and covered with fine hairs on the both sides (Figure 6). This species has not yet been botanically described.

Figure 6. *Plectranthus* sp. 'Hahn Tableland'.

Ornamental *Plectranthus*

Ornamental *Plectranthus* species investigated in this study is known, but has not yet been identified.

1.3. Traditional medicinal uses of *Plectranthus* species

Traditional medicine has been used in many cultures for thousands of years to prevent and treat different medical conditions. Medicinal herbs were found in the personal effects of an "ice man", whose body was frozen in the Austrian Alps for more than five thousand years.

Even nowadays in some Asian and African countries traditional medicine represents the primary health care and it is getting more and more popular in western society as well.

According to WHO 70% to 80% of the population in many developed countries has used some form of alternative or complementary medicine.

Herbal treatments are the most popular form of traditional medicine and are very profitable in the international marketplace. Revenue from sales of herbal products reached 5 billion US$ in 2003-2004 in Western Europe, and 14 billion US$ in 2005 in China.

Plant metabolites have a long history of use as herbal remedies, including opium, digitoxine, vinblastine. According to the WHO, approximately 25% of modern drugs used in the United States have been derived from plants which confirms that chemistry of natural products is very interesting and a promising research field.

Plants of *Plectranthus* genera are well-known remedies in traditional medicine of many countries. They have been used for different digestive, skin and respiratory conditions, fever and infections, genital-urinary conditions, pain and muscular-skeletal conditions. For example, leaves of *P. igniarius* are used in Kenya to treat inflamed eyes. Numerous species including *P. amboinicus, P. barbatus, P. caninus, P. esculentu*s are reported to have cytotoxic and anti-tumour activity and can be used in the treatment of cancer. *Plectranthus barbatus* is one of the most used *Plectranthus* species. For example, its essential oil exhibits anti-allergic activities through passive cutaneous anaphylaxis inhibition. It is also used for various digestive disorders like nausea, stomachache, and in Brazil even to treat gastritis. The essential oil of *P.sylvestris* is used to treat skin diseases. *P. mandalensis* is used in Malawi to treat depression and in India *P. vettiveroides* is used as a stimulant [1].

Some of the plants and their usage are listed in Table 1.

Except in traditional medicine these plants are also used as ornamentals (*P. glabratus, P. forsteri)*, kitchen spices (*P. amboinicus,* also known as Mexican oregano)*,* food (*Plectranthus esculentus)* or food additives.

Table 1. Traditional uses of some *Plectranthus* species.

Species	**Part used**	**Area used**	**Used for**
P. barbatus	leaves	Africa, Brazil	stomachache
P. floribundus	root tuber	Nigeria	food
P. amboinicus	seed oil	Polynesia	acute oedematous otitis acuta
P. asirensis	whole leaf	Saudi Arabia	Antiseptic wound-dressing
P. sylvestris	volatile oil	Rwanda	skin diseases
P. caninus	root extract	Kenya	coughs
P. vettiverioides	plant remedy	Indian ayurvedic	vomiting and nausea
P. congestus		Australia	internal complaints

1.4. Chemistry and biological activity of the components

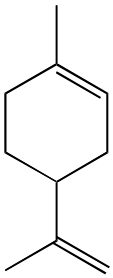
This broad spectrum of traditional uses makes it interesting to investigate the chemistry of the genus, especially its secondary metabolites as they play an important role in protecting the plant against herbivores and microorganisms or in attracting pollinating or seed dispersing animals, and therefore have variety of biological activities [5].

Essential oils are big and diverse group of secondary metabolites that represent a mixture of terpenoid molecules. Terpenoids are made in a plant via *mevalonic acid* biosynthetic pathway (sesquiterpene) and via the xylulose-pyruvate-pathway (mono- and diterpenes) and usually stored in resin ducts, glandular trichomes or oil cells. Because of their volatile and lipophilic nature essential oils could be easily absorbed in human body via respiratory system, or via skin. Once absorbed they bind to the target molecules. Different constituents of an essential oil could have different target molecules and therefore different activity, which could make the oil very interesting in therapeutic sense, since it could fulfil various effects simultaneously [6]. Although they are widely in use, especially in cosmetic, flavour and fragrance industry, their therapeutic effects (anti-inflammatory, anti-tumour, antioxidant) and mechanism of action have not yet been well investigated.

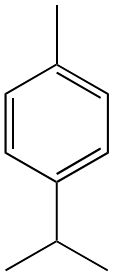
The genus *Plectranthus* belongs to Nepetoideae subfamily, the biggest of all Lamiaceae subfamilies, comprising ca. 3500 species in 105 genera. This subfamily showes specific secondary metabolites chemistry that is different to the other Lamiaceae subfamilies. The

essential oils contain monoterpenoids instead of monoterpenoid iridoid glycosides. Phenylpropanoid chemistry produces components that are more non-polar comparing to other families. An explanation why these changes took place could be found in wide spectra of activities in their essential oils (antifungal, anti-insecticidal) and phenolic components (antioxidant) offering these species advantages in natural selection during evolution [7]. *Plectranthus* is an essential-oil rich genus with > 0.5% volatile oil on a dry weight basis [8]. Secondary metabolites that can be found in the oils include mono-, sesqui- and diterpenes and phenolics.

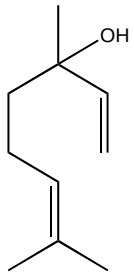
Mono- and sesquiterpenes are usually the major components of the volatile oil. The therapeutic effects of some of these compounds are well-known. Limonene is found to be tumour preventive, linalool sedative and anti-infectious, pinene and *p*-cymene to relieve muscular aches [6]. But still only a few investigations have been carried out on the relationship of biological activity of monoterpenoids and traditional uses of the plants.



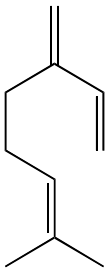
limonene



***p*-cymene**

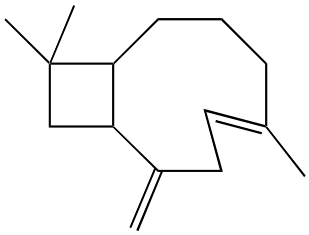


linalool

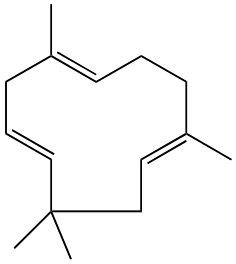


myrcene

Some of the sesquiterpenes that have been reported in the genus are β-caryophyllene in *P. barbadus* and *P. sylvestris,* and humulene in *P. barbatus*, and 15-hydroxyspathulenol in *P. fruticosus*. They are an interesting group of components with various therapeutic properties such as antitumor [9], anti-inflammatory [10] and antiviral [11] but yet not well studied in this genus.



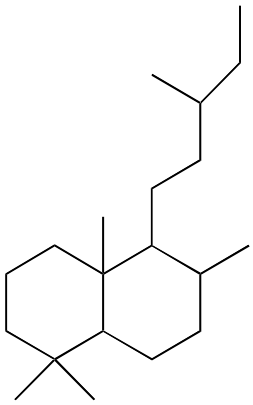
β-caryophyllene



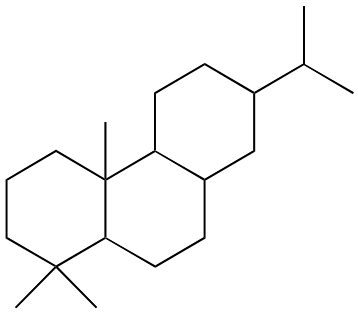
α-humulene

Diterpenoids are the major and very common group of second metabolites in the species. They are usually highly modified abietanoids. The other major group contains mainly labdane

diterpenoids. Phyllocladanes, *ent*-kaurenes and a *seco*-kaurene were also found in the genus. Clerodane diterpenoids, which are present in *Salvia*, are not found in *Plectranthus*.



Labdane type



Abietane type

The majority of studies on phytochemical profiles of *Plectranthus* oils were concentrated on activity and isolation of diterpenes. Royleanone and coleone are abietane diterpene found in *P. grandidentatus*, and were reported to have activity on T-, B-lymphocyte proliferation [12]. In the same plant horminone (7,12-dihydroxy-8,12-abietadiene-11,14-dione) is reported to be antibacterial against *Staphylococcus aureus* [13]. Antiviral activity against Herpes simplex type II was found in acetone extract of the root of *P. hereroensis* due to the presence of 16-acetoxy-7α,12-dihydroxy-8,12-abietadiene-11,14-dione [14]. Ferrungiol, sugiol, 6,7-dehydroroyleanone are reported to have anti-SARS-CoV activity [15].

Labdane diterpenes were found in *P. barbatus* and *P. ornatus* and include forskolin and plectrornatin. Forskolin was found to directly activate adenyl cyclase hereby influence cAMP concentration [1]. This could explain traditional uses of this plant in diverse gastric and muscular conditions. It is also found to have antimetastatic activity against lung cancer in mice [16].

Phenolic compounds and their antioxidative activity in *Plectranthus* genus have not been well studied. In *Plectranthus ambiguus* 5,6-dihydroxy-7,4-dimethoxyflavone (ladanein) [17] has been reported. Grayer et al. reported two non-flavonoid phenolics –Nepetoidin A (1) and B (2) that are shown in Figure 7. They seem to be present in nearly every Nepetoideae species. Nepetoidin B showed strong antioxidant activity and both components had antifungal properties [7].

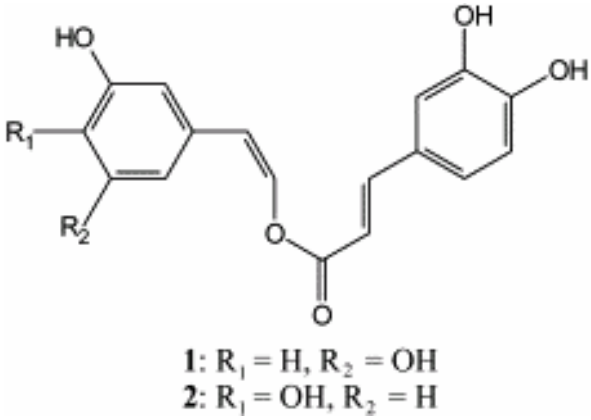


Figure 7. Nepetoidin A (1) and B (2).

Lukhoba and Abdel-Mogib both concluded that not much research has been done on chemistry of *Plectranthus*, especially on biologically active mono- and sesquiterpenes. All this makes any further investigation in taxonomy, chemistry and pharmacology of this genus very interesting and worthwhile.

1.5. *Plectranthus* sp. 'Hahn Tableland'

There has been not a lot research done on chemistry on *Plectranthus sp.* 'Hahn Tableland'. Rosikari et al. have worked on insecticidal activity of the crude methanol extract and its water, ethyl-acetate and hexane partitions. Interestingly, they have noticed two compounds in methanol extract that were not present in other 26 *Plectranthus* species investigated in that study. UV and mass spectra of these compounds seemed to be similar to the patterns of flavonoid glycoside. These extracts showed high toxicity against *Tetranychus. urticae* and *Heliothrips haemorrhoidalis* and they were more toxic than extract of *P.graveolens*. Activity against *T.urticae* was attributed to the non-polar constituents of the extract. As a fresh residue it had an ovicidal and oviposition inhibition effect. The phenomenon that the ethyl acetate partition was only mildly toxic to *T. urticae* but highly toxic to cells was observed in *P.* sp. 'Hahn Tableland' and *P. graveolens* as well and may be due to the presence of abietane diterpenes [4].

Marangattil has done some preliminary investigation in chemical composition and antioxidant and cytotoxic activity of the plant`s volatile oil. GC profile showed mostly monoterpenes: carvacrole, eucarvone, limonene, 3-carene, α-pinene, α-phellandrene and *p*-cymene. The oil did not seem to have significant antioxidant or cytotoxic activity, but some fractions showed better response against free radicals and P388 cells. Because of time and material constraints, but also because of specific nature of monoterpenes found in the oil, she did not manage to isolate any pure component at the time.

1.6. *Plectranthus nitidus*

To the knowledge of the author of this thesis, there have been no studies published neither on phytochemistry or pharmacology of this species.

1.7. Pharmacological assays

Pharmacological assays were chosen accordingly to the previous reported cytotoxic, antioxidant and inflammatory activities in the genus.

1.7.1. Antioxidant activity

Antioxidants are chemicals that have scavenging activity against reactive nitrogen and oxygen species (RNS and ROS). RNS and ROS cause oxidative stress in the human body, damaging cellular membrane, proteins and DNA, and therefore play an important role in aging and various pathological processes. Although the body has antioxidant enzymes (superoxide dismutases, catalases, peroxidases, glutathione-system and others) that can neutralize these reactive species, their capacity can get overloaded. Antioxidants act supportively because of their ability to either prevent or break the radical chain reaction [18]. Antioxidants are not only interesting as food supplements, but are also extensively used as preservatives in food and cosmetic industry, as stabilizers in fuels and lubricants and used to prevent oxidation processes in different polymers like plastic and rubbers.

To measure the antioxidant activity the *Oxygen Radical Absorbance Capacity* (ORAC) method was used, which is suitable for measuring antioxidant capacities in biological samples. The ORAC method evaluates substances' antioxidant capacity with and without lag phases of their antioxidant capacities. This is especially beneficial when samples contain components with various slow and fast acting antioxidants. Another benefit of this method is that it directly estimates the chain-breaking antioxidant activity comparing to some other assays.

1.7.2. Cytotoxic activity

Cytotoxic components can induce cell death by either inducing necrosis or apoptosis. Cells undergoing necrosis typically undergo rapid swelling and lose membrane integrity and release their cytoplasmic contents (ATP, LDH etc.) into the surrounding culture medium. Cells undergoing rapid necrosis *in vitro* do not have sufficient time or energy to activate apoptosis process and therefore release apoptosis factors into media.

In this study a certain amount of released ATP was measured. Advantage of measuring this agent is that it`s generated in only few minutes comparing to some other signalling

components (MTS or resazurin), which makes this assay more convenient, but also the probability of an interaction of the test compound with assay chemistry is reduced.

There are numerous factors that have to be determined when choosing a cytotoxicity assay: type of cells used, type of control components, dose of the samples and duration of exposure. In this study P388 mammalian cell line was used, and curcumin and chlorambicil were the controls.

2. Materials and methods

2.1. Oil distillation

All of the samples were collected in Lismore, in July 2009. Leaves were removed from the twigs and the oils were obtained using steam distillation for 48 hours. Samples of *Plectranthus sp.* 'Hahn Tableland' and ornamental *Plectranthus* were distilled in two batches, due to a large sample amount, and therefore two batches of oils were obtained. Molecular sieve was added to the first batch of *P.*sp. 'Hahn Tableland' to remove water from the oil, and a change of colour was observed from yellow to brownish-yellow. Each batch of *Plectranthus* oils was treated separately in succeeding analysis.

Figure 8. Apparatus of the steam water distillation

Table 2. Samples material

Code	Scientific name	Plant material (g)	Comment	Weight of essential oil(g)	Colour of essential oil
P1	*P. graveolens*	1124.3		3.7715	Red
P2	*P. suaveolens*	102.2		0.608	Orange
P3	*P. nitidus*	49.8		0.2139	Red
P4A	*P. sp.* Hahn Tableland	2884.8	1st batch	4.6318	Yellow-brownish
P4B	*P. sp.* Hahn Tableland		2nd batch	4.5671	Yellow
P6A	Ornamental *Plectranthus*	3600.0	1st batch	0.6787	Yellow
P6B	Ornamental *Plectranthus*		2nd batch	1.8069	Yellow

2.2. Phytochemistry

2.2.1. Gas Chromatography – Mass Spectroscopy (GC-MS)

GC-MS was used to get the information about the chemical profiles of the volatile oils, as well as their detailed mass spectra and retention times of individual peaks within Total Ion Chromatogram (TIC).

2.2.1.1. GC-MS technical information

The system used was Agilent 6890 with an Agilent 7673 Injector and Agilent 5973 Network Mass Selective Detector (MSD). The column was SGE BPX5 capillary column. Samples were dissolved in 100% Methanol (HPLC grade purity, purchased from LAB-SCAN) at the concentration of ≈10mg/mL.

Method used was ELENA1 with the following parameters:

Oven:
Initial Temperature: 50°C
Rate: 8°C/min
Final Temperature: 300°C
Run Time: 42.25min

Injector (back inlet):
Injection volume: 1μL
Mode: Split
Split Ratio: 50:1
Gas Type: Helium

Capillary Column – SGE BPX5:
Film thickness: 50.0m x 220μm x 1μm
Initial flow: 1.2 mL/min
Mode: constant flow
Pressure: 26.58 psi (1.83 bar)
Average velocity: 31 cm/sec
Gas: Helium

MSD:
Transfer temperature: 280°C
Source temperature: 230°C
Quadrupole temperature: 150°C
Ionisation Voltage: 70eV
Scanning mass range: 35–350m/z

2.2.1.2. Identification of components

The identification of the compounds was made by comparing the fragmentation patterns:

1. With reference standard (if available)

2. With the ones in Database Library. Libraries used were ADAMS, WILEY275 and NIST 98. If the match quality was under 80%, 60%, 40% respectively, the next database would be scanned.

3. With Kovats Index in the Adams book of identification of essential oils by GC-MS [19].

Reference standards used were as listed in Table 3:

Table 3. Reference standards.

Ref. Standard	**Supplier**	**Purity**
p-Cymene	Aldrich	99%
(1R)-(-)-Fenchone	Aldrich	98%
Linalool	Aldrich	97%
(1R)-(+)-α-Pinene	Aldrich	98%
(S)-(-)-Limonene	Aldrich	96%
α –Bisabolol	Fluka	95%
Ocimene	Fluka	97%
Carvacrol	Aldrich	98%
± Camphor	Aldrich	99%
Thymol	Aldrich	98%
(1S)-(+)-3-Carene	Aldrich	99%
(R)-(-)-Carvone	Aldrich	98%
(1S)-(-)-Verbenene	Aldrich	94%
(-)-Carveol	Aldrich	97%

Standards were dissolved in methanol at the concentration of ≈ 10mg/mL.

For all of the compounds their Kovats index (KI) was calculated. Calculation was needed because retention times may vary as column age or if other parameters were used and therefore the KI can be index based on series of references. Two standard solutions of different hydrocarbons were used and their accuracy in calculating the Kovats index (KI) for the reference substance (limonene) compared. Standard1 contained n-nonane, n-undecane, n-hexodecane, n-eicosane and n-tetracosane. Standard2 contained n-heptane, n-octane, n-nonane, n-decane, n-undecane, n-dodecane, n-tridecane, n-tetradecane, n-pentadecane, n-hexadecane, n-heptadecane, n-octadecane, n-nonadecane, n-eicosane, n-heneicosane, n-

docosane, n-tetracosane. Standard2 showed closer value to the one from the Adams library. For that reason it was used for calculating Kovats index for components found in the oil samples.

In Table 4 are shown Retention times (RT-Adams) and KI (KI-Adams) from Adams Library, and Retention times (RT) obtained from GC-MS, as well as molecular weight (MW) of the component.

Table 4. Hydrocarbons used in calculating KI.

	Compound	**n**	**RT**	**MW**	**KI-Adams**	**RT-Adams**	**KI calculated**
C7	n-Heptane	7	6.98	100.21	700	2.23	701
C8	n-Octane	8	9.28	114,23	801	3.17	727
C9	n-Nonane	9	11.65	128.2	900	4.93	792
C10	n-Decane	10	13.94	142.29	1000	7.77	853
C11	n-Undecane	11	16.09	156.31	1100	11.48	909
C12	n-Dodecane	12	18.11	170.34	1200	15.77	965
C13	n-Tridecane	13	19.99	184.365	1300	20.19	1019
C14	n-Tetradecane	14	21.76	198.39	1389	24.10	1075
C15	n-Pentadecane	15	23.42	212.42	1500	28.82	1130
C16	n-Hexadecane	16	24.98	226.45	1589	32.44	1187
C17	n-Heptadecane	17	26.45	240.48	1700	36.74	1245
C18	n-Octadecane	18	27.84	254.51	1800	40.40	1307
C19	n-Nonadecane	19	29.17	268.53	1900	43.92	1369
C20	n-Eicosane	20	30.42	282.56	2000	47.33	1432
C21	n-Heneicosane	21	31.62	269.59	2100	50.47	1502
C22	n-Docosane	22	32.77	310.61	2200	53.54	1780
C24	n-Tetracosane	24	35.41	338.65	2400	59.33	1798

Kovats Index was calculated using the formula [19]:

$$KI(x) = 100\,Pz + [(\log RT(x) - \log RT(Pz)) / (\log RT(Pz+1) - \log RT(Pz))]$$

2.2.2. Solid-Phase-Extraction (SPE)

SPE was used to fractionate the volatile oil for further investigation. STRATA C18-E pre-packed column with 55μm particle and 70Å pore size was used. The capacity of the column

was 10g and bed volume (BV) of 20 mL. The chamber used to collect the fractions was SUPELCO (Cat. No 5-7030) and vacuum pump was from BUCHI.

The quality of the solvents used was HPLC purity grade from LAB-SCAN. Mili-Q water was obtained by water filtration using a Millipak® 40 (0.22µm pore size). Column was first regenerated with 2x2BV of 100% methanol and then equilibrated with 2x2BV 50% methanol. Elution was carried out using vacuum. Approximately 1g of oil was weighed on a Sartorius analytical balance and then diluted in approximately equal volume of 50% methanol and transferred into the column. Fractions were collected into 20mL glass vials and dried under nitrogen. Fractions were obtained as follows:

	Mobile phase	Volume BV(20ml)
1	50% MeOH	2BV
2	75% MeOH	4BV
3	100% MeOH	9BV

After the methanol from the fractions was evaporated further extraction was made from the residual water using either chloroform or hexane.

2.2.3. Liquid Chromatography- Mass Spectrometry (LC-MS)

LC-MS was used to obtain additional information about mass spectra and UV-absorbance of components. System used was Agilent 1100 Series HPLC with binary pump, aut*o*-injector, Diode Array Detector (DAD) and Mass Spectrometer Detector (MSD). DAD was equipped with UV and VIS lamps, and it was set to store the signals at 210, 280, 360 and 500nm wavelength. MSD was operating in atmospheric pressure chemical ionization (APCI) mode. Gas and Vaporizer temperature in the spray chamber were set at 350°C, and nebulizer pressure was 60 psig[1] (4.14 bar). Column was Phenomenex® Luna 3 µ C18 (2) 100 Å (100 x 4.6mm, 3 micron) with the temperature set at 40°C. Flow rate was 0.5 mL/min. Solvents were purchased from LAB-SCAN with HPLC purity grade. Mili-Q water was purified and filtered by a Millipak® 40 (0.22µm pore size). Mobile Phases were water (Solvent A) and acetonitrile (Solvent B), both contained 0.005% TFA (trifluoric acid) to enhance peak shape. Soniclean sonicator was used to homogenize and degas the solvents.

[1] Pound-force per square inch gauge is a unit of pressure relative to the surrounding atmosphere.

Method: ELENA1

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Pressure (bar)
0.00	50	50	400
3.00	50	50	400
10.00	5	95	400
18.00	5	95	400
20.00	50	50	400
25.00	50	50	400

2.2.4. High Performance Liquid Chromatography (HPLC)

2.2.4.1. Analytical HPLC

Analytical HPLC was used to develop the chromatographic method, which was subsequently used in preparative HPLC. By developing the method, separation of the peaks were improved in order to obtain single components.

Preparative HPLC was carried out on an Agilent 1100 system, using Phenomenex® Luna 5µ C18 column (5 micron packing, 150 x 4.6mm internal diameter). Column temperature was set at 40°C. Detector was Diode Array Detector (DAD) with UV/Vis Lamp. Signals on 210nm, 280nm, 360nm and 560nm were stored. Mobile phases were mili-Q water (Solvent A), 100% methanol (Solvent B) or 95% methanol (Solvent C) in mili-Q water with 0.05% TFA. Timetables of different methods that were used are shown below.

Method: ELENA1

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)	Pressure (bar)
0.00	50	50	1	400
3.00	50	50	1	400
10.00	5	95	1	400
18.00	5	95	1	400
20.00	50	50	1	400
25.00	50	50	1	400

Method: ELENA2

Isocratic flow for 20min with 100% solvent (0.005&TFA/95%MeOH).

Method: ELENA3

Timetable:

Time (min)	Solvent A (%)	Solvent C (%)	Flow (mL/min)	Pressure (bar)
0.00	40	60	1	400
2.00	40	60	1	400
8.00	0	100	1	400
18.00	0	100	1	400
20.00	40	60	1	400
25.00	40	60	1	400

Method: ELENA4

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)	Pressure (bar)
0.00	40	60	1	400
2.00	40	60	1	400
12.00	0	95	1	400
20.00	0	95	1	400
24.00	40	60	1	400
30.00	40	60	1	400

Method: ELENA5

Timetable

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)	Pressure (bar)
0.00	50	50	1	400
3.00	50	50	1	400
15.00	5	95	1	400
18.00	5	95	1	400
20.00	50	50	1	400
25.00	50	50	1	400

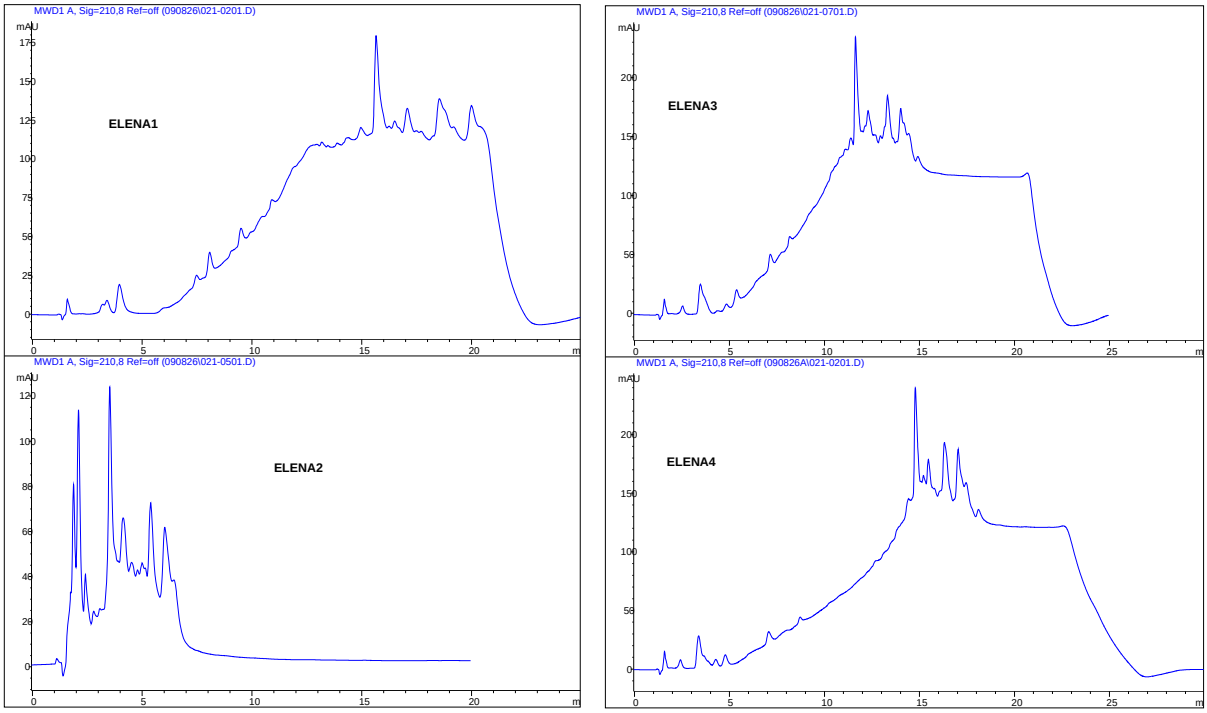


Figure 9. P4A-G1 fraction run with methods ELENA1-ELENA4.

2.2.4.2. Preparative HPLC

Preparative HPLC was used for further fractionation of the individual fractions obtained by SPE. Fractionation was carried out on a Gilson® unit equipped with Gilson® Pump 322, Gilson® UV/Vis-156 dual wavelength detector and FC204 fraction collector. Phenomenex® Luna 5µ C18 100 Å column (150 x 21.2 mm, 5 micron) was used with a Phenomenex® Sepra C18 – E (50 µm particle size, 65Å) guard column. Detector signals used were 210nm and 280nm wavelengths. Mobile Phases were miliQ water (Solvent A) and 100% methanol (Solvent B) with 0.05% TFA.

Column was first washed with isopropanol for 45 minutes, and then equilibrated with Solvent B for another 10 minutes with a flow rate of 15 mL/min.

Three different methods have been used in preparative HPLC – “Elena5” to fractionate P4B-D and P4B-C, “Elena6” for P4B-I, and “ELENA-H” for P4B-H and P3.

Prep. HPLC method: Elena5

From P4B-H 48 fractions were collected in 0.16min time interval and from P3 67 fractions in time interval of 0.24min.

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)
0.00	50	50	15
15.00	40	60	15
24.00	30	70	15
26.00	30	70	15
27.00	50	50	15

HPLC method: Elena6

Total of 47 fractions is collected in 0.17min time interval.

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)
0.00	40	60	15
5.00	5	95	15
24.00	5	95	15
25.00	40	60	15

HPLC method: Elena-H

Timetable:

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)
0.00	25	75	15
5.00	5	95	15
18.00	5	95	15
20.00	25	75	15

From P4B-H we have collected 48 fractions in 0.16min time interval, and from P3 there were 67 fractions in time interval of 0.24min.

2.2.5. Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance Spectroscopy has been used to obtain structural information about molecules. The system was a Bruker Avance DRX-500 and TopSpin 1.8 software was used for results analysis. The experiments were carried out in deuterated chloroform using the solvent peak as a reference. The NMR spectra were recorded at 500.13 MHz for ^{1}H and at 125.77 MHz for ^{13}C. The chemical shifts were expressed in parts per million (ppm) as δ values and the coupling constants (*J*) in Hertz (Hz). Multiplicities were shortened as: s (singlet), d(doublet), dd (doublet of doublets), m (multiplets) and br (broad).

The structures were mainly established by three spectroscopic techniques:

- one-dimensional spectroscopy
- two-dimensional homonuclear correlation spectroscopy (COSY)
- two-dimensional heteronuclear correlation spectroscopy:
    - HSQC (Heteronuclear Single Quantum Correlation)
    - HMBC (Heteronuclear Multiple-Bond Correlation)

2.3. Pharmacology

Pharmacology assays were used to examine the essential oils and their fractions on different activity. The assays used were:

- Antioxidant activity – Oxygen Radical Absorbance Capacity (ORAC)
- Cytotoxic activity

2.3.1. Oxygen Radical Absorbance Capacity (ORAC)

To measure antioxidant activity of volatile oils and their fractions the Oxygen Radical Absorbance capacity assay was used. It directly measures the antioxidant capacity of the sample, using fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) as the fluorescent probe. Any reaction of fluorescein with peroxyl radical will be detected as quenching of fluorescence. Antioxidants prevent these reactions and therefore preserve fluorescence. This effect is measured by assessing the area under the fluorescence decay curve (AUC) of the sample and compared to that of the blank. Trolox (a water soluble vitamin E analogue) was used as a standard to obtain a standard curve, which will be used to extrapolate the ORAC values from. As a positive control epicatechin, a polyphenolic antioxidant plant metabolite was used.

2.3.1.1. Reagents and materials

All samples were dissolved in methanol at the concentration of ≈10 mg/mL.

Phosphate buffer stock solution was prepared by dissolving 17.25g of NaH_2PO_4 and 86.25g of Na_2HPO_4 in 1000 mL of Milli-Q water, and pH was adjusted to 7.4.

To prepare phosphate buffer working solution (75 mM) 100 mL of the phosphate buffer stock solution (750 mM) were added to 900 mL of Milli-Q water and the pH was adjusted to 7.4.

Fluorescein stock solution (4.9×10^{-3} M) was prepared by dissolving 116 mg of fluorescein ($C_{20}H_{12}O_5 \cdot 2Na$) in 63.2 mL of 75 mM phosphate buffer (pH 7.4). After that 5 µL of fluorescein stock solution (4.9×10^{-3} M) was added to 16.5 mL of the 75mM phosphate buffer (pH 7.4). All three solutions were stored in a fridge.

Trolox standard stock (0.01 M) was prepared by weighing 0.25 g of Trolox and dissolving it in 50 mL of 75 mM phosphate buffer (pH 7.4). Volume was filled up to 100 mL with 75 mM phosphate buffer (pH 7.4).

Epicatechin stock was prepared as 0.50 mg/mL solution in a phosphate buffer.

Both solutions were stored in -20^{o}C freezer..

For AAPH ([2,2'-azobis(2-amidino-propane)dihydrochloride]) working solution (20 mM) 0.2 g of AAPH was dissolved in 25 mL of 75 mM phosphate buffer (pH 7.4). This solution was prepared prior to use.

2.3.1.2. Procedure

Dilutions were prepared in a clear 96-well dilution plate (JRH flat bottomed) as shown in Table 5. The final concentration of trolox was 0.1mM, 0.05mM, 0.025mM, 0.0125mM and epicatechin: 250µg/mL, 1.25µg/mL,0.625µg/mL, 0.3125µg/mL.

Table 5. Strategy of diluting the samples/controls used in ORAC assay

1	2	3	4	5	6	7	8	9	10	11	12
Blank: Buffer 1	conc1: 5 µL Sample + 245 µLBuffer1 / 5 µL Trolox/Epicatechin + 995 µL Buffer1 + 170 mL AAPH										Control: Buffer 1 + 170mL AAPH
	conc2: 50 µL Sample(conc1) / 100 µLTrolox/Epicatechin + 100 µL Buffer2 + 170mL AAPH										
	conc3: 50 µL Sample(conc2) / 100 µLTrolox/Epicatechin + 100 µL Buffer2 + 170mL AAPH										
	conc4: 50 µL Sample(conc3) / 100 µLTrolox/Epicatechin + 100 µL Buffer2 + 170mL AAPH										

- Buffer1: Phosphate Buffer Working Solution (75 mM)
- Buffer2: 10% Phosphate Buffer in Methanol

The actual assay was conducted in a black 96-well Optiplate (Perkin Elmer). To each well 20 µL of sample/buffer and 10 µL fluorescein was added. APPH solution (170mL) was added to each well, except into the blank-column (170mL phosphate buffer 75mM was added instead) and the fluorescence was measured.

The chamber were the measurement was carried out was preheated to 37 ^{o}C and the plate was shaken 10 sec before reading. Wallac Victo 3 reader (Perkin Elmer) reads the plate 35 times a minute. Two replicates were done for every sample and the results represents the average value of both replicates of all concentration considered.

2.3.2. Cytotoxicity

This assay provides information about in-vitro cytotoxicity of the samples against mammalian cell line P388. It is based on the fact that ATP level rapidly decreases in the cells that undergo necrosis or apoptosis. The light that was emitted in the reaction of ATP with D-Luciferin in presence of firefly (*Photinus pyralis*) luciferase was measured.

$$\text{ATP} + \text{D-Luciferin} + \text{O2} \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO2} + \text{light}$$

The light is proportional to the ATP-concentration. It was measured using the Luminescence ATP Detection Assay System ATPLite – PerkinElmer.

2.3.2.1. Reagents and materials

Culture medium was prepared by dissolving 350mg of D-glucose (Sigma) in 86mL colour free DMEM (Gibco), 10 mL horse sera (Gibco), 2 mL Pen/Strep (5000 U/mL and 5000 μg/mL) and 2mL (Gibco) L-glutamine. All the reagents were warmed up in a Heto SBD 50 water bath at 37°C for about 20min, mixed up in the Sterile hood and filtered using Milipore Stericup® and Steritop™ vacuum-driven disposable filtration system with pore size 0.22 μm. Cell line used was P388 purchased from American Type Culture Cell Collection. Cells were grown under humidified conditions with 5% CO_2 at 37%. The small amount was transferred into the glass vial using a small transfer transfer pipette. Cells were counted in the ActDiff Cell Counter and diluted in a culture medium to the concentration of $0.1x10^6$ cells/mL and volume of 20 mL .

An ATPLite-PerkinElmer kit that was used for this assay contained mammalian cell lysis solution, substrate buffer solution, luciferase/luciferin lyophilized solution and lyophilized ATP standard. Chlorambucil and curcumin were used as controls and stored as a solution in sterilized DMSO at the concentration 60mg/mL.

2.3.2.2. Procedure

The whole procedure and preparation work was carried out in the Haereus Sterile hood. Samples were all prepared at the concentration of approximately 10 mg/mL in methanol. Dilutions were made in preparation plate (JRH flat bottomed plate). Controls (chlorambucil and curcumin) were diluted as well in following concentrations:

Chlorambucil (μg/mL):

- 500
- 250
- 125
- 62.5
- 31.25
- 15.625

Curcumin (μg/mL):

- 100
- 50
- 25
- 12.5
- 6.25
- 3.125

Sample/standard was transferred to the white cell culture plate in amount of 10 μL and 90 μL of cell dilution was added to every well. Two plates were made. Plates were incubated at 37°C for 24 hours. The ATPLite – PerkinElmer Kit components were equilibrated to the room temperature. To make up a substrate solution ~ 5.2 mL of the substrate buffer solution was added to a vial of the lyophilised substrate solution.

To develop the plate, 50 μL of mammalian cell lysis solution was added to each well. The plate was shaken for 5 minutes on the Wallac 1296-003

A Delfia plateshaker (700 rpm) was used to lyophilize the cells and stabilize ATP. After that 50 μL substrate solution were added to each well and placed again on the plate shaker for 5 minutes. Then the plate was wrapped in the aluminium foil to protect from light exposure and after 10 minutes the luminescence was measured using the Wallac Trilux 1450 Microbeta.

3. Comparison of the oil profiles

3.1. Phytochemical comparison of the oil profiles

Oil samples were dissolved in 100% methanol at a concentration of approximately 10mg/mL and profiled by GC-MS. Identification of compounds was made as described in Section 2.2.1.2.

3.1.1. *Plectranthus graveolens* (P1)

P.graveolens oil was vibrant red coloured with a strong aromatic smell. GC-MS analysis of the oil showed that it contained mono-, sesqui and diterpenes. The compounds are listed in Table 6 and the chromatogram of the oil is shown in Figure 10. Two major components were found in the region of diterpenes with 21.07% and 42.83% relative distribution. Both components had *m/z* of 288.

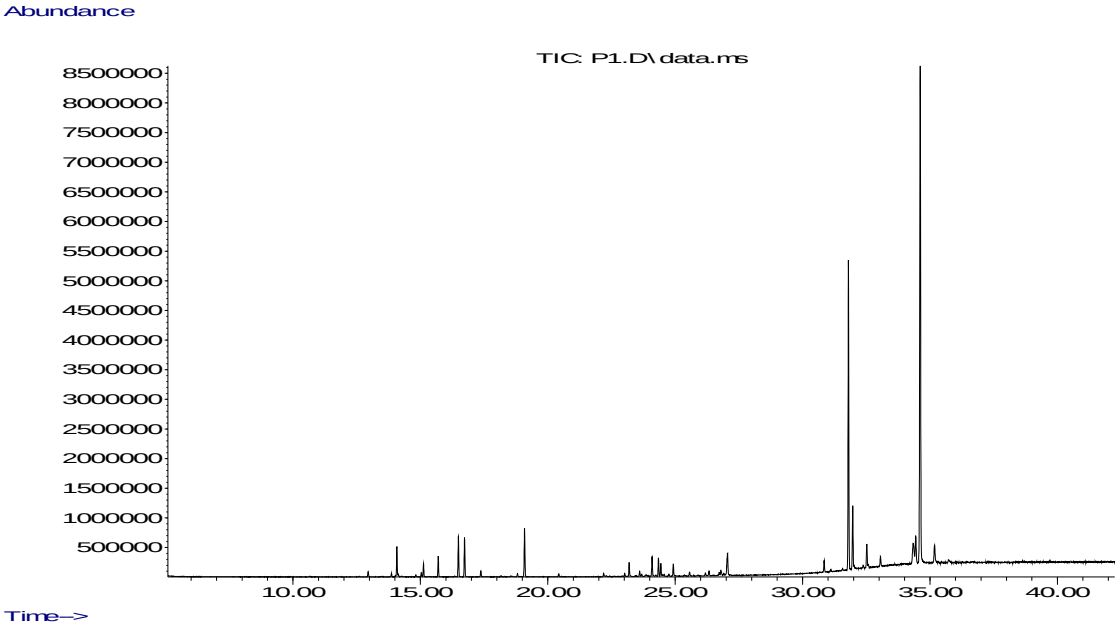


Figure 10. Total Ion Chromatogram of freshly distilled oil of *Plectranthus graveolens* P1.

Table 6. Chemical profile of the *Plectranthus graveolens.*

Compound	RT (min)	Area (%)	MW (g/mol)	KI-Adams	KI calculated	Cas #	Identification
3-Octanol	14.08	1.80	129	991	1007	000589-98-0	B
Limonene	15.12	0.88	136	1029	1057	000138-86-3	A,B
γ-Terpinene	15.70	1.38	136	1059	1083	000099-85-3	B
Unidentified, m/z 143	16.49	2.71	-	-	1121	-	-
Fenchone	16.74	2.80	152	1086	1133	001195-79-5	A,B
endo-Fenchyl acetate	19.09	3.47	196	1220	1253	004057-31-2	B
β-Sesquiphellandrene	23.20	0.95	204	1522	1487	020307-83-9	B
β-Bisabolene	24.09	1.39	204	1505	1544	000495-61-4	B
Sesquicineole	24.35	1.26	204	1516	1560	090131-02-5	B
β-Sesquiphellandrene	24.44	0.79	204	1522	1566	020307-83-9	B
Sesquiabinene hydrate	24.93	0.81	222	1544	1597	058319-05-4	B
Italicene	27.05	2.31	204	1405	1744	094535-52-1	B
ent-Primara-8,15-diene	30.85	0.83	272	-	2036	021561-92-2	B
Unidentified, m/z 288	31.80	21.07	-	-	2116	-	-
Unidentified, m/z 272	31.97	4.81	-	-	2131	-	-
Phenantrene	32.52	1.46	270	-	2179	019407-28-4	B
Unidentified, m/z 272	33.06	0.80	-	-	2211	-	-
Unidentified, m/z 281	34.34	3.10	-	-	2260	-	-
Unidentified, m/z 281	34.44	2.73	-	-	2264	-	-
Unidentified, m/z 288	34.62	42.83	-	-	2271	-	-
Unidentified, m/z 286	35.18	1.38	-	-	2291.589	-	-

"A" - Identification has been done by comparison with the reference standard

"B" – Identification has been done by comparison with the GC-MS database library

3.1.2. *Plectranthus suaveolens* (P2)

Only six components were detected in GC-MS in the orange oil of *P. suaveolens* as it is shown in Table 7. They included mono- and sesquiterpenes. Major compounds were β-cedrene, 1,10-di-epi-cubenol and camphor with 51.49%, 18.22% and 20.08% relative distribution, respectively. The chromatogram of the oil is shown in Figure 11.

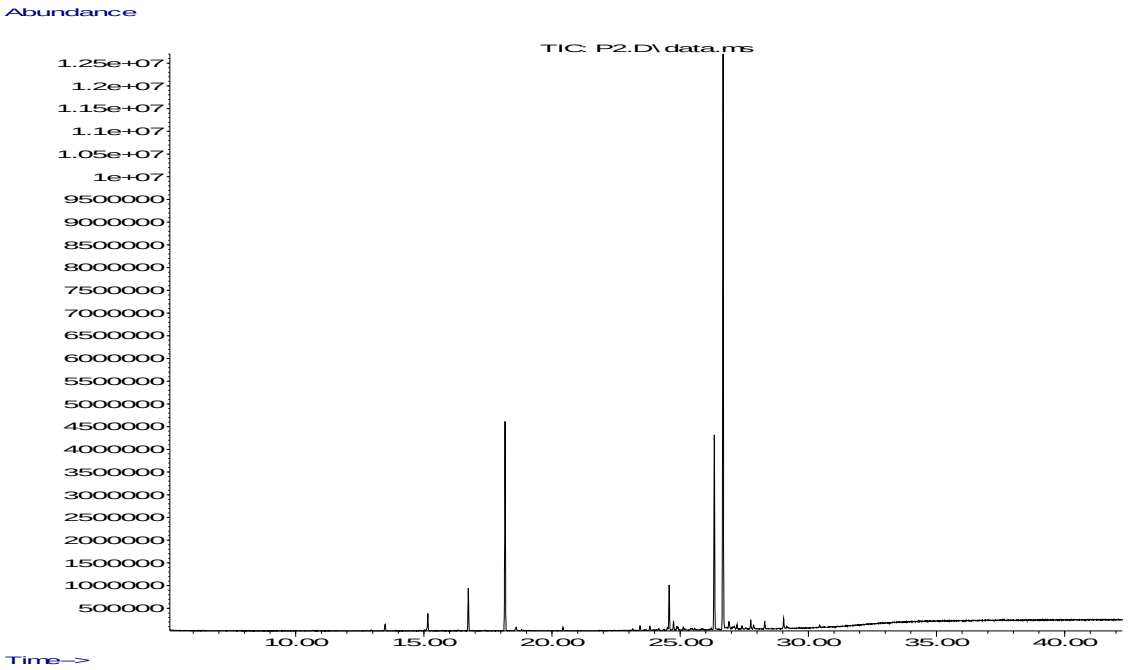


Figure 11. Total Ion Chromatogram of freshly distilled oil of *P. suaveolens* P2.

Table 7. Chemical Profile of *Plectranthus suaveolens.*

Compound	RT (min)	Area (%)	MW (g/mol)	KI-Adams	KI calculated	Cas #	Identification
E-β-Ocimene	15.16	1.69	136.13	1044	1058	003779-61-1	B
Fenchone	16.74	4.15	152.12	1083	1133	001195-79-5	A,B
Camphor	18.17	20.08	152.12	1141	1203	000076-22-2	A,B
γ-Cadinene	24.57	4.38	204.19	1513	1574	039029-41-9	B
1,10-di-epi-Cubenol	26.33	18.22	222.2	1618	1692	073365-77-2	B
β-Cedrene	26.67	51.49	204.19	1419	1716	000546-28-1	B

"A"- Identification has been done by comparison with the reference standard

"B" – Identification has been done by comparison with the GC-MS database library

3.1.3. *Plectranthus nitidus* (P3)

The oil of *Plectranthus nitidus* had a similar red colour to *P. graveolens* but not that strong smell. The crystallization process was observed in the vial where the oil was kept. Its GC-MS chromatogram showed an interesting profile since it contained only diterpenes. The composition of the oil is shown in Table 8 and the chromatogram in Figure 12. Major components had *m/z* of 288 with 58.74% and 14.02% relative distribution. RT (retention time) and molecular weight of these two compounds are the same as RT and molecular weight of the major compounds in *P. graveolens* (P1). In comparing the profiles of these two oils it can be seen that all diterpenes present in P1 are also present in P3. Percentages of relative distribution of these components followed the same pattern in both, *P.graveolens* and *P.nitidus*.

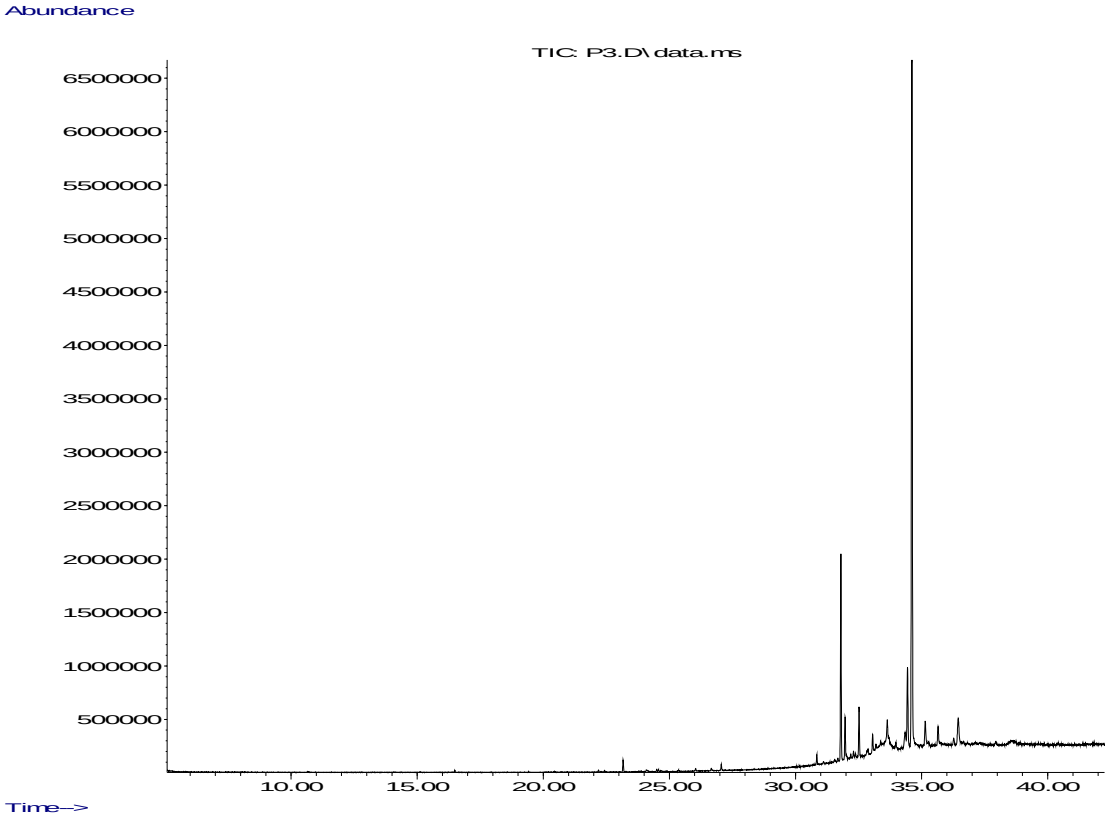


Figure 12. Total Ion Chromatogram of freshly distilled oil of *Plectranthus nitidus* P3.

Table 8. Chemical Profile of *Plectranthus nitidus.*

Compound	RT (min)	Area (%)	MW (g/mol)	KI-Adams	KI calculated	Cas #	Identification
Abietatriene	31.80	14.02	288	-	2116	-	B
Unidentified, m/z 272	31.97	3.30	-	-	2131	-	-
8,11,13-Abietatriene	32.52	3.60	270	2056	2179	088530-52-3	B
Unidentified, m/z 272	33.06	1.29	-	-	2211	-	-
Unidentified, m/z 270	33.64	1.54	-	-	2234	-	-
Unidentified, m/z 281	34.34	2.08	-	-	2260	-	-
Trypethelone	34.44	7.43	272	-	2264	081750-95-0	B
Unidentified, m/z 288	34.62	58.74	-	-	2271	-	-
Unidentified, m/z 286	35.15	2.38	-	-	2290	-	-
Unidentified, m/z 207	35.66	1.59	-	-	2309	-	-
Unidentified, m/z 348	36.46	4.04	-	-	2338	-	-

"B" – Identification has been done by comparison with the GC-MS database library

3.1.4. *Plectranthus* sp. 'Hahn Tableland'

3.1.4.1. First distillation batch (P4A)

The GC-MS profile of the oil from the first distillation batch of *P.*sp 'Hahn Tableland' revealed only monoterpenes. The composition of this oil represented a mixture of monoterpene molecules with or without oxygen-containing functional groups and it is shown in Table 9. Major component was δ-3-carene (42.34% relative distribution), followed by limonene (15.8%). Most of the compounds were isomers that had molecular weight of 134, 136 or 150 g/mol. Components that contained oxygen in their structure were coming out of the column at the end, which could be explained by their less volatile nature [6]. The chromatogram of the oil is shown in Figure 13.

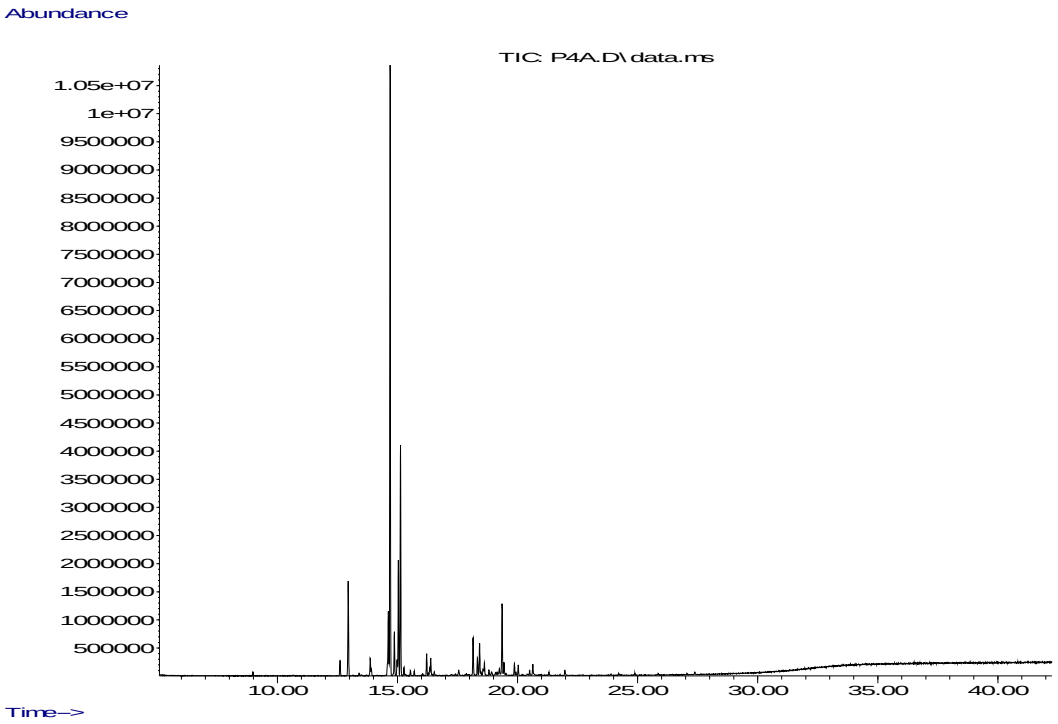


Figure 13. Total Ion Chromatogram of freshly distilled oil of *P. sp.* 'Hahn Tableland' P4A.

Table 9. Chemical Profile of *Plectranthus sp.* 'Hahn Tableland' P4A.

Compound	RT (min)	Area (%)	MW (g/mol)	KI-Adams	KI calculated	Cas #	Identification
α-Thujene	12.61	1.18	136	930	944	002867-05-2	B
α-Pinene	12.95	7.44	136	939	959	000080-56-8	A,B
Myrcene	13.87	0.83	136	990	997	000123-35-3	B
α-Phellandrene	14.62	5.72	136	1002	1033	000099-83-2	B
δ-3-Carene	14.70	42.34	136	1011	1037	013466-78-9	A,B
o-Cymene	14.87	3.25	134	1026	1045	000527-84-4	B
Sylvestrene	14.98	1.09	136	1030	1050	001461-27-4	B
p-Cymene	15.04	8.27	134	1026	1053	000527-84-4	A,B
Limonene	15.13	15.80	136	1029	1057	000138-86-3	A,B
Terpinolene	16.22	1.580	136	1088	1107	000586-62-9	B
Linalool	16.39	1.16	154	1096	1116	000078-70-6	A,B
Unidentified, m/z 91	18.15	2.78	-	-	1202	-	-
Unidentified, m/z 94	18.33	1.19	-	-	1212	-	-
Unidentified, m/z 150	18.42	2.27	-	-	1217	-	-
Unidentified, m/z 150	19.36	5.09	-	-	1268	-	-

"A"- Identification has been done by comparison with the reference standard

"B" – Identification has been done by comparison with the GC-MS database library

3.1.4.2. Second distillation batch (P4B)

The second batch of distillation gave light yellow oil with very similar composition to the first batch. Monoterpenes were again the only constituents and they had very similar percentage of relative distribution comparing to P4A. Major component δ-3-carene is present here in 39.08% and limonene 15.79%. The components are listed in Table 10 and chromatogram is shown in Figure 14.

Some differences have been noticed between the two distillation batches. In P4A two components were found that were not present in P4B. They had RT 12.61 and 14.98 min and were identified as thymol and sylvestrene, respectively. In P4B another two components were found that were not present in P4A. They had RT 18.62 and 20.64 min and the latter was identified as carvacrol.

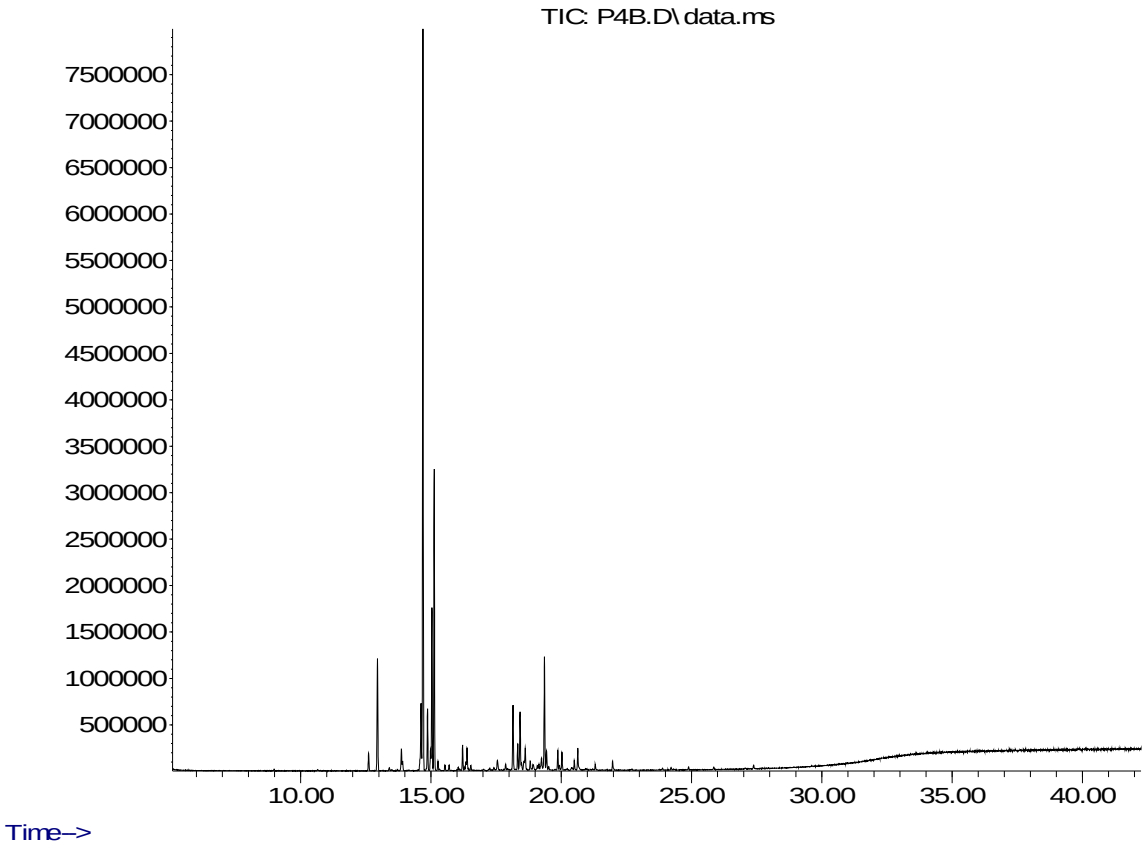


Figure 14. Total Ion Chromatogram of freshly distilled oil of *P. sp.* 'Hahn Tableland' P4B.

Table 10. Chemical profile of *Plectranthus sp.* 'Hahn Tableland' P4B.

Compound	RT (min)	Area (%)	MW (g/mol)	KI-Adams	KI calculated	Cas #	Identification
α-Pinene	12.95	6.78	136	939	959	000080-56-8	A,B
Myrcene	13.87	0.74	136	990	997	000123-35-3	B
α-Phellandrene	14.62	4.39	136	1002	1033	000099-83-2	B
δ-3-Carene	14.69	39.08	136	1011	1037	013466-78-9	A,B
o-Cymene	14.87	3.45	134	1026	1045	000527-84-4	B
p-Cymene	15.04	8.86	134	1026	1053	000527-84-4	A,B
Limonene	15.13	15.79	136	1029	1057	000138-86-3	A,B
Terpinelone	16.22	1.42	136	1088	1107	000586-62-9	B
Linalool	16.39	1.16	154	1096	1116	000078-70-6	A,B
Unidentified, m/z 91	18.15	3.74	-	-	1202	-	-
Unidentified, m/z 94	18.33	1.42	-	-	1212	-	-
Unidentified, m/z 150	18.42	3.67	-	-	1217	-	-
Unidentified, m/z 150	18.62	1.92	-	-	1228	-	-
Unidentified, m/z 150	19.36	6.18	-	-	1268	-	-
Carvacrol	20.64	1.41	150	1299	1338	000499-75-2	A,B

"A"- Identification has been done by comparison with the reference standard

"B" – Identification has been done by comparison with the GC-MS database library

3.1.5. Ornamental *Plectranthus*

3.1.5.1. First distillation batch (P6A)

Ornamental *Plectranthus* oil had a light yellow colour, similar to one of P4B. Its chemical profile is shown in Table 11 and it contained mono- and sesquiterpenes. Major components were monoterpenes δ-3-carene, camphor and carvacrol with 20.68%, 12.46% and 23.47% relative distribution. All sesquiterpenes were isomers with MW (molecular weight) 204 g/mol, except trans-calamene that had MW 202 g/mol. The chromatogram is shown in Figure 15.

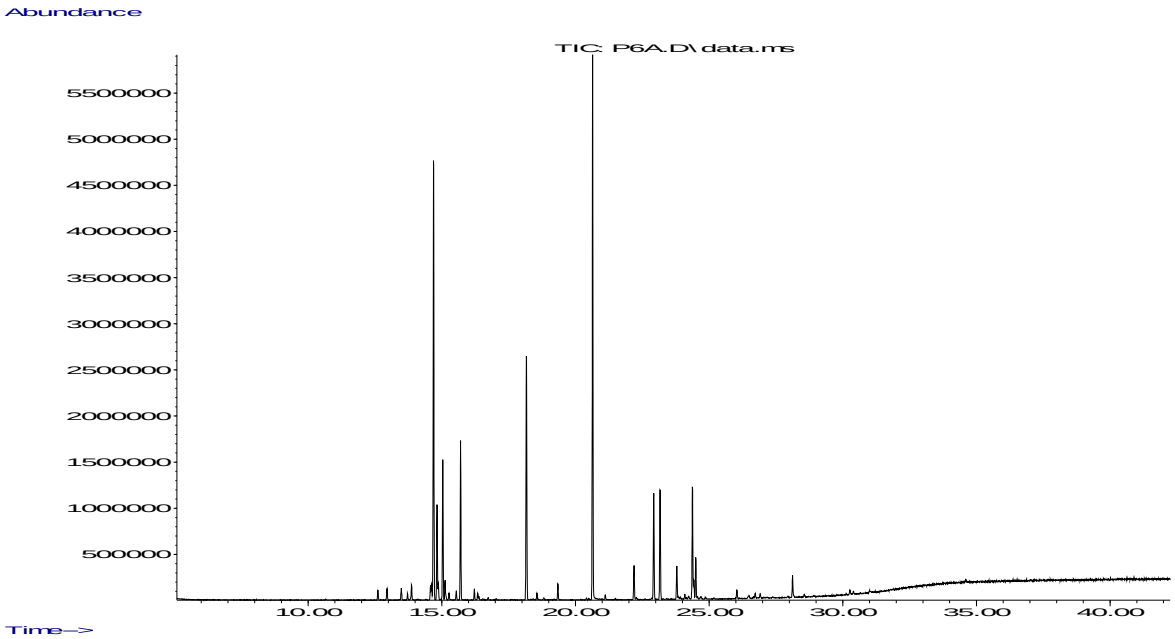


Figure 15. Total Ion Chromatogram of freshly distilled oil of ornamental *Plectranthus.*

Table 11. Chemical Profile of ornamental *Plectranthus* P6A.

Compound	**RT (min)**	**Area (%)**	**MW (g/mol)**	**KI-Adams**	**KI calculated**	**Cas #**	**Identification**
Myrcene	13.87	0.76	136	990	197	000123-35-3	B
δ-3-Carene	14.69	20.68	136	1011	1037	013466-78-9	A,B
δ-2-Carene	14.82	3.75	136	1002	1043	000554-61-0	B
p-Cymene	15.04	6.39	134	1026	1053	000527-84-4	A,B
Limonene	15.13	1.09	136	1029	1057	000138-86-3	A,B
γ-Terpinene	15.70	7.23	136	1059	1083	000099-85-4	B
Camphor	18.17	12.46	152	1146	1203	000076-22-2	A,B
Carvacrol, methylether	19.34	0.75	164	1244	1267	006379-73-3	B
Carvacrol	20.64	23.47	150	1299	1338	000499-75-2	A,B
α-Copaene	22.19	1.67	204	1376	1427	003856-25-5	B
α-cis-Bergamotene	22.93	5.12	204	1412	1471	018252-46-5	B
Z-Caryophyllene	23.17	5.62	204	1408	1485	000118-65-0	B
α-Humulene	23.79	1.57	204	1454	1521	006753-98-6	B
α-Guaiene	24.38	5.56	204	1439	1555	003691-12-1	B
α-Selinene	24.43	0.81	204	1498	1557	000473-13-2	B
δ-Amorphene	24.51	1.93	204	1512	1562	189165-79-5	B
Unidentified, m/z 202	28.12	1.14	-	-	1821	-	-

"A"- Identification has been done by comparison with the reference standard

"B" – Identification has been done by comparison with the GC-MS database library

3.1.5.2. Second distillation batch (P6B)

The oil from the second distillation batch, P6B, had very similar profile to P6A. The compounds are listed in Table 12 and chromatogram is shown in Figure 16. Myrcene was the only component detected in P6A that was not in P6B and its distribution was only 0.76%. Also some differences in percentages of relative distribution of the main components were present. Carvacrol had relative distribution of 29.33% in P6B and 23.47% in P6A. On the other hand, δ-3-carene was in smaller amount (15.57%) in P6B compared to P6A (20.68%).

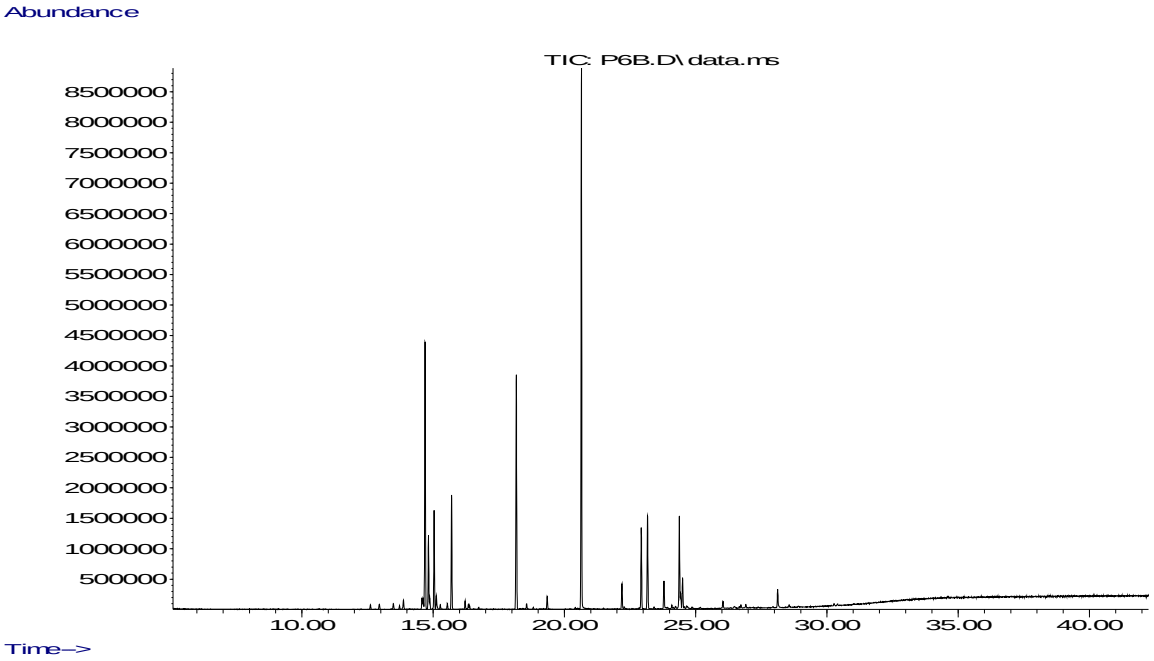


Figure 16. Total Ion Chromatogram of freshly distilled oil of ornamental *Plectranthus.*

Table 12. Chemical Profile of ornamental *Plectranthus* P6B.

Compound	RT (min)	Area (%)	MW (g/mol)	KI-Adams	KI calculated	Cas #	Identification
δ-3-Carene	14.69	15.57	136	1011	1037	013466-78-9	A,B
δ-2-Carene	14.82	3.60	136	1002	1043	000554-61-0	B
p-Cymene	15.04	5.57	134	1026	1053	000527-84-4	A,B
Limonene	15.12	0.89	136	1029	1057	000138-86-3	A,B
γ-Terpinene	15.70	6.43	136	1059	1083	000099-85-4	B
Camphor	18.17	14.49	152	1146	1203	000076-22-2	A,B
Carvacrol, methylether	19.34	0.76	164	1244	1267	006379-73-3	B
Carvacrol	20.65	29.33	110	1299	1338	000499-75-2	A,B
α-Copaene	22.19	1.55	204	1376	1427	003856-25-5	B
α-cis-Bergamotene	22.93	4.84	204	1412	1471	018252-46-5	B
E-Caryophyllene	23.17	5.82	204	1419	1485	000087-44-5	B
α-Humulene	23.79	1.67	204	1454	1521	006753-98-6	B
β-Selinene	24.38	5.73	204	1490	1555	017066-67-0	B
α-Selinene	24.43	0.79	204	1498	1557	000473-13-2	B
δ-Amorphene	24.51	1.79	204	1512	1562	189165-79-5	B
Unidentified, m/z 202	28.12	1.18	-	-	1821	-	-

"A"- Identification has been done by comparison with the reference standard

"B" – Identification has been done by comparison with the GC-MS database library

3.1.5.3. Comparison of the P6A and P6B after three months period

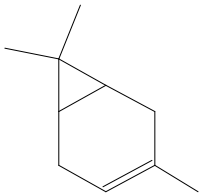
During this study oils were kept in the closed glass vials in the laboratory. They were thus exposed to the daylight and room temperature. After three months a change in colour between the two oils was observed. P6A was still yellowish but became a fraction turbid and P6B was light greenish coloured. GC-MS analysis was done to see if the composition has changed. The results are listed in Table 13.

Table 13. Chemical composition of P6A, P6B and change in percentage of distribution.

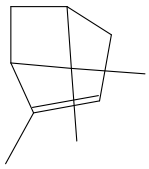
Compound	RT(min)	P6A		P6B	
		Area2 (%)	Area1-Area2 (%)	Area2 (%)	Area1-Area2 (%)
δ-3-Carene	14.68	16.57	4.11	13.82	1.75
o-Cymene	14.85	1.27	New	1.01	New
p-Cymene	15.03	15.00	-8.61	11.34	-5.77
γ-Terpinene	15.70	7.23	0	2.35	4.08
Camphor	18.16	12.97	0.51	14.68	-0.19
Carvacrol	20.64	20.23	3.24	20.80	8.53
α-Copaene	22.17	1.56	0.11	1.60	-0.05
α-cis-Bergamotene	22.91	4.60	0.52	4.72	0.12
E-Caryophyllene	23.14	1.57	4.05	3.47	0.59
β-Selinene	24.36	5.68	0.12	6.07	-0.34
δ-Amorphene	24.48	1.61	0.81	1.75	0.04
Caryophyllene oxide	26.01	3.15	New	2.07	New
Unidentified, m/z 202	28.10	1.11	0.03	1.19	-0.01

The last column in Table 13 is showing difference in percentage of distribution between the fresh oil (Area1) and the one stored for three months (Area2). The relative distribution of some components has significantly changed, and two new components had been identified. The percentage of relative distribution of δ-3-carene has dropped 20% in P6A, whereas in P6B has not changed a lot. On the other hand carvacrol has degraded more in P6B (decrease by over 20%) compared to P6A (approximately 13%). The component that drastically changed its amount is *p*-cymene with about 100% increase in both of the oils. New components that have been detected are caryophyllene oxide and *o*-cymene. The first is probably the oxidation product of E-caryophyllene, since decrease of its amount was approximately the same as increase of E-caryophyllene oxide.

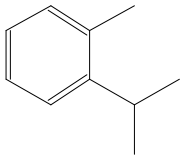
The non-stable nature of monoterpenes has been reported. Tammela et al. have reported decreasing in δ-3-carene and α-pinene content of ageing pine seeds [20].



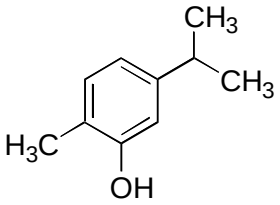
δ-3-carene



α-pinene



o-cymene



carvacrol

The presence of two rings in structure of both compounds probably contributed to their instability. An increased content of *o*- and *p*-cymene suggests possible degradation of δ-3-carene and α-pinene to *o*- and *p*-cymene. This leads to the conclusion that some monoterpenes were unstable and therefore should be kept very carefully, in the tightly sealed vials, and not exposed to any source of free energy like light or temperature.

3.2. Pharmacology of the *Plectranthus* oils

3.2.1. ORAC

Samples were dissolved in 100% methanol in approximate concentration of 10mg/mL. The results of the ORAC assay are listed inTable 14. P6A and P6B had the highest antioxidant activity, followed by P3 and P4B. The high activity of ornamental *Plectranthus* is probably due to its high percentage of carvacrol that has already been reported as highly antioxidant [21]. Interestingly P6B showed noticeably higher value compared to P6A, which is again probably due to the higher relative distribution of carvacrol (29.33%, Table 12). There was a significant difference between P4A and P4B values, but there was no significant difference observed between the GC-MS profiles of P4A and P4B (Figure 17). However there is a possibility that some oxidation process happened after adding molecular sieve to the first oil batch which resulted in smaller antioxidant activity.

Table 14. ORAC values of *Plectranthus* oils.

Sample	**ORAC value (µmol TE/g)**	**RSD (%)**
P1	584 ± 52	9.01
P2	699 ± 54	7.69
P3	1351± 362	26.79
P4A	531.358 ± 89 4	16.80
P4B	1111 ± 168	15.15
P6A	1560 ± 356	22.84
P6B	2215 ± 377	17.00

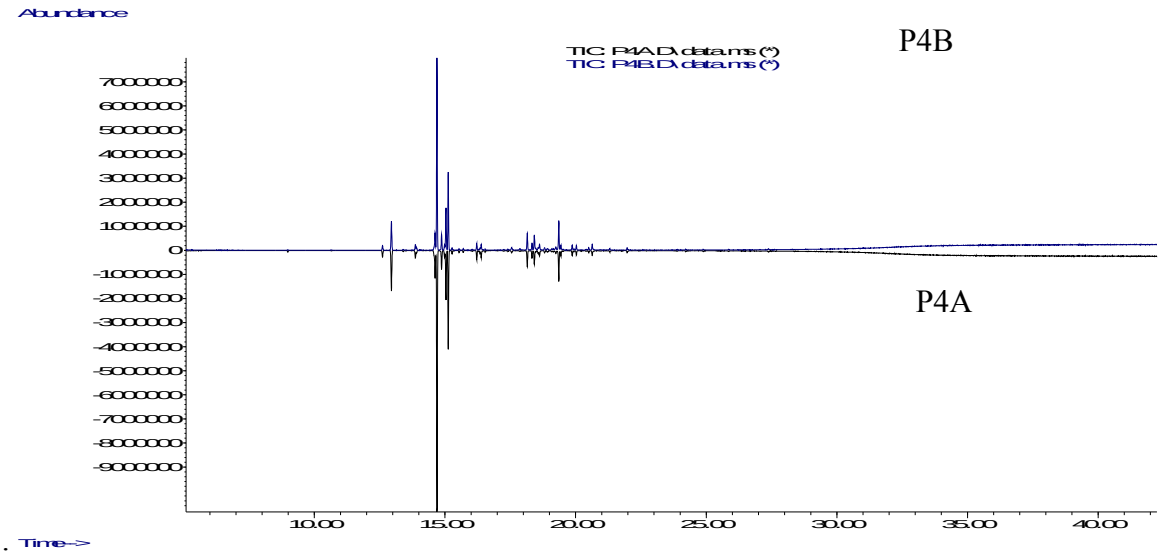


Figure 17. Overlaid TIC of P4A and P4B.

3.2.2. Cytotoxicity assay

Cytotoxic activity against P388 mammalian cell line was represented as IC_{50} value and shown in Figure 18. P3 had the highest cytotoxic activity, followed by P1 and P2. Low IC_{50} value can be contributed to the presence of diterpenes in P3, since they are the components in this oil and the major components in P1. Cytotoxic activity of diterpenes in this genus has already been reported. Forskolin, a diterpene from Indian plant *Coleus forskohlii* (synonym for *Plectranthus barbatus*) has reduced tumour colonization in the lungs [16]. Another diterpene in the same plant was found to be highly active against breast and uterine cell lines [22]. The fact that P3 was much more active than P1 is probably because it contained only diterpenes whereas P1 oil had other terpenes. Detailed cytotoxic results are listed in Appendix 1.

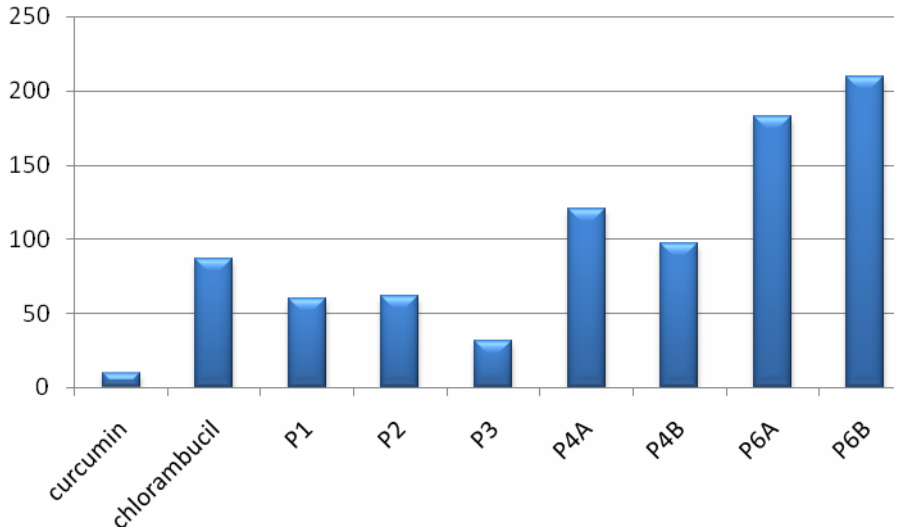


Figure 18. IC_{50} (µmol/mL) values of *Plectranthus* volatile oils (P1-P6).

4. *Plectranthus* sp. 'Hahn Tableland': P4B

4.1. Fractionation

P4B oil was fractionated using SPE column as described in Chapter 2.2.2. Four batches of fractionations of 1g each have been carried out and fractions were collected as shown in Table 15 and fractionation was done as is shown in Figure 19.

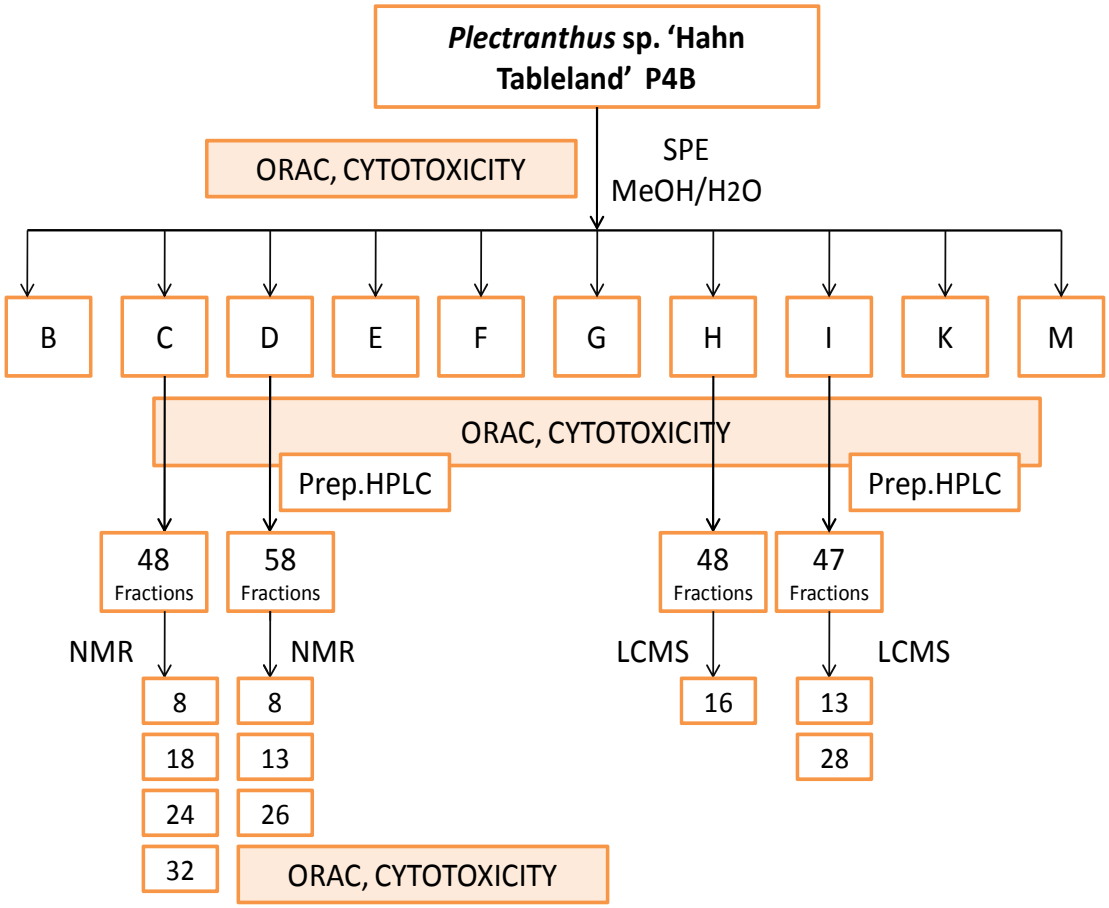


Figure 19. Schematic representation of the fractionation of P4B.

Table 15. SPE fractionation strategy and yields of the individual fractions (batches I, II, III and IV).

Crude Oil (g)		**Batch I** **0.9313**		**Batch II** **0.9513**		**Batch III** **0.9545**		**Batch IV** **0.9700**	
Fraction Code	**Mobile Phase, MeOH in H_2O (%)**	**Weight (g)**	**Yield (%)**	**Weight (g)**	**Yield (%)**	**Weight (g)**	**Yield (%)**	**Weight (g)**	**Yield (%)**
B	50	0.0018	0.19	0.0028	0.29	0.012	1.26	0.001	0.10
C	75	0.0694	7.45	0.0782	8.22	0.1844	19.32	0.0934	9.63
D	75	0.1467	15.75	0.1693	17.80	0.0725	7.60	0.0858	8.85
E	75	0.0119	1.28	0.0141	1.48	0.0042	0.44	0.0044	0.45
F	75	0.0096	1.03	0.025	2.63	0.0036	0.38	0.0033	0.34
G	100	0.0174	1.87	0.0334	3.51	0.0043	0.45	0.014	1.44
H	100	0.0205	2.20	0.0293	3.08	0.039	4.09	0.0411	4.24
I	100	0.0254	2.73	0.0211	2.22	0.0186	1.95	0.0198	2.04
J	100	0.003	0.32	0.0038	0.40	0.0018	0.19	0.0023	0.24
K	100	0.0056	0.60	0.005	0.53	0.0121	1.27	0.0231	2.38
L	100	0.1994	21.41	0.4956	0.00	0.1828	19.15	0.0969	9.99
M	100	0.3912	42.01	0.2199	23.12	0.0226	2.37	0	0.00

Fractions B, C, D, E, F, and G were extracted with chloroform and I, J and K with hexane. No extraction was made for fractions L and M. The extracts were concentrated to dryness under nitrogen and dissolved in 100% methanol for further analysis. GC-MS and LCMS profiling was done for all of the fractions and after analysis, fractions that were labelled with the same letter from all four groups had been combined. Fractions J and K were combined together and L and M were combined resulting to 10 pooled fractions.

4.2. GC-MS and LCMS analysis of the fractions

4.2.1. P4B-B fraction

Fraction P4B-B showed no response in GC-MS analysis, but from LCMS profile one large peak was observed at the RT of 2.6 min with *m/z* 201.1 (Figure 20).

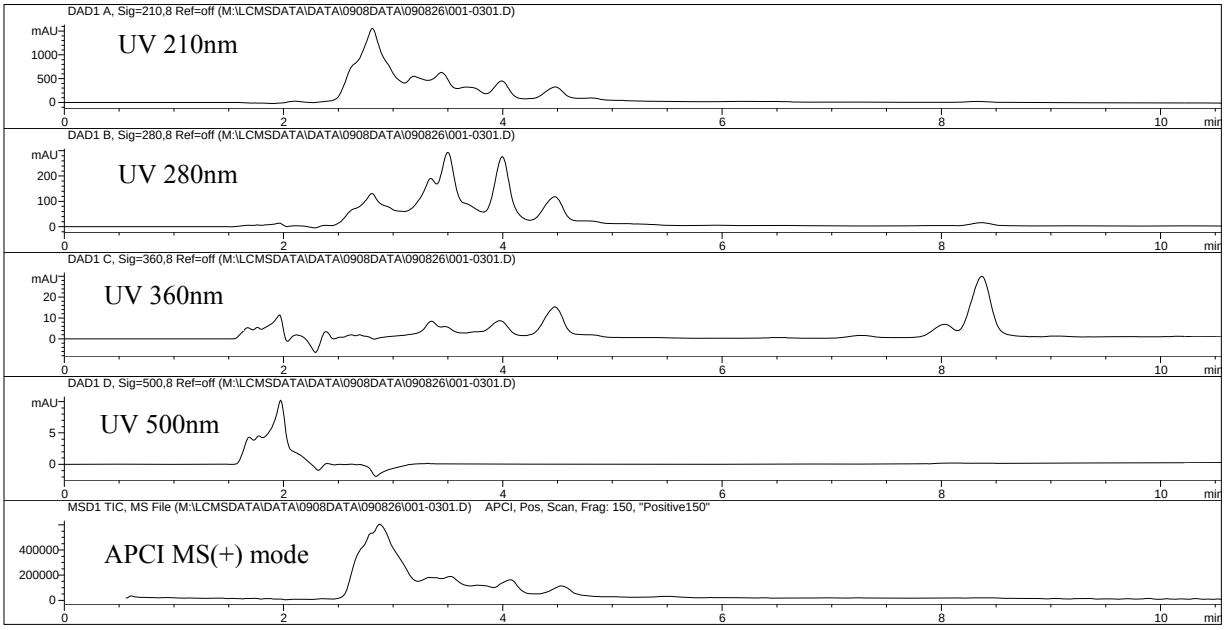


Figure 20. UV spectra at 210, 280, 360 and 500nm wavelength and LCMS profile of P4B-B fraction.

4.2.2. P4B-C fraction

GC-MS analysis of fraction P4B-C showed one large peak at 19.35 min (Figure 21) and the composition was determined by comparison with database. Compounds found in the P4B-C fraction are listed in Table 16. The major component of fraction C at 19.35 min was chrysanthenone (41.69% relative distribution). Other components were *p*-cymen-8-ol (27.55% relative distribution) and 3-caren-2-one (10.29%), which was not observed in the crude oil and could be an oxidized product of δ-3-carene.

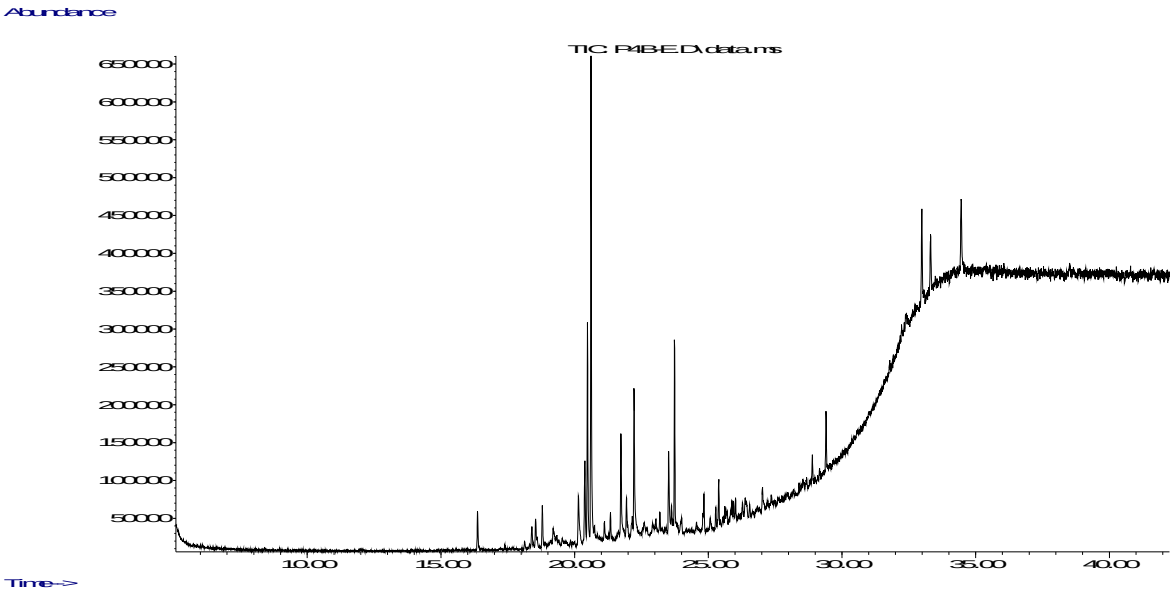


Figure 21. Total ion chromatogram of the P4B-C fraction.

Table 16. Chemical composition of the P4B-C fraction.

Compound	RT (min)	Area (%)	Match (%)	KI-calculated	KI Adams	Cas #
Unidentified, m/z 119	18.14	4.44	42	1202	-	-
p-Cymen-8-ol	18.42	27.55	87	1217	1182	1197-01-9
Unidentified, m/z 150	18.56	1.53	78	1225	-	-
Unidentified, m/z 150	18.62	8.07	68	1202	-	-
Chrysanthenone	19.35	41.69	83	1267	1127	473-06-3
Eucarvone	19.43	2.36	94	1271	1150	503-93-5
3-Caren-2-one	20.02	10.29	95	1320	1248	53585-45-8
Unidentified, m/z 150	21.29	3.80	86	1374	-	-

4.2.3. P4B-D fraction

P4B-D fraction showed the most complex profile (Figure 22) with 21 components that are summarized in Table 17. Numerous compounds could not be identified by comparison with library database. The major components of this fraction had RT of 18.14 and 19.34 min with *m/z* 119 and 150, respectively. The latter (RT 19.34 min) was also observed as the major component in the P4B-C fraction. The components that could be identified with library database were linalool (4.76% relative distribution), *p*-cymen-8-ol (3.19% relative distribution), trans-carveol (5.75% relative distribution), eucarvone (2.89% relative distribution), carvone (6.22% relative distribution), thymol (4.89% relative distribution), carvacrol (12.40% relative distribution) and 2-caren-4-one (3.89% relative distribution).

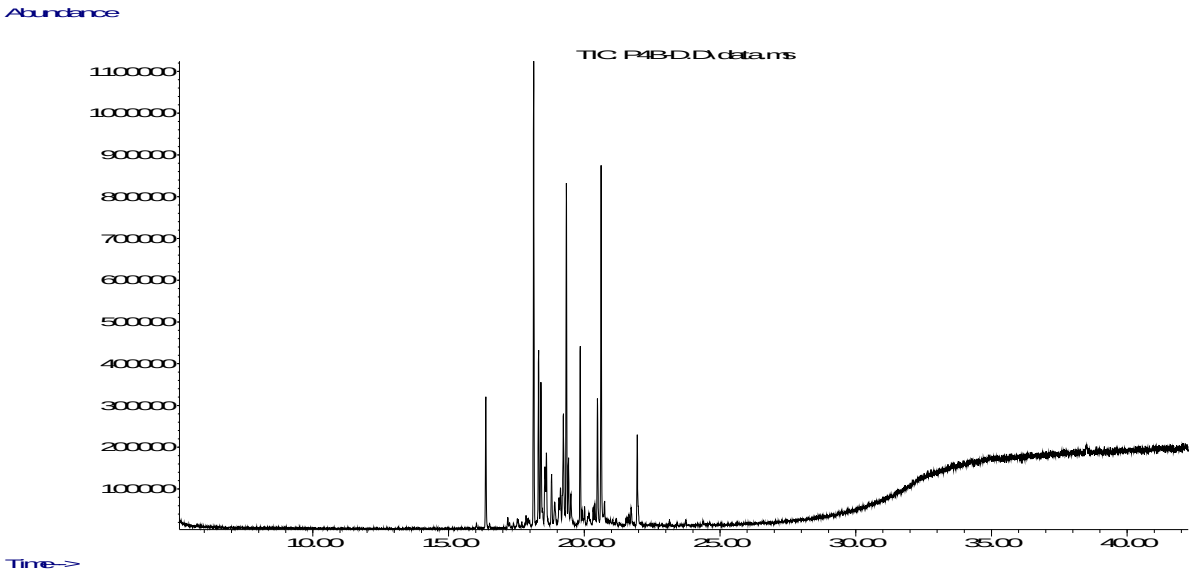


Figure 22. Total ion chromatogram of the P4B-D fraction.

Table 17. Chemical composition of the P4B-D fraction.

Compound	RT (min)	Area (%)	Match (%)	KI-calculated	KI Adams	Cas #
Linalool	16.37	4.76	96	1115	1096	78-70-6
Unidentified, m/z 119	18.14	16.39	59	1202	1166	1686-2--0
Unidentified, m/z 91	18.32	6.74	64	1212	-	-
Unidentified, m/z 150	18.41	6.15	83	1217	-	-
Unidentified, m/z 148	18.55	2.94	46	1224	-	-
p-Cymen-8-ol	18.61	3.19	87	1228	1182	1197-01-9
Unidentified, m/z 136	18.80	2.19	64	1238	-	-
Unidentified, m/z 164	18.92	1.56	50	1244	-	-
Unidentified, m/z 152	19.07	1.16	46	1252	-	-
Unidentified, m/z 148	19.13	1.72	70	1255	-	-
trans-Carveol	19.23	5.75	86	1261	1216	99-48-9
Unidentified, m/z 150	19.34	12.41	78	1267	-	-
Eucarvone	19.42	2.89	86	1271	1150	503-93-5
Unidentified, m/z	19.51	1.43	60	1275	-	-
Carvone	19.85	6.22	89	1293	1243	99-49-0
Unidentified, m/z 165	20.33	0.70	38	1320	-	-
Unidentified, m/z 150	20.39	0.91	76	1323	-	-
Thymol	20.49	4.89	90	1329	1290	89-83-8
Carvacrol	20.62	12.40	93	1337	1299	499-75-2
Unidentified, m/z 167	20.75	0.78	50	1344	-	-
2-Caren-4-one	21.95	3.89	83	1412	-	-

4.2.4. P4B-E fraction

The TIC of P4B-E is shown in Figure 23. The database showed carvacrol (32.66% relative distribution) as the major component in P4B-E, followed by *Z*-ethyl cinnamate (12.24% relative distribution) and an unknown component with RT 20.48 min (14.79% relative distribution). This fraction showed an interesting profile because all of the compounds had RT>20min, which was the part of chromatogram that has not been observed in the profile of the crude oil. This suggests a low proportion of these compounds in the oil. However, no further fractionation was carried out because of the low yield. Composition of the fraction is shown in Table 18.

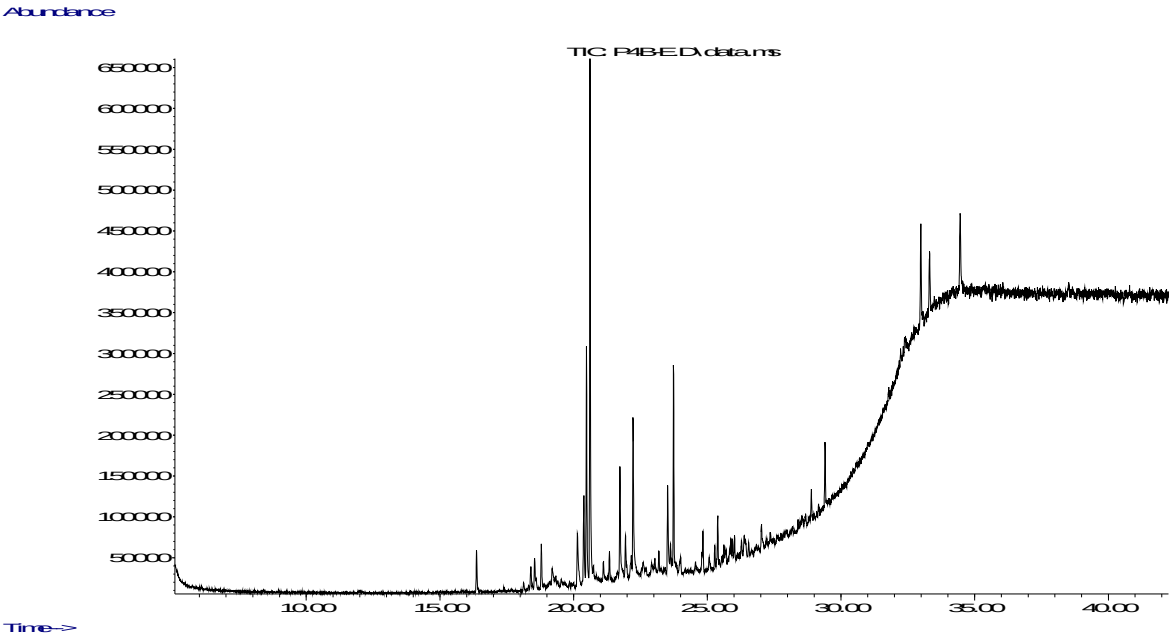


Figure 23. Total ion chromatogram of the P4B-E fraction.

Table 18. Chemical composition of the P4B-E fraction.

Compound	RT (min)	AREA (%)	Match (%)	KI-calculated	KI Adams	Cas #
Thymol	20.38	6.45	93	1323	1290	89-83-8
Unidentified, m/z 150	20.48	14.79	90	1329	-	-
Carvacrol	20.61	32.66	95	1336	1299	499-75-2
Unidentified, m/z 163	21.73	7.75	22	1399	-	-
Unidentified, m/z 159	22.22	12.80	38	1429	-	-
Unidentified, m/z 153	23.52	6.65	38	1507	-	-
Z-Ethyl cinnamate	23.74	12.24	97	1521	1377	4610-69-9
Unidentified, m/z 281	32.98	6.66	50	2209	-	-

4.2.5. P4B-F fraction

The GC-MS profile of fraction F showed two sesquiterpenes in approximately the same amount (Figure24). They were identified as spathulenol and intermedeol by comparison with the database.

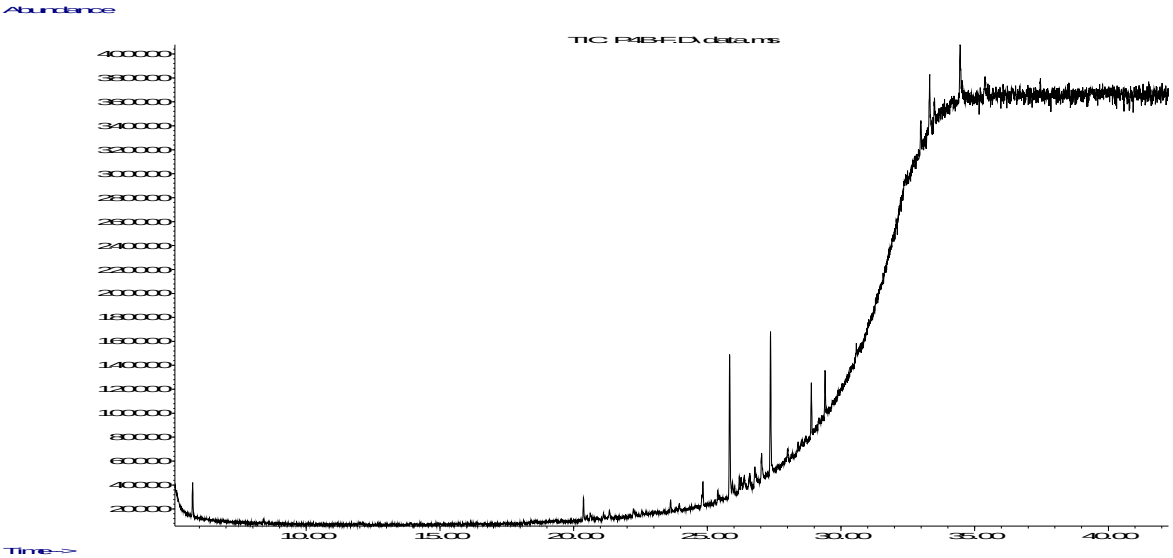


Figure 24. Total ion chromatogram of the P4B-F fraction.

Table 19. Chemical composition of the P4B-F fraction.

Compound	RT (min)	AREA (%)	Match (%)	KI-calculated	KI Adams	Cas #
Spathulenol	25.84	49.65	99	1659	1578	6750-60-3
Intermedeol	27.36	50.35	97	1766	1666	6168-59-8

4.2.6. P4B-G fraction

The chromatogram of the fraction P4B-G is shown in Figure 25. Fraction P4B-G contained spathulenol and intermedeol, but also two other unidentified components with RT of 24.88 and 27.05 min. The compounds found in the fraction are listed in Table 20.

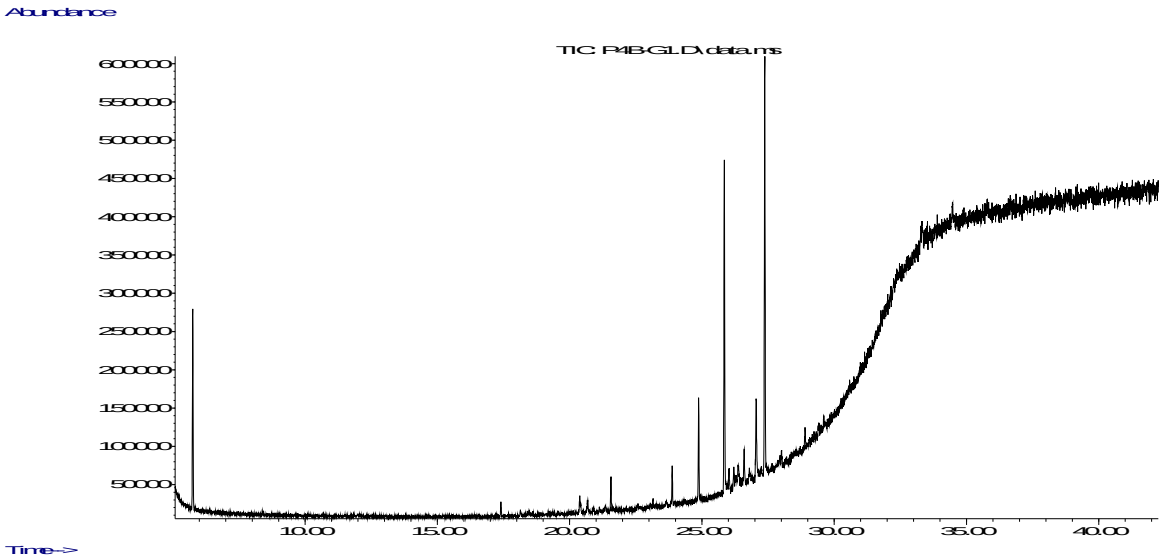


Figure 25. Total ion chromatogram of the P4B-G fraction.

Table 20. Chemical composition of the P4B-G fraction.

Compound	RT (min)	AREA (%)	Match (%)	KI-calculated	KI Adams	Cas #
Unidentified, m/z 147	24.88	8.95	47	1594	-	-
Spathulenol	25.85	29.6	95	1655	1578	6750-60-3
Unidentified, m/z 207	27.05	9.60	72	1744	-	-
Intermedeol	27.38	36.37	97	1767	1666	6168-59-8

4.2.7. P4B-H fraction

The chromatogram of the P4B-H fraction is shown in Figure 26. Spathulenol and intermedeol were also present in high percentage in this fraction, as well as *o*-cymene and unidentified component with RT 24.88 min. During SPE fractionation this fraction was collected as a yellow-orange band. Composition of the fraction is shown in Table 21.

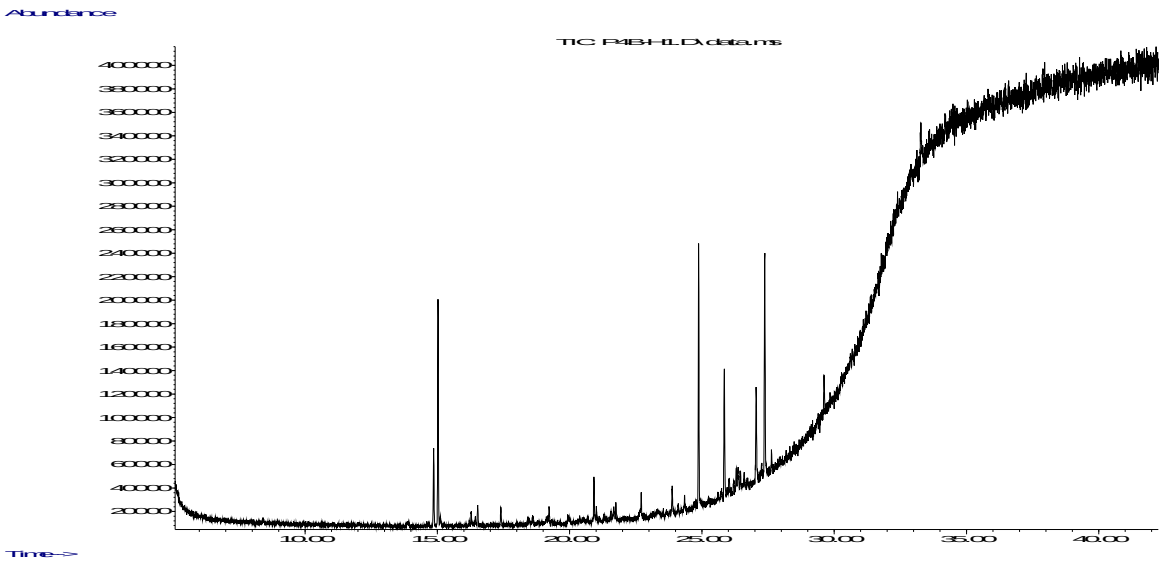


Figure 26. Total ion chromatogram of the P4B-H fraction.

Table 21. Chemical composition of the P4B-H fraction.

Compound	RT (min)	Area (%)	Match (%)	KI-calculated	KI Adams	Cas #
o-cymene	15.02	24.87	90	1052	1026	527-84-4
Limonene	15.11	10.53	81	1056	1029	138-86-3
Unidentified, m/z 147	24.88	29.35	47	1594	-	-
Spathulenol	25.85	17.27	96	1655	1578	6750-60-3
Intermedeol	27.38	28.50	83	1767	1666	6168-59-8

4.2.8. P4B-I fraction

P4B-I contained δ-3-carene and limonene as major components, which are also the main components in the crude oil. Components are listed in Table 22 and the chromatogram of the fraction is shown in Figure 27. The major component of the oil, δ-3-carene, was only found in this fraction. Considering the fact that fraction I had relative low yield (<3% in all four fractions) and the non-stabile nature of δ-3-carene it was thought that this compound must have decomposed during the fractionation process.

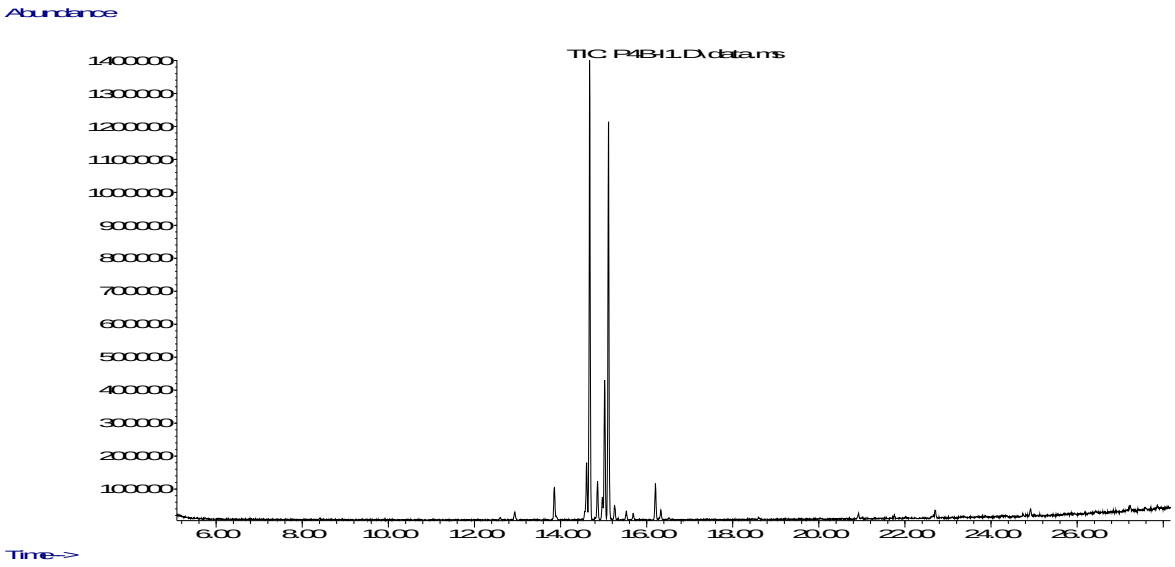


Figure 27. Total ion chromatogram of the P4B-I fraction.

Table 22. Chemical composition of the P4B-I fraction.

Compound	RT	AREA %	Match %	KI-calculated	KI Adams	Cas #
α-Phellandrene	14.61	6.03	83	1033	1002	99-83-2
δ-3-Carene	14.68	39.48	94	1036	1011	13466-78-9
o-Cymene	14.86	3.60	90	1045	1026	527-84-4
Limonene	15.12	34.65	91	1056	1029	138-86-3
Terpinolene	16.21	3.41	87	1106	1088	586-62-9

4.2.9. P4B-J and P4B-K fractions

GC-MS profile from the fractions P4B-J and P4B-K (Figure 28) showed a sesquiterpene, β-selinene, as the major compound (Table 23). Other minor peaks were also present in this fraction, but they were not integrated due to the low proportion.

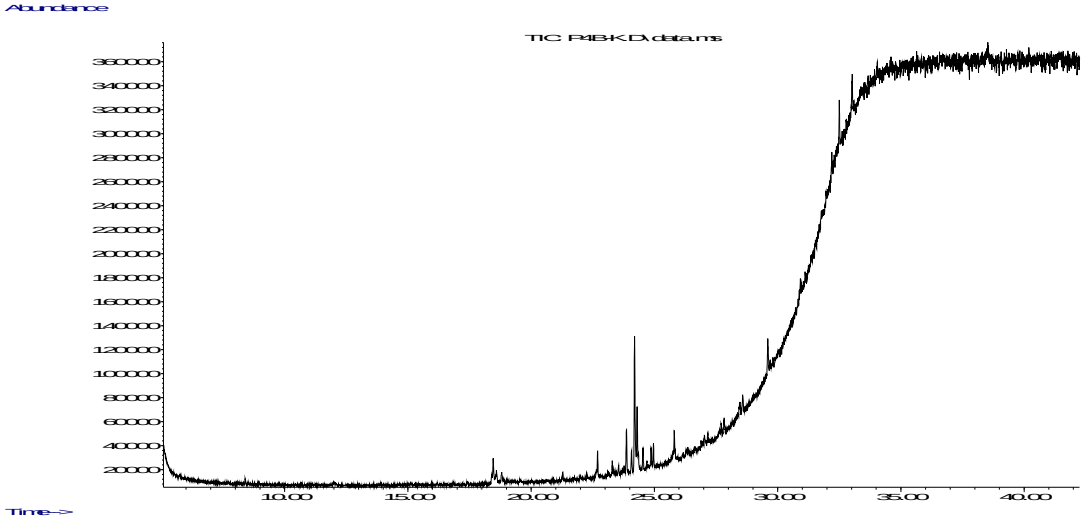


Figure 28. Total ion chromatogram of the P4B-J and P4B-K fractions.

Table 23. Chemical composition of the P4B-J and P4B-K fractions.

Compound	RT (min)	AREA (%)	Match (%)	KI-calculated	KI Adams	Cas #
β-Selinene	24.20	100.00	81	1551	1490	17066-67-0

4.2.10. P4B-L and P4B-M fractions

Similar to the P4B-B, fractions L and M did not show any response in the GC-MS, but the LCMS profile (Figure 29) showed two components with the same fragmentation pattern of *m/z* of 230.

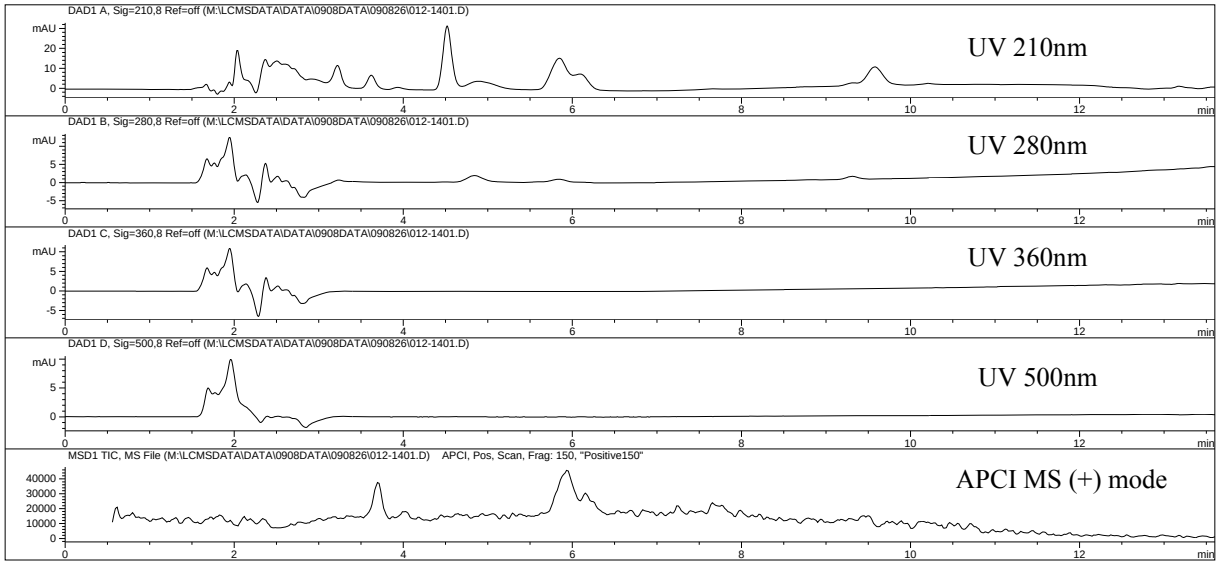


Figure 29. UV spectra at 210, 280, 360 and 500nm wavelength and LCMS profile of P4B-K and P4B-M fractions.

4.3. Pharmacology of the P4B subfractions

4.3.1. ORAC

The results of the ORAC assay on the P4B fractions are summarized in Table 24. Fraction D had the highest antioxidant activity with ORAC value of 2368 μmol TE/g, which is much higher than any activity found in the crude oil. The reason could be due to the presence of carvacrol. Fraction E showed also a high activity which could be explained by the high amount of carvacrol and thymol, that were already reported to have high antioxidant activity [23].

Table 24. ORAC results of the P4B fractions.

Sample	ORAC value (µmol TE/g)	RSD (%)
P4B-B	72 ± 25	34.80
P4B-C	989 ± 91	9.19
P4B-D	2368 ± 100	8.41
P4B-E	1600.65 ± 181	11.31
P4B-F	642 ± 80	12.42
P4B-G	562 ± 69	12.37
P4B-H	546 ± 130	23.84
P4B-I	593 ± 191	32.25
P4B-K	335 ± 107	31.91
P4B-M	101 ± 20	19.49

4.3.2. Cytotoxicity assay

The results of the cytotoxic assay are shown in Figure 30. Fractions E, F and G showed the highest activity with IC_{50} 36, 29 and 38 µmol/mL, respectively. Carvacrol and thymol, which were the major components in fraction E have been reported to have cytotoxic properties [24]. Fractions F and G contained spathulenol and intermedeol as major components, and intermedeol is already reported as cytotoxic [25]. Cytotoxic effects of monoterpenes in general have also been reported and their use as possible future anti-cancer drugs [26]. Fractions B, J+K and L+M did not show any activity against P388 cell line. Detailed cytotoxic results are listed in Appendix 1.

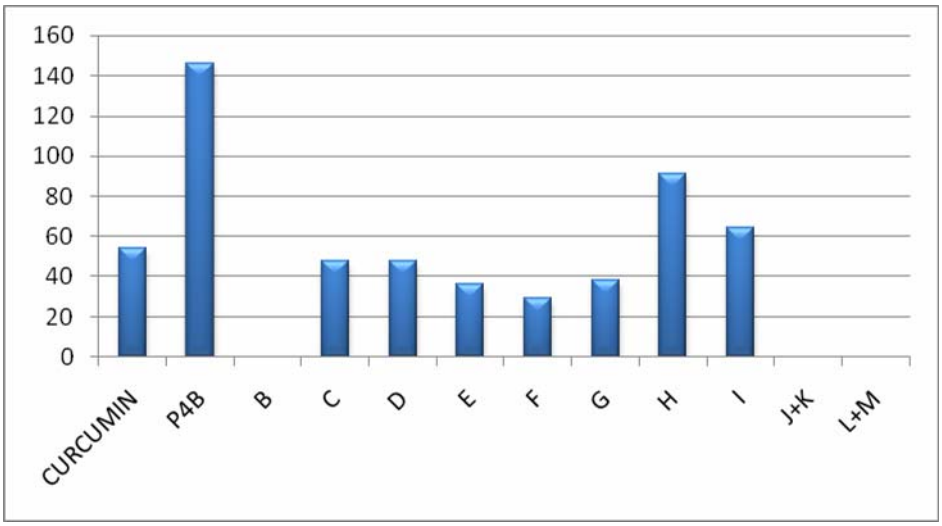


Figure 30. IC_{50} (µmol/mL) values of the P4B-D fractions.

4.4. Preparative HPLC

The fractions C, D, H and I were further fractionated by preparative HPLC. Fraction D showed highest antioxidant activity, whereas fraction C had interesting chemical profile containing 3-caren-2-one, the possible oxidation product of the major component from the crude oil. Fraction H was collected as a band from SPE column, and contained intermedeol and spathulenol, sesquiterpenes that are possibly responsible for cytotoxic activity of the fractions F, G and H. Fractions E and F showed high cytotoxic activity but were not fractionated further because of lack of material. Fractionations have been carried out by the preparative HPLC using the methods described in the Chapter 2.2.4.2.

4.4.1. Fraction P4B-C

The chromatogram of the P4B-C fraction, showing spectra of 210 and 280 nm and solvent gradient of the mobile phases, is shown in Figure 31 Figure 31. From the total of 48 fractions 17 were dried under nitrogen and extracted with chloroform. Altogether 300mg of P4B-C were fractionated and the yields of the obtained fractions are shown in Table 25. After extraction a GC-MS analysis was done. Detailed data on composition of each fraction are shown in Appendix 2. Pure fractions were subjected to NMR analysis.

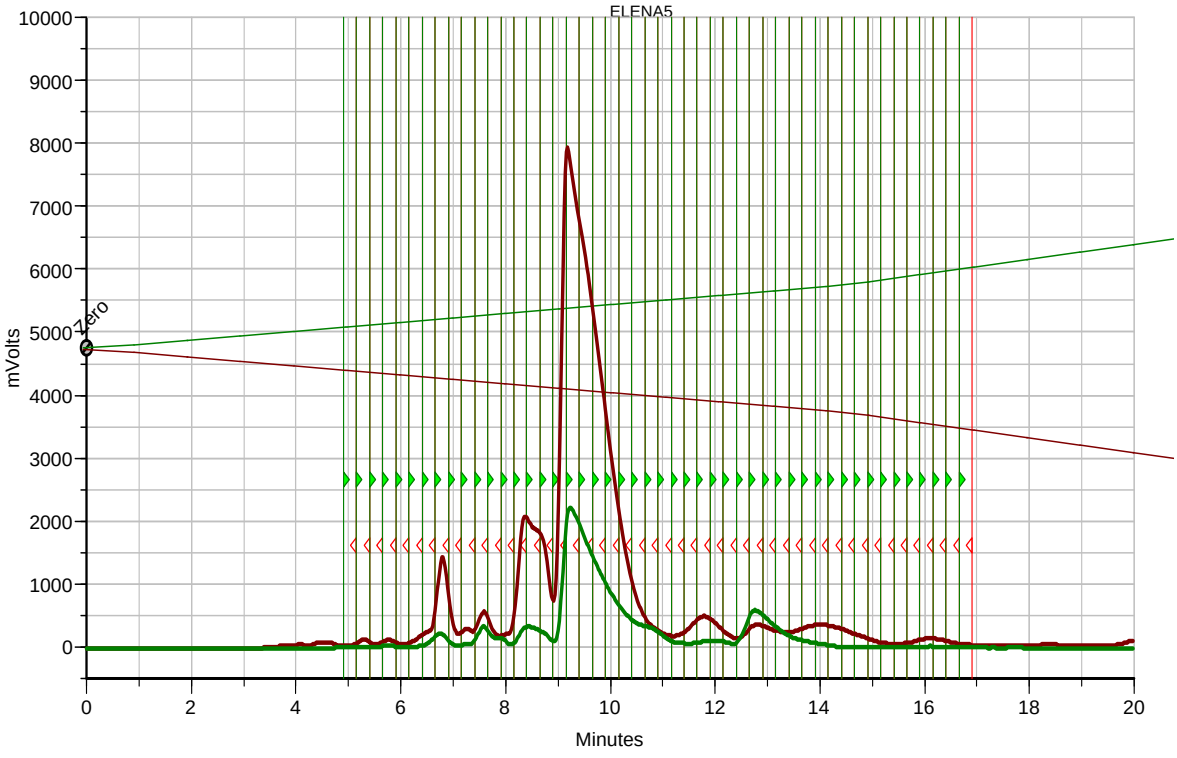


Figure 31. Prep. HPLC Chromatogram of the P4B-C fraction (Method: ELENA5).

Table 25. Yields of the P4B-C fractions.

Fraction	Weight (g)	Yield (%)
FR.7	0.0027	0.90
FR.8	0.0035	1.17
FR.11	0.0023	0.77
FR.14	0.0089	2.97
FR.15	0.0050	1.67
FR.16	0.0046	1.53
FR.18	0.0270	9.00
FR.19	0.0260	8.67
FR.20	0.0100	3.33
FR.21	0.0090	3.00
FR.22	0.0032	1.07
FR.23	0.0032	1.07
FR.24	0.0015	0.50
FR.27	0.0026	0.87
FR.28	0.0026	0.87
FR.32	0.0049	1.63
FR.37	0.0140	4.67

4.4.1.1. Identification of P4B-C-14 as 3-caren-2-one

The ^{1}H and ^{13}C NMR data of the fraction P4B-C-14 are shown in Table 26 and the compound was elucidated as 3-caren-2-one with molecular formula $C_{10}H_{14}O$ and molecular weight of 150.22 g/mol, which agrees with *m/z* of 150 g/mol found in GC-MS (Figure 33). RT was 20.02 min. The structure is shown in Figure 32. This compound was not found in the crude oil and it is possibly the oxidation product of the δ-3-carene, the major component from the oil, since δ-3-carene can be easily oxidized [27].

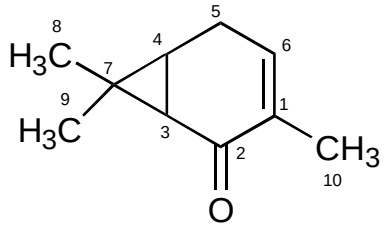


Figure 32. Structure of P4B-C-14 (3-caren-2-one, CAS Registry Number: 53585-45-8).

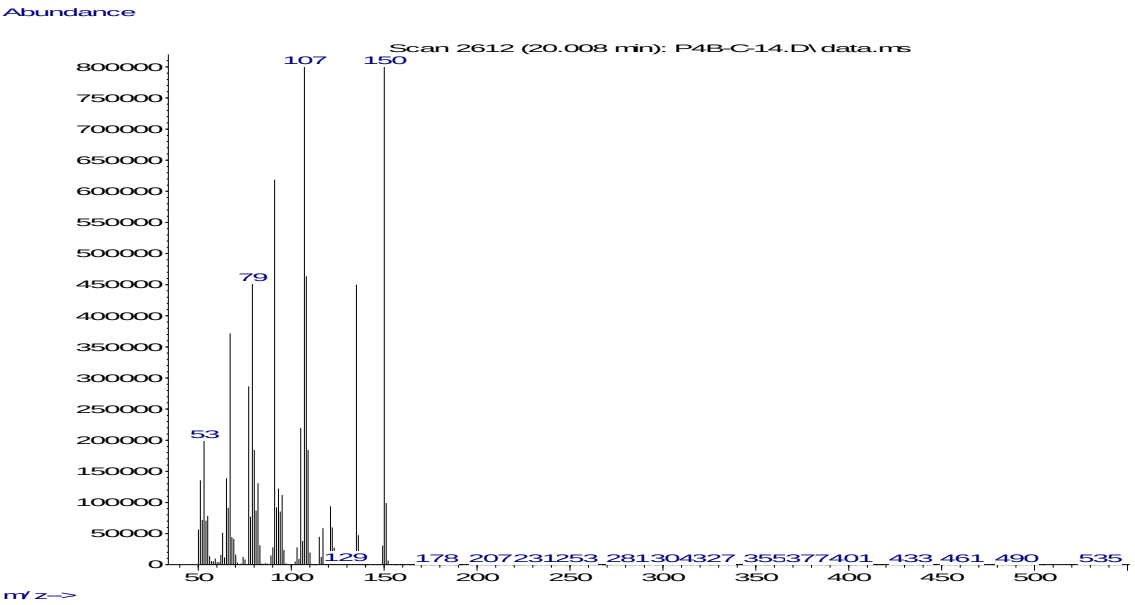


Figure 33. GC-MS profile of the fraction P4B-C-14.

Table 26. The ^{1}H and ^{13}C NMR data for P4B-C-14 (3-caren-2-one).

Position	**Chemical Shift, ppm**	
	^{1}H	**^{13}C**
1	-	135.3
2	-	196.8
3	1.64 *dd* (1.6, 7.85)	34.6
4	1.43 *t* (8.1/8.0)	26.6
5	2.70 2.45 *dt* (1.75)	23.3
6	6.40 *m*	142.7
7	-	22.1
8	1.08 *s*	14.5
9	1.19 *s*	28.7
10	1.76	16.3

4.4.1.2. Identification of P4B-C-18 as *m*-cymen-8-ol

The ^{1}H and ^{13}C NMR data of the fraction P4B-C-18 are shown in Table 27. The compound was identified as *m*-cymen-8-ol with molecular formula $C_{10}H_{14}O$ and molecular weight of 150.22 g/mol which corresponds with *m/z* of 150 from GC-MS profile (Figure 35). The structure is shown in Figure 34. This compound has been already found in *Artemisia pontica* [28] but has not been reported in *Plectranthus* genus before.

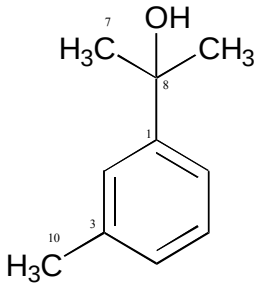


Figure 34. Structure of P4B-C-18 (m-cymen-8-ol, CAS registry number: 5208-37-7).

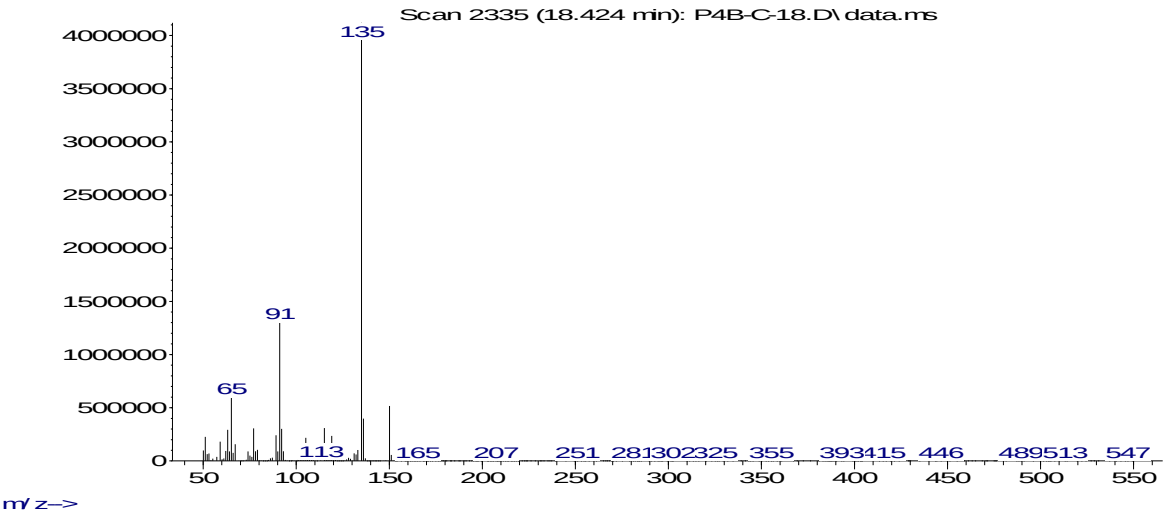


Figure 35. GC-MS profile of the P4B-C-18 fraction.

Table 27. ^{1}H and ^{13}C NMR data for P4B-C-18 (m-cymen-8-ol)

Position	Chemical Shift, ppm	
	^{1}H	^{13}C
1	-	149.3
2	7.32 br *s*	125.3
3	-	138.0
4	7.06 br *d* (7.25)	127.6
5	7.23 *t* (7.5, 7.65)	128.3
6	7.28 br *d* (8.0, 9.4)	121.6
1`	1.58 *s*	32.0
2`	-	72.7
3-CH_3	2.37 *s*	21.8

4.4.2. Fraction P4B-D

The chromatogram of the P4B-D fraction is shown in Figure 36. From the total of 58 fractions collected, 14 were dried under nitrogen, extracted with chloroform and analysed in GC-MS. Yields of the fractions are shown in Table 28. Detailed data on composition of each fraction are shown in Appendix 2. Pure fractions were subjected to NMR analysis.

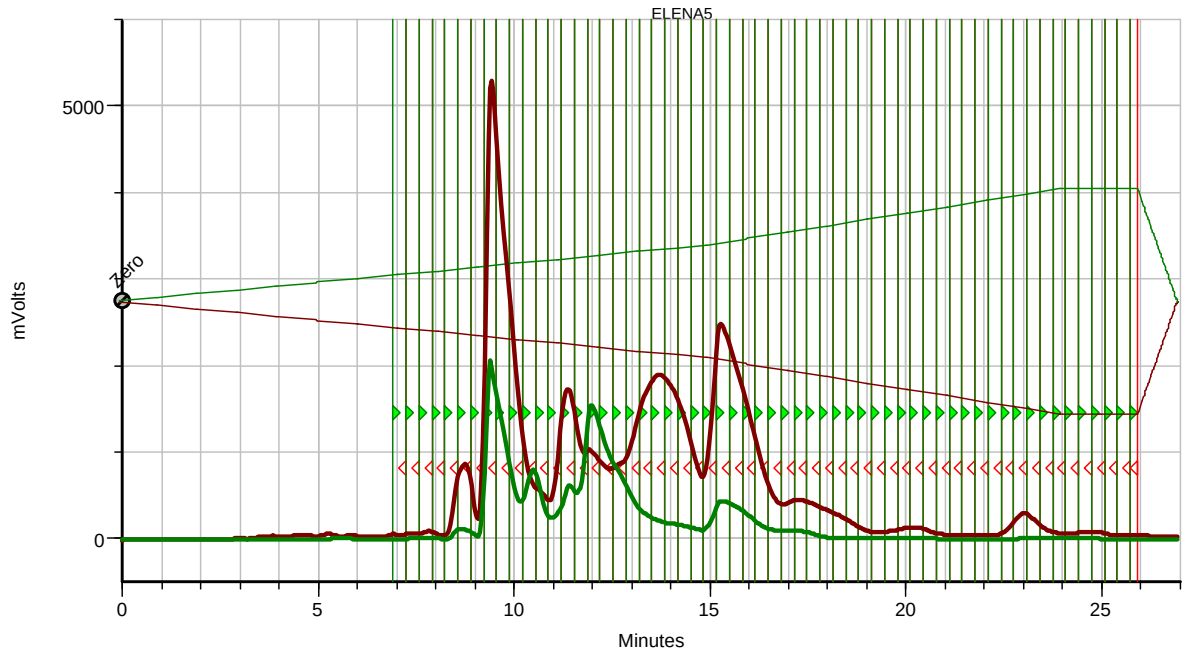


Figure 36. HPLC Chromatogram of the P4B-D fraction (Method: ELENA5).

Table 28. Yields of P4B-D subfractions.

Fraction	Weight (g)	Yield (%)
FR.8	0.0151	4.08
FR.9	0.0088	2.38
FR.13	0.016	4.32
FR.14	0.0162	4.38
FR.15	0.015	4.05
FR.16	0.0192	5.19
FR.19	0.0124	3.35
FR.20	0.0137	3.70
FR.21	0.0116	3.14
FR.22	0.0068	1.84
FR.25	0.0113	3.05
FR.26	0.009	2.43
FR.27	0.0083	2.24
FR.28	0.0087	2.35

4.4.2.1. Identification of P4B-D-8 as 3,6,6-trimethyl-2,4-cycloheptadien-1-one

The compound was isolated from the fractions P4B-D-8 and P4B-C-18 and elucidated as 3,6,6-trimethyl-2,4-cycloheptadien-1-one with molecular formula $C_{10}H_{14}O$ and molecular weight of 150.22g/mol. The structure is shown in Figure 37 and the ^{1}H and ^{13}C NMR data in

Table 29. The compound has not been reported in this genus before but it was already isolated from the oxidised oil of *Piper nigrum*.

Since the fractions P4B-D-8 and P4B-C-18 were not pure and there was no component with the same RT in either of them, it was not possible to define the RT time of this component or to explain this finding.

To the knowledge of the author of this thesis no activity was reported for this compound. Thus, eucarvone (2,6,6-trimethyl-2,4-cycloheptadien-1-one), a compound with very similar structure, has been reported to have very high insecticidal activity which could also be a property of 3,6,6-trimethyl-2,4-cycloheptadien-1-one [29].

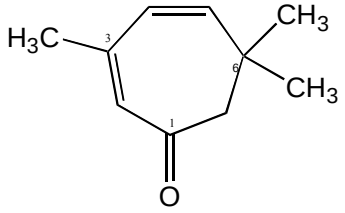


Figure 37. Structure of P4B-D-8 (3,6,6-Trimethyl-2,4-cycloheptadien-1-one, CAS Registry Number: 2767-18-2).

Table 29. ^{1}H and ^{13}C NMR data for P4B-D-8 (3,6,6-trimethyl-2,4-cycloheptadien-1-one).

Position	**Chemical Shift, ppm**	
	^{1}H	**^{13}C**
1	-	199.7
2	6.04 *d* (1.3)	129.8
3	-	147.8
4	5.75 *dd* (1.4, 11.85)	126.7
5	6.07 *d* (1.85)	150.1
6	-	32.9
7	2.60 s	54.1
3-CH_3	2.01 *d* (1.3)	27.4
6- CH_3	1.11 s	27.1

4.4.2.2. Identification of P4B-D-14 as 5-isopropenyl-2-methyl-2-cyclohexen-1-one (Carvone)

The ^{1}H and ^{13}C NMR data of the fraction P4B-D-14 are shown in Table 31 and the structure in Figure 38. The compound was elucidated as 5-isopropenyl-2-methyl-2-cyclohexen-1-one with molecular formula $C_{10}H_{14}O$ and molecular weight of 150.22 g/mol which corresponds to *m/z* of 150 from GC-MS profile (Figure 39).

Carvone has been reported as carminative agent [30], and also as antibacterial and antifungal [31].

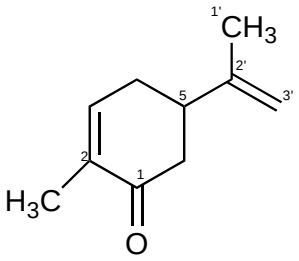


Figure 38. Structure of the P4B-D-14 (5-isopropenyl-2-methyl-2-cyclohexen-1-one, CAS Registry Number: 99-49-0).

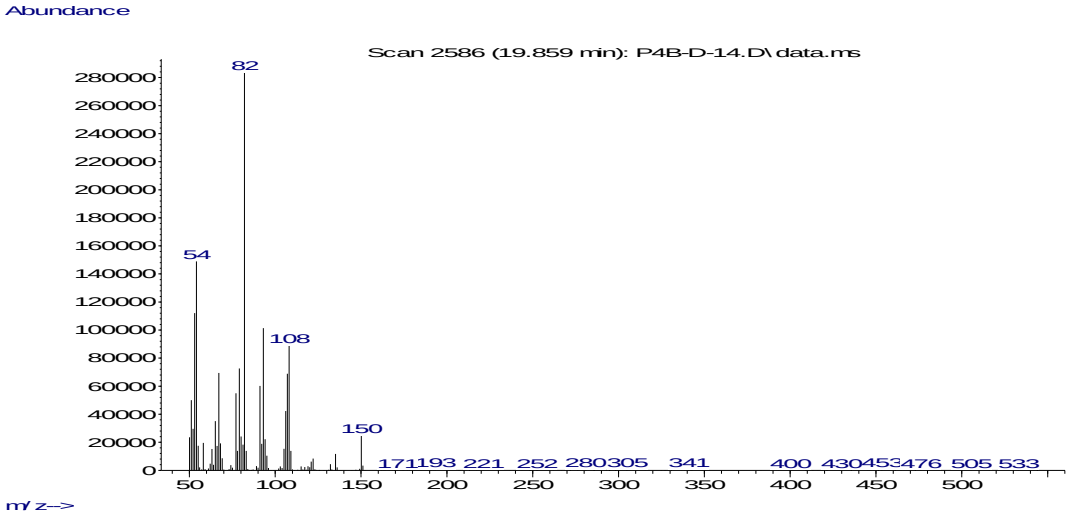


Figure 39. GC-MS profile of the fraction P4B-D-14.

Table 30. ^{1}H and ^{13}C NMR data for P4B-D-14 (5-isopropenyl-2-methyl-2-cyclohexen-1-one).

Position	Chemical Shift, ppm		Literature value [32]
	^{1}H	^{13}C	^{13}C
1	-	200.0	198.4 s (0.608)
2	-	135.7	135.4 s (0.625)
3	6.75	144.8	144.0 d (0.828)
4	2.45 *m* 2.335 *dd* (13.3, 16)	31.5	31.4 t (1.0)
5	2.69 *m*	42.7	42.7 d (0.739)
6	2.59 *ddd* (1.55, 3.75, 16.0) 2.35 *dd* (13.3, 16.0)	43.4	43.2 t (0.769)
1’	1.76 br *s*	20.7	20.5 q (0.98)
2’	-	146.9	146.8 s (0.576)
3’	4.81 *t* (1.15, 1.1) 4.76 br *s*	110.7	110.5 t (0.874)

4.4.2.3. Identification of P4B-D-25 and P4B-D-27 as 5-isopropyl-2-methylphenol (Carvacrol)

The ^{1}H and ^{13}C NMR data are shown in Table 32 and the compound was elucidated as 5-isopropyl-2-methylphenol. Structure is shown in Figure 40 and GC-MS profile in Figure 41. The compound had RT 20.64 min, a molecular formula $C_{10}H_{14}O$ and molecular weight 150.22 g/mol, which corresponds to *m/z* from GC-MS profile. Both P4B-D-25 and P4B-D-27 fractions had relative high ORAC values. The fraction P4B-D-25 had a value of 4480 µmol TE/g and P4B-D-27 value of 6959 µmol TE/g. The two fractions were only relatively pure which explains different antioxidant activity. Carvacrol has already been reported to have a high antioxidant activity, and this is probably due to its phenolic structure [21].

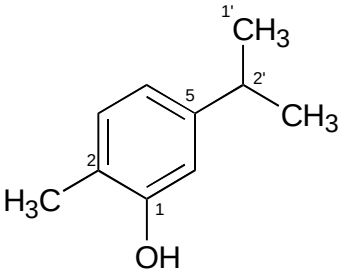


Figure 40. Structure of the P4B-D-25 (5-isopropyl-2-methylphenol,CAS Registry Number: 499-75-2).

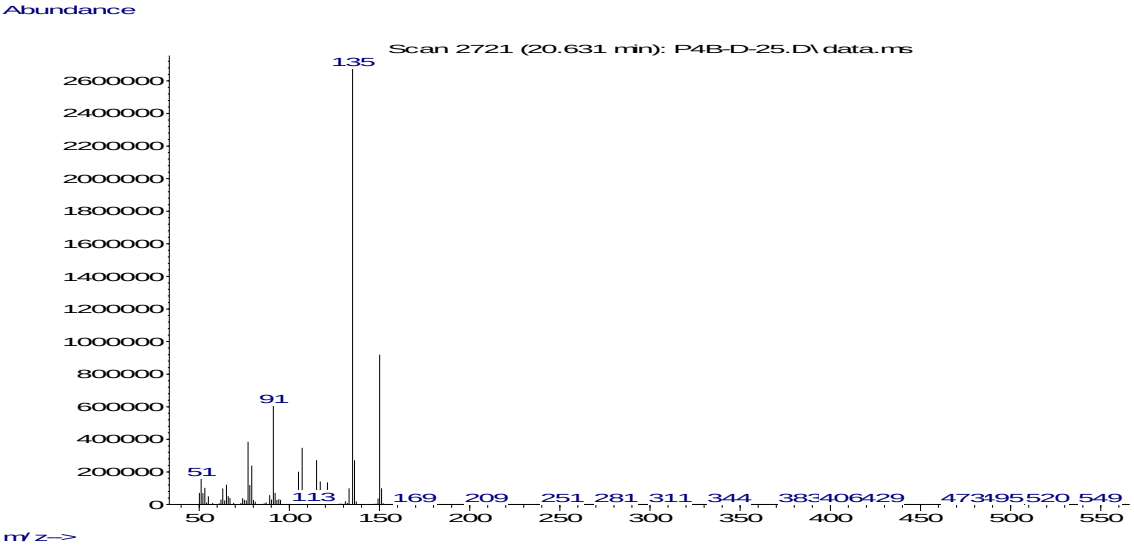


Figure 41. GC-MS profile of the fraction P4B-D-25.

Table 31. ^{1}H and ^{13}C NMR data for P4B-D-25 (5-isopropyl-2-methylphenol).

Position	**Chemical Shift, ppm**		**Literature value [32]**
	^{1}H	**^{13}C**	**^{13}C**
1	-	153.9	153.5 *s* (0.13)
2	-	121.1	121.5 *s* (0.08)
3	7.04 *d* (7.65)	131.0	131.0 *d* (0.54)
4	6.73 *dd* (1.5, 7.65)	118.9	119.1 *d* (0.49)
5	-	148.6	148.4 *s* (0.08)
6	6.67 *d* (1.4)	113.2	113.5 *d* (0.33)
2-CH_3	2.23 *s*	15.5	15.4 *q* (0.27)
2`	2.84 (6.9)	33.9	33.7 *d* (0.36)
1`		24.2	23.9 *q* (1.0)

4.4.2.4. Identification of P4B-D-16 as *m*-mentha-4,6-dien-8-ol

Compound isolated from the fraction P4B-D-16 was elucidated as *m*-mentha-4,6-dien-8-ol with RT of 18.15 min, a molecular formula $C_{10}H_{16}O$ and molecular weight of 152.24 g/mol which corresponds to *m/z* from the GC-MS profile (Figure 43). The structure is shown in Figure 42 and the ^{1}H and ^{13}C NMR data in Table 33. The compound was found in the crude oil in the amount of 3.74%. It was not found in the Dictionary of Natural Products [33] and to the knowledge of the author of this thesis, no activity of this compound was reported.

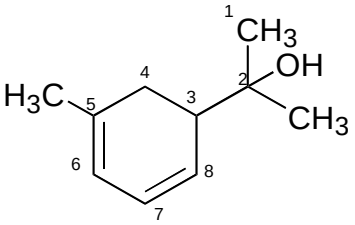


Figure 42. Structure of the m-mentha-4,6-dien-8-ol.

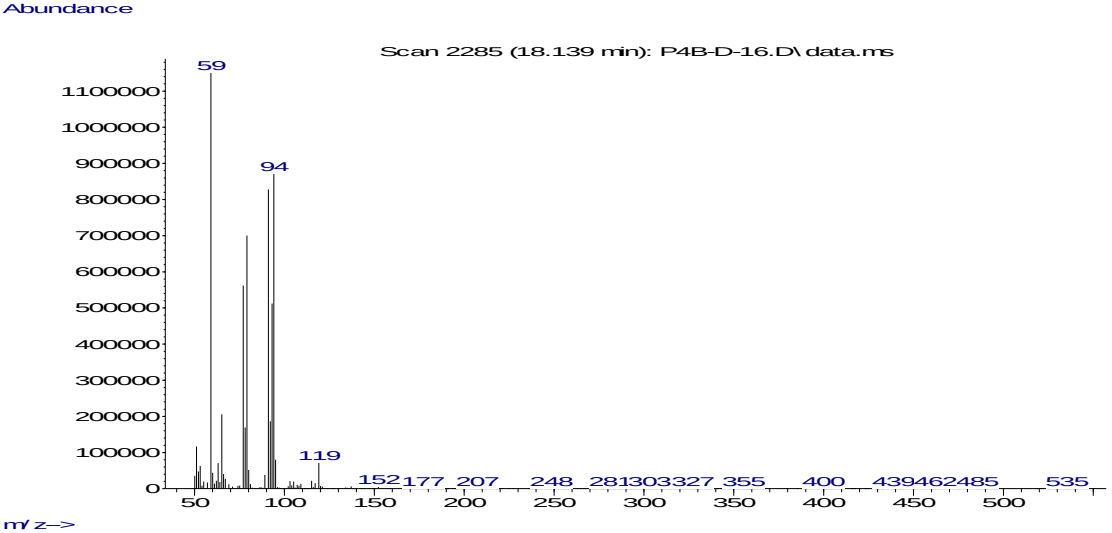


Figure 43. GC-MS profile of the fraction P4B-D-16.

Table 32. ^{1}H and ^{13}C NMR data for P4B-D-16 (m-mentha-4,6-dien-8-ol).

Position	Chemical Shift, ppm	
	^{1}H	^{13}C
1	1.21	27.3
2	-	73.5
3	2.37	45.4
4	2.17, 1.25	30.1
5	-	136.0
6	5.63	118.7
7	5.94	126.1
8	5.63	123.5
5-CH_3	23.5	23.5

4.4.2.5. Pharmacology of the P4B-D fractions

ORAC assay was done for all extracted P4B-D fractions. Results are shown in Table 29. The highest antioxidant activity had fractions 14, 26 and 27. Fractions 26 and 27 contained carvacrol as a major compound, but also a high percentage (>20%) of another compound that could not be identified. Carvacrol was also the major component in fraction 25, but its ORAC value was not as high. This could suggest that the unidentified component in 26 and 27 contributed a lot to their high activity. However, because of the lack of material no further fractionation has been carried out.

High ORAC value of the fraction 14 could not be explained only from its GC-MS profile, but UV profiles (Figure 44) suggest the presence of an aromatic compound, which could be responsible for the antioxidant activity. The compound was found in the region of monoterpenes and it had $[M+1]^{+}$ of 151.2. Cytotoxic activity was not observed in the P4B-D fractions.

Table 33. ORAC results of P4B-D fractions.

Sample	ORAC value µmol TE/g	RSD (%)
P4B-D	2535 ± 410	16.17
P4B-D-8	139 ± 43	31.16
P4B-D-9	474 ± 76	16.01
P4B-D-11	2493 ± 207	8.31
P4B-D-13	735 ± 137	18.64
P4B-D-14	8008 ± 1139	14.22
P4B-D-15	2667 ± 2150	8.06
P4B-D-16	874 ± 179	20.43
P4B-D-19	1221 ± 186	15.26
P4B-D-20	2065 ± 168	8.12
P4B-D-21	995 ± 192	19.27
P4B-D-25	4480 ± 764	17.04
P4B-D-26	7203 ± 2061	28.62
P4B-D-27	6959 ± 1322	19.00
P4B-D-28	2834 ± 820	28.95

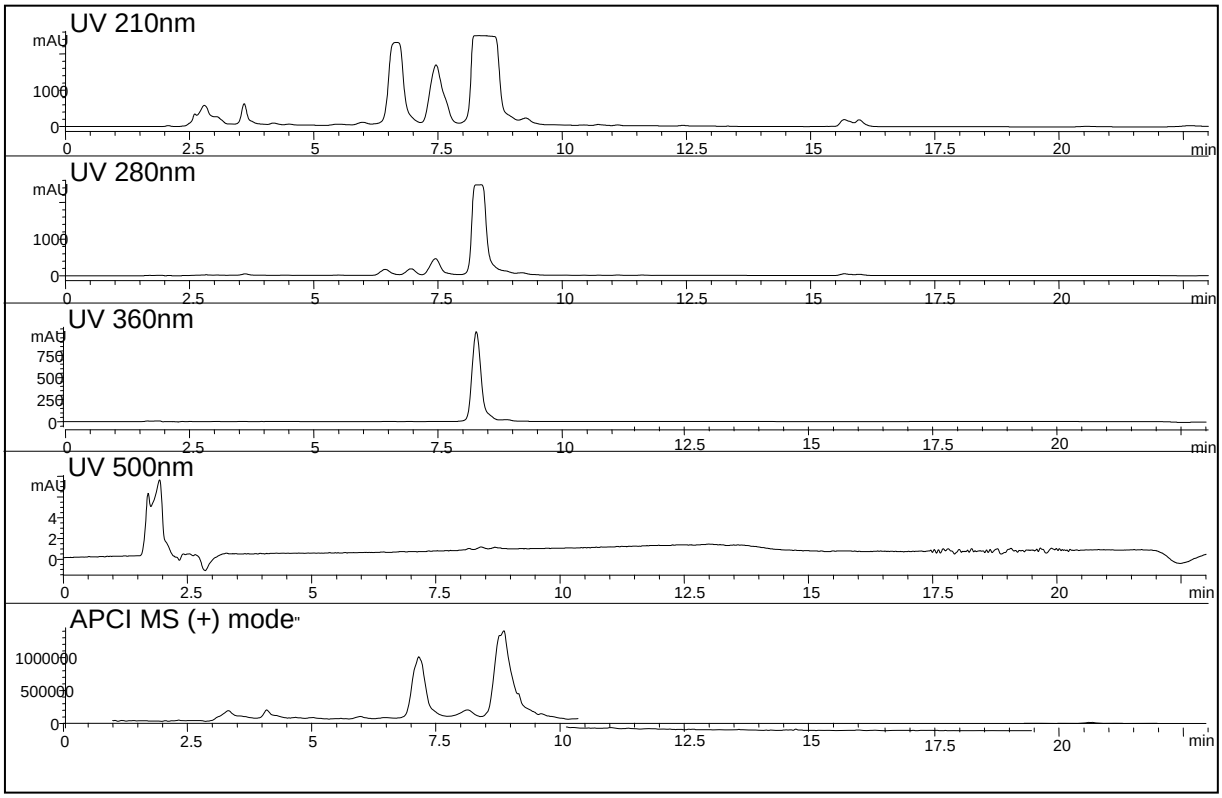


Figure 44. UV spectra at 210, 280, 360 and 500nm and LCMS profile of the P4B-D-14.

4.4.3. Fraction P4B-H

The UV spectra of the P4B-H fraction at 210 and 280nm and solvent gradient of the mobile phases are shown in the chromatogram (Figure 45). Altogether 48 fractions were collected, dried under nitrogen, extracted with hexane and analysed in LCMS. The LCMS analysis of the fraction P4B-H-16 (which corresponds to the first large peak in the chromatogram) showed a component with a molecular peak 223.2 which correspond to the $[M+1]^+$ of intermedeol. Unfortunately, isolation of this component was not successful due to the small amount of P4B-H fraction.

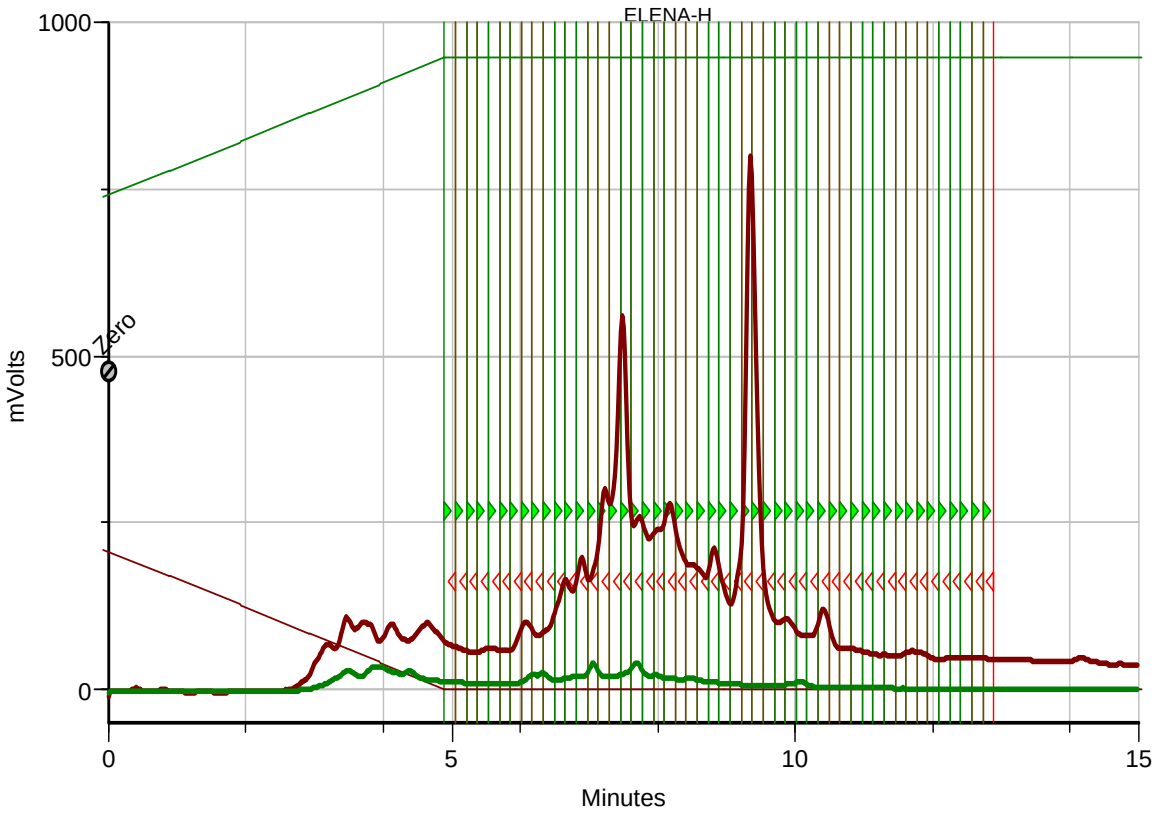


Figure 45. Prep. HPLC Chromatogram of the P4B-H fraction (Method :ELENA-H).

4.4.4. Fraction P4B-I

The chromatogram of the P4B-I fraction showed UV spectra at 210 and 280nm and solvent gradient of the mobile phases (Figure 46). GC-MS analysis of the fractions did not show any volatile compounds but the LCMS profiles of fractions P4B-I-13 and P4B-I-26 were unexpected. The UV at 210nm and TIC of P4B-I-13 is shown in Figure 47. UV at 210nm suggests absence of chromophores and the TIC gave $[M+1]^+$ of 279.2 which correspond to a fatty acid with molecular formula of $C_{18}H_{20}O_2$. The chromatographic profile of P4B-I-28 (Figure 48) showed component with $[M+1]^+$ of 392.3, which is characteristic for a plasticizer. These findings could suggest a contamination of the sample.

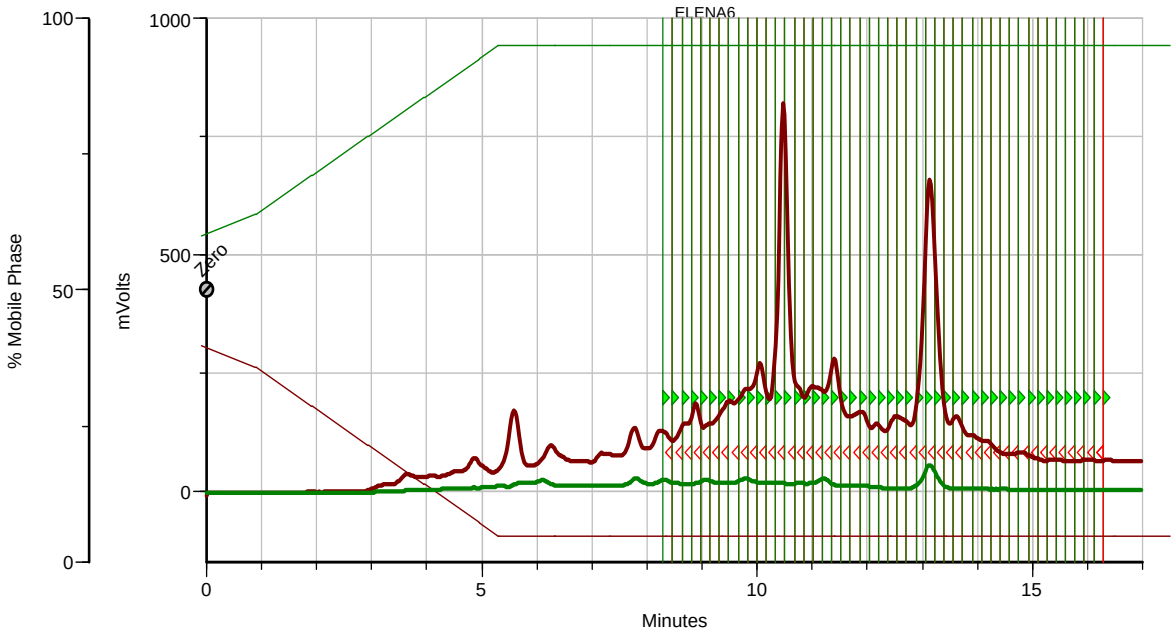


Figure 46. Prep. HPLC of the P4B-I fraction (Method: ELENA6).

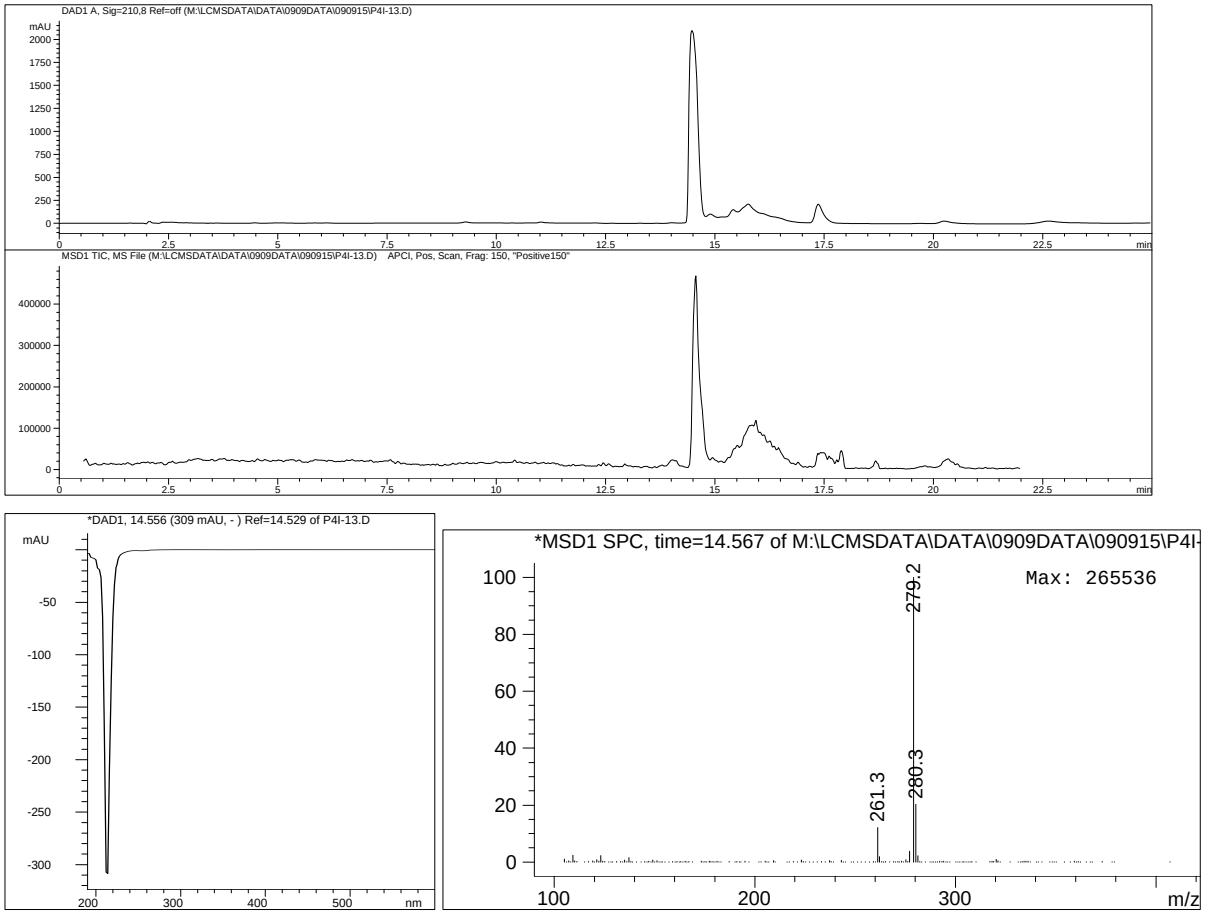


Figure 47. UV spectrum at 210nm and LCMS profile of P4B-I-13.

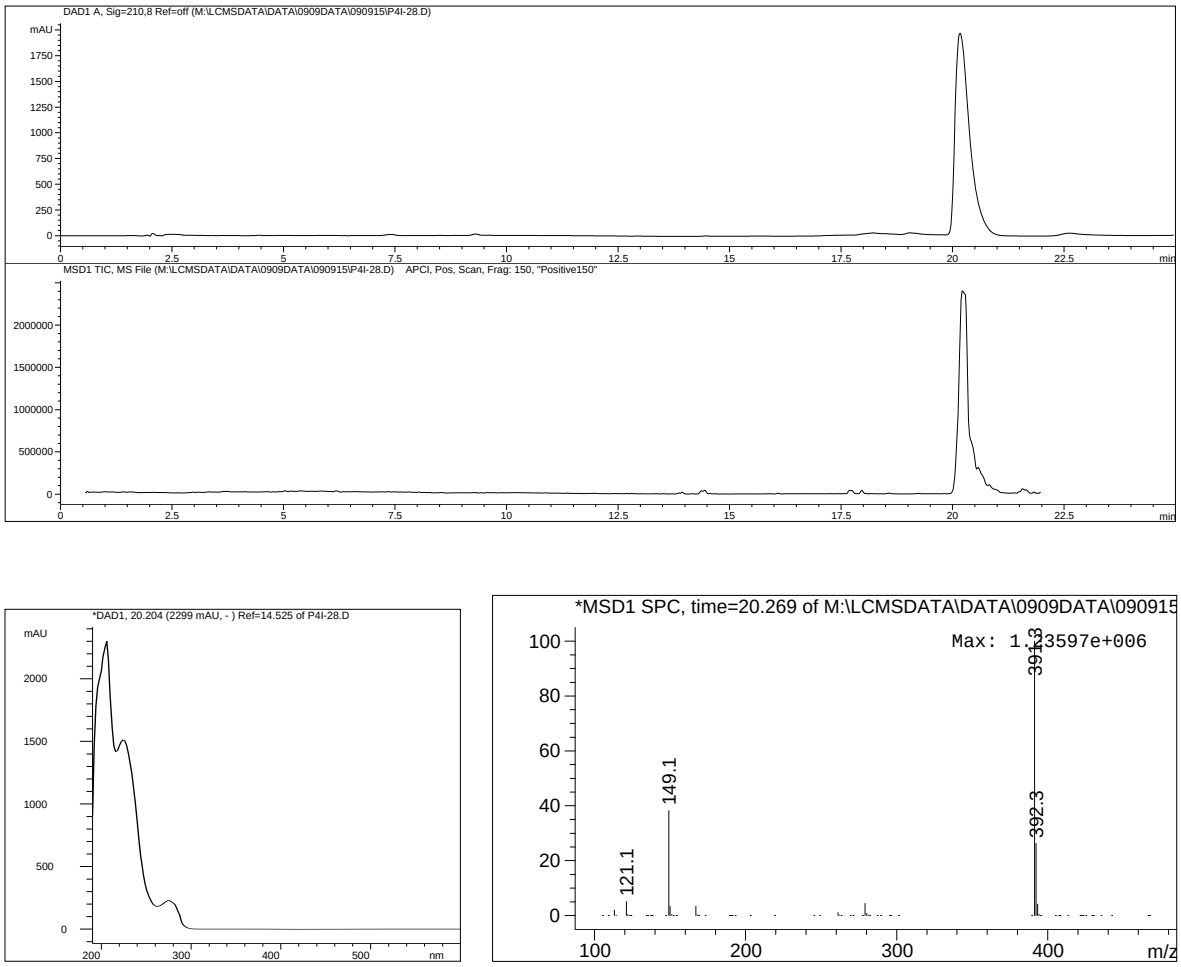


Figure 48. UV spectrum at 210nm and LCMS profile of P4B-I-28.

5. *Plectranthus nitidus* (P3)

Plectranthus nitidus oil was intensely red in colour, more viscous than the rest of the oils and growth of red crystals was observed in the vial. The crystals were separated from the rest of the oil and washed with cold methanol. Methanol was evaporated under nitrogen and the crystals were dried in the freeze drier over night. The rest of the oil was diluted in 100% methanol and fractionated by preparative HPLC.

5.1. Preparative HPLC of *P. nitidus* oil (P3)

The HPLC chromatographic profile of *P. nitidus* oil (P3) is shown in Figure 49. A total of 67 fractions were collected. Fractions were mostly yellow coloured, except for two fractions that were intense red in colour (P3-31, P3-32) and another two that were reddish-orange (P3-26, P3-27). Preliminary LCMS analysis was carried out for all the fractions. Analysis showed that fractions P3-31 and P3-32 were the same as the red crystal that was separated at the beginning and that the other reddish compound from fractions P-26 and P-27 was different. Nine relatively pure fractions were selected, dried under nitrogen and extracted with chloroform. Yields are shown in Table 34. The fractions and the red crystal were analyzed by ^{1}H NMR spectroscopy. Analysis showed that the red crystals and fraction P3-36 were pure enough for further NMR analysis.

Overlaid UV chromatograms at 210nm wavelength of the crude oil, P3-31 (that was the same as the red crystal) and P3-36 are shown in Figure 50. The overlaid LCMS profiles are shown in Figure 51. (The single chromatograms of the nine extracted fractions and the crystal (P3-31) are shown in Appendix 3.) The UV profiles were characteristic of diterpenes, a fact which is in correlation with GC-MS finding. Molecular ions of the fractions showed *m/z* of 345.2 $[M+1]^+$ for fraction P3-20; 288.2 and 331.2 $[M+1]^+$ for fractions P3-26 and P3-27; 315.2 $[M+1]^+$ for fractions P3-31 and P3-33 and 272.3 $[M+1]^+$ for fraction P3-36.

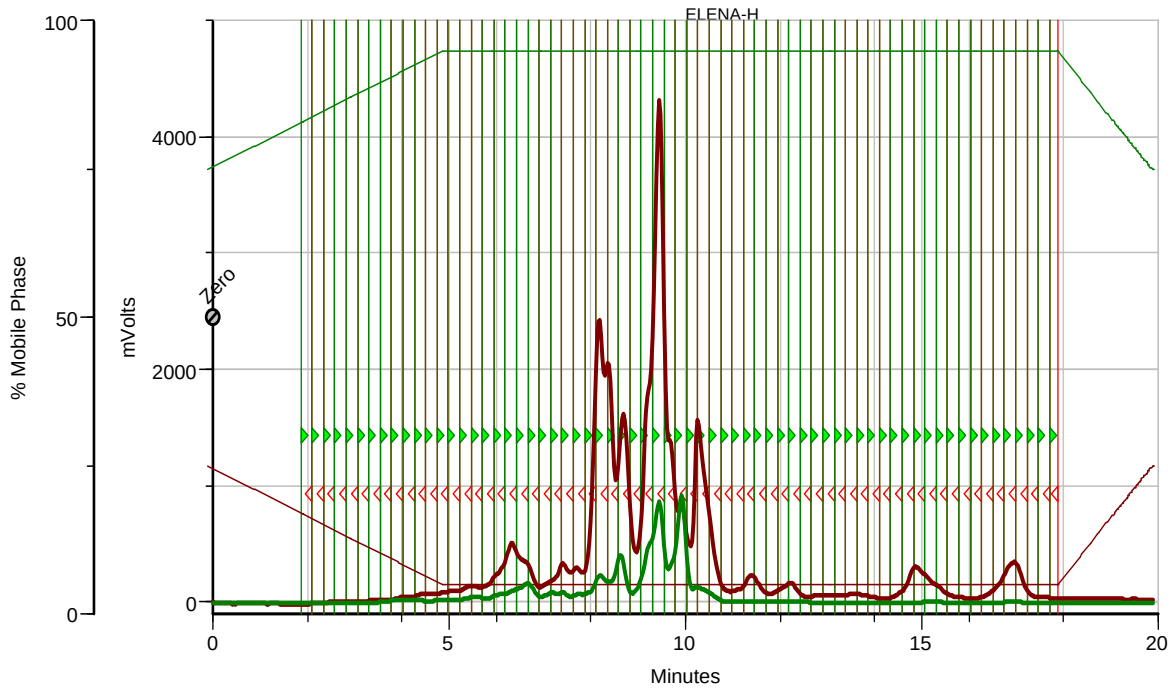


Figure 49. Prep. HPLC of the P3 oil (Method:ELENA-H).

Table 34. Yields of the P3-Fractions.

Fraction	**Yield (g)**
P3-20	0.0016
P3-25	0.0031
P3-26	0.0090
P3-27	0.0113
P3-29	0.0035
P3-35	0.0065
P3-36	0.0074
P3-37	0.0020
P3-40	0.0022

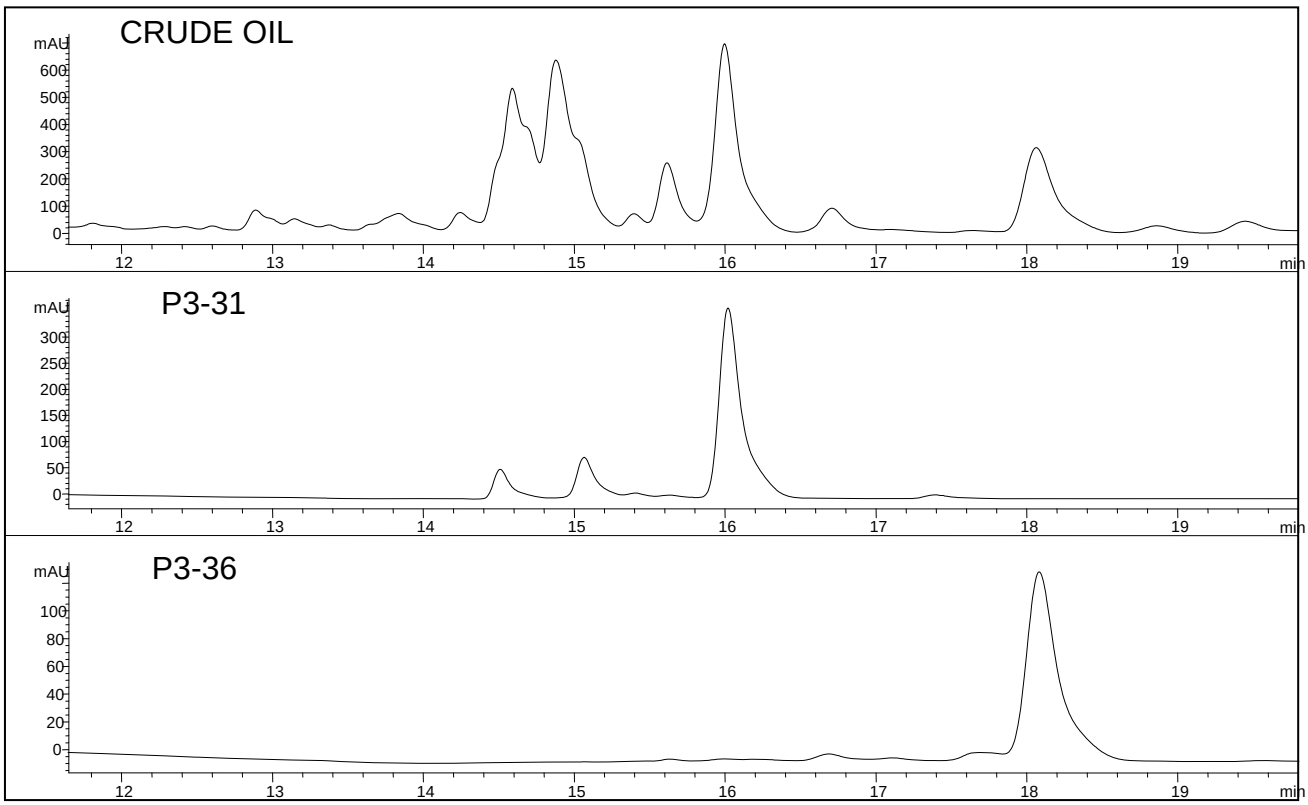


Figure 50. Overlaid UV spectra at 210nm wavelength of the crude oil, P3-31 and P3-36.

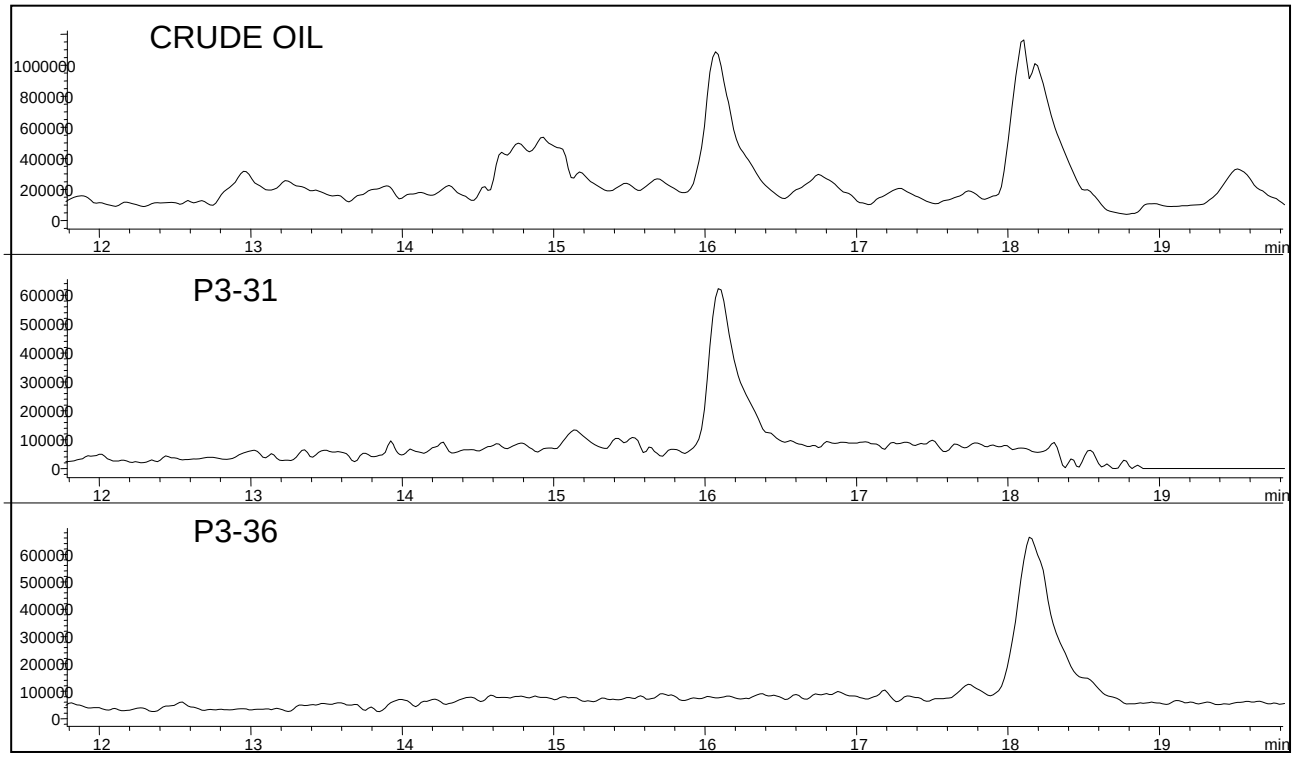


Figure 51. Overlaid LCMS spectra of the crude oil, P3-31 and P3-36.

5.1.1. Identification of P3 crystal as 12-hydroxy-6,8,12-abietatriene-11,14-dione

The red crystals of the *P.nitidus* oil were run on the NMR and the ^{1}H and ^{13}C NMR spectra were compared with the ones of the red crystals that were isolated from *P. graveolens* by Leema Marangattil. The comparison showed that the crystals were the same and the compound was identified as 12-hydroxy-6,8,12-abietatriene-11,14-dione (Figure 52). The molecular formula is $C_{20}H_{26}O_3$ and the MS data of $C_{20}H_{26}O_3$ [M+1]=315.2 agreed to this molecular formula (Figure 53).

Marangattil reported significant cytotoxic activity of this compound against P388 mammalian cell line (personal communication). This was the major compound and it is responsible for the red colour of the oil.

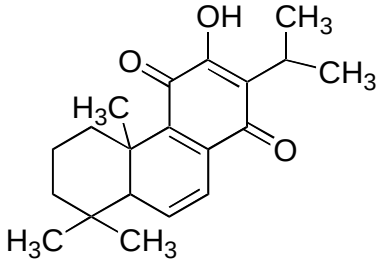


Figure 52. Structure of P3 crystal (12-hydroxy-6,8,12-abietatriene-11,14-dione).

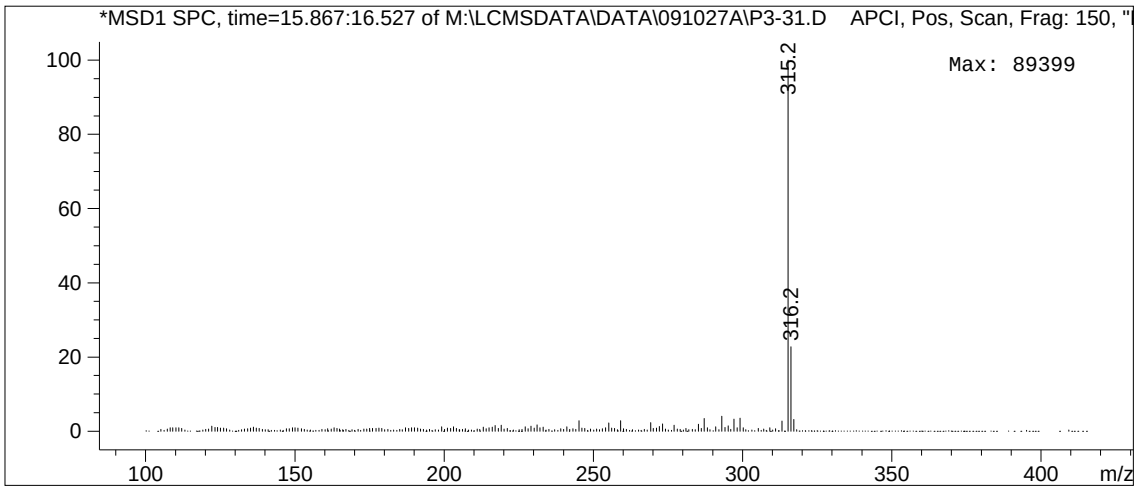


Figure 53. LCMS profile of the P3 crystal.

5.1.1.1. Identification of P3-36 as 7,9(11)-abietadiene-13-ol

The ^{1}H and ^{13}C NMR data of P3-36 are shown in Table 35. The compound was elucidated as 7,9(11)-abietadiene-13-ol, with molecular formula of $C_{20}H_{32}O$ and molecular weight of 288.47 g/mol. The structure is shown in Figure 54 and the LCMS profile in Figure 55. This compound was one of the major components of the oil. It was yellow coloured and had oily consistence. It was not found in the Dictionary of Natural Products [33] and could possibly be novel. However further NMR analysis is needed to confirm the structure and thus the identification was deemed as tentative.

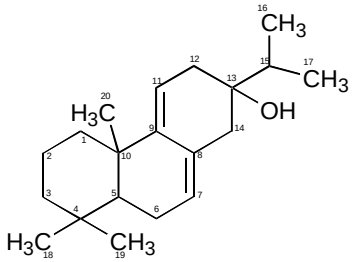


Figure 54. Structure of the P3-36 (7,9(11)-abietadiene-13-ol).

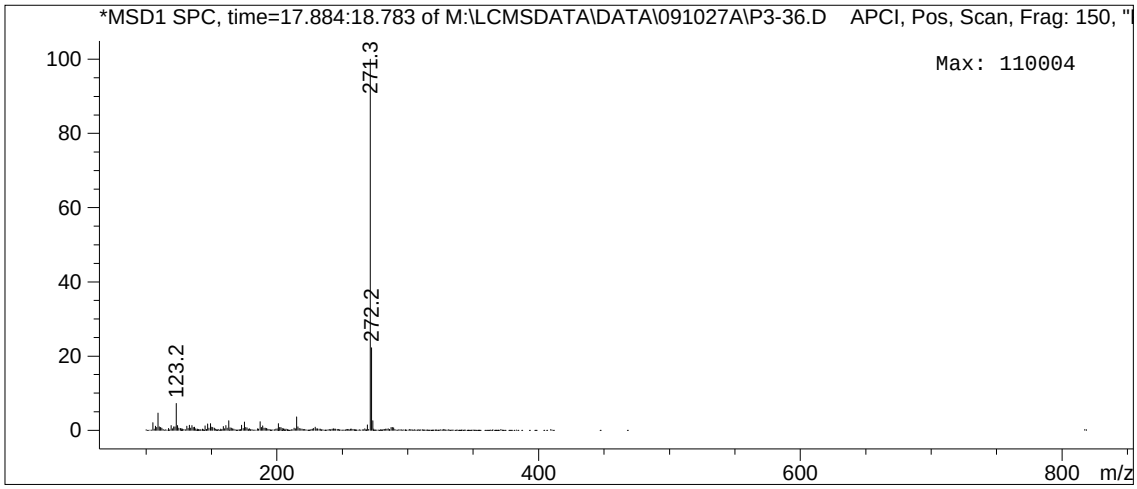


Figure 55. LCMS prfile of the fraction P3-36.

Table 35. The ^{1}H and ^{13}C NMR data for P3-36 (13-hydroxyl-7,9-abietadiene).

Position	Chemical Shift, ppm	
	^{1}H	**^{13}C**
1	1.91 br *d* 12.75 1.31 *t* 3.95, 13.0	37.5
2	1.64 1.54	19.2
3	1.43 br *dd* 1.65, 13.05 1.17 *td* 3.9/3.7, 13.55	42.4
4	-	33.42
5	1.22 *dd* 4.2, 11.8	49.3
6	2.01-2.17 *m*	24.2
7	5.59 br *d* 5.6	126.1
8	-	130.2
9	-	148.0
10	-	
11	5.43 br	115.3
12	2.28-2.39 *m* 2.01-2.17 *m*	36.3
13	-	72.5
14	2.28-2.39 *m*	41.2
15	1.74 heptet 6.9	35.2
16	0.96 *d* 3.5	17.2
17	0.94 *d* 3.5	17.0
18	0.93 *s*	22.4
19	0.89 *s*	33.39
20	1.0 *s*	22.3

6. Summary and recommendations

The chemical profiles of the *Plectranthus* oils are summarized in Table 36. Although all of the profiled species belong to the same genus and are common for the eastern Australian coast, there is no correlation in their chemical profiles. Pharmacological results (Figure 56) showed that ornamental *Plectranthus* had the highest antioxidant activity, probably due to its high carvacrol content and *Plectranthus nitidus* had the highest cytotoxic activity which could be attributed to diterpenes that were present in the oil.

Table 36. Summarized table of the *Plectranthus* species oil profiles

	P. graveolens	***P. suaveolens***	***P. nitidus***	***P.*sp. 'Hahn Tableland'**	**ornamental *Plectranthus***
Monoterpene	+	+		+	+
Sesquiterpene	+	+			+
Diterpene	+		+		

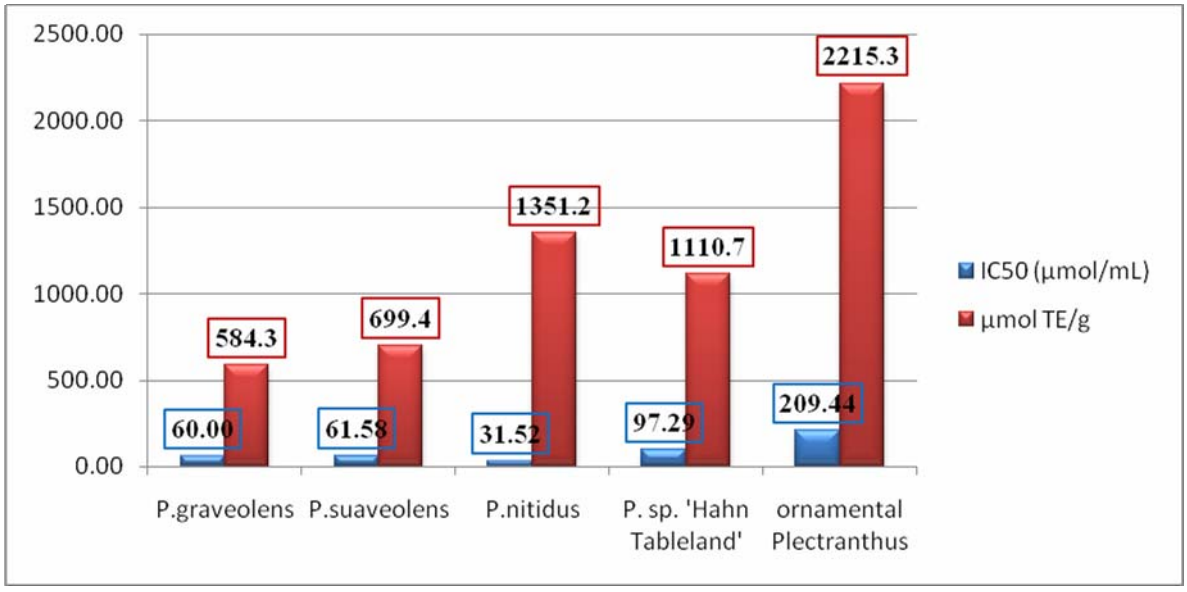


Figure 56. Summarized pharmacology results of the *Plectranthus* oils.

Plectranthus sp. 'Hahn Tableland' was fractionated and the fractions were screened for antioxidant and cytotox activity. Six compounds were isolated from the fractions P4B-D and P4B-C (Table 37). Out of 5 compounds that could not have been identified in the crude oil two were isolated and identified. They are m-mentha-4,6-dien-8-ol, with RT 18.15 min and

3.74% relative distribution in the crude oil and m-cymen-8-ol with RT 18.42 min and 3.67% relative distribution in the crude oil. Another isolated compound was 3-caren-2-one that was not present in the crude oil and is possibly oxidation product of the major component from the oil, δ-3-carene. Carvacrol was isolated and found to be responsible for the highest antioxidant activity of the P4B-D fraction. Compounds m-cymen-8-ol, *m*-mentha-4,6-dien-8-ol, 3,6,6-trimethyl-2,4-cycloheptadien-1-one have not been reported in this genus before.

Table 37. Isolated compounds from the P4B oil.

Fraction	**Compound**	**Molecular formula**	**MW (g/mol)**
P4B-C-14	3-Caren-2-one	$C_{10}H_{14}O$	150.22
P4B-C-18	m-Cymen-8-ol	$C_{10}H_{14}O$	150.22
P4B-C-18, P4B-D-8	3,6,6-Trimethyl-2,4-cycloheptadien-1-one	$C_{10}H_{14}O$	150.22
P4B-D-14	Carvone	$C_{10}H_{14}O$	150.22
P4B-D-16	m-Mentha-4,6-dien-8-ol	$C_{10}H_{16}O$	152.24
P4B-D-25, P4B-D-27	Carvacrol	$C_{10}H_{14}O$	150.22

The volatile oil of the *P*. sp. 'Hahn Tableland' has shown moderate antioxidant and cytotoxic activity, but further analysis on its antimicrobial properties would have been relevant, since its components (limonene, carvone,, carvacrol**)** were already reported as antimicrobial [34], [35]. Also α-pinene and δ-3-carene were reported to have antifungal properties [36].

Plectranthus nitidus oil was fractionated by preparative HPLC and at the time of writing two compounds were identified by NMR. The red crystals were identified as 12-hydroxy-6,8,12-abietatriene-11,14-dione and that was also the major compound of the oil. Another compound was 13-hydroxyl-7,9-abietadiene, but its structure has to be reconfirmed. It has not been reported in Dictionary of Natural Products and could possibly be novel. Crude oil showed magnificent cytotoxic activity and further analysis on the pure compounds is planned.

This was the first study on chemistry and pharmacological activity of this species. In future work the method of separation should be improved in order to get more pure compounds. Also investigation of cytotoxic activity on different cell lines could give more information about cytotoxic activity of this plant. Anti-insecticidal activity was reported in *P.graveolens* [4], and it was attributed to its diterpenes content. Since *P. nitidus* has very similar diterpenes profile to *P. graveolens* it could also have similar anti-insecticidal properties and is worth investigating.

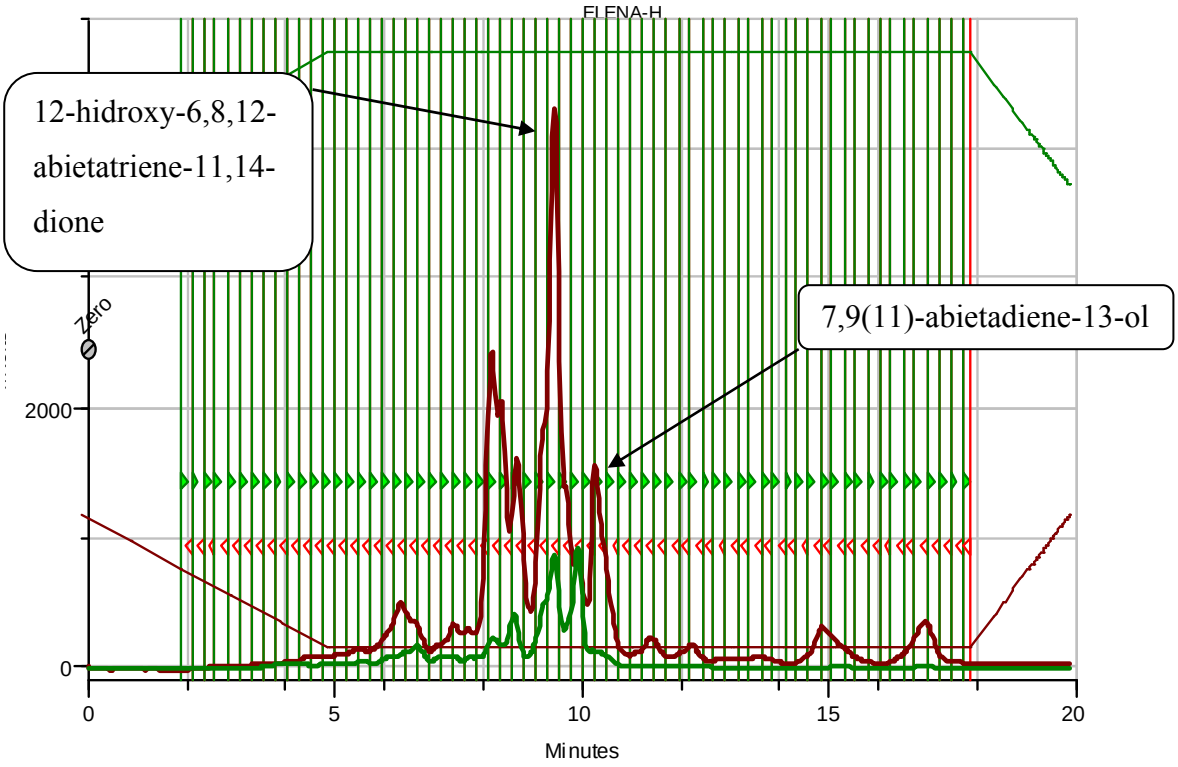


Figure 57. Identified compounds from *P.nitidus* oil shown on the UV chromatogram at 210nm wavelength.

This study was based upon analysis of the volatile oils of the *Plectranthus* species. The examination of the leaf extracts with different solvents would have been relevant and could possibly reveal new, more polar components that were not present in the volatile oil.

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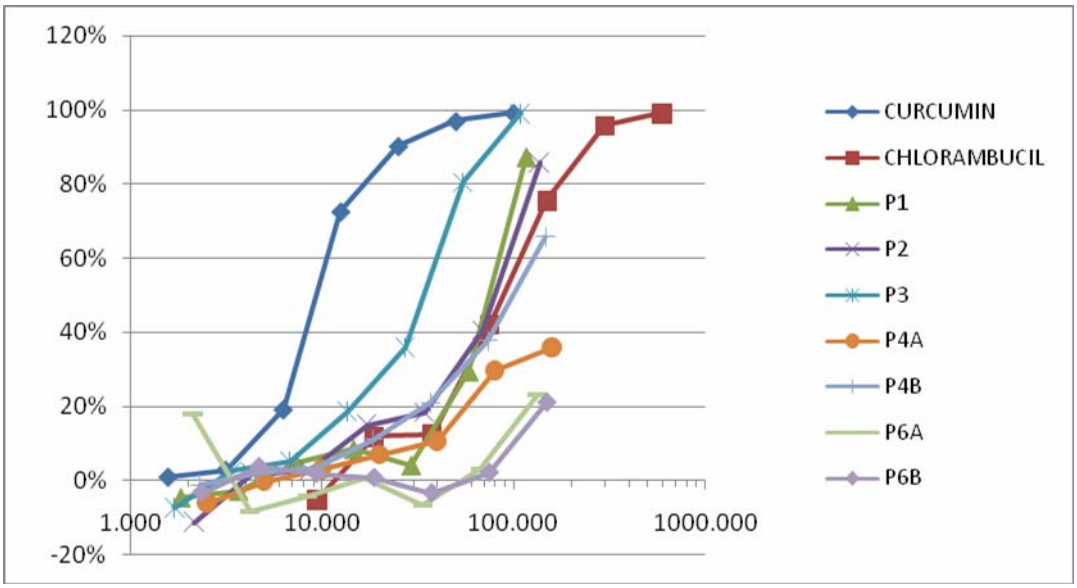
Appendix 1

Detailed cytotoxic results



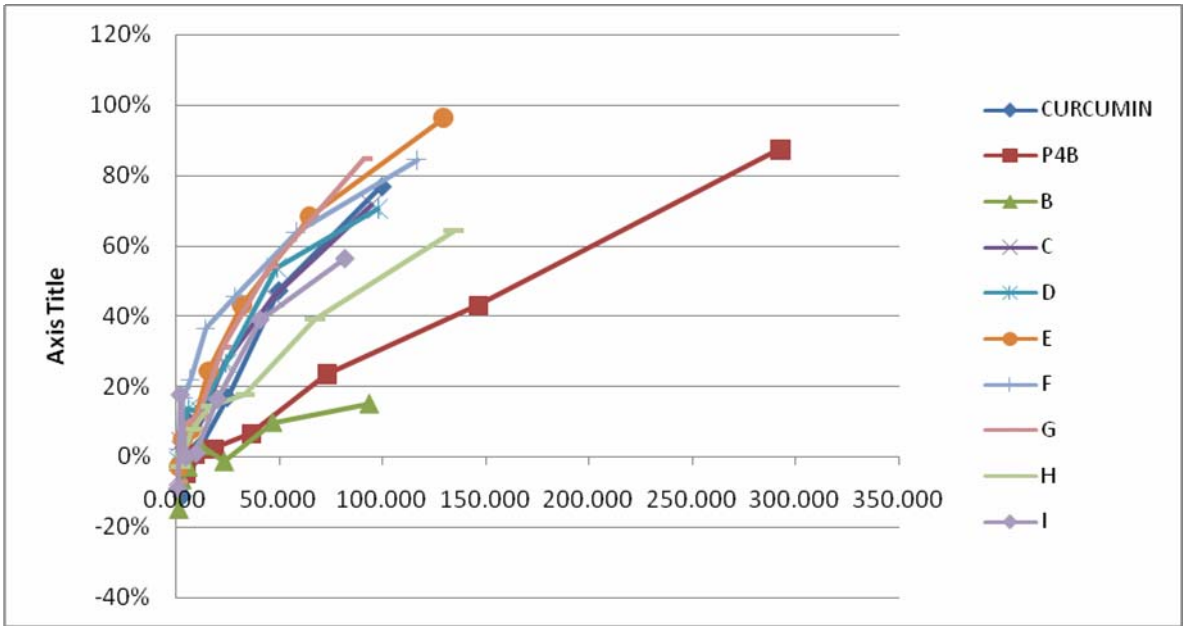
Appendix Figure 1. Cytotoxic results of *Plectranthus* oils (P1-P6).

Appendix Table 1. Cytotoxic results of *Plectranthus* oils.

Sample	Conc. (µg/mL)	Inhib. Rep1 (%)	Inhib. Rep2 (%)	Average Inhibition	IC50 (µg/mL)
curcumin	100.000	99	99	99	
	50.000	95	99	97	
	25.000	90	91	91	
	12.500	67	78	73	**9.53**
	6.250	23	16	20	
	3.125	10	-14	-2	
	1.563	21	-19	1	
chlorambucil	600.000	99	99	99	
	300.000	96	96	96	
	150.000	74	78	76	
	75.000	48	36	42	**86.89**
	37.500	18	7	13	
	18.750	15	8	12	
	9.375	-9	-2	-6	
P1	117.000	86	88	87	
	58.500	16	43	30	
	29.250	0	8	4	
	14.625	10	7	9	**60**
	7.313	7	3	5	
	3.656	6	-11	-3	
	1.828	7	-16	-5	
P2	137.000	98	74	86	
	68.500	49	32	41	
	34.250	22	15	19	
	17.125	28	2	15	**61.58**
	8.563	7	-2	3	
	4.281	16	-12	2	
	2.141	11	-34	-12	
P3	109.000	100	99	100	
	54.500	80	81	81	

Sample	Conc. (µg/mL)	Inhib. Rep1 (%)	Inhib. Rep2 (%)	Average Inhibition	IC50 (µg/mL)
	27.250	37	35	36	
	13.625	28	10	19	**31.52**
	6.813	14	-4	5	
	3.406	9	-4	3	
	1.703	4	-18	-7	
P4A	160.000	61	11	36	
	80.000	33	27	30	
	40.000	16	5	11	
	20.000	1	13	7	**120**
	10.000	10	-4	3	
	5.000	8	-8	0	
	2.500	3	-15	-6	
P4B	147.000	70	61	66	
	73.500	43	32	38	
	36.750	14	28	21	
	18.375	11	12	12	**97.29**
	9.188	4	3	4	
	4.594	5	-1	2	
	2.297	4	-7	-2	
P6A	134.000	26	20	23	
	67.000	7	-1	3	
	33.500	-9	-4	-7	
	16.750	11	-10	1	**182.63**
	8.375	6	-14	-4	
	4.188	3	-19	-8	
	2.094	-3	38	18	
P6B	150.000	18	24	21	
	75.000	-14	18	2	
	37.500	-10	3	-4	
	18.750	-2	3	1	**209.44**
	9.375	8	-4	2	

Sample	Conc. (µg/mL)	Inhib. Rep1 (%)	Inhib. Rep2 (%)	Average Inhibition	IC50 (µg/mL)
	4.688	13	-6	4	
	2.344	3	-10	-4	



Appendix Figure 2. Cytotoxic results of P4B fractions.

Appendix Table 2. Cytotoxic results of P4B fractions.

Sample	Conc. (µg/mL)	Inh. Rep1 (%)	Inh. Rep2 (%)	Average Inhibition	IC50 (µg/mL)
CURCUMIN	100.000	74%	80%	77%	
	50.000	41%	53%	95%	
	25.000	16%	18%	92%	
	12.500	6%	1%	81%	**54**
	6.250	4%	-8%	49%	
	3.125	-4%	-19%	31%	
	1.563	-3%	-16%	7%	
P4B	147.000	70%	61%	66%	
	73.500	43%	32%	38%	
	36.750	14%	28%	21%	
	18.375	11%	12%	12%	**146**

Sample	Conc. (µg/mL)	Inh. Rep1 (%)	Inh. Rep2 (%)	Average Inhibition	IC50 (µg/mL)
	9.188	4%	3%	4	
	4.594	5%	-1%	2	
	2.297	4%	-7%	-2	
B	94.000	46%	50%	48%	
	47.000	18%	34%	26%	
	23.500	-3%	1%	-1%	
	11.750	-7%	-12%	-10%	**>200**
	5.875	7%	-21%	-7%	
	2.938	-5%	-11%	-8%	
	1.469	-20%	-25%	-23%	
C	95.000	68%	64%	66%	
	47.500	37%	33%	35%	
	23.750	14%	12%	13%	
	11.875	-5%	-11%	-8%	**48**
	5.938	-3%	-5%	-4%	
	2.969	-6%	-21%	-14%	
	1.484	-20%	-30%	-25%	
D	98.000	64%	66%	65%	
	49.000	49%	40%	45%	
	24.500	11%	13%	12%	
	12.250	-5%	-4%	-5%	**48**
	6.125	-2%	-5%	-4%	
	3.063	-6%	-30%	-18%	
	1.531	-7%	-36%	-22%	
E	130.000	96%	96%	96%	
	65.000	60%	63%	62%	
	32.500	19%	43%	31%	
	16.250	6%	11%	9%	**36**
	8.125	-11%	-13%	-12%	
	4.063	-16%	-14%	-15%	
	2.031	-26%	-24%	-25%	

Sample	Conc. (µg/mL)	Inh. Rep1 (%)	Inh. Rep2 (%)	Average Inhibition	IC50 (µg/mL)
F	117.000	76%	82%	79%	
	58.500	51%	51%	51%	
	29.250	26%	27%	27%	
	14.625	15%	13%	14%	**29**
	7.313	-4%	-7%	-6%	
	3.656	-4%	-21%	-13%	
	1.828	-32%	-33%	-33%	
G	91.000	83%	81%	82%	
	45.500	43%	48%	46%	
	22.750	20%	17%	19%	
	11.375	-2%	-6%	-4%	**38**
	5.688	-1%	-13%	-7%	
	2.844	-6%	-18%	-12%	
	1.422	-19%	-35%	-27%	
H	135.000	55%	61%	58%	
	67.500	20%	37%	29%	
	33.750	-4%	10%	3%	
	16.875	-8%	7%	-1%	**91**
	8.438	-9%	-8%	-9%	
	4.219	-21%	-12%	-17%	
	2.109	-26%	-16%	-21%	
I	82.000	50%	49%	50%	
	41.000	22%	38%	30%	
	20.500	-1%	8%	4%	
	10.250	-11%	-17%	-14%	**64**
	5.125	-11%	-19%	-15%	
	2.563	-1%	11%	5%	
	1.281	-16%	-35%	-26%	
K	108.000	10%	34%	22%	
	54.000	13%	28%	21%	
	27.000	9%	28%	19%	

Sample	Conc. (µg/mL)	Inh. Rep1 (%)	Inh. Rep2 (%)	Average Inhibition	IC50 (µg/mL)
	13.500	5%	-6%	-1%	**>200**
	6.750	11%	-2%	5%	
	3.375	9%	18%	14%	
	1.688	2%	5%	4%	
M	140.000	10%	34%	22%	
	70.000	13%	28%	21%	
	35.000	9%	28%	19%	
	17.500	5%	-6%	-1%	**>200**
	8.750	11%	-2%	5%	
	4.375	9%	18%	14%	
	2.188	2%	5%	4%	

Appendix 2 -

Detailed composition of the P4B-C and P4B-D fractions

Appendix Table 3. Chemical composition of the P4B-C fractions.

Fraction	Component	RT(min)	AREA (%)	Match (%)
P4B-C-7	*o*-Cresol	15.65	26.14	98
	Eucarvone	19.33	9.44	81
	Unidentified	20.53	4.94	45
	Eucarvone	21.28	59.48	90
P4B-C-8				
	o-Cresol	15.65	12.48	98
	Eucarvone	19.33	3.70	80
	Eucarvone	21.29	69.04	90
	Unidentified	21.34	2.86	50
P4B-C-11				
	Verbenone	19.30	67.81	97
P4B-C-14				
	cis-Linalool oxide	16.32	2.44	91
	Car-3-en-2-one	20.03	89.40	97
P4B-C-15				
	p-Methyl-acetophenone	18.45	16.12	97
	p-Methyl-acetophenone	18.79	17.52	97
	Unidentified	19.81	2.37	72
	Car-3-en-2-one	20.01	59.72	98
P4B-C-16				
	p-Cymen-8-ol	18.40	4.23	87
	p-Methyl-acetophenone	18.45	23.00	95
	Cryptone	18.88	7.49	95
	Car-3-en-2-one	20.00	10.98	97
	Unidentified	20.21	9.55	50

Fraction	Component	RT(min)	AREA (%)	Match (%)
	trans-Carvone oxide	20.96	27.47	95
	6-Hydroxy-carvotanacetone	21.21	7.59	78
P4B-C-18				
	Unidentified	18.24	5.27	45
	p-Cymen-8-ol	18.43	33.71	83
	p-Cymen-8-ol	18.61	1.41	90
	Eucarvone	19.36	54.94	74
P4B-C-19				
	p-Cymen-8-ol	18.43	24.08	83
	Unidentified	18.56	14.14	59
	p-Cymen-8-ol	18.61	5.50	83
	Eucarvone	19.36	51.24	74
P4B-C-20				
	Unidentified	18.24	2.71	45
	p-Cymen-8-ol	18.41	20.10	83
	Unidentified	18.55	16.90	59
	p-Cymen-8-ol	18.61	8.34	90
	Eucarvone	19.35	51.2	83
P4B-C-21				
	p-Cymen-8-ol	18.4	13.41	90
	Unidentified	18.55	21.33	59
	o-Cymen-8-ol	18.61	9.98	90
	Eucarvone	19.34	46.1	83
	Eucarvone	19.41	5.31	81
P4B-C-22				
	p-Cymen-8-ol	18.4	11.24	87
	o-Cymen-8-ol	18.6	19.7	90
	Unidentified	19.12	4.16	64
	Chrysanthenone	19.33	39.9	83
	Eucarvone	19.41	25	76
P4B-C-23				
	p-Cymen-8-ol	18.4	4.46	90

Fraction	Component	RT(min)	AREA (%)	Match (%)
	Unidentified	18.55	14.73	59
	o-Cymen-8-ol	18.6	11.4	90
	Eucarvone	19.33	14.81	87
	Eucarvone	19.41	49.91	87
P4B-C-24				
	Eucarvone	19.41	100	70
P4B-C-27				
	Carvone	19.85	65.85	90
	p-Cymen-7-ol	20.68	9.49	97
P4B-C-28				
	Carvone	19.85	63.74	95
	p-Cymen-7-ol	20.68	9.23	83
	Eucarvone	21.95	19.73	68
P4B-C-32				
	p-Mentha-1,5-dien-8-ol	18.14	97.04	80
P4B-C-37				
	p-Mentha-1,5-dien-8-ol	8.31	36.65	83
	Unidentified	19.06	11.14	64
	trans-Carveol	19.22	52.21	97

Appendix Table 4. Chemical composition of the P4B-D fractions.

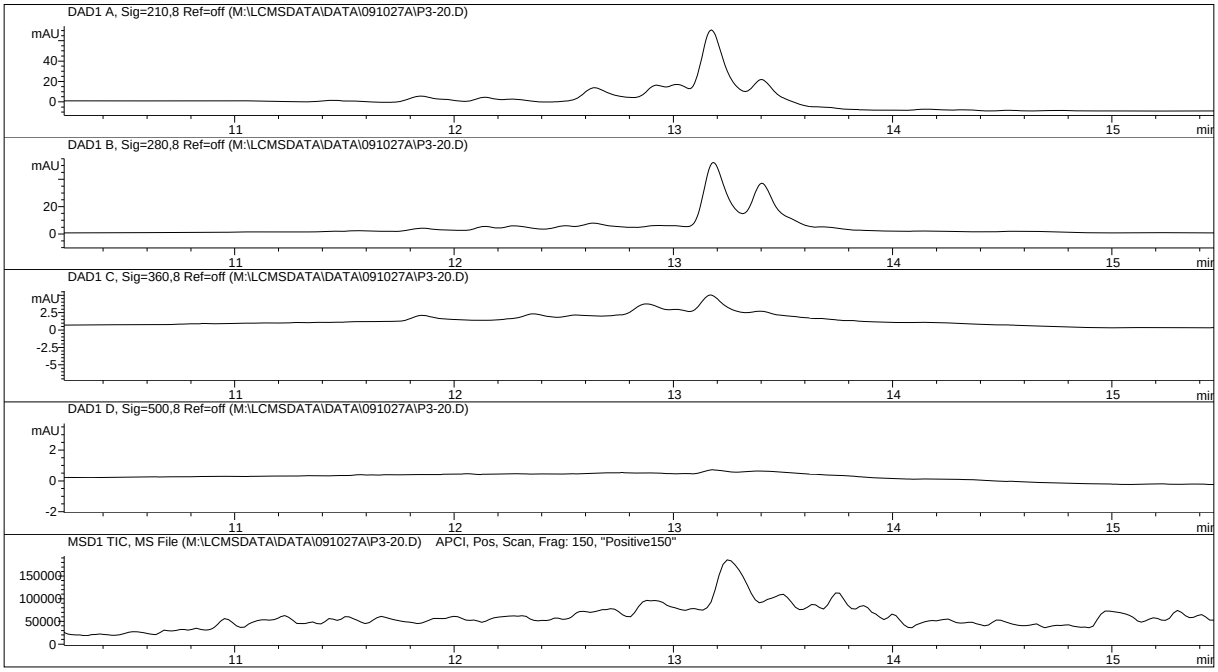
Fraction	Component	RT(min)	AREA (%)	Match (%)
P4B-D-8	Carvone	19.85	63.98	94
	Unidentified	20.68	5.79	64
	Eucarvone	21.95	10.07	90
P4B-D-9				
	Eucarvone	19.36	75.81	87
P4B-D-13				
	p-Cymen-8-ol	18.41	22	83

Fraction	Component	RT(min)	AREA (%)	Match (%)
	Unidentified	18.24	5.5	45
	4-Ethyl-3,4-dimethyl-2,5-cyclohexadien-1-one	19.36	67.2	72
	Eucravone	21.28	2.84	90
P4B-D-14				
	Carvone	19.86	50.84	90
	Eucarvone	21.96	39.45	90
P4B-D-15				
	Unidentified	18.15	84.44	59
	o-Piperitone	21.96	7.32	80
P4B-D-16				
	Unidentified	18.15	93.67	64
P4B-D-19				
	trans-Carveol	19.23	19.13	95
	p-Mentha-1,5-dien-8-ol	20.75	4.37	72
	p-Mentha-1(7),2-dien-8-ol	20.99	11	72
P4B-D-20				
	trans-Carveol	19.24	26.86	96
	Unidentified	19.51	4.83	62
P4B-D-21				
	trans-Carveol	19.23	65.66	95
	cis-Carveol	19.51	34.34	96
P4B-D-22				
	trans-Carveol	19.23	49.43	86
	Unidentified	19.51	50.57	59
P4B-D-25				
	Carvacrol	20.64	88.33	96
P4B-D-26				
	Carvacrol acetate	20.49	20.25	94
	Carvacrol	20.64	79.75	95
P4B-D-27				
	Carvacrol	20.49	30.79	94

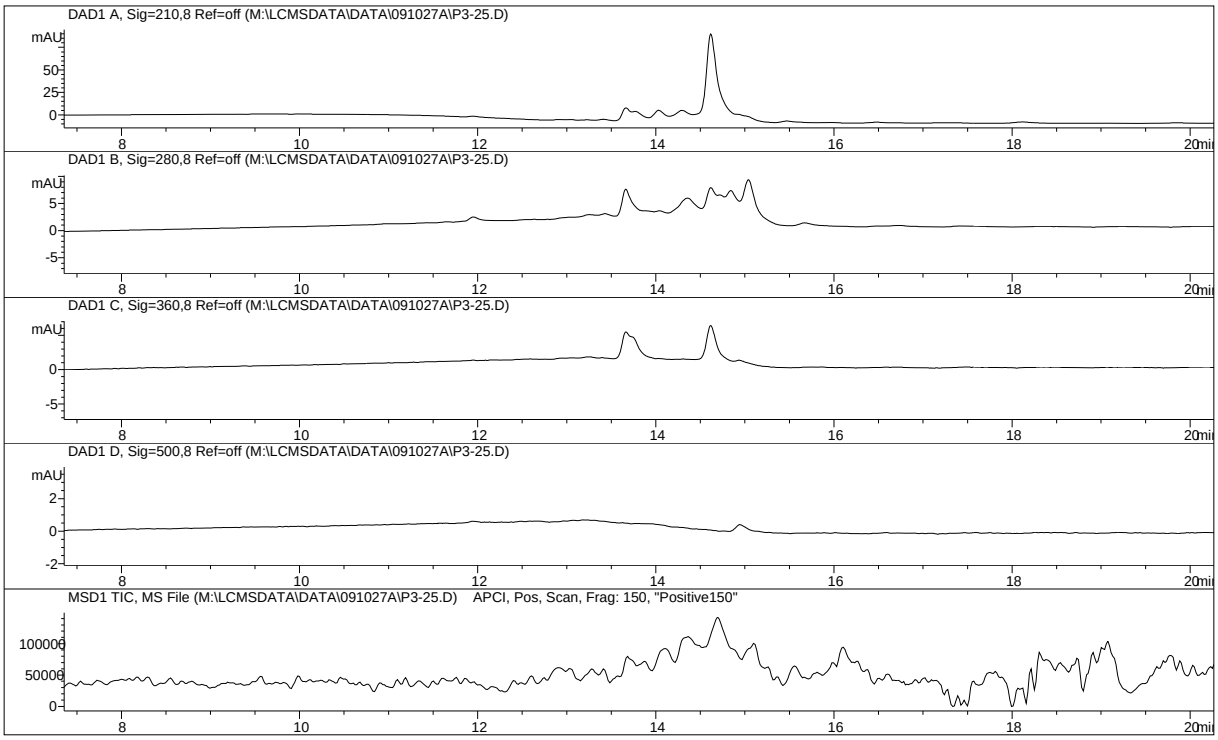
Fraction	Component	RT(min)	AREA (%)	Match (%)
	Carvacrol	20.63	69.21	93
P4B-D-28				
	Unidentified	18.8	16.35	64
	Carvacrol	20.49	37.54	91
	Thymol	20.62	46.11	83

Appendix 3 -

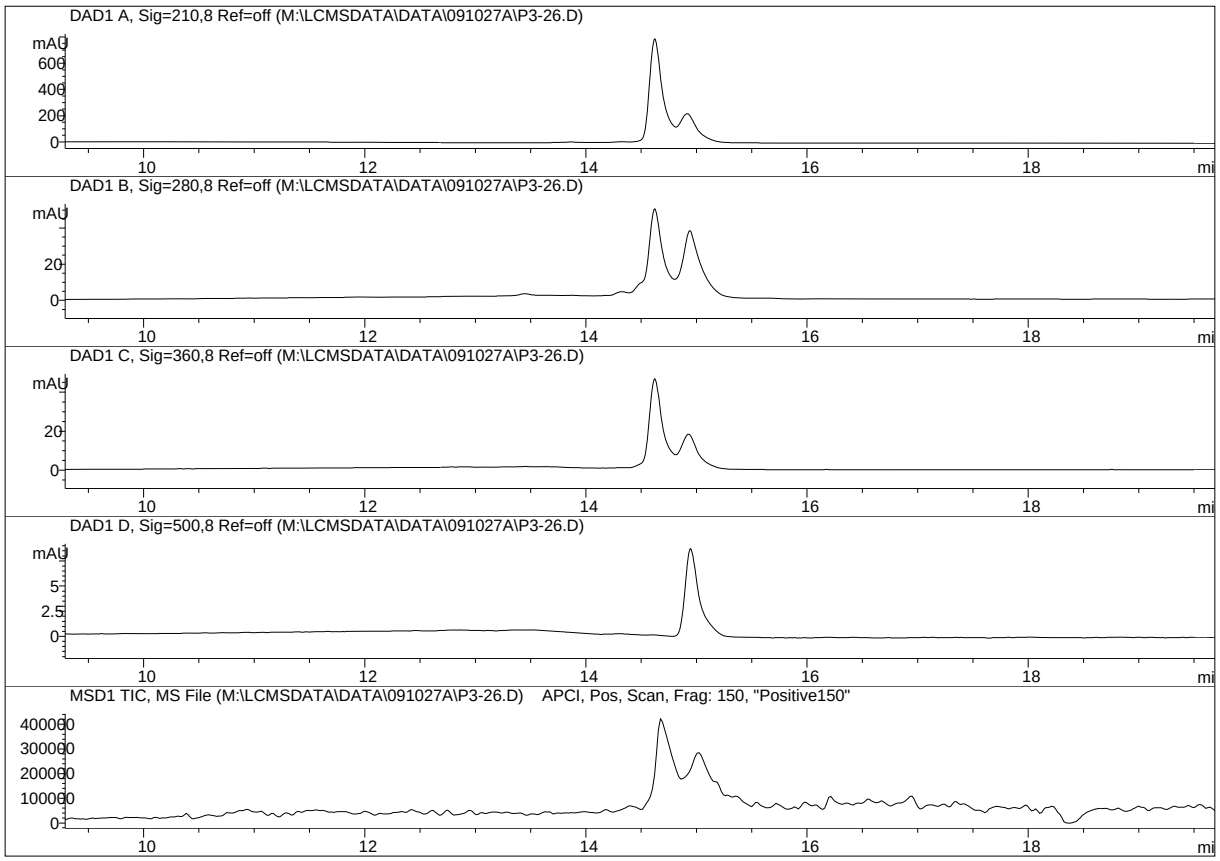
Chromatographic profiles of the *P. nitidus* fractions



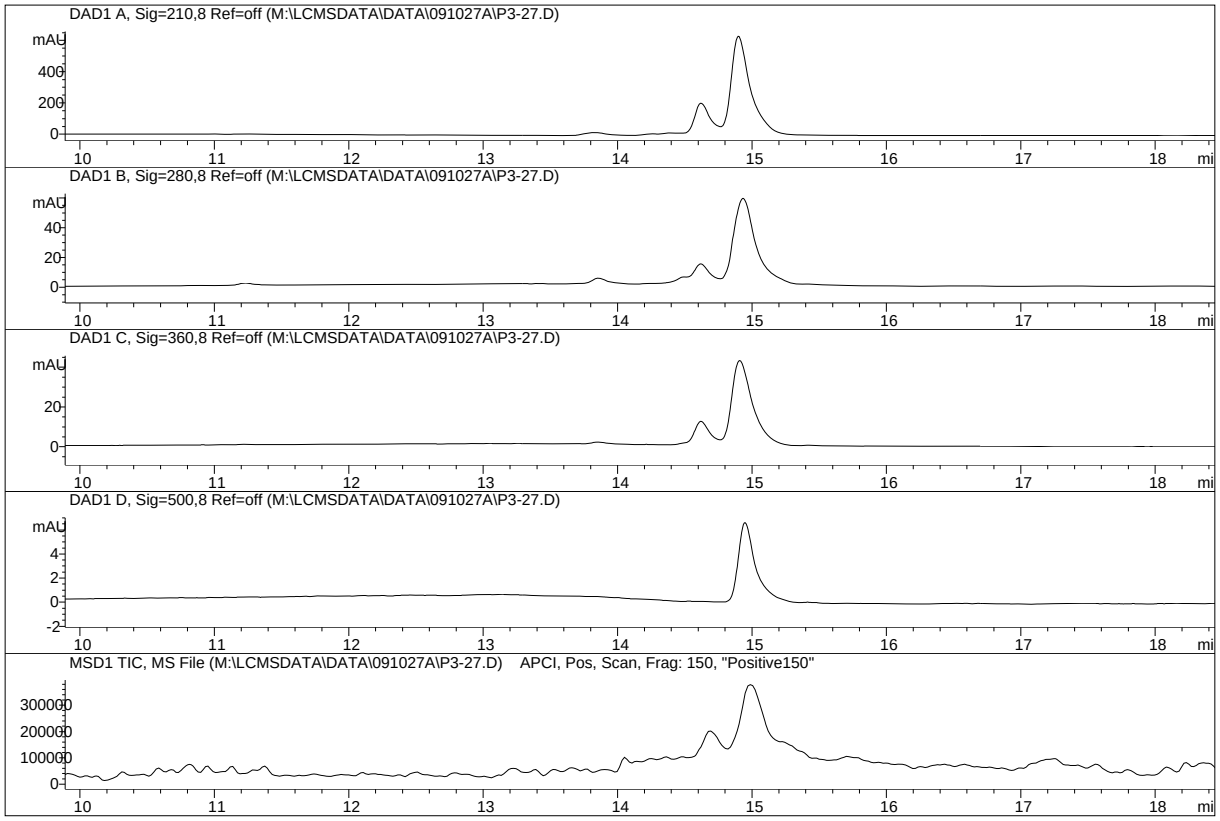
Appendix Figure 3. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-20.



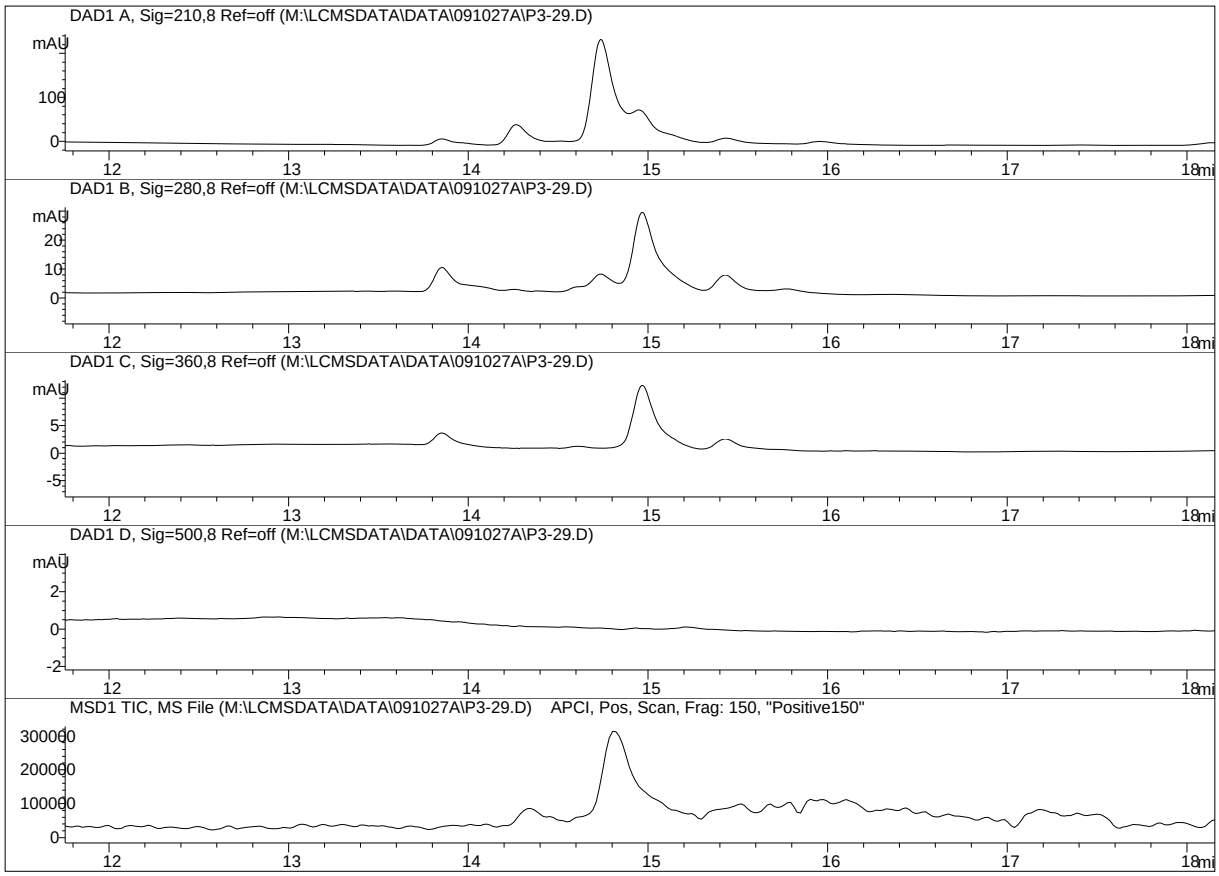
Appendix Figure 4. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-25.



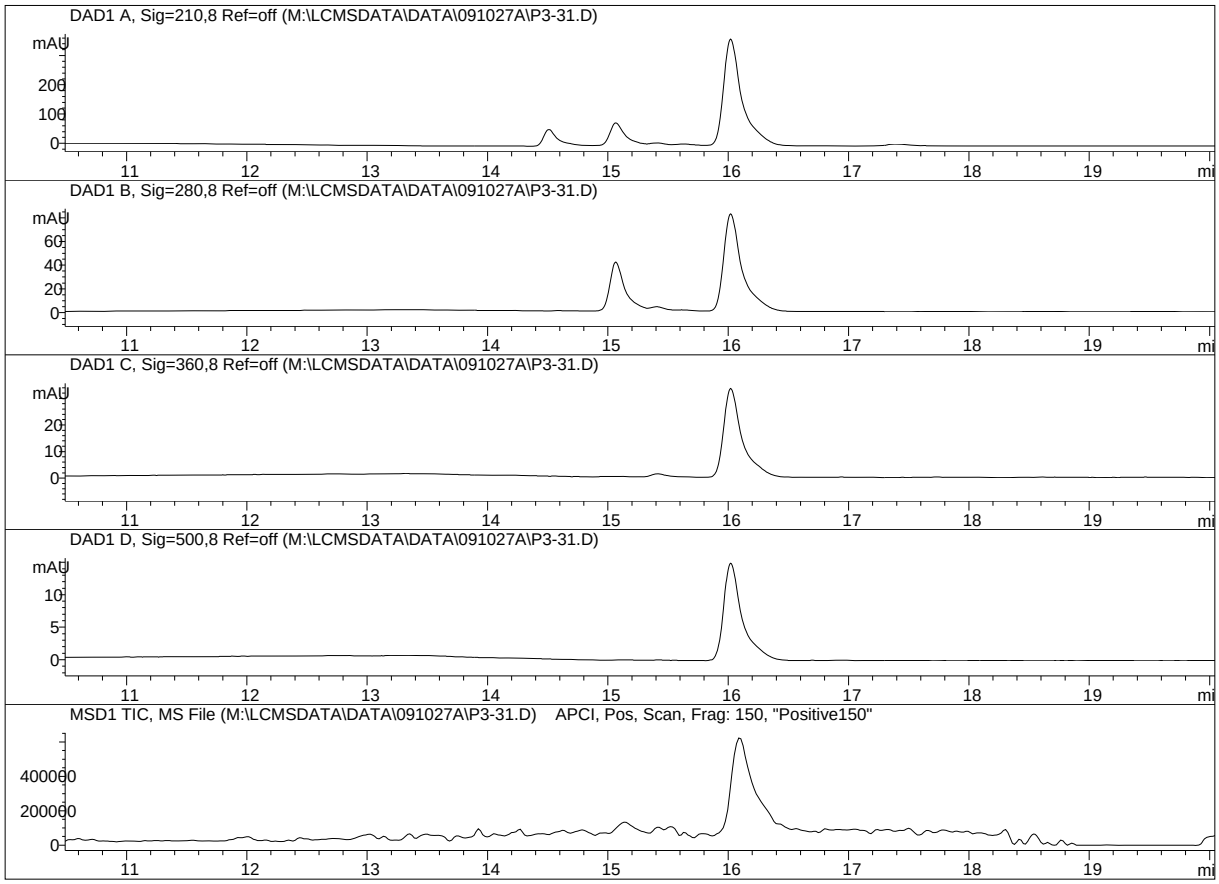
Appendix Figure 5. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-26.



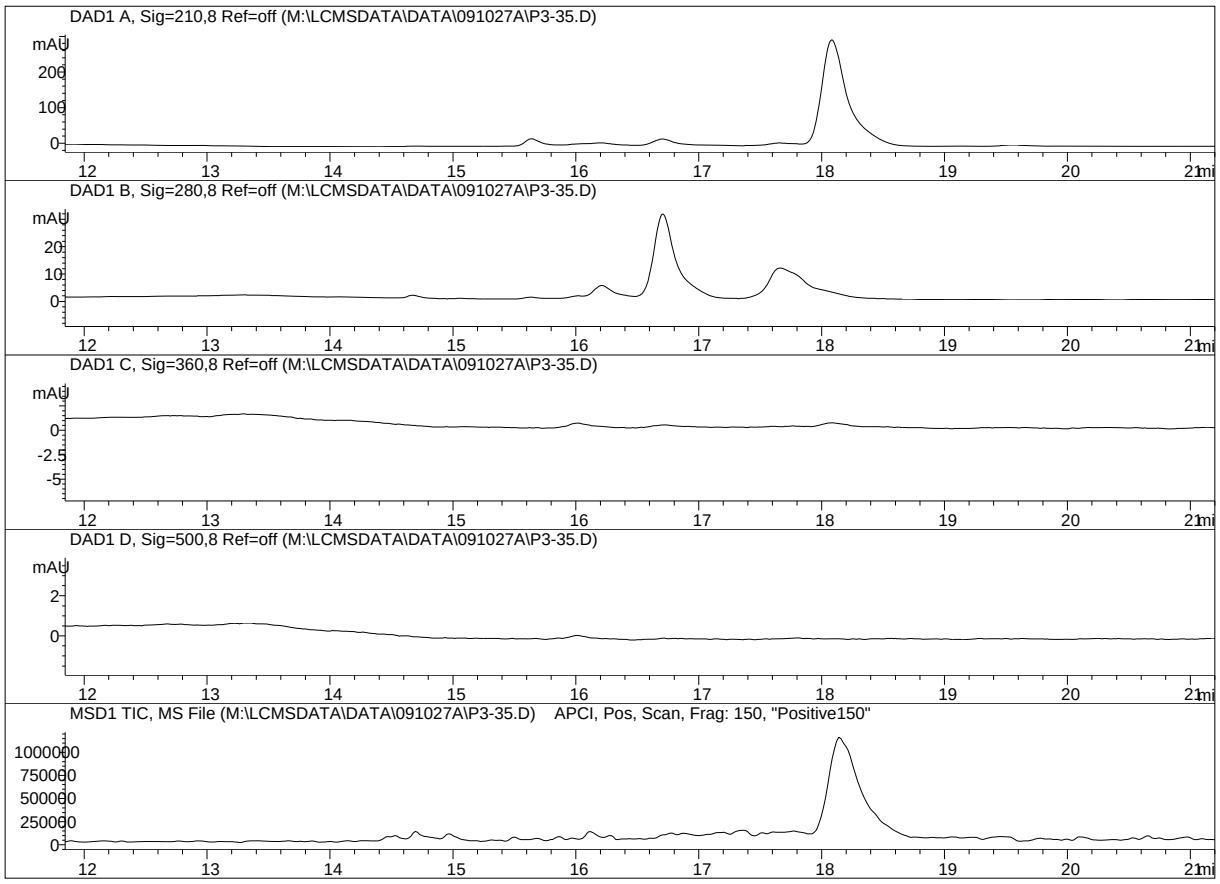
Appendix Figure 6. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-27.



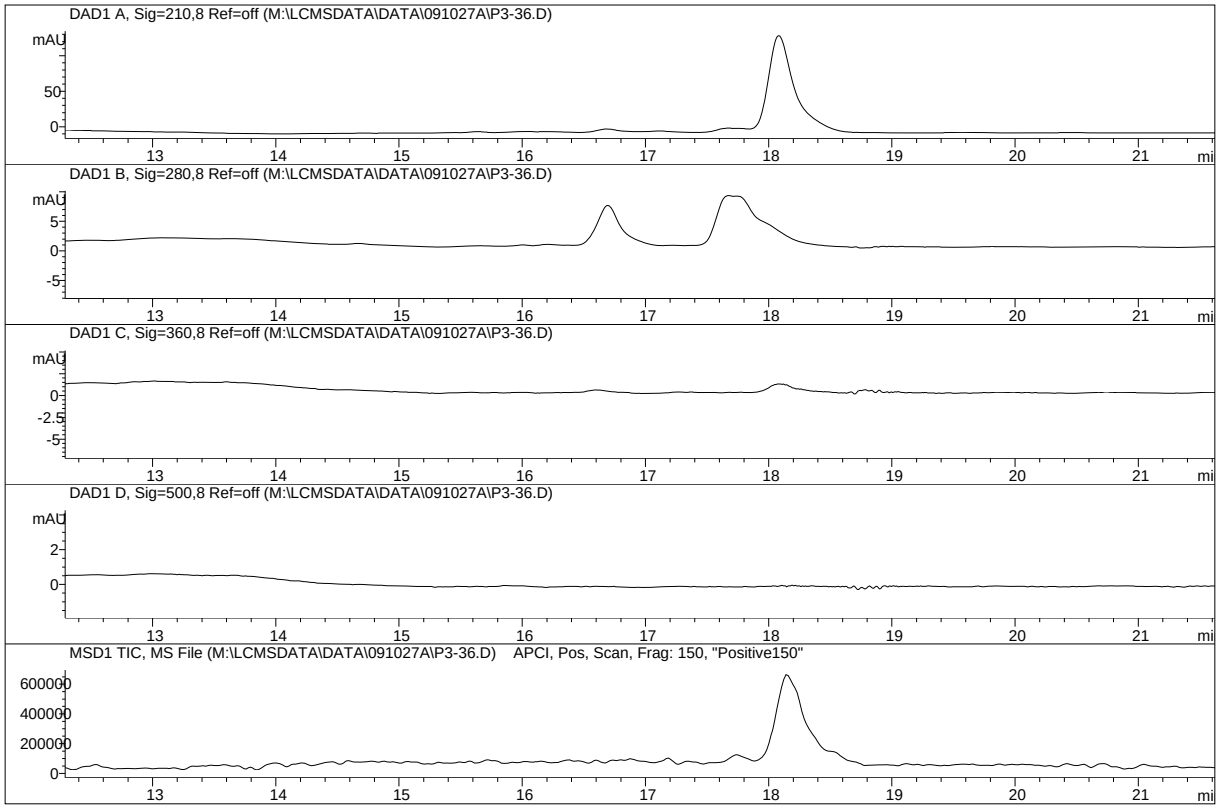
Appendix Figure 7. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-29.



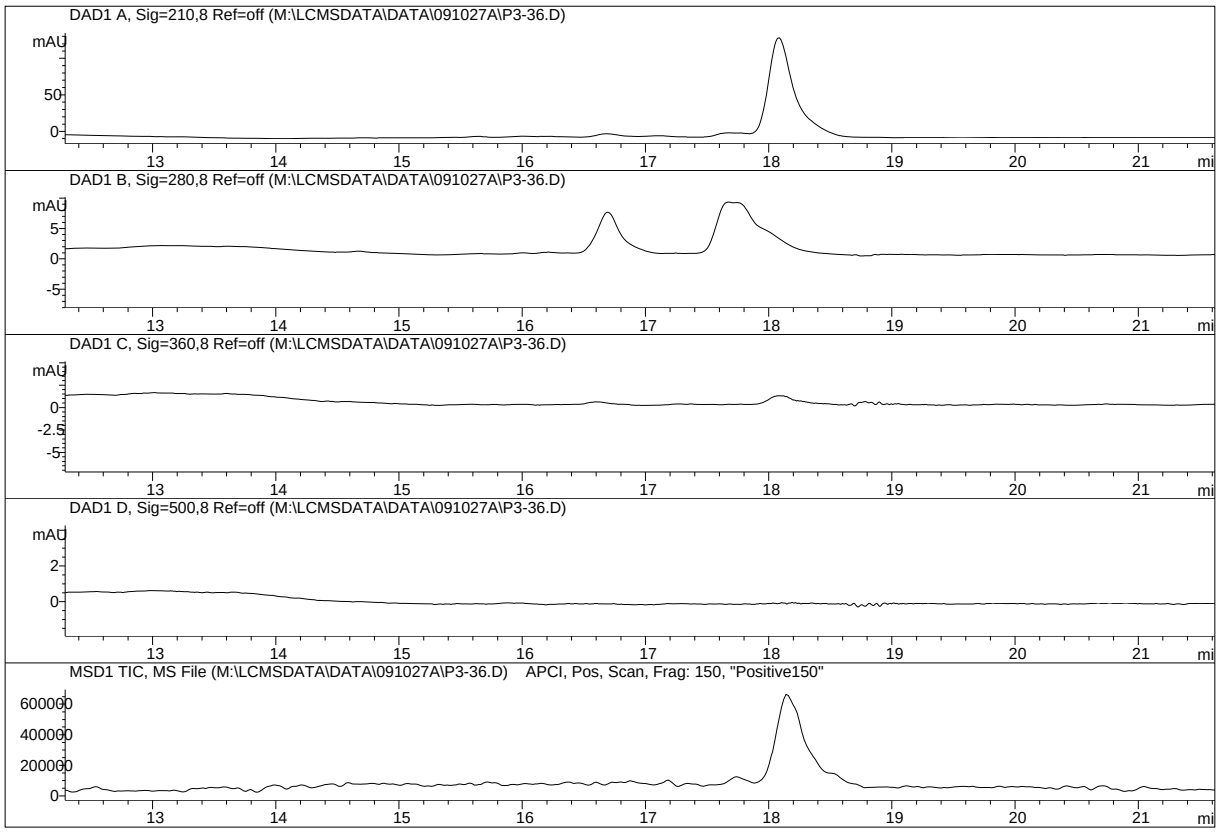
Appendix Figure 8. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-31.



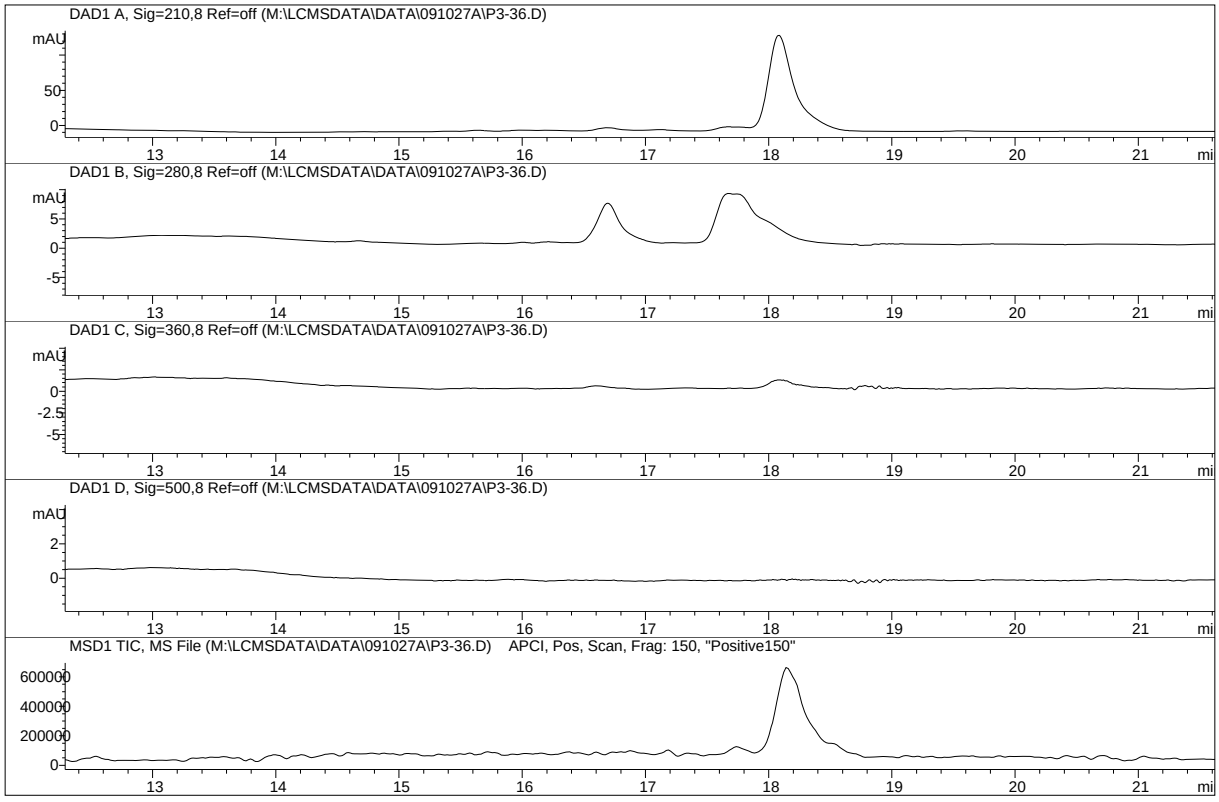
Appendix Figure 9. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-35.



Appendix Figure 10. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-36.



Appendix Figure 11. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-37.



Appendix Figure 12. UV profile at 210, 280, 360, 500nm and LCMS profile of the P3-40.

Curriculum Vitae

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07/07	Freiwilliges Camp in der Turkei, Genctur Agentur

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10/04	„Risk management and pharmacy profession", **EPSA-ESCP** Studenten-Symposium in Prag, Tschechien
09/04 – 02/05	Buchführungskurs
03/04	„Wie kann man ein guter Manager in der Pharmazie werden", Fakultät der Organisationswissenschaft, Belgrad
11/03	„Leadership training program", Kurt R. Richter; IEEE Organisation
08/02	**Universität Siegen,** Deutschintensivsprachkurs
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Deutsch	Fließend im Wort und Schrift
Englisch	Fließend im Wort und Schrift
Französisch	Maturaniveau