Strathclyde Institute of Pharmacy and Biomedical Sciences

STUDIES ON THE ACTIVITY OF SELECTED PLANTS AGAINST BIOFILMS OF *PSEUDOMONAS AERUGINOSA* STRAIN PA14

by

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A thesis presented in fulfilment of the requirement for the degree of

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"This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which leads to the award of a degree."

Signed: 

Date: 1st April 2015

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Abstract

This thesis describes the activity of selected plants against biofilms of *Pseudomonas aeruginosa* strain PA14. The activity of plant extracts and subsequently purified compounds was evaluated using a stepwise separation process called bioassay-guided fractionation and used a microtitre plate based assay. Active extracts (showing more than 50 % biofilm inhibition, BFI) were further investigated for the presence of active compounds. The fractionation process involved the use of chromatographic techniques. Compounds were identified using NMR, GC-MS and LC-MS.

Out of a total 129 extracts screened for antibiofilm activity using microtitre plate method, 44 extracts showed more than 50 % biofilm inhibition, whilst 85 extracts were found to increase biofilm formation. Four active extracts, **(E333)**, **(E341)**, **(H338)** and **(M338)** were selected for further investigation. A process of bioassay-guided fractionation was used to purify the phytochemicals present in each active extracts.

This study led to the discovery of four bioactive compounds, namely **(E333F1S1)**, **(E341)**, **(HA6)** and **(M338B)** with antibiofilm activity against *P. aeruginosa* PA14. The active fraction **(E333F1S1)** from *Ribes nigrum* leaf was found to contain mixtures of alkanes such as *n*-nonadecane, 2-methylnonadecane, 2-methylicosane and 2 -methyloctacosane. The active extract of *Sambucus nigra* flower **(E341)** contained a mixture (2:1 ratio) of oleanolic and ursolic acid. Comparable activity was found at the same ratio (2:1) when these compounds were tested as a mixture of pure compounds. No significant difference ($p < 0.05$) in activity compared to a positive

control was observed when these compounds were combined in different ratios. Only weak antibiofilm activity was observed when each compound was tested on its own.

The LC-MS analysis on *Coriandrum sativum* seed active fractions, **(HA6)** revealed the presence of putative compounds such as 10-undecenal, dodecanal, 2-hexyl furan, linalool oxide, caryophyllene oxide and 4-ethylcamphor. When linalool oxide was tested, it exhibited a strong activity but reduced activity when in mixtures. Both activities were significantly different ($p < 0.05$) compared to positive control. Another active fraction of *Coriandrum sativum* seed, **(M338B)** showed the presence of putative compounds such as fatty acids, carboxylic acid, carboxylate and tetraone.

This study led to the discovery of potential bioactive compounds from selected plant as antibiofilm agents against *P. aeruginosa* PA14. The bioactive compounds were from *Ribes nigrum* leaf (e.g. mixture of alkanes), *Sambucus nigra* flowers (e.g. mixture of ursolic acid and oleanolic acid) and *Coriandrum sativum* seeds (e.g. mixture of oxygenated monoterpenes, carboxylic acid, carboxylate, tetraone, glycerol, carbohydrates and fatty acids).

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List of Abbreviations

BFF	: Biofilm formation
BFI	: Biofilm inhibition
CC	: Open Column Chromatography
CD_3OD	: Deuterated methanol
CV	: Crystal Violet
COSY	: Correlation Spectroscopy
DBE	: Double bond equivalence
DEPT	: Distortionless Enhancement by Polarisation Transfer
DMSO	: Dimethyl sulfoxide
DMSO-d_6	: Deuterated dimethyl sulfoxide
DW	: Dry weight
EPS	: Exopolysaccharides
ESI	: Electrospray Ionisation Mass Spectroscopy
EtOAc	: Ethyl acetate
GC	: Gas Chromatography
GF	: Gel Filtration
HREI-MS	: High Resolution Electron Impact-Mass Spectrometry

HRESI-MS : High Resolution Electrospray Ionisation -Mass Spectrometry

HMBC : Heteronuclear Multiple Bond Coherence

HMQC : Heteronuclear Multiple Quantum Coherence

LB : Luria-Bertani

LC : Liquid Chromatography

LLP : Liquid-Liquid Partition

MDR : Multi Drug Resistance

MeOH : Methanol

MH : Mueller-Hinton

MS : Mass Spectrometry

NMR : Nuclear Magnetic Resonance

(1D: one dimensional, 2D: two dimensional)

PA14 *: Pseudomonas aeruginosa* strain PA14

RP : Reverse Phase column chromatography

TLC : Thin layer chromatography

UV : Ultraviolet light

VLC : Vacuum Liquid Chromatography

CHAPTER 1 - INTRODUCTION

1.1 General considerations

The use of plant extracts in traditional folk medicine for the treatment of many diseases has been well-documented. Plant extracts have demonstrated a range of biological effect including antimicrobial (Witkowska *et al.*, 2011; Suzutani *et al.*, 2003) and antibiofilm (Budzynska *et al.*, 2011) activity. Many of the bioactive phytochemicals in such active plant extracts however remain to be identified.

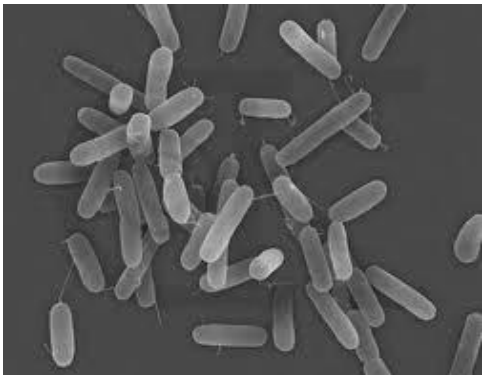
Studies on the activity of plant-derived natural products could provide some novel antibiofilm compounds to fulfil the urgent need for new therapies for *Pseudomonas*-related infections. *Pseudomonas* is a bacterium which is pathogen of plants and an opportunistic pathogen of humans that has an important clinical relevance in chronic infections due to the formation of biofilms.

1.2 *Pseudomonas aeruginosa*

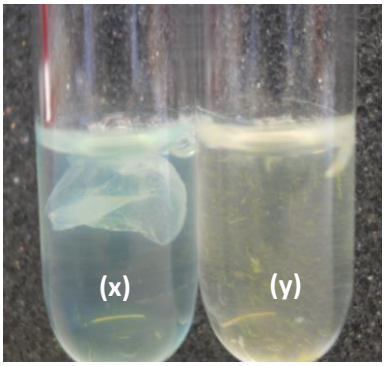
Pseudomonas aeruginosa is a Gram-negative bacterium, rod shaped with a single polar flagellum (Figure 1). *P. aeruginosa* is ubiquitous in environmental water, soil and on plants. In nature, *P. aeruginosa* can be a planktonic cell and a sessile colony of biofilms. This free-living bacterium and its biofilm can attach to many surfaces or substrates such as plants and animals and then multiply in a moist environment.

P. aeruginosa is a nosocomial pathogen that can alter the microenvironment and cause chronic infections with persistent inflammation and tissue damage. *P. aeruginosa* can cause variety of skin infections, both localised and diffused and tendency to colonise the surfaces in a biofilm form. This bacterium is also known as the primary cause of death in immune-compromised patients including those with cystic fibrosis and undergoing cancer chemotherapy.

The resistance of *P. aeruginosa* towards many antibiotics is afforded by its Gram-negative outer membrane. The membrane acts as a permeability barrier and makes the cells impervious to therapeutic concentration of antibiotics. This bacterium is the most prevalent infective agents as it possessed a large numbers of virulence factors and antimicrobial genes (Church *et al.*, 2006).



(a)



(b)

Figure 1: Morphology of *Pseudomonas aeruginosa*. Image of 1(a) showing *Pseudomonas aeruginosa* cells as a rod shaped with single flagellum observed under Scanning Electron Micrograph (SEM). Images in 1(b) are showing the formation of *Pseudomonas aeruginosa* strain PA14 biofilm in M63 medium (x) with an uninoculated M63 medium (y) as a negative growth control. Figure 1(a) was adapted from Pseudomonas Genome Database, (Winsor *et al.*, 2011).

1.2.1 Biofilms and quorum sensing

Biofilms are defined as a structured consortium of bacterial cells embedded in a self-produced polymeric matrix, which consists of polysaccharides, protein and DNA (Hoiby *et al.*, 2001). The exopolysaccharides (EPS) constitute the scaffolding components for bacteria aggregating in the biofilm and act as scavengers of free oxygen radicals and binding to many classes of antibiotics. Thus this matrix provides structural stability and protection to the biofilm. The genes that are responsible for *P. aeruginosa* biofilm production have been identified as *cup*A genes (Kuppusamy and Murugan, 2010).

P. aeruginosa is able to produce a mature biofilm within 5 to7 days *in vitro*. Growth, protein synthesis, metabolic activities and oxygen concentration in biofilms are stratified. At the surface of a biofilm, a higher level of oxygen and metabolic activity is observed compared to the centre (Werner *et al.*, 2004). This explains the reduced susceptibility of biofilms to antibiotics as the slower growing bacteria in the centre will be less sensitive to the antibiotics (Boles and Singh, 2008).

The growth of a *P. aeruginosa* biofilm is associated with an increasing level of mutations as well as with quorum sensing mechanisms. Mutation frequency of biofilm-growing bacteria is significantly higher compared to planktonic growing isogenic bacteria and there is an increased horizontal gene transmission in biofilms (Driffield *et al.*, 2008; Molin and Tolker-Nielsen, 2003).

Bacteria communicate by means of synthesising and reacting to signal molecules. The term of quorum-sensing (QS) indicates that this system allows bacteria to sense when a critical number of cells are present in a limited space in the environment. The

bacteria will respond by activating certain genes that then produce virulence factors such as enzymes or toxins. In Gram- negative bacteria, N-acyl-L-homoserine lactone are the commonly described QS molecules whilst small peptides are produced by many Gram-positive bacteria (Bjarnsholt and Givskov, 2007). The QS effect influences the development of cells within biofilms (Parsek and Greenberg, 2005).

1.2.2 Pseudomonal infections and drug resistance

The occurrence of resistant isolates of *P. aeruginosa* is a problematic in many hospitals (Tam *et al.*, 2010). *P. aeruginosa* can break through the host's defences to initiate an infection, this causes serious infections to critically ill and hospitalised patients (Kuppusamy and Murugan, 2010; Martinez *et al.*, 2009). The chronic infection was due to increase tolerance of *P. aeruginosa* biofilm to antibiotics, disinfectants and host immune system (Smith, 2005; Costerton *et al.*, 2003) that initiated resistance of *P. aeruginosa* to most antimicrobial (Tart and Wozniak, 2008) and antibiotic (Kuppusamy and Murugan, 2010) therapies.

Antibiotics as such β–lactams are only active against dividing the cells of *P. aeruginosa* but not against the cells in biofilms (Anwar and Costerton, 1990). Factors such as oxidative stress has contributed to mutability in biofilms (Driffield *et al.*, 2008). A high percentage of hyper-mutable *P. aeruginosa* isolates was found in cystic fibrosis patients (Mandall *et al.*, 2003) that were associated with β-lactam, (e.g. inhibiting bacterial cell wall biosynthesis) and ciprofloxacin (e.g. inhibiting bacterial cell division) (Winsor *et al.*, 2011); colistin (e.g. solubilising bacterial outer

membrane) (Johansen *et al.*, 2008) and tobramycin (e.g. prevent translation of protein and resulted in cell death) (Islam *et al.*, 2009).

1.3 Plants as a source of new drug leads

Plants have a long history of being used as traditional remedies to treat diseases and for example can be used as topical antiseptics (Hernández *et al.*, 2009). Medicinal plants contain natural products which can provide templates for new drug candidates. Such products can be discovered following a systematic process called bioassay-guided fractionation (Potterat and Hamburger, 2008).

Plant natural products display a range of pharmacological properties, including antioxidant, immunostimulant, anticoagulant, hypocholesterolemic and antimicrobial activity (Lang and Buchbauer, 2012; Panghal *et al.*, 2011; Brandt *et al.*, 2004), antiviral (Suzutani *et al.*, 2003) and some antibiofilms (Quave *et al.*, 2012; Budzynska *et al.*, 2011). For example, sulfoxides and flavonoids from *Allium* species can protect against cardiovascular diseases (Griffiths *et al.*, 2002) and glucosinolates from *Brassica* species have anticancer activity (Smith *et al.*, 2003).

Many drugs currently on the market are either plant natural products or are based on natural product templates. These include morphine from opium, quinine from *Cinchona*, artemisinin, salicylic acid, rivastigmine, *Digitalis* glycosides and reserpine (Beutler, 2009). Morphine is a drug with sedative and analgesic properties (Sneader, 2001), quinine and artemisinin are used as antimalarial agents (Wells, 2011; Huttinger *et al.*, 2010), rivastigmine acts a cholinesterase inhibitor used in the treatment of dementia (Winblad and Machado, 2008), salicylic acid is used as pain

reliever (An and Mou, 2011) and *Digitalis glycosides* are used in the treatment of heart disease (Katz *et al.*, 2010).

1.4 Natural antibiofilm agents and the need for antibiofilm drug leads

Most antibiotics have been discovered with assays using planktonic bacterial cultures, often omitting the fact that bacteria can form biofilms (Lindsay and Von Holy, 2006). As a result, current antibiotics do not adequately combat infections caused by biofilm-forming microorganisms.

The antibiofilm activity of plants has been reported for garlic (*Allium sativum)* (Ankri and Mirelman, 1999), rosemary (*Rosmarinus officinalis)* (Bozin *et al.*, 2007), mustard tree (*Salvadora persica)* (Hattab, 1997), neem (*Azadirachta indica),* mango (*Mangifera indica)* (Prashant *et al.*, 2007) and *Adrographis paniculata* (Murugan *et al.*, 2011).

Bioassay guided fractionation is a method that can be used to isolate compounds with antibiofilm activity in plant extracts. The chemical diversity, chirality and various functional groups present in natural products make them an ideal source of new antibiofilm compounds (Sandasi *et al.*, 2009; Jiang *et al.*, 2009).

In this study, some medicinal plants used in traditional medicine for the treatment of infectious diseases have been selected to be investigated for the presence of compounds with activity against *P. aeruginosa* biofilms. The selected plants for this study were from berry crops, herbs and spices.

1.5 Berry crops

Berries are rich source of various bioactive compounds (Mullen *et al.*, 2002). Berry crops have been used as folk remedy such as fruits of cloudberry and raspberry to treat diarrhoea, problems with night vision and urinary infections (Puupponen-Pimia *et al.*, 2005), blackcurrant leaves to soothe sore throat and to reduce fever (Kuiken *et al.*, 2012) and leaves of dwarf elder to treat inflammatory diseases (Schwaiger *et al.*, 2011). A wide range of phytochemicals in berries have beneficial effects on cardiovascular diseases (Maatta-Riihinen *et al.*, 2005), some cancers (Vinson *et al.*, 2001) and act as antiadhesive and antimicrobial agents against pneumococci (Huttunen *et al.*, 2010). Berry phytochemicals regulate the activity of metabolising enzymes, nuclear receptors, transcription, subcellular signalling pathways and repair DNA oxidative damage (Seeram *et al.*, 2006).

Phenolic phytochemicals present in berries often act as defence compounds in response to stress (Mullen *et al.*, 2002). In plant tissues, phenolics occur as glycosides or complex polymerised molecules with high molecular weights (e.g. polymeric tannins) and these compounds have been identified as strong antioxidants. Typically, berry phenolics include flavonoids, tannins and phenolic acids (Table 1). The genus *Vaccinium* such as bilberry, blueberry and cranberry contains predominantly anthocyanins (Cesoniene *et al.*, 2009) and possess bacterial anti-adhesive properties (Seeram, 2008). The genus *Rubus* such as blackberry, black raspberry, red raspberry, strawberry, artic bramble and cloudberry contain predominantly ellagitannins (Seeram, 2008) which possess strong antibacterial activity (Puupponen-Pimia *et al.*, 2005).

Anthocyanins in cranberry have shown antimicrobial activity against a wide range of Gram-negative and Gram-positive bacteria (Cesoniene *et al.*, 2009) and the bacterial antiadhesive properties of cranberry have been attributed to its oligomeric proanthocyanidins which possess an A-type structural linkage (Howell, 2007). Ellagitannins are complex phenolic polymers typically found in cloudberry and raspberry. These compounds can inhibit bacterial growth by destabilising the cytoplasmic membrane, inhibiting extracellular enzymes, and depriving microbes from the substrates required for their growth.

Studies have also indicated that bioactive compounds in berries have anti-adhesive activity against a wide range of pathogens. Ellagitannins related to anti-adherence of bacteria to epithelial cells which is a prerequisite for colonisation and infection of a few pathogens such as *Salmonella, Escherichia* and *Staphylococcus species* (Puupponen-Pimia *et al.*, 2005). However, there is limited evidence in the literature on the activity of compounds found in berry crops against *P. aeruginosa* strain PA14 biofilms.

Table 1: Phenolic compounds previously isolated from berries

Phenolic compounds			Fruits	References
Flavonoids	Anthocyanidins	cyanidin, pelargonidin, delphinidin, peonidin, petunidin, malvidin	elderberry, redcurrant, blueberry, bilberry, blackcurrant, strawberry, raspberry,	(Del Rio *et al.*, 2010) (Seeram, 2008) (Puupponen-Pimia *et al.*, 2005)
	Flavonols	quercetin, myricetin, kaempferol	bilberry, cranberry, lingonberry, blackcurrant, bog whortleberry	(Cesoniene *et al.*, 2009) (Del Rio *et al.*, 2010) (Puupponen-Pimia *et al.*, 2005)
	Flavanols (Catechins)	(+)-catechin, (-)-epicatechin, gallocatechin, epigallocatechin	Cranberry	(Del Rio *et al.*, 2010)

Table 1: (continued). Phenolic compounds previously isolated from berries

Phenolic compounds			Fruits	References
Tannins	Condensed tannins	pro-anthocyanidins	blueberry, cranberry	(Puupponen-Pimia *et al.*, 2005) (Del Rio *et al.*, 2010)
	Hydrolysable tannins	ellagitannins, gallotannins	blackberry, black and red raspberry, strawberry	(Del Rio *et al.*, 2010) (Seeram, 2008)
Phenolic acids	Hydroxycinnamic acids	caffeic acid, p-coumaric acid, ferulic acid	present in low concentrations	(Del Rio *et al.*, 2010)
	Hydroxybenzoic acids	protocatechic acid, p-hydroxy benzoic acid, gallic acid	blackberry, strawberry, raspberry	(Del Rio *et al.*, 2010)

1.5.1 *Ribes nigrum*

Table 2: Botanical description of *Ribes nigrum*

Kingdom	Plantae (plants)
Division	Angiosperms
Class	Eudicots
Order	Saxifragales
Family	Grossulariaceae
Genus	*Ribes*
Species	*Ribes nigrum*

1.5.1.1 Botanical description

Ribes nigrum or known as blackcurrant is a medium sized shrub and can grow up to 1.5 m. The leaves are 3 to 5 cm broad and long with five palmate lobes and a serrated margin. They are rich source of natural antioxidants (Tabart *et al.*, 2011) and have been used in European traditional medicine for rheumatic disease (Garbacki *et al.*, 2004). The fruits are up to 12mm in diameter, purple-black in colour, sweet, aromatic, edible and seed-containing berries. All parts of the plant are strongly aromatic (Gopalan *et al.*, 2012).

Figure 2: *Ribes nigrum* fruits and leaf. The image was adapted from the website http://www.en.wikipedia.org/wiki/Blackcurrant. 'Blackcurrant. Wikipedia. The Free Encyclopedia'. Accessed October 2013.

1.5.1.2 Traditional uses

The fruits and leaves of *Ribes nigrum* have been used as traditional medicine for the treatment of a variety of ailments both in Asia and Europe (Suzutani *et al.*, 2003; Garbacki *et al.*, 2002). The fruit traditionally was used to soothe sore throats and to reduce fevers due to viral infection (Kuiken *et al.*, 2012). Decoctions of the leaves, bark and roots were used as traditional remedies for treatment of rheumatic diseases (Sasaki *et al.*, 2013). Traditional medicinal plants of *R. nigrum* have been reported as a rich source of bioactive compounds (Haasbach *et al.*, 2014).

1.5.1.3 Previous biological work

The potent antimicrobial, anti-inflammatory, antiviral and antioxidant effects of *R. nigrum* have been attributed to its constituents such as anthocyanins, flavonols, phenolic acids and polyunsaturated fatty acids (Bonarska-Kujawa *et al.*, 2014; Skold *et al.*, 2002). The phenolic compounds from *R. nigrum* leaves have a strong antioxidant effect on cell membranes (Bonarska-Kujawa *et al.*, 2014; Cyboran *et al.*, 2011). Phenolic compounds from the berries have also revealed activity against Gram-positive and Gram-negative bacteria (Zimmer *et al.*, 2014; Puupponen-Pimia *et al.*, 2001). Phenolic compounds can block the swarming motility of bacteria (O'may and Tufenkji, 2011). Work on the activity of *R. nigrum* leaves against *P. aeruginosa* strain PA14 biofilms has yet to be undertaken.

1.5.1.4 Previous phytochemical work

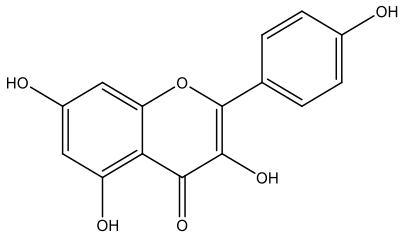
Ribes nigrum is a rich source of bioactive compounds, mostly phenolics (Gopalan *et al.*, 2012). The juice is a potent free scavenger and chelator of iron. The seed oil is rich with vitamin E and several unsaturated fatty acids such as alpha-linolenic acid and gamma-linolenic acid (Lu *et al.*, 2002) that are able to reduce skin lesion caused by atopic dermatitis (Gopalan *et al.*, 2012). Several phytochemicals such as polyphenols, flavonoids, polyunsaturated fatty acids, carbohydrates, non-volatile organic acids, tannins and lignans have been isolated from different parts of *R. nigrum* (Haasbach *et al.*, 2014; Jaworska *et al.*, 2011; Bordonaba and Terry, 2008).

The leaves are rich in flavonols, anthocyanidins and carotenoids (Tabart *et al.*, 2011). Flavonols have been identified as kaempferol-3-*O*-rutinoside while anthocyanins are delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside (Vagiri *et al.*, 2012). Lignans such as epoxylignans, tetrahydrofuran-type sesquilignans and spirocyclic dilignan have been identified in the leaves (Sasaki *et al.*, 2013).

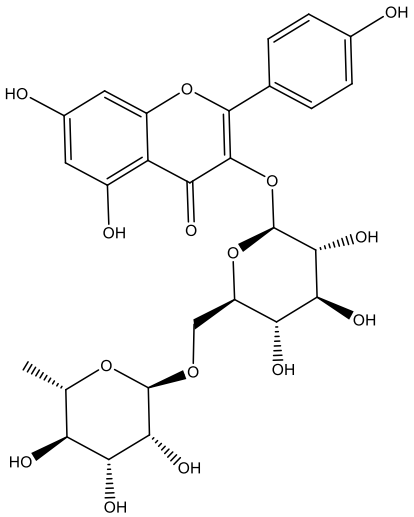
Kaempferol-3-O-rutinoside (Vagiri *et al.*, 2012) and prodelphinidin (Garbacki *et al.*, 2002) have been found in the leaves of *R. nigrum*. A previous study revealed that kaempferol glucoside was able to attenuate the growth and biofilm formation of *S. aureus* and *S. epidermis* strains (Bubulica et al., 2012). In contrast, kaempferol itself was unsuccessfull at inhibiting the *Candida tropicalis* biofilm formation (Rajasekharan *et al.*, 2014).

Table 3: Flavonoids previously isolated from *Ribes nigrum* leaves

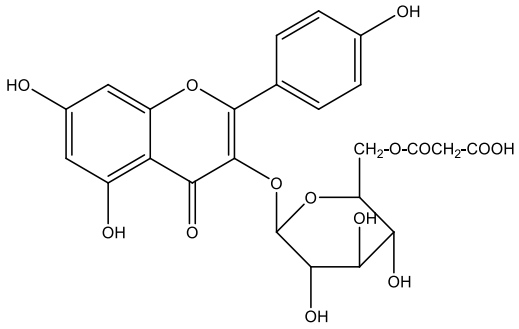
Compounds	References
Kaempferol **(R1)**	(Sasaki *et al.*, 2013)
	(Oszmianski *et al.*, 2011)
Kaempferol-3-*O*-rutinoside **(R2)**	(Vagiri *et al*., 2012)
Kaempferol-3-*O*-(6”-*O*-malonyl)-β-D-glucopyranoside **(R3)**	(Pieri *et al*., 2002)
Isorhamnetin glycosides **(R4)**	(Liu *et al.*, 2014)
Myricetin **(R5)**	(Tabart *et al.*, 2011)
	(Oszmianski *et al.*, 2011)
Quercetin **(R6)**	(Tabart *et al.*, 2011)
	(Oszmianski *et al.*, 2011)



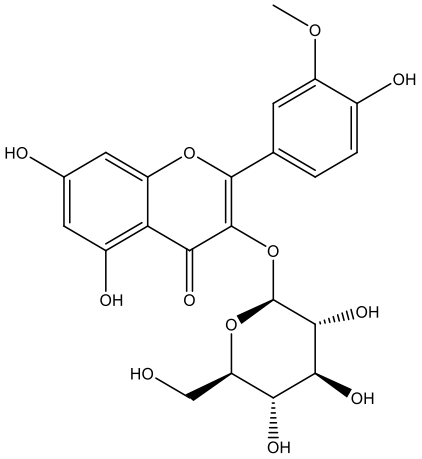
Kaempferol **(R1)**



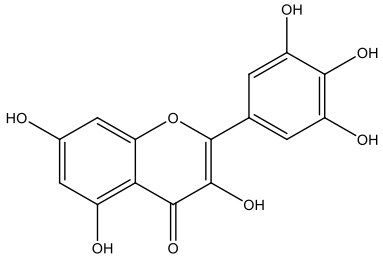
Kaempferol-3-*O*-rutinoside **(R2)**



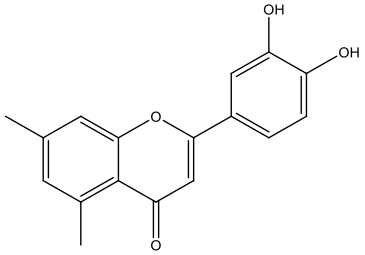
Kaempferol-3-*O*-(6'-*O*-malonyl)-β-D-glucopyranoside **(R3)**



Isorhamnetin-3-glucosides **(R4)**



Myricetin **(R5)**

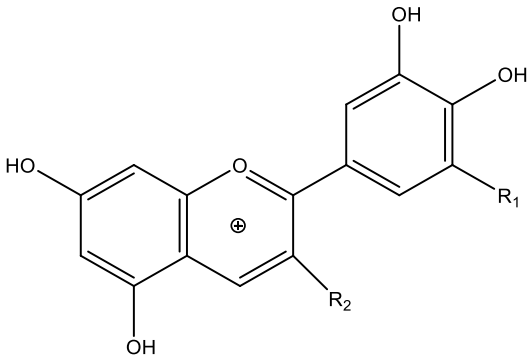


Quercetin **(R6)**

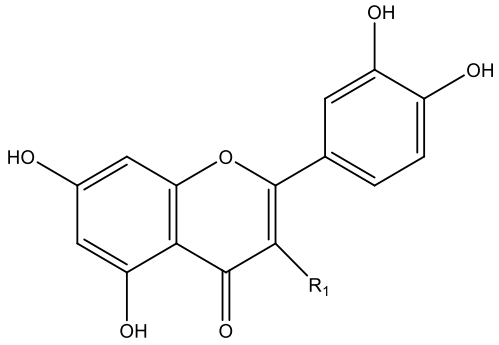
Figure 3: Flavonoids previously isolated from *Ribes nigrum* leaves

Table 4: Phenolic compounds previously isolated from *Ribes nigrum* leaves

Compounds		References
Flavanols	Catechin **(R7)**	(Vagiri *et al.*, 2012)
		(Tits *et al.*, 1992)
	Epicatechin **(R8)**	(Vagiri *et al.*, 2012)
		(Tits *et al.*, 1992)
	Gallocatechin **(R9)**	(Tits *et al.*, 1992)
	Epigallocatechin **(R10)**	(Vagiri *et al.*, 2012)
		(Tits *et al.*, 1992)
	Quercetin-3-O-galactoside **(R11)**	(Bonarska-Kujawa *et al.*, 2014)
		(Oszmianski *et al.*, 2011)
	Quercetin-3-(6”-malonyl)-	(Bonarska-Kujawa *et al.*, 2014)
	glucoside **(R12)**	(Oszmianski *et al.*, 2011)
	Quercetin-3-O-glucosyl-6”-acetate	(Bonarska-Kujawa *et al.*, 2014)
	(R13)	(Oszmianski *et al.*, 2011)
Antho-	Cyanidin-3-O-glucoside **(R14)**	(Vagiri *et al.*, 2012)
cyanidins		
	Cyanidin-3-O-rutinoside **(R15)**	(Vagiri *et al.*, 2012)
	Delphinidin-3-O-glucoside **(R16)**	(Vagiri *et al.*, 2012)
	Delphinidin-3-O-rutinoside **(R17)**	(Vagiri *et al.*, 2012)
	Chlorogenic acid **(R18)**	(Bonarska-Kujawa *et al.*, 2014)
		(Vagiri *et al.*, 2012)

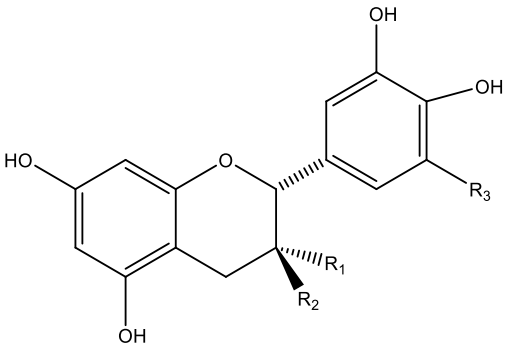


		R_1	R_2	R_3
Catechin	**(R7)**	H	OH	H
Epicatechin	**(R8)**	OH	H	H
Gallocatechin	**(R9)**	H	OH	OH
Epigallocatechin	**(R10)**	OH	H	OH

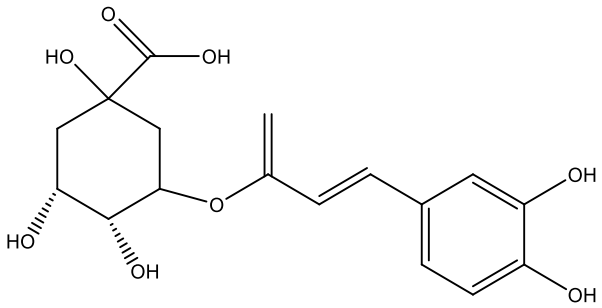


		R_1
Quercetin-3-*O*-galactoside	**(R11)**	glucose
Quercetin-3-(6”-malonyl)-glucoside	**(R12)**	(6”-malonyl)-glucose
Quercetin-3-*O*-glucosyl-6”-acetate	**(R13)**	glucosyl-6”-acetate

Figure 4: Phenolic compounds previously isolated from *Ribes nigrum* leaves



		R_1	R_2
Cyanidin-3-*O*-glucoside	**(R14)**	H	glucose
Cyanidin-3-*O*-rutinoside	**(R15)**	H	rutinose
Delphidin-3-*O*-glucoside	**(R16)**	OH	glucose
Delphidin-3-*O*-rutinoside	**(R17)**	OH	rutinose

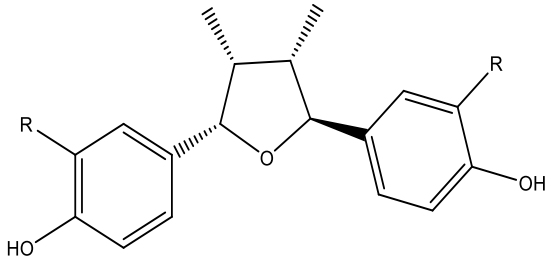


Chlorogenic acid **(R18)**

Figure 4: (continued). Phenolic compounds previously isolated from *Ribes nigrum* leaves

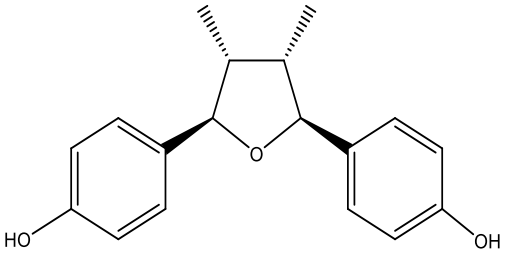
Table 5: Lignoids previously isolated from *Ribes nigrum* leaves

Compounds	References
Larreatricin **(R19)**	(Sasaki *et al.*, 2013)
3,3'-dimethoxylarreatricin **(R20)**	(Sasaki *et al.*, 2013)
3,3'-didemethoxynectandrin B **(R21)**	(Sasaki *et al.*, 2013)
Roseside **(R22)**	(Sasaki *et al.*, 2013)
Icariside B1 **(R23)**	(Sasaki *et al.*, 2013)
Methyl ρ-coumarate **(R24)**	(Sasaki *et al.*, 2013)
Ribesin A-H **(R25a-h)**	(Sasaki *et al.*, 2013)

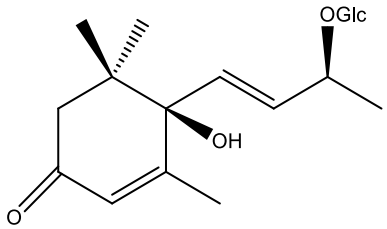


Larreatricin **(R19)**, R=H

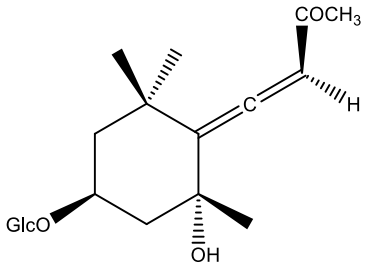
3,3’-dimethoxylarreatricin **(R20)**, R=OCH_3



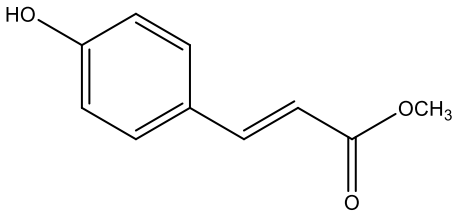
3,3’-didemethoxynectandrin B **(R21)**



Roseside **(R22)**

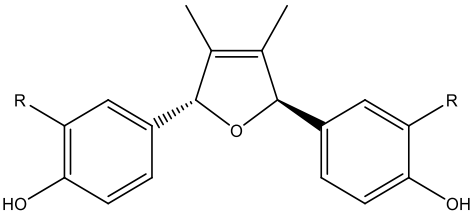


Icariside B1 **(R23)**



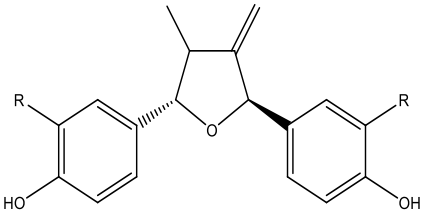
Methyl ρ-coumarate **(R24)**

Figure 5: Lignoids previously isolated from *Ribes nigrum* leaves



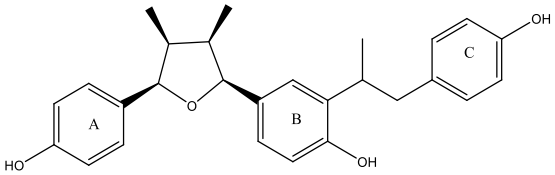
Ribesin A **(R25a)**, R=H

Ribesin B **(R25b)**, R=OCH_3

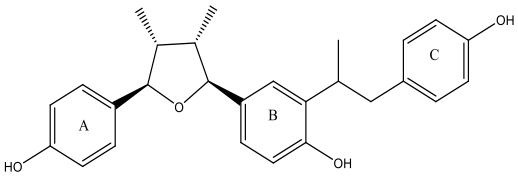


Ribesin C **(R25c)**, R=H

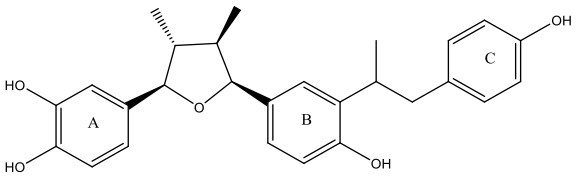
Ribesin D **(R25d)**, R=OCH_3



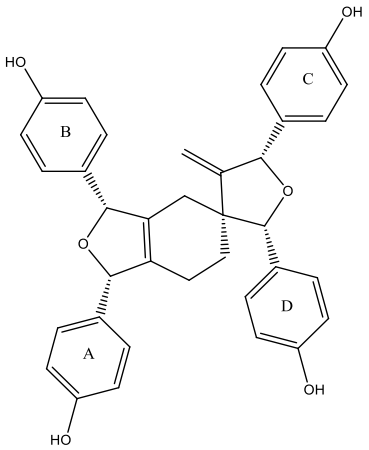
Ribesin E **(R25e)**



Ribesin F **(R25f)**



Ribesin G **(R25g)**



Ribesin H **(R25h)**

Figure 5: (continued). Lignoids previously isolated from *Ribes nigrum* leaves

1.5.2 *Sambucus nigra*

Table 6: Botanical description of *Sambucus nigra*

Kingdom	Plantae (plants)
Division	Angiosperms
Class	Eudicots
Order	Dipsacales
Family	Adoxaceae
Genus	*Sambucus*
Species	*Sambucus nigra*

1.5.2.1 Botanical description

Sambucus nigra or known as elderberry is a wide and small shrub growing up to 6 m tall. The leaflets are 5–12 cm long and 3–5 cm broad, with a serrated margin arranged in opposite pairs and pinnate with five to seven leaflets. The hermaphrodite flowers are borne in large, flat corymbs 10–25 cm diameter in late spring to middle summer. The individual flowers are ivory white, 5–6 mm diameter with five petals and generally pollinated by flies. The fruit is a glossy dark purple to black with 3 to 5 mm diameter and produced in drooping clusters in late autumn (Vikramaditya and Sharma, 1999).

(a) (b)

Figure 6: The fruits (a) and flowers (b) of *Sambucus nigra*. The images were adapted from the website http://www.en.wikipedia.org/wiki/Sambucus_nigra. '*Sambucus nigra*. Wikipedia. The Free Encyclopedia'. Accessed October 2013.

1.5.2.2 Traditional uses

The uses of *Sambucus nigra* range from food flavouring, alcoholic drinks to medical purposes. The bark, leaves, flowers, fruits, and root extracts from *S. nigra* have been used as folk medicine to treat respiratory infections, skin injuries, diabetes wounds and as a diuretic (Barros *et al.*, 2012). In Cyprus, the boiled *S. nigra* flowers are a well-known treatment in traditional medicine for ocular infections (Aristoteli and Willcox, 2006). The flowers and leaves are also used for pain relief, swelling or inflammation and as a diaphoretic or expectorant (Mairura, 2007). The unripe fruits, bark, leaves and seeds are used as an emollient ointment in homeopathy (Vikramaditya and Sharma, 1999) and as explained in Monograph of *Sambucus nigra* (2005).

1.5.2.3 Previous biological work

The flowers of *S. nigra* have been used as expectorant to relieve dry cough and remedy for inflammation by colds and fever (Lopez-Garcia *et al.*, 2013; Barros *et al.*, 2012). A study published in the Journal of Alternative Complementary Medicine revealed that *S. nigra* is effective for treating patients with Influenza B (Zakay-Rones *et al.*, 1995). Polyphenols from *S. nigra* flowers incorporated onto atelocollagen matrices have been evaluated a candidates to promote keratinocytes cell growth and proliferation of tissues (Lopez-Garcia *et al.*, 2013). The ribosome-inactivating proteins (RIP) from *S. nigra* were found excellent candidates for the construction of immunotoxins and conjugates for cancer therapy (Ferreras *et al.*, 2011).

1.5.2.4 Previous phytochemical work

Sambucus nigra contain a variety of bioactive flavonoids, vitamins and lectins (Veberic *et al.*, 2009). Sambucus lectins such as ebulins and nigrins exhibit a ribosome-inactivating activity (Ferreras *et al.*, 2011; Girbes *et al.*, 2003). *S. nigra* is a poisonous plant to animals with all parts, except flowers and ripe berries, containing mildly poisonous compounds (Jimenez *et al.*, 2014).

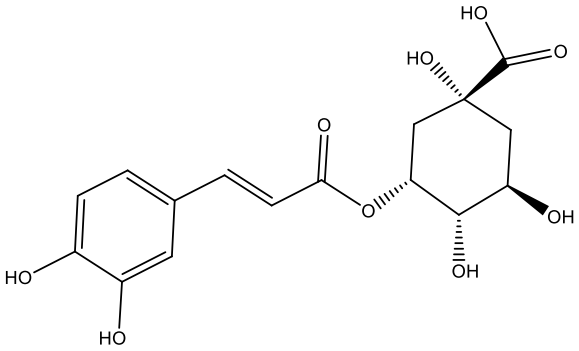
S. nigra flower extracts consist of pyranoid and furanoid compounds from linalool oxide, hotrienol and linalool (Joulain, 1987). Other compounds are hydrocarbons, ethers and oxides, ketones, aldehyde, alcohols, esters and acids (Toulemonde and Richard, 1983) and flavonoid rutin (3-rhamnoglucoside) (Davídek 1961).

Flavonols such as rutin, isoquercitrin and astragalin are present in *S. nigra* fruits whilst anthocyanins such as cyaniding-3-sambubioside and cyaniding-3-glucoside are the most abundant phytochemicals present in the fruits (Veberic *et al.*, 2009).

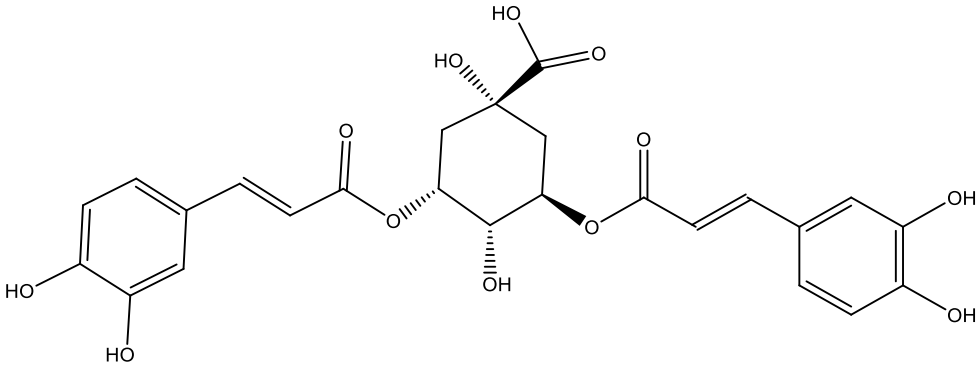
Rutin is the most abundant flavonol contained in the leaves and flowers of *S. nigra* (Dawidowicz *et al.*, 2006). The flowers of *S. nigra* contain a high amount of phenolic acids such as hydroxycinnamic acids and dihydroxybenzoic acids (Lopez-Garcia *et al.*, 2013; Barros *et al.*, 2012).

Table 7: Phenolic compounds previously isolated from *Sambucus nigra*

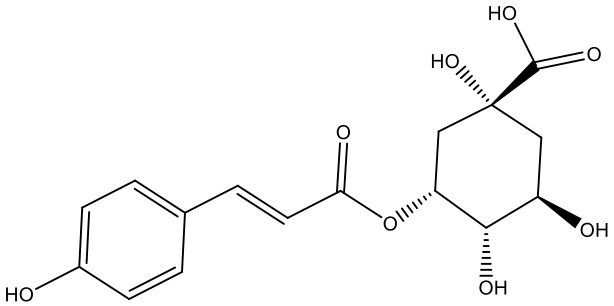
		Compound	References
Phenolic acids	flower	5-*O*-caffeoylquinic acid **(S1)**	(Barros *et al.*, 2012)
	flower	3,5-*O*-dicaffeoylquinic acid **(S2)**	(Barros *et al.*, 2012)
	flower	5-*O*-*p*-coumaroylquinic acid **(S3)**	(Barros *et al.*, 2012)
Anthocyanins	fruit	Cyanidin 3-sambubioside **(S4)**	(Veberic *et al.*, 2009)
	fruit	Cyanidin 3-glucoside **(S5)**	(Veberic *et al.*, 2009)
Flavonols	flower fruit	Quercetin-3-*O*-rutinoside **(S6)**	(Barros *et al.*, 2012), (Rieger *et al.*, 2008), (Veberic *et al.*, 2009)
	flower	Quercetin-3-*O*-glucoside **(S7)**	(Barros *et al.*, 2012)
	flower	Kaempferol-3-*O*-rutinoside **(S8)**	(Barros *et al.*, 2012)
	flower	Isorhamnetin-3-*O*-rutinoside **(S9)**	(Barros *et al.*, 2012)
	flower	Isorhamnetin-3-*O*-glucoside **(S10)**	(Barros *et al.*, 2012)



5-*O*-caffeoylquinic acid **(S1)**

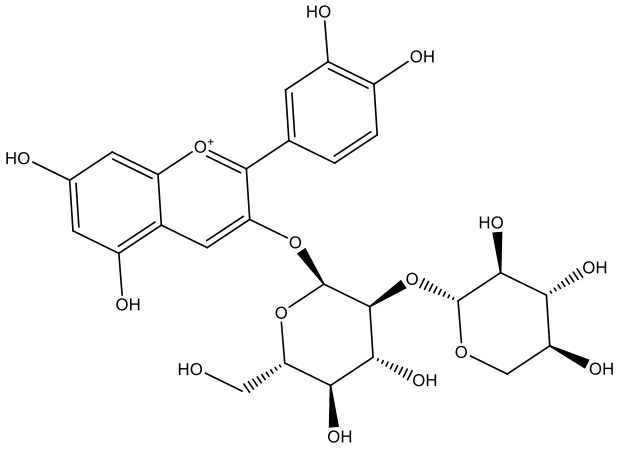


3,5-*O*-dicaffeoylquinic acid **(S2)**

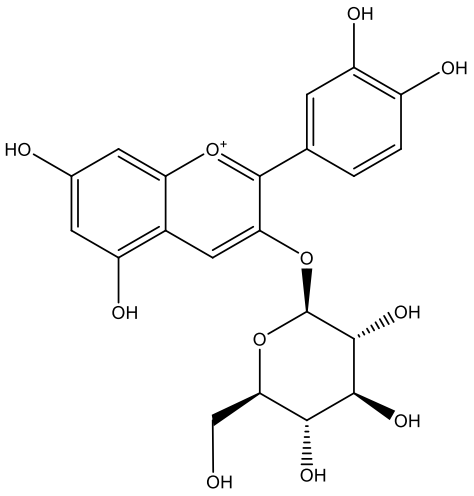


5-*O*-*p*-coumaroylquinic acid **(S3)**

Figure 7: Phenolic compounds previously isolated from *Sambucus nigra*

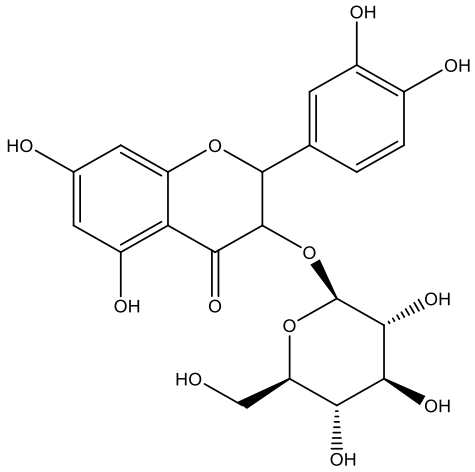


Cyanidin-3-sambubioside **(S4)**

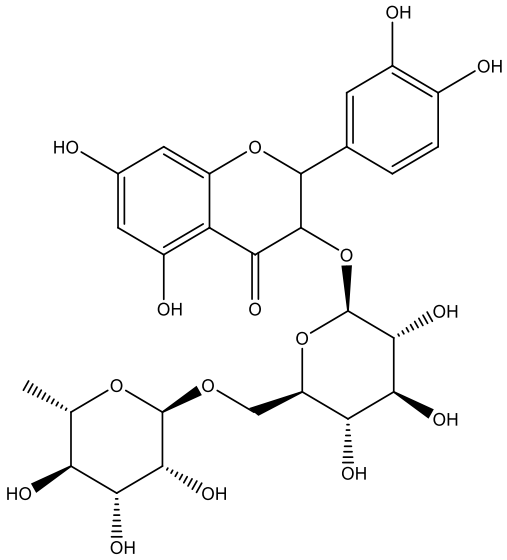


Cyanidin-3-glucoside **(S5)**

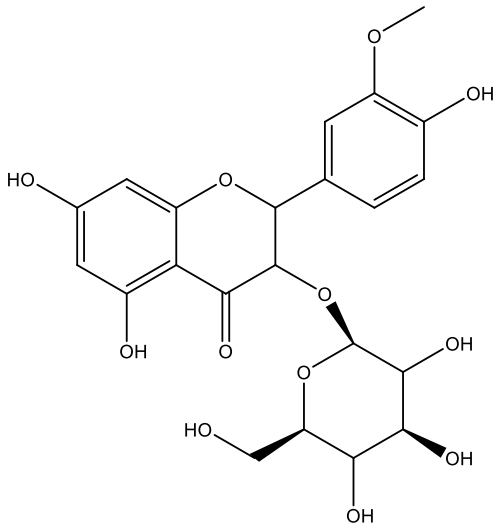
Figure 8: Anthocyanins previously isolated from *Sambucus nigra*



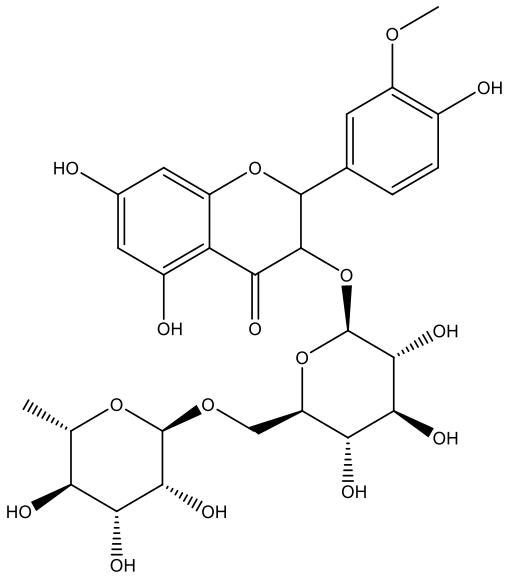
Quercetin-3-*O*-glucoside **(S6)**



Quercetin-3-*O*-rutinoside **(S7)**

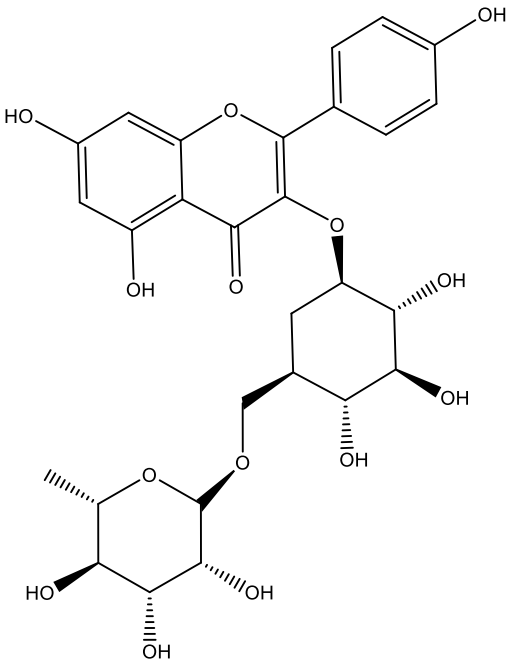


Isorhamnetin-3-*O*-glucoside **(S8)**



Isorhamnetin-3-*O*-rutinoside **(S9)**

Figure 9: Flavonols previously isolated from *Sambucus nigra*



Kaempferol-3-O-rutinoside **(S10)**

Figure 9: (continued). Flavonols previously isolated from *Sambucus nigra*

1.6 Herbs and spices

Herbs and spices have been traditionally exploited as aroma and flavour enhancers, in food preservation as well as for their medicinal properties. There are no clear distinction between culinary herbs and spices in the scientific literature and some plants can even be both.

Herbs and spices containing simple phenols, phenolic acids, coumarins, terpenoids and alkaloids at which possess antimicrobial activity (Bergonzelli *et al.*, 2003; Cowan, 1999). Many spices work synergistically in displaying antimicrobial (Billing and Sherman, 1998) and have either a broad or narrow spectrum antibacterial effect (Liu and Nakano, 1996). For example, garlic extracts exhibit bactericidal activity similar to antibiotics against *Escherichia coli, Salmonella* and *Vibrio cholera* (Arora and Kaur, 1999).

Herbs and spices tend to inhibit Gram-positive bacteria more than Gram-negative bacteria. Sporulating Gram-positive bacteria such as *Bacillus* spp. also appear to be more sensitive to herbs and spices in slightly acidic or salty media (Yano *et al.*, 2006). In most cases, the mechanisms of action for the antimicrobial activity of herbs and spices are not completely understood (Cowan, 1999; De *et al.*, 1999) as they contain several different phytochemicals that may involve multiple modes of action against bacteria (Lambert *et al.*, 2001).

The potential mechanisms of antimicrobial activity of herbs and spices include interference with the phospholipid bilayer of the bacterial membrane resulting in a greater permeability, loss of cellular components, impaired enzyme systems required for the production of energy and structural components, and inactivation or

destruction of genetic material (Liu and Nakano, 1996). The classification of phytochemicals in herbs and spices and summary of bioactive compounds in culinary herbs and spices is presented in Table 8 and Table 9, respectively.

Table 8: Classification of phytochemicals in herbs and spices

Group	Compounds[a]	Plant of example(s)
Polyphenols-	Hesperitin	Peppermint
Flavonoids:	Naringenin	Rosemary
Flavanones	Eriodictyol	Peppermint
Flavones	Apigenin	Parsley, thyme
	Luteolin	Oregano, parsley, peppermint, rosemary
Flavonols	Quercetin, kaempferol	Basil, coriander, cumin, fennel
	Isorhamnetin	Dill, parsley, tarragon
	Myrcetin	Dill, oregano, parsley
Phenolic acids:	Gallic acid	Thyme
Hyroxybenzoic acid	Vanillic acid	Sage
derivatives	Salicylic acid	Cumin
Hydroxycinnamic	Caffeic acid	Fennel
acid derivatives	ρ-coumaric acid	Cumin
Terpenes:	Limonene	Cumin, thyme, rosemary, caraway, mint, dill,
Monoterpenes		celery seed, sage, coriander, fennel,marjoram
	Citral	Thyme, sage
	Camphor	Thyme, sage, rosemary, marjoram, fennel,
		coriander, basil
	Menthol	Peppermint, basil
	Perillyl alcohol	Spearmint, sage
Sesquiterpenes	Humulene, caryophyllene	Turmeric, coriander
Diterpenes –	Retinol	Paprika, red pepper, chilli powder
Retinoids		
Triterpenes –	Glycyrrhizin	Licorice
Saponins		
Tetraterpenes	Carotenoids	Mustard, fennel, cumin, coriander, sage
Vanilloids:	Curcumin	Turmeric, ginger, mustard
	Gingerol	Ginger
	Paradol	Ginger oleoresin
	Capsaicin	Paprika, red pepper
Organosulfur:	Diallyldisulfide	Garlic
	Allicin	Onion

[a] Table is based on adapted sources from (Jakobsen *et al.*, 2012; Msaada *et al.*, 2009; Tiwari *et al.*, 2009; Kaefer and Milner, 2008; Shan *et al.*, 2007)

Table 9: Bioactive compounds in culinary herbs and spices

Plants	Bioactive compounds	Reference(s)
Basil	Eugenol, apigenin, limonene, ursolic acid, methyl cinnamate, 1,8-cineole, α-terpinene, anthocyanins, β-sitosterol, carvacrol, cintronellol, farnesol, geraniol, kaempferol, menthol, ρ-coumairc acid, quercetin, rosmarinic acid, rutin, safrole, tannin, catechin	(Kaefer and Milner, 2008) (Witkowska et al., 2013)
Caraway	Carvone, limonene, α-pinene, kaempferol	(Kaefer and Milner, 2008)
Cinnamon	Cinnamic aldehyde, 2-hydroxycinnamaldehyde, eugenol	(Kaefer and Milner, 2008) (Witkowska et al., 2013)
Cloves	Eugenol, isoeugenol, gallic acid	(Kaefer and Milner, 2008) (Witkowska et al., 2013)
Coriander	Quercetin, caffeic acid, cineole, geraniol, borneol, 1,8-cineole, α-terpinene, β-carotene, β-pinene, β-sitosterol, cinnamic acid, ferrulic acid, γ-terpinene, kaempferol, limonene, myrcene, ρ-coumaric acid, quercetin, tannin, thymol	(Kaefer and Milner, 2008) (Msaada *et al.*, 2009); (Witkowska et al., 2013)
Cumin	α-pinene, β-pinene, γ-terpinene, ρ-cymene, cuminaldehyde, carvone, 1,8-cineole, β-carotene, β-sitosterol, caffeic acid, carvacrol, carvaol, geranial, kaempferol, limonene, ρ-coumairc acid, quercetin, tannin, thymol	(Kaefer and Milner, 2008) (Witkowska et al., 2013)

Table 9: (continued). Bioactive compounds in culinary herbs and spices

Plants	Bioactive compounds	Reference(s)
Fennel	α-pinene, β-carotene, limonene, quercetin, benzoic acid, β-sitosterol, caffeic acid, cinnamic acid, ferulic acid, fumaric acid, kaempferol, myristicin, 1,8-cineole, ρ-coumairc acid, quercetin, rutin, vanillic acid, vanillin	(Kaefer and Milner, 2008) (Witkowska *et al*., 2013)
Garlic	Allicin, diallyldisulfide, ally isothiocyanate	(Kaefer and Milner, 2008) (Harjai *et al.*, 2010) (Jakobsen *et al.*, 2012)
Ginger	Zingiberone, zingiberene, ingerol, paradol, curcumin, shagoal	(Kaefer and Milner, 2008) (Kim and Park, 2013)
Marjoram	Eugenol, limonene, ursolic acid, 1,8-cineole, α-pinene, α-terpinene, carvacrol, farnesol, geraniol, ρ-cymene, rosmarinic acid, sterols, thymol, apigenin	(Kaefer and Milner, 2008) (Witkowska *et al*., 2013)
Onion	Quercetin, dipropyldisulfides	(Kaefer and Milner, 2008) (Witkowska *et al*., 2013)
Paprika	α-tocopherol, capsaicin, dihydrocapsaicin, lutein, β-carotene, ascorbic acid, Vitamin E	(Kaefer and Milner, 2008) (Witkowska *et al*., 2013)
Parsley	Apigenin, luteolin, kaempferol, myricetin, quercetin, caffeic acid	(Kaefer and Milner, 2008) (Witkowska *et al*., 2013)
Pepper, black	Piperidine, piperine, limonene, α-pinene, β-pinene	(Kaefer and Milner, 2008) (Witkowska *et al*., 2013)

Table 9: (continued). Bioactive compounds in culinary herbs and spices

Plants	Bioactive compounds	Reference(s)
Pepper, red	Capsaicin, α-tocopherol, lutein, β-carotene, ascorbic acid, Vitamin E	(Kaefer and Milner, 2008)
Peppermint	Limonene, menthol, eriodictyol, hesperitin, apigenin, luteolin	(Kaefer and Milner, 2008)
Rosemary	Carnasol, carnosic acid, cineole, geraniol, α-pinene, β-carotene, apigenin, limonene, naringin ,luteolin, caffeic acid, rosmarinic acid, rosmanol, vanillic acid	(Witkowska *et al*., 2013) (Lang and Buchbauer, 2012)
Sage	α-pinene, β-sitosterol, citral, farnesol, ferulic acid, gallic acid, geraniol, limonene, cineole, perillyl alcohol, β-carotene, catechin, apigenin, luteolin, saponin, ursolic acid, rosemarinic acid, carnosic acid, vanillic acid, caffeic acid, thymol, eugenol	(Witkowska *et al*., 2013)
Tarragon	Luteolin, isorhamnetin, kaempferol, quercetin, caffeic acid	(Kaefer and Milner, 2008) (Cowan, 1999)
Thyme	Thymol, carvacrol, cineole, α-pinene, apigenin, β-carotene, eugenol, limonene, ursolic acid, luteolin, gallic acid, caffeic acid, rosmarinic acid, carnosic acid, hispidulin, cismaritin	(Kaefer and Milner, 2008) (Shan *et al.*, 2007)
Turmeric	Curcumin, curcuminoids	(Kaefer and Milner, 2008) (Witkowska et al., 2013)

1.6.1 *Coriandrum sativum*

Table 10: Botanical description of *Coriandrum sativum*

Kingdom	Plantae (plants)
Division	Magnoliophyta (flowering plants)
Class	Magnoliopsida (dicotyledons)
Order	Apiales
Family	Apiaceae (the carrot family)
Genus	*Coriandrum*, L
Species	*Coriandrum sativum* L.

1.6.1.1 Botanical description

Coriandrum sativum is an annual, herbaceous plant originally native to the Mediterranean and Middle Eastern regions. *C. sativum* is widely cultivated in North Africa, Central Europe (Sriti et al., 2010) and Asia (Singh and Barrett, 2006). The plant is 25-60 cm in height with thin, spindle-shaped roots, erect stalk, alternate leaves and small, pinkish-white flowers (Figure 10). The seeds can either be grounded and used as spice or distilled to produce an essential oil used in condiments and liquors. The leaves are extensively used in cooking whereas the seeds of *C. sativum* are popular for its oil and as a culinary spice (Momin *et al.*, 2012).

(a)

(b)

Figure 10: *Coriandrum sativum* dried seeds (a) and fresh leafy plant (b)

1.6.1.2 Traditional uses

Coriandrum sativum is of great economic importance. The leaves, seeds and the oil are used as flavouring agents, adjuvant, cosmetic, perfumes and have some medicinal uses. Since ancient times the plant has been used as a traditional remedy. In Chinese, Ayurveda and Greco-Arab folk medicine (Momin *et al.*, 2012; Patwardhan *et al.*, 2005), *C. sativum* has been used to treat gastrointestinal disorders such as digestive problems, flatulence, vomiting and diarrhoea (Sahib *et al.*, 2013).

1.6.1.3 Previous biological work

The essential oil (EO) from coriander seed has demonstrated activity against both Gram-negative and Gram-positive food-borne bacteria including *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* (Delaquis *et al.*, 2002) and also inhibited *Campylobacter jejuni* on meat (Rattanachaikunsopon and Phumkhachorn, 2010). Similarly, EO from coriander leaves has showed activity against both Gram-negative bacteria such as *E. coli, Salmonella typhi, Klebsiella pneumonia, Proteus mirabilis,* Gram-positive bacteria such as *S. aureus* and *Bacillus spp.* and the yeast *Candida albicans*. The EO however did not show any activity against non-clinical isolates of *Pseudomonas aeruginosa* (Matasyoh *et al.*, 2009).

C. sativum essential oil consists of non-phenolic monoterpenic compounds with the main constituents being monocyclic non-phenolic (e.g. 1,8-cineole, pulegone) and bicyclic monoterpenes (e.g. camphor, borneol, α-pinene) (Lang and Buchbauer, 2012). The limonene, α-pinene, *3*-carene and *n*-octyl acetate had shown antibiofilm activity against already existing biofilms (Schillaci *et al.*, 2008) and carvacrol was

active against *S. aureus* and *S. epidermis* biofilms (Nostro *et al.*, 2009; O'toole and Kolter, 1998). A strong antimicrobial activity was reported from the oxygenated compounds such as 1,8-cineole and bornyl acetate, and hydrocarbon compounds such as α-pinene, camphene and limonene (Schmidt *et al.*, 2010). A bactericidal effect was reported for *C. sativum* oil against the Gram-negative bacterium *Camphylobacter jejuni*, however the active phytochemicals were not revealed (Rattanachaikunsopon and Phumkhachorn, 2010).

The ability of coriander essential oil to disrupt the microbial cell membrane is the main reason for lethal action against the microorganisms (Skaltsa *et al.*, 2003; Carson and Riley, 1995, 1993). Ketones, aldehydes and alcohols are present in the essential oil of *C. sativum*. They display an antimicrobial activity associated with their compound's functional groups and their hydrogen-bonding capability (Skaltsa *et al.*, 2003).

1.6.1.4 Previous phytochemical work

An essential oil of *C. sativum* contains fatty acids and terpenoids which contribute to its aroma and flavour (Sahib *et al.*, 2013). The seeds contain linalool, geranyl acetate and petroselinic acid whereas the aerial parts contain phenolic compounds, apigenin, catechin, coumaric acid, aliphatic alkenals and aldehyde (Momin et al., 2012). The fruits also contain linalool, geranyl acetate and other compounds such as camphor, humulene, caryophyllene, borneol and menthol (Msaada *et al.*, 2009).

The major compound of *C. sativum* seeds is the terpene alcohol, linalool also known as coriandrol (Momin et al., 2012). The (S)-(+)-linalool isomer is present and

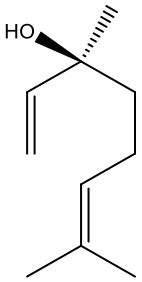
perceived as sweet and floral, the other isomer (R)-(-)-linalool or licareol has a more woody and lavender-like odour. Other alcohols such as geraniol, terpinen-4-ol and alpha-terpineol are found in the seeds (Naquvi *et al.*, 2012). Other parts of *C. sativum* such as the roots, leaves and stems contain some phenolic compounds (Tang *et al.*, 2013).

Phenolic compounds and terpenoids have showed activity against bacteria, fungi, viruses and protozoa (Lang and Buchbauer, 2012; Tiwari *et al.*, 2009; Suzutani *et al.*, 2003). Their mechanism of action has not been fully explained but it has been suggested that it involves an alteration of membrane permeability resulting in a leakage of intracellular materials (Thomadsen *et al.*, 2010). Compounds isolated from *C. sativum* to date are compiled in Table 11 to 14 and Figure 11 to 14. The *n*-hexane extract from *C. sativum* seed crude oil consists of neutral lipid, glycolipid and phospholipid showed a strong antioxidant property (Ramadan *et al.*, 2003). Lipopolysaccharides (Bandara *et al.*, 2010) and sulphated polysaccharides (Sato *et al.*, 1998) have been reported with antibiofilm activity (Rendueles *et al.*, 2013).

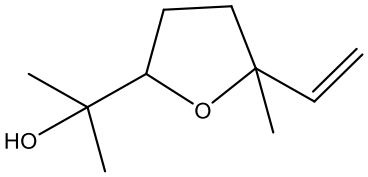
Research also revealed two aliphatic lactone namely coriander lactone and hydroxyl coriander lactone from the methanol extract of *C. sativum* seeds (Naquvi *et al.*, 2012). Other phytochemicals detected are glyceryl-1,2-dioctadec-9,12-dienoate-3-octadex-9-enoate; glyceryl-1,2,3-trioctadecanoate; *n*-nonadecanyl-n-docos-11-enoate and oleiyl glycoside.

Table 11: Terpene alcohols previously isolated from *Coriandrum sativum*

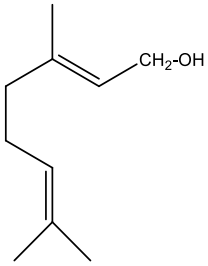
Compound	Plant part	References
Linalool **(C1)**	Seed	(Silva et al., 2011)
	Fresh plant	(Burdock and Carabin 2009)
		(Matasyoh *et al*., 2009)
Linalool oxide **(C2)**	Seeds	(Msaada *et al.*, 2009)
Geraniol **(C3)**	Seeds	(Chung *et al.*, 2012)
	Leaves	(Lo Cantore *et al*., 2004)
Nerol **(C4)**	Seeds	(Msaada *et al.*, 2009)
α-terpineol **(C5)**	Seeds	(Lo Cantore *et al*., 2004)
Terpinen-4-ol **(C6)**	Seeds	(Gil *et al.*, 2002)
Dihydrocarveol **(C7)**	Seeds	(Lo Cantore *et al*., 2004)



(*S*)-(+)-linalool **(C1)**

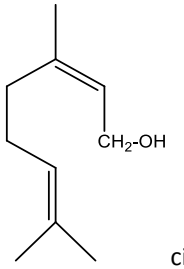


Linalool oxide **(C2)**



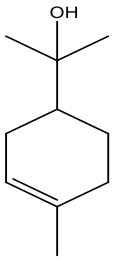
trans isomer

Geraniol **(C3)**

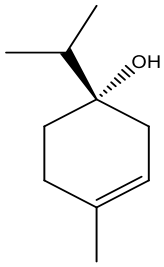


cis isomer

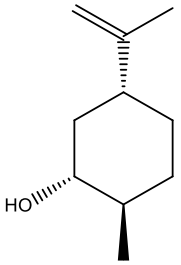
Nerol **(C4)**



α-terpineol **(C5)**



Terpinen-4-ol **(C6)**

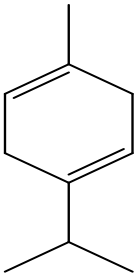


Dihydrocarveol **(C7)**

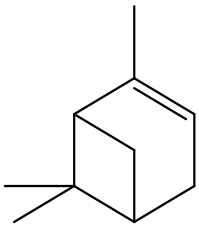
Figure 11: Terpene alcohols previously isolated from *Coriandrum sativum*

Table 12: Monoterpenes and sesquiterpenes previously isolated from *Coriandrum sativum*

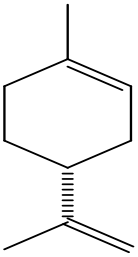
Compound	Plant part	References
γ-terpinene **(C8)**	Seeds	(Burdock and Carabin, 2009)
		(Lo Cantore *et al.*, 2004)
	Leaves	(Grosso *et al.*, 2010)
α-pinene **(C9)**	Seeds	(Matasyoh *et al.*, 2009)
		(Lo Cantore *et al*., 2004)
Limonene **(C10)**	Seeds	(Grosso *et al*., 2010)
		(Lo Cantore *et al*., 2004)
Camphene **(C11)**	Seeds	(Grosso *et al*., 2010)
		(Lo Cantore *et al*., 2004)
r-cymene **(C12)**	Seeds	(Grosso *et al*., 2010)
		(Lo Cantore *et al*., 2004)
Myrcene **(C13)**	Seeds	(Grosso *et al*., 2010)
		(Lo Cantore *et al*., 2004)
Caryophyllene **(C14)**	Seeds	(Lo Cantore *et al*., 2004)



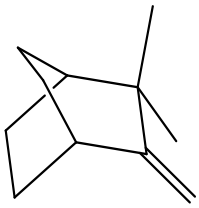
γ-Terpinene **(C8)**



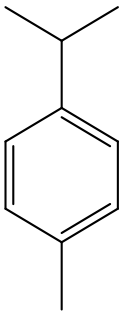
Pinene **(C9)**



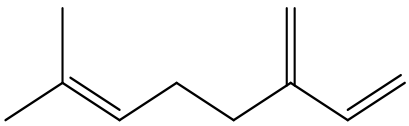
Limonene **(C10)**



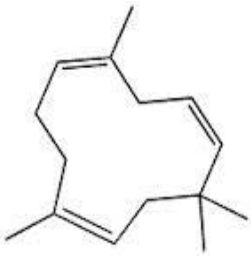
Camphene **(C11)**



Cymene **(C12)**



Myrcene **(C13)**

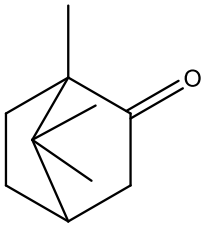


Caryophyllene **(C14)**

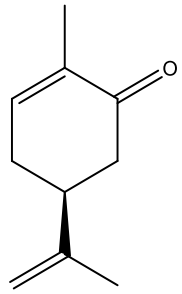
Figure 12: Monoterpenes previously isolated from *Coriandrum sativum*

Table 13: Monoterpenes ketones and esters previously isolated from *Coriandrum sativum*

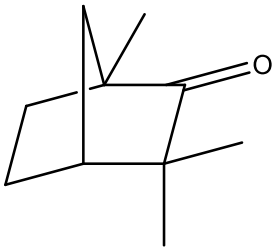
	Compound	Plant part	References
Ketones	Camphor **(C15)**	Seeds	(Grosso *et al.*, 2010) (Msaada *et al.*, 2009)
	Carvone **(C16)**	Seeds	(Lo Cantore *et al.*, 2004)
	Fenchone **(C17)**	Seeds	(Lo Cantore *et al*., 2004)
Esters	Geranyl acetate **(C18)**	Seeds	(Sahib *et al*., 2013) (Burdock and Carabin, 2009) (Msaada *et al.*, 2009)
	Linalyl acetate **(C19)**	Seeds	(Sahib *et al.*, 2013)



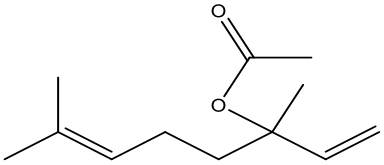
Camphor **(C15)**



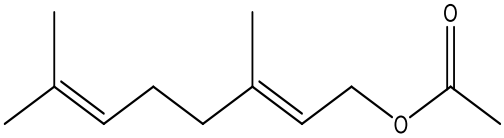
Carvone **(C16)**



Fenchone **(C17)**



Linalyl acetate **(C19)**

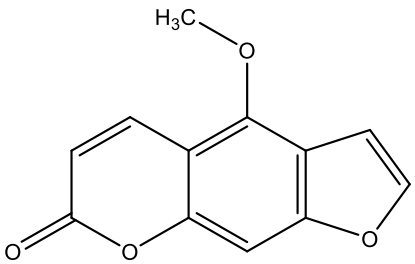


Geranyl acetate **(C18)**

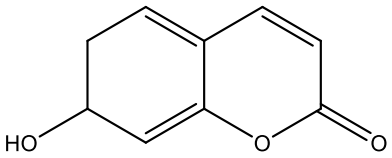
Figure 13: Monoterpenes ketones and esters previously isolated from *Coriandrum sativum*

Table 14: Coumarins previously isolated from *Coriandrum sativum*

Compound	Plant part	References
Bergapten **(C20)**	Seeds	(Rieger *et al.*, 2008) (Ramadan *et al.*, 2003)
Umbelliferone **(C21)**	Seeds	(Ramadan *et al.*, 2003)



Bergapten **(C20)**



Umbelliferone **(C21)**

Figure 14: Coumarins previously isolated from *Coriandrum sativum*

1.7 Aims and objectives

The aim of this study was to identify novel compounds from a selection of medicinal plants including berry crops, spices and herbs, with activity against biofilms formed by the clinical *Pseudomonas aeruginosa* strain PA14.

The objectives of the work were:

- To extract compounds from a selection of medicinal plants and screen them using antimicrobial and antibiofilm analyses for activity against clinical bacteria of *Pseudomonas aeruginosa* strain PA14.
- To fractionate selected extracts with antibiofilm activity into small fractions and isolate the active compounds using bioassay-guided fractionation methods and chromatographic techniques.
- To elucidate and identify the molecular structure of the active compounds with novel antibiofilm activity using spectroscopic techniques.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Solvents and chemicals

2.1.1 Solvents and chemicals used in phytochemical work

Solvents for extraction and chromatographic analyses were purchased either from Fisher Scientific UK or VWR UK and solvents for NMR analyses were purchased from Sigma-Aldrich UK. Chemicals and consumables were purchased from Sigma-Aldrich UK and VWR UK. Pure compounds were purchased from Sigma-Aldrich UK or unless stated otherwise.

Solvents - extraction and chromatographic analyses

Acetone (HPLC grade)

Acetic acid glacial (Analytical grade)

n-Butanol (HPLC grade)

Ethyl acetate (HPLC grade)

n-Hexane (HPLC grade)

Methanol (HPLC grade)

Dichloromethane (HPLC grade)

Sulfuric acid (Analytical grade)

n-Pentane (HPLC grade)

Solvents - NMR analyses

Deuterated chloroform, $CDCl_3$ (99.9 % purity)

Deuterated methanol, CD_3OD (99.9 % purity)

Chemicals and consumables

p-Anisaldehyde (Sigma-Aldrich, UK)

Lipophilic Sephadex® LH-100 (; Sigma-Aldrich, UK)

Silica gel 60 (column grade, 0.063-0.20 mm; Merck, Germany)

Silica gel 60H (TLC grade; 200-300 mesh; Merck, Germany)

TLC silica gel 60 F_{254} Aluminium sheets (20 x 20 cm; Merck, Germany)

Filter paper QL 100 (qualitative, 125 mm diameter; Fisher, UK)

Pure compounds

(-) - Linalool, $C_{10}H_{18}0$ ($\geq$ 95 % purity GC; sum of enantiomers)

Linalool oxide, $C_{10}H_{18}O_2$ ($\geq$ 97 % purity GC; mixture of isomers)

Oleanolic acid, $C_{30}H_{48}O_3$ (> 97 % purity; Alfa Aesar, UK.)

Petroselinic acid, $C_{18}H_{34}O_2$ (> 99 % purity)

Ursolic acid, $C_{30}H_{48}O_3$ (> 90 % purity)

2.1.2 Solvents and chemicals used in microbiological work

Solvents and chemicals used in microbiological work were purchased from Sigma-Aldrich UK and VWR UK unless otherwise mentioned.

Solvents

Dimethyl sulfoxide, DMSO ($\geq$ 99.9 % purity)

Ethanol (HPLC grade)

Chemicals

Ammonium sulphate, $(NH_4)_2SO_4$

Crystal violet (Dye content $\geq$ 90 %)

Casein hydrolysate (Fluka, UK)

Gentamicin sulphate (salt)

D-Glucose (Fisher, UK)

Magnesium sulphate, $MgSO_4$

Potassium dihydrogen orthophosphate, KH_2PO_4

Potassium phosphate, $K_2HPO_4.3H_2O$

Sodium Chloride, NaCl ($\geq$ 99 % purity)

2.2 Media and consumables used in microbiological work

Media and consumables were purchased from Sigma-Aldrich UK and VWR UK unless stated otherwise.

Media

Agar (granular powder; Fisher UK)

Luria-Bertani, (LB) broth and agar

M63 medium (enriched media)

Mueller-Hinton (MH) agar (Merck, Germany)

Tryptone (Fluka, UK)

Yeast extract powder (Sigma- Aldrich UK)

Consumables

Breath-easy sealing membrane (sterilised, 6x3.25 inches, Sigma-Aldrich UK)

96 well ELISA microtitre plates (flat bottom; Greiner Bio-one, Switzerland)

2.3 Plant materials

The dried plant materials were either purchased from a commercial supplier (G. Baldwin and Co., UK) or kindly supplied by Dr Xianmin Chang (Orkney College UHI, Kirkwall, UK) and Mr Nigel Stangroom (Stangroom Bros Ltd, UK). The fresh plant materials were collected from Crarae Garden, (National Trust for Scotland, Inveraray, UK) by Dr Nicholas P. Tucker.

The fresh plant materials were left to dry at 25 ºC and then ground into a fine powder using an analytical mill (IKA A11 BASIC, China). All plant materials were packed in a paper envelope and stored in a dry cabinet in the Natural Product Research Laboratories, Strathclyde Institute of Pharmacy and Biomedical Sciences. The origin of all plant materials is presented in Table 15. The botanical names including other details for all selected medicinal plants are listed in Table 16 (see Chapter 3.3).

Table 15: Origin of plant materials

Plant material*	Source	Type
327 and 355	Stangroom Bros Ltd., UK	Dried powder
328 to 354,	G. Baldwin and Co., UK	Dried powder
356 to 362	Orkney College, UK	Fresh fruit
SF	McLaren's Nurseries, UK	Fresh fruit
BW, PDF, PDL, RF, RAA and 1213	Crarae Garden, UK	Fresh plant crop

*code used.

2.4 Extraction of plant materials

Each plant material (500 g DW) was extracted using *n*-hexane (1 L), ethyl acetate (1 L) and methanol (1 L) for 1 h at 25 ºC using a sonicator (DECON F5100b, UK). During sonication the bubbles produced by acoustic cavitation aid the solvent to disrupt plant cell wall which releases phytochemicals (Wu *et al.*, 2001). All extracts were filtered using a qualitative filter paper (125mm; Fisher UK) and then concentrated under reduced pressure using a rotary evaporator (Buchi R-205, Switzerland) at 40 ºC and later dried under a flow of nitrogen gas. All extracts were kept sealed in glass containers and stored at – 20 °C prior to analysis.

2.5 Preparation of samples

The term "sample" in this study refers to extracts, fractions or purified compounds from plant materials. In preparation for an assay, each sample was weighed and dissolved in DMSO to obtain a final concentration of 1 mg/mL. Each sample was kept sealed in a sterile glass vial and stored at - 80 °C prior to analysis.

2.6 Microorganisms

The clinical *Pseudomonas aeruginosa* strain PA14 (Lee *et al.*, 2006), isolated from a patient with a burn was obtained from Dr. Nicholas P. Tucker. The stock culture of *P. aeruginosa* PA14 was stored in glycerol beads and kept at – 80 ºC. A fresh culture of *P. aeruginosa* PA14 was prepared from a bead of stock culture subsequently inoculated on LB agar prior to analysis.

2.7 Preparation of *P. aeruginosa* PA14 cultures

A fresh culture of *P. aeruginosa* PA14 was initiated on LB plates and cultured overnight at 37 °C. A loop-full of *P. aeruginosa* PA14 culture from LB agar was inoculated into 5 mL of sterilised LB broth in a sterilised universal bottle. The broth was left in shaking incubator at 37 °C for an overnight. Later, 100 µL of overnight culture was then inoculated into 50 ml of sterilised LB broth in a sterilised glass conical flask. The inoculated broth was then incubated at 37°C with shaking until the reading reach the optical density (WPA Biowave Cell Density Meter, UK) approximately 0.25 at 600 nm.

2.8 M63 medium

This medium comprised:

5.3 g of potassium dihydrogen orthophosphate

13.9 g of potassium phosphate anhydrous

2.0 g of ammonium sulphate

The following (sterilised) reagents were subsequently added:

1.0 mM of magnesium sulphate

0.6 % (w/v) of glucose

0.5 % (w/v) of casamino acids

The medium was prepared using the above recipe then dissolved in 1 L of distilled water. The solution was sterilised at 121ºC for 15 minutes. The sterilised reagents were added into the recipe prior to start the antibiofilm analysis. Each sterilised reagents was added into the sterilised solution and then shaken slowly to homogenise the solution thus complete the preparation of M63 medium.

2.9 Broth and agar media

Preparation of Luria-Bertani (LB) broth and agar and Mueller-Hinton (MH) agar were prepared as follows.

The Luria-Bertani (LB) broth and agar comprised:

10.0 g of tryptone

5.0 g of yeast extract

5.0 g of sodium chloride

The Mueller-Hinton (MH) agar comprised:

2.0 g of beef extract

17.5 g of digest casein hydrolysate

1.5 g of starch

17.0 g of bactoagar

Broth or liquid medium of both was prepared by dissolving the relevant amounts of media in 1 L of distilled water, followed by autoclaving at 121ºC and 15 psi for 15 minutes. Agar or solid medium was prepared similarly as above but with addition of 1 % (w/v) bactoagar to liquid medium before sterilisation. In a sterile laminar flow hood, petri dishes were labelled and 20 ml of molten agar was aseptically poured into each petri dish allowing it to set and dry off excess liquid.

2.10 Determination of antibacterial activity

The antibacterial activity of each sample against *P. aeruginosa* PA14 was measured using a disc diffusion method on a Mueller-Hinton (MH) agar. Sample at final concentration of 1 mg/mL (see Chapter 2.5) was used in the analysis with DMSO as a negative control. The antibacterial activity using disc diffusion method is described below.

Briefly, 200 µL of fresh *P. aeruginosa* PA14 (PA14) cultures were pipetted on MH agar and spread using a sterilised cotton bud to produce a uniform PA14 lawn. The PA14 lawn was then embedded with sterilised filter discs (6mm diameter) and 10 µL of each sample (1 mg/mL) was pipetted onto each disc and incubated overnight at 37ºC.

The antibacterial activity was shown by the presence of clear zones of inhibition surrounding disc on the MH agar plate. A clear zone indicated that the tested sample had antibacterial activity against the growth of PA14. The antibacterial activity was measured as the diameter (Ø, mm) of the zone of inhibition, illustrated in Figure 15.

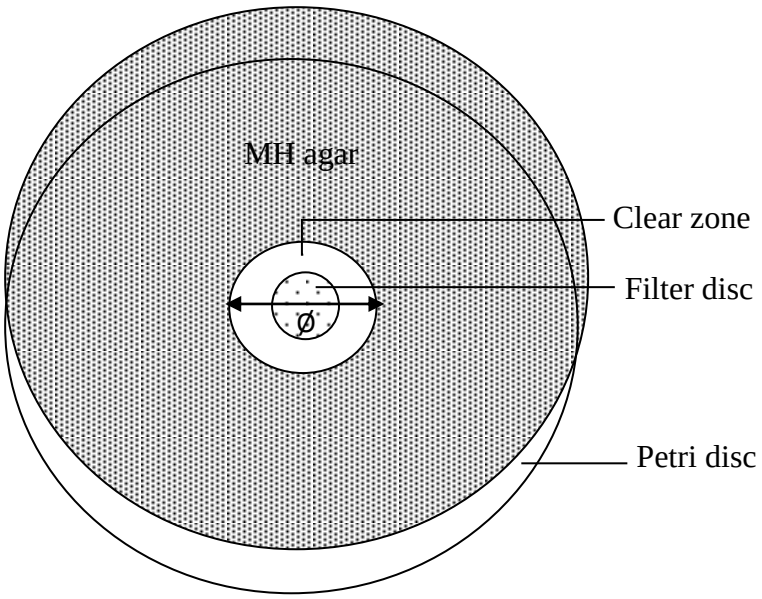


Figure 15: Schematic diagram of the disc diffusion method used. The antibacterial activity is showed by the presence of clear zone (Ø, mm) surrounding the filter disc that embedded on the Mueller-Hinton (MH) agar.

2.11 Determination of antibiofilm activity in a test tube

The antibiofilm activity was first evaluated in a sterilised glass test tubes as described previously by (O'toole and Kolter, 1998) with a few modifications. In the analysis, adherence biofilm on the wall of test tube was quantified using a crystal violet staining assay and antibiofilm activity was measured as percentage (%) change in biofilm formation. The method and calculation are described as below.

Twenty five microlitres of fresh *P. aeruginosa* PA14 culture was inoculated into 50 ml of M63 medium in a sterilised conical flask. The flask was shaken slowly to homogenise the inoculated M63 medium. Each sterilised glass test tube was loaded with 1 ml of inoculated M63 medium followed by 50 µl of sample. The test tube was capped and incubated at 37^{o}C overnight without shaking.

Samples are prepared in triplicate with a negative control is an uninoculated M63 medium. After overnight (18-24 hours) incubation, suspension in the test tube was carefully discarded and the test tube was stained with 1 ml of 0.3 % (v/v) of crystal violet (CV) solution for 5 min. at room temperature. The CV in test tube was discarded and washed 3 times with water. The test tube was put upside down on a tube rack and air dried to remove excessive water. Ethanol at 1.5 mL was pipetted into the test tube and vortex for 30 seconds to immobilise the CV stained. The absorbance of CV stained solution was measured using NanoDrop 2000 (Thermo Scientific, USA) at 582 nm using a 1 mm cuvette with ethanol as a blank.

$$\text{Antibiofilm activity (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\ \%$$

2.12 Determination of antibiofilm activity in a microtitre plate

The antibiofilm activity using crystal violet staining assay was then evaluated using a microtitre plate method. The microtitre plate format was developed in response to constraints and several weaknesses in the test tube format. The antibiofilm method using microtitre plate format was developed based on the works of (O'toole, 2011; Knezevic and Petrovic, 2008; Pitts *et al.*, 2003) with few modifications to meet the basic conditions (e.g. concentration, time and temperature) as in the test tube format. The antibiofilm analysis using microtitre plate format is illustrated using a schematic diagram of 96 well ELISA microplates (Greiner Bio-One, Switzerland) as shown in Figure 16.

The microtitre plate was given a code to represent the analysis and wells for treatment, control and blank were earlier designed in the microplate reader software (Microplate reader Spectra Max190, USA) before performing the analysis. The microtitre plate consists of 8 rows **(A- H)** and 12 columns **(1-12)** with the first three columns **(1-3)** were allocated for blank and controls and the remaining columns **(4-12)** were allocated for samples. The first four rows **(A-D)** were allocated for sample with treatment **(T)** and the remaining four rows **(E-H)** were allocated for sample without treatment **(Cs)**.

The location for the blank and control was rather flexible and can be designed as required. The location for sample was suggested to be made in the same row between sample with treatment **(T)** with sample without treatment **(Cs)** in order to minimise experimental error. Analysis was performed on duplicate plates which gave values for a total of 6 replicate samples per analysis.

The developed method also enabled two activities to be measured on a single analysis, simultaneously. The activities are (1) percentage of biofilm formation and (2) percentage of biofilm inhibition, which was estimated before and after the crystal violet assay staining, respectively. Both activities are elaborated in detail below.

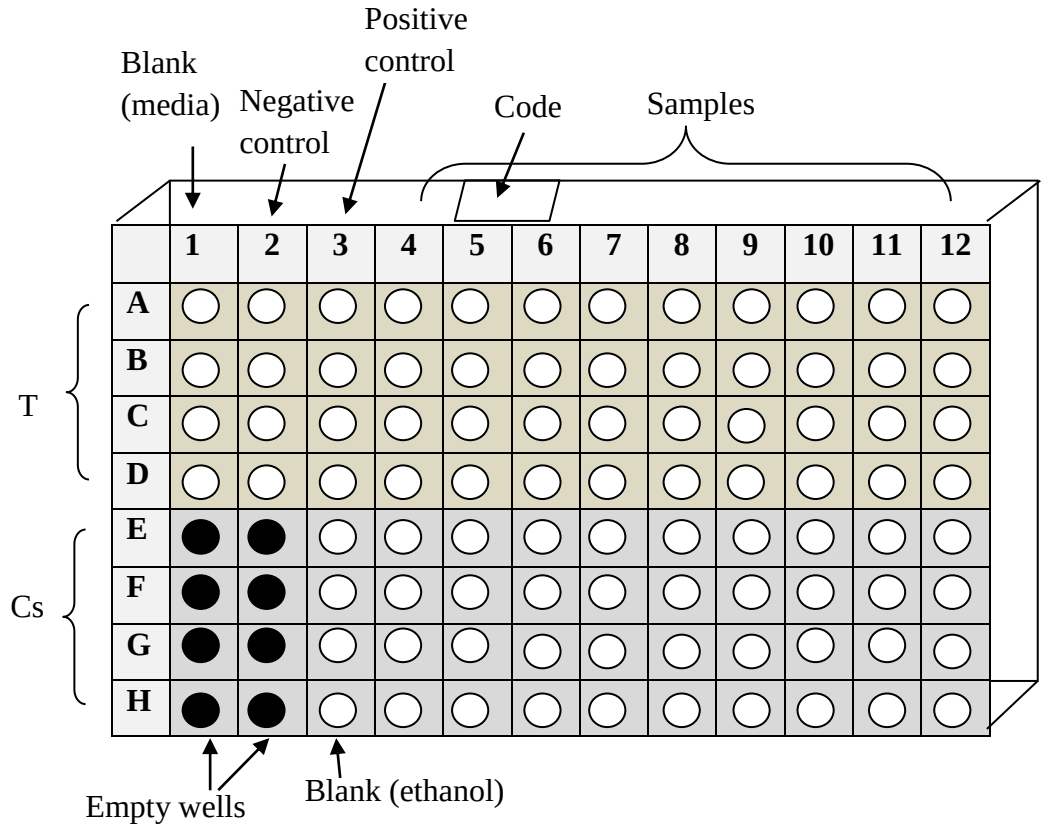


Figure 16: Schematic diagram of the microtitre plate method used. Each row represent different sample at which (T) is a sample with treatment and (Cs) is a sample without treatment. Naming of well is explained as A_1 represent location of well at row A and column 1 whereas G_8 represent location of well at row G and column 8.

2.12.1 Antibiofilm activity as percentage of biofilm formation, BFF

Using multi-channel micropipettes, 100 µl of inoculated M63 medium was loaded in each well of treatment, **(T)** (e.g. wells A_{4-12} - well D_{4-12}) whereas 100 µl of uninoculated M63 medium was loaded in each well of sample without treatment, **(Cs)** (e.g. wells E_{4-12} - H_{4-12}). Sample at 5 µL was pipetted into each row of well, **(T** and **Cs)** (e.g. wells A_4 - H_4) followed by other samples until all rows are completed. The same exercise was performed on duplicate plates which were prepared for each analysis. Fresh M63 medium is used as a blank **(B_M)** and inoculated M63 medium consisting of DMSO is used as a negative control, **(Cn)** whereas Gentamicin is used as a positive control, **(Cp)**. The microtitre plate was laminated using a gas permeable sealing membrane (Sigma-Aldrich) and incubated without shaking at 37 °C, overnight.

Later, the microtitre plates were removed from the incubator and the sealing membrane was slowly peeled off. The blank, **(B_{Media})** was loaded into the blank-wells and the absorbance was measured at 600nm using microplate reader (SpectraMax 190, USA). Data was recorded and saved. Only readings from three wells were recorded and one outlier reading was discarded. The duplicate plate was treated in the same manner. Data present is the average of a total of six readings taken from duplicate plates.

Data collected only for observation (data not reported) between biofilm formation (changes in growth) and biofilm inhibition (percentage of adherence biofilm) in the following method. The changes in biofilm formation of *P. aeruginosa* PA14 was calculated as percentage of biofilm formation as described below.

$$\text{Biofilm formation, BFF (\%)} = \frac{(T - B_M) - (Ct - B_M)}{(Cn - B_M)} \times 100\ \%$$

Where;

B_M: blank (fresh M63 medium)

T: treatment (inoculated M63 medium with sample)

Ct: treatment control (uninoculated M63 medium with sample); and

Cn: negative control (inoculated M63 medium with DMSO)

2.12.2 Antibiofilm activity as percentage of biofilm inhibition, BFI

This analysis was a continuation from the above method (see Chapter 2.12.1). The plate containing antibiofilm assay was blotted vigorously onto stacks of tissue towels to discard the suspensions. Each well was stained with 0.3% (v/v) crystal violet and immobilised for 10 min. Plates are again blotted vigorously onto stacks of tissue towels to discard the crystal violet solution. The plate was rinsed 3 times by submerging the plate into a container filled with tap water and again blotted vigorously on stacks of tissue towels to remove the excessive water. After the plate dried, 150 *u*l of ethanol was added into each well and shaken at 150-170 rpm for 2 min to immobilise the stain. Absorbance was measured at 582nm using microplate reader (SpectraMax 190, USA), data was recorded and saved.

The duplicate plate was treated in the same manner as mentioned above. Similarly, only triplicate readings were taken and one outlier reading was discarded. Data present is the average of a total of six readings taken from duplicate plates.

In the earlier method, the crystal violet staining assay using the test tube format was designed to measure the total amount of biofilm biomass. However, it was found that the total biomass was contributed by the intrinsic value from the biofilms and probably from uncertain amounts of biomass from the plant sample. Therefore in order to consider the intrinsic values in each sample, the antibiofilm activity using crystal violet staining assay in microtitre plate method was designed to use a robust control **(Cr)** which assumed that a background level of staining needed to be accounted for in the calculation (Pettit Rk Fau - Weber *et al.*, 2005).

The antibiofilm activity was measured after overnight biofilms were treated with samples. Ethanol was used as blank **(B_E)** and gentamicin was used as positive control **(Cp)**. Absorbance for each well was initially corrected by subtracting the value of absorbance from the treatment **(T)** with the blank **(B_E)**. Values from treatment control **(Ct)** were added to negative control **(Cn)** and determined to be a robust control **(Cr)**. The percentage (%) of biofilm inhibition for *P. aeruginosa* PA14 was summarised below.

Percentage biofilm inhibiton, BFI (%) = $\left[1 - \frac{(T - B_E)}{Cr}\right] \times 100$

Where;

B_E: blank (ethanol)

T: treatment (inoculated M63 medium with sample)

Ct: treatment control (uninoculated M63 medium with sample)

Cn: negative control (inoculated M63 medium with DMSO); and

Cr: robust control (total values from corrected treatment control and corrected negative control; where $Cr = [(Ct - B_E) + (Cn - B_E)]$

2.13 Bioassay-guided fractionation

A stepwise separation process called bioassay-guided fractionation **(BGF)** was used to isolate pure compounds from plant extracts with activity against *P. aeruginosa* PA14 biofilm. The isolation of antibiofilm agents was guided by the antibiofilm activity of extracts observed in the microtitre plate method (see Chapter 2.12.2). A schematic protocol for bioassay-guided fractionation is shown in Figure 17.

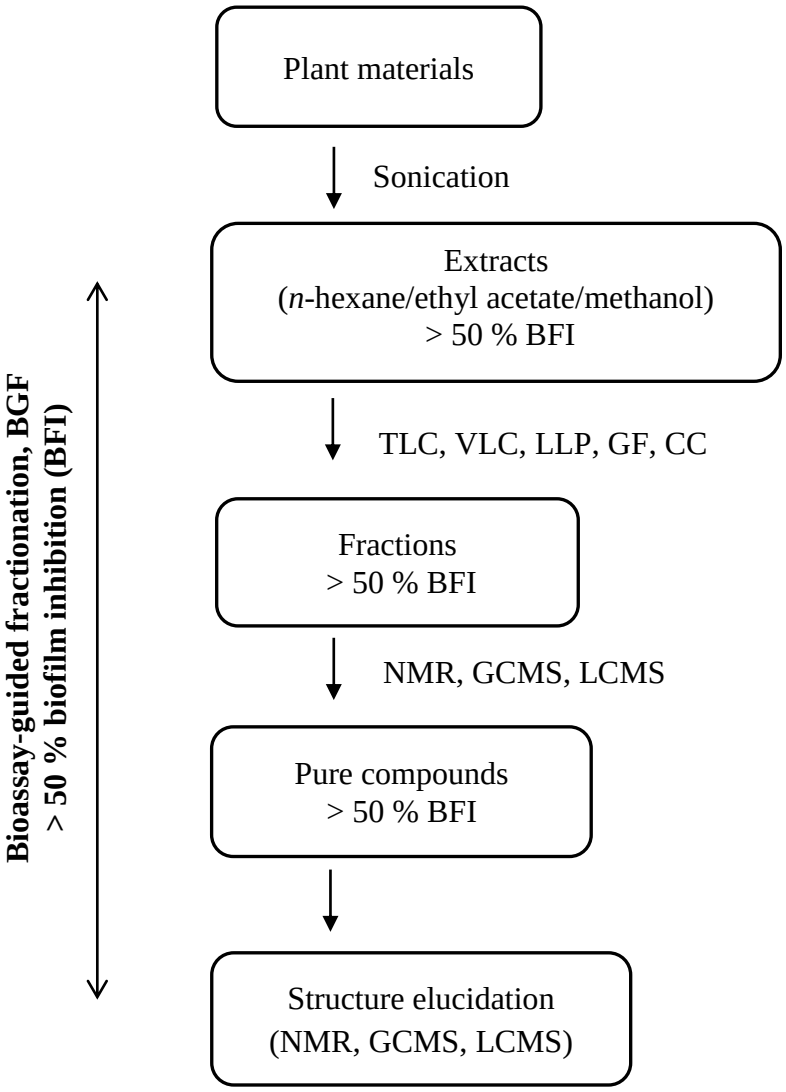


Figure 17: Schematic diagram of the bioassay-guided fractionation method. This method is used for the discovery of phytochemicals with inhibitory activity against *P. aeruginosa* PA14 biofilms.

2.14 Separation and detection of phytochemicals

2.14.1 Thin Layer Chromatography

Thin layer chromatography (TLC) was done on aluminium-supported silica gel 60 F_{254} TLC plates (Merck). Samples were dissolved in an appropriate solvent and applied as bands approximately 1 cm above the bottom edge of a TLC plate and then placed in an ascending direction in the TLC tank containing a mobile phase. Binary solvent systems such as *n*-hexane/ethyl acetate, ethyl acetate/methanol or otherwise mentioned were used as mobile phases.

In TLC, the relative rates of migration of compounds are affected by their individual affinity for the silica. Separation occurs when one compound is more strongly adsorbed by the silica than the others. Polar compounds move slowly compared to non-polar compounds. After development, the solvent front on the TLC plate was marked and the plate was dried immediately in the fume hood. Compounds were visualised under short and long waves UV light at λ_{254} nm and λ_{365} nm, respectively. The TLC plates were then sprayed with *p*-anisaldehyde-sulfuric acid reagent to visualise the functional group of compounds from the colour of reaction mixture.

2.14.2 Vacuum Liquid Chromatography

Vacuum liquid chromatography (VLC) was performed using a sintered glass funnel containing a compressed layer of silica gel 60H (Merck). An appropriate solvent was passed through the column bed to confirm uniformity of the column bed before loading the sample. Sample was dissolved in a small amount of solvent and mixed with silica gel 60 (Merck) to produce a free flowing powder, which was then loaded

as a thin layer onto the top of the column bed. A piece of filter paper was put on top of the sample to avoid disturbing the top of the column while eluting.

In general, 100-500 g of packing material was used per gram of sample. Each non-polar sample (e.g. hexane extract) was fractionated using a mobile phase with decreasing polarity starting from 100% hexane to 100% ethyl acetate. The fractionation continued with mixtures of ethyl acetate:methanol with increasing polarity up to 50% methanol. Eluents for each fraction were collected into a round-bottomed flask and then concentrated under vacuum.

2.14.3 Size Exclusion Chromatography

Size exclusion chromatography was performed using slurry of Sephadex® LH-100 (Sigma) in a thick-wall glass frit column with a sintered glass disc at its bottom. The slurry was prepared a day before fractionation and was left overnight to allow sufficient time for the Sephadex® LH-100 to swell. Once ready, the slurry was poured into the glass column.

A glass rod was used to stir the slurry to remove any entrapped air. A small amount of glass wool was kept at the top of the column to avoid any disruption of adsorbent while eluting. Sample was dissolved in appropriate solvent and then loaded onto the top of the packed column by using a long glass pipette. The exit valve was opened to allow the sample solution to be adsorbed on the packed column and then closed. Eluents were collected in glass vials in a small volume and concentrated under nitrogen flow. Sephadex® LH-100 is used to purify small molecular weight compounds from their native mixture which include mechanisms of adsorption and partition. According to their size, as compounds migrate with the solvent through the

sorbent, the small molecules become included into the gel matrix and larger molecules are excluded and migrate at a greater rate (Cannell, 1998).

2.14.4 Column chromatography on silica gel

Column chromatography on silica gel was performed using Silica gel 60 (Merck) in a thick-wall glass column. The silica gel was prepared as pourable slurry using a suitable solvent. Some glass wool was initially plugged at the end of the column to support the packing material. The slurry was left to settle down until it reached the desired height. Samples were dissolved in a suitable solvent or optionally mixed with a small amount of silica gel 60 and dried to obtain a free-flowing powder and then loaded onto the column. Eluted fractions were collected in glass vials and then concentrated under a nitrogen flow.

2.14.5 Liquid-liquid partition

Liquid-liquid partition was performed on the extract using a separating funnel by adding solvents with different polarity. This method was applied to the methanol extracts. The extracts are first dissolved in 2.5 % (v/v) of ethanol and then partitioned with dichloromethane (DCM). The step was repeated with *n*-butanol using a separating funnel resulting in DCM and *n*-butanol fractions. The DCM and *n*-butanol fractions were dried over anhydrous sodium sulphate then evaporated using a rotary evaporator. The remaining aqueous fraction was freeze dried and stored at – 20 ºC.

2.15 Structure elucidation of isolated phytochemicals

2.15.1 Nuclear Magnetic Resonance (NMR) spectroscopy

The structures of purified compounds were identified using NMR. A ^{1}H-NMR spectrum was performed to determine the chemical shifts, integration, multiplicity and coupling constants of protons present in a molecule. The ^{1}H-NMR also allowed the determination of molar ratios if two components were present in a mixture. The structure elucidation of some compounds required 2D-NMR analyses such as COSY, HMBC and HSQC.

Non-polar samples (10 mg) were dissolved in $CDCl_3$ whereas polar samples were dissolved in DMSO-d_6 unless otherwise mentioned. All spectra were recorded either using a JEOL JNM-LA400 FT spectrometer operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR, or a Bruker Avance AV3 400 spectrometer operating at 400 MHz. A Bruker Avance AV500 instrument was also used operating at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR in some cases. All spectral data obtained were processed with MestReNova, v. 7.

2.15.2 Gas Chromatography (GC) – Mass Spectroscopy (MS)

The identification of purified compounds was performed using a gas chromatograph, (GC) (Finnigan LTQ Orbitrap, UK) equipped with a mass selective (MS) detector (Thermo Finnigan Surveyor) and a Rtx-1capillary column (Thames Restek UK) at 30 m x 0.25 mm x 0.25 µm diameter. The injector was operated at 250 ºC with an oven temperature at 100 ºC, ramping of 20 ºC/min. to final temperature of 320 ºC with helium as carrier gas at constant flow rate. Samples were dissolved in either

methanol or ethyl acetate unless otherwise mentioned to give concentration of 1 mg/mL. The sample was analysed in a full scan MS using electron impact mode at 70 eV for EI-MS in positive mode.

2.15.3 Liquid Chromatography (LC) – Mass Spectroscopy (MS)

Liquid chromatography (Thermo Accela) equipped with mass selective (MS) detector (LTQ Orbitrap) and an ACE-C_{18} (Hichrom Ltd., UK) capillary column at 150 x 4.6 mm x 3 µm diameter and gradient solvent system with flow rate at 0.3 mL/min. In this study, LC-MS was performed to measure the mass-to-charge (m/z) ratio of charged particles from complex mixtures. Samples were dissolved in methanol, unless otherwise mentioned, to give a final concentration of 1 mg/mL.

2.16 Software and data processing

The spectra generated from GC-MS were compared with standard spectral library using the manufacturer-supplied software (Thermo Fisher Scientific Inc.) and the built up NIST database from the National Institute of Standards (NIST 11 MS library and AMDIS v.2.70 , USA). Data obtained from LC-MS and GC-MS were processed using Thermo Xcalibur v.2 (Thermo Fisher Scientific Inc.) to generate the MS based chromatograms for the identified compounds.

MZmine 2.10 (Pluskal *et al.*, 2010) is a software use to process, visualise and analyse mass spectrometry based on molecular profile data for LC-MS data. The generated peak list from both positive and negative ESI modes were identified by searching for

the accurate mass from PubChem Compound website and Dictionary of Natural Products database. Errors for chemical formulas were assigned within ± 3 ppm mass error.

2.17 Statistical analyses

Statistical analysis was done using Minitab ® 16.1.1 Statistical Analyses, (U. K.). All data values were expressed as mean ± SD and statistically analysed using one-way ANOVA with significant difference at *P* values of < 0.05. The Dunnett's Method was used to compare each sample mean with the mean of a control. Unless stated, Tukey's Method was used to compare all possible pairwise difference of means at the same time.

CHAPTER 3 - RESULTS AND DISCUSSION

3.1 Activity of plant extracts against *Pseudomonas aeruginosa* strain PA14

3.1.1 Yields of extracts

A total of 129 extracts were produced from 43 different plant materials. Yields of extracts, expressed in relation to dry weight of the plant material (%, w/w) are listed in Table 16. The yields of *n*-hexane (non-polar) extracts ranged between 0.2 to 11.4 % and the yields of ethyl acetate (medium polarity) extracts ranged between 0.1 to 12.0 %.

The yields for methanol (polar) extracts ranged between 1 to 58.2 % were the highest yields obtained for most plants. Yields of methanol extracts above 50 % (w/w) were found for *Vaccinium corymbosum* **(M356)** and *Vaccinium macrocarpon* **(M359)** fruits.

Plants consist of various groups of bioactive compounds such as tannins, lignans, alkaloids, glycosides and terpenoids. As amphiphilic compound, methanol capable to dissolve both polar and certain of nonpolar compounds and this contributed to the high percentage of compounds consist in the methanol extracts compared to others such as *n*-hexane and ethyl acetate extracts.

Table 16: Selected medicinal plants and percentage yields of extracts

Code	Latin name	Plant part	Yields of extract (% of dry material)		
			Hexane	Ethyl acetate	Methanol
327	*Coriandrum sativum*	Leaf	130 mg (1.3)	90 mg (0.9)	680 mg (6.8)
328	*Pimpinella anisum*	Seed	750 mg (7.5)	160 mg (1.6)	500 mg (5.0)
329	*Melissa officinalis*	Fruit	900 mg (9.0)	140 mg (1.4)	600 mg (6.0)
330	*Ocimum basilicum*	Leaf	110 mg (1.1)	150 mg (1.5)	570 mg (5.7)
331	*Laurus nobilis*	Leaf	200 mg (2.0)	180 mg (1.8)	1020 mg (10.2)
332	*Vaccinium myrtillus*	Fruit	40 mg (0.4)	50 mg (0.5)	1190 mg (11.9)
333	*Ribes nigrum*	Leaf	160 mg (1.6)	300 mg (3.0)	870 mg (8.7)
334	*Carum carvi*	Seed	1140 mg (11.4)	450 mg (4.5)	460 mg (4.6)
335	*Apium graveolens*	Seed	620 mg (6.2)	370 mg (3.7)	530 mg (5.3)
336	*Matricaria recutita*	Aerial	130 mg (1.3)	90 mg (0.9)	710 mg (7.1)
337	*Cinnamomum verum*	Bark	20 mg (0.2)	210 mg (2.1)	930 mg (9.3)

Table 16: (continued). Selected medicinal plants and percentage yields of extracts

Code		Plant part	Yields (% of dry material) of extract		
			Hexane	Ethyl acetate	Methanol
338	*Coriandrum sativum*	Seed	1090 mg (10.9)	140 mg (1.4)	290 mg (2.9)
339	*Cuminum cyminum*	Seed	170 mg (1.7)	290 mg (2.9)	820 mg (8.2)
340	*Sambucus nigra*	fruits	1070 mg (10.7)	110 mg (1.1)	3430 mg (34.3)
341	*Sambucus nigra*	Flower	190 mg (1.9)	180 mg (1.8)	100 mg (1.0)
342	*Foeniculum vulgare*	Seed	1140 mg (11.4)	480 mg (4.8)	700 mg (7.0)
343	*Allium sativum*	Bulb	200 mg (2.0)	30 mg (0.3)	820 mg (8.2)
344	*Zingiber officinale*	Root	250 mg (2.5)	210 mg (2.1)	250 mg (2.5)
345	*Origanum majorana*	Leaf	110 mg (1.1)	240 mg (2.4)	890 mg (8.9)
346	*Mentha piperita*	Leaf	100 mg (1.0)	190 mg (1.9)	800 mg (8.0)
347	*Olea europaea*	Leaf	80 mg (0.8)	390 mg (3.9)	1150 mg (11.5)
348	*Citrus sinensis*	Flower	70 mg (0.7)	50 mg (0.5)	2140 mg (21.4)

Table 16: (continued). Selected medicinal plants and percentage yields of extracts

Code		Plant part	Yields (% of dry material) of extract		
			Hexane	Ethyl acetate	Methanol
349	*Rubus idaeus*	Leaf	90 mg (0.9)	140 mg (1.4)	480 mg (4.8)
350	*Rheum palmatum*	Root	40 mg (0.4)	70 mg (0.7)	2230 mg (22.3)
351	*Rosmarinus officinalis*	Leaf	660 mg (6.6)	1200 mg (12.0)	1060 mg (10.6)
352	*Salvia officinalis*	Leaf	320 mg (3.2)	480 mg (4.8)	580 mg (5.8)
353	*Fragaria ananassa*	Leaf	150 mg (1.5)	90 mg (0.9)	980 mg (9.8)
354	*Juglans regia*	Leaf	140 mg (1.4)	150 mg (1.5)	670 mg (6.7)
355	*Coriandrum sativum*	Stalk	110 mg (1.1)	10 mg (0.1)	580 mg (5.8)
356	*Vaccinium corymbosum*	Fruit	100 mg (1.0)	70 mg (0.7)	5820 mg (58.2)
357	*Sambucus nigra*	Fruit	820 mg (8.2)	300 mg (3.0)	1400 mg (14.0)
358	*Rubus arcticus*	Fruit	280 mg (2.8)	220 mg (2.2)	3970 mg (39.7)
359	*Vaccinium macrocarpon*	Fruit	149 mg (1.5)	286 mg (2.9)	5755 mg (57.6)

Table 16: (continued). Selected medicinal plants and percentage yields of extracts

Code		Plant part	Yields (% of dry material) of extract		
			Hexane	Ethyl acetate	Methanol
360	*Gaultheria shallon*	Fruit	340 mg (3.4)	140 mg (1.4)	3050 mg (30.5)
361	*Aronia melanocarpa*	Fruit	120 mg (1.2)	130 mg (1.3)	3230 mg (32.3)
362	*Hippophae rhamnoides*	Fruit	810 mg (8.1)	400 mg (4.0)	3230 mg (32.3)
SF	*Sorbus thuringiaca fastigiata*	Fruit	265 mg (2.7)	210 mg (2.1)	4474 mg (44.7)
BW	*Berberis wilsoniae*	Fruit	471 mg (4.7)	748 mg (7.5)	2543 mg (25.4)
PDF	*Photinia davidiana*	Fruit	131 mg (1.3)	259 mg (2.6)	1845 mg (18.5)
PDL	*Photinia davidiana*	Leaf	107 mg (1.1)	312 mg (3.1)	1875 mg (18.8)
RF	*Rhododendron falconeri*	Leaf	72 mg (0.7)	164 mg (1.6)	474 mg (4.7)
RAA	*Rhododendron arboreum*	Leaf	67 mg (0.7)	250 mg (2.5)	673 mg (6.7)
1213	*Cotoneaster simonsii*	Fruit	364 mg (3.6)	175 mg (1.8)	2804 mg (28.0)

3.1.2 Screening of plant extracts against *P. aeruginosa* PA14

3.1.2.1 Antibacterial activity of plant extracts

The screening for antibacterial activity was performed on solid media using a disc diffusion assay (DDA) method (see Chapter 2). A total of 30 plant extracts from 10 plant materials were tested for antibacterial activity against *P. aeruginosa* PA14. The plants were selected due to reports on their antimicrobial activity as published in the literature (see Table 1, Appendix 2).

The antibacterial activity is reported in Figure 18. Results showed that all plant extracts exhibited nearly a similar effect showing that all extracts had relatively low inhibitory activity against *P. aeruginosa* PA14. The antibacterial activity of gentamicin (control) against *P. aeruginosa* PA14 had produced a distinct zone (39.3 mm) of inhibition on disc diffusion agar, while negligible (7.3 – 9.7 mm) zones of inhibition revealed by most of the plant extracts.

Results indicated that the most active extracts in inhibiting the growth of *P. aeruginosa* PA14 were **(E349)** and **(H329)**, which were the ethyl acetate extract of *Rubus idaeus* leaves and the *n*-hexane extract of *Melissa officinalis* fruits. Both extracts inhibited the growth of *P. aeruginosa* PA14 on solid media of MH agar at the same value of 11.3 mm.

Other extracts that showed antibacterial activity were the ethyl acetate and *n*-hexane extracts from *Citrus sinensis* flower **(348)** and *Vaccinium myrtillus* fruits **(332)** and the *n*-hexane extracts of *Ribes nigrum* leaves **(333)**. All extracts from *Coriandrum sativum* stalks **(355)**, the *n*-hexane and ethyl acetate extracts from its seeds **(338)**, the

ethyl acetate extract from its leaf **(327)** and all extracts from *Sambucus nigra* flowers **(341)** showed activity.

R. idaeus leaves have been used traditionally to treat diarrhoea, sore throats, fever and gastrointestinal problems. Our results revealed that extracts from *R. idaeus* leaves could inhibit *P. aeruginosa* PA14. The antibacterial activity could be due to the activity of phenolic compounds (Gudej, 2003) which are known to be helpful in treating bacterial infections (Patel *et al.*, 2004). Other *Rubus* species such as *R. ulmifolius* have been reported as having activity against biofilm formation of *Staphylococcus aureus* (Kumar *et al.*, 2011).

Antibacterial activity against *P. aeruginosa* PA14 was also found for extracts from *M. officinalis* fruits. Reports have showed that the essential oil of *M. officinalis* could eradicate biofilms of *S. aureus* and *E. coli* (Budzynska *et al.*, 2011) and inhibit the attachment of *Herpes simplex* virus *in vitro*. (Astani *et al.*, 2012). Other active plants such as *V. myrtill*us have showed strong activity against *Salmonella* and *Staphylococcus* spp. (Puupponen-Pimia *et al.*, 2005). This antibacterial activity could be due to their high amount of anthocyanins with powerful antioxidant capacity (Jaakola and Hohtola, 2010).

The essential oil of *C. sativum* has showed strong activity against *Campylobacter jejuni* (Rattanachaikunsopon and Phumkhachorn, 2010), *Staphylococcus* and *Eschericia* spp. (Lixandru *et al.*, 2010). Monoterpene alcohols in *C. sativum* (Duman *et al.*, 2010) have showed strong antimicrobial activity against *Staphylococcus aureus* (Awen *et al.*, 2011).

Results in Figure 19 showed that the positive control (gentamicin, 1 mg/mL) had activity on *P. aeruginosa* PA14 at 39.3 mm. In comparison to gentamicin, the tested plant extracts showed weak antibacterial activity. In the disc diffusion method, both motile and sessile states of *P. aeruginosa* PA14 were present and swarming motility of *P. aeruginosa* PA14 was also found in some cases (Figure 20).

The *P. aeruginosa* PA14 can reverse between motile and sessile states depending on a multitude of physiological changes (Gellatly and Hancock, 2013) and could be a reason for the weak antibacterial activity found against *P. aeruginosa* PA14. A weak antibacterial activity was also reported on *Pseudomonas fluorescens* which displayed the least antibacterial activity against a number of extracts from herbs and spices (Witkowska et al., 2013).

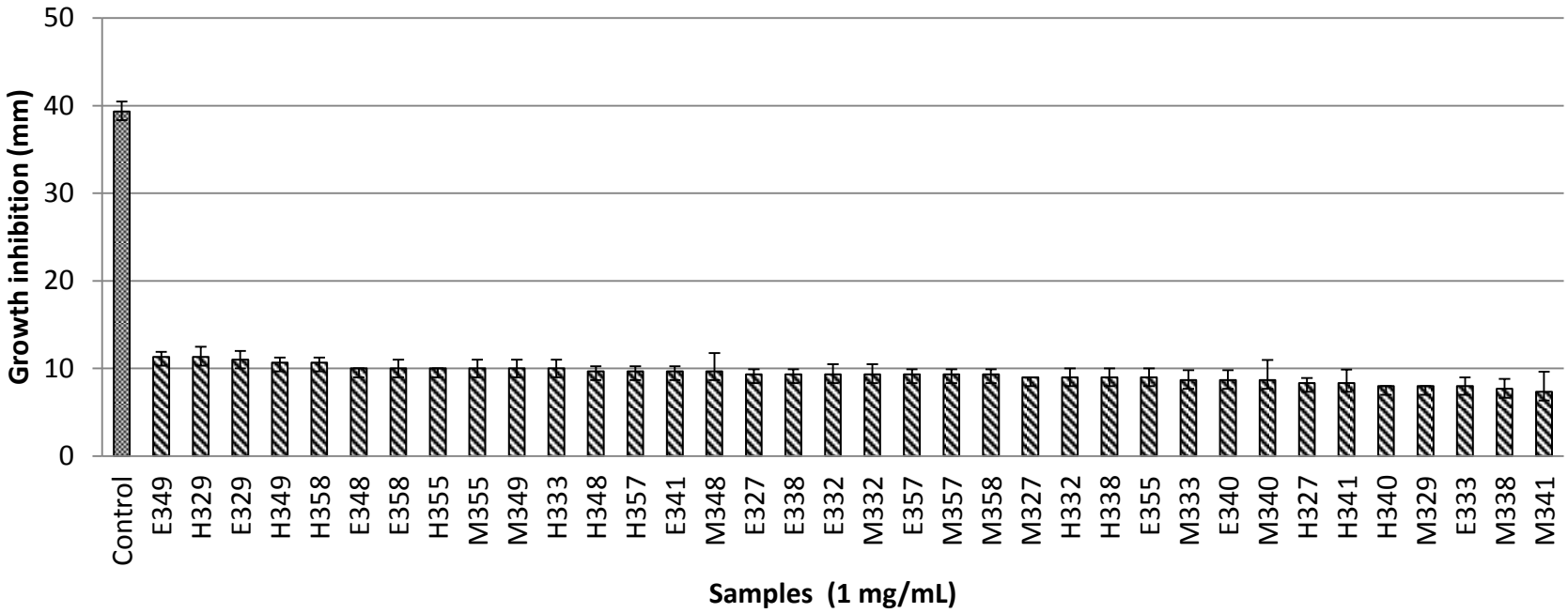
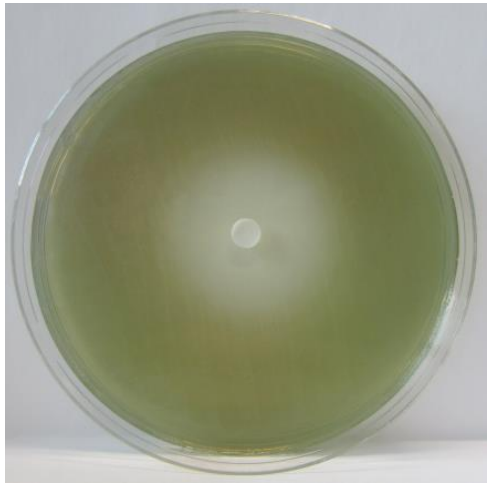
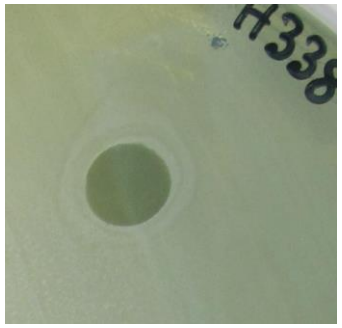


Figure 18: Antibacterial activity of selected plant extracts against the growth of *P. aeruginosa* PA14 using a disc diffusion assay (DDA). Extracts coded as H, E and M are referring to *n*-hexane, ethyl acetate and methanol extracts, respectively. The positive control (Control) is gentamicin at a concentration of 1 mg/mL. Error bars represent the mean $\pm$ SD of three replicates.

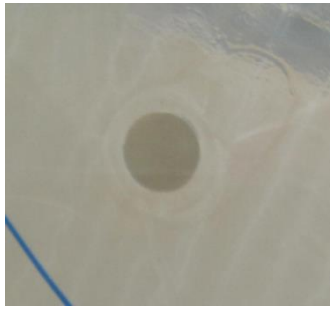


(a) Gentamicin (39.3 mm)



(b) H338 (9.0 mm)

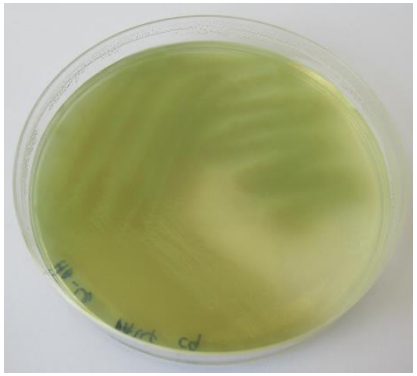
(c) M338 (7.7 mm)



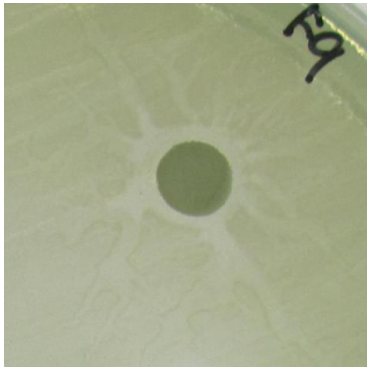
(d) E333 (8.0 mm)

(e) E341 (9.7 mm)

Figure 19: Selected samples with antibacterial activity against *P. aeruginosa* PA14. Sample with codes H, E and M are referring to *n*-hexane, ethyl acetate and methanol extracts, respectively. The concentration of positive control (gentamicin) and each sample are at 1 mg/mL



(a) Culture of *P. aeruginosa* PA14 on LB agar



(b) Swarming motility of *P. aeruginosa* PA14 on MH agar

(c) *P. aeruginosa* PA14 sessile biofilm in M63 media

Figure 20: *P. aeruginosa* PA14 fresh culture on solid medium (a), swarming motility on solid medium (b) and sessile biofilms in liquid medium (c)

3.1.2.2 Antibiofilm activity of plant extracts – preliminary study

The antibiofilm activity of plant extracts against *P. aeruginosa* PA14 was first assessed using a crystal violet staining assay in a test tube format (see Chapter 2.11). A total of 30 plant extracts (see Chapter 3.3.1) were subjected to preliminary screening. Figure 21 shows results of antibiofilm activity presented as percentage of changes in biofilm formation inhibition (BFF). Results revealed that samples **(E341)**, (**M332**), **(H332)**, **(H338)**, **(E332)**, **(E349)** and **(E329)** inhibited the formation of *P. aeruginosa* PA14 biofilms at 58.2, 61.3, 67.4, 71.7, 91.4, 95.6 and 100.6 % of BFF, respectively. The samples were coded as H, E and M, referring to *n*-hexane, ethyl acetate and methanol extracts, respectively.

Results also revealed that in total only 16.7 % of plant extracts (Group A) inhibited *P. aeruginosa* PA14 biofilm formation whereas majority 83.3 % of plant extracts (Group B) enhanced the biofilm. Results in Group B also showed that all three extracts from **(327)** and **(355)** plants enhanced the formation of *P. aeruginosa* PA14 biofilms. The reason for this effect is possibly due to the reaction of *P. aeruginosa* PA14 cells on the stress response from the compounds that influenced the *P. aeruginosa* PA14 cells to adhere to surfaces and resulted in more formation of biofilms (Parsek and Greenberg, 2005).

Previous studies have revealed that *Sambucus nigra* flowers **(341)** and *Vaccinium myrtillus* **(332)** contain some polyphenols which can interfere with bacterial quorum-sensing in *Escherichia coli* and *Pseudomonas putida* (Huber *et al.*, 2003). The presence of anti-adhesive compounds with lower molecular weights (< 10kDa) able to inhibit the binding of *Streptococcus pneumoniae* to human bronchial cells has also

been reported in *V. myrtillus* (Huttunen *et al.*, 2010). Previous studies have also reported the presence of terpinen-4-ol in *Melissa officinalis* fruits **(329)** a potent inhibitor of *Staphylococcus aureus* and *E. coli* biofilms on the surface of medical biomaterials (Budzynska *et al.*, 2011). Compounds such as 2-hexen-1-ol, 3-hexen-1-ol and cyclodecane detected in *Coriandrum sativum* **(338)** have previously been reported active against the formation of *Candida albicans* oral biofilms (Furletti *et al.*, 2011).

To date, there is no report on the activity of *C. sativum* against the formation of *P. aeruginosa* PA14 biofilms. To the best of our knowledge, this is the first report concerning such activity of *C. sativum* seeds. In this study, extract of *C. sativum* seeds had antibacterial against *P. aeruginosa* PA14 (see Chapter 3.3.1) and inhibited the formation of its biofilms. Limited information was found on the antibiofilm activity of the phytochemicals in the genus *Rubus*. A previous study revealed that ellagic acid and its derivatives found in the root of *Rubus ulmifolius* significantly inhibited the formation of catheter-associated *Staphylococcus aureus* biofilms (Quave *et al.*, 2012). The extract of *R. idaeus* has activity against *E. coli* (Rauha *et al.*, 2000).

The screening of plant extracts for antibiofilm activity using a test tube format had several drawbacks. The method did not allow for the analysis of a large number of samples simultaneously. A high throughput method in a microtitre plate format was subsequently developed to replace the test tube format. The microtitre plate format was more time efficient and reduced variations between experiments as more replicates could be done per analysis compared to the test tube format. The advantages and limitation of both methods are listed in Table 17.

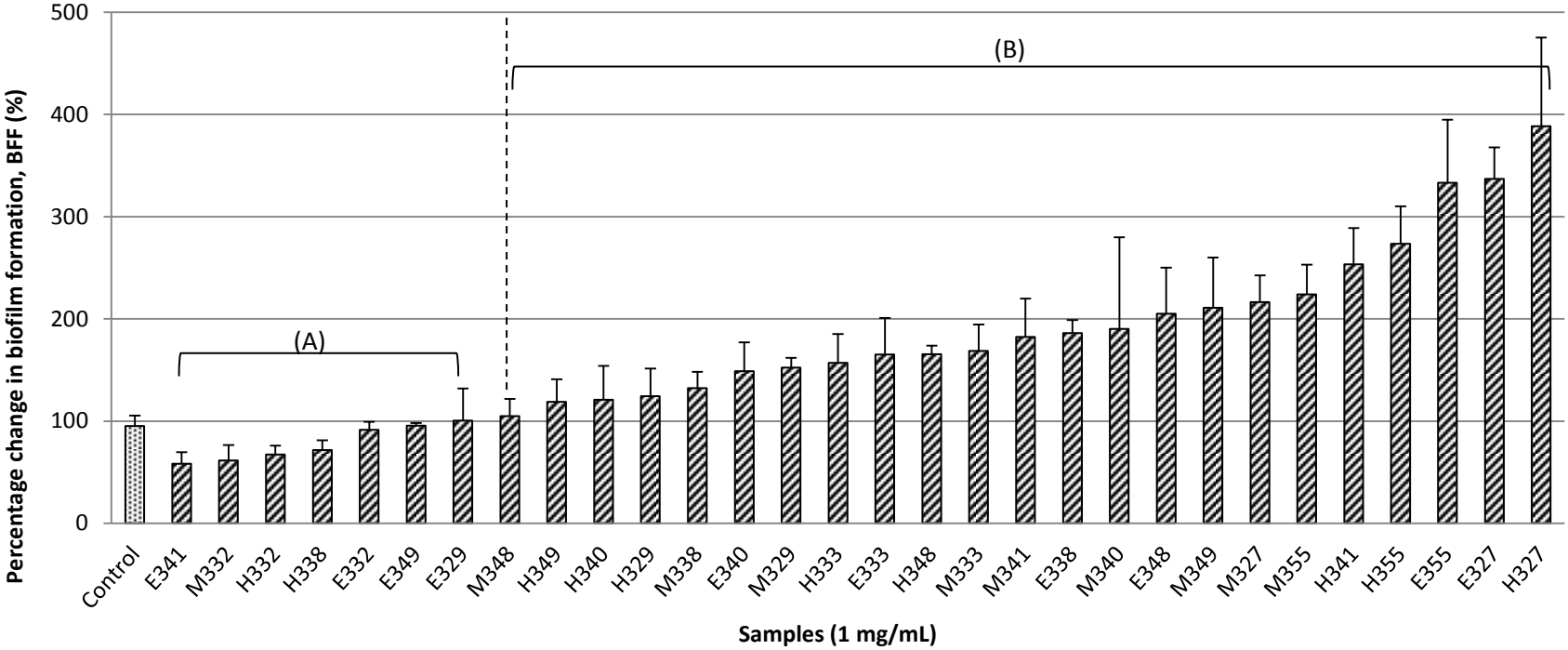


Figure 21: Activity of plant extracts against *P. aeruginosa* PA14 biofilms using a crystal violet staining assay in the test tube method. The antibiofilm activity was measured as percentage change in biofilm formation, BFF (%). Group (A) refers to samples with less than 100 % BFF whereas Group (B) refers to samples exceeding 100 % BFF. Samples coded as H, E and M are referring to *n*-hexane, ethyl acetate and methanol extracts, respectively. The negative control (Control) is an assay with DMSO only. Error bars represent the mean $\pm$ SD of three replicates.

Table 17: Advantages and limitations of the crystal violet staining assay method in a test tube and microtitre plate format

Attributes	Test tube	Microtitre plate
Number of samples	Limited. Up to 20 samples including controls and replicates. Subjected to availability of test tubes and incubator. Conventional method. Tedious analysis.	Flexible. More than 64 samples including controls and replicates. The number of sample can be increased using more than one plate. High throughput method with efficient time consuming.
Sample per assay	Minimum of 10 mg. Required a large amount of sample with high volume of solvent per analysis.	Small amount as minimum of 0.5 mg. Cost effective as less amount of sample and solvent required per analysis.
Experimental design	Manually designed with each single assay was analysed using a cuvette in NanoDrop absorbance meter.	Experiment was designed in microplate reader software and assay was analysed using Microplate reader Spectra Max190.
Replicates	Few. Up to three replicates.	Can be up to six replicates.
Variability in mean value	High standard deviation (SD) of mean value due to less replicates per analysis.	Low SD of mean value as more replicates per analysis.
Reproducible and sustainability	Low reproducible results as less replicates per sample was used. Washable and sterilised glass test tubes were used in analyses. Cross contamination may occur.	Highly reproducibly results. Used large replicates and control for each sample. Analyses done in sterilised and disposable plate.

3.1.2.3 Antibiofilm activity of plant extracts - high throughput method

A total of 129 plant extracts were screened for antibiofilm activity using the crystal violet assay in a microtitre plate format. Figure 22 shows the results for the antibiofilm activity of plant extracts presented as percentage of biofilm inhibition, BFI. Antibiofilm activity of plant extracts (1 mg/mL) with more than 50 % BFI are presented in Figure 22(a) whereas the remaining extracts showing less than 50 % BFI are presented in Figure 22(b).

Figure 22a also shows results of three groups of extracts (A, B and C) which are categorised by their activity in comparison to the positive control. Table 18 shows Group A with the most active extracts (110 - 120 % BFI) comprising **(M355)**, **(M340)**, **(H343)**, **(M343)**, (**M335)**, **(H335)**, **(E332)**, **(H338)**, (**E333)**, **(H357)** and **(E357)**, Group B with moderately active extracts (100 - 110 % BFI) consisting of **(M332)**, **(M338)**, **(E340)**, **(E343)**, **(H55)**, **(E355)**, **(M347)** and **(M330)**, and the remaining Group C with fairly active extracts (80- 100 % BFI). A literature search on promising extracts was performed to select those for investigation in this study.

Statistical analyses using Dunnett's Method shows that all extracts with more than 50 % BFI (see Figure 22a) are not significantly different ($p < 0.05$) from **Control** (Gentamicin). Thus the antibiofilm activities of those extracts are similar to **Control**. In Tukey's Method, **(H338)** and **(E333)** are significantly different ($p < 0.05$) which indicated that the activity of both are different and significantly higher than **Control** (Appendix 4.1). Thus, the antibiofilm activity of **(H338) and (E333)** was much higher than **Control** in inhibiting the biofilm of *P. aeruginosa* PA14.

Results indicated that the methanol extract of *C. sativum* stalk **(355)** from Group A was the most active at 74.3 % BFI. Further information from the supplier (Stangroom Bros Ltd., UK) indicated that the plant had not been organically grown and had been chemically treated with azoxystrobin and copper to prevent blight caused by *Pseudomonas* (Harrison *et al.*, 2008). This sample was excluded from further study.

Other active extracts from Group A were *Allium sativum* bulbs **(343)**, *Sambucus nigra* fruits **(340 and 357)**, *V. myrtillus* fruits **(332)**, *Apium graveolens* seeds **(335)** and *C. sativum* seeds **(338)** with more than 68 % BFI. Antibiofilm activity against *P. aeruginosa* PAO1 has been studied for *Allium sativum* **(343)** (Jakobsen *et al.*, 2012). Indeed, ajoene (Figure 23) in *Allium sativum* was identified as a primary quorum sensing inhibitor and attenuated the virulence factor in *P. aeruginosa* PA01 thus eradicating the biofilm formation (Jakobsen *et al.*, 2012; Harjai *et al.*, 2010; Bjarnsholt *et al.*, 2005).

Sambucus nigra fruits **(340 and 357)** were obtained from different sources. Both extracts were found active with > 62 % BFI against the formation of *P. aeruginosa* PA14 biofilms. A literature search on the antibiofilm activity of *S. nigra* fruits was scarce as most studies had reported on the direct antibacterial activity of this plant. However, other *Sambucus* species had been previously studied. This included the bark of *S. williamsii* which possessed antibacterial and antibiofilm activities against the *P. aeruginosa* (ATCC 27853) with (+)-medioresinol (Figure 24) isolated as the active compound. (+)-Medioresinol also showed antibiofilm activity on other Gram-negative bacteria such as *E. coli* and Gram-positive bacteria such as *S. aureus, Enterococcus faecium* and *Propionicbacterium acnes* (Hwang *et al.*, 2013).

S. nigra flowers **(341)** and fruits **(340 and 357)** showed activity with > 55 % BFI against the *P. aeruginosa* PA14 biofilms. No information could be found on the antibiofilm activity of *S. nigra* flowers. The antibiofilm activity of *S. nigra* fruits and flowers found in this study was therefore a good indication that the plant was a candidate for further investigation.

The extracts of *V. myrtillus* fruits **(332)** were also found active against the *P. aeruginosa* PA14 biofilm (showing between 56.0 to 69.4 % BFI). A literature search provided limited information on the antibiofilm activity of *V. myrtillus* fruits. A previous study on the fruits of *V. macrocarpon* revealed an inhibitory effect against *Candida* spp. biofilms attributed to high molecular weight polyphenols (Girardot *et al.*, 2012).

The identification of polyphenols such as cyanidin-3-*O*-glucoside, cyanidin-3-*O*-sambubioside, cyanidin-3-5-*O*-diglucoside, cyanidin-3-*O*-sambubioside-5-O-glucoside and rutin in the methanol extract of *V. myrtillus* fruits (Rieger *et al.*, 2008) probably contributed to the antibiofilm activity. Study on *V. virgatum* had revealed the activity of their hydroethanolic extracts against the *P. aeruginosa* and *S. epidermidis* biofilms (Zimmer *et al.*, 2014).

To our knowledge, this is the first report on the antibiofilm activity of *S. nigra* fruits and flowers of and *V. myrtillus* fruits against *P. aeruginosa* PA14 biofilm. The antibiofilm activity of *S. nigra* and *V. myrtillus* fruits were discovered at a late stage in this study and the difficulties in obtaining high amounts of plant material (limited sources and seasonal constraints) explains why these extracts were not selected for

bioassay-guided fractionation in this study. Unlike *S. nigra* fruits, *S. nigra* flowers were available in high amounts.

Ribes nigrum leaf **(333)** showed antibiofilm activity against *P. aeruginosa* PA14. A high activity was demonstrated for the ethyl acetate extract (68.3 % BFI) and the methanol extracts (59.2 % BFI). Results revealed that *R. nigrum* possessed both antibiofilm and antibacterial activity. To our knowledge, this is the first report on the antibiofilm activity of *R. nigrum* leaves against the formation of *P. aeruginosa* PA14 biofilm and hence supporting further investigation on their active phytochemicals.

A high activity was observed for the *n*-hexane extracts of *C. sativum* seeds **(H338) (**69.2 % BFI) and the methanol extracts **(M338)** (63.3 % BFI). *C. sativum* seed extracts were categorised in groups A and B (see Table 18). *C. sativum* is well known for its antibacterial activity (Silva *et al.*, 2011; Wong and Kitts, 2006; Kubo *et al.*, 2004). The only report on antibiofilm activity has been for the essential oil of fresh *C. sativum* against *Candida albicans* biofilm (Furletti *et al.*, 2011). To date there has been no study on *C. sativum* seeds against the formation of *P. aeruginosa* PA14 biofilm. This warranted further investigation on the active phytochemicals in *C. sativum*.

The extracts of *A. graveolens* seeds **(335)** were also found to be active against *P. aeruginosa* PA14 biofilm formation. The methanol and *n*-hexane extracts were very active (around 70 % BFI) but the ethyl acetate extract was slightly lower (57.2 % BFI). A literature search revealed that essential oil of *A. graveolens* consists of β-pinene, camphene, cumene, limonene, α-thuyene, α-pinene, β-phellendrene, ρ-cymene, γ-terpinene, sabinene and terpinolene that possessed antibacterial activity

against *Escherichia coli*, *P. aeruginosa* and *Staphylococcus aureus* (Baananou *et al.*, 2012). To our knowledge, this is the first report on the activity of *A. graveolens* seeds against *P. aeruginosa* PA14 biofilms. This activity was discovered at a late stage in this study and it was not possible to investigate it any further.

Interestingly, more plant extracts increased rather than inhibited the formation of *P. aeruginosa* PA14 biofilm as shown in Figure 22b. Results indicated that a large number of plant extracts (65.9 % of all extracts) had enhanced biofilm formation by compared to only 34.1 % inhibiting biofilm formation. This finding was supported by recent work of (Cho *et al.*, 2013) which identified that 98.5 % from 522 plant extracts could increase biofilm formation in *P. aeruginosa* and *E. coli*.

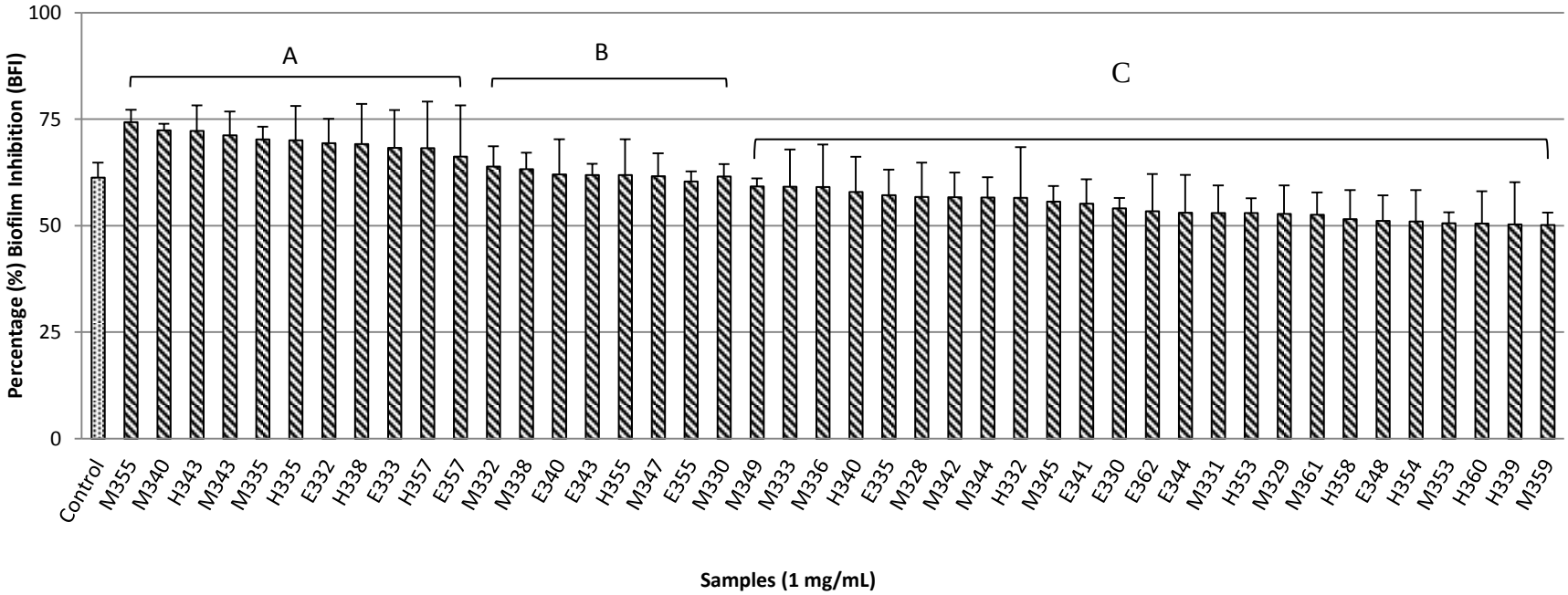


Figure 22 (a): Activity of plant extracts against *P. aeruginosa* PA14 biofilm using a crystal violet staining assay in the microtitre plate format. Bar charts represent sample with more than 50 % biofilm inhibition (BFI). Sample coded as H, E and M are referring to *n*-hexane, ethyl acetate and methanol extracts, respectively. Gentamicin (1 mg/mL) was used as a positive control (Control). Error bars represent the mean $\pm$ SD of six replicates. Activity of active samples are categorised according to their activity compared to positive control. The Group A (very active), B (moderate) and C (fairly active) are in the range of 120 % $\geq$ A $\geq$ 110 $\geq$ B $\geq$ Control=100 $\geq$ C $\geq$ 80 % activity (see Table 18).

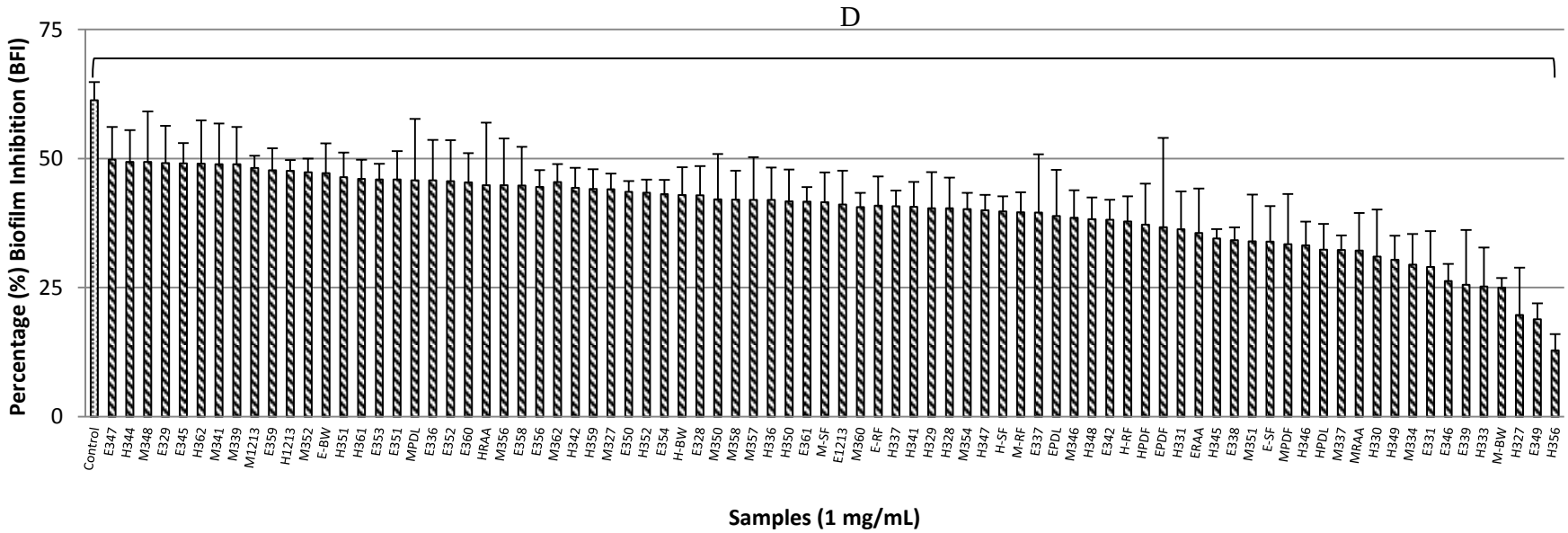
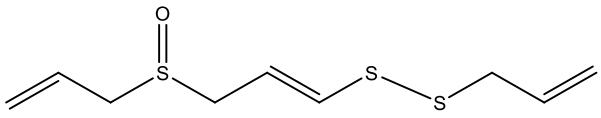


Figure 22(b): (Continued). Activity of plant extracts against *P. aeruginosa* PA14 biofilm using a crystal violet staining assay in the microtitre plate format. Bar charts represent sample with less than 50% biofilm inhibition (BFI) namely Group D (less or non- active) as compared to Control (see Table 18). Sample coded as H, E and M are referring to *n*-hexane, ethyl acetate and methanol extracts, respectively. Gentamicin (1 mg/mL) is used as a positive control (Control). Error bars represent the mean $\pm$ SD of six replicates.

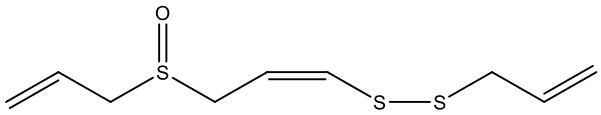
Table 18: Classification of active plant extracts into groups according to their activity compared to the positive control

Group of active extracts		***Activity of extract over positive control (%)**
Group A	Very active	110 - 120
Group B	Moderately active	100 - 110
Group C	Fairly active	80 - 100
Group D	Less or non-active	50 - 10

* Activity of positive control (Gentamicin at 1mg/mL) was taken as 100 % activity to categorise the extracts into groups. Active plant extract are those with more than 50 % BFI activity (see Figure 21b).



(E)-Ajoene



(Z)-Ajoene

Figure 23: Ajoene, mixture of E- and Z- isomers

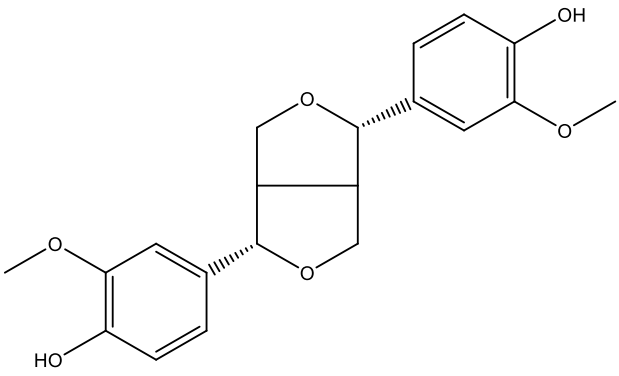


Figure 24: (+)-Medioresinol

3.2 Antibiofilm activity of *Ribes nigrum* leaf3.2.1 Fractionation of the ethyl acetate extract from *Ribes nigrum* leaf

The fractionation scheme followed for the purification of active phytochemicals from *Ribes nigrum* leaf is shown in Figure 25. Both the ethyl acetate **(E333)** and methanol **(M333)** extracts were active against *P. aeruginosa* PA14 biofilm. Only the most active extract, **(E333)** at 68.3 % of biofilm inhibition (BFI) was selected to be further fractionated. By using VLC, **(E333)** was fractionated into eight fractions and all fractions were tested for antibiofilm activity.

The most active fraction, **(E333F1)** with 54.3% BFI was further fractionated using Sephadex® column to afford a total of three fractions. Fraction **(HA30F1S1)** (68.5 % BFI) was further purified using silica column to give seven fractions. Upon screening for activity, none of the latest fractions obtained showed activity and the fractionation process had to be terminated.

Figure 25 also shows that the TLC profile of **(HA30F1S1)** active fraction is consists of mixture of compounds. The loss of activity in the purified fractions **(HA30F1-F7)** were probably as results to the loss of certain amounts of certain compound(s) in the mixture, as small molecules compounds had a tendency to entrap within the silica gel during fractionation process using column chromatography on silica gel.

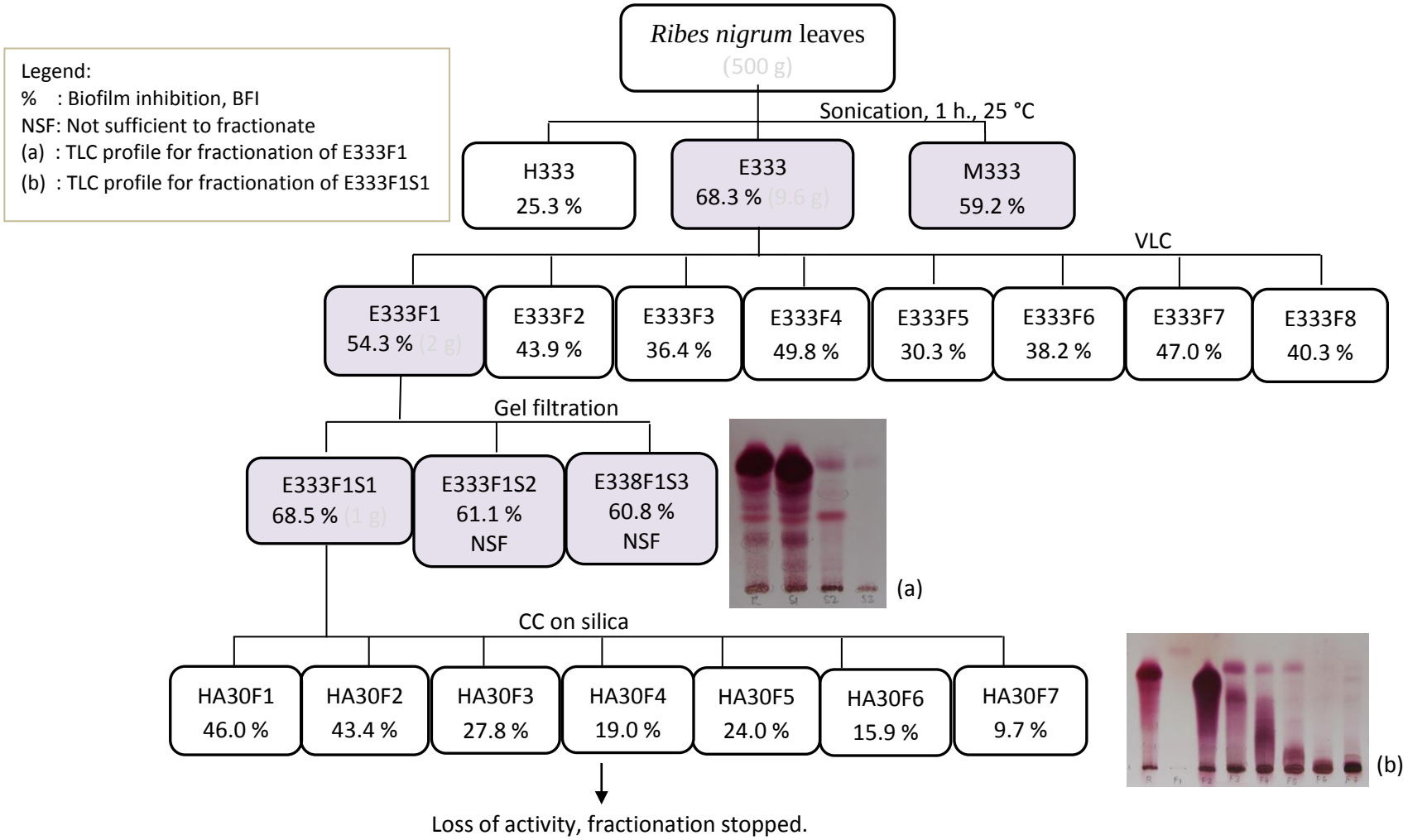


Figure 25: Fractionation scheme of the ethyl acetate extract from *Ribes nigrum* leaves

3.2.2 Characterisation and identification of antibiofilm active products from (HA30F1)

The profile of spots on TLC plate helped us to guide the fractionation of compounds (see Figure 25). Only one of the final fractions **(HA30F1)** was further investigated by ^{1}H NMR (Figure 26). The spectrum indicated the presence of aliphatic carbons with methyl group (CH_3) at 0.86 ppm, a broad methylene envelope (CH_2 groups) at 1.27 ppm and a singlet peak of CH_2 present at 1.55 ppm. The sample was further subjected to GC-MS analysis (Figure 27).

The GC trace and fragmentation pattern obtained for each peak afforded a mixture of alkanes in **(HA30F1)**. The identity of compound was assigned by comparison of mass spectra and retention indices of a series of alkanes. Experimental data were compared with corresponding mass spectral data using NIST Mass Spectral Search Program (NIST MS Search ver. 2.0, 2011) and from literature data. Library search matching of mass spectral for each compound are presented in Appendix 3. The identified four compounds are *n*-nonadecane **(RN1)**, 2-methylnonadecane **(RN2)**, 2-methylicosane **(RN3)** and 2-methyloctacosane **(RN4)** as summarised in Table 19.

Mass spectral of **(RN1-4)** indicated that lower mass alkyl fragments (e.g. C_4-C_6) are more intense (e.g. *m/z* 57, 71, 85) than higher mass fragments (> C_6, e.g. *m/z* 99, 113, 127 *etc*.). The peak height is corresponding to subsequent fragments after the C_4 peak decrease in an exponential fashion to a minimum at [M-C_2H_5]. The base peak at *m/z* 57 is corresponding to the C_4H_9 and separated by 14 mass units resulting from the loss of CH_2 group. The fragment of [M-CH_3] exhibited a weak peak in a smaller compound and absent in long chain compounds at which due to relative instability of

the methyl radical. While the prominent peaks (C_nH_{2n+1}, *m/z* 57, 71, 85) with the decaying intensity of these peaks indicates those compounds are an alkane (Figure 28-31).

Mass spectrum for **(RN1)** in Figure 28 showed an exponentially decreases of fragment masses with an increase of mass (*m/z*) which an indication for a straight chain alkane (Beauchamp, 2009). The molecular ion, [M^+] at *m/z* 268 indicates that this compound is *n*-nonadecane and the mass ions are identified as in literature (Huneck and Snatzke, 1965). Mass spectra for **(RN2)**, **(RN3)** and **(RN4)** also contain peaks at C_nH_{2n+1}, however lacks of smooth exponential decay at *m/z* 71 and *m/z* 85 that suggest that the chain is branched (Figure 29-31).

The intensity of the peak at m/z 71 indicates a fragment at C_4 fragment suggesting a methyl group located on the second carbon (Dunnivant and Ginsbach, 2011). Spectral also shows the loss of largest alkyl fragment at the branched site is favoured in stabilised the radical resulted to a more stable secondary carbonation. Smaller [M^+] is presence in branched alkanes and also may be absent in highly branched compounds. Thus, the molecular ion, [M^+] at *m/z* 282 **(RN2)**, *m/z* 296 **(RN3)** and *m/z* 408 **(RN4)** indicate that these compounds are 2-methylnonadecane, 2-methylicosane and 2-methyloctacosane, respectively and the mass ions are identified as in literature (Krkošová *et al.*, 2007).

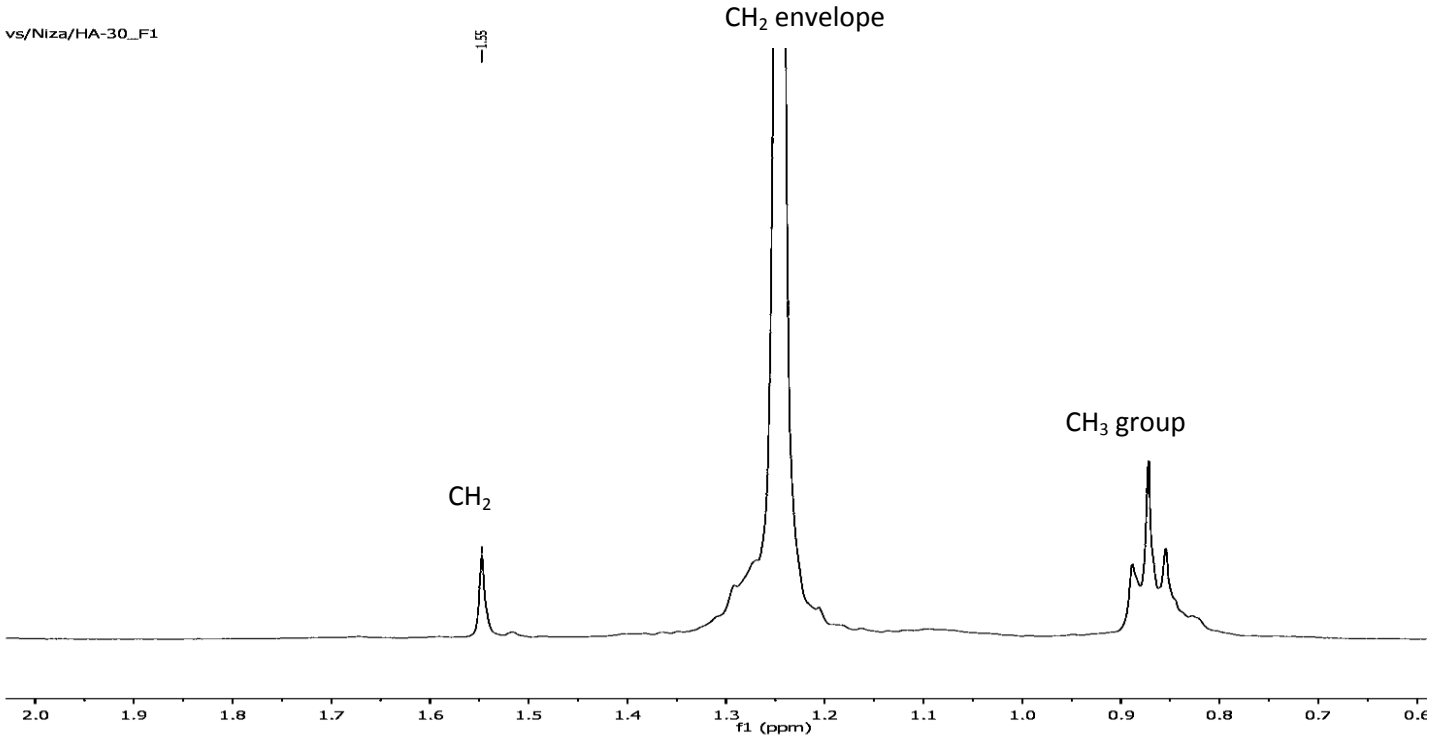


Figure 26: ^{1}H (400MHz) NMR spectrum (selected expansion) of **(HA30F1)** in $CDCl_3$

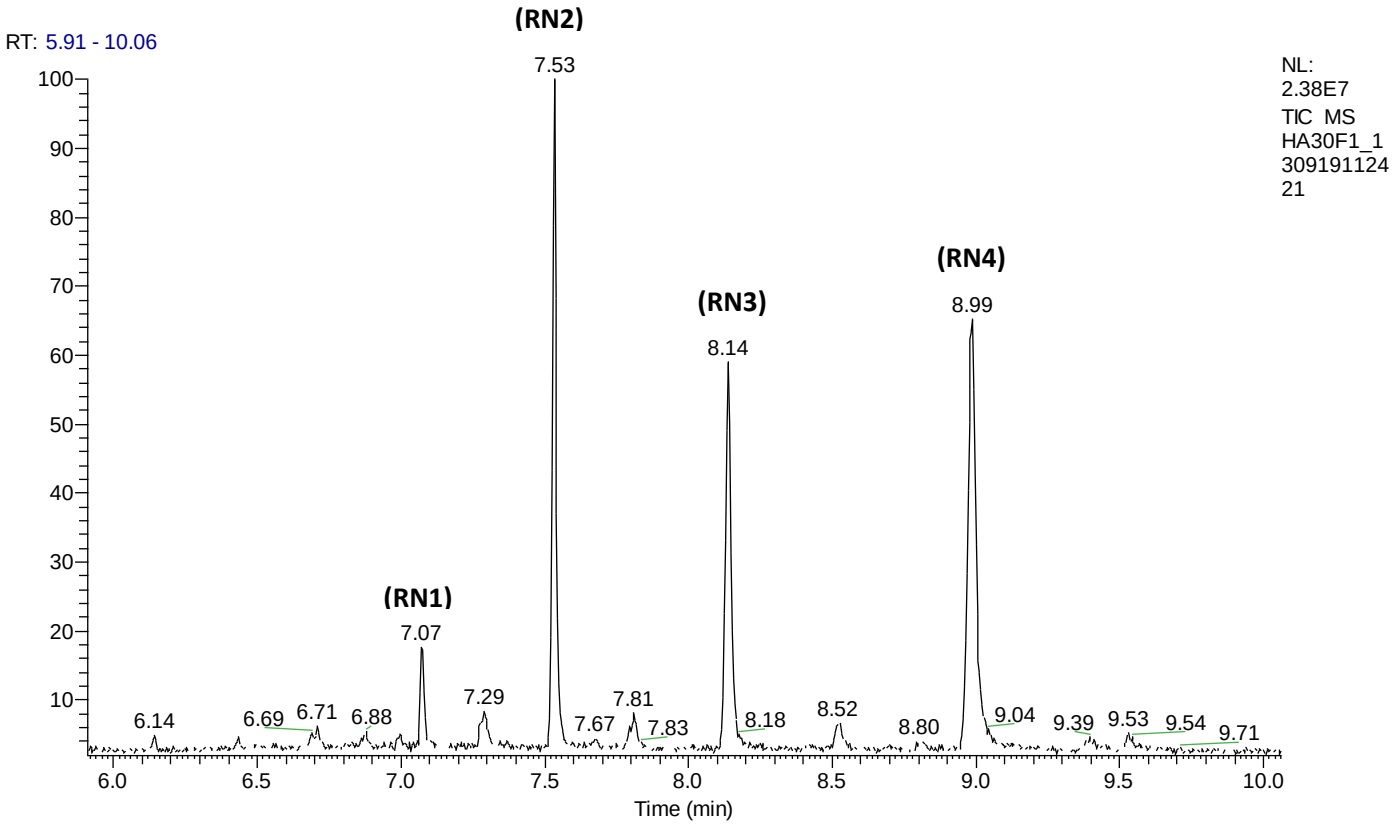


Figure 27: GC-MS (selected expansion) of trace in **(HA30F1)**

Table 19: Trace compounds in **(HA30F1)** identified by GC-MS

Peak	Retention time, t (min)	Compounds	Molecular formula	Molecular weight (g/mol)
(RN1)	7.07	*n*-nonadecane	$C_{19}H_{40}$	268.52
(RN2)	7.53	2-methylnonadecane	$C_{20}H_{42}$	282.55
(RN3)	8.14	2-methylicosane	$C_{21}H_{44}$	296.57
(RN4)	8.99	2-methyloctacocosane	$C_{29}H_{60}$	408.47

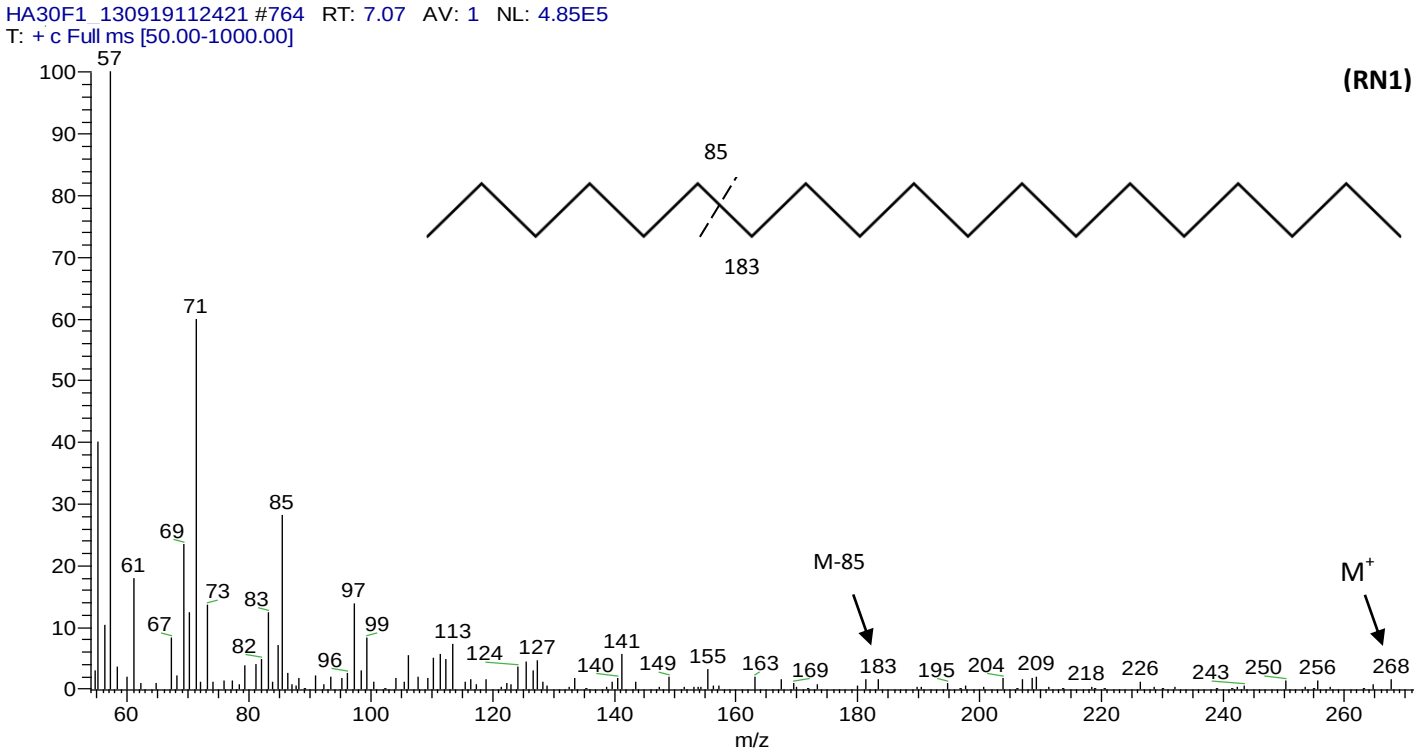


Figure 28: Spectrum of alkane **(RN1)** in **(HA30F1)** identified as *n*-nonadecane

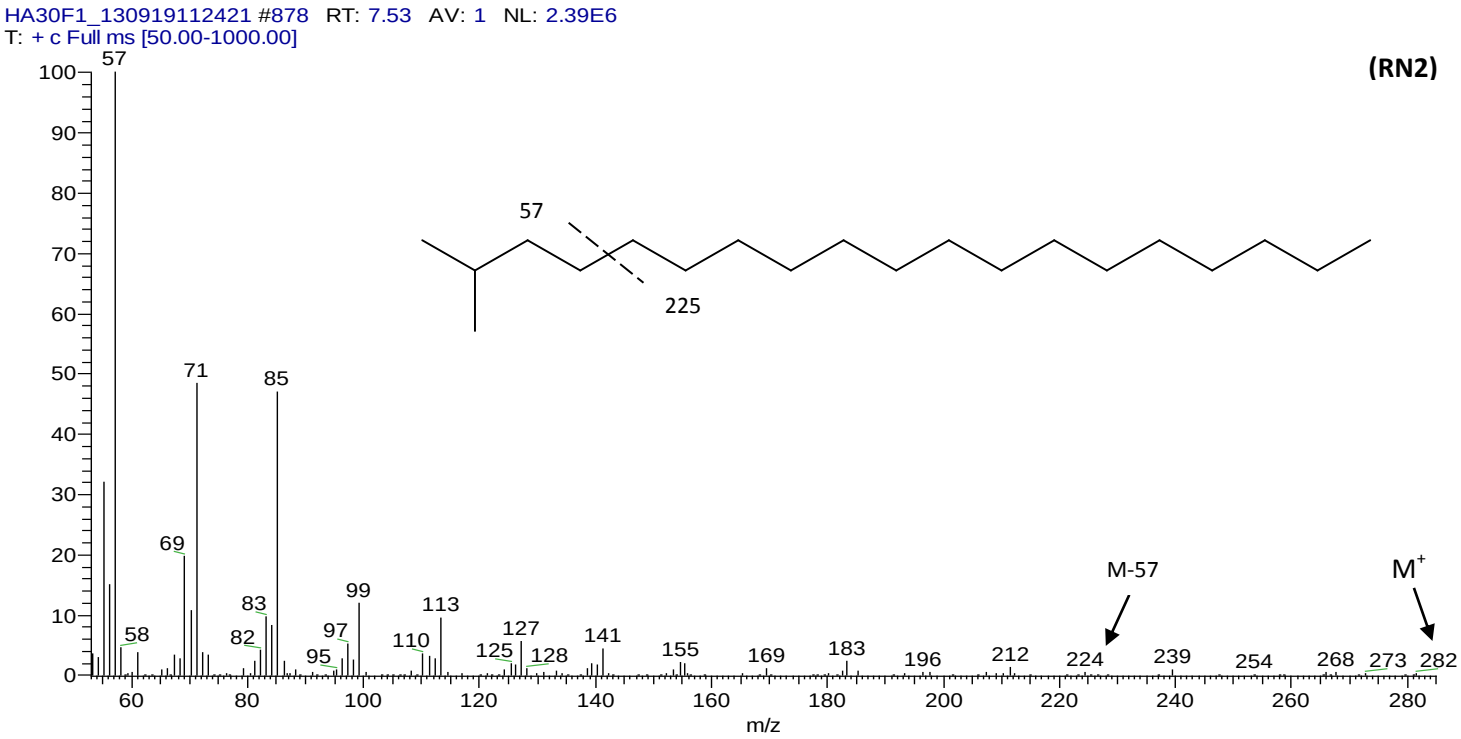


Figure 29: Spectrum of alkane **(RN2)** in **(HA30F1)** identified as 2-methylnonadecane

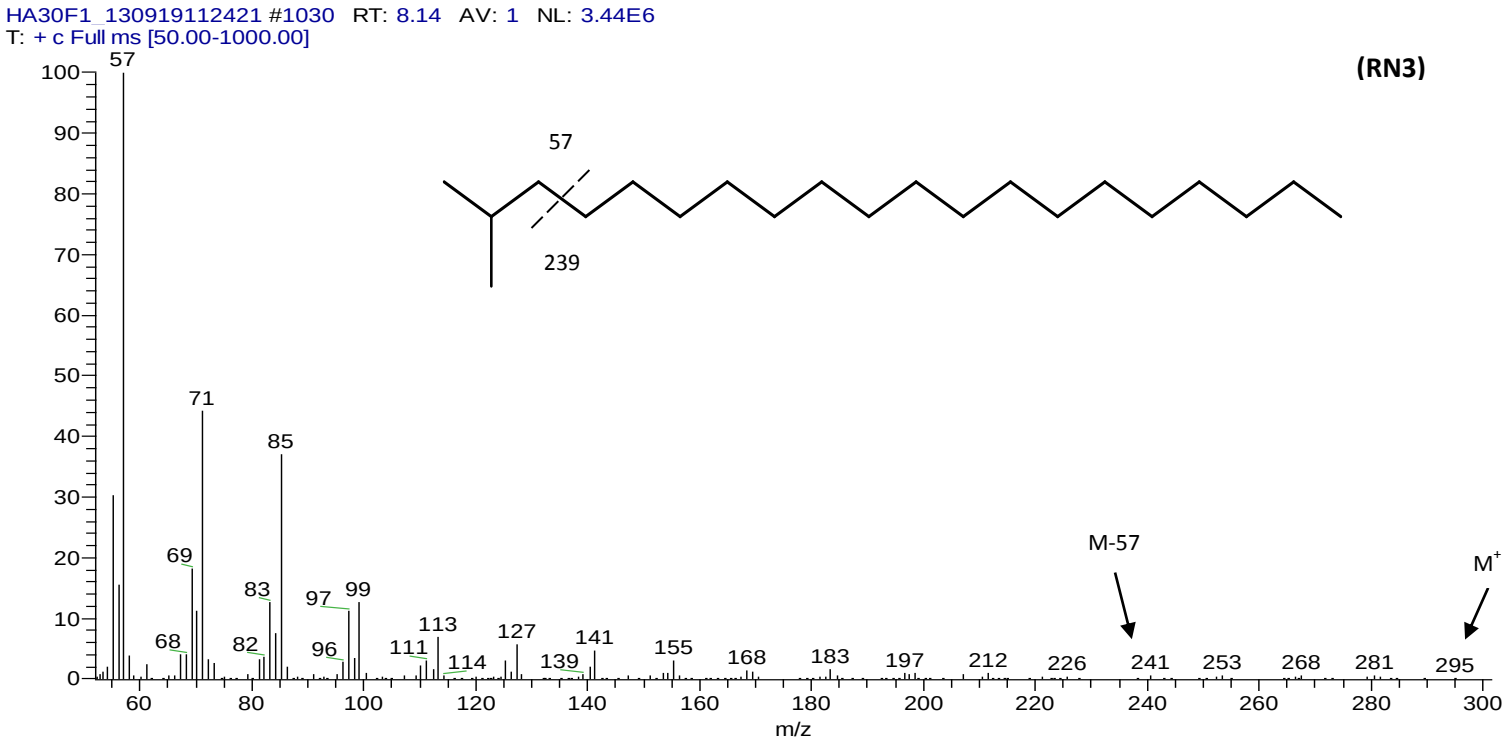


Figure 30: Spectrum of alkane **(RN3)** in **(HA30F1)** identified as 2-methylicosane

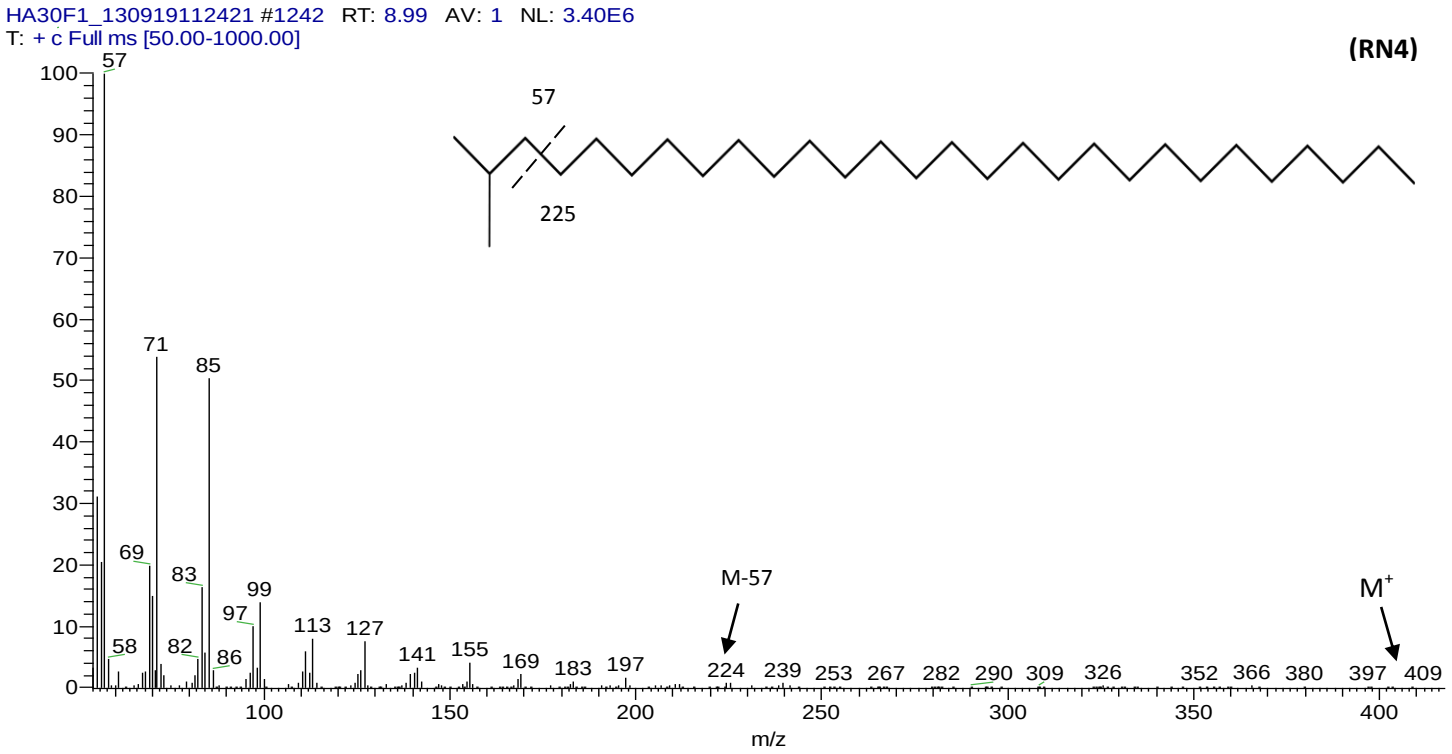


Figure 31: Spectrum of alkane **(RN4)** in **(HA30F1)** identified as 2-methyloctacosane

3.3 Antibiofilm activity of *Sambucus nigra* flowers

3.3.1 Characterisation of (E341) as the active antibiofilm product from *Sambucus nigra* flowers

The antibiofilm activity of extracts from *Sambucus nigra* flowers are shown in Table 20. The ethyl acetate extract of *S. nigra* flower **(E341)** was obtained as a white powder. Upon TLC analysis, the extract showed as a major pink colour spot (R_f 0.55) after spraying with anisaldehyde-H_2SO_4. This suggested the presence triterpene or triterpenic mixture. Further ^{1}H and ^{13}C NMR analyses (Figures 32 and 33) revealed that **(E341)** was indeed a mixture of two very common pentacyclic triterpenes, namely ursolic acid and oleanolic acid with ursolic as the major compound at a 2:1 ratio. The NMR analyses of mixture ursolic and oleanolic acid at 2:1 ratio was also reported in rosemary and sage leaves (Kontogianni *et al.*, 2009).

HRESI-MS analysis (Figure 34) of **(E341)** in the negative mode indicated a quasi-molecular ion peak $[M-H]^-$ at m/z 455.3531, suggesting a molecular ion $[M]^+$ of 456.3603 and corresponding to the molecular formula of $C_{30}H_{48}O_3$ (DBE = 7). Ursolic acid has been previously found in other *Sambucus* species such as *Sambucus chinensis* (Liu, 1995), *Sambucus ebulus* (Schwaiger et al., 2011) and *Sambucus adnata* (Sasaki *et al.*, 2011).

This is the first finding that revealed the presence of ursolic and oleanolic acids (Figure 35) in the flowers of *S. nigra*. Ursolic and oleanolic acid have only been reported so far in the bark (Huneck and Snatzke, 1965) and leaves (Inoue and Sato, 1975) of *S. nigra.* These results are also the first report on the activity of ursolic and oleanolic acids (as a 2:1 mixture) against *P. aeruginosa* PA14 biofilm.

Table 20: Activity of *Sambucus nigra* flower extracts and compounds against *P. aeruginosa* PA14 biofilms

Extracts of *S. nigra* flower	Activity of biofilm inhibition, BFI (%)	Antibiofilm active compounds
(H341)	40.7	n/d
(E341)	55.2	Mixtures of ursolic acid and oleanolic acid (2:1 ratio)
(M341)	49.0	n/d

n/a : not detected. Extracts with symbols of H, E and M are respectively referring to the *n*-hexane, ethyl acetate and methanol extracts of *S. nigra* flower.

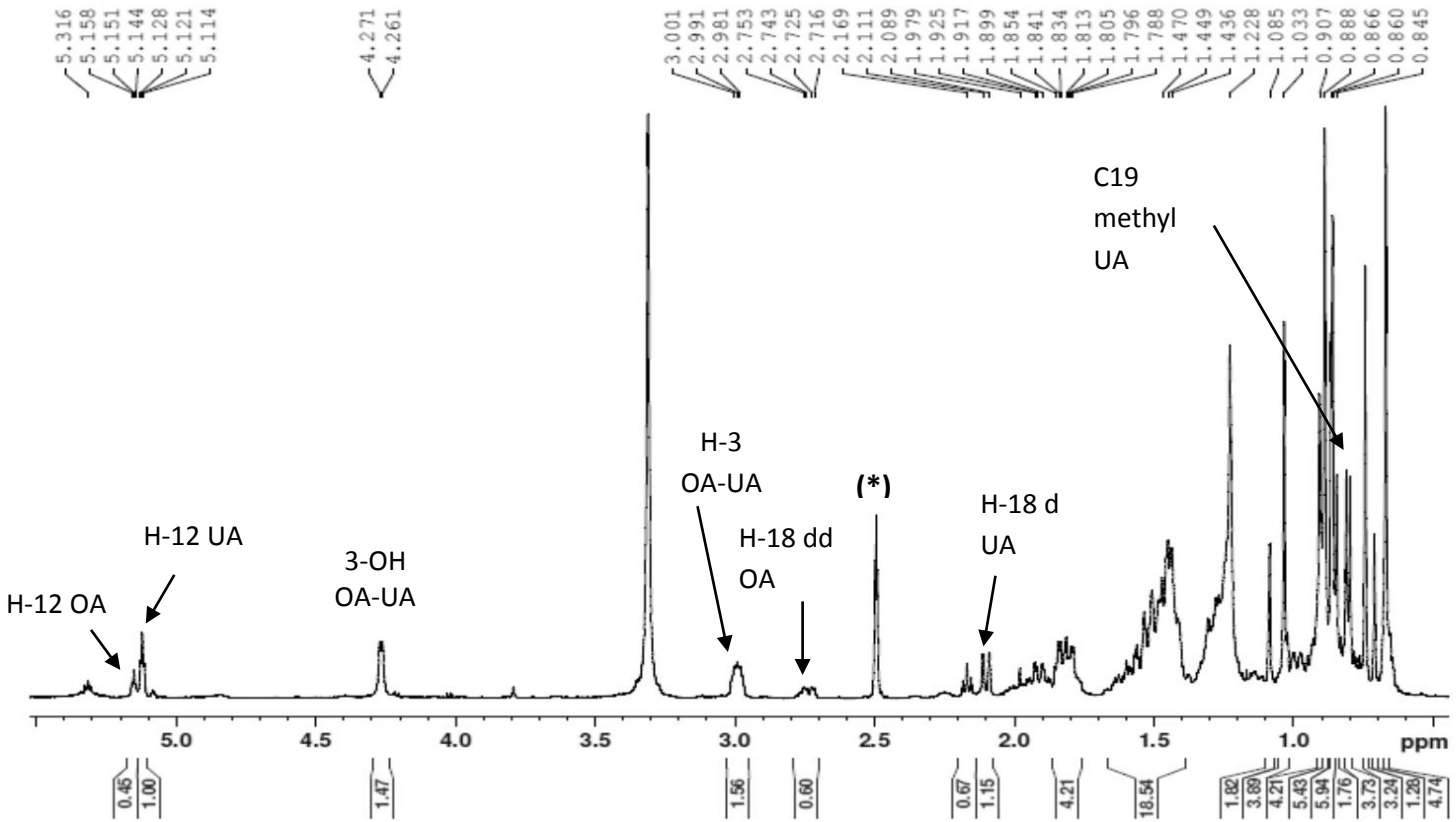


Figure 32: ^{1}H (500MHz) NMR spectrum (selected expansion) of **(E341)** in DMSO-d_6 (*). Stereochemistry of ursolic acid and oleanolic acid) are shown in Figure 35. Codes of (UA) and (OA) are referring to ursolic acid and oleanolic acid, respectively.

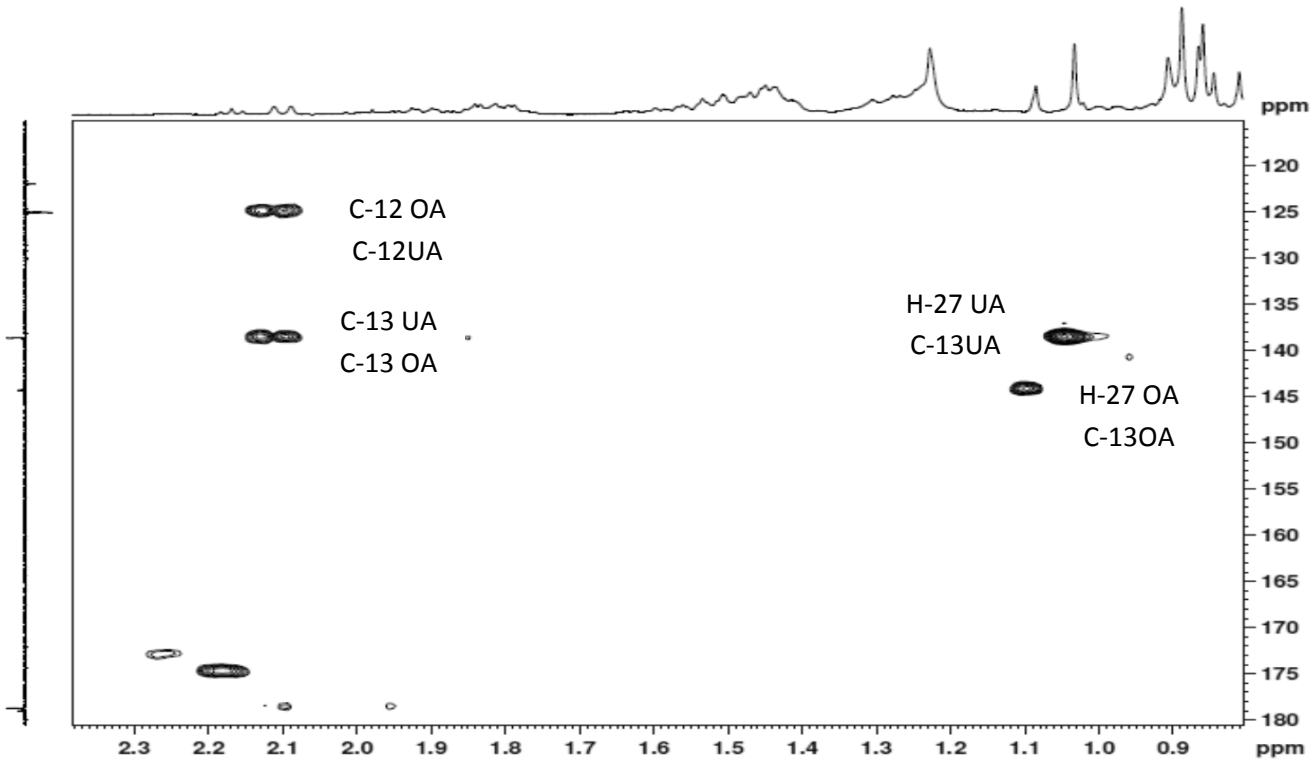


Figure 33: HMBC spectrum (selected expansion) of **(E341)**

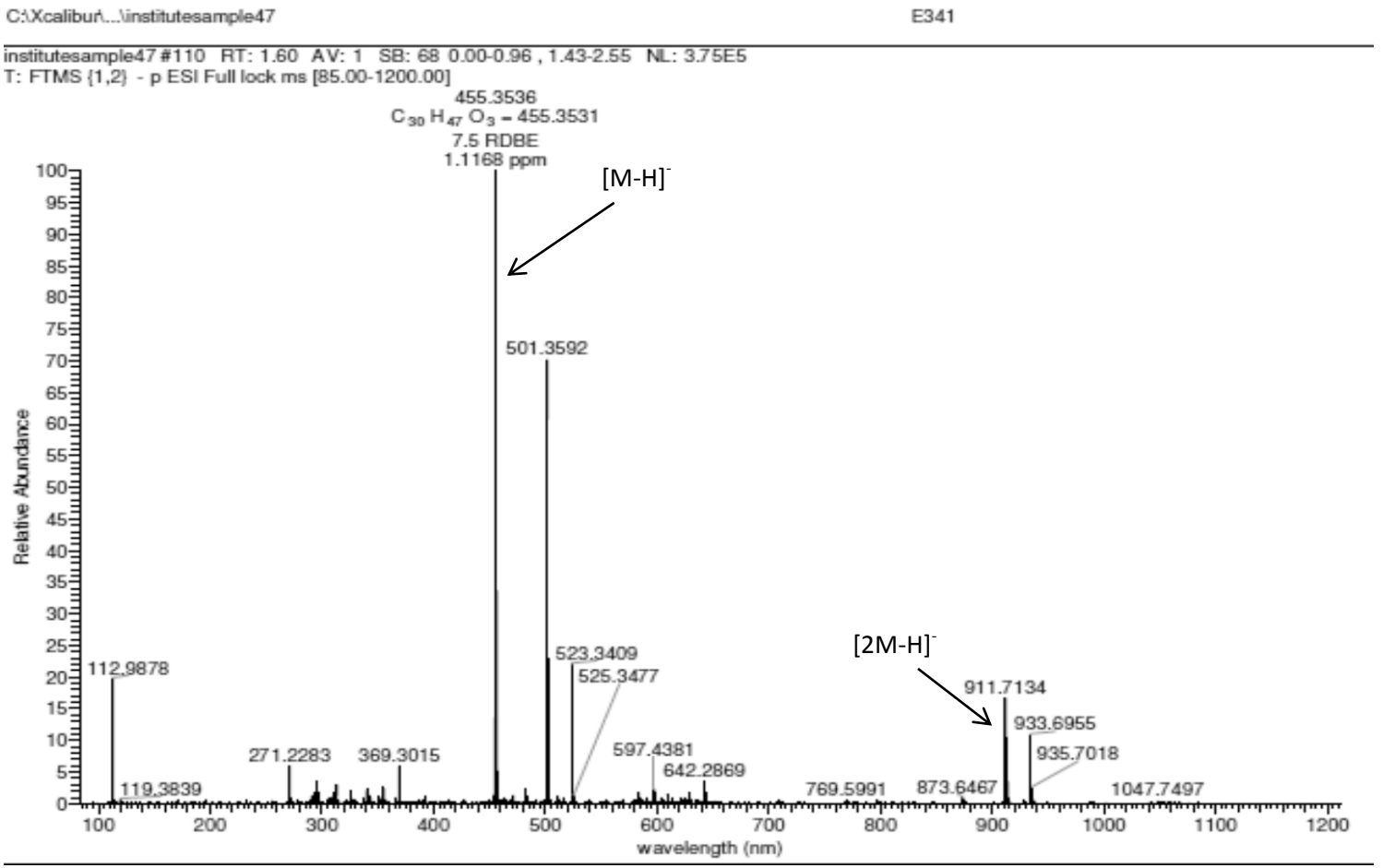
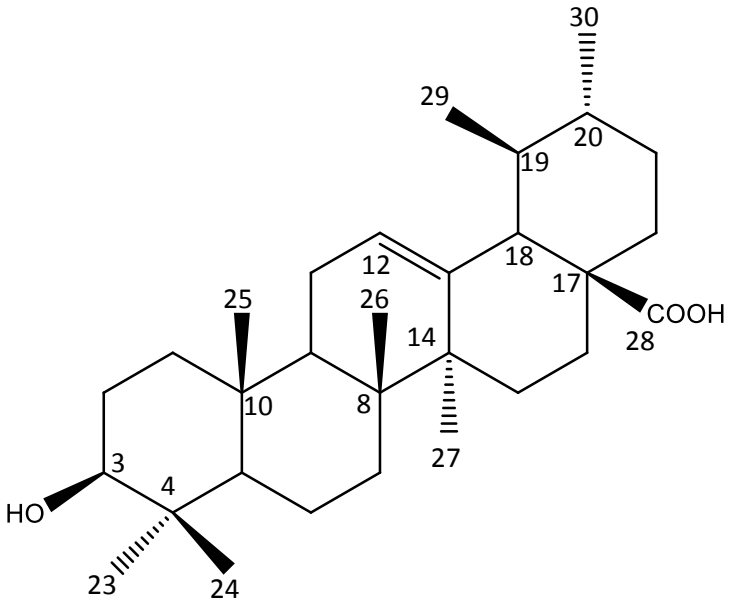
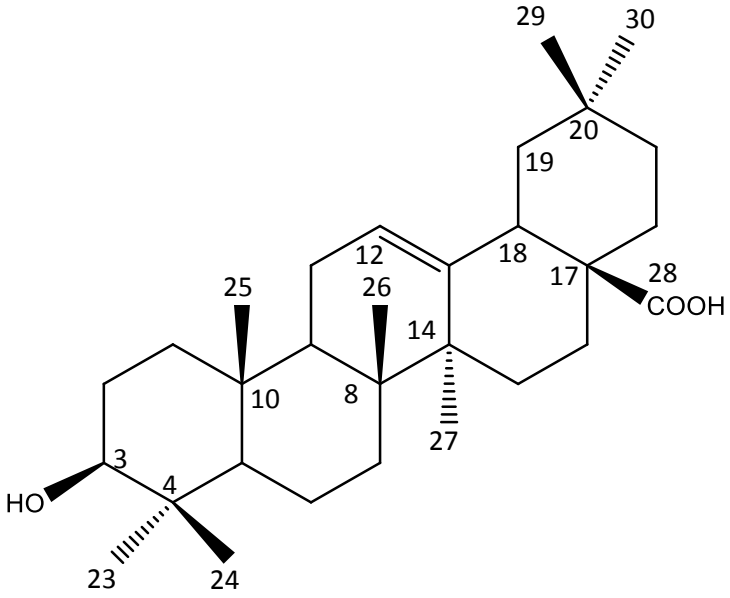


Figure 34: HREI-MS spectrum of **(E341)**



Ursolic acid



Oleanolic acid

Figure 35: Structures of ursolic acid and oleanolic acid

3.4 Antibiofilm activity of *Coriandrum sativum* seeds

3.4.1 Fractionation of the *n*-hexane extract from *Coriandrum sativum* seed

C. sativum seeds were fractionated into three main extracts (Figure 36). The **(H338)** and **(M338)** extract showed 69.2 and 63.3 % BFI, respectively, indicating a potent activity against *P. aeruginosa* PA14 biofilm. The most active extract, **(H338)** was first investigated and afforded nine fractions. All fractions were tested against *P. aeruginosa* PA14 biofilm. Only fraction **(H338F3)** showed activity at 57.6 % BFI.

Further fractionation of **(H338F3)** produced 11 fractions namely **(H338F3S1)** to **(H338F3S13)**, however only **(H338F3S10)** and **(H338F3S12)** showed strong antibiofilm activity at 60.8 and 67.9 % BFI, respectively. The other four fractions were inactive **(H338F3S1, -S2, -S11** and **–S13)** while the remaining fractions **(H338F3S2-S89)** enhanced biofilm production.

Fraction **(H338F3S13)** and **(H338F3S12)** showed nearly similar TLC profiles. They were therefore pooled and recoded as **(HA14)**. Fractionation of **(HA14)** further produced four fractions, namely **(HA14F1-F4).** Antibiofilm screening however, showed that all fractions did not inhibit more than 50 % of *P. aeruginosa* PA14 biofilm and the fractionation had to be terminated.

The fractionation of the second most active fraction, **(H338F3S10)**, produced six fractions, **(HA1-6)**. Three fractions, namely **(HA2, HA3** and **HA6)** showed antibiofilm activity at 51.7, 52.2 and 62.5 % BFI, respectively. Two fractions were inactive **(HA4** and **HA5)** and one fraction **(HA1)** could not be screened due to insufficient amount obtained.

No significant ($p < 0.05$) of antibiofilm activity could be seen for **(HA6)**, **(HA3)** and **(HA2)** from Positive Control (Gentamicin). The antibiofilm activity of those fractions was as active as Positive Control. The other fractions, **(HA4)** and **(HA5)** were significantly different ($p < 0.05$) with Positive Control (Gentamicin), indicating that those fractions were not antibiofilm active.

The antibiofilm activity of **(HA6)** and Positive Control was found highly significantly different ($p < 0.05$) at which indicated that the activity of both are significantly similar (see Tukey Method, Appendix 4.2). The insufficient amounts of **(HA6)** obtained however hindered further fractionation. Thus, **(HA6)** is the most active fraction against *P. aeruginosa* PA14 biofilm (62.5 % BFI). **(HA6)** was therefore subjected to LC-MS analysis.

3.4.2 Fractionation of the methanol extract from *Coriandrum sativum* seeds

Figure 37 shows the fractionation scheme obtained for the methanol extract from *C. sativum* seeds. The methanol extract **(M338)** was partitioned into DCM **(M338D)**, *n*-butanol **(M338B)** and water **(M338W)** extracts. Antibiofilm screening showed that all extracts exhibited strong antibiofilm activity. The most active extract was **(M338D)** with 65.4 % BFI followed by **(M338W)** and **(M338B)** at 62.1 and 53.4 % BFI, respectively.

Fractionation of **(M338D)** extract gave a total of six fractions, **(M338DF1-F6)**. Two fractions **(M338DF2** and **-F6)** were found inactive while the remaining **(M338DF3, -F4** and **-F5)** enhanced biofilm production. Only fraction **(M338DF1)** was active (51.6 % BFI) against *P. aeruginosa* PA14 biofilm.

Fractionation of **(M338DF1)** afforded five fractions, **(HA15F1-F5)**. Three fractions were found inactive **(HA15F3, -F4** and **-F5)** and two fractions **(HA15F1 and –F2)** were obtained with insufficient amount for further screening. Other active extracts such as **(M338B)** was subjected to LC-MS analysis and their antibiofilm compounds were identified using MZmine software.

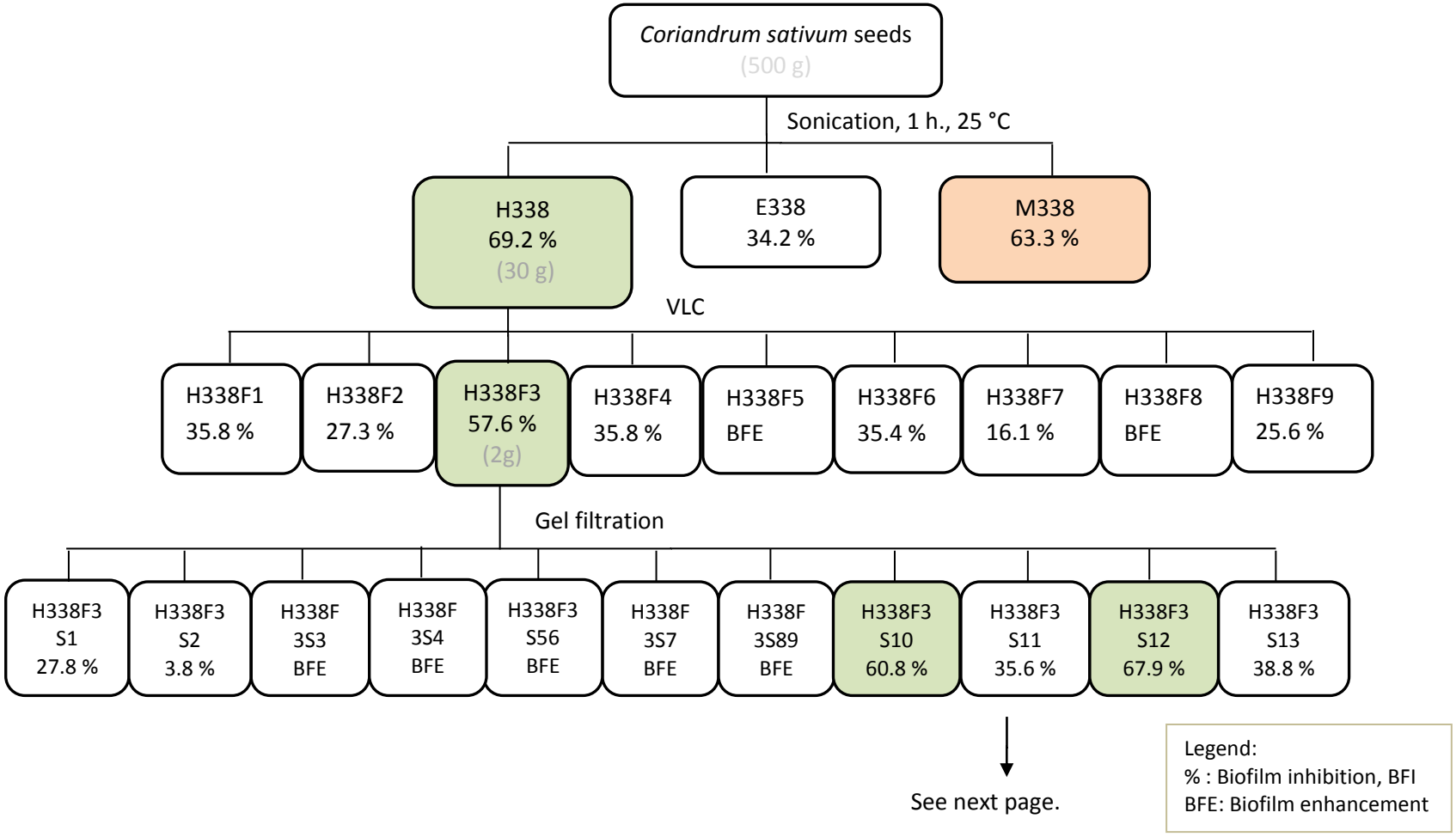


Figure 36: Fractionation scheme of the *n*-hexane extract of *Coriandrum sativum* seeds

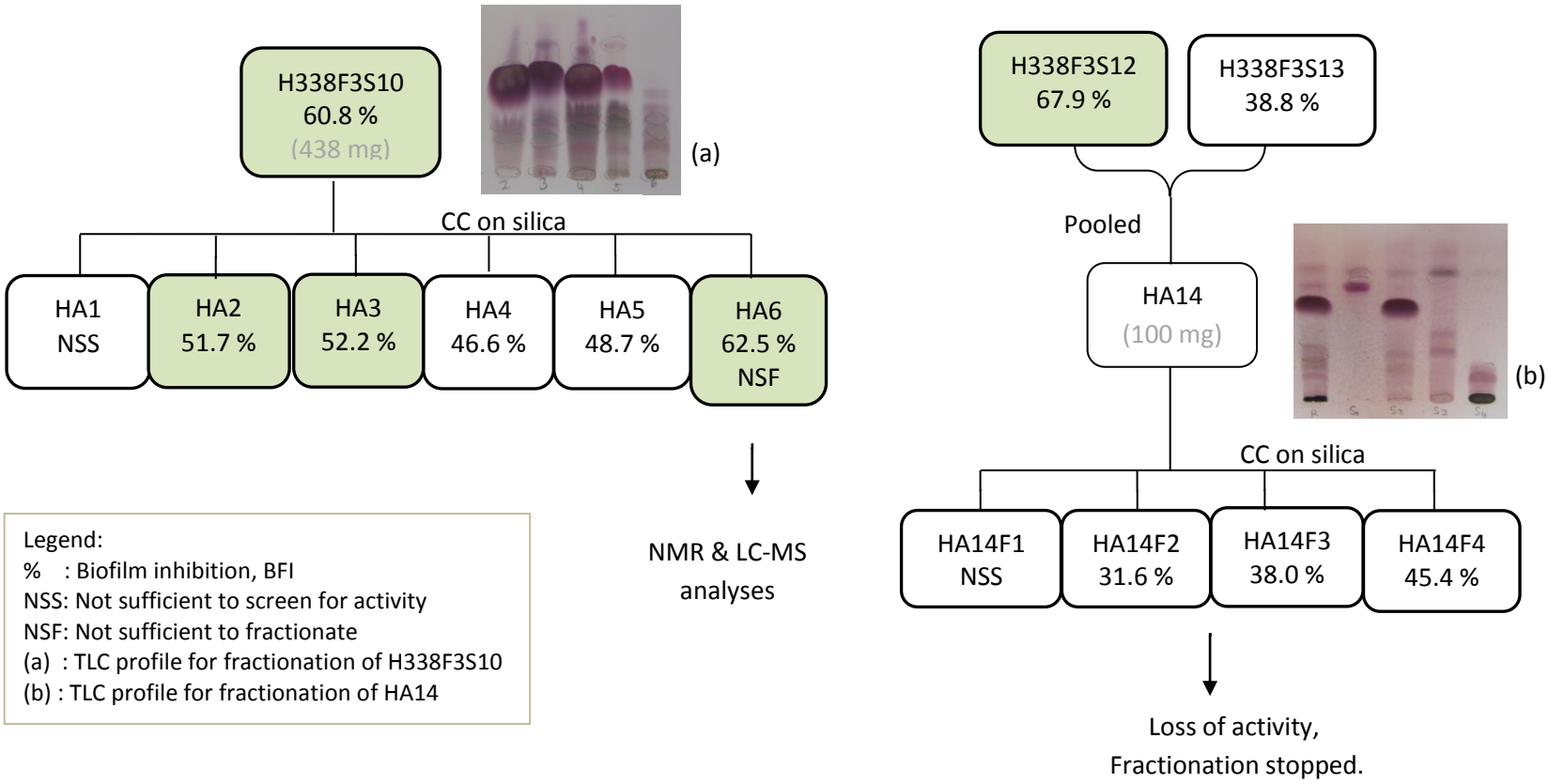


Figure 36: (continued). Fractionation scheme of the *n*-hexane extract of *Coriandrum sativum* seeds

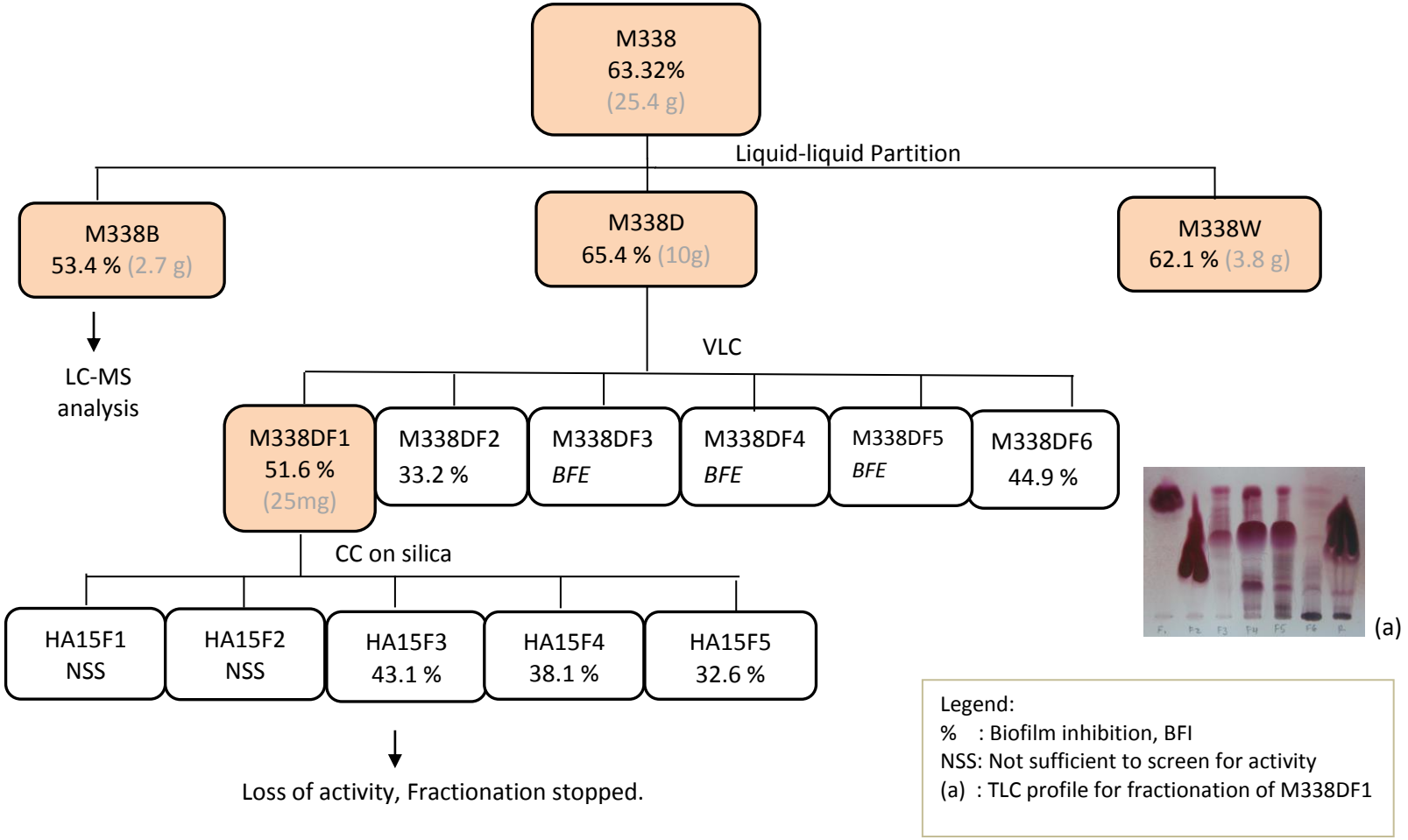


Figure 37: Fractionation scheme of the methanol extract of *Coriandrum sativum* seeds

3.4.3 Characterisation and identification of antibiofilm active products

3.4.3.1 Characterisation of (HA6)

(HA6) was obtained as a white powder isolated from the *n*-hexane extract of *C. sativum* seeds. On TLC, it showed three strongly absorbing spots under UV light (λ_{254} nm). After spraying with *p*-anisaldehyde-sulfuric acid reagent and heating, a spot on the baseline gave a brown colour while other two spots (R_f 0.24 and 0.32) took a greenish colour. Three spots (R_f 0.16, 0.40 and 0.46) that were not previously detected under UV light exhibited a purple colour (see Figure 36).

In its ^{1}H NMR spectrum (Figure 38), **(HA6)** presented some resonances in the region of 0 - 2.0 ppm attributable to protons of aliphatic carbons; 2.0- 3.3 ppm attributable to protons on carbons next to aromatic, carboxylic, carbonyl or other electron-drawing groups; 3.3- 6.5 ppm attributable to protons on carbons next to oxygen atoms (e.g. methoxy groups and aldehydes) and protons associated with oxygen (e.g. alcoholic, phenolic and carboxylic groups) or nitrogen (e.g. amines) and 6.5- 9.0 ppm attributable to protons associated with aromatic carbon atoms (Lu *et al.*, 2004). Signal assignments on the ^{13}C NMR spectrum of **(HA6)** are presented in Figure 39. Both spectra and assignment of ^{1}H and ^{13}C NMR data for **(HA6)** are presented in Table 21.

(HA6) was subjected to LC-MS analysis in the positive and negative ESI mode (Table 22-23, Figure 40) and trace of compounds were identified using MZmine software. Possible metabolites identified in the ESI positive mode (Table 22) include 10-undecenal, linalool oxide (pyranoid and furanoid), 4-ethylcamphor, dodecanal, 2-hexylfuran and caryophyllene oxide. Possible metabolites identified in the ESI

negative ionisation mode of **(HA6)** include 12(13)-EpOME and (E)-12,13-dihydroxy-11-methoxyoctadec- 9-enoic acid (Table 23).

Amongst these possible compounds detected in **(HA6)**, compounds known to be present in *C. sativum* (based on literature reports) are camphor and dodecanal (Chung *et al.*, 2012), linalool oxide (Galata *et al.*, 2014), 10-undecenal (Donega *et al.*, 2014), 2-hexylfuran (Pluskal *et al.*, 2010) and caryophyllene oxide (Sahib *et al.*, 2013).

Camphor oil has showed antibiofilm activity against *P. aeruginosa* infected burnt mice (Raghad, 2012). It is possible that camphor disrupts *P. aeruginosa* PA14 cell membrane. A previous study has demonstrated that simple phenolics could penetrate into the periplasmic space and cytoplasmic membrane of *P. aeruginosa* and could increase the cell membrane permeability, leading to a leakage of cellular components and cell damage (Witkowska et al., 2013). In addition, it is known that *P. aeruginosa* PA14 contains some specific catabolic genes to use camphor as a source of energy for its growth which would favour the penetration of camphor in the cells (Bhuvaneswari, 2013).

The antibiofilm activity of dodecanal could probably due to reduced hydrophobicity on the cell surface of *P. aeruginosa* PA14. Hydrocarbons such as a mixture of C_{12} and C_{16} can reduce the hydrophobicity of the cell surface of *P. fluorescence* and *P. putida* (Kaczorek and Olszanowski, 2011). To our knowledge this is the first report on antibiofilm activity of dodecanal against *P. aeruginosa* PA14 biofilms.

Aldehyde derivatives (e.g. glutaraldehyde) are capable of removing biofilms of *P. aeruginosa* ATCC 27853 on infected medical equipment (Balsamo *et al.*, 2012). We

hypothesized those aldehyde derivatives such as 10-undecenal identified in this study supposedly could possess similar activity against *P. aeruginosa* PA14 biofilms.

Oxygenated monoterpenes are reported as active antibiofilm agents. The oxygenated monoterpenes of linalool oxide which majorly present in *Mentha piperita* had showed antibiofilm activity against biofilm of *Enterococcus faecalis* (Benbelaid *et al.*, 2014). To date no reports have been published on the activity of linalool oxide in *C. sativum* seeds against *P. aeruginosa* PA14 biofilms. This finding was interesting to further explored, thus subsequent studies were performed and the findings were presented in the last chapter of this thesis.

As for 2-hexylfuran, the heterocyclic ring of furanone is capable of interfering with QS signalling molecules in *P. aeruginosa*. The compound penetrates into microcolonies, blocking cell signalling and quorum sensing in biofilm cells, thus reducing the production of virulence factors and finally causing loss of bacterial biomass due to bacterial detachment (Hentzer *et al.*, 2002).

Caryophyllene oxide also possessed antibiofilm activity. The compound which is present in the essential oil of *Murraya koenigii* showed QS inhibitory and antibiofilm activity against *P. aeruginosa* PA01 (Bai A and Vittal, 2014). To our knowledge this is the first report on activity of caryophyllene oxide in *C. sativum* seeds against biofilm of *P. aeruginosa* PA14.

12(13)-EpOME (called (E)-12,13-epoxy-9-octadecenoic acid, vernolic acid or isoleukotoxin) (Pluskal *et al.*, 2010) is a lipophilic compound and first characterised as an epoxy oleic acid (Takeda *et al.*, 2010). Many epoxide-containing lipids are bioactive (Hou, 2008) and serve as signalling molecule to their host organisms

(Newman *et al.,* 2005). The compound is presents in a seed oils as triacylglycerols and concentrated in plant cutin and suberin. To our knowledge this natural epoxy fatty acid was first reported in *C. sativum* seeds. The role of this epoxide-containing lipid has not been studied on *P. aeruginosa* PA14 elsewhere yet, thus this compound is interesting as an excellent model of lipid signalling in antibiofilm activity against *P. aeruginosa* PA14 biofilm in near future.

(E)-12,13-dihydroxy-11-methoxyoctadec- 9-enoic acid is an oxygenated unsaturated fatty acids product from the bioconversion of linoleic acid. This product is a potential antimicrobial agent that showed antibacterial activity including against food-borne pathogens (Hou, 2008). To our knowledge, this is the first time (E)-12,13-dihydroxy-11-methoxyoctadec- 9-enoic acid has been identified in *C.sativum* seeds. The antibiofilm activity of this compound against *P. aeruginosa* PA14 has not been reported elsewhere. In depth study therefore could investigate the mechanism beneath the inhibition of biofilm using this compound in near future.

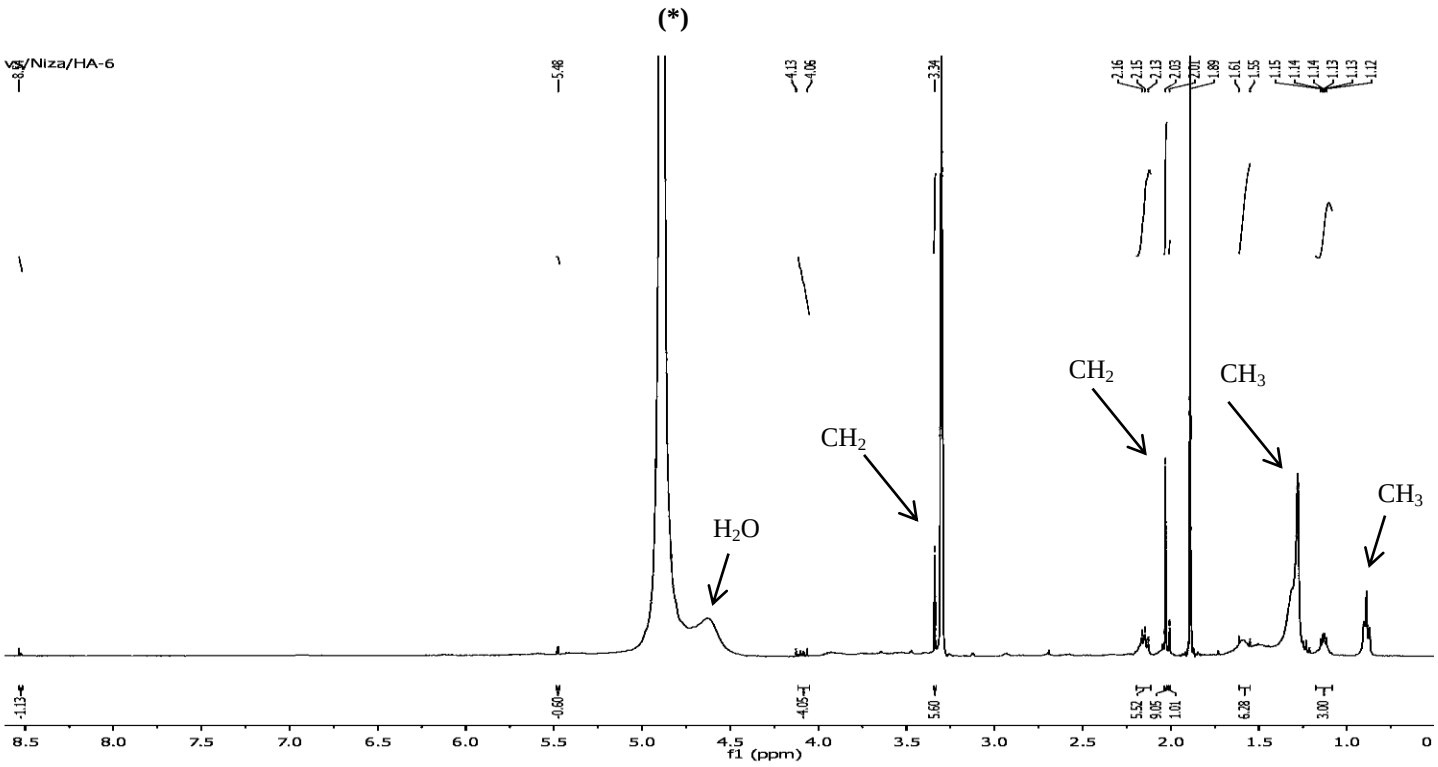


Figure 38: ^{1}H (400 MHz) NMR spectrum of **(HA6)** in CD_3OD (*)

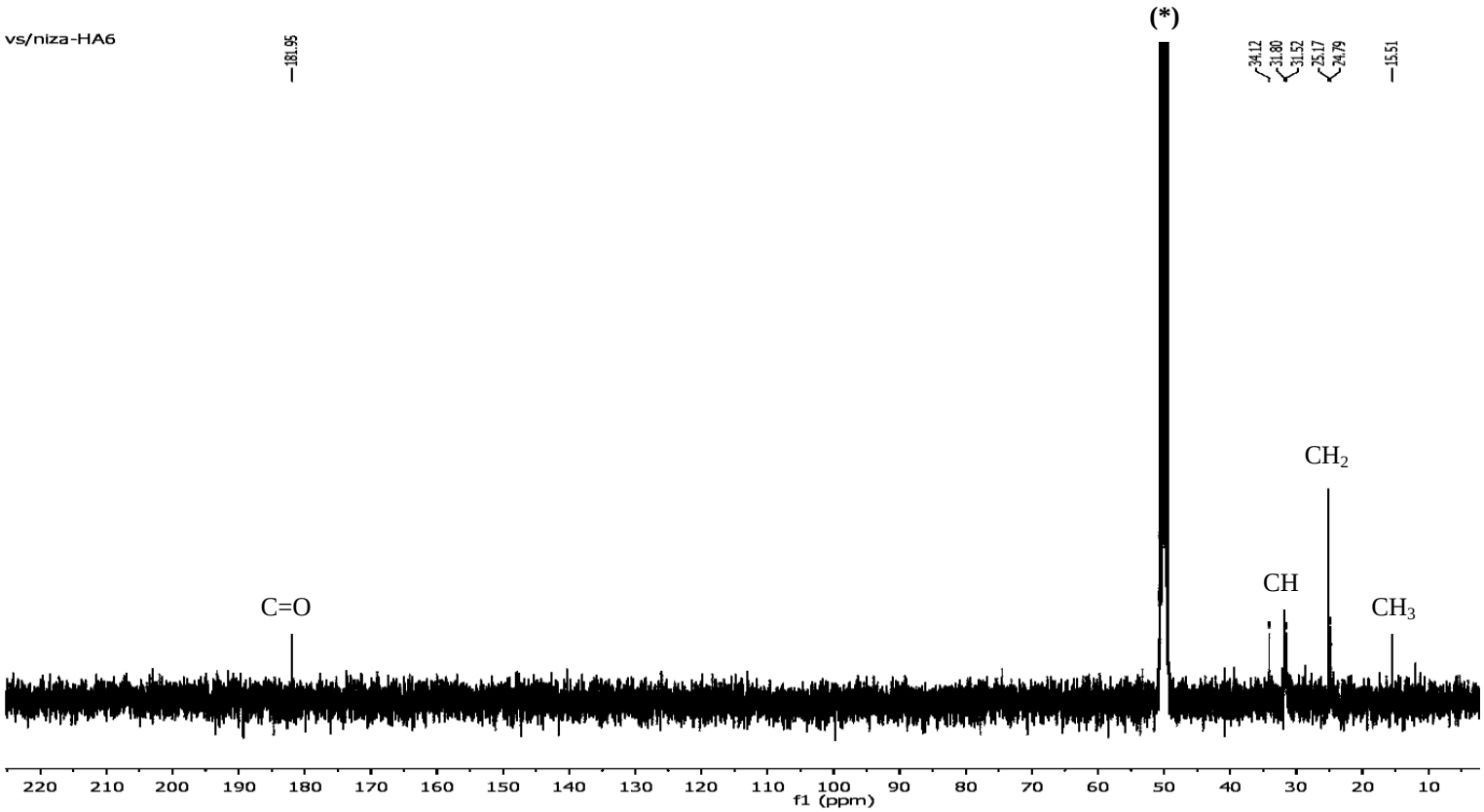


Figure 39: ^{13}C (100 MHz) NMR spectrum of **(HA6)** in CD_3OD (*)

Table 21: ^{1}H and ^{13}C NMR peak assignments for **(HA6)**

δ_H (*J* Hz)	Proton types	δ_C (*J* Hz)	Carbon types
0.86	- CH_3	15.51	
1.26	- $(CH_2)_n$	24.79	RCH_2-
1.89	- CH_{2-}	25.17	
2.02..2.04	- CH_3	31.82	
2.11..2.20	- CH2-	34.12	-CH_2-
2.68..2.69	- OH	181.95	C=O
3.32..3.35	- CH_{2-}		
3.34	- CHOH		
4.26..4.28	- CH-		
5.48	- CH=CH -		
8.52..8.54	Ar-H		

All spectra were recorded in CD_3OD with δ_H at 3.31 ppm and δ_C at 49.00 ppm.

Table 22: Compounds identified in **(HA6)** using positive mode ESI-MS

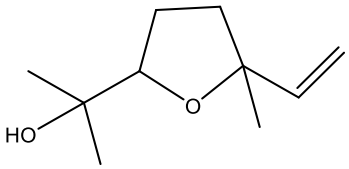
Compound	Formula	Calculated mass	Theoretical mass	Mass difference	Error (ppm)
2-hexylfuran **(C22)**	$C_{10}H_{16}O$	152.1200851	152.1201152	-0.0000301	-0.1981329
Linalool oxide **(C2)**	$C_{10}H_{18}O_2$	170.1306442	170.1306799	-0.0000357	-0.2099563
Linalool oxide **(C23)**	$C_{10}H_{18}O_2$	170.1307357	170.1306799	0.0000558	0.3278656
10-undecenal **(C24)**	$C_{11}H_{2O}O$	168.1514114	168.1514154	-0.0000040	-0.0237881
4-Ethylcamphor **(C25)**	$C_{12}H_{20}O$	180.1513809	180.1514154	-0.0000345	-0.1915056
Dodecenal **(C26)**	$C_{12}H_{24}O$	184.1827072	184.1827156	-0.0000084	-0.0453897
Caryophyllene oxide **(C27)**	$C_{15}H_{24}O$	220.1826919	220.1827156	-0.0000237	-0.1074562

Table 23: Compounds identified in **(HA6)** using negative mode ESI-MS

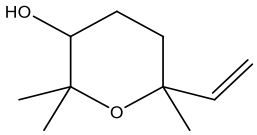
Compound	Formula	Calculated mass	Theoretical mass	Mass difference	Error (ppm)
12(13)-EpOME **(C28)**	$C_{18}H_{32}O_3$	296.2351395	296.2351438	-0.0000043	-0.0145254
(E)-12,13-dihydroxy-11-methoxyoctadec- 9-enoic acid **(C29)**	$C_{19}H_{36}O_5$	344.2562729	344.2562730	-0.0000001	-0.0002326

2-hexylfuran **(C22)**

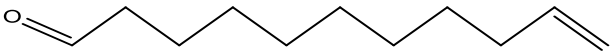
Linalool oxide (furanoid) **(C2)**



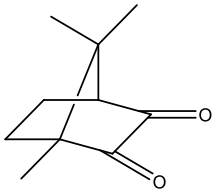
Linalool oxide (pyranoid) **(C23)**



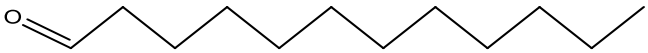
10-undecenal **(C24)**



4-Ethylcamphor **(C25)**



Dodecanal **(C26)**



Caryophyllene oxide **(C27)**

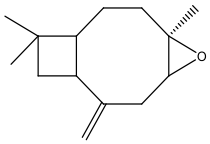
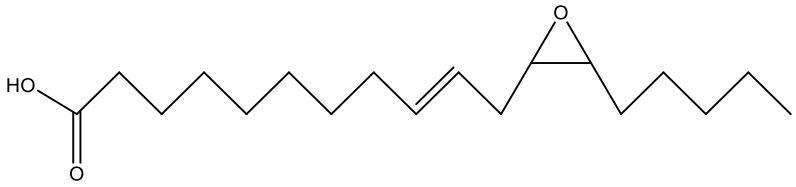
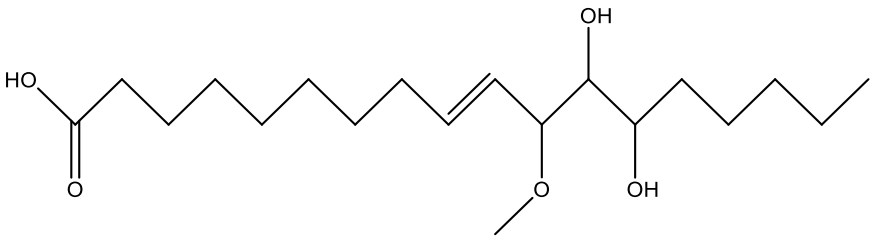


Figure 40: Putative compounds identified by LC-MS analysis in **(HA6)** and previously found in *Coriandrum sativum* seeds



12(13)-EpOME **(C28)**



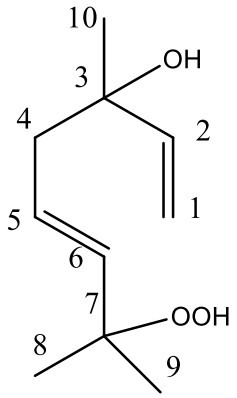
(E)-12,13-dihydroxy-11-methoxyoctadec- 9-enoic acid **(C29)**

Figure 40: (continued.) Putative compounds identified by LC-MS analysis in **(HA6)** and previously found in *Coriandrum sativum* seeds

3.4.3.2 Characterisation of (H338F3S11)

(H338F3S11) is fraction isolated from the *n*-hexane **(H338)** extract of *C. sativum* seeds. The ^{1}H and ^{13}C NMR data obtained for this fraction revealed the presence of 7-hydroperoxy-3,7-dimethyl-octa-1,5-3-ol, also known as linalool hydroperoxide (Figure 41, **C32**), (Skold *et al.*, 2002).

Signals assignment in NMR spectra for **(H338F3S11)** are summarised in Table 24 and the spectra are presented in Figures 42 - 48. To the best of our knowledge, this is the first time linalool hydroperoxide **(C32)** is described in *C. sativum* seeds. Unfortunately, this compound was not obtained in sufficient amounts in **(H338F3S11)** to allow for its isolation and subsequent screening (as a pure compound) for antibiofilm activity.



(C32)

Figure 41: Linalool hydroperoxide **(C32)** identified in **(H338F3S11)**

Table 24: NMR peak assignments (500 and 100 MHz, $CDCl_3$) for **(H338F3S11)**

Position	^{1}H δ_H	^{13}C δ_C	DEPT135 δ_C	COSY δ_H-δ_H	HMBC δ_H-δ_C
	5.05	112.25	(CH_2)	2	
1	5.17				
2	5.95	144.76	(CH)	1	
3	-	**72.73**		-	
4	2.36	45.22	(CH_2)	5	
	2.27				
5	5.72	126.71	(CH)	5,6	
6	5.59	137.91	(CH)	5	
7	-	**82.12**	-	-	
8	1.32	24.38	(CH_3)	-	6, 7, 9
9	1.32	24.25	(CH_3)	-	6, 7, 8
10	1.29	27.70	(CH_3)	-	2, 3, 4

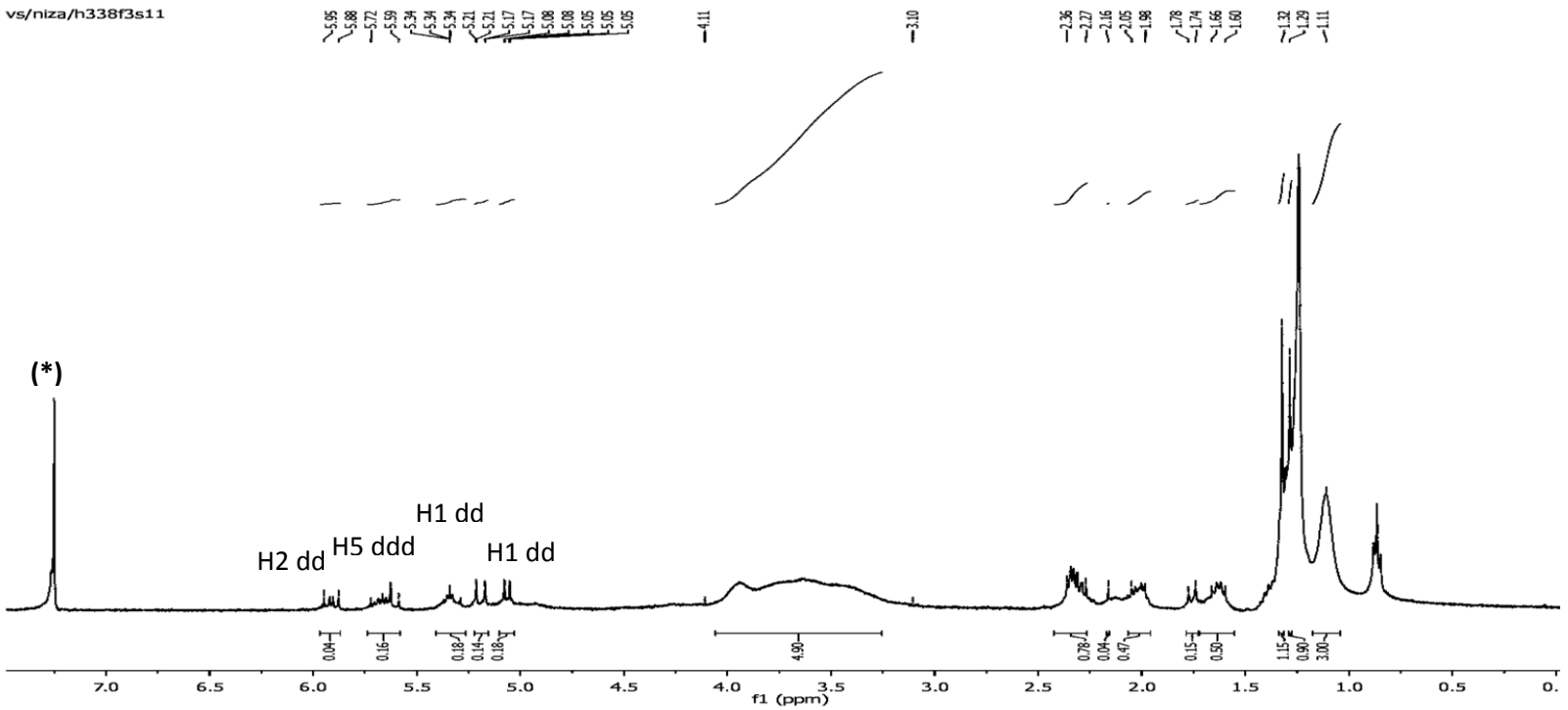


Figure 42: ^{1}H (500MHz) NMR spectrum of **(H338F3S11)** in $CDCl_3$ (*)

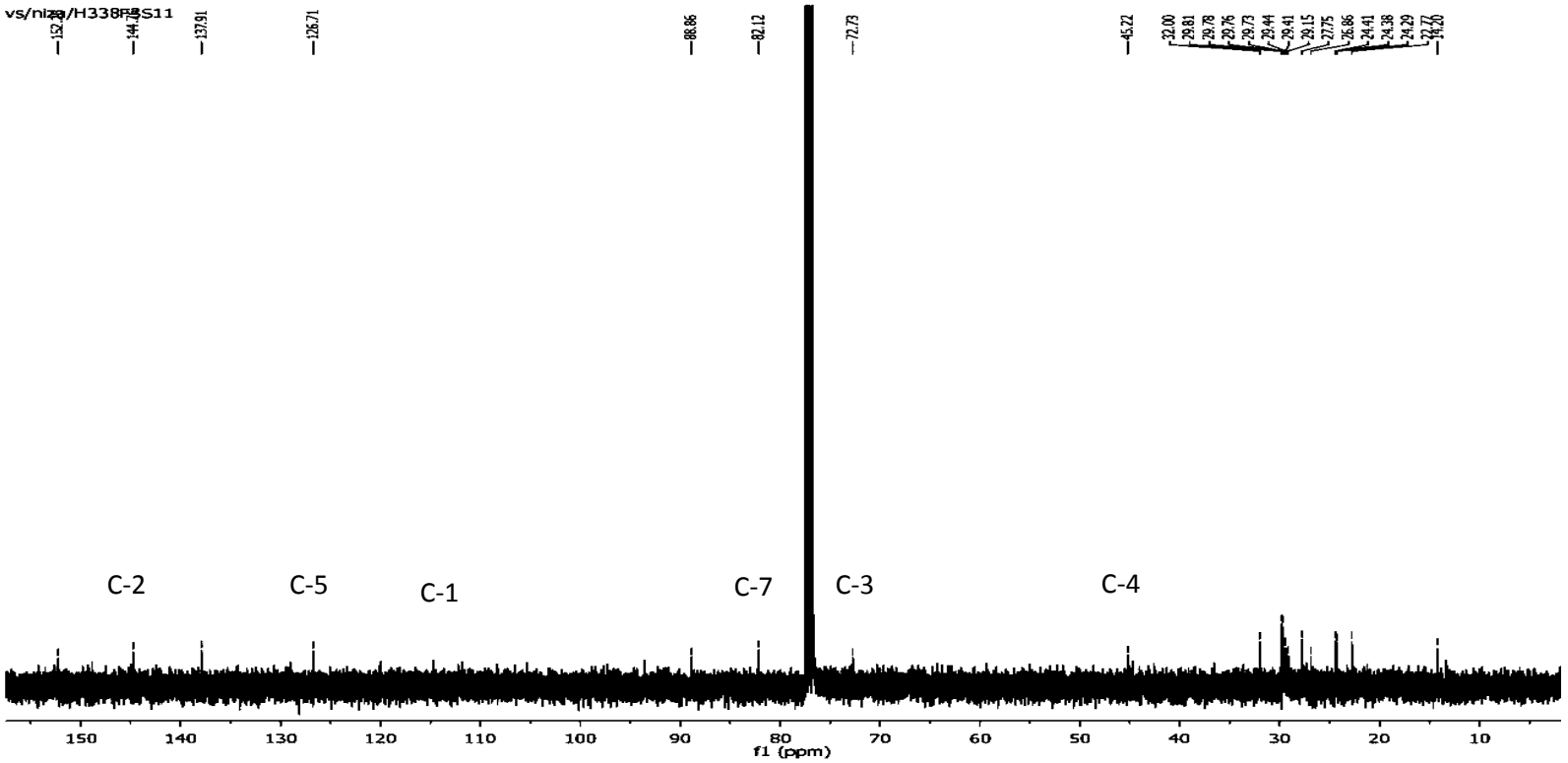


Figure 43: ^{13}C (100MHz) NMR spectrum of **(H338F3S11)** in $CDCl_3$

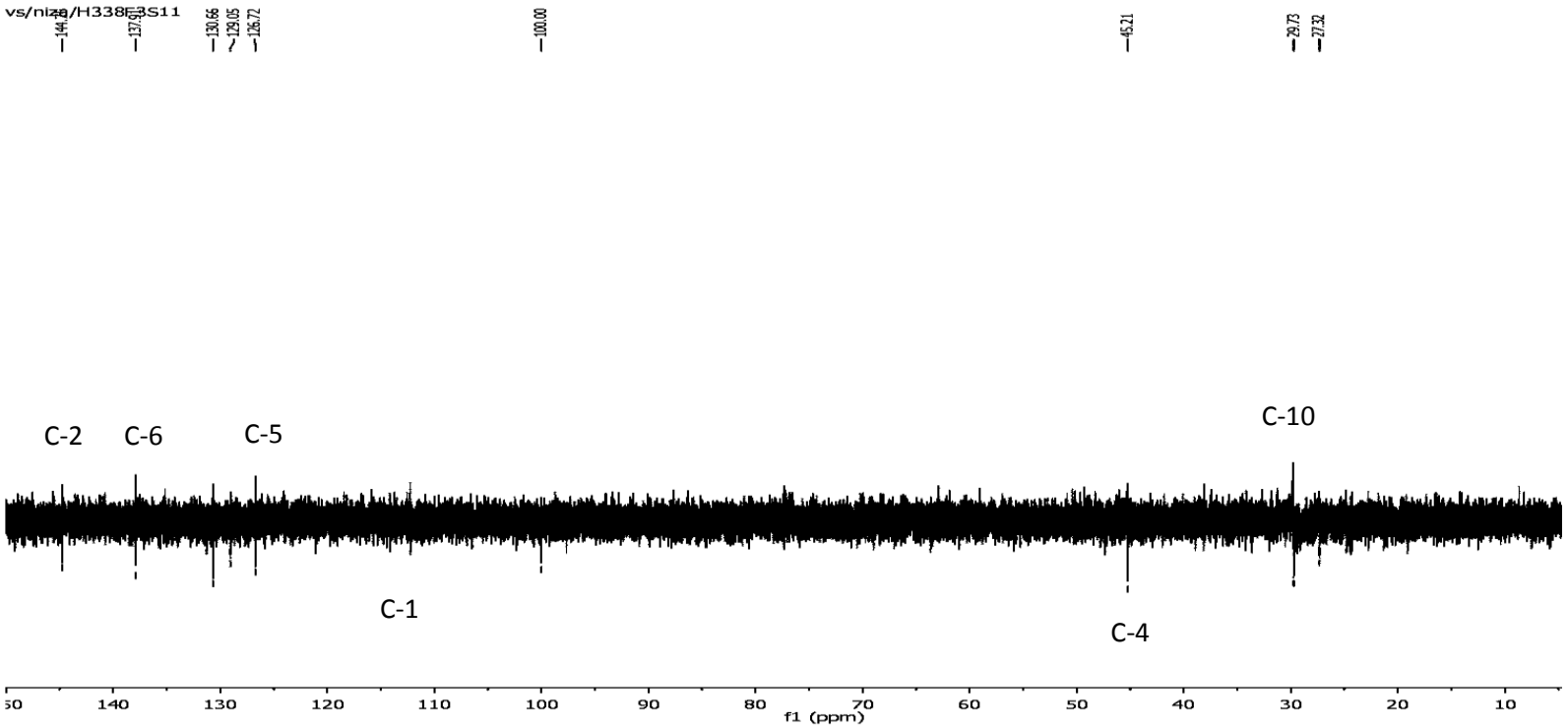


Figure 44: DEPT135 spectrum of **(H338F3S11)** in $CDCl_3$

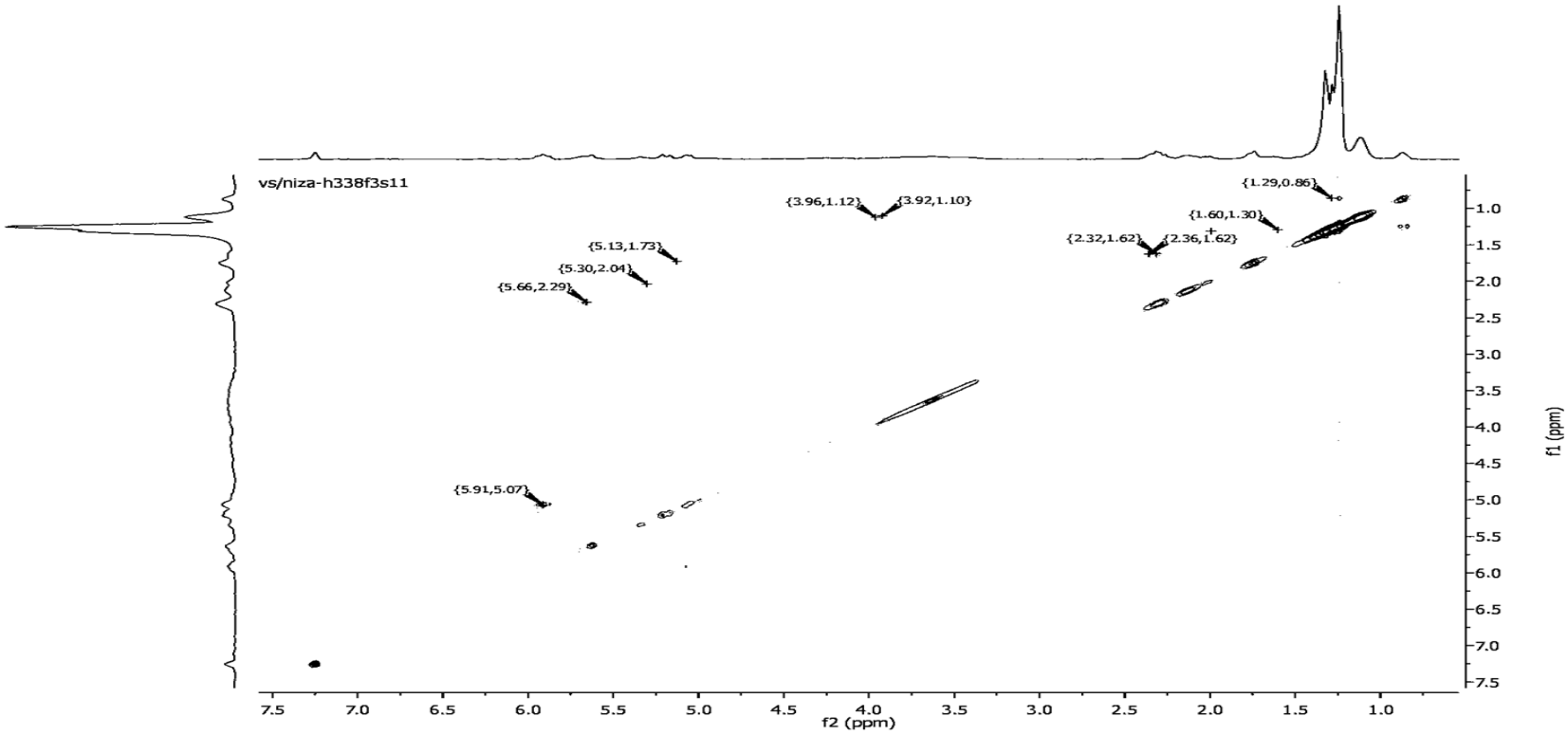


Figure 45: COSY spectrum of **(H338F3S11)** in $CDCl_3$

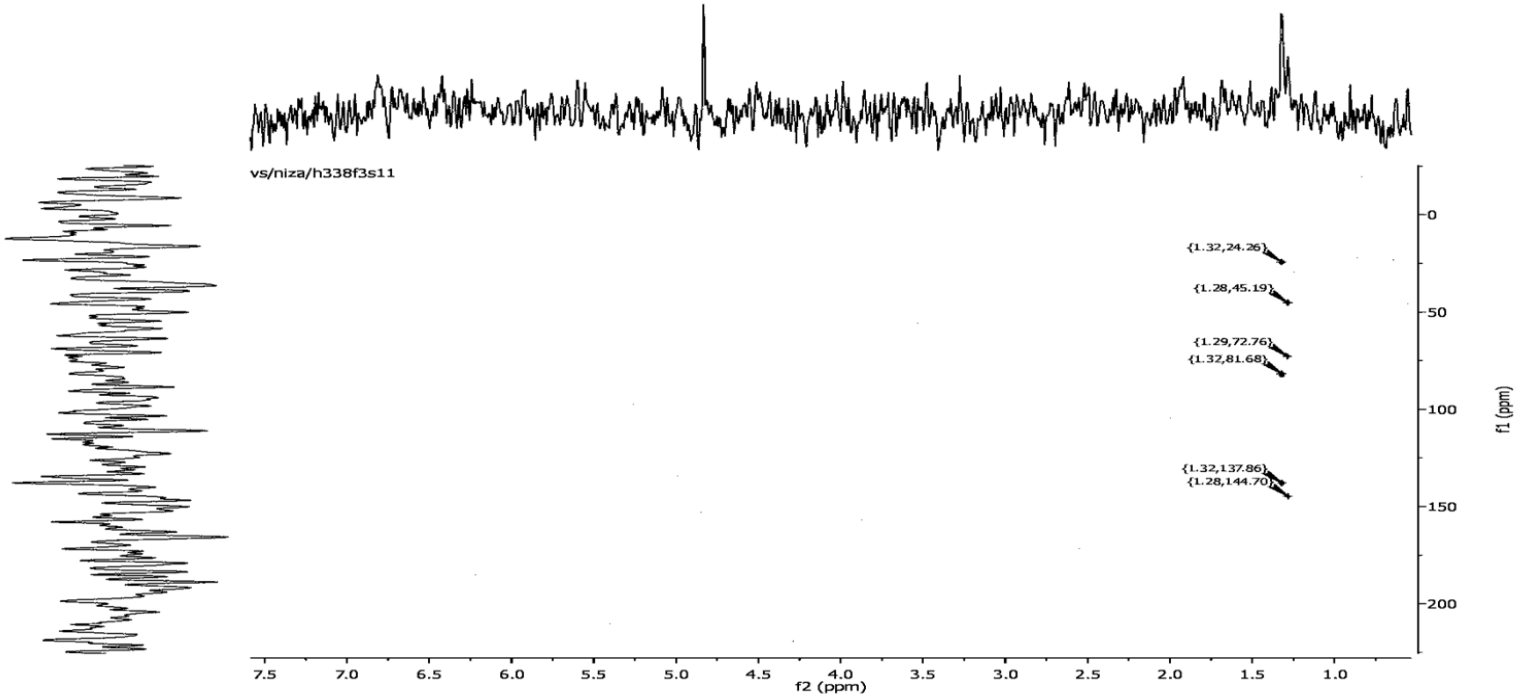


Figure 46: HMBC spectrum (selected expansion) of **(H338F3S11)** in $CDCl_3$

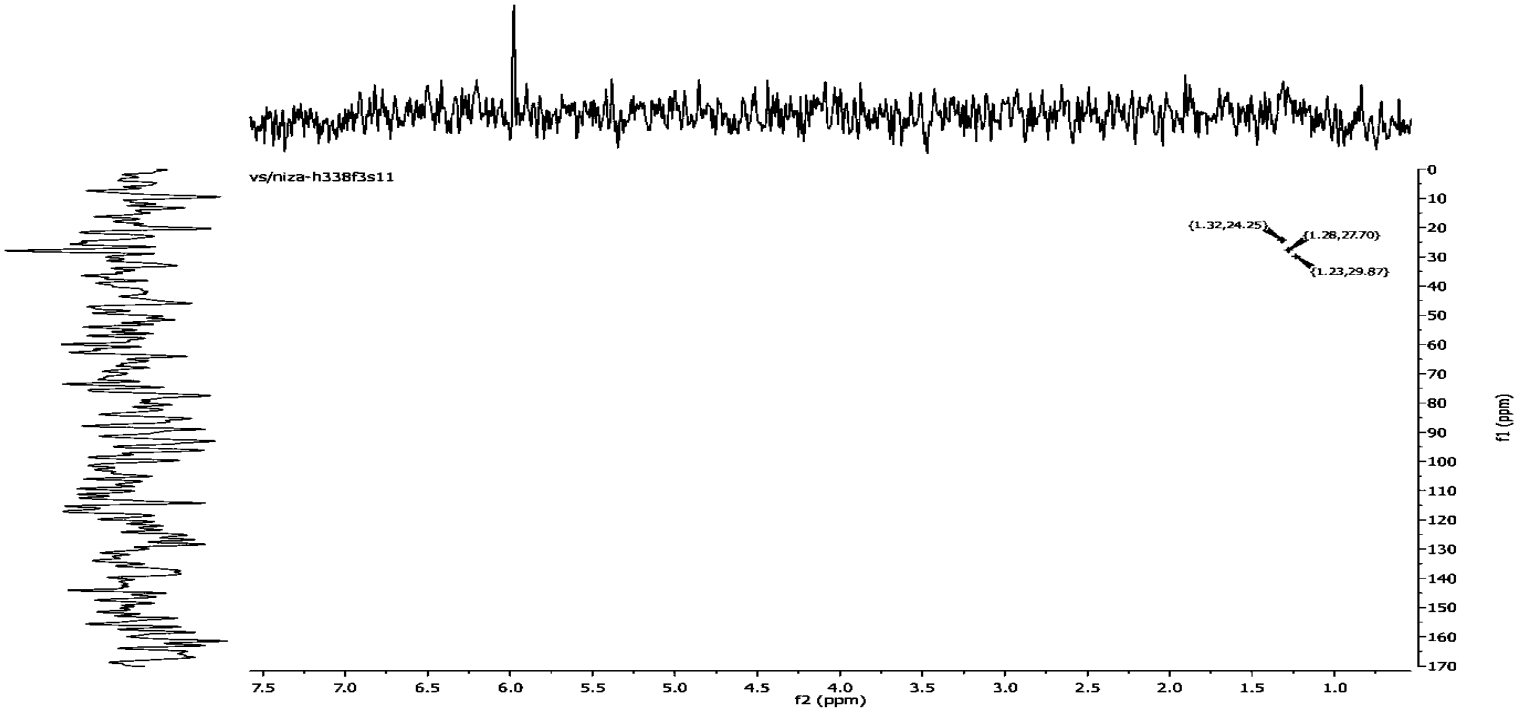


Figure 47: HMQC spectrum (selected expansion) of **(H338F3S11)** in $CDCl_3$

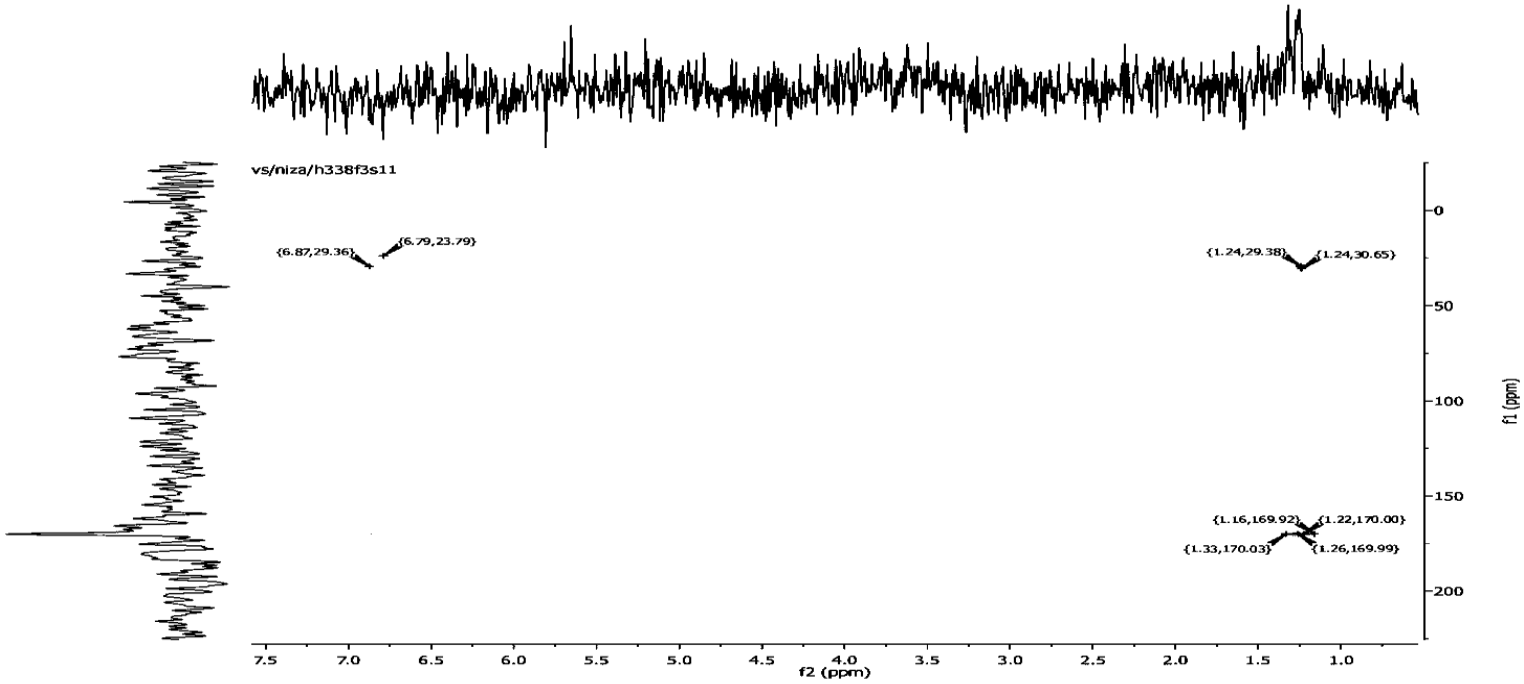


Figure 48: HSQC spectrum (selected expansion) of **(H338F3S11)** in $CDCl_3$

3.4.3.3 Identification of (M338B)

(M338B) was isolated from the purification of the methanol extract of *C. sativum* seeds. **(M338B)** was subjected to LC-MS analysis in the negative ESI mode and trace of compounds were identified using MZmine software (Table 25). Possible metabolites identified are include fatty acids such as saturated long chain and hydroxyl fatty acids **(P1, P2 and P3)** (e.g. C_{24}-C_{33}), carboxylate **(P4)** (e.g. $C_{31}H_{40}0_6$), carboxylic acid **(P5)** (e.g. $C_{28}H_{36}0_4$) and tetraone **(P6)** (e.g. $C_{28}H_{34}0_4$). All compounds putatively in **(M338B)** of *C. sativum* seeds are shown in Figure 49.

Fatty acids can affect genes that regulate the virulence genes in *P. aeruginosa* (Liu *et al.,* 2014). It is presumed that long chain fatty acids such as compounds **(P1)**, **(P2)** and **(P3)** can affect the QS signal and expression of virulence determinant (exoenzyme S/T), resulting in reduced *P. aeruginosa* PA14 biofilm formation**. It has been reported that** uneven-numbered saturated methyl-branched chain fatty acids can affect the growth of biofilms (Benamara *et al.,* 2011). Fatty acids also play an important role as one of a built up component for *N*-acyl homoserine lactone (AHL), an autoinducer of *P. aeruginosa* (Joulain, 1987).

Carboxylate **(P4)** may act as a signal molecule to N-acyl-L-homoserine lactone (AHL) (Cheng *et al.,* 2014) for *P. aeruginosa* PA14 that contributed to biofilm inhibition. A carboxylate group functionalised with iron oxide strongly disrupted biofilm formation and retarded the growth of *S. aureus* (Leuba *et al.,* 2013). Carboxylic acids have showed activity against biofilm of *Staphylococcus epidermidis* (Pan *et al.,* 2010) and carboxylic acid **(P5)** presumably had a similar action against the biofilm of *P. aeruginosa* PA14. Tetraone is a plant natural product and

microbiologically active (Jin *et al.*, 1998) exhibiting a strong antibacterial activity against *P. aeruginosa* (Ibrahim *et al.,* 2013) at which compound **(P6)** could be reacted against *P. aeruginosa* PA14 biofilm.

Putative compounds in **(M338B)** consist of hyper-branched molecules that are more effective as disrupters in the biofilm of *P. aeruginosa* PA14 possibly because they were interacting with a larger biofilm surface area (Burdock and Carabin, 2009). On the other hand, compounds which contained an aromatic ring are able to inhibit the formation of *P. aeruginosa* biofilms (Grosso *et al.*, 2010). Fatty acids such as 4-phenylbutanoic acid were found to reduce the hydrophobicity index and production of EPS in biofilms and inhibited the biofilm formation (Lo Cantore *et al.*, 2004). Thus, presumably the fatty acids present in **(M338B)** could react similarly against the biofilm of *P. aeruginosa* PA14.

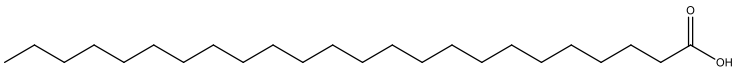
Table 25: Compounds identified in **(M338B)** using negative mode ESI-MS

Compound (Possible identities)	Predicted molecular formula	Calculated mass	Theoretical mass	Mass difference	Error (ppm)	References (compound structure)
Saturated long chain fatty acids **(P1)** (e.g. tetracosanoic, pentaco sanoic and hydroxylpenta cosanoic acids)	$C_{25}H_{50}O_3$	398.3771530	398.3759958	0.0011572	2.9047935	CSID:4472209, http://www.chemspider.com/Chemical-Structure.4472209.html (accessed Mar 6, 2015)
Hydroxy fatty acid **(P2)** (e.g. 2-hydroxyhexacosa noic acid)	$C_{26}H_{52}O_3$	412.3924424	412.3916459	0.0007965	1.9314649	CSID:77285, http://www.chemspider.com/Chemical-Structure.77285.html (accessed Mar 6, 2015)
Fatty acid **(P3)** (e.g. 4-[1-[3-(3-hydroxypro poxy)propoxy]-6,10,14,18-tetramethylnonadecan-4-yl]oxy-4-oxobutanoic acid)	$C_{33}H_{64}O_7$	572.4660202	572.4652048	0.0008154	1.4244359	CID 85758232 http://pubchem.ncbi.nlm.nih.gov/compound/85758232 (accessed Mar 6, 2015)

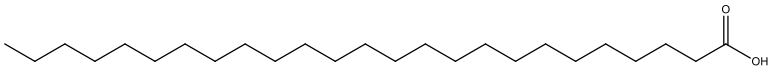
Table 25: (continued.) Compounds identified in **(M338B)** using negative mode ESI-MS

Compound (Possible identities)	Predicted molecular formula	Calculated mass	Theoretical mass	Mass difference	Error (ppm)	References (compound structure)
Carboxylate **(P4)** (e.g. 2-[2-(Dodecyloxy) ethoxy] ethyl 9,10-dioxo-9,10-dihydro-1-anthracene carboxylate)	$C_{31}H_{40}O_6$	508.2814347	508.2824892	-0.0010545	-2.0746337	CSID:277769, http://www.chemspider.com/Chemical-Structure.277769. html (accessed Mar 6, 2015)
Carboxylic acid **(P5)** (e.g. 10-hydroxy-2,4a,6a,6a, 14a-pentamethyl-11-oxo-1, 3,4,5,6,13,14,14b-octahydro picene-2-carboxylic acid)	$C_{28}H_{36}O_4$	436.2601181	436.2613598	-0.0012417	-2.8463213	CID 2661, http://pubchem.ncbi.nlm.nih.gov/compound/2661. (accessed Mar 6, 2015)
Tetraone **(P6)** (e.g. 1,16-diphenylhexa decane-3,5,12,14-tetraone)	$C_{28}H_{34}O_4$	434.2444474	434.2457098	-0.0012624	-2.9070178	CSID:227866, http://www.chemspider.com/Chemical-Structure.227866. html (accessed Mar 6, 2015)

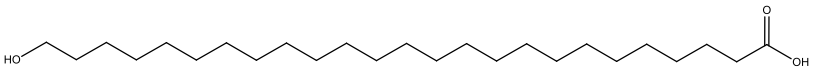
(P1)



Tetracosanoic acid, $C_{24}H_{48}O_2$

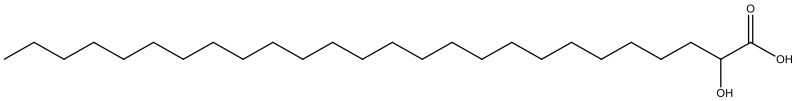


Pentacosanoic acid, $C_{25}H_{50}O_2$



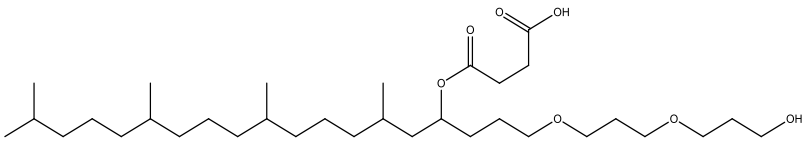
Hydroxylpentacosanoic acid, $C_{25}H_{50}O_3$

(P2)



2-Hydroxyhexacosanoic acid, $C_{26}H_{52}O_3$

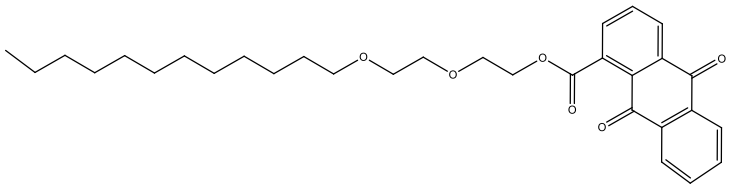
(P3)



4-[1-[3-(3-hydroxypro poxy)propoxy]-6,10,14,18-tetramethylnonadecan-4-yl]oxy-4-oxobutanoic acid, $C_{33}H_{64}O_7$

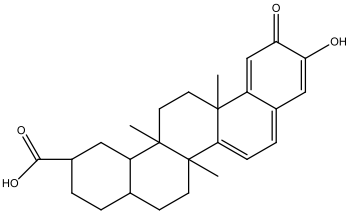
Figure 49: Putative compounds identified by LC–MS analysis in **(M338B)**

(P4)



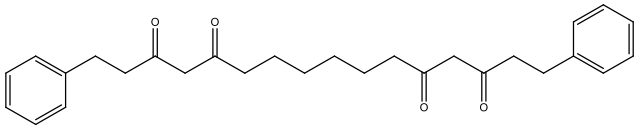
2-[2-(Dodecyloxy)ethoxy] ethyl 9,10-dioxo-9,10-dihydro-1-anthracene carboxylate, $C_{31}H_{40}O_6$

(P5)



10-hydroxy-2,4a,6a,6a, 14a-pentamethyl-11-oxo-1, 3,4,5,6,13,14,14b-octahydro picene-2-carboxylic acid, $C_{28}H_{36}O_4$

(P6)



1,16-diphenylhexadecane-3,5,12,14-tetraone, $C_{28}H_{34}O_4$

Figure 49: (cont.) Putative compounds identified by LC–MS analysis in (**M338B)**

3.5 Antibiofilm activity of commercially available pure natural products

This chapter focuses on the activity of selected natural products against the *P. aeruginosa* PA14 biofilm. The selection was based on our previous observations. The compounds were purchased from a commercial supplier (see Chapter 2) and tested as single compounds and as part of mixtures of compounds.

The five selected pure natural products were ursolic acid, oleanolic acid, petroselinic acid, linalool and linalool oxide. The latter is not commercially available. Linalool was used instead as the starting material for an oxidation process to afford linalool oxide and other derivatives. The results are discussed in detail later in the final section of this chapter. Results of their antibiofilm activity against *P. aeruginosa* PA14 are presented in Figure 50.

Results indicated that linalool oxide was the most potent compound against *P. aeruginosa* PA14 biofilm (73.8 %, BFI). To date, this is the first report of the antibiofilm activity of linalool oxide against the *P. aeruginosa* PA14 biofilm. Other active compounds were linalool (64.3 %, BFI) and petroselinic acid (57.0 %, BFI), while ursolic acid and oleanolic acid were found not active (38.8 and 46.2 % BFI, respectively). The antibiofilm activity of linalool oxide and linalool were slightly higher than Gentamicin (61.3 %, BFI) which is a positive control for this study.

Statistical analyses using Dunnett's Method shows that linalool oxide **(LOx)** is significantly different ($p < 0.05$) from the Positive Control (Gentamicin) and indicated that the activity of **(LOx)** is stronger than Gentamicin. A statistical comparison of the selected pure natural products using Tukey's Method, found that the activity of **(LOx)** is significantly different ($p < 0.05$) which indicated that the

activity of **(LOx)** is the strongest among all of the compounds tested (Appendix 4.3). Thus, **(LOx)** was identified as the most active compound against *P. aeruginosa* PA14 biofilm.

When ursolic acid and oleanolic acid were tested as mixtures of different ratios, they showed high antibiofilm activity at ratios of 1:2 (62.8 % BFI), followed by 1:1 (62.6 %, BFI) and 2:1 (60.5 %, BFI). The 1:2 mixture inhibited the *P. aeruginosa* PA14 biofilm more than the 1:1 mixture of linalool and linalool oxide.

Statistical analysis using Tukey's Method showed that the antibiofilm activity for all the mixtures of ursolic acid and oleanolic acid was not significantly different ($p < 0.05$). Results also indicated that the effect of antibiofilm activity in all mixtures of ursolic acid and oleanolic acid were similar to the Positive Control (Appendix 4.3). In contrast, ursolic acid and oleanolic acid alone were not active (38.8 and 46.2 % BFI, respectively) when used as a single pure compounds.

Results suggested that ursolic acid and oleanolic acid showed an additive effect in inhibiting the *P. aeruginosa* PA14 biofilm when present in a mixture. The combination of ursolic acid and oleanolic acid exhibited an additive effect rather than synergistic effect as both acids possess a similar molecular structure of pentacyclic triterpenes with different slightly at positions C_{19} and C_{20} and both shared a same molecular formula ($C_{30}H_{48}O_3$). The additive effect has been previously observed on combination of plant essential oils and extracts with similar compositions (Witkowska *et al*., 2013).

Results revealed that linalool and linalool oxide alone were very active against the *P. aeruginosa* PA14 biofilm (64.3 and 73.8 % BFI, respectively), however the activity

slightly decreased when present as a mixture (1:1 ratio). Mixing of linalool oxide with linalool resulted in a slight loss of activity. Results indicated that the combination of linalool and linalool oxide in a mixture had produced a synergistic negative effect on the activity against the *P. aeruginosa* PA14 biofilms. The study of synergistic negative effect was also observed on the organic mixture of Selenium and Mercury in fish reproduction (Penglase *et al.*, 2014). The negative synergistic effect exhibited by the mixture of linalool and linalool oxide was presumably due to the reactivity of the compounds that promote important structural changes in the mixture. Thus, the changes in molecular complexity of the mixture could affect the hydrogen-donating ability and causes a steric hindrance that would reduce the effect of activity in the mixtures (Queiros *et al.*, 2009).

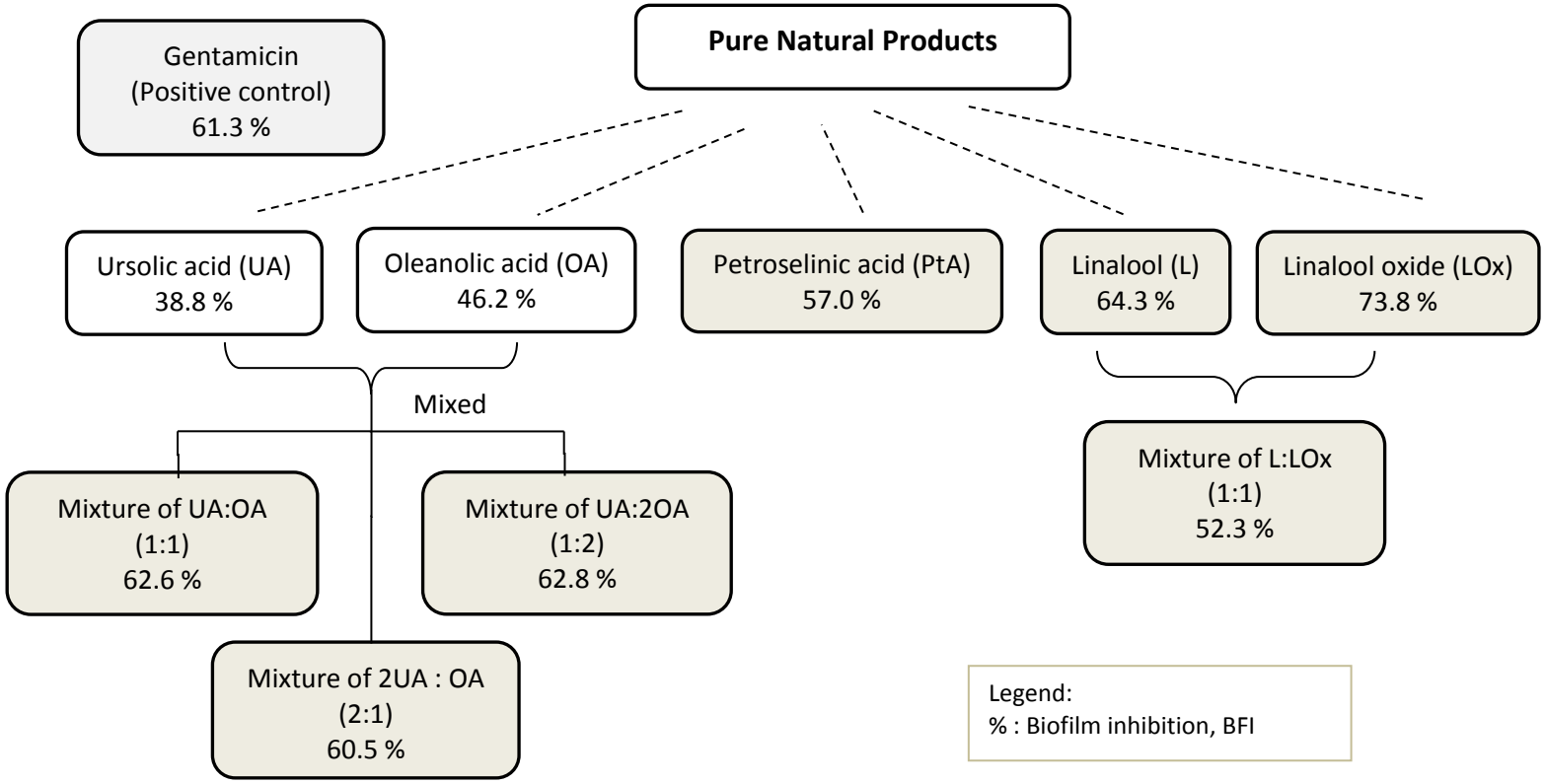


Figure 50: Antibiofilm activity of selected pure natural products against *P. aeruginosa* strain PA14 biofilms

3.5.1 Antibiofilm activity of linalool oxidation products

The autoxidation of linalool is known to occur when linalool is exposed to air (Skold *et al.*, 2002; Sköld *et al.*, 2004). The linalool autoxidation was conducted to obtain an oxidised product which is assumed to consist of active compounds that present in the active extracts. The oxidised product is then fractionated into few selected fractions and later tested against the *P. aeruginosa* PA14 biofilm.

The autoxidation process was monitored weekly based on observation on TLC profile and by comparing the R_f values to linalool and linalool oxide. The oxidation was stopped at the 12^{th} week when the TLC profile of the end product **($L_{10/9}$)** was found similar to both references. The oxidation end product, **($L_{10/9}$)** was then named as **(HA50)**. Figure 51 shows the autoxidation of linalool to produce **(HA50)** at which fractionated to obtain few fractions **($HA50P_n$)**. The TLC plate showed profiles of linalool oxide **(LOx)**, linalool **(L)** and **($L_{10/9}$)** which is **(HA50)**.

Fractionation of **(HA50)** afforded four selected fractions namely **(HA50P5)**, **(HA50P8)**, **(HA50P11)** and **(HA50P14)**. Results indicated that these fractions exhibited less than 50 % BFI activity, which showed that all fractions were not active against the *P. aeruginosa* PA14 biofilms. Fractionation of **(HA50)** failed to produce any active fraction, presumably due to lack, absent or loss of any active compounds in the fraction that could affect the activity. Spectra of **(HA50P5)**, **(HA50P8)**, **(HA50P11)** and **(HA50P14)** are attached in the appendices section (Appendix 5, Figure 1-6).

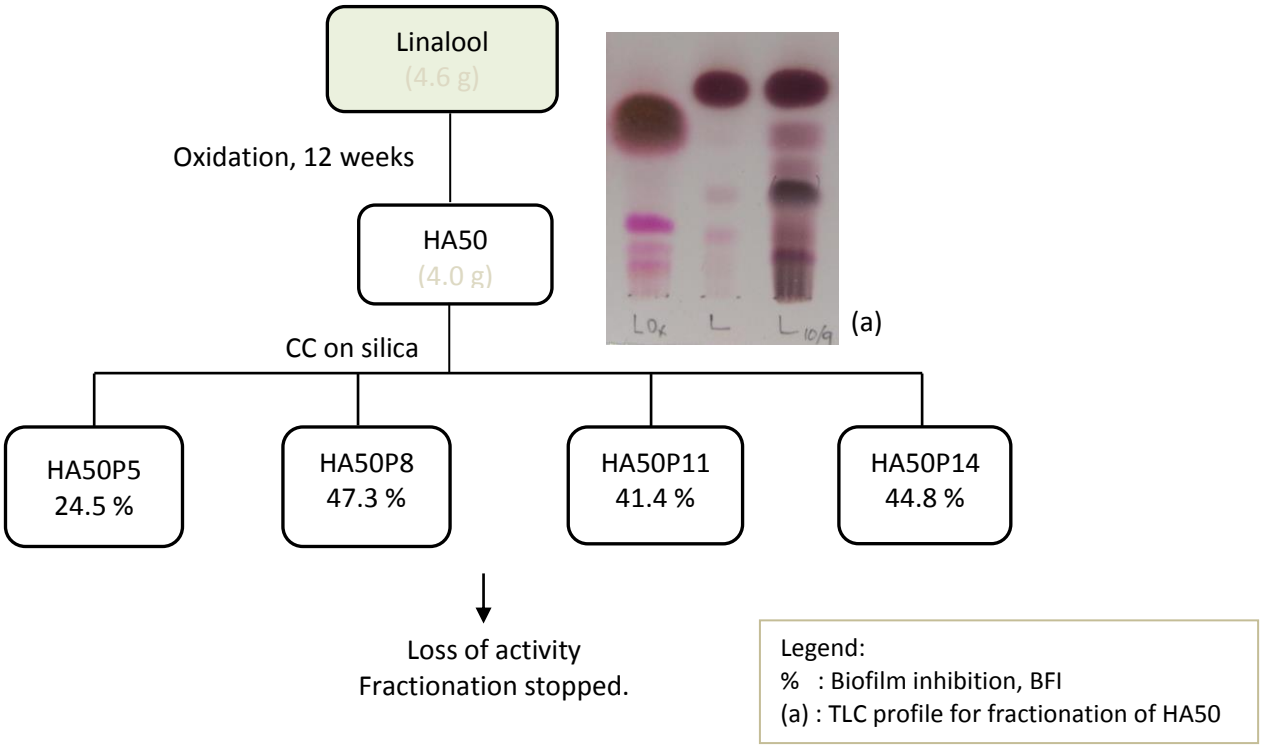


Figure 51: Linalool oxidation and fractionation scheme for selected fractions from **(HA50)**. Oxidation of linalool produced **(HA50)** which is later fractionated into small fractions and only four selected fractions were tested against the *P. aeruginosa* PA14 biofilms.

CHAPTER 4 - CONCLUSION

Our study highlighted the potential of some selected plants to inhibit biofilms of *P. aeruginosa* strain PA14. Out of a total of 129 extracts screened for activity, 44 extracts showed more than 50 % biofilm inhibition (see Figure 22a) while a total of 85 extracts had increased biofilm formation (see Figure 22b). Such activity could be due to the presence of different types of major or trace compounds in the extracts as observed in a previous study (Inoue and Sato, 1975).

The isolation and purification of phytochemicals with antibiofilm activity was carried out in a stepwise manner using a process of bioassay-guided fractionation. The latter was performed with an assay using a microtitre plate format (see Table 17). A total of four active extracts, namely **(E333)**, **(E341)**, **(H338)** and **(M338)** were selected for further investigation (see Table 18).

Ribes nigrum leaf extract **(E333)** showed antibiofilm activity, however the activity was lost upon further fractionation. The reason could possibly due to a loss of synergistic activity between mixtures of compounds present in the active extract (see Figure 25). **(HA30F1)** from the active fraction **(E333F1S1)** afforded for alkanes such as *n*-nonadecane, 2-methylnonadecane, 2-methylicosane and 2–methyloctacosane (see Figures 28-31). It was suggested that degradation of hydrocarbon chain of fatty acids in fraction **(E333F1S1)** could contribute to the presence of these alkanes in **(HA30F1)**. While *Pseudomonas* sp. is also capable of degrading *n*-alkanes (Hua *et al.*, 2014) and using them as a source of carbon and energy (Singh *et al.*, 2012).

Bioassay-guided fractionation of *Sambucus nigra* flower extract **(E341)** led to the identification of a mixture of ursolic and oleanolic acids as active agents in a (2:1) ratio (see Figure 35). The antibiofilm activity of selected pure natural products, ursolic and oleanolic acids alone exhibited a weak activity (see Figure 49). A mixture of ursolic and oleanolic acids however showed a comparable activity with **(E341)** when tested using a same ratio (2:1), (see Table 20). No significant differences ($p < 0.05$) in the activity compared to positive control were found when ursolic and oleanolic acids were tested in mixture using different ratios (see Appendix 4.3).

The investigation of *Coriandrum sativum* seed extract **(H338)** led to the identification of two active fractions **(H338F3S12)** and **(H338F3S10),** (see Figure 36). Only **(H338F3S10)** was successfully fractionated further to afford six fractions including three that were active **(HA2)**, **(HA3)** and **(HA6)**. One of the active fractions **(HA6)** was subjected to LC-MS analysis and results revealed that it may contain 10-undecenal, dodecanal, 2-hexyl furan, linalool oxide, caryophyllene oxide and 4-ethylcamphor (see Figure 40). One compound, characterised as linalool hydroperoxide (see Figure 41) was identified in another active fraction but could not be tested on its own due to the in sufficient amounts obtained.

An active fraction of *Coriandrum sativum* seed **(M338B)** was also analysed by LC-MS and found to contain putative fatty acids, such as saturated long chain and hydroxyl fatty acids (e.g. tetracosanoic acid, pentacosanoic acid, hydroxyl pentacosanoic acid and 2-hydroxyhexacosanoic acid); carboxylic acid (e.g. 10-hydroxy-2,4a,6a,6a,14a-pentamethyl-11-oxo-1,3,4,5,6,13,14,14b-octahydropicene-2-carbocylic acid); carboxylate (e.g.2-[2-(Dodecyloxy)ethoxy]ethyl 9,10-dioxo-9,10-dihydro-1-anthracene carboxylate) and tetraone (e.g.1,16-diphenylhexadecane-

3,5,12,14-tetraone), (see Figure 49). Notably unsaturated fatty acids have been reported to afford the antibiofilm activity and effect of fatty acids on disruption of *P. aeruginosa* biofilms (Awen *et al.*, 2011).

LC-MS analysis on active fraction **(HA6)** led the identification of linalool oxide (see Figure 40) and when tested as a pure compound, the activity of linalool oxide was found to be significantly different ($p < 0.05$) compared to the positive control (Gentamicin). Linalool oxide exhibited a strong (73.8 % BFI) antibiofilm activity when on its own. However, in a mixture with linalool at a 1:1 ratio, the activity of the mixture was slightly lower (52.3 % BFI) than activity of both compounds alone (see Figure 50).

To summarise, this study led to the discovery of five bioactive compounds that have potential as antibiofilm compounds against *P. aeruginosa* PA14 biofilms. The bioactive compounds are **(E33F1S1)** (e.g. mixture of alkanes); **(E341)** (e.g. a mixture of ursolic acid and oleanolic acid); **(HA6)** (e.g. a mixture of oxygenated monoterpenes and epoxy fatty acids) and **(M338B)** (e.g. a mixture of fatty acids, carboxylic acids, carboxylate and tetraone).

FUTURE WORK

This study offers other studies which deserve further consideration include the following:

- Fractionation of other active plant extracts (e.g. *V. myrtillus* and *A. graveolens)* to isolate and characterise other antibiofilm-active compounds.
- Evaluation of the activity of isolated compounds either alone or in mixed-ratios.
- Evaluation of the activity of identified compounds against biofilms of other *Pseudomonas* and Gram-negative bacteria.
- Study of those active compounds on the bacterial stress response using transcriptomic analysis.
- Effect of active compounds to corresponding *P. aeruginosa* PA14 metabolic pathways using Omnilog assays.
- Physical effects on cell-to-cell and cell-to-surface interactions of *P. aeruginosa* PA14 with those active compounds using adhesion assays.

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APPENDICES

Appendix 1: Preparation of *p*-anisaldehyde-sulfuric acid spray reagent

The reagent was used for detection of phenols, sugars, steroids and terpenes. The spray was prepared with a solution of freshly prepared 0.5 mL of *p*-anisaldehyde in 10 mL glacial acetic acid, 85 mL methanol and 5 mL concentrated sulfuric acid.

After spraying the TLC plate and heat to 105 °C until maximum visualization of spots will produced component with violet, blue, red, grey or green spots.

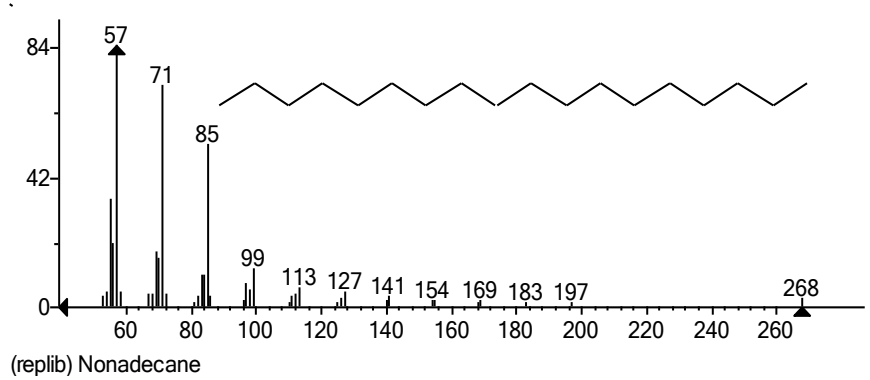
Appendix 2:

Table 1: Selection of plants tested for antimicrobial activity including the screening method used

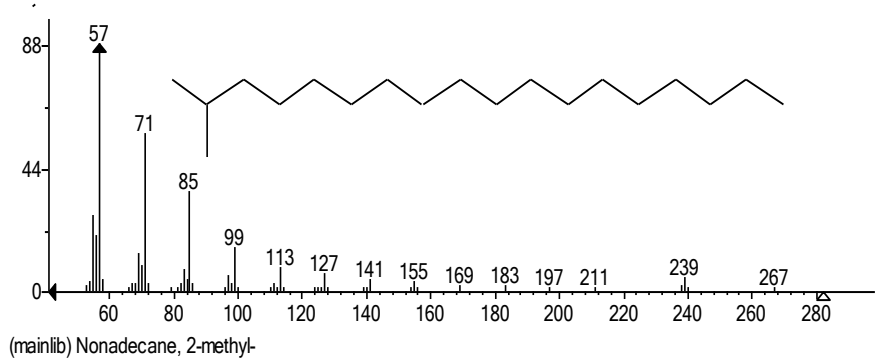
Plants	Microbial species tested	Screening method	References
Coriandrum sativum	*Escherichia coli, Pseudomonas spp.*	Disc diffusion assay	Lo Cantore *et al*., 2004
	Camphylobacter jejuni	Disc diffusion assay	Rattanachaikunsopon, P. and Phumkhachorn, P., 2010
Melissa officinalis	*Escherichia coli*	Microtitre broth dilution assay	(Uzun *et al.*, 2004)
Vaccinium myrtillus	*Streptococcus pneumoniae*	Microtitre broth dilution assay	(Huttunen *et al.*, 2011)
Ribes nigrum	*Listeria. monocytogenes and Pseudomonas aeruginosa* ATTC 27853	Agar dilution assay	(Miladinovic *et al.*, 2014)
Apium graveolens	*Listeria innocua, Escherichia coli, Staphylococcus aureus* and *Pseudomonas fluorescens*	Agar dilution assay	Witkowska, A.M. *et al*., 2013
Citrus sinensis	*Bacillus cereus, Listeria monocytogenes, Escherichia coli, Staphylococcus enteritidis* and *Proteus mirabilis*	Disc diffusion assay	Irkin, R. and Korukluoglu, M. 2009.
Rubus idaeus and *Rubus spp.*	*Bacillus subtilis, Escherichia coli*	Disc diffusion assay	(Rauha *et al.*, 2000)

Appendix 3: NIST MS Search 2.0 library matching for compounds present in **(HA30F1)**

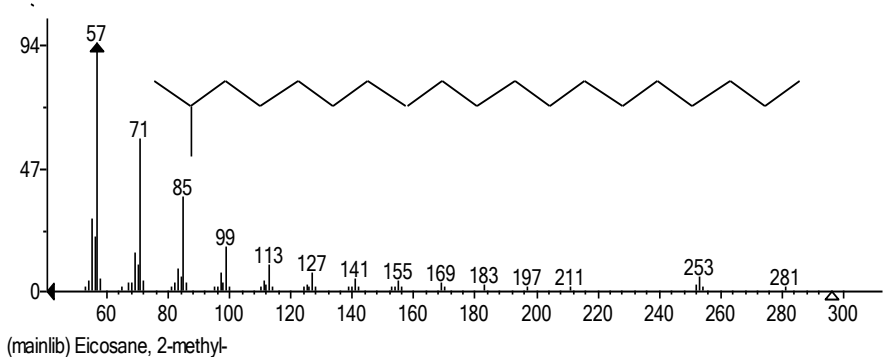
3.1 RN1 at t = 7.07 min



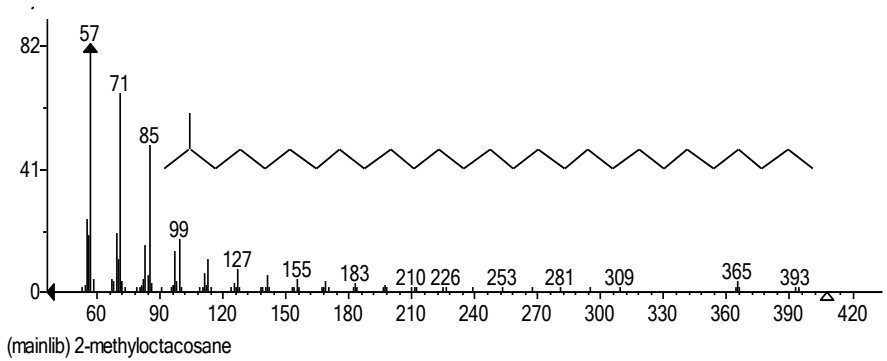
3.2 RN2 at t = 7.53 min



3.3 RN3 at t = 8.14 min



3.4 RN4 at t = 8.99 min



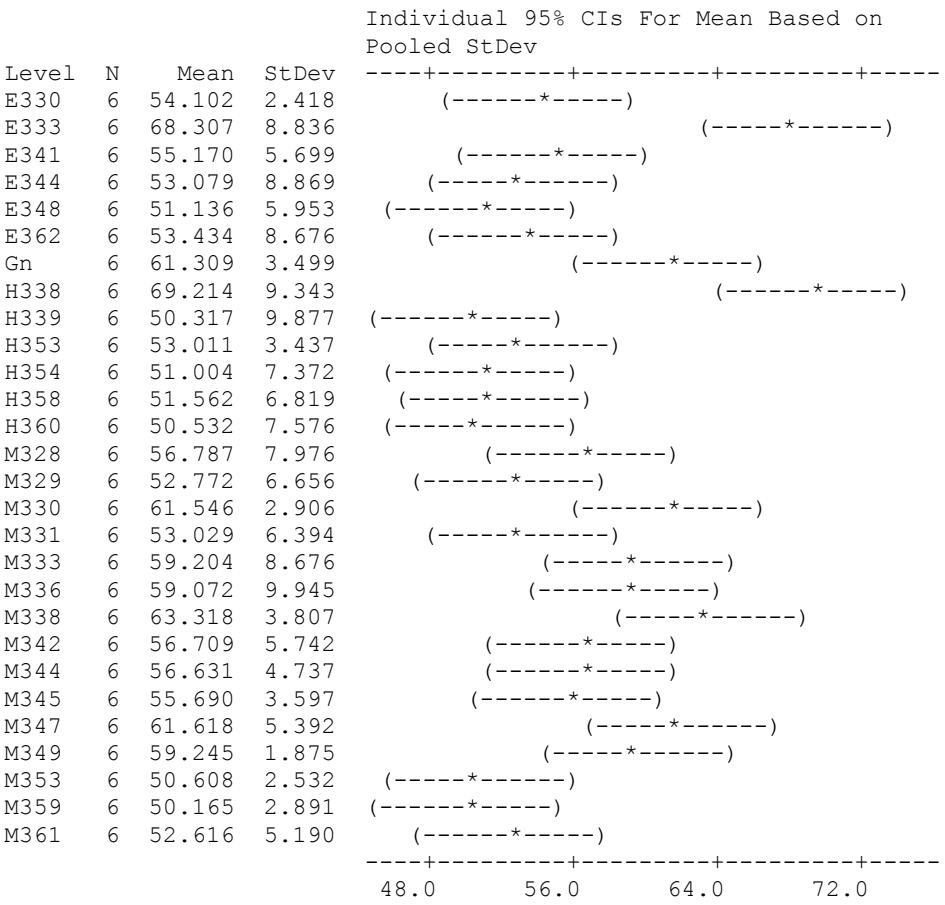
Appendix 4: Statistical analyses

4.1 One way ANOVA using Dunnett's and Tukey methods for screening of antibiofilm activity in plant extracts

One-way ANOVA: BFI versus sample

```
Source   DF       SS     MS     F      P
sample   27   4525.7  167.6  4.04  0.000
Error   140   5807.3   41.5
Total   167  10333.0

S = 6.441   R-Sq = 43.80%   R-Sq(adj) = 32.96%
```




```
Pooled StDev = 6.441
```

Grouping Information Using Dunnett's Method

Level	N	Mean	Grouping
Gn (control)	6	61.309	A
H338	6	69.214	A
E333	6	68.307	A
M338	6	63.318	A
M347	6	61.618	A
M330	6	61.546	A
M349	6	59.245	A
M333	6	59.204	A
M336	6	59.072	A
M328	6	56.787	A
M342	6	56.709	A
M344	6	56.631	A
M345	6	55.690	A
E341	6	55.170	A
E330	6	54.102	A
E362	6	53.434	A
E344	6	53.079	A
M331	6	53.029	A
H353	6	53.011	A
M329	6	52.772	A
M361	6	52.616	A
H358	6	51.562	A
E348	6	51.136	A
H354	6	51.004	A
M353	6	50.608	A
H360	6	50.532	A
H339	6	50.317	A
M359	6	50.165	A

Means not labeled with letter A are significantly different from control level mean.

Dunnett's comparisons with a control
Family error rate = 0.05
Individual error rate = 0.0029
Critical value = 3.03
Control = level (Gn) of sample

Grouping Information Using Tukey Method

sample	N	Mean	Grouping
H338	6	69.214	A
E333	6	68.307	A
M338	6	63.318	A B
M347	6	61.618	A B
M330	6	61.546	A B
Gn	6	61.309	A B
M349	6	59.245	A B
M333	6	59.204	A B
M336	6	59.072	A B
M328	6	56.787	A B
M342	6	56.709	A B
M344	6	56.631	A B
M345	6	55.690	A B
E341	6	55.170	A B
E330	6	54.102	B
E362	6	53.434	B
E344	6	53.079	B
M331	6	53.029	B
H353	6	53.011	B
M329	6	52.772	B
M361	6	52.616	B
H358	6	51.562	B
E348	6	51.136	B
H354	6	51.004	B
M353	6	50.608	B
H360	6	50.532	B
H339	6	50.317	B
M359	6	50.165	B

Means that do not share a letter are significantly different.

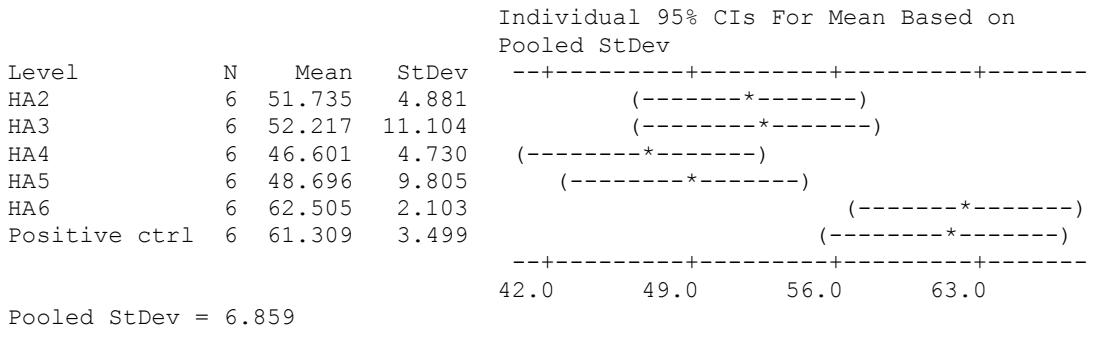
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of sample

4.2 Statistical analyses using Dunnett's and Tukey methods in screening for antibiofilm activity in **(HA2)** – **(HA6)**

One-way ANOVA: BFI versus Fractions

```
Source     DF      SS     MS     F      P
Fractions   5  1300.8  260.2  5.53  0.001
Error      30  1411.5   47.1
Total      35  2712.3

S = 6.859   R-Sq = 47.96%   R-Sq(adj) = 39.28%
```




```
Pooled StDev = 6.859
```

Grouping Information Using Dunnett's Method

Level	N	Mean	Grouping
Positive ctrl	6	61.309	A
HA6	6	62.505	A
HA3	6	52.217	A
HA2	6	51.735	A
HA5	6	48.696	
HA4	6	46.601	

Means not labeled with letter A are significantly different from control level mean.

Dunnett's comparisons with a control
Family error rate = 0.05
Individual error rate = 0.0125
Critical value = 2.66
Control = level (Positive ctrl) of Fractions

Grouping Information Using Tukey Method

Fractions	N	Mean	Grouping
HA6	6	62.505	A
Positive ctrl	6	61.309	A
HA3	6	52.217	A B
HA2	6	51.735	A B
HA5	6	48.696	B
HA4	6	46.601	B

Means that do not share a letter are significantly different.

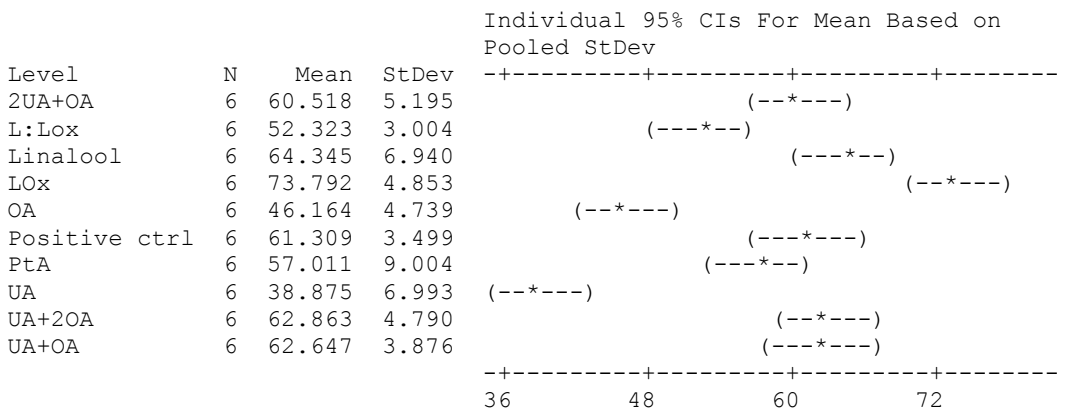
Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Fractions

4.3 Statistical analyses using Dunnett's and Tukey methods in screening for antibiofilm activity in commercially available pure compounds

One-way ANOVA: BFI versus Fractions

```
Source     DF      SS     MS      F      P
Fractions   9  5347.5  594.2  19.14  0.000
Error      50  1551.8   31.0
Total      59  6899.3

S = 5.571   R-Sq = 77.51%   R-Sq(adj) = 73.46%
```




```
Pooled StDev = 5.571
```

Grouping Information Using Dunnett's Method

Level	N	Mean	Grouping
Positive ctrl	6	61.309	A
LOx	6	73.792	
Linalool	6	64.345	A
UA+2OA	6	62.863	A
UA+OA	6	62.647	A
2UA+OA	6	60.518	A
PtA	6	57.011	A
L:Lox	6	52.323	
OA	6	46.164	
UA	6	38.875	

Means not labeled with letter A are significantly different from control level mean.

Dunnett's comparisons with a control
Family error rate = 0.05
Individual error rate = 0.0075
Critical value = 2.79
Control = level (Positive ctrl) of Fractions

Grouping Information Using Tukey Method

Fractions	N	Mean	Grouping
LOx	6	73.792	A
Linalool	6	64.345	A B
UA+2OA	6	62.863	B C
UA+OA	6	62.647	B C
Positive ctrl	6	61.309	B C
2UA+OA	6	60.518	B C
PtA	6	57.011	B C
L:Lox	6	52.323	C D
OA	6	46.164	D E
UA	6	38.875	E

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Fractions

Appendix 5: NMR spectra of **(HA50)** fractions

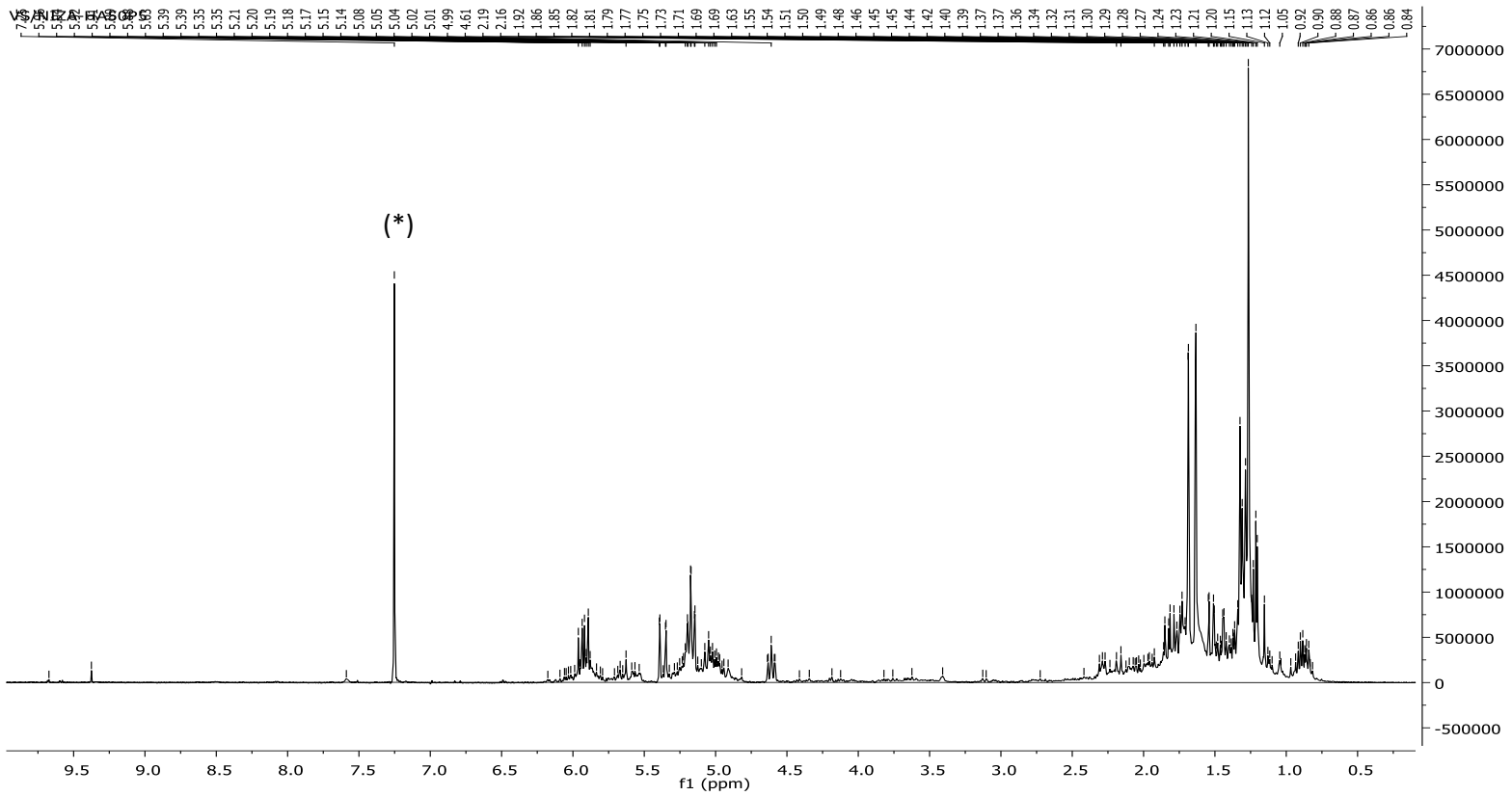


Figure 1: ^{1}H (500MHz) NMR spectrum of **(HA50P5)** in $CDCl_3$(*)

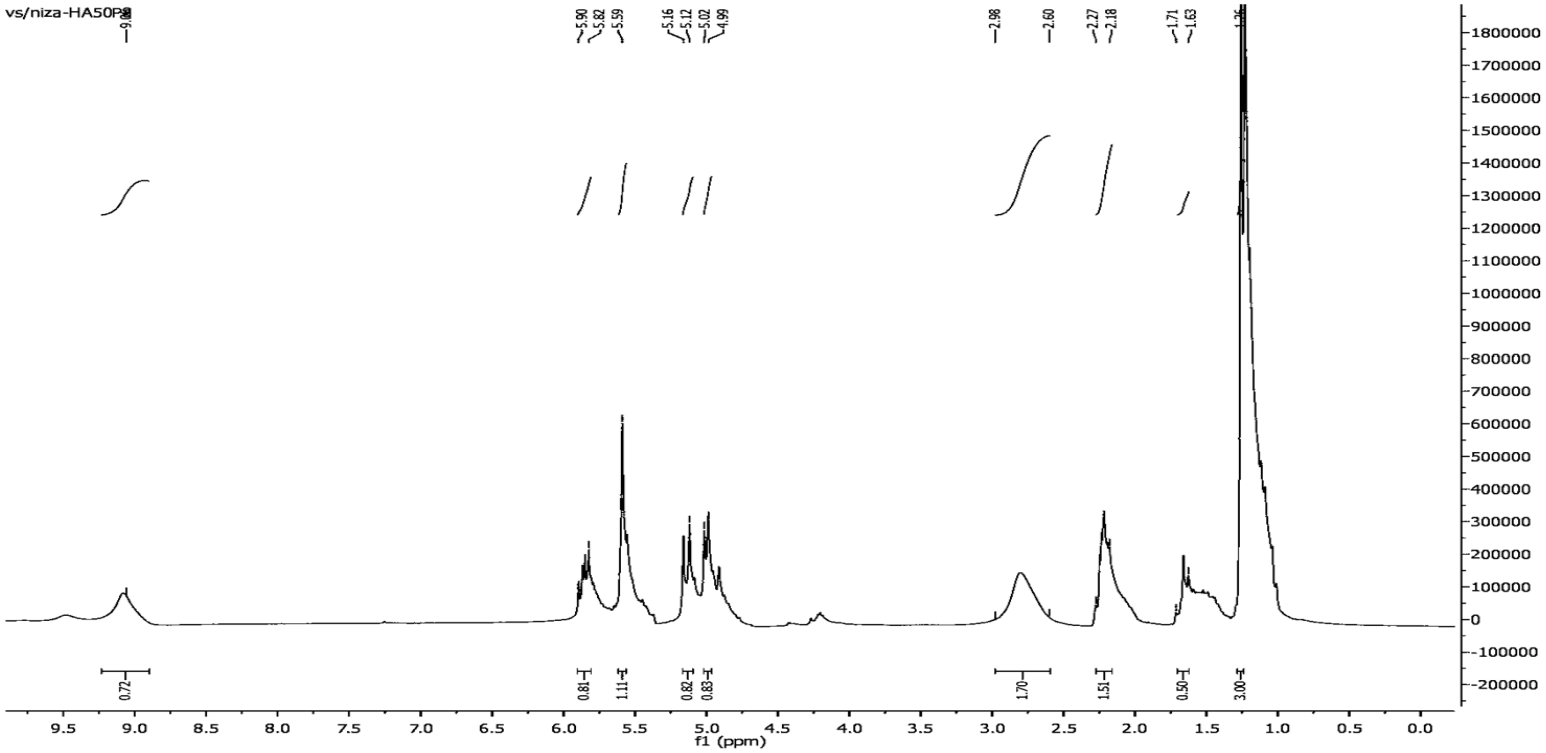


Figure 2: ^{1}H (500MHz) NMR spectrum of **(HA50P8)**

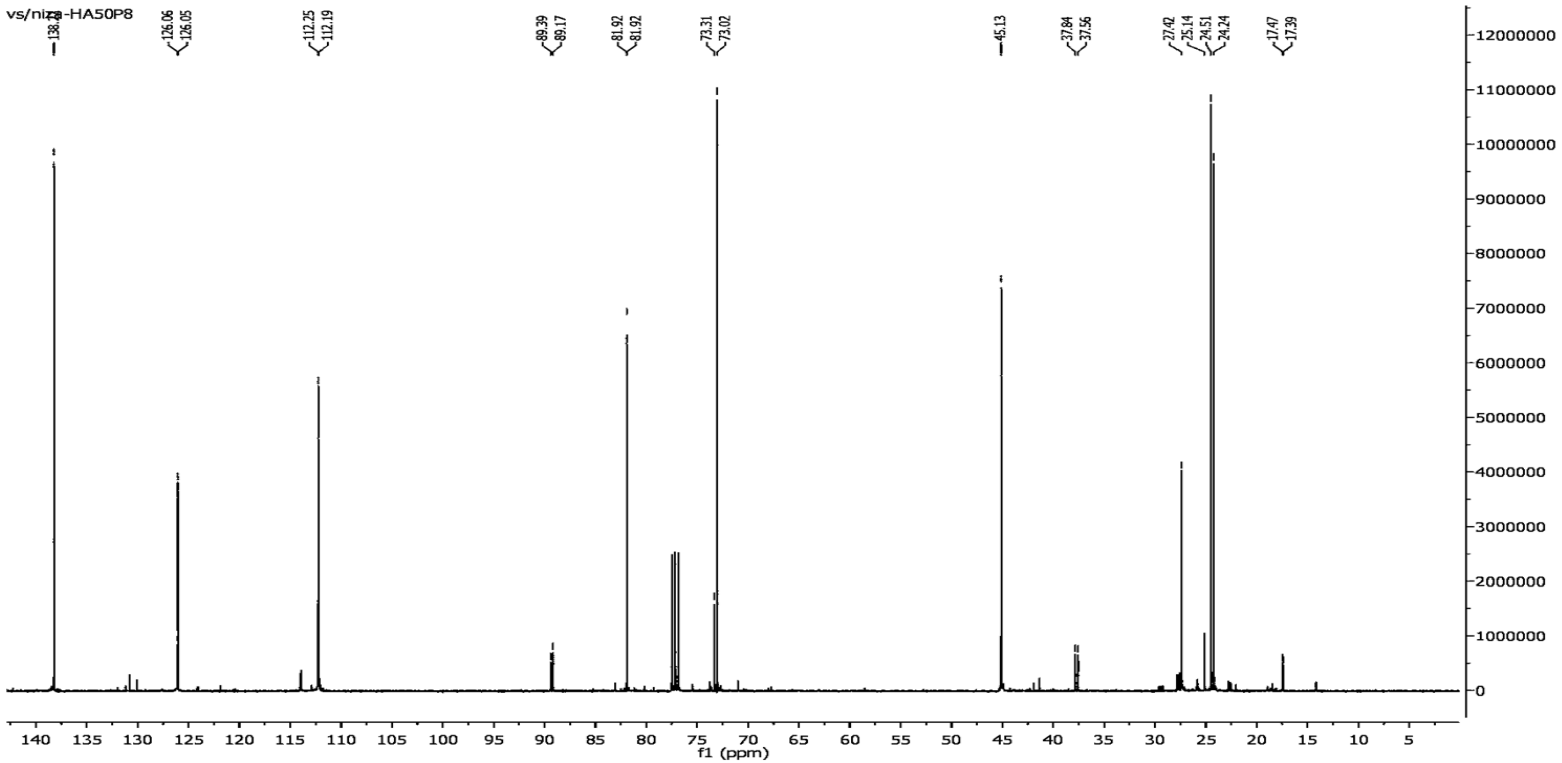


Figure 3: ^{13}C (100MHz) NMR spectrum of **(HA50P8)**

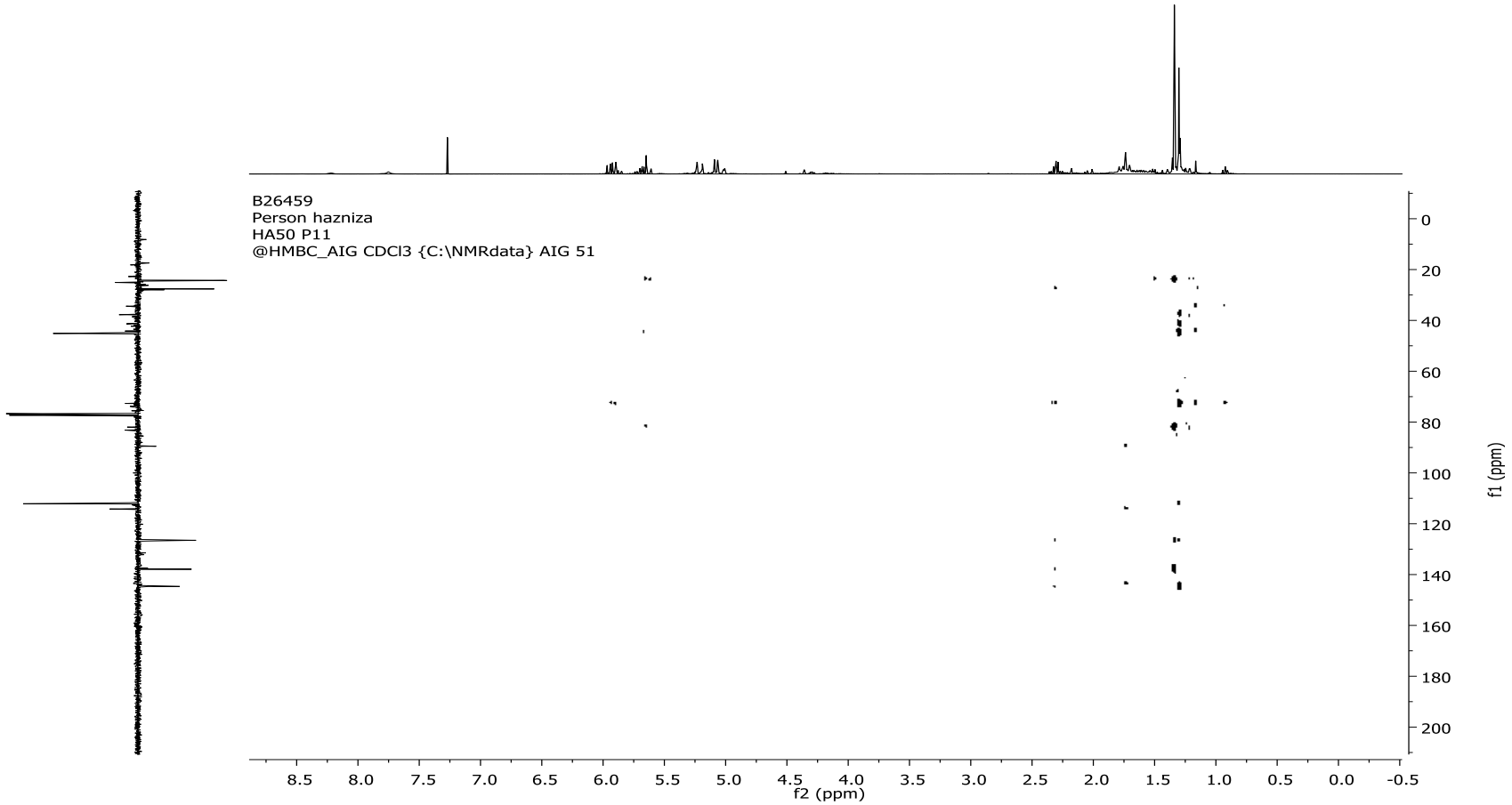


Figure 4: HMBC NMR spectra of **(HA50P11)**

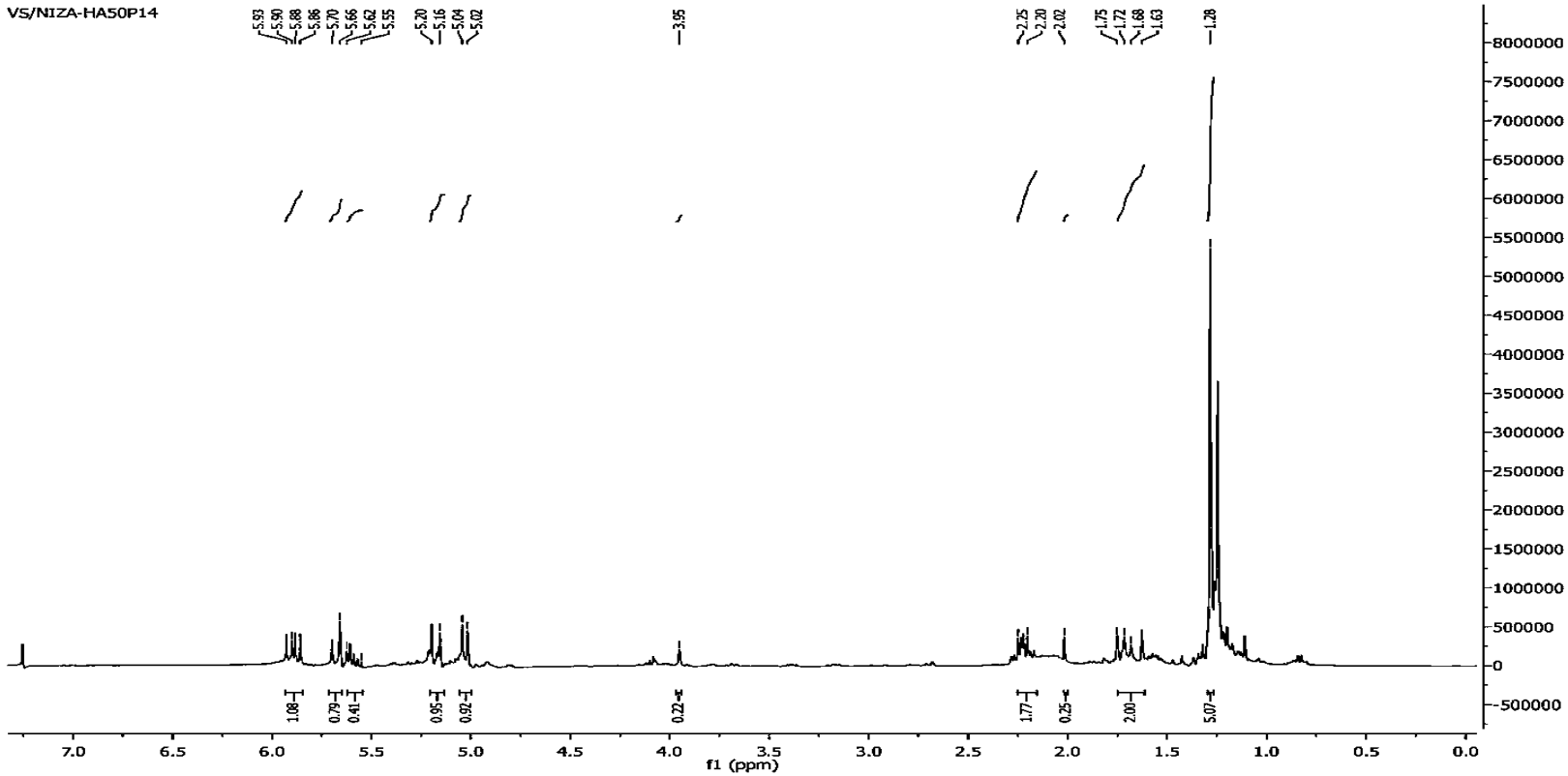


Figure 5: ^{1}H (500MHz) NMR spectrum of **(HA50P14)** in $CDCl_3$(X)

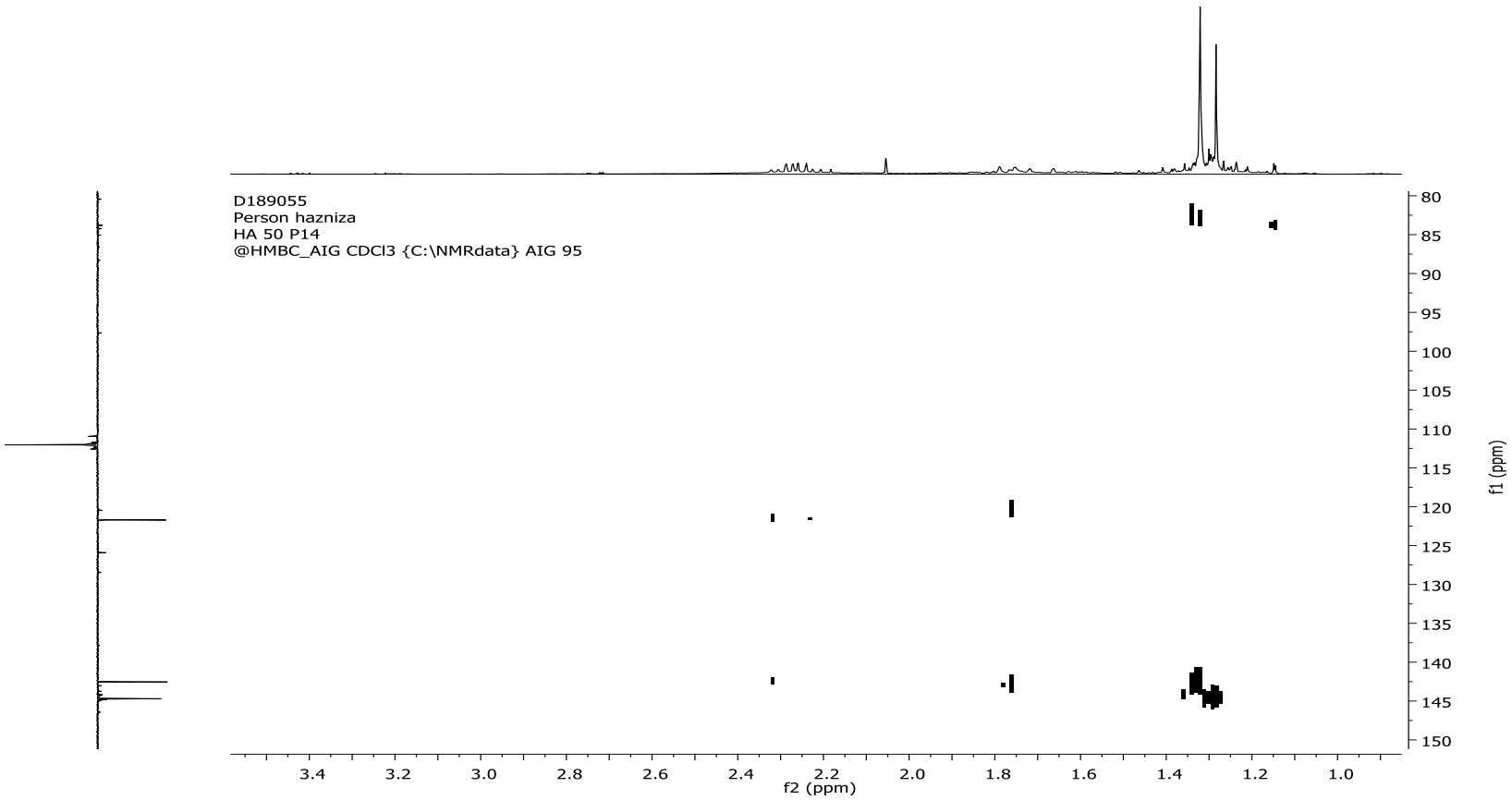


Figure 6: HMBC NMR spectra (selected expansion) of **(HA50P14)**