Chemical and Bio-analytical Studies on *Pergularia tomentosa* and Species from the *Mentha* Genus

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By

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Abstract

In this study samples of plant species *Pergularia tomentosa*; family Asclepiadaceae were collected from the Sultanate of Oman and extracted with organic solvents. These extracts were screened for their relative total phenol content, total antioxidant and anti-microbial properties. This species of plants has been traditionally used in Oman and much of the Middle East for the treatment of skin diseases, as abortive agents and as a laxative. The ethanolic extract of the plant was shown to possess anti-oxidant activity (inhibition of DPPH• radical), which is related to their phenolic content and also anti-bacterial and anti-viral properties. Partial purification of these extracts (and associated anti-oxidant activity) has also been achieved by a combination of column chromatography and HPLC. Although complete structural elucidation of the compounds present within the extracts has not been possible, NMR analysis has indicated the presence of certain functional groups. Antioxidant activities of the extracts have been determined by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) assay and phospho-molybdate method. Folin-Ciocalteu method was used to determine total phenolic contents. In order to detect anti-bacterial activities of extracts a preliminary screening study was performed by using Varioskan spectrophotometer (Transgalactic Ltd).

The second objective of this thesis describes the overall phenotypic relationships of the mint species and their interspecific hybrids and to provide some additional vision as to the development of the cultivated mint plants. Protocols for the extraction (microwave) and analysis (GC-MS) of the essential oil content within fresh and historical (dried) mint leave samples have been developed. By applying these methods to samples from both parental and hybrid mint species and carrying out a Principal coordinate analysis (PCoA) analysis we have been able to determine the relationship between essential oil composition and parental genus.

*To my **mum** (big heart),*

You not just brought me into this world,

But also moulded me into the person I am today.

I owe all I am to you and only you.

DAD,

Although Alzheimer's disease has robbed your mind, but I can see a peace upon your face and I am sure that you smile on your eyes mean that you remember me. You may have thought I did not see, Or that I had not heard, Life lessons that you taught to me, but I got every word. Thank you.

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Firdos and her family, for been there to listen and help me in whichever way they can and for Share a truth, pain and never left my side.
Hanan Al kindi for been my echo wisdom.

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Love,

Sharifa

List of Contents

Abbreviations and Symbols

AIDS	Acquired immune deficiency syndrome
B.p	Boiling point
^{o}C	Degrees Celsius
CAM	Complementary and alternative medicine
CAT	Catalase
COSY	Correlation spectroscopy
cm^{-1}	Per centimeter
DAST	(Diethylamino)sulfur trifluoride
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarisation transfer
DMAP	4-Dimethylaminopyridine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH•	2,2-diphenyl-1-picrylhydrazyl

EC_{50}	Half maximal effective concentration
EI	Electron impact
EtOAc	Ethyl acetate
EtOH	Ethanol
ESI-MS	Electrospray ionization mass spectrometry
E23	E. coli strain
g	Grams
GPX	Glutathione peroxidase
h	Hours
HCl	Hydrochloric acid
Hz	Hertz
HMQC	Heteronuclear Multiple Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
M	Molar
M^+	Molecular ion

MeOH	Methanol
MHz	Megahertz
ml	Milliliter
mm	Millimeter
mmol	Millimoles
mol $^{-1}$	Per mole
MS	Mass spectrometry
m.p.	Melting point
nAChR	Nicotinic acetylcholine receptor
NEt_3	Triethylamine
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
O•	Superoxide anion
•OH	Hydroxyl radical
ON	Overnight

PA14	Pseudomonas aeruginosa strain
PBS	Phosphate Buffered Saline
Petrol	Petroleum fraction 40 : 60 ° C
ppm	Parts per million
P.tomentosa	Pergularia tomentosa
Rf	Retention factor
ROS	Reactive oxygen species
RT	Room temperature
SOD	Superoxide dismutase
Sq. km	Square kilometer
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
TFAA	Trifluoroacetic anhydride
TLC	Thin layer chromatography
Ubi-hq	ubiquinol

Ub-q	ubiqinone
Vit C	Vitamin C
Vit E	Vitamin E
WHO	The world health organization

CHAPTER 1

Chapter 1 Introduction

1.1 Challenges in ethnobiology and ethnomedicine

Ethnomedicine is the anthropological and scientific study of traditional medicine. A large number of biological resources derived from animals and plants or their parts have remained in use as medicines by humans since prehistoric times. Many such medicinal uses are documented quite early but still many such remedies are transferred from one generation to next via the oral communication. In the modern era ethnomedicine has taken a more scientific direction with major focus on extraction of pharmacological agents followed by the studies of their effects on different body systems in useful models including humans.

Ethnomedicinal studies are also important to understand people's perceptions and understandings about natural resources, health and well-being. Ethnomedicine also provides answers to questions such as "how people's perceptions and preferences change in accordance with the changes in the socio-cultural, political and environmental factors in different eras. In the modern era, ethnomedicine has taken an important position on national and international political agendas and has become an attractive option for stakeholders in environment and health sectors"[1](Pieroni, 2006).

In 2007, The European Traditional Medicine, an international conference, provided an opportunity to discuss emerging challenges in ethnobiology and ethnomedicine where the latest research in this field, the wide range of therapeutic potentials, paradigmatic aspects, ethical issues, and methodological approaches were on the agenda (Firenzuoli and Gori, 2007a).[2] In 2009, the World Health Organization held a congress on traditional medicine in Beijing, China. This international meeting worked on the adoption of the Beijing Declaration to promote safety and effectiveness of

traditional medicine. The congress called WHO member states and all stakeholders to foster efforts for the integration of traditional, complementary and alternative medicine into health systems(WHO, 2009).[3]

1.2 Traditional medicine

Traditional medicine is an area of practice and skill utilisation of a vast knowledge, beliefs and experiences for the treatment and prevention of human pathological conditions and mental ailments. Many aspects of traditional medicine are highly restricted to some cultures but their utility is now being widened. In some Asian and African regions, about 80% of the human population relies on traditional medicine and over 100 countries have adopted regulations for the uses of herbal drugs and products. However, the availability of poor-quality and adulterated medicinal products have posed serious safety risks (Ameh and Obodozie, 2010).[4]

Traditional Chinese medicine, Indian Ayurveda, Unani and Siddha types of curative practices are among the most famous traditional medicines and almost all parts of the world, including Europe and America, have a large number of believers of several schools of thought. In Europe, traditional herbalisms have many similarities with Asian medicinal philosophy of 'humoral-energetic doctrines' that is based on the qualities such as temperature and moisture and elements like fire, air, earth and water (Firenzuoli and Gori, 2007b; Kim *et al*., 2011).[5,6] Philosophical foundation of traditional medicine rests on the principle that pathological conditions result from single or multiple imbalances in physical, psychological, social and spiritual aspects and therefore curative efforts should account for relationships between body, mind and spirit. Thus, the aim of therapy is treatment of whole body not an isolated symptom or disease (Kim *et al*., 2011).[6] Others such as traditional Sasang constitutional medicine, traditional Amazonian medicine and traditional Iranian medicine also have similar

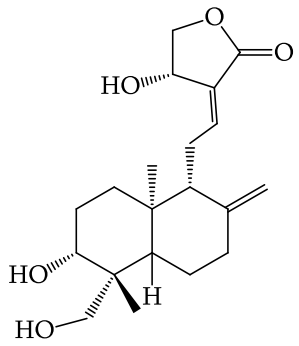
foundations and philosophies. Traditional medicine encompasses recognized ancient wisdom. Several factors like beliefs, knowledge, prejudices, culture, and the way of thinking of patients and provider's skills and experience count much towards the outcome of the treatment. One important aspect of traditional medicine is its globalization i.e. its spread from indigenous cultures to more widespread populations. Acupuncture is one of the best examples in this regard(Cardini *et al*., 2006).[7]

1.3 Medicines from Plants

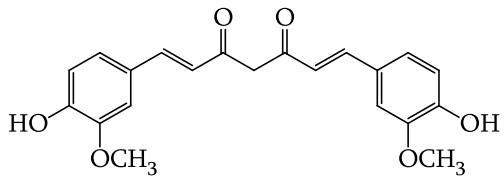
There is a long list of medicinally active chemicals derived from plants. Many of these chemicals are well known for their pharmacological and therapeutic properties but many have not yet been studied scientifically. The numbers of plant-synthesized aromatic compounds are almost limitless of which over 12,000 have been isolated so far and this is estimated to be just 10% of the total (Cowan, 1999).[8] Extraction of quinine from cinchona plant and artemisinin from Artemisia as antimalarial drugs are two of the most important discoveries in ethnomedicine (Graz *et al*., 2011).[9] Similarly, *Polyalthia* sp. (family Annonaceae), *Haplophragma adenophyllum* (Bignoniaceae), *Annona reticulata* (Annonaceae) and *Aegle marmelos* (Rutaceae) have shown strong anti-tuberculosis activities in bioassays (Elkington *et al*., 2009).[10]

There are approximately 1200 species of plants that are used to treat diabetes and about 100 are proven to possess antidiabetic properties. Many flowering plant families e.g. Amaryllidaceae, Fabaceae, Rutaceae, Solanaseae, Cucurbataceae and Ranunculaceae synthesize a range of phytochemicals which are found to be effective in overcoming the symptoms of diabetes (Kar *et al*., 2003; Modak *et* ., 2007; Hsu *et al*., 2009; Xie *et al*., 2011; Khan et al., 2012).[11] Leaf extracts from *Vitex negundo* leaves are antitussive and antiasthmatic, antiinflammatory, anticonvulsant, antimicrobial,

anticancer, and hypoglycaemic (Nagarsekar *et al*., 2011; Vinuchakkaravarthy *et al*., 2011).[12] A tropical plant, *Centella asiatica,* of family Apiaceae is famous for its hepatoprotective, cardioprotective, wound healing, anticancer, antiinflammatory, anti-sporiatic, anti-ulcer, antidiabetic, antibacterial, antiviral, antifungal, antioxidant, anticonvulsant, cytotoxic, sedative, and immunostimulant effects (Orhan, 2012).[13]

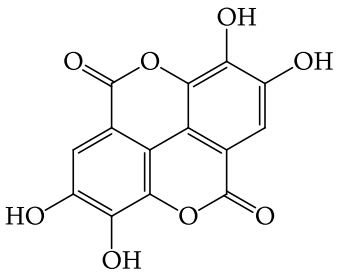


Andrographolide is a diterpene lactone phytochemical produced in *Andrographis paniculata.* It is commonly used to treat colds, diarrhoea, fever, and inflammation.This compound exhibits antiinflammatory, antiprotozoal, antibacterial, and has immunostimulating properties.It also possesses antioxidant, antithrombotic, hepatoprotective, cardioprotective, and analgesic properties. It also inhibits platelet aggregation and stimulates cell differentiation (Batkhuu *et al*., 2002; Anju *et al*., 2012).[14] A flavonoid,

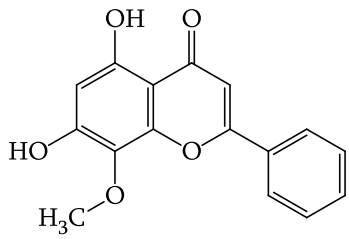


curcumin found in *Curcuma* species of family Zingiberaceae is an antiinflammatory, antiviral, antibacterial, antifungal, antispasmodic, anti-tumour, photoprotective, hepatoprotective and neuroprotective compound. This compound has been found to be mosquitocidal, antivenomous, active against high blood pressure, arthritis, cancer, HIV and irritable bowel

syndrome (Kawamori *et al*., 1999; Itthipanichpong *et al*., 2003; Dohare *et al.,* 2008; Funk *et al.,* 2010; Rogers *et al*., 2010; Saraf and Kaur, 2010; Rahimi and Abdollah, 2012).[15] Euphorbiaceae family usually contains milky latex which contains fatty acids, terpenoids, phenolics and alkaloids that can be used for pharmaceutical, pesticide and in the rubber industry (Mwine and Damme, 2011a & b).[16,17] Tannins and cyanidanol have astringent value and use as antimicrobial treatments while triterpenoids offer antiinflammatory and anticarcinogenic benefits. Quercetin is hepatoprotective and epicatechin is hypoglycaemic. Lectins from *Cannabis sativa* exhibits heamagglutination and thus helps in controlling bleeding (Cowan, 1999).[8]

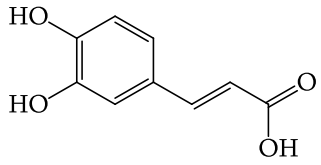


Ellagic acid (4,4',5,5',6,6'- hexahydroxydiphenic acid 2,6,2',6'-dilactone) is phenolic compound naturally found in woody plants, grapes, berries, and nuts. Ellagic acid obtained from several plants such as *Fragaraia nubicola, Rubus ulmifolius, Phyllanthus urinaria* etc. is an antioxidant and has antimicrobial, antiinflammatory, antiproliferative and antiangiogenic properties (Huang *et al.*, 2011; Quave *et al*., 2012).[18]

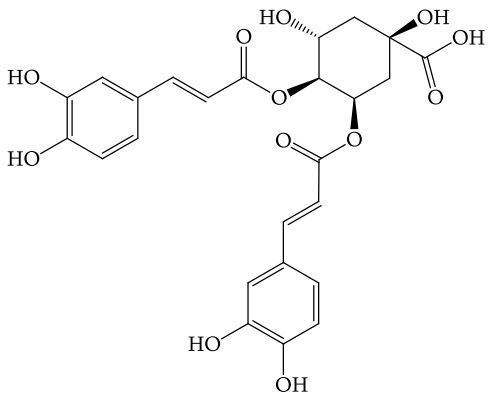


Wogonin, a flavonoid (5,7-dihydroxy-8-methoxyflavone), is a potent antiviral,

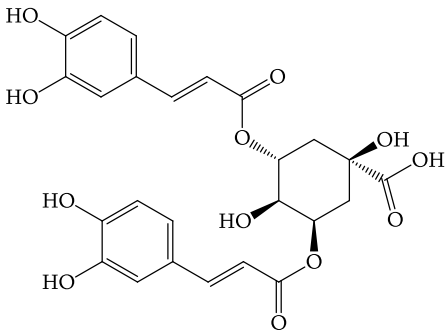
anxiolytic, antioxidant, anticarcinogenic, and neuroprotective compound which is also found to be effective against rheumatoid arthritis. It is synthesized in *Scutellaria* (family Lamiaceae) plant roots (Polier *et al*., 2011; Chen *et al*., 2012).[19] Many plants offer strong antioxidant benefits through the use of one or more of their parts or ingredients. Extracts from *Rubus coreanus* (Raspberry), *Rubus Scizostylus, Schisandra chinensis and Terminalia chebula* have been reported to exhibit powerful antioxidant activities in multiple assays (Ko *et al*., 2008).[20] *Aconitum spicatum* (Ranunculaceae) contains caffeic acid



and its derivatives such as 4,5-dicaffeoylquinic acid and 3,5-dicaffeoliquinic



4,5-dicaffeoylquinic acid



3,5-dicaffeoylquinic acid

acid suppress nitric oxide (NO) production and also possess analgesic and antiinflammatory properties (Park *et al*., 2009).[21] A paste from the extracts of this plant in combination with *Terminalia chebula* (Combretaceae) offers antipyretic and analgesic merits. Root powder of *Ptentilla fulgens* is used as tooth paste. Plants of family Meliaceae contain several phytochemicals with

curative potentials such as the anti-malaria properties of oleic acid and the gedunin exhibited by nimbidin flavonoids. (Kunwar *et al.,* 2010).[22] An annual herb, *Ageratum conyzoides*, exhibits antiinflammatory, analgesic, anti-tusive, anti-itch properties and is used for pain, fever, leprosy, purulent ophthalmia, flatulence, intestinal colic, rheumatism, pneumonia, infections, asthma, sleeping sickness as well as several other diseases and conditions (Rahman *et al*., 2012).[23] Several plants are used for skin problems of which notables are Wild tobacco (*Acnistus arborescens*), Neem (*Azadirachta indica*), Needle glass (*Bidens alba*), Senna (*Cassia alata*), Congolala (*Eclipta prostate*) and Man better man (*Achyranthes indica*) (Lans, 2007).[24] Emilia sonchifolia is also an annual herb used to treat asthma, inflammation, wound healing, as a cytotoxic agent, anti-nociceptive agent. Known compounds of this plant include similar, stigmasterol, palmitic acid and beta-sitosterol (Rahman *et al.,* 2012).[23]

Over 100 species of American palm are used medicinally for several conditions such as pain, skin problems, digestive problems, infections, inflammation, neoplasm, circulatory and blood conditions, neurological disorders, respiratory and urinary problems and reproductive dysfunction (Sosnowska and Balslev, 2009).[25] Many plant extracts have been found to be efficacious against several gut problems such as irritable bowel syndrome. These include *Aloe vera, Curcuma species, Cynara scolumus, Fumaria officinalis, Hypericum perforatum, Maranta arundinacea, Mentha peperita Paeonia lactiflora* and *Plantago psyllium (Rahimi and Abdollahi, 2012).*[26] Carotenoids a large category of natural compounds found in the plants with many metabolic effects and diets rich in carotenoids are usually proclaimed for reduced risk of chronic diseases. Among these, α- and β- carotene, β-cryptoxanthin, lutein, zeaxanthin and lycopene are common forms. Lycopene is found in reddish fruits and vegetables such as tomatoes, pink grape fruit, watermelon, papaya and guava. It has proven anticancer effects and curative properties in cardiovascular diseases, hypertension, osteoporosis,

neurological disorders, male infertility and many others (Chauhan *et al*., 2011).[27] A mushrooms (*Antrodia camphorata*) is traditionally used in treating influenza, cold, headache, fever, twisted tendons, muscle damages, and many mental conditions. This mushroom contains 39 terpenoids, several benzenoids, lignans, benzoquinones, maleic or succinic acid derivatives and sterols (Geethangili and Tzeng, 2009).[28]

1.4 Omani traditional edible herbs

Traditional medicine in The Sultanate of Oman is derived from Hippocratic-Greek practices in which applied herbal, spiritual and mechanical methods and gained popularity because of their demonstrated safety and traditional values (Shenoy *et al*., 2009).[29] A number of herbs are used to cure several conditions of which two are discussed below:

1.4.1 *Pergularia tomentosa* (Family: Asclepiadaceae)

Pergularia tomentosa, called in Arabic as Hassaniya, is a scandent or climber and grows on woody root-stock distributed in Mauritania, Mali, Nigeria, Congo, North Africa, Arabia, India and Pakistan.[3030] It is a twining milky shrub with dense pubescence over the branches. Leaves are pubescent and cordate with lower velvety surface. Flowers are pediculate and are found in umbelloid cymes with pubescent sepals and dull white corolla.[31] The extract of these plants are shown to have antifungal (Bekheet *et al*., 2011),[32] antimicrobial, anticancer and antioxidant (Mothana *et al.,* 2010) [33] and hypoglycaemic properties (Shabana *et al*., 1990) [34] and exhibits cytotoxicity mediated by its ingredients such as Cardenolide (Piacente *et al*., 2009). [35] This plant has also been reported to cause poisoning and mortality in cattle (Abiola *et al.*,1993).[36]

1.4.2 *Ziziphus hajarensis* (Family: Rhamnaceae)

Endemic to Hajar Mountains of northern Oman, *Ziziphus hajarensis* belongs to family Rhamnaceae and is locally called qusum. Medicinally important phytonutrients of this plant include flavanoids, Gallocatechin, cyclopeptide alkaloids, flavanol glycosides, and quercetin glycosides, which have shown moderate antioxidant activity as plant extracts (Marwah *et al.,* 2006).[37]

1.5 Traditional herbs and health effects

Approximately 50% of licensed drugs registered in the last three decades are natural products (Newman and Cragg, 2007 in Kennedy and Wightman, 2011).[38] Use of herbal medicine for healing is as old as human civilization and appears to be again blooming worldwide triggered by perceived problems with modern medicine, which had flourished over previous decades. However, herbal medicines, which are usually preferred because of safety and spirituality, have been challenged for adverse side effects in some cases, though mainly because of contamination and adulteration. Revival of this component of medicine in the modern era is far more scientific than in the past and this may have contributed to its upsurge (in the modern era) (Mosihuzzaman, 2012)[39] Considerable evidence has been gathered in epidemiological studies to associate the consumption of polyphenol-rich fruit, vegetables, wine, tea, soy and other plant products with the prevention of cancer, stroke, coronary heart disease, osteoporosis, and in overcoming oxidative stress (Scalbert and Williamson, 2000). [40] Spices obtained from a large number of plants are beneficial in several ways. There is reasonable evidence for the role of spices in affecting proliferation, angiogenesis, apoptosis, and immunocompetence and therefore a spicy diet may have cancer risk lowering effect (Kaefer and Milner, 2011). [41] Health effects of many other groups of phytochemicals are discussed throughout this review.

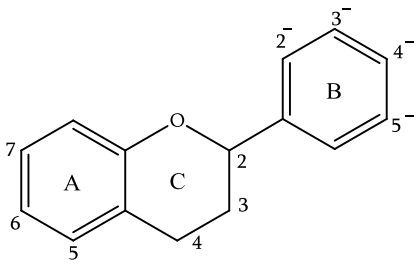
1.6 Phytochemicals in plants

Plants have evolved secondary biochemical pathways for the synthesis of chemicals in response to environmental stimuli such as herbivory, pathogen deterrence and nutrient shortage. These secondary metabolites do not interfere with primary metabolism but have significant survival value (Kennedy and Wightman, 2011).[42] Several phytochemicals mediate their health effects in humans by acting through specific genes or by acting on a number of metabolic pathways. An overview of common phytochemicals has been given in Table 1.2.

1.6.1 Phenolic compounds – Flavonoids

Phenolic compounds are widely distributed in the plant kingdom with over 10,000 types identified so far. Basically, phenolic compounds have toxicity-mediated feeding deterrence function in different ecological niches but they also render symbiotic and attractant functions. These compounds share at least one aromatic hydrocarbon ring along with one or more hydroxyl groups in their structure. Synthesis of a vast majority of phenolic compounds utilise the phenylpropanoid pathway (Kennedy and Wightman, 2011).[42] Phenolic acids are the esters of polyols such as glucose. Caffeic acid is a phenolic compound normally found esterified forms such as chlorogenic acid in coffee and vegetables. Tannins are derivatives of phenolic acids (Scalbert and Williamson, 2000).[40]

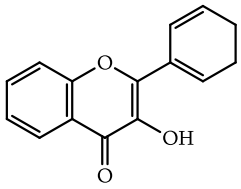
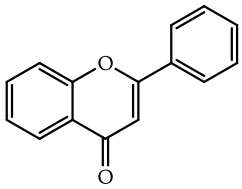
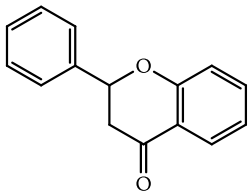
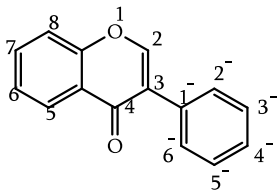
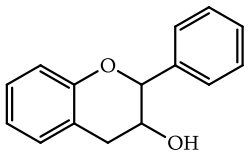
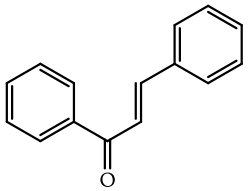
Among the phenolic compounds, flavonoids are the largest group of over 6000 types. These are benzopyran derivatives phenyl substituted chromones and consist of a15-carbon basic skeleton which contain two 6-carbon rings with a 3-carbon bridge acting as third ring. Modifications of this skeleton



Basic flavonoid skeleton

molecule give rise to several classes of flavonoids such as flavones, isoflavones, flavonols, flavonones, chalcones, flavan-3-ols and anthocyanins (Kennedy and Wightman, 2011).[42] Purified flavonoids are found to affect several aspects of atherogenesis including Low Density Lipoprotein (LDL) oxidation, chemotaxis, cell adhesion, inflammation, smooth muscle cell proliferation and platelet aggregation and thus several flavonoids are identified as having positive effects on cardiovascular disease prevention. Flavonoids inhibit a number of CYP isoforms and thus have potentials for altering drug properties (Egert and Rimbach, 2011).[43]

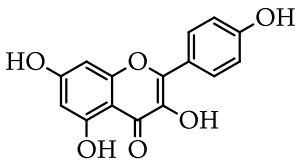
Table 1.1 Classification of flavonoids

Class	Basic structure	Common type	Food
Flavonol		Quercetin,kaempferol, myricetin,	Onion, curly kale, broccoli, blueberries, cherry tomatoes, red wine, tea,
Flavone		Luteolin, apigenin	Green leafy spices, parsley,
Flavonone		Hesperetin,naringenin, eriodictyol	Citrus fruit
Isoflavone		Genistein,diadzein, glycitein	Soy beans, soy food, legumes
Flavan-3-ols		Catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin3-gallate, eipgallocatechin3gallate,	Tea, red grapes, red wine,cocoa, chocolate, apricot
Anthocyanidin		Malvidin,cyanidin, delphindin, pelargonidin,	Red-, purple-, and blue-berries, black grapes, leafy and root vegetable

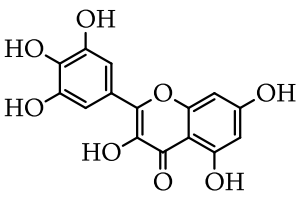
Reproduced from Erdman *et al*., 2007[44]

1.6.2 Flavonols

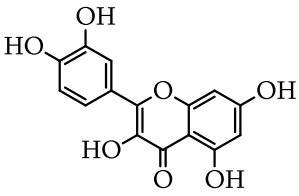
Major sources of dietary flavonols are fruit, vegetables, tea and red wine. However, dietary intake of flavonols is influenced by species, growth rate, season, light availability, ripeness, food preparation and processing (Aherne and O'Brien, 2002).[45] Decreased cardiovascular disease mortality has been reported in free living populations having high dietary intake of flavonols from fruits, vegetables, tea and red wine (Huxley and Neil, 2003).[46] Quercetin is an important flavonols which is recommended at a daily dose of 1g but normal dietary intake remains 10–100 mg (Erdman *et al*., 2007).[44] Other important flavonols include kaempferol and myricetin.



Kaempferol



Myricetin

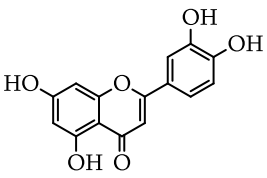


Quercetin

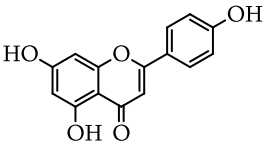
Quercetin and kaempferol inhibit calcium channel blockers such as nifedipine and felodipine (Egert and Rimbach, 2011).[43]

1.6.3 Flavones

Flavones are generally less abundant in fruits and vegetables as compared to flavonols. Parsley and celery are the primary sources of flavones. Important flavones include luteolin and apigenin.

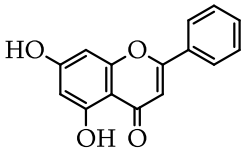


Luteolin



Apigenin

Luteolin (5,7,3',4'-tetrahydroxyflavone) is usually found in parsley, celery, peppers, onion leaves, cabbage, broccoli, and apple skin. It is used to treat hypertension, inflammatory conditions, and cancer because of its antiinflammatory, anticarcinogenic, antioxidant, apoptosis promoting, antiangiogenesis and anti-metastasis properties (Lin *et al*., 2008).[47] Chrysin



Chrysin

(5,7-dihydroxyflavone) is a potent aromatase inhibitor and thus enhances testosterone levels in men. As such chrysin is very poorly bioavailable but its methylated forms are stable and highly bioavailable (Ta and Walle, 2007).[48]

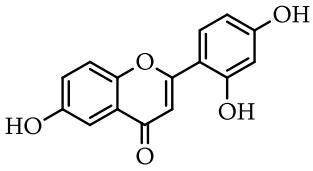
1.6.4 Flavanones

The primary sources of flavanones are citrus fruit, which contain naringenin, eriodictyol and hesperidin. Naringin is citrus flavonoid-glycoside, which produces the bitter taste in grapefruit. Naringin is hydrolyzed into naringenin by gut microflora. Naringenin possess antioxidant, antitumor and radioprotective activities and thus affects cancer, cardiovascular diseases, hyperlipidemia, neurological disorders and skin disorders (El-Mahdy *et al.,* 2008).[49] Hesperidin exhibits antioxidant, antiinflammatory, anticarcinogenic, antimicrobial and radioprotective activities (Hosseinimehr and Nemati, 2006).[50]

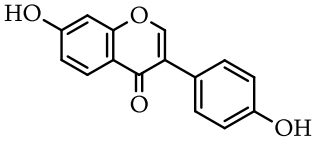
1.6.5 Isoflavones

Of this general class of compounds isoflavones are the predominant constituent of the diet. Most common in human use are genistein (4',5,7-

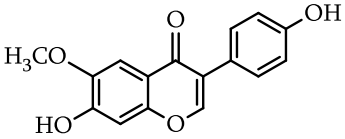
trihydroxyisoflavone), diadzein (4',7-dihydroxyisoflavone), and glycitein (7,4'-dihydroxy-6-methoxyisoflavone). These chemicals possess estrogenic activity by bindng with estrogen receptors ER-β. Genistein has shown significant antiproliferative and apoptotic activity and thus acts as chemopreventive agent (Saldanha and Tollefsbol, 2012).[51] Genistein has been found to improve blood flow by affecting vasodilation and this effect can be reversed by NO synthase inhibition (Walker *et al*., 2001).[52]



Genistein

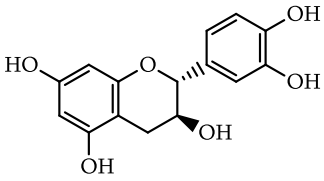


Daidzein

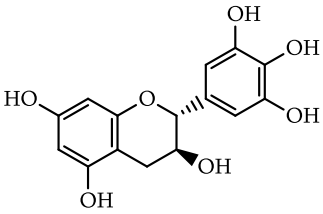


Glycitein

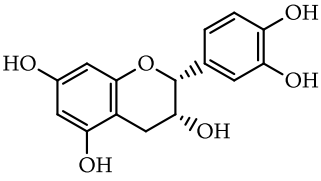
1.6.6 Flavan-3-ols



Catechin



Gallocatechin



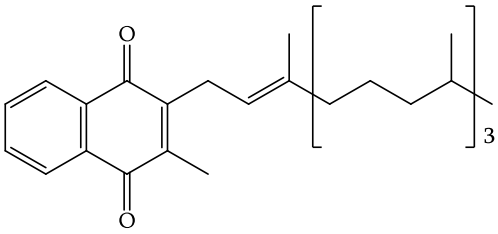
Epicatechin

Flavan-3-ols like catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, are found in grapes and other food sources, enhance the release of nitric oxide (NO) and decrease superoxide production during platelet aggregation and thus limit this process. These phytochemicals also reduce the release of proinflammatory mediators from platelets and thus inhibit inflammation in arteries. These compounds also suppress LDL / HDL oxidation (Erdman *et al.,* 2007).[44] Those lipoprotein receptors, which play an important role in control of cholesterol in human

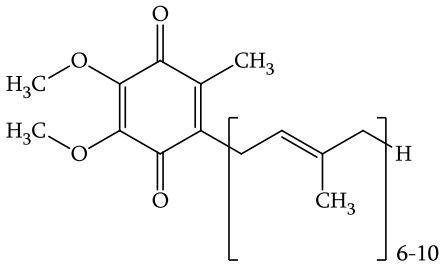
body that can increase risk of heart disseaes (atherosclerosis), Low-density lipoproteins (LDL) is the major carrier of cholesterol in the blood, if LDL level increased promoting athersclorosis by oxidizing the LDL in artery walls and narrowing it with cholesterol particles while HDL act to reverse cholesterol transport and inhibit LDL-induced cytotoxicity.

1.6.7 Anthocyanidin

Anthocyanidins exhibit a range of pro-health effects including antioxidant, antiinflammatory, antimicrobial, anticarcinogenic, neuroprotective, vision improving, and apoptosis inducing properties. Anthocyanidins such as pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides are found to be strong inhibitors of nitric oxide (NO) production *in vitro* without having harmful effects(Wang and Mazza, 2002). [53] Anthocyanins, cyanidins and delphinidins potently inhibit platelet derived growth factors (PDGF) induced vascular endothelial growth factor (VEGF) expression in vascular smooth muscle cells by preventing p38 mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways (Oak et al., 2006).[54]



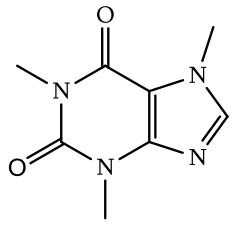
Phylloquinone(Vitamin K)



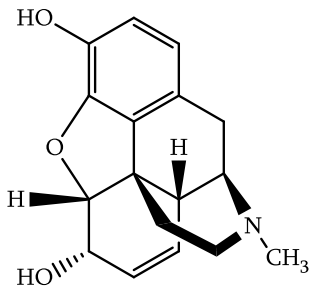
Ubiquinone (Coenzyme Q)

1.6.8 Alkaloids

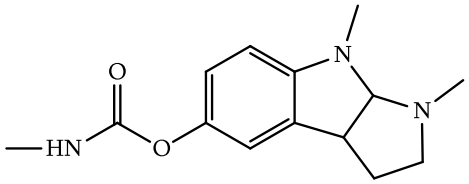
Alkaloids are a diverse class of cyclic nitrogenous compounds found in about 20% of plant species. Alkaloids exist in many categories such as indole, benzylisoquinoline, tropane, pyrrolizidine, or purine alkaloids. For over 5000



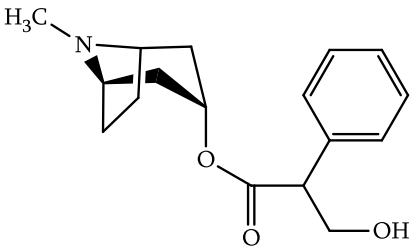
Caffeinc



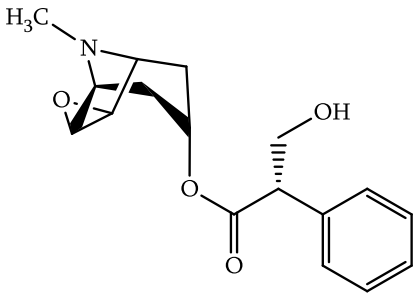
Morphine



Physostigmine



Atropine



Scopolamine

years alkaloids have been in use as poisons, neurotoxins, and psychedelics. Caffeine, cocaine, ephedrine, morphine, nicotine, atropine, physostigmine, scopolamine are common alkaloids that affect many health conditions including behaviour[42] (Kennedy and Wightman, 2011). Bioprotective alkaloids in plants and plant-endophyte associations include pyrrolizidines, ergot alkaloids, indole diterpenes, pyrrolopyrazines. Such alkaloids increase fitness of plants and host plants against the exploitation by animals and make them more resistant and competent to drought. Ergot alkaloids are the main sources of toxicities to livestock[55] (Bush *et al.*, 1997). The majority of polyhydroxylated alkaloids such as pyrrolidine, piperidine, pyrrolizidine etc inhibit glycosidic bond hydrolysis by interfering with glycosidases, which have highly important functions in primary metabolism. Various polyhydroxylated alkaloids are used for therapeutic uses such as anticancer, antiviral, and antidiabetic agents, immune stimulants and in treating glycosphingolipid lysosomal storage diseases (Watson *et al*., 2001).[56]

1.6.9 Quinones

Quinones are aromatic compounds that are usually derived from benzene or naphthalene The major groups of natural 2-methyl-3-oligoisoprenyl quninones are vitamin K, coenzyme Q (ubiquitones), and tocopherol quinones which are produced from the oxidation of vitamin E. Ubiquitone is a prenylated benzoquinone, which plays basic role in electron shuttle in the electron transport chain. It also participates in the mitochondrial oxidation of dihydroorotate, in the biosynthesis of pyrimidines (Lederer, 1964).[57] Antiproliferative and cytotoxic properties of many quinones and hydroquinones derived from sponges have created promising opportunities for the development of antitumor agents and many quinones exhibit significant anti-tuberculosis, antimalarial, antifungal and cardiotonic activities (Gordaliza, 2012).[58]

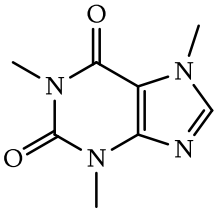
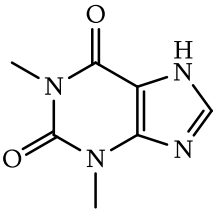
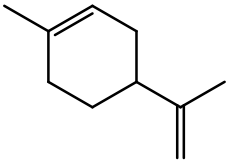
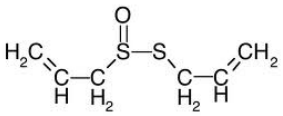
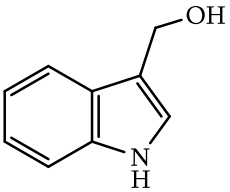
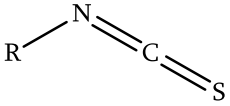
1.7 Phytochemicals and health effects

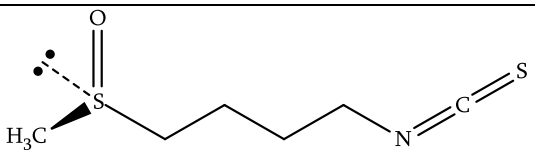
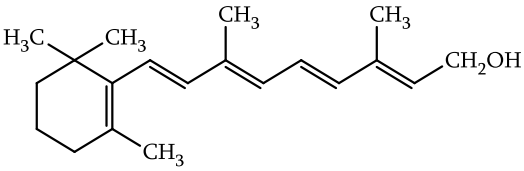
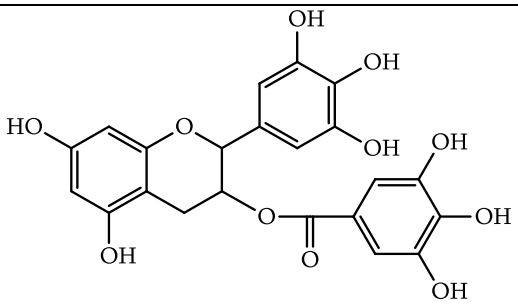
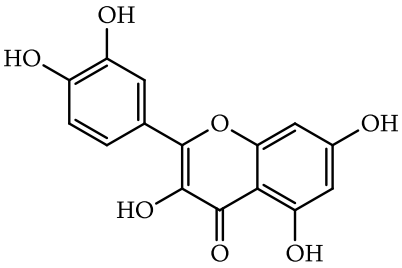
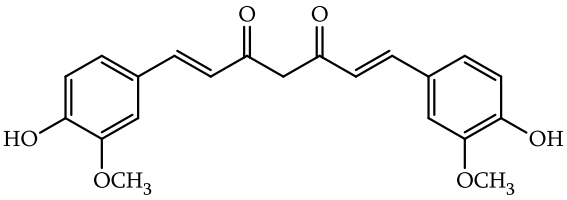
Phytochemicals are in use for almost every pathological condition and many of their efficacious roles are well documented in animal models as well as in humans. Results of *in vitro* experiments cannot be treated as preclinical evidence for clinical trials as *in vitro* tests are conducted under completely controlled conditions while a host of factors are needed to be taken into account before testing a bioactive chemical on humans. On the other hand, *in vivo* experiments can substantiate the findings of *in vitro* studies as a multitude of factors can alter the path of bioactive compound under test (Saldanha and Tollefsbol, 2012).[51]Therefore, ethnomedicinal scientific evaluations are highly desirable. Preliminary studies have provided impetus for well controlled and randomized trials to evaluate the efficacies and as more evidence gathers, scientific and medical knowledge of the therapeutic potentials of phytochemicals will become conclusive.

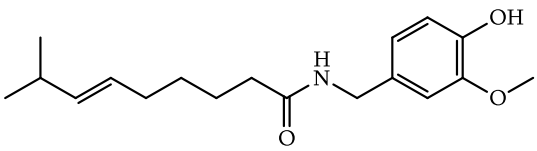
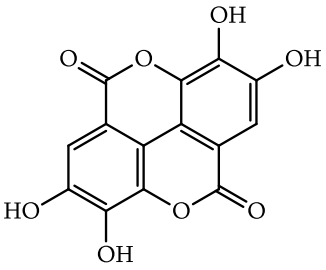
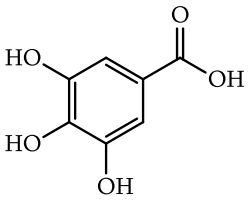
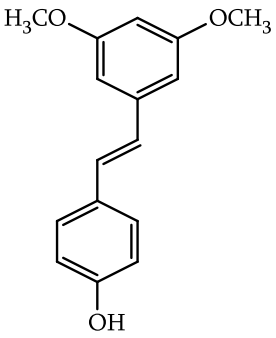
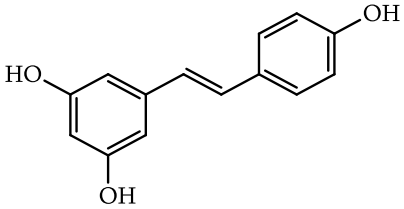
1.8 Biological activities

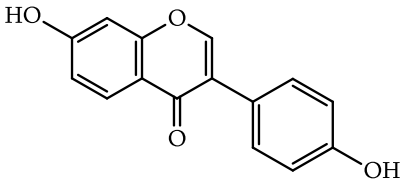
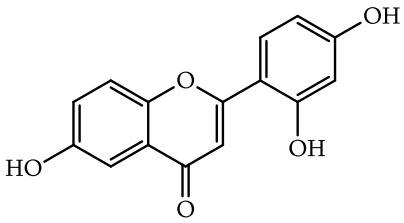
Phytochemicals are not evenly found in all plant tissues and food preparation and processing may also affect their bioavailability. For example, in apples, quercetin is found in the skin, which contains no other flavonol. Polyphenols in wheat grain are contained mainly in the seed coat and outer layers and so are lost during grain flouring. In many cases such as with polyphenols it is very difficult to estimate average daily intake because of poorly standardized analytical methods. Bioavailability studies have revealed that different polyphenol compounds vary from each other with respect to their intact presence in urine after dietary intake (Scalbert and Williamson, 2000).[40] It is low for quercetin and rutin (a glycoside of quercetin) but higher for catechins of green tea, isoflavones of soy, flavonones of citrus fruits and anthocyanidins of red wine. The maximum concentration of flavonoids appears in blood about 2 hours after ingestion. Polyphenols are absorbed after being partially degraded by gut microflora and therefore their maximum concentration in blood is reached quite late (e.g., rutin in-take follows quercetin maximum blood concentration in about 9 hours). Their fast elimination is due to conjugation reactions (de Vries *et al.,* 1998).[59] Biological activities of phytochemicals are thus affected by a number of factors and scientific knowledge of these components can improve their efficacious properties.

Table 1.2 Important phytochemicals and their beneficial effects

Class	Bioactive agent	Food source	Formula	Beneficial effects
Alkaloid	Caffeine	Tea, coffee, cacao		Alertness, improves brain function
	Theophylline	Tea, coffee, cacao		Antihypertensive, antiinflammatory, bronchodilator, cardiovascular benefit, reduces signs of vitamin B6 deficiency
Monoterpenes	Limonene	Citrus, mandari, lime, grapefruit		Gallstone dissolution, Gastroesophageal reflux disorder relief, anticancer
Organosuphides	Allicin	Garlic		Immunomodulatory, antiinflammatory, antioxidant, antibacterial
	Indole-3-carbinol	Cabbage		Antioxidant, Antiinflammatory, Antihypertensive, Hypocholesterolemic
	Isothiocyanates	Broccoli		Antioxidant, anticarcinogenic

Class	Bioactive agent	Food source	Formula	Beneficial effects
	Sulforaphane	Broccoli		Antiinflammatory, antioxidant
Carotenoids	Carotene, Lycopene	Tomatoes		Curative properties in neurological disorders, infertility, hypertension, cardiovascular diseases and cancer
Flavonoids	Epigallocatechin-3-gallate	Green tea		Neuroprotective against neurodegenerative diseases, antioxidant
	Quercetin	Black tea		Antioxidant, curative in neurological disorders, osteoporosis, cardiovascular diseases, cancer
	Curcumin	Turmeric		Neuroprotective, anticancer, hypolipidic

Class	Bioactive agent	Food source	Formula	Beneficial effects
Phenolic acids	Capsaicin	Chili peppers		Neuromodulator, Curative in nasal rhinitis and pruritus
	Ellagic	Black berries, raspberries		Anticarcinogenic
	Gallic acid	Pomegranates. Nuts		Neuroprotective, cardioprotective, antioxidant, antimutagenic, anticarcinogenic
Stilbenes	Pterostilbene	Blueberries, grapes		Antioxidant,
	Resveratrol	Almonds, blueberries, grapes		Antioxidant, antiinflammatory, hypolipidic, antiplatelet aggregation,

Class	Bioactive agent	Food source	Formula	Beneficial effects
Isoflavones	Daidzein	Soy		Antidiabetic, catecholamine stimulator, estrogenic / antiestrogenic,
	Genistein	Soy		Neuroprotective, curative in bone loss, cardioprotective, anticarcinogenic

Modified from Saldanha and Tollefsbol, 2012[51]

CHAPTER 2

Chapter 2 Free Radicals, Oxidative Stress, Skin Related Diseases And Antioxidants

2.1 Free radicals, endogenous defence and the role of oxidative stress in human

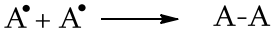
2.1.1 Definition of free radicals

"Any species (atoms, molecules or ions) capable of independent existence that contain one or more unpaired electrons are known as free radicals.[60,61,62,63-64] These species can be atoms, molecules or ions having unpaired electrons in their orbitals. In other words, they possess an open shell configuration[65], where an open shell is a valence shell[66], which is not completely filled with electrons, or all the electrons contained in it are not in chemical bonding with other atoms or molecules. Free radicals may carry positive, negative or zero charge.[67,68] The free electrons present in the open shells make these radicals highly reactive species. These free radicals play a considerable role in the processes of polymerization[69]and combustion[70,71], and many chemical reactions of plasma chemistry[72,73], atmospheric chemistry[74,75]and biochemistry.[76,77,78,79] In living organisms, intermediary metabolism[80] of many biological compounds takes place via these reactive species. In a process referred to as redox signalling[81,82], free radicals serve as messengers.

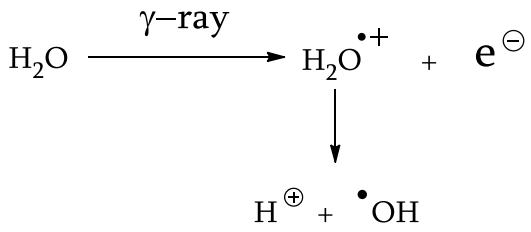
In short, free radicals play a very important role in a number of chemical reactions, which occur in our body. Usually, our body exploits a number of free radical physiological and biochemical reactions, and keeps them under tight control. [83,84] But, unfortunately, these processes may go out of control leading to a chain of reactions that are damaging to surrounding tissue. They therefore play constructive as well as destructive roles in the body.

2.1.2 Origins and target of free radicals

Each day, our body produces numerous free radicals during metabolism which in turn react with other molecules in several of ways.[60, 83 and 84]Mitochondria are the most important cellular source of free radicals and deal with regulatory and toxic reactions accomplishing the processes like cell cycle, proliferation and cell death. Mitochondria produce primary free radicals such as superoxide anion and nitric oxide along with hydrogen peroxide and peroxynitrite as termination products.[85,86] It has been shown that electron transport chain directly produces superoxide anion radical and then hydrogen peroxide which in turn gives rise to hydroxyl radical.[86,87]Free radicals are highly reactive species that can attack and damage almost each and every molecule contained in living cells[60, 88, and 89]. As free radicals contain unstable or free electrons in their shells, they tend to be more reactive than non-radical species, although they possess a wide range of reactivity. When two radicals come together, they share their unpaired electrons through the formation of a covalent bond.



A radical has tendency to steal an unpaired electron from the other molecules in order to pair, generating others free radical. Free radicals react via chain reactions, one radical produces another one, and so on. Chemists and biochemists have studied the production of free radicals by high energy radiations and their damage to the living tissues. When living tissues are exposed to radiation (say γ radiations), most of the energy is absorbed by the water molecules found in the tissue cells. The radiation splits water molecules and creates two free radicals from each molecule of water.



Where $H^{\bullet}$ is hydrogen radical and $^{\bullet}OH$ is hydroxyl radical. Hydroxyl free radical is highly reactive and it is can attack any of the molecules found in the living cells causing a damage to the system. The hydroxyl radical gives birth to a chain of reactions in the cell and if it attacks DNA, it causes chemical changes in the DNA bases and even breaks the linkages, activating oncogenes and carcinogenesis.[90] However, hydroxyl radical production is, paradoxically, the major mechanism to kill cancer cells during radiotherapy.[91]

Studies have revealed that proteins are the initial cell targets of free radicals.[92]Exposed to hydroxyl free radicals, proteins were shown to be oxidized before lipids. In an early study carried out by Du and Gebicki, protein peroxides were shown to be produced before lipid peroxidation and DNA damage.[93]

2.1.3 Source of free radicals

As mentioned above, the source of free radicals is our body itself as well as the environment. Mitochondrial electron transport chain is an adventitious source of free radicals during normal metabolism[94] in addition to other physiological factors that also contribute to free radicals production.[95] Thus, sources of free radicals can be either internal or external. Enzymatic reactions occurring in the body serve as a source of free radicals. These reactions are involved in the electron transport chain, phagocytosis, cytochrome P450 systems and prostaglandin synthesis.[96] Mitochondria, phagocytes, peroxisomes, xanthine oxidase, arachidonate pathways, ischemia, inflammation and exercise are described as internal sources of free radical generation.[96,97,98, 99]

Similarly, non-enzymatic reactions of oxygen with organic compounds and the reactions initiated by ionizing radiations serve as an external source of free radicals.[100,101,102,103,104] Additionally, cigarette smoke, environmental pollutants, ozone, drugs, pesticides, UV light, industrial solvents and electronic pollutants (extra low frequencies from computers, power lines, television, microwaves, etc.) are some other external sources of free radicals.[105,106,107,108,109,110,111,112] Physiological factors like stress, emotions, anxiety and other disease conditions add to the generation of free radicals and put the body under oxidative stress.[113]

2.1.4 Formation of reactive oxygen species and action *in vivo*

Reactive oxygen species (ROS) are chemically highly reactive molecules with one unpaired electron in their valance shell derived from molecular oxygen.[114,115] ROS are generated during normal metabolism of oxygen by endogenous systems as well as exposure to physiochemical and patho-physiological conditions, and play a key role in cell signalling and homeostasis. ROS include superoxide, hydroxyl radical, hydrogen peroxide, peroxyl radical, organic hydroperoxide, singlet oxygen and ozone[114,115,116,117][Table 2.1]. These reactive oxygen species are produced in the body with controlled mechanisms. Enzymes such as alpha-1-microglobulin, catalases, superoxide dismutases (SOD), glutathione peroxidases and lactoperoxidases defend living cells against oxidative damage by ROS.[118,119,120,121,122] If these mechanisms get out of control, free radicals can adversely affect proteins, lipids, DNA and may lead to a number of human diseases. Damages to proteins and lipids lead to loss of enzyme activity and lipid peroxidation respectively. Mutagenesis and carcinogensis may take place due to DNA oxidation.[122,123,124]

Table 2.1 Oxygen Reactive Species (ROS)

Reactive Oxygen Species	Symbol	Reactivity
Superoxide	$O_2^{-}\bullet$	Produced in mitochondria and cardiovascular system
Hydroxyl radical	•OH	Highly reactive, generated in iron overload and attack every molecule of the living cells
Hydrogen peroxide	H_2O_2	By product of several body reactions and gives rise to potent $^{\bullet}OH$
Peroxyl radical	$RO_2^{\bullet}$	Reactive and generated during oxidative damage to lipids, proteins, DNA and sugar
Organic hydroperoxide	ROOH	Reacts with transient metal ions
Singlet oxygen	$^1O_2$	Highly reactive, generated in photosensitization and other chemical reactions
Ozone	$\bullet O_3$	An environmental pollutant, reacts with living molecules and yields singlet oxygen

Among ROS, the hydroxyl radical is the most reactive and potent damaging species which can affect each and every molecule found in the living cells. *In vivo*, it has been shown that hydroxyl radical can lead to formation DNA-protein linkages, base damages, of DNA strand breaks, protein fragmentation and lipid peroxidation.[125,126] The process of lipid peroxidation is initiated by the abstraction of a hydrogen atom from a molecule of polyunsaturated fatty acid (PUFA). Lipid peroxidation leads to the generation of carbon-centred radicals which give rise to peroxyl radicals by reacting with O_2. Peroxyl radical further oxidizes membrane proteins and adjacent PUFA side chains, thus initiating a chain reaction.[127] Moreover, peroxyl radical can abstract $H^{\bullet}$ from the same PUFA, giving rise to cyclic peroxides.[128] Free radical often mediates lipid auto-oxidation, which takes

place in a single chain reaction via the three processes initiation; propagation and termination as shown below (see Figure 2.1).

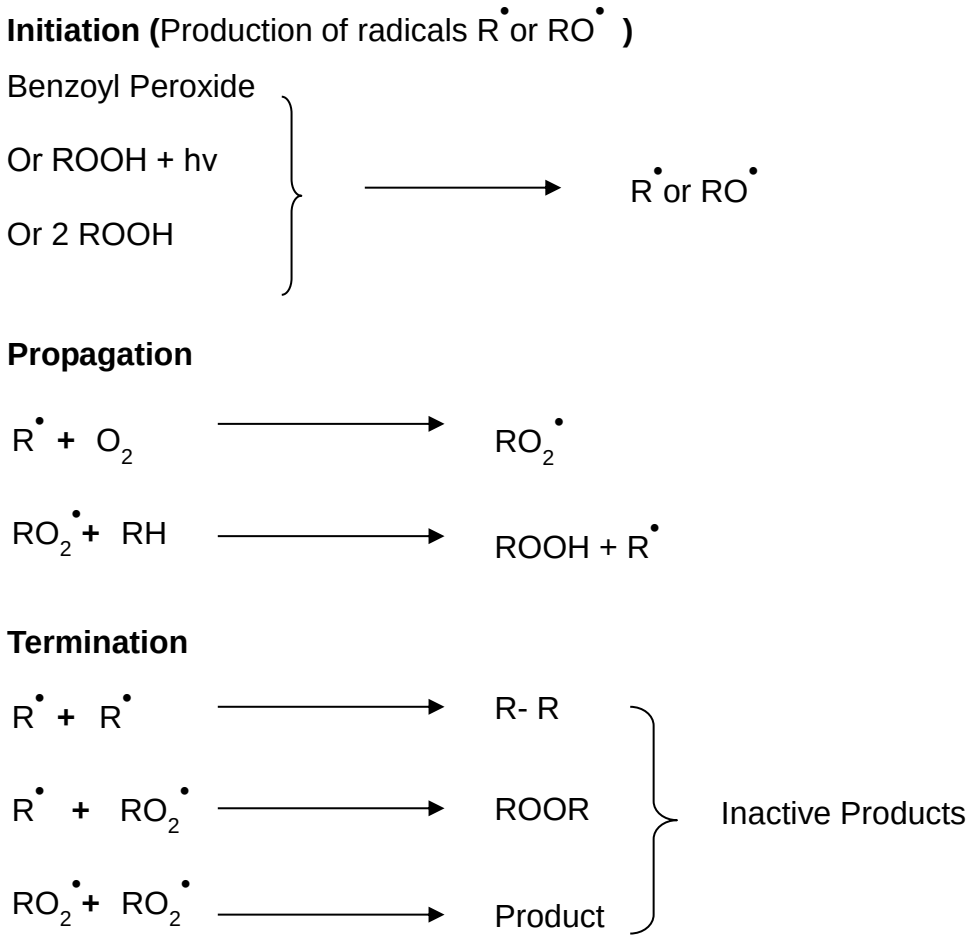


Figure 2.1 The initiation, propagation, and termination processes of the autoxidation chain reaction for lipids was taken from (book, Lundberg. Autoxidation and Antioxidants) [129]

The overall effect of lipid peroxidation results in decreased membrane fluidity, increased membrane leakiness, damaged proteins and inactivation of several receptors, enzymes, and ion channels. Continued lipid peroxidation leads to loss of membrane integrity and rupture of central vascular membranes. In addition, isoprostanes (IPs) are some of the end products of lipid peroxidation and are formed from PUFAs with at least three double

bonds (e.g. linolenic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, etc.). Isoprostanes have direct damaging effect on the cell structures. Researchers have found increased IPs in many human diseases and in animals exposed to a range of toxins.[130]

2.1.5 Oxidative stress, skin-related diseases and antioxidants

Sies (1991) defined oxidative stress as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage".[131] It is also known as oxidative damage that is described as "biomolecular damage when reactive species attack on the constituents of living organisms."[132] Oxidative damage can occur due to oxidative stress, failure to replace the damaged biomolecules, decreased antioxidant levels in the body (e.g. decreased MnSOD due to mutations), lack of dietary antioxidants and other essential dietary constituents such as copper, zinc, iron and magnesium. Children with Kwashiorkor and iron overload suffer from oxidative stress due to low glutathione (GSH) levels and inability to make enough transferrin.[133,134] Exposure to elevated oxygen, ROS- producing toxins (e.g. paraquat, a weed-killer) and over activation of natural systems can also lead to oxidative stress.[135, 136]

The body cells and tissues may adapt themselves by up-regulation or by enhancing the defence system. In this way, the cells can protect themselves from the oxidative damage, completely or to some extent or over protect by making themselves resistant to the insults. These adaptations may involve the available antioxidants, ROS producing systems, protective mechanisms and changes in oxidative damage targets.[137] Moderate stress halts the cells and stops their division to produce new ones. Severe oxidative stress, especially damage to DNA, leads to cell death by apoptosis or necrosis or by both.[138] What is the relationship between free radicals and the skin diseases? How do free radicals affect our skin? In fact, free radicals can come from internal, external and physiological sources and factors, and they can affect our body in many ways. We all know that our skin is constantly and directly subjected to the external environment. In simple words, our body skin is exposed to everything contained in our environment such

as air, solar radiation, different pollutants, mechanical and chemical insults, and many other factors which lead to the generation of free radicals and ROS in our body.[139,140] Hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{-\bullet}$), nitric oxide ($NO^{\bullet}$), thyl ($RS^{\bullet}$) and peroxyl ($RO_2^{\bullet}$) are important free radicals for living organisms, though they damage our body in the long run. Similarly, some other species like peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), and ozone (O_3) also play an important role in the free radical reactions occurring in living organisms. ROS are formed and degraded in all the aerobic organisms and may put the body into oxidative stress when a balance between the formation of ROS and their degradation gets disturbed.

Ionizing radiations, stress, drug abuse, poor nutrition, environmental pollution and substandard life style are the causes of external skin damage. Ultraviolet radiation (UVR) is the most damaging environmental factor that causes 80% UV-induced ROS in the skin and develops oxidative stress when prooxidant-antioxidant imbalance occurs.[140,141,141,142] Molecular response initiates in the skin with the photochemical generation of ROS i.e. superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen.[143] Penetrating the skin, UVR reaches the sub-dermal cells and is absorbed by DNA, forms photoproducts and inactivates DNA functions. The UVR can cause damage by different mechanisms. Usually, there are two ways: direct absorption of the incident light and sensitization mechanisms. Direct absorption leads to excited state of the target molecule and subsequent reactions leding to damage while sensitization mechanisms involve intermediary photosensitizers. Excited photosensitizers produce free radicals by electron transfer and hydrogen abstraction processes or singlet oxygen by energy transfer with O_2.[144] Thus, these chemical reactions and free radicals lead to damage to DNA and generate different products such as strand breaks, sister chromatid exchange, DNA-protein crosslinks, abasic sites, and sugar damage. Genetic mutations and other damages lead to cancer and several skin diseases.

Our skin contains a network of protective antioxidants including enzymatic antioxidants (i.e. glutathione peroxidase, superoxide dismutase, catalase, etc.) and non-enzymatic low-molecular weight antioxidants (i.e. vitamin E, vitamin C, GSH, uric acid, ubiquinol, etc.) .[145] Ascorbate, carotenoids, and sulfhydryls present in the skin also work as potent antioxidants. Glucose, uric acid, ascorbic acid, pyruvate, bilirubin and glutathione present in plasma are water soluble antioxidants while alphatocopherol, ubiquinol-10, *β*-carotene, lycopene, zeaxanthin, lutein and alpha-carotene are known as lipid soluble antioxidants. It has been shown that outer layer of skin (the epidermis) possesses higher concentrations of protective antioxidants than those kept in the inner layer of skin (the dermis). [146] Antioxidants play an important role in keeping the skin healthy with their protective effect against toxic free radicals.[147,148,149]

2.1.8 Aging

Aging is explained as decrements in maximum function and accumulation of genetic mutations.[150] It is a universal, intrinsic and progressive phenomenon. These are over 300 theory about the aging phenomenon, of which many are related to the changes that accumulate with the passage of time[151], however, none of these theories has yet been officially accepted by gerontologists. Among the favoured theories, the free radical theory of aging (FRTA), developed in 1954, has attracted the attentions of researchers in the field and to the interest of mainstream biological research.[152,153] Now it has been developed and turned into the mitochondrial theory of aging.[154]

For the last two or three decades, increasing evidence has convinced many scientists that oxidants play a key role in the phenomenon of aging. Involvement of free radicals in the aging process also reflects their important role in the origin and evolution of life. Actually, aging is a complex mechanism that involves free radical DNA damage, oxidative stress, autoantibodies and role of antioxidants. FRTA hypothesizes a single common process in which ROS are responsible for age-related alterations at cellular and tissue levels. In fact, aging and death of living beings arise from the accumulation of excessive ROS and modified biological molecules in the body.[94, 155]

Herman (1972) revised FRTA after the recognition that the mitochondria are a major source of free radical reactions occurring in the cells. Also, it was concluded that life span depends upon the rate of oxidative damage to mitochondria. As a matter of fact, the mitochondria itself is a major source of free radical generation and its DNA is the key target of oxidative damage. Thus, it can be concluded that imbalance between antioxidants and on-going accumulation of free radicals is responsible for aging. Carmeli *et al* (2002) described the function of oxidants, antioxidants and biomolecules in a golden triangle, all the three factors being at each apex[156]By and large, all the three elements maintain equilibrium; however, continuous and excess generation of ROS may overwhelm and exceed the natural
antioxidant defence leading to oxidative stress and cellular functional impairment.[157]
FRTA implies that interventions aimed at the inhibition of free radical reactions should lead to the reduction in the rate of aging, age-related changes and disease pathogenesis.[158] Additionally, if antioxidant therapy intends to improve oxidative stress, as adopted in Western World, there must be convincing evidence.

2.1.9 Effects of antioxidants on skin disease

The skin is the outermost organ of our body and it is constantly exposed to both endogenous and environmental hazards (e.g. solar radiations, UVR, pollutants, chemical factors, etc.) and so directly or indirectly to free radicals[140-143]. In other words, our skin is always under assault. However, Nature has provided our body with an exceptional and splendid protective system of antioxidants that work to combat with these assaults. Antioxidants play a key role in the prevention of several skin diseases. They protect the cells against oxidative injury and prevent the generation of oxidative products. Topical preventive antioxidant therapy has been recommended to cope with photoaging and UV-induced skin cancer.[159]The enzymes catalysing the reactions to convert collagen to its triple helix structure require vitamin C as a co-factor; thus helping with the development and maintenance of blood vessels, cartilage and scar tissues.[160,161] Humbert *et al* (2003) conducted a study on the effect of a cream containing vitamin C on photoaged skin which came out with positive results.[162] Oral

supplementation with nutrient antioxidants is claimed to improve and prevent several skin disease especially those mediated by UVR. Studies have been conducted on antioxidants such as β-carotene, ascorbic acid and tocopherol in order to prevent sunburn, photodermatoses and photocarcinogenesis, however, clinical data and convincing evidence is yet to be presented.[163] Along with antioxidants like vitamin E, vitamin C and glutathione, selenium and zinc also work as potential antioxidants and protect cell structures from being damaged by ROS.[164] In summary, antioxidants protect our skin from a number of skin diseases (e.g. photosensitivity, malignancy, etc.) and intend to improve the photodamaged skin.

2.1.10 Biological Defence Systems

Our body is provided with several biological defence mechanisms including specific enzymes and essential nutrients e.g. carotenoids, vitamin C, Vitamin E, etc. and non-essential components e.g. plasma proteins, uric acid, etc. Each of which act as antioxidants which combat noxious free radicals and protect our body.[165] Among specific biological defence enzymes, superoxide dismutase, glutathione peroxidase and catalase are of prime importance. These enzymes, in fact, provide first line defence against free radicals in organisms.

As a matter of fact, ROS play a key role in the cell growth, maturation, progression and death. Low concentration of ROS, according to some studies, may be beneficial in cell signalling as well as in bactericidal activity against harmful microorganisms. However, high concentrations of these noxious species lead to aging, failure in endocrine functioning, immunity and many cause diseases like cancer and ischemia.[128,166] Biological defence system therefore promotes the expression and regulation of antioxidant enzymes in order to cope with higher concentrations of free radicals and the resulting damaging stress.

Superoxide dismutase (SOD) is found in all aerobic cells and catalyses the reaction of superoxide ion to oxygen and hydrogen peroxide. These enzymes use metal ions e.g. copper, iron, zinc, manganese, etc. as co-factors; therefore, absence or lack of these metal ions in the body can hamper the function of these enzymes. Catalases are found

in peroxisomes and convert hydrogen peroxide to oxygen and water.[167] Similarly, glutathione peroxidase catalyses the oxidation reaction of GSH with hydrogen peroxide; thus removing hydrogen peroxide from the body.[168]

$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$

The enzyme glutathione peroxidase (GSH) contains selenium at the active site reduces water and hydroperoxides using glutathione as a reducing agent i.e.

$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ or

$ROOH + 2GSH \rightarrow ROH + GSSG + H_20$

Superoxide dismutase (SOD) requires copper as cofactor to catalyse the reaction below i.e.

$Cu^{+2}\text{-SOD} + O_2^{\bullet -} \rightarrow Cu^{+1}\text{-SOD} + O_2$

And then

$Cu^{+1}\text{-SOD} + O_2^{\bullet -} + 2H^+ \rightarrow Cu^{+2}\text{-SOD} + H_2O_2$.

Catalase converts H_2O_2 into H_2O and O_2 by using iron as cofactor.

In the same way, antioxidant vitamins decrease lipid peroxidation after exercise as well as protect our body against several disease conditions.[169] Because of these properties, vitamin supplementations are being recommended now a days.

2.2 Therapeutic use of antioxidants

2.2.1 Definition of Antioxidants:

The National Centre for Biotechnology Information (NCBI) defines antioxidants as “naturally occurring or synthetic substances that inhibit or retard the oxidation of a substance to which it is added; protecting the living tissues from being damaged by harmful effects of oxidation”.[170]Superoxide dismutase, cellular glutathione peroxidase, catalase, vitamins (e.g. vitamin A, C and E) and minerals work as antioxidants in our body. These are natural sentries against free radicals and oxidative stress.

2.2.2 Antioxidant defence mechanisms

Research on antioxidants using animal models has brought to light that superoxide dismutase, cellular glutathione peroxidase and catalase are beneficial to reduce ischemia-reperfusion injury of heart and brain, cold-induced brain oedema, hyperoxia and drug toxicities (e.g. adriamycin, paraquat, etc.).[171] Decreased incidence of these conditions in animal models reflects inhibition or reduction in the activity of ROS.

Antioxidants are classified according to their mode of action i.e. scavenger, preventive and enzymatic antioxidants. Scavenger antioxidants include vitamins such as vitamin A, vitamin C and vitamin E, substances having thiol group, bilirubin and urate. Preventive antioxidants largely include proteins like albumin, transferrin, lactoferrin, haptoglobin and caeruloplasmin. These antioxidants work to sequester transition metal ions in order to prevent Fenton reactions.

$$H_2O_2 + Fe^{2+} \text{----> } Fe^{3+} + OH^{\bullet} + OH^{-}$$

The exact mechanisms of action of antioxidants are yet to be identified; however, many antioxidants may act by more than one mechanism. Carotenoids (e.g. beta-carotene) contain long chains containing double bonds in their structure and thus are capable of attracting and quenching free radical attack. However, clear evidence about the reaction of carotenoids and ROS is still lacking. Vitamin C[172] is characterized as a powerful scavenger of superoxide, singlet oxygen and hydroxyl radicals, and protects against the highly reactive oxidant hypochlorous acid (HOCl).[173] Vitamin C reduces vitamin E radicals formed at membrane surfaces during scavenging of ROS. In this way, vitamin C has the ability to regenerate vitamin E.[204,174] Moreover, in gingival crevicular fluid (GCF), vitamin C prevents activation of neutrophil derived collagenase,[175] and ascorbate deficiency results in gingival bleeding.[176,177] Similarly, vitamin E (α-tocopherol) is found embedded in cell membrane phospholipids and acts as a major scavenger antioxidant by neutralizing the free radicals.[178] Uric acid is a relatively strong scavenger antioxidant especially for water soluble free radicals (e.g. HOCl, $^1O_2$, etc.). Uric acid also has capability to bind with copper and iron ions, and higher

concentrations of uric acid breakdown products have been reported in patients suffering from rheumatoid arthritis.[179]

Glutathione in its reduced form works as an important scavenger antioxidant and reduces pro-inflammatory cytokines by blocking hydrogen peroxide-mediated reactions. In the same manner, via its thiol groups, albumin acts an extracellular antioxidant molecule.[180]

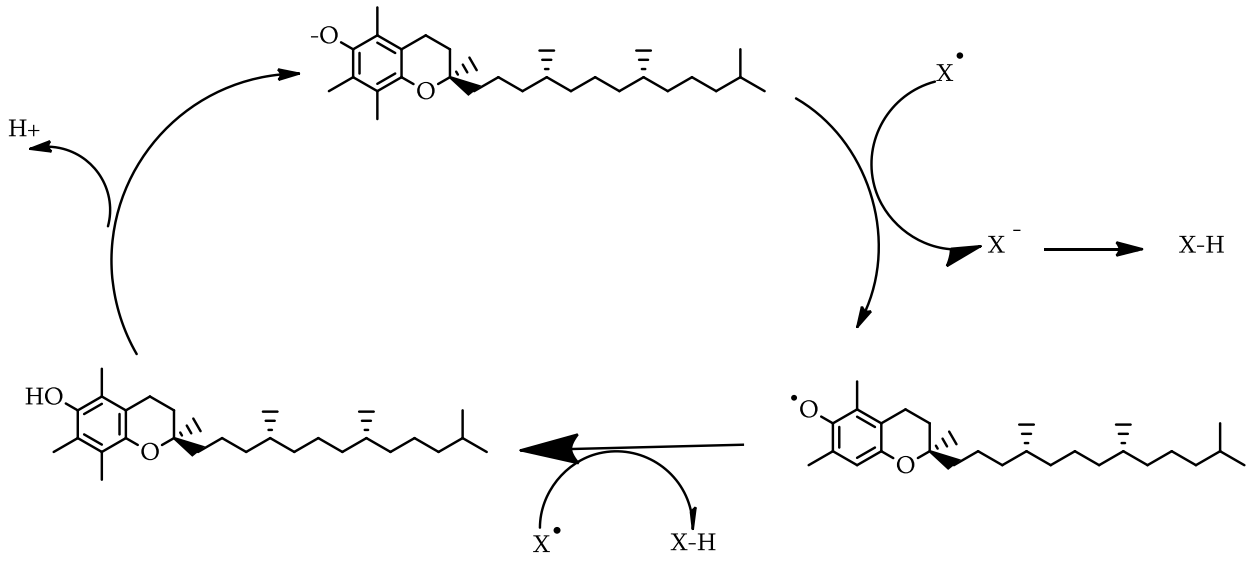
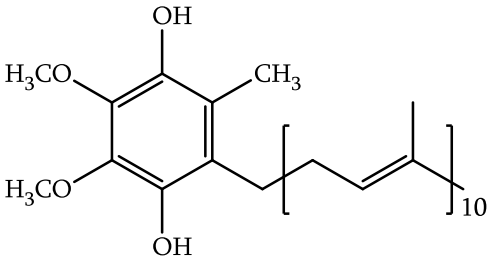
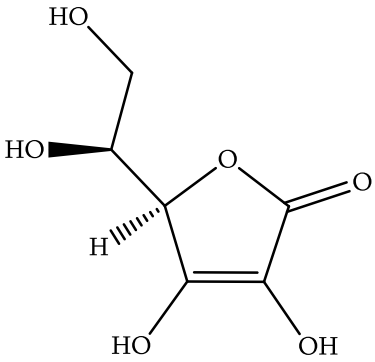
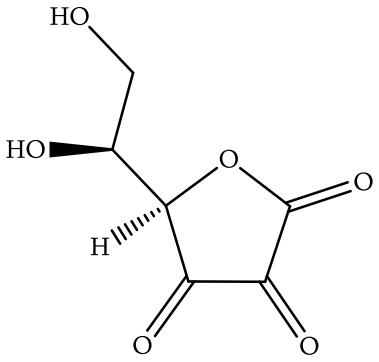


Figure 2.2 Chemical structures of α-tocopherol when react with DPPH• radical, X•=DPPH•



Coenzyme Q_{10}





Ascorbic acid (reducing form of Vitamin C) **Ascorbic acid (oxidized form of Vitamin C)**

Figure 2.3 Example of coenzyme and vitamin that act as antioxidant in the body

While the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) serve as primary line of defense in destroying free radicals .

$$O_2 + e^- \rightarrow \cdot O_2^- \quad [1]$$

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad [2]$$

$$H_2O_2 + \cdot O_2^- \xrightarrow{CAT} OH^- + \cdot OH + O_2 \quad [3]$$

$$H_2O_2 + H_2O_2 \rightarrow 2\,H_2O + O_2 \quad [4]$$

SOD first reduces the superoxide radical (O_2) to form hydrogen peroxide (H_2O_2) and oxygen (O_2)

$$H_2O_2 + 2\ \text{glutathione} \xrightarrow[\text{Glutathion peroxidase}]{\text{Catalase}} \text{oxidized glutathione} + H_2O \qquad [5]$$

Catalase and GPX then work simultaneously with the protein glutathione to reduce hydrogen peroxide and ultimately produce water (H_2O). The oxidized glutathione is then reduced by another oxidant enzyme glutathione reductase. They repair oxidized DNA, degrade oxidized protein, and destroy oxidized lipids, various other enzymes act as a secondary antioxidant defense mechanism to protect us from further damage.[129]

2.2.3 Antioxidant and lifespan

Antioxidants are usually investigated as a promising measure for extending lifespan as they reduce free radicals, which cause oxidative damage to living tissues. Hence, there is much interest in developing antioxidants supplements extracted from plants, though, at present no convincing evidence for the benefits of this has been presented. Additionally, lifespan research is expensive and exhausting to conduct[181]; however, animals of phylum Rotifera are promising models for lifespan studies because of their short lifespan of only two-weeks and typical pattern of aging.[182]

Studies regarding over-expression of antioxidant enzymes e.g. copper zinc superoxide dismutase, catalase, or their combination, etc. performed on model animals reflect no extension in lifespan.[183]However, a study on house flies (*Musca domestica*) by Sohal and co-workers (1986)[184] suggested that longer life expectancy is associated with relatively higher concentrations of antioxidants and lower levels of ROS. Similarly, another study revealed that the species with longer lifespan are less prone to oxidative stress and the species with higher metabolic rate have shorter lifespan.[185] Another study by Andziak and associates (2005)[186] on antioxidants failed to explain disparate longevity between mice and naked mole-rats.

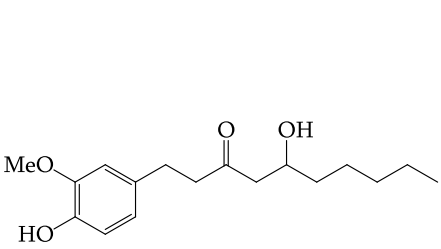
A review on vitamin supplements by BBC News stated “Taking antioxidant supplements neither increased, nor reduced, the risk of early death”.[187] In a nutshell, although antioxidants fight against oxidative stress and protect living tissues from

harmful effects of free radicals, there is no convincing evidence that antioxidants increase lifespan, and it requires an extensive research and a multidisciplinary approach to reach a satisfactory conclusion on this issue.

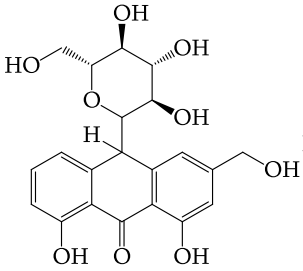
2.2.4 Phenolic compounds as antioxidants

Phenolic compounds are a diverse group of molecules widely distributed in plants as aromatic secondary metabolites. These compounds possess antioxidant properties and are capable of scavenging toxic free radicals that cause oxidative damage to the living cells and tissues. Among phenolic metabolites, flavonoids, anthraquinones, stilbenoids, and their derivatives, extracted from plants, play an importance role in our healthy life as they are thought to prevent cardiovascular diseases and cancer.[188,189,190]Flavonoids are potent antioxidants and chelating agents found in fruits, vegetables, tea, coca and wines. They block lipid peroxidation, chelate redox-active metals and modify processes involving ROS. In vitro studies have shown antioxidant and scavenging characteristics of anthraquinones in hepatocytes.[191]

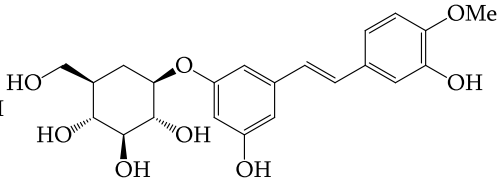
Phenolic compounds are able to donate electrons and make stable phenoxy radicals on interacting with ROS. Using chemical models and some biological models e.g. lipid peroxidation systems, liver microsomes, etc. scavenging effects of phenolic compounds on superoxide, hydroxyl radicals, 2,2-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) and 2,2-diphenyl-1-picrylhydrazyl radicals have been studied.[192,193] Phenolic compounds can adopt different mechanisms in order to exert their antioxidant effects. 6-Gingerol serves as strong scavenger antioxidant in lipid while barbaloin poses many protective actions on proteins. Similarly, rhapontin is thought to be a potent inhibitor of lipid peroxidation. In a combination, these compounds exert synergistic effect and may have prophylactic or therapeutic value against ROS in atherosclerosis.[167]



6-gingerol



barbalion



rhapontin

2.3 Aim of the research project

The overall objective of the present study is to investigate, determine and quantify the phytochemical properties of species of plants have been traditionally used in Oman and much of the Middle East (*Pergularia tomentosa*; family Asclepiadaceae) for their relative total phenol content, total antioxidant and anti-microbial activity. In the first section of this study, discussed in Chapter 3, Omani traditional herb aqueous ethanol extracts were screened for their antioxidant activity and total phenolic content using three methods, DPPH• assays, evaluation of total antioxidant capacity by phosphomolybdenum method and to determine the total phenolic content by the Folin-Ciocalteu colorimetric method. While the next aim, which is presented in Chapter 4, was to investigate the antibacterial and antifungal properties of crude *P.tomentosa* ethanolic extract and some of its isolated fractions against E23, PA14, *S.aureus* and *C.albicans*. Methods are used to assess the antibacterial activity and antifungal properties are disk well diffusion, Broth dilution method (CFU) and Time course of bacterial growth. Interest in selected mint species of and their essential oil content along with the study of the inheritance characteristics of compounds and their interspecific hybrids exhibited by several types of mint species and origins (either from fresh mint plants or stored dried mint) has led to the final objective of this study, which is presented in Chapter 5. A comparison of the effect of hybridization of these plants on the mint oil composition and properties in terms of quality and quantity by using microwave extraction is presented. The resulting compounds were analysed using gas chromatography-mass spectrometry (GS-MS).

CHAPTER 3

Chapter 3 Antioxidant approach to wound healing and the role of *Pergularia tomentosa* and *Ziziphus hajarensis*

3.1 Aims and objectives

The objectives of the current investigation was to separate, characterise and identify the major chemical components of *Pergularia tomentosa.* Plants were extracted by combining traditional and utilised spectroscopic characterisation to identify components. The investigation is also intended to screen plant extracts for their antioxidant activity and total phenolic content by three methods, DPPH• assays, evaluation of total antioxidant capacity by phosphomolybdate method and to determine the total phenolic content of these selected plant extracts by the Folin-Ciocalteu colorimetric method. There is no previous research on phenolic content and associated antioxidant properties of medicinal plants traditionally used in Oman. Therefore, this study attempts to explore the scientific basis of these plants as curatives and compare medicinal usage and chemical activity of these plants with others of Omani origin. Finally, it is also intended to study and improve the analytical methodology used in the isolation and characterisation of traditional herbal medicinal plants of Oman.

The choice of plants investigated in this study was based on two object factors: firstly, in this area of science there is no published study in Sultanate of Oman, secondary, the main aim of this project was to isolate and identify wound-healing chemicals from the following two plants from the region discussed below.

3.1.1 *Pergularia tomentosa*

Pergularia tomentosa belongs to the family Asclepiadaceae and is well known in the Sultanate of Oman as Ghalaka "الغلقه". The plants are small in height (25 cm), usually with many leaves and branches having pale green-white stems that are

mostly ascending. The leaves very short and stiff see Figure 3.1. The flowers of the plant are very small and occur in few axillary umbellate cymes. Milky latex can be isolated from the *P. tomentosa* tree and dried and this preparation has been used for the treatment of skin sores (as well as other skin disorders) and a purgative and expectorant.[194]

Figure 3.1 *Pergularia tomentosa*, taken from (A) arabianwildlife.uaeinteract.com, (B) www.wildflowers.co.il and (C) www.adigicam.com

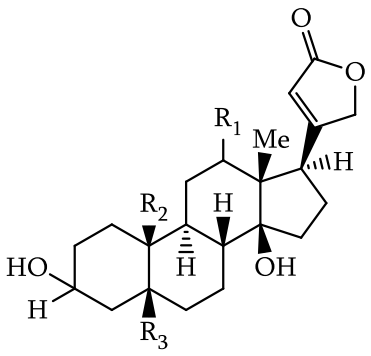
3.1.2 *Ziziphus hajarensis*

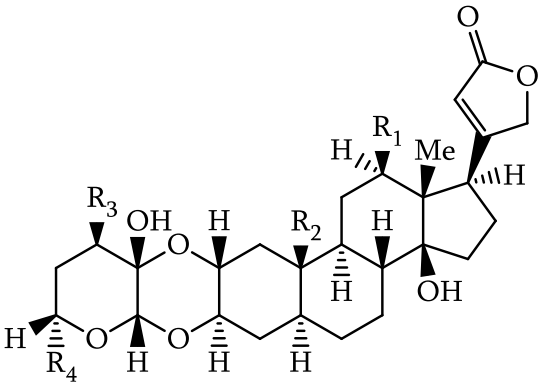
This plant, commonly known as Qusum, belongs to the family Rhamnaceae and is an endemic tree species from the Hajar Mountains in the Sultanate of Oman, The interest in the plant is due in part to the fact that some species of the genus *Ziziphus* have been shown to have an anti-inflammatory action in gum and mouth disease and have been applied on blisters and various surface wounds.

Figure 3.2 *Ziziphus H.*, taken from www.planetefleurs.fr

Very little research has been previously reported on the plants *Pergularia tomentosa* and *Ziziphus hajarensis.* One of the few reports on *P. tomentosa* was published by Hamed and *et al.* [251] The authors investigated these plants for

their potential anti-cancer properties. They dried and powdered the roots of plant samples and mixed then with EtOH-H_2O (4:1) with extensive maceration at room temperature. The alcoholic extracts were combined and the volatiles removed *in vacuo*. The resulting crude extract was partitioned between hexane-H_2O, $CHCl_3$-H_2O, and *n*-BuOH-H_2O (1:1); to obtain hexane, $CHCl_3$ and *n*-BuOH. The *n*-BuOH extract was then purified by alumina chromatography using a $CHCl_3$-MeOH gradient elution. The authors isolated calactin (**5**) and ghalakinoside (**4**) see Figure 3.3 from the fraction eluted with $CHCl_3$-MeOH (8:2). More polar fractions $CHCl_3$-MeOH (7:3) were combined and further purified by HPLC using a reverse phase C-18 column and eluted with CH_3CN (20-25%) to obtain compounds **1**-**3** (see Figure 3.3). The structures of these compounds were elucidated by extensive spectroscopic methods including 1D- and 2D-NMR experiments as well as ESI-MS analysis. This study has shown that the isolated cardenolides caused cell death of Kaposi's sarcoma cells.





NO	R_1	R_2	R_3
1	H	CH_2OH	α-H
2	OH	Me	β-H

NO	R_1	R_2	R_3	R_4
3	H	CHO	α-H, β-OH	Me
4	OH	CH_2OH	α-OH, β-H	CH_2OH
5	H	Me	α-H, β-OH	Me

Figure 3.3 The chemical structure of the cardenolide glycosides isolated from the roots of *Pergularia tomentosa*, (1) 3'-*O*-*β*-D-glucopyranosylcalactin, (2) 12-dehydroxyghalakinoside, (3) 6'-dehydroxyghalakinoside, (4) ghalakinoside and (5) calactin [195]

Secondly, these plants have an ethno-pharmacologically good reputation indicating their traditional utilisation in the treatment of some skin disease, where they have been proved to be efficient in the treatment of various disorders. Furthermore, they have been described as being anti-inflammatory agents. Due to their traditional widespread utilisation and evidence of active components, these plants are also considered to be efficient for the treatment of free radical-related disorders.

3.2 Antioxidant activity and total phenolic constituents from *Pergularia tomentosa*

3.2.1 Preparation of plant material:

Plant material was collected and extracted on two separate occasions in January 2010 and January 2011. The plants were collected from Ibra in the Sultanate of Oman. The Department of Chemistry, Sultan Qaboos University, authenticated the identity of the plants. Air-drying as a form of preservation was carried out for *Pergularia tomentosa* and *Ziziphus H.* (at 50 °C in the dark for two weeks to prevent photo-degradation of bioactives). The dried plant material was then ground down using commercial miller to give 2 kg of both plant stems and leaves was prepared and used.

3.2.2 Methods

3.2.2.1 Extraction and Isolation

Crude plant extracts *(Pergularia tomentosa)*

Dried stem and leaves of plant material (2 kg) were powdered and extracted by standing with different solvents ranging from non-polar to polar. Initially, the plant materials were extracted in 8 litres of petroleum ether for four days and the plant materials were re-extracted twice with 7.5 L ethyl acetate for five days and 6 L ethanol for one week respectively. The supernatant was filtered using 0.22 µm

membrane (MILLIPORE Millex GP, Ireland) before being reduced to dryness in vacuo using a Rotavapor (Buchi Rotavapor R200, Japan) at 67 °C, 100 rpm to afford dark greenish gummy ethanolic residue (30 g). After evaporation of the solvent the solid residues were weighed and stored in sealed vials in a freezer until further investigation for potential antioxidant properties could be carried out. The overall procedure was performed twice. Firstly, it was conducted on January 30, 2010 to produce 28.9 g extract by a Masters student in the Department of Chemistry at Sultan Qaboos University. The second time it was carried out on the January 7, 2011 and produced 30 g extract. The extracts were transferred to the UK for further evaluation.

3.2.3 Separation and Identification of compound from the plants extracts:

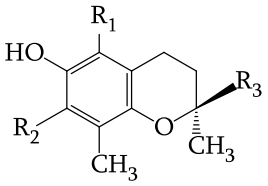
Ethanolic extracts *P. tomentosa* sample were loaded on to normal phase silica columns that were eluted with a range of solvents from non-polar (petroleum), to polar (EtOH), and the separated compounds were analysed and detected by thin layer chromatography (TLC) visualised under UV illumination, then sprayed with a visualizing agent (PMA in EtOH). This technique has the advantage of combining separation of extract components with identification of fractions with bioactivity but it is not a suitable method when there is a synergy between two or more compounds (Hamad *et al*. 2006).[196]

3.2.4 High Performance Liquid Chromatographic analysis (HPLC)

In order to determine the number of constituents present in each fraction, ethanolic semi-purified fractions of extracts were analysed by both normal and reversed phase high performance liquid chromatography (HPLC) in EtOAc: EtOH (1:1). The poor resolution made it impossible to distinguish the separated components and we abandoned this technique as an analytical method for *P. tomentosa*.

The preparative (normal and reversed) phase high performance liquid chromatography of the ethanolic crude extract and fractions from column chromatography (flash columns) was carried out in order to determine constituents both qualitatively and quantitatively. Preparative HPLC of the fraction (collected from flash column) was carried out using an ACE-5-C18 column (250 mm x 7 mm) at room temperature. Using mobile phase ethyl acetate: hexane, (20:80 %, v/v) at a flow rate of 1 ml/min, with the injection volume of 1ml. The UV detector monitored at 280 nm. The sample was prepared in ethyl acetate: hexane, 50:50, v/v.at a concentration of 0.66 mg/ml, and the column eluted isocratically with a run time of 30 minutes. The partially purified compounds were obtained by combining the fractions which exhibit peaks with the same retention time. These were evaporated and the masses of the purified compounds noted.

Three different antioxidant assays were used in this study as they measure different aspects of antioxidant activity. Two *in vitro* assay models were used in this study to estimate the antioxidant power of the *P. tomentosa* extract : a DPPH• assay (Chen, Wang, Rosen and Ho, 1999)[197] and the phosphomolybdenum method (Prieto, Pineda and Aguilar, 1999).[198] The total phenolic in plant extract was estimated according to Folin-Ciocalteu in alkaline medium, using α-tocopherol as a standard. The components were further screened for antimicrobial and antifungal activities using Agar diffusion assays and time courses of growth by using Varioskan spectrophotometer.

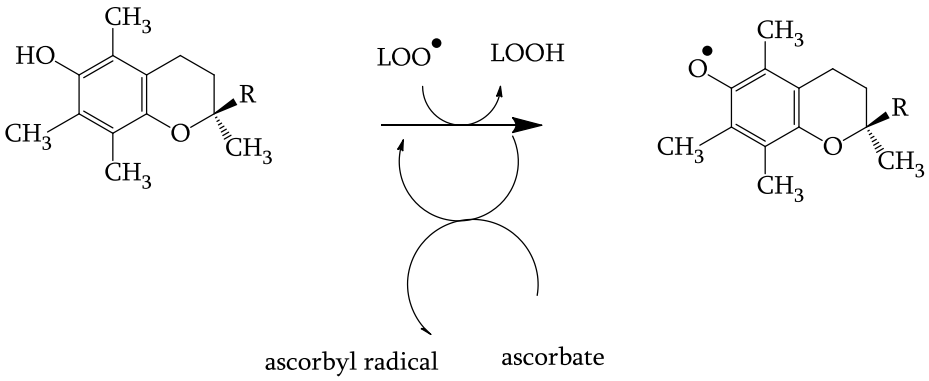


Vitamin E (Tocopherol)

(A)

	R_1	R_2	R_3
α –Tocopherol	CH_3	CH_3	$C_{16}H_{33}$
β –Tocopherol	CH_3	H	$C_{16}H_{33}$
γ –Tocopherol	H	CH_3	$C_{16}H_{33}$
δ –Tocopherol	H	H	$C_{16}H_{33}$

(B)



(C)

Figure 3.4 a) showing the vitamin E molecule b) R group c) mechanism

Figure 3.4 show antioxidant activities and recycling of vitamin E. LOO• is a lipid peroxyl radical. α-tocopherol is oxidized to a tocopheroxyl radical in reaction with a free radical and then may be regenerated and radical recombine with other radicals to produce inactive products.

3.2.5 DPPH• scavenging activity

DPPH• Figure 3.5 is a stable free radical capable of abstracting a hydrogen atom from suitable chemical compounds. The ability of antioxidants to scavenge this DPPH• radicals is a convenient assay technique.

DPPH•, 517nm (purple in colour)

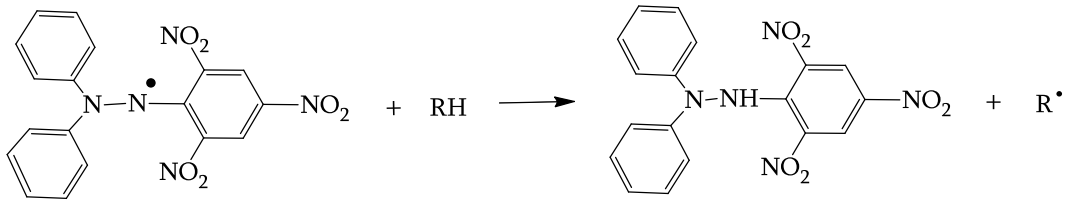


Figure 3.5 Scheme for scavenging the DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical by anantioxidant, RH: antioxidant.

To evaluate antioxidant activity an ethanolic solution of a known concentration of DPPH• /EtOH (a deep violet colour, with a maximum absorbance at 517 nm) to which test compounds (TC-H) are added. A positive test is demonstrated by reduction of the DPPH• radical to the yellow-coloured diphenylpicrylhydrazine resulting in a decrease in absorbance. De-colourisation is stoichiometric with respect to the number of electrons taken up (see equation below).

(DPPH)•+ TC-H → DPPH-H + TC• (Blois, 1958)[199]

The antioxidant compounds that can scavenge the DPPH• radical are expected to supress lipid peroxidation.

3.3 DPPH• scavenging activity

The methodology was adapted from the method of Chen *et al* (1999). The stable free radical DPPH• was dissolved in ethanol to give a 0.06 mg/ml solution and the test solution was prepared by diluting the stock solution (crude extract or

isolated pure compounds, 3.1 mg) with ethanol to the desired concentration. Using the procedure described by Chen *et a.l* (1999),[249] a portion of 2 ml of 100 µM DPPH• solutions in ethanol was mixed with either 2 ml of 100 µg/ml of plant extract, or 2 ml of 100 µg/ml α-tocopherol (as standard) or 2 ml of ethanol (as control). The reaction mixtures were allowed to incubate in the dark at room temperature for 15 min. The absorbance was recorded at 517 nm against the blank (ethanol). The assay was carried out in triplicate.

Calculation:

To calculate the DPPH• "Free Radical Scavenging Activity" (FRSA), the absorbance of the control (2 ml of DPPH• solution in ethanol was mixed with 2 ml of ethanol and the absorbance of the solution was recorded after 15 min) was subtracted from the absorbance of the reaction mixture. The resultant figure was then divided by the absorbance of the control and expressed as the percentage decrease in the absorbance of the reaction mixture compared with that of the standard used to calculate the antioxidant activity, as percentage inhibition (%IP),

Percentage inhibition (%IP) =[(At =0 – At =15)] /(At =0) x100

Where At=15: absorbance of the test sample after 15 min; At=0: absorbance of the control after 15 min. The antioxidant activity of each test solution was also as an IC50 value (concentration in mg/ml or µM/ml required to reduce the DPPH• radical by 50%), which was determined from the log dose-inhibition curve. By experimentation it was shown that this method, widely used in the literature, shows non-linearity with respect to the amount of the crude plant/tincture extracts. The fit to a logarithmic curve was superior to that produced by the linear graph. All results were based on how much each test solution reduced the DPPH• 15 min after adding the test solution and after overnight. The assay was carried out in triplicate to ensure reliability and reproducibility of results. The reaction below illustrated the primary reaction, Z representing the DPPH• and AH for donor molecule.

Z•+AH → ZH + A•

This presumably occurs via two steps (i) electron transfer (redox step), (ii) protonation.

3.4 Determination of total phenolics

3.4.1 Material

Folin-Ciocalteu reagent, α-tocopherol, sodium bicarbonate, sodium phosphate and ammonium molybdate were purchased from Sigma-Aldrich. Analytical grade solvents, was obtained from Fisher Chemical Company.

3.4.2 Determination of Total Phenolics

The methodology was adapted from the method of Singleton and Rossi (1965) [252] the total phenolic content was expressed as α-tocopherol equivalents in mg/g of ethanol plant extract. Briefly 400 µl of plant extract were taken in test tubes, 1.0 ml of Folin-Ciocalteu reagent (diluted 10 folds with distilled water) and 0.8 ml sodium bicarbonate to solution (7.5% w/v) were added and mixed, respectively. The reaction mixture was allowed to incubate in the dark at room temperature for 30 min and 120 min. The absorbance was recorded at 765 nm against the blank that contained 400 µl of ethanol in place of sample (plant extract) and 400 µl of α-tocopherol as standard. The assay was carried out in triplicate. The total phenolic content was expressed as α-tocopherol equivalents (mg of α-tocopherol /g of ethanol extract).

Calculation**:**

Total phenolics as α-tocopherol equivalents (mg/g of ethanol extract) =

[(A spl / Astd)] x (Conc std /Conc spl)] x 100

Where Aspl = absorbance of the test mixture after 15 min or 120 min A std = absorbance of the standard α-tocopherol sample after 15 min or 120 min Conc std = concentration of α-tocopherol (as standard) = 0.05mg/ml; Conc spl = concentration of test mixture = 0.05 mg/ml. The assay was carried out in triplicate.

3.4.3 Evaluation of total antioxidant capacity

Total antioxidant capacity of the plant extracts was estimated by the phosphomolybdate method, based on the procedure of Prieto *et al*. (1999),[250] using α-tocopherol as a standard. The reagent solution contains 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The absorbance was recorded at 695 nm against the blank that contained a mixture of ethanol and the reagent solution. The assay was carried out in triplicate. The antioxidant activity was expressed as α-tocopherol equivalents in mg/g of ethanol plant extract. The assay was carried out in triplicate.

3.5 Results and discussion

In this study we aimed to purify and identify the active components in two edible plants in used in Oman for their antioxidant properties and antimicrobial activities. The common use of these medicinal herbs was found to be in skin disorders and to aid dermal wound healing. Due to this combination of antioxidant properties with antimicrobial activity this enhances the importance of those remedies for the management many diseases such as cancer and AIDS. As described above, among the easier, rapid and sensitive techniques to survey antioxidant activity of a specific compound or plant extracts are the DPPH• scavenging assay, phosphomolybdate method and Folin-Giocalteu procedure Table 3.1 and Figure 3.6.

3.5.1 Examining the crude EtOH extraction with different solvent

Anti-oxidant activities and total phenolics of plant extracts

Plant Name	Extract with	DPPH assay *(a)	Total phenolics as α-tocopherol equivalents(b)	Phosphomolybdate assay as α-tocopherol equivalents(c)
*P. tomentosa**	Petroleum Ether	0.4%	-	-
*P. tomentosa**	EtOAc	0.8%	-	-
P. tomentosa	Petroleum Ether	2.7%	-	-
P. tomentosa	EtOAc	6.0%	-	-
P. tomentosa	EtOH	47.4%	143.2%	248.8%
Ziziphus H.	Petroleum Ether	24.0%	-	-
Ziziphus H.	EtOH	75.2%	140.7%	232.7%

Table 3.1 Antioxidant activities and total phenolic contents of plant extracts (a) tested at 50 µg/ml concentration (b) tested at 10 µg/ml conc. And results presented as α-tocopherol equivalents (mg/g), (c) tested at 18 µg/ml conc. and results presented as α-tocopherol equivalents (mg/g), A.*_P. tomentosa_ (sample sent by courier), B. _P. tomentosa_ (sample brought by the author).

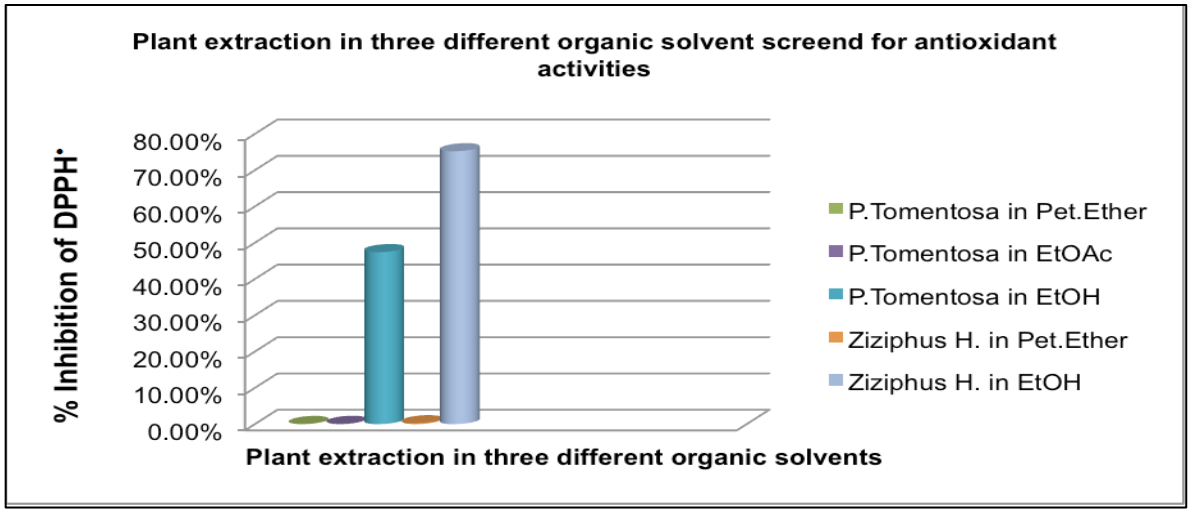


Figure 3.6 Antioxidant activity (% Inhibition of DPPH•) of plant extraction obtained with different solvents. The aqueous EtOH extracts showed better antioxidant activities.

The antioxidant activity of crude extracts of (*P. tomentosa* and *Z. hajarensis***)** was determined using the 2,2-diphenyl-1-picrylhdrazyl (DPPH•) radical scavenging method, as described by Chen *et al*. (1999).[249] The ethanol extracts for both plants showed very high antioxidant activity towards the DPPH• referred to [Table 3.1 and Figure 3.6] with %IP (percentage inhibition) values > 47% and 75% for *Pergularia tomentosa* and *Z. hajarensis* respectively at an effective test concentration of 50 µg/ml for both plants the activity was predominately found in the ethanolic extract. The phosphomolybdate method usually detects antioxidants such as α-tocopherol, ascorbic acid, some phenolics and cartenoids (Prieto *et.al.* 1999).[250] Since, the ability to donate hydrogen and electrons is known to be higher in such compounds as ascorbic acid and α-tocopherol they are easily detected by these two methods in general. Ethanolic extracts in *P. tomentosa* and *Z. hajarensis* have also been shown to have very strong antioxidant in the phosphomolybdate assay (see Table 3.1 and Figure 3.6). The ethanolic extract (only these extracts were tested based on the DPPH• assay results) of both above plants showed high levels of the total phenolics as α-tocopherol equivalents (mg/g). Further analysis of the ethanolic extract of *P. tomentosa* and *Z. hajarensis*. using different concentrations of the plant extracts showed that this inhibition of DPPH• was concentration dependent and so related to the compounds present in the extract. (See Figure 3.7)**.**

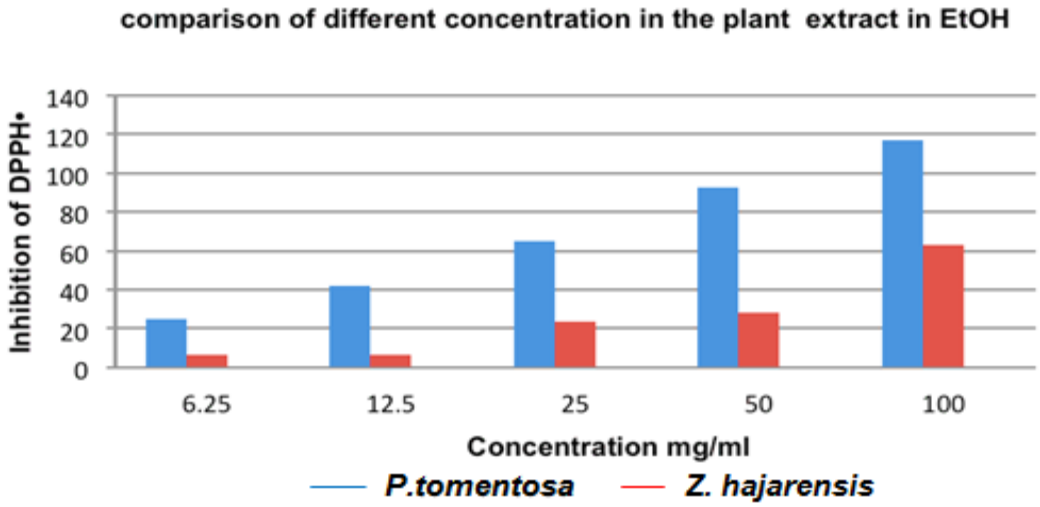


Figure 3.7 The bar chart represents changes in the antioxidant activity the % IP (percentage inhibition) of DPPH• by different concentrations of the plant extract.

Interestingly, when examine the effect of time on the DPPH• assays with the in the ethanolic extract of *P. tomentosa* and *Z. hajarensis*. We observed that the highest levels of antioxidant activity were seen after allowing the test mixture to stand 24 hrs. In addition the % IP (percentage inhibition) of DPPH• and total antioxidant capacity by the phosphomolybdate assay slightly increased when the test was left without sample withdrawal for 24 hrs. Figure 3.8 and Table 3.2.

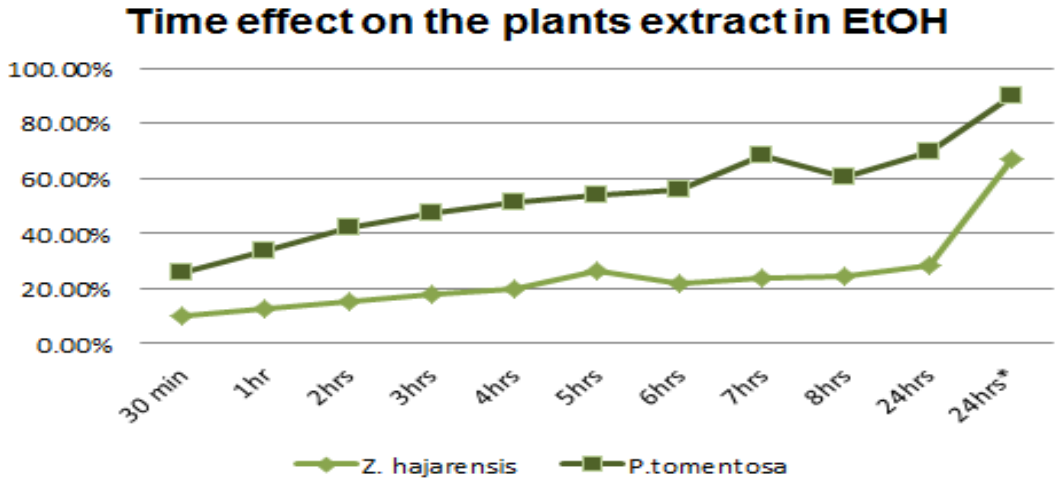


Figure 3.8 Antioxidant activity (% Inhibition of DPPH•) of *P. tomentosa and Z. hajarensis* 24 hrs after treatment. 24 hrs*=tested after 24 hrs without sample withdrawal

Table 3.2 Total antioxidant capacity by the phosphomolybdate assay of the two plants extracts from *P. tomentosa* and *Z. hajarensis* after 24 h treatment.

Phosphomolybdenum assay	*P.tomentosa*	*Z. hajarensis*
30 min	172.2	116.0
1 h	277.6	202.7
2 h	236.9	189.2
3 h	205.3	166.0
4 h	152.8	141.3
5 h	165.5	131.4
6 h	181.3	130.8
7 h	156.6	124.4
8 h	151.0	113.7
24 h	129.1	115.0
24 h, without sample withdrawal	189.8	127.5

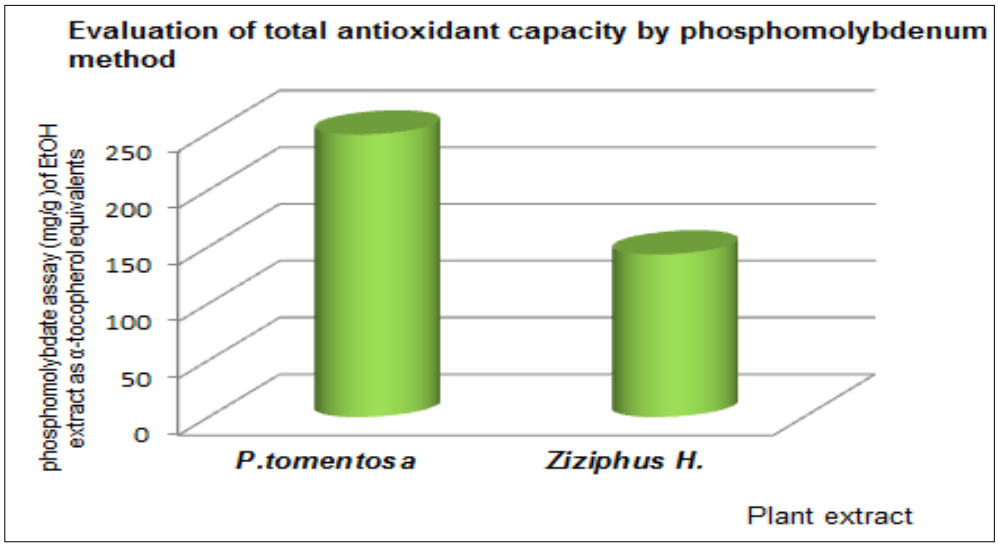


Figure 3.9 Total antioxidant capacity by phosphomolybdate assay as α-tocopherol equivalents (mg/g of EtOH extract) of selected plant extract in EtOH. *P. tomentosa* contains higher antioxidant activities in the phosphomolybdate assay than *Z. hajarensis*

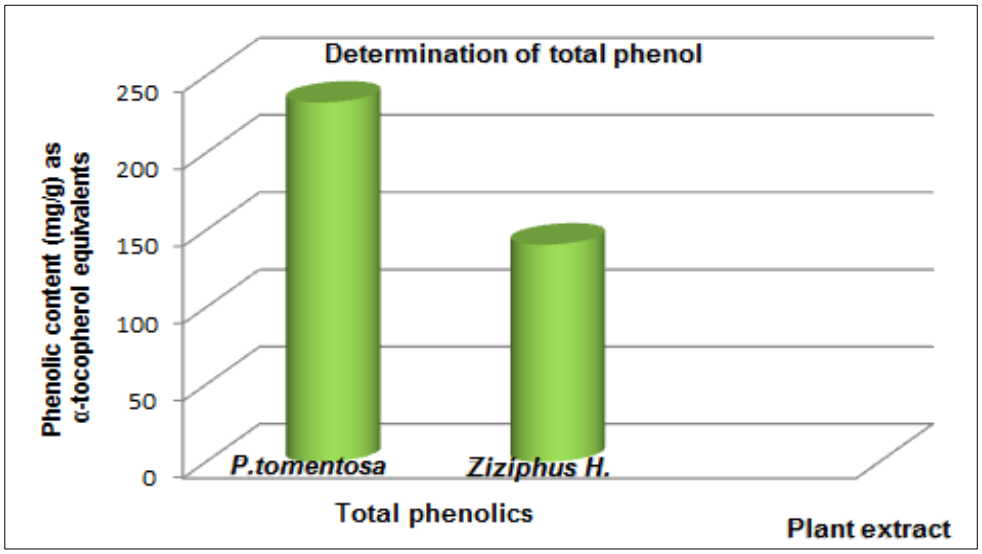


Figure 3.10 Phenolic contents mg/g of EtOH extract (as α-tocopherol equivalents) of selected plant extract in EtOH. *P.tomentosa* contains higher amounts of total phenolics than Ziziphus H.

Figure 3.9 and 3.10 show that *P. tomentosa* has higher phenol content than *Ziziphus H.* which encouraged us to select the *P. tomentosa* species for further investigation for the antioxidants activities and different microbial tests. In general all three antioxidant tests (DPPH• assay, phosphomolybdate assay methods and total phenolic in the Folin-Ciocalteu assay) not linearity related results it chemical structure depend of different phenolic in the antioxidant molecular (Statue-Gracia *et al.,* 1997),[200] thus further study should be done to clarify the situation and it clearly indicates the importance of using various radical assays and oxidation systems to determine the antioxidant activities and phenolics content at the same time as evaluating the anti-oxidant potential of plant extracts. Most isolated plant extracts with potent DPPH• scavenging contain compounds related to flavonoid and polyphenols.[201] Ascorbic acid, some phenolics, α-tocopherol and carotenoids are`detected by phosphomolybdate assay according to (Prieto *et al.,*1999).[198]While Folin-Ciocalteu assay depend on the chemical structure of the antioxidant gives different responses to different phenolic compounds (Statue-Gracia *et al*., 1997).[200]

3.5.2 Attempted isolation of *P.tomentosa* ethanolic extract by HPLC

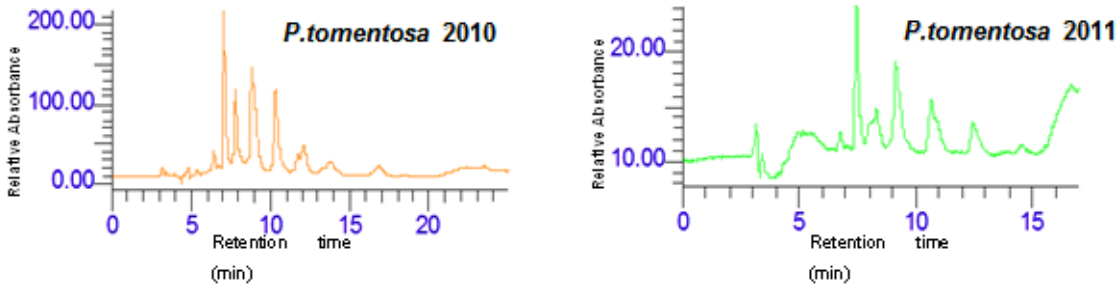


Figure 3.11 HPLC analysis of the crude *P. tomentosa* to attempt separates some of compounds

An initial attempt was made to separate the constituents of the ethanolic extract from *P. tomentosa* by HPLC Figure 3.11. Unfortunately the poor resolution made it impossible to distinguish the separated components and so this technique was abandoned as an isolation method for *P. tomentosa*.

3.6 Isolation, Identification and structural elucidation of constituents of *Pergularia tomentosa* Family *Asclepidaceae*

3.6.1 Separation by Column Chromatography

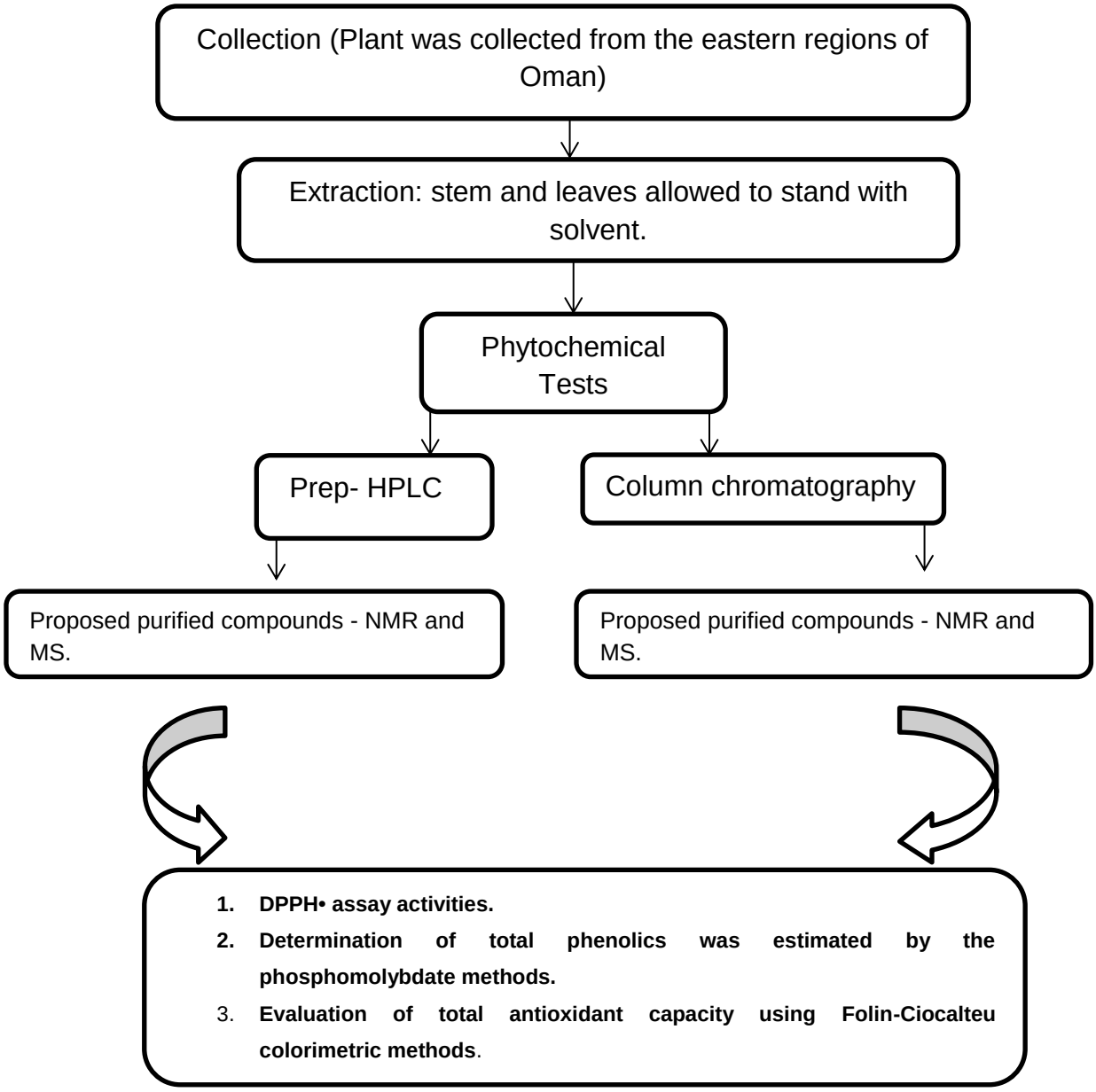


Figure 3.12 Flow Chart of Separation techniques and Test Used in Chronological Order for the ethanolic steam and leaf extract of *P. tomentosa*

We initially sought to separate the crude ethanolic extract using normal phase

column chromatography. The first column was developed under gentle pressure with appropriate solvent mixtures, Initial purification was carried using 5.8 g of crude extract and using silica chromatography (approximately 30 cm column width and 46 cm length) eluting with a gradient partitioned between [{(100%) Petrol}, {(1:1) Petrol:EtOAc}; {(100%) EtOAc}; {(8:2) EtOAc:EtOH};{(1:1) EtOAc:EtOH}; to end with (100%) EtOH}, to afford 5.3 g of various fractions; 91% yield]. After each fraction was collected, an appropriate solvent was added to the top of the column without pressure until the surface was well covered. The pressure was gently reapplied. The fractions were collected and evaporated to remove all traces of solvent. Thin layer chromatography (TLC) using phosphomolybdic acid reagent for detection was performed to ascertain the number of compounds present in each fraction. The result of TLC analysis showed that the column chromatography had only been partially successful in separating the various components in the mixture with considerable overlap between all the fractions. The details of fractions obtained from the column chromatography are given in the Table 3.3

Table 3.3 illustrates the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of spots	R_f value MeOH: Diethyl ether: Pet. (1:1:8)	% Mass (% of 5.8g crude extract)	Description
Fraction 1	Petrol (100%)	5	0.83,0.77,0.69,0.20,0.18	1.1%	Light yellow solid product
Fraction 2	Petrol:EtOAc (1:1)	6	0.83,0.76,0.66,0.34,0.19,0.10	41.6%	Light yellow gummy product
Fraction 3	EtOAc (100%)	4	0.83,0.77,0.58,0.33	3.4%	Light green gummy product
Fraction 4	EtOAc:EtOH (8:2)	3	0.83,0.33,0.28	3.5%	Dark green oily product
Fraction 5	EtOAc:EtOH (1:1)	3	0.83,0.28,0.19	18.4%	Light green oily product
Fraction 6	EtOH (100%)	3	0.83,0.33,0.28	22.8%	Yellow oily product

Table 3.4 Total phenolic content and DPPH• radical-scavenging activity of isolated fractions of *P. tomentosa*

Plant Preparation	Solvent system	DPPH• assay (%IP)a	Total phenolics as α-tocopherol equivalents (mg/g of EtOH extract)b	
		15min	30min	120min
α- tocopherol		96.7%	Abs=0.109	Abs=0.109
Fraction 1	Petrol (100%)	38.4%	6.2%	29.17%
Fraction 2	Petrol:EtOAc	24.4%	14.7%	55.91%
Fraction 3	EtOAc (100%)	41.8%	28.1%	47.8%
Fraction 4	EtOAc:EtOH (8:2)	43.0%	20.8%	46.3%
Fraction 5	EtOAc:EtOH (1:1)	47.5%	22.1%	39.1%
Fraction 6	EtOH (100%)	43.9%	3.0%	29.1%
crude EtOH extract		47.4%	82.4%	143.2%

Table 3.4 Antioxidant activities and total phenolic contents of plant extracts (a) tested at 50 µg/ml concentration (b) tested at 10 µg/ml conc. And results presented as α-tocopherol equivalents (mg/g). Maximum reproducibility of DPPH• ±0.01 and for total phenolics ±0.01

The various combined fractions were still found to be mixtures with various impurities by NMR spectroscopy. The results of DPPH• reduction as antioxidant activities and total phenolics by Folin-Ciocalteu colourimetric assay shown in Table 3.4 from the six different fractions, (from petrol. to EtOH) are summarized in Table 3.3. As can be seen the % yield for fraction No. 2 (Petrol:EtOAc 1:1) was the highest at 41.6% followed by fraction 6 (EtOH 100%) 22.8% and fraction 5 (EtOAc:EtOH 1:1) 18.4% compared with fractions 1, 3, and 4 which showed less than 4% of the total eluted percentage. Results of antioxidant and total phenolics of six fraction considered as the group with a moderate capacity of scavenging activity against the DPPH• free radical in a range between (47.5% - 24.4%) and total phenolics after 2 hours in a range (55.9% - 29.1%) using α- tocopherol as the standard. Generally, this examination of the antioxidant and total phenolics value of the above extracts showed that they exhibited moderately effective compared with standard α- tocopherol Table 3.4.There was no appreciable concentration of either activity of either activity into any of the fractions collected.

3.7 Effective of environmental and varieties on the *P.tomentosa*

Some environmental factors that have been reported [202] may affect the phytochemical content of plant materials such as climate alteration, type of soil, harvest time and handling process. Though, not much information is however of these factors with particular report for *P. tomentosa.* In this study, *P. tomentosa* analysed from two batches that were collected from two different times (Jan 2010 and Jan 2011) and between extracts filtered with Millipore and non-filtered samples were examined. Both batches were investigated and compared for their DPPH• inhibition activity and total phenolics content (Table 3.5).

Table 3.5 Total phenolic content, and DPPH• radical-scavenging activity of isolated fractions of *P. tomentosa*

Plant Preparation	DPPH• assay (%IP)a		Phosphomolybdate assay as α-tocopherol equivalents (mg/g of EtOH extract)b		Total phenolics as α-tocopherol equivalents (mg/g of EtOH extract)c	
	15min	ON	30min	120min	30min	120min
α- tocopherol	91.8	91.8				
Plant extract 2011*	33.8	43.8	22.1	16.6	49.9	179.7
Plant extract 2011	29.3	39.3	27.6	27.3	28.4	176.6
Plant extract 2010 *	29.3	40.5	30.4	22.5	74.3	188.9
Plant extract 2010	10.6	22.7	30.9	27.3	41.8	134.3

Table 3.5 Antioxidant activities and total phenolic contents of plant extracts (a) tested at 50 µg/ml concentration (b) tested at 10 µg/ml conc and results presented as α-tocopherol equivalents (mg/g), (c) tested at 18 µg/ml conc. and results presented as α-tocopherol equivalents (mg/g), maximum reproducibility of DPPH• ±0.01 and for total phenolics ±0.01 * indicate filtered sample.

The level of total phenolics in crude ethanolic extract was measured with three assay methods (i) DPPH• assay, (ii) determination of total phenolics was estimated by the phosphomolybdate method and (iii) evaluation of total antioxidant capacity

using the Folin-Ciocalteu colorimetric method. There was some variability between the same *P. tomentosa* ethanolic extract harvested at different times (2010 & 2011) and between extracts filtered with Millipore and non-filtered samples as shown in above Table 3.5. The results shown in the table display some minor variations between the different samples and testing conditions. Thus the total phenolics test and DPPH• assays give higher values when tested after 120 min and ON (overnight) respectively then when tested after 30 min and 15 min. These results match those run previously (section 3.5 Table 3.1). There is some increase in activity seen with the filtered samples that is expected as it will remove insoluble material. In general there were no significant differences between the antioxidant activates and the level of total phenolics either in term of the harvested time or in purification methods prior to assay. This validates the initial result we had seen with the original samples.

3.8 Separation by Column Chromatography

Pergularia tomentosa species harvested on 2011 was extracted initially as per section 3.2.2.1 to afford a dark greenish gummy residue 30 g. 1.1 g from the residue was purified as described in section 3.6.1, using silica chromatography (approximately 7cm column width and 30 cm length) to afford 0.9 g; 81% yield. The details of fractions obtained from the column chromatography are given in the Table 3.6.

Table 3.6 illustration of the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of spots	R_f Value MeOH: Diethyl ether: Pet. (1:1:8)	% Mass (% of 1.1g crude extract)	Description
Fraction 1	Petrol (100%)	5	0.83, 0.76, 0.69, 0.20, 0.19	1.3%	White ppt
Fraction 2	Petrol:EtOAc (1:1)	6	0.83, 0.76, 0.67, 0.36, 0.20,0.12	1.6%	Dark green gumy product
Fraction 3	EtOAc (100%)	4	0.83,0.76,0.58,0.34	21.7%	Dark green gumy product
Fraction 4	EtOAc:EtOH (8:2)	3	0.83, 0.34, 0.29	8.3%	Dark green gumy product
Fraction 5	EtOAc:EtOH (1:1)	3	0.83, 0.29, 0.20	49.3%	Dark green solid product

The Table 3.6 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, Rf values and the colour and form of the products

Table 3.6 represents the various fractions collected from silica chromatography of the *P. tomentosa* extract. The EtOH:EtOAc 1:1 eluent gave the maximum yield of compounds (fraction 5, 49.3%) compared to fractions 3 and 4 collected from moderately polar solvents EtOAc 100% and EtOAc:EtOH 8:2 (21.7% and 8.3% respectively). It was demonstrated solvent extraction could be used to separate the organic extract of *P. tomentosa* in to three major fractions and two minor ones (yield less than 2%), using solvents of different polarity an initial fractionation was reached. In comparison with the original column (Table 3.3) the slightly different distribution of material is probably due to more careful chromatography conditions in this second separation.

3.9 Determination of total phenolics

The antioxidant activity of the plant extract was estimated using a slight modification to reach the maximum scavenging activity values as antioxidant capacity of the collected fractions Table 3.7.

Table 3.7 demonstrated the antioxidant activity in separated fractions

DPPH• assay (%IP)			
	15 min	**60 min**	**O/N**
Control at zero 0.460	**(0.526)**		**(0.579)**
α- tocopherol	**84.2%**	**88.4%**	**89 %**
Fraction 1	**0.00**	**0.00**	**0.00**
Fraction 2	**0.00**	**0.00**	**0.00**
Fraction 3	**8.9%**	**15.3%**	**19.4%**
Fraction 4	**6.6%**	**7.4%**	**10.9%**
Fraction 5	**21.9%**	**27.3%**	**44.2%**

Antioxidant activities of isolated fractions, tested at 50 µg/ml concentration maximum reproducibility of DPPH• ±0.01.

All five fractions were found to be mixtures with various impurities when analysed by NMR spectroscopy. The results of DPPH• reduction to indicate antioxidant

activities are shown above in Table 3.7. Some of these isolated fractions showed no inhibitory activity such as fraction 1 and 2 (less polar compounds). The results of fraction 5 show higher capacity of scavenging activity against DPPH• free radical and the colour of the solution mixture gradually changed from light purple (21.9%), after 15min to yellow (44.2%) when left overnight. Fraction 3 and fraction 4 exhibited low DPPH• values. In conclusion, the isolated fractions (1 and 2) from the ethanolic extract harvested in 2010 show high inhibitory activity in similar assay compared to fraction 1 and 2 harvested in 2011. This may be due to the slight differences in fractions e.g. In the original column fraction 2 contained now in fraction 3 better separation. While the antioxidant activity of remaining isolated fractions harvested in 2010 or 2011 were almost have similar DPPH• inhibition activities.

3.10 HPLC normal phase

The above results demonstrate very elegantly that the fraction 3 and fraction 5 show most of the antioxidant activity in accordance with the amount of phenolics present. Accordingly further purification was attempted by normal phase preparative scale HPLC utilizing EtOAc (30%) and hexane (70%), flow rate 10 ml/min, λ 240 nm and 280 nm, concentration 1 mg/ml for fraction 3 and fraction 5. HPLC unfortunately showed very poor separation and was an unsuccessful technique for both fractions. As mentioned earlier due to the difficulty of separating multi-component mixtures founds in some plant materials, no further analysis was carried out in this study using this procedure.

3.11 Schematic representation of *Pergularia tomentosa* extracts approach for isolation of plant constituents (2010)

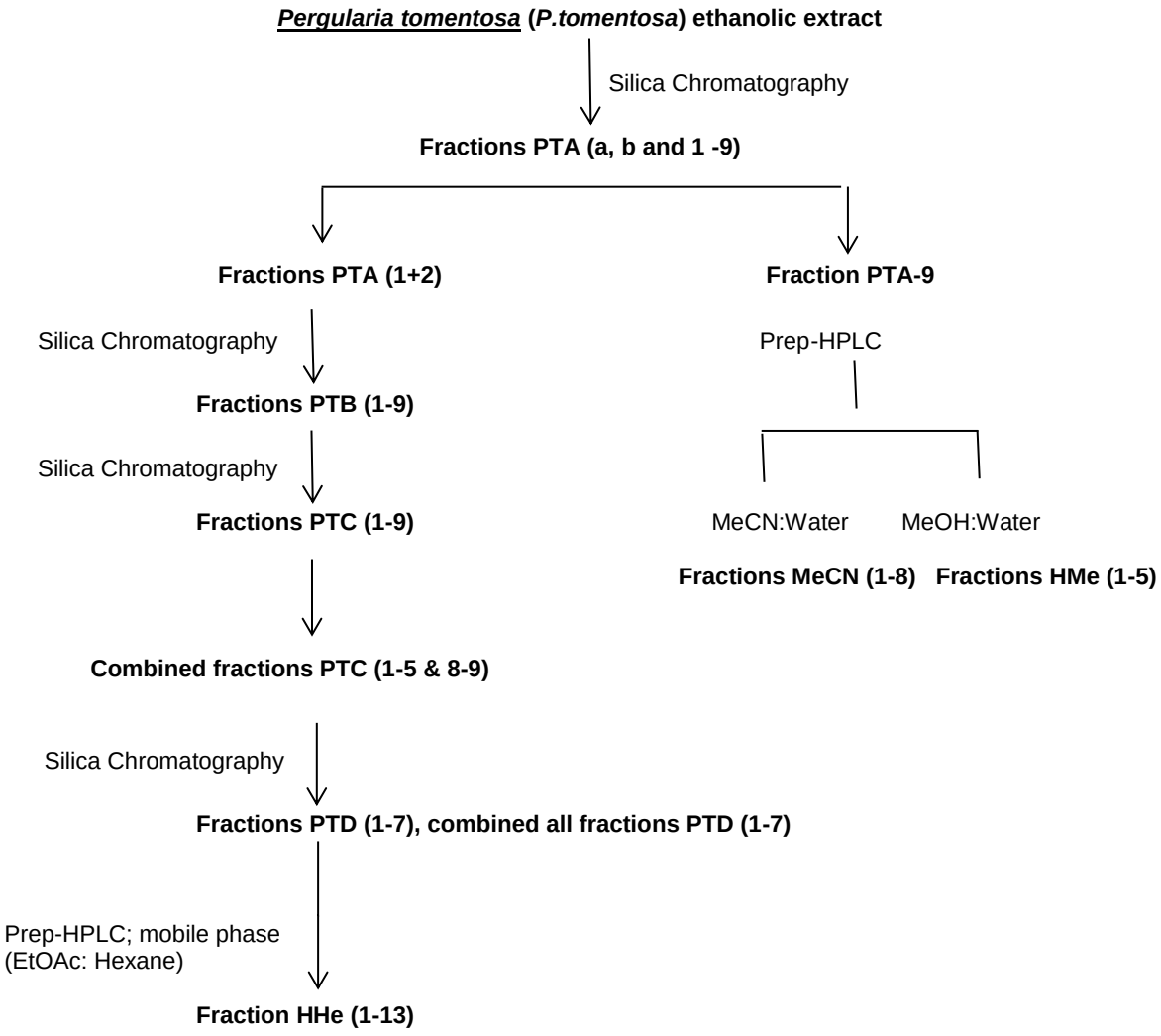


Figure 3.13 Flow Chart-Analytical methodology used for isolation and identification of active constituents of *Pergularia tomentosa* ethanolic extracts

3.12 Separation by Column Chromatography

Pergularia tomentosa species harvested in 2010 was extracted initially as per section 3.2.2.1 to afford a dark greenish gummy residue 28 g. A small sample 2.1 g from the residue was purified as described in section 3.6.1, using silica chromatography (approximately 10.5 cm column width and 31 cm length) to afford 1.8 g of material (over 11 fractions; PTA a-b,1-9); 85.7% yield. The details of fractions obtained from the column chromatography are given in the Table 3.8.

Table 3.8 illustration of the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of Spots	R_f MeOH: Diethyl ether: Pet. (1:1:8)[a] MeOH: DCM: Pet. (1:10:1)[b]	% Mass (% of 2.1g crude extract)	Description
PTA-a	Petrol (100%)	1	0.83	0.4%	Colorless product.
PTA-b	Petrol (100%); Petrol:EtOAc (1:1)	1	0.83	0.4%	White ppt product
PTA-1	Petrol:EtOAc (1:1)	5	0.83, 0.76,0.68,0.17,0.02	3.3%	Dark green gumy product.
PTA-2	EtOAc (100%)	4	0.83, 0.56, 0.20,0.17	15.7%	Dark green gumy product.
PTA-3	EtOAc (100%)	5	0.83, 0.66, 0.34, 0.19, 0.02	1.2%	Dark green gumy product.
PTA-4	EtOAc (100%)	6	0.83,0.69,0.32,0.24,0.17,0.10	0.6%	Dark green oily product.
PTA-5	EtOAc (100%)	3	0.83, 0.76, 0.59, 0.37	0.4%	Dark green gumy product.
PTA-6	EtOAc:EtOH (8:2)	3	0.83, 0.34, 0.29	4.1%	Dark green solid product.
PTA-7	EtOAc:EtOH (1:1)	2	0.83, 0.29	2%	Dark green gumy product.
PTA-8	EtOAc:EtOH (1:1)	3	0.83, 0.29, 0.14	10.3%	Dark green gumy product.
PTA-9	EtOH (100%)	3	0.83, 0.37, 0.29	43.2%	Green-whitish solid product.

Table 3.8 Analysis of the fractions isolated from the extraction with the different solvent systems. The number of components yield, R_f DPPH• values and the appearance of residues are recorded. a =TLC used for fractions (PTA-a –PTA-3), b=TLC used for fractions (PTA-4 – PTA-9).

The separation was carried out in order to validate the earlier results and to obtain sufficient quantities of the fractionated components to conduct the necessary phytochemical analysis of their polyphenolic content. Table 3.8 above, represents the various fractions collected from the silica chromatography of the *P.tomentosa* extract. It can be seen that the majority of material (43.2%) was eluted with EtOH compared to fractions collected from moderate polar solvents EtOAc 100% and EtOAc:EtOH 8:2 (15.7% and 10.3% respectively). It was demonstrated solvent extraction could be used to separate the organic extract of *P.tomentosa* in to five major fractions (PTA-1, 2, 6, 8, 9) and seven minor ones (yield less than 2%) using solvents of different polarity. Overall the attempt to separate the organic extract of *P.tomentosa* gave nine fractions in total yield of 85.7% .All of the fractions isolated from this initial column were analysed by ^{1}H NMR spectroscopy and some representative spectra are shown in Figure 3.14. Whilst all of the fractions are clearly mixtures (also evidenced by TLC analysis), some structural information can be elucidated from the NMR analysis. Thus the complex set of signals seen around 4.5-5.5 ppm (e.g. fraction PTA-1 and PTA-2) are indicative of sugar moieties. Moreover, the signals in range between 1 ppm to 2.5 ppm suggest long hydrocarbons according to previously reported in the literature. [203,204,205,206] In addition, ^{13}C NMR support possibility of long chain hydrocarbon present in the fraction. Further, the proton signals in the region 6 to 7 may suggest an aromatic residue is present.

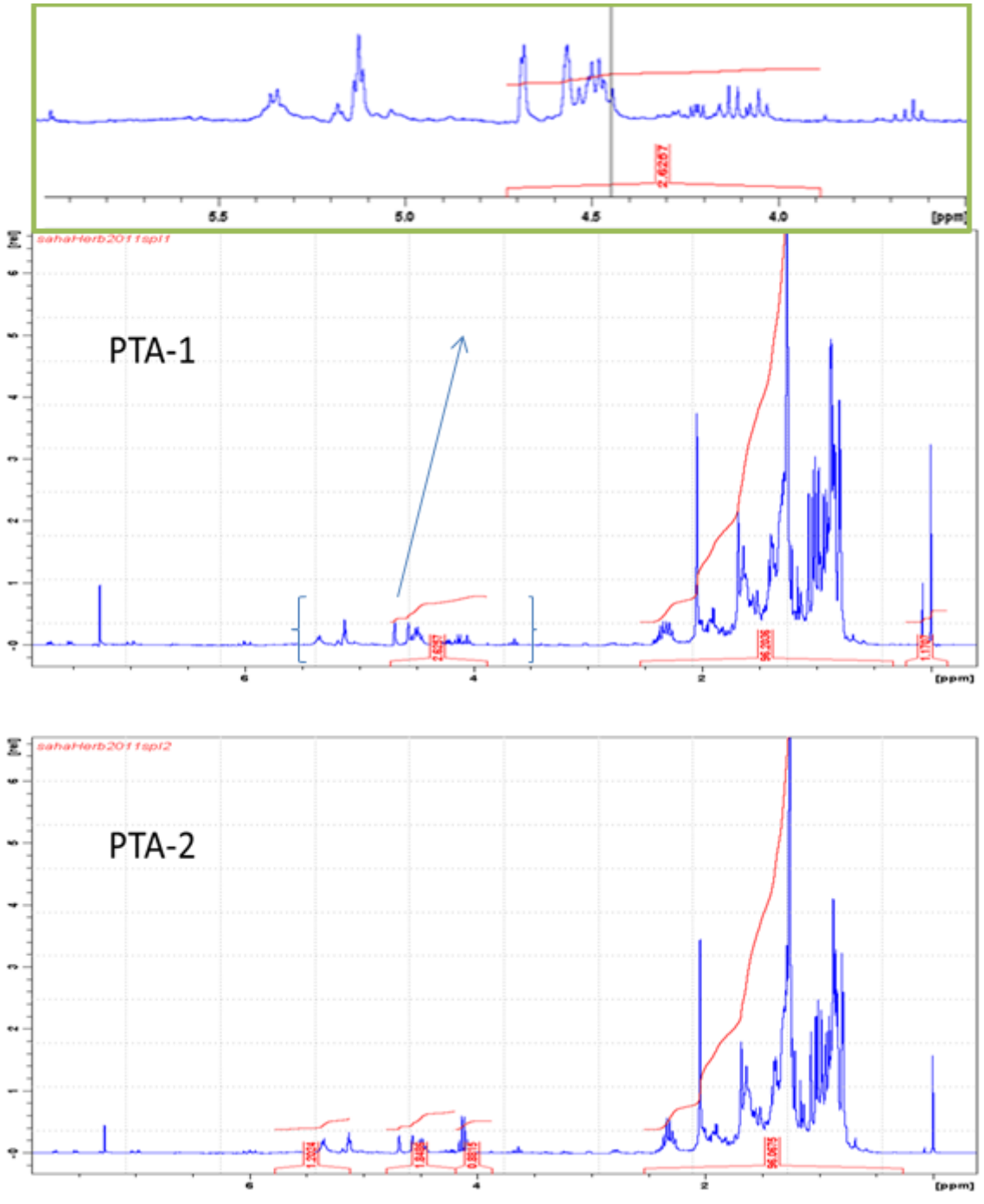


Figure 3.14 NMR of fraction PTA-1 and PTA-2

Unfortunately with these mixtures it proved impossible to do a further analysis of the spectra. Also the NMR spectrum showed a lot of impurities present, which made identification hard.

3.12.1 DPPH• radical scavenging activities of some of the isolated fractions, PTA-8 and PTA-9

DPPH• scavenging activities of fraction PTA-8 and PTA-9 are shown in Table 3.9. compared to the crude extract both of the fractions showed reduced DPPH• radical scavenging activity and significant increase of fraction PTA-9 in total phenolics, while fraction PTA-8 showed slightly decreased in total phenolics.

Table 3.9 Total phenolic content, and DPPH• radical-scavenging activity of isolated fractions of *P. tomentosa*

Plant Preparation Fractions	DPPH• assay (%IP)a		Total phenolics as α-tocopherol equivalents (mg/g of EtOH	
	15 min	ON	30 min	120 min
α- tocopherol	91.5	92.6%		
PTA8 (EtOAC:EtOH,	10.1%	14.8%	82.7%	140.2%
PTA9 (EtOH, 100%)	15.4%	21.0%	199.2%	400.7%

Table 3.9 Antioxidant activities and total phenolic contents of plant extracts (a) tested at 50 µg/ml concentration (b) tested at 10 µg/ml conc. And results presented as α-tocopherol equivalents (mg/g), maximum reproducibility of DPPH• ±0.02 and for total phenolics ±0.01

3.12.2 Prep-high performance liquid chromatography

The NMR spectra of all fractions from the initial column showed that they were mixture. Further purification was done on fraction PTA-9 based on the quantity of material isolated from the column (43.2%). Initially we examined the purification of fraction PTA-9 using prep-HPLC reverse phase using two different mobile phase system as shown in Table 3.10 and Table 3.11, in order to determine the number of constituents present. Using a short column, (Waters spherisorb, SS OD S_2, 20 X 250 mm, prep-column) PTA-9 was separated into it components. First, operating an gradient mobile phase as seen below (Table 3.10) at flow rate of 10 ml /min, concentration 10.3 mg/ml, with injection volume of 2.5 ml. The detection was carried out at 280 nm.The sample was dissolved in the mobile phase: [MeCN:

Water (20:80,v/v)]. The analysis time was 55 minutes. Since retention times varied between each run and the peak shapes changed from run to run this led us to collect our samples fractions according to time periods. The fractions from this HPLC separation were labelled HAc-1 to 8 (see Table 3.12).

Table 3.10 HPLC conditions for attempted separation of fraction PTA-9

Time (min)	0.0	2.0	37.0	45.0	50.0	55.0
Solvent (MeCN)	20	20	100	100	20	20
Solvent (Water)	80	80	0	0	80	80

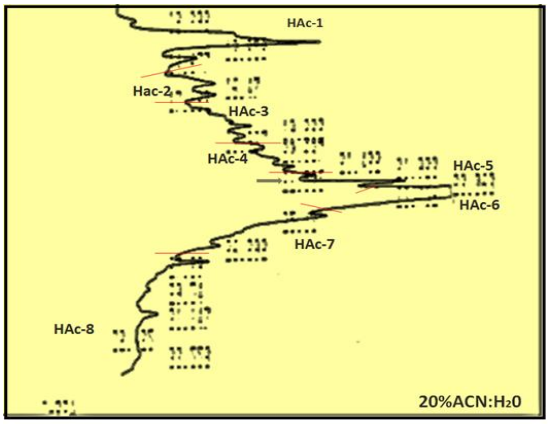


Figure 3.15 Prep-HPLC analysis of the separation of fraction PTA-9, mobile phase: [MeCN: Water (20:80,v/v)].

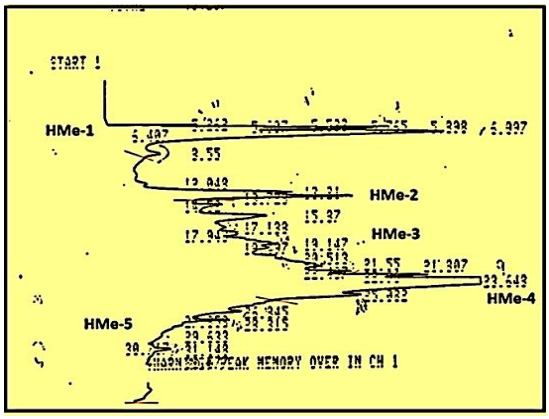


Figure 3.16 Prep-HPLC analysis of the separation of fraction PTA-9, in mobile phase: [MeOH:Water (5:95,v/v)].

Table 3.11 conditions for attempted separation of fraction PTA-9

Time (min)	0.0	5.0	30.0	35.0	40.0	50.0
Solvent (MeOH)	5	5	100	100	5	5
Solvent (Water)	95	95	0	0	95	95

An additional separation of PTA-9 was tried using MeOH : Water as the mobile phase. Thus, the process of isolation of compound PTA-9 through the same column was repeated by using a gradient mobile phase as shown in (Table 3.11). The flow rate was 8 ml /min, and the concentration of the sample was 10 mg/ml, and injection volume of 2.5 ml. The detection was carried out by UV monitoring at 280 nm. The sample was dissolved in the mobile phase: [MeOH : Water (5:95, v/v)]. The analysis time was 50 minutes. Since retention times varied between each run and the peak shapes changed from run to run this led us to collect our samples fractions according to time periods. The fractions from this HPLC separation were labelled HMe -1 to 5 (see Table 3.12). All the fractions collected from these two HPLC separations were subjected to the standard DPPH• assay and the results are shown in Table 3.12.

Table 3.12 DPPH• radical scavenging activity of isolated fractions *of P. tomentosa*

Plant Preparation	DPPH• assay (%IP)	
	15 mint	O/N
α- tocopherol	91.4%	93.9%
(Prep-HPLC) reverse phase 20% MeCN: water		
HAc-1	11.8%	29.4%
HAc-2	1.7%	14.2%
HAc-3	9.3%	27.3%
HAc-4	3.4%	10.8%
HAc-5	-	-
HAc-6	8.5%	19.8%
HAc-7	1.3%	13.1%
HAc-8	3.8%	14.0%
(Prep-HPLC) reverse phase 5% MeOH: water)		
HMe-1	7.4%	15.8%
HMe-2	30.2%	60.2%

Plant Preparation	DPPH• assay (%IP)	
HMe-3	10.2%	24.6%
HMe-4	13.5%	26.7%
HMe-5	6.3%	15.2%

Table 3.12 Antioxidant activities of isolated fraction by Prep-HPLC. All result were tested at 50 µg/ml concentration, maximum reproducibility of DPPH• ±0.01.

All fractions obtained from prep-HPLC for both mobile phases show reduction in absorbance of DPPH• free radical. According to Table 3.12 fraction HMe-2 reveals the highest antioxidant activities (60.2%), while the other fractions shows varying inhibition in range between [(10.8%-29.4%) with MeCN : Water (20:80,v/v) and (15.2%-60.2%) with 5% MeOH : Water]. Previous reports (Djilas, S *et al*, 2008)[207] have established that many flavonoid and polyphenols which can be isolated from plant material show potent DPPH• free radical reactivity therefore Table 3.12 shows various data that would be consistent with the presence of several types of flavone and polyphenol.

To confirmed the above results second time examined fractions HMe-1 to 5 for their DPPH• free radical activity (Table 3.13).

Table 3.13 DPPH• radical scavenging activity of isolated fractions *of P. tomentosa*

Plant Preparation	DPPH• assay (%IP)	
	15 min	O/N
α- tocopherol	91.0%	94.0%
(Prep-HPLC) reverse phase 5% MeOH : Water		
H_1Me-1	7.3%	16.2%
H_1Me-2	40.8%	63.6%
H_1Me-3	9.1%	26.1%
H_1Me-4	12.6%	27.6%
H_1Me-5	5.1%	16.0%

Table 3.13 Antioxidant activities of isolated fraction by Prep-HPLC. All result were tested at 50 µg/ml concentration, maximum reproducibility of DPPH• ±0.01.

As illustrated in Table 3.13 the fraction H_1Me-2 has the greatest antioxidant activity

(DPPH• inhibition) compared with the remaining fractions between (16.0% - 27.6%). The above results demonstrate very elegantly that the fraction HMe-2 and H_1Me-2 showed the highest antioxidant activity. This activity may be un-contributed with the total amount of phenolics present and so both these fractions were investigated using the Folin-Ciocalteu assay. The results are shown in Table 3.14.

Table 3.14 Total phenolic contents of isolated fractions of *P. tomentosa*

Plant Preparation	**Total phenolics as α-tocopherol equivalents (mg/g of EtOH extract)**	
(Prep-HPLC) reverse phase 5% MeOH:H_2O		
	30 m	**120 m**
α- tocopherol	**-**	**-**
HMe-2	**61.6%**	**190.7%**
H_1Me-2	**88.6%**	**198.0%**

Table 3.14 represent total phenolic content of isolated fractions by using Prep-HPLC. All result tested at 10 µg/ml conc. And results presented as α-tocopherol equivalents (mg/g), maximum reproducibility of total phenolics ±0.01.

Both fractions HMe-2 and H_1Me-2 Table 3.14 show a higher level of total phenolic as measured in α-tocopherol equivalents with stronger antioxidant activities according to the DPPH• assay.

3.12.3 Separation by Column Chromatography (PTA-1 & PTA-2)

The fraction PTA-1 and PTA-2 (0.5 g) from Table 3.8 were combined and re-columned using silica chromatography (approximately 4.5 cm column width and 25 cm length) to afford 0.4 g; 80% yield. The details of fractions obtained (PTB-1 to PTB-9) from the column chromatography are given in the Table 3.15. In total nine fractions were collected from this column and analysed by TLC and ^{1}H NMR spectroscopy. The TLC analysis again indicated incomplete separation of sample into its components and this was confirmed by NMR spectroscopy which showed a mixture of compounds.

Table 3.15 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of Spots	R_f Value EtOAc: Pet. (1:9)[a] EtOAc: Pet. (1:1)[b]	Mass % (% of 0.5 g crude extract)
PTB-1	EtOAc :Petrol (2:98)	2	0.81, 0.76	1%
PTB-2	EtOAc :Petrol (5:95)	4	0.81, 0.77, 0.69, 0.57	37%
PTB-3	1. EtOAc :Petrol (10:90) 2. EtOAc :Petrol (20:80)	3	0.76, 0.70, 0.57	8.7%
PTB-4	EtOAc :Petrol (20:80)	3	0.76, 0.70, 0.61	2.9%
PTB-5	EtOAc :Petrol (20:80)	2	0.67, 0.61	9.3%
PTB-6	EtOAc :Petrol (20:80)	2	0.69, 0.61	6%
PTB-7	EtOAc :Petrol (20:80)	4	0.80, 0.74, 0.69, 0.61	5.1%
PTB-8	1. EtOAc :Petrol (20:80) 2. EtOAc :Petrol (50:50)	3	0.87, 0.80, 0.69	5.3%
PTB-9	1. EtOAc :Petrol(70:30) 2. EtOAc (100%) 3. EtOAc:EtOH (50:50) 4. EtOAc:EtOH (90:10) 5. EtOAc:EtOH (80:20) 6. EtOAc:EtOH (30:70) 7. EtOH (100%)	6	0.87, 0.80, 0.69, 0.56, 0.31, 0.19	11%

The Table 3.15 indications similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, R_f values and the colour of the products. a =TLC used for fractions (PTB-1 –PTB-8), b=TLC used for fraction (PTB-9).

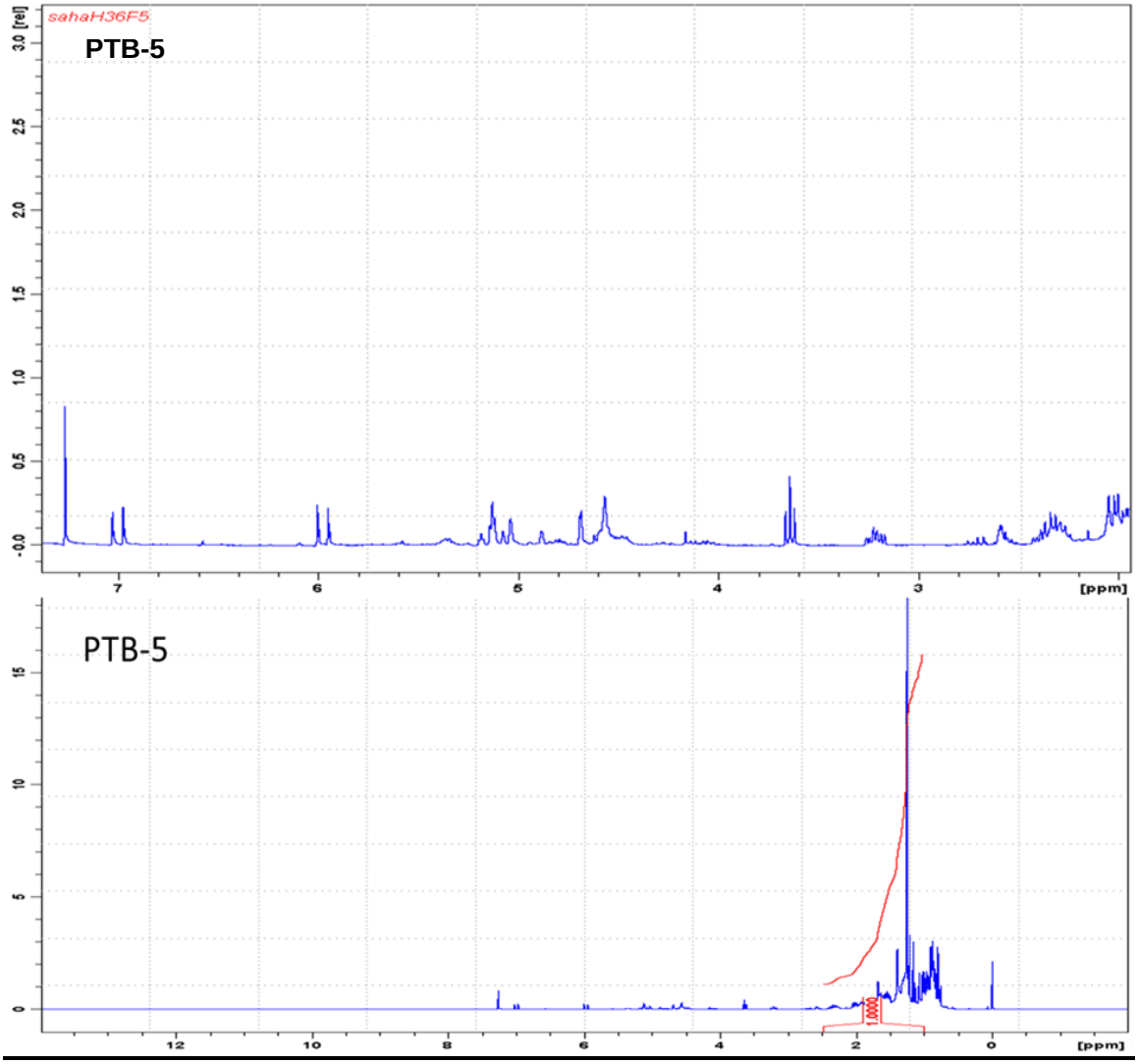


Figure 3.17 NMR of fraction PTB-5

Although the NMR spectra were difficult to interpret due to incomplete separation a number of interesting structural fractions could be assigned to the fractions. A representative spectrum (for fraction PTB-5) is shown in Figure 3.17. Whilst there is obviously many signals in the upfield hydrocarbon region (from 1 ppm – 2 ppm) which are difficult to analyse some more distinct signals can be made out in the downfield regions. Thus we can see a pair of doublets

at 5.97 ppm (d, J=15.5 Hz) and 6.96 ppm (d, J=15.5 Hz) with a coupling constant indicating a trans double bond. The large difference in chemical shift between the two signals also suggests that this may from part of an α, β-unsaturated carbonyl system. These is also a triplet at 3.64 ppm (J=6.6 Hz) which could seem to indicate the presence of a CH_2-CH-X (X= O or N) group in one of the molecules present. There are also a number of multiple between 4.4 ppm – 5.2 ppm which could represent a sugar. However, overall it is difficult to make too many conclusions from this data.

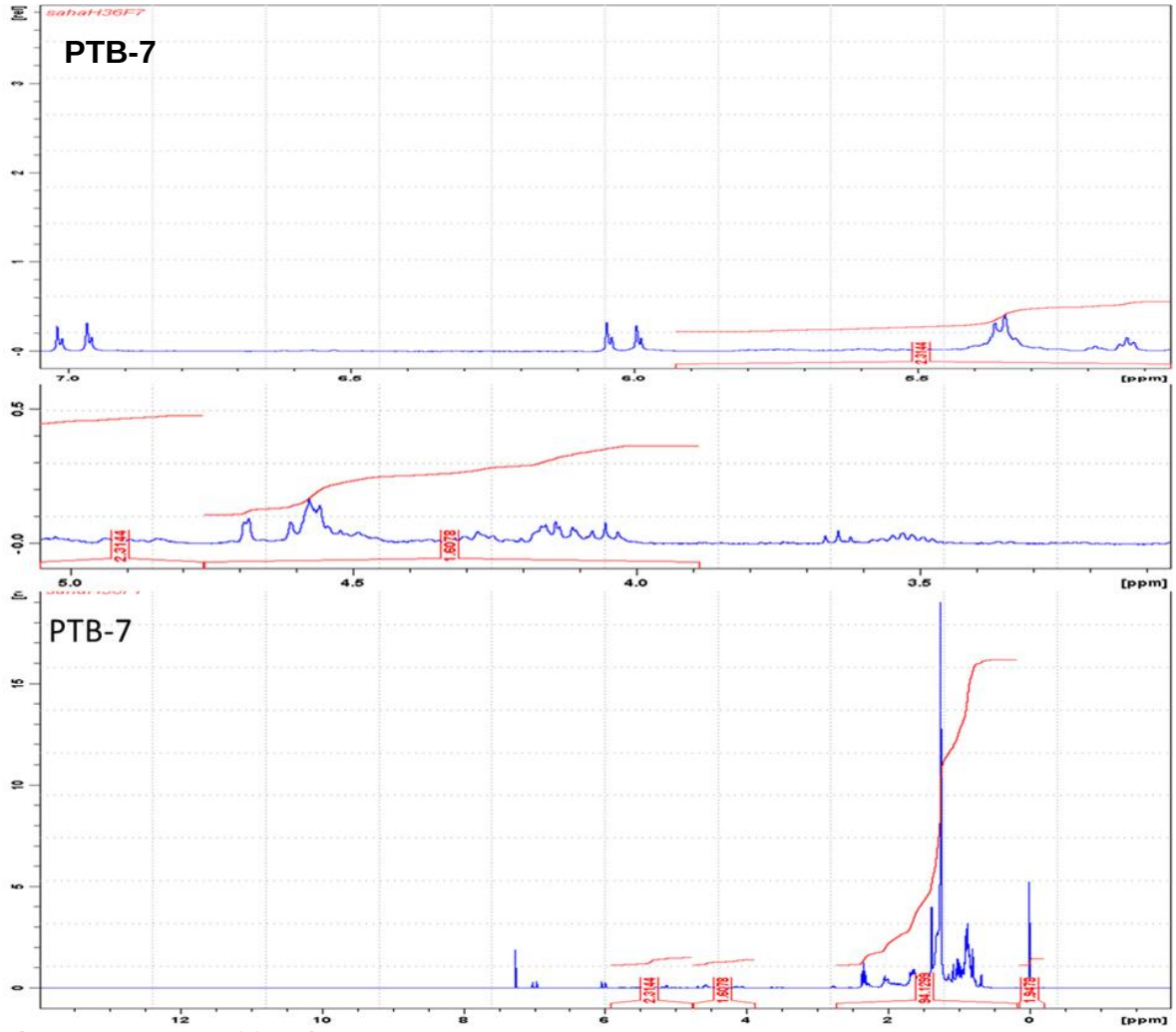


Figure 3.18 NMR of fraction PTB-7

Fraction PTB-7 showed pattern of signals appeared as double of doublets of at 6.02 ppm (dd, 15.7, 2.5 Hz) and 6.99 ppm (dd, 15.7, 2.5 Hz) were similar to PTB-5

which suggested that this may form part of an α, β-unsaturated carbonyl system due to trans double bond. In addition, signals present in chemical shift in range between 1 ppm to 2.5 ppm may indicate long chain hydrocarbon. Whilst many signals found at region 4.69 ppm to 4.13 ppm which may represent a sugar compounds. Though, it is difficult to make an absolute assignment of these signals due to the incomplete separation of sample.

The ^{1}H NMR spectrum of fraction PTB-6 and PTB-8 showed mixture of components. Although NMR spectra give similar spectra as PTB-5 which may indicated same compound with more mixture on it.

3.12.4 Separation by Column Chromatography (PTB-5 to PTB-8)

The fractions (PTB-5 to PTB-8) from Table 3.15 were combined (0.2 g) and using silica chromatography (approximately 3 cm column width and 25 cm length) to afford 0.14 g; 70% yield. The details of fractions obtained from this stage of column chromatography are given in the Table 3.16. In total nine fractions were collected from this column and analysed by TLC and ^{1}H NMR spectroscopy. The TLC analysis again unfortunately shows some mixture and incomplete separation of sample into its components and this was confirmed by NMR spectroscopy which showed a mixture of compounds. Fraction PTC-7 represents the cleanest spectrum from the other fractions.

Table 3.16 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of Spots	R_f Value EtOAc: Pet. (1:1)	Mass % (% of 0.2 g crude extract)	Description
PTC-1	EtOAc :Petrol (5:95)	4	0.81, 0.44,0.36, 0.26	9.9%	Colourless product.
PTC-2	EtOAc :Petrol (5:95)	4	0.42, 0.36, 0.24, 0.18	19%	White ppt product
PTC-3	EtOAc :Petrol (5:95)	3	0.42,0.36, 0.18	2%	Dark green gummy product.
PTC-4	EtOAc :Petrol (5:95)	3	0.42, 0.36, 0.29	8.6%	Dark green gummy product.
PTC-5	EtOAc :Petrol (5:95)	4	0.36, 0.29, 0.24,0.18	4.6%	Dark green gummy product.
PTC-6	EtOAc :Petrol (5:95)	3	0.29, 0.24, 0.11	0.7%	Dark green oily product.
PTC-7	EtOAc :Petrol (5:95)	4	0.35, 0.29, 0.24, 0.11	6%	Dark green gummy product.
PTC-8	EtOAc :Petrol (5:95)	5	0.86, 0.66, 0.24, 0.22, 0.10	15%	Dark green solid product.
PTC-9	[{(5%;10%; 20%; 40%; 60%; 80% and 100%) EtOAc: Petrol}, {(5%; 10%; 20%; 40%; 60% and 80%) EtOH: EtOAc].	2	0.86, 0.22	26%	Dark green gummy product.

The Table 3.16 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, R_f values and the colour of the products.

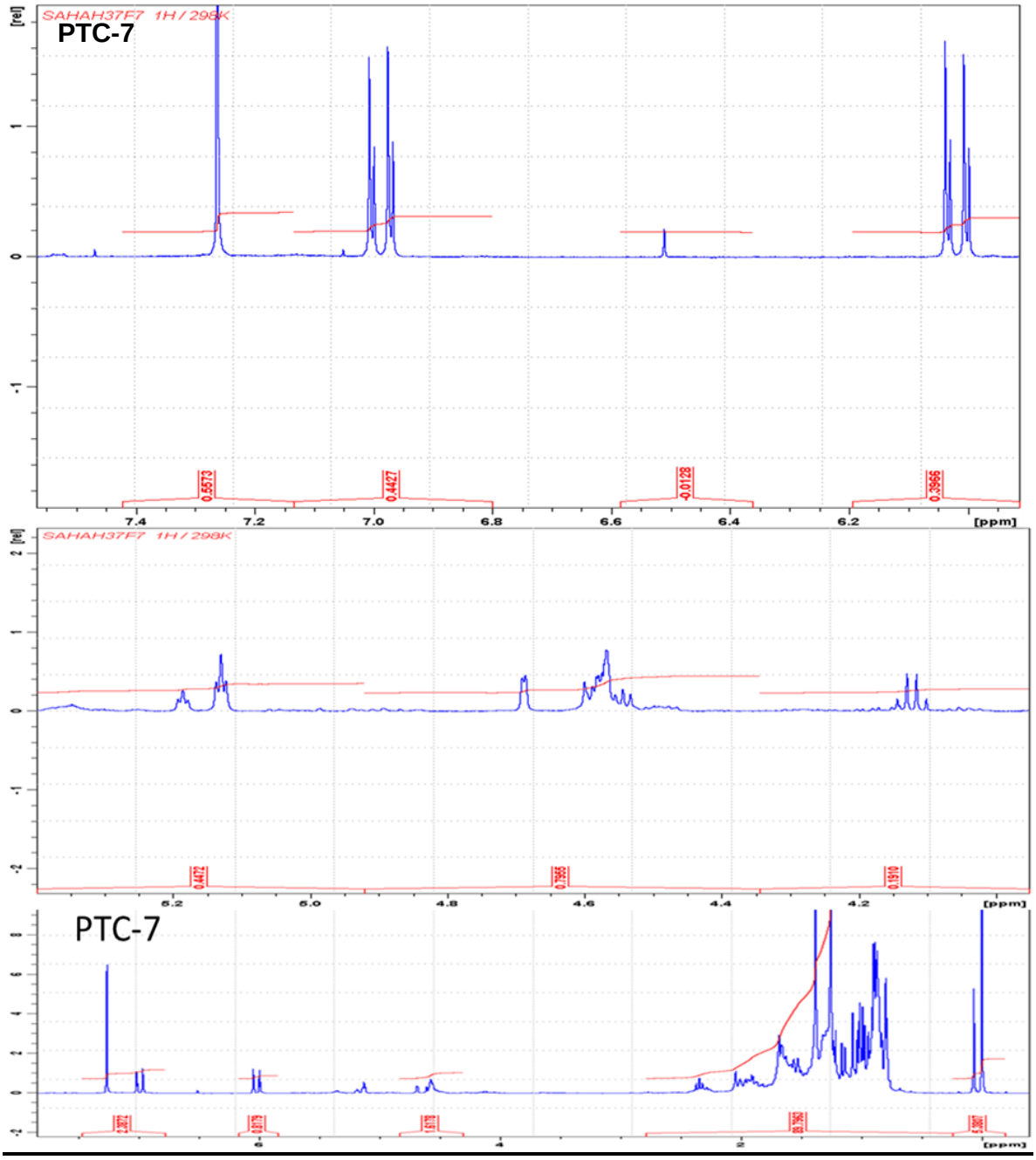


Figure 3.19 NMR of fraction PTC-7

In the above example ^{1}H NMR of fraction PTC-7 spectrum is shown in Figure 3.19. The signals at 1 ppm to 2 ppm appeared in the upfield of hydrocarbon region are very difficult to integrate and make absolute assignment of these signals. While some separate signals at 6.02 ppm (dd, J=15.6 ,15.5 Hz) and

6.98 ppm (dd, 15.6 ,15.5 Hz) can see a pair of double of doublets with a coupling constant falls into expiated trans double bond. In addition to the large difference in chemical shift between two signals can suggests that this maybe from part of an α, β unsaturated carbonyl system.

Fraction PTC-7 from silica-column was observed to possess some promising peaks in the NMR spectrum and therefore was subjected to DPPH• assay to check the antioxidant activities present in this fraction Table 3.17.

Table 3.17demonstrated the total phenolic content in separated fractions

Plant Preparation	DPPH• assay (%IP)	
	15 mint	O/N
α- tocopherol	91.44%	93.94%
(Prep-HPLC) reverse phase 5% MeOH: H2O		
PTC-7	0.63%	4.23%

Fraction PTC-7 obtained from silica chromatography show a very low antioxidant activities as DPPH• assay inhibition Table 3.17 (4.23%). Although fraction PTC-6 showed NMR signals similar as PTC-7 which can suggest having same compound with isomers.

3.12.5 Separation by Column Chromatography [PTC-(1-5) and PTC-(8- 9)]

PTC-7 and PTC-6 showed some NMR promising separation led us to combine most fractions from Table 3.16 [PTC-(1-5) to PTC-(8-9), 0.11 g] and using silica chromatography (approximately 3 cm column width and 25 cm length) to afford 0.10 g; 91% yield. The details of fractions obtained from this stage of column chromatography are given in the Table 3.16. In total seven fractions were collected from this column and analysed by TLC and ^{1}H NMR spectroscopy. The details of fractions obtained from the column chromatography are given in Table 3.18.

Table 3.18 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of Spots	R_f Value EtOAc: Pet. (1:1)	Mass % (% of 0.11g crude extract)	Description
PTD-1	1. EtOAc :Petrol (1:99) 2. EtOAc :Petrol (2:98) 3. EtOAc :Petrol (5:97)	2	0.77, 0.70	11%	Colourless product.
PTD-2	EtOAc :Petrol (3:97)	3	0.77,0.68, 0.58	6.7%	White ppt product
PTD-3	EtOAc :Petrol (4:96)	4	0.72,0.67,0.50, 0.45	11%	Dark green gummy product.
PTD-4	EtOAc :Petrol (4:96)	4	0.73, 0.68, 0.52, 0.43	4.7%	Dark green gummy product.
PTD-5	EtOAc :Petrol (3:97)	2	0.59, 0.35	18%	Dark green oily product.
PTD-6	1. EtOAc :Petrol (4:96) 2. EtOAc :Petro (5:95) 3. EtOAc :Petrol (10:90)	6	0.78, 0.70, 0.62 0.60, 0.52, 0.36	19%	Dark green gummy product.
PTD-7	EtOAc :Petrol (20:80)	5	0.78, 0.68, 0.65, 0.58, 0.36	28%	Dark green solid product.

The Table 3.18 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, R_f values and the colour of the products.

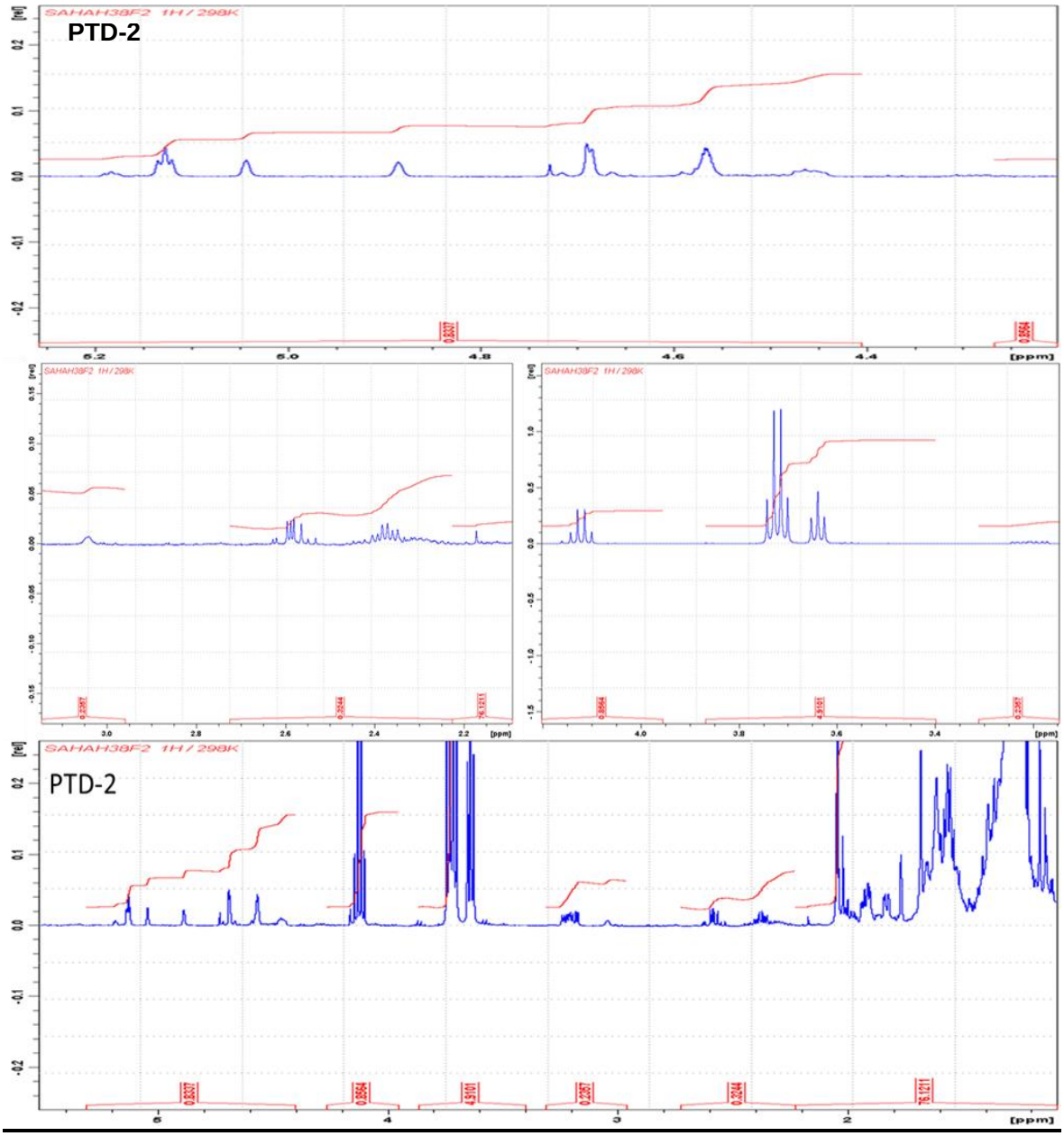


Figure 3.20 NMR of fraction PTD-2

The TLC analysis again indicated incomplete separation and this was confirmed by the NMR spectra which showed mixtures of components. A representative spectrum (for fraction PTD-2) is shown in Figure 3.20. In region between 1 ppm to

2.5 ppm shows many signals in the upfield long chain hydrocarbon which are difficult to analysis, while some distinct signals can be made out in the downfield regions. Thus one can notice triplet at 3.64 ppm (J=6.7 Hz), which would seem to indicate the presence of a CH_2-CH-X (X= O or N) group in one of the molecules present. They are also a number of signals between 4.12 ppm – 5.2 ppm which could represent a sugar. However, overall it is difficult to make an absolute assignment of these signals.

Table 3.19 Antioxidant activities and total phenolic contents of various fractions of the *P. tomentosa*

Plant Preparation	DPPH• assay (%IP)a		Total phenolics as α-tocopherol equivalents (mg/g of EtOH extract)b	
	15min	ON	30 min	120 min
α- tocopherol	91.5%	92.6%	-	-
PTD-1(EtOAc:Pet.(1%,2%,3%)	0.4%	2.0%	67%	86%
PTD-2 (EtOAc :Pet. (3:97)	5.3%	8.5%	113%	170%
PTD-3 (EtOAc, 100%)	1.3%	1.7%	73.2%	137.3%
PTD-4 (EtOAc, 100%)	0.5%	0.9%	79.8%	97.7%
PTD-5 (EtOAc, 100%)	-	-	26.4%	46.48%
PTD-6 (EtOAc:ETOH, 8:2)	2.0%	3.0%	168.7%	181.5%
PTD-7 (EtOAc:ETOH, 1:1)	4.1%	7.3%	95.5%	162.8%

Table 3.19 Antioxidant activities and total phenolic contents of plant extracts (a) tested at 50 µg/ml concentration (b) tested at 10 µg/ml conc. And results presented as α-tocopherol equivalents (mg/g), maximum reproducibility of DPPH• ±0.01 and for total phenolics ±0.002.

Table 3.19 shows antioxidant activities in the DPPH• assay after 15 min and overnight stand. The fraction PTD-6 has the greatest of total phenolic contents as α-tocopherol equivalents (181.5% / 120 min) followed by fraction PTD-2 and PTD-7 (170% / 120 min and 162.8% / 120 min) compared with the other fractions (PTD-3 and PTD-4). The lowest total phenolic contents were found in PTD-5 (46.5% / 120 min). These results support the notion that total phenolic contents not

necessarily be positively correlated with antioxidant activities. According to what is shown in the Table 3.19 PTD-7 >PTD-6 and overall, all fractions are shown a minimum antioxidant activity (DPPH• inhibition).

According to the NMR spectra of all fractions from the column (section 3.12.5, Table 3.18) showed that they were still mixture. Further purification was done on combined fractions [PTD-(1-5),] all fractions were combined and were subjected to prep-HPLC normal phase in order to determine the number of constituents present in fractions. Concentration of 10 mg /ml and injection volume of 2.5 ml was utilized. Detection was carried out at 280 nm as shown below (Table 3.20) at flow rate of 1 ml/min and the analysis time was 50 min. The samples were dissolved in mobile phase: [EtOAc: hexane (20:80,v/v)]. Since retention times varied between each run and the peak shapes changed from run to run this led us to collect our samples fractions according to time periods. The fraction from this HPLC separation were labelled HHe-1 to 13 (see Table 3.21).

Table 3.20 HPLC condition for analysis of plant extracts; *λ* 280nm

Time (minutes)	Flow Rate (ml/min)	% of solvent
Prerun	1.0	A=80% B= 20%
20.0	1.0	A=80% B= 20%
10.0	1.0	A=70% B= 30%
10.0	1.0	A=60% B= 40%
10.0	1.0	A=00% B= 100%

Table 3.21 DPPH• radical-scavenging activity of isolated fractions of *P.tomentosa* eluted from column chromatography

Plant Preparation	DPPH• assay (%IP)	
	15 mint	O/N
α- tocopherol	92.2%	93.7%
HHe-1	17.9%	19.7%
HHe-2	9.6%	12.3%
HHe-3	11.9%	13.1%
HHe-4	14.2%	19.1%
HHe-5	16.1%	18.4%
HHe-6	17.0%	25.4%
HHe-7	17.9%	26.5%

Plant Preparation	DPPH• assay (%IP)	
HHe-8	13.3%	13.6%
HHe-9	10.6%	12.8%
HHe-10	13.6%	17.9%
HHe-11	27.8%	28.6%
HHe-12	29.9%	32.9%
HHe-13	31.3%	33.3%

Table 3.21 Antioxidant activities of isolated fractions, tested at 50 µg/ml concentration maximum reproducibility of DPPH• ±0.1

All fraction obtained from prep-HPLC show a reduction in absorbance of DPPH• free radical. According to (Table 3.21) all 13 fractions exhibited similar moderate level of antioxidant activity as %inhibition either after 15 min or after overnight standing, in rang between (9.6% to 31.3%) after 15 min and (12.76% to 33.27%) after overnight. As reported by (Rice-Evans, 2001)[208] that antioxidant activity of flavonoid is structure depending. Ability of the phenolic hydroxyl group to act as antioxidants by hydrogen donating free radicals depends on the reduction potential of radicals and their chemical structure. It seems that all above isolated fractions contains a mixture of several polyphenol and flavonoid compounds which make it difficult to attribute antioxidant activity to specific structural compounds.

3.13 Schematic representation of *P.tomentosa* extracts (2011) approach for isolation of plant constituents.

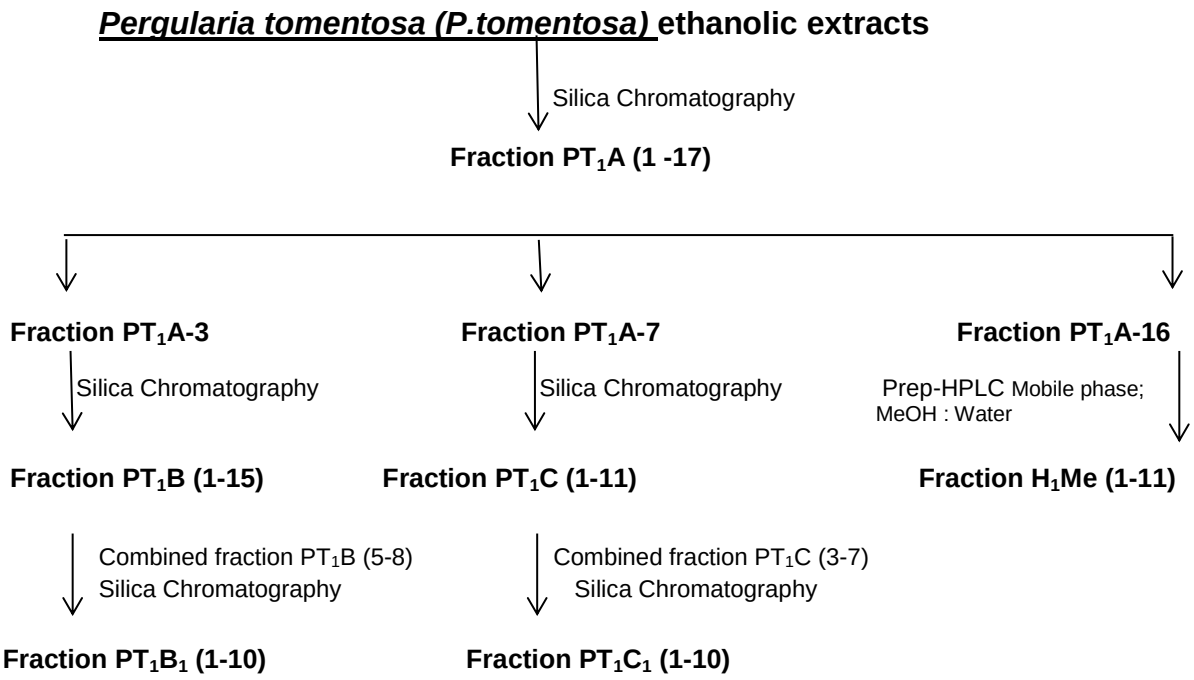


Figure 3.23 Flow Chart-Analytical methodology used for isolation and identification of active constituents of *P. tomentosa* ethanolic extracts.

3.14 Separation by Column Chromatography

All isolated fractions which were analysed above either by column chromatography or Prep-HPLC produced a poorly separated compounds, and this was confirmed by antioxidant and total phenolics properties results that had in general similar antioxidant activities as DPPH• inhibition and total phenolic between all isolated fractions Therefore, the attempt to separate the crude ethanolic extract (collected in 2011) harvested in 2011 was extracted secondly, as per (section 3.2.2.1). 5.1 g from the residue was purified as described in section 3.6.1, using silica chromatography (approximately 30 cm column width and 46 cm length) to afford 4.4 g; 86.6% yield. The details of fractions obtained from the column chromatography are given in the Table 3.22.

Table 3.22 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of Spots	R_f Value MeOH: Diethyl ether: Pet. (1:1:8)[a] MeOH: DCM: Pet. (1:10:1)[b]	Mass % *	Description
PT_1A-1	Petrol (100%)	1	0.84	0.12%	Colourless product.
PT_1A-2	Petrol (100%)	1	0.84	0.33%	White ppt product
PT_1A-3	Petrol (100%)	4	0.84, 0.69, 0.29,0.20	19.3%	Dark green gummy product.
PT_1A-4	Petrol (100%)	2	0.29,0.20	0.1%	Dark green gummy product.
PT_1A -5	1. Petrol (100%) 2. Petrol:EtOAc(1:1)	4	0.71, 0.29, 0.20,0.09	0.31%	Dark green gummy product.
PT_1A -6	Petrol:EtOAc(1:1)	4	0.71, 0.29, 0.24,0.20	0.12%	Dark green oily product.
PT_1A -7	Petrol:EtOAc(1:1)	4	0.71, 0.29, 0.24,0.20	3.7%	Dark green gummy product.
PT_1A -8	Petrol:EtOAc(1:1)	2	0.33, 0.22	1.2%	Dark green solid product.
PT_1A -9	Petrol:EtOAc(1:1)	1	0.22	1.5%	Dark green gummy product.
PT_1A -10	Petrol:EtOAc(1:1)	-	B	1.4%	Dark green gummy product.
PT_1A -11	1. Petrol:EtOAc(1:1) 2. EtOAc (100%)	-	B	0.29%	Green-whitish solid product.
PT_1A -12	1. EtOAc (100%) 2. EtOAc:EtOH(8:2)	-	B	2.3%	Green-whitish solid product.
PT_1A -13	EtOAc:EtOH(8:2)	-	B	2.6%	Green-whitish solid product.
PT_1A -14	EtOAc:EtOH(1:1)	-	B	3.0%	Green-whitish solid product.
PT_1A -15	EtOAc:EtOH(1:1)	-	B	2.2%	Green-whitish solid product.
PT_1A -16	1. EtOAc:EtOH(1:1) 2. EtOH (100%)	-	B	31.9%	Green-whitish solid product.
PT_1A -17	EtOH (100%)	-	B	16.2%	Green-whitish solid product.

B: bottom, The Table 3.22 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, R_f values and the colour of the products. * (% of 5.1 g crude extract).a =TLC used for fractions (PT_1A-1 – PT_1A-12), b=TLC used for fraction (PT_1A-13- PT_1A-17).

3.14.1 Antioxidant activities and total phenolics of isolated fractions

DPPH• scavenging activities of above fractions PT_1A-1 to PT_1A-17 are shown in Table 3.22. Most of the fractions extract in comparison to their crude extract (section 3.7.) and earlier isolated PTA-8 & PTA-9 (section 3.11.1) show significant DPPH• radical scavenging activity. Both EtOH fraction (PTA-9 and PT_1A-17) show similar total phenolic activity and considered higher then crude *P. tomentosa*. Though **PT_1A-11** possessed the highest DPPH• inhibition activity of 94%, ON and can be following in this order, PT_1A-14> PT_1A-16> PT_1A-10> PT_1A-12> PT_1A-15> PT_1A-13> PT_1A-9> PT_1A-17> PT_1A-8> PT_1A-4, 5 and 6> PT_1A-7> PT_1A-1+ PT_1A-2. Moreover, **PT_1A-11**show the highest activity in total phenolic also (2298.6% equivalent to α-tocopherol) follow by order, PT_1A-17> > PT_1A-4, 5 and 6> PT_1A-7> PT_1A-10> PT_1A-15> PT_1A-9> PT_1A-13> PT_1A-14> PT_1A-8> PT_1A-16> PT_1A-4, 5 and 6> PT_1A-1+ PT_1A-2=PT_1A-3. In generally, this study confirmed that the antioxidant activities are independent of total phenolic contents therefore, different responses to different phenolic compounds depend on chemical structure.

Table 3.23 show total phenolic content and DPPH• radical-scavenging activity of isolated fractions of *P.tomentosa*

Plant Preparation	DPPH• assay (%IP)a		Total phenolics as α-tocopherol equivalents (mg/g of EtOH extract)b	
	15m	O/N	30m	120m
α- tocopherol	88.1%	93.0%		
[PT_1A-1+ PT_1A-2]	20.1%	9.3%	87.3%	95.1%
PT_1A-3	5.0%	9.3%	87.3%	95.1%
[PT_1A-4+ PT_1A -5+ PT1A -6]	15.7%	24.0%	120.8%	248.6%
PT_1A -7	9.8%	14.1%	118.6%	164.2%
PT_1A -8	3.5%	64.5%	57.8%	99.5%
PT_1A -9	4.1%	70.9%	72.4%	125.8%
PT_1A -10	45.1%	80.2%	94.0%	161.2%
PT_1A -11	25.7%	94.0%	220.8%	2298.6%
PT_1A -12	23.0%	79.7%	37.0%	73.9%
PT_1A -13	32.9%	74.2%	67.5%	123.7%
PT_1A -14	49.3%	88.6%	57.7%	121.9%
PT_1A -15	65.2%	77.7%	82.1%	144.1%
PT_1A -16	67.9%	83.5%	47.7%	99.2%
PT_1A -17	58.0%	68.6%	183.1%	553.8%

Antioxidant activities and total phenolic contents of plant extracts (a) tested at 50 µg/ml concentration (b) tested at 10 µg/ml conc. And results presented as α-tocopherol equivalents (mg/g). Maximum reproducibility of DPPH• ±0.04 and for total phenolics ±0.01

3.14.2 Separation by Column Chromatography (PT_1A-3)

Fraction PT_1A-3 (0.7 g) from Table 2.23 was carried out by using silica column chromatography (approximately 5.3 cm column width and 27 cm length) to afford 0.6 g; 85.7% yield. The details of fractions obtained from the column chromatography are given in the Table 3.24. In total 15 fractions were collected from this column and analysed by TLC and ^{1}H NMR spectroscopy. The TLC analysis again indicated incomplete separation and this was confirmed by NMR spectroscopy which showed a mixtures of components.

Table 3.24 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	No. of Spots	R_f Value Diethyl ether: Pet. (1:9)	Mass % (% of 0.7g crude extract)
PT_1B-1	Diethyl Ether: Petrol (1:99)	1	0.75	3.7%
PT_1B-2	Diethyl Ether: Petrol (1:99)	4	0.68, 0.60, 0.56 0.47	32%
PT_1B-3	Diethyl Ether: Petrol (1:99)	1	0.47	2.9%
PT_1B-4	Diethyl Ether: Petrol (1:99)	2	0.47, 0.35	3.1%
PT_1B-5	Diethyl Ether: Petrol (1:99)	2	0.47, 0.35	3.5%
PT_1B-6	Diethyl Ether: Petrol (1:99)	2	0.44, 0. 30	3.2%
PT_1B-7	Diethyl Ether: Petrol (1:99)	2	0.44, 0.30	5.2%
PT_1B-8	Diethyl Ether: Petrol (2:98)	-	-	2.5%
PT_1B-9	1. Diethyl Ether: Petrol (2:98) 2. Diethyl Ether: Petrol (3:97)	1	0.23	10.6%
PT_1B-10	1. Diethyl Ether: Petrol (3:97) 2. Diethyl Ether: Petrol (4:96)	-	-	0.9%
PT_1B-11	Diethyl Ether: Petrol (5:95)	-	-	2.3%
PT_1B-12	Diethyl Ether: Petrol (5:95)	2	0.09, 0.05	2.3%
PT_1B-13	Diethyl Ether: Petrol (10:90)	1	0.05	3.7%
PT_1B-14	Diethyl Ether (100%)	-	-	13.9%
PT_1B-15	Diethyl Ether:EtOH (1:1)	-	-	3.1%

The Table 3.24 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots and yields, R_f values.

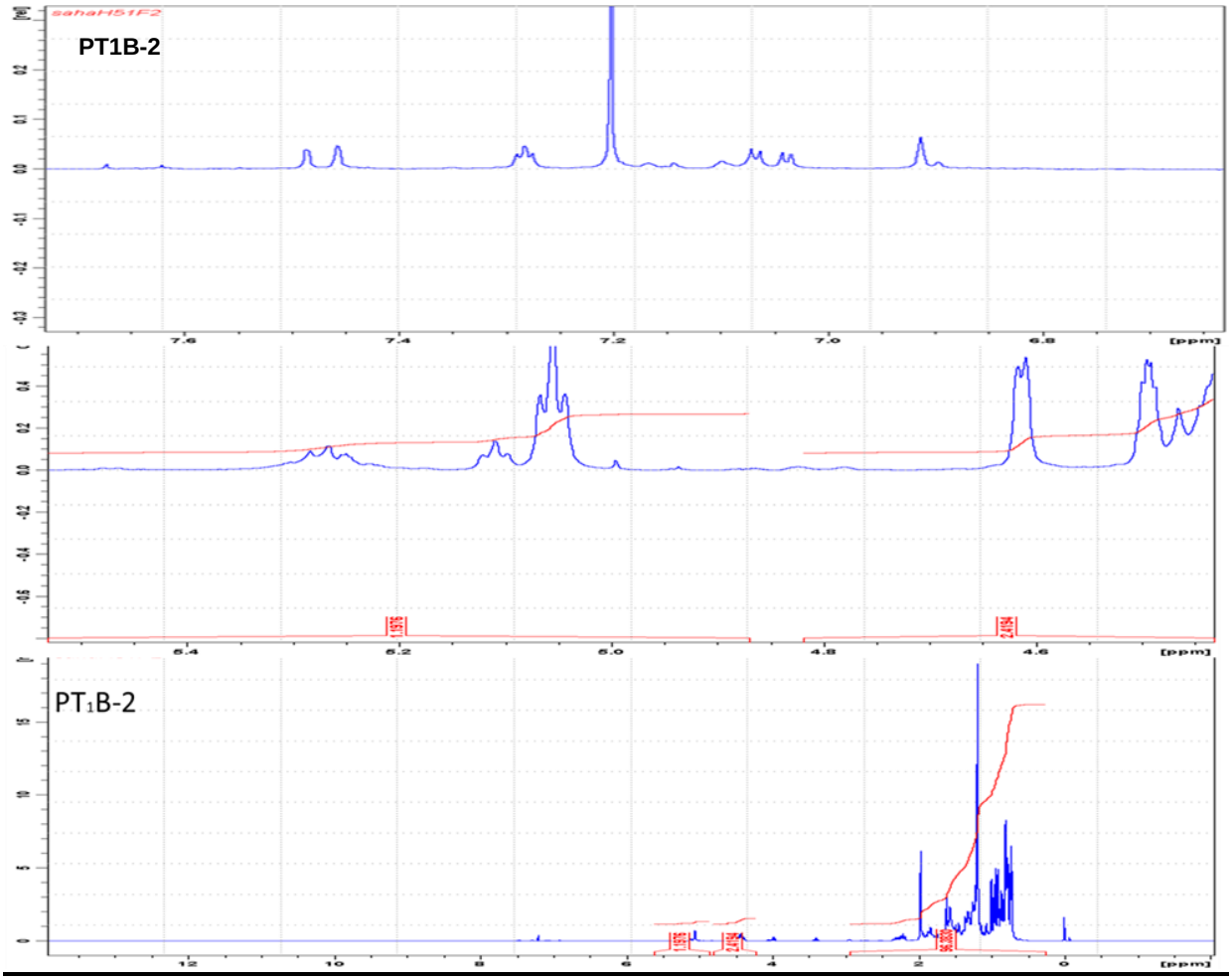


Figure 3.24 NMR of fraction PT_1B-2

Though the NMR spectra still show incomplete separation of the sample there number of interesting signals could be assigned to the fraction (see Figure 3.24). as seen in region between 6.90 ppm to 6.96 ppm there are a number of multiplets seemed which could represent an aromatic (Ar-H) moiety in addition Strong signals at 7.77 ppm confirmed that. There are also a number of signals between 4.36 ppm – 5.28 ppm which could represent a sugar. However, signals were found in chemical shift δ around 1.7, which proposed the presence of allylic functional group (C=C-CH3). Furthermore, the range between 2.0 to 2.7 ppm is favourable region for long

chain alkyl groups. Same as previously fractions it is difficult to make the expect conclusion from this data.

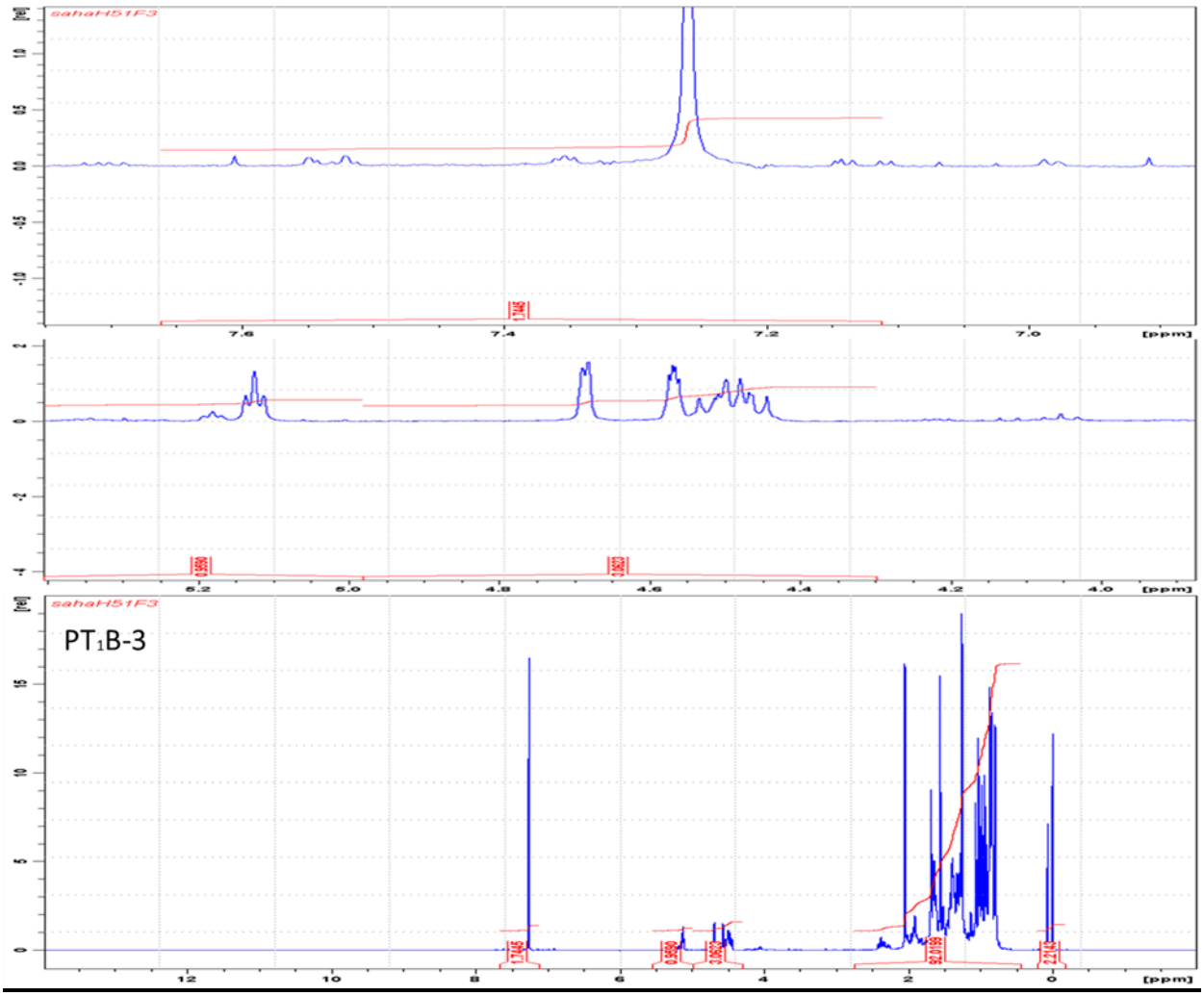


Figure 3.25 NMR of fraction PT1B-3

Figure 3.25 represents fraction PT_1B-3, the ^1H NMR spectrum showed similar signals as (Figure 3.24, fraction PT_1B) with more impurities. As before the fraction required further purification by silica chromatography.

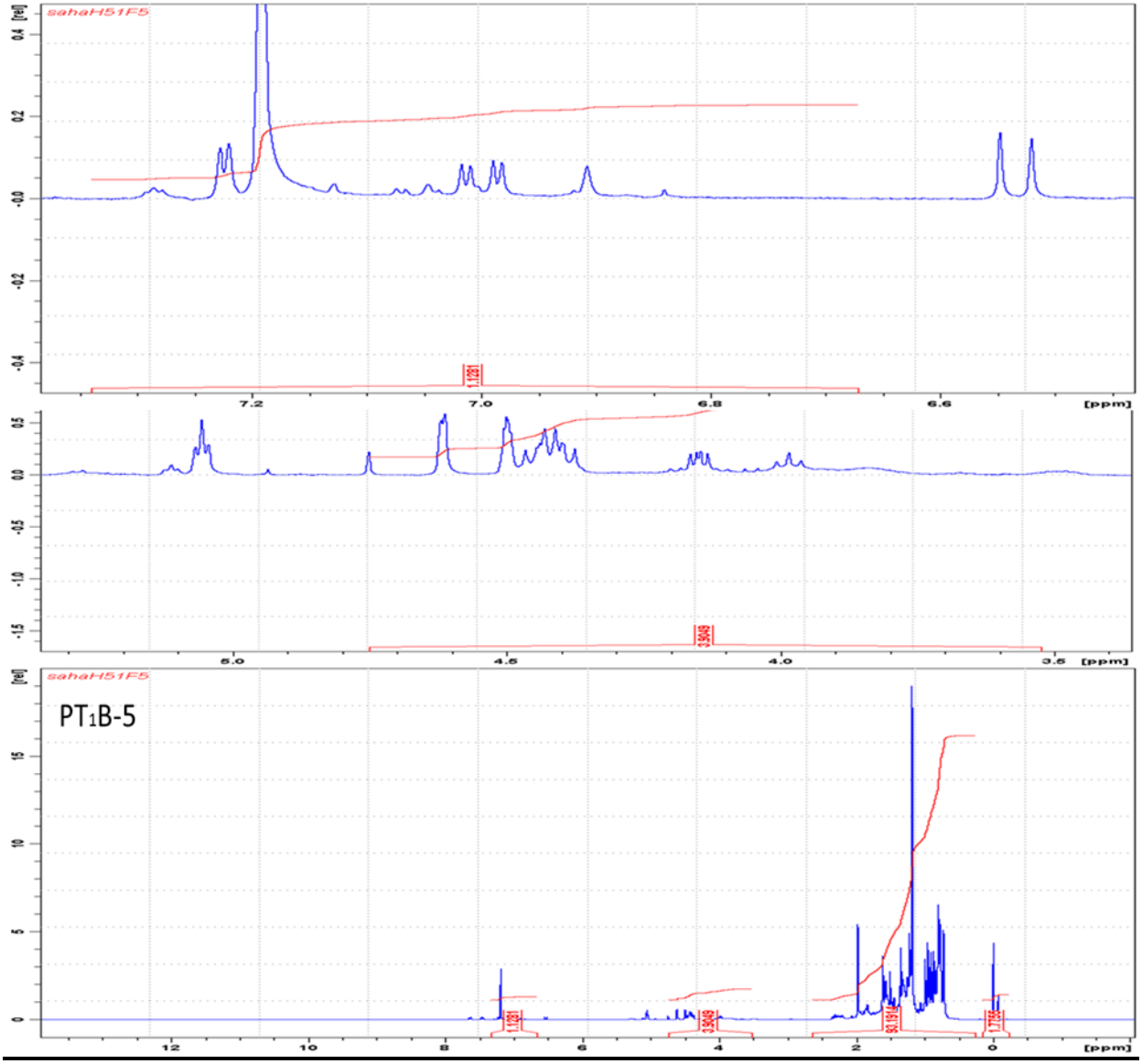


Figure 3.26 NMR of fraction PT1B-5

Fraction PT1B-5 Figure 3.26 though the separation still incomplete between the sample and it show some interesting signals in downfield could be assigned to the fractions. As representative in the spectrum we can see a pair of doublet and pair of double of doublets at [6.53 ppm (d, J=8.4 Hz), 6.99 ppm (dd, J=8.4, 2.3 Hz), 7.06 ppm (d, J=2.3 Hz) and 7.22 ppm (br. d, J=2.3 Hz)] with a coupling constant indicating a cis double bond. The strong signals occur at above region also can indicate an aromatic (Ar-H) moiety. While the triplet at 3.98 ppm (t, J=6.2Hz), could be indicate

the presence of a CH_2-CH-X (X= O or N). Moreover, the range between 2.0 ppm to 2.7 ppm is a favourable region for long chain hydrocarbon. Furthermore, signals found in region 1.7 ppm proposed the presence of allylic functional group (C=C-CH3).

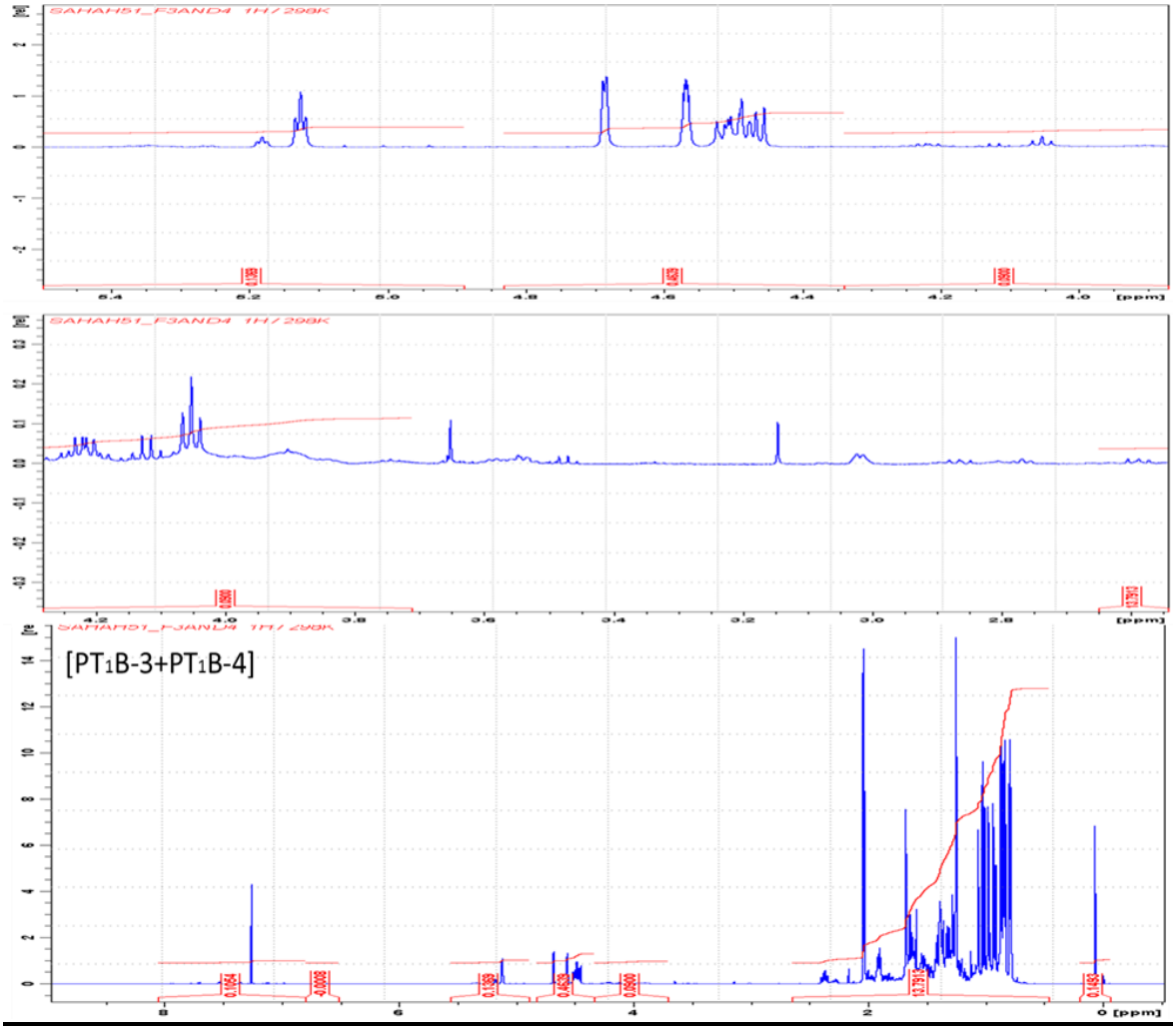


Figure 3.27 NMR of fraction PT1B-3 and PT1B-4

Although the NMR spectra were difficult to interpret due to incomplete separation a number of signals could be assigned to the fractions. A representative spectrum (for combined fractions PT_1B-3 + PT_1B-4) is shown in Figure 3.27. Though, there are clearly many signals in the upfield hydrocarbon region (from 1 ppm – 2 ppm) which

are very difficult to analyse. Also, there is obviously many signals in downfield region 6.53 ppm to 7.28 ppm probably can indicate an aromatic (Ar-H) moiety. While pair of triplet at 5.12 ppm (t, J=3.6 Hz) and 5.18 ppm (t, J=3.6 Hz) could expose a sugar compound, e.g. glucose. In addition, signals found in chemical shift δ around 1.7 proposed the presence of an allylic functional group (C=C-CH3).

3.14.3 Separation by Column Chromatography (PT_1B-5 - PT_1B-8)

The fractions PT_1B-(5-8) from Table 3.24 were combined (0.6 g) and using further silica chromatography (approximately 4.5 cm column width and 25 cm length) to afford 0.5 g, 83% yield. The details of fractions obtained from the column chromatography are given in the Table 3.25.

In total ten fractions were collected from this column and analysed by TLC and ^1H NMR spectroscopy. The TLC analysis unfortunately again indicated incomplete separation of the sample into its components and this was confirmed by the NMR spectrum which showed a mixture of component.

Table 3.25 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	R_f EtOAc: Pet. (1:1)	Mass % (% of 0.6 g crude extract)
PT_1B_1-1	EtOAc:Petrol (1:99)	-	9.8%
PT_1B_1-2	EtOAc:Petrol (1:99)	0.8	0.18%
PT_1B_1-3	EtOAc:Petrol (1:99)	0.8	16.1%
PT_1B_1-4	EtOAc:Petrol (1:99)	0.8	14.3%
PT_1B_1-5	EtOAc:Petrol (1:99)	0.55	3.6%
PT_1B_1-6	EtOAc:Petrol (2:98)	-	7.1%
PT_1B_1-7	EtOAc:Petrol (5:95)	-	11.6%
PT_1B_1-8	EtOAc:Petrol (10:90)	-	10.7%
PT_1B_1-9	EtOAc:Petrol (10:90)	-	8.9%
PT_1B_1-10	EtOAc:Petrol (10:90)	-	12.9%

The Table 3.25 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, R_f values.

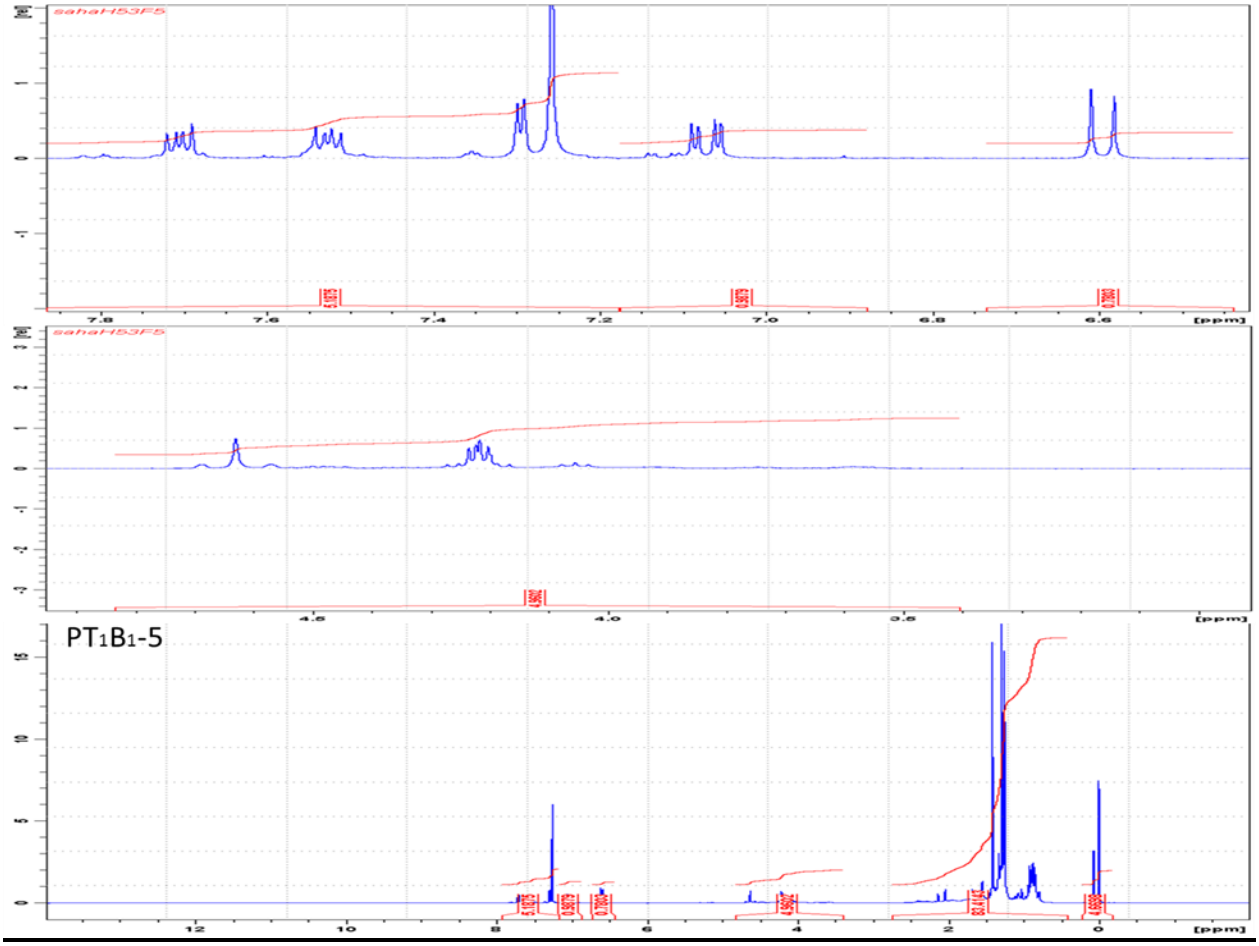


Figure 3.28 NMR of fraction PT_1B_1-5

Figure 3.28 revealed incomplete separation of sample (e.g. fraction PT_1B_1-5) show signals at 6.59 ppm (d, J=8.2 Hz) and 7.07 ppm (d, J=8.2 Hz) appeared as double of doublets with a coupling constant indicating a cis double bond in carbonyl system. Also signals at 7.13 ppm (br. d, J=2.6 Hz), 7.29 ppm (br. d, J=2.6Hz) suggest other carbonyl group. While Figure 3.29 (for fraction PT_1B_1-9) 7.06 ppm (br. d, J=4.8 Hz), 7.11 ppm (br. d, J=4.8 Hz), 7.19 ppm (br. d, J=4.8 Hz), 7.39 ppm (br. d, J=5.4 Hz), 7.52 ppm (br. d, J=5.4 Hz), 7.70 ppm (d, J=5.4 Hz) show similar signal as fraction PT_1B_1-5. Accordingly the results of ^{1}H NMR plus TLC analysis showed that the two column (Table 3.24 and Table 3.23) had only been partially successful in separation the various components (PT1A-3, section 3.13.2) in the

mixture with considerable overlap between all the fraction. Accordingly with available data, it is difficult to make an absolute assignment of these signals or to do a further analysis of the spectra.

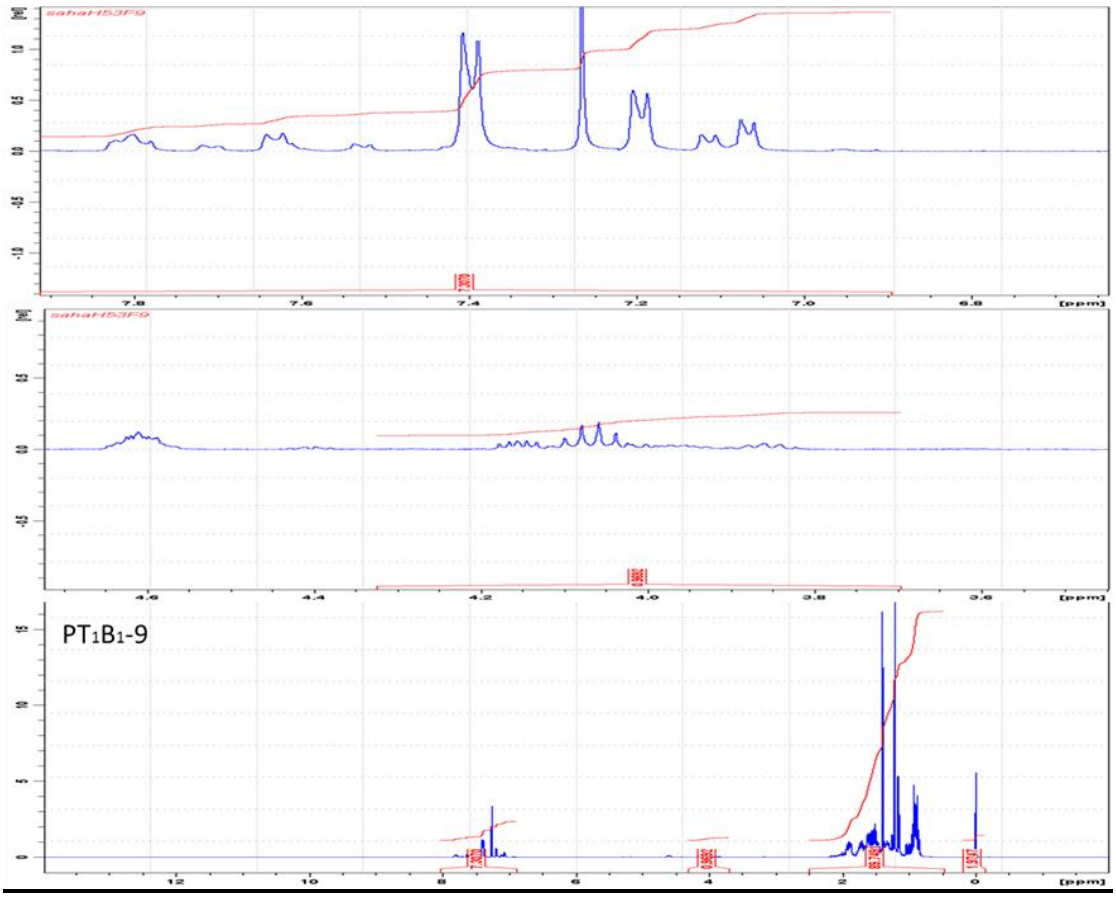


Figure 3.29 NMR of fraction PT_1B_1-9

3.14.4 Separation by Column Chromatography (PT_1A-7)

Fraction PT_1A-7 (from section 3.14, Table 3.22) was also further chromatographed on silica gel (approximately 3 cm column width and 25 cm length) to afford 0.17 g, 85% yield. The details of fractions obtained from the column chromatography are given in the Table 3.26. In total eleven fractions were collected from this column and analysed by TLC and ^{1}H NMR spectroscopy. The TLC analysis still indicated incomplete separation of sample into its components and this was confirmed by NMR spectroscopy which showed a mixture of compounds.

Table 3.26 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	R_f MeOH:Diethyl ether:Pet.(1:2:8)	Mass % (% of 0.2 g crude extract)
PT_1C-1	MeOH: Diethyl Ether: Petrol (1:2:8)	-	3.1%
PT_1C-2	MeOH: Diethyl Ether: Petrol (1:2:8)	0.42,0.33	8.9%
PT_1C-3	MeOH: Diethyl Ether: Petrol (1:2:8)	0.42,0.33	3.7%
PT_1C-4	MeOH: Diethyl Ether: Petrol (1:2:8)	0.42,0.33	4.7%
PT_1C-5	MeOH: Diethyl Ether: Petrol (1:2:8)	0.42,0.33	7.9%
PT_1C-6	MeOH: Diethyl Ether: Petrol (1:2:8)	0.33, 0.30	2.1%
PT_1C-7	MeOH: Diethyl Ether: Petrol (1:2:8)	0.33, 0.30	2.7%
PT_1C-8	MeOH: Diethyl Ether: Petrol (1:2:8)	0.33, 0.30, 0.18	17.8%
PT_1C-9	MeOH: Diethyl Ether: Petrol (1:2:8)	0.30, 0.18	3.7%
PT_1C-10	MeOH: Diethyl Ether: Petrol (1:2:8)	0.05	9.9%
PT_1C-11	MeOH: Diethyl Ether: Petrol (1:2:8)	B	26.7%

The Table 3.26 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, R_f values. B=bottom.

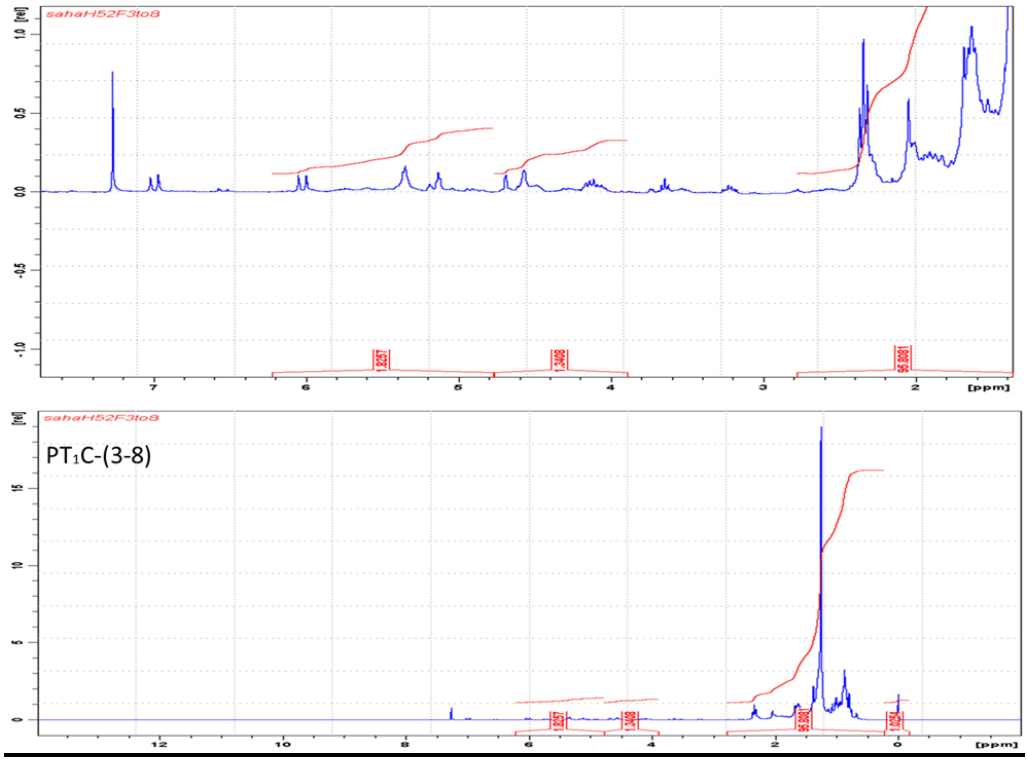


Figure 3.30 NMR of fraction PT1C (3-8)

All of the fractions isolated from this column (Table 3.26) were analysed by ^{1}H NMR spectroscopy. Unfortunately all of the fractions are clearly mixtures (also evidenced by TLC analysis). Combination of fractions PT_1C-(3-8) and analysis by ^{1}H NMR spectroscopy Figure 3.30 it proved impossible to do a further analysis of the spectrum.

3.14.5 Separation by Column Chromatography (PT_1C-3 to PT_1C-8)

The NMR spectra of all fractions from the column (see 3.14.4, fraction PT_1A-7), even though combined most fractions [Figure 3.30, PT_1C (3-8; 0.4 g)] showed that they were still a mixture. Further purification was done using silica chromatography (approximately 3 cm column width and 25 cm length) to afford 0.35 g; 87.5% yield. The details of fractions obtained from the column chromatography are given in the Table 3.27. In total ten fractions were collected from this column and analysed by TLC and ^{1}H NMR spectroscopy.

Table 3.27 illustration the separated fractions eluted from column chromatography

Fraction	Solvent system	R_f EtOAc: Pet. (1:1)	Mass% (% of 0.4 g crude extract)
PT_1C_1-1	EtOAc:Petrol (2.5%)	0.54,0.46, 0.36	2.6%
PT_1C_1-2	EtOAc:Petrol (2.5%)	0.52, 0.46, 0.34, 0.28	2.6%
PT_1C_1-3	EtOAc:Petrol (2.5%)	0.52,0.46, 0.28	2.6%
PT_1C_1-4	EtOAc:Petrol (2.5%)	0.52, 0.46, 0.39	2.6%
PT_1C_1-5	EtOAc:Petrol (2.5%)	0.46, 0.39, 0.24, 0.28	30.8%
PT_1C_1-6	EtOAc:Petrol (5%)	0.39, 0.24, 0.11	7.7%
PT_1C_1-7	EtOAc:Petrol (5%)	0.45, 0.39, 0.24	7.7%
PT_1C_1-8	EtOAc:Petrol (5%)	0.86, 0.66, 0.24	7.7%
PT_1C_1-9	EtOAc:Petrol (5%)	0.81, 0.44, 0.36	17.9%
PT_1C_1-10	EtOAc:Petrol (1%)	0.42, 0.36, 0.24	7.7%

The Table 3.27 shows similarities and differences among the fractions in terms of the use of different solvents, number of spots, yields, R_f values.

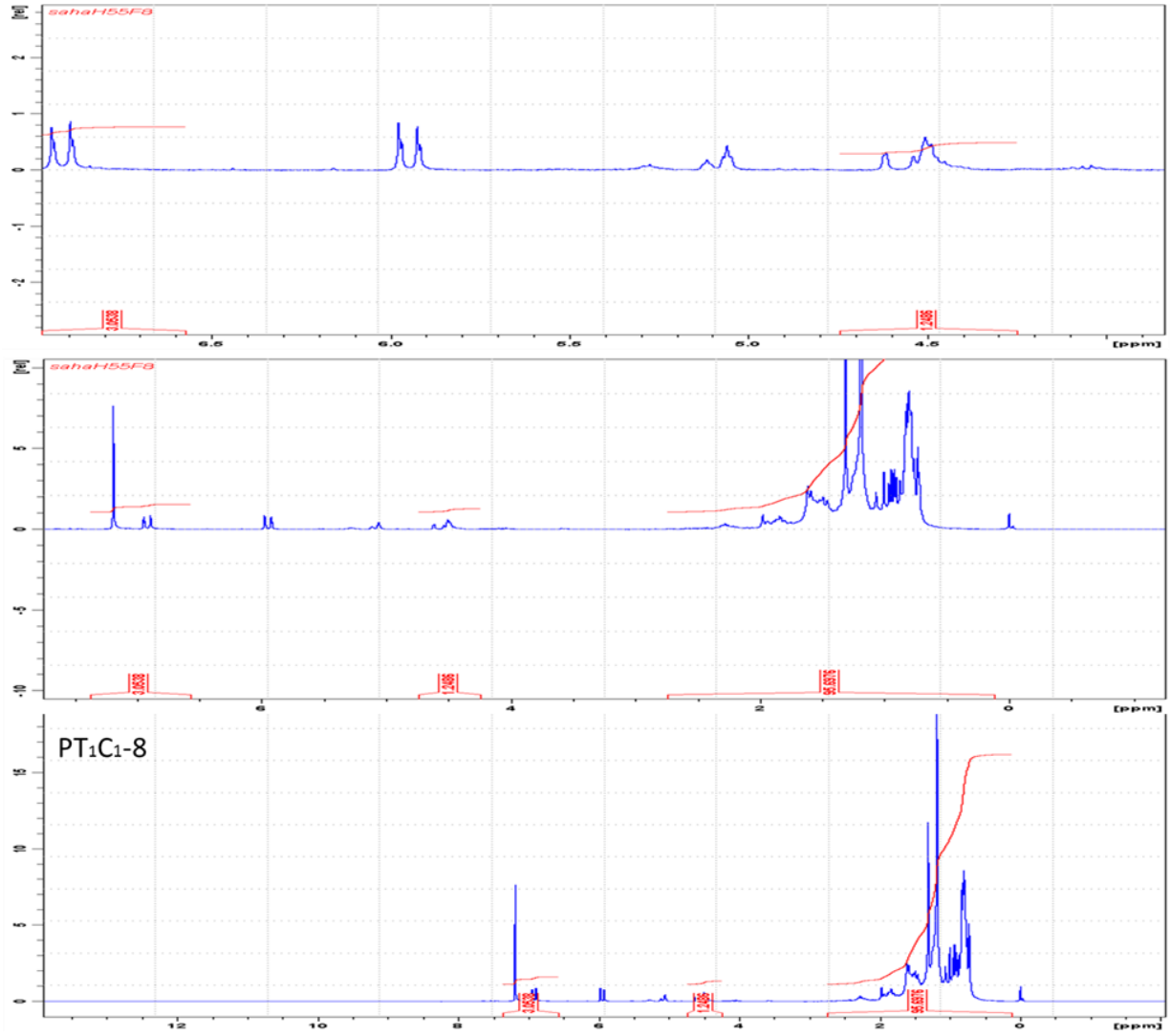


Figure 3.31 NMR of fraction PT1C1-8

Although the NMR spectra were difficult to interpret due to incomplete separation a number of interesting structural features could be assigned to the fractions. A representative spectrum (for fraction PT_1C_1-8) is shown in Figure 3.31. Whilst there are obviously many signals in the upfield hydrocarbon region (from 1 ppm – 2 ppm) which are difficult to analyse some more distinct signals can be made out in the downfield regions. Thus we can see a pair of doublets at 5.94 ppm (d, J=15.7 Hz) and 6.91 ppm (d, J=15.7 Hz) with a coupling constant indicating a trans double bond. The large difference in chemical shift between the two signals also suggests that this

may from part of an α, β-unsaturated carbonyl system. These is also a triplet at 5.05 ppm (J=3.6 Hz) which could seem to indicate the presence of a CH_2-CH-X (X= O or N). Also singlet at 4.62 ppm may represent sugar. However, overall it is difficult to make too many conclusions from this data.

3.14.6 Separation by Prep-HPLC (PT_1A-16)

In order to determine the number of the constituents present in fraction PT_1A-16 (from section 3.14, Table 3.22) prep-HPLC reverse phase of the semi-purified fractions PT_1A -16 was carried out using a short column (water spherisorb, $SSODS_2$, 20 X 250 mm, pre-column) using a gradient mobile phase (Table 3. 28) at a flow rate of 8 ml / min, concentration of 10 mg/ml and an injection volume of 2.5 ml. Detection was carried out at 280 nm. The sample was dissolved in the mobile phase (MeOH: Water 5:95,v/v). The analysis time was 50 minutes. Since retention times varied between each run and the peak shapes changed from run to run this led us to collect our samples fractions according to time periods. The fractions from this HPLC separation were labelled H_2Me-1 to 11 (see Table 3.28). All the fractions collected from these HPLC separations were subjected to the standard DPPH• assay and total phenolics determined by Folin-Ciocalteu colourimetric assay. The results are shown in Table 3.29

Table 3.28 HPLC conditions for attempted separation of fraction PT_1A-16

Time (min)	0.0	5.0	30.0	35.0	40.0	50.0
Solvent (MeOH)	5	5	100	100	5	5
Solvent (Water)	95	95	0	0	95	95

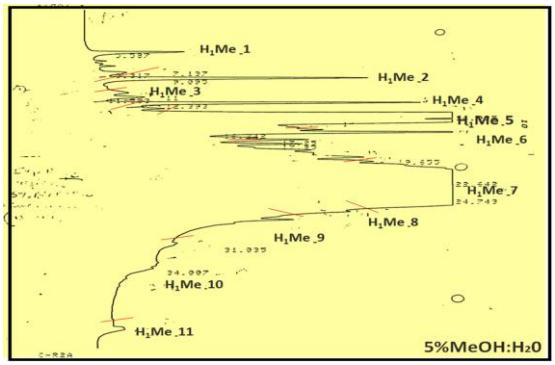


Figure 3.32 Prep-HPLC analysis of the separation of fraction PT_1A-16

3.14.7 Antioxidant activities and total phenolics of isolated fractions H_2Me (1 to 11)

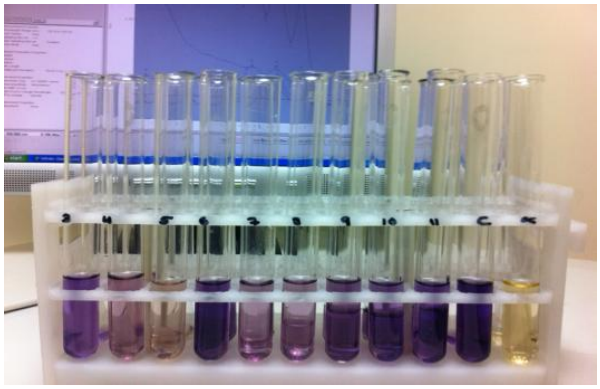


Figure 3.33 The DPPH• assay: free radical reduced by antioxidant in fractions e.g. (H_2Me- (2 to 11) change the colour from purple to yellow (with varying degree depending on the quantum of antioxidants in each fraction). The α-tocopherol is used as antioxidant standard.

Table 3.29 demonstrated total phenolic content and DPPH• radical-scavenging activity of isolated fractions of *P. tomentosa*

DPPH• assay (%IP)			Total phenolics as α-tocopherol equivalents (mg/g) of collected fraction	
(Prep-HPLC) reverse phase 5% MeOH:H_2O)				
	15 min	O/N	30 min	120 min
Control at zero 0.446	(0.526)	(0.579)	(0.0125)	(0.0145)
α- Tocopherol	91.8%	93.8%	(0.0125)	(0.0145)
H_2Me-1	7.2%	23.8%	1584%	1717.2%
H_2Me-2	17.5%	42.3%	1298%	1481.5%
H_2Me-3	27.0%	40.8%	1448%	2372.4%
H_2Me-4	36.1%	50.1%	255.7%	1889.7%
H_2Me-5	49.4%	66.8%	435.3%	1313.3%
H_2Me-6	12.4%	9.9%	576%	2937.9%
H_2Me-7	34.4%	48.0%	1992%	2200%
H_2Me-8	30.4%	49.4%	1328%	2048.3%
H_2Me-9	10.8%	29.4%	1440%	1222.8%
H_2Me-10	17.9%	00.00%	1112%	986.2%
H_2Me-11	11.6%	1.4%	1193.2%	1080.4%

Antioxidant activities and total phenolic contents of plant extracts (a) tested at 50 µg/ml concentration (b) tested at 10 µg/ml conc. And results presented as α-tocopherol equivalents (mg/g). maximum reproducibility of DPPH• ±0.07 and for total phenolics ±0.06.

As illustrated in (Table 3.29, Figure 3.33) confirmed that the fraction H_2Me-5 has the greatest antioxidant activity (DPPH• inhibition) 66.8% followed by fraction H_2Me-4 (50.1%). Moderate antioxidant activity was observed for H2Me-(2, 3, 7 and 8) in the range of (40.8% to 49.4%), followed by H_2Me-1 and H_2Me-9 in the range (23.8% - 29.4%). The lowest level of antioxidant activity was detected for the fractions H_2Me-6 and H_2Me-11 in the range (1.4% - 9. 9%) The H_2Me-11 fraction showed no % inhibition after overnight stand in DPPH• assay. Total phenolic contents (mg of α-tocopherol equivalents / g fraction extracted) were investigated using the Folin-Ciocalteu colorimetric method. The results are shown in Table 3.29. Fractions (H_2Me-6, H_1Me-3, H_2Me-7 and H_2Me-8) after 2 hours reveal high levels of total phenolics as α-tocopherol equivalents (mg/g) almost double than other fractions (Table 3.29). Generally, these studies confirmed that the antioxidant activities are independent of

the total phenolic contents. Wheres, the Folin-Ciocalteu colourimetric assay show different responses to different phenolic compounds according to chemical structure.

3.15 Conclusion

Phytochemical analysis of the *P. tomentosa* in this study has given the perspective to provide humans with a wide range of antioxidant compounds as well as to have a mixture of antioxidants compounds that may be effective in several physiology activities. As mentioned earlier from previous researcher isolated compounds from plants improved the medicines due to synergistically character of those natural extractions. Antioxidant capacity assessment is assay dependent as reported by (Rice-Evans,2001)[208] The specificity and sensitivity of one assay is not necessary signal its validity and complementary in extract assess for all phenolic compounds and antioxidants in the plant extract. Therefore, to get better evaluation and assessment of antioxidant activity data need to share several methods together.

In conclusion, the present studies on crude *P. tomentosa* and its isolated fractions demonstrates that the extracts of ethanolic *P. tomentosa* and its fractions tested contained a selection of different type of phenolic compounds identified to have antioxidant activity and also determined of total phenolic content by Folin-Ciocalteu method. Crude *P. tomentosa* extracts exhibit relatively higher antioxidant potentials as compared to *Ziziphus H.*, which may be acting therapeutically, as antioxidants potential assessment of phytochemicals. However, the findings of these studies provide scientific support to the traditional use of these plants in healing wounds and other skin disorders in various parts of the world including Oman by virtue of their antioxidant potentials.

As a future research all fractions that found to be mixtures need to be separated to eliminate the minor components of the mixtures.

3.16 Material and Apparatus

Thin layer chromatography of compounds was conducted on silica plate, *Merck*® silica gel 60 F254 thin layer chromatography plates. Column chromatography to purify compounds was carried out using *Fluka*® silica gel 60. The fraction was applied to the plate using a pulled glass capillary. TLC plates were run in various solvent systems depending on the polarity of the sample. The compounds were detected under short UV (254 nm) and long UV (365 nm) wavelengths and in daylight, and then sprayed with a visualising agent, 3 g PMA (phosphomolybdic Acid) in 100 ml absolute ethanol. TLC plates were used to monitor the progress of the fractionation and to pool similar fractions.

- Low-resolution electrospray mass spectra were recorded using a Micromass Quattro-LC using electrospray ionisation from solution and a Quadrupole analyser.
- High-resolution fast atom bombardment spectra were recorded using a Kratos™ Concept IIH 2-sector mass spec, using xenon as the fast atom bombardment gas and *m*- nitro benzyl alcohol as the matrix (unless otherwise stated) with 8 kV as accelerating voltage and a resolution of @2000[10 % valley] for nominal spectra. Accurate mass determination was achieved using a 'peak matching' technique against polyethylene glycol as reference and a resolution of @10000 [10 % valley].
- Other high-resolution spectra (electrospray) were recorded using a Waters™Xevo Q-time of flight instrument using electrospray ionisation from solution. The instrument was pre-calibrated using sodium iodide and had a "lock mass" of leucine enkephalin. This enabled a mass accuracy of @2ppm at a resolution of @10000 [full width at half maximum].
- Gas chromatography mass spectrum (electron ionisation) spectra were recorded using a *Perkin Elmer*® Turbo Mass, using a pre-calibrated single quad analyser, with electron ionisation giving nominal mass spectra, with searchable NIST™

database. Gas chromatography (flame ionization detector) spectra were recorded using a *Perkin- Elmer*® Clarius 500 Gas Chromatograph instrument.

- Nuclear magnetic resonance spectra were recorded using one of the instruments below:
  - *Bruker Biospin*® DPX300 ™ 5mm QNP probe (1H/13C) with Z axis gradient, XWIN- NMR v 3.5-pl0, 1H=299.90 MHz, 13C=75.41 MHz
  - *Bruker Biospin*® DRX400 ™ 5mm QNP probe (1H/13C), XWIN-NMR v 3.5-pl6, 1H=400.13 MHz, 13C=100.61 MHz
  - *Bruker Biospin*® AV500 ™ 5mm BBO probe with Z axis gradient, TOPSPIN v 2.1, 1H=500.13 MHz, 13C=125.76 MHz
  - 1H NMR Chemical shifts are reported in ppm using TMS or the residual proton signal from the solvent as an internal reference.
- High performance liquid chromatography analyses were performed using a *Perkin Elmer*® Series 200 System, operating with *Perkin Elmer*® TotalChrom software. The columns used were CHIRALCEL OD-H, CHIRALCEL OJ, CHIRALPAK AD and CHIRALPAK AS manufactured by *Daicel Technologies ™.* All were 250 mm × 4.6 mm. The wavelength parameters used were a maximum wavelength of 250 nm and minimum wavelength of 300 nm the viewing wavelength being 240 nm and 280 nm
- Prep-High performance liquid chromatography (SHIMADZU LC-4A Liquid Chromatograph), is the most useful instrument for the separation and identification of multi-complex mixtures of small fragments in the plant extract. The columns used for Reversed phase were [(Water Spherisorb, SS ODS_2, 250× 20 mm) and (Ascentis™ SI, 5 micron manufactured by SUPELCO, Cat NO. 581515-u, Col.79868-01, BL: 5937, 25 cm × 4.6 mm 250 mm × 4.6 mm)]. The wavelength parameters used were a maximum wavelength of 250 nm and minimum wavelength of 300 nm the viewing wavelength being 280 nm**.**
- Ultra – violet/ Visible Spectroscopy (UV-VIS Spectroscopy), UV absorption spectra were obtained on a SHIMADZU UV-2401PC, UV-VIS Recording Spectrometer.

- All materials used were of analytical grade. Petroleum ether 40-60, ethyl acetate, ethanol, 2,2-diphenyl 1-1-picrylhyrazyl (DPPH•,100mg), δ-tocopherol and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich, Alfa Aesar Lancaster and Strem. Solvents were dried using an Innovative Technology Inc. Pure solvent purification station. Removal of solvents *in vacuo* was done using a Buchi rotary evaporator followed by drying with a high vacuum pump. Solvents for chromatography were routinely basified with ammonia and solvents used for extraction were re-distilled.
- All reactions were carried out in oven-dried glassware under dry nitrogen unless stated otherwise.
- DPPH• and α-tocopherol were purchased from Sigma-Aldrich. Analytical grade material was obtained from Fisher Chemical Company.

CHAPTER 4

Chapter 4 Biological Testing of the *Pergularia tomentosa* Crude Extracts-and Isolated Fractions

4.1 Material and Methods

4.1.1 Materials

4.1.1.1 Bacterial Strains.

The details of the bacterial strains used in this thesis are summarized in the table below.

Table 4.1 Bacterial strains used in this thesis

Strain	Relevant characteristics	Source or reference
Staphylococcus aureus	Newman	Freestone *et al.* (2008) [209]
Pseudomonas aeruginosa	UCBPP-PA14	Lee *et al.* (2006) [210]
Escherichia.coli	Enteropathogenic *E. coli* strain E2348/69,serotype O127:H6	Freestone *et al*. (1999) [211]

4.1.1.2 Bacterial Culture Media

The basic media used to grow bacterial strains in this part of experiments are found below.

Luria broth (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract (oxoid), and 0.5% (w/v) NaCl, adjusted to pH 7.0 with 1M NaOH.

Luria agar (LA) LB solidified with 1.5% (w/v) agar.

Yeast extract Peptone (YPD medium) 2% Bacto peptone, 1% yeast extract, 2% glucose, 80 µg/ml uridine.

4.1.2 Sterilization of Media

The liquid media constituents for bacterial culture were sterilised by autoclaving at 121 ºC, 15 lb/inch^2 for 15 minutes and then stored at room temperature until required.

For plate culture of bacteria, LA was melted in a microwave oven, and then allowed to cool to 50 ºC before pouring (approximately 20-25 ml/plate) into sterile 90 mm plastic petri dishes. After setting, the plates were dried at 37 ºC, and stored at 4 ºC.

4.1.3 Bacterial strains preservation

For long term preservation, bacterial strains were growth overnight in LB at 37 °C with shaking and then mixed 1:1 with cryoprotectant medium (50% (v/v) glycerol), and multiple aliquots of the suspension stored at ˉ80 °C. For short term working (2-3 months), stability of the bacterial strains used was maintained by re-plating on LA every 3-4 weeks and the stock replaced from frozen culture stocks.

4.1.4 Fungal strain culture

Candida albicans strain SC5314 plated on YPD plate (containing 2% glucose, 80 µg/ml uridine).

4.1.5 Chemicals

All chemicals material used were of analytical grade and purchased from Sigma-UK or Fisher Scientific-UK, unless stated otherwise.

4.2 Methods

All experiments that required reproducible replication were performed at least in triplicate unless stated otherwise.

4.2.1 Time course of bacterial growth in LB (*S. aureus,* PA14 and E23) in the presence of plant extracts

An inoculum of the bacteria strains was grown overnight in LB at 37 °C, in a shaking incubator set at 180 rpm A 1:100 dilution of the inoculum was added in 20 µl triplicate volumes to a 96 well culture plate. Incubation of the cultures was carried in a Varioskan spectrophotometer (Transgalactic Ltd), set at 37 °C and 180 rpm. The growth as measured by increase in optical density at 600 nm was monitored for 24 hours, with readings taken every 15 minutes. Growth curves were plotted from

triplicate experiments using three independent cultures using Graph Pad Prism software.

4.2.2 Preparation of Plant Material

Plant material was collected and extracted for bioactive compounds on two separate occasions in January 2010 and January 2011. The plants were collected from Ibra in the Sultanate of Oman. The department of Chemistry, Sultan Qaboos University, authenticated the identity of the plants. Air-drying as a form of preservation was carried out for *Pergularia tomentosa* at 50 °C in the dark for two weeks to prevent photo-degradation of bioactives. The dried plant material was then ground down using commercial miller to give 2 kg of both plant stems and leaves.

4.2.2.1 Extraction & Isolation

Crude dried plant extracts *(Pergularia tomentosa*) (2 kg) were extracted by standing with different solvents (from non-polar to polar). This extraction started with Pet. Ether, 8 L for 3-4 days, then followed by EtOAc, 7.5 L for 5 days and ended with EtOH, 6 L for 7 days. The solvents were partly evaporated using a rotary evaporator at 69 °C and any residual solvents removed from the resulting residue under high vacuum. All extracted material was stored in a ˉ18 °C freezer until use.

4.2.3. Antimicrobial Activity Investigations

Crude plant extracts were screened for antimicrobial activities against EPEC *Escherichia coli* (E23*), Pseudomonas aeruginosa* (PA14) *and Staphylococcus aureus.* The activity assay was performed by dissolving the crude plant extracts in DMSO to a concentration of 56 mg/ml for crude extract and 5 mg/ml for isolated fractions from column chromatography. Overnight cultures of the different bacteria grown in LB were diluted (1:100 dilutions) in LB and time courses of growth responsiveness to the extracts at 37 ^{o}C were carried out as described in Section 4.2.1. Growth curves shown were derived from triplicate time courses using three independent cultures.

4.2.4 Disc-well Diffusion Method to Assess Antimicrobial and Antifungal Properties of the Crude *P.tomentosa* extract and isolated fractions

Due to scarcity of material following the solvent extractions, only a small number of isolated compounds could be tested for the presence antimicrobial and antifungal activities. The activity of plant extracts against either bacterial or fungal strains (E23, PA14, *S. aureus* and *C. albicans*) was typically assessed using the disk diffusion method. The inhibition of growth in these tests was expressed as a percentage of the growth of control colonies in the absence of the proposed inhibitor. The disk diffusion assay involved the preparation of a petri dish containing 15-25 ml LA. A 250 µl volume the test cultures were spread on the agar surface and allowed to dry. Sterile filter paper discs (6 mm prepared using a hole puncher) were then placed on the surface of the agar plate and distributed in a way to not overlap between two different test samples. The plant materials under test were dissolved in dimethylsulfoxide (DMSO) at a concentration of 56.0 mg/ml and 10 µl of the extract were pipetted to the centre of each paper disc. Up to 4 paper discs were placed on each 90 mm plate to prevent overlapping on the zone of inhibition area and possible errors in measurement. The plates were inverted and incubated at 37 °C for 24 h after which the diameters of the zones of growth inhibition around the disc were measured and recorded in millimetres (mm).

4.3 Statistical Analysis

All experimental results were expressed as mean ± standard deviation (SD) where n = the number of samples. Analysis was carried out using one-sample t-test has been used to compare one group to a hypothetical value (P value). Also, t test for unpaired group, while one-way ANOVA analysis was used to compare three or more groups t-test or one-way and two-way analysis of variance (ANOVA). Values at $p < 0.0001$ were considered statistically significant (ns = not significant) as compared to the relevant control. Analysis was carried out using Excel 2010 or Prism version 5 for

windows (GraphPad Software, San Diego California USA). All individual experiments were run in triplicate and repeated on at least 3 separate occasions.

4.4 Results and Discussion

4.4.1 Introduction

Pergularia tomentosa is a perennial herbaceous plant that is; commonly used as an abortive, for treatment of skin diseases and a depilatory.[212] Nigerians use it to tan the skin and to poison arrowheads for hunting.[213] A previous study has reported molluscicidal activity of extracts from this plant[214] and persistent hypoglycemic effects caused by *P.tomentosa* have also been reported by Shabana *et al.*[34] A study by Hamed *et al.*[196]revealed apoptotic activity of *P. tomentosa* for Kaposi sarcoma. Similarly, Martin *et al*[215] reported cytotoxic activity of *P. daemia* against cell lines derived from an ovarian cancer. In the same manner, Piacente *et al.*[35] reported marked cytotoxic activity of *P. tomentosa* extracts caused by inhibition of an essential cellular Na+/K+-ATPase. These studies bring to light the awareness that species of *P. tomentosa* are of considerable importance in the field of human health care.

4.4.2 Antioxidant approach to wound healing and the role of *P.tomentosa*

Previously the quest for natural remedies has drawn researchers' attention to herbal preparations.[216,217] Polyphenolic bioflavonoids like proanthocyanidins are obtained from plants or plant extracts.[267] Sen *et al.* [267] and Khanna *et al.* [218] have reported the combination of biologically active polyphenolic bioflavonoids and resveratrol help vascular endothelial growth factor (VEGF) expression and facilitate angiogenesis and wound healing by working as antioxidants. Traditional Peruvian medicine uses bark of *Uncaria tomentosa* (cat's claw) for the treatment of inflammation.[219] Suntar *et al*.[220] studied *Rubus sanctus Schreber* (Rosaceae) and demonstrated its role in wound healing, supporting its ethnobotanical usage. All these studies reveal that herbal preparations can be used as antioxidant agents that are beneficial for wound healing.

Previous studies on *P. tomentosa* have shown it has potent antioxidant properties. Piacente *et al*.[35] reported that *P. tomentosa* inhibits Na^+/K^+ ATPase activity and displays cytotoxic properties preventing the morphologic changes seen in cancer cell lines. Antioxidants protect cells from free radical damage that is associated with cancer development.[221] Additionally, plant extract from the leaves of the species of this herb shows antioxidant activity *in vitro*.[222] Similarly, Marwah *et al.*[37] have reported that *Z. hajarensis* contains antioxidant activity (due to its phenolic content) but at levels less than other plant species used therapeutically such as as *Becium dhofarense, Pulicaria crispa, Anogeissus dhofarica, Ficus lutea, Allophylus rubifolius, Moringa peregrina and Acalypha indica*. In short, research done on these two plants species shows that they display antioxidant properties and may help protect the body from the harmful effects free radicals, leading to useful wound healing properties.

4.4.3 Antibacterial activities

The increasing antibiotic resistance of bacteria has stimulated researchers discover new ways to prevent bacterial growth and infections. There is a need of new strategies and targets to avoid this overwhelming resistance. A number of chemical agents broadly classified as antimicrobial agents and including disinfectants and antiseptics, can be used to control the growth of microorganisms. These agents work in different ways to control or stop bacterial growth and no single agent is effective in all situations. Different antimicrobial agents are required for different microbes depending upon their pH, solubility, toxicity, organic material and cost.[223] Inhibition of specific virulence factors is appearing as a new strategy for targeting specific bacteria, as virulence factors are necessary to establish an infection within a host [224,225] Various methods exist with which to examine the inhibition of bacterial growth by chemical agents. The agar diffusion method has also been employed to assess bacterial growth inhibition as it allow testing of multiple antimicrobial test samples on a single plate of the target microbe.[223] In this method, microbes are uniformly swabbed onto a nutrient agar plate and the disks containing chemical are added to the surface of agar. The plates are then incubated for a set period of time and any

inhibition of growth is determined by measuring the zone of non-growth around the impregnated discs.

Increasing bacterial resistance is an evident challenge to modern health care. This has led to the need to develop new compounds to control microbial growth that are suitable for human or animal use. These emerging challenges have stirred researchers to explore herbal extracts for their anti-bacterial properties. Ethno pharmacologists, microbiologists, botanists and chemists are therefore working together to discover and develop phytochemicals that are effective for the treatment of resistant infections. Plants are being tested for their antibacterial properties and a number have been established and used for this purpose.[8] Additionally, some of the plant species with potentially the most potent inhibitory effects on pathogen growth are on the verge of extinction, and so there is an urgent need to establish their antibacterial effectively as soon as possible, so that steps can be taken to preserve the plant species itself.[226] Pevious studies have revealed that plant extracts can be an excellent source of stable and biologically active antimicrobial agents. Additionally, the studies discussed above have also indicated that extracts from *Pergularia tomentosa* can show significant antioxidant and cytotoxic properties[35,37] Therefore the aim of this current research was to examine the potential anti-bacterial and anti-fungal properties of *P. tomentosa* obtained from Oman.

4.4.4 Fungal infections

Few studies have examined the ability of plant extracts to inhibit the growth of fungal pathogens such as *C.albicans.* Infections by this yeast are common and can cause significant distress among dermatology patients, and life threatening systemic infections in the immune-compromised such as HIV patients. Amphotericin B was the first antifungal major agent which came into use in the 1950s. However, anti-fungal can have significant side effects on humans because both host and microbe are eukaryotes and share similar biology. Plant extracts are being explored as possible anti-fungal, and the active principles were found to be secondary metabolites, which have been isolated from different parts of plants.[227] Thus another aim of this study

was to investigate the therapeutic ability of *P. tomentosa* extracts on growth of the pathogenic yeast *C. albicans*.

4.5 Antibacterial and antifungal activates of *Pergularia tomentosa* species and their isolated fractions.

Natural products especially from plants are now days being investigated as a possible source of novel antimicrobial agents.[228] Natural sources have previously been used to develop antibiotics e.g. streptomycin and penicillin [229] and so exploring natural plants as a resource is attractive they tend to be associated with fewer side effects.

4.5.1 Evaluation of antimicrobial and antifungal activity of *Pergularia tomentosa* species and its isolated fractions

This chapter aims to evaluate the antibacterial and antifungal properties of crude *P.tomentosa* and some its isolated fractions. Several methods are available to screening microbial and fungal activities, such as, biofilm formation and adherence and electron microscopic observation,[230] but these can be limited due to long processing time, high cost of some reagents and equipment and so in this study the effects on growth (time courses in liquid culture media and disc diffusion assays on culture plates), were used for evaluating the antimicrobial effects on test bacterial pathogens: a gram-positive species (*S. aureus*) and two gram-negatives (PA14 and E23) plus a fungus, *C. albicans*. The crude *P. tomentosa* extract and some of the fractions purified from it were extracted and isolated as described in Chapter 3 and diluted to working concentrations in DMSO as appropriate.

4.6 Anti-bacterial and anti-fungal properties of the crude plant extract

Ethanolic extracts of *P. tomentosa* were initially selected to investigate the plants antibacterial and antifungal properties based on our previous studies (see Chapter 3,

section 3.3). This was because it had been found that the majority of the anti-oxidant and phenolic content of the plant was located in the ethanolic extract, with very low levels present in both the petroleum ether and ethyl acetate extracts.

4.6.1 Disc diffusion analysis of the antifungal and antibacterial activity of crude extract of *P. tomentosa* on selected bacterial pathogens and *C.albicans*

Starter inocula of the selected bacterial and fungal cultures were grown overnight in LB and 250 µl of the culture was spread in agar surface and allowed to dry as described in section 4.2.4. After placing the filter papers on the agar plate, 10 µl of the of 56 mg/ml preparation of crude *P. tomentosa* extract solution were pipetted to the centre of each paper disc. For the negative solvent control an equivalent volume of DMSO was used. Finally all petri-dish plates were inverted and incubated at 37 °C for 24 h. After incubation, the diameters of the zones of complete microorganism inhibition were measured the results and recorded in millimetres (mm). A typical set of results is shown in figure 4.1

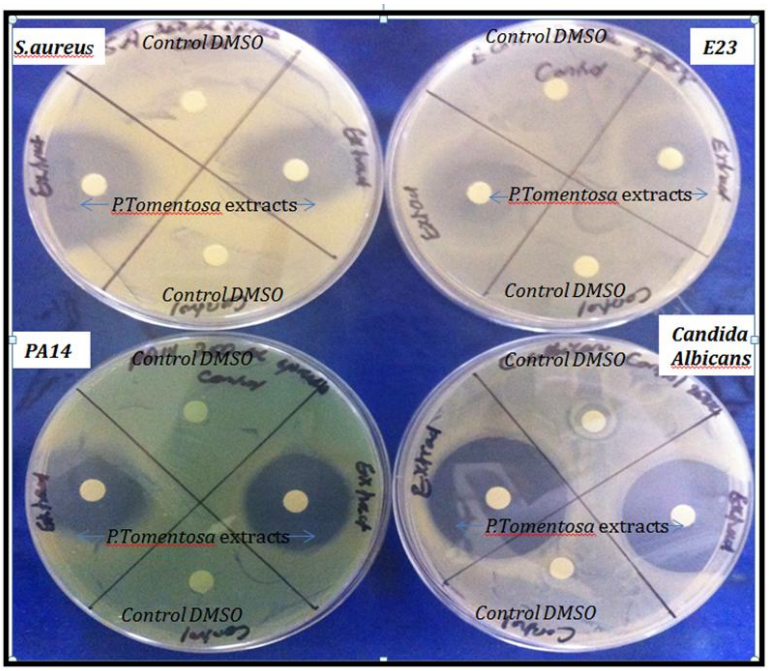


Figure 4.1 Antimicrobial activities of *P.tomentosa* extract against *S. aureus*, E23, PA14 and *C. albicans* using the well - diffusion method

Figure 4.1 shows that inhibition of growth is occurring against all the microorganisms tested. In the figure shown the largest zone of inhibition of 57 mm was measured against *C. albicans*, followed by a similarly large zone (approx. 50 mm) against *S. aureus* and PA14 49.5 mm and the smallest inhibition zone (24 mm) was observed with E23. The solvent DMSO, which was used to diluted the extract, showed only a minor inhibition against *C. albicans* and PA14 while it had no effect on E23 and *S. aureus*.

A similar experiment was conducted to investigate the solubility of the ethanolic *P.tomentosa* extract in distilled water. However, this method of dilution abolished the antimicrobial activity, probably due to the insolubility plant extract materials in water. Thus all further anti-bacterial and anti-fungal testing was done using solutions of the plant material (and purified components thereof) in DMSO.

4.6.2 Microbial growth in the presence of *P. tomentosa* extracts

Although disc diffusion is a common screening methodology to investigate anti-bacterial and anti-fungal properties, determination of the compound effects on growth profiles is also important. The objective of this study was to determine and the effects of the *P. tomentosa* on extract on microbial growth levels using a Miles & Misra or drop-plate colony count (Miles *et al.*, 1938, Herigsted *et al.*, 2001).[231,232]This study was performed to determine the ability of the ethanolic extract to inhibit bacterial and fungal liquid culture growth from a very low inoculum. Overnight cultures were serially diluted 10-fold in LB to give a cell density of around 100 CFU (colony forming units)/ml. Then, 100 µl of plant extract (56 mg/ml in DMSO) and 10 µl of the culture was added for each and the final volume made up to 1000 µl (1 ml) with LB – this gave a final concentration of 5.6 mg/ml of the plant extract. For the negative control pure DMSO was used at the same volume 100 µl. All tubes were incubated at 37 °C for 24 h. After incubation cultures were mixed, and then serially diluted in PBS in 10-fold dilution steps, followed by spot plating of 6 x 10 µl culture dilutions onto LA. Plates were allowed to dry and then incubated inverted at 37 °C for 24 h to allow for colony growth. The colonies present on the agar plates were counted and from the

serial dilution used the number of the colony forming unit per ml microorganism (bacterial and fungal) count were calculated as shown below.

Calculation of colonies forming units (CFU)

$$\text{CFU/ml} = \frac{\text{number of colonies x dilution factor}}{\text{Volume of culture plate (ml)}} \times 1000 \text{ ml}$$

Typical results are shown in figure 4.2

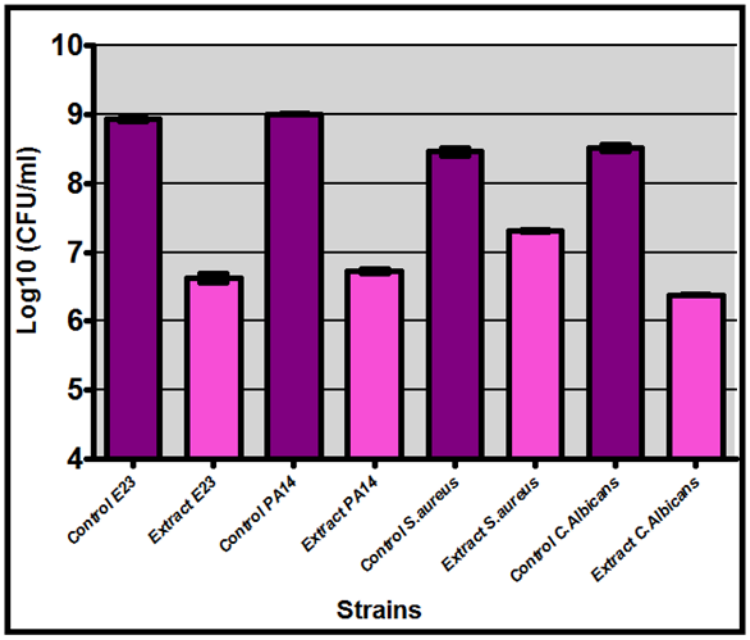


Figure 4.2 Effect of *P.Tomentosa* extract on the growth of the gram-negative and gram-positive bacteria (E23, PA14, *S.aureus*) and fungus (*C.albicans*). Key: the purple histogram represents the control of the experiment, and the pink shows the effect on growth of the test extract.

Figure 4.2 confirmed that the *P.tomentosa* extract showed significant antibacterial activity against *S.aureus,* E23, PA14 and *C.albicans*. The growth of E23, *P.aeruginosa and C.albicans* showed a 750 fold reduction and that of *S.aureus* a 100-fold reduction in growth. The results from this study agree in general with those from the disc diffusion analysis in the finding that the plant extract was able to inhibit

growth against all four of the organisms. However the relative order of inhibition is slightly different – the disk diffusion analysis suggested least inhibition with E23 whilst the CFU analysis indicated that *S.aureus* was least affected. This discrepancy is presumably due to the slightly different nature of the two tests – growth on a solid v's liquid medium and the non-quantitative nature of the disk diffusion analysis.

4.6.3 Dose response analyses of *P. tomentosa* effects on microbial growth

Due to the promising effect of crude ethanolic *P. tomentosa* extracts as anti-bacterial and anti-fungal agents, an extension of the antimicrobial screening was carried out by carrying out dose response analyses on the microbes using a time course of growth method. According to the results illustrated in Figure 4.2 there was strong growth inhibition of all the organisms at a 5.6 mg/ml concentration of the *P. tomentosa* extract. Therefore, concentrations ranging from 56 mg/ml to 1.8 mg/ml (Figure 4.4) of the extract were made up in order to determine whether growth inhibiton could be observed at lower concentrations.

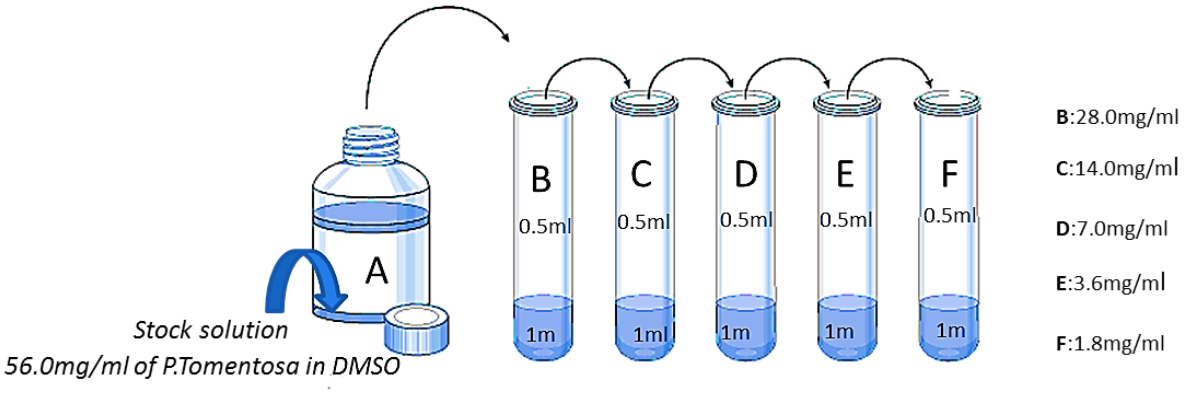


Figure 4.3 shows the dilution process for *P.tomentosa extract in DMSO*

Starter inoculums of selected bacterial strains E23, PA14 and *S.aureus* were grown overnight at 37 °C in LB in a shaking incubator set at 180 rpm . A 1:100 dilution of the overnight inoculate growth (10 μl) was then added to different dilutions of the crude extract (100 μl each), in 890 μl of LB. This gave a final concentration of the extract of 5.6, 2.8, 1.4, 0.7, 0.36 and 0.18 mg/ml in the assays. For the negative solvent control

an equivalent volume of DMSO was used. The supplemented and control cultures, LB, DMSO mixture with LB were added to 96 well plates in 200 µl triplicate volumes. Incubation of the cultures was carried out in a varioskan spectrophotometer, set at 37 °C and shaking at 180 rpm. The growth responses (changes in optical density at 600 nm) were observed for 24 h, with readings taken every 15 min. The time points were plotted using the Graph-Pad prism data analysis package and are shown in Figures 4.5 to 4.7.

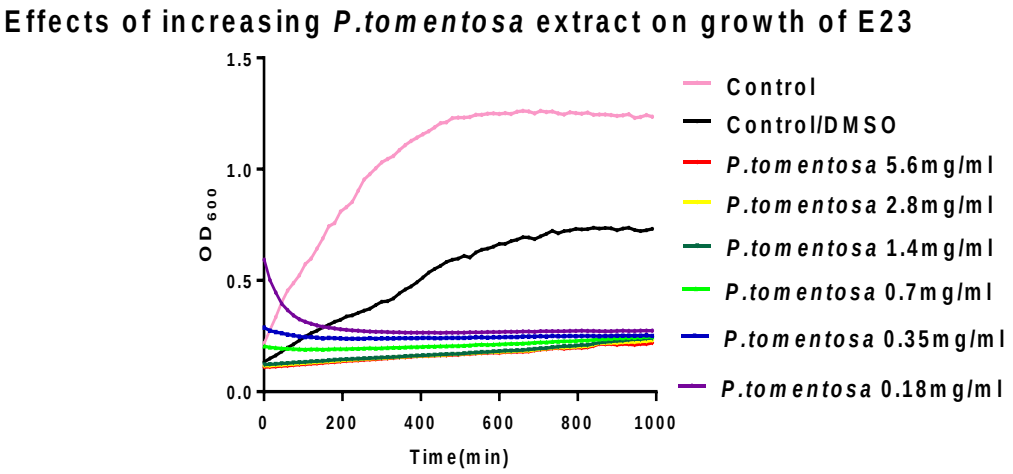


Figure 4.4 The antibacterial activity of different concentration of crude extract against E23

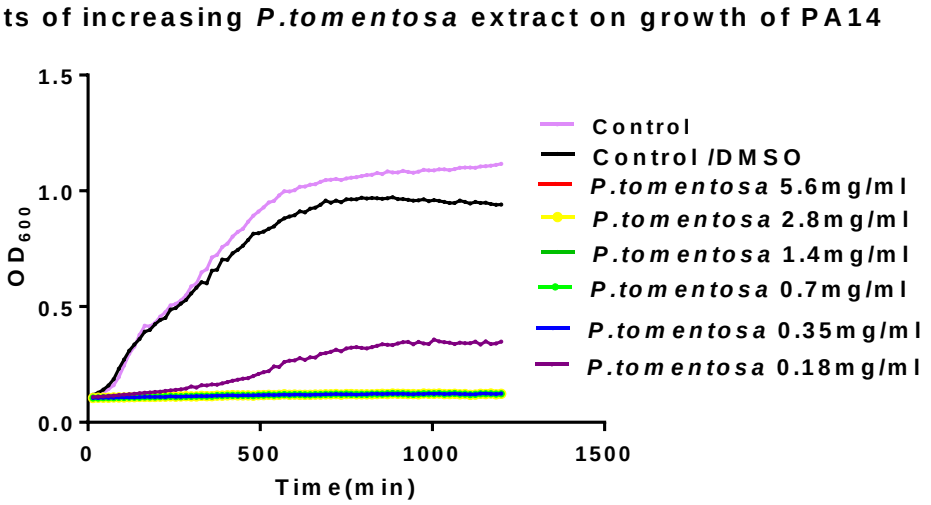


Figure 4.5 The antibacterial activity of different concentration of crude extract against PA14

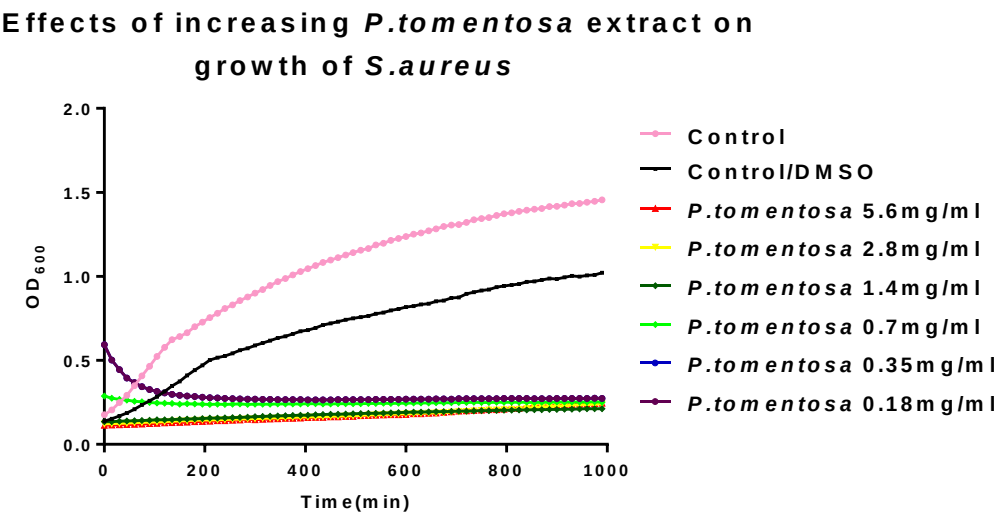


Figure 4.6 The antibacterial activity of different concentration of crude extract against *S.aureus*

The time course growth graphs show the microbial growth inhibition range of *P. tomentosa* extract levels. The values shown are the mean of triplicate assays. As can be seen in Figure 4.5, Figure 4.6 and Figure 4.7, in all selected bacterial strains, the ethanolic plant extract had a significantly high inhibitory effect against E23, PA14, and *S. aureus*, ($p<0.0001$) at all concentrations from 5.6 mg/ml down to 0.18 mg/ml when compared to DMSO which was used as a solvent control.

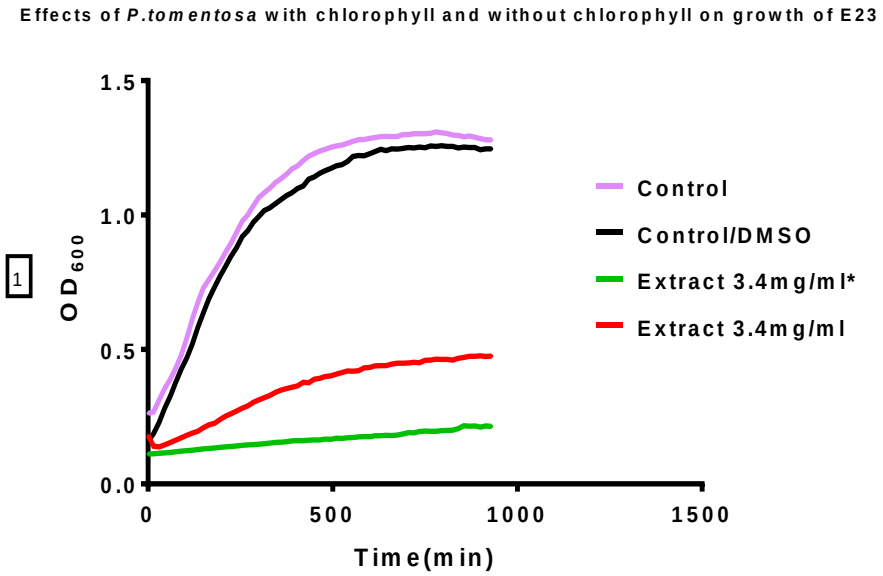
4.6.4 The effect of *P.tomentosa* (ethanolic extract) with chlorophyll and without chlorophyll on antimicrobial growth:

The crude *P. tomentosa* was analysed further by removal of the chlorophyll from the plant extract to determine if the presence of chlorophyll was involved in the inhibition of the microbes tested. To remove the chlorophyll, a 54.8 mg portion of the ethanolic extract was dissolved in 25 ml of boiling alcohol and approximately two medium size spatulas of activated charcoal were added. The resulting mixture was refluxed for a few minutes and filtered whilst hot by vacuum filtration using a Buchner flask and filter paper (Whatman No.1). The

filtrate was then concentrated under reduced pressure yielding 34 mg of material; this method is known as Chlorophyll (Talcum filtration). This chlorophyll free extract was then dissolved in DMSO to give a 34 mg/ml concentration and a separate solution of the crude extract (with chlorophyll still present) was also prepared at 34 mg/ml for testing. Starter inoculate of E23 and *S.aureus* were grown overnight in LB as described for Figures 4.7-4.8 and 1:100 dilutions were added to different volumes of the crude extract both containing chlorophyll and having had it removed and the final volume made to 1000 μl with LB. The amounts of extract tested (and the corresponding final concentration of the sample) were 100 μl (3.4 mg/ml), 50 μl (1.7 mg/ml), 25 μl (0.85 mg/ml), 10 μl (0.34 mg/ml) and 5 μ (0.17 mg/ml). For the negative control, DMSO at the same volumes of the plant extract was added to cultures. The culture plus extract or DMSO were then added in 200 μl triplicate volumes to 96 well plates. Incubation of the cultures was carried out in a varioskan spectrophotometer, set at 37 °C and shaking at 180 rpm. The growth responses (increase in optical density at 600 nm) were observed for 24 h, with readings taken every 15min. The time points were analysed using Graph-Pad prism.

Some typical results from these time growth studies are shown in Figures 4.7- 4.8. It can be seen that in all cases the extract having had chlorophyll removed still inhibited growth of the microorganisms to a greater level than that seen with the control (DMSO) samples. Additionally, in all cases the inhibition seen with the sample having had chlorophyll removed was greater than seen with a similar concentration of the crude extract. This indicated that the majority of material that had been removed by the hot filtration procedure was not responsible for any growth inhibition. These experiments also allowed us to investigate the minimum inhibitory concentration (MIC) of the chlorophyll free samples. The bottom graphs in figures 4.7 & 4.8 shows that the MIC of the *P. tomentosa* extract for E23 was 0.85 mg/ml which was

higher than that measured for *S. aureus* at 0.34 mg/ml.



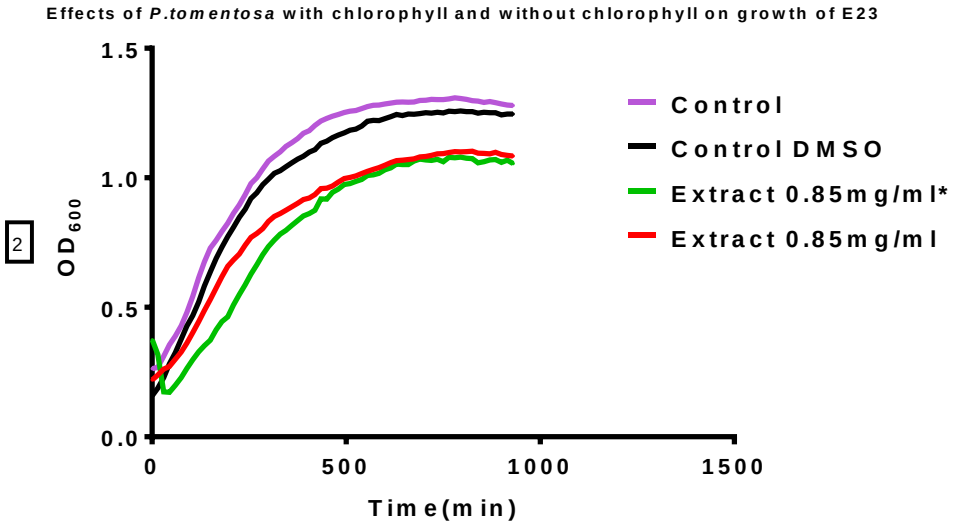


Figure 4.7 Time course of growth of E23 in *P.tomentosa* extract in LB media in the presence and absence of chlorophyll.* chlorophyll absence.

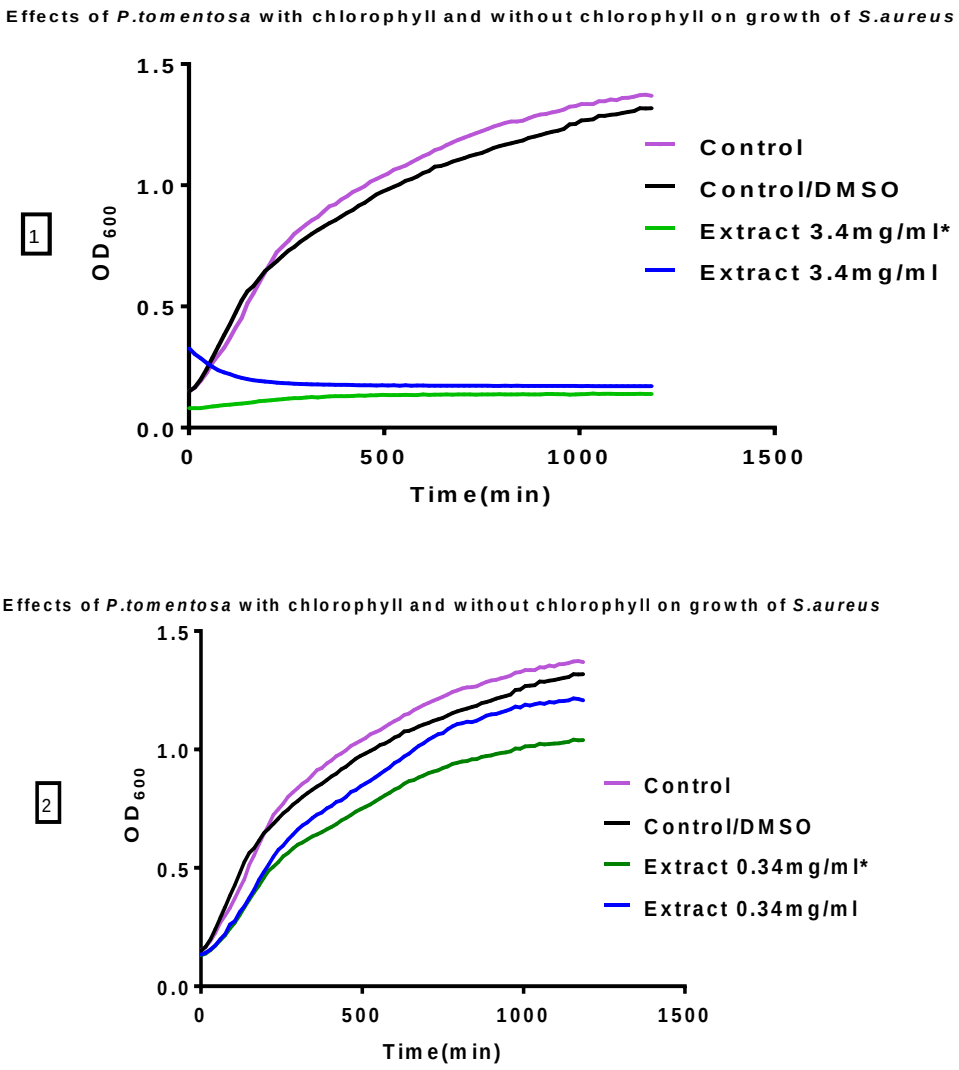


Figure 4.8 Time course of growth of *S.aureus* in *P.tomentosa* extract in LB media in the presence and absence of chlorophyll.* chlorophyll absence.

Furthermore, this experiment also estimated the minimum inhibitory concentrations (MIC) of the extract. The above experiments had indicated that chlorophyll was not the only component of the crude extract that was responsible for the inhibition of bacterial growth. Also, that fact that chlorophyll free samples showed greater inhibition than those of the same concentration but still containing chlorophyll indicated that the treatment with activated charcoal had selectively removed non-active components from the mixture. In order to check if any active components had actually been

removed in the process the chlorophyll-free extract was tested alongside the original crude extract at the initial concentration of 5.6 mg/ml (34 mg of chlorophyll-free material had been obtained from 56 mg of initial extract). The results are shown in Figures 4.9 & 4.10 and indicated that in both instances the chlorophyll-free extract inhibited bacterial growth as very similar levels to the original extract (P. value <0.0001.), thus confirming that very little 'active' antimicrobial components had been removed in the process. The observed antimicrobial properties against bacterial strains is possibly due to the polyphenols within the plants extract. As these experiment sample material was limited, it was not possible to carry out a similar investigation using PA14.

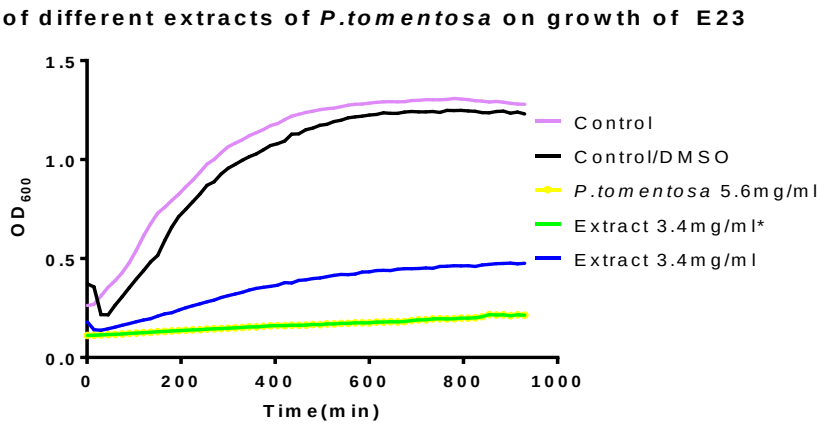


Figure 4.9 The time course growth of *E23* in LB with and without chlorophyll. Growth was carried out at 37 °C in a Varioscan spectrophotometer. Growth inhibition was monitored by taking optical density readings at 600 nm every 15 minutes.* chlorophyll absence.

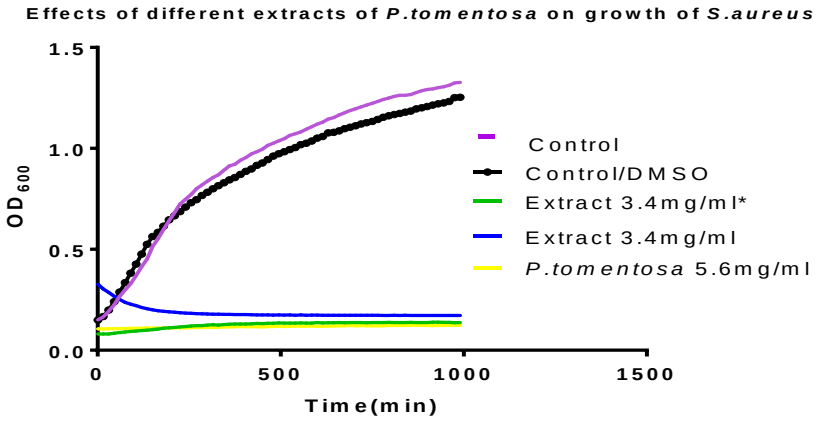


Figure 4.10 The time course of growth of *E23* in LB with and without chlorophyll. Growth was carried out at 37 °C in a Varioscan spectrophotometer. Growth inhibition was monitored by taking optical density readings at 600 nm every 15 minutes.* chlorophyll absence.

4.7 Assessment of the antimicrobial of the *P.tomentosa* fractions isolated by Column Chromatography

Having shown that the ethanolic extract from *P.tomentosa* displayed good anti-bacterial and anti-fungal properties that were not associated with chlorophyll the next step was to carry out similar investigations with some of the different fractions that had been isolated using column chromatography (as outlined in Chapter 3, Table 3.3). Similar methods to those used for the crude plant extract used to determined antimicrobial properties, by using the time course growth method. Due to the fact that we had much less material from to work with from the separation process a final concentration of 0.1 mg/ml of each of the fractions were prepared. However, the results outlined above which indicated that the crude extracts showed anti-microbial activity down to 0.18 mg/ml and below meant that testing at these lower concentrations was not envisaged to be a problem. Six different fractions from the ethanolic *P.tomentosa* extract that had been isolated from the initial column chromatography (see section Chapter Table 3.3) were tested for activity against E23 and *S. aureus* and the results are shown in Figure 4.11 and 4.12.

CRUDE *P.tomentosa* → Silica chromatography → 6 isolated fractions

Start extraction from non-polar solvent (pet.spirit) to polar solvent (EtOH)

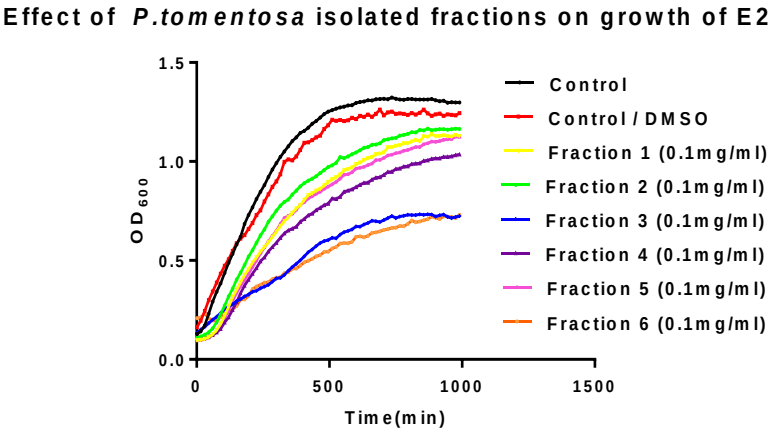


Figure 4.11 The antibacterial activity of six different isolated fractions of *P. tomentosa* extract tested against E23. The cultures were incubated for 24 h at 37 °C in a Varioscan spectrophotometer and growth levels measured by monitoring optical density (A600 nm) every 15 min.

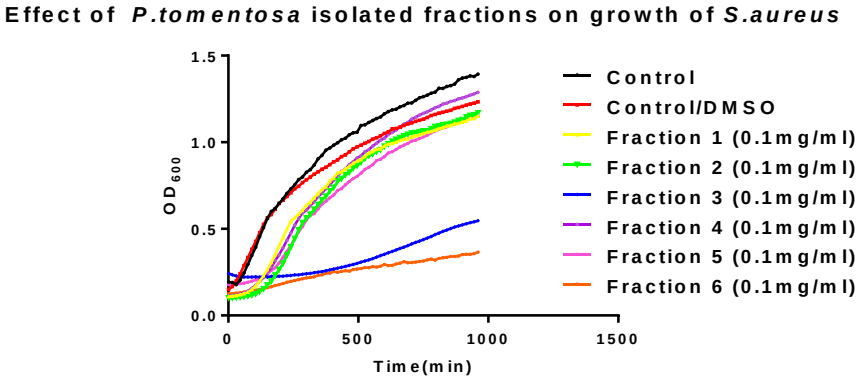


Figure 4.12 The antibacterial activity of six different isolated fractions *P. tomentosa* extract tested against *S. aureus*. The cultures were incubated for 24 h at 37 °C in a Varioscan spectrophotometer and growth levels measured by monitoring optical density (A600 nm) every 15 min.

As can be seen from Figure 4.11 all 6 of the *P. tomentosa* fractions examined were able to inhibit the growth of E23 but at different levels. The most active (inhibitory) samples were found to be fractions 3 & 6, but all the other

fractions showed levels of inhibition over those seen with the control at this low concentration 0.1mg/ml. Similar results were also observed with *S. aureus* where again fractions 3 & 6 showed the highest activity by some margin whilst three of the remaining four fractions also showed modest growth inhibition (fraction 4 showed no inhibition compared to the control), were higher comparable with the results for the growth inhibition of the fraction 3 and fraction 6 were higher comparable with the other fractions (1, 2, 4, and 5). Over the same concentrated range fraction 3 and 6 showed high inhibitory effects against *S. aureus*, compared to other 4 remaining fractions (1, 2, 4 and 5) Figure 4.11 and Figure 4.12. Therefore, it was investigated that this isolated fractions from *P. tomentosa* extracts showed poorest inhibitory effects except fraction (3 and 6) against E23 and *S.aureus*. Examining the anti-oxidant and total phenolics analysis which was discussed in Chapter 3 (Table 3.4) it can be seen that all six fractions tested here displayed similar levels in the DPPH• assay (24.4-47.5% sample range; 41.8% for fraction 3, 43.9% for fraction 6)-and total phenolics (29.1-55.9% sample range; 47.8% for fraction 3, 29.1% for fraction 6). There doesn't seem to be any obvious correlation between the anti-microbial and anti-oxidant activity.

Further analyses were carried out in the concentration ranges between (0.001 mg/ml – 0.1 mg/ml), on the same fractions to investigate the MIC of the antibacterial activity of fractionated *P. tomentosa* extract against E23 and *S.aureus.* Some representative results are presented in figures 4.13 & 4.14 (not all results are shown for conciseness).

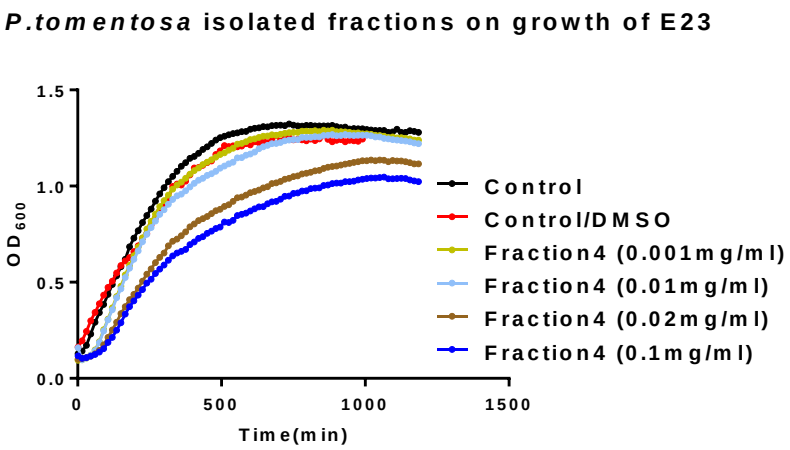


Figure 4.13 The antibacterial activity of fractionated *P. tomentosa* extract tested against E23. The cultures were incubated for 24 h at 37 °C in a Varioscan spectrophotometer and growth levels measured by monitoring optical density (A600 nm) every 15 min.

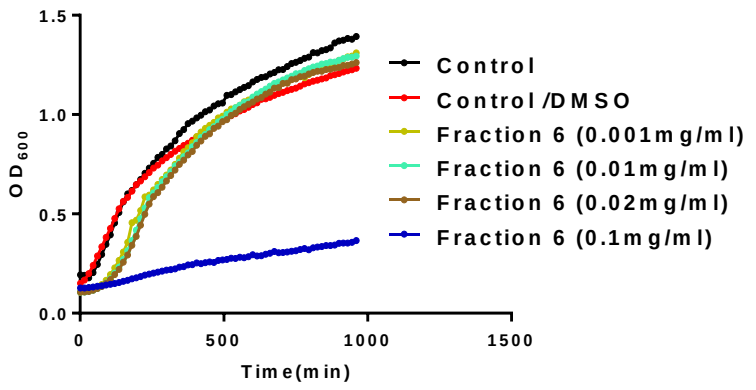


Figure 4.14 The antibacterial activity of fractionated *P. tomentosa* extract tested against *S.aureus*. The cultures were incubated for 24 h at 37 °C in a Varioscan spectrophotometer and growth levels measured by monitoring optical density (A 600 nm) every 15 min.

For all 6 of the fractions the ability to inhibit the growth of E23 were concentration dependent as shown in the representative graphs for fraction 4 (Figure 4.13) The results for the growth inhibition of the 6 fractions were comparable with, or slightly higher than, the DMSO as control at lower concentrations (< 0.02 mg/ml), *i.e.* fraction 4 in a Figure 4.13. Over the same concentrated range fraction 3 and fraction 6 showed high inhibitory effects

against *S.aureus*, *i.e.* fraction 6 in Figure 4.14. Therefore, it was concluded that these isolated fractions from *P. tomentosa* extracts presented poor inhibitory effects at lower concentrations against the selected bacterial strains and this was fractions dependent, accordingly, the results for the MIC of the 6 fractions were different between each fractions.

Table 4.2 Comparison of antibacterial activity of fractionated *P.tomentosa* extract tested against E23 and *S.aureus* in minimum inhibitory concentration (MIC) assay (n=3)

Organism	MIC					
	isolated fractions from *P. tomentosa* extracts					
	F1	F2	F3	F4	F5	F6
E23	0.02mg/ml	0.02mg/ml	0.02mg/ml	0.02mg/ml	0.01mg/ml	0.01 mg/ml
S.aureus	0.01mg/ml	0.01mg/ml	0.01mg/ml	0.1mg/ml	0.001mg/ml	0.001mg/ml

4.8 The effect of *P. tomentosa* extract on growth of gram-negative and gram-positive bacteria (samples in 2011).

Due to the fact that environmental and other factors (*e.g.* climate change, soil nature, harvesting time and handling process) may effect or change the phytochemical content in the plants,[233] we also tested material extracted from *P. tomemtosa* that had been collected at a different time and location to the initial plant material. Experiments were performed as described above in section 4.6.3 using material isolated from these new plant samples. Growth inhibitions against selected bacterial strains were carried out at a concentration of 2.8 mg/ml in DMSO.The results from testing the crude ethanolic extracts are shown in Figures 4.15 & 4.16.

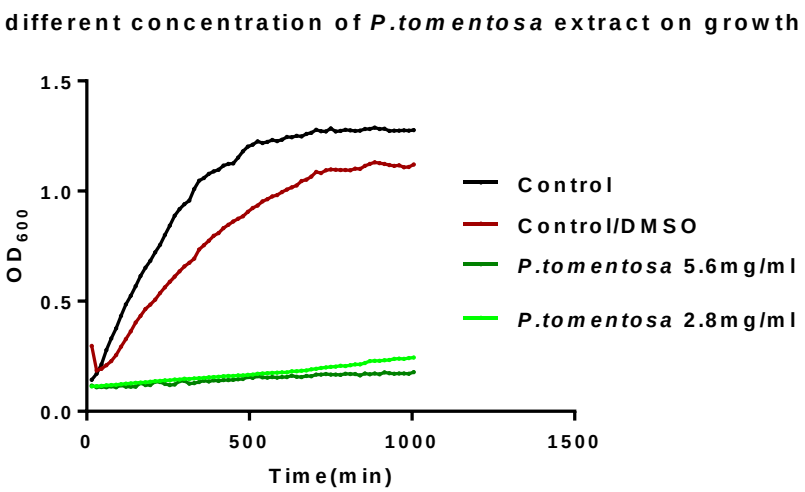


Figure 4.15 represents inhibition of crude extract against E23 (growth was carried out at 37°C in a Varioscan spectrophotometer; inhibition on bacteria strains was monitored by taking optical density reading at 600 nm every 15 minutes. The values shown are the mean of triplicate assays.

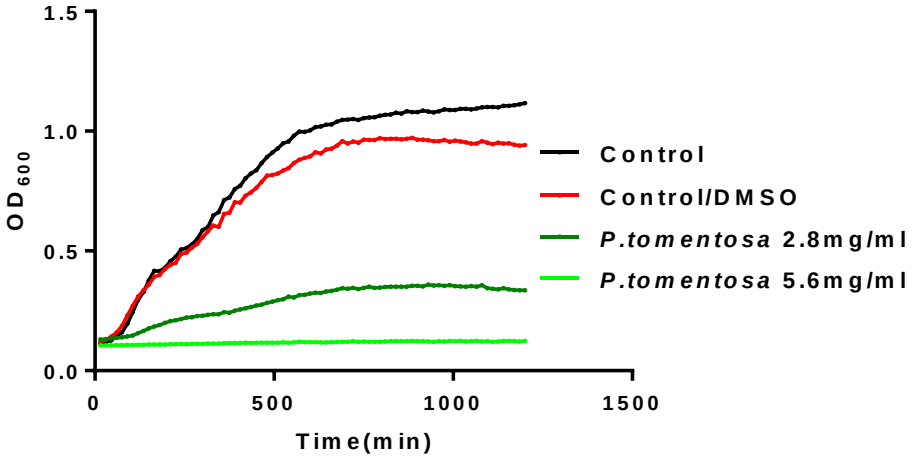


Figure 4.16 represents inhibition of crude extract against PA14 (growth was carried out at 37 °C in a Varioscan spectrophotometer; inhibition on bacteria strains was monitored by taking optical density reading at 600 nm every 15 minutes. The values shown are the mean of triplicate assays

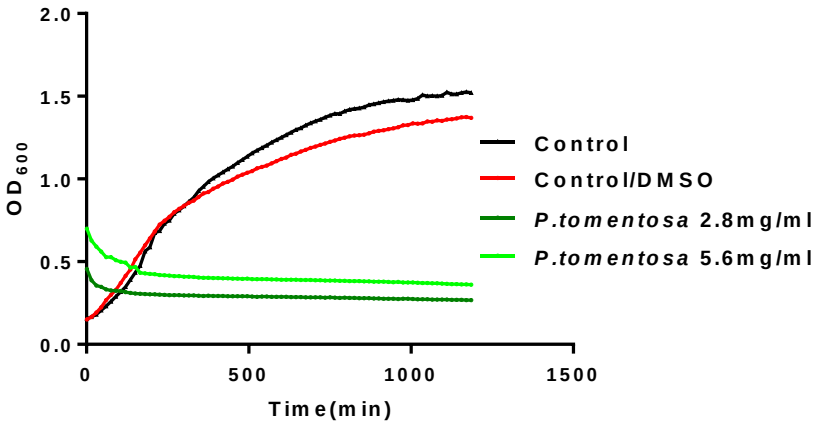


Figure 4.17 represents inhibition of crude extract against *S.aureus* (growth was carried out at 37 °C in a Varioscan spectrophotometer; inhibition on bacteria strains was monitored by taking optical density reading at 600 nm every 15 minutes. The values shown are the mean of triplicate assays.

The graphs show the results obtained with the crude ethanol extract isolated from this 2nd crop of plant material (tested at 2.8 mg/ml – light green in Figure 4.15, dark green in Figure 4.16 and 4.17). On the same graphs we have also superimposed the inhibition profiles we had obtained from our previous tests with the crude ethanol extract from the original material (tested at 5.6 mg/ml – light green). It should be noted that the tests with the 'old' and 'new' material were carried out at separate time points and are only shown on the same graph to allow a comparison to be made.

From the results with E23 it can be seen that both samples show essentially complete inhibition of bacterial growth (as indicated by the flat-line graph) at the concentrations tested. A similar result is also observed against *S. auerus*. This suggests that the anti-microbial properties we are observing are indeed inherent to the plant species and do not depend significantly on the time and place of sample collection. The results against PA14 are broadly similar but the absence for a flat line inhibition with the 2.8 mg/ml new sample suggests that this concentration, whilst still having significant inhibitory properties, is

not sufficient for complete bacterial growth inhibition.

4.9 Assessment of the antimicrobial activity of various fractions isolated from the crude extract of *P. tomentosa*

We next investigated the anti-microbial properties of the 17 fractions that had been isolated after the 1st column chromatography of this new plant extract (fractions PT_1A-1 to PT_1A-17, see Chapter 3; Table 3.23).

Crude *P.tomentosa* $\xrightarrow[\text{Start extraction from non-polar solvent (Pet.spirit) to polar solvent (EtOH)}]{\text{Silica chromatography}}$ 17 isolated fractions, PT_1A-(1-17)

The previous results from the anti-oxidant and total phenolic studies (Chapter 3, Table3.24) had indicated none of these fractions had exceptional levels of either of these properties and so we selected fractions for anti-microbial testing based solely on the amount of isolated material (only three fractions where we had isolated a significant amount of material were tested - PT_1A-3, PT_1A-7 and PT_1A-16). The time course growth method was used to assess bacterial growth inhibition. Thus, 1mg/ml of each of the three selected fractions (PT_1A-3, PT_1A-7 and PT_1A-16) were serially diluted in the range between (0.05 mg/ml – 0.1 mg/ml). Inhibition against E23, PA14 and *S. aureus* was tested (growth carried out at 37 °C in a Varioscan spectrophotometer; inhibition on bacteria strains was monitored by taking optical density reading at 600 nm every 15 minutes. The values shown are the mean of triplicate assays). Some representative results obtained are shown in Figures 4.18-4.20.

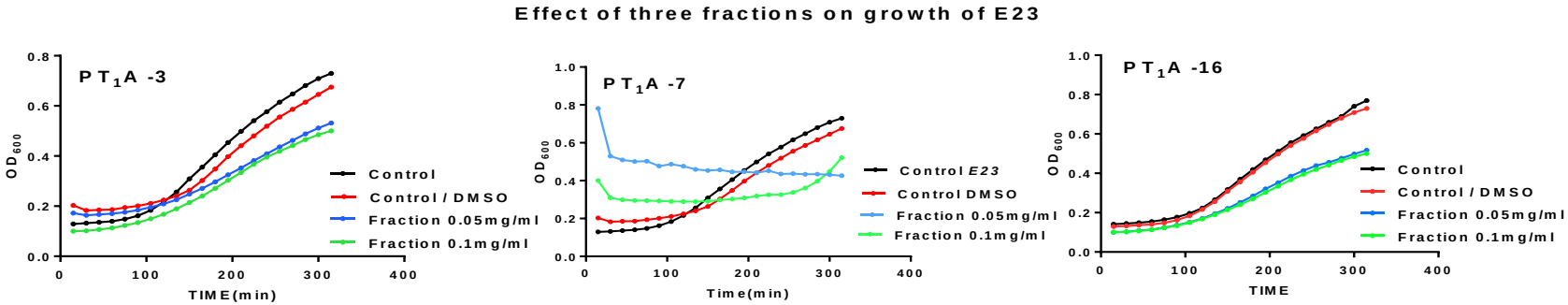


Figure 4.18 The antibacterial activity of three fractions (PT_1A-3, PT_1A-7, and PT_1A-16) against E23

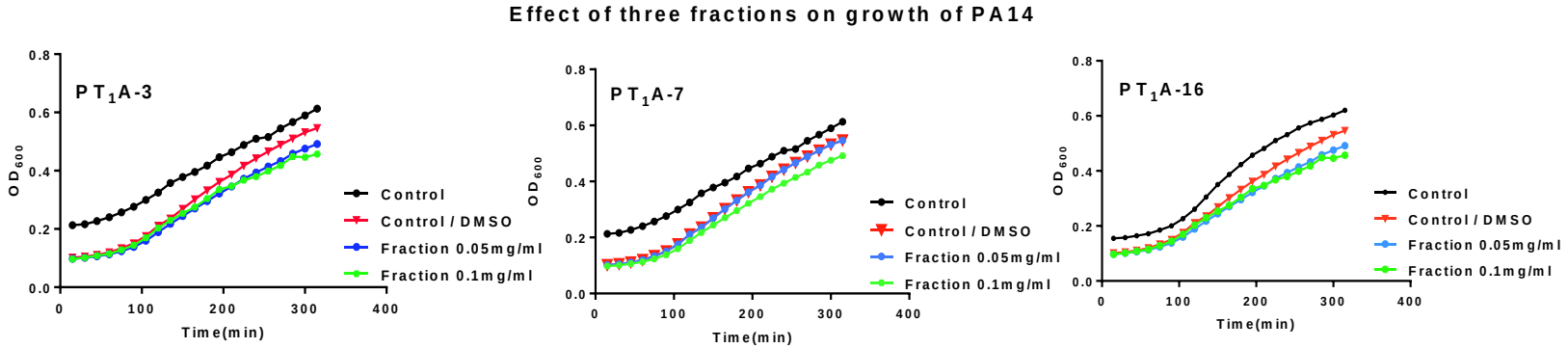


Figure 4.19 The antibacterial activity of three fractions (PT_1A-3, PT_1A-7, and PT_1A-16) against PA14

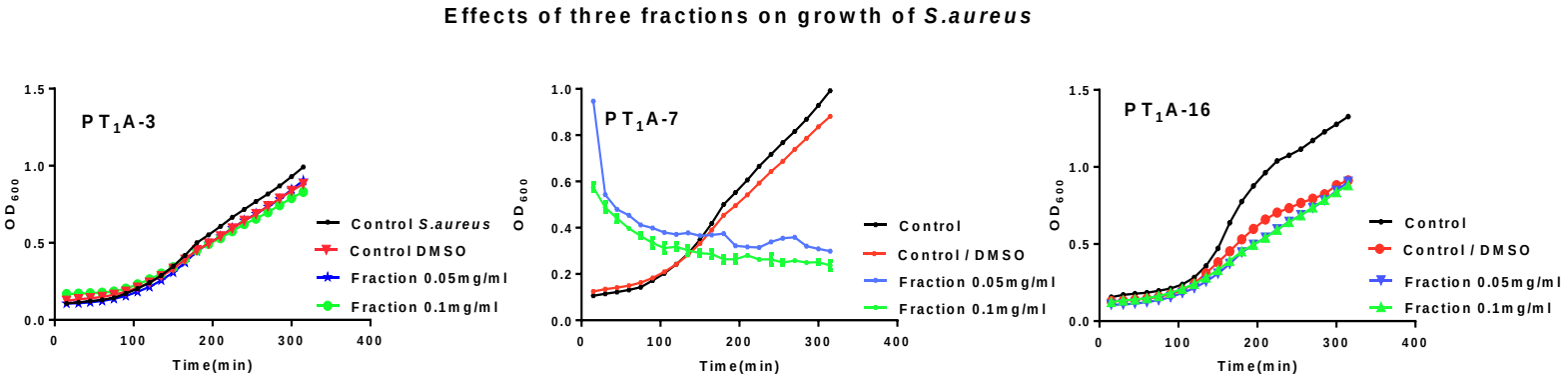


Figure 4.20 The antibacterial activity of three fractions (PT_1A-3, PT_1A-7, and PT_1A-16) against *S.aureus*

Effects of the fractionated extract (PTA_1-3, PTA_1-7 and PTA_1-16), on microbial growth Inhibition were conducted by using the time course of growth method. Fraction PTA_1-7 showed considerable variation in the levels of growth inhibition of E23 compared to the other two fraction PTA_1-3 and PTA_1-16 which showed a slight growth inhibition ($p<0.0001$) Figure 4.18 represents fraction PT_1A-7 with optical density approximately at 0.05 mg/ml and 0.1 mg/ml show 0.33 and o.48 respectively. A similar effective of growth inhibition was observed in Figure 4.20 for both 0.05 mg/ml and 0.1 mg/ml concentrations, and showed a minor growth inhibition against PA14. Fractiion PT_1A-7 showed a strong growth inhibition against *S.aureus* compared to fraction PT_1A-3 and PT_1A-16, which produced minor growth inhibition. Figure 4.19 demonstrated that fraction PTA_1-16 of both concentrations gives similar effects to the control DMSO.

4.10 Assessment of more isolated fractions on microbial growth

A further eleven fractions that were isolated by column chromatography from the ethanolic *P. tomentosa* extract (Chapter 3, Table 3.27) were also investigated. In the main the fractions were selected for testing due to the fact that some of them had promising peaks in their NMR spectra that gave some potential indication as to their structure (see discussion Chapter 3, section 3.12.4). The fractions that were tested were - (PT_1C (1-7) and PT_1C (9-11).

Crude *P.tomentosa* $\xrightarrow{1}$ 17 isolated fractions **PT_1A-7** $\xrightarrow{2}$ 11 isolated fractions **PT_1C-(1-11)**

1. Silica chromatography start extraction from non-polar solvent (per.spirit) to polar solvent (EtOH). (see Chapter 3, Table 3.22).
2. Fraction PT_1A-7 isolated by silica chromatography, start extraction from gradient from [{(1:2:8); (1:4:5); (1:6:3) MeOH: diethyl ether: petrol}, to end with {(100%) MeOH . (see Chapter 3, Table 3.26)

Time courses of growth were used for assessment of antimicrobial effects; 1mg/ml of isolated fractions (PT_1C (1-7), PT_1C (9-11), were used in the range between 0.05mg/ml – 0.1mg/ml. The antimicrobial properties of the isolated fractions were tested against E23, PA14, and *S.aureus*; growth was carried out at 37 °C in a Varioscan spectrophotometer. The values shown are the mean of triplicate assays.

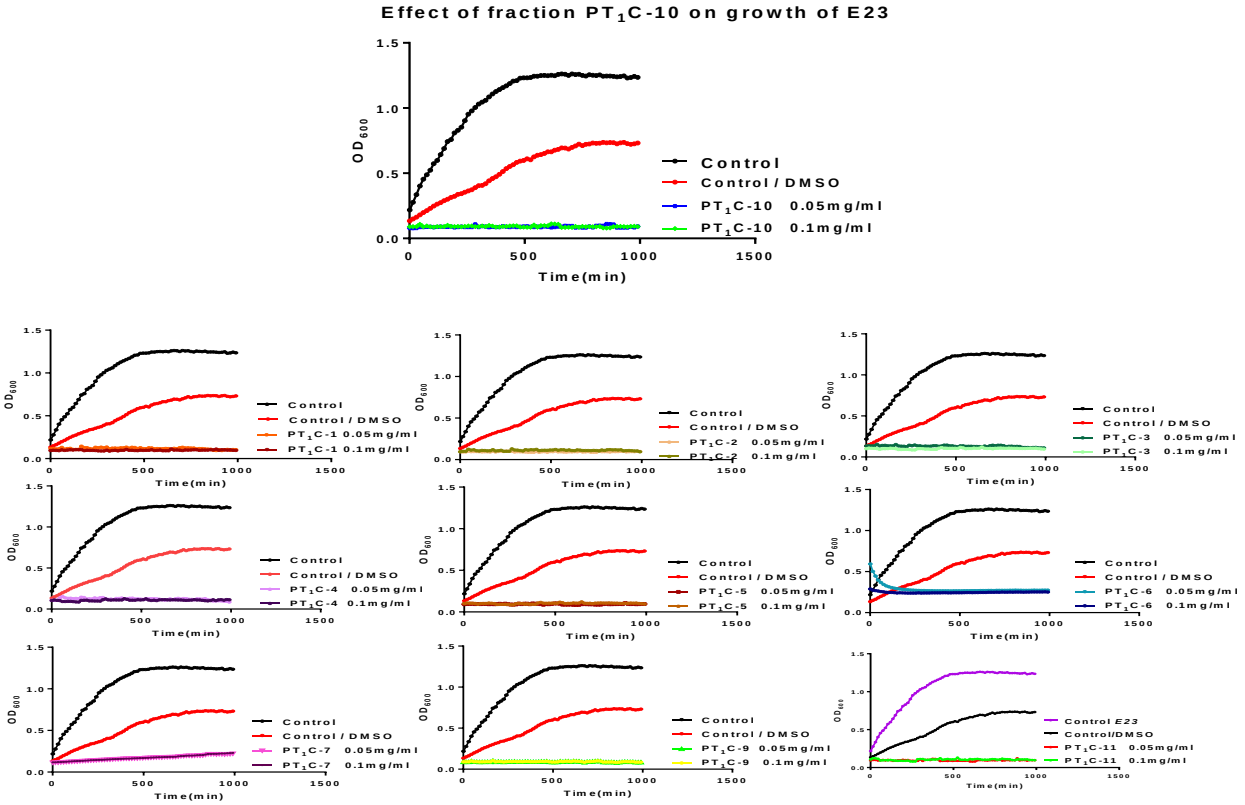


Figure 4.21 showed the effect of isolated fraction [(PT_1C-1-7) & PT_1C (9-11)] against E23

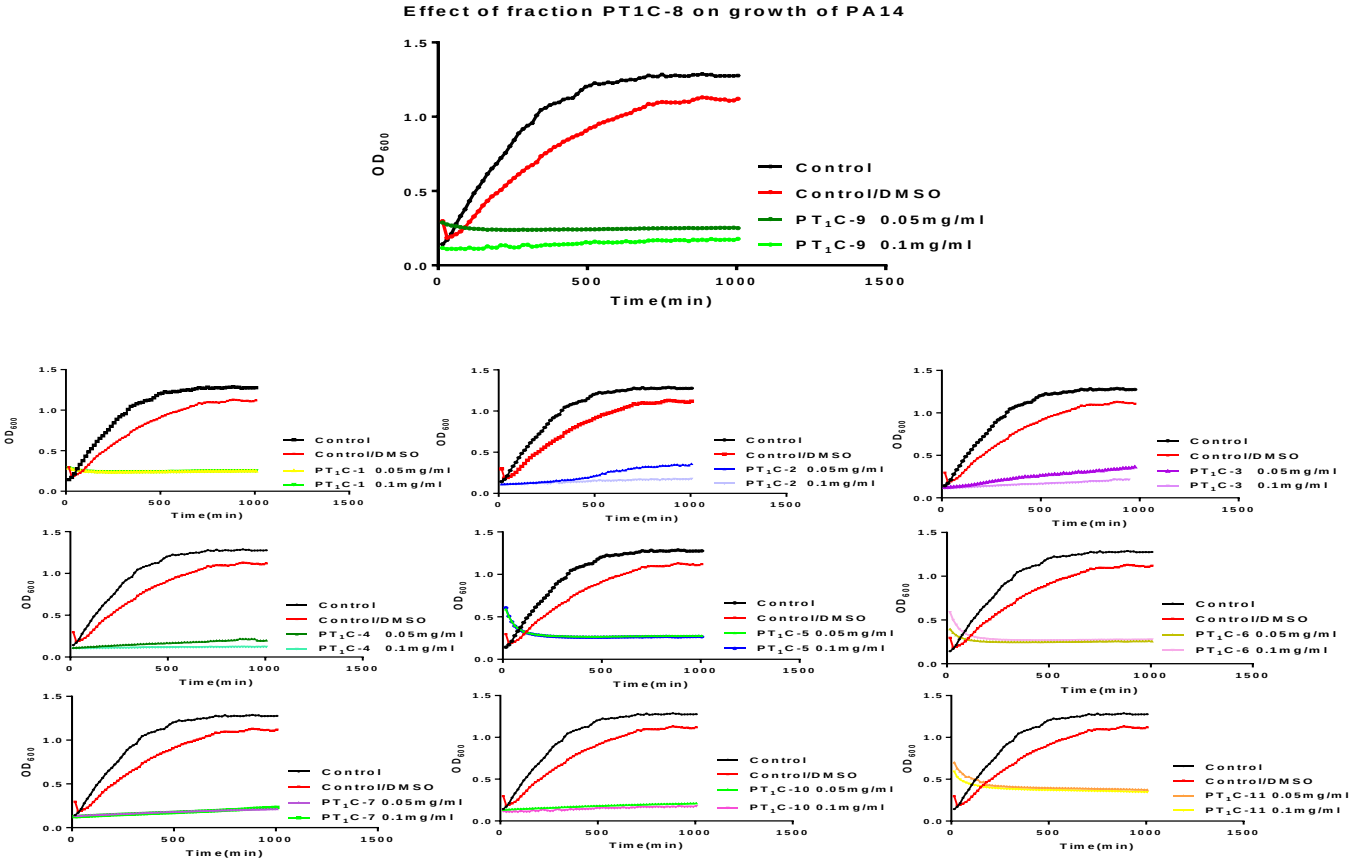


Figure 4.22 showed the effect of isolated fraction [(PT_1C-1-7) & PT_1C (9-11)] PA14

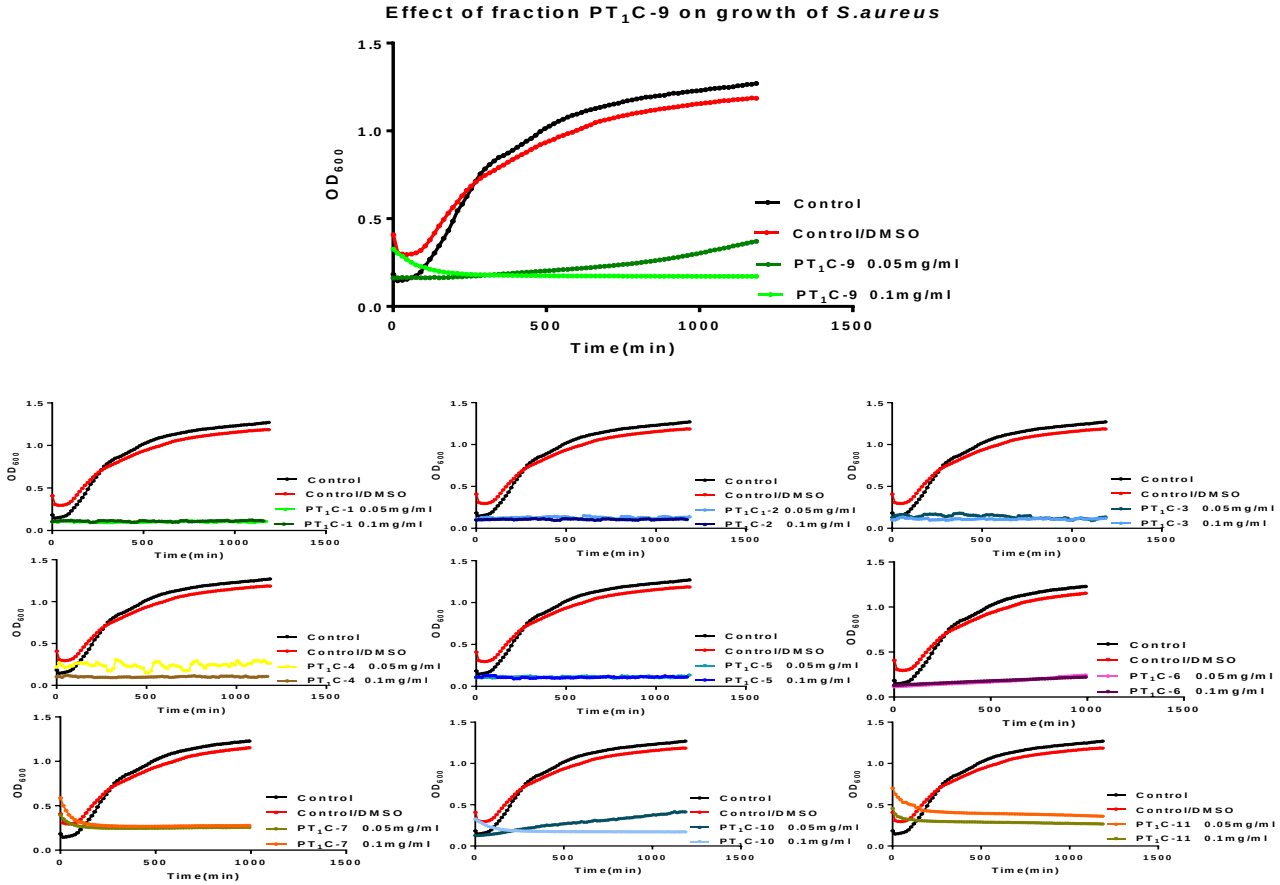


Figure 4.23 showed the effect of isolated fraction [(PT_1C-1-7) & PT_1C (9-11)] against *S.aureus*

As shown in Figures 4.21-4.23 growth inhibition was found against gram negative and gram positive bacteria. All the isolated fractions showed significant growth inhibition of the bacteria compared to the DMSO. Accordingly, this indicated that the effect of this isolated compound was as effective as the crude ethanolic 5.6 mg/ml *P. tomentosa* extract.

4.11 Disk-well Diffusion tests for fractions collected from prep-HPLC (PT_1A-16)

A final set of growth inhibition studies were carried out using the disk-well diffusion method and fractions that had been isolated by prep-HPLC (fractions H_2Me (1-11), Table 3.28).

Cultures of E23, PA14, *S.aureus* and *C.albicans* were spread onto Luria agar surfaces and allowed to dry. According to the disc well diffusion method discussed earlier (section 4.2.4), filter paper discs were then applied on the surface and 50 µl of 1 mg / 0.5 ml of a DMSO solution of 11 fractions *P. tomentosa* isolated by prep-HPLC were pipetted to the centre of each paper disc. For a negative control DMSO was used. Finally all petri-dish plates were inverted and incubated at 37°C for 24h. After incubation, the diameters zones of complete microorganism inhibition were measured the results and recoded in millimetres (mm).

Crude *P.tomentosa* $\xrightarrow{1}$ 17 isolated fractions $\xrightarrow{2}$ 11 isolated fractions

PT_1A-16 **H_1Me-(1-11)**

1. silica chromatography start extraction from non-polar solvent (pet.spirit) to polar solvent (EtOH). (See Chapter 3, Table 3.22)
2. Prep-HPLC analysis of the separation of fraction PT_1A-16 in mobile phase: [MeOH:Water (5:95,v/v)]. (See Chapter 3, section 3.12.6)

Table 4.3 after semi-purification of fraction PT_1A-16 using prep-HPLC, Antibacterial and antifungal (well, d = 5.0 mm) activity of fraction named as H_2Me (1 to 11) in agar dilution disc diffusion assay

Prep–HPLC Fraction	Diameter (mm) of inhibition zone			
	E23	PA14	*S. aureus*	*C. albicans*
H_2Me-1	22	17	23	27
H_2Me-2	22	15	25	24
H_2Me-3	25	14	29	30
H_2Me-4	28	14	22	31
H_2Me-5	20	11	10	19
H_2Me-6	27	16	22	33
H_2Me-7	29	17	27	34
H_2Me-8	30	18	27	35
H_2Me-9	34	30	30	37
H_2Me-10	29	28	21	36
H_2Me-11	36	33	43	37
DMSO	≥1	2	≥1	≥0.5

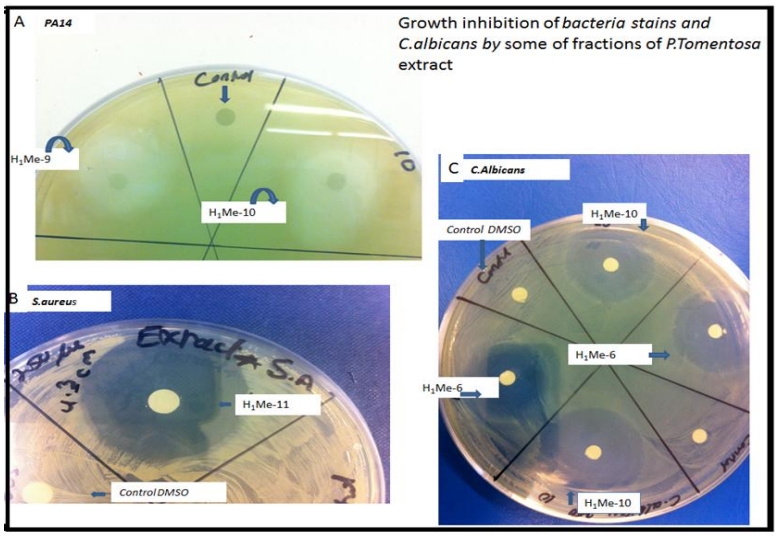


Figure 4.24 Examples of Disc diffusion results shown in Table 4.2. Effect of growth inhibition of some collected fraction obtained from Prep-HPLC against bacteria strains and fungal activities at concentration 1 mg/ 0.5 ml of fractions.

The results of antibacterial activity and fungal inhibition evaluated by disc diffusion method were show in Table 4.2 and Figure 4.24. All different fractions H_2Me- (1-11), had substantial inhibitory effects on all strains of bacterial and fungi tested.

4.12 Discussion:

According to the WHO around 20,000 medicinal plants species are used in the treatment different types of sickness.[234] Among these, several types are used to cure conditions, which have microbial associations in their etiology. World-wide, researchers are investigating the activities of identified plant compounds or plant extracts on microorganism infections. Moreover, traditional healers have offered therapeutics of a multifactorial nature and more than 70% of world populations consult traditional folk medical practitioners for remedy.[235] This chapter describes isolation and characterisation of bioactive compounds within ethanolic extracts of *P. tomentosa* using different isolation techniques including column chromatography, HPLC and Prep-HPLC and the properties these fractions had as antibacterial and anti-fungal agents. This is the first reported evidence of the efficacy of ethanolic extracts of *P. tomentosa* against human pathogenic microbes. The *P. tomentosa* has been previously isolated and identified as a plant with wound-healing potentials, though a little research has been previously reported. Hamed *et al*.[236], who investigated this plant for its potential anti-cancer properties, published one of such studies on *P. tomentosa*. This study has shown that the isolated cardenolides are cytotoxic to Kaposi's sarcoma cells. The cardenolide glycosides isolated from the roots of *P. tomentosa* included 3'-*O*-â-D-glucopyranosylcalactin, 12-dehydroxyghalakinoside, 6'-dehydroxyghalakinoside, ghalakinoside and calactin.

Successful prediction of plant compounds from medicinal herbal material is largely dependent on the type of solvent used in the extraction procedure. Most of the homoeopathy practitioner's use distilled water as the solvent for extraction. Water in medicinal plant extraction is considered inapplicable solvent compared to alcoholic solvents.[237] According to scientific investigations, plant compounds extracted by alcoholic solvents shows higher

activities than their aqueous extracts.[238] The leaf and stem ethanolic extracts of *P. tomentosa* show antimicrobial activity against some microorganisms at a test concentration approximately 1 mg/ml in DSMO. The poor solubility of the *P. tomentosa* extracts particularly those solvent available for use in bioassay without any effect on the microorganisms led us to use control in each step to differentiate the activities of the extracts from DMSO.

Current studies have shown antifungal and antimicrobial activities of *P. tomentosa.*[32,265,282] Antibacterial activity of ghalakoside obtained from *P. tomentosa* has been put forth by Mahalel.[282] Dangoggo *et al*.[239] reported that methanolic extracts obtained from *P. tomentosa* leaves show antibacterial activity against *Pseudomonas aeruginosa*. Similarly, Bekheet *et al*.[32] studied antifungal activity of the extracts obtained from *Ficus sycomorus* and *P. tomentosa*.

In this study, *P. tomentosa* extracts have shown antibacterial activity against *S. aureus*, E23, *P.aeruginosa* and antifungal effects against *C. albicans*. In order to estimate the minimum inhibitory concentrations (MIC) of the extract further dilutions were tested with DMSO. Column chromatography (TLC) with petroleum, EtOAc and ethanol was performed to separate the fractions of *P. tomentosa* that were subsequently used to test their anti-microbial activities against the microbial cultures. After silica column chromatography for crude extract DPPH• assay, total phenolic contents were subjected to Folin-Ciocalteu method to screen for antimicrobial activities for fractions PT_1A (3, 7 and 16) according to the quantity chosen. The fractions were assessed by prep-HPLC that showed different concentrations had different levels of activity against the microbial strains.

Although in this study, crude plant (*P. tomentosa*) extracts were tested on the microorganisms at different concentrations using TLC and Prep-HPLC. The *P. tomentosa* extracts inhibited the growth of microbes in a dose dependent manner. This study supports the findings of Dangoggo et al. (2002), Mahalel

et al., (2012), Bekheet et al. (2011) and Hamed et al. (2006) regarding the antimicrobial activity of *P. tomentosa*. enhanced. Purified fractions showed similar activity against bacteria and *C. albicans,* thus supporting the traditional usage of *P. tomentosa* to heal different type of skin disorders. Further research will be required to isolate and identify the phytochemicals of medicinal importance from this plant and to determine the molecular basis of their antimicrobial effects.

Chapter 5

Chapter 5 Investigation of the Inheritance of Bio-markers (mainly volatile oils) in Different Varieties of Mint

The current research was carried out to understand the value of secondary metabolites of plants for their characteristic aromatic or therapeutic features and the importance of these compounds for the chemical industry. We chose to investigate the inheritance characteristics of the various volatiles components found in hybrids of mint species with the aim of determining which secondary metabolite products were passed down through the cross breeding process. The mint species was chosen for several reasons, (i) they are widely distributed around the world, (ii) all mint species are fast growing, (iii) harvesting of mint leaves can be undertaken at any time and (iv) extraction of the essential oils can be performed from fresh or dried mint leaves. The research outlined in this chapter involved the following phases of work:

- Standardizing mint extraction, sampling and analysis of volatile oils using a microwave and comparing this process with traditional methods such as steam distillation.
- Studying the effect of microwave extraction with seven different mint species cultivated in the botanic garden of the University of Leicester [English lamb mint, Moroccan mint, Tashkent spearmint, *Mentha* spearmint, common mint, garden mint (*Mentha Spicata*) and black peppermint (*Mentha Piperita*)].
- Studying the effect of plant hybridization on the composition of volatile oils found in the offspring plants in comparison to the parental species.

- This chapter briefly introduces the concept of microwave irradiation as a technique for the extraction of volatile components from mint. The resulting compounds were analysed using gas chromatography-mass spectrometry (GS-MS) to determine which secondary metabolite compounds were present.

5.1 Introduction

Biomarkers refer to biological molecules found in living species (or their tissues) that can reliably represent the signs of normal or abnormal conditions. These biomarkers can be used to help monitor the body's response to treatment strategies for the disease processes.[240] The National Institutes of Health defines biomarkers as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[241] The world Health Organization (WHO) defines biomarkers as "any substance, structure, or process that can be measured in the body or its products that influence or predict the incidence of outcome or disease".[242] Conveniently biomarkers can be either the biomarkers of exposure or the biomarkers of the disease.[243] They can also be used to link specific environmental exposure to the outcome of condition or disease. Epidemiologists, physicians and scientists use various biological markers to study the disease processes in the human body. They help to diagnose and manage different infections, diseases and cardiovascular and genetic disorders.[244]

Mentha or mint species are small perennial herbaceous plants, well known for their essential oils contents. Mint species are cultivated as well as found in the wild. Essential oils are aromatic and volatile liquids which are obtained from different plants and mint species. These oils are of prime importance in the plant's defence. The metabolites of these essential oils work as antibiotic

agents.[245] This is why these oils are used for medical therapeutic purposes. Mint extracts are widely used to treat gastrointestinal problems and the spectrum of their use is much wider. They relieve pains and ease aches. Aromatherapy utilizes essential oils to relieve stress, anxiety, depression, pains and aches.[246] The effects of essential oils in terms of Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida albicans* infections have been previously studied.[247]

5.2 Essential Oils

Essential oils are concentrated hydrophobic aromatic and volatile liquids obtained from plants by distillation. For centuries, these essential oils have been used in medicine and the cosmetic industries, and as condiments for food. The earlier use of the essential oils started with the preparation of medicines and then spread to the cosmetic industry in the nineteenth century.[293] About three thousand essential oils have been identified of which three hundred types are being used as flavors and cosmetics.[248]

The metabolites contained in these essential oils play an important role in the defence mechanisms of the plants. In human beings, these compounds turn out to have antimicrobial activity.[249,250] Mimica-Dukic *et al*. (2003) studied the antioxidant activity of three *Mentha* species (*M. aquatica L., M. longifolia L. and M. piperita L*.).[251] They reported that the essential oils contained in these species had strong antibacterial activity against *E. coli* strains. Monoterpene ketones in these essential oils were the powerful scavenging compounds against DPPH• and •OH radicals and where able to reduce DPPH• radicals to the neutral DPPH-H form and this activity was dose dependent. Similarly, Oumzil *et al*. (2002) reported the antibacterial and antifungal activity of essential oils extracted from *M. suaveolens.*[252] Moreover, along with antibacterial and antifungal activity of essential oils, antiviral,[253]

antiparasitic,[254] insecticidal[255] and antioxidant[256] properties have also been reported.

Plants produce several kinds of metabolites in response to different assaults. These metabolites may always be present in the plants or may be produced to cope with the environmental effects. It is difficult to identify the most active compounds with antibacterial activity as essential oils are reported to be a mixture of up to 45 different constituents and the composition of the oil may differ with the season of harvest and the method used to extract them.[257,258]The essential oils extracted from the plants contain low molecular weight organic compounds with different antimicrobial activities. In terms of chemical structures, these compounds can be divided into 4 groups: terpenes, terpenoids, phenylpropenes, and others. Figure 5.1. shows the structures of some essential constituents.[259]

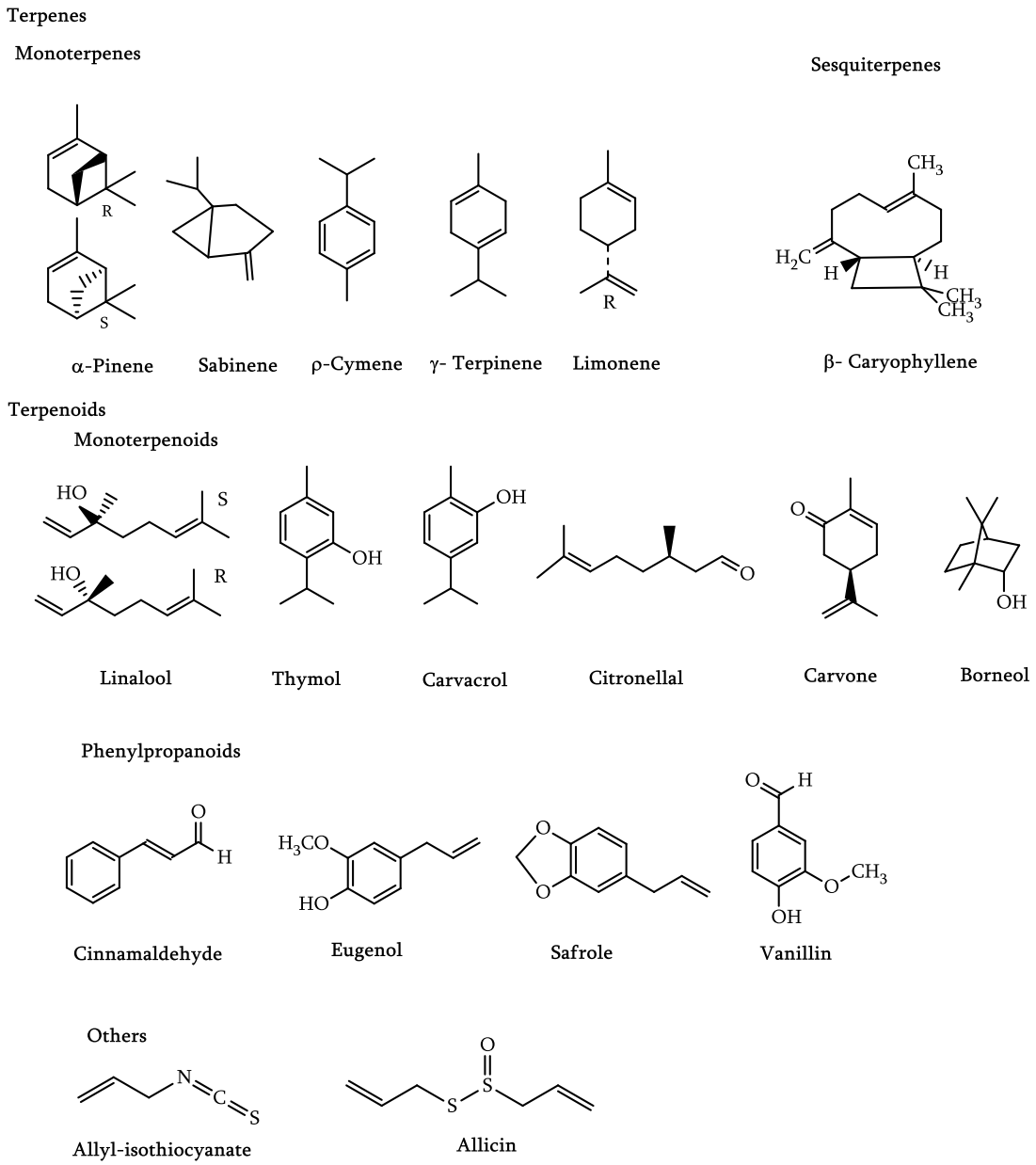


Figure 5.1 Chemical structures of selected essential oil constituents (Hildegard *et al*., 2012)[260]

Terpenes are hydrocarbons produced in the cytoplasm of plant cells through the mevalonic acid pathway. The key structure of these compounds is made of isoprene (C_5H_8) units, the process being catalyzed by cyclases.[261] They can be found in the form of monoterpenes, diterpenes, triterpenes and so on. However, monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$) are the main

components. Terpenes have been reported to have low antibacterial activity.[245,262]Terpenoids are biochemically-modified terpenes where various enzymes add oxygen or move or remove methyl groups from the terpene compounds. The antibacterial activity of terpenoids depends upon the functional groups linked to them. The terpenoids with hydroxyl groups have been reported to play important role in terms of antimicrobial activity.[263,264] Phenylpropenes are also organic compounds synthesized from phenylalanine in the plants. These compounds make-up a small part of essential oils and their antibacterial activity is attributed to their side chains, double bonds and functional groups.

5.3 Mint essential oil composition

The species of mint belongs to the genus *Mentha* of the family Lamiaceae. Various parts of these species are distilled to obtain essential oils that are used in the medical and cosmetic industry, as foods, flavors and beverages. This essential oil contains a large number of chemicals such as menthol, menthone, carvone, menthofuran, linalool, linalyl acetate, 1,8-cineol, limonene, pulegone etc.[265] Argentina, Australia, New Zealand, Brazil, China and India are the major areas of the world where these mint essential oils are extracted.[266] It has been reported that the quality, quantity and composition of the essential oils contained in the mint species differ with the method of extraction, time of the harvest, age of the crop, methods of storage, season and environment.[267] However, time of the harvest, place and genotype play the most important role in the variations of essential oil composition.

The composition of peppermint essential oil from peppermint (*Mentha piperita L.*) has been well studied. According to the International Pharmacopoeia, the composition of peppermint oil quoted in a paper by Alankar [268] is given in Table 5.1. The structural formulae for some of the peppermint oil constituents are shown in Figure 5.2.

Table 5.1 Various constituents of peppermint oil

Constituents	Percentage (%)
Limonene	1.0-5
Cineole	3.5-14
Menthone	14.0-32
Menthofuran	1.0 -9
Isomenthone	1.5-10
Menthyl acetate	2.8-10
Isopulegol	Max. 0.2
Menthol	30.0-55
Pulegone	Max. 4
Carvone	Max. 1

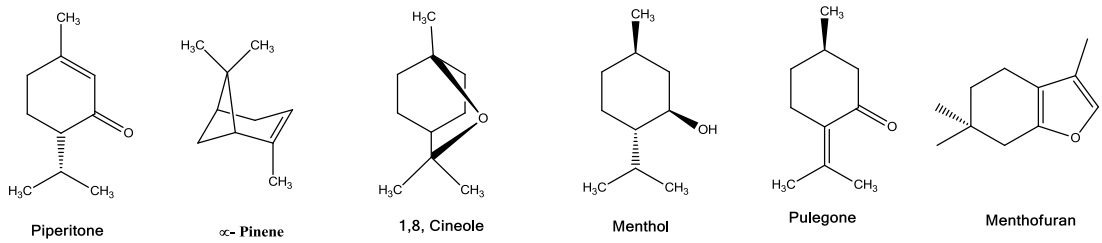


Figure 5.2 Structural formulae for some constituents of peppermint oil (Alankar, 2009)[314]

5.4 Traditional Extraction Techniques of Essential Oils

A number of extraction methods are used to obtain essential oils from the plants depending on the type of the botanical material. Distillation, expression (known as cold pressing a special technique for citrus peel oils) and solvent extraction (enfleurage, maceration, solvent, supercritical carbon dioxide by using CO_2 etc.) are some traditional methods that have been used. Steam distillation is the most common method [269] of essential oil extraction where the botanical material is immersed in water and distilled, with the essential oils contained in the aqueous distillate separating out upon cooling. Enfleurage[314] is the oldest method of oil extraction in which a thin layer of odourless fat placed on glass is used to absorb the oil contained in petals. A brief description of some traditional extraction techniques for the extraction of essential oils is given below.

5.5 Distillation

This is the most common method used to extract essential oils from plants. In this method, plant materials such as leaves, flowers, petals, roots, bark or seeds are boiled to generate steam in a specialized container (distillation apparatus) and the vapours are collected and cooled to obtain the essential oil.[270] Most of the plant materials need a single distillation process. The condensed product of distillation is called hydrosol or plant water essence. Water distillation, steam distillation and hydro-diffusion are some of the distillation techniques.

5.6 Expression

The expression method for the extraction of essential oils refers to a “cold pressed method”. Most of nut and seed oils are obtained through this method. Nuts and seeds from the plants are pressed under high mechanical pressure that gives good quality essential oils. Citrus oils are also obtained through this method of extraction. Sponge expression and machine abrasion are techniques used for expression extraction method.

5.7 Solvent extraction

Plants or plant materials which contain very low quantities of essential oils are often subjected to solvent extraction. In this method, hexane, solid oils and supercritical CO_2 are used to extract the essential oils from the plants. Maceration and enfleurage are the other techniques of solvent extraction. In the maceration technique, flowers are immersed in hot oil that absorbs the essence. Enfleurage is one of the oldest methods where petals are spread on to the thin fatty layer and left for few days. The fatty layer absorbs the essential oils contained in the petals. Then the fat layer is washed with alcohol that separates the oil from the fat layer. Alcohol evaporates leaving the oil behind.

5.8 Microwave technology - an overview

Extraction of diverse biological materials including essential oils by using microwaves has proven to be a superior technological development with its specific parameters that can be addressed for particular plants and their ingredients. When solutions are subjected to microwaves, the polar molecules within the mixture get excited and orientate in the opposite direction relative to the applied field. The frequency of change of polarity of the electric field determines the molecular rotation frequency (Saoud *et al*., 2006).[271] Microwaves are a form of electromagnetic waves which range in frequency from 300 MHz to 300 GHz. Microwave technology heat escalation can be very fast e.g. at 4.9 GHz heat escalation is estimated at 10 ˚C/sec. Microwaves cause an electric field that rotates water molecules very fast (microwaves with 2450 MHz frequency can cause water molecular swing at 2.45 X 10^9 times per second) thus creating intense heat. Since water is an essential component of biological materials, its contents affects heating. The dielectric constant indicates how well microwaves can heat a particular material. The dielectric constant for water at 20 ˚C is 78.5 as compared to 32.6 of methanol, 24.3 of ethanol, 20.7 of acetone and 1.89 of hexane (Ramanadhan, 2005).[272]

A domestic microwave oven generates microwaves from alternating current starting from 50Hz and escalates up to 2450 MHz. The transformer in the oven converts the normal voltage into 4000–6000 volts for this purpose. Further heating efficiency is achieved by heat reflecting cavity walls and a rotating platform. There are several methods for the extractions of biological materials using microwaves and their use in agricultural technology is increasing day by day. These include the CEM solvent extractor, microwave assisted extractor, microwave reflux, Sub-500W microwave extractor, Drydist model of milestone, solvent free extractor and closed vessel mono-model of CEM Corporation (Ramanadhan, 2005).[272]

Solvent free microwave extraction combines microwave heating with dry distillation at atmospheric pressure without adding water or solvent. This method has been reported to yield higher quantities of oxygenated compounds in garden mint with the yield and aromatic profile of the essential oils remaining comparable to the hydrodistillation method (Lucchesi *et al*., 2004).[273] Several factors affect the microwave assisted extraction process and optimization of experimental conditions are critical in order to reduce time and cost and improve the outcomes as has been seen in a study in which the yield of trans-β-ocimene is decreased when using microwave assisted extraction upon increasing irradiation over 700 W (Asghari *et al*., 2012).[274]

5.9 Inheritance characteristics of the various volatiles in mint hybrid species

Hybridization has played a very important role in evolution as plant life on earth is chiefly based on this process. These days we are familiar with post-hybridization events such as genetic changes and epigenetic alterations which substantiate hybridization (Tucker, 2011).[275] Genetic improvement in *Mentha* plants can yield a higher quantity and quality of essential oil contents. Therefore, selection of accessions should be based on a reliable tool for effective mint breeding. Cluster analysis is one such tool that helps in assessing genotypic and phenotypic characteristics among a set of accessions or populations from which parent species for hybridization are selected. Multivariate analyses including principal components are among the valuable statistical tools that can be used to examine and classify genotypic characteristics of chemical constituencies in *Mentha* plants (Zeinali *et al*., 2004).[276]

Global cultivation of *Mentha* plants originates from two primary constituent essential oils and four hybrid polyploid species. (-)-Menthol is the primary

constituent of peppermint (*Mentha species* x *M. piperita* L.) and Chinese cornmint (*M. arvensis* x *M. longifolia*) or Japanese peppermint (*Mentha canadensis*). (-)-Carvone is the primary constituent of Scotch spearmint (*M. species* x *M. gracilis*) and American spearmint (*Mentha species* x *M. villosonervata*). English Mitcham peppermint originated as a hybrid of *M. aquatica* and *M. spicata* (Tucker, 2011).[275] Several hybrids have been raised in the last few decades after the hybrids raised by crossing *M. piperita* x *M. arvensis* and *M. piperita* x *M. sachalinesis* were found to be more bushy and capable of yielding higher amounts of essential oils (Lutkov *et al.*, 1966).[277] Genetic breeding can also be supported by metabolic engineering *e.g*. peppermint is a sterile hybrid (*M. aquatica* x *M. spicata*). The monoterpene essential oil biosynthetic pathway is well understood in this plant which has facilitated the development of metabolic engineering (Lange et al., 2011).[278]

5.10 The aim of study

This study was intended to evaluate the practicalities of a microwave-assisted separation procedure for small amounts of mint leaves. Compared to other thermal treatments, a microwave-based procedure is considered more uniform and faster for the extraction of species. Selected mint species were to be subjected to a procedure of extraction and analysis of their essential oil content along with the study of the inheritance characteristics of compounds in selected inter-specific hybrids of mint. Specifically, the aim of this study was to extract volatile oil from several types of mint species and origins (either from fresh mint plants or stored dried mint), analyse the component volatile oils and compare the effect of hybridization of these plants on the mint oil composition and properties in terms of quality and quantity.

5.12 Materials and Methods

Figure 5.3 Images above shows the selected different species of mint cultivated in Leicester University botanic garden.

5.12.1 Plants materials

The plant species used in this study project were collected fresh from the botanic garden of the University of Leicester (Figure 5.6). Leaves of the cultivated plants of different mint species including English lamb mint, Moroccan mint, Tashkent spearmint, *Mentha* spearmint, common mint, garden mint (*Mentha Spicata*) and black peppermint (*Mentha Piperita*) were harvested during different period in 2011. Additionally, some selected dried hybrid inter-species plants from storage of the botanical garden of the University of Leicester, were studied to compare the effect of the hybridization on composition of volatile oils.

5.13 *Mentha* species and hybrids

Thirty-three dried species of mint (*Mentha)* were selected from the Botanical library. The names of dried mint species and hybrids investigated in this study are given in Table 5.2 together with their collection history. These plants represented mint hybridization diversity and were provided by the Botanical Garden of the University of Leicester.

Table 5.2 accession data. Species, Collectors and Collection History

NO	Species	Collectors	Collection History
1	*M.longifolia (L)*	Hudson& C.R.Fraser Jenkins	16/09/1970
2	*M.longifolia (L)*	J.W.Franks	07/08/1952
3	*M.longifolia (L)*	S.Lewalle(10830)	16/07/1983
4	*M.suaveolens*	R.P.Libbey	25/09/1978
5	*M.suaveolens*	R.P.Libbey	10/09/1979
6	*M.suaveolens*	O.Polunin(18529)	04/07/1975
7	*M. spicata*	H.Jibbons	20/09/1932
8	*M. spicata*	B.molesworth Allen (7164)	13/07/1971
9	*M. spicata*	PHA.Sneath	19/08/1986
10	*M. arvensis (L)*	T.G.Tutin(61128)	19/08/1961
11	*M.arvensis (L)*	E.K.Howood	30/08/1949
12	*M. arvensis(L)*	I.M.Roper	29/07/1914
13	*M. aquatica(L)*	A.I.primavesi	14/08/1982
14	*M. aquatica (L)*	Miss P.J.Wittle	19/08/1962
15	*M. aquatic (L)*	D.A.Ritchie;	08/09/1958
16	*M.x villosa*	T.J.Tuten(5172)	23/08/1951
17	*M.x villosa*	Hudon,T.H.Wawe &,S.H.Bickham	01/10/1904
18	*M.x villosa*	Haines	12/09/1930
19	*M.x verticillata*	Jean.green	03/09/1989
20	*M.x verticillata*	F.J.Taylor	03/09/1950
21	*M.x verticillata*	H.Kibbens	07/08/1925
22	*M.x villosonervata*	L.B.Hall	10/08/1919
23	*M.x villosonervata*	JW.white	08/1932
24	*M.x villosonervata*	E.C.Wallace	05/09/1953
25	*M.x piperita*	Lousaly	30/08/1948
26	*M.x piperita*	S.H.Bickham	01/09/1902
27	*M.x rotundifolia*	S.H.Bickham	20/08/1909
28	*M.x rotundifolia*	HKibbons	30/08/1929
29	*M.x suavis*	Coll.SHB	05/10/1904
30	*M.xCarinthiaca*	R.Graham	12/08/1949
31	*M.x gracillis*	Coll.alee	01/09/1904
32	*M.x gracillis*	Sabranky	08/1912
33	*M.x gracillis*	SHB	09/09/1910

Group of individuals transpired in cluster analysis based on phytochemical of essential oil constituent.

5.14 Modified microwave oven

Fresh plant materials were placed in to a modified GHz microwave reactor (CEM Discover-s-class system with addition gas kit accessory), with a maximum delivered power of 300 W. The temperature was adjusted according to suitability of the organic solvent used, to isolate mint oils 0.5 g of fresh dried material, were heated for 10 min with addition of 4 ml water + 2 ml hexane.The extraction mixture was left to settle to obtain two immiscible layers (water, a lower layer and hexane, an upper precipitate containing layer). The hexane layer subjected to GC-MS for further analysis.

5.15 Quality and quantity of essential oils

Compounds were extracted and identified in mint essential oils using two techniques (1) steam distillation using Clevenger's apparatus, (2) Microwave assisted extraction. The individual compounds were separated by analytical GC and identified by comparison of their retention time to those of authentic pure samples obtained from commercial sources, as well as by comparing the retention indices of individual components with a library database. The percentage compositions of the samples were computed from the GC peak areas.

The advantage of using microwave heating as an alternative to other modes of heating is it is quicker, saves energy, uses smaller quantities of solvent, higher extraction rate and can be carried out on a smaller scale.

5.16 GC analysis

The carrier gas was helium. The column inlet pressure was 100 kPa, the flow rate was 1.0 ml/min, and the temperature of the injector and detector was 275 $^{\circ}$C. The oven temperature was programmed from 40 to 240 $^{\circ}$C with rate of 8 $^{\circ}$C/min and volume injected 2.0 µl of solution of oil in hexane, split 50:1

mode, 250 °C injector temp and detector temp 270 ˚C. HP-INNOwax column (29.95 m).

5.17 Chemicals used

All solvents used were HPLC grade.

5.18 Preparation of the essential oil from mints

5.18.1 Modified microwave

Harvested leaves of seven different types of mint were collected and frozen with liquid nitrogen and ground to a fine powder. Extracts were obtained by mixing 0.5 gm of dry frozen leaves part powder with 4 ml water and 2 ml hexane in a microwave tube that was tightly closed before being subjected to the microwave reactor and run for 10 minutes in microwave at 150 W, 68.7 °C (boiling point for hexane, 70 °C) and 250 PSI extracted the volatile oil. The hexane solution was sampled by syringe to run the analysis by GC-MS. For a rigorous comparison, the condition for microwave extraction were kept constant (same plant material, quantity, solvent volume), and the analyses were run in the same day. e.g. GC-MS parallel on analysis for both steam distillation and microwave. Most constituents were identified by gas chromatography by comparison of their retention indices with those for authentic samples of individual components. Extractions were performed in duplicate and mean values were reported.

5.18.2 Steam distillation

2.07g of freshly harvested samples of all the leaves of mint were distilled using Clevengers apparatus for four hours to extract the essential oil. 1.2 ml of oil was collected from the condenser outlet stream. The collected samples were taken and 3 ml of hexane added, the resulting solutions dried with anhydrous magnesium sulphate, filtered and analysed by GS-MS.

5.19 Results and Discussion

Initially we decided to investigate a microwave-based method for the extraction of essential oils from mint leaves. The reasons behind this were partly to do with the reported efficiency of this method for the extraction of essential oils but also because the final goal of this study would require analysis of very small (approx.0.1 g) historical samples of the cross bred mint species and the small scale of these samples would make steam distillation an experimental impossibility. In the initial experiment we took 0.5 g of fresh mint leaves from the garden mint species added 2 ml hexane and heated the resulting mixture at 70 °C for 10 min in the microwave. Analysis of the resulting hexane extract by GC-MS (and comparison to known standards of some of the mint essential oil components) Table 5.3 showed that this extraction procedure had generated fewer essential oils when compared with those found either by the hydro-distillation (HD) or by solvent free microwave extraction (SFME).[279] The results, however, showed that there was no remarkable loss of the major essential oils components such as limonene and carvone, even though the amount of essential oil extracted by the microwave method was lower compared to HD and SFME. Also some essential oils that have been reported to be obtained from HD and SFME [279] (such as β-myrcene, octane-3-ol, γ-terpinene, cis-sabinene hydrate, terpinolene, thymol, eugenol, β-bourbonene, β-elemene, α-humulene, sesquiterpene1, γ-muurolene, bicyclogermacrene and calamenene.) were from garden mint extracts isolated from this intial microwave experiment.

Table 5.3 comparison of essential oils extracted with different techniques

NO.	Identified compound	Garden mint M.W.	Garden mint * SFME	Garden mint * HD	Mode of identification
1	*α – Pinene*	0.36	0.1	0.8	GC-MS
2	*Camphene*	1.02	0	0	GC-MC
3	*β -Pinene*	0.28	0.4	1.4	GC-MS
4	*Sabinene*	0.18	0.3	1	GC-MC
5	*α-Phellandrene*	0.28	0	0	GC-MS
6	*Limonene*	3.41	9.7	20.2	GC-MC
7	*1,8-Cineole*	0.8	1.5	0	GC-MS
8	*Cis-Ocimene*	0.28	0	0	GC-MC
9	*p-Cymene*	0.26	0	0	GC-MS
10	*Menthone*	1.13	0	0	GC-MC
11	*Menthofurane*	0	0	0	GC-MS
12	*Iso-Menthone*	0	0	0	GC-MC
13	*Menthylaacetate*	0	0	0	GC-MS
14	*Iso--Menthol*	0.27	0	0	GC-MC
15	*I-Menthol*	0.16	0	0	GC-MS
16	*Linalool*	0	0.4	0.4	GC-MC
17	*Linalyl*	0	0	0	GC-MS
18	*Bornyl*	0	0	1.2	GC-MC
19	*β-Caryophyllene*	0.15	3.5	3.4	GC-MS
20	*Terpinen-4-ol*	1.32	0.4	2.6	GC-MC
21	*Pulegone*	0.54	0	0	GC-MS
22	*α-Cayophyllene*	0	0	0	GC-MC
23	*α-Terpineol*	0.76	0.5	2.1	GC-MS
24	*Germacrene D*	2.17	0	0	GC-MC
25	*Piperitone*	0.39	0	0	GC-MS
26	*Carvone*	41.44	64.9	52.3	GC-MC
27	*Geranyl Acetate*	0	0	0	GC-MS
Etraction time (min)		**10**	**30**	**270**	

In the next experiment we followed the same initial protocol but with the added step that the mint leaves were first frozen in liquid nitrogen and then ground into small pieces using a pestle and mortar.Then 0.5 g of the resulting powdered mint leaves were mixed with 4 ml water and 2 ml hexane and the

mixture heated for 10 min in the microwave (same settings as above). After microwave extraction the GC-MS analysis showed the amount of essential oils recovered from that just chopped in to small pieces without frozen in liquid nitrogen by increasing peak area and total number of components.

Having shown that this freezing procedure considerably increased the amount of essential oils extracted (presumably by increasing the surface area of the mint leaf in contact with the hexane) all further extractions used this as the first step. We next investigated how the ratio of water to hexane affects the extraction process. Thus we tried an extraction process in which different amounts of water and hexane were added to the mint leaves. Table 5.4 summarize the experimental design.

Table 5.4 Summary of microwave extraction with variation in water/hexane ratio

No.	sample	Water	Hexane
1.	0.5g	3ml	3ml
2.	0.5g	4ml	2ml
3.	0.5g	5ml	1ml
4.	0.5g	2ml	4ml
5.	0.5g	1ml	5ml
6.	0.5g	-	6ml

The experimental results (GC-MS traces), for black mint as an illustration are shown in Figure 5.7. These GC traces show a moderate spectrum of essential oils in 5:1 mixture of water: hexane and weak spectrum of essential oil all other water: hexane ratios, whilst extracting mint with only hexane gave a very poor GC trace. The results from these experiments suggested that additional water was an essential component of the mixture required for efficient extraction of the essential oils from the mint leaves and that we could not just use the water present inside the frozen leaves themselves. Similar results were seen with all the 7 mint species under investigation and hence this protocol was used throughout the rest of the studies described in this chapter.

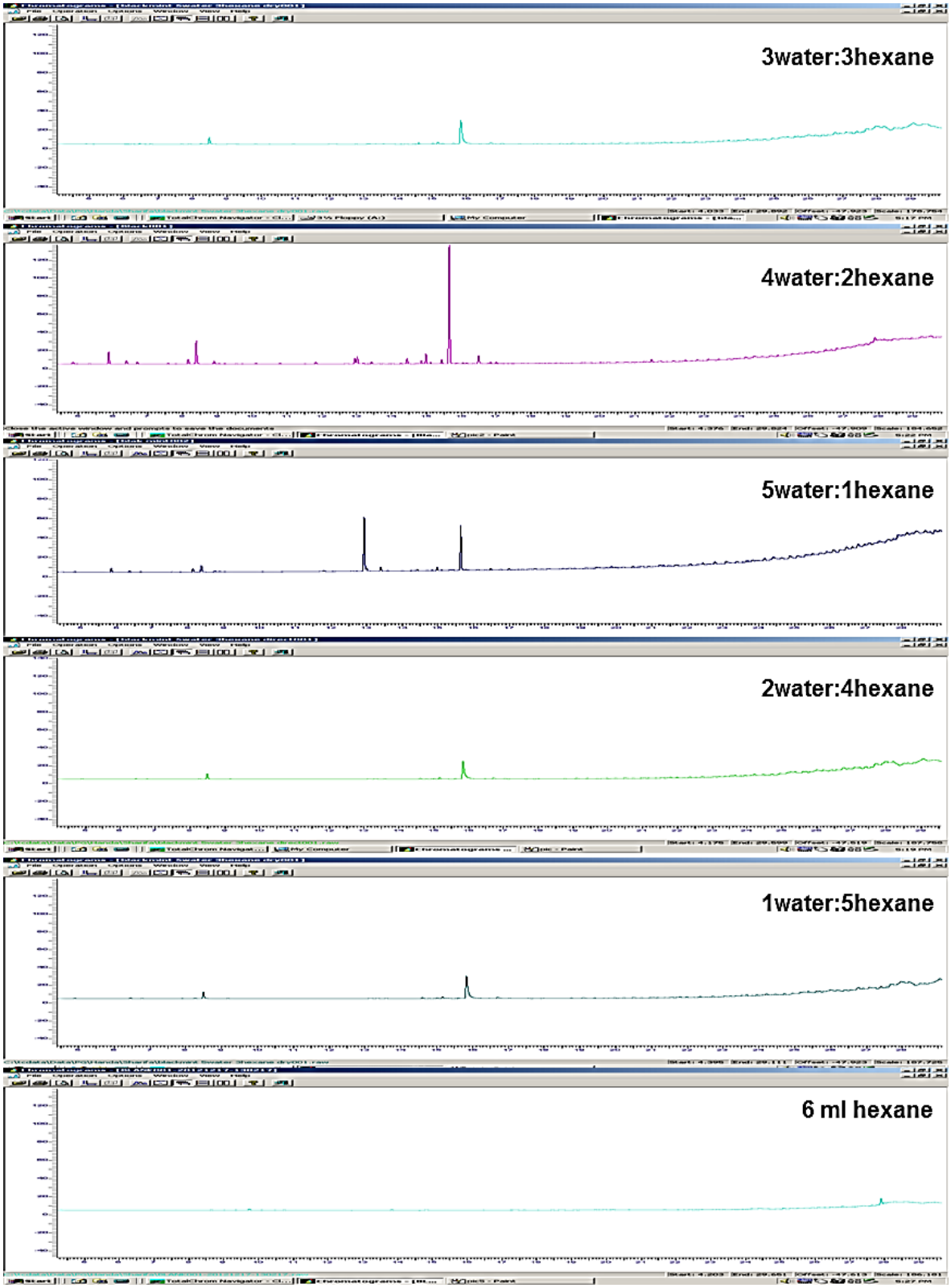


Figure 5.4 Comparison of best extraction yields with variation in water/hexane ratio mixture *e.g.* black peppermint (*Mentha Piperita*)

After much experimentation we decided upon an optimised procedure which involved extraction of 0.5 g of mint leaves (that had been frozen and crushed using mortar and pestle) with a 2:1 mixture of water: hexane (4 ml: 2 ml) using microwave heating for 10 min.These conditions gave a good combination of extraction efficiency and also speed of analysis.

Having determined an optimised microwave extraction procedure the next step was to compare the essential oil profile from this method to more conventional steam distillation techniques.Thus 2.0 g of fresh mint leaves were extracted using steam distillation and analysed by GC-MS. Two representative GC trace spectra from the microwave and steam distillation extractions are shown below.

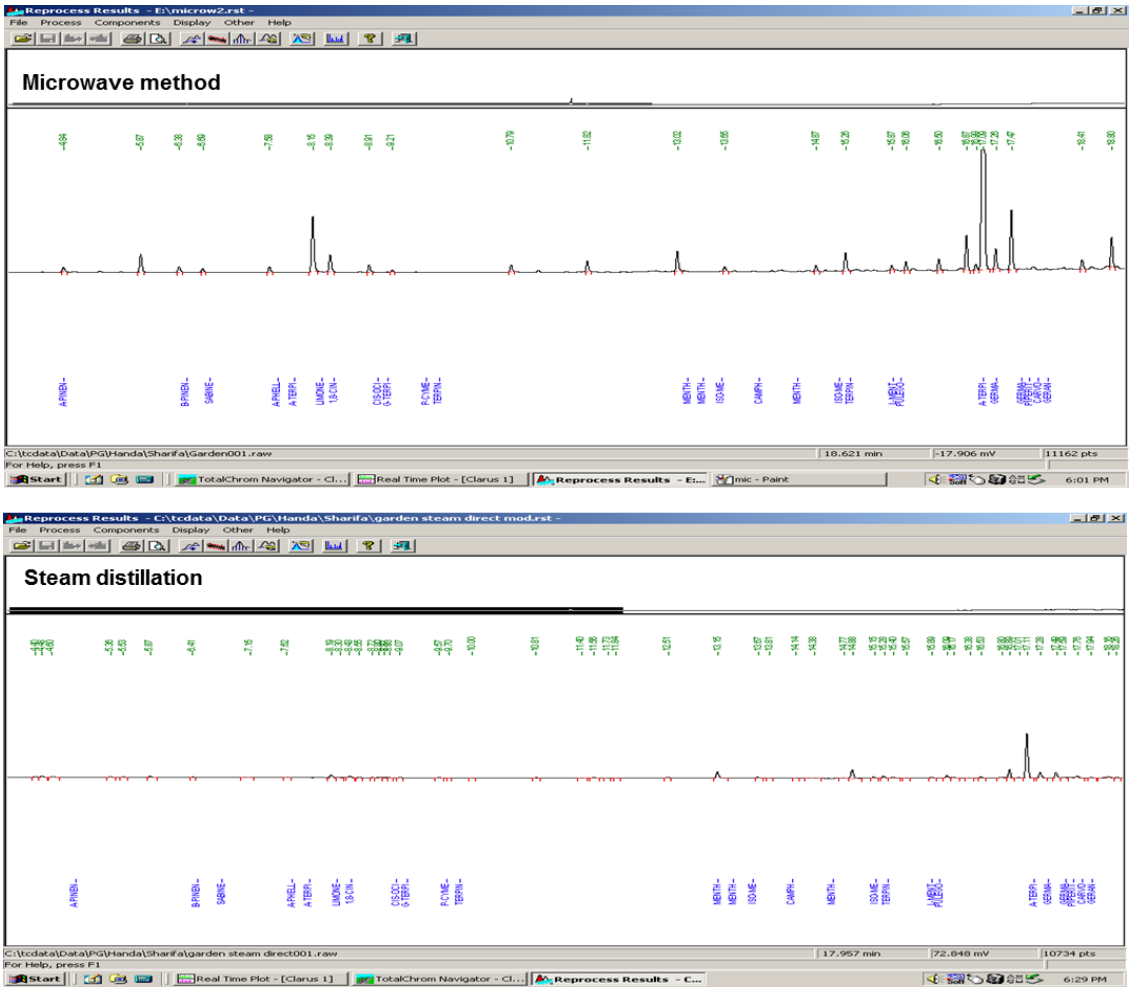


Figure 5.5 Comparison of microwave method vs steam distillation on essential oils from garden mint

These spectra show a comparison between the microwave method with one of conventional techniques (steam distillation), for the extraction of essential oil from garden mint species. As can be seen from the respective GC traces extraction of essential oils using a microwave garden was much more efficient than steam distillation in term of rapidity (10 min vs 360 min), energy saving, product yield, a cleaner GC-MS trace and better isolation as indicated by GC-MS.

5.20 Graphs showing analysis of Essential oil

Having established that our microwave extraction procedure produced both reproducible results and results comparable to those already reported in the literature we next turned out attention to examining different mint species using this method.To gain an approximate indication of the purity of these extracts we calculated the percentage of the total area of the GC trace for peaks that we could identify as known essential components of mint (identified by comparison to known standards that we ran under the same conditions) to the total area of the whole GC trace (including peaks for non-identifiable components). We also carried out steam distillation extractions with these species to further compare the two methods.The results are presented in Figure 5.9

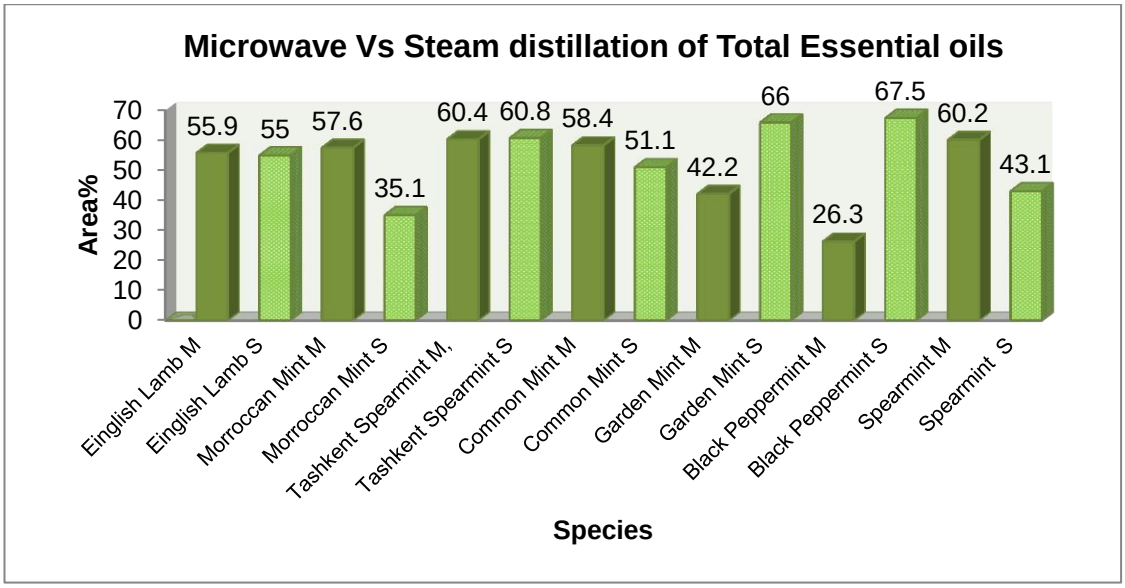


Figure 5.9 represent the comparison between two isolation technquies method M:microwave, S:steam distulation.

Comparison of the results shown in Figure 5.9 clearly indicates that for each of the seven different species that were examined the purity of essential oil extracted (as indicated by the percentage of identifiable components) was measurably higher for the microwave extraction as opposed to the steam distillation metod. For microwave extractions the maximum purity of oil was obtained from Black mint (67.5%) and the minimum purity of oil was measured for Einglish mint (55.6%) whilst for steam distillations the maximum oil purity was found with Tashkent mint (60.4%) and the minimum measured was for Black spearmint (26.3%). This indicates that the microwave extraction offers clear advantages method in terms of purity of the oils extracted , as well as a better isolation time, essential oil composition and environmental benefits.

The results from these experiments with the seven different mint species are presented numerically in table 5.5 and graphically in figure 5.10 as shown below.

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Table 5.5 the main compounds identified in different mint species

			٪ **Area**							
NO.	Identified compound	R.T	English	Moroccan	Tashkent	Common	Garden	Black	Spearmint	Mode of identification
1	α – Pinene	5.43	0.19	4.82	0.3	0.35	0.36	0.46	0.46	GC-MS
2	Camphene	5.94	2.88	2.73	0.82	2.03	1.02	2.64	2.56	GC-MC
3	β -Pinene	7.08	0.39	0.37	0.31	0.6	0.28	0.77	0.75	GC-MS
4	Sabinene	7.39	0.83	0.16	0.2	0.4	0.18	0.4	0.45	GC-MC
5	α-Phellandrene	8.29	0.86	0.28	0.83	0.43	0.28	0.1	0.49	GC-MS
6	Limonene	8.91	6.5	2.72	5.56	3.93	3.41	1.46	4.53	GC-MC
7	1,8-Cineole	9.08	0.95	0.43	1.08	4.77	0.8	5.28	4.41	GC-MS
8	Cis-Ocimene	9.6	0.3	0.42	0	0.38	0.28	0.17	0.67	GC-MC
9	*p* -Cymene	10.3	0.9	0.6	0	0.44	0.26	0	0.35	GC-MS
10	Menthone	13	0.53	0.16	0	2.39	1.13	0.64	2.1	GC-MC
11	Menthofurane	13.4	0	0	0	0	0	1.06	0	GC-MS
12	Iso-Menthone	13.66	0	0	0	0.55	0	0.33	0	GC-MC
13	Menthylaacetate	13.98	0	0	0	0.4	0	1	0.41	GC-MS
14	Iso--Menthol	14.12	0	4.34	0.26	0.28	0.27	0.28	0.16	GC-MC
15	l-Menthol	14.47	0.2	0	0.39	0	0.16	0.22	2.09	GC-MS
16	Linalool	14.95	0	0	0.42	0	0	0	0	GC-MC
17	Linalyl	15.18	0	0	0.14	0	0	0	0	GC-MS
18	Bornyl	15.69	0	0	0.16	0	0	0	0	GC-MC
19	β-Caryophyllene	15.95	0	0	0	0	0.15	0	0	GC-MS
20	Terpinen-4-ol	16.34	0	0	0.29	1.69	1.32	0.74	0.18	GC-MC
21	Pulegone	16.54	0.69	1.07	0.15	1.2	0.54	0.31	0.51	GC-MS
22	α-Cayophyllene	16.79	0	0.51	0.34	0.32	0	0	0	GC-MC
23	α-Terpineol	17.19	0	1.42	2.07	0.85	0.76	0.5	1.16	GC-MS
24	Germacrene D	17.25	0.78	0	0.49	0.19	2.17	1.77	0.09	GC-MC
25	Piperitone	17.34	0.35	0.58	0.31	0.39	0.39	0.35	0.07	GC-MS
26	Carvone	17.61	36.37	31.26	49.61	36	41.44	28.9	33.5	GC-MC
27	Geranyl Acetate	17.86	3.83	0	0	0	0	0	0	GC-MS

R.T: Retention Time linear. Identification Compounds listed in order of elution in GC-MS

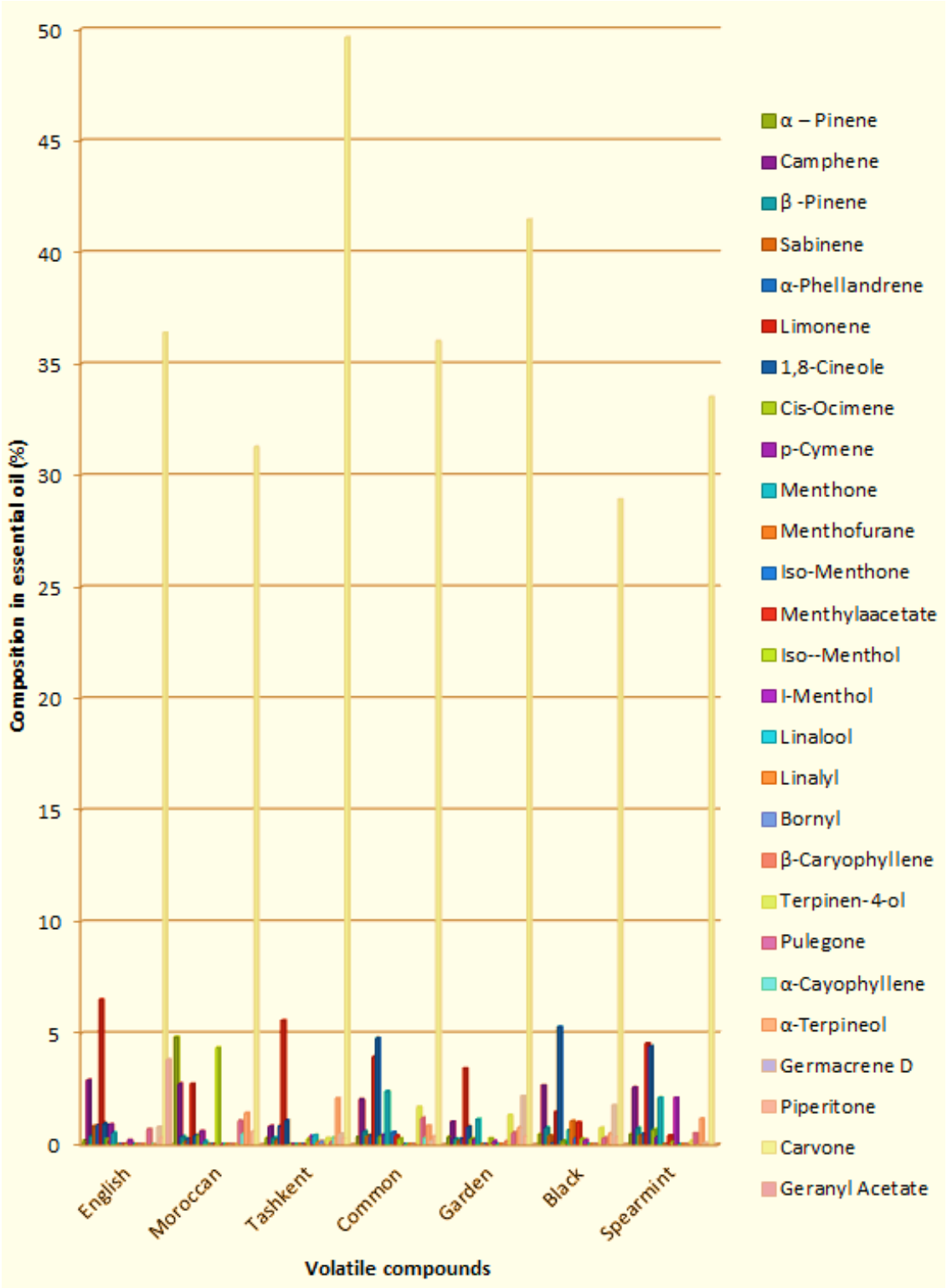


Figure 5.10 represents the composition variation of essential oil between seven

different harvested mints from botanic garden – Leicester University

5.21 Interspecific mint genetic diversity

Having established an efficient and reliable method for the extraction and analysis of volatile components from mint leaves we next turned our attention to examining the essential oils compositions of various cross-bred mint species.The operation and data collection was carried out using the microwave method and GC-MS analysis described above (section 5.18.1). Table 5.6 summarizes the *Mentha* species that were studied in this experiment – the eight cross-bred species under investigation are shown together with their five different parental sources. In total 33 different samples were analysed (see table 5.2)

Table 5.6 represent list of 33 *Mentha* accession used in this study (five parents species and eight crossbred species). *M.* is used to indicate parental species and *M.x* hybrid species.

No.	Parent species		Crossbred species
1	*M.aquatica* 96	*M.arvensis* 72	*M.x verticillata*
2	*M.aquatica* 96	*M.spicata* 36	*M.x piperita*
3	*M.suavelens* 24	*M.spicata* 36,48,84	*M.x villosa*
4	*M.suavelens* 24	*M.longifolia*	*M.x rotundifolia*
5	*M.longifolia*	*M.spicata* 36,48,84	*M.x villosonervata*
6	*M.suavelens* 24	*M.arvensis* 72	*M.x carinthiaca*
7	*M.spicata* 36	*M.arvensis* 72	*M.x gracilis*
8	*M.aquatica* 96	*M.suavelens* 24	*M.x suavis*

With reference to the analysis of the volatile components for the dried mint species shown in Table 5.6, no changes were made to the microwave procedure except for scaling down the amount of dried mint from 0.5 g to 0.1

g (with an appropriate scaling of the amounts of water-hexan solvent used), due to limited historical samples of the cross bred mint species available from the Botanic Department, Leicester University. Some of these species were found to be of insufficient quantity for analysis (less than 0.1 g). In addition, for some species only a single batch was available for analysis and therefore these experiments could not carried out in triplicate and so the results for these samples discussed below are for single extractions/analysis. The experiments sought to investigate the amount of genetic diversity of 27 known *Mentha* accessions from different geographic inheritances, representing five species (*M.aquatica* 96, *M.arvensis*72, *M.longifolia*, *M.spicata* 36,48, 84 and *M.suavelens* 24) and eight hybrids (*M.x carinthiaca, M.x gracilis, M.x piperita, M.x rotundifolia, M.x suavis, M.x verticillata, M.x villosa* and *M.x villosonervata)*. Initially the volatile oil composition of the 5 parental species were analysed and the results are given in table 5.4 Subsequently, the 8 species derived from various cross-breding of these parental plants were also analysed and the results are given in Table 5.5. An analysis (using principal coordinate analysis) and discussion of these results is presented below.

Table 5.4 the Percentage of each of the main chemical components of the essential oils obtained by microwave extraction from five different parental species

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M.longifolia	2.9	2.3	2.5	2.3	0.3	0.3	2.7	1.6	8.1	3.2	0.687	0.3	0.7	0.7
M.suaveolens	2.3	1.4	2.0	1.5	0.3	0.3	2.2	1.2	6.7	2.7	0.6	0.2	0.00	0.6
M.spicata	2.5	5.2	3.6	2.6	0.9	17.6	3.2	1.8	9.8	3.8	0.7	0.6	1.2	1.0
M.arvensis	4.7	1.5	5.2	2.6	0	1.9	5.8	3.6	18.6	6.6	1.3	0.6	0.8	0.9
M.Aquatica	4.8	2.0	4.8	6.1	1.8	1.0	5.7	2.8	14.7	8.0	1.3	0.00	0.9	3.5
R.T	**4.53**	**4.8**	**5.95**	**6.34**	**7.55**	**8.27**	**8.7**	**9.09**	**9.77**	**10.77**	**11.14**	**12.44**	**14.05**	**14.77**

Species	15	16	17	18	19	20	21	22	23	24	25	26	27
M.longifolia	0.8	1.4	0.7	1.4	1.8	0.7	1.0	0.1	9.6	26.3	2.6	0.6	2.1
M.suaveolens	0.3	1.2	0.3	0.7	0.9	0.5	2.2	1.0	4.3	42.4	2.1	0.3	1.6
M.spicata	1.1	1.7	0.4	4.9	1.8	0.6	0.7	1.3	1.8	1.4	1.5	0.4	1.2
M.arvensis	0.9	1.7	1.3	1.2	1.3	0.8	1.4	0.00	2.8	0.5	4.5	0.8	2.2
M.Aquatica	5.4	2.1	5.0	2.3	1.4	1.0	0.00	0.00	4.0	0.00	1.9	1.1	3.7
R.T.	**14.87**	**15.43**	**15.55**	**15.9**	**16.16**	**16.39**	**16.51**	**16.67**	**16.75**	**17.03**	**17.1**	**17.59**	**17.77**

1= α-Pinene, 2= Camphene, 3= β-Pinene, 4= Sabinene, 5= α- Phellandrene, 6= Limonene, 7= 1,8-Cineole, 8= γ-Terpinene, 9= Terpinoline, 10= ρ-Cymene, 11= Cis-Ocimene, 12= Menthone, 13= Menthyl Acetate, 14= Linalool, 15= Linalyl, 16= Terpinen-4-ol, 17= iso—Menthol, 18= Pulegone, 19= *I-Menthol,* 20= α-Caryphylene, 21= α-Terpineol, 22= α-Terpinene, 23= Germacrene B, 24=carvone, 25= Geranyl Acetate, 26= Germacrene D, 27=pipertone

Table 5.5 the Percentage of each of the main chemical components of the essential oils obtained by microwave extraction from eight different hybrids species

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M.x villosa	3.0	1.4	3.5	1.9	0	6.26	3.8	2.2	12.2	5.1	0.9	0.4	0.8	0.8
M.x verticillata	2.8	2.03	3.1	6.0	1.5	16.3	3.4	2.0	10.9	4.5	0.8	0.3	0.55	3.6
M.x villosonervata	2.4	1.3	2.8	2.0	0.8	2.5	3.0	1.6	9.5	3.7	0.8	0.3	0.4	1.2
M.x piperita	2.2	1.1	2.7	1.41	0	7.9	3.2	1.5	8.8	3.6	0.7	00.0	0.1	0.6
M.x rotundifolia	4.7	3.3	5.4	2.2	0	0.6	5.9	3.1	18.6	7.3	1.2	1.7	0.7	0.8
M.x suavis	2.4	2.1	0	2.5	0	2.6	8.5	4.6	26.7	9.7	1.9	00.0	0.00	00.0
M.x carinthiaca	1.7	3.3	0	6.4	0	9.2	5.0	00.0	15.8	14.8	1.2	0.6	0.00	0.6
M.x gracillis	0.8	1.9	0	5.1	0	12.2	7.5	3.6	22.0	9.0	1.4	0.00	0.00	5.0
R.T	**4.53**	**4.8**	**5.95**	**6.34**	**7.55**	**8.27**	**8.7**	**9.09**	**9.77**	**10.77**	**11.14**	**12.44**	**14.05**	**14.77**

Species	15	16	17	18	19	20	21	22	23	24	25	26	27
M.x villosa	1.7	0.9	0.7	0.8	1.4	0.6	1.8	0.9	1.5	27.7	1.5	0.2	1.1
M.x verticillata	4.6	1.4	0.2	0.9	1.8	0.4	1.8	0.00	1.5	3.1	0.7	0.8	1.2
M.x villosonervata	2.9	1.2	22.5	1.5	2.6	0.5	0.8	0.00	2.7	22.5	4.7	1.6	0.8
M.x piperita	0.6	1.2	37.4	1.30	0.7	0.4	0.3	0.00	1.4	0.8	0.8	0.3	0.8
M.x rotundifolia	0.6	2.1	8.1	0.3	1.3	0.8	1.0	0.00	2.5	0.8	3.9	0.6	2.0
M.x suavis	1.2	0.00	0.00	20	1.7	0.00	0.00	0.00	4.5	0.5	0.9	2.0	2.5
M.x carinthiaca	00.0	0.00	0.7	2.7	1.0	0.9	2.1	0.00	3.6	0.6	0.3	0.3	2.5
M.x gracillis	1.8	0.00	00.00	1.2	1.6	0.00	0.4	2.6	3.0	3.8	2.7	0.3	2.3
R.T.	**14.87**	**15.43**	**15.55**	**15.9**	**16.16**	**16.39**	**16.51**	**16.67**	**16.75**	**17.03**	**17.1**	**17.59**	**17.77**

1= α-Pinene, 2= Camphene, 3= β-Pinene, 4= Sabinene, 5= α- Phellandrene, 6= Limonene, 7= 1,8-Cineole, 8= γ-Terpinene, 9= Terpinoline, 10= ρ-Cymene, 11= Cis-Ocimene, 12= Menthone, 13= Menthyl Acetate, 14= Linalool, 15= Linalyl, 16= Terpinen-4-ol, 17= iso—Menthol, 18= Pulegone, 19= *I-Menthol,* 20= α-Caryphylene, 21= α-Terpineol, 22= α-Terpinene, 23= Germacrene B, 24=carvone, 25= Geranyl Acetate, 26= Germacrene D, 27=pipertone.

5.21.1 Relationships between parents and hybrids with Principal coordinate analysis (PCoA)

Principal coordinates analysis (also known as classical multidimensional scaling), based on projection, uses spectral decomposition to approximate a matrix of similarities or distances of data objects by a reduction of the dimensions on the basis of some general of total variation. The PCoA calculates the coordinates of the data objects in a way that shows the best first order approximation according to the original interobject distances provided in the axis 1 and axis 2 to give the best second order approximation, then the analysis proceeds so on till all principle coordinates are obtained as 100%. Due to complications in the clarification of the compositional data based on the raw quantities, further data analysis was directed using the different essential oil compositions from the parents species and hybrids species using principle coordinate analysis (PCoA), according to Nei and Li"s [280] in order to construct an analysis based on the matrix developed from both the components shown in Figures 5.11, 5.13, 5.15, 5.17, 5.19, 5. 2, 5.23 and 5.25 where groupings of taxa similar to the hierarchical cluster analysis. The numerical results of the analysis of the volatile oil constituent are summarized in the Table 5.4.and Table 5.5. The results of these PCoA analyses for each of the eight hybrid species are discussed in turn below.

5.21.1.1 Relationship between (*M.arvensis X M.aquatica*) and hybrid *M.x verticillata*

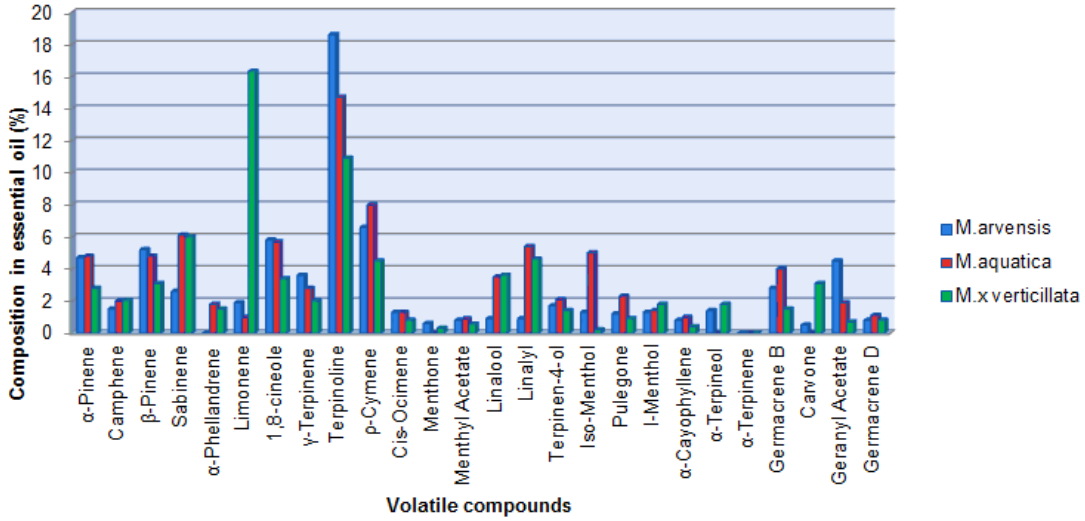


Figure 5.10 The 3-D clustered column graph showing the relationship between species (*M.arvensis X M.aquatica*) and hybrid M.x *verticillata.*

In total nine historical mint leaf samples were analysed for this comparison 3 from *M.arvensis* species (10,11,12) 3 from *M.aquatica* species (13, 14, 15) and 3 from *M.x verticillata* species (19, 20, 21)(the numbers refer to the samples as indicated in table 5.2) . The results from this analysis are shown in Figure 5.10 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically. The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak – the average percentage over the 3 samples are given in each case. From the graphical analysis, some clear indications can be observed. Thus, limonene is present as a major component in the hybrid *M.x verticillata* (16.3%) whilst in the two parental species limonene is found in minor quantities; *M.arvensis* (1.9%) and *M.aquatica* (1.0%). Similarly, l-menthol and carvone show slightly increased levels in the hybrid (1.8% and 3.1% respectively) compared to the parent species *M.arvensis* and *M.aquatica* (1.3% and1.4% for l-menthol and 0.5% and 0.00 for carvone respectively). Some of the essential oil components in this hybrid show a slight decrease in

overall composition, such as (α- Pinene, β-Pinene, 1,8-Cineole, terpinoline, cis-ocimene, ρ-cymene, menthone, menthyl acetate, terpinen-4-ol, i-menthone, pulegone, linalyl, germacrene B, germacrene D, piperitone. Whilst camphene, sabinne, α-phellandrene, γ-terpinene, linalool and geranyl acetate are found in the hybrid at percentage levels that lie in the range between each of the parental species, although the values are slightly closer to *M. arvensis* than *M. aquatica.*

Whilst a qualitative analysis of the data presented graphically in Fig 5.10 leads to the interesting observations outlined above a more complete analysis of the data can be provided by a PCoA. The results for this analysis using the data from the two parents *M.arvensis* and *M.aquatica* plus the hybrid *M.x verticillata* are shown in Figure 5.11. The variation of the first three principal coordinates accounted was 95% of the total variance. Accounting of the first axis was 59.5% of the variation separating the hybrid from the two parents. Additionally, 24.7% of the variation and distinguished distinct for each three species could be accounted for on the second axis. Finally a 3rd axis accounts for another 10.8% variation bringing the total to over 95% on these three axes. However, looking at the individual results in more detail the scatter plot shows some considerable variation in the components found in each of the 3 samples analysed for each species. Thus, two of the samples for the hybrid (samples 19 & 21) can be found on the right of the plot whilst the 3rd sample for this species (sample 20) is much closer in similarity to the parental species. Additionally, one of the samples (sample 13) of the parental species also seems to have quite different analytical results from the other two samples for *M.aquatica*.

The two different analytical methods (the percentage of the main constituents of the essential oil extracted and PCoA data) indicate similar results, hybrid *M.x verticillata* present as intermediate between each of the two parents *M.aquatica* and *M.arvensis.*

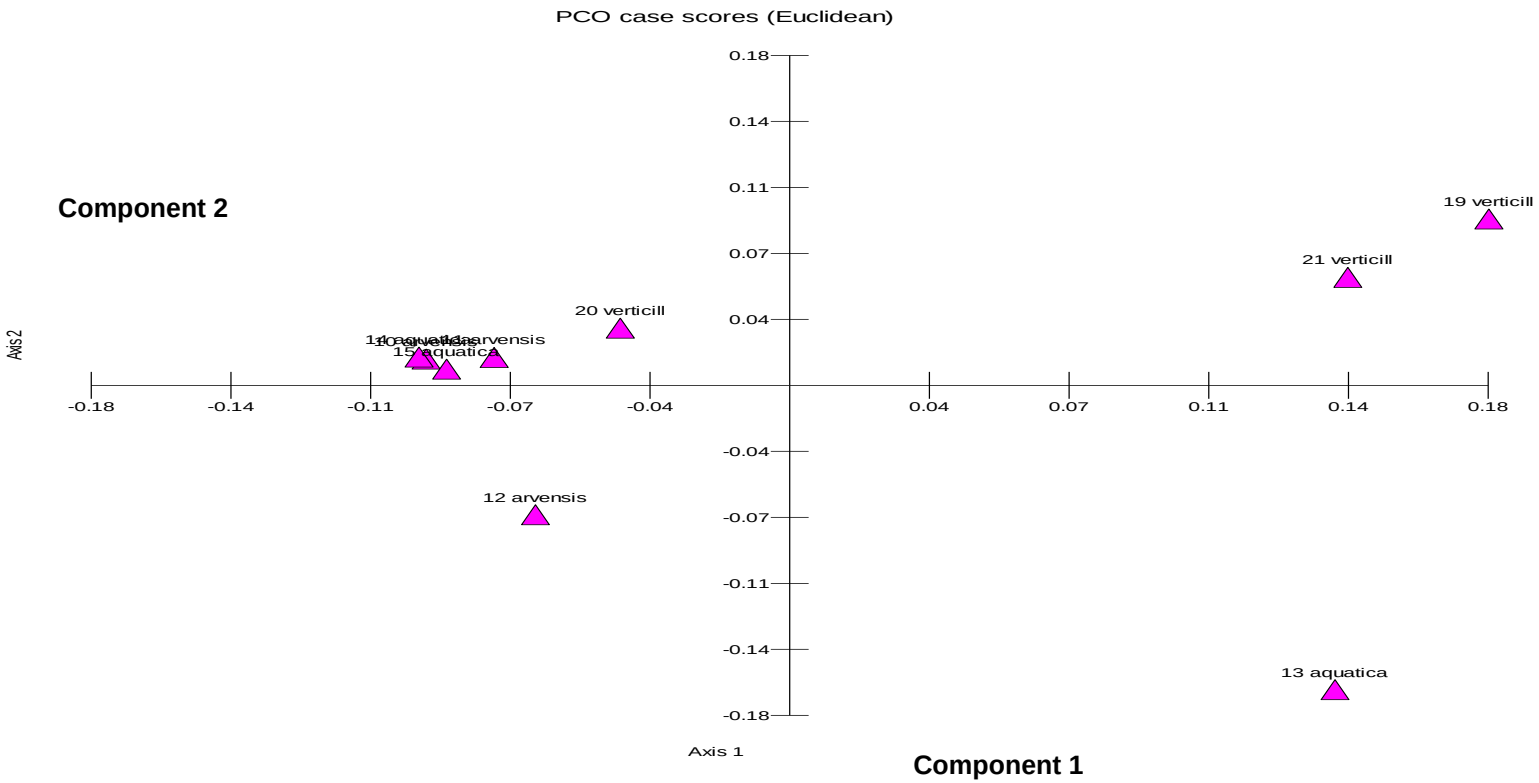


Figure 5.11 The principle coordinates scatter plot represent the relationship between species (M.*arvensis* X M.*aquatica*) & hybrid *verticillata*, based on oil constituents

	Axis 1	Axis 2	Axis 3
Eigenvalues	0.109	0.045	0.020
Percentage	59.5	24.7	10.8
Cum. Percentag	59.549	84.306	95.186

5.21.1.2 Relationship between (*M.spicata X M.aquatica*) and hybrid *M.x piperita*

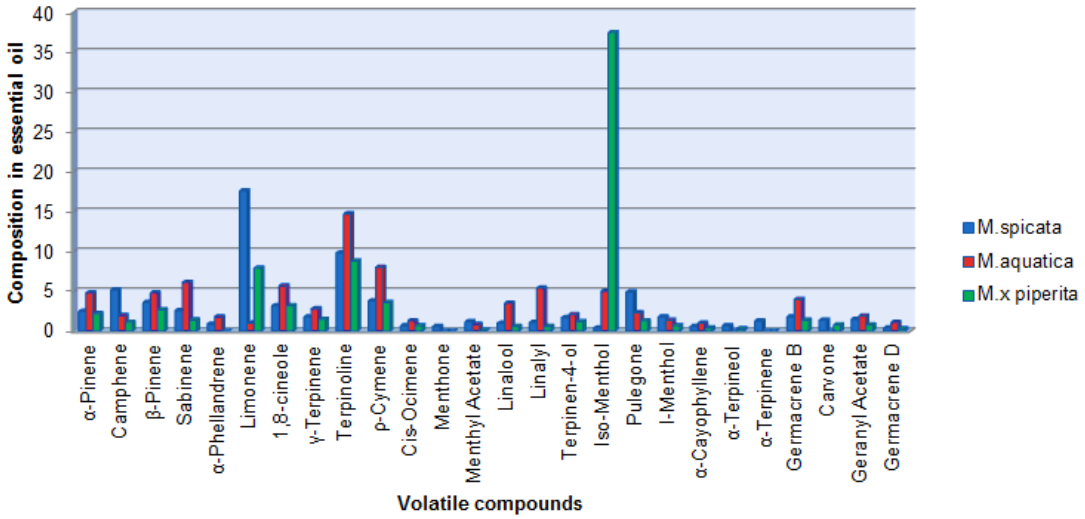


Figure 5.12 The 3-D clustered column graph showing the relationship between species (*M.spicata* X *M.aquatica*) and hybrid *M.x piperita.*

In total eight historical mint leaf samples were analysed for this comparison 3 *M.spicata* species (7, 8, 9) *3 M.aquatica* species (13, 14, 15) and 2 from *M.x piperita* species (25, 26) (the numbers refer to the samples as indicated in table 5.2*).* The results from this analysis are shown in Figure 5.12 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically. The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak the average percentage over the 2-3 samples are given in each case. From the graphical analysis some clear indications can be detected. It can be ssen that the major component of the volatile oil of hybrid *M.x piperita* was iso-menthol (37.4%) in comparison to the parent species. *M.spicata* (0.4%) and *M.aquatica* (5.0%). The α-terpene, menthone was found only in *M.spicata* and was absent in both *M.aquatica* and *M.x piperita.* While some components of essential oil in this hybrid showed a slight decrease in

chemical composition, such as α-pinene, camphene, β-pinene, sabinene, γ-terpinene, terpinoline, ρ-cymene, cis-ocimene, menthyl acetate, linalool, linalyl, terpinen-4-ol, pulegone, α-caryophyllene, α-terpineol, germacrene B, geranyl acetate, germacrene D, piperitone. For limonene and 1,8-cineole the percentage composition was found to be in the range between the parental species (*M.spicata* and *M.aquatica*). The presence of carvone, in relatively low percentage (0.77%), may be due to parental characteristic that shows the absence of carvone in the *M.spicata*.

The scatter plot from the principle coordinate analysis is shown in Figure 5.13. The analysis showed that three axes could account for 96.5% of the total variance in these data points that separated *M.x piperita* from (*M.spicata* and *M.aquatica*) with over 89% of this being accounted for in the first two axes. The PCoA results data show that essential oil composition of the hybrid occupied an intermediate position between each of the two parents. Thus examining the data for hybrid samples 25 & 26 shows that they show resemblance to both parental species (samples 8 & 13).

The two different analytical methods (the percentage of the main constituents of the essential oil extracted and PCoA data) indicate that the hybrid *M.xpiperita* present as intermediate between each of the two parents with slight character towered *M.aquatica* more than *M.spicata.*

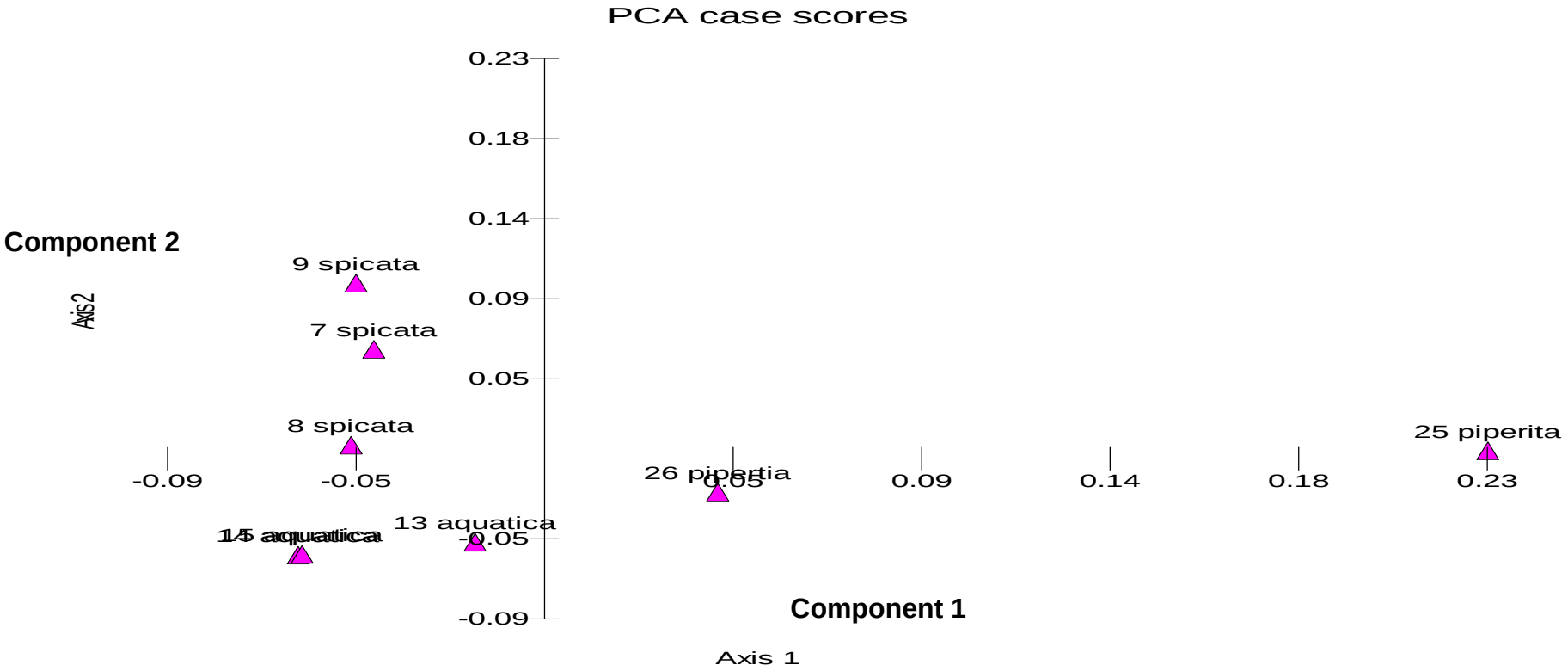


Figure 5.13 the principle coordinates scatter plot represent the relationship between species (*M.spicata s X M.aquatica*) and hybrid *piperita* based on oil constituents.

	Axis 1	Axis 2	Axis 3
Eigenvalues	0.068	0.023	0.008
Percentage	66.6	22.4	7.5
Cum. Percentage	66.6	89.1	96.6

5.21.1.3 Relationship between (*M.suaveolens X M.spicata*) and hybrid *M.x villosa*

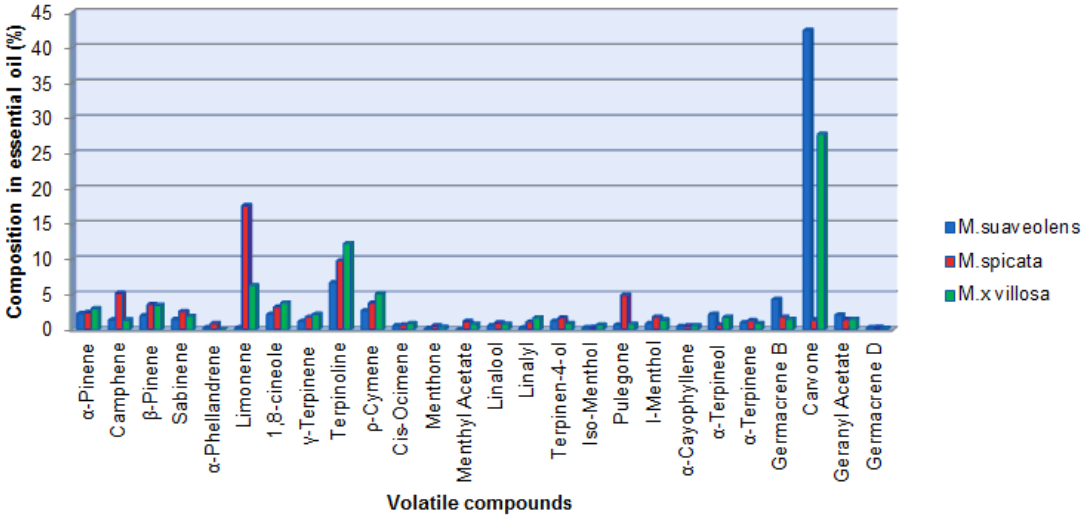


Figure 5.14 The 3-D clustered column graph showing the relationship between species (*M.suaveolens X M.spicata*) and hybrid *M.xvillosa*

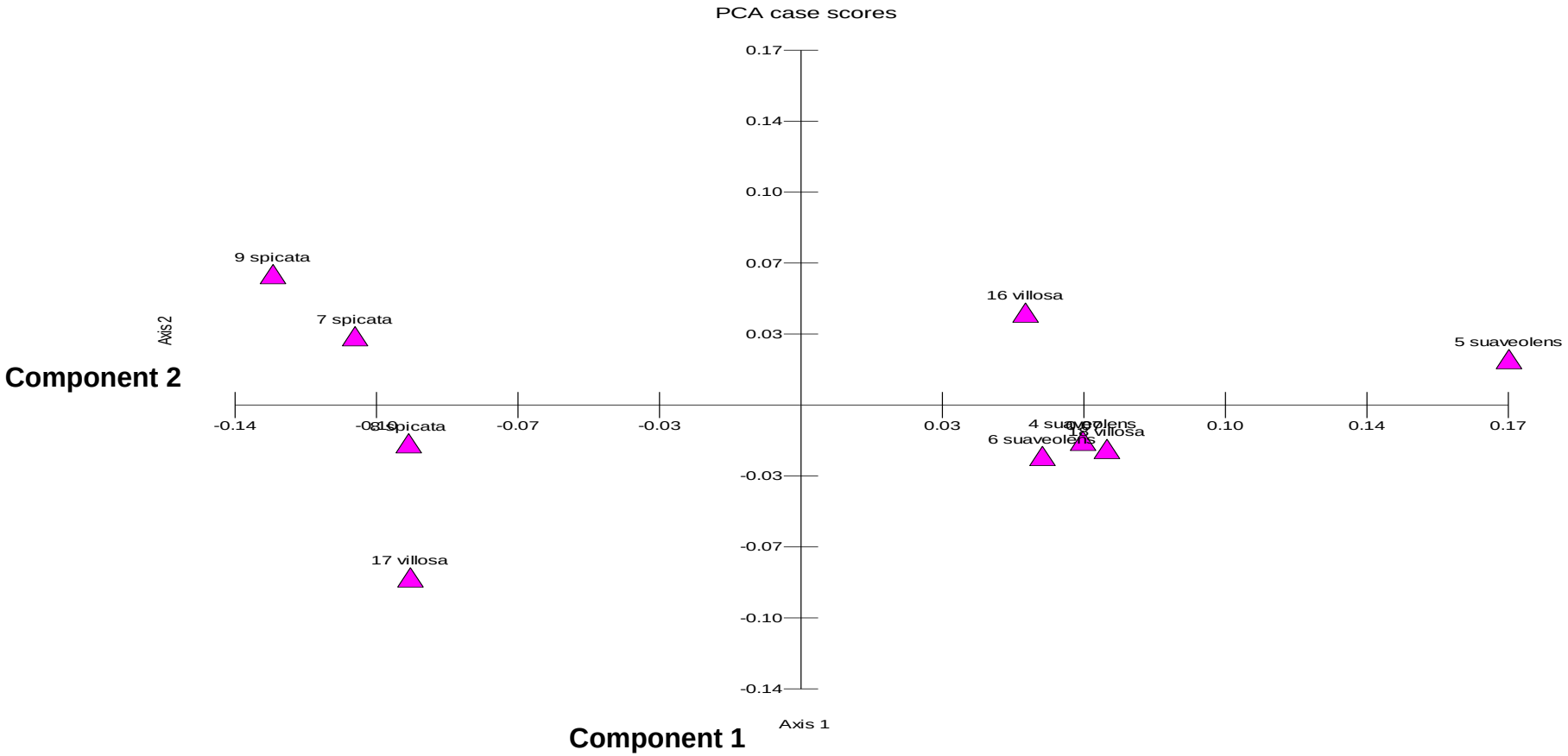
In total nine historical mint leaf samples were analysed for this comparison 3 *M.spicata* species (7, 8, 9) 3 *M.suaveolens* species (4, 5, 6) and 3 *M.xvillosa* species (16, 17, 18) (the numbers refer to the samples as indicated in table 5.2). The results from this analysis are shown in Figure 5.14 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically. The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak the average percentage over the 3 samples are given in each case. From the graphical analysis some clear indications can be detected**.** Figure 5.14 shows that the α-Phellandrene not detected in the hybrid *M.x villosa* compared to the parental species (*M.suaveolens* and *M.spicata)* which contain low but significant levels of α-phellandrene. However the essential oils compounds obtained from hybrid *M.x.vellosa* show improvements in some of the essential oil percentages such as α- pinene, α-terpinene, terpinoline, ρ-

cymene, cis-ocimene, linalyl, iso-menthol. While some of the components of the essential oil in this hybrid show a slight reduction in percentage, such as, β-pinene, terpinen-4-ol, α-terpinene, germacrene B, germacrene D and piperitone. For the following componenets, camphene, sabinene, limonene, 1,8-cineole, menthone, menthyl acetate, linalool, pulegone, i-menthol, α-caryophyllene, α-terpineol, carvone and geranyl acetate the percentages found in the hybrid were in the range between the two parents *M.suaveolens* and *M.spicata* .

The scatter plot from the principle coordinate analysis (Figure 5.15) shows that only two axes were required to account for 95.5% of the total variance that separated *M.x villosa* from the parents (*M.suaveolens* and *M.spicata*).

The scatter plot distantly placed *M.x villosa* between two parents as intermediate. Though in this hybridization the *M.x villosa* (sample 18), can be found on the right of the plot move more toward two samples of one parent *M.suaveolens* (samples 4, 6) and less possibility for the hybrid *M.x villosa (*sample 17*),* to back cross to other parent *M.spicata* (sample 8) found on the left below of the plot more than upper left of the plot for two samples (9, 7) of parents *M.spicata*. Additionally, one of the samples (sample 5) of the parental species also seems to have quite different analytical results from the other two samples for *M.suaveolens.*

The two different analytical methods (the percentage of the main constituents of the essential oil extracted and PCoA data) indicate similar results, though hybrid *M.x villosa* located more towered *M.suaveolens* but still present as intermediate between each of the two parents in axis 2.



	Axis	Axis 2
Eigenvalues	0.09	0.016
Percentag	81.5	14.3
Cum. Percentage	81.595	95.983

Figure 5.15 The principle coordinates scatter plot represent the relationship between (*M.suaveolens X M.spicata*) and hybrid M.x *villosa*, based on oil constituents.

5.21.1.4 Relationship between (*M.suaveolens* X *M.longifolia*) and hybrid *M.x rotundifolia*

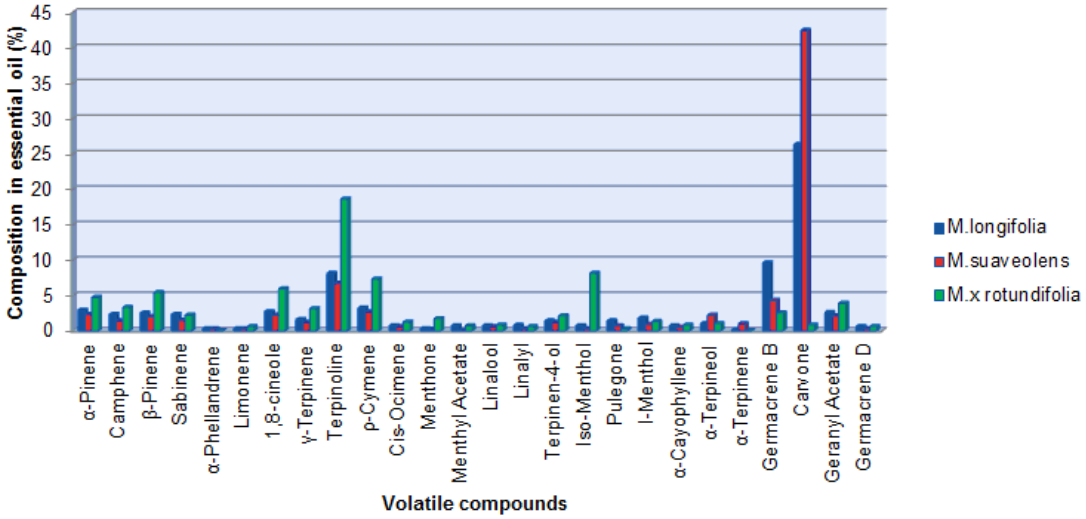


Figure 5.16 The 3-D clustered column graph showing the relationship between species (*M.suaveolens X M.longifolia*) and hybrid *M.x .rotundifolia*

In total eight historical mint leaf samples were analysed for this comparison 3 from *M.longifolia* species (1, 2, 3) 3 from *M.suaveolens* species (4, 5, 6) and 2 from *M.x rotundifolia* species (27, 28) (the numbers refer to the samples as indicated in table 5.2). The results from this analysis are shown in Figure 5.16 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically. The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak the average percentage over the 2-3 samples are given in each case. From the graphical analysis some clear indications can be drawn. The most striking observation is the significant reduction in the percentage composition for carvone in the hybrid (0.8%) when compared to both parent species *M.suaveolens* (26.3%) and *M.longifolia* (42.4%). Similarly, two of the essential oil constituents (α-phellandrene and α-terpinene) are not detected

in the hybrid whilst being present in both parents and some components in this hybrid show a slight reduction in chemical composition such as pulegone, germacrene B, and piperitone. In contrast, several essential oils compounds are present in the hybrid in increased percentages; these include α-pinene, camphene, β-pinene, limonene, 1,8-cineole, γ-terpinene, terpinoline, ρ-cymene, cis-ocimene, menthone, terpinen-4-ol and iso-menthol geranyl acetate. The componenets sabinene, linalool, linalyl, i-menthol, α-caryophyllene, α-terpineol, germacrene D and menthyl acetate are found in the hybrid in percentage compositions that are in the range between both species *M.suaveolens* and *M. longifolia.*

The scatter plot in principle coordinates for the data is given in Figure 5.17 and shows that 96.6% of the total variance of the hybrid *M.x rotundifolia* from the parents (*M.suaveolens* and *M.longifolia*) can be accounted for using the first two axes. The PCoA plots in these species are difficult to identify because the two parents are very similar and not separated very well. However, it does seem that the position of the two hybrid *M.x rotundifolia* samples (27 and 28*)* were more closely related to *M.longifolia* (samples 2 and 3) then *M.suaveolens* (samples 4 and 6*).* Additionally, one of the samples from each of the two parental species (*M.suaveolens* sample 5 and *M.longifolia* sample 1) also seem to have quite different analytical results from the other two samples that were tested from each of these parental plants.

The two different analytical method show quite different results, according to the percentage of the main constituents of the essential oil extracted the hybrid located as intermediate between two parents while as PCoA data hybrid *M.x rotundifolia* found to be more towered *M.longifolia* then *M.suaveolens.*

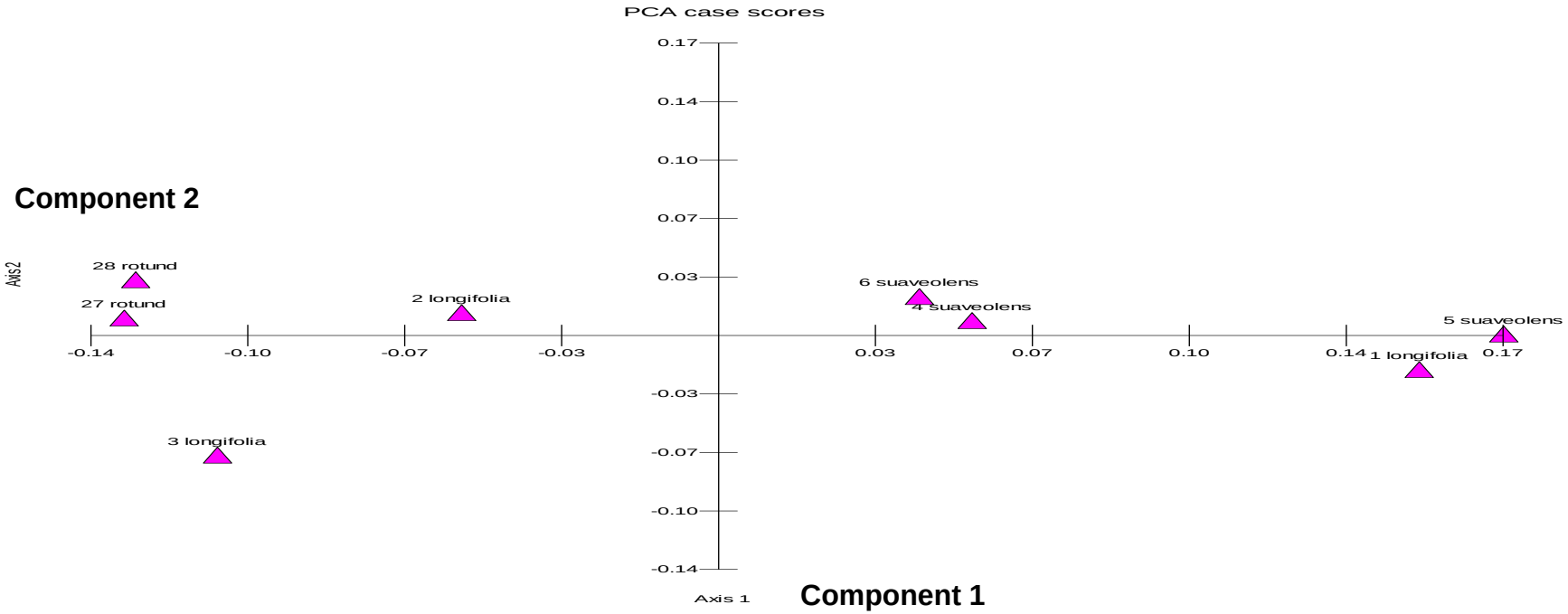


Figure 5.17 The principle coordinates scatter plot represent the relationship between (*M.suaveolens X M.longifolia*) and hybrid *M.rotundifolia*, based on oil constituents.

	Axis 1	Axis 2
Eigenvalues	0.106	0.007
Percentage	90.4	6.2
Cum. Percentag	90.4	96.6

5.21.1.5 Relationship between (*M.spicata X M.longifolia)* and hybrid *M.x villosonervata*

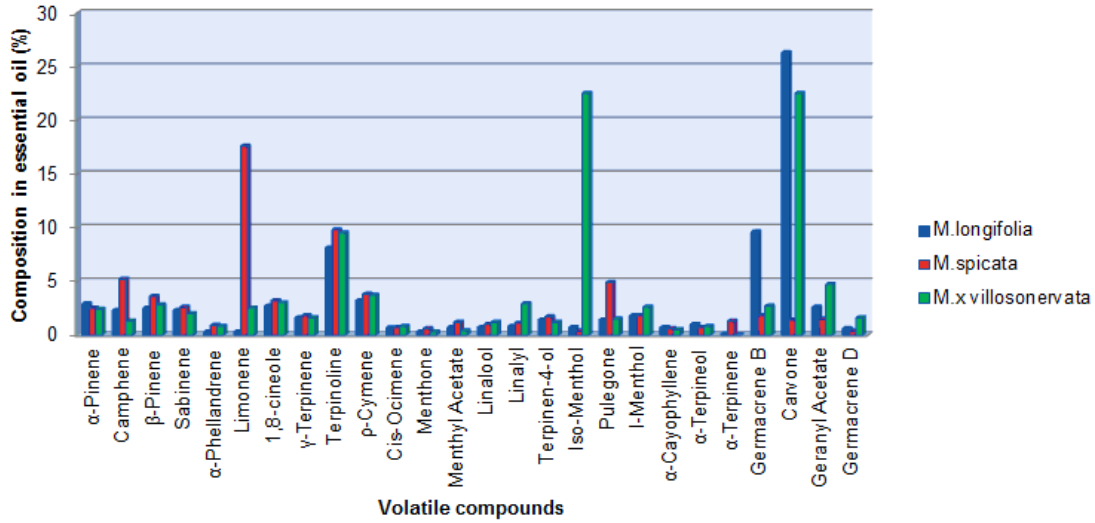


Figure 5.18 The 3-D clustered column graph showing the relationship between species (*M.spicata* X *M.longifolia)* and hybrid *M.x villosonervata*.

In total nine historical mint leaf samples were analysed for this comparison 3 from *M.longifolia* species (1, 2, 3) 3 from *M.spicata* species (7, 8, 9) and 3 from *M.x villosonervata* species (22, 23, 24) (the numbers refer to the samples as indicated in table 5.2). The results from this analysis are shown in Figure 5.18 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically. The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak the average percentage over the 3 samples are given in each case. Analysis of the results indicates that the essential oils compounds obtained from hybrid *M.x. villosonervata* show improvements in some of the essential oil percentages such carvone and iso-menthol (22.5% for carvone and 22.5% for iso-menthol) compare to the parents, carvone in hybrid show similar to one of the parents *M.longifolia* (26.3%) and quite different from the other *M.spicata* (1.4%), while iso-menthol in both parents present in minor

quantities. The levels of limonene found in the hybrid (2.5%) are much closer to those found in *M.longifolia* (0.3%) than those present in *M.spicata* (17.6%). Other essential oils present in the hybrid samples have slightly increased percentages when compared to both parents – examples are linalool, linalyl, i-menthol geranyl acetate and germacrene D. Conversely, some of the essential oil in this hybrid shows a slight reduction in chemical composition such as, α-pinene, camphene, *sabinne*, γ-terpinene, menthyl acetate, terpinen-4-ol, α-caryophyllene, α-terpineol, and piperitone. The remaining essential oils are found at levels that lie within the range between the two parent species, such as β-pinene, α-phellandrene, 1,8-cineole, terpinoline, ρ-cymene, cis-ocimene, menthone, pulegone, germacrene B and carvone.

The scatter plot in principle coordinates is shown in Figure 5.19 and shows that 98.1% of the total variance could be accounted for in 3 axes, with over 94% found in the first two of these. Examination of the results presented in the PCoA analysis further confirms the trends seen in the graphical analysis (Figure 5.18) which suggested that the essential oil composition of the hybrid most closely resembles that of the *M.longifolia* parent rather than *M.spicata.*

The two different analytical method show quite different results, according to the percentage of the main constituents of the essential oil extracted the hybrid located as intermediate between two parents while as PCoA data hybrid *M.x villosonervata* found to be more towered *M.longifolia* then *M.spicata.*

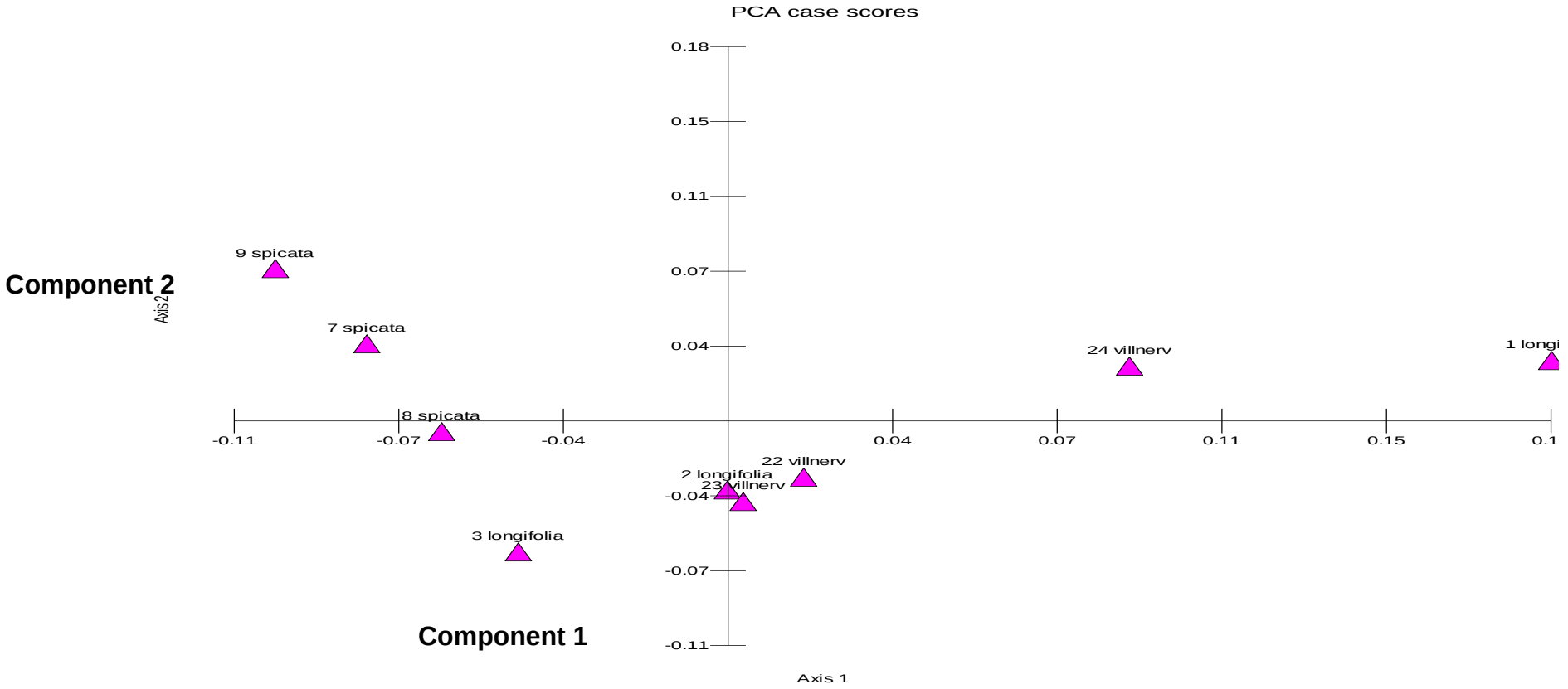


Figure 5.19 The principle coordinates scatter plot represent the relationship between (*M.spicata X M.longifolia*) and hybrid *villosonervata,* based on oil constituents.

	Axis 1	Axis 2	Axis 3
Eigenvalue	0.501	0.086	0.023
Percentage	80.6	13.8	3.7
Cum. Percentage	80.7	94.5	98.2

5.21.1.6 Relationship between (*M.suaveolens X M.arvensis*) and hybrid *M.xCarinthiaca*

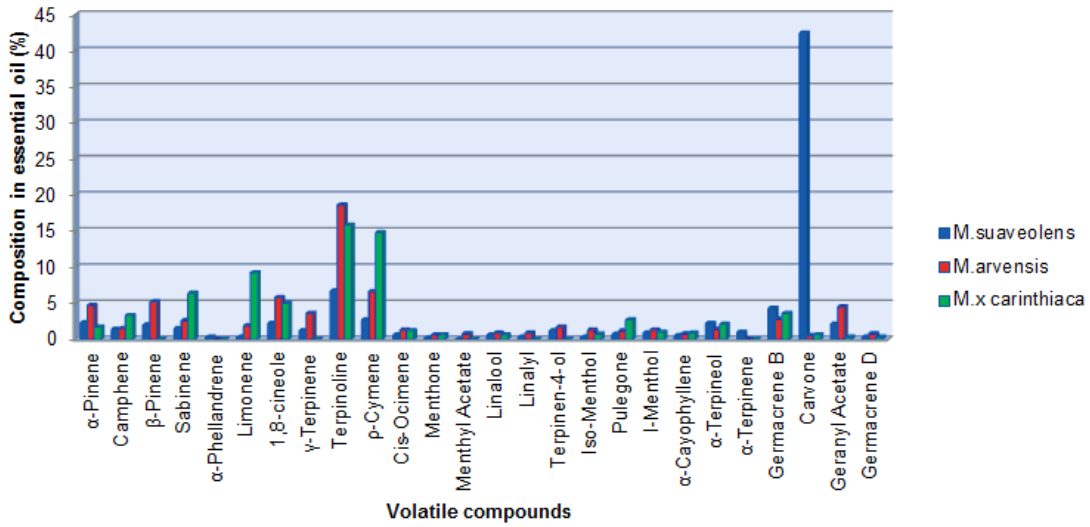


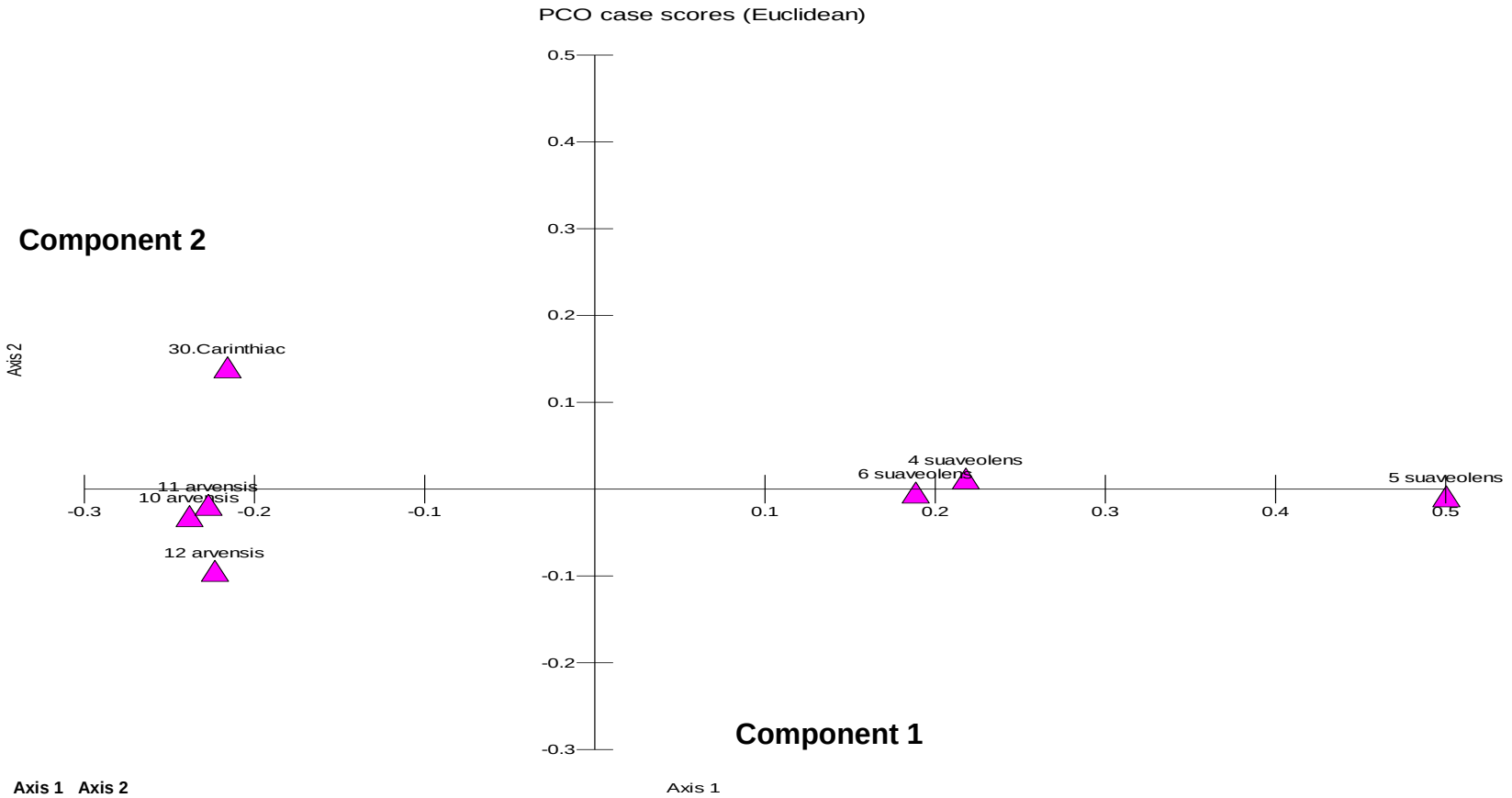
Figure 5.20 The 3-D clustered column graph showing the relationship between species (*M.suaveolens* X *M.arvensis*) and hybrid *M.x Carinthiaca*

In total seven historical mint leaf samples were analysed for this comparison, 3 from *M.suaveolens* species (4, 5, 6), 3 from *M.arvensis* species (10, 11, 12) and a single sample from the *M.x Carinthiaca* hybrid (30) (the numbers refer to the samples as indicated in table 5.2). The results from this analysis are shown in Figure 5.20 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically. The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak the average percentage over the 3 samples are given in each case. It should be noted that with only one sample of the hybrid available the results from the extraction and GC-MS analysis need to be examined with caution. However, from the graphical analysis it can be seen that some of the essential oil components were not detected in the hybrid such as β-pinene, γ-terpinene, linalyl, and terpine-4-ol even though they were present in both parental species. Similarly, some of the components were only present

in one of the parental species but again were missing in the hybrid – this included α-phellandrene and menthyl acetate. In addition some of oil constituents show significant increase for the hybrid, such as limonene (9.3%) and ρ-cymene (14.8%) compare to *M.suaveolens and M.arvensis* (0.3%, 1.9%) for limonene and (2.6%, 6.6%) for ρ-cymene. Moreover, camphene, sabinene, menthone, α-caryophyllene and piperitone were found at slightly higher levels in for the hybrid *M.x carinthiaca*. While some components in the same hybrid showed a slight reductionsuch as, α-pinene, germacrene B, geranyl acetate and germacrene D.

The scatter plot in the principle coordinates analysis (Figure 5.21) accounted for 96.5% of the total variance in the first two axes. Only one hybrid sample was analysed and the PCoA analysis indicates that this sample showed essential oil characteristics closely related to the *M.arvensis* parent (sample 10, 11, 12) and less close in composition to the *M.suaveolens* samples (4, 5, 6). However, based on the variations of the essential oil composition seen in the different samples from each species that have been discussed above care must be taken into reading too many conclusions into a single sample result.

The two different analytical method show similar results, according to the percentage of the main constituents of the essential oil extracted and PCoA data the hybrid *M.x carinthiaca* located as intermediate between two parents although more toward *M.arvensis* then *M.suaveolens.*



	Axis 1	Axis 2
Eigenvalue	0.649	0.036
Percentag	91.4	5.1
Cum. Percentage	91.4	96.6

Figure 5.21 The principle coordinates scatter plot represent the relationship between (*M.suaveolens X M.arvensis*) and hybrid Carinthaica, based on oil constituents.

5.21.1.7 Relationship between (*M.spicata X M.arvensis*) and hybrid *M.x gracillis*

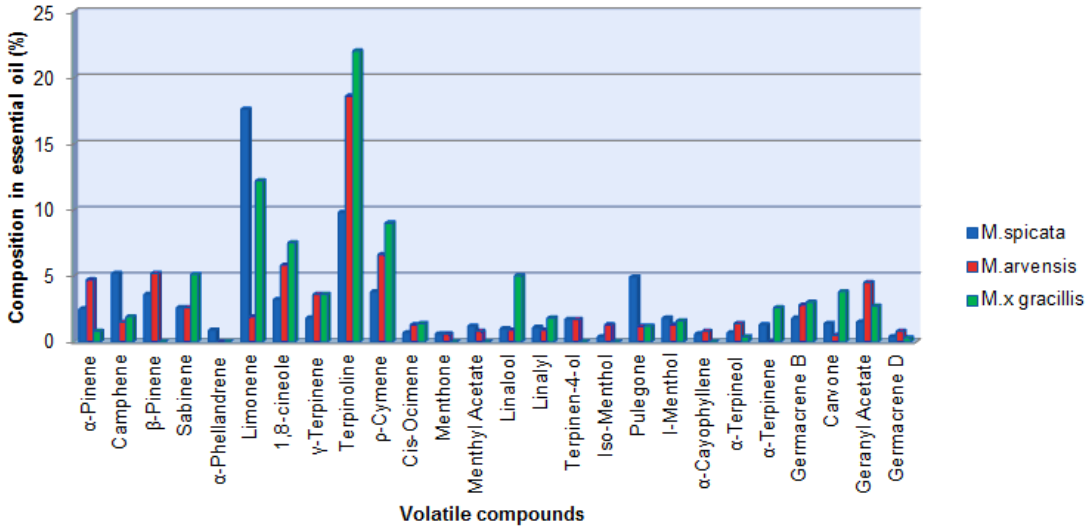


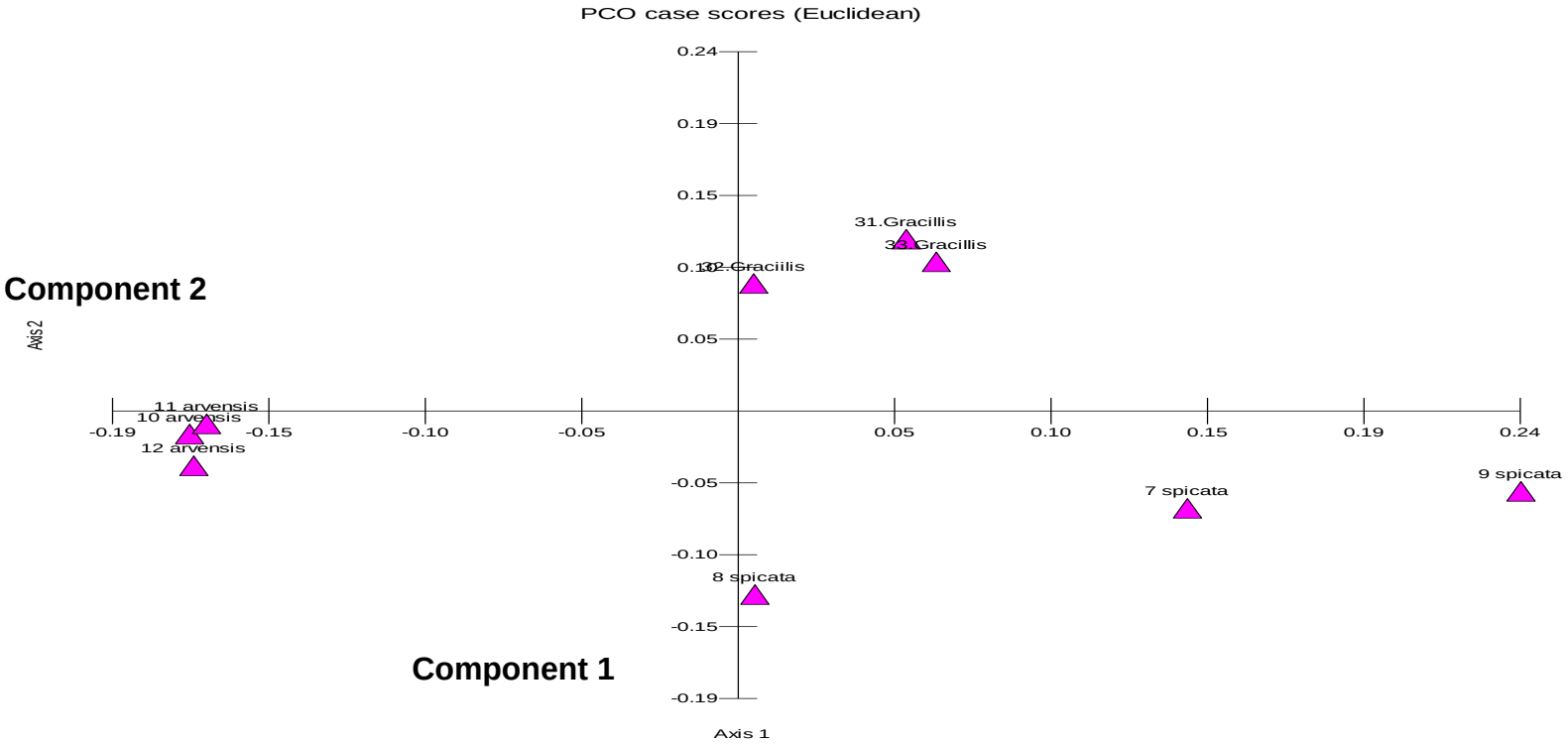
Figure 6.22 The 3-D clustered column graph showing the relationship between species (*M.spicata* X *M.arvensis*) and hybrid *M.x gracillis*

In total nine historical mint leaf samples were analysed for this comparison; 3 from *M.spicata* species (7, 8, 9), 3 from *M.arvensis* species (10, 11, 12) and 3 from the hybrid *M.x gracillis* species (31, 32, 33) (the numbers refer to the samples as indicated in table 5.2). The results from this analysis are shown in Figure 5.22 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically.The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak the average percentage over the 3 samples are given in each case. From the graphical analysis we can see that a number of components (such as β-pinene, menthone, menthyl acetate, terpinen-4-ol, iso-menthol, α-caryophyllene) are not present in the hybrid species even though both parental species have significant percentages of these compounds. Similarly α-phellandrene was not detected in the hybrid but is present in one of the parents *M.arvensis*. In addition, some of the essential oil components were

found in *M.x gracillis* at slightly higher levels than in both parents, such as sabinene, 1,8-cineole, terpinoline, ρ-cymene, cis-ocimene, linalool, linalyl, α-terpinene, germacrene B, carvone, germacrene D and piperitone. However, the levels of α-pinene, pulegone and α-terpineol in this hybrid were reduced. Finally, some of essential oli components were found for *M.x gracillis* at a similar overall percentage to the two parents, such as camphene, limonene, γ-terpinene, i-menthol and geranyl acetate.

The scatter plot analysis of the principle coordinates is given in Figure 5.23 and indicated that although 4 axes were required to account for 98% of the overall variation that the first two of these axes made up 83.3% of the total. Examination of this plot indicates that the three hybrid *M.x gracillis* samples (31, 32, 33) do not have a very strong resemblance to either parent and that any similarity seems to be closer to *M.spicata* (samples 7, 8, 9) rather than *M.arvensis* (samples 10, 11, 12).

The two different analytical method show similar results, according to the percentage of the main constituents of the essential oil extracted and PCoA data the hybrid *M.x gracillis* located as intermediate between two parents although more toward *M.spicata* then *M.arvensis* in axis 2.



Eigenvalue	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.171	0.05	0.025	0.015
Percentage	62.8	20.5	9.2	5.6
Cum. Percentage	62.8	83.3	92.5	98.1

Figure 5.23 The principle coordinates scatter plot represent the relationship between (*M.spicata X M.arvensis*) and hybrid M.x *gracillis*, based on oil constituents.

5.21.1.8 Relationship between (M.*suaveolens* X *M.aquatica*) and hybrid *M.x suavis.*

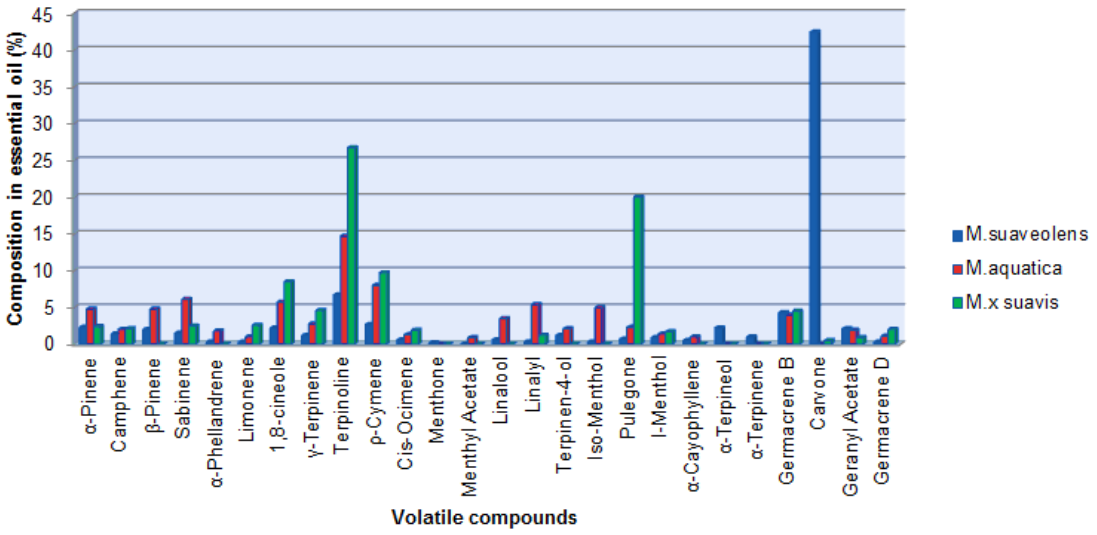


Figure 5.24 The 3-D clustered column graph showing the relationship between species (*M.suaveolens* X *M.aquatica*) and hybrid *M.x suavis.*

In total seven historical mint leaf samples were analysed for this comparison; 3 from *M.suaveolens* species (5 , 6, 7), 3 from *M.aquatica* species (13, 14, 15) and 1 from the hybrid *M.x suavis* species (29) (the numbers refer to the samples as indicated in table 5.2). The results from this analysis are shown in Figure 5.24 where the percentage of each of the main constituents of the essential oil extracted from each parent and the hybrid species are indicated graphically.The percentages are calculated from the GC trace and are the percentage for each component as indicated by the area under the GC peak the average percentage over the 1-3 samples are given in each case. Only one sample from the hybrid could be analysed and so any analysis has to be taken with this proviso, however a number of findings can be suggested. A number of compounds which are present in both parental species are not detected in the hybrid such as, β-pinene, α-phellandrene, linalool, terpinen-

4-ol, iso-menthol, α-caryophyllene. Similarly menthone, α-terpineol and menthyl acetate are also absent from the hybrid species even though they are found in one of the parental species. Some of essential oil components are present in *M.x suavis* at slightly percentage increased levels compared to the parental speciessuch as limonene, γ-terpinene 1,8-cineole, ρ-cymene, cis-ocimene, α-terpinene, germacrene B, pulegone and germacrene. The essential oil terpinoline showed a significant increase in percentage terms (26.7%) compared to *M.suaveolens and M.aquatica* (6.7% and 14.7% respectively). Geranyl acetate shows a small reduction. Finally, the percentage levels of α-pinene, camphene, sabinene, linalyl, i-menthol carvone, and piperitone found in the hybrid are within the range of the two parent species.

The scatter plot analysis is given in Figure 5.25 and shows that almost 98% of the variance can be accounted for in the first two axes. The scatter plot placed the *M.x suavis* oil composition (sample 29) more toward two of the *M.aquatica* parent samples (14, 15) And much further away from the three *M.suaveolens* samples (4, 5, 6).

The two different analytical method show similar results, according to the percentage of the main constituents of the essential oil extracted and PCoA data the hybrid *M.x suavis* located as intermediate between two parents although more toward *M.aquatica* then *M.suaveolens* according to PCoA data results.

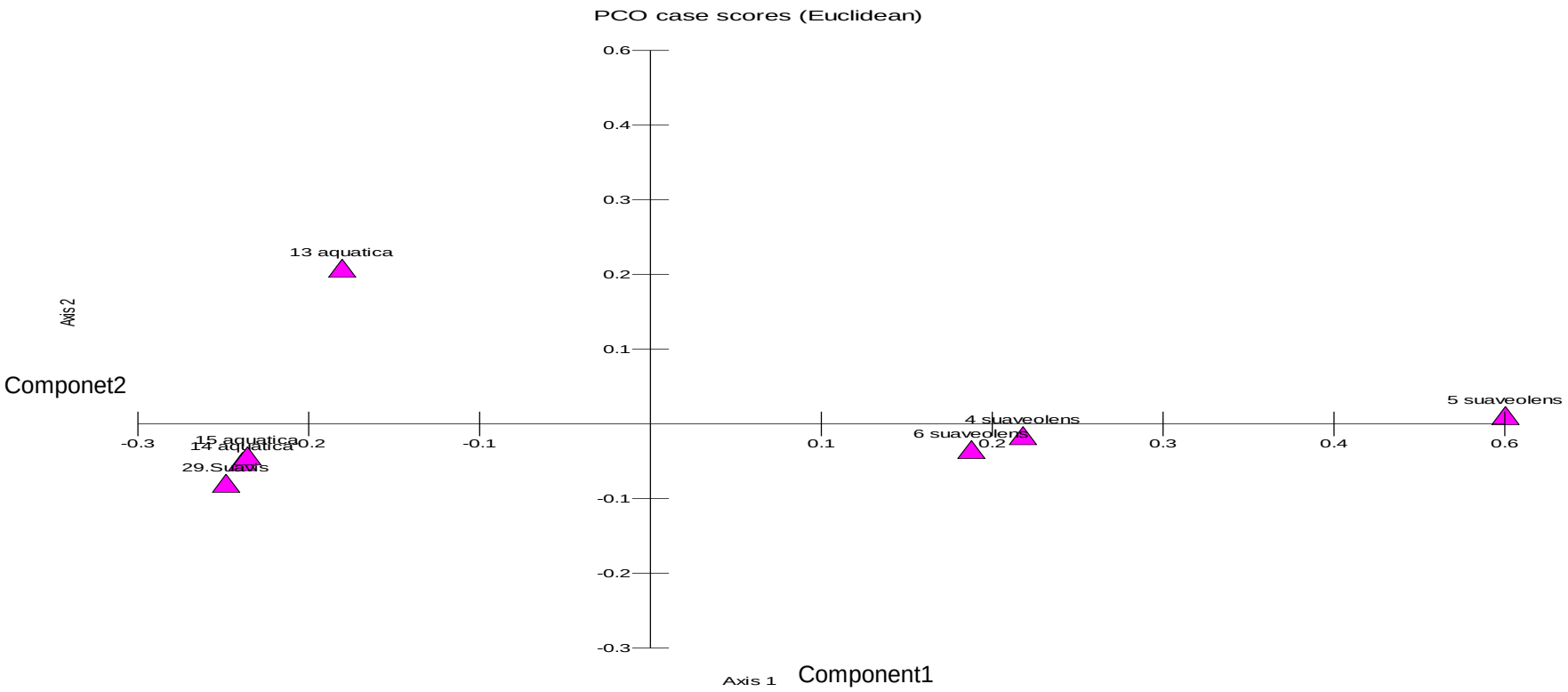


Figure 5.25 The principle coordinates scatter plot represent the relationship between (*M.suaveolens X M.aquatica*) and hybrid *suavis*, based on oil constituents.

	Axis 1	Axis 2
Eigenvalues	0.654	0.068
Percentage	88.7	9.2
Cum. Percentage	88.7	97.9

5.21.2 Using un-weighted pair-group method with an arithmetic average (UPGMA)

UPGMA trees (known also as Group-average) are a method of showing relationships between each of the 33 mint samples that we analysed (Table 5.2) through principle component analysis (PCA). The relationships within each species are constructed and reflect phenotypic similarities by phylogenetic distances. Figure 5.26 represents the taxa with high similarity in their essential oil constituents clustered together and the taxa with less similarity grouped separately. The pairwise genetic distance of the accession were calculated to cluster, the data between the *Mentha* accession using the un-weighted pair-group method with an arithmetic average (UPGMA). It can been seen from to Figure 5.26 that this analysis divides the accession into one minor cluster containing only 25 *M.x piperita* and one major cluster which is in turn divided into four sub-clusters or branches: (I) *32.M.x gracillis, 33.M.x gracillis, 31.M.x gracillis, 30.M.x carinthaica, 21.M.x verticillata,19.M.x verticillata, 8.M.spicata, 9.M.spicata and 7.M.spicata* created to the first branch (II) *13.M.aquatica, 3.M.longifolia,* 28.*M.x rotundifolia, 26.M.x piperita,* 27.*M.x rotundifolia, 12.M.arvensis, 29.M.x suavis, 20.M.x verticillata, 11.M.arvensis,15.M.aquatica, 17.M.x Villosa, 14.M.aquatica, 10.M.arvensis, 23.M.x villosonervata, 22.M.x villosonervata* and *2.M.x longifolia* made up the second branch. While the third (III) branch belongs to *16.M.x Villosa, 24.M.x villosonervata, 18.M.x villosa, and 6.M.x suaveolens and 4.M.suaveolens,* were the fourth branch joined to *5.M.suaveolens* and *1.M.longifolia.* In general twenty-four compounds were identified in the essential oils of the five different parental mint species (Table 5.4) and eight hybrid species derived from them (Table 5.5).There was great variation in the essential oils identified from the above species. α-phellandrene rich essential oils have been recorded only for two hybrid *M.x verticillata* (6.0%) and *M.x villosonervata* (2.0%). While β-pinene not detected in *M.x suvais, M.x carinthiaca* and *M.x gracillis.* In addition α-terpinene rich volatile oils were recorded only for *M.x*

Villosa and *M.x gracillis* (0.9%, 2.6%, respectively). The major oil constituents in the essential oil of *M.x piperita* was iso-menthol (37.4%). Finally carvone rich volatile oil was detected in *M.x villosa* and *M.x villosonervata* (27.7% and 22.5%). However, much more data from different mint inhabitants' is needed before the inherent control and explanation of the essential oil compounds in the naturally occurring hybrids is clearly understood.

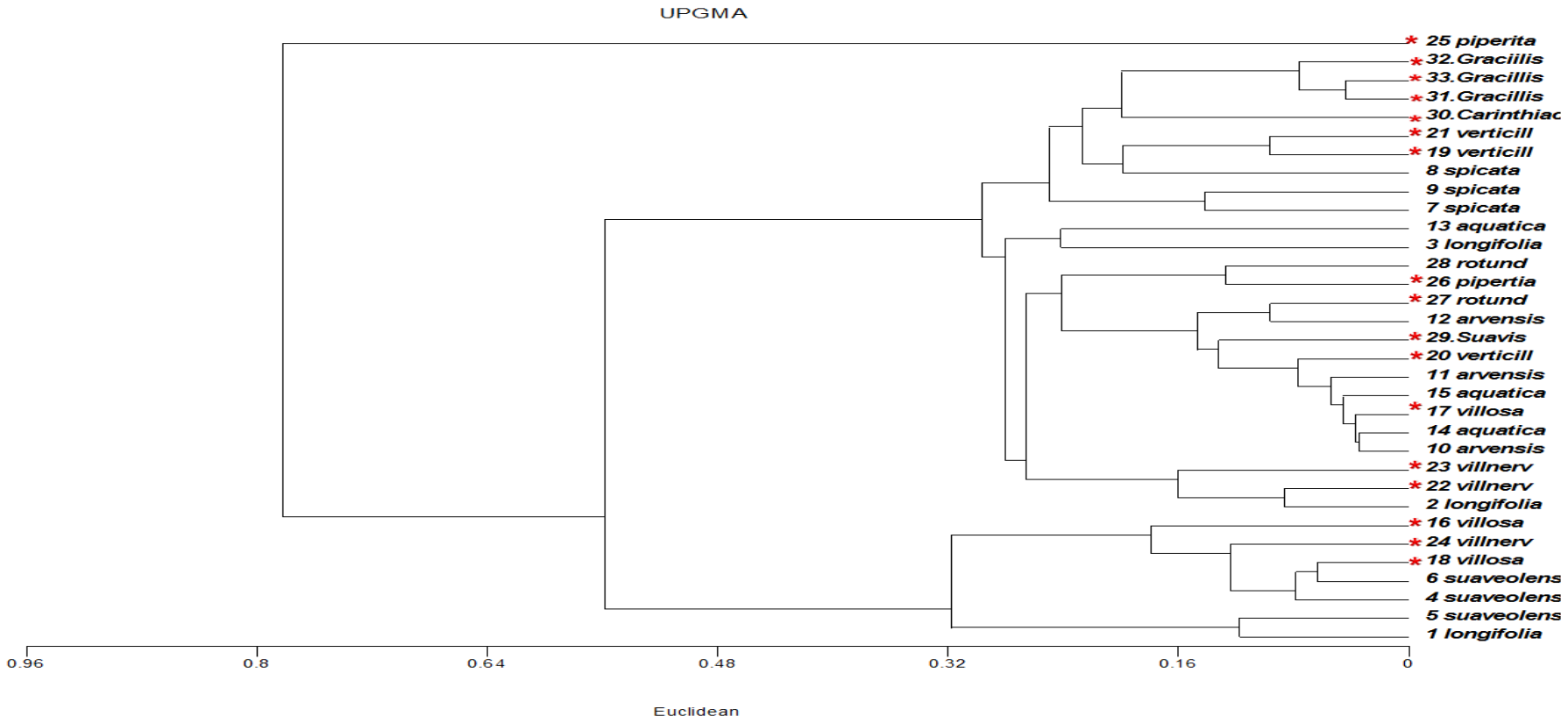


Figure 5.26 Cluster analysis (UPGMA Phenogram) of 33 *Mentha* accession based on Nei and Li's (1979) distance.* Indicates hybrid species

5.22 Discussion

The microwave extraction method was found to be one of the promising techniques for the extraction of essential oils from plants as this process would preserve the original qualities of the plant. Microwave is a special type of extraction process for temperature sensitive materials like oils, resins, hydrocarbons, etc. which are insoluble in water and may decompose at their boiling point (which can be prevented using steam distillation method).

Analysis using gas chromatography-mass spectrometer was found to be the best method to identify even the minor components of particular oil along with major components.

Extraction of essential oils using the microwave, method can be used on a small scale as well as in large scale to make various finished products which includes body oils, cosmetic lotions, bath oils, hair rinses, soaps, perfumes and room sprays.

The aim of this project was to characterize the mint oil recovery with respect to its chemical components (relative ratio of the individual essential oil constituents in individual species or hybrids). The aspect of the mint oil was considered when the quality of the essential oils was evaluated as a percentage composition. The percentage composition of essential oils from different mint species selected in this study associated varied in quantity of dominant components with comparable literature data. Accordingly, volatile oils extracted from Leaves of the cultivated plants of different types of mint [(*English Lamb mint, Moroccan mint, Tashkent Spearmint, Mentha spearmint, Common mint, Garden Mint* Family *Mentha Spicata)* and *Black peppermint* Family*: Mentha Piperita*], were varied in range (>1% to 49.6%) from total oil extracted. The composition of the oil extracted varies in certain constituents more than others. In all analyses conducted carvone was the largest percentage component observed in seven selected mints [Tashkent

Spearmint (49.6%), Garden Mint (41.4%), English Lamb mint (36.4%), Common mint (36%), Mentha spearmint(35.5%), Moroccan mint(31.3%) and finally Black peppermint(28.9%)]. follow by 1,8-cineol (5.3%) in Black peppermint .

From literature it is well established that essential oils show important biological activities such as antioxidant, antimicrobial as well as anti-fungal properties. It also is used in pharmaceutical production as flavourings agent in cough syrup or oral preparation such as, toothpaste and mouthwash. By GC-MS these dried species were analysed for their major constituents and these were identified by using a combination of the retention times of authentic commercial standards of individual components. Plus oils of identified composition in accordance with previous literature it has been shown that the volatile oil plants contain a complex mixture of compounds of low molecular weight. From five *Mentha* species and eight hybrids studied in this research appearance a wide compositional diversity within the same class genus has been demonstrated. Furthermore, the differences and similarity between essential oil constituents reveal either within or between same genus and inheritance species explore that the interspecific chemical difference shows eugenics or even novel compounds which can improves the quantity and quality of mint in pharmaceutical production as well as in food as preservative.[281,282,283]

5.23 General Conclusion & Future Work Recommendation

This research chose to establish relationship between the herbal medicinal plants as secondary metabolism and the therapeutic efficacy of selection traditional plants used by herbalist in treatment of several types of disease .The main conclusion that can explore in this thesis is separation, characterisation of ethanolic crude of *P.tomentosa* extract and its semi purified fractions were tested for antioxidant properties. Start purified the extraction with column chromatography and using instrumental analytical

technologies such as Prep-HPLC, NMR. In addition of three different antioxidant assays were used in this study as they measure different aspect of antioxidant activity. The two in vitro assay models were used in this study to estimate the antioxidant power of *Pergularia tomentosa* extract. The two methods were the DPPH• assay (Chen, Wang, Rosen and Ho, 1999)[249] and the phosphomolybdenum method (Prieto, Pineda and Aguilar, 1999).[250] The total phenolic in plant extract was estimated according to Folin-Ciocalteu in alkaline medium, using α-tocopherol as a standard. While methods employed in this study for antimicrobial and antifungal, Time course of growth which can explore microbial activities for most types of bacteria, gram-positive (*S. aureus*) and gram-negative (PA14 and E23). Disc diffusion method to assess antimicrobial and anti-fungal property of crude extracts and isolated fractions There is no direct proof the known chemical compound in the *P.tomentosa* extract but it was clear that there is moderate antioxidant activity could be attributed to the presence of phenolic compounds such as flavonoid….

According to the very complex nature of plant extracts and inability to impure and identified compounds, analytical HPLC cannot considered as an identification tool in this studies. However HPLC was used to monitor the analytical gradient method for *P.tomentosa* extracts and its isolated fractions appropriate phase system for further separation in Prep-HPLC led to run different antioxidant activities and antimicrobial effective limited samples it was not possible test each individual fractions for all above tests .its suggested that appropriate future work should be focussed on the isolation of these compounds. And because the importance of synergistic inhibition effects that these secondary metabolites might have on microorganisms, cover the microbiological and antifungal activity for this pure isolated compounds should been tested. Plus extend the testing to further biological activities such as Cytotoxicity (brine shrimp lethality test, anti-tumour assay) and anti-inflammatory has been reported previous flavonoid have a variety of medicinal activities including antimicrobial, anti-inflammatory, analgesic, anti-

cancer and anti-diabetic.[284] Other general conclusion that can be explore from this research lie within the studies on supporting analytical methodology for secondary metabolite compounds like mint plants. Used microwave technology in a lab scale to preform extraction of volatile oil by using microwave technique that considered faster, energy saver and environment friendly. This technique can also been used to study the different nature of volatile oil composition and yield between mints from different hybrid. According to the earlier results mention in Chapter 6 were prove that oil can be extracted from either direct harvested or extracted from dried plants which were stored in a good conditions for many years, using less quantity and energy compared to the traditional steam distillation method from 120 minute to 10 minute extraction time .

It would be very interesting in future to investigate whether hybrid volatile oils from aromatic and medicinal plants can developed to have a better quantity and quality of total oil. Microwaves have been found to be an interesting alternative to conventional methods for volatile oil extraction for flavours, perfumes and pharmaceutical aroma.

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