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CHEMICAL TRANSFORMATIONS AND PHYTOCHEMICAL STUDIES OF BIOACTIVE COMPONENTS FROM EXTRACTS OF *ROSMARINUS OFFICINALIS* L.

By

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science and Agriculture at the University of Fort Hare

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TABLE OF CONTENTS

Declaration

I, the undersigned, declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Chemistry in the Faculty of Science and Agriculture, School of Science, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other university in partial or entirety for the award of any degree.

Name: _____

Signature: _____

Date: _____

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Dedication

This Thesis is

Dedicated to my late parents, Mr. and Mrs. Z.A Otuneye

May your gentle souls continue to rest in perfect peace (Amen)

List of Tables

List of Figures

Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
BEA	benzene/ethyl acetate/ammonia
BINAP	1,2'-bis(diphenylphosphino)-1,1'-binaphthyl
BHT	butylated hydroxytoluene
CEF	chloroform/ethyl acetate/formic acid
CFU	colony-forming unit
COSY	correlation spectroscopy
DNPH	dinitrophenyl
DPPH	1,1-diphenyl-2-picrylhydrazyl
EMW	ethyl acetate/methanol/water
EO	essential oil
GC MS	gas chromatography/mass spectrometry
HD	hydrodistillation
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple bond correlation
INT	*p*-iodonitrotetrazolium chloride
IR	infrared spectroscopy
MAE	microwave-assisted extraction
MBC	minimum bactericidal concentration
MHz	Megahertz
MIC	minimum inhibitory concentration

MS	mass spectroscopy
NADH	nicotinamide adenine dinucleotide (reduced form)
NMR	nuclear magnetic resonance spectroscopy
PMS	potassium metabisulfite
PTFE	polytetrafluoroethylene
rpm	revolutions per minute
SFME	solvent-free microwave extraction
TAE	tannic acid equivalents
TLC	thin layer chromatography
UV-VIS	ultraviolet visible spectroscopy

Abbreviations for Journal Titles

AB	Annals of Botany
ABB	Archives of Biochemistry and Biophysics
ABC	Analytical Biochemistry
ABR	Advances in Botanical Research
ABT	African Journal of Biotechnology
AC(A)	Applied Catalysis A: General
ACA	Analytica Chimica Acta
ACN	American Journal of Clinical Nutrition
ACP	Atmospheric Chemistry and Physics Discussions
ACR	Accounts of Chemical Research
AE	Atmospheric Environment
AEM	Applied Environmental Microbiology
AFS	Agricultural and Food Science in Finland
AH	Acta Horticulturae
AHJ	American Heart Journal
AJB	American Journal of Botany
AJC	Australian Journal of Chemistry
AJH	African Journal of Pharmacy and Pharmacology
AJP	African Journal of Plant Science
AMB	Applied Microbiology and Biotechnology
AO	Acta Otolaryngologica

AP	The Annals of Pharmacotherapy
APL	Australian Plants
BAB	Brazilian Archives of Biology and Technology
BF	Biofactors
BH	Botanica Helvetica
BJM	Brazilian Journal of Microbiology
BP	Biochemical Pharmacology
BR	Biological Research
BSE	Biochemical Systematics and Ecology
BST	Biochemical Society Transactions
BT	Bioresource Technology
BUR	Burns
CAC	Catalysis Communications
CAR	Carcinogenesis
CAT	Catalysis Today
CI(L)	Chemistry and Industry (London)
CID	Clinical Infectious Diseases
CIM	Central Institute of Medicinal and Aromatic Plants
CIR	Circulation
CJC	Canadian Journal of Chemistry
CMC	Current Medicinal Chemistry
CMI	Clinical Microbiology and Infection

CP	Crop Protection
CPB	Chemical and Pharmaceutical Bulletin
CRV	Chemical Reviews
CT	Cosmetics and Toiletries’
EG	Experimental Gerontology
EJB	Electronic Journal of Biotechnology
EJG	European Journal of Gastroenterology and Hepatology
EP	European Patent
EST	Environmental Science and Technology
FC	Food Chemistry
FCR	Food Control
FCT	Food and Chemical Toxicology
FFJ	Flavor and Fragrance Journal
FM	Food Microbiology
FPE	Fluid Phase Equilibria
FRB	Free Radical Biology and Medicine
FRR	Free Radical Research
FT	Fitoterapia
GBC	Global Biogeochemical Cycles
IBB	International Biodeterioration and Biodegradation
ICP	Industrial Crops and Products
IJF	International. Journal of Food Science and Nutrition

IJM	International Journal of Food Microbiology
IJP	Iranian Journal of Pharmaceutical Research
IJS	International Journal of Mass Spectrometry and Ion Processes
JA	Journal of the American Chemical Society
JAB	Journal of Applied Bacteriology
JAC	Journal of Atmospheric Chemistry
JAF	Journal of Agricultural and Food Chemistry
JAM	Journal of Applied Microbiology
JAO	Journal of the American Oil Chemists' Society
JBC	Journal of Biological Chemistry
JC	Journal of Chromatography
JCC	Journal of Computational Chemistry
JCR(S)	Journal of Chemical Research (Synopsis)
JCT	Journal of Chemical Technology and Biotechnology
JEO	Journal of Essential Oils Research
JEP	Journal of Ethnoparmacology
JFA	Journal of the Science of Food and Agriculture
JFE	Journal of Food Engineering
JFI	Journal of Food Science
JFP	Journal of Food Protection
JFS	Journal of Food Safety
JGR	Journal of Geophysical Research

JHS	Journal of Horticultural Science and Biotechnology
JMA	Journal of Medical and Aromatic Plants Science
JMC(A)	Journal of Molecular Catalysis: A: Chemistry
JMC(B)	Journal of Molecular Catalysis: B: Enzymatic
JMS(T)	Journal of Molecular Structure, THEOCHEM
JN	Journal of Nutrition
JNP	Journal of Natural Products
JOC	Journal of Organic Chemistry
JPC	Journal of Physical Chemistry
JPP	Journal of Pharmacy and Pharmacology
JSF	Journal of Supercritical Fluids
JSS	Journal of Separation Science
LAM	Letters in Applied Microbiology
LID	Lancet Infectious. Diseases
LRO	Laryngo-Rhino-Otologie
LS	Live Sciences
LWT	Lebensmittel, Wissenchaften und Technologien
M	Methods
MB	Microbiology
ME	Methods in Enzymology
MI	Miscellaneous
MRC	Magnetic Resonance in Chemistry

NFJ	Nigerian Food Journal
NJB	Nigerian Journal of Basic Applied Science
NP	Neuropsychologia
NPR	Natural Products Reports
NR	Nutrition Reviews
OJV	Onderstepoort Journal of Veterinary Research
OS	Organic Synthesis
PA	Phytochemical Analysis
PB	Pharmaceutical Biology
PC	Phytochemistry
PCE	Physics and Chemistry of the Earth, Part C: Solar, Terrestrial & Planetary Science
PCS	Proceedings of the Chemical Society
PF	Perfumer and Flavorist
PH	Pharmazie
PM	Pharmacognosy Magazine
PME	Planta Medica
PP	Plant Physiology
PR	Phytotherapy Research
PSR	Physiology Research
PT	Pharmacotherapy
PWS	Pharmaceutish Weeklad Scientific Edition

RCM	Rapid Communications in Mass Spectrometry
RCR	Russian Chemical Reviews
RIE	Rivista Italiana EPPOS
RMI	Rev. Microbiol. Ind. San et Environ.
RNP	Records of Natural Products
SAB	South African Journal of Botany
SCI	Science
SH	Scientia Horticulturae
SYN	Synthesis
TC	Topics in Catalysis
TFS	Trends in Food Science and Technology
TL	Tetrahedron Letters
XB	Xenobiotica
ZLU	Zeitschrift für Lebensmitteluntersuchung und -Forschung
ZN(C)	Zeitschrift für Naturforschung C - A Journal of Biosciences

ABSTRACT

Variations in the yield, chemical composition, antibacterial, and antioxidant properties of the essential oils of *Rosmarinus officinalis* L. cultivated in Alice, Eastern Cape of South Africa over a period of 12 months using the solvent-free microwave extraction and traditional hydrodistillation methods were evaluated. The GC-MS analyses of the essential oils revealed the presence of 33 compounds with 1,8-cineole, α-pinene, camphor, verbenone, bornyl acetate and camphene constituting about 80% of the oils throughout the period of investigation, with the solvent-free microwave extraction method generally yielding more of the major components than the hydrodistillation method. Each of the major components of the oils varied in quantity and quality of yield at different periods of the year. The method of extraction and time of harvest are of importance to the quantity and quality of essential oil of *Rosmarinus officinalis*. Higher amounts of oxygenated monoterpenes such as borneol, camphor, terpene-4-ol, linalool, α-terpeneol were present in the oil of SFME in comparison with HD. However, HD oil contained more monoterpene hydrocarbons such as α-pinene, camphene, β-pinene, myrcene, α-phellanderene, 1,8-cineole, *trans*-β-ocimene, γ-teprinene, and *cis*-sabinene hydrate than SFME extracted oil. Accumulation of monoterpene alcohols and ketones was observed during maturation process of *Rosmarinus* leaves.

Quantitative evaluation of antibacterial activity, minimum inhibitory concentration values were determined using a serial microplate dilution method. The

essential oils obtained using both methods of extraction were active against all the bacteria tested at a concentration of 10 mg mL^{-1}. The minimum inhibitory concentrations for the SFME extracted oils ranged between 0.23 and 1.88 mg mL^{-1}, while those of the HD extracted oils varied between 0.94 and 7.5 mg mL^{-1}, thus suggesting that the oil obtained by solvent free microwave extraction was more active against bacteria than the oil obtained through hydrodistillation.

The antioxidant and free radical scavenging activity of the obtained oils were tested by means of 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^+$) assay and β-carotene bleaching test. In the $DPPH^+$ assay, while the free radical scavenging activity of the oil obtained by SFME method showed percentage inhibitions of between 48.8 % and 67 %, the HD derived oil showed inhibitions of between 52.2 % and 65.30 % at concentrations of 0.33, 0.50 and 1.0 mg mL^{-1}, respectively. In the β-carotene bleaching assay, the percentage inhibition increased with increasing concentration of both oils with a higher antioxidant activity of the oil obtained through the SFME than the HD method.

Thin layer chromatography (TLC) was used to analyze the chemical composition of the extracts using three eluent solvent systems of varying polarities *i. e.* CEF, BEA and EMW and sprayed with vanillin-sulfuric acid. The chemical composition of the different extracts was similar with the exception of methanol and water extracts which had only one or two visible compounds after treating with vanillin-spray reagent. To evaluate the number of antibacterial compounds present in the fractions, bioautography was used against two most important nos-

ocomial microorganisms. *S. aureus* (Gram positive) and *E. coli* (Gram negative). Nearly all the crude serial extraction fractions contained compounds that inhibited the growth of *E. coli.* The hexane extract had the most lines of inhibition followed by ethyl acetate.

Bioassay-guided fractionation against *E. coli* was used to isolate antibacterial compounds. The largest number of antibacterial compounds occurred in the hexane fraction. Furthermore we tried to complete the characterization by extracting and studying other biologically important plant metabolites such as phenolic compounds to evaluate the antioxidant capacity of *Rosmarinus* extracts.

198 pages, 8 Tables, 24 Figures, 332 References.

I. INTRODUCTION

Natural compounds such as secondary plant metabolites are of interest from the research and practical points of view. Many of these metabolites as well as products of their transformation possess very useful medicinal properties. For example, oriental medicine has long been using camphor, which is a secondary plant metabolite. It was recommended as an inhalation remedy during plague epidemics in the Middle Ages in Europe (07RCR655). A number of individual natural compounds were also introduced into medicinal practices in the 19th century. These are quinine, morphine, strophanthin, *etc*. In the 1950's, drugs based on alkaloids vinblastine and vincristines were introduced into antitumor therapy (07RCR655).

Essential oils are volatile, natural complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. They are very complex natural mixtures which can contain about 20-60 components at quite different concentrations (08FCT446). They are soluble in alcohol but to a very limited extent in water and could be mixtures of esters, aldehydes, alcohols, ketones, and terpenes (08MI1). The extraction product can vary in quality, quantity, and in composition according to climate, soil composition, plant organ, age, and vegetative cycle stage (03JAF7115, 06JAF4364). In order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of plant which has been growing on

the same soil, under the same climate and harvested in the same season (05FCT1141, 10MI2).

Essential oils are widely used in the cosmetic industry, especially, in the production of various cologne waters, bathing lotions, hair lotions, shampoos, and as components of disinfectants and insecticides (85PF1). Phenolic components, present in the essential oils, are known to possess antimicrobial activity and some are generally recognized as safe substances; therefore they are used to prevent post-harvest growth of native and contaminant bacteria (91MI2, 02LWT720). Antimicrobial activity of essential oils obtained from oregano, thyme, sage, rosemary, clove, coriander, garlic and onion against both bacteria and fungi is noticeable (95MI2). Also, the chemical composition, antibacterial, antioxidative and radical–scavenging properties of the essential oils of *Cuminum cyminum* and *Rosmarinus officinalis* obtained by steam distillation were reported (07FC(102)898). Plant essential oils have been used for thousands of years for food preservation, pharmaceuticals, alternative medicine and natural therapies (96MI1, 97JAM759), and have been known to consist of volatile, lipophilic substances that are mainly hydrocarbons or monofunctional compounds derived from the metabolism of mono- and sesquiterpenes, phenypropanoids, amino acids (lower mass aliphatic compounds), and fatty acids (long-chain aliphatic compounds). They are commonly found as components of essential oils from aromatic plants (74PC868, 94PC183, 94PC641, 95PC1115, 03FFJ106). Un-

like fatty oils, essential oils do not leave a grease stain when dabbed on filter paper.

Essential oils are to be distinguished from the so-called distillates which are ethanol-containing products that are obtained from plant materials by distillation with ethanol or with ethanol-water mixtures. Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. They are very complex natural mixtures which can contain 20-60 components at quite different concentrations (08FCT446) and has usually been isolated by traditional hydrodistillation, steam distillation or organic solvent extraction methods. Losses and degradation of some volatile compounds due to long extraction times, degradation of unsaturated or ester compounds through thermal or hydrolytic effects are the principal disadvantages of these extraction methods (97JFE47, 04FC587). For example, monoterpenes are well known to be vulnerable to chemical changes under steam distillation conditions and even conventional solvent extraction is likely to involve losses of more volatile compounds during the removal of the solvent (05JSS273). Recently, the supercritical fluid extraction of rosemary with CO_2 has been a subject of a lot of research (05JSF(35)197) and has become a valid alternative to the conventional extraction procedures mainly because the dissolving power of the extracting medium can be adjusted by regulating the pressure and temperature conditions. Today, an alternative method for extracting natural products by using microwave energy has been developed (03EP1,

04JC(A)(1043)323). Solvent-free microwave extraction (SFME) is based on the combination of microwave heating and distillation performed at atmospheric pressure. SFME appeared to be particularly attractive for the isolation of essential oil from rosemary (95FFJ101). Some of the advantages of this method over HD includes rapidity in attaining the extraction temperature of 100^{o}C for the first essential oil droplet, high yield of essential oil, lower energy requirement, and high purity of the oil extracted (04JC(A)(1043)323). In order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of plant, which has been growing on the same soil, under the same climate and harvested in the same season (96MI1, 05FCT1141).

There are some chemical transformations of the components of essential oils either during the growth of the plants and or during the distillation of the oil due to the heat of distillation, thus questioning the validity of the naturalness of essential oil as the heat of distillation and other warm extraction methods causes changes in the natural chemical composition of the oil, resulting in substances not found in the plant (06MI1). Information like this is necessary for the full understanding of the nature of these oils and also to enable scientists give well-informed advice to the users of essential oils.

Essential oils of *Rosmarinus officinalis*, known as rosemary oils, are obtained by steam distillation of the fresh leaves and twigs, and the yields range from 0.5 to 1.0 % (87CIM185). It is an almost colorless to pale-yellow oil with a

characteristic, refreshing and pleasant odor. Major constituents described for the oil are α-pinene, 1,8-cineole, and camphor (01MI2).

Significant variations in the chemical composition of this oil have been reported with relation to the geographic origin (86FFJ137, 91JEO11, 92FFJ81, 93JEO613, 95PF49, 97MI2, 99JEO27, 10RMI120). Times of harvest, condition of the twigs and leaves, distillation equipment, and management have also been reported to play an important role in the overall quality of the oil (87CIM185).

A fundamental basis for resource studies lies in the identification of medicinal molecules, determination of the chemical composition and elucidation of the structure of particular components. Often, chemical compounds isolated from plants turn out to be novel. Today a significant part of the search for biologically-active compounds is associated with modifications of the known plant metabolites aimed at either enhancing the original activity of the chemical compound or obtaining derivatives that exhibit other activities.

Hitherto, the main methods used to obtain essential oils from plant materials are hydrodistillation, steam distillation, water distillation, maceration, empyreumatic (or destructive) distillation and expression (02MI3). Among these methods, hydrodistillation has been the most common approach to extract the essential oils from medicinal plants. However, in order to reduce the extraction time and possibly improve the extraction yield, in order to enhance the quality of the extracts and also to reduce the operation costs, new approaches such as

microwave-assisted extraction and pressurized solvent extraction have also been employed (02PA105, 06TFS300).

Some recently published studies have successfully utilized solvent-free microwave extraction for the extraction of essential oils from medicinal plants (04JC(A)(1025)93, 04JC(A)(1025)105, 04JC(A)(1043)323, 10FC308). For a solvent-free microwave method for the extraction of essential oils from three aromatic herbs (basil, garden mint, and thyme), the amount of essential oil obtained with this method was more comparable, both from qualitative and quantitative points of view to those obtained with hydrodistillation (04JC(A)(1025)93).

Although several workers have reported variations in chemical compositions of essential oils due to their origin, environmental condition, and the developmental stage of collected plant materials, no such information is available on the seasonal variation of the composition of oil from *Rosmarinus officinalis*. Our aim was to establish if there were a link between the chemical variability of the essential oil and the season during the annual phonological cycle of this plant. This information will help in understanding of the chemical transformations that take place in the oils of this plant during the year.

Rosmarinus officinalis L. is a perennial herb with ever-green needle-like leaves that belongs to the *Lamiaceae* family. The *Lamiaceae* is a large family, rich in aromatic species that are used as culinary herbs, folk medicines, fragrances and many of this family possess essential oils that are secreted by

glandular trichomes (06SAB378). Previous studies have shown that rosemary essential oil had antimicrobial, antioxidant, anti-carcinogenic, cognition-improving and certain glucose level lowering properties, which make it useful as a natural animal feed additive (99IJF413, 01FCT907, 07PR989).

Many compounds have been isolated from rosemary, including flavones, diterpenes, steroids, and triterpenes. Of these, the antioxidant activity of rosemary extracts has been primarily related to two phenolic diterpenes: carnosic acid and carnosol (96JAF131). The main compounds responsible for the antimicrobial activity are α-pinene, bornyl acetate, camphor, and 1,8-cineole (00JAF2576, 02FFJ15, 03CP39). Despite the natural abundance of some compounds in the essential oil of *Rosmarinus officinalis*, no study has been reported on their chemical transformations into other useful compounds. Neither have there been reports on comparative analyses of the effects of the extraction methods of SFME and HD on the chemical composition and biological activities of the essential oil of the plant, which are the overall aims of this study. The specific objectives include:

- To cultivate *Rosmarinus officinalis* harvested in the vicinity of the University of Fort Hare, Alice, South Africa in the greenhouse.
- To harvest the plant at regular intervals (monthly), distil its essential oil and analyze these oils using GC MS.

- To study the effects of hydrodistillation and solvent-free microwave extraction methods on the chemical compositions of the essential oil of *Rosmarinus officinalis*.
- To assess the antibacterial activities as well as determine the minimum inhibitory concentrations and minimum bactericidal concentrations; and the rate of kill of bacteria by the essential oil of the plant.
- To assess the antioxidant properties of essential oil of the plant obtained by hydrodistillation and solvent-free microwave extraction.
- To assess the phytochemical and polyphenolic antioxidant activity of leaf extracts of *Rosmarinus officinalis* L.
- To isolate, purify, and identify bioactive compound of the leaf extracts.
- To perform FT-IR spectral analysis for functional group identification, ^{1}H NMR for proton and ^{13}C NMR for carbon environment, and elemental analysis for the isolated organic compounds.
- To assess the chemical transformations in the essential oils of the plant using the dominant chemical components as target compounds.

II. REVIEW OF LITERATURE

A. ESSENTIAL OILS

Essential oils are mixtures of volatile compounds, mostly terpenes and their oxygenated derivatives, cyclic hydrocarbons and their alcohols, aldehyde derivatives (high vapor pressure and low water solubility) produced in small quantities as secondary metabolites from aromatic or medicinal plants (85SCI1154). Volatile compounds are defined as the odorant, aroma, flavor chemical compounds, which are transported to the olfactory system in a sufficiently high concentration in order to interact with olfactory receptors (03MI1).

Essential oils are complex and highly variable mixtures of components that belong to two groups: terpenoids and aromatic compounds of which monoterpenes form the major components (98MI2). Terpenoids are compounds derived from isoprene units contain oxygen in various functional groups. Aromatic compounds are derivatives containing the benzene ring. Both are accumulated in all types of vegetable organs: flowers (bergamot tree), leaves (mint, eucalyptus), barks (cinnamon), woods (sandalwood), root (vetiver), rhizomes (ginger), fruits (anise), and seeds (nutmeg) (99MI1). It was observed that essential oils when they occur in various organs in the same plant have different composition profiles (85SCI1154).

The term 'essential' used in essential oils indicates that the oil carries distinctive scent (essence) of the plant (07MI1). Certain cold pressed oils are considered to be essential oils but are rather cold pressed carrier oils such as ol-

ives, grape seed and coconut oil which compose mainly of fatty acid triglycerides without fragrance components of essential oils (85SCI1154). It was suggested that essential oil must be isolated by physical means from the aromatic medicinal plants to be a true essential oil (93PF1).

The physical methods used are distillation (steam or water), expression (known as cold pressing, a unique feature for citrus peel oils), and maceration used specifically for a very limited number of essential oil plants. The most delicate fragrance and flavor components from flowers are extracted by concentration using solvent wash to obtain concentrate which are purified to yield essential oils (99MB1). Concentrate is a substance containing volatile essential oils and a fatty waxy material obtained by the extraction of plant tissue with lipophilic solvents (03PC3). The components of essential oils are known to be active against a wide variety of microorganisms, including Gram negative and Gram positive bacteria (96JAF1202). Gram negative bacteria have been reported to be more resistant than Gram positive bacteria to the inhibitory effects of essential oils because of the lipopolysaccharides present in their outer membrane (87IJM161). However, there is a view (88JFS97) that Gram positive bacteria are more resistant than Gram negative bacteria to the antibacterial properties of plant essential oils, which is in contrast to the hypothesis (87IJM161, 87IJM165).

Moreover, some essential oils of aromatic plants indigenous to the Eastern Cape region of South Africa have been reported to possess not only unique fra-

grance properties but also broad antimicrobial activities against both Gram negative and Gram positive bacteria as well as fungi (01JEP(74)217). These oils have been used in medicinal field, such as inhalation therapy to treat acute and chronic bronchitis and acute sinusitis (83AO157). Essential oil has also been used for respiratory tract infections, and as a good medicine for colds, anti-inflammatory effect on the trachea and to reduce asthma (97LRO23). Such therapies can sometimes turn out to be ineffective due to the rapid development of bacterial resistance. However, plant oils account for a source of very promising natural ingredients for producing new antimicrobial drugs in spite of the fact that their antibacterial action has been proven to be remarkably milder than the commercial synthetic drugs (05RIE3).

In two essential oils of *Ravensara aromatica* and *Eugenia caryophyllus* no bacteria or virus could survive (02MI). This is a significant breakthrough since there are many life-threatening viruses and drug-resistant bacteria. In the past two decades, many studies have reported the inhibitory and bactericidal effects of essential oils tested against a wide variety of microorganisms in various media and food products. However, comparison of the efficacy of various oils is complex because their composition varies among the different plant genera as well as within the same genus. This variation in composition of active components may be as a result of different geographic origin, climate, processing, and variety differences (88JFS97).

B. Secondary metabolites

Secondary metabolites are present in all higher plants, usually in high structural diversity with varied biological activity to protect plants against viruses, bacteria, fungi, and most importantly against herbivores (03PC3). The aroma of spices, fragrance of flowers, tinctures of eucalyptus, lavender, and basil are examples of secondary metabolites. Many secondary metabolites such as cyanogenic glycosides, glucosinolates, terpenes, saponins, tannins, anthraquinones, and polyacetylenes, influence the growth and development of neighboring plants. Limonene (monoterpene), one of the components of secondary metabolites, has shown deterrent and insecticide properties (01AFS243).

The chemical compounds from secondary metabolites are phenomenally varied; they have been classified in essentially six categories based on biosynthetic pathways (02NPR181). These are acetate, shikimate, mevalonate, and deoxyxylulose phosphate, alkaloids, proteins, and carbohydrates pathways. The synthesis of terpenoids is the most predominant among the chemical compounds, and is the cause of aroma in plant which is synthesized from mevalonate and deoxyxylulose phosphate pathways.

C. Extraction of essential oils

The method used in extracting the oil from plants depends on the plant material as well as the type of end product that is desired (05IJP175). Plants that are liable to immediate deterioration are processed quickly after harvesting. Seeds and roots, on the other hand, can easily be stored for a period of four to

six hours before processing (99LAM291). Essential oils can be obtained from plants by steam distillation, maceration, cold pressing, and solvent extraction. However, the method of steam distillation is most commonly used for commercial production (85SCI1154).

1. Steam distillation

Steam distillation is the most common method of essential oils production and involves the placing of the plant material into a still where pressurized steam passes through the plant material. The heat from the steam causes globules of oil in the plant to burst and the oil evaporates. Both the essential oils vapor and the steam passes through the top of the still into a water cooled pipe where the vapors are condensed back to liquids, the essential oil thus separates from the water and floats on the top (06MI2). A number of factors have been reported to determine the final quality of a steam distilled essential oil. Apart from the plant material itself, most important are time, temperature, pressure, and the quality of the distillation equipment. Some of the molecules of the oil (EO) are fairly delicate structures which can be altered or destroyed by adverse heating process.

2. Maceration

Maceration is defined as the method of preparation of extract by soaking the part of plants in warm water, vegetable oil or organic solvent for the rupturing of the membrane in order to release essential oil. Thus the oil is cleared from the plant material and decanted (10PM234). Maceration as a method has been

ascertained to create more of infused oil rather than an essential oil. Examples of oils produced by maceration are oils from onion, garlic, wintergreen, and bitter almond.

3. Cold pressing

Cold pressing is a method used to extract the essential oils from orange, lemon, grapefruit, and bergamot. The rinds separated from the fruit are grounded and pressed; the watery mixture of essential oil and liquid is obtained from the process and is separated. The oils extracted using this method have a relatively short shelf life (99MB1).

4. Solvent extraction

Solvent extraction is mostly used in the extraction of essential oils from citrus. Addition of a solvent such as carbon dioxide has been used in a newly developed method for the extraction of oil under pressure of liquefaction. Also, alcohol as an organic solvent has been used to extract the essential oil from the plant material. The resulting solution is filtered and distilled to obtain a pure form of the essential oil. The pure essential oils obtained are depressurized to avoid missing some important components of the oils (99MB1). Thus, the method has been considered not appropriate for the extraction of essential oils since small amount of the oil can be left behind during extraction which could cause allergies and affect the immune system.

D. COMPOSITION OF ESSENTIAL OILS

The composition of essential oils are mainly monoterpenes and sesquiterpenes with the general formula $(C_5H_8)_n$ (01MI2). Oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters, ethers, ketones, phenols, and oxides. These components are grouped into major and minor components. The major components of the economically interesting essential oils have been summarized. Likewise numerous publications have presented data on the composition of various essential oils.

Major components of EO's can constitute up to 85 % of the essential oil, whereas other components are presented only in trace amounts (96JAF1327). There is some evidence that minor components play a critical role in antibacterial activity by producing a synergistic effect between other components. The composition of essential oils from a particular species of plant can differ between harvesting seasons and geographical areas (99JFP1017). For example, oils produced during or immediately after flowering period possess the strongest antimicrobial activity. In addition, the seeds of coriander (*Coriandrum sativum* L.) have shown a different composition of essential oils obtained from the immature leaves of the same plant (02IJM101). The study of essential oil components has been undertaken by researchers to produce more useful data about their mechanism of action, which is influenced by variation in the composition of their components. The phenolic structures of some of the essential oil seem to play an outstanding role in altering the permeability of bacterial mem-

brane by joining to the amine and hydroxylamine groups of the proteins resulting in the death of the bacteria cells (94JAB626). Table 1 describes selected essential oils and their major antimicrobial components.

Table 1

Plants and their Bioactive Components

Plant source	Bioactive component	Basic structures
Oreganum and thyme	Carvacrol	Phenol
Clove	β-Caryophyllene	Cyclic sesquiterpene
Cassia and cinnamon	Cinnamic aldehyde	Aromatic aldehyde
Citrus peel	Limonene	Cyclic terpene
Sweet basil and sage	Linalool	Acyclic terpene alcohol
Rose and geranium	Rhodinol	Acyclic terpene alcohol
Rosemary	α-Pinene	Bicyclic terpene
Oreganum and thyme	Thymol	Phenol

E. Factors affecting composition of essential oils

The impact of environmental factors such as, harvesting time, relative humidity, irradiance, photoperiod, and the method of extraction, location, plant cultivation techniques, soil structure, and climate heavily influence the composition and quality of essential oils (93JEP167). These factors have been used to ascertain why exact specification of the component of essential oils is not acceptable.

1. Harvest time

Harvesting time is one of the most important factors influencing the quality of essential oils (01IJF187). The amount of essential oils composition is strongly dependent on developmental stage of the plant (ontogeny). Harvesting a crop early or late resulted in a low yield of leaves as well as the essential oil content,

the immature crop or over mature resulting in a poor yield of herb and oil content. It has been concluded that in order to obtain high yield of essential oils it is better to harvest plant essential oil after flowering to obtain oil that contains a high amount of essential oils (93JEP167). Besides the timing of harvest, the number of harvests per year greatly influences the yield, and the composition of essential oil (84JAF1191).

2. Method of extraction

Influence of the method of extraction on oil composition and the variability of the constituents of essential oil explains why the composition of the product obtained by steam distillation is often different from that which is initially present in the secretory organs of the vegetable (99MI1). Distillation parameters such as the time of day and stage of growth when the plant is picked, the part of the plant that are distilled, the length of distillation, method of distillation, affect the constituents of essential oils and hence their quality and medicinal effects (84JAF1191). The length of time taken for distillation is important. The oil of lavender as reported required about an hour and a half for distillation. If distillation is shortened only slightly, 18 to 20 percent of the essential oil's chemical constituents can be missing (99MI1).

3. Temperature

Fragile aromatic molecules of an essential oil are easily destroyed or altered by high temperatures and so, the distillation process must use low-temperature methods (86AB729). High temperature seems to cause harshness in the oil, the

pH, electropositive and electronegative balances of the essential oils are greatly affected by the effect of temperature. The distilling process for lavender and cypress should not exceed 118.3°C, however, high temperature was found to decrease the oil yield.

F. METHODS OF ADMINISTRATION OF ESSENTIAL OILS

Administration of essential oils can be carried out using various methods such as aromatic baths compresses, massage "neat" directly on the skin, inhalation and orally. The method is used depending on the need or condition for application (95MI1). Many French practitioners have found that taking the oils internally is highly effective (96MI2).

Inhalation might be preferred over topical application if the goal is to induce weight loss to balance mood and emotions. In other cases, topical application would produce better results, as in the case of muscle or spinal injuries. Peppermint oil, taken orally has been shown to be very effective for indigestion (90MI1). More so, the same results can be produced when massaged on the stomach. In some cases, all three methods of application (topical, inhalation and ingestion) are interchangeable and may produce similar benefits (89PF21).

G. PROPERTIES OF ESSENTIAL OILS

1. Antimicrobial properties

The antimicrobial properties of essential oils have been well recognized for many years (99JAM985). They have been found applicable as naturally occurring antimicrobial agents in pharmacology, pharmaceutical botany, phyto-

pathology, medical and clinical microbiology, and food preservation. Essential oils, such as aniseed, lavender, lemon, orange, lime, rosemary, and basil, have been traditionally used by people for different purposes in different parts of the world. These essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties. Some of them have been used in cancer and HIV treatments, while others have been used in food preservation, aromatherapy and fragrance industries (94FM334). Some researchers have concluded that cinnamon, clove and rosemary oils had antibacterial and antifungal activity (05MI1). Anti-inflammatory activity has been found in basil, anti-diabetic property in cinnamon oil, and antioxidant property in lemon and rosemary oils. Orange has shown anticancer activity; lavender oil - antibacterial and antifungal activity. The antibacterial properties of these compounds are in part associated with their lipophilic character, leading to accumulation in membranes and energy depletion in membrane-associated event.

2. Antibacterial properties

Plant essential oils exhibit antibacterial activity by interfering and destabilizing the phospholipids bilayer of the cell membrane, enzyme systems, and genetic material of bacteria (95JAF2839). In addition, a number of constituents of EO (98JAB211) exhibit significant antibacterial properties when tested separately by interfering at different level of mechanism. It is evident that EO's are more strongly antibacterial than is accounted for by the additive effect of their major antimicrobial components as a result of their minor components that ap-

pear, to play a significant role synergistically (94RIE13). Some essential oils components such as carvacrol, thymol and eugenol (phenolic compound) have been proved antibacterial. Cinnamic aldehyde as a non phenolic compound has been ascertained by scientists to possess anti-infectious action (98JAB211). Aldehyde components of essential oils are somewhat antibacterial; the most widely used are neral and geranial, citronnellal and cuminal. The antibacterial action of ethers is certain, but irregular; terpenes have been proven interesting, but are mostly diffused into the air (95JAF2839).

3. Antiviral properties

Essential oils from different plant families have now been demonstrated to have antiviral properties (03MI2). Interestingly, different plant families exhibit varying degree of effectiveness depending on the viral strain and structure. This is due to the particular molecular structures found in each type of oil, which penetrate physical entities to varying degrees (05MI1).

Researchers have shown that a number of essential oils have antiviral activity against *Herpes Simplex* I, *Herpes Zoster* (shingles), some strains of influenza virus, adenovirus, glandular fever, viral enteritis, viral enterocolitis, and HIV-1 (01PH343). Specific essential oils act against specific viruses e.g., *Houttynia cordata*, was shown to have remarkable effect against HIV-1 (03MI2). Specific components of essential oils have been isolated and found to have antiviral properties. These include anethole, carvone, beta-caryophyllene, citral, eugenol, limonene, linalool, and linalyl acetate (79MI1).

It has been suggested that the antiviral effect of an essential oil is due to particular components of the oil such as monoterpenols (cineole, ketones, and cryptone) and monoterpenals. Viruses are highly sensitive to aromatic molecules and that some severe viral infections may show a vast improvement (94PME237). The synergy of this oil such as cineole-monoterpenol has been used to treat viral infections of the respiratory tract; ketones and cryptone components of essential oils have shown a capacity to fight naked viruses (98PC1515).

Several methods of antiviral action have been proposed for essential oils and essential oil components. Some essential oils interfere with surface glycoprotein in the viral envelope, thus preventing attachment of the virus to the host cells. Other essential oils are believed to attack viruses in the host cells, possibly at the level of the cell membrane (79MI1). Some oils exhibiting antiviral effects belong to the following essential oils; anise, tea tree, juniper, eucalyptus, lavender, rosemary, clove, thyme, lemon grass and cinnamon bark (01PH343).

4. Antifungal properties

Fungal infections have been increasing in recent years due to a growing number of high-risk patients, particularly immune-compromised hosts (person with deficient immunologic mechanism) (04PT4S). *Candida* is the fourth-most-common isolate in nosocomial bloodstream infections in the USA. It was reported to be the cause of candidosis as the most common invasive fungal infection

in critically ill non-neutropenic patients (03LID772). The increase in fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies (04PT4S). Essential oils from many plants are known to possess antifungal activity (03CMC813), however, only limited information exists about activity toward human fungal pathogens. The molecular groups of essential oils with the strongest antibacterial action have been proven active against fungi. Fundamental studies revealed the anti-fungal activity of alcohols and sesquiterpenic lactones (04ZN(C)75).

5. Antioxidant properties

Lipid peroxidation is a complex process occurring in aerobic cells and reflects the interaction between molecular oxygen and polyunsaturated fatty acids (90FRB515). It involves in asthma, inflammation, arthritis, neurodegeneration, Parkinson's disease, mongolism, and dementia as a result of the effect of free radical formation (00NP252). Free radicals are any species that contain one or more unpaired electrons causes food deterioration and promotion of carcinogenesis (99EG293).

In the past few years, there has been a growing interest in the search for natural antioxidants (94MI2, 06AHJ100, 10MOL6905). The antioxidant properties of many herbs and spices have been reported to be effective in this respect (90MI1). Many terpenoids have been identified as potent antioxidants (00JAF4156). The plants belonging to the *Lamiaceae* family possess appreciable

antioxidant activity (94MI4). It was also found that oregano essential oil, rich in thymol and carvacrol, has a considerable antioxidant effect on the process of lard oxidation (93ZLU20).

H. Mechanism of action of essential oils

The pathways by which microorganisms are inhibited by essential oils seem to involve different modes of action. The most frequent inhibition involves the phenolic components of the oils which sensitize the phospholipids bilayer of the cell membrane causing an increase of permeability and consequently leakage of vital intracellular constituents (95JAF2839). Mostly essential oils used by inhalation, oral administration or application to the skin, act by means of their lipophilic fraction reacting with the lipid bilayer of the cell membranes, and as a result, modify the activity of the calcium ion channels. They saturate the membranes and show effects similar to those of local anesthetics (98JAB211) by interacting with the cell membranes of bacteria through their physicochemical properties on molecular level and thus influence their enzymes, ion channels, and receptors (96IJF83).

Some components of essential oils including eugenol are highly active against microorganisms such as *Bacillus cereus*. Members of this class are known as either bactericidal or bacteriostatic agents depending on their concentration (99AEM4606). These components are strongly active despite their relatively low solubility in water (96CT69). The solubility of essential oils and their terpenoid compounds should therefore be taken into consideration when study-

ing their action on the membrane-catalyzed functions within the cell wall that acts as a physical barrier (87PWS193).

The hydrophobicity of essential oils enables them to partition the lipids of the cell membrane and mitochondria, rendering cell membranes permeable and leading to leakage of cell contents. Due to this effect the antimicrobial activity of essential oils has been proven to be enhanced by physical conditions such as low pH, low temperature and low oxygen levels (87PWS193).

1. Mechanism of inhibition by changes in membrane function

The mechanism of action of antimicrobial activity of essential oils is the disruption of the cytoplasmic membrane, which leads to a decrease in the proton motive force of the bacterial cell (02AEM1561). The energy for cell processes is generated from a proton-motive force which is established by the separation of proton across the cytoplasmic membrane, which then creates an excess of hydrogen ions and positive charges outside the membrane. The concentration and electrical differences between the inside and outside of the cell across the cytoplasmic membrane produces the proton-motive force which includes the electrical potential. Protons pass through the cell through a specific proton channel, *i. e.*, ATPase. ATPase is an enzyme that catalyses the conversion of ADP to ATP as proton passes through the channel to provide energy for the cell (95IBB317). Moreover, the interaction of essential oil compounds such as carvacrol with the bacterial membrane affects the structure of the cytoplasmic membrane, which may increase the passive proton flux across the membrane. The leakage of pro-

tons decreases the proton gradient and therefore dissipates the proton motive force. Some essential oil components such as eugenol, carvacrol, and thymol have been reported to partition into the cytoplasmic membrane (99AEM4606). The mechanism of action of carvacrol was investigated when it was added to a *Bacillus cereus* cell pellet suspended in buffer. Viable cell numbers decreased exponentially when vegetative *B. cereus* cells were incubated for 30 min with 150-451 mg/L of carvacrol. This reduction was related to a change in the membrane potential.

2. Mechanism of inhibition by changes in protein content

Components of essential oil appear to act on cell proteins embedded in the cytoplasmic membrane (89JEO119). Possible mechanisms have been suggested to affect enzymes (ATPases) located in the cytoplasmic membrane bordered by lipid molecules. Lipophilic hydrocarbon molecules could accumulate in the lipid bilayer and distort the lipid-protein interaction. Direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (94JAB626). This could be an indication that essential oils act on the enzymes involved in the energy regulation or synthesis of structural components (05MI1).

I. GC-MS ANALYSIS OF ESSENTIAL OILS

Gas chromatography is a method of separation which employs a gas mobile phase and either a solid or liquid adsorbed on a solid as a stationary phase. It is capable of separating very complex mixtures, and the selectivity can be adjusted to separate any given pair of solutes by judicious choice of the stationary

phase (01MI1). It becomes clear that analysis of essential oils is difficult because of the incredible complexity of hundreds of different chemical components in their percentage content contained in single oil. However, this is made possible by using GC and GC coupled to mass spectrometric detection system (GC-MS) to ascertain quality and purity of most essential oils. Chromatography efficiencies are highly needed to deliver baseline separation and quantitative determination of the critical groups of components.

J. Application of essential oils

Essential oils have a wide variety of applications. Essential oils are used in the production of cosmetics, as food additives, in medicine, antiseptic, and in health care.

1. In cosmetics

The use of essential oils in the production of cosmetics and related products may have several advantages such as a pleasant aroma, assuring protection against microorganisms and in some instances enhancing the derma-cosmetic properties and preservation of the cosmetic products (98JAM368). Scalp problems like dandruff and seborrheic dermatitis caused by *Malasseizia furfur* could be cured using tea tree oil due to the presence of terpinen-4-ol which has been proved effective. However, the application of essential oils as antimicrobial agents in cosmetic preparations is often discouraged because of their milder action compared to classical preservatives (84MI1), their odor and color which

might add to the final product and a potential loss of antimicrobial action due to their volatility and lipophilicity (99JEP1).

2. In food

Essential oils can serve as food additives or preservatives that support processing and production and maintain or impart the properties of food (02MI2). Their functions in food industry include the following: improvement of the nutritional value of food, *e. g.*, vitamins, minerals, and other nutrients; maintenance of palatability and wholesomeness of food, *e. g.*, preservatives, antioxidants, and sequestrates; provision of leavening or control of acidity/alkalinity, *e. g.*, leavening agents, acidifiers, alkaline, buffers, and acid regulators; enhancement of flavor and color. As a preservative agent, essential oils serve as either antimicrobials or antioxidants. Essential oil as antimicrobial prevents the growth of moulds, yeasts, and bacteria. As antioxidants, they prevent the foods from becoming rancid, browning and the development of black spots. Antioxidant properties of essential oils suppresses the reaction that occurs when foods combine with oxygen in the presence of light, heat, and some metals to minimize the damage to some essential amino acids and the loss of some vitamins.

3. In health care

Essential oils have been used for centuries in healing, altering of moods, and enhancement of consciousness (98AP680). It affects the emotional, mental, spiritual, and physical state of a human being. When diffused, the oils reach the brain by means of the olfactory system. The impulses are then transported to

the limbic system and to the olfactory sensory center at the base of the brain where they pass between the pituitary and pineal gland when absorbed, and work to stimulate the body's natural healing system. Data have shown that aromatic compounds can exert strong effects on the brain, especially on the hypothalamus and the limbic system (the seat of emotions). Some essential oils can dramatically increase oxygenation and activity in the brain that cause increase of ozone and negative ions, inhibit bacterial growth and can alter non-toxic chemicals by interfering with their molecular structure.

K. *ROSMARINUS OFFICINALIS* ESSENTIAL OILS

Rosemary (*Rosmarinus officinalis*) (Fig. 1) is a woody, perennial herb with fragrant evergreen needle-like leaves. It is native to the Mediterranean region, belonging to the member of the mint family *Lamiaceae*. The leaves are evergreen with dense woolly hairs and the flowers are variable in color. *Rosmarinus officinalis* oil is used as a flavor agent in the perfumery industry (aromatherapy). It helps to overcome mental and physical fatigue, by stimulating blood circulation (96EJG1227). It provides support in stressful situations and helps promote mental concentration and meditation processes. Recently, rosemary oil has been tried in cancer therapy. The established chemical content of the oils investigated in the present study also agrees with the results of reviewed analyses of different essential oils (03LAM162). The major constituents of *Rosemary officinalis* include 1,8-cineole, camphor, verbenone, α-pinene, bornyl acetate, and camphene.

Figure 1: *Rosmarinus officinalis*

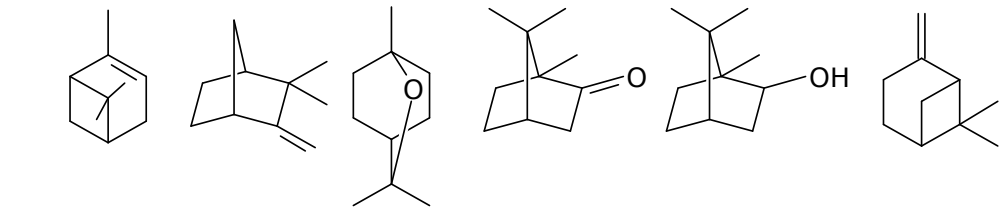
A fundamental basis for plant resource studies lies in the identification of medicinal molecules, determination of the chemical composition and elucidation of the structure of particular components (07RCR655). Often, secondary metabolites isolated from plants represent novel classes of chemical compounds. Today the search for biologically active compounds is mainly associated with modifications of the known plant metabolites aimed at either enhancing the original activity of the chemical compound or obtaining derivatives that exhibit other activities. Commercially it provides several important essential oils to the fragrance industry for soaps, perfumes, skin lotions and other cosmetics products.

The method of essential oil extraction affects their chemical composition and biological activity (05JSS273). Plants, especially herbs and spices, have many phytochemicals which are potential sources of natural antioxidants. These include phenolic diterpenes, flavonoids, tannins and phenolic acids (06LWT308).

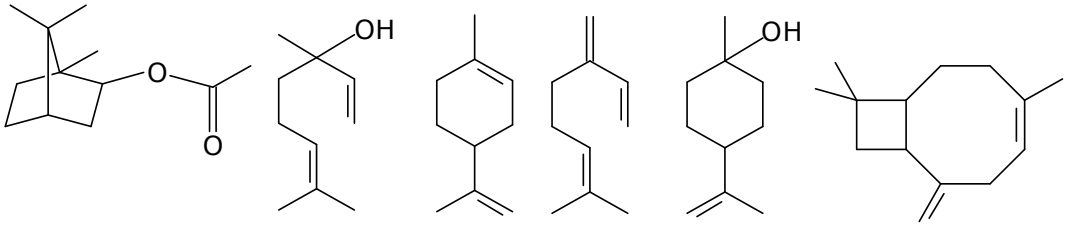
Some antioxidants have been widely used as food additives to provide food protection against oxidative degradation by free radicals. Spices which are used in different types of food to improve flavors are well known for their antioxidant properties (95TFS271). Recently, there has been increasing interest in the use of natural antioxidants such as tocopherols, flavonoids, and extracts from rosemary (*Rosmarinus officinalis* L.) for food preservation (00FC(71)229, 04FRB838, 04FT50, 05LWT61). According to these authors, this natural antioxidant does not present undesired health problems that may arise from the use of synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and propyl gallate, which have side effects (92XB257, 04FC551). Antioxidants are compounds that neutralize chemically active products of metabolism, such as free radicals which can damage the body. It has been documented that plant phenols, with their potential to act as antioxidant potentials; play a major role in the prevention of cancer, cardiovascular and neurogenerative diseases which are believed to be caused by oxidative stress (07MI2).

Whilst the general antioxidant potential of *Rosmarinus officinalis* essential oil have been reported before (07FC(102)898), there is no information on the possible effect of the method of extraction of its essential oils on the antioxidant property of this herb. The biological and phytochemical properties of essential oils extracted through different methods have been found to depend on the extraction method (10FC308).

It is widely accepted (10MI1) that basic constituents of the *Rosmarinus officinalis* essential oil are α-pinene **1**, camphene **2**, 1,8-cineole **3**, camphor **4**, and bornyl alcohol **5**. Their content is in the range between 10 and 15%. Additional components of the volatile and essential oils are β-pinene **6**, bornyl acetate **7**, linalool **8**, limonene **9**, β-myrcene **10**, β-terpineol **11**, and caryophyllene **12**. Certainly, the qualitative and quantitative composition of essential and volatile oils is a function of geographic zone, climate conditions, part of the plant, storage before extraction, drying conditions (11JFE219), method of extraction, including the fast-controlled pressure drop as a version of the solvent-free method (05JFE9), and other factors.



1 **2** **3** **4** **5** **6**



7 **8** **9** **10** **11** **12**

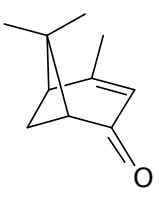
In Table 2 below, the data for different locations and extraction methods are given. Apart from the substances listed in the leading reference source (10MI1), verbenone **13**, linalool oxide **14**, piperitone **15**, *p*-cymene **16** can sometimes be mentioned as sizeable components.

Table 2

Composition of Essential Oil of *Rosmarinus officinalis* in Various Geographic Zones

Country	Distillation method	Components	Reference
Algeria	Hydrodistillation	α-Pinene **1** 44.1% Camphor **4** 7.82% Verbenone **13** 6.37% Camphene **2** 6.14% Limonene **9** 5.48%	08FC(114)355
	Microwave hydrodiffusion and gravity	α-Pinene **1** 43.6% Camphor **4** 8.60% Verbenone **13** 7.65% Camphene **2** 6.48% Limonene **9** 5.533%	
Brazil	Hydrodistillation	α-Pinene **1** 42.8% 1,8-Cineole **3** 18.4% Camphene **2** 5.40% Verbenone **13** 5.10%	05BAB1035
Chile	Spinning cone column	Camphor **4** 14% β-Myrcene **10** 14% 1,8-Cineole **3** 14%	10FCR615
China	Hydrodistillation	1,8-Cineole **3** 27.3% α-Pinene **1** 19.4% Camphor **4** 14.3% Camphene **2** 11.5% β-Pinene **6** 6.71%	08FC(108)1019
Greece	Hydrodistillation	1,8-Cineole **3** 53.5% Bornyl alcohol **5** 9.6% α-Pinene **1** 9.2% β-Terpineol **11** 5.1% Oxygenated terpenes 73.8% Monoterpenes 19.8%	08JAF7254
Iran	Hydrodistillation	Piperitone **15** 23.7% α-Pinene **1** 14.9% Linalool **8** 14.9% 1,8-Cineole **3** 7.43%	07FC(102)898
Italy	Hydrodistillation	α-Pinene **1** 23% Camphene **2** 7.6% Bornyl alcohol **5** 16%	04JAF3530

		Camphor **4** 4.5% Verbenone **13** 9.4% Bornyl acetate **7** 10.4%	
Pakistan	Hydrodistillation	1,8-Cineole **3** 38.5% Camphor **4** 17.1% α-Pinene **1** 12.3% Limonene **9** 6,23% Camphene **2** 6.00% Linalool **8** 5.70% Oxygenated terpenes 67.0% Monoterpenes 26.0%	10BJM1517
Poland	Hydrodistillation	α-Pinene **1** 33.3% Bornyl acetate **7** 14.8% Camphene **2** 13.8% 1,8-Cineole **3** 12.3%	10JFE253
Serbia	Hydrodistillation	Limonene **9** 21.7% α-Pinene **1** 13.5% Camphor **4** 21.6% Linalool oxide 14 10.8%	07JAF7879
Spain	Hydrodistillation	1,8-Cineole **3** 23.6% Camphor **4** 22.1% α-Pinene **1** 14.7% Verbenone **13** 1.97%	09AH167
Tunisia	Hydrodistillation	1,8-Cineole **3** 40.0% Camphor **4** 17.9% α-Pinene **1** 10.3% Camphene **2** 6.30%	10FCT3144
Turkey	Hydrodistillation	1,8-Cineole **3** 61.4% α-Pinene **1** 10.2% Camphor **4** 5.8%	07FC(100)553



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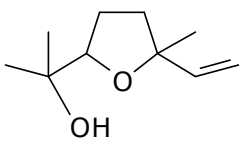

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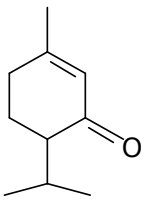

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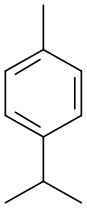

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Seasonal changes have certain trends, such as general increase of the essential oil yield during the flowering period (08JAF7254), which contrasts *Rosmarinus officinalis* from some other herbs. This period is usually long enough and sometimes all year long. Leaves from the apical part of a plant are the principal source of useful substances of the essential oil followed by leaves of the intermediate and bottom parts and the flowers. Stems contain negligible amounts of the essential oil, and it is recommended to discard them before extraction (02JAF3512). Different species of *Rosmarinus officinalis* are classified into two general chemotypes: 1,8-cineole and α-pinene-bornyl alcohol chemotype (03JAF6158). These two types studied for the plants in Tuscany area (Italy) differ drastically in content (%) of principal components as shown in Table 3 compiled from the data (02JAF3512), and the α-pinene type is regarded as more productive and useful in terms of various applications. It contains considerably more camphene, myrcene, and linalool than the 1,8-cineole-bornyl alcohol type. The 1,8-cineole-bornyl alcohol type predominates in Morocco, Tunisia, Turkey, Greece, Yugoslavia, Italy, and France. Cooler areas are richer in terms of camphor and verbenone, while hotter areas contain high amounts of 1,8-cineole and α-pinene.

Another classification according to chemotype includes 1,8-cineole-like (high content of 1,8-cineole), camphor-like (content of camphor exceeding 20%), and verbenone-type (content of verbenone exceeding 15%) (10BSE659). Some other chemotypes are permissible in this classification, *e. g.*, α-pinene-type (It-

aly and Morocco), myrcene-type (Portugal, Argentine, Brazil), and mixed types, such as those with equal content of 1,8-cineole and camphor (India) or 1,8-cineole and α-pinene (Lebanon). Earlier classification (86FFJ137) proposes six chemotypes based on predominance of bornyl alcohol, bornyl acetate, camphor, camphene, 1,8-cineole, or α-pinene, respectively. However, the general consensus is to regard three basic types of essential oils obtained from *Rosmarinus officinalis* (02FFJ15): those with more than 40% of 1,8-cineole (Morocco, Tunisia, Turkey, Greece, Yugoslavia, Italy, France, and others), with approximately equal ratios (20–30%) of 1,8-cineole, α-pinene, and camphor (France, Spain, Italy, Greece, Bulgaria, and others), and those with domination of myrcene (Argentina, Portugal, Spain, and others). Some unique oils contain as a basis camphor – 1,8-cineole - bornyl alcohol (Cuba), and 1,8-cineole – bornyl alcohol – *p*-cymene (Turkey).

Table 3

Composition of Essential Oil of two Chemotypes of *Rosmarinus officinalis*

Component	% Content in α-Pinene Type	% Content in 1,8-Cineole – Bornyl Alcohol Type
α-Pinene	20.6	12.3
1,8-Cineole	6.6	37.9
Camphor	9.1	5.7
β-Pinene	1.5	6.4
Bornyl alcohol	15.5	7.9

The influence of the extraction method emerged especially as the microwave distillation methods appeared in practice. Microwaves efficiently interact with

more polar molecules and ensure greater extraction of the polar compounds, in our case oxygenated terpenes compared to monoterpenes (08FC(114)355). A systematic study of the extraction methods exists, and some of the results obtained for *Rosmarinus officinalis* collected in the University of Messina (Italy) (05JSS273) are presented in Table 4. Hydrodistillation and microwave-assisted distillation give rise to the best yields of the useful products, and the trend of emerging β-pinene is of interest.

Table 4

Composition of Essential Oils of *Rosmarinus officinalis* Obtained by Various Extraction Methods

Compound	Solvent extraction	Microwave-assisted hydrodistillation	Hydrodistillation	Supercritical fluid extraction
α-Pinene	12.1	8.1	8.6	2.3
β-Pinene	4.5	6.3	4.8	2.3
1,8-Cineole	50.8	45.8	56.9	35.6
Camphor	2.5	5.9	5.9	3.7

L. PLANT PRODUCTS AND THEIR TRANSFORMATION

A fundamental basis for plant studies lies in its taxonomy, determination of the chemical composition of the plant and the elucidation of the structure of its components. Often, secondary metabolites isolated from plants represent novel classes of chemical compounds. Also, many plant metabolites and some products of their transformation possess very useful properties. Of the secondary plant metabolites whose chemically transformed components have found uses economically are the essential oils (07RCR655). Essential oils are commonly ex-

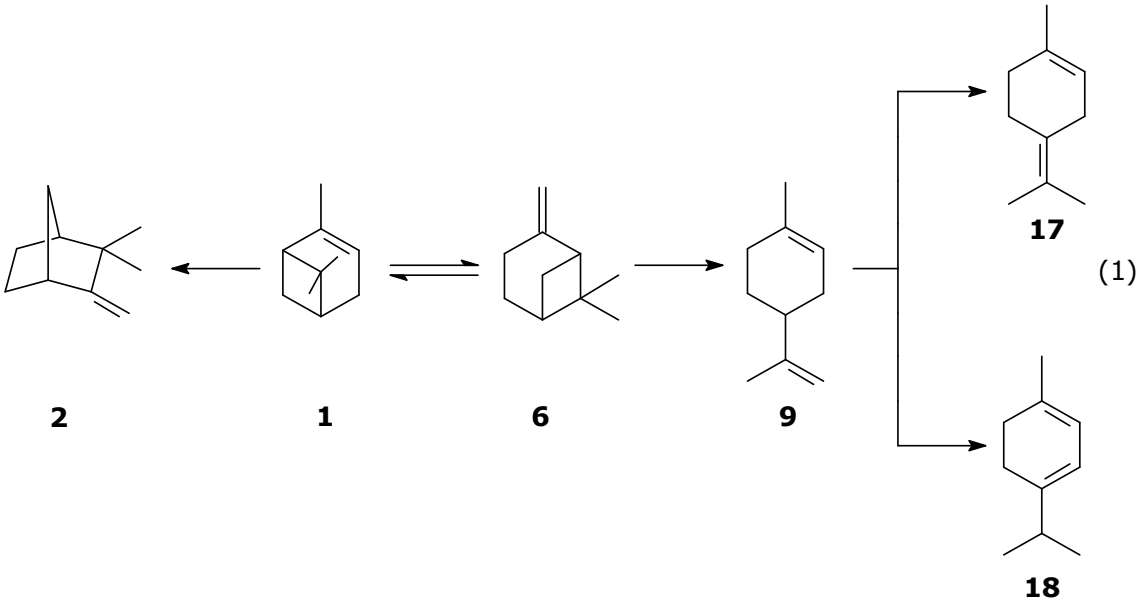
tracted from plants using steam distillation or hydrodistillation, and recently, microwave-assisted extraction. Whichever method used, there is always some chemical transformation of components in the final products. In the further few sections general synthesis and properties of the principal components of essential oils of *Rosmarinus officinalis* are considered.

1. Pinenes

α- and β-pinenes are bicyclic terpenes. They are prepared by distillation of turpentine oils.

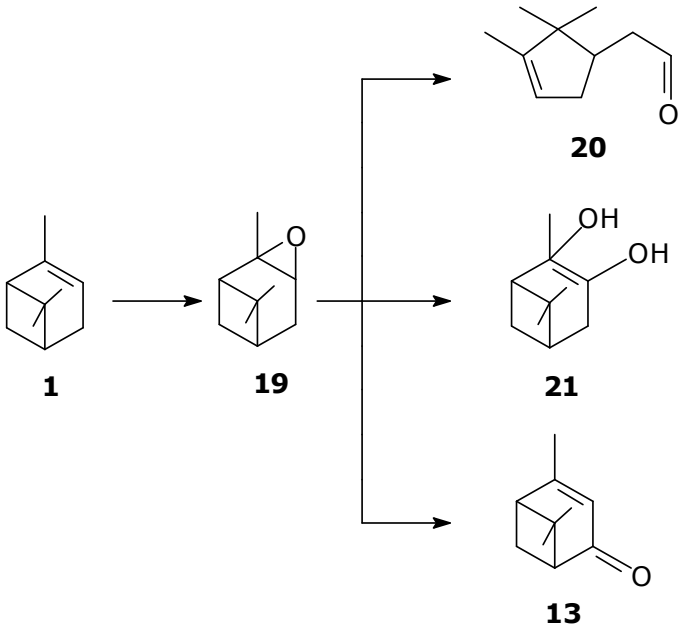
α-Pinene **1** and β-pinene **6** being in isomerization equilibrium may be transformed also by isomerization route to camphene **2** and limonene **9**, respectively (07CRV2411, 08JMS(T)81). The latter is able to isomerize to terpinolene **17** and α-teprinene **18** as shown in equation 1. Several routes are known leading to various monocyclic and tricyclic terpenes. However, camphene and limonene remain the main products, and selectivity of their formation depends on the nature of the acidic heterogeneous catalyst. The route leading to camphene and other similar products is the ring-expansion route finally leading to such a valuable substance as camphor. The β-pinene route leads to monocyclic terpenes. The processes are heterogeneously catalyzed by various acidic oxide catalysts including mesoporous molecular sieves with different Si/ Al ratios (10BT7224). Acidic β-zeolites give preference to camphene **2** (05AC(A)261), especially with Brønsted acidic surface sites (05JMC(A)253). Generally weak surface acids favor camphene, while strong acids are selective with respect to the monocyclic

terpenes (96CJC113). Tin(IV)-, titanium(IV)- and zirconium(IV)-polyphosphate catalysts, in contrast, favor the limonene route (96JMC(A)251).



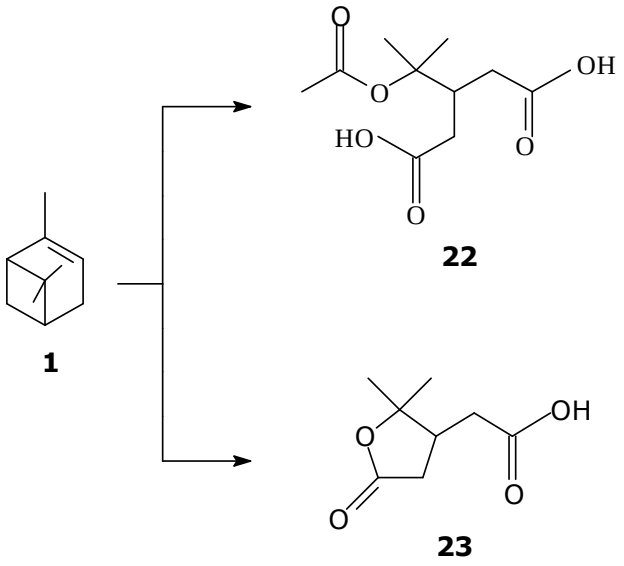
(1)

Epoxidation of α-pinene **1** using *t*-butyl hydroperoxide or cumyl hydroperoxide is also a heterogeneously catalyzed reaction leading to a mixture of products, α-pinene oxide **19**, campholenic aldehyde **20**, 1,2-pinanediol **21**, and verbenone **13**, as depicted in the chain of transformations of equation (2) (03AC(A)309).



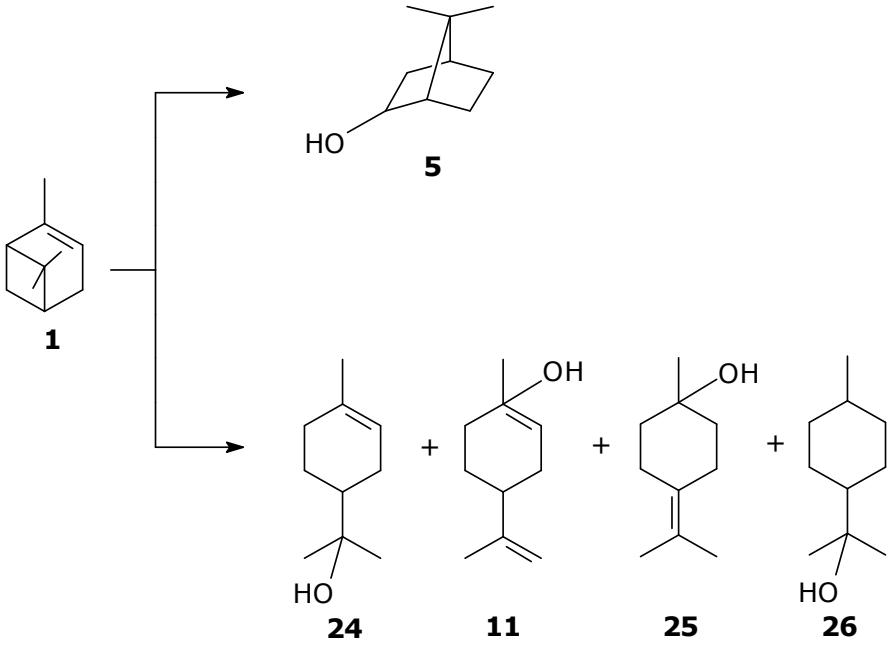
(2)

Atmospheric photooxidation and ozonolysis of α-pinene **1** give diaterpenylic acid acetate **22** and terpenylic acid **23** as shown in equation 3 (09EST6976). The hydroxyl radical addition to the double bond of α- or β-pinene initiates their degradation (98ACR574). The same function belongs to β-hydroxyalkoxy radicals (01JA4228). Thus α-pinene with hydroxyl radicals yields formaldehyde, acetaldehyde, acetone, campholene aldehyde and pinon aldehyde, and β-pinene – formaldehyde, nopinone, acetaldehyde, acetone, *trans*-3-hydroxypinone, peril aldehyde, and myrtanal (02ABC630).



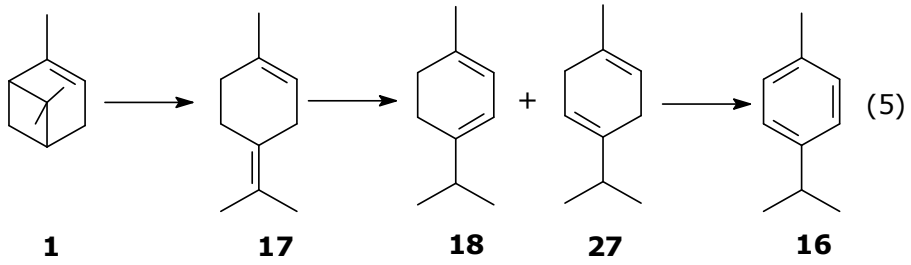
(3)

Hydration of α-pinene **1** may go *via* two routes depending on the way of isomerization, either to the hydrated product of camphene bornyl alcohol **5**, or hydrated products of terpinene - α- **24**, β- **11**, and γ-terpineols **25** as well as 1,8-terpine **26** (equation 4) (05CAT296).



(4)

Dehydrogenation of α-pinene accompanied by isomerization and hence dehydroisomerization occurs in the presence of the platinum or palladium catalysts supported on the surface of silica, alumina, or zeolites (01AC(A)(215)111). The first step is isomerization of α-pinene **1** to terpinolene **17**, then dehydrogenation to α- **18** and γ-terpinenes **27** takes place, and finally *p*-cymene **16** and other products are afforded (equation 5). Hydrogenation of α-pinene gives pinane, an important substance in the flavor and fragrance industry (97MI1). Pyrolysis of α-pinene results in ocimene and alloocimene while that of β-pinene gives myrcene. β-Pinene adds formaldehyde to yield nopole.

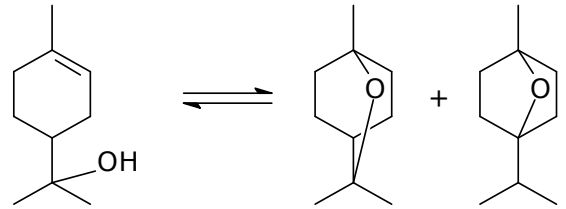


2. 1,8-Cineole

1,8-Cineole (1,3,3-trymethyl-2-oxabicyclo[2.2.2]octane, eucalyptol, 1,8-epoxy-*p*-menthane) is the monoterpene cyclic ether. Its molecule has C_s symmetry, and its dipole moment is in the mirror plane of the oxygen-containing ring. The electron density evolves over the oxygen ring (07JPC(B)3167).

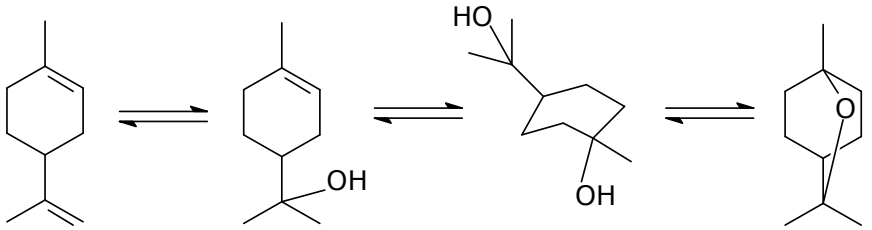
1,8-Cineole **3** can be prepared from α-terpineol **24** in the presence of heteropolyacid supported on silica $H_3PW_{12}O_{40}/SiO_2$ along with 1,4-cineole **28** (equation 6) (06JMC(A)99). In natural conditions, 1,8-cineole **3** can be formed from limonene **9** through the steps of α-terpineol **24** and 1,8-terpine **26** (equation 7)

(05JAF1633). Steps are slow and reversible but the final yield of 1,8-cineole may be substantial.



(6)

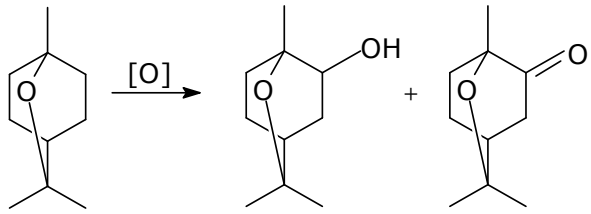
24 **3** **28**



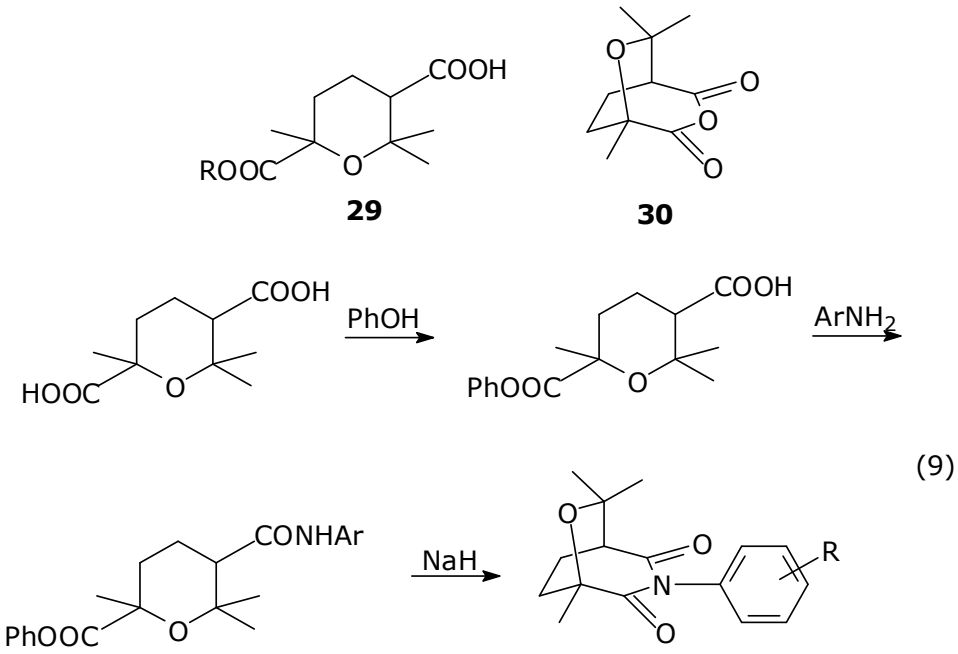
(7)

9 **24** **26** **3**

Biooxidation leads to 2-hydroxo- and 2-oxo-1,8-cineole, potential synthons for organic chemistry (equation 8) (06EJB28, 09JMC(B)173). Chemically, the oxidation process can be extended to cineolic acid **29** (R = H) and its monomethyl ester **29** (R = Me) as well as anhydride **30** (74AJC1143). Cineolic acid **29** (R = H) can be transformed into cyclic N-phenylimides by a sequence of reactions (9) involving carboxylic group protection and cyclization (97JCR(S)228). Here R = H, 4'-OMe, 4'Cl, 4'-Br.

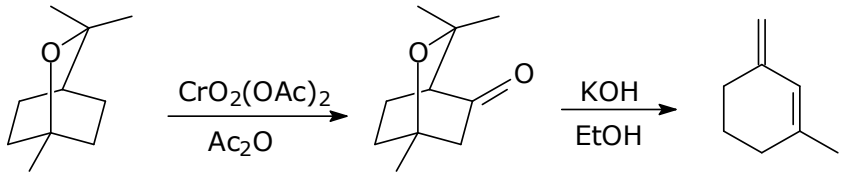


(8)



(9)

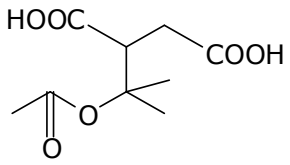
Another chain of transformations starts with chemical oxidation of 1,8-cineole **3** using hydrogen peroxide in the presence of manganese(III) porphyrin (96TL1893) or chromyl acetate (87JOC1505) to yield 3-keto-1,8-cineole **31**. Treatment of the product by potassium hydroxide in ethanol yields seudenone **32** (equation 10) (00ICP53).



3 **31** **32**

(10)

In atmospheric conditions, oxidation of 1,8-cineole proceeds even further to diaterebic acid acetate **33** and diaterpenylic acid acetate **34** (09EST280).



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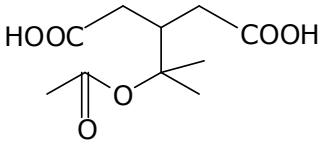

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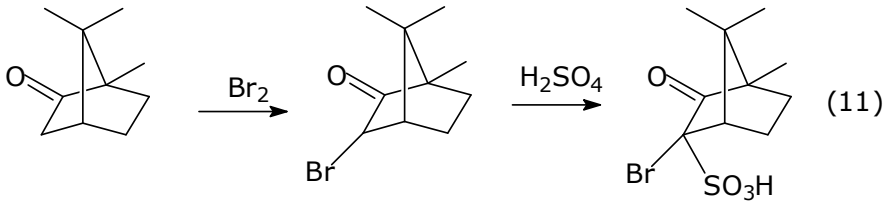
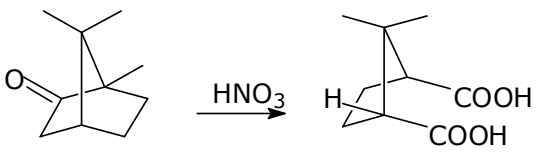
3. Camphor

Camphor, 1,7,7-trimethylbicyclo[2.2.1]heptan-2-one is a terpenoid with the chemical formula $C_{10}H_{16}O$. It is produced from oil of turpentine as well as from α-pinene, which readily rearranges into camphene. Wagner-Meerwein rearrangement of the latter and further acetylation yields *iso*-bornyl acetate, which hydrolyzes into iso-borneol. Finally, dehydrogenation of the product leads to camphor.

Camphor can be brominated (equation 11), oxidized using nitric acid into camphoric acid **35** (equation 12), transformed into *iso*-nitroso derivative (equation 13), and reduced using sodium borohydride into iso-bornyl alcohol (equation 14).



(11)



(12)

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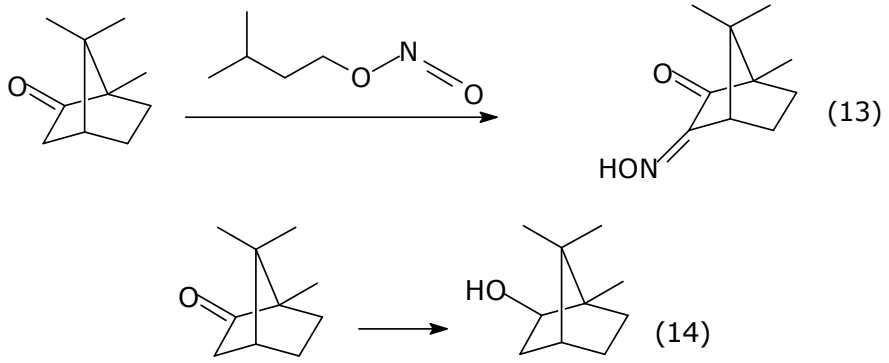
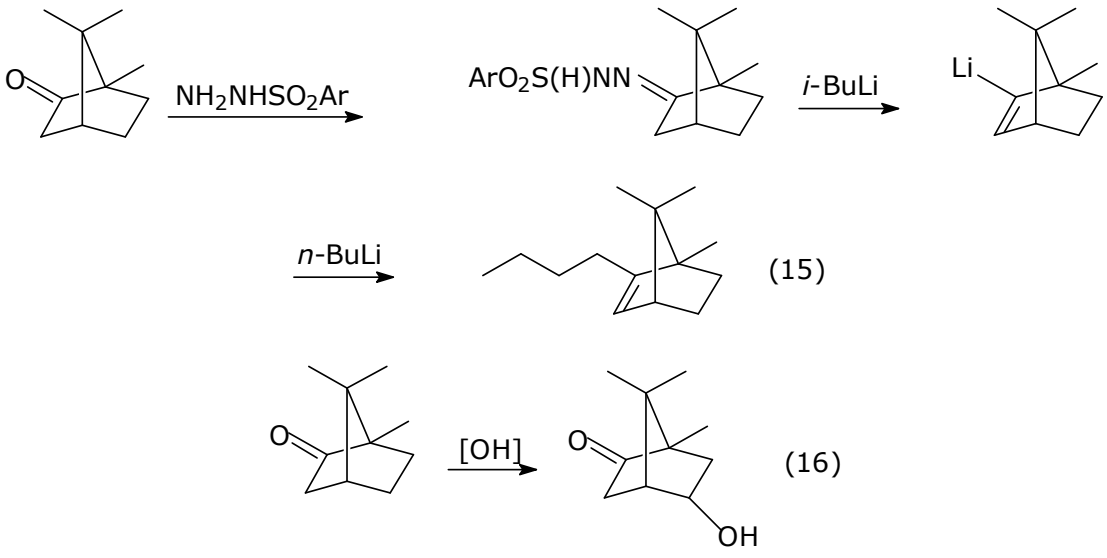
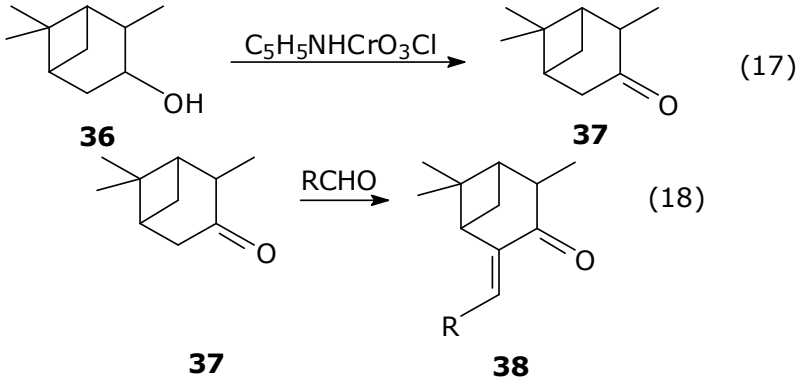


Another way of derivatization of camphor is Shapiro reaction sequence (15). The process includes (83OS141) interaction with tri-*i*-propylbenzene sulfonyl-hydrazone to yield camphor derivative, then with *i*-butyl lithium and then n-butyl bromide to give 2-n-butyl bornene as a final product through the stage of 2-lithiobornene. Biohydroxylation of camphor leads to 5-hydroxycamphor (equation 16) (95JBC28042, 06JCC1324).



Oxidation of *iso*-pinocampheol **36** (3-pinanol) using pyridinium chlorochromate gives 2,6,6-trimethylbicyclo[3.1.1]heptan-3-one (3-pinanone) **37** (equation 17) (03FFJ441). This 3-pinanone enters aldol condensation with various al-

dehydes giving rise to pinocamphone enones **38** (equation 18). 2-Hydroxy-3-pinanone is one of the products of the atmospheric reaction of α-pinene with hydroxyl radicals (03ACP1, 05ACP1053).



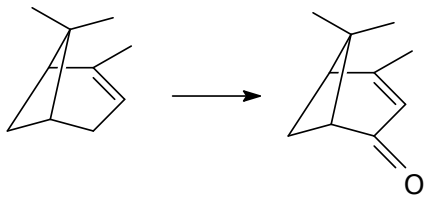
(17)

(18)

36 **37** **37** **38**

4. Verbenone

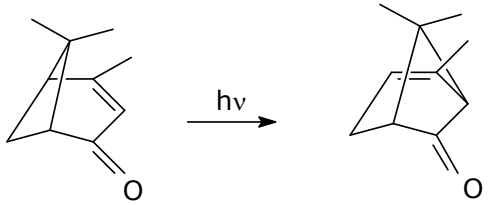
Verbenone is a bicyclic ketone terpene. It is sparingly soluble in water but miscible with most organic solvents. Synthesis of verbenone **13** is based on oxidation of α-pinene **1** (equation 19). The process can also go biooxidation route (07EJB3458).



(19)

1 **13**

In the process of photochemical rearrangement verbenone **13** can be transformed to chrysanthenone **39** (equation 20):



(20)

13 **39**

5. Bornyl acetate

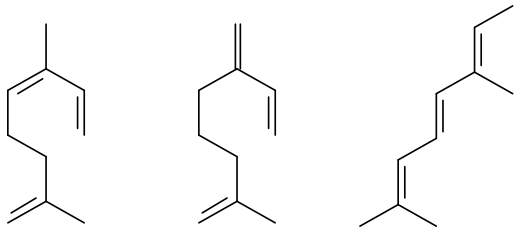
Industrially α-pinene is treated with liquid mineral acids, and the resulting borneol is esterified with acetic anhydride in the presence of the mineral acidic catalysts (01AC(A)(209)269). In the laboratory esterification is achieved by direct reaction with acetic acid in the presence of acidic ionic liquids (08CAC1634).

Bornyl acetate (bicyclo[2,2,1]-heptan-2-ol-1,7,7-trimethyl-acetate), with hydroxyl radical of the atmosphere gives 1,7,7-trimethyl-bicyclo[2.2.1]-heptan-2-one, 4,7,7-trimethyl-5-acetyloxy-bicyclo[2.2.l]-heptan-2-one, and 1,7,7-trimethyl-6-acetyloxy-bicyclo[2.2.1]-heptan-2,3-dione (99PCE537). The observed biotransformations are generally regio- and stereoselective hydroxylation, acetate hydrolysis and oxidation of alcohols to carbonyl compounds, which leads to various new hydroxybornyl acetates (05JCT567).

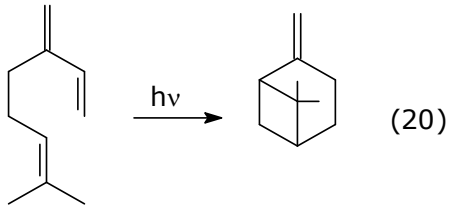
M. CHEMICAL TRANSFORMATION IN PLANTS

Chemical transformation is the modification of known plant metabolites in order to enhance the original activity of the chemical compound or obtain derivatives that exhibit other activities. Often secondary metabolites isolated from plants represent novel classes of chemical compounds and these plant metabolites and products of their transformations possess very useful properties. Many natural products become very useful only after their transformations into other valuable products. One major group of secondary plant metabolites whose chemically transformed components have found uses economically are the es-

sential oils. Monoterpenes such as *cis*-α-ocimene **39**, α-myrcene **40**, and 4-*trans*-6-*trans*-alloocimene **41**, which are chemically transformed products of essential oils, have pleasant odors; hence they are synthesized on a large scale for use in perfumery industry (05AJB931). At elevated temperatures, β-myrcene **10** is converted to β-pinene **6** (equation 20) (64JA1892, 64PCS17, 69JOC3587).



39 **40** **41**

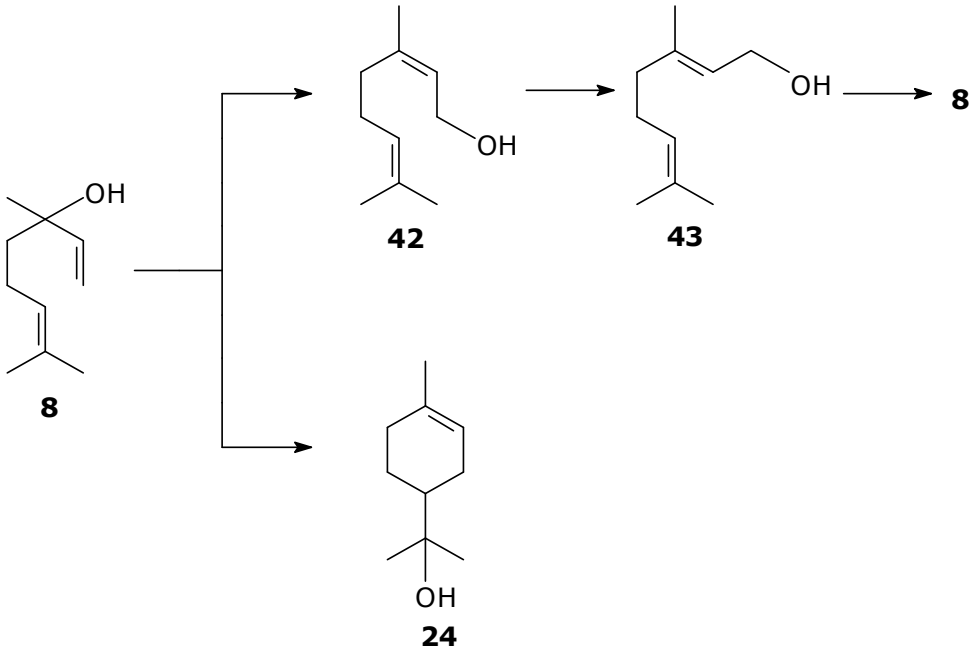


10 **6** (20)

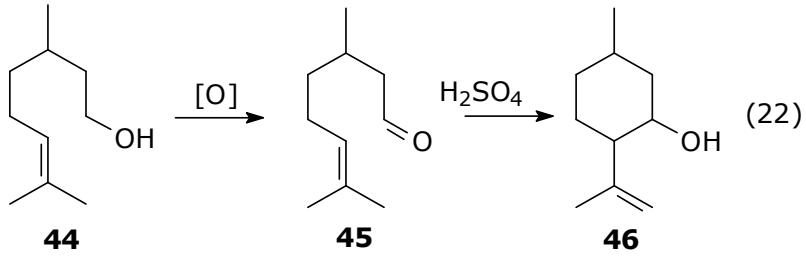
Kinetic studies have shown that linalool **8**, geraniol **42**, and nerol, **43** inter-convert in acids *via* an allylic rearrangement to yield linalool **8** and α-terpineol, **24**, respectively (equation 21) (76JOC2419).

On oxidation, citronellol **44** gives citronellal **45**, while citronellal **45** cyclizes to give isopulegol **46** in good yield with sulfuric acid (equation 22) (64CI(L)263). On partial hydrogenation of geraniol **42**, the monounsaturated alcohol citronellol **44** was obtained (equation 23). Condensation of citronellal **45** with acetone gives, *via* aldol-type pathway, dihydropseudoionone **47**, which

readily cyclizes to dihydroionone **48**, a substance with a fresh smell of flowers (equation 24) (04TC169).



(21)



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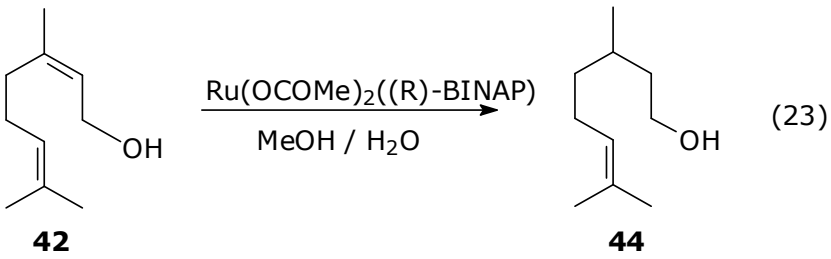

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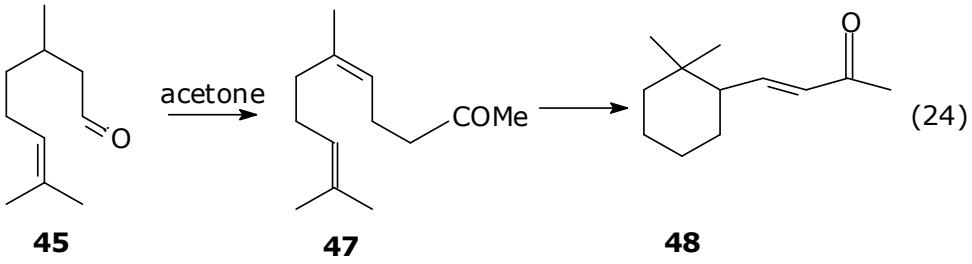


45 **47** **48** (24)

Plants emit a wide range of volatile organic compounds whose common precursor is isoprene (92GBC389). Isoprene emission is generally related to photosynthesis because both processes are light and carbon dioxide dependent (91CIR333). Monoterpenes are released from specialized organs such as resin ducts, oil glands and glandular trichomes (91MI3). In many cases, monoterpenes are emissions found in field studies (96PP267).Temperature is the main factor affecting the emission of monoterpenes (80PP801, 91MI3). Terpenes have the general formula $(C_5H_8)_n$ and are biosynthesized from isoprene units in the form of isopentyl pyrophosphate. The parent terpenes and their oxidation products such as epoxides, alcohols, aldehydes, and ketones constitute one of the largest class of organic compounds found in biological systems, monoterpenes (n=2) are more volatile than their sesquiterpenoid (n=3) homologues. The involatile diterpenes such as squalene (n=4) and larger terpenoids have important biological activities, *e. g.*, some are hormones or precursors to hormones. Many mono- and sesquiterpenoid compounds found naturally in plant essential oils are sought after fragrances and flavorings due to their distinctive pleasant odors and taste notes (91MI1, 94MI1). Verbenol, verbenone, and myrtenol are derived from the oxidation of α-pinene (96AMB822, 03JA705). Myr-

tenol arose from oxidation of C_{10} group. Monoterpenes are biogenic volatile organic compounds that play an important role in atmospheric chemistry (97JAC189, 99JAC207, 06JGR7302, 07AE4877).

N. Antioxidant Activity

1. Definition of antioxidant

Oxygen is essential to human cells but could in the presence of free radicals lead to oxidative destruction of the cell, hence the need for cell protection by antioxidants. It is estimated that there are about 4,000 compounds in foods that have antioxidant activities. The most studied of these antioxidant compounds in the past include vitamin C (ascorbic acid), vitamin E, beta-carotene, selenium, and carotenoids (99FC323).

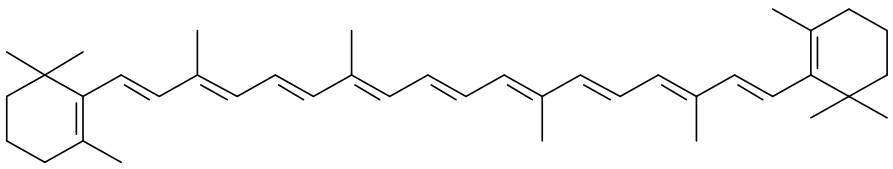
Antioxidants acts as radical scavengers inhibit lipid peroxidation and other free radical-mediated processes and therefore protect the human body from several diseases attributed to the reactions of radicals. Numerous substances have now been suggested to act as antioxidants. Various phenolic antioxidants such as flavonoids, tannins, coumarins, xanthenes, and more recently, procyanidins have been shown to scavenge radicals in dose-dependent manner and therefore are viewed as promising therapeutic drugs for the radical pathologies (01JEP(77)31). Antioxidants are also used in food industry and are sometimes added to foods containing fats or oils to prevent them from becoming rancid and discolored. Increase in the intake of antioxidants have been reported to enhance thyroid hormone action, normalize zinc and copper levels, normalize

blood sugar levels, restore proper cell oxygen level, restore libido, normalize blood clotting, protect against breast fibrocysts, help prevent breast and endometrial cancer, increase the chance of survival of the embryo and maintain secretory endometrium (03CMC1795).

2. Natural antioxidants

a. Beta carotene and carotenoids

Carotenoids are a group of pigments (red, orange and yellow), found in plant foods. The most prevalent carotenoids are β-carotene **49**, lycophene **50**, lutein **51**, and zeaxanthin **52** (99FC323). Carotenoids are a collection of about 600 nutrients and are not considered vitamins themselves, even though β-carotene is converted in the body to vitamin A **53** (97ZN(C)823). However, some of the other above mentioned carotenoids are re-emerging as providing health protection better than β-carotene. Experts now believe that carotenoids work best along with other phytochemicals and vitamins (98JN1845). Carotenoids act as antioxidants in many ways. β-Carotene helps to reduce risk of cancer and strengthens the immune system; lutein and zeaxanthin promote eye health by protecting the retina from harmful radiation, and lycophene helps reduce risk of prostate cancer. Carotenoids are found in deeply colored fruits and vegetables (88PC969).



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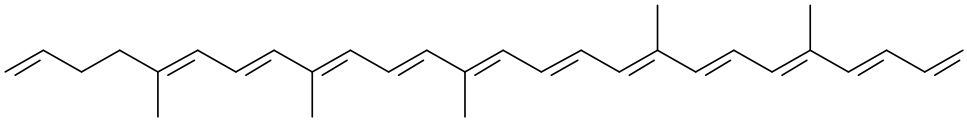

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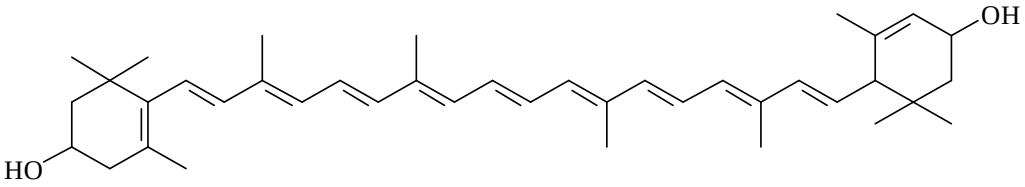

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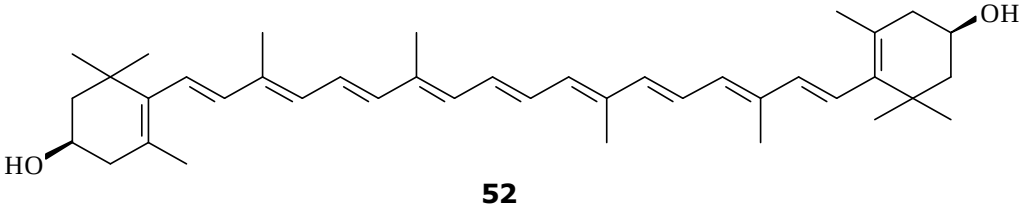

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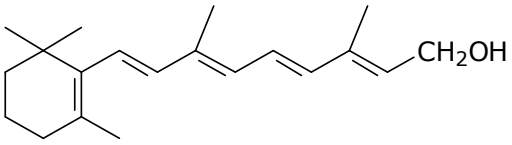

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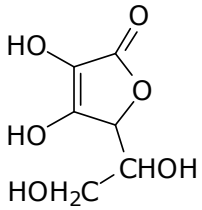
β-Carotene is primarily found in fruits and vegetables such as carrots, sweet potatoes, mangoes, and in dark green vegetables such as spinach and turnip green (98FRR247). Lutein and zeaxanthin are also found in pumpkin and dark green leafy vegetables such as spinach and kale. Lycopene is found in tomatoes, water melon and red pepper.

b. Ascorbic acid (Vitamin C)

Vitamin C **54** is a water-soluble vitamin. When the concentration of ascorbic acid in the blood is too high, it is eliminated through the urine. Vitamin C works with vitamin E **55** to block the damaging reactions that are caused by free radicals (95ACN797). Vitamin C is equally powerful scavenger of air pollutants and

an antioxidant; it has been reported to prevent free radical-related diseases such as cancer, as well as reversing the disease conditions. In addition to its antioxidant property, vitamin C has also been reported to be essential for the production of collagen (an important protein that forms the body connecting tissues, *e. g.*, bones, cartilage, tendons, and ligaments). It is also essential for the absorption of minerals such as iron (00JEP343). Other diseases that vitamin C protects against include: heart disease, obesity, and weight loss, osteoarthritis, high blood pressure and diabetes. Sources of vitamin C include various fruits and vegetables such as oranges, grape fruits, mangoes, the dark green vegetables, red cabbage, and chili peppers.



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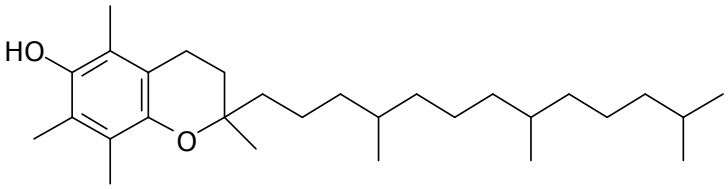

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c. Vitamin E

This is a fat-soluble vitamin. It is absorbed in the gastro-intestinal tract with the aid of dietary fats and bile. Fat-soluble vitamins are stored in the fatty compartments of the body and the liver. The major role of vitamin E is in the protection of the red blood cells and also in the stabilization of the cell membranes. This vitamin also has antioxidant property (02FPE879).

Most of the research on vitamin E as an antioxidant is related to free radical-induced heart diseases. This condition was found to be reversed with vitamin E

ingestion. Vitamin E also acts as cell stabilizer by preventing the oxidation of low-density lipoprotein cholesterol (LDL-Cholesterol). This is known to reduce risk of atherosclerosis and heart attack (98PR288). Vitamin E is found in nuts, seeds (especially sun flower seeds), vegetable oils, wheat germ, liver, egg yolk, soya beans and sweet potatoes.

d. Selenium

Selenium is a trace element which is available in plant food. It is necessary for different cellular functions. Selenium has been reported to have antioxidant activity (00BF141). It is a co- factor of several enzymes that are free-radical scavengers and also aids in cell growth because it helps maintain tissue elasticity by preventing excessive cell damage. The role of selenium in the protection against prostate cancer is a subject of investigation (02FPE879). Selenium can be found in vegetables such as cucumber, garlic, mushrooms, onions, whole grain products like brown rice, wheat germ and animal products such as liver, chicken, egg yolk, sea foods, milk, yeast.

3. New antioxidants

Spices and herbs are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases resulting from lipid peroxidation (00BF141). The medicinal properties of folk plants are mainly attributed to the presence of flavonoids, but they may also be influenced by other organic and inorganic compounds, such as coumarins, phenolic acids and antioxidant micronutrients, *e. g.*, Cu, Mn, Zn

(01JEP(77)31). Flavonoids and other polypenols belong to the recently popular phytochemicals, chemicals derived from plant materials with potentially beneficial effects on human health. The compounds are known as secondary plant metabolites, a designation indicating that most of these substances have been regarded as non essential and therefore secondary in function. Yet over the years they have been found to be an important part of the human diet and are considered to be active principles in some medicinal plants. The antioxidant activity of flavonoids is efficient in trapping superoxide anion (O_2^-), hydroxyl (OH), peroxyl (ROO), an alkoxyl (RO) radicals. The flavonoids that contain multiple OH substitution have very strong antioxidant activities against peroxyl radicals (99FRB193).

It is now believed that there are hundreds of other antioxidants that occur naturally in foods and beverages. Most of them are not considered nutrients, but phytochemicals (meaning plant chemicals). Phytochemicals are now being discovered as powerful disease fighters. Flavonoids, polyphenols and anthocyanins are some of the names found in relation to the new antioxidants (98NR317).

a. Polyphenols

Polyphenols are a class of phytochemicals found in high concentrations in wine, grapes and a wide variety of other plants and have been associated with heart disease and cancer prevention (09ACA293). In general terms, phenolic compounds or polyphenols have a similar basic structural chemistry including

an aromatic or phenolic ring structure. It is also important to note that at least 8,000 phenolic compounds are responsible for the brightly colored pigments of many fruits and vegetables, but they protect plants from diseases and ultra violet light and help prevent damage to seeds until they germinate (96JNP205, 98NPR265).

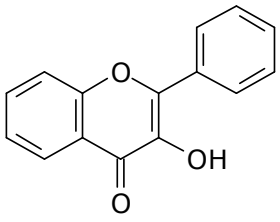
Polyphenols can form complexes with reactive metals such as iron, zinc, and copper, reducing their absorption. At first glance, this may seem to be a negative side effect (reducing nutrient absorption), but excess levels of such elements (metal cations) in the body can promote the generation of free radicals and contribute to oxidative damage of cell membranes and cellular DNA (00JPP938). In addition to chelating effect on metal cations, polyphenols also function as potent free radical scavengers within the body, where they can cause cellular damage.

Natural polyphenols can range from simple molecules such as phenolic acid to large highly polymerized compounds such as tannins. Conjugated forms of polyphenols are the most common, where various sugar molecules, organic acids, and lipids (fats) are linked with the phenolic structure. Differences in this conjugated chemical structure accounts for different chemical classification and variation in the modes of action and health properties of various compounds. It has been claimed that polyphenols help in cancer prevention, protection from heart disease, hypertension, antibiotic/antiviral activity, anti-inflammation ac-

tivity, protection and strengthening of blood high dietary intakes of phenolics and reduced risk of cardiovascular diseases and cancer (98PB63).

b. Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized according to chemical structure in flavonoid **56**, flavone **57**, flavanone **58**, isoflavone **59**, anthocyanidin **60**, catechin **61**, and chalcone **62** (00BF141). Over 4,000 flavonoids have been identified, many of which are widely distributed in plant foods and they include: (1) lignins (nuts, whole grain cereals); (2) proanthocyanins (grapes, pine bark); (3) anthocyanins / anthocyanidins (brightly-colored fruits and vegetables, berries); (4) isoflavones - Genistein/daidzein (soybeans); (5) catechins (tea, grapes, wine); (6) tannins (tea, nuts); and (6) quercetin (grapes, wine, onions).



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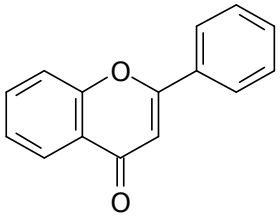

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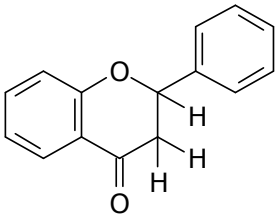

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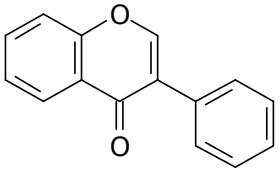

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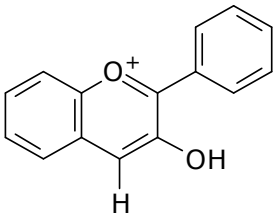

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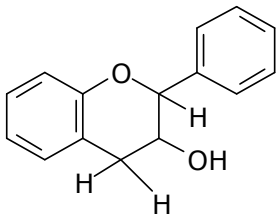

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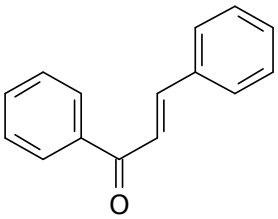

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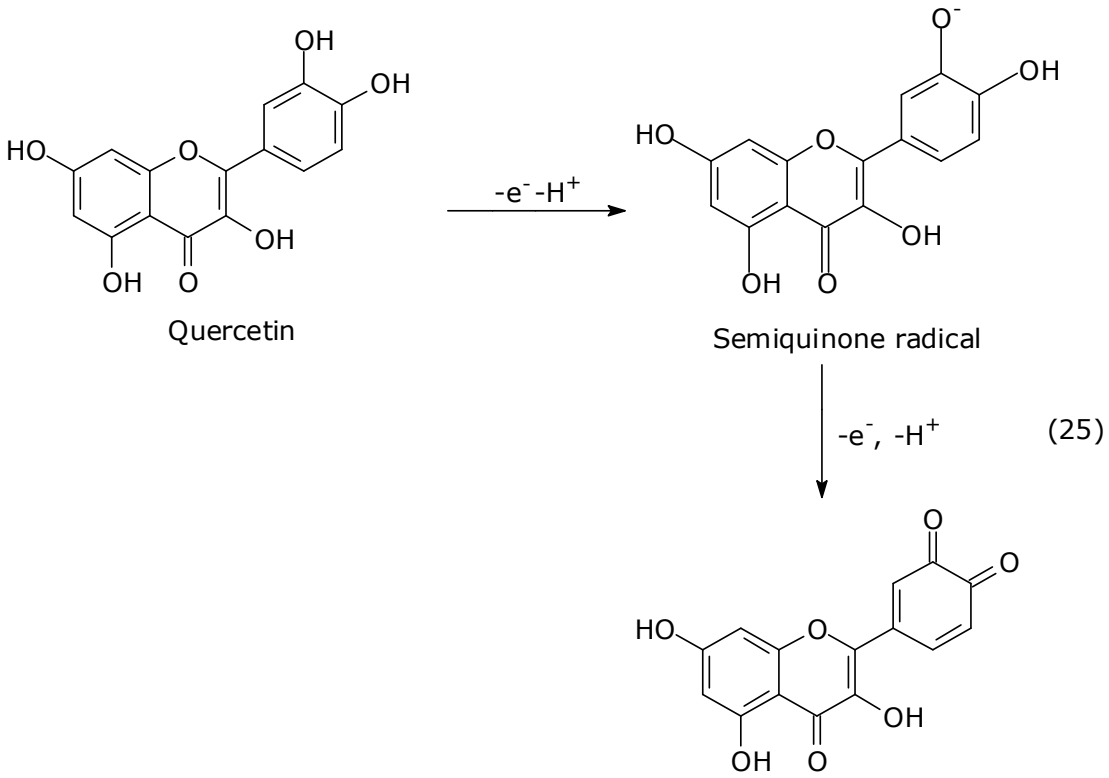
The antioxidant activity of flavonoids found in hops and beer confers surprisingly potent activity exceeding that of red wine, tea, or soya beans. The flavo-

noids have aroused considerable interest recently because of their potential beneficial effects on human health. They have been reported to have antiviral, antiallergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities (00JPP938).

Flavonoids generally consist of two benzene rings (ring A and B) linked by an oxygen containing heterocyclic ring (C). It should be noted that the chalcone **62** is considered to be a member of the flavonoid family, despite lacking the heterocyclic ring C, the fused A and C rings are often collectively termed the flavonoid nucleus. Flavonoids behave as antioxidant through the mechanism of H-donation (96FRB35) as depicted in equation (25) below:



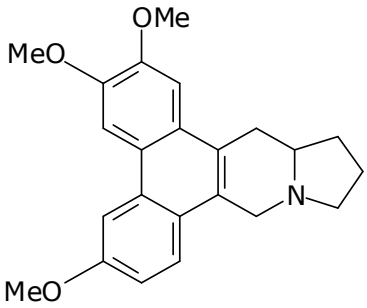
(25)

c. Free radicals

Free radicals are chemically unstable atoms or molecules that cause extensive damage to cells as a result of imbalance between the generations of reactive oxygen species (ROS) and the antioxidant enzymes (87NJB13). Molecular oxygen is an essential component for all living organisms, where it helps in the process of oxidation which is a basic component of aerobic life and of our metabolism. Hence radicals are produced either naturally or by some biological dysfunction (00JFA985). Unpaired electrons which are centered in atoms of oxygen or nitrogen are called reactive oxygen species (ROS) or reactive nitrogen species (RNS) and their excess have a harmful effect, such as peroxidation of the membrane lipids, aggression to tissue proteins and membranes, or damage to DNA and enzymes (87NJB13). Therefore, they can be related to pathologies such as arthritis, hemorrhagic shock and coronary diseases, cataract, cancer, AIDS, as well as age-related degenerative brain disorders (00JFA985). Beneficial effects of antioxidants on promoting health are believed to be achieved through several possible mechanisms, such as directly reacting with and quenching free radicals, chelating transition metals, reducing peroxides, and stimulating the antioxidant defense enzyme system.

Currently there is a great interest in the study of antioxidant substances mainly due to the findings of the free-radicals effects in organisms. The phenolic constituents found in plants have attracted considerable attention for being the main constituents of antioxidant activity, antofine **63**, ficuseptine **64**, and

galegine **65**. The antioxidant activity of phenolics is mainly due to their redox properties, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelation potential. The antioxidant activities of phenolics play an important role in adsorption or neutralization of free radicals (05JEP32).



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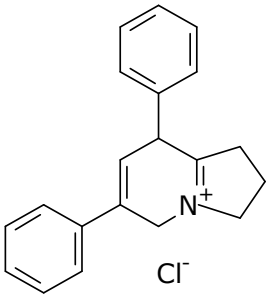

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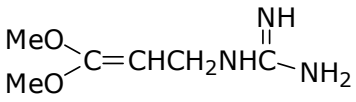

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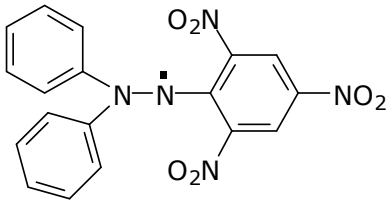
4. Diphenylpicrylhydrazyl (DPPH) free radical

Our bodies are made up of billions of molecules held together by electromagnetic forces. These chemical bonds are created by paired electrons. Free radicals are unstable molecules that have lost an electron and are unbalanced (07BST1147). One free radical can damage a million or more molecules in a chain reaction referred to as radical propagation, which leads to oxidation or what is referred to as oxidative stress. Not all free radicals produced by the body are harmful and indeed free radicals produced by the immune system help to destroy viruses and bacteria (88BP989). Others are involved in producing vital hormones and activating enzymes that are needed for life itself. The problem arises where there are excessive free radicals in the body which can dam-

age the cells and tissues and the over abundance of the free radical create even more radicals in the body (96FRB35).

1,1-Diphenyl-2-picrylhydrazyl **66** is a black solid; a cell-permeable stable free radical at room temperature that acts as hydrogen radical scavenger. It is usually protected from light and can also be used as a screening tool for detecting free radical scavenging activity of antioxidants (95LWT25).



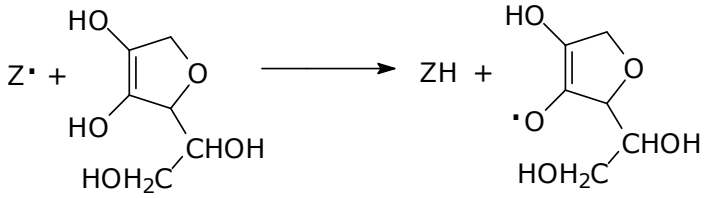
66

DPPH is characterized as a stable free radical by virtue of the decolorization of the spare electron over the molecule as a whole, so that the molecules do not dimerize, as would be the case with most other free radicals. The decolorization also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 520 nm (95LWT25). When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom it gives rise to the reduced form with the loss of this violet color. Representing the DPPH radical by Z and the donor molecule by AH, the primary reaction is

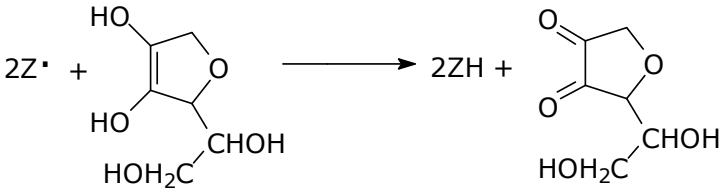
$$Z + AH \rightarrow ZH + A \qquad (26)$$

Here ZH is the reduced form and A is free radical produced in this first step. The latter radical undergoes further reactions, which control the overall stoichiometry, that is the number of molecules of DPPH reduced (decolorized by the

molecule of a reductant). The reaction (26) is intended to provide a link with the reactions taking place in an oxidizing system, such as the autooxidation of a lipid or other unsaturated substance: the DPPH molecule Z is thus intended to represent free radicals formed in the system whose activity is to be suppressed by the substance AH (97ZN(C)823). If, however, the molecule has two adjacent sites for hydrogen abstraction which are internally connected, as is the case with ascorbic acid (vitamin C), then there may be a further hydrogen abstraction reaction after the first one, as shown below (equations 27 and 28). This leads to a 2:1 stoichiometry that is two molecules of DPPH being reduced by one molecule of ascorbic acid.



(27)



(28)

One parameter that has been introduced for interpretation of the results from the DPPH method is the effective concentration or IC_{50}. This is defined as the concentration of substrate that causes 50% loss of DPPH activity color (05LS1319). The IC_{50} parameter has the draw back in that the higher the antioxidant activity, the lower the IC_{50} (01PR127).

5. Some tropical plants with antibacterial and antioxidant properties

Plant is a rich source of medicine, from which a host of bioactive molecules are produced, most of which probably evolved as chemical defenses against predation or infection (78MI1, 88FT354). A representative of medicinal plants that are commonly used in the treatment of antibacterial and antioxidant diseases in the tropics include: *Caricca papaya* L. (Fam. *Caricacea*), *Sida rhombifolia* L. (Fam. *Malvaceae*), *Peperomia pellucid* (Fam. *Piperaceae*), *Phyllanthus amarus* (Fam. *Euphorbiaceae*), *Ficus septic* Burm (Fam. *Moraceae*), *Piper aduncum* L. (Fam. *Piperaceae*) and *Galega officinalis* L. (fam. *Fabaceae*).

O. STRUCTURAL ELUCIDATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS ISOLATED FROM *ROSMARINUS OFFICINALIS* L.

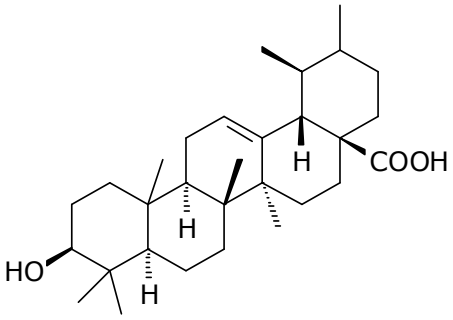
Infections due to microorganisms constitute serious health problems, especially in developing nations of West Africa. A wide range of antibiotics and anti-infective drugs are available nowadays, but their use is limited by a number of factors, such as high cost, low potency, toxic side effects, and emergence of resistance strains. Therefore, the search for new and more potent antimicrobial agents especially from plant sources is necessary. Carnosic acid, found in rosemary, may shield the brain from free radicals, lowering the risk of strokes and neurodegenerative diseases like Alzheimer's and Lou Gehrig's (07MI3).

Rosemary may have some anti-carcinogenic properties; a study where a powdered form of rosemary was given to rats in a measured amount for 2

weeks showed reduction in the binding of a certain carcinogen by 76%, and greatly reduced the formation of mammary tumors (05MI2).

Rosemary contains a number of potentially biologically active compounds, including antioxidants such as carnosic acid and rosmarinic acid. Other bioactive compounds include camphor (up to 20% in dry rosemary leaves), caffeic acid, ursolic acid, betulinic acid, rosmaridiphenol, and rosmanol. Rosemary in culinary or therapeutic doses is generally safe, and a toxicity study of the plant on rats has shown hepatoprotective and antimutagenic activities (99IJF413).

However, the compounds responsible for antibacterial activities in this plant have been identified in only few cases. A diterpene, rosmanol (7α,11,12-trihydroxyabieta-8,11,13-trien-20-oic acid 20,6-lactone) have been isolated from the flowers of this plant (85PC1853). Three new flavonoid glucuronides, luteolin 3'-O-β-D-glucuronide, luteolin 3'-O-(4"-O-acetyl)-β-D-glucuronide, and luteolin 3'-O-(3"-O-acetyl)-β-D-glucuronide, together with hesperidin, were also isolated from 50% aqueous MeOH extract of the leaves of rosemary (94PC1463). Chemical structures of some pure compounds isolated from extracts of *Rosmarinus officinalis* L. (07JAF1718) include the following: 3-β-hydroxy-urs-12-en-28-oic acid (ursolic acid) **67**, 3-β-hydroxy-olean-12-en-28-oic acid (oleanoic acid) **68**, 3-β-hydroxy-ursan-12,20(30)-dien-28-oic acid (micromeric acid) **69**, 3-β-hydroxy-ursan-12,20(30)-dien-28-oic acid methyl ester (micromeric acid methyl ester) **70**.



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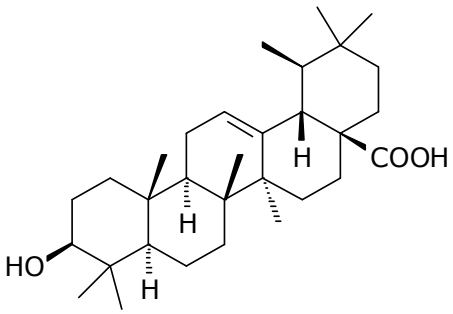

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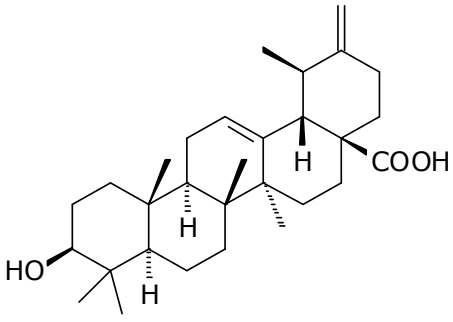

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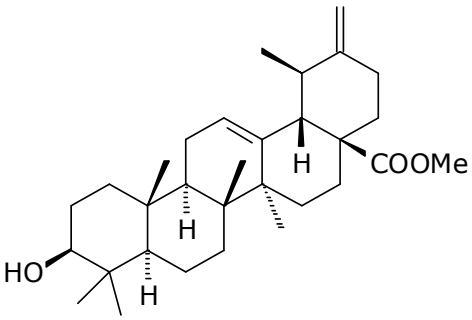

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III. Materials and Methods

A. Cultivation

Rosmarinus officinalis was cultivated in the greenhouse of the University of Fort Hare farm. Individual plants were grown in polythene bags. The soil was collected from the university research farm, dried for about 48 h, sieved through a 2-mm wire mesh (93MI1) and homogenized before filling the polythene bags. All plants were adequately watered as required. Harvesting was done only after the first two weeks following transplanting.

B. Harvesting for essential oil distillation

Following the first two weeks after transplanting, the leaves were harvested monthly. After each harvesting the fresh leaves was weighed and the essential oil distilled using the solvent free microwave-assisted extract method as well as hydrodistillation method.

C. Solvent-free microwave extraction

Essential oils are commonly extracted from plants using steam distillation, hydrodistillation, and recently microwave-assisted extraction. With an increasing interest in avoiding organic solvents in the extraction of compounds from medicinal plants, microwave-assisted distillation has been shown to be a feasible alternative approach. However, much more attention has been given to the use of microwave-assisted distillation because of economy of time, simplified manipulation, ease of use, and higher purity of the final oil.

Solvent free microwave extraction was carried out with a Milestone Dry DIST (2008) (Fig. 2) microwave apparatus. The multimode microwave reactor has a twin magnetron (2,800 W, 2,450 MHz) with a maximum delivered power of 1000 Win 10 W increments. A rotating microwave diffuser ensures homogeneous microwave distribution throughout the plasma-coated PTFE cavity (35 cm × 35 cm × 35 cm). The temperature is monitored by a shielded thermocouple (ATC-300) inserted directly into the corresponding container. Temperature is been controlled by feedback to the microwave power regulator. In a typical SFME procedure, 100 g of the leaves of *Rosmarinus officinalis* were placed in the reactor. The essential oil is collected after the extraction.

Figure 2: Dry DIST Microwave Extractor.

D. HYDRODISTILLATION

This is a traditional method of extraction. It is a process whereby the plant material is placed in water and heated directly in a Clevenger type distillation apparatus (Figure 3). The essential oils whose boiling points normally range up

to 300°C are distilled with steam and both are condensed and separated. The distillation is usually done at atmospheric pressure, although vacuum processes are used if the oil is subject to hydrolysis (11MI1). Since most essential oils are prepared by this method, and have a specific gravity less than water some essential oils processed in this fashion may have a small amount of water at the base of the flask; this might be a disadvantage. Hence it is necessary to dry the essential oil over anhydrous sodium sulfate. However, the advantage of this method over isolation methods such as solvent extraction is that the isolates obtained do not include non-volatile matter, which may interfere with chromatographic analysis. It should also be noted that the yield of essential oils using hydrodistillation process is very small (96NFJ78).

Figure 3: Clevenger hydrodistiller

E. GC-MS ANALYSES AND IDENTIFICATION OF COMPONENTS

GC-MS is standard equipment used to analyze essential oils. The GC-MS analyses was carried out using Hewlett-Packard HP 5973 mass-spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column. The fol-

lowing conditions were used: initial temperature 70°C, maximum temperature 325°C, equilibration time 3 min, ramp 4°C / min, final temperature 240°C; inlet: split less, initial temperature 220°C, pressure 8.27 psi, purge flow 30 mL/min, purge time 0.20 min, gas type helium; column: capillary, 30 m × 0.25 mm, film thickness 0.25 μm, initial flow 0.7 mL/min, average velocity 32 cm/s; MS: EI method at 70 eV.

The components of the oils were identified by matching their mass spectra and retention indices with those of the Wiley 275 library (Wiley, New York) in the computer library and literature (87MI2). The yield of the oil was calculated per gram of the plant material, while the percentage composition was calculated from summation of the peak areas of the total oil composition.

F. ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

Two Gram-positive and two Gram-negative bacteria species used in this study were obtained from the culture collection of the applied and environmental microbiology research group at the University of Fort Hare. The bacteria species include: *Escherichia coli* (ATCC 8739); *Bacillus subtilis* (ATCC 10702); *Klebsiella pneumoniae* (ATCC 10031), and *Staphylococcus aureus* (ATCC 6538). Each species was maintained on nutrient agar plates and recovered for testing by sub-culturing in nutrient broth (Biolab No.2) for 24 h. At the end of the incubation, the cultures were centrifuged at 4000 rpm for 15 min to pellet the cells. The supernatant were discarded and the pellets washed twice with sterile normal saline, re-suspended in the saline and standardized to OD_{660nm} 0.1.

The test bacteria were screened for susceptibility to the differently extracted essential oils using the standard agar cup well diffusion method (87IJM165), using a cut-off screening concentration of 10 mg mL^{-1} of the oils in hexane (04JAF2485, 09RNP52). For this purpose, 100 µL of the standardized bacterial suspension was inoculated in 20 mL of molten nutrient agar maintained at 45°C to give a cell population of approximately 10^5 cells. The molten agar was swirled gently to ensure proper mixing of the bacterial suspension and then poured into sterile 90-mm Petri dishes and allowed to set. After setting, wells were bored into the agar using a 6-mm diameter cup borer. Each plate of one test organism contained three wells, one each for the two oil samples and the other for the hexane control. This was done in duplicate. One hundred milliliter of the test samples were introduced into the wells and allowed to stand for 60 min to enable complete diffusion into the media, after which the plates were incubated at 37 °C for 24 hours. At the end of the incubation period the plates were observed for zones of inhibition. Sizes of the zones were compared to those of the hexane control by subtracting the diameter of the zones of inhibition of the hexane control from that of the hexane plus oil to ascertain activity. All analyses were carried out in triplicates.

All four test bacteria were susceptible to the essential oils, hence the minimum inhibitory concentration of the oil against the test bacteria were determined using standard colorimetric broth microdilution technique (07M325). A 96-well microtitre plate was used, and each well contained 100 µL of double-

strength Mueller Hinton broth, 50 µL of test organism or sterile normal saline (for controls), and 50 µL of oil (of known concentrations) or hexane diluents (as control). The final concentrations of the oils in the wells ranged from 0.0 to 7.5 mg mL^{-1}. Each treatment was performed in duplicate and the plates were incubated at 37°C for 24 h. At the end of the incubation period, the turbidity of the cultures was determined using a microplate reader, and MIC was estimated as the least concentration of the oil that inhibited bacterial growth relative to the hexane control. For MBC determination, the contents of the wells showing growth inhibition were streaked onto the surface of nutrient agar plates and incubated at 37°C for 24 h. After the incubation, the plates were observed for growth. The MBC was estimated as the least concentration of the extract where no visible growth was observed.

G. TIME-KILL ASSAY

Determination of the rate of kill of the crude extract was done following time-kill procedure (05ABT946). Inocula were prepared following the described guidelines of European Committee on Antimicrobial Susceptibility Testing (03CMI467). The resultant suspension was diluted 1:100 with fresh sterile broth and used to inoculate 50 mL volumes of Mueller Hinton broth incorporated with extract at MIC and 2 × MIC to a final cell density of approximately 5 × 10^5 cfu/mL. The flasks were incubated at 37°C on an orbital shaker at 120 rpm. A 500-µL sample was removed from cultures at 0, 6, and 12 h, and transferred to 4.5 mL of Mueller Hinton broth and recovery medium containing 3%

Tween-80 to neutralize the effects of the crude extracts carry-overs from the test suspensions. The suspension was then diluted serially, and 100 µL of the diluted samples were plated in triplicate on Mueller Hinton agar plates and incubated at 37 °C for 24 h. Controls included extract-free Mueller Hinton broth seeded with the test inoculum.

H. Antioxidant activity of essential oils

1. DPPH radical scavenging activity

The method (05JAF2433) was used for the determination of scavenging activity of DPPH free radical in the essential oils. A solution of 0.135 mM DPPH^{o} in methanol was prepared and 1.0 mL of this solution was mixed with 1.0 mL of oil prepared in methanol containing 0.33, 0.5 and 1.0 mg/mL of the essential oil and standard drugs (BHT). The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the plant essential oils to scavenge DPPH^{o} radical was calculated by the following equation:

$$\text{DPPH radical scavenging activity} = \frac{\text{Abs}_{\square\square\square\square\square\square\square\square} - \text{Abs}_{\square\square\square\ \square\square\square}}{\text{Abs}_{\square\square\square\square\square\square\square\square}} \times 100$$

Here $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + sample oil /standard.

2. β-Carotene bleaching assay

This method evaluates the capacity of the oil to reduce the oxidative loss of β-carotene in a β-carotene - linoleic acid emulsion (84JAO928). β-Carotene (10

mg) was dissolved in 10 mL of chloroform ($CHCl_3$). An aliquot (0.2 mL) of this solution was added into a boiling flask containing 20 mg of linoleic acid and 200 mg of Tween 40. The chloroform was removed using a rotary evaporator at 40°C and distilled water (50 mL) was slowly added to the residue with vigorous agitation. The obtained emulsion was added to a tube containing 0.2 mL of essential oil. The absorbance was immediately measured at 470 nm against blank, consisting of an emulsion without β-carotene and the test emulsion was incubated in a water bath at 50°C for 50 min, when the absorbance was measured again. Control samples contained 10 μL of water instead of essential oils. BHT, a stable antioxidant was used as the positive control. The antioxidant activity (%) of the oil was evaluated in terms of the bleaching of the β-carotene using the following formula:

$$\% \text{ inhibition} = \frac{A_t - C_t}{C_0 - C_0} \times 100$$

where A_t and C_t are the absorbances measured for the oil and control respectively, after incubation for 50 min, and C_0 are the absorbance values for the control measured at zero time during the incubation for 50 min, and C_0 are the absorbance values for the control measured at zero time during the incubation.

I. Isolation and characterization of bioactive compounds from leaf extracts of *Rosmarinus officinalis* L.

1. Introduction

Isolation and characterization of the bioactive compounds in a plant leads to the possible synthesis of a more potent drug with reduced toxicity. The pure

compound is required to assess the possible toxicity or side effects (82MI1). Chemicals from plants may possess complex structures that are not available in synthetic compound libraries. There are estimated to be 250,000 plant species in the world, and about 5-15% of these species have been tested for potentially useful biologically active compounds (08JEP559). Chromatographic techniques such as thin layer chromatography (TLC), gas chromatography (GC), and high performance liquid chromatography (HPLC) are frequently used for the analysis of plant medicines. TLC is an important method for the isolation, purification and confirmation of natural products, this method has been proved to be reproducible and accurate (04BT31).

2. Plant collection

The leaves of *Rosmarinus officinalis* were collected from the wild around University of Fort Hare campus in August 2010. The plant was identified in the botany department of the University of Fort Hare. A voucher specimen was deposited at the university herbarium.

3. Plant preparation

The leaves were dried at room temperature for about two weeks in the phytomedicine research laboratory at the University of Fort-Hare. The dried leaves were then ground to fine powder using a grinder. The fine powder was stored in closed bottles at room temperature in the laboratory until needed.

4. Extraction and partitioning

The ground powder (1 kg) was extracted with absolute methanol using cold extraction method (10OJV220). The methanol extract was filtered and concentrated using a rotary evaporator (Buchi Labotec rotavapor Model R-205, Switzerland) at 40°C. The concentrated extract was transferred into pre-weighed beakers, dried under stream of air and weighed. The combined extract was partitioned between *n*-hexane and water. The water fraction was further partitioned between ethyl acetate and *n*-butanol. This extraction procedure resulted in four fractions, *n*-hexane, ethyl acetate, *n*-butanol, and water fractions (Fig. 4).

5. Chromatographic analysis

Chemical constituents of the extracts were analyzed by thin layer chromatography (TLC) using aluminum backed TLC plates (Merck, 60 F_{254}). The TLC plates were then developed with three eluent systems; ethyl acetate/methanol, water (10:1.35:1) (EMW Polar); chloroform/ethyl acetate/ formic acid (10:8:2) (CEF, intermediate polarity) and benzene/ethanol/ammonium hydroxide (18:2:0.2) (BEA, non-polar). Aliquots of 10 μL (100μg) of the extracts were loaded with a micropipette on each of the three TLC plates. The developed chromatograms were air-dried in the fume cupboard and thereafter visualized under UV light (254 and 360 nm Camac Universal UV lamp). For further detection of chemical compounds, the plates were sprayed with a mixture of vanillin-sulfuric acid (0.1 g vanillin: 28 mL methanol: 1 mL sulfuric acid) and anisalde-

hyde mixture (0.5 mL anisaldehyde: 10 mL glacial acetic acid: 85 mL methanol: 5 ml sulfuric acid). The plates were heated at 110°C to optimal color development.

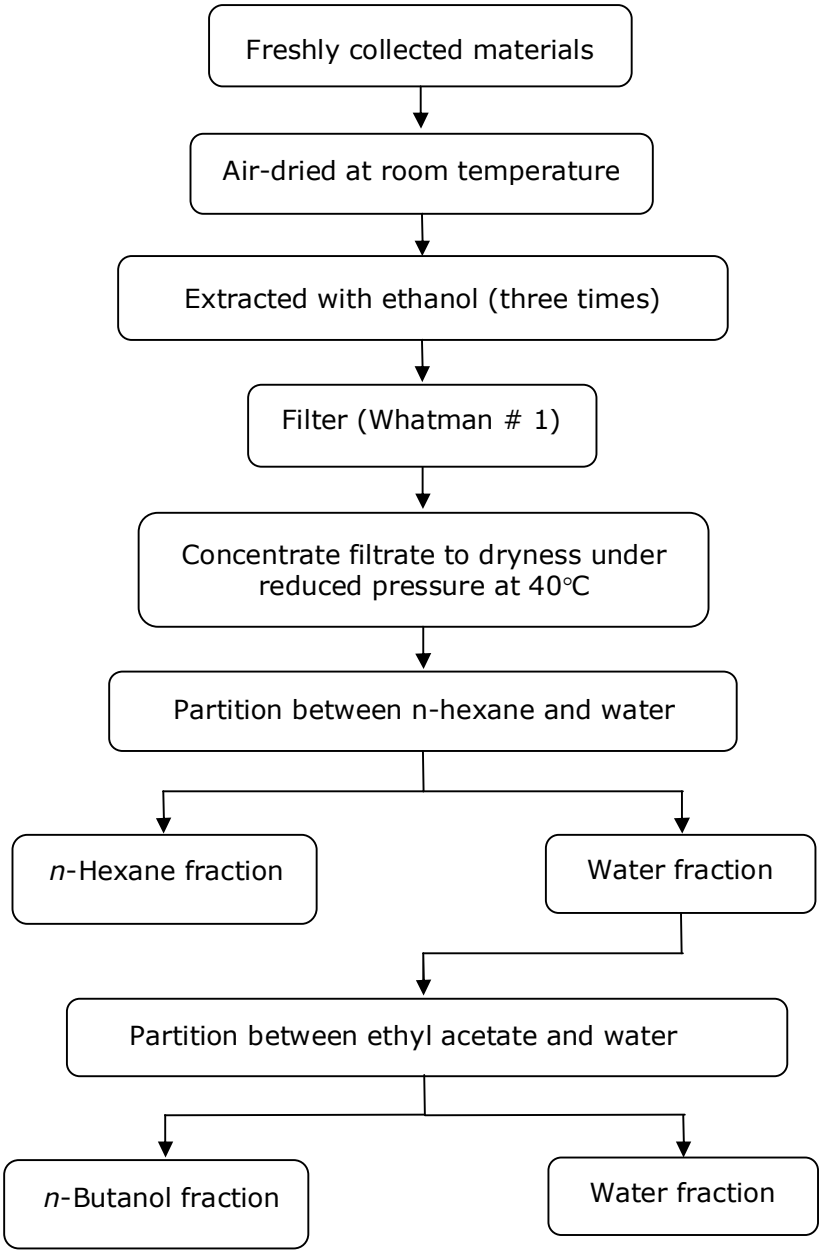


Figure 4: Extraction and Partitioning Method Employed for Isolation of Compounds from *Rosmarinus officinalis* L

J. BIOLOGICAL ACTIVITY METHODS

1. Bacterial cultures

The test bacteria used in this assay include *Staphylococcus aureus* (ATCC 29212) (Gram-positive) and *Escherichia coli* (ATCC 25922) (Gram-negative). Bacterial cultures were maintained on nutrient agar slants at refrigeration temperature until needed. These bacteria were separately inoculated in sterile Mueller-Hinton (MH) broth in 250-mL Erlenmeyer flasks and incubated at 37^{o}C for 16 hours.

2. Bioautographic assay of plant extracts

A modified autobiographic assay method (89BH203) was followed. A thin-layer chromatography (TLC) plate (Merck silica gel F_{254}) was spotted with 100 µg of each of the plant extracts (*n*-hexane, ethyl acetate, water and *n*-butanol fractions), and dried in a stream of air before developing in mobile phases of varying polarities (BEA, CEF, and EMW). Ten milliliters of a dense fresh bacterial suspension prepared above was added into two centrifuge tubes and centrifuged at 3500 rpm for 20 minutes to concentrate the bacteria. The supernatant was discarded and the pellet was re-suspended in 2-4 mL of fresh MH broth with a vortex shaker.

The chromatograms were then sprayed with the concentrated cultures of *the* bacteria until completely moist with the aid of a spraying gun. The plates were incubated at 37°C for 24 hours in a closed container, to which 20 mL of distilled water had been added to provide a humid environment, to encourage the

growth of the bacteria. At the end of the incubation period, the plates were sprayed with a 2 mg mL^{-1} solution of *p*-iodonitro-tetrazolium chloride and incubated for further 30 minutes. The inhibition of bacterial growth by the compounds on the TLC plates was observed as white spots against a deep-red background (Sigma-Aldrich, USA) (72JC182). The emergence of purple-red color resulting from the reduction of INT to its respective formazan was a positive indicator of cell viability. Clear zones indicated growth inhibition of the compounds present in the extract with specific R_F value.

3. Vacuum liquid chromatography (VLC)

The active *n*-hexane fraction (16 g) was obtained from the partitioning of the crude extract of *Rosmarinus officinalis* L. and adsorbed onto a small amount of silica gel, allowed to dry and applied uniformly on the top of the packed VLC column. Elution was carried out with solvents of increasing polarity, EtOAc in hexane (0-100%) and then methanol in EtOAc (0-15%) to yield 15 fractions (200 mL each). Each fraction was tested for activity. The two fractions obtained from 40% and 60% EtOAc in hexane showed antimicrobial activity and were further purified (VLC fractions A and B).

4. Purification of VLC fraction A

The fraction was concentrated to dryness (150 mg), dissolved in chloroform and applied to a Sephadex LH-20 column using chloroform as eluent. Thirty fractions of 5 mL each were collected. Fractions 19-28 were the same and showed antibacterial activity. These fractions were combined and concentrated

to dryness (42 mg), dissolved in chloroform and subjected to TLC, using 20% methanol in chloroform as the solvent. Spots of compounds were visualized under UV-light (254 and 366 nm). The plate was sprayed with *E. coli* for antibacterial activity and one spot was active. Preparative TLC plates were made and the active band was scraped from the plate and eluted with chloroform. After drying (22 mg), the sample was washed with methanol and the residue dissolved in chloroform to yield one pure compound (12 mg) (Figure 5). These samples were prepared in deuterated chloroform and analyzed by NMR on a Bruker AMX 400-MHz machine.

K. PHYTOCHEMICAL SCREENING AND POLYPHENOLIC ANTIOXIDANT ACTIVITY OF LEAF EXTRACTS OF *ROSMARINUS OFFICINALIS* L

Leaves of *Rosmarinus officinalis* were collected in June 2010 from the University of Fort Hare farm, Alice in Eastern Cape Province of South Africa. The plant materials were compared with voucher specimen earlier collected from same spot and deposited in the Griffin's herbarium of the plant science building of the University of Fort Hare in Alice. The plant materials were further confirmed by professor Don Grierson of the Botany Department. The leaves were separated carefully to remove all unwanted dirt and pulverized in a mill and then stored in an airtight container.

1. Preparation of extract

The prepared plant (200 g) was extracted in methanol and acetone on shaker (Stuart Scientific Orbital shaker, UK) for 48 hours. The extract was filtered

using a Buchner funnel and Whatman No. 1 filter paper to give 40 g (acetone) and 45 g (methanol) dry extracts, respectively. The resulting extracts were dissolved in distilled water to give desired concentration used in this study.

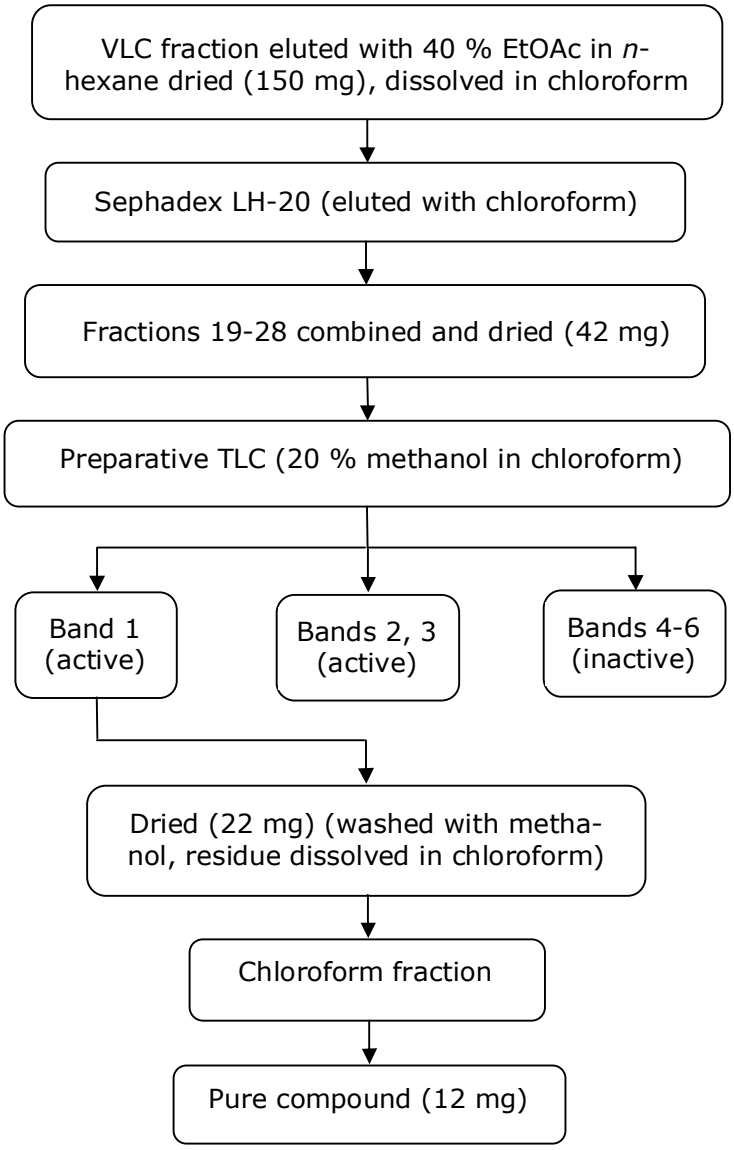


Figure 5: Isolation of a bioactive component from the leave extract of *Rosmarinus officinalis*

2. Chemicals

All chemicals used in this study were of the highest purity (>99.0%). Ferric chloride, HCl, Dragendorff s reagent, magnesium metal strips, methanol, gallic acid, commercial saponin were purchased from BDH, England; blood agar from Biolab, South Africa; and chloroform, H_2SO_4, Folin-Ciocalteu reagent, Na_2CO_3; Vanillin, aluminum chloride, potassium acetate, phosphate buffer, $K_3Fe(CN)_6$, trichloroacetic acid, 2-thiobarbituric acid (TBA), potassium thiocyanate, butylated hydroxyl toluene (BHT), 2, 2-diphenyl-1-Picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid, potassium persulfate, sodium nitroprusside, hydrogen peroxide, sulfanilic acid, glacial acetic acid, naphthylethylenediamine dichloride, potassium metabisulfite (PMS), NADH were all purchased from Merck, USA.

3. Phytochemical screening of the plant extract

A small portion of the dry extract was used for the phytochemical tests for compounds which include tannins, flavonoids, alkaloids, saponins, and steroids in accordance with the methods (98MI1, 08MI1) with little modifications.

4. Test for tannins

Exactly 1.0 g of plant extract was dissolved in 10 mL of distilled water and filtered (using Whatman No 1 filter paper). A blue coloration resulting from the addition of ferric chloride reagent to the filtrate indicated the presence of tannins in the extract.

5. Test for alkaloids

Exactly 0.5 g of the plant extract was dissolved in 5 mL of 1% HC1 on steam bath. A milliliter of the filtrate was treated with few drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloid.

6. Test for flavonoids

About 0.2 g of the extract was dissolved in 2 mL of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange coloration was indicative of the flavonoids.

7. Test for saponins

Freshly prepared 7% blood agar plate was used and wells were made in it. The extract in methanol was applied with distilled water and methanol used as negative control while commercial saponin solution was used as a positive control. The plates were incubated at 35°C for 6 h. Complete haemolysis of the blood around the extract was indicative of the presence of saponin.

8. Test for steroids

About 0.5 g of the extract was dissolved in 3 mL of chloroform and filtered. Concentrated H_2SO_4 was carefully added to the filtrate to form lower layer. A reddish-brown color at the interface was taken as positive for steroid ring.

9. Determination of total phenolic composition

The amount of phenolic compound in the aqueous leaf extract of *Rosmarinus officinalis* was determined with Folin-Ciocalteu reagent using the method (90JAF1565) further modified (05FCT(90)891, 08ABT1891). To 0.5 mL of each sample (three replicates) of plant extract solution (1 mg/mL) was added 2.5 mL of 10 % Folin-Ciocalteu reagent and 2 mL of Na_2CO_3 (2% w/v). The resulting mixture was incubated at 45°C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Results were expressed as milligrams of gallic acid (0-0.5 mg/mL) dissolved in distilled water.

10. Estimation of total flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination. One milliliter (1 mL) of sample was mixed with 3 mL of methanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water and allowed to remain at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with UV-visible spectrophotometer. The content was determined from extrapolation of calibration curve which was prepared using gallic acid solution (0-0.8 mg/mL) in distilled water. The concentration of flavonoid was expressed in terms of mg/mL.

11. Determination of total proanthocyanidins

Total proanthocyanidins was determined based on the procedure (98BUR225). The mixture of 3 mL of vanillin-methanol (4% v/v), 1.5 mL of hy-

drochloric acid was added to 0.5 mL (1 mg/mL) of aqueous extract and vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidin content was expressed as gallic acid equivalent (mg/mL) from the standard curve.

12. Determination of reducing power

The reducing power of the extract was evaluated according to the method (86JN307). The mixture containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$ (1%w/v) was added to 1.0 mL of the extract dissolved in distilled water. The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 mL of TCA (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 ml of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700 nm against blank sample.

L. Antioxidant assay of plant extracts

The antioxidant activity of the aqueous plant extract was determined using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. The FTC method was used to measure the amount of peroxide at the beginning of peroxidation while TBA method was used to measure free radicals present after peroxide oxidation.

1. Ferric thiocyanate (FTC) method

The standard method (91CPB120) was used for the FTC determination. The absorbance of the resulting mixture (red color) was measured at 500 nm every 24 h until the absorbance of the control reached its maximum. Butylated hydroxyl toluene (BHT) was used as positive controls. The mixture without the plant extract was used as the negative control.

2. Thiobarbituric acid (TBA) method

The method (59ABB355) modified (93JFI1407) was used for the determination of free radicals present after peroxide oxidation of aqueous leaf extract. The final sample concentration of 0.02% (w/v) from the same samples prepared for FTC assay was used. Two milliliters of 20 % trichloroacetic acid and 2 mL of 0.67% thiobarbituric acid were added to 1 mL of sample solution followed the FTC method. The mixture was placed in a boiling water bath for 10 min and then centrifuged after cooling at 3000 rpm for 20 min. The absorbance activity of the supernatant was measured at 552 nm and recorded after it has reached its maximum.

3. 2, 2'-Aazino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) scavenging activity

The method of Re *et al.* (99FRB1231) was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS solution and 2.4 mM potassium persulfate solution in equal amount and allowed to react for 12 h at room temperature in

the dark. The resulting solution was later diluted by mixing 1 mL of freshly prepared $ABTS^+$ solution followed by the measurement of absorbance at 734 nm after 7 min. The percentage of scavenging inhibition capacity of $ABTS^+$ of the extract was calculated and compared with butylated hydroxytoluene (BHT).

4. Scavenging activity of nitric oxide

The method (64MI1) was adopted to determine the nitric oxide radical scavenging activity of methanol and acetone extracts of *Rosmarinus officinalis*. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions determined by the use of Griess reagents. To 2 mL of 10 mM sodium nitroprusside dissolved in 0.5 mL phosphate buffer (pH 7.4) was added 0.5 mL of plant extract at various concentrations (0.025-0.5 mg/mL). The mixture was incubated at 25°C. After 150 min, 0.5 mL of incubation solution was withdrawn and mixed with 0.5 mL of Griess reagent: 1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthylethylenediamine dichloride (0.1% w/v)). The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. The amount of nitric oxide radical was calculated following the equation:

$$\% \text{ NO inhibition} = \frac{A_{\square} - A_{\square}}{A_{\square}} \times 100$$

Here A_o is the absorbance before reaction and A_i is the absorbance after the reaction has taken place. The percentage of scavenging inhibition capacity of

NO of the extracts was calculated and compared using butylated hydroxytoluene (BHT) and rutin as standards.

5. Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was determined by the method (89CAR1003). Plant extract (4 mL) prepared in distilled water at various concentrations was mixed with 0.6 mL of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the plant extract without H_2O_2. The percentage of scavenging inhibition capacity of hydrogen peroxide of the extracts was calculated using tannic acid as reference standard.

M. SPECTROSCOPIC TECHNIQUES

Spectroscopy is the study of the interaction of electromagnetic radiation (EMR) with the matter. Nuclear magnetic resonance (NMR) is the study of interaction of radio frequency (RF) of the EMR with unpaired spins in an external magnetic field. Such interactions are important and lead to structural information of a compound under investigation. NMR spectroscopy is the most useful analytical tool available to the chemist. It is routinely used to study the chemical structure of simple molecules by using one-dimensional technique (1D-NMR), compounds that are more complicated require the use of two-dimensional technique (2D-NMR).

1D-NMR is used to quantify the number of protons that are present in a compound of interest. Proton NMR (^{1}H-NMR) is a plot of signals arising from absorption of RF during an NMR experiment by the different protons in a compound. The area under the plots provides information about the number of protons present in the molecule (88MI1). The position of a proton signal reveals information regarding the chemical and electronic environment of the proton and the spinning pattern provides information about the number of neighboring protons (88MI2). Similar to proton NMR carbon NMR (^{13}C- NMR) is a plot of signals arising from the different carbons as a function of chemical shift. The signals in ^{13}C-NMR experiments normally appear as singlet because of the decoupling of the attached protons. The range of the chemical shift values differs between the ^{1}H (normally 0 to 10) and ^{13}C- NMR (normally 0 to 230) that arise from the two nuclei having different numbers of electrons around their corresponding nuclei as well as different electronic configuration (88MI1).

2D-NMR experiments are concerned with the structural elucidation of natural products and include homonuclear ^{1}H, ^{1}H- correlated spectroscopy (COSY) and ^{1}H, ^{13}C-heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC). COSY is a plot that shows coupling among neighboring protons. It provides information on the connectivity of the different groups within the molecule (87MI1). HMQC experiments provide correlation between proton and their attached heteronuclei through the heteronuclear scalar coupling. This experiment is used to eliminate protons signals not coupled with

the heteronuclei. HMQC further provides information regarding the number and chemical shifts of methyl, methylene and methine groups (88MRC501). HMBC experiment on the other hand, detects long-range coupling between proton and carbon (two or three bonds away). The HMBC, in conjunction with COSY thus enables the elucidation of the skeleton of the compound.

1. Nuclear magnetic resonance

Vacuum liquid chromatography (VLC) and column chromatography (CC) experiments were performed using silica gel 60 (particle size 0.063-0.200 mm, Merck). Preparative TLC was carried out using silica gel 60 $PF_{(254+366)}$ precoated glass plates (Merck); analytical TLC performed on silica gel 60 PF_{254} precoated alumina sheets (Merck); visualization of compounds was done under UV-lamp (254 and 266nm) and also using vanillin-sulfuric acid and anisaldehyde spray.

The ^{1}H-NMR, ^{13}C-NMR, COSY, DEPT, HMQC, HMBC spectra (in deuterated chloroform) were obtained on a Bruker Avance DPX 400 (400 MHz); melting points were recorded on Stuart scientific (SMPI) apparatus. Compounds were weighed (20 mg) and dissolved in deuterated chloroform (Merck) as a reference signal to a final volume of 2 mL. The mixture was transferred to NMR tubes (Milmad, Economy) and sent to the Department of Chemistry, Rhodes University for analysis.

2. Mass-spectroscopy

The isolated compounds were further analyzed by mass spectroscopy (MS) at Stellenbosch University. Antibacterial bioautographic assay-guided phytochemical investigation of the *n*-hexane fraction of *Rosmarinus officinalis* led to the isolation of a compound. The compound was identified by spectroscopic data (IR, ^{1}H-NMR, ^{13}C-NMR, COSY, DEPT, HMQC and HMBC) measurement and by comparison with literature.

3. Infrared spectroscopy

IR-spectroscopy deals with the infra red region of the electromagnetic spectrum, that is light with a longer wavelength and lower frequency than visible light. It covers a range of techniques, mostly based on absorption spectroscopy. As with all spectroscopic techniques, it can be used to identify and study chemicals. A common laboratory instrument that uses this technique is a Fourier-transform infra red (FTIR) spectrometer.

The infrared portion of the electromagnetic spectrum is usually divided into three regions; the near-, mid- and far- infrared, named for their relation to the visible spectrum. The higher energy near-IR, approximately 14000–4000 cm^{-1} (0.8–2.5 μm wavelength) can excite overtones or harmonic vibrations. The mid-infrared, approximately 4000–400 cm^{-1} (2.5–25 μm) may be used to study the fundamental vibrations and associated rotational-vibrational structure. The far-infrared, approximately 400–10 cm^{-1} (25–1000 μm), lying adjacent to the microwave region, has low energy, and may be used for rotational spectrosco-

py. The names and classifications of these sub-regions are conventions, and are only loosely based on the relative molecular or electromagnetic properties.

IV. Results and Discussion

A. Essential oil yield

The hydrodistillation and solvent-free microwave extraction of essential oils of *Rosmarinus officinalis* gave yellowish oils with yields in the range from 0.1% to 1.82%. The yield of the oil was affected by seasonal changes as shown in Fig. 6. Generally, the harvest time and seasons have influence on the total yield and compositions of the essential oils of the plant. Throughout the period of our observation, the main components in the oils were 1,8-cineole, α-pinene, camphor, verbenone, borneol, bornyl acetate and camphene. The composition of the rosemary extracts was qualitatively similar to those obtained by other authors (93JEP167, 05FT450, 05JSF(36)40, 05FC(91)621, 07FC(102)898), but with different quantitative composition. However, differences in rosemary oil composition have already been reported (05FC(91)621).These differences in the chemical composition of the oils could be attributed to climatic effects on the plants (07FC(102)898). Factors to be considered when observing differences in essential oil components include: genotype and environmental differences of the species, sample extraction time and the extraction technique used to obtain the rosemary oil or extract. The highest oil yields were obtained in November (spring), with 1.76 % and 1.82 % from hydrodistllation and solvent-free microwave extraction, respectively, while the lowest oil yields were obtained in January (summer), HD (0.10 %) and SFME(0.19 %). The high essential oil yield in spring may be as a result of the high rainfall compared to

other seasons. The low yield in summer may be as a result of the water deficit in the plants during these season; water deficit in plants may lead to physiological disorders, such as reduction in photosynthesis and transpiration which may cause changes in the yield and composition of their essential oils (05SH387). Water deficit in aromatic plants such as *Rosmarinus officinalis* L. decreased the essential oil yield (00JMA659) as was observed in our studies.

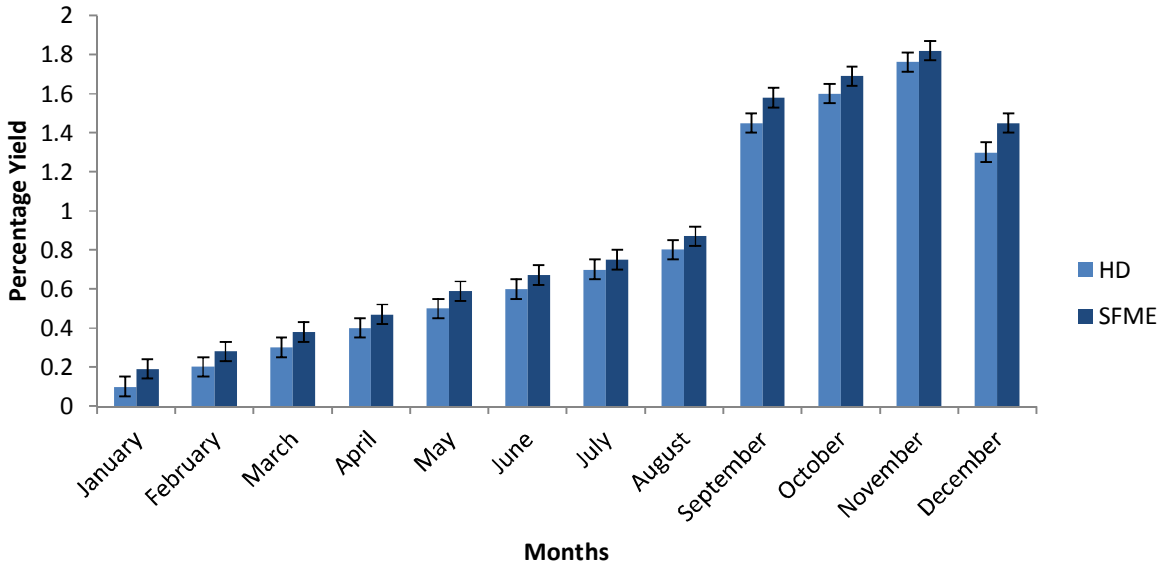


Figure 6: Yields of Essential Oils from Fresh Leaves of *Rosmarinus officinalis* L. Collected Monthly Using HD and SFME from January 2009 through December 2009

B. IDENTIFICATION AND QUANTIFICATION OF *ROSMARINUS OFFICINALIS* ESSENTIAL OIL

A total of 23 compounds were identified accounting for 99.60% of the volatile constituents in the leaves (Table 2). Oxygenated monoterpenes were the dominant compounds comprising 66.61-70.01% of the oil, and they consisted mainly of camphor (10.02-20.60%), borneol (5.74-16.31%), verbenone

(11.04-23.76%), geraniol (0.13-0.70%), linalool (1.18-7.96%) and 1,8-cineole (7.05–17.10%). The percentage of monoterpene hydrocarbons was 33.49-49.65% and these consist mainly of α-pinene (7.83-19.20%), camphene (3.13-13.53%), myrcene (0.01-1.80%), and α-phellandrene (0.31-0.59%). The percentage of sesquiterpene hydrocarbons was 0.73-0.86% and the major constituent were β-caryophyllene (0.43-1.91), α-humulene (0.30-0.89%). The percentage of oxygenated sesquiterpene was (0.26-0.32%) and it consists of caryophyllene oxide (0.01–0.58%). Another major oxygenated monoterpene compound is bornyl acetate (5.01–15.85%).

C. MONTHLY VARIABILITY OF THE MAJOR COMPONENTS

The results show that the content of the essential oil varied throughout the months and seasons (Table 5, 6). The major components of the oils were oxygenated monoterpenes which comprise mainly of α-pinene, 1,8-cineole, verbenone, camphor, camphene, borneol, and bornyl acetate. The composition of 1,8-cineole extracted using solvent-free microwave extraction method revealed that highest composition of this compound was obtained from plants harvested in December (18.64 %) while the lowest was observed from the oils harvested in August (5.23 %). However, there were little differences in the percentage composition of the 1,8-cineole extracted from plants harvested in the other months using hydrodistillation (Table 5, 6). The relative high composition of the compound in the essential oil of *Rosmarinus officinalis* throughout the year may be a measure of good quality in the commercial sector. 1,8-Cineole is a well

known remedy for the discomfort of bruises, sprains and pulled muscles, because it stimulates blood circulation near the point of application, recent clinical research has demonstrated 1,8-cineole's effectiveness in reducing inflammation and pains and in promoting leukemia cell death (06ABT1890).

There was appreciable variation in the percentage composition of α-pinene with respect to the month of harvest using hydrodistillation and microwave extraction (Table 5, 6). It increased sharply in the months of April and September. However, the percentage of essential oil obtained by the SFME method was generally lower than the oils extracted by HD, with both methods of extraction showing opposite monthly variations. α- pinene is a bicyclic monoterpene. It is used against rheumatism but is best used by aromatherapists and as a tonic of the mucus membrane of the respiratory system. Pinene has pleasant fragrance and is believed to have diuretic properties (94MI3).

Highest composition of camphor was obtained from the oils of plant harvested in August (21.48 %) and the lowest was obtained in April (15.68 %). Again, there was appreciable fluctuation in the composition of the compound throughout the sampling period. The lowest percentage composition was obtained in April in both methods of extraction. Camphor is a waxy, white or transparent solid with a strong, aromatic odor. It is a terpenoid and is found in wood of camphor laurel and used as insect repellant (94MI3).

Similar trend was obtained in the percentage composition of verbenone, camphene, and bornyl acetate throughout the sampling period. Verbenone was

another major compound of the essential oils extracted from *Rosmarinus officinalis* using HD and SFME in this study. Verbenone is used for insect control and because of its pleasant aroma, essential oils rich in verbenone are used in perfumery, aromatherapy, herbal teas, spices and herbal remedies; it also has antimicrobial properties (05JFP790).

Bornyl acetate is an oxygenated monoterpene. It was one of the major compounds found in the esential oils of *Rosmarinus officinalis* in this study. Bornyl acetate soothes and relieves pain (anodyne) and also releases or eases muscular spasms, cramps or convulsions (95FT291).

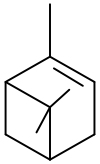
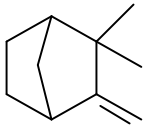
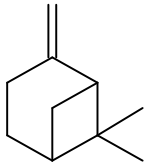
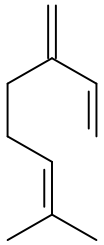
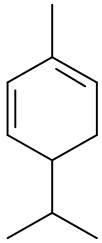
Camphene is used widely in the synthesis of camphor, perfumes and pesticides. It is a terpene that repels mammalian predators and generally repellent to insect pests (00ABR77).

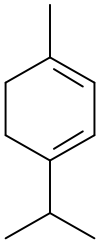
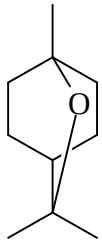
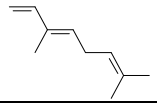
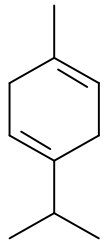
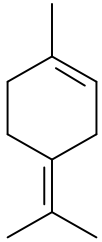
The month of January falls in the summer period in South Africa. Low essential oil yield in summer might be attributed to the high temperature and partial evaporation of some constituents of the oil. Our results were in agreement with the conclusion that growing season has a major effect on the essential oil yield in *Rosmarinus officinalis* (00JHS520). The leaves of lemon in Pakistan exhibited maximum essential oil yield in November, which falls in spring (02JAF147). The leaves of lemon exhibited maximum essential oil yield during November (autumn). Also, there is considerable difference in the essential oil contents of *Mentha spicata* and *Mentha pulegum* leaves with respect to seasonal variations, respectively (97JAF4831, 06JEO469, 09AJP217).

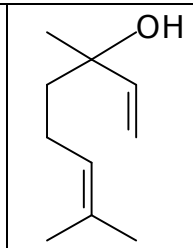
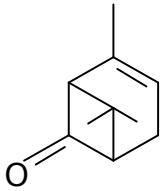
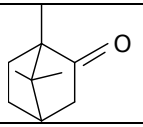
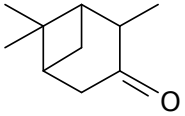
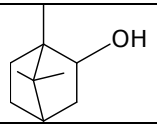
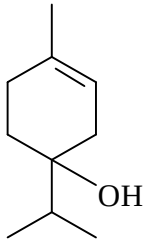
A substantial variation in the yield of essential oil from the aerial parts of *Artemisia verlotiorum* was also reported throughout the year (04JEP263). Some other studies in the literature revealed that plants exhibited remarkable fluctuation in essential oil contents with the progress of the seasons (07SAB441).

From the chemical viewpoint, the results of seasonal variations of the composition of essential oils are especially interesting. Observations of the hydrodistilled essential oils (Table 5) are more objective and therefore we first concentrate on them. Generally one can notice that the overall composition of the essential oil undergoes drastic changes throughout the year being α-pinene-cineole type in April and verbenone-camphor type in August. This change in chemotype is parallel to the climatic changes in Eastern Cape. In the end of summer – beginning of autumn weather is hot, windy, and dry. As a result, the amount of the products of hydrolysis (camphor, bornyl acetate, bornyl alcohol) and oxidation (verbenone) decreases and essential oils of poor quality with a high content of minor and unidentified compounds are at their maximum. As autumn proceeds, temperatures become comfortable for life support, water practically does not evaporate and readily available for hydrolysis, oxygen content in air becomes optimal (with a light content of ozone), and essential oils in August contain maximum amounts of most useful components listed above.

Table 5: Monthly Chemical Composition (%) of the Essential Oils from *Rosmarinus officinalis* (Hydrodistillation)

Compound	Structure	Jan	Feb	Mar	Apr	May	June	July	Aug	Sep	Oct	Nov	Dec
α-Pinene		11.73	12.23	13.32	21.93	13.28	11.56	7.20	14.13	12.73	12.61	12.07	12.60
Camphene		5.70	6.34	7.94	9.93	7.71	3.48	3.39	6.35	5.85	4.87	5.81	4.83
β-Pinene		1.12	1.57	1.69	1.80	1.50	6.22	1.12	1.51	2.42	1.94	2.09	1.29
β-Myrcene		1.25	1.48	1.55	2.03	1.58	0.26	0.92	1.04	1.11	0.91	1.40	1.14
α-Phellandrene		0.41	0.43	0.49	0.59	0.34	-	0.22	-	0.27	-	0.36	0.44

α-Terpinene		0.65	0.60	0.70	0.82	0.65	-	0.28	0.37	0.44	0.37	0.55	0.49
1,8-Cineole		11.91	15.43	15.84	16.35	13.51	12.66	14.49	15.51	14.79	12.78	13.66	13.65
trans-β-Ocimene		0.11	0.26	-	0.35	-	-	-	-	-	-	-	0.05
γ-Terpinene		0.98	0.93	1.13	1.40	0.94	4.26	0.60	0.65	1.04	0.84	1.23	0.90
Terpinolene		1.23	1.26	1.37	1.48	1.15	3.27	0.59	0.71	0.97	0.83	1.31	0.93

Linalool		2.02	2.91	2.25	1.49	3.03	-	2.08	1.53	1.94	1.93	3.39	1.24
Chrysan-thenone		0.51	0.71	0.72	0.13	0.64	1.91	0.76	0.37	0.42	0.44	0.46	-
Camphor		16.57	16.99	18.02	12.43	16.23	21.28	24.51	19.08	17.51	18.25	18.05	19.94
Bicy-clo[3.1.1] heptan-3-one		2.46	2.56	2.66	1.64	1.36	-	2.07	1.66	1.64	2.97	-	2.12
Bornyl alco-hol		5.74	6.74	7.66	4.19	7.54	3.43	7.82	7.46	7.42	5.59	8.01	4.07
Terpinen-4-ol		1.42	1.61	1.81	1.07	1.44		1.49	1.28	1.26		1.93	1.38

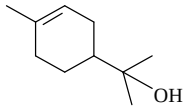
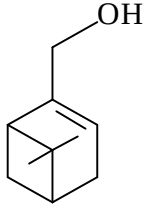
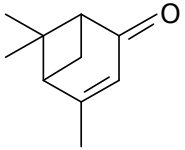
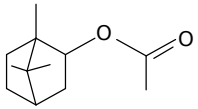
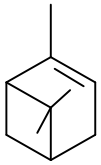
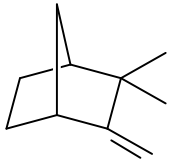
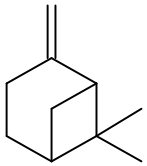
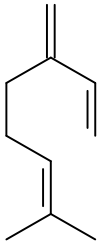
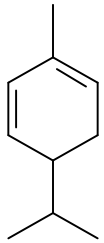
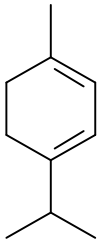
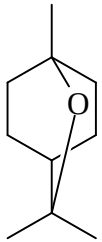
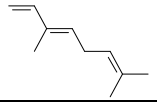
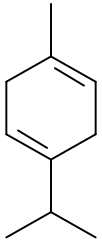
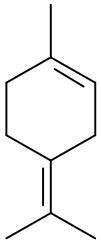
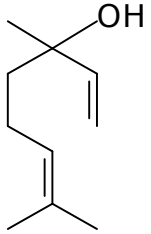
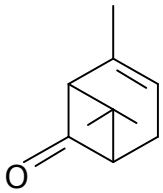
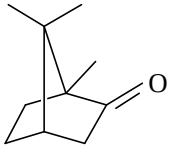
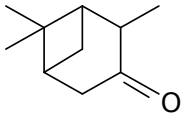
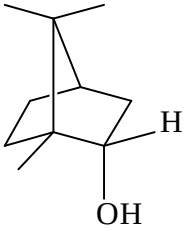
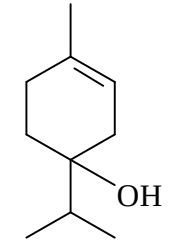
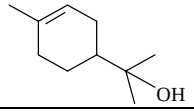
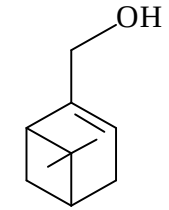
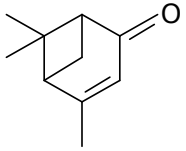
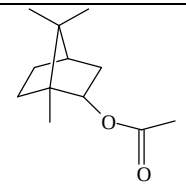
α-Terpineol		2.00	2.48	2.50	1.44	2.12		1.87	1.66	1.60	1.83	2.30	1.67
Myrtenol		1.37	1.32	1.12	1.11	2.04		1.76	1.31	0.84	0.34		0.57
Verbenone		17.43	15.05	12.66	10.15	13.60	14.41	11.94	12.14	13.97	19.42	14.76	18.77
Bornyl acetate		9.19	6.51	6.00	5.80	8.55	9.05	9.30	10.19	12.09	13.09	10.21	10.37
Minor or non-identified components	Total	6.30	3.21	0.57	3.87	2.79	8.21	7.59	3.05	1.69	0.99	2.41	3.55

Table 6: Monthly Chemical Composition (%) of the Essential Oils from *Rosmarinus officinalis* (Microwave Distillation)

Compound	Structure	Jan	Feb	Mar	Apr	May	June	July	Aug	Sep	Oct	Nov	Dec
α-Pinene		8.14	7.83	7.65	7.19	7.16	6.46	3.96	9.35	16.03	3.31	8.36	8.92
Camphene		4.19	3.93	4.07	5.72	3.43	5.49	2.81	3.10	6.01	2.62	3.54	2.53
β-Pinene		1.06	0.74	1.11	2.14	0.65	1.70	0.41	1.75	3.20	0.97	1.56	1.25
β-Myrcene		1.18	0.84	1.91	3.95	0.71	2.70	0.41	1.75	1.45	0.56	0.65	0.80

α-Phellandrene		0.32	-	0.56	-	-	0.74	0.11	0.38	-	-	-	0.31
α-Terpinene		0.43	-	0.45	0.77	0.28	0.37	0.14	0.20	0.43	0.17	-	0.45
1,8-Cineole		10.56	9.78	9.55	9.45	9.19	9.05	7.14	5.23	18.64	7.32	8.08	12.95
trans-β-Ocimene		-	-	0.32	0.25	0.64	0.28	0.05	0.10	-	-	-	-
γ-Terpinene		1.06	0.64	0.99	1.62	0.56	1.36	0.30	0.30	1.16	0.51	0.54	1.08

Terpinolene		1.32	0.88	1.34	2.41	0.88	1.87	0.41	0.40	1.04	0.58	0.55	1.25
Linalool		3.00	2.03	1.44	4.34	2.28	0.44	1.61	1.86	1.28	2.19	1.73	2.49
Chrysan-thenone		1.15	0.67	1.11	1.85	1.15	1.29	0.75	1.91	0.80	0.68	0.57	0.82
Camphor		16.89	19.72	17.56	15.68	19.39	19.41	19.08	21.48	16.86	17.49	16.22	18.89
Bicy-clo[3.1.1] heptan-3-one		2.74	2.50	1.67	1.03	0.97	-	1.82	1.75	1.81	1.78	3.55	2.87

Bornyl alcohol		5.86	7.69	9.64	10.65	9.19	15.11	7.70	8.10	4.48	6.27	4.86	0.12
Terpinen-4-ol		1.56	1.54	1.52	-	1.49	-	1.40	1.50	0.77	1.18	-	1.56
α-Terpineol		2.26	2.04	2.00	-	2.63	-	2.31	1.75	0.95	2.04	2.57	-
Myrtenol		1.09	1.56	1.68	-	2.21	2.01	2.13	1.75	0.61	1.16	0.34	0.07
Verbenone		21.80	23.92	24.66	11.68	21.68	12.33	26.24	22.68	12.60	27.49	26.56	21.22
Bornyl acetate		11.62	10.54	10.11	7.18	12.15	8.93	14.93	11.64	10.94	14.64	16.66	11.08

Minor or non-identified components	Total	3.77	3.15	0.66	14.09	3.37	10.46	6.29	3.02	0.94	9.04	3.66	11.34

It is thus highly recommended to collect essential oils of *Rosmarinus officinalis* in August when the plant offers optimal composition in terms of its antimicrobial, antibacterial, antioxidant, and other useful properties.

Kinetic analysis of the data presented is senseless because of complexity of the system and natural conditions of its existence. However, some kinetic estimates of disappearance of α-pinene could give some insight into the direction and nature of the occurring chemical transformations. Therefore, reaction rates were estimated using graphical differentiation of kinetic curves by writing a subroutine in MATHCAD programming language. Then logarithms of the rates were arranged against logarithms of the respective α-pinene concentrations and fitted to the best linear dependence using linear regression approach within the same software package. The slopes of such linear functions are partial reaction orders. The data for other components are also treated but give little information, because partial reaction orders are inevitably close to unity and well fit to the predominating hydrolysis processes. α-Pinene, however, may enter isomerization reaction with the expected zero order, and the data for this component may be quite instructive.

Numerical material of Table 5 may best of all be described in the form of the scheme of chemical transformations depicted in Fig. 7. Detailed inspection of the table reveals the following trends.

a. As α-pinene isomerizes, concentration of β-pinene decreases due to its further consumption. As α-pinene oxidizes to verbenone, β-pinene may accu-

mulate up to substantial, anomally high, levels (6.22% in June). Verbenone is at minimum in April and maximum in June and towards the end of the year. Increase of the α-pinene content occurs at the expense of 1,8-cineole or switching-off the isomerization route in certain seasons. Indeed, 1,8-cineole has a maximum content in April and minimum in June. Camphene follows the trends of α-pinene reaching its maximum in April and minimum in July. In contrast, camphor keeps minimum levels in April and maximum in July.

b. Bornyl acetate is at minimum in April and rapidly grows starting from August. Bornyl acetate may block formation of borneol and camphor when conditions for hydrolysis are retarded. Thus borneol and α-terpineol have minimum content in April.

c. The content of α-pinene is maximum in April, minimum in July, and in this case the partial reaction order of pinene is 1.14 (close to one) with correlation coefficient of the "ln v *vs*. ln [A]" linear dependence (correlation coefficient 0.963), which confirms predomination of the hydrolysis pathway (expected partial reaction order is strictly one) and oxidation reactions. For oxidation, a complex mechanism is expected, which can lead to the fractional partial reaction order for α-pinene.

d. When α-pinene starts its second descend in concentration, this time the partial reaction order is estimated at 0.88 (correlation coefficient 0.971), which

is readily interpreted as a combination of the isomerization (expected zero order) and hydrolytic (expected first order) reactions.

During the favorable season late summer - early autumn, α-pinene takes the ring-expansion and oxidation routes, and essential oils are enriched with especially valuable components and acquire optimal properties making the plant of applied interest. In the hot, windy, and dry month of April, predominant is the isomerization route, which does not spoil the plant but makes it less attractive. The results for this month reveal a number of foreign compounds contaminating the plant, which is especially seen from microwave results briefly discussed below. Otherwise, *Rosmarinus officinalis* has a balanced composition of basically α-pinene, 1,8-cineole, camphor, verbenone, bornyl alcohol, and bornyl acetate.

Microwave data show that strong preference is given to the extraction of components with functional carbonyl, carboxyl, and hydroxyl groups, perhaps due to stronger interaction of the oxygen lone pairs in such groups with microwave dipoles (Table 6). Such an interaction is much weaker for non-oxygenated terpenes even containing double bonds and for 1,8-cineole containing an oxygen atom of the ether type. These components seem underextracted. On the other hand, trends for camphor, verbenone, bornyl alcohol, and bornyl acetate are normal and correspond to those obtained by hydrodistillation method. Another feature is sometimes a substantial content of numerous minor and unidentified components, normally polar, and influencing the overall picture. However, during favorable season, essential oils are pure and of high quality. Due

to heavier presence of valuable components during the whole year, oils obtained by microwave method would be of better value.

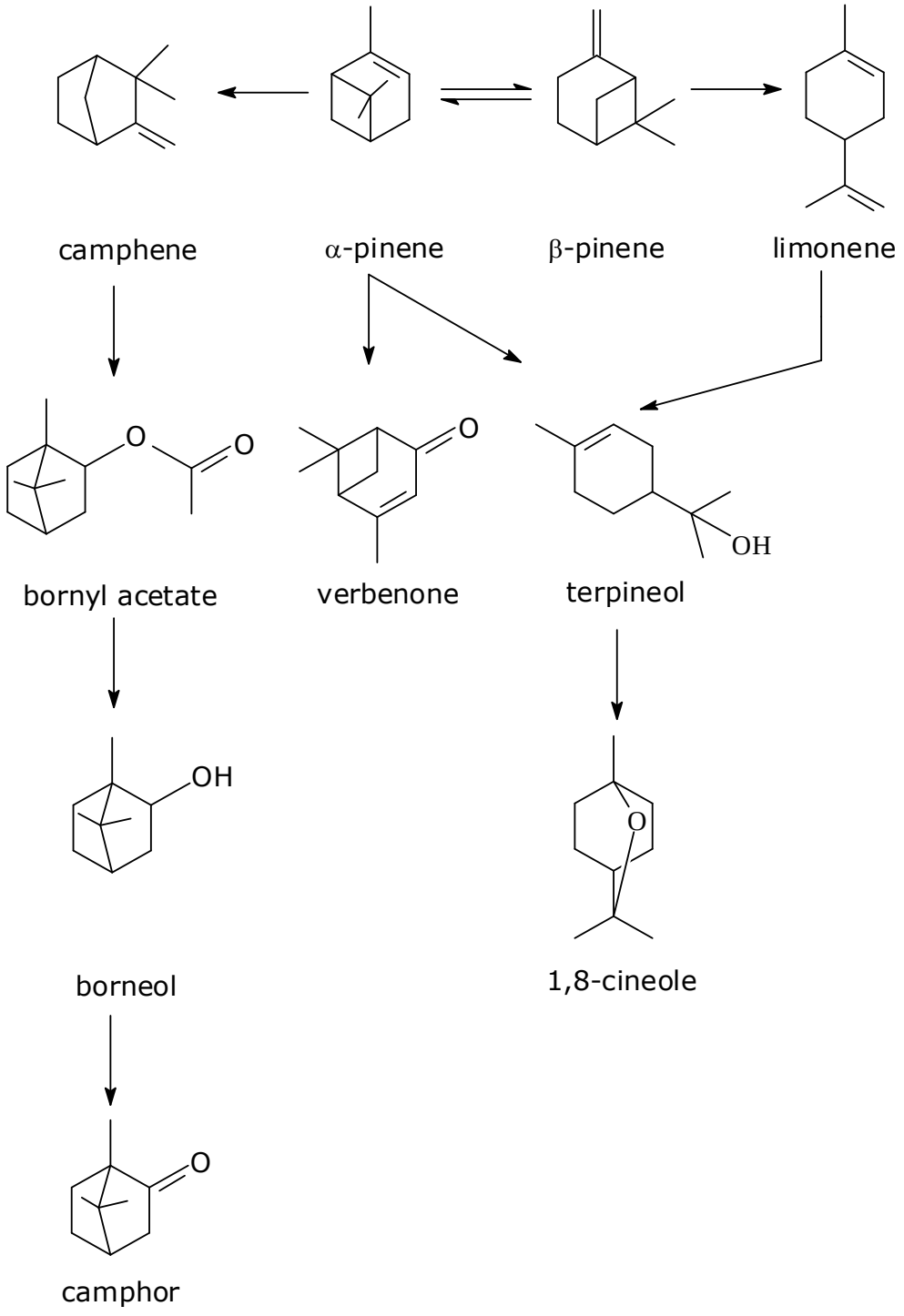


Figure 7: Possible Chemical Transformations in *Rosmarinus officinalis*

D. COMPARATIVE EVALUATION OF ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS EXTRACTED BY HYDRODISTILLATION AND SOLVENT-FREE MICROWAVE EXTRACTION

Generally, essential oils of *Rosmarinus officinalis* have shown broad spectra of activity against the tested microorganisms. The antimicrobial property of the essential oil of *Rosmarinus officinalis* can be attributed to the presence of α-pinene, 1,8-cineole, camphor, verbenone, and borneol with borneol being the most potent followed by camphor and verbenone (05JFP790). The quantities of these compounds were very high in our oils. The essential oils of *Rosmarinus officinalis* L. harvested in February, 2010 were collected, extracted using SFME and hydrodistillation methods and analyzed using GCMS were screened against two Gram-positive (*Staphylococcus aureus*, and *Bacillus subtilis*) and two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria strains.

The results of the effect of the essential oil from *Rosmarinus officinalis* obtained through HD and SFME on tested bacterial strains are shown in Table 7. The oils inhibited the growth of both the Gram-positive and Gram-negative bacteria at MIC values ranging between 0.23 mg mL^{-1} and 7.5 mg mL^{-1} (Table 8). There was, however, more activity on Gram-positive bacteria than Gram-negative bacteria. This result was in agreement with many other studies reported on the other plant species (02JEP51). The higher resistance among Gram negative bacteria than Gram positive bacteria could be due to the differences in the cell membranes of these bacterial groups. Indeed, the external membrane of Gram negative bacteria renders their surfaces highly hydrophobic

(98LAM118), whereas the lipophilic ends of the lipoteichoic acids of the cell membrane of Gram positive bacteria may facilitate penetration by hydrophobic compounds (99AEM4606, 00JAM170).

Table 7

Antibacterial Activities of Essential Oil of Rosemary Obtained by Hydrodistillation and Microwave Distillation Methods

Test Bacteria	Antibacterial activity of oil	
	Hydrodistillation	Microwave distillation
Escherichia coli ATCC 8739	+	+
Staphylococcus aureus ATCC 6538	+	+
Streptococcus feacalis ATCC 29212	-	-
Bacillus cereus ATCC 10702	-	+
Klebsiella pneumonia ATCC 10031	+	+
Proteus vulgaris ATCC 6830	-	-
Bacillus subtilis (LIO)	+	+
Salmonella sp. (LIO)	-	-
Micrococcus kristinae (LIO)	-	-

Table 8

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Profiles of the Essential Oils of Rosemary Against Four Susceptible Bacteria

Test Bacteria	**MIC and MBC (in parenthesis) (mg/ml)**	
	Hydrodistillation	**Microwave distillation**
Escherichia coli ATCC 8739	7.5 (7.5)	1.88 (7.5)
Staphylococcus aureus ATCC 6538	3.75 (7.5)	0.47 (1.88)
Klebsiella pneumonia ATCC 10031	0.94 (7.5)	0.23 (7.5)
Bacillus subtilis (LIO)	1.88 (7.5)	1.88 (7.5)

Klebsiella pneumoniae was more susceptible to the essential oil obtained by SFME (0.23 mg mL^{-1}) than the oil obtained by HD. Generally, the essential oil obtained by SFME showed more activity than HD oil against *Escherichia coli* and *Staphylococcus aureus*. The lowest MBC value was obtained from the SFME oil against *Staphylococcus aureus* while the same values were observed for *Bacillus cereus, Escherichia coli* and *Klebsiella pneumoniae.* However, the essential oil of *Rosmarinus officinalis* has been reported to be weakly inhibitory against *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes* as compared to other oils (05JAF6939). In addition, oxygenated sesquiterpenes such as caryophyllene oxide, α-humulene, and pentasiloxane were not found in our hydrodistilled essential oil.

Monoterpene hydrocarbons are less valuable than oxygenated compounds in terms of their contribution to the fragrance of the essential oil, oxygenated compounds being highly odoriferous and, hence, most valuable, and we propose that this could explain why we have more antibacterial activities in SFME than HD essential oil in line with the suggestion (09FC355).

The essential oils of this plant show that it has monoterpenes such as α-pinene, β-pinene, myrcene 1,8-cineole, and borneol as the major components. These compounds possess strong antibacterial and antimicrobial activities (03PR1005, 08FCT346). These chemical components exert their antimicrobial activity on microorganisms through the disruption of bacteria membrane integrity (89JEO119). Another important characteristic of essential oils is their hy-

drophobicity, which enables them to penetrate into the lipid components of bacterial cell membrane and mitochondria, disrupting the cell structure and rendering them more permeable resulting in leakages of critical molecules from within the cell and eventual death of the bacteria cells (94JBC8022, 98IBB261). In this study, however, the oils obtained by SFME was more active than the oil obtained by HD against the tested microorganisms.

E. *IN VITRO* TIME-KILL ASSAY OF *ROSMARINUS* ESSENTIAL OILS OBTAINED BY HYDRODISTILLATION AND SOLVENT-FREE MICROWAVE EXTRACTION METHODS

Staphylococcus aureus and *Escherichia coli* were selected to represent Gram positive and Gram negative bacteria respectively amongst the test bacteria that were susceptible to the essential oils. The profile of the rate of kill of these test bacteria are as articulated in Figure 8.

The hydrodistilled essential oil resulted in approximately 4.3–5.3 $\log_{10}$ reduction in *Staphylococcus aureus* counts in 6 h of exposure, and approximately 8.9 $\log_{10}$ reduction in 12 h of exposure. Complete elimination of the pathogen was achieved in 9 hours at the MIC and in 12 hours for the 2×MIC. The rate of kill characteristics of the SFME-derived oil against *Staphylococcus aureus* does not appear to be different from that of the HD derived oil. The biocidal activity of the essential oils appears to be more pronounced against *Escherichia coli* as complete elimination of the bacteria was achieved in 3 hours at both the MIC and 2×MIC with approximately between 8.3 and 8.8 $\log_{10}$ reductions in the bacterium counts at 6 and 12 hours of reaction.

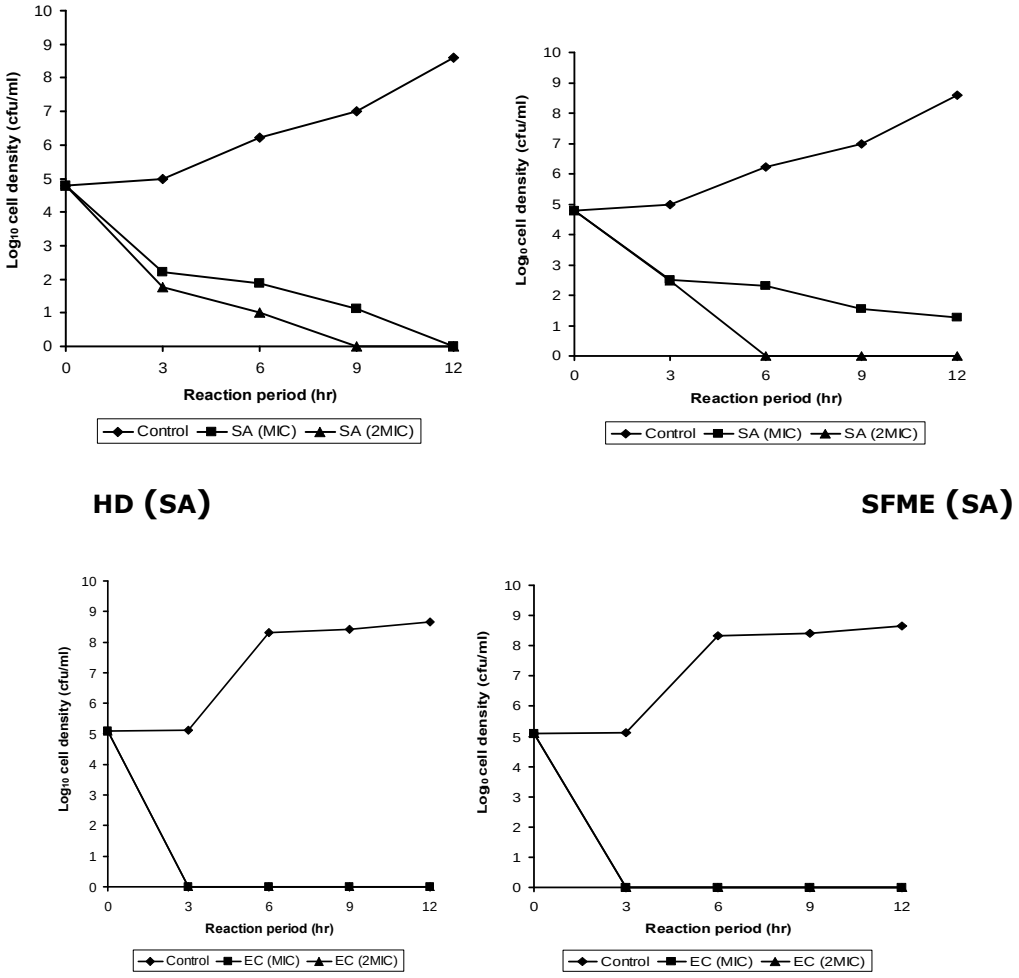


Figure 8: Profiles of the Rate Of Kill of *Staphylococcus aureus* (SA) and *Escherichia coli* (EC) by Essential Oils of Rosemary Obtained by Hydrodistillation (HD) and Microwave Distillation (MD) Methods

The essential oils, irrespective of the method of extraction, proved to be bactericidal against both *Staphylococcus aureus* and *Escherichia coli* achieving a >99.99% (3 log10) reduction in counts after 12 hours of exposure to the oils. A greater than 99.9% killing activity in 24 hours is generally used as a standard

of measurement of bactericidal efficacy (00CMI503). Also, although the essential oils appears to more biocidal against *Escherichia coli* by virtue of the shorter period to achieve complete elimination of the bacteria, method of extraction of the oils does not appear to affect the activity. This comparatively higher biocidal activity of the essential oil against the Gram negative bacteria is worth of noting. Gram negative bacteria are known to be inherently more resistant to the antimicrobial compounds than Gram positive bacteria (09BR339), and this resistance characteristic has been reported to be related to the structure of the cell wall of the bacteria (98CIDS32). Hence, the essential oils of this plant appear to be a potential source of antibacterial compounds that could be of importance in the treatment of infection caused by Gram negative bacteria.

F. ANTIOXIDANT ACTIVITIES OF ESSENTIAL OILS OBTAINED BY HYDRODISTILLATION AND SOLVENT-FREE MICROWAVE EXTRACTION

The principle of antioxidant activity is based on the availability of electrons to neutralize free radicals. In this study, the antioxidant activity of *Rosmarinus officinalis* oil was evaluated by two complementary tests: scavenging of $DPPH^+$ free radicals and the β-carotene bleaching test. The results are as shown in Figures 9 and 10.

The free radical scavenging activity of essential oil of *Rosmarinus officinalis* obtained by SFME revealed percentage inhibitions of 48.80%, 61.60%, and 67.00%, while that of the HD oil showed percentage inhibitions of 52.20%, 55.00%, and 65.30% both at concentrations of 0.33 mg/mL, 0.50 mg/mL, and

1.0 mg/mL, respectively. These results show that activity increases as the concentrations of the oils were increased, at least within the limit of the test concentrations of the oils (Fig 8). The SFME essential oil showed a slightly higher DPPH-radical scavenging activity (IC_{50} = 0.34mg/mL) than the hydrodistilled essential oil (IC_{50} = 0.46 mg/mL), whereas for BHT IC_{50} was 0.22 mg/mL at concentration of 0.33 mg/mL.

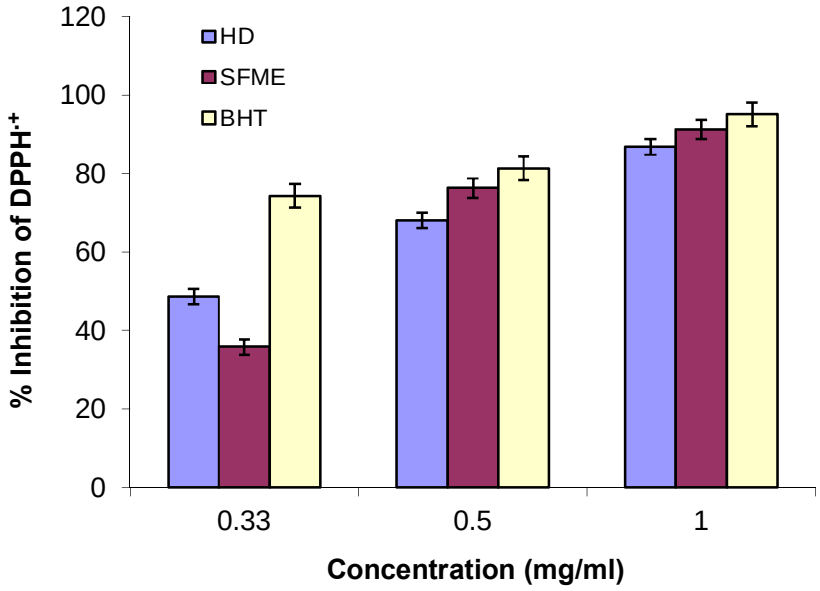


Figure 9: Free Radical-Scavenging Activity of *Rosmarinus officinalis* Essential Oil Evaluated by the 1,1-Diphenyl-2-picrylhydrazyl

The result of lipid peroxidation inhibitory activity of the essentials oils, assessed by the β-carotene bleaching test are shown in Figure 10. β-Carotene usually undergoes rapid discoloration in the absence of an antioxidant. This is because the oxidation of β-carotene and linoleic acid generates free radicals (01FC285). The linoleic acid free radical formed upon the abstraction of a hy-

drogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecule, hence β-carotene is oxidized losing its orange color which is then monitored spectrophotometrically (03FC(82)593, 10ICP152). The results obtained from this assay are similar to the data obtained from DPPH test. The percentage inhibitions were 48.62, 68.06 and 86.79% for hydrodistilled oil; and 35.87, 76.29 and 91.19% for solvent-free microwave-extracted oil at concentrations of 0.33, 0.5, and 1.0 mg/mL, respectively. Although both oils prevented the bleaching of β-carotene, the SFME-extracted oil had a slightly higher activity than the HD-extracted oil. These results are also consistent with the results obtained from DPPH test. The concentrations providing 50% inhibition were 0.338, 0.282, and 0.22 mg/mL for HD, SFME and BHT, respectively. It was noted that the antioxidant activities of the tested samples were dependent on their concentrations. The IC_{50} of SFME was however lower than that of HD essential oil.

In plant essential oils, oxygenated monoterpenes and monoterpene hydrocarbons are mainly responsible for the antioxidant potential (00FC(69)167). According to our data, oxygenated monoterpenes and monoterpene hydrocarbons were the main components of *Rosmarinus officinalis* essential oil (Table 5, 6).

Many reports of the investigations on the activity of *Rosmarinus officinalis* have shown that there are biologically active compounds in rosemary essential oil that exhibit cytotoxic, antioxidant, anti-carcinogenic and cognition-enhancing properties (94PC1463, 96JAF131, 03JC(A)119). These compounds that have

the potential to influence glucose level in diabetic patients, modify rumen microbial fermentation and enhance bone re-sorption and do not enhance immune response (04PC3249, 08PP1254). Essential oils, despite their wide uses and fragrances, constitute effective alternatives to synthetic compounds produced by chemical industry without showing the same side effects as the latter (08PSR12).

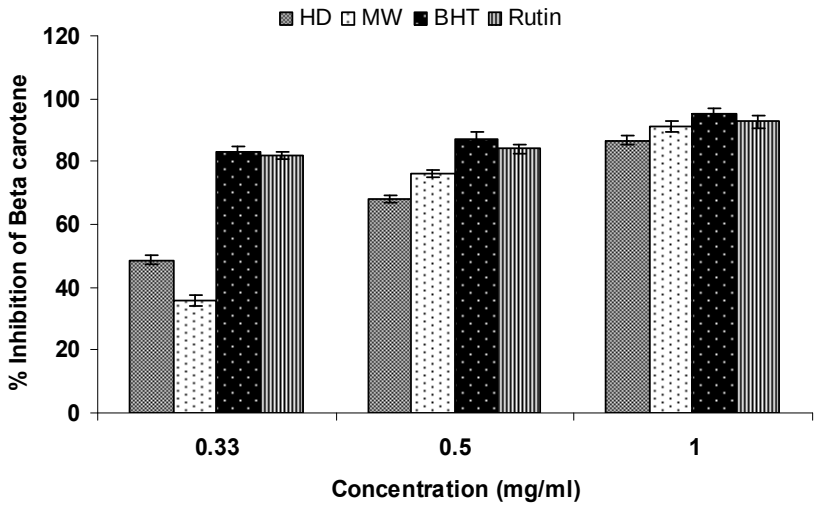


Figure 10: Antioxidant Activity of *Rosmarinus officinalis* L. Essential Oil Determined by β-Carotene Bleaching Test

G. THE ANTIOXIDANT POTENTIAL AND PHYTOCHEMICAL CONSTITUENTS OF *ROSMARINUS OFFICINALIS* L

The results of total polyphenolics content obtained in this study revealed the high content of phenols and flavonoids in both acetone and methanol leaves extract of *Rosmarinus officinalis* L. Higher amounts of phenols and flavonoids

were present in methanolic extract compared with acetone extract. However, the level of flavonols and proanthocyanidins were significantly lower in methanol extract but significantly higher in acetone extract as shown in Figure 11. Among these compounds, proanthocyanidins was the least followed by flavonols in both solvent extracts. Polyphenolic compounds investigated in this study are known to have antioxidant activity which may be responsible for the activities observed in this plant. These activities have been reported to be due to their ability to quench or neutralize free radicals (99LWT269). In addition, the presence of conjugated ring structures and carboxylic group in these compounds may enhance their antioxidant activities, which have been shown to inhibit lipid peroxidation (95FRR375). In the present study, the high levels of phenols and flavonoids content might be responsible for the strong activity observed against ABTS, DPPH, nitric oxide (NO), and hydrogen peroxide radicals. The results obtained from this study strongly suggest that polyphenolics content are important components of *Rosmarinus officinalis* and could be attributed to some of its pharmacological activities. The relationships between total phenolic content and antioxidant properties of many plants have been investigated in previous studies (98JAF4113, 01JAF5165, 04JEO145, 05LWT565). Some studies obtained good positive linear correlations; others obtained poor linear correlations or even could not explain the relationship between total antioxidant activity and phenolic content (07FC(103)778).

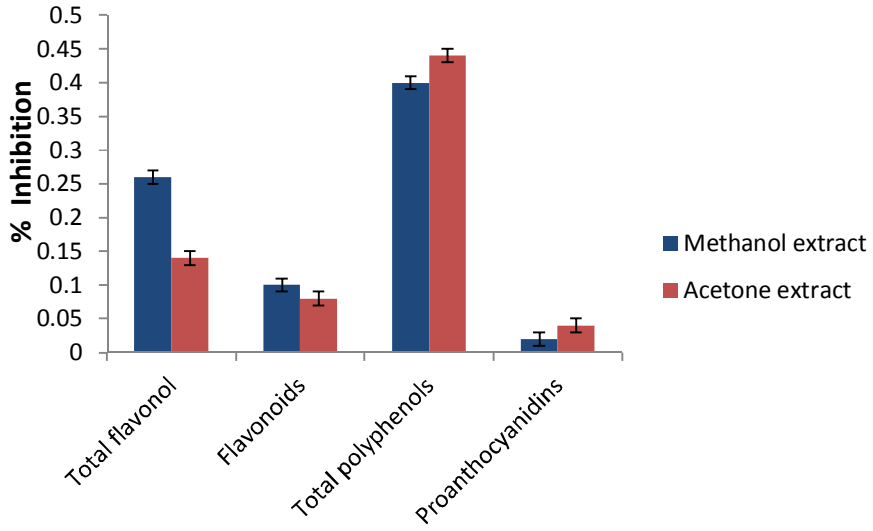


Figure 11: Polyphenolic Contents of Acetone and Methanol Extracts of *Rosmarinus officinalis* L

Figure 12 shows the concentration-response curve of DPPH radical scavenging activity of acetone and methanolic extracts of *Rosmarinus officinalis* was similar to that of tannic acid used as reference drug in this study. It was found that the scavenging activity of methanolic extract was higher than that of acetone extract and tannic acid. At a concentration of 0.5 mg/mL, the percentage inhibition of methanol, acetone extract and tannic acid reached 78.1, 76.8 and 75.6%, respectively. The antioxidant activity of this plant against DPPH radicals is considered to be due to proton-donating ability and thus could serve as free radicals inhibitors or scavengers and possibly primary antioxidants (09RNP23). Through the contents of phenolics and flavonoids in acetone extract, however, it has identical antioxidant activities compared with methanolic extracts, which may be compensated by the increased level of flavonols and proanthocyanidins.

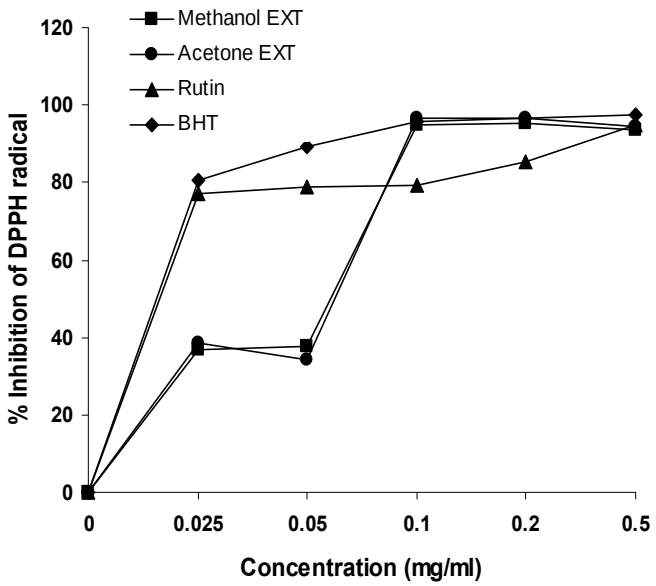


Figure 12: Inhibition of DPPH Radical by extracts of *Rosmarinus officinalis* L

The methanol and acetone extracts of *Rosmarinus officinalis* were fast and effective scavenger of the ABTS radicals (Figure 13) which is comparable to that of BHT used as standard drug. At 0.5 mg/mL, the extracts exhibited high percentage inhibition of ABTS radical. The percentage inhibition was 78.15, 79.14 and 78.40% for the methanol, acetone extract, and BHT, respectively. Both acetone and methanolic extracts exhibited similar results, which was comparable to BHT. The activity observed in the acetone extract despite lower contents of phenols and flavonoids may be due to the high contents of proanthocyanidins and flavonols. The scavenging of $ABTS^{+}$ by the plant extract was found to be similar to that of DPPH radical. This result contradict the report (98JAF4869), which demonstrated that some compounds with $ABTS^{+}$ scavenging activity may not exhibit DPPH scavenging activity. Meanwhile, the results obtained in this study corroborate with the report (09RNP23) on the effect of

methanolic leaves and stem extracts of *Celtis africana* against ABTS radical. This results in further strengthening the capacity of *Rosmarinus officinalis* extract to scavenge various radicals in different systems and thus could be used as therapeutic agents for the treatment of free radicals induced diseases.

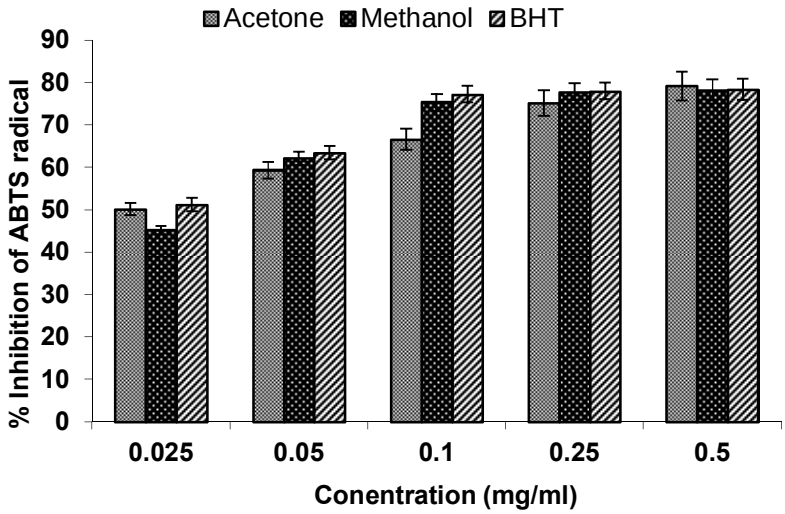


Figure 13: Inhibition of ABTS Radical by Extracts of *Rosmarinus officinalis* L

The scavenging activity of methanol and acetone extract of *Rosmarinus officinalis* compared to BHT and vitamin C for hydrogen peroxide is shown in Figure 14. The results indicated a concentration-dependent activity against H_2O_2 with percentage inhibition of 78.1, 75.6, and 76.8% for methanol, acetone and tannin, respectively at 0.5 mg/mL. Hydrogen peroxide is a highly important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radicals in the cell (03FC(83)371). The scavenging activities of the methanol and acetone extract of this plant were the same with the tannic acid which was used as the refer-

ence compound. Similar observation was reported (10AJH70) on the aqueous extract of *Strychnos henningsii*. The observed result could be due to the presence of phenolics compounds, which have been reported to donate electrons to H_2O_2 and thus neutralize it to water (06FCT198).

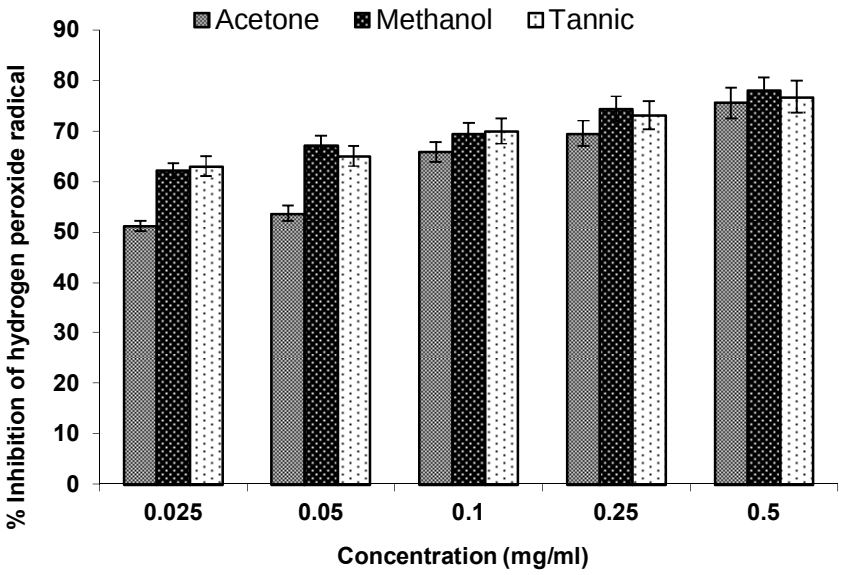


Figure 14: Inhibition of Hydrogen Peroxide Radical by Extracts of *Rosmarinus officinalis* L

Nitric oxide (NO) is a reactive free radical generated from sodium nitroprusside in aqueous solution at physiological pH and reacts with oxygen to form nitrite. Both methanol and acetone extracts showed similar results but significantly lower when compared with the rutin and BHT (standard drugs). The plant extract inhibits nitrite formation by directly competing with oxygen, nitric oxide and other nitrogen oxides such as NO_2 N_2O_4 and N_2O_3 in the reaction

(94ME462). The percentage inhibition of NO by methanol, acetone, BHT, and rutin were 76.3, 73.2, 98.8 and 90.5%, respectively at 0.5 mg/mL (Figure 15).

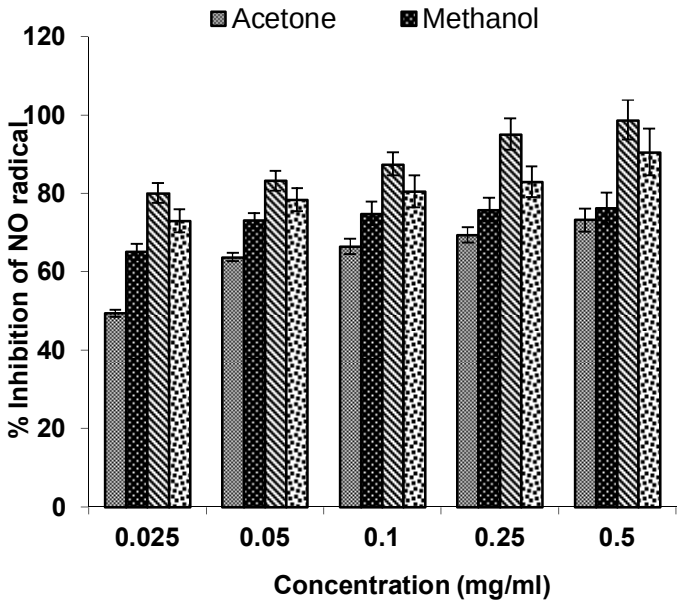


Figure 15: Inhibition of NO Radical by Extracts of *Rosmarinus officinalis* L

H. ISOLATION OF A BIOACTIVE COMPOUND FROM LEAF EXTRACTS OF *ROSMARINUS OFFICINALIS* L

Compounds responsible for antibacterial activities in this plant have been identified in only few cases. A diterpene, rosmanol (7α,11,12-trihydroxyabieta-8,11,13-trien-20-oic acid 20,6-lactone) have been isolated from the flowers of this plant (85PC1853). Three new flavonoid glucuronides, luteolin 3'-O-beta-D-glucuronide, luteolin 3'-O-(4"-O-acetyl)-beta-D-glucuronide, and luteolin 3'-O-(3"-O-acetyl)-beta-D-glucuronide, together with hesperidin, were also isolated from 50% aqueous MeOH extract of the leaves of rosemary (94PC1463).

Since Rosmarinus is known by the indigenous people of Africa for therapeutic properties one of the main objectives of this study was to focus on this plant found predominantly in the Eastern cape province of South Africa and by means of identification of the plant constituents, be able to relate the bacterial activities on the basis of literature precedents, to the compound isolated.

I. STRUCTURAL ELUCIDATION AND CHARACTERIZATION OF THE BIOACTIVE COMPOUND ISOLATED FROM *ROSMARINUS OFFICINALIS* L

The antibacterial activity of *Rosmarinus officinalis* was observed in the *n*-hexane extract. The active compound was found predominant in the *n*-hexane extract and thus this extract was used for the isolation of target compound. Various fractions were generated from the *n*-hexane extract and were analyzed as shown in Fig. 4 and 5. The active compound was found to reside in the fraction eluted by 30 % hexane in 70 % ethyl acetate. The molecular ion is m/z 263. The ^{1}H-NMR and ^{13}C-NMR spectra gave a total of 18 carbon atoms (three appearing as double signals, the proton NMR shows 17 protons (2 x CH_3, 1 x CH_2, 9 x ArH (or proton attached to heteroatom).

1. Infrared spectra

The infrared spectrum of the pure compound (Fig. 16) shows the bands in the region 3551-2889 cm^{-1} due to the N-H and C-H vibrations in the spectrum. Carbonyl band is at 1678 cm^{-1} and aromatic bands at 1638, 1616, and 1521 cm^{-1}.

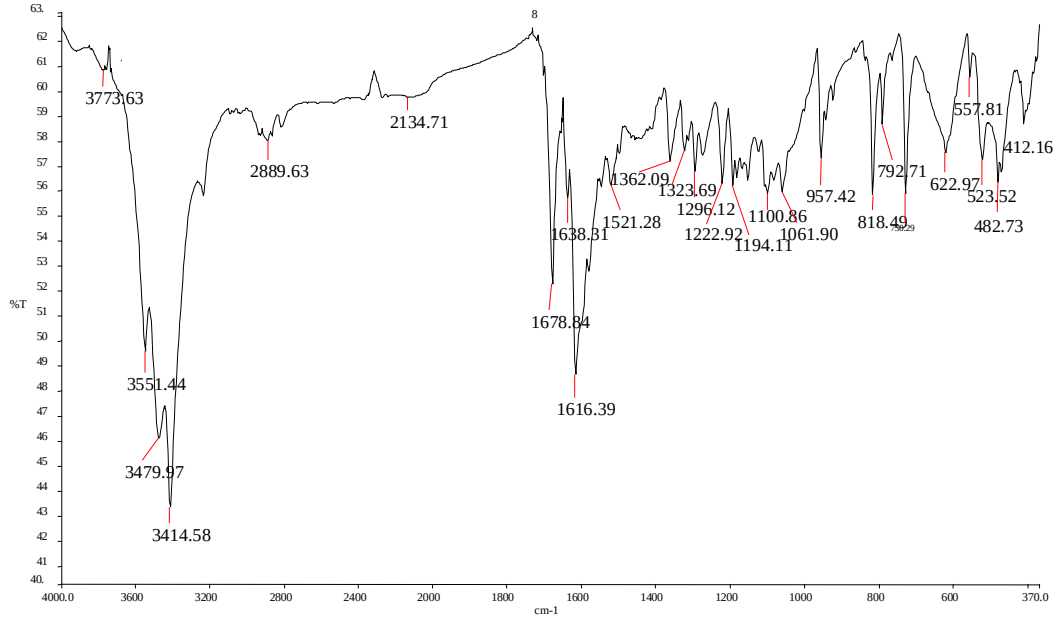
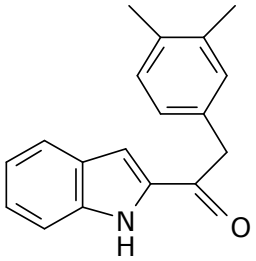


Figure 16: The IR Spectrum of Compound **71**

2. NMR-spectroscopy

The structure of the pure compound was established by ^{1}H and ^{13}C NMR, COSY, DEPT, HSQC and IR. The proton NMR spectrum (Fig. 17, 18) of **71** contains five peaks at δ7.88 (d, J = 6.8 Hz, 1H), 7.58 (s, 1H), 7.48(br, 4H), 7.36 (s, 1H), and 6.63 (d, J = 7.6 Hz, 2H). The COSY spectrum revealed the correlation of the proton peaks (Fig. 19). The aromatic proton doublets at δ 6.63 (d, J=7.6Hz, and δ 7.87 (d,J = 6.8 Hz) were due to a 3,4 disubstituted phenyl ring for protons H-2 and H-5, H-6, respectively, while the signals at proton singlets at d 7.58 ppm, 7.36 ppm, and broad singlets at δ7.48 ppm accounted for the indolyl ring protons H-1', H-3' and H-4', H-5', H-6' and H-7' respectively. At the aliphatic region of the proton NMR, two broad singlet peaks at δ 3.80 ppm integrating for two protons and δ 2.92 ppm integrating for six protons were assigned to the CH_2 and the two methyl protons of a 3,4-dimethyl substituted

phenyl ring. DEPT 135 confirmed the presence of the CH_2-group, it showed a negative sign (Fig. 21). The ^{1}H- ^{1}H COSY spectrum were used to assign the proton signals, while ^{1}H-^{13}C correlation HSQC was further used to assign the carbonyl carbon and other C–H bonds (Fig. 20). Hence the compound was identified as 1-(1H-indol-2-yl)-2-(3,4-dimethylphenyl)ethanone **71**.



71

The ^{13}C NMR of compound **71** shows expected peaks at δ194.57 assigned to the carbonyl (C=O) peak (Fig. 21, 22). The C-N bonds were found at δ 151.43 and δ 149. 85 ppm, while the methine and the quaternary carbons are found in the range δ 139.14 – δ 112.21 ppm. The two methyl carbons are assigned to the peak at 40.28 ppm while the methylene carbon was assigned to the peak at 33.05 ppm.

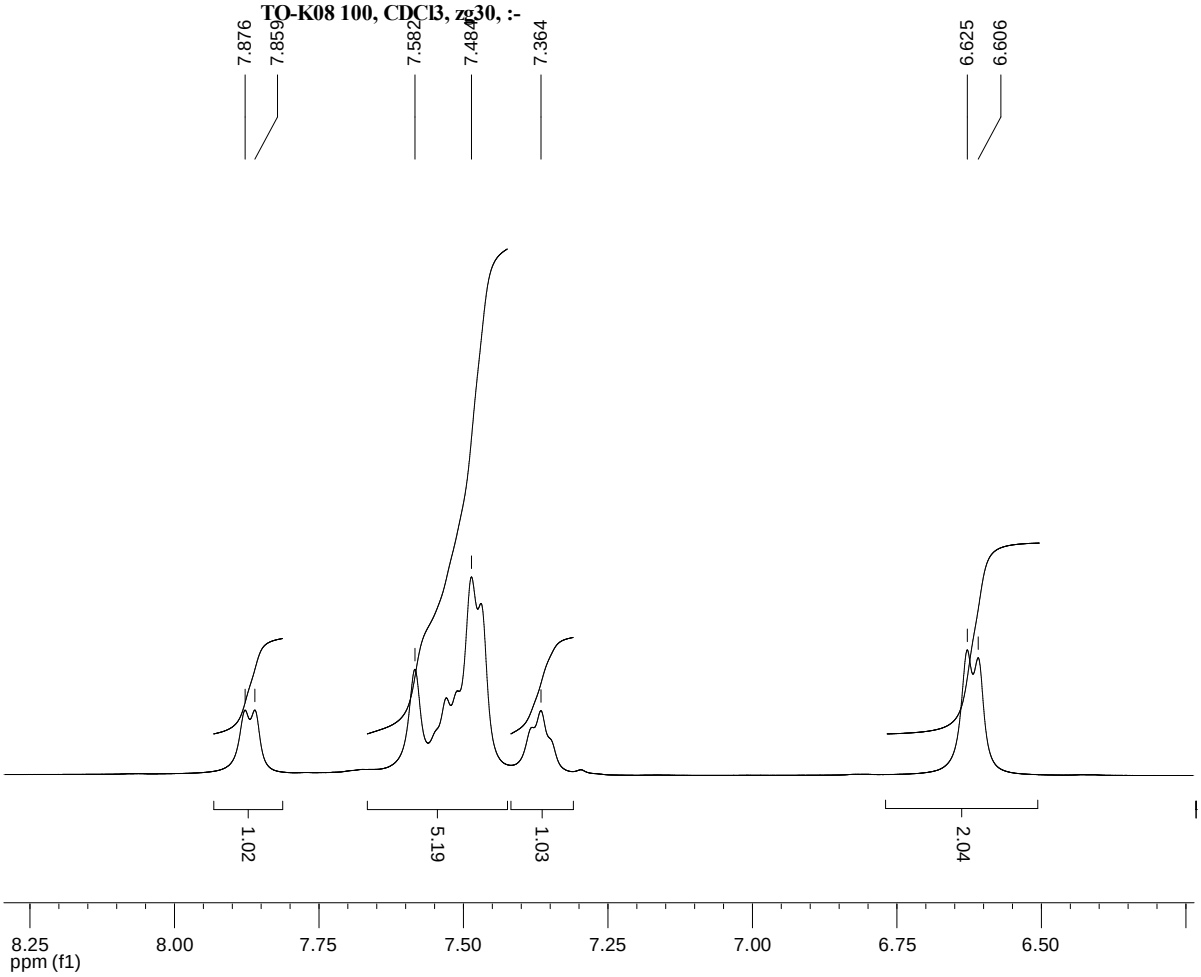


Figure 17: The ^{1}H-NMR expanded spectrum of compound **71** (aromatic region).The compound was dissolved in deuterochloroform and analyzed with 400 MHz for protons

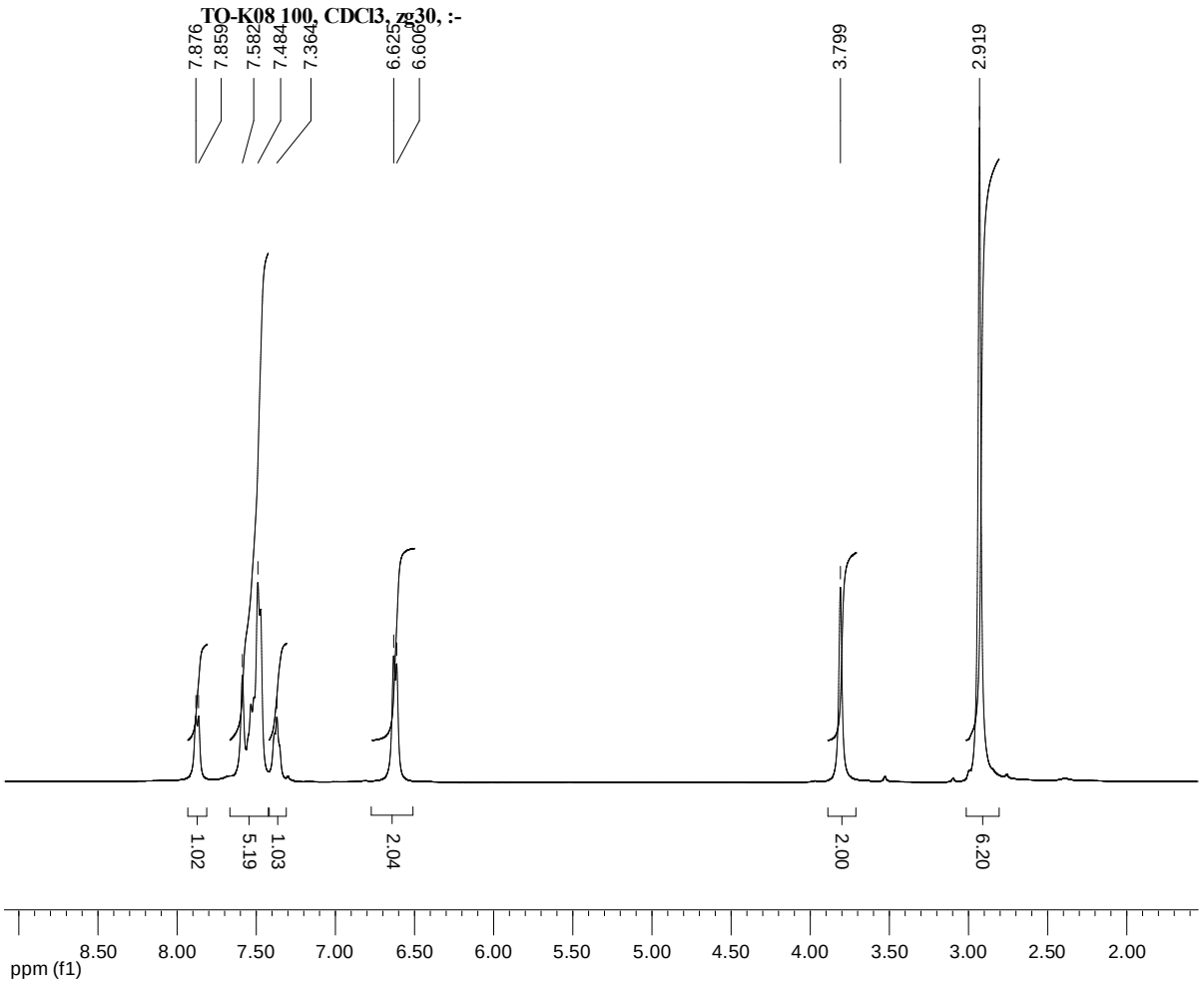


Figure 18: The ^{1}H-NMR expanded spectrum of compound **71** (aliphatic region). The compound was dissolved with deuterochloroform and analyzed with 400 MHz for protons

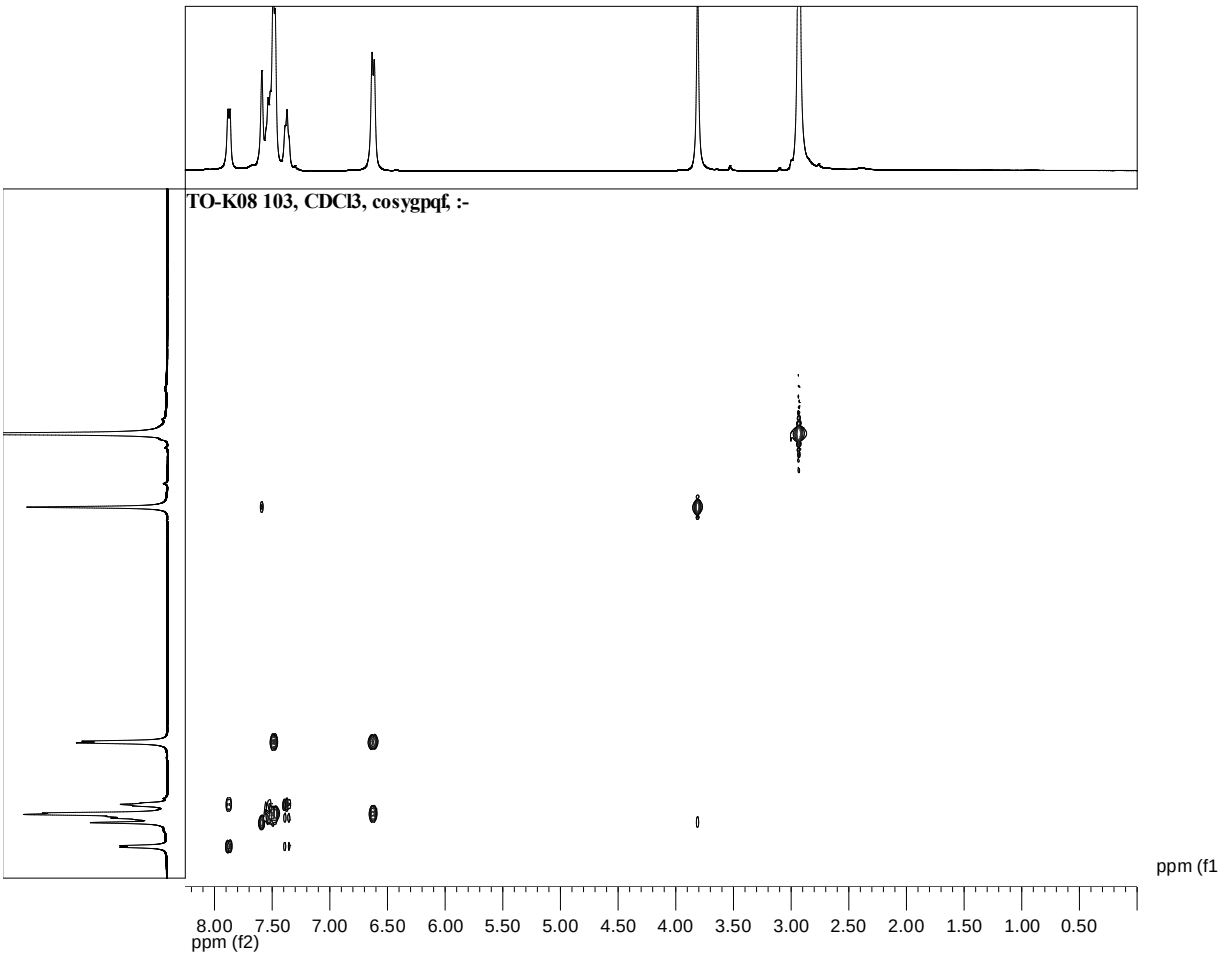


Figure 19: The COSY-NMR expanded spectrum of compound **71**. The compound was dissolved in deuterochloroform and analyzed with 400 MHz NMR

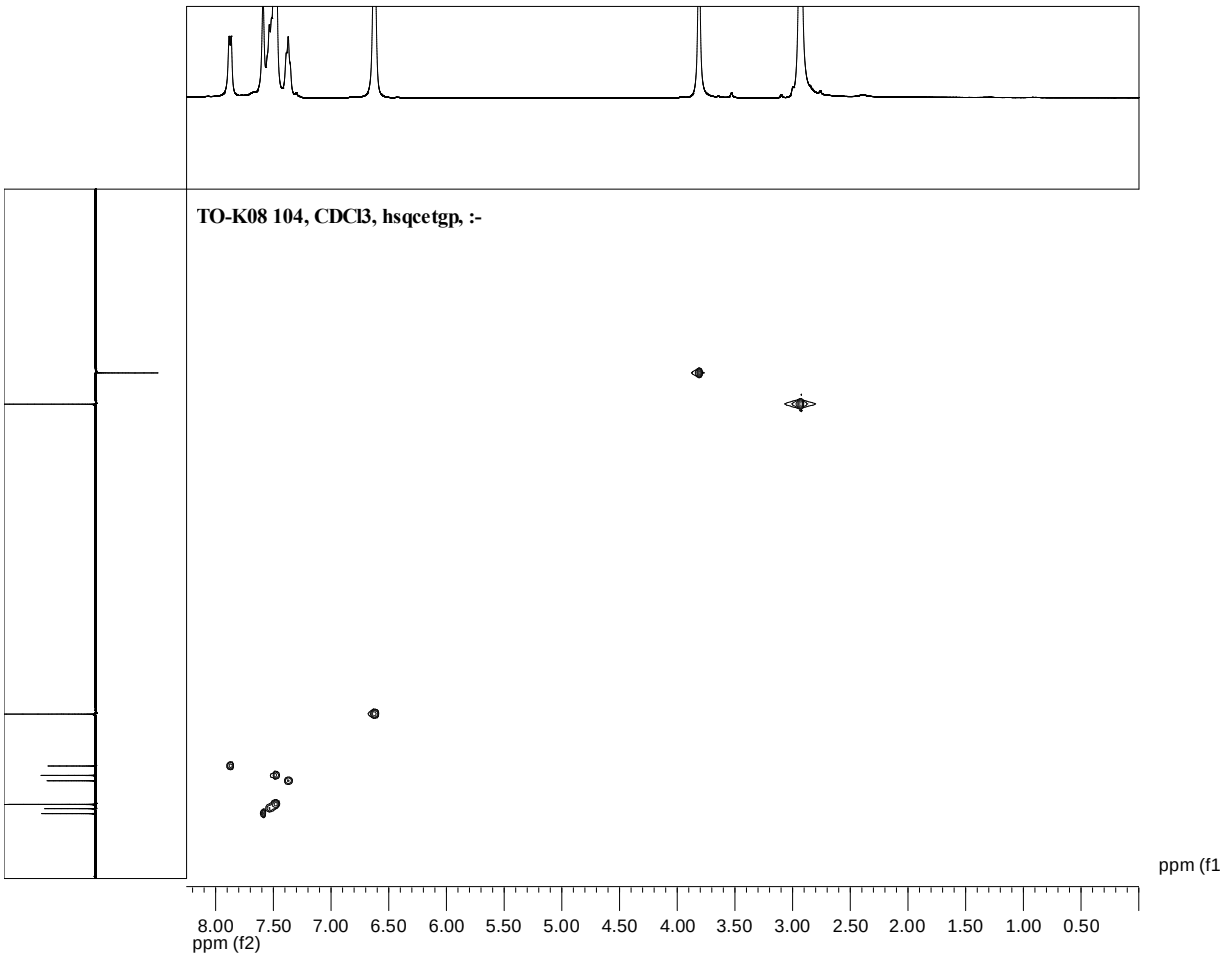


Figure 20: The HSQC-NMR expanded spectrum of compound **71**. The compound was dissolved in deuterochloroform and analyzed with 400 MHz NMR

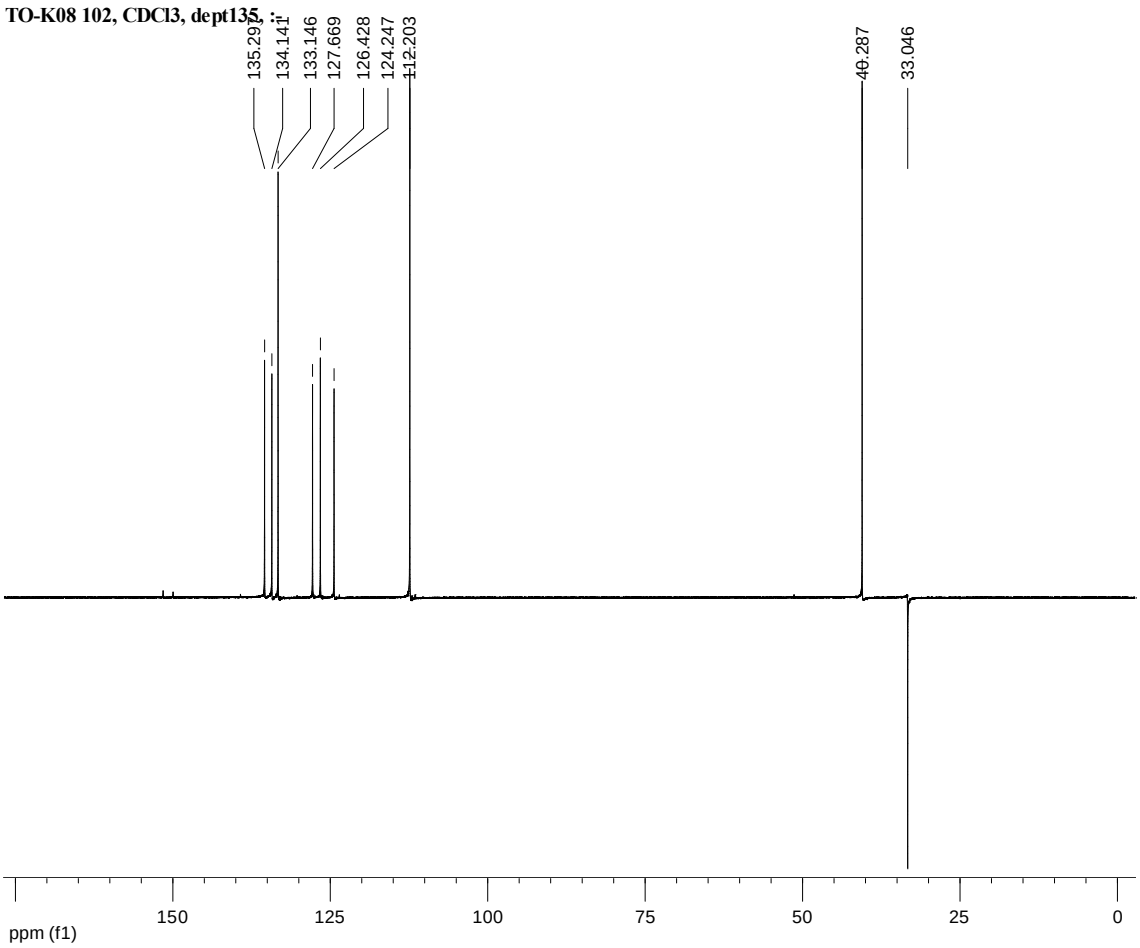


Figure 21: DEPT 135 NMR expanded spectrum of compound **71**. The compound was dissolved in deuterochloroform and analyzed with 400 MHz NMR for DEPT

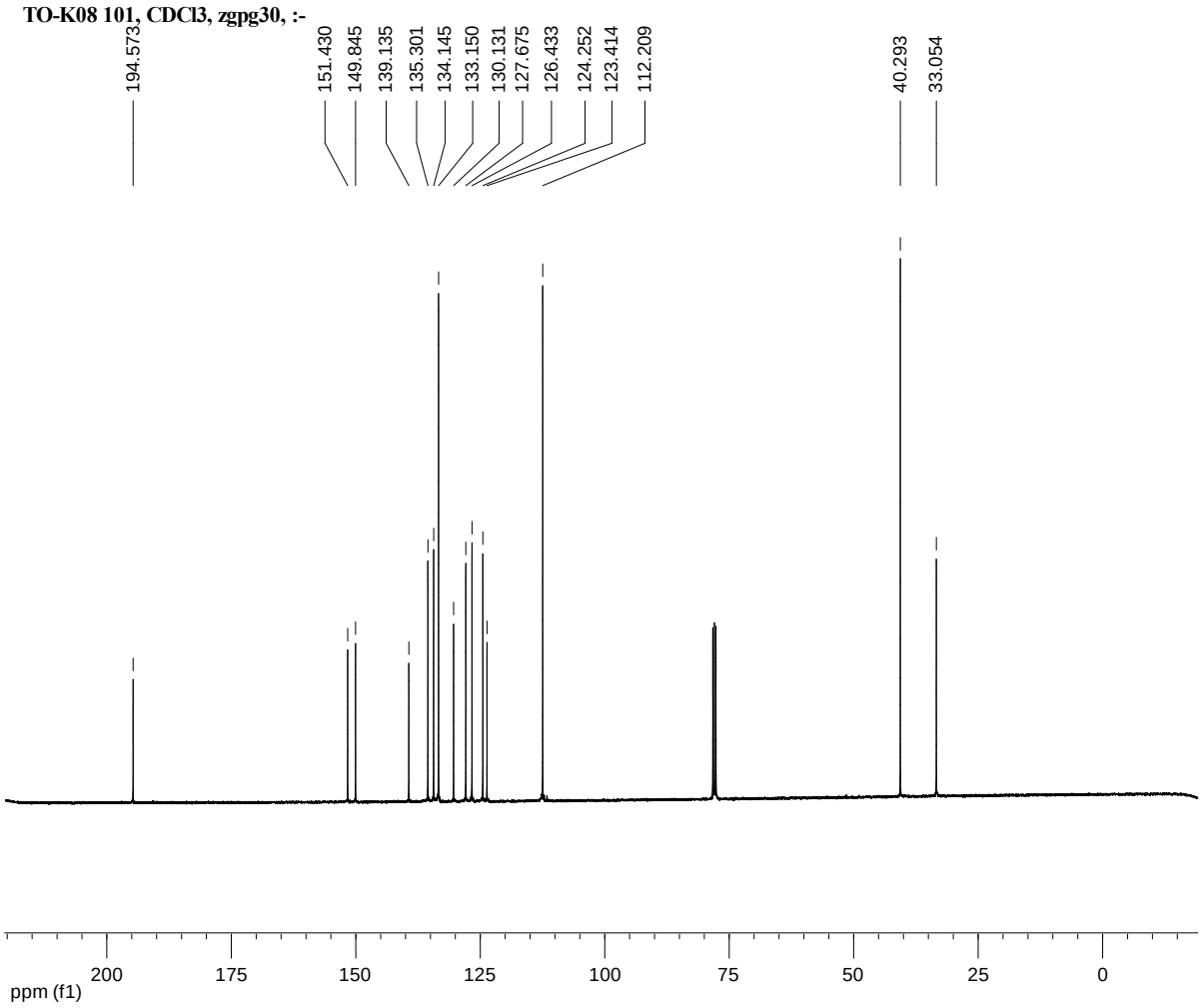


Figure 22: ^{13}C-NMR spectrum. The compound was dissolved in deuterochloroform and analyzed with 400 MHz NMR for DEPT

The mass spectrum (Fig. 23) confirms the identity of the compound. The molecular ion peak was found at 263 corresponding to the molecular formula $C_{18}H_{17}NO$. The fragments formed include m/z 117 for indole ring $(C_8H_7N)^+$ and in parallel the products of splitting of the heteroring. Fragmentation pattern is shown in Fig. 24 and corresponds to the analogous proposed patterns for the derivatives of indole (96JIS97, 02RCM346, 07SYN1559). Splitting of the heteroring itself is normally not discussed.

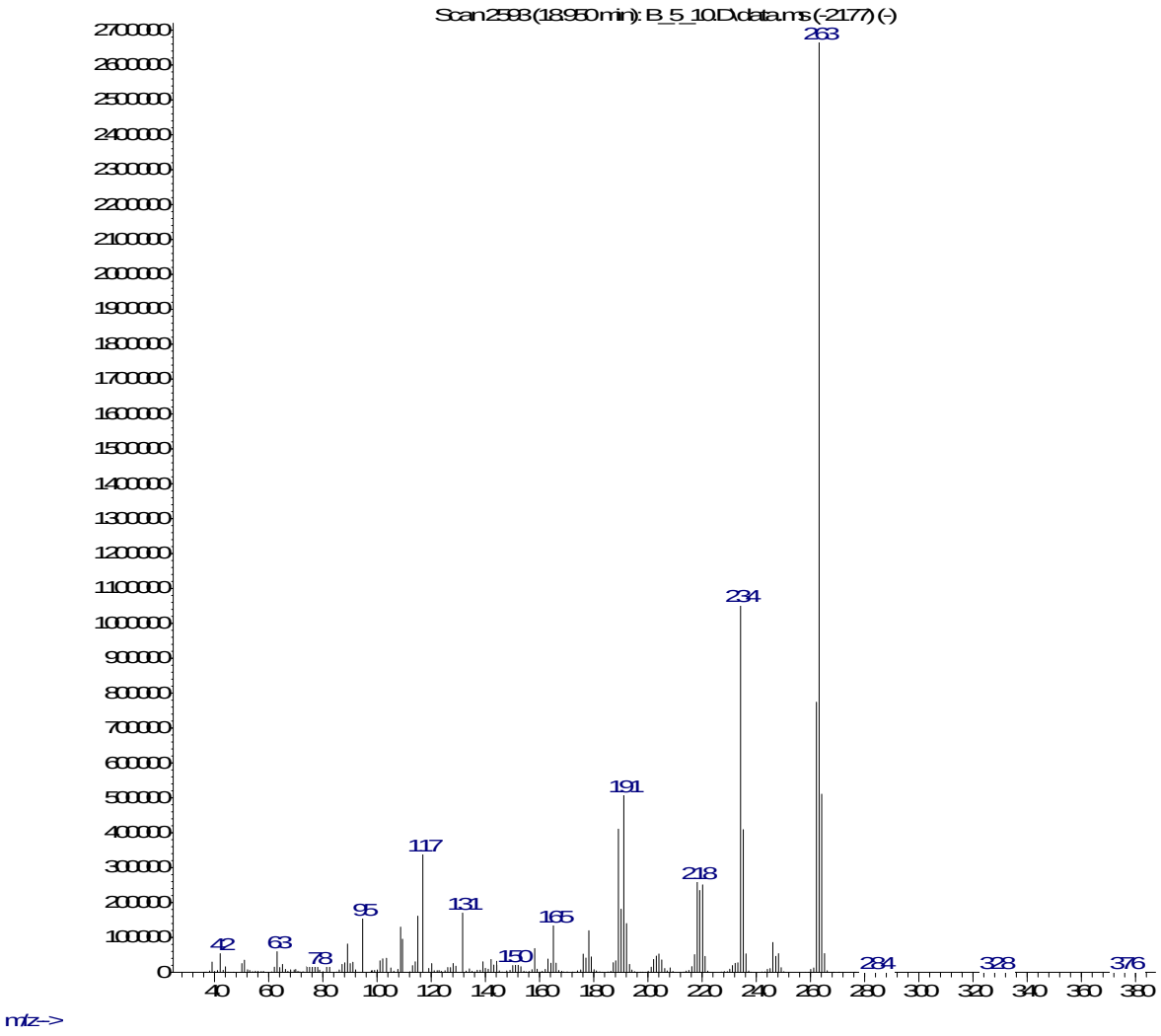


Figure 23: The Mass-Spectrum of Compound **71** under Identification

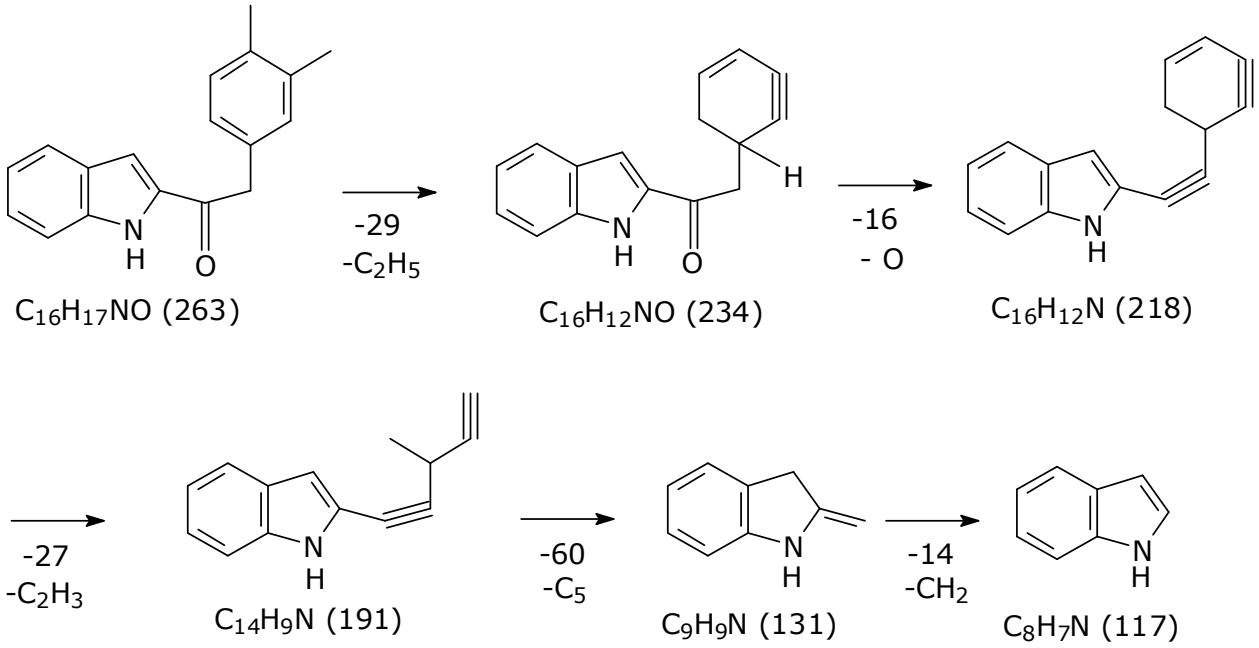


Figure 24: The Fragmentation Pattern of Compound **71** under Identification

V. Conclusion

1. Main components in the essential oils of *Rosmarinus officinalis* are 1,8-cineole, α-pinene, camphor, verbenone, bornyl alcohol, and bornyl acetate. The highest oil yields were obtained in November (spring), while the lowest oil yields were obtained in January (summer) as a result of the high rainfall in spring and the water deficit in summer. The overall composition of the essential oil undergoes drastic changes throughout the year being α-pinene-cineole type in April and verbenone-camphor type in August. This change in chemotype is parallel to the climatic changes in Eastern Cape. It is recommended to collect essential oils of *Rosmarinus officinalis* in August when the plant offers optimal composition in terms of its antimicrobial, antibacterial, antioxidant, and other useful properties. During the favorable season late summer - early autumn, α-pinene takes the ring-expansion and oxidation routes, and essential oils are enriched with especially valuable components. In the hot, windy, and dry month of April, predominant is the isomerization route, which does not spoil the plant but makes it less attractive.
2. The oils inhibited the growth of both the Gram positive and Gram negative bacteria at MIC values ranging between 0.23 mg mL^{-1} and 7.5 mg mL^{-1}, activity on Gram positive bacteria being higher. The essential oil obtained by SFME showed more activity than HD oil against *Escherichia coli* and *Staphylococcus aureus*. The biocidal activity of the essential oils appears to be more pronounced against *Escherichia coli*. The essential oils of this plant appear to

be a potential source of antibacterial compounds that could be of importance in the treatment of infection caused by Gram negative bacteria.

3. The free radical scavenging activity of essential oil of *Rosmarinus officinalis* obtained by SFME and HD revealed high inhibitions. The results of total polyphenolics content obtained in this study revealed the high content of phenols and flavonoids in both acetone and methanol leaves extract of *Rosmarinus officinalis* L. High levels of phenols and flavonoids content might be responsible for the strong activity observed against ABTS, DPPH, nitric oxide (NO), and hydrogen peroxide radicals. The scavenging of $ABTS^{+}$ by the plant extract was found to be similar to that of DPPH and other radicals.
4. The antibacterial activity of *Rosmarinus officinalis* was observed in the *n*-hexane extract. The active compound was found predominant in the *n*-hexane extract and thus this extract was used for the isolation of target compound. Spectroscopically the compound was identified as 1-(1H-indol-2-yl)-2-(3,4-dimethylphenyl)ethanone, which is a new component responsible for antibacterial and antioxidant properties.

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