CHEMICAL AND BIOACTIVE STUDIES ON BYPRODUCTS FROM SPICE PROCESSING

A thesis submitted to the

UNIVERSITY OF MYSORE

For the award of the Degree of

Doctor of Philosophy

IN

CHEMISTRY

By

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DECLARATION

I hereby declare that the thesis entitled "**CHEMICAL AND BIOACTIVE STUDIES ON BYPRODUCTS FROM SPICE PROCESSING**" submitted herewith for the degree of **DOCTOR OF PHILOSOPHY** of the **UNIVERSITY OF MYSORE** is the result of the work done by me at **Central Food Technological Research Institute**, Mysore under the guidance of **Dr. L. JAGAN MOHAN RAO**, (Guide) Senior Scientist, Plantation Products Spices and Flavour Technology Department and **Dr. K. K. SAKARIAH**, (Co-Guide) Former Head and Deputy Director, Human Resource Development, CFTRI, during the period 1999-2003.

I further declare that the results of the work have not been previously submitted for any degree or fellowship.

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CERTIFICATE

*This is to certify that the Ph. D. thesis entitled "**CHEMICAL AND BIOACTIVE STUDIES ON BYPRODUCTS FROM SPICE PROCESSING**" submitted by Mr. G. K. Jayaprakasha for the award of degree of **DOCTOR OF PHILOSOPHY** of the University of Mysore is the result of the work carried out by him under my guidance and supervision during the period 1999-2003 at Central Food Technological Research Institute, Mysore.*

[Dr. L. Jagan Mohan Rao]
Co-Guide

26th Aug. 2003

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CERTIFICATE

This is to certify that the Ph. D. thesis entitled "**CHEMICAL AND BIOACTIVE STUDIES ON BYPRODUCTS FROM SPICE PROCESSING**" submitted by Mr. G. K. Jayaprakasha for the award of degree of **DOCTOR OF PHILOSOPHY** of the **UNIVERSITY OF MYSORE** is the result of the work carried out by him in the Human Resource Development Department, Central Food Technological Research Institute, Mysore, India, under my guidance and supervision during the period 1999-2003.

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G. K. Jayaprakasha

PREFACE

The spice *Cinnamomum zeylanicum* belongs to Lauraceae family. The genus *Cinnamomum* is representing about 350 species, which occur in Asia and Australia. Cinnamon leaves and bark possess aromatic and medicinal properties as well as food applications. However, there are no reports on chemical composition of unconventional parts like buds, flowers, fruits and fruit stalks and their bioactivities.

Curcumin is industrially produced using turmeric oleoresin as the starting material. The mother liquor / turmeric spent oleoresin ≈ 70-80% after isolation of curcuminoids has no commercial value at present and it is known as curcumin removed turmeric oleoresin (CRTO). It has a composition of oil, resin and left over curcuminoids. There are no reports on recovery of left over curcuminoids from CRTO, as well as the bioactivities of the volatile oil and their chemical composition.

Therefore, a basic study was carried out with the following objectives.

Part A. Cinnamon (Unconventional parts) – Farm byproduct

(1). Isolation and analysis of volatile oils from cinnamon buds, flowers, fruits and fruit stalks by GC and GC-MS.
(2). Isolation of bioactive fractions from cinnamon unconventional parts.
(3). Antioxidant, antimicrobial and antimutagenic activities of solvent extracts.
(4). Separation and identification of bioactive compounds.

Part B. Turmeric (CRTO) – Industrial byproduct

(1). Isolation, fractionation and identification of antibacterial, antifungal and antimutagenic fraction from CRTO.
(2). Recovery and purification of left over curcuminoids from CRTO.
(3). Development of HPLC method for the quantification of curcuminoids.
(4). Antioxidant activity of individual curcuminoids from CRTO.

The experimental results obtained were compiled in this thesis consisting of six chapters. The tables, structures, chromatograms (GC, GC-MS and HPLC) and spectra (MS and NMR) are given at the end of each sub-chapter. References and summary are given at the end of part A and B.

CONTENTS

PART – A

PREFACE

The spice *Cinnamomum zeylanicum* belongs to Lauraceae family. The genus *Cinnamomum* is representing about 350 species, which occur in Asia and Australia. Cinnamon leaves and bark possess aromatic and medicinal properties as well as food applications. However, there are no reports on chemical composition of unconventional parts like buds, flowers, fruits and fruit stalks and their bioactivities.

Curcumin is industrially produced using turmeric oleoresin as the starting material. The mother liquor / turmeric spent oleoresin ≈ 70-80% after isolation of curcuminoids has no commercial value at present. It has a composition of oil, resin and left over curcuminoids. There are no reports on recovery of left over curcuminoids from spent turmeric oleoresin, as well as the bioactivities of the volatile oil and their chemical composition.

Therefore, a basic study was carried out with the following objectives.

Part A. Cinnamon (Unconventional parts) – Farm byproduct

(1). Isolation and analysis of volatile oils from cinnamon buds, flowers, fruits and fruit stalks by GC and GC-MS.
(2). Isolation of bioactive fractions from cinnamon unconventional parts.
(3). Antioxidant, antimicrobial and antimutagenic activities of solvent extracts.
(4). Separation and identification of bioactive compounds.

Part B. Turmeric (Spent Turmeric Oleoresin) – Industrial byproduct

(1). Isolation, fractionation and identification of antibacterial, antifungal and antimutagenic fraction from CRTO.
(2). Recovery and purification of left over curcuminoids from CRTO.
(3). Development of HPLC method for the quantification of curcuminoids.
(4). Antioxidant activity of individual curcuminoids from CRTO.

The experimental results obtained were compiled in this thesis consisting of six chapters. The tables, structures, chromatograms (GC, GC-MS and HPLC) and spectra (MS and NMR) are given at the end of each sub-chapter. References and summary are given at the end of part A and B.

NOTES FOR EXPERIMENTAL

The reagents and solvents used in the present work were purified and dried according to standard methods (Fieser and Fieser, 1967; Ongley et al., 1973; Vogel, 1989). Melting points were determined in capillaries using melting point apparatus (Gallenkamp, England) and were uncorrected. Optical rotations were determined on Perkin-Elmer 243 Polarimeter at 25 °C and at 589 nm.

Thin layer chromatography (TLC) was done using silica gel-G, E-Merck, Germany. TLC spots were detected by exposure to iodine vapour and (or) by charring with 10% sulfuric acid in methanol. UV-Visible spectral measurements were done using Genesys-5 UV-visible spectrophotometer (Milton Roy, New York, USA). All the extracts were concentrated using flash evaporator (Büchi, Switzerland) at 40 °C. Gas-chromatography (GC) analyses were carried out on Shimadzu GC-15A (Kyoto, Japan) equipped with a FID detector. Peak areas were computed by a Shimadzu C-R4A Chromatopac data processor. HPLC analyses were done on Hewlett Packard Quaternary pump (Model HP 1100 Series, Hewlett-Packard, California, USA), fitted with Zorbax analytical (Hewlett-Packard, CA, USA) C_{18} column (250 $\times$ 4.6 mm I.D), 5 μm particle size. The injection system (Rheodyne) used was a 20 µl sample loop. A HP 1100 Series variable wavelength detector was used. The compounds were quantified using HP CHEMSTATION software.

GC-MS analyses were carried out using a Shimadzu GC-17A (Kyoto, Japan) chromatograph equipped with a QP-5000 (Quadrapole) Mass Spectrometer. Elemental analyses were recorded on Varioel elemental analyser, (Elementar Americas, Inc. NJ, USA). Mass spectra were recorded using JMS HX-110/110A (JEOL, Tokyo, Japan), LCQ Classic (Theroelectron, Waltham, MA) Reflex II (Bruker Daltonics, Billerica, MA) and Apex II 70e (Bruker Daltonics, Billerica, MA). IR spectra were recorded on a Bruker-IFS 25 spectrometer using KBr discs. NMR spectra were recorded on Bruker AM 400, Bruker Avance 600 and 800 instruments (Karlszruhe, Germany). TMS was used as the internal standard.

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Fieser, L.F.; Fieser, M. Reagents for Organic Synthesis, Wiley, New York, 1967.

Ongley, P.A. Organicum, Practical Handbook of organic Chemistry, edited Engl. Trans. B. J. Hazzard, by Addison-Wesley, Massachussets, 1973.

Vogel, A.I. A Text Book of Practical Organic Chemistry, Longman, London, 5th edition, 1989.

LIST OF ABBREVIATIONS

°C:	Degree centigrade
μl:	micro liter
AcOH:	acetic acid
BHA:	Butylayated hydroxyanisole
BHT:	Butylayated hydroxytoluene
Br.s:	broad singlet
CI:	Chemical ionization
cm:	Centimeter
d:	doublet
DPPH:	2,2-diphenyl-1-picrylhydrazyl
dd:	double doublet
DQFCOSY:	Double Quantum Filtered Correlated Spectrum
EtOAc:	Ethyl acetate
g:	gram (s)
GC-EI-MS:	Gas Chromatography-Electron Ionization - Mass Spectrometry
GC-FID:	Gas Chromatography-Flame Ionization Detector
GC-MS:	Gas-chromatography-Mass spectrometry
h:	hour
HMBC:	Heteronuclear Multiple-Bond Correlation Spectrum
HPLC-UV:	High-performance liquid chromatography – Ultraviolet
HSQC:	Heteronuclear Single-Quantum Correlation Spectrum
Hz:	Hertz
IR:	Infrared
LC-MS:	Liquid chromatography – Mass spectrometry
J:	Coupling constant
m:	multiplet
MAE:	Microwave assisted extraction
MeOH:	Methanol
min:	minute
mm:	millimeter
MP:	Melting point
nm:	Nanometer
NMR:	Nuclear Magnetic resonance
NCI:	Negative ion chemical ionization
NPD:	Nitrogen-phosphorus detector
ppm:	Parts per million
O-FID:	Oxygen flame ionization detector

s: singlet
SEFT: Spin-Echo Fourier Transform Spectra
SFE: Supercritical fluid extraction
TBA: Thiobarbituric acid
TLC: Thin-layer chromatography
TMS: Tetramethylsilane
TOF: Time of flight
UV: Ultra-violet

MATERIALS

Plant Materials

The buds, flowers and fruits stalks of *C. zeylanicum* were collected from Karkala (coastal Karnataka). The species were identified and voucher specimen were deposited at the Manasagangotri herbarium, MGH No. 3A/96/01, 2/96 and 4A/96/02. The cinnamon fruits were collected from Karkala (coastal Karnataka), (sample A) and from Kottayam (South Kerala) (sample B). The species were identified and voucher specimens were deposited at the Manasagangotri herbarium (MGH NO.1/96 and 2A/96), Department of Botany, University of Mysore, Mysore. Two batches of turmeric spent oleoresin (i.e., Curcumin removed turmeric oleoresin - CRTO) was obtained from M/S Flavours and Essences (P) Ltd, Mysore, Karnataka state during the year 1998 and 1999.

Chemicals

β-Carotene, catechin, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxyanisole (BHA) were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Diaion HP-20 and HP-20SS were obtained from M/s Mitsubishichemical Co., Curcumin was procured from E-Merck (Mumbai, India). The solvents used for extraction were Laboratory Reagent grade and distilled before use. Other solvents / chemicals used were of analytical or HPLC grade and obtained from E-Merck, Mumbai, India. Silica gel (60-120 mesh size) was obtained from BDH (Mumbai, India). Cinnamtannin B-1 was obtained from Dr. T. Satake, Kobe Gakuin University, Kobe, Japan.

Organisms

Prof. B. N. Ames, University of Berkeley, California kindly supplied *Salmonella typhimurium* strain, TA-100. Bacterial cultures (viz., *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa)* and fungal cultures (viz., *Aspergillus flavus, Aspergillus parasiticus, Fusarium moniliforme* and *Penicillium digitatum)* were obtained from the Department of Food Microbiology, CFTRI, Mysore.

Synopsis

In recent times, natural food additives are in great demand due to consumers' preference and health concerns associated with the use of synthetic additives. Agricultural and industrial by-products / wastes are interesting sources of natural additives. By-products of plant food processing represent a major disposal problem for the industry concerned and at the same time, they are also promising sources of food additives, which may be used, because of their desirable functional properties. A number of by-products have been previously studied as potential source of food additives.

In the background of present knowledgebase, a systematic investigation on the chemical as well as bioactivity studies of the volatiles and non-volatile fractions from unconventional parts of *Cinnamomum zeylanicum* (viz., buds, flowers, fruits, fruit stalks - farm by-products) and spent turmeric oleoresin (industrial by-product) were undertaken and the findings of these studies are presented in Part A and B respectively.

The objectives of the present investigation in part A for cinnamon unconventional parts were (i) Isolation and identification of volatile oils by GC and GC-MS from the unconventional parts of cinnamon such as buds, flowers, fruits and fruit stalks, (ii) Isolation and identification of the major flavour compound, (iii) Successive extraction of cinnamon fruits with solvents of increasing polarity, (iv) Separation and structure elucidation of bioactive compounds from cinnamon fruits and (v) Antioxidant, antimicrobial and antimutagenic activities of extracts of cinnamon fruits. Similarly, objectives in part B for spent turmeric oleoresin were (i) Isolation and identification of antibacterial, antifungal, antioxidant and antimutagenic fractions from spent turmeric oleoresin, (ii) Development of a method for the isolation of curcuminoids mixture and individual curcuminoids from spent turmeric oleoresin, (iii) Development of an analytical method for the determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin from different varieties of turmeric rhizomes and (iv) Antioxidant activity of curcumin, demethoxycurcumin and bisdemethoxycurcumin.

PART – A
Studies on the Unconventional Parts of Cinnamon

A brief introduction is presented on the isolation and identification of natural products. It describes isolation procedures of volatiles and non-volatiles by different methods. Chromatographic techniques for separation of individual components and their structure determination techniques using different spectroscopic techniques have also been discussed. The review of literature

covers aspects relating to the chemical composition and bioactivities of *Cinnamomum zeylanicum* (*C. zeylanicum*) as well as scope of further research and the protocol of the work. Besides, the general pathways of biogenesis leading to the volatile aromatic compounds with reference to mono and sesquiterpenoids and their oxygenated compounds and other selected components present in the cinnamon volatile oils have also been discussed. The results of the present study on unconventional parts of cinnamon are presented in the following paragraphs:

The hydro-distilled volatile oil of the *C. zeylanicum* buds was analysed using GC and GC-MS for the first time. Thirty-four compounds representing ≈ 98% of the oil were characterized. It consisted of terpene hydrocarbons (78.0%) and oxygenated terpenoids (9.0%). α-Bergamotene (27.4%) and α-copaene (23.1%) were found to be the major compounds.

The hydro-distilled volatile oil of cinnamon flowers was analysed by GC and GC-MS for the first time. It consisted of 23% hydrocarbons and 74% oxygenated compounds. A total of twenty-six compounds constituting ≈ 97% of the oil were characterized. (*E*)-Cinnamyl acetate (41.9%), *trans*-α-bergamotene (7.9%) and caryophyllene oxide (7.2%) were found to be the major compounds.

The hydro-distilled volatile oil of the *C. zeylanicum* fruits grown in Karnataka and Kerala were analysed using GC and GC-MS. It consisted of hydrocarbons (32.8% and 20.8%) and oxygenated compounds (63.7% and 73.4%). Thirty-four compounds representing more than 94% of the oil were identified. (*E*)-Cinnamyl acetate and □-caryophyllene were found to be the major compounds. This is the first report on the chemical composition of the volatile oil of fruits of *C. zeylanicum.*

The hydro-distilled volatile oil from cinnamon fruit stalks was analysed using GC and GC-MS. It consisted of 44.7% hydrocarbons and 52.6% oxygenated compounds. Twenty-seven compounds constituting ≈ 95.9% of the volatile oil were characterized. (*E*)-Cinnamyl acetate (36.59%) and caryophyllene (22.4%) are found to be the major compounds.

The findings obtained from the comparison of the chemical composition of the volatile oils obtained from different parts (*viz.,* buds, flowers, fruits and fruit stalks) of *C. zeylanicum* revealed, that seven volatile compounds were common in all the four parts of *C. zeylanicum* volatile oils.

The major compound *viz.,* (*E*)-cinnamyl acetate from volatile oil of *C. zeylanicum* was isolated and identified. Volatile oil was fractionated using column chromatography to get pure compound. The structure of the isolated compound was identified on the basis of ^{1}H NMR and Mass spectra.

Solvent extraction, separation, purification and identification of chemical constituents from cinnamon fruit extracts have been presented. The cinnamon

fruits powder was successively extracted with hexane, benzene, ethyl acetate, acetone, methanol and water. Water extract was found to possess maximum phenolics and antioxidant activity. Hence, it was used for fractionation using Diaion HP-20SS, Diaion HP-20, Sephadex LH-20 columns. After repeated column chromatography, five compounds were isolated and purity was analysed by HPLC. The purified compounds (1-5) were identified as 3,4-dihydroxybenzoic acid (protocatechuic acid), epicatechin- (2β→O→7, 4β→8)-epicatechin- (4β→8)-epicatechin (cinnamtannin B-1), [2,3-dihydro-3-(hydroxymethyl)-5-(3-hydroxypropyl)-7-(methoxy) benzofuranyl]-2-methoxyphenyl 4'-O-□-D-glucopyranoside (2S-trans) (Urolignoside)**,** 3-[[6-O-α-L-rhamnopyranosyl)-β-D-glucopyranosyl] oxy]-2-(3,4-dihydroxy phenyl)-5,7 dihydroxy-4H-1benzopyran-4-one (rutin) and quercetin-3-O-α-L-rhamnopyranoside with the help of NMR (one and two dimensional) and mass spectra.

Besides, antioxidant, antibacterial and antimutagenic activites of cinnamon fruit extracts have been presented. The antioxidant activity of ethyl acetate, acetone, methanol and water extracts along with BHA were screened for antioxidant activity using β-carotene-linoleate and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) model systems. All the extracts showed good antioxidant activity. Hexane, benzene, ethyl acetate, methanol and water extracts were screened for antibacterial activity by pour plate method. All the extracts showed a broad spectrum of antibacterial activity. Hexane extract was found to be most effective of all. Ethyl acetate, methanol and water extracts of cinnamon inhibited the mutagenicity of sodium azide in *Salmonella typhimurium* (TA-100), which ranged from weak to strong inhibition depending upon the concentration of extract per plate.

PART – B

Studies on Volatile oil and Curcuminoids from Spent Turmeric Oleoresin

The recent literature on chemistry and biological activities of *Curcuma longa* have been presented under introduction. Isolation and identification of antibacterial, antifungal and antioxidant and antimutagenic fractions from volatiles of spent turmeric oleoresin have been presented.

Curcumin, the yellow colour pigment of turmeric, was produced industrially from turmeric oleoresin. The mother liquor / spent turmeric oleoresin / curcumin removed turmeric oleoresin (CRTO) after isolation of curcumin from turmeric oleoresin, contained approximately 40% oil. The CRTO oil was extracted and broadly fractionated into three fractions. These fractions were tested for antibacterial activity by pour plate method against *Bacillus cereus, Bacillus coagulans, Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Fraction II eluted with 5% ethyl acetate in hexane was found to be most active fraction. The CRTO oil, fractions I and II were analysed by GC and GC-MS for their chemical composition. Thirteen chemical

constituents were identified. *ar*-Turmerone, turmerone and curlone being the major compounds present in these fractions.

The CRTO oil was subjected to fractional vacuum distillation to get two distillates. CRTO oil, distillate I and II were tested for antifungal activity against *Aspergillus flavus, A. parasiticus, Fusarium moniliforme* and *Penicillium digitatum* by spore germination method. Distillate II was found to be more active. Twenty-five chemical constituents from CRTO oil, distillates I and II were identified by GC and GC-MS. *ar*- Turmerone (52.6%), turmerone (11.5%) and curlone (8.5%) were major compounds present in the distillate II along with other oxygenated compounds.

CRTO oil was broadly fractionated using silica gel column chromatography to obtain three fractions and fifteen chemical constituents were identified by GC-MS. CRTO oil contained *ar*- turmerone (31.3%), turmerone (15.1%) and curlone (9.7%), whereas fraction III had *ar*-turmerone (44.5%), curlone (19.2%) and turmerone (10.9%) as the major compounds. Also, oxygenated compounds were enriched in fraction III. CRTO oil and its fractions were tested for antioxidant activity and antioxidant capacity, using the β-carotene-linoleate model system and the phosphomolybdenum method, respectively. The fraction III showed maximum antioxidant capacity. These fractions were also used to determine their protective effect against the mutagenicity of sodium azide by means of the Ames test. CRTO oil, fractions I and II exhibited good antimutagenicity, of which fraction III was the most effective.

Isolation of curcuminoids from CRTO was standardised. After removal of oil, left over CRTO was extracted with medium polar solvent and the extract was concentrated to 50%. Curcuminoids were precipitated by adding non-polar solvent. The purity of isolated curcuminoids mixture was analysed by HPLC. Curcumin, bisdemethoxycurcumin and demethoxycurcumin were separated by column chromatography, identified from their IR, ^{1}H, ^{13}C NMR and mass spectra.

An improved HPLC method was developed for the separation and estimation of curcumin, bisdemethoxycurcumin and demethoxycurcumin. HPLC separation was performed on a C_{18} column using three solvent system, *viz.,* methanol, 2% acetic acid, and acetonitrile, using detection at 425 nm. Four different varieties of turmeric were analysed to determine the percentage of three curcuminoids. The quantities of curcumin, demethoxycurcumin, and bisdemethoxycurcumin were found to be 1.06 - 5.65, 0.83 - 3.36 and 0.42 - 2.16%, respectively.

Antioxidant activity of curcumin, demethoxycurcumin and bisdemethoxycurcumin were determined by phosphomolybdenum and linoleic acid peroxidation methods. Antioxidant capacity of curcumin, bisdemethoxycurcumin and demethoxycurcumin were found to be 3144 ± 140, 2744.8 ± 48 and 2864.1 ± 44 μmol / g ascorbic acid equivalents, respectively at 50 μg/ml concentrations. The antioxidant activities of the curcumin,

bisdemethoxycurcumin and demethoxycurcumin were found to be 81.98, 73.0 and 81.77%, respectively using linoleic acid peroxidation method.

The significant findings of the complete study have been presented in a comprehensive way under *Summary* and *Conclusions.*

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PART – A

Chapter - 1

A Preview of Natural Products, Isolation and their Identification

INTRODUCTION

The term 'Natural products' in the broadest sense should represent all the chemical compounds, occurring in nature (Krishnaswamy, 1999). However, by convention and practice, the term is now used to refer only to the organic compounds occurring in nature. The boundaries are further defined by restricting the term to the secondary metabolites, leaving out the primary metabolites and whose biochemical functions are more or less well known and therefore covered under biochemistry. However, with the growing knowledge about the biological functions of these compounds (which were not long ago, dismissed as waste products of metabolisms!), natural products are emerging from the cocoon of the organic chemistry. This development was indeed, predicted several years ago by Lord Todd. Sir Robert Robinson had also anticipated or advocated the emergence of this trend as is clear from his following words: *"The structures are points of reference on a chart or milestones on a road, but it is the surrounding country which is of greatest interest. Not what the molecules are, but what they will do, how they may be formed and what transposition they may undergo or be induced to undergo these are the significant problems".* In consonance of this view, the large numbers of different compounds prominently exhibit their common traits while the differences among them bring out the diversity (Krishnaswamy, 1999).

Different criteria have been used in literature (Luckner, 1984) for the definition of secondary metabolism. In 1891, Kossel applied the term 'secondary' to certain cell constituents. Later, Czapak adopted the same term in 1921. It was however, not before 1950, that the books of Paech and Booner brought the term 'secondary products' into broader use. The formation of secondary products was first assumed to be a monopoly of higher plants. But later, biochemical studies led to the detection of the secondary products in microbial cultures and in animals. In the seventies, secondary metabolism was shown to be an important expression. This is the basis of the chemical heterogeneity and of the restricted distribution of the secondary products as well as the finding that the producer organism rather than the producer cell benefits from them. Considering the expression of secondary metabolic pathways as a process of cell differentiation, offers the possibility of defining secondary metabolism in terms of molecular biology and may promote a better understanding of the most interesting general aspects of this field (Luckner, 1984).

The characteristic aroma of a food material is due to the volatile flavour components, which are generally low molecular weight compounds; contain carbon, hydrogen and oxygen / nitrogen / sulphur atom. Flavour is a complex appreciation of the total sensations perceived whenever food or drink is consumed. Flavour is the sensation caused by those properties of any substance taken into the mouth that stimulates one or both of the senses of taste and smell or also the general pain, tactile and temperature receptors in the mouth.

Flavour helps human beings and animals to recognize the food and evaluate its edibility and it stimulates secretions needed for digestion. The volatile components of flavours generally referred to, as aroma components are responsible for their characteristic odour. By virtue of their volatile nature they play a significant role in the reproductive processes of both plants and animals (Srinivasa Rao, 1990).

The most important natural sources of flavours are the essential oils derived from spices, fruits and flowers, extractives and exudates (resins) from certain other plant sources and certain animal secretions *e.g.,* Musk. Essential oils are aromatic substances that are widely used in the perfume industries, in the pharmaceutical sector and in the food and human nutrition field. These are mixtures of more than 200 compounds (Shaw, 1979), that can be grouped basically into two fractions, a volatile fraction, that constitutes 90–95% of the whole oil and contains monoterpenes and sesquiterpene hydrocarbons and their oxygenated derivatives, along with aliphatic and aromatic aldehydes, alcohols and esters and a non-volatile residue, that constitutes from 5 to 10% of the whole oil and contains hydrocarbons, fatty acids, higher terpenoids, sterols, carotenoids, waxes, coumarins, psoralens, alkaloids and flavonoids. Moreover, since terpenes are mostly unsaturated compounds, these are decomposed by heat, light and oxygen to produce undesirable compounds, which can give off-flavours and off-aromas. The oxygenated fraction (along with sulphur / nitrogen containing fraction) is highly odoriferous and is mainly ressible for the characteristic flavours.

EXTRACTION OF SECONDARY METABOLITES FROM PLANT MATERIALS

Extraction of the secondary metabolites from the plant materials is a trial and error excise in which different solvents are tried under a variety of conditions such as time and temperature. There are three conventional types of extraction techniques. In order of increasing technological difficulty, these involve the use of solvents, steam and supercritical fluids. In recent times, world wide, water extraction is practised while making coffee or tea. Basically, pre-treated plant material is put into contact with hot water, which takes up the flavour compounds and tasting / colouring agents. After filtration, the extract is ready for consumption. In case of the isolation of certain compounds from plant material by means of liquid extraction, some technological problems have to be overcome (Starmans and Nijhuis, 1996). First the plant material has to be pre-treated in order to obtain reasonable extraction yields. Another problem is the need for special solvents to be used in the extraction procedure (Bracco et al., 1981). More recently, attention has broadened towards the isolation of specific compounds that can be used in the food industry. Of particular interest is the isolation of aromas and fragrances from plants and fruits (Cu et al., 1989; Rajagopalan and Cheryan, 1995).

Once extracted from the plant, the bioactive component has to be separated from the extract. It involves further solvent extractions and extensive chromatography keeping in view of the particular properties of the desired compound, such as acidity, alkalinity, polarity and molecular size and structure. Some times, this may involve simple crystallisation of the compound from the

crude extract, *e.g.,* isolation of the glycoside dianellin from *Dianella caerulea.* In some cases, the isolation can be assisted by the preparation of suitable derivatives, imparting more easily manageable properties of the desired compound, *e.g.,* isolation of the swainsonine as its triacetate from *Rhizoctonia leguminicola* is representative of such an approach. Final purification to provide compounds of suitable purity for structural analysis may be accomplished by appropriate techniques such as crystallisation, sublimation or distillation (Colegate and Molyneux, 1993).

ISOLATION OF FLAVOURS FROM NATURAL SOURCES

Essential oils are usually isolated from the odoriferous part of the plant by the process of steam distillation, mechanical expression, solvent extraction and enfluerage. The choice of the process depends on the nature of the plant material, the quantity and the site of the oil present and relative stability of the various components.

The isolation, concentration and purification of essential oils have been important processes for many years. The common methods used so far are mainly based on solvent extraction and steam distillation. The drawbacks linked to these techniques have led to the searching for new alternative extraction processes. Another important aspect in the essential oils industry is the improvement of the quality of the oil. The commercial methods used earlier to obtain essential oils are chromatographic separation (Kirchner and Miller, 1952), fractional vacuum distillation (Vora et al., 1983) and selective solvent extraction

(Owusu et al., 1986). All these methods have important drawbacks, such as low yields, formation of by-products (owing to the time of exposure to high temperatures) and the presence of toxic organic residues in the extracts. The implementation of new techniques is thus mandatory in this context.

Conventional Techniques

Techniques for the extraction of essential oils have been traditionally based on the use of discontinuous, continuous and hybrid approaches. The discontinuous techniques include the use of either organic solvents (sometimes assisted by ultrasound) or water, whilst steam distillation and vacuum distillation are continuous methods. Some methods involving both continuous and discontinuous approaches, such as distillation–extraction and Soxhlet extraction have also been reported (Ayala and Castro, 2001).

Discontinuous Techniques

Solvent extraction has long been used for the isolation of essential oils from natural products. This technique uses either pure organic solvents or mixtures. Organic solvent extraction assisted by ultrasounds, also known as sonication, is another technique widely used for the isolation of essential oils / extracts from plants. Thus, sonication methods based on 20-30 min extraction with methanol–chloroform mixtures have been used for the isolation of white clove essential oil prior to detection of selenium compounds (Alsing-Pedersen and Larsen, 1997) and cyanogenic glucosides (Lino and Noronha da Silveira, 1997).

The use of organic solvent extraction has its main shortcomings, solvent residues in the extract, with the subsequent toxicological risk and the long extraction time required in most cases for achieving efficient extractions. In addition, organic solvents have a low selectivity. Thus, apart from the desired substances, high molecular weight, non-volatile components, such as fatty oils, resins, waxes and colouring matters, are co-extracted. The non-feasibility of automation of the technique is another important drawback to be taken into account.

Water extraction (under ambient conditions, without the application of an auxiliary energy source) has proved to be an effective technique for the isolation of essential oil from citrus as a step prior to GC–FID analysis (Lancas and Cavicchioli, 1990). The main shortcomings of this alternative lie again in its slowness (because an equilibrium must be established between the plant material and extractant), its non-quantitative nature (because partitioning must be reached between the solid and liquid phases) and its infeasibility for automation.

Continuous Techniques

Steam distillation has been together with solvent extraction; the most widely used conventional technique for the extraction of essential oils from plants. This technique has been applied extensively as a step prior to compositional studies of essential oils, such as in the case of curcuma (Zwaving and Bos, 1992), marjoram (Komaitis et al., 1992) and of rosemary, sage and lavender (Guillén et al., 1996).

Hybrid (continuous–discontinuous) Techniques

The simultaneous combination of steam distillation and solvent extraction, usually implemented by a Likens–Nickerson apparatus or its analogues, has also been widely used for the isolation of essential oils whose composition was analysed by GC–MS, as in the case of lavender (Figueiredo et al., 1995).

RECENT TECHNIQUES

Microwave-assisted extraction (MAE) together with both supercritical fluid extraction (SFE) and continuous subcritical water extraction (CSWE) are considered here as recent alternatives for the isolation of highly valuable essential oils.

Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) (Paré et al., 1994) is a simple technique that provides a novel way of extracting soluble products into a fluid, from a wide range of materials, helped by microwave energy. The technique can be applied to both liquid phase extraction (when a liquid is used as solvent) and gas phase extraction (when a gas acts as extractant). The extraction process in liquid phase extraction (used for the isolation of essential oils from plants) is based on a basic physical principle, namely the difference in the ability to absorb microwave energy depending on the chemical nature of the molecules/ compounds from the species being subjected to microwave irradiation. The parameter generally used as a measure of this physical property is the dielectric constant. Thus, liquid phase extraction assisted by microwaves is based on the fact that it is possible to immerse the matrix to be extracted into a solvent that is

characterised both by a low dielectric constant and a relative transparency to microwaves. The first applications of the technique dealt with the extraction of essential oils from plant products (Paré et al., 1994). The kinetics of the microwave extraction of rosemary leaves in hexane, ethanol or hexane-ethanol mixtures, as well as the influence of factors such as the source of the leaf, the microwave energy, duration of irradiation and sample load, on the rate of extraction of the compounds have been reported (Chen and Spiro, 1995). In more recent research, MAE has been coupled with liquid chromatography for the determination of herbicides in plant tissue (Stout et al., 1996).

Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a relatively recent extraction technique based on the enhanced solvating power of fluids above their critical point (Luque de Castro et al., 1994; Taylor, 1996). Its usefulness for sample extraction is due to the combination of gas-like mass transfer properties and liquid-like solvating characteristics with diffusion coefficients greater than those of a liquid. The majority of analytical SFEs have been focused on the use of CO_2, because of its preferred critical properties, low toxicity and chemical inertness. Super critical carbon dioxide (SC-CO_2) extraction has also been used recently for the extraction of essential oils from plants, in an attempt to avoid the drawbacks linked to conventional techniques. Such is the case with the extraction of volatile oil in camomile flower heads (Vuorela et al., 1990). The effects of the parameters like time, temperature and pressure, have been studied for the extraction of volatile compounds from lavandin and thyme (Oszagyan et al., 1996) and from lavender and rosemary (Walter et al., 1994; Reverchon et al.,

1995). SC-CO_2 extraction is also a suitable technique for enhancing the quality of essential oils obtained by conventional extraction methods, by means of fractionation and deterpenation.

Continuous Subcritical Water Extraction

Continuous subcritical water extraction (CSWE), a technique based on the use of water as an extractant in a dynamic mode, at temperatures between 100 and 374 °C (critical point of water, 221 bar and 374 °C) and a pressure high enough to maintain the liquid state, is emerging as a powerful alternative for solid sample extraction (Luque de Castro and Jimenez Carmona, 1998). The most outstanding feature of this leaching agent is the easy manipulation of its dielectric constant (ε). In fact, this can be changed within a wide range by changing the temperature under moderate pressure. Thus, at ambient temperature and pressure, water has a dielectric constant of *ca*. 80, making it an extremely polar solvent. This parameter is drastically lowered by raising the temperature under moderate pressure. The use of this technique in the field of essential oil isolation is recent and very promising. Thus, subcritical water under pressure between 125 and 175 °C has been shown to extract the oxygenated fragrance and flavour compounds from rosemary, whilst the monoterpenes are extracted slowly and only very small amounts of the sesquiterpenes, waxes and lipids are removed. The extraction rate of the process is determined by the partition of the compounds between the plant material and the water and not by the rate of diffusion of the compounds out of the plant material. It can also be inferred that the composition and quality of the oil can be adjusted by controlling the amount of water relative to the mass of plant material (Basile et al., 1998).

Similar trends are observed for the isolation of marjoram essential oil with water. An in-depth study of the variables affecting the extraction process was carried out in this case. The temperature was found to be the key variable. Its influence was studied between 100 and 175 °C and a value of 150 °C was the optimum, because it provided the best quality essential oil (in terms of the amount of oxygenated compounds) (Jiménez Carmona et al., 1999).

SEPARATION AND STRUCTURE DETERMINATION

The wide range of spectroscopic techniques such as Ultra violet (UV), Infrared (IR), Gas Chromatography - Mass spectroscopy (GC-MS), Liquid Chromatography - Mass spectroscopy (LC-MS) Nuclear Magnetic Resonance (NMR) and mass spectroscopy (MS) form the backbone of modern structural elucidation studies. Prior to the availability of such advanced techniques, ambiguities existed in the determination of structures. The process of spectroscopic determination should be closely allied to familiarity with the scientific literature. If the compound has already not been described, it may be very similar to reported compounds and that will assist in the interpretation of the data for the unknown. In this regard, an awareness of the co-extractives from the plant may also be of value.

The degradation process involved the treatment of the unknown compound with functional group specific reagents or degradation of the compound in a predictable manner until a compound of known structure is obtained. Backtracking should then provide a structure for the unknown compound. Apart

from being a time consuming and exacting art, this method can be loaded with difficulty and ambiguity. If structural analysis data are ambiguous and the compound not amenable to an X-ray diffraction study, then chemical synthesis from precursors of known structure and stereochemistry is usually sufficient to prove or disprove an established structure. The chemical literature abounds with examples of unambiguous synthesis, which are vital in finally establishing a structure for an unknown compound (Colegate and Molyneux, 1993).

Ultra-Violet Absorption Spectroscopy

The ultra violet spectrum is measured over the range of 200-400 nm. This is used to study nature of the chromophore present in the molecules; e.g. It is useful to know the properties of colourless solutions and for determination of rancidity in oils by measuring the absorbance at different wavelengths, which indicates the nature of conjugated double bonds formed.

Infrared Absorption Spectroscopy

Infrared (IR) spectra are obtained where light energy in the IR region at a given frequency is absorbed by a molecule, thereby increasing the vibrations of bonds between the atoms in the molecule. IR spectrum is scanned over a range of wavelength of 2.5 - 15 μm. It can give the information regarding the nature of functional groups present in the molecules; e.g., double bonds, carbonyls, hydroxyls, amines, nitriles, amides. In addition, it can give the nature of molecule whether it is aromatic or aliphatic.

Gas Chromatography

The development of gas chromatography (GC) in the mid 1960s and subsequent application to flavour research has resulted in many compounds being identified. GC is ideally suited to flavour studies, since it has excellent separation powers and extreme sensitivity (picogram detection levels). Mostly capillary columns with dimethyl polysiloxanes (methyl silicone) non-polar and carbowax 20M polar phases are used. Carbowax 20M phases include DB-Wax, BP-20, PEG-20M and HP-20, while methyl silicone phases include SE-30, SF-96, OV-1, OV-101, BP-1, CP-SIL 5CB, SP-2100, DB-1, DB-5 and HP-1 (Davies, 1990). Capillary column GC has become the standard for work in the flavour area. Resolving power of a capillary column is multiple folds better than the best-packed column.

Capillary columns provide very reproducible retention times in view of their high resolving power. This is essentially helpful in computerised, pattern recognition of GC profiles and subsequent identifications based on Kovats indices (Davies, 1990; Stashenko et al., 1996). The fused silica columns offer exceptional durability and chemical inertness. A typical fused silica column can detect components in less than 100 ng of concentration in a volatile mixture.

One of the most important developments in gas chromatography has been the introduction of enantioselective capillary columns with high separation efficiency of stereoisomers in the mid-1960s by Gil-Av et al. (1966). The first optimum performance was reached in the development of Chirasil-Val, a methyl–

polysiloxane phase containing about 6% branched aliphatic side chains with L-valine in diamide linkage and similar polymeric chiral diamide stationary phases which possessed high thermal stability (Konig, 1987). Finally, the introduction of capillary columns using different hydrophobic cyclodextrin derivatives with a higher thermal stability considerably improved the separation facilities (Kubeczka et al., 1991; Konig, 1998).

In gas chromatography, FID is the most common detection method used. Nitrogen-containing compounds can be identified by nitrogen–phosphorus detection (NPD) (Stashenko et al., 1996). Another possibility is the use of oxygen flame ionization detection (O-FID) for the selective determination of oxygenates (Kubeczka et al., 1991, Betts, 1994). This application seems to be particularly useful if very small samples have to be analyzed, e.g., from tissue cultures or oil glands.

Gas chromatography and Mass spectrometry

With the advent of high-resolution capillary GC using fused-silica columns, separation of complex mixtures of volatile organic compounds was possible and the number of compounds isolated from essential oils has increased in recent years. Against this background identification, only by GC retention data and Kovats retention indices alone has become uncertain. Presently, the combination of gas chromatography and mass spectrometry in the electron impact mode (GC–EI-MS) is a well-established technique for the routine analysis of essential oils. This technique offers the possibility to gain additional information by mass spectra (Kubeczka et al., 1991). Many a times, it has to be

emphasized, that identification of volatile organic compounds based only on mass spectra is not completely justifiable. Molecular rearrangement and isomerisation processes of unsaturated hydrocarbons result in very similar mass spectra lacking characteristic fragmentation patterns. Only combined data of retention times, Kovats or Sadler retention indices (Richmond and Pombo-Villar, 1997) and mass spectral data offer the possibility of an unambiguous identification of volatile organic constituents.

Nevertheless, sometimes-mass spectra scanned may not give entire information; these may miss the molecular ions; e.g., esters. In this case, the application of the chemical ionisation (CI) mode using various reagent gases in GC-MS, gives valuable additional information. For sesquiterpenes, it was reported that the use of ammonia was by far superior to isobutane. An additional method is negative ion chemical ionisation (NCI) with OH^- as the reactant ion (Hendriks et al., 1985; Cazaussus et al., 1988).

GC-MS is a very useful tool for the analysis of complex mixtures, but some times identification is limited when a single chromatographic peak contains several compounds so that the recorded mass spectra is difficult to interpret. There are several techniques to solve this problem. One is MS–MS (tandem mass spectrometry) which, when coupled with GC, allows separation and analysis of each component from such complex peaks. Moreover, the presence of minor constituents can also be confirmed (Cazaussus et al., 1988; Sellier, et al., 1991).

The bench-top quadrupole mass spectrometers have been around longer than the ion trap MS, which was introduced in the late 1980s. As the quadrupole is also a bit cheaper than the ion trap, it is the most popular in the perfume laboratory. However, ion trap mass spectrometers offer a number of positive features, when compared to the quadrupole mass spectrometers (Hubschmann, 2001) like higher sensitity in the full-scan mode; switching between EI and chemical ionisation (CI) without conversion and ability to perform MS/MS experiments.

Consequently, structure elucidation is required to obtain more insight into the new compound. In this task, MS also plays a crucial role, as often the quantities are too low to enable straightforward identification via NMR. The EI-MS spectrum can be very informative, but is often not sufficient by itself to provide a positive identification. Chemical ionization can be used to determine the molecular weight of the unknown, especially for molecules that exhibit extensive fragmentation during EI. In addition, accurate mass measurements with sector or Time of flight (TOF)-MS instruments can be employed to obtain the molecular formula (Hubschmann, 2001).

The principal limiting factor upon the use of the method is the volatility of the compounds and modern commercial GC-MS systems are designed almost exclusively for the analysis of volatile compounds.

Gas Chromatography–FT-IR Spectroscopy

GC-MS is considered to be the method of choice in the identification of volatile compounds. However, in distinguishing isomers, which often occur in the terpene group, capillary GC-FT-IR coupling offers a useful supplementation. Advantages of FT-IR spectroscopy are high resolution and sensitivity. The more time-consuming interpretation and the absence of a database of reference vapour phase spectra may be reasons why this technique is not generally used for the analysis of volatile compounds. However, a GC-FT-IR-MS instrument is available, whereby simultaneously IR and mass spectra can be obtained. Thus, the unambiguous identification of critical isomeric sesquiterpenes is possible (Herres, 1987).

Nuclear Magnetic Resonance (NMR)

NMR is a phenomenon, which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin.

^{1}H NMR

The ^{1}H nucleus is the most commonly observed nucleus in NMR spectroscopy. Hydrogen is found throughout most organic molecules and, fortunately for chemists, the proton has high intrinsic sensitivity as well as being almost 100% abundant in nature, all of which make it a favourable nucleus to observe. The proton spectrum provides a wealth of information with regard to the chemical shifts, multiplicity and coupling constants and is the starting point for most structural determinations. In addition to these data, the area of a

resonance, usually presented as its relative integral, relates to the number of protons giving rise to the signal, providing further information as to a possible structural fragment. However, for most routine acquisitions, the accuracy of such integrals is not rational and may commonly show errors of ten percent or more. This level of accuracy is usually sufficient to decide whether it is one or two protons that are giving rise to a particular resonance, but are unsuitable to get an accurate determination of say, the relative proportion of two isomers in a mixture (Harwood and Claridge, 1997).

Homonuclear decoupling in ^{1}H NMR

The appearance of multiplet fine structure on NMR resonances is due to the presence of scalar coupling with another nucleus. In proton spectroscopy, such coupling will usually occur over two or three bonds (geminal or vicinal coupling, respectively) and its presence provides direct evidence of connectivity within a structure. If the connectivity between all atoms in a structure is known, the gross structure is, therefore, defined. Coupling partners can often be identified by direct analysis of multiplet fine structure. If proton A shows a coupling to proton B of 7 Hz, then the coupling from B to A is also 7 Hz. However, it is often not possible to identify coupling partners in this way; the multiplet may be too complex to determine all coupling constants; one or both, protons may be hidden under another resonance or many multiplets may display a coupling similar in magnitude to the one of interest. Homonuclear decoupling (which belongs to a class of experiments known as *double-resonance* experiments) offers a simple and effective means to identify coupled protons. The idea is to selectively saturate one multiplet in the spectrum with a radio frequency during acquisition.

This causes a loss of all couplings with the saturated proton and, hence, the multiplet structure of its partners will change (Gunther, 1995). The multiplet structures of the resonance's in this area are quite complex and do not lend themselves to direct analysis. A simple homonuclear decoupling experiment with saturation of the proton on a selected carbon unambiguously identifies the protons on adjacent carbons by removal of the vicinal couplings.

2D ^{1}H-^{1}H Correlation Spectroscopy (COSY)

The COSY experiment provides a means of identifying mutually coupled protons and is the most widely used 2D experiment. It finds use when the homonuclear decoupling experiment is unsuitable, for example in complex spectra, where selective decoupling is not possible because of resonance overlap. The COSY experiment is a very efficient way of establishing links when a large number of coupling networks need to be identified, as it maps all correlations with a single experiment and is now more frequently used than homonuclear decoupling (Friebolin, 1991; Williams and Fleming, 1995).

The experiment presents a two-dimensional contour map, each dimension representing proton chemical shifts and the contours representing signal intensity (just as contours are used to map mountains heights). The diagonal (running bottom left to top right) shows peaks that correspond with those in the usual ^{1}D spectrum, and contain no new information. The peaks of interest are the off-diagonal or cross peaks. Each of these represents a coupling between the protons that are correlated by the cross peak. The spectrum is symmetrical

about the diagonal, as the coupling from proton A to B will always be matched with one from B to A (Friebolin, 1991; Williams and Fleming, 1995).

Double Quantum Filtered COSY (DQF-COSY)

The double quantum filtered experiment detects a phenomenon known as a double (or two) quantum coherence. Since two (and higher) quantum coherence's can only be observed in first and higher order spin systems, the resulting spectra are somewhat simplified. Peaks on the diagonal are greatly reduced in intensity (since these represent single quantum transitions) with the consequent clarification in this region and much reduced *t*1 noise. A further advantage of DQF-COSY, is that in the phase sensitive mode, both diagonal and cross peaks can be adjusted to have a pure absorption line shape. Since this spectrum was recorded with a particular frequency the resolution (2.0 Hz/point) is quite high for a 2-D spectrum and the coupling patterns are apparent in both the diagonal and cross peaks. As the frequency range becomes larger and the resolution smaller, the structure of the coupling may not be resolved and it is more common for the cross peaks to look like single spots. This is especially true with magnitude mode (non phase sensitive) spectra where the intensity is positive everywhere (Braun et al., 1998).

^{13}C NMR

The ^{13}C NMR spectrum offers further characterisation of the molecule as it relates directly to the carbon skeleton. The resonance of carbon-13 nucleus is observed, as carbon-12 has no nuclear spin and is "NMR silent". Unfortunately,

^{13}C has a lower intrinsic sensitivity than the proton and has only 1.1 % natural abundance. The low abundance means that direct ^{13}C -^{13}C coupling is usually never seen in the spectrum, so carbon-carbon connectivity assignments are not possible directly. Each carbon may be coupled to a number of protons in the molecule, typically over one, two or three bonds such that the resulting carbon resonances have complex structure, which further reduces signal intensity by spreading the resonance. The spectrum is, therefore, usually recorded with broadband decoupling of all protons. This removes multiplicity in carbon resonances, so that the doublet, triplet and quartet patterns indicative of CH, CH_2 and CH_3 groups, respectively, are not seen and each carbon resonance appears a singlet (so increasing sensitivity). Typically one signal is observed for each carbon atom in the molecule, as resonance overlap is rare. The broadband decoupling produces saturation of the proton resonances and this generates a nuclear overhauser enhancement of the carbon signal, further increasing signal intensity. The chemical shift of each resonance is again indicative of environment, and it is possible to identify certain functional groups for which there is no direct evidence in the proton spectrum e.g. carbonyls and quaternary carbons (Friebolin, 1991).

Distortionless Enhancement by Polarisation Transfer (DEPT)

DEPT is used as a means of enhancing signal intensity and for editing spectra. As stated above, broadband proton decoupling removes multiplicity in carbon resonances, but the DEPT sequence allows one to establish the nature of the carbon atom, whilst still acquiring broadband decoupled spectra, by making use of changes in signal intensities under differing experimental

conditions. Three DEPT spectra are required for a full analysis and are termed DEPT-45, DEPT-90 and DEPT- 135 (the number indicates the flip angle of the editing proton pulse in the sequence). The signal intensities in these spectra are as follows:

	CH	CH_2	CH_3
DEPT-45	+ve	+ve	+ve
DEPT-90	+ve	zero	zero
DEPT-135	+ve	-ve	+ve

Non-proton-bearing carbons are not seen in DEPT spectra, because the technique relies on polarisation transfer, that is, in this case, the transfer of proton magnetisation onto the *directly bound* carbon. Analysis of the three spectra reveals the carbon multiplicities directly, or these may be combined appropriately to yield sub-spectra containing CH or CH_2 or CH_3 resonances only. Often, it is not necessary to acquire all three experiments to assign multiplicities. Methyl resonances are often easily identified as they frequently resonate at lower frequency, so a DEPT-135 alone may be sufficient to distinguish between methine and methylene protons (Friebolin, 1991; Williams and Fleming, 1995).

2D ^{1}H-^{13}C Heteronuclear Multiple-Quantum Correlation (HMQC)

The HMQC experiment is a *heteronuclear* correlation technique that offers a means of identifying single bond H-C connectivities within a molecule. The results are displayed in a similar manner to those from COSY, with one dimension of the 2D map representing ^{13}C chemical shifts and the other representing ^{1}H chemical shifts. Crosspeaks in the contour plot define to which

carbon a particular proton (or group of protons) is attached, and it is therefore possible to map ^{13}C assignments from known ^{1}H assignments. The technique relies on magnetisation transfer from the proton to its directly attached carbon atom, and back onto the proton (for higher sensitivity) and so no responses are to be expected for non-protonated carbons or for protons bound to other heteroatoms. Usually, one peak is observed at the frequency of each protonated-carbon resonance, although occasionally two are seen and this is indicative of a diastereotopic CH_2 group (Friebolin, 1991; Williams and Fleming, 1995).

The HMQC experiment is also useful in the analysis of complex ^{1}H spectra as it provides dispersion of the proton spectrum along the ^{13}C dimension. Thus, a mass of overlapping multiplets in the proton spectrum can be spread apart by differences in ^{13}C chemical shifts, so allowing the ^{1}H chemical shift of each multiplet to be recognised. ^{1}H -^{1}H COSY and ^{1}H -^{13}C HMQC represent the primary 2D techniques of organic chemistry (the HSQC experiment provides equivalent data to HMQC and is often seen as an alternative in the literature).

2D Heteronuclear Single-Quantum Correlation experiment (HSQC)

HSQC permits to obtain a 2D heteronuclear chemical shift correlation map between directly bonded ^{1}H and X-heteronuclei (commonly, ^{13}C and ^{15}N). It is widely used, because it is based on proton-detection, offering high sensitivity when compared with the conventional carbon-detected 2D HETCOR experiment. Similar results are obtained using the 2D HMQC experiment (Williams and Fleming, 1995).

2D ^{1}H - ^{13}C Heteronuclear Multiple-Bond Correlation (HMBC)

This experiment is closely related to HMQC and operates in essentially the same manner. In this case, however, the sequence timings are optimised for much smaller coupling constants and therefore seek the correlations across more than one bond that arise from so-called long-range couplings. These ^{1}H - ^{13}C couplings typically occur with significant intensity over only 2 and 3 bonds (^{n}JCH usually <10 Hz), but may be apparent over 4 bonds in conjugated systems. Such experiments contain a mass of data on the molecular skeleton and can be extremely powerful tools in structure elucidation. The sequence also produces correlations across heteroatoms other than carbon. Those prove useful when attempting to link fragments identified, for example, from ^{1}H -^{1}H correlation spectra, and which show no ^{1}H -^{1}H couplings between the fragments themselves. Correlations can also be observed to quaternary centres and can be used, for example, in the assignment of carbonyl resonances. Note however, that as this experiment relies on correlations through small couplings, it is significantly less sensitive than the HMQC experiment (Williams and Fleming, 1995).

PART- A

Chapter - 2

Chemical examination of the volatiles from the unconventional parts of Cinnamon

2.0. A BRIEF INTRODUCTION ON *CINNAMOMUM ZEYLANICUM* AND SCOPE OF INVESTIGATION AND OBJECTIVES

The genus *Cinnamomum,* comprises several hundreds of species, which are distributed in Asia and Australia. These are evergreen trees and shrubs and many species are aromatic. *Cinnamomum zeylanicum,* the source of cinnamon bark and leaf oils, is an indigenous tree of Sri Lanka, although most oil now comes from cultivated areas. Smaller areas of wild trees are also found in south-western parts of India. *Cinnamomum cassia*, the source of internationally traded cassia oil, occurs wild as a bush in the mountains of Southern China, but is now cultivated for oil production, mainly in the provinces of Kwangsi and Kwangtung. The other cassia occurs wild on the islands of Sumatra and Java in Indonesia (*Cinnamomum burmannii*); in Vietnam (*Cinnamomum loureirii*) and India and Nepal (*Cinnamomum tamala*) (The Wealth of India, 1992).

The species from *Cinnamomum* genus yields volatile oil on distillation. The most important *Cinnamomum* oils in world trade are those from *C. zeylanicum* bark and leaf oils, *C. cassia* (cassia oil) and *C. camphora*. However, a number of other *Cinnamomum* species are distilled on a smaller scale and the oils are used either locally or exported to regional markets (The Wealth of India, 1992).

Botanical and common names

- *Cinnamomum verum* Presl (syn. *C. zeylanicum* Nees) True or Ceylon cinnamon
- *C. cassia* Presl Cassia, Chinese cinnamon, "Cassia lignea"

- *C. burmannii* Blume Indonesian cassia
- *C. loureirii* Nees Vietnamese cassia
- *C. tamala* (Buch.-Ham.) Nees & Eberm.Indian cassia.

CHEMICAL CONSTITUENTS OF CINNAMON VOLATILE OIL

Krishna et al. (1946) reported the chemical composition of *C. zeylanicum* leaves, which contains 70-90% of eugenol. It was concluded that, this forms an alternative source of clove oil. Paillot-Cressole and Duquenois (1952) reported 0.75-2.35% essence content from barks of *C. zeylanicum*, which were collected from Madagascar. Schantz et al. (1962) reported the separation and identification of cinnamaldehyde and linalool from *C. zeylanicum* oil by TLC.

Genin et al. (1971) reported the development of a gas chromatographic determination of the chemical composition of the essential oils of spices which include cinnamon, permitting rapid analysis and identification of the compounds. Rogers et al. (1971) reported that essential oil of cinnamon bark obtained from Sri Lanka contained cinnamaldehyde (60-70%) and cinnamyl acetate (8-10%) along with other minor constituents. The root bark of *C. zeyalnicum* grown in Ghana was reported to contain the highest amount of oil (2.8%) with camphor as the major compound (Angmor et al., 1972). Straus and Wolstromer (1974) reported that the major component of cinnamon bark oil as *(E)*-cinnamaldehyde. The gas chromatographic analysis of the volatile oils of the leaves, stem-bark and root-bark of commercial cinnamon grown in Sri Lanka revealed interesting variations among the oils (Wijesekera et al., 1974). All the three oils possessed the same array of monoterpene hydrocarbons though in different proportions.

The main constituents of leaf, stem bark and root bark oils were found to be eugenol, cinnamaldehyde and camphor, respectively (Figure 2.1), while α-ylangene, methyl and ethyl cinnamate in leaf oil, benzyl benzoate in bark oil and terpinene-4-ol in root-bark oil were reported for the first time (Figure 2.1).

Angmor et al. (1975) studied the composition of oils obtained from a single tree of *C. zeylanicum* growing in Kumasi, Ghana, during 4 years 1971-1974. Gas chromatography of volatile oils from bark samples subjected to steam distillation immediately after collection indicated a higher proportion of cinnamyl acetate in young bark than aged. 'Fermented' barks, i.e. simulating commercial preparation gave oils with generally lower cinnamyl acetate and increased cinnamaldehyde contents, particularly in older barks. It was concluded that, due to the presence of hydrolytic and oxidative enzymes in bark and their activation during commercial bark preparation may be accounted for the small proportion of cinnamyl acetate (about 3.0%) in commercial volatile oil. However, oil distilled from fresh bark samples of Ghanaian trees was shown to contain a high proportion of cinnamyl acetate (6-26%). Isogai et al. (1976) isolated two compounds named as cinnzeylanine and cinnzeylanol from dried bark of *C. zeylanicum* (Figure 2.1). Lawrence (1977) reported the notes on intra specific chemical differences on the oils of cinnamon, clove, costus root, dill, ginger, laurel leaf, origanum, patchouli, sage and tangerine.

The analysis of *C. zeylanicum* leaf, stem bark, and root bark oils indicated 72 compounds, out of which 32 compounds were reported for the first time in

cinnamon oils (Senanayake et al., 1978). All three oils had a similar array of compounds but in varying proportions. The new compounds reported, were 11 monoterpenes, 4 sesquiterpenes, 2 aliphatic, and 15 aromatic compounds. Senanayake (1978) reported the distribution and chemical composition of *C. zeylanicum, C. cassia* and *C. camphora*. GC studies on commercial samples of steam distilled oils of leaf, stem bark and root bark of *C. zeylanicum* revealed the presence of more than 75 volatile compounds in each type of oil. Volatile composition of leaf and root bark oils was unaffected with age, while that of stem bark oil varies slightly with age, the stem bark oil from two and half year old plants having more terpenoids and less cinnamic aldehyde. *C. cassia* oil possessed predominantly aromatic (benzenoid) compounds, such as cinnamic aldehyde, whereas *C. camphora* oil has mainly terpenoid compounds, like camphor and cineole. The biosynthetic pathways of the major volatiles of *C. zeylanicum* are examined (Senanayake, 1978). All parts of the *C. zeylanicum* plant are capable of synthesising the major volatiles found in the cinnamon oils. However, the leaf is the predominant site of eugenol and cinnamaldehyde synthesis.

The essential oil obtained from cinnamon leaves has a high content of eugenol, whereas the cinnamon bark has low eugenol content. Determination of the eugenol content of cinnamon bark oil by GLC using a non-polar stationary phase provides a method for the detection of adulteration with leaf oil (Analytical Methods Committee, 1981). Tateo and Chizzini (1989) reported the extraction of coarsely ground cinnamon with supercritical CO_2 at 50 °C using 300, 400, 500

and 600 bar pressure. Oleoresin and essential oil were produced from the same ground material by refluxing with CH_2Cl_2 and water distillation, respectively. Volatile composition of the supercritical extracts, oleoresin and essential oil were determined by GC. The supercritical extracts did not differ significantly from each other, the major component being cinnamaldehyde, varying from 77.2-81.5% and eugenol from 1.6-2.3%. The oleoresin and volatile oil contained 83.19 and 75.72% cinnamaldehyde, respectively.

Vernin et al. (1990) reported twenty-five components from Sri Lankan cinnamon bark oil using GC-MS analysis with *(E)*-cinnamaldehyde as the major compound (72 - 82%). Cheng and Yu (1991) reported twenty-two compounds from bark oil of cultivars of *C. zeylanicum* using GC-MS with *(E)*-cinnamaldehyde (31.2%), *p*-cymene (12.0%) and α-phellandrene (8.9%) as the major compounds (Figure 2.1 and 2.2). It seems that there is a wide range of variation in chemical composition of bark obtained from trees harvested from the forest. The TLC analysis of essential oils from leaf, stem-bark and root-bark of the genus *Cinnamomum* from Sri Lanka showed cinnamaldehyde, eugenol, linalool, α-terpineol, acetyleugenol and cineole as the main constituents (Figures 2.1 and 2.2) (Sritharam et al., 1994). There were some variations in the main constituents of leaf and stem-bark oils among the species. Major constituents in oils of stem-barks were cinnamaldehyde in *C. citriodorum* and *C. verum*, linalool in *C. dubium* and *C. ovalifolium*, α-terpineol in *C. litseaefolium* and eugenol in *C. capparucoronde* and *C. rivulorum*. In the oils isolated from the leaf, the principal constituents were eugenol (*C. capparucoronde, C. rivulorum, C. sinharajanse*

and *C. verum*), linalool (*C. ovalifolium*) and citronellal (*C. citriodorum*) (Figures 2.1 and 2.2). The presence of citronellal in *Cinnamomum* species was reported for the first time. Camphor was found to be the major component in the root-bark oils of all the species examined.

Vernin et al. (1994) analysed the *C. zeylanicum* leaves volatile oils by GC and GC-MS, where they identified *p*-cymene (21.35%) and eugenol (16.7%) as major components. Ehlers et al. (1995) identified the volatile constituents in cinnamon bark oil with (*E*)-cinnamaldehyde as the major compound (56.0%). Forty-seven chemical constituents were identified using GC and GC-MS analysis of essential oils from cinnamon leaf grown in Bangalore and Hyderabad (India) with eugenol (81.4-84.5%) as the major component (Mallavarapu et al., 1995). These two samples differed with respect to relative amounts of linalool, *(E)*-cinnamaldehyde, *(E)*-cinnamyl acetate, β-caryophyllene and benzyl benzoate (Figures 2.1 and 2.2). Yield of volatile oil content of the Hyderabad sample (4.7%) was higher than that of the Bangalore (1.8%).

Quirin and Dick (1995) reported the supercritical CO_2 extraction of vanilla, cinnamon and paprika. It was concluded that, varying supercritical CO_2 extraction pressures could result in fractions rich in selected ingredients; concentration of desired ingredients due to the high solvent selectivity. Using GC and GC-MS analysis, Nath et al. (1996) reported seventeen components representing 97.0 and 98.9% of volatile oils from leaf and stem bark, respectively, obtained by hydrodistillation of *C. zeylanicum* grown in

Brahmaputra Valley, India. Benzyl benzoate was the main component of both oils, however, levels were higher in stem bark oil (84.7%) than in leaf oil (65.4%).

Prakash Rao et al. (1996) reported volatile oil constituents from cinnamon leaves grown in India with linalool (1.07%), β-caryophyllene (1.96%), benzyl benzoate (0.20%) and eugenol (84.5%) as markers for their study. Miller et al. (1996) reported chromatographic profiles of semi - volatile compounds from true cinnamon and cassia by headspace solid-phase micro extraction. GC equipped with ion trap MS was used to identify the major volatile compounds in cassia and true cinnamon. Extracts isolated by solid-phase microextraction contained relatively higher concentrations of terpenes than the solvent-assisted supercritical fluid extracts, but these compounds were of little value in distinguishing the botanical origin of authentic cinnamon and cassia samples. The latter were easily distinguished by the presence of eugenol and benzyl benzoate in true cinnamon that was absent in cassia and the presence of coumarin and δ-cadinene in cassia that was either absent or low in concentration in true cinnamon. It is concluded that headspace solid-phase microextraction provides a rapid and simple method for establishing the botanical origin of commercial cinnamon.

The leaves and barks were harvested from 4 year old *C. zeylanicum* trees which were subjected to a single organic manuring (M) or to chemical fertilization (C) (Koketsu et al., 1997). Volatile oils were isolated by steam distillation and headspace analysis of volatiles was carried out by GC and GC-MS. The yields

of essential oils in bark from M and C trees were 0.17% and 0.27%, respectively and in leaves were 2.17% in M and 2.16% in C samples. A total of 21 compounds were quantified; cinnamaldehyde and eugenol were the major aroma compounds in bark (54–58% and 11–14% of total aroma compounds, respectively) and eugenol predominated in leaves (55-59% and 94-95%, depending on tree) followed by safrole. However, the fertilization methods were not found to have a significant effect on concentration of cinnamon essential oils or its composition.

The GC and GC-MS analysis of cinnamon oil of Madagascar origin indicated the major compounds (*E*)-cinnamaldehyde (41.3%) (Mollenbeck et al., 1997). Furthermore, using chiral GC, the enantiomeric distribution of linalool and terpinen-4-ol were determined to be (3R)-(-)-linalool (95%), (3S)-(+)-linalool (5%) and (4R)-(-)-terpinen-4-ol (69%) and (4S)-(+)-terpinen-4-ol (31%). Cinnamon bark oil produced from Madagascar cinnamon was analysed by Chalchat and Valade (1998), where (*E*)-cinnamaldehyde (52.2%) and camphor (15.2%) were found to be the major compounds. Some commercial samples of cinnamon bark oil of Sri Lankan origin were analysed using GC and GC-MS by Jirovetz et al. (1998) and the major compound was found to be (*E*)-cinnamaldehyde (61.4-75.6%).

A commercial oil of cinnamon that was screened for its antimicrobial and antioxidant properties by Baratta et al. (1998) was reported to possess the (*E*)-cinnamaldehyde (68.4%) as the major compound. The occurrence of traces of

dihydrolinalool and high level of limonene (13.2%) infer that the oil used in this screening study was adulterated.

Jirovetz et al. (2001) reported the chemical composition of an essential oil produced by hydrodistillation of leaves of *C. zeylanicum* collected from South India using GC and GC-MS and analysis. Thirty-three constituents of the oil were identified, the predominant component being linalool, which accounted for 85.7% of the essential oil. Eugenol (3.1%) and β-caryophyllene (2.4%) were the second and third most abundant compounds, respectively. This was the first report that cinnamon leaf oil was found to contain linalool as the major compound. Raina et al. (2001) have determined the composition of *C. zeylanicum* leaf oil obtained from plants cultivated in Little Andaman (India), and compared it with the composition of cinnamon leaf oil cultivated in Southern India. Forty-seven compounds were identified with eugenol, linalool and piperitone are the predominant compounds at 76.6, 8.5 and 3.3%, respectively. Oil composition was considered similar to that produced in the traditional area of cinnamon cultivated in Southern India, particularly in terms of its eugenol and linalool concentration.

BIOLOGICAL ACTIVITIES

Antioxidant Activities

Na Mi Kim et al. (1993) reported the effects of extraction under different conditions *viz.,* temperature, time, quantity of the solvent used and number of cycles of extractions on antioxidative properties of dried cinnamon extracts.

Twelve solvents were used for extraction - such as distilled water, 70% ethanol, 70% methanol, methanol, ethanol, butanol, ethyl acetate, acetone, chloroform, ethyl ether, petroleum ether and hexane. Extracts obtained with water and 70% ethanol as solvents were reported to possess highest antioxidant activity. These solvents were used for further study to optimise the extraction conditions. Antioxidative activity and percentage of total phenol were found to be highest of the extract obtained when the material was extracted for 8 h at temperature 80 °C.

Na Mi Kim et al. (1994) attempted the extraction of dried cinnamon by enzymatic hydrolysis using cellulase, hemicellulase, pectinase, β-glucosidase and tannase or lipase at 0-2.0% concentration for 2 h, followed by water extraction at 80 °C for 2 h. Enzymatic hydrolysis resulted a slight increase in cinnamic aldehyde content of extracts.

Seok et al. (1995) reported the superoxide dismutase (SOD) activity of natural antioxidants by measuring the inhibition of pyrogallol autoxidation that was catalysed by the superoxide radical. Cinnamon oil, γ-oryzanol, extract of rosemary leaf, L-α-lecithin, and L-α -cephalin exhibited activity, although the activity.

Sung et al. (1999) reported the *in vitro* antioxidative activities of 33 plant-derived essential oils and 37 phytochemicals, using inhibition of linoleic acid autoxidation, generation of superoxide anions and radical scavenging by 1,1-

diphenyl-2 picrylhydrazyl (DPPH). Antioxidative activities were compared to those of the widely used plant-derived antioxidants pyrogallol and quercetin, and to the synthetic antioxidant BHT. At 0.01% concentration essential oils from cassia (*C. cassia*) roots, mint leaves, ginkgo nuts (*Ginkgo biloba*) and clove flowers were highly antioxidative. Of the phytochemicals used, eugenol and isoeugenol at 0.01% showed potent antioxidative activity, similar to that of pyrogallol, quercetin and BHT. It was concluded that cinnamon root, mint leaf, ginkgo nuts and clove flower-derived materials may be a good source of alternative antioxidants for use in the food industry.

The antioxidant activity of essential oils and hexane extracts from rosemary, ginger, cinnamon and lemongrass was studied using the Schaal oven test (Dang et al., 2001). Antioxidant activity was found to be in the order of rosemary extract > ginger extract > cinnamon essential oil > cinnamon extract > rosemary essential oil.

Badei et al. (2002) reported the possible use of spices such as cardamom, cinnamon or cloves as antioxidants in cookies. Sensory thresholds of these spices and their essential oils in cookies were determined. Stability of lipids, refractive index, acid value, peroxide value and TBA value in experimental batches of cookies were monitored during storage for up to 8 months at ambient temperature. All the spices and their essential oils increased oxidative stability of lipids in cookies. However, essential oils were more effective than the corresponding spices.

Antimicrobial Activities

Volatile oils and aqueous alcohol extracts of infusions, of the spices were tested with thirteen Gram-negative and Gram-positive bacteria and thirteen yeasts and moulds. It was observed that the essential oils of mustard and cinnamon and extracts of juniper had pronounced antimicrobial effect (Galli et al., 1985).

The development of active packaging materials for the preservation of meat products and the efficacy of several approved antibacterial agents (fatty acids and essential oils) to control the growth of meat spoilage organisms were studied by Ouattara et al. (1997). Minimum inhibitory concentration of the fatty acids and essential oils were determined against *Brochothrix thermosphacta, Carnobacterium piscicola, Lactobacillus curvatus, L. sake, Pseudomonas fluorescens* and *Serratia liquefaciens*. Generally, these strains were also able to overcome the effects of essential oils after 24 h exposure. The most effective essential oils were pimento, cinnamon, clove and rosemary; at least 5 species of the bacteria tested were inhibited by a 1/100 dilution of these oils. Results demonstrated a relationship between the inhibitory effect of essential oils and the presence of cinnamaldehyde and eugenol. Ying Rong Yong et al. (1998) reported the antimicrobial and antioxidant effects of adding ginger, cinnamon, anise or coriander powders or essential oils of spices to Chinese-style sausages. Sausages with essential oil additions showed significantly lower microbial counts during storage than controls or those containing spice powders. Both spice oils and powders reduced TBA values in sausages, ginger and cinnamon essential oils having the greatest antioxidant activities.

In model food system, essential oils *Pelargonium* showed potent antimicrobial activity, which was comparable with or greater than, that of commercial oil of geranium and thyme at 250 ppm and clove, cinnamon and coriander at 500 ppm. Activity was higher against yeasts than bacteria. It is concluded that *Pelargonium* essential oils can potentially be used in food processing, as antimicrobial (Lis Balchin et al., 1998). Pao Chuan Hsieh (2000) reported the antimicrobial compounds from edible medicinal plant, herb and spice extracts. Antimicrobial activities of cinnamon, star anise, *Crataegi fructus* and *Menthae pocanadensis* extracts were tested against 15 microorganisms. Results showed that cinnamon extract possessed stronger antimicrobial activity than other spice extracts.

Montes and Carvajal (1998) reported the effects of 11 plant essential oils for maize [corn] kernel protection against *Aspergillus flavus*. Tests were conducted to determine the optimal levels of dosages for maize protection, effects of combinations of essential oils, and residual effects and toxicity of essential oils to maize plants. Essential oils of *C. zeylanicum*, *Mentha piperita, Ocimum basilicum, Origanum vulgare, Teloxys ambrosioides, Syzygium aromaticum* and *Thymus vulgaris* caused a total inhibition of fungal development on maize kernels. Thymol and o-methoxy cinnamaldehyde significantly reduced maize grain contamination. The optimal dosage for protection of maize varied from 3 to 8%. Combinations of *C. zeylanicum* with the remaining oils gave efficient in growth inhibition.

Smith Palmer et al. (2001) reported the antibacterial effect of bay, clove, cinnamon and thyme essential oils against *Listeria monocytogenes* and *Salmonella enteritidis* inoculated into low fat and full fat soft cheese, stored at 4 and 10 °C, respectively, for 14 days. These showed that all four oils at a concentration of 1% reduced *L. monocytogenes* to less than or equal 1.0 $\log_{10}$ cfu/ml in the low fat cheese, whereas only clove oil achieved this reduction in the full fat cheese. These results suggest that fat composition of the cheese is a factor that affects effectiveness of the oils as antimicrobial. All 4 oils reduced *Salmonella enteritidis* to less than or equal 1.0 $\log_{10}$ cfu/ml from day 4 onwards in the low fat cheese. However, of the 4 oils, thyme oil was unable to produce this result in the full fat cheese. It is concluded that certain plant essential oils had the potential to be used as natural food preservatives, owing to their inhibitory effects against *L. monocytogenes* and *Salmonella enteritidis.*

The antimicrobial activity of various combinations of extracts of corni fructus, cinnamon and Chinese chive were tested against 15 foodborne microorganisms, of pathogenic and spoilage potential (Hsieh et al., 2001). Results showed that the combined extract of corni fructus, cinnamon and Chinese chive in the ratio 8:1:1 v/v/v exhibited strong and broad-spectrum of antimicrobial activity. Significant antimicrobial activity of the combined extract was observed in all foods and beverages tested. It was proposed that the combined extract may be of potential use as a natural additive in foods acting both as a seasoning and antimicrobial agent.

Recently, Mejlholm and Dalgaard (2002) reported the antimicrobial effect of nine essential oils on *Photobacterium phosphoreum* and the effect of oregano oil on the shelf life of modified atmosphere-packed (MAP) cod fillets. These authors also studied the antimicrobial effect of essential oil in a liquid medium and in product storage trials. Results showed that oils of oregano and cinnamon had the strongest antimicrobial activity, followed by lemongrass, thyme, clove, bay, marjoram, sage and basil oils. All the essential oils had an inhibitory effect, particularly those extracted from thyme, rosemary and cinnamon, and to a lesser extent, oregano. The results indicate the possibility of using essential oils to inhibit growth of undesirable and pathogenic microorganisms, and thereby extend shelf life of foods.

In conclusion, cinnamon bark oil possesses the delicate aroma of the spice with a sweet and pungent taste. The major constituent of volatile oil is *trans*-cinnamaldehyde, but other minor components also contribute to the overall characteristic odour and flavour. It is employed mainly in the flavouring industry where it is used in meat and fast food seasonings, sauces and pickles, baked goods, confectionery, cola-type drinks, tobacco flavours and in dental and pharmaceutical preparations. Perfumery applications are far fewer than in flavours because the oil has some skin-sensitising properties, but it has limited use in selected perfumes. Cinnamon leaf oil has a warm, spicy, but rather harsh odour, lacking the rich body of the bark oil. Its major constituent is eugenol. It is used as a flavouring agent for seasonings and savoury snacks. It is added to soaps and insecticides as a fragrance. High eugenol content makes the oil as a

valuable natural source for this chemical for subsequent conversion to *iso*-eugenol, another flavouring agent (The Wealth of India, 1992).

Cassia oil is distilled from a mixture of leaves, twigs and fragments of bark of *Cinnamomum cassia*. *trans*-Cinnamaldehyde is the major constituent of cassia oil and it is used mainly for flavouring cola-type drinks, with smaller amounts used in bakery products, sauces, confectionery and liquors (The Wealth of India, 1992). The major incentive to cultivation of *C. zeylanicum* and *C. cassia* has been their value as spice crops, for which world demand is considerable.

Indonesian cassia (*C. burmanii*) is much more important as a spice than as a source of oil and enters international trade along with Chinese cassia. Oleoresin of cinnamon is produced for flavouring purposes, and used in North America. Bark is also used for medicinal purposes, particularly in the Peoples Republic of China (Ji et al., 1990).

The above literature review showed that, there are number of studies on chemical composition of cinnamon leaves, root bark and stem bark volatile oils and their bioactivities. However, there are no reports on the chemical composition of unconventional parts from *Cinnamomum zeylanicum* and their biological activities.

AIMS AND SCOPE OF THE PRESENT STUDY

There is a rapidly growing body of literature covering the role of plant secondary metabolites in food and their potential effects on human health.

Furthermore, consumers are increasingly aware of diet related health problems, therefore demanding natural ingredients that are expected to be safe and health promoting. By-products of plant food processing represent a major disposal problem for the industry concerned, but they are also promising sources of compounds which may be used because of their favourable technological or nutritional properties. Due to increasing production, disposal represents a growing problem since the plant material is usually prone to microbial spoilage, thus limiting further exploitation. On the other hand, costs of drying, storage and shipment of by-products are economically limiting factors.

Natural antioxidants are in great demand now a days due to both consumers preference and health concerns associated with the use of synthetic antioxidants such as BHT and BHA (Madhavi and Salunkhe, 1995). The agricultural and industrial residues offer untapped source of natural additives (Azizah et al., 1999; Jayaprakasha et al., 2001; Moure et al., 2001; Chidambaramurthy et al., 2002a, b; Singh et al., 2002). A number of byproducts have been previously studied as potential sources of antioxidants. As reviewed by Moure et al., (2001), the extracts from vegetable materials of residual origin showed antioxidant activity, in some cases comparable to that of synthetic antioxidants and their extraction and use could be an alternative for obtaining natural antioxidants. Even when the natural extracts are less efficient, the use of some of them as food antioxidants can be advantageous. Moreover, the search for cheap, renewable and abundant sources of antioxidant compounds is attracting worldwide interest. Natural antioxidants often shown antioxidant powers lower than those of synthetic ones, but these are not law-limited in quantity (Moure et

al., 2001). Much research is needed in order to select raw materials; those of residual origin are especially promising due to their lower costs.

Literature survey revealed that, there are no reports on chemical composition of unconventional parts of cinnamon *viz.,* buds, flowers, fruits (Figure 2.3) and fruit stalks (a farm by-product) and curcumin removed turmeric oleoresin (industrial by-product i.e. from curcumin production) (Figure 2.4) (Literature review on turmeric have been presented in Part B, Chapter 1). In this background of present knowledgebase, a systematic investigation on the chemical as well as bioactivity studies of the volatiles and non-volatile fractions from unconventional parts of *Cinnamomum zeylanicum* (viz., buds, flowers, fruits, fruit stalks - farm by-products) and spent turmeric oleoresin (industrial by-product) were undertaken and the findings of these studies are presented in Part A and B respectively.

The following plan of the work was proposed and undertaken. The protocols of the study are as follows.

PART - A

- Isolation of volatile oils from the unconventional parts of cinnamon such as, buds, flowers, fruits and fruit stalks by hydro-distillation.

- GC and GC-MS analysis of volatile oils for determining their chemical composition.

- Successive extraction of cinnamon fruits using different solvents with increasing polarity. `

- Separation and identification of pure compounds by column chromatography and their structure elucidation by spectroscopic methods.

- Antioxidant, antimicrobial and antimutagenic activities of solvent extracts.

PART - B

- Isolation and identification of antibacterial, antifungal, antioxidant and antimutagenic fractions from spent turmeric oleoresin.

- Development of a method for the isolation of curcuminoids mixture and individual curcuminoids from spent turmeric oleoresin.

- Development of an analytical method for the determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin from different varieties of turmeric.

- Antioxidant activity of curcumin, demethoxycurcumin and bisdemethoxycurcumin.

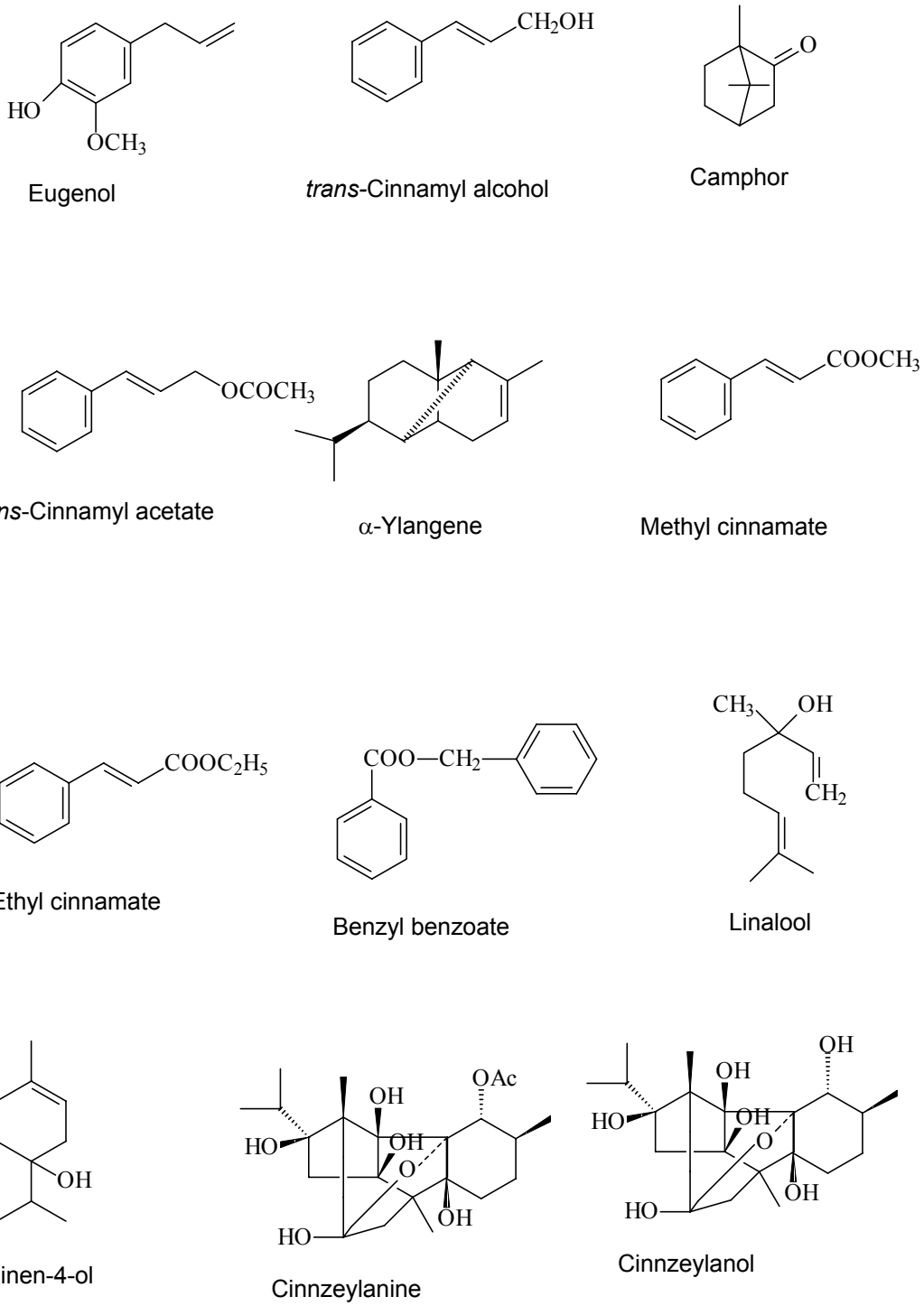
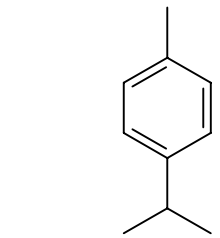
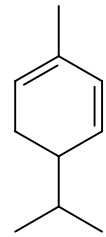


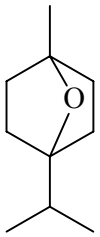
Figure 2.1. Structures of cinnamon volatiles reported (Wijesekera et al., 1974; Angmor et al., 1975; Isogai et al., 1976).



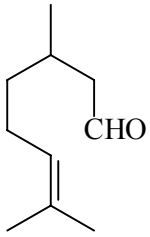
p-Cymene



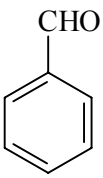
α–Phellandrene



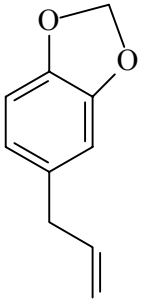
(1,4)-Cineole



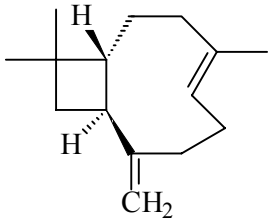
Citronellal



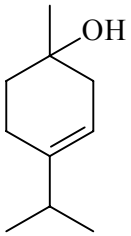
Benzaldehyde



Safrole



Caryophyllene



Terpinen-1-ol

Figure 2.2. Structures of cinnamon volatiles (Cheng and Yu, 1991; Sritharam et al., 1994; Ehlers et al., 1995).

Figure 2.3. ***Cinnamomum zeylanicum*** **fruit samples.**

Figure 2.4. Curcumin removed turmeric oleoresin / spent turmeric oleoresin sample.

2.1. STUDIES ON CHEMICAL COMPOSITION OF VOLATILE OIL FROM BUDS

INTRODUCTION

The genus *Cinnamomum* comprises of 250 species, which are distributed in Asia and Australia. These are evergreen trees and shrubs and most of the species are aromatic. *Cinnamomum zeylanicum* (*C. zeylanicum*), the source of cinnamon bark, leaf and their essential oils, is an indigenous tree of Sri Lanka. Many species of cinnamon yield a volatile oil on distillation. The most important cinnamon oils in world trade are those from *C. zeylanicum*, *C. cassia* and *C. camphora*. However, a number of other cinnamon species are distilled on a much smaller scale and the oils used either locally or exported (The Wealth of India, 1992). *C. zeylanicum*, the cinnamon is native to tropical Asia. The tree occurs in South India up to altitudes of 500 meters, but mostly below 200 meters. The tree flowers in January and fruits ripen during May-August (The Wealth of India, 1992). Cinnamon leaf and bark are used as spices and in the production of essential oils. Leaves have a hot taste and emit a spicy odour when crushed. There are no reports in the literature on chemical composition of cinnamon buds volatile oil. The objective of the present study is to determine the chemical composition of the various other parts of *C. zeylanicum*. In this chapter systematic study of chemical composition of the volatiles of buds, flowers, fruits and fruit stalks are presented. Their oils are compared and possible biogenesis is extrapolated. Development of a process for isolation of a major flavour compound [*viz.,* (*E)*-Cinnamyl acetate] is also presented.

MATERIALS AND METHODS

Plant Material

The buds of *Cinnamomum zeylanicum* were collected from Karkala, Karnataka State, India. The species was identified and voucher specimen was deposited at the Manasagangotri herbarium (MGH NO.3A/96/01), Department of Botany, University of Mysore, Mysore, India.

Isolation of Volatile Components

Fresh buds of *C. zeylanicum* (100 g, moisture content 84%) were manually separated and coarsely powdered in a mixer grinder with 200 ml of ice-cold distilled water for 2 min and transferred to 1000 ml round bottom flask. The Clevenger apparatus and along with a condenser was assembled and the mixture was subjected to hydro-distillation for 4 h. The yield (v/w) of volatile oil was 0.2 ml. The volatile oil was dried over anhydrous sodium sulphate and stored at 4 °C in a refrigerator until analysis.

Gas Chromatographic (GC) Analysis of the Volatile Oil

The GC analysis of the volatile oil was performed on a Shimadzu GC 15A (Shimadzu, Kyoto, Japan) chromatograph equipped with a FID detector, using a SE-30 column (10′ × 1/8′′) made up of 3% SE-30 on 80/10 superIcoport packing, methyl silicon phase type. The oven temperature was programmed from 60 °C for 5 min to 225 °C at the rate of 2 °C / min at which temperature the column was maintained for 3 min. The injector port temperature was 225 °C, the detector temperature was 250 °C and nitrogen was used as the carrier gas at a flow rate

of 40 ml/min. 0.5 μl of volatile oil sample was injected to GC and peak areas were computed by a Shimadzu C-R4A chromatopak data processor.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of the volatile oil was carried out to determine the total chemical composition of which included the percentage content of the major as well as the minor constituents. The experiment was carried out using a Shimadzu GC -17A chromatograph equipped with a QP-5000 (Quadrupole) mass spectrometer (Shimadzu, Kyoto, Japan). The injector port temperature was 225 °C, the detector temperature was 250 °C, and the split ratio was 1:25. The oven temperature was initially maintained at 60 °C for 2 min and then increased to 225 °C at the rate of 2 °C/min at which temperature the column was maintained for 5 min. Helium was used as the carrier gas at a flow rate of 1 ml/ min and a fused silica capillary column SPB-1 (30 m × 0.32 mm i. d., 0.25 μm film thickness) coated with poly(dimethylsiloxane) was used. Mass spectra were recorded under electron impact ionisation at 70 eV electron energy with a mass range from 40 to 400 at a rate of one scan / second. The sample was diluted 25 times with acetone, and 1 μl was injected.

RESULTS AND DISCUSSION

The *C. zeylanicum* buds volatile oil (0.2%) was obtained by Clevenger hydro-distillation and subjected to GC and GC-MS analysis to determine chemical composition. Cinnamon buds volatile oil was analysed using SPB-1 column and

Total Ion Chromatogram (TIC) of GC-MS is presented in Figure 2.5. Retention indices for all the compounds were determined according to the Kovats method (Jennings and Shibamoto, 1980) using *n*-alkanes as standards. Kovats retention indices were calculated using *n*-paraffins (C_6-C_{26}) as references.

KI = 100 N + 100 n [log t^1 R (A) – log t^1 R (N)] / [log t^1 R (N + n) – log t^1 R(N)],

Where t^1 R(N) and t^1 R (N+ n) are the adjusted retention times of n-paraffin hydrocarbons of carbon number N and (N + n) that are respectively smaller and larger than the adjusted retention time of the unknown t^1 (R).

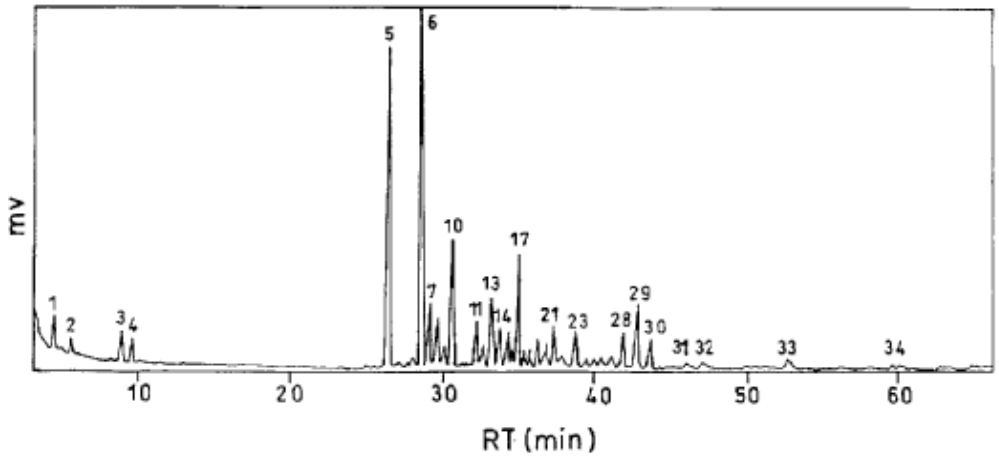


Figure 2.5. GC-MS total ion chromatogram of cinnamon buds volatile oil.

The compounds were identified by comparison of Kovats indices with those reported in the literature (Jennings and Shibamoto, 1980; Davies, 1990), wherever possible, by co-injection with an authentic sample and by matching their fragmentation patterns in mass spectra with those stored in NIST62-LIB library and published mass spectra (Einar Strenhagen et al., 1974; Ten Noever Bravw et al., 1988; Adams, 1989).

A total of thirty-four compounds were identified, which constituted ≈ 96% of the volatile oil (Table 2.1). Mevalonic acid and shikimic acid metabolites account for ≈ 87% and ≈ 2.4%, respectively. Straight chain compounds were present to the extent of ≈ 6.45%. Mass spectra of selected major constituents are presented in Figures 2.6, 2.7 and 2.8. The identity of major compounds *i.e*. α-Bergamotene and α-copaene was confirmed by mass spectral fragmentation. α-Bergamotene showed m/z (% abundance): 93 (100%), 119 (86%), 69 (46%), 41 (50.4%), 55 (28.4%), 107 (25.2%), 204 (4.4%). α-Copaene showed m/z (% abundance): 105 (100%), 119 (95%), 41 (80%), 161 (50%), 93 (48%), 55 (43%), 81 (35%), 204 (10) (Figure 2.6).

Sesquiterpenes were the major compounds (≈ 85%) among the mevalonic acid metabolites. The monoterpene portion was very low (< 2%). In addition to the major sesquiterpenes *viz.,* α-bergamotene, α-copaene the minor compounds α-humulene, α-muurolene and δ-cadinene were identified. Spathulenol and globulol were the major sesquiterpene alcohols. Caryophyllene oxide was the only oxide and present in very low percentage. Straight chain compounds were represented by three different classes of functional groups *viz* alcohols, aldehydes and acids. Two compounds were found from each class. Aromatic ester *viz.* (*E*)-cinnamyl acetate (Figure 2. 9) was present to an extent of 2.4% along with a trace amount of benzyl benzoate.

In other words, buds of *C. zeylanicum* contained twenty-six terpenoids (86.4%), of which 15 compounds are hydrocarbons (75.2%) and 11 compounds

are oxygenated compounds (9.89%). Of the 15 hydrocarbons, one compound (*i.e*. α-pinene) is bicyclic monoterpene, eight are bicyclic sesquiterpenes with normal ring (C-6) size and one compound is larger ring (C-10) size. Three compounds represent tricyclic sesquiterpenes *i.e.* α-copeane, aromadendrene and viridiflorene. Macrocyclic sesquiterpenes are represented by α-humulene and germacrene-D. Of the 11 oxygenated compounds, linalool belongs to acyclic monoterpene alcohol. Two compounds belonged to monocyclic sesquiterpenes alcohol, four compounds are bicyclic sesquiterpenes alcohol and three compounds are tricyclic sesquiterpenes alcohol. Macrocyclic sesquiterpenes is represented by only one compound (caryophyllene oxide).

CONCLUSION

Thirty-four constituents were identified from the volatile oil from buds of *C. zeylanicum* by GC-MS for the first time. The volatile oil of buds of *C. zeylanicum* contains α-bergamotene (27.4%) and α-copaene (23.1%) as the major compounds. This was different from oils of other parts of *C. zeylanicum* such as leaf (Mallavarapu et al., 1995), root bark and stem bark (Senanayake et al., 1978). However, there were some similarities, as it contains many other compounds that were present in other oils as well.

Table 2.1. Composition of the cinnamon buds volatile oil

Peak No	RT (min)	Compound	Peak Area (%)	KI	Identification by
1	3.07	Heptanal*	1.09	--	MS
2	4.52	α-Pinene	0.87	953	RI, MS, CI
3	9.10	Nonanal	1.09	1046	RI, MS
4	9.30	Linalool	0.91	1053	RI, MS, CI
5	26.45	α-Copaene	23.05	1366	RI, MS
6	28.76	α-Bergamotene	27.38	1399	RI, MS
7	29.12	*(E)*-Cinnamyl acetate	2.41	1430	RI, MS, CI
8	29.30	Aromadendrene	1.79	1435	RI, MS
9	30.10	α-Cadinene	0.56	1440	RI, MS
10	30.90	α- Humulene	6.19	1448	RI, MS
11	32.12	Germacrene-D	2.10	1455	RI, MS
12	32.32	Valencene	0.66	1458	RI, MS
13	33.17	Viridiflorene	3.29	1470	RI, MS
14	33.60	α-Muurolene	1.70	1480	RI, MS
15	34.18	γ-Cadinene	1.57	1487	RI, MS
16	34.43	1S-*cis*-Calamenene	0.42	1492	RI, MS
17	34.97	δ-Cadinene	5.97	1509	RI, MS
18	35.32	α-Calacorene	0.38	1520	RI, MS
19	35.63	β-Guaiene*	1.91	1533	MS
20	35.80	Ledol	1.29	1542	RI, MS
21	36.80	Spathulenol	2.02	1552	RI, MS
22	37.78	Caryophyllene oxide	0.41	1558	RI, MS
23	38.10	Globulol	2.10	1569	RI, MS
24	39.50	τ-Cadinol	Tr.	1611	RI, MS
25	40.10	τ-Muurolol	Tr.	1620	RI, MS
26	40.40	Torreyol	Tr.	1623	RI, MS
27	41.60	α-Cadinol	Tr.	1631	RI, MS
28	42.43	□-Bisabolol	1.26	1652	RI, MS
29	43.10	Tetradecanol	4.27	1666	RI, MS
30	43.90	*epi*-α-Bisabolol	1.90	1690	RI, MS
31	46.10	Benzyl benzoate	Tr.	1707	RI, MS, CI
32	47.00	Tetradecanoic acid*	Tr.	1778	MS
33	52.10	Hexadecanol	Tr.	1863	RI, MS
34	59.80	Hexadecanoic acid*	Tr.	1978	MS
			Total = 96.59%		

KI: Kovats index on SPB-1 capillary column.
MS: Mass spectra.
CI: Co-injection with authentic sample.
Tr: Less than 0.01%
***:** Identified tentatively

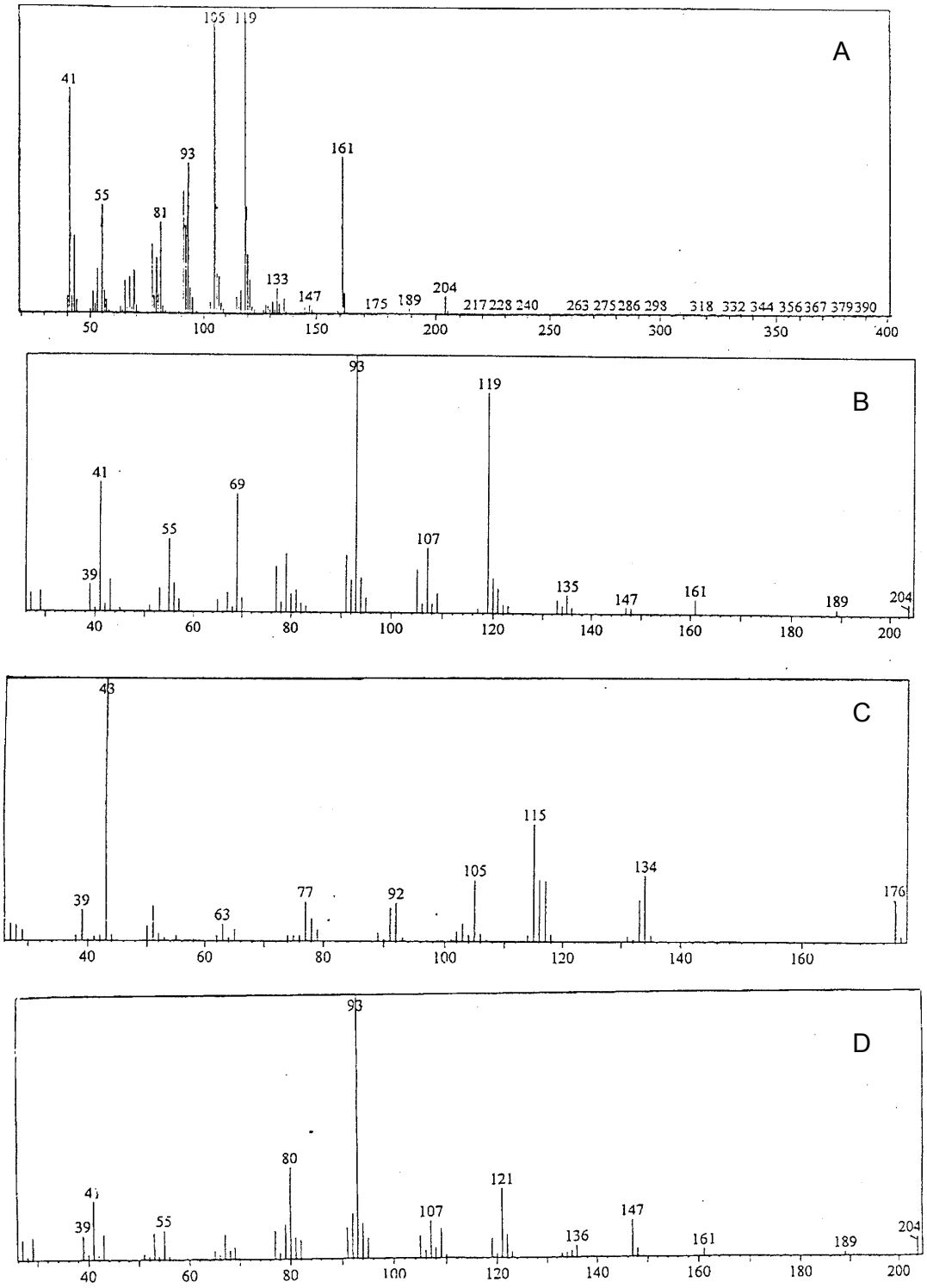


Figure 2.6. Mass spectra of (A). α-Copaene, (B). α-Bergamotene, (C). *(E)*-Cinnamyl acetate and (D). α-Humulene, from cinnamon buds volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].

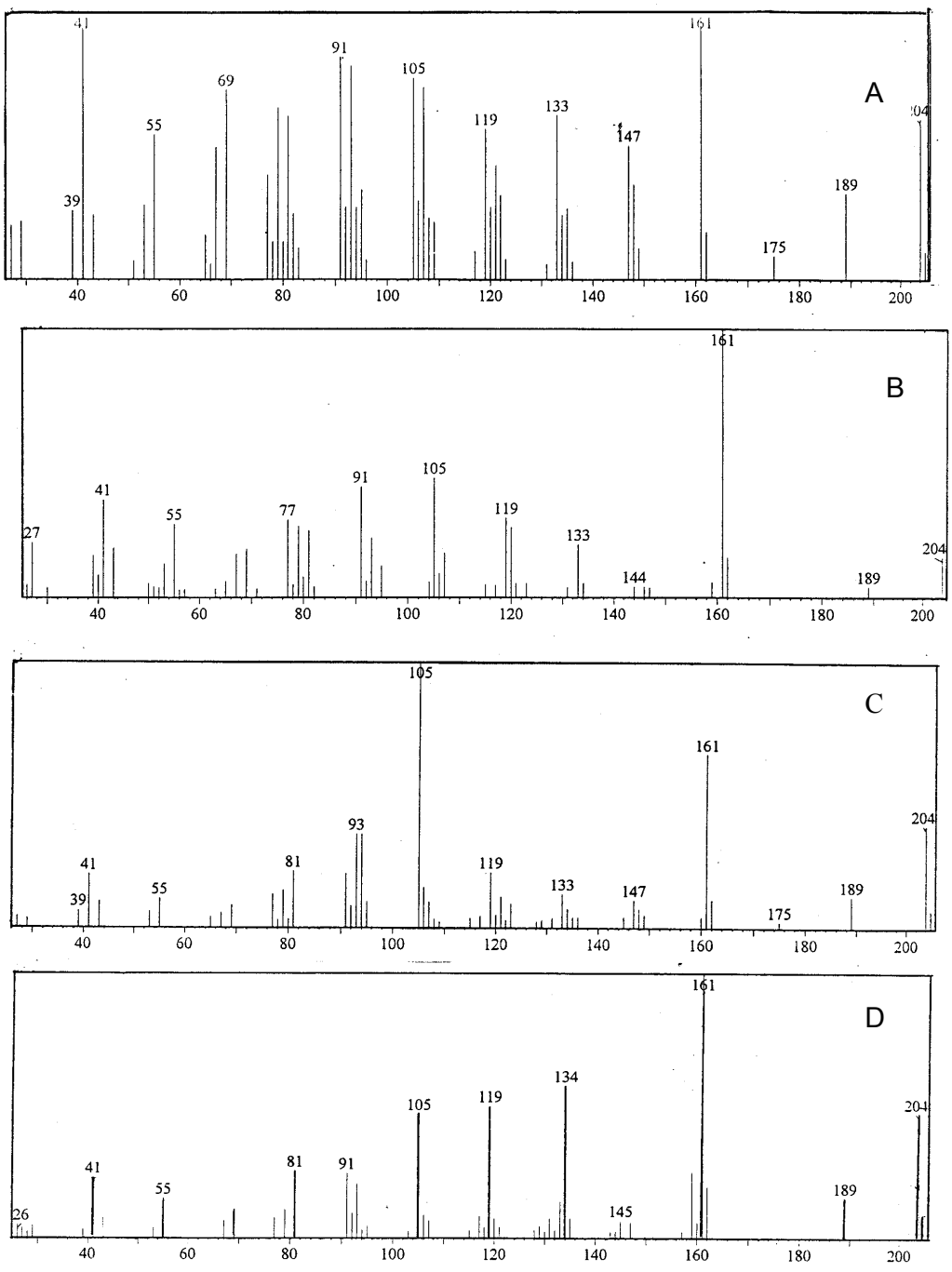


Figure 2.7. Mass spectra of (A). Aromadendrene, (B). Germacrene-D, (C). α-Muurolene and (D). δ-Cadinene, from cinnamon buds volatile oil,
[X-axis: m/z, Y-axis: Relative abundance (%)].

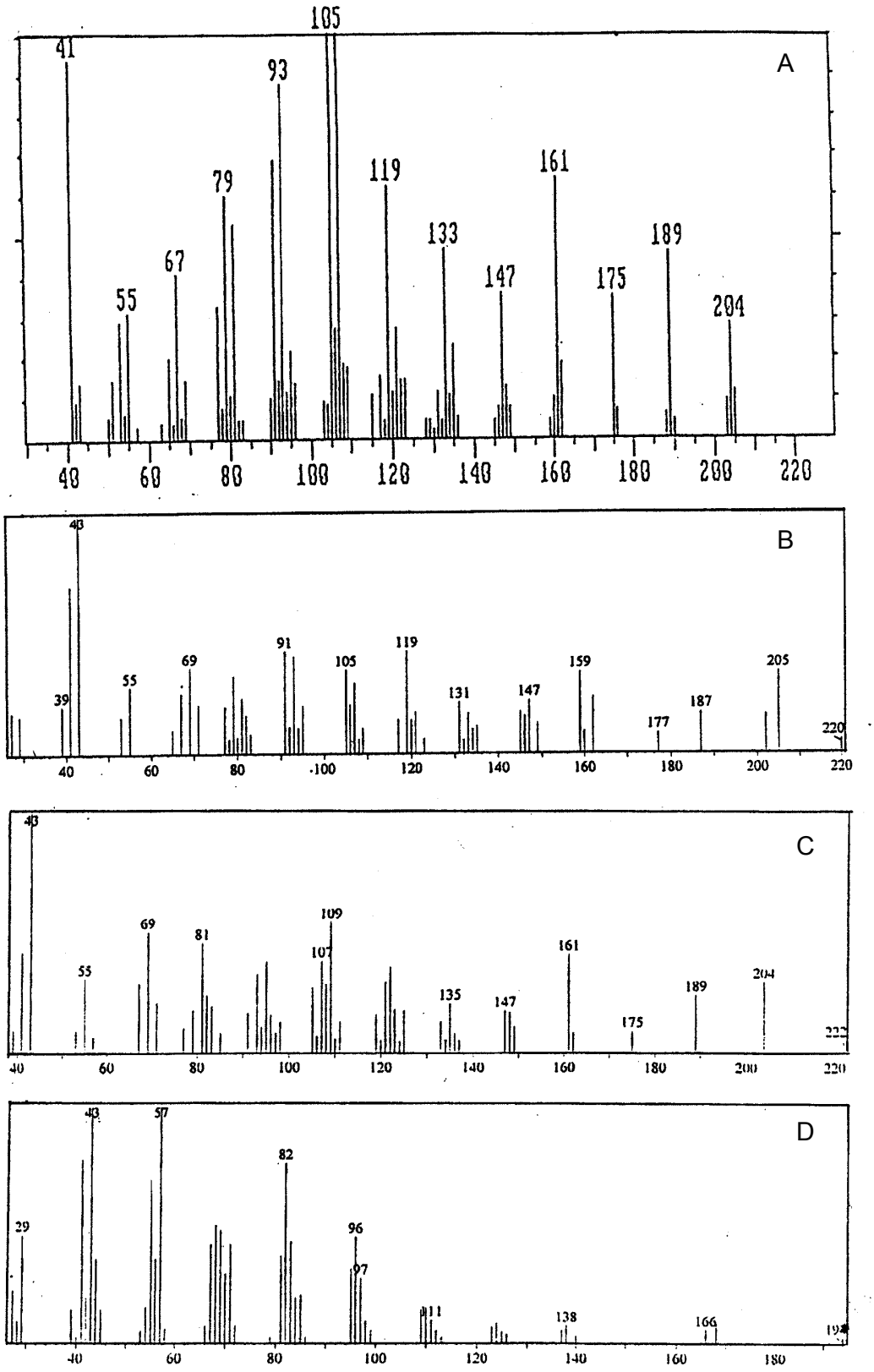
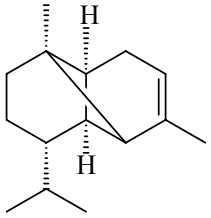
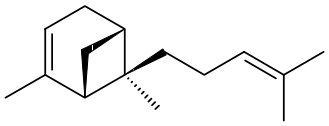


Figure 2.8. Mass spectra of (A). Viridiflorene, (B). Spathulenol, (C). Globulol and (D). Tetradecanol, from cinnamon buds volatiles,

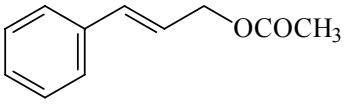
[X-axis: m/z, Y-axis: Relative abundance (%)].



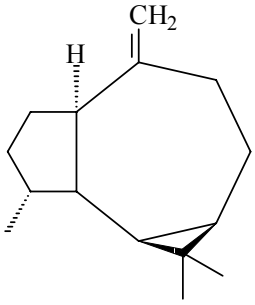
α-Copaene



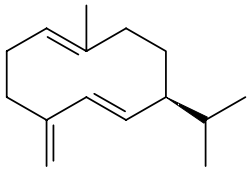
α-Bergamotene



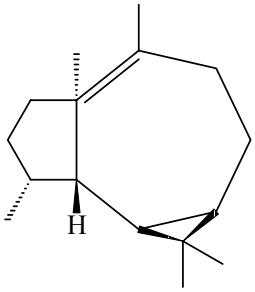
(*E*)-Cinnamyl acetate



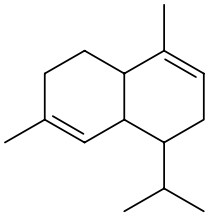
Aromadendrene



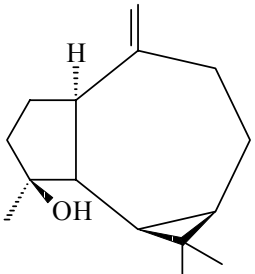
Germacrene-D



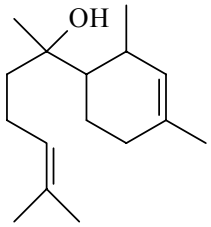
Viridiflorene



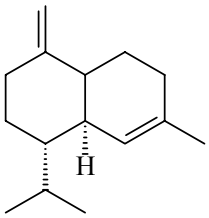
α-Muurolene



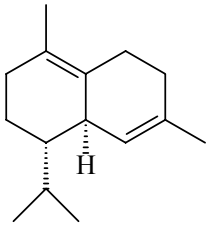
Spathulenol



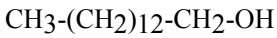
epi-α-Bisabolol



γ-Cadinene



δ-Cadinene



Tetradecanol

Figure 2.9. Structures of important flavour constituents from cinnamon buds volatile oil.

2.2. STUDIES ON CHEMICAL COMPOSITION OF VOLATILE OIL FROM FLOWERS

INTRODUCTION

Literature survey revealed no information on the chemical composition of volatile oil of flowers of *C. zeylanicum*. Therefore a systematic investigation was undertaken on *C. zeylanicum* flowers with regard to the chemical composition of the volatile oil. The results of this study are presented and discussed in this section.

MATERIALS AND METHODS

Plant Material

The flowers of *C. zeylanicum* (syn. *Cinnamomum verum* J. S. Presl) were collected from Karkala, South Canara District, Karnataka state, India. The species was identified and voucher specimen was deposited at the Manasagangotri herbarium (MGH NO.2/96), Department of Botany, University of Mysore, Mysore, India.

Isolation of Volatile Oil

Flowers (80 g; Moisture content 86%) of *C. zeylanicum* were ground along with 170 ml of ice-cold distilled water using a mixer grinder for 2 min and subjected to hydro-distillation in a Clevenger-type apparatus for 4 h. The yield of volatile oil was 0.4 ml (v/w). The oil was dried over anhydrous sodium sulphate

and kept at 4-5 °C in a refrigerator for further analysis. The light yellow oil possessed a sweet floral odour.

GC Analysis

GC analysis was carried out as per conditions mentioned in Part A, Chapter 2.1. Page No. 46.

GC-MS Analysis

GC-MS analysis was carried out as per conditions mentioned in Part A, Chapter 2.1, Page No. 47.

Identification of Compounds

Retention indices for all the compounds were determined using *n*-alkanes as standards (Jennings and Shibamoto, 1980). The compounds were identified by comparison of retention indices with those reported in the literature (Jennings and Shibamoto, 1980; Davies, 1990), wherever possible, by co-injection with an authentic specimen and by matching their mass spectral fragmentation patterns with those stored in the spectrometer database, using the NIST62 MS library (Supplied by Shimadzu Corporation, Japan) or comparison of MS data with those reported in literature (McLafferty et al., 1980; Jennings and Shibamoto, 1980; Ten Noever Bravw et al., 1988; Adams, 1989).

RESULTS AND DISCUSSION

The volatile oil was obtained by conventional hydro-distillation of *C. zeylanicum* flowers in a Clevenger-type apparatus, which gave a yield of 0.5% (v/w). The volatile oil was subjected to GC and GC-MS analysis for qualitative and quantitative identification of its chemical composition. The total ion chromatogram (Figure 2.10) indicated at least 26 constituents, which constituted ≈ 96% of the volatile oil. The chemical composition of the flower oil is presented in Table 2.2. A total twenty-six compounds were identified with *(E)*-cinnamyl acetate (42%) was the major compound.

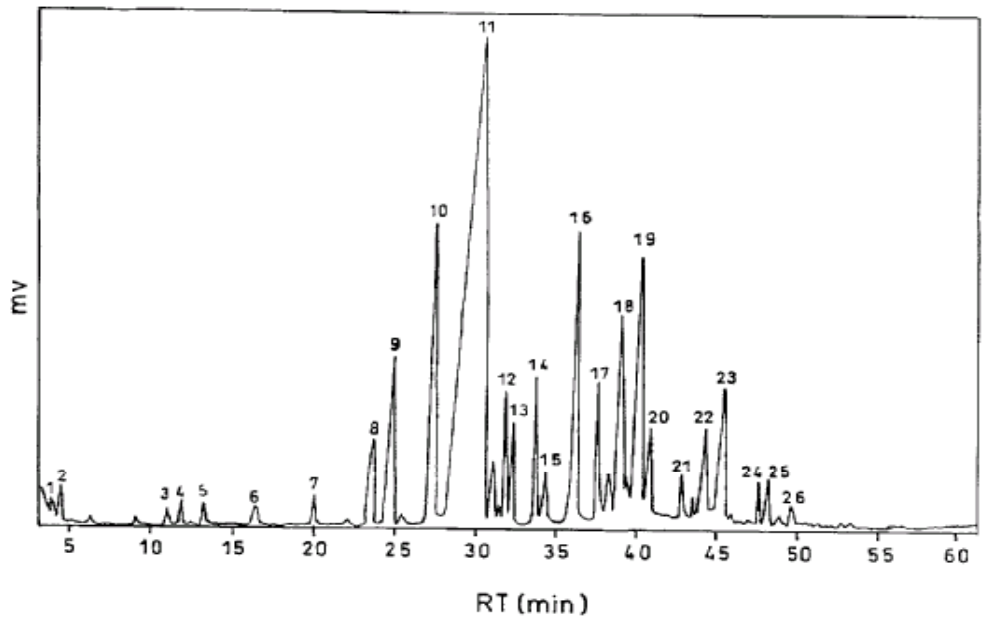


Figure 2.10. GC-MS total ion chromatogram of cinnamon flowers volatile oil.

Shikimic acid and mevalonic acid metabolites account for 49% and 38.5%, respectively. Approximately nine percent was contributed from straight chain compounds. Figures 2.11, 2.12 and 2.13 shows the mass spectra of twelve

major compounds present in cinnamon flower volatile oil. Esters, such as *(E)*-cinnamyl acetate, benzyl benzoate, 3-phenyl propyl acetate and 2-phenylethyl benzoate, accounted for 48%. Other compounds from the shikimic acid metabolites are benzaldehyde, dihydrocinnamaldehyde, *(E)*-cinnamaldehyde and *(E)*-cinnamyl alcohol. The fragmentation pattern of *(E)*-cinnamyl acetate was m/z (% abundance), 43 (100%), 115 (44%), 134 (25%), 105 (23.2%), 176 (16%) and 92 (14.4%) (Figure 2.11).

Sesquiterpenes accounted for 38% of the flower volatile oil among the mevalonic acid metabolites. The monoterpene portion was very low (<0.5%). α-Bergamotene, α-copaene, α-humulene and δ-cadinenes were the major sesquiterpene hydrocarbons (Figure 2.14). α-Cadinol and globulol were the major sesquiterpene alcohols. Caryophyllene oxide was the only oxide and present in considerable amount (7.3%). Minor monoterpenes were represented by borneol and α-terpineol. Cadalene was the lone aromatic hydrocarbon might be from the mevalonic metabolites. Straight chain compounds were represented by four different classes of functional groups *viz.* hydrocarbons (*n*-heptadecane), aldehydes (tetradecanal), ketones (2-hexadecanone) and alcohols ((*Z*)-Hex-3-en-1-ol, pentadecanol).

Of the thirteen terpenoids, six compounds were hydrocarbons (19.1%) and seven compounds were oxygenated compounds (19.4%). Of the six hydrocarbons, three were bicyclic sesquiterpene with normal ring (C-6) size, one compound (α-copane) was tricyclic sesquiterpene and two compounds were

macrocyclic sesquiterpenes (α-humulene and germacrene-D). Of the seven oxygenated compounds, α-terpineol and borneol represent monocyclic and bicyclic monoterepene alcohols, respectively. Acyclic sesquiterpene alcohol was represented by nerolidol. *epi*-Bisabolol, α-cadinol, and globulol belonged to monocyclic, bicyclic and tricyclic sesquiterpene alcohols respectively, along with one macrocyclic oxide (i.e. caryophyllene oxide).

CONCLUSION

The volatile oil from the flowers of *C. zeylanicum* contained *(E)*-cinnamyl acetate as the major compound. This was different from oils of other parts of *C. zeylanicum*, since the major compounds were eugenol in the leaf oil (Mallavarapu et al., 1995), (*E*)-cinnamaldehyde in stem bark oil, camphor in root-bark oil (Senanayake et al., 1978). However, there were some similarities, as it contained many other compounds, which were present in other oils as well. The major compound *i.e.,* (*E*)-cinnamyl acetate could be used for the replacement of synthetic (*E*)-cinnamyl acetate in perfumery, because of its excellent sensory and fixative properties. The results of the present study were indicative of the potential for utilization of flowers in perfumery. This was the first report on the chemical composition of the flowers volatile oil of *C. zeylanicum.*

Table 2.2. Chemical composition of the volatile oil of *C. zeylanicum* flowers

Peak No	RT (min)	Compound	Peak area (%)	KI	Identification by
1	3.14	(*Z*)-Hex-3-en-1-ol *	0.10	---	MS
2	3.93	Benzaldehyde	0.35	940	MS, RI, CI
3	11.10	Hydrocinnamaldehyde	0.18	1128	MS, RI
4	11.97	Borneol	0.17	1150	MS, RI
5	13.26	α-Terpineol	0.15	1179	***MS, RI***
6	16.52	*(E)*-Cinnamaldehyde	0.38	1243	MS, RI, CI
7	20.09	*(E)*-Cinnamyl alcohol	0.49	1300	MS, RI, CI
8	23.78	3-Phenylpropyl acetate	1.99	1363	MS, RI
9	24.97	α-Copaene	3.03	1381	MS, RI
10	27.54	*trans*-α-Bergamotene	7.97	1424	MS, RI
11	29.28	*(E)*-Cinnamyl acetate	41.98	1440	MS, RI, CI
12	32.37	α-Humulene	2.40	1450	MS, RI
13	32.98	Germacrene-D	1.31	1514	MS, RI
14	33.79	δ-Cadinene	2.97	1529	MS, RI
15	34.38	**Nerolidol**	0.95	1539	MS, RI
16	36.40	Caryophyllene oxide	7.29	1574	MS, RI
17	37.68	Globulol	3.80	1595	MS, RI
18	39.09	Tetradecanal	5.05	1621	MS, RI
19	40.20	α-Cadinol	6.35	1642	MS, RI
20	41.30	Cadalene	1.39	1662	MS, RI
21	42.90	*epi*-α-Bisabolol	0.73	1690	MS, RI
22	44.39	*n*-Heptadecane	2.14	1718	MS, RI
23	45.56	Benzyl benzoate	3.19	1741	MS, RI, CI
24	48.23	Pentadecanol	0.71	1791	MS, RI
25	48.98	2-Hexadecanone*	0.71	1805	MS
26	49.63	2-Phenyethyl benzoate*	0.44	1818	MS
			Total: 96.22%		

RI: Retention Index.
KI: Kovats indices.
MS: Mass spectra.
CI: Co-injection with authentic sample.
*: Identified tentatively

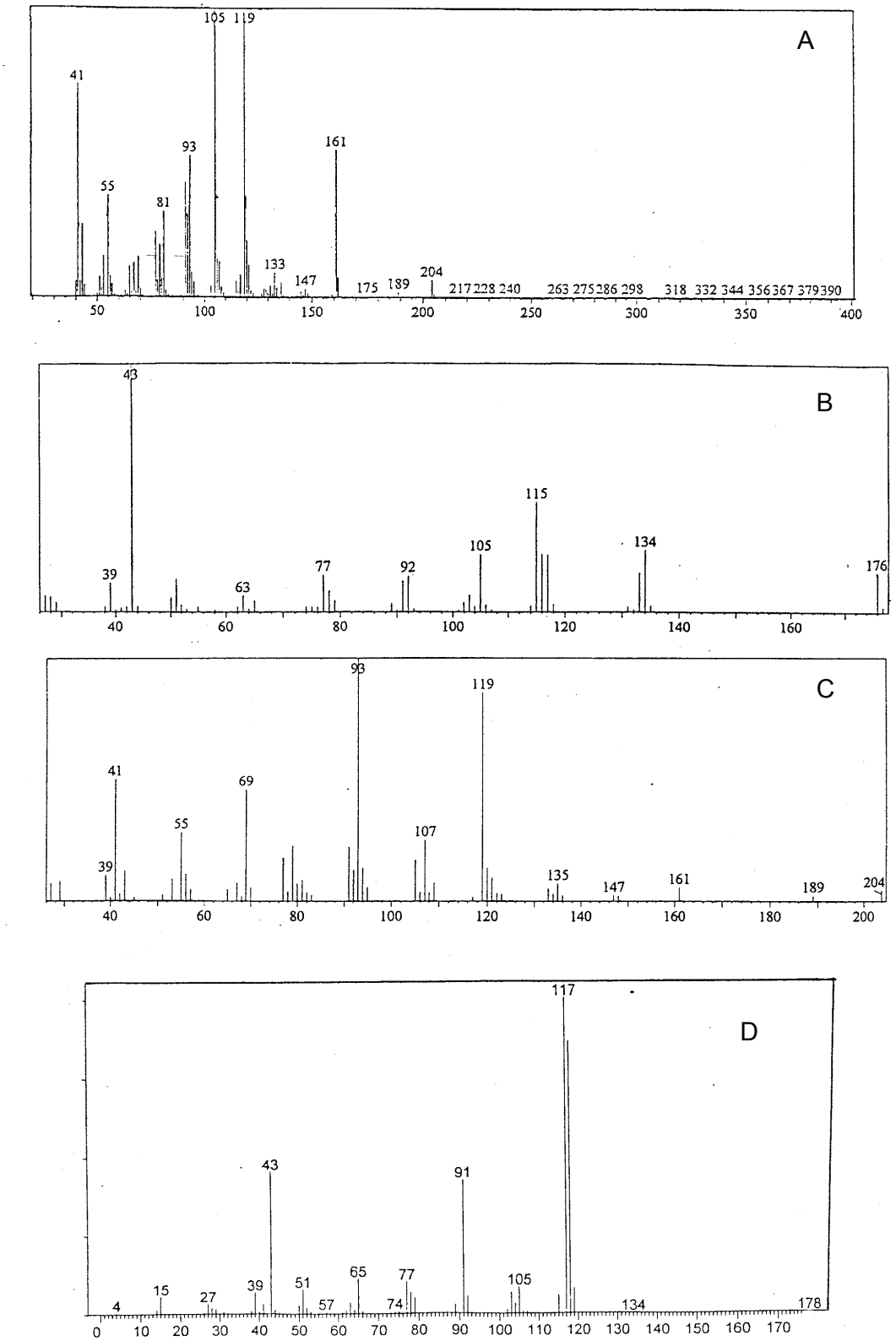


Figure 2.11. Mass spectra of (A). α-Copaene, (B). *(E)*-Cinnamyl acetate, (C). α-Bergamotene and (D). 3-Phenyl propyl acetate, from cinnamon flower volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].

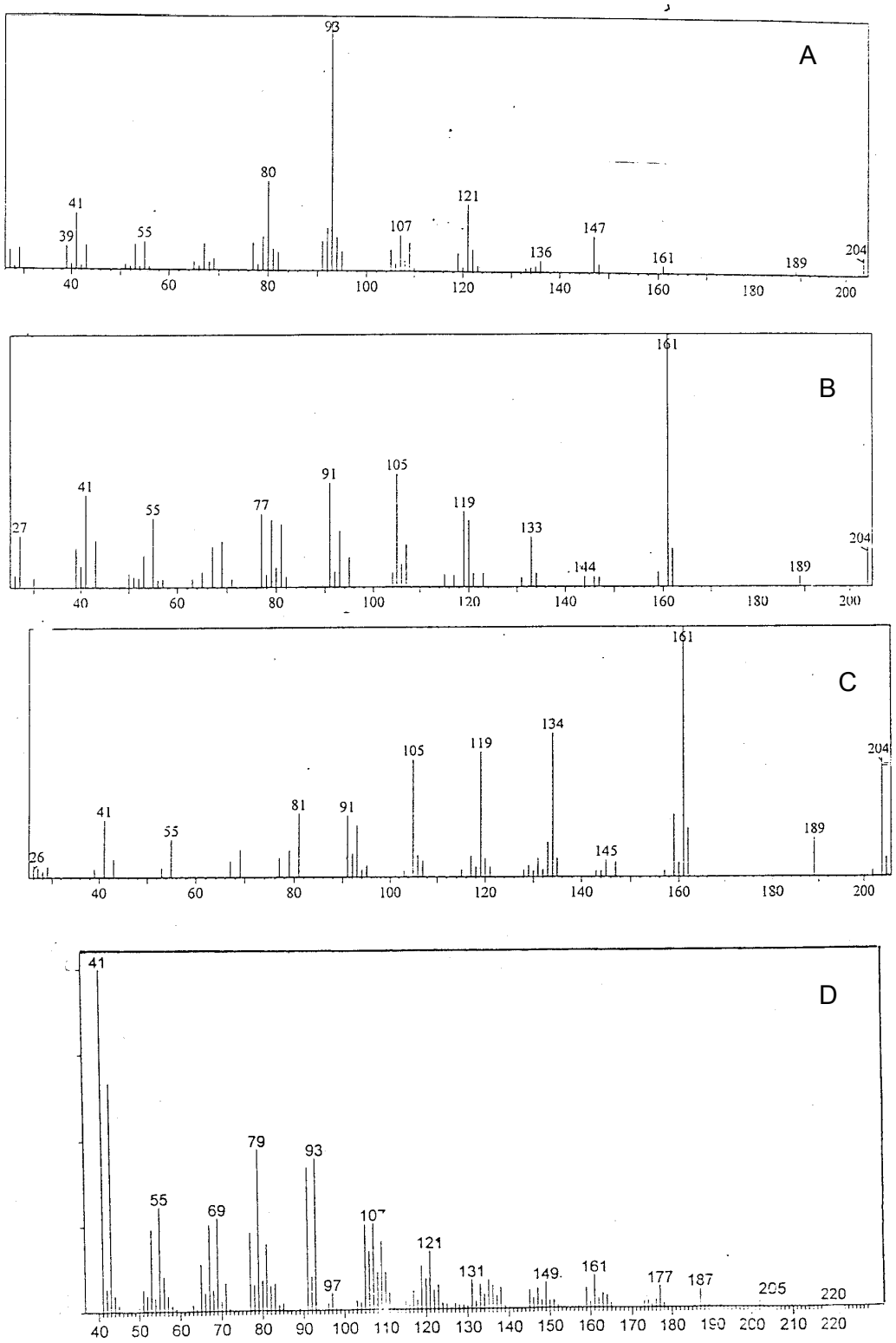


Figure 2.12. Mass spectra of (A). α-Humulene, (B). Germacrene-D, (C). δ-Cadinene and (D). Caryophyllene oxide, from cinnamon flower volatile oil,
[X-axis: m/z, Y-axis: Relative abundance (%)].

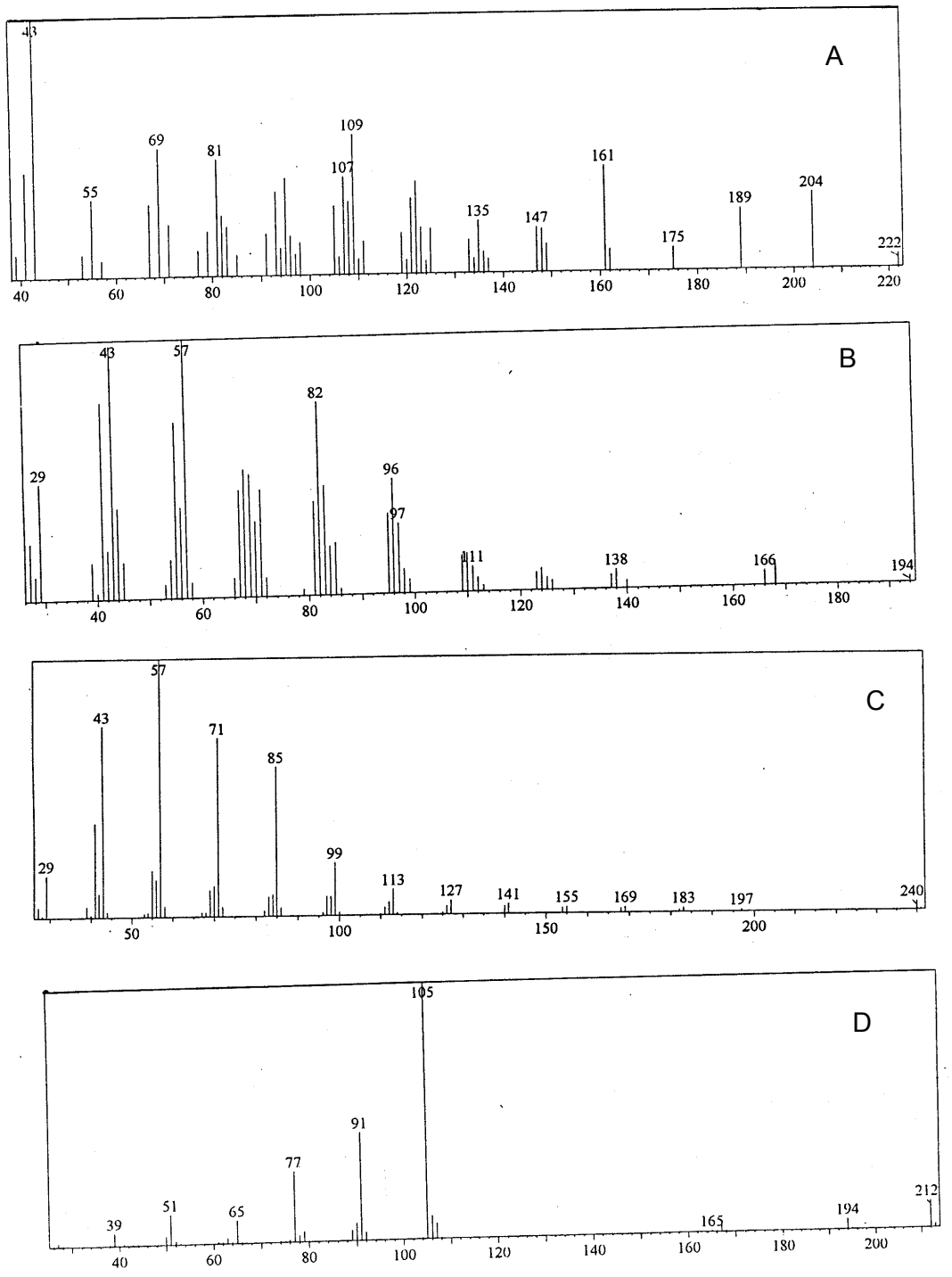
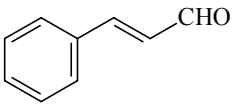
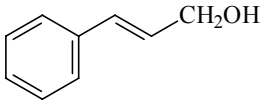


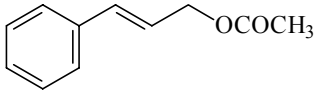
Figure 2.13. Mass spectra of (A). Globulol, (B). Tetradecanol, (C). *n*-Heptadecane and (D). Benzyl benzoate, from cinnamon flower volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].



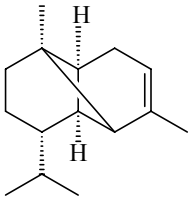
(*E*)-Cinnamaldehyde



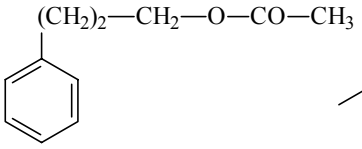
(*E*)-Cinnamyl alcohol



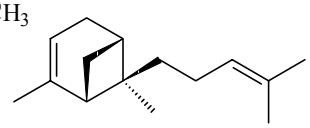
(*E*)-Cinnamyl acetate



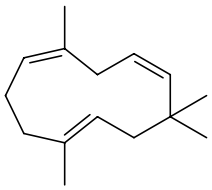
α-Copaene



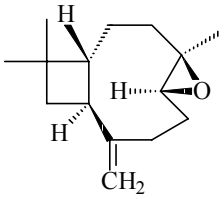
3-Phenylpropyl acetate



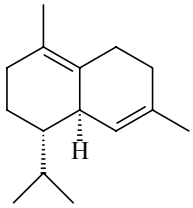
trans-α-Bergamotene



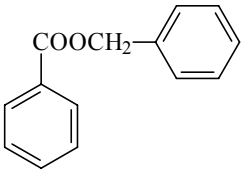
α-Humulene



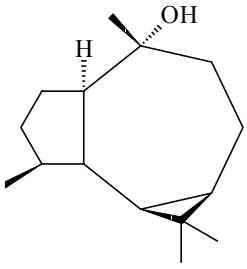
Caryophyllene oxide



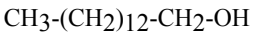
δ-Cadinene



Benzyl benzoate



Globulol



Tetradecanol

Figure 2.14. Structures of flavour components identified in cinnamon flower volatile oil.

2.3. STUDIES ON CHEMICAL COMPOSITION OF VOLATILE OIL FROM FRUITS

INTRODUCTION

The cinnamon tree flowers in January and the fruits ripen during May-August. Literature survey revealed that, there are no reports on the chemical composition of *C. zeylanicum* fruit volatile oil. Hence, a systematic investigation on the chemical composition of cinnamon fruits volatile oil was under taken. The results of the GC-MS analysis of the volatile oil constituents, which were collected at two different locations of South India was presented in this section.

MATERIALS AND METHODS

Plant Material

The fruits of *C. zeylanicum* were collected from Karkala, Karnataka state, India, (sample A) and Kottayam, Kerala state, India (sample B). The species were identified and voucher specimens were deposited at the Manasagangotri herbarium (MGH NO.1/96 and 2A/96), Department of Botany, University of Mysore, Mysore, India.

Isolation of Volatile Components

Cinnamon fruit samples A and B (100 g each; Moisture content 83 and 85%) were coarsely ground separately with 210 ml of ice cold distilled water using mixer grinder and transferred to two round bottom flasks of 1000 ml capacity. The Clevenger apparatus and condenser were fixed and subjected to hydro-

distillation for 4 h each separately. The yield of volatile oil was 0.8 ml and 1.0 ml respectively from samples A and B. The volatile oils were dried over anhydrous sodium sulphate and stored at 4 °C for further analysis. The light yellow oil possessed a sweet floral odour.

Gas Chromatographic Analysis of the Volatile Oil

GC analysis of volatile oils was carried out as per conditions mentioned in Part A, Chapter 2.1. Page No.46.

Gas chromatographic - Mass Spectrometric (GC-MS) Analysis

GC-MS analysis was carried out as per conditions mentioned in Part A, Chapter 2.1. Page No. 47.

Identification of Volatile Constituents using Retention Indices and Mass Spectra

Retention indices for all the compounds were determined according to the Kovats method using n-alkanes as standards (Jennings and Shibamoto, 1980). The compounds were identified by comparison of Kovats indices (Jennings and Shibamoto, 1980; Davies, 1990) and by co-injection with an authentic specimen. Also, by matching their fragmentation patterns in mass spectra with those of NIST62-LIB library and published mass spectra (Jennings and Shibamoto, 1980; Adams, 1989).

RESULTS AND DISCUSSION

The volatile oils were obtained by hydro-distillation of *C. zeylanicum* fruits obtained from two regions of South India (sample A and B) in a Clevenger - type apparatus, which gave an oil yield (v/w) of 0.8 and 1.0% respectively. Both the volatile oil samples A and B were analysed using GC and the chromatograms were presented in Figures 2.15 and 2.16 respectively. Figures 2.17 and 2.18 depict the GC-MS total ion chromatogram of cinnamon fruit volatile oil of samples A and B respectively. Thirty-four components were identified in sample A, which constituted to 96.6% and thirty-two compounds were identified in sample B that constituted to 94.2%. The chemical composition of fruits volatile oil was presented in Table 2.3. Mass spectra of twelve major compounds are presented in Figures 2.19 - 2.21.

Oxygenated compounds were major and present to the extent of 63.7% and 73.4% in sample A and sample B respectively. Five types of oxygenated compounds were present *viz.*, esters (48.6% in A and 61.2% in B); alcohols (9.3% in A and 10.1% in B); aldehydes (4.6% in A and 0.8% in B); oxides (0.91% in A and 1.11% in B) and phenols (0.3% in A and 0.2% in B). Several compounds represented esters and alcohols. However, aldehydes, oxides and phenols were represented by one compound each *viz*. *trans* and *cis* isomers of cinnamaldehyde, caryophyllene oxide and eugenol respectively. Among the esters, *(E)*-cinnamyl acetate was the major compound and constituted to the extent of 42.4% in A and 54.2% in B, followed by 3-phenylpropyl acetate and benzyl benzoate. Sample A contained two aliphatic esters *viz.* methyl palmitate

and methyl oleate. In alcohols except cinnamyl alcohol, all others are terpenic alcohols.

Samples A and B of cinnamon fruits volatile oil contained 25 and 24 terpenoids, respectively, which constituted to 40.0% and 29.9% of the volatile oil. Sample A of cinnamon fruit volatile oil contained twenty-five compounds, of which 14 compounds were hydrocarbons (32.8%) and 11 compounds were oxygenated compounds (7.95%). Sample B of cinnamon fruits volatile oil contained twenty-four compounds of which 13 compounds were hydrocarbons (20.8%) and 11 compounds were oxygenated compounds (9.16%).

Non-oxygenated compounds were present to the extent of 32.8% in sample A and 20.8% in sample B. Two classes of terpenes were present *viz.* monoterpenes (5.1% in A and 0.9% in B) and sesquiterpenes (27.7% in A and 19.9% in B). Among the monoterpenes β-phellandrene (1.6% in A and 0.37% in B) and α-pinene (1.57% in A and 0.12% in B) were the major components. Monoterpene alcohols were represented by borenol and α-terpineol. Among the sesquiterpenes β-caryophyllene was the major compound (13.73% in A and 9.2% in B); followed by α-humulene, α-copaene, δ-cadinene, germacrene-B, and γ-cadinene. The major sesquiterpenic alcohols represented by torreyol (2.71% in A and 3.42% in B) and α-cadinol (1.01% in A and 1.46% in B) (Figure 2. 22).

Of the fourteen-terpene hydrocarbons, two compounds of each belonged to acyclic, monocyclic and bicyclic monoterpenes in sample A. Among sesquiterpenes, two compounds were bicyclic with normal ring (C-6) size, while

the other two compounds are larger ring (C-10) size. α-Copane was represented by tricyclic sesquiterpene and three compounds were macrocyclic sesquiterpenes. Of the eleven oxygenated compounds, bicyclic alcohol and acyclic sesquiterpene alcohol are represented by one compound each *i.e.* borneol and nerolidol. Monoterpene alcohol is represented only one compound *i.e*. α-terpineol. Five compounds belonged to bicyclic sesquiterpene alcohol; tricyclic sesquiterpene alcohol and macrocyclic oxide is represented by one compound each.

CONCLUSION

The volatile oil from fruits of *C. zeylanicum* contained *(E)*- cinnamyl acetate and β-caryophyllene as the major compounds. The volatile oil of fruits *C. zeylanicum* has a compositional profile that was distinctly different from that of other parts of the plant such as leaf (Mallavarapu et al., 1995), root bark and stem bark (Senanayake et al., 1978). This was the first report of GC and GC-MS analysis of cinnamon fruit volatile oil and its chemical composition.

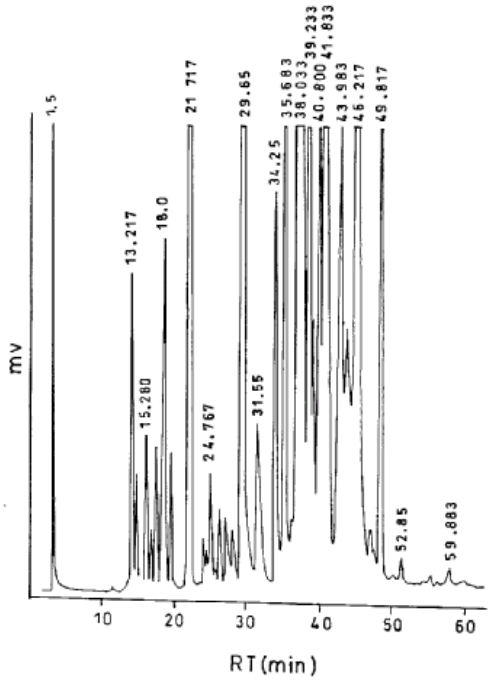


Figure 2.15. GC chromatogram of cinnamon fruit volatile oil from sample A (Karkala)

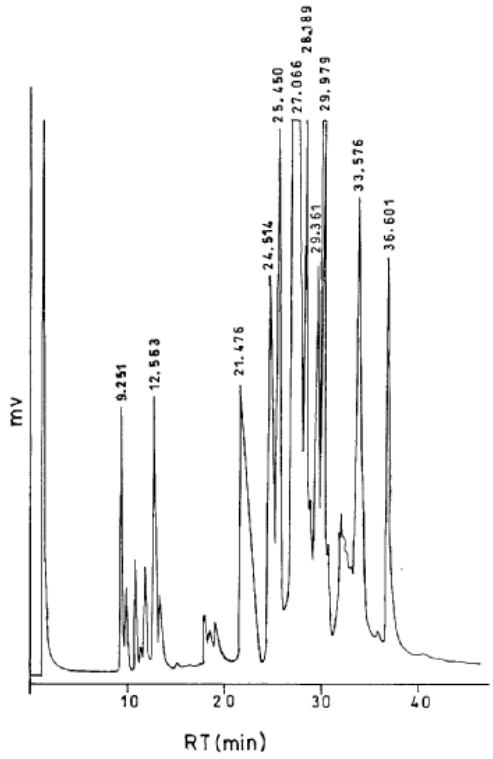


Figure 2.16. Gc chromatogram of cinnamon fruits volatile from sample B (Kottayam)

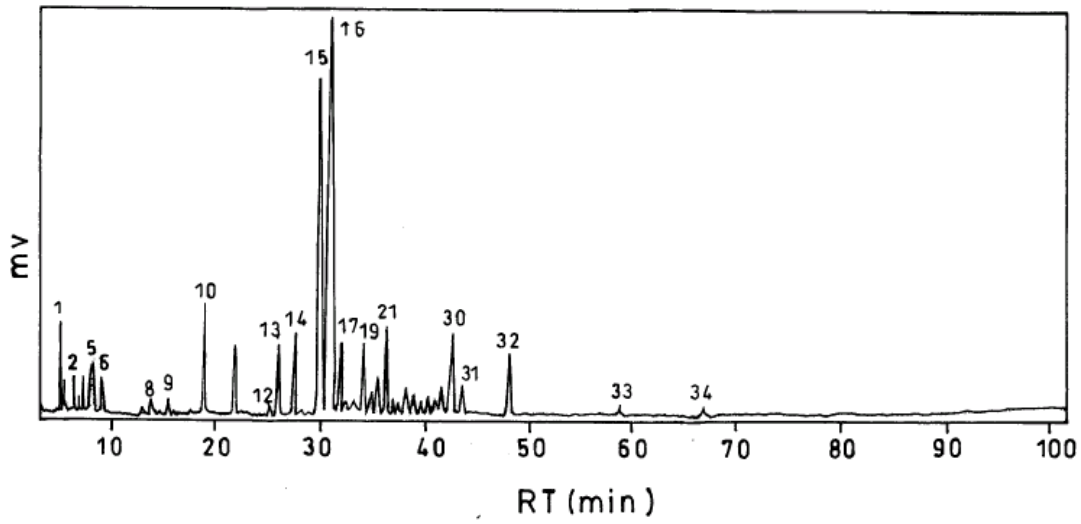


Figure 2.17. GC-MS total ion chromatogram of cinnamon fruits volatile oil from sample A (Karkala).

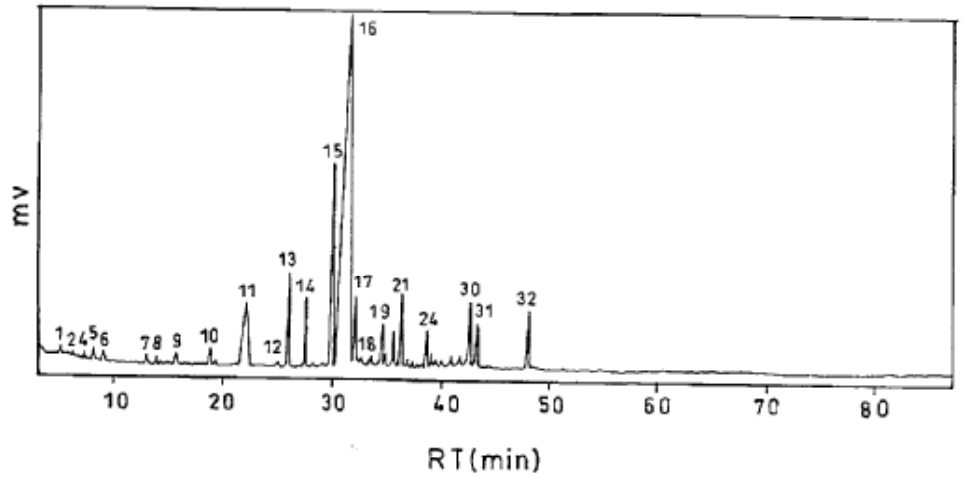


Figure 2.18. GC-MS total ion chromatogram of cinnamon fruits volatile oil from sample B (Kottayam).

Table 2.3 Percentage composition of the fruit volatile oil of cinnamon sample A and B.

Peak No	RT (min)	**Compound**	Peak area		KI	Identification by
			A	B		
1	5.1	α-Pinene	1.57	0.12	953	***MS, RI, CI***
2	6.2	β-Pinene	0.59	0.09	981	MS, RI
3	6.8	Myrcene	0.17	--	993	***MS, RI***
4	7.2	α-Phellanrene	0.69	0.13	1001	MS, RI
5	8.1	β-Phellandrene	1.60	0.37	1026	MS, RI
6	8.9	*(E)*-Ocimene	0.51	0.16	1048	MS, RI
7	13.9	*cis*-Cinnamaldehyde	0.34	0.19	1161	MS, RI
8	14.3	Borneol	0.39	0.27	1167	MS, RI
9	15.7	α-Terpineol	0.32	0.26	1193	MS, RI
10	19.0	*(E)*-Cinnamaldehyde	4.26	0.58	1257	MS, RI, CI
11	21.7	Cinnamyl alcohol	2.24	2.04	1304	MS, RI, CI
12	25.2	Eugenol	0.34	0.17	1354	MS, RI, CI
13	25.9	3-Phenylpropyl acetate	2.91	4.26	1363	MS, RI
14	27.5	α-Copaene	3.10	1.82	1388	MS, RI
15	29.9	β-Caryophyllene	13.73	9.21	1425	MS, RI, CI
16	30.5	*(E)*-Cinnamyl acetate	42.38	54.18	1434	MS, RI, CI
17	31.9	α-Humulene	3.11	2.79	1456	MS, RI
18	33.4	Germacrene-D	0.78	0.65	1477	MS, RI
19	34.4	Germacrene-B	2.01	1.65	1491	MS, RI
20	35.5	γ-Cadinene	1.57	1.18	1508	MS, RI
21	36.3	δ-Cadinene	3.03	2.28	1521	MS, RI
22	36.9	β-Guaiene	0.35	0.35	1533	MS, RI
23	38.2	Nerolidol	0.23	0.36	1555	MS, RI
24	38.8	Caryophyllene oxide	0.91	1.11	1565	MS, RI
25	39.1	Globulol	0.50	0.64	1571	MS, RI
26	39.6	Germacrene-D-4-ol	0.34	0.31	1578	MS, RI
27	40.2	10-*epi*-Eudesmol	0.42	0.53	1588	MS, RI
28	41.0	Cubenol	0.31	0.29	1602	MS, RI
29	41.7	τ-Cadinol	0.81	0.51	1616	MS, RI
30	42.5	Torreyol	2.71	3.42	1630	MS, RI
31	43.1	α-Cadinol	1.01	1.46	1642	MS, RI
32	48.1	Benzyl benzoate	2.65	2.78	1731	MS, RI, CI
33	58.7	Methyl palmitate*	0.34	--	1937	MS
34	66.8	Methyl oleate*	0.33	--	2104	MS
		Total:	**96.55%**	**94.16%**		

RI: Retention Index.
KI: Kovats indices
MS: Mass spectra.
CI: Co-injection with authentic sample.
*: Identified tentatively

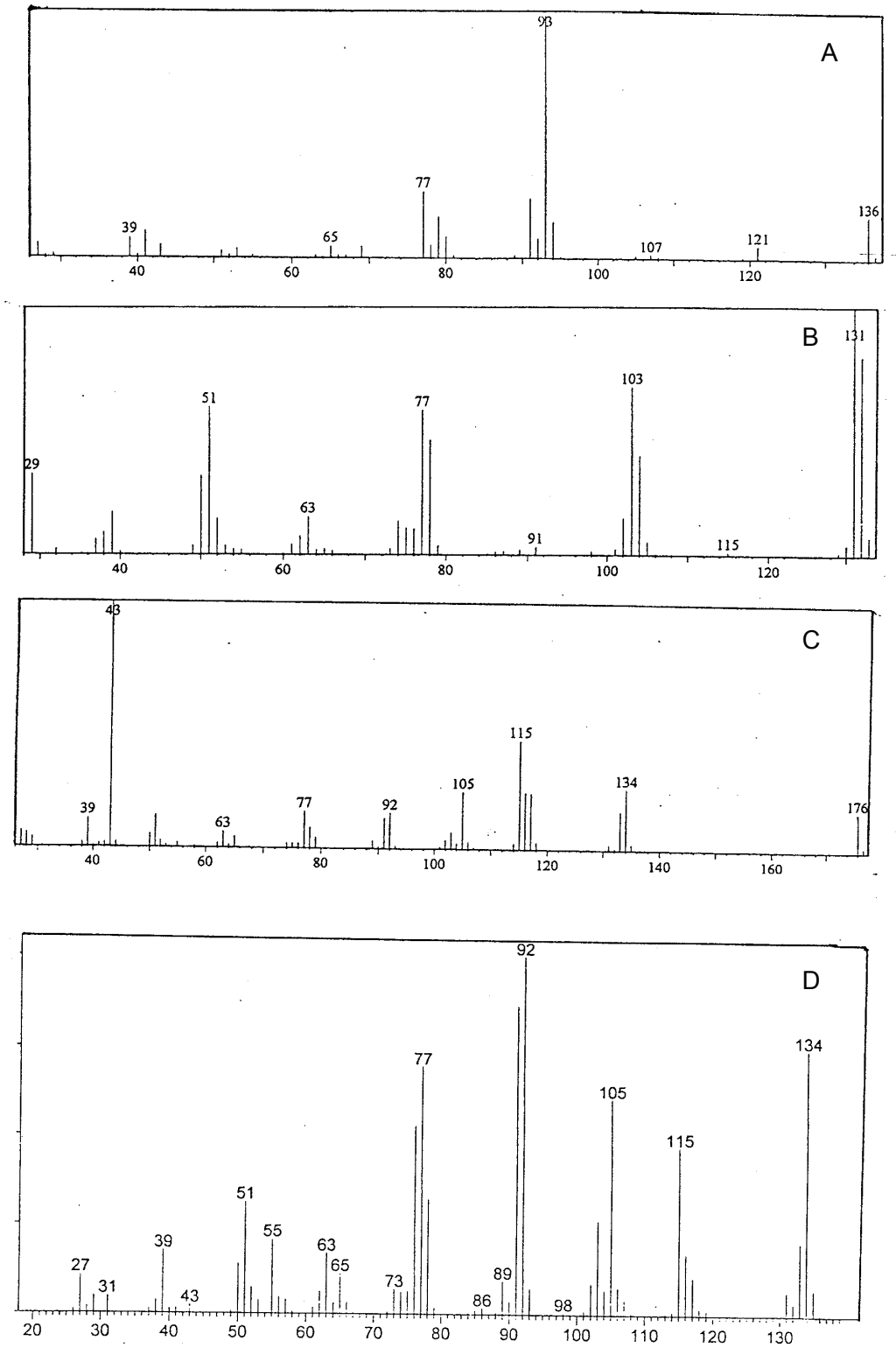


Figure 2.19. Mass spectra of (A). β-Phellandrene, (B). *(E)*-Cinnamaldehyde, (C). *(E)*-Cinnamyl acetate and (D). Cinnamyl alcohol, from cinnamon fruits volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].

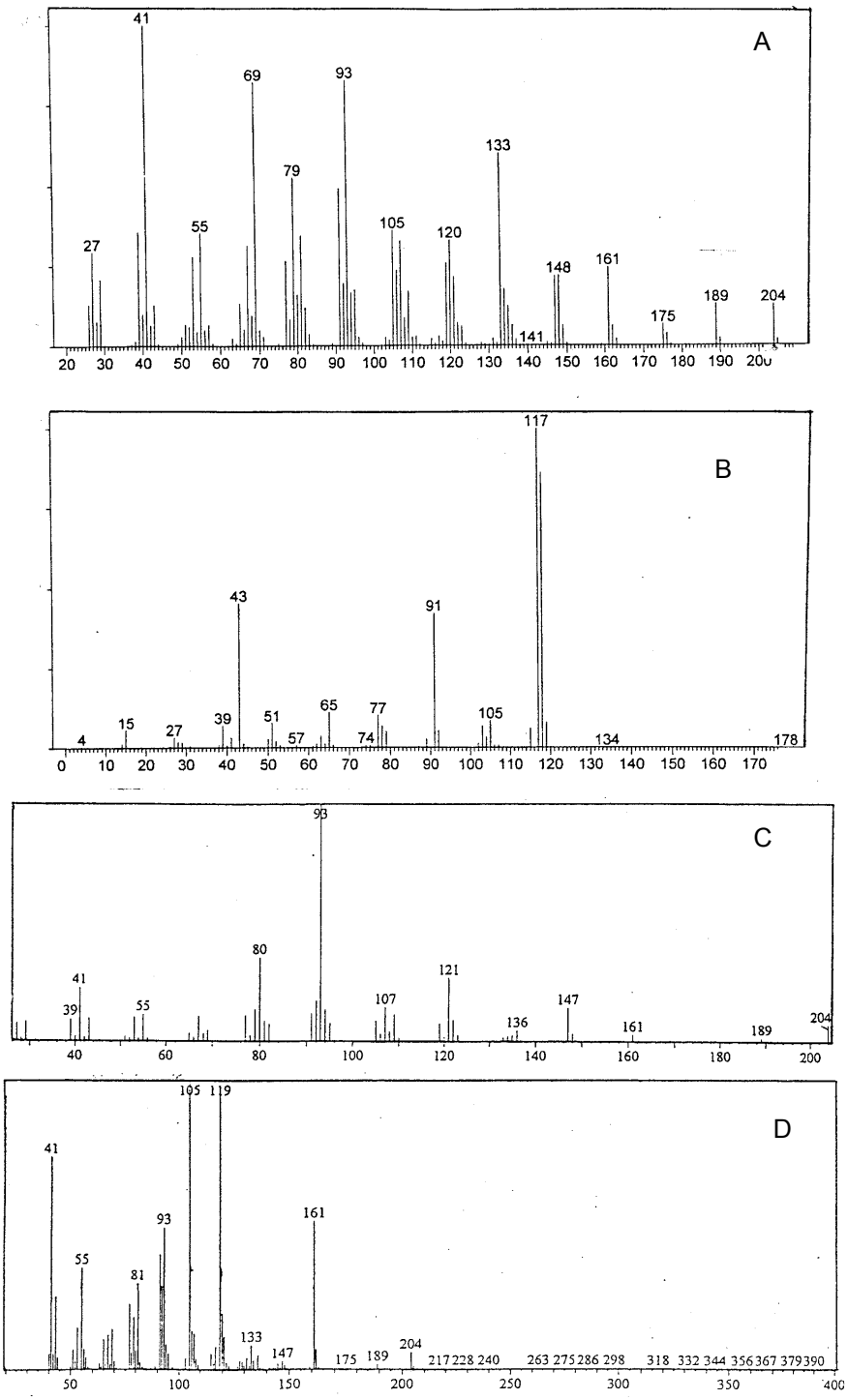


Figure 2.20. Mass spectra of (A). β-Caryophyllene, (B). 3-Phenyl propyl acetate, (C). α-Humulene and (D). α-Copaene, from cinnamon fruits volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].

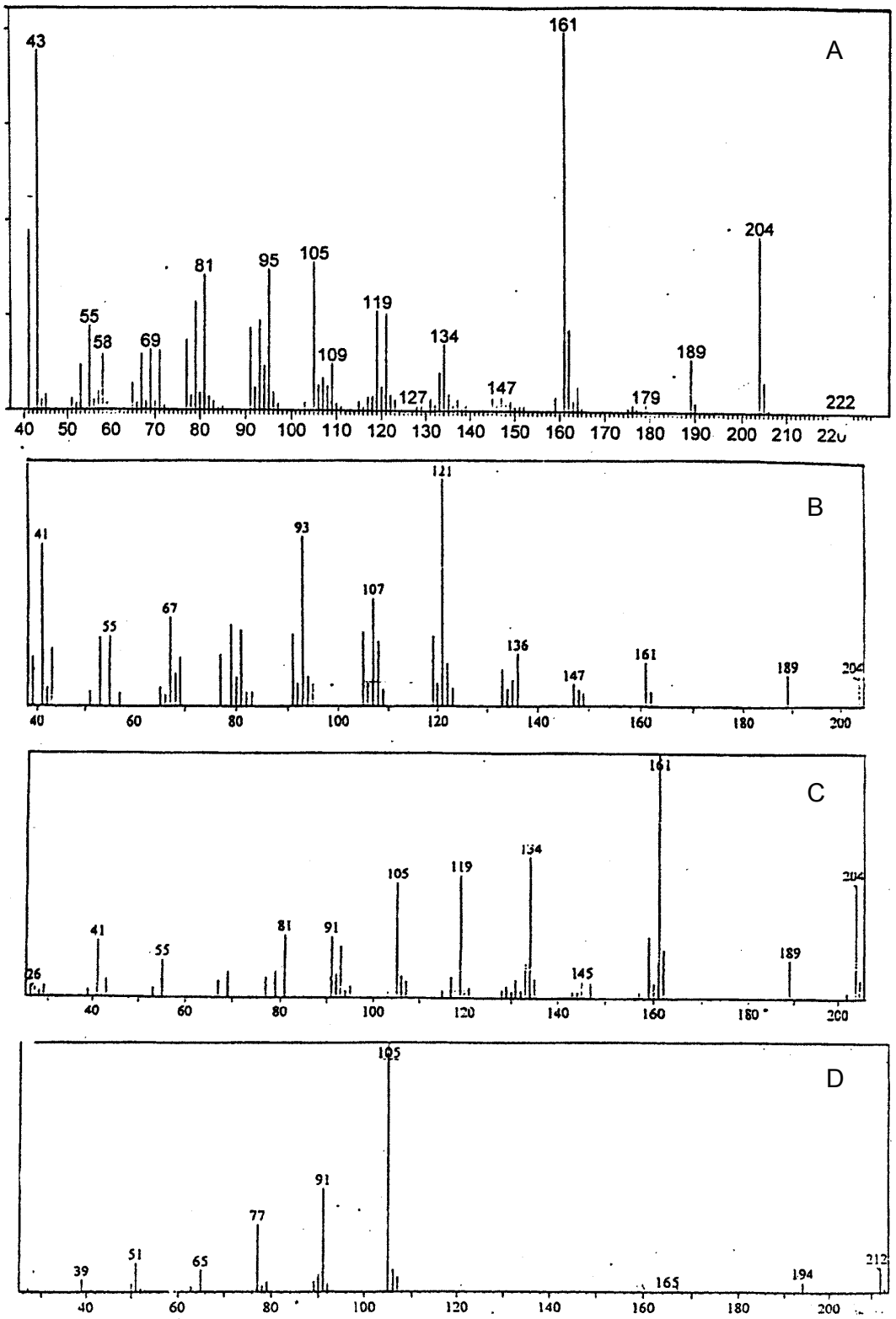


Figure 2.21. Mass spectra of (A). τ-Cadinol, (B). Germacrene-B, (C). δ-Cadinene and (D). Benzyl benzoate, from cinnamon fruits volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].

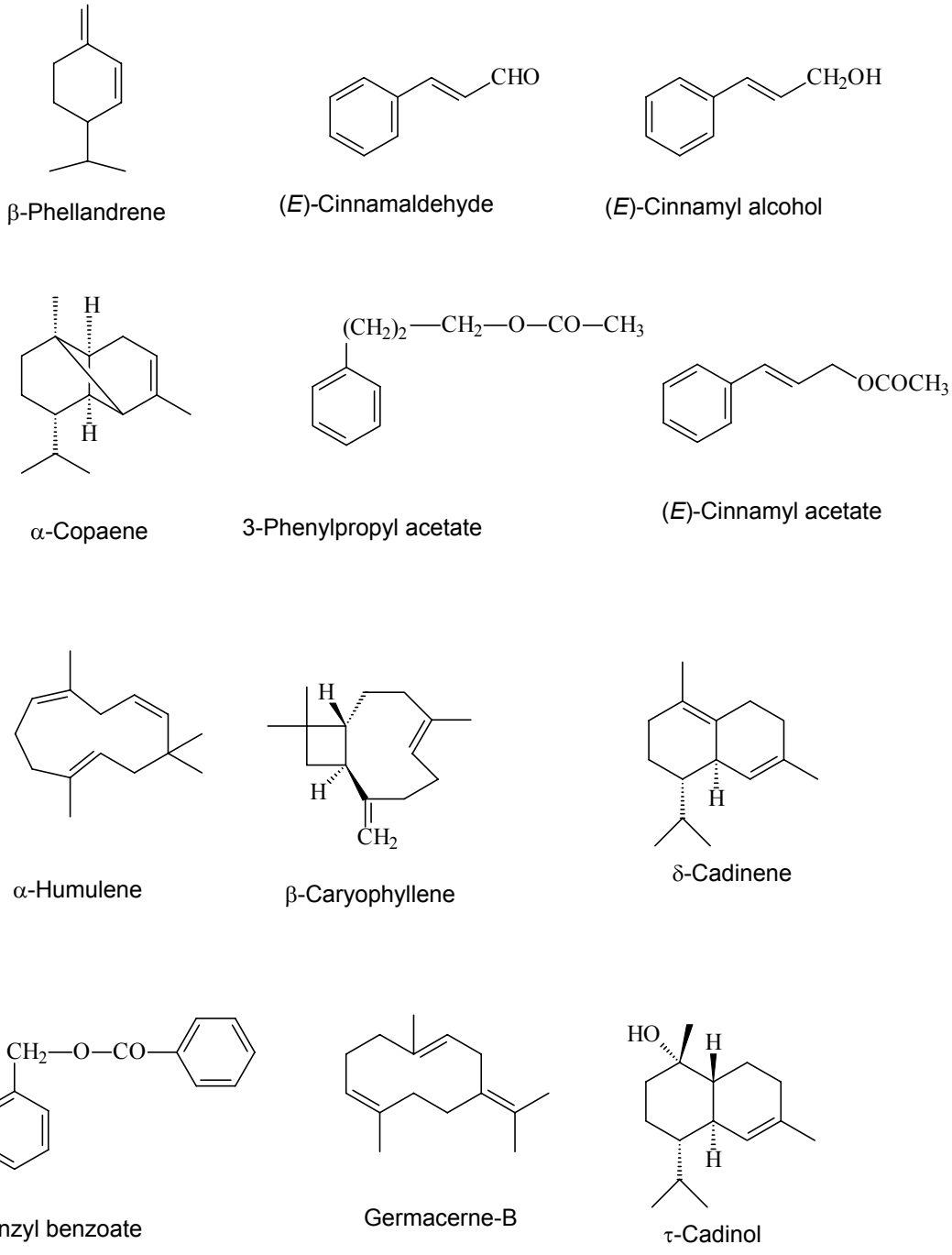


Figure 2.22. Structures of major components identified in cinnamon fruits volatile oil from samples A and B.

2.4. STUDIES ON CHEMICAL COMPOSITION OF VOLATILE OIL FROM FRUIT STALKS

INTRODUCTION

Natural products and naturally derived compounds may have applications as food additives *viz.,* antioxidants, antimicrobials and flavouring agents. Some spices are known to contain essential oil that possesses these activities, such as eugenol in cinnamon bark is antimicrobial. The limitations in the use of naturally derived preservatives are due to associated flavors, which can alter the taste of food. Therefore, understanding of these essential / volatile oils can lead to new technologies for their use in maintaining the quality of foods. Cinnamon is rich of volatile oil. Literature survey revealed no report on the chemical composition of cinnamon fruit stalks volatile oil. Hence, studies were undertaken on the composition of fruit stalks' volatile oil and the results are reported in this sub-chapter.

MATERIALS AND METHODS

Plant Material

The fruit stalks of *Cinnamomum zeylanicum* were collected from Karkala (South Canara district, Karnataka state, India) during fruiting stage. The fruit stalk is a dark purple 1.1 to 4.0 cm long. The plant material was identified and voucher specimen was deposited at the Manasagangotri herbarium (MGH NO.4A/96/02), Department of Botany, University of Mysore, Mysore, India.

Isolation of Volatile Components

Cinnamon fruit stalks (50 g, Moisture content 81%) were coarsely powdered using mixer grinder with 105 ml of ice-cold water for 2 min and it was transferred to 500 ml of round bottom flask. Then the Clevenger apparatus and condenser were fixed and subjected to hydro-distillation for 4 h. The yield of volatile oil was 0.2 ml. The oil was dried over anhydrous sodium sulphate and stored at 4 °C until analysed. The light yellow oil possessed a sweet floral odour.

Gas Chromatography - Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was carried out as per conditions mentioned in Part A, Chapter 2.1, Page No. 47.

RESULTS AND DISCUSSION

The cinnamon fruit stalks were subjected hydro-distillation to obtain volatile oil (yield 0.4%, v/w). It was analysed on GC-MS to determine its chemical composition. GC-MS total ion chromatogram of volatile oil is presented in Figure 2.23. Retention indices for all the compounds were determined according to the Kovats method using n-alkanes as standards (Jennings and Shibamoto, 1980). The compounds were identified by comparison of Kovats indices (Jennings and Shibamoto, 1980; Davies, 1990) and by co-injection with an authentic specimen. Also, by matching their fragmentation patterns in mass spectra with those of

NIST62- library and published mass spectra (Jennings and Shibamoto, 1980; Ten Noever Bravw et al, 1988; Adams, 1989).

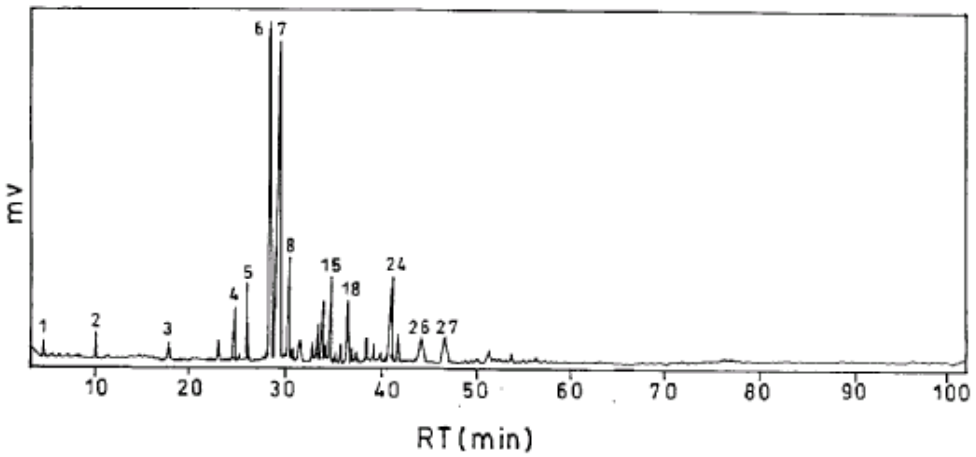


Figure 2.23. GC-MS total ion chromatogram of cinnamon fruit stalks volatile oil.

In the volatile oil of fruit stalks of *C. zeylanicum,* twenty-seven chemical constituents were identified (Table 2.4). These compounds constituted to ≈ 96% of the volatile oil. Oxygenated compounds were the major components and present to an extent of ≈ 53%. Mass spectra of major twelve compounds are presented in Figures 2.24 – 2.26. Three types of oxygenated compounds were present *viz.* esters (39.50%); aldehydes (0.34%); and alcohols (12.76%). Several compounds represented esters and alcohols. However, aldehydes were represented by only one compound *i.e.,* (*E*)-cinnamaldehyde. Among the esters, *(E)*-cinnamyl acetate was the major compound and constituted to an extent of 36.59%, followed by 3-phenylpropyl acetate and benzyl benzoate. In alcohols, all were terpenic alcohols. Acyclic monoterpene alcohol was represented by only one compound *i.e*. linalool. Among the nine sesquiterpenes alcohols, τ-

cadinol and ledol were the major compounds and present to an extent of 4.90 and 2.55% respectively.

Non-oxygenated compounds were present to an extent of 43.36%. Two classes of terpenes were present *viz.* monoterpene (0.40%) and sesquiterpene (42.98%). Among the monoterpenes, only β-pinene was present. Among the sesquiterpenes, β-caryophyllene was the major compound present to an extent of 22.36%; followed by α-humulene, α-copaene, δ-cadinene, germacrene-B, germacrene-D and γ-cadinene (Table 2.4; Figure 2. 27).

The cinnamon fruit stalks volatile oil contained twenty-three terpenoids. Of the thirteen hydrocarbons, bicyclic monoterepne was represented by β-pinene. Two compounds *i.e*. γ-curcumene and *cis*-bisabolene belonged to monocyclic sesquiterpenes. Four compounds belonged to bicyclic sesquiterpene with normal ring (C-6) and two compounds were larger ring (C-10) size. α-Copane was represented the tricyclic sesquiterpene hydrocarbon and three compounds were macrocyclic sesquiterpenes. Of the ten oxygenated compounds, one compound (linalool) belonged to monocyclic sesquiterpene alcohol, one compound (nerolidol) represented the acyclic sesquiterpene, five compounds were bicyclic sesquiterpene alcohols and three compounds belonged to tricyclic sesquiterpene alcohols.

CONCLUSION

Cinnamomum zeylanicum fruit stalks volatile oil contained *(E)*-cinnamyl acetate (36.59%) and caryophyllene (22.4%) as major compounds. Therefore the former compound can be a natural substitute for synthetic *(E)*-cinnamyl acetate used as a flavouring agent in confectioneries and liquors.

Table 2.4. Chemical composition of *C. zeylanicum* fruit stalks volatile oil

Peak No	**RT (min)**	*Compound*	**Peak area (%)**	KI	**Identification by**
1	4.4	β-Pinene	0.40	950	RI, MS
2	10.07	Linalool	0.70	1051	RI, MS
3	17.53	*(E)*-Cinnamaldehyde	0.34	1240	RI, MS, CI
4	24.35	3-Phenylpropyl acetate	1.45	1359	RI, MS
5	25.90	α-Copaene	3.02	1378	RI, MS
6	28.40	Caryophyllene	22.36	1420	RI, MS
7	29.50	*(E)*-Cinnamyl acetate	36.59	1430	RI, MS, CI
8	30.28	α-Humulene	5.49	1451	RI, MS
9	31.73	Germacrene-D	0.53	1476	RI, MS
10	32.78	Germacrene-B	0.96	1489	RI, MS
11	33.10	Valencene	0.55	1490	RI, MS
12	33.23	α-Muurolene	1.29	1491	RI, MS
13	33.88	γ-Cadinene	2.27	1505	RI, MS
14	34.00	*cis*-γ-Bisbolene	0.52	1510	RI, MS
15	34.65	δ-Cadinene	4.70	1518	RI, MS
16	34.95	γ-Curcumene*	0.56	1522	MS
17	35.28	β-Guaiene*	0.73	1529	MS
18	36.50	Ledol	2.55	1540	RI, MS
19	36.92	Nerolidol	0.30	1553	RI, MS
20	37.42	Spathulenol	0.40	1555	RI, MS
21	38.37	Globulol	0.75	1568	RI, MS
22	39.08	10-*epi*-Eudesmol	0.65	1581	RI, MS
23	40.03	***Cubenol***	0.20	1610	RI, MS
24	40.83	τ-Cadinol	4.90	1626	RI, MS
25	41.00	Torreyol	0.65	1629	RI, MS
26	41.12	α-Cadinol	1.66	1638	RI, MS
27	46.45	Benzyl benzoate	1.46	1690	RI, MS, CI
			Total:	**95.98%**	

RI: Retention Index.
KI: Kovats indices.
MS: Mass spectra.
CI: Co-injection with authentic sample.
*: Identified tentatively

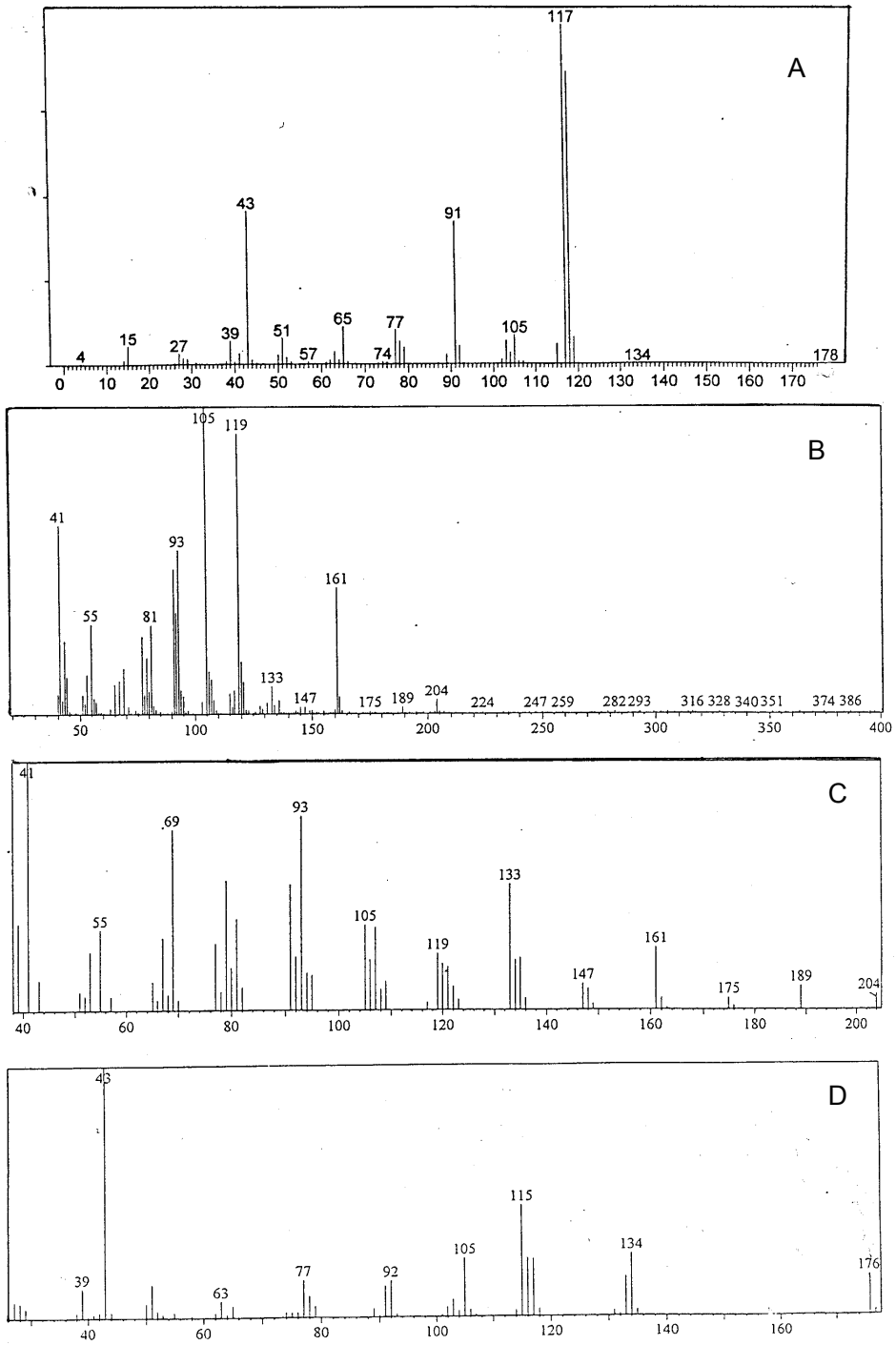


Figure 2.24. Mass spectra of (A). 3-Phenyl propyl acetate (B). α-Copaene, (C). Caryophyllene and (D). *(E)*-Cinnamyl acetate, from cinnamon fruit stalks volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].

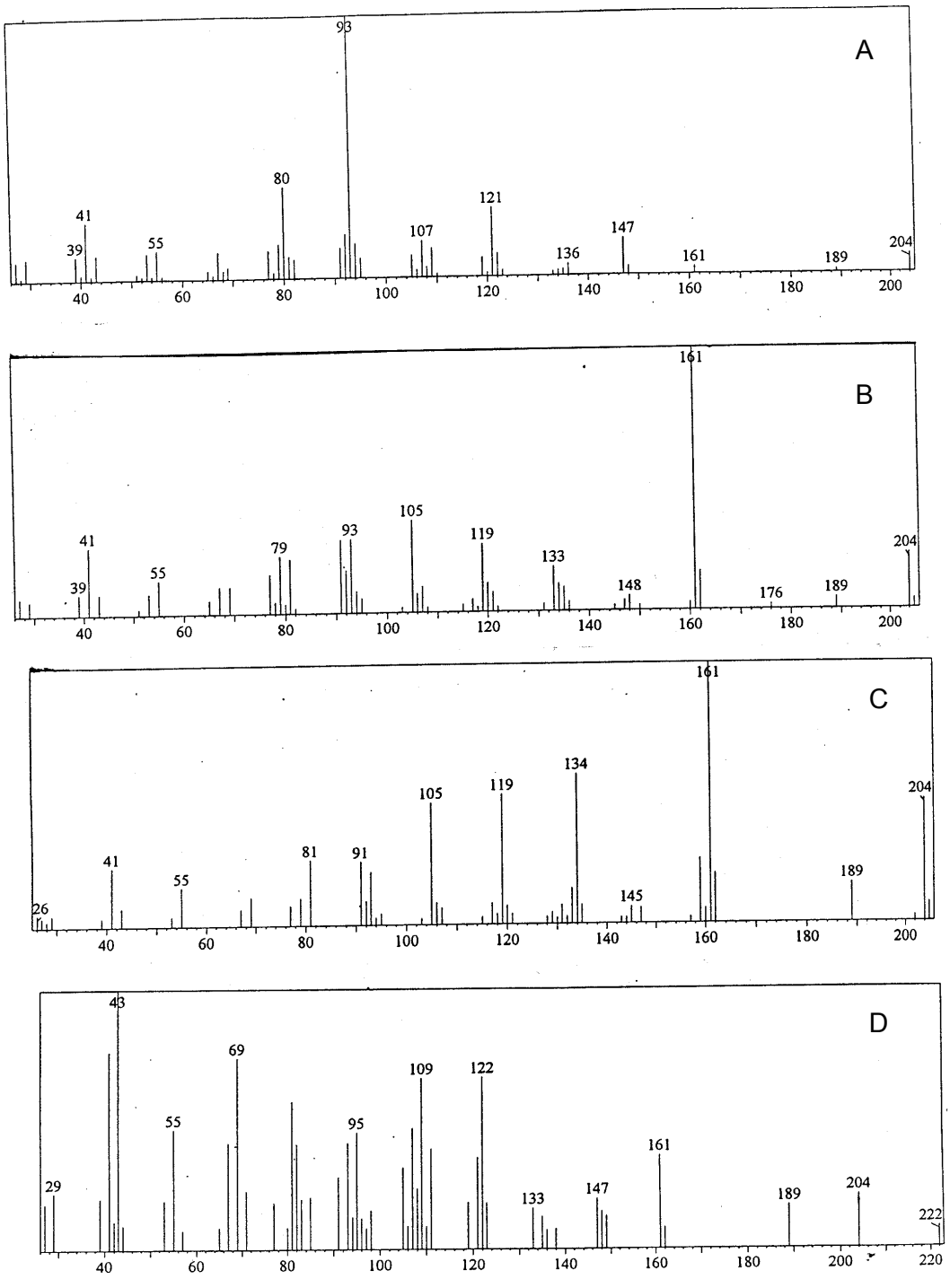


Figure 2.25. Mass spectra of (A). α-Humulene, (B). γ-Cadinene, (C). δ-Cadinene and (D). Ledol, from cinnamon fruit stalks volatile oil,

[X-axis: m/z, Y-axis: Relative abundance (%)].

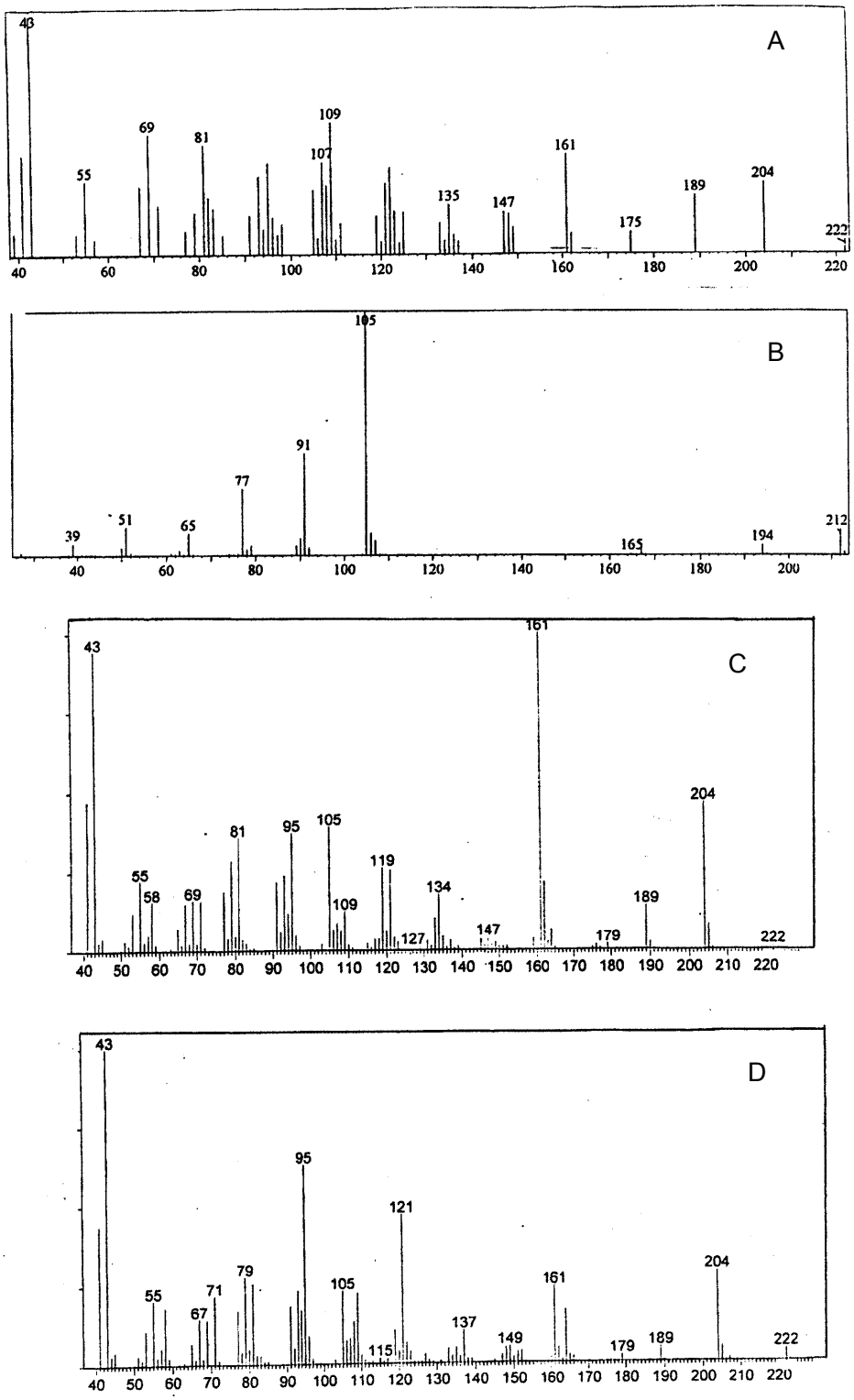


Figure 2.26. Mass spectra of (A). Globulol, (B). Benzyl benzoate, (C). τ-Cadinol and (D). α-Cadinol, from cinnamon fruit stalks volatile oil, [X-axis: m/z, Y-axis: Relative abundance (%)].

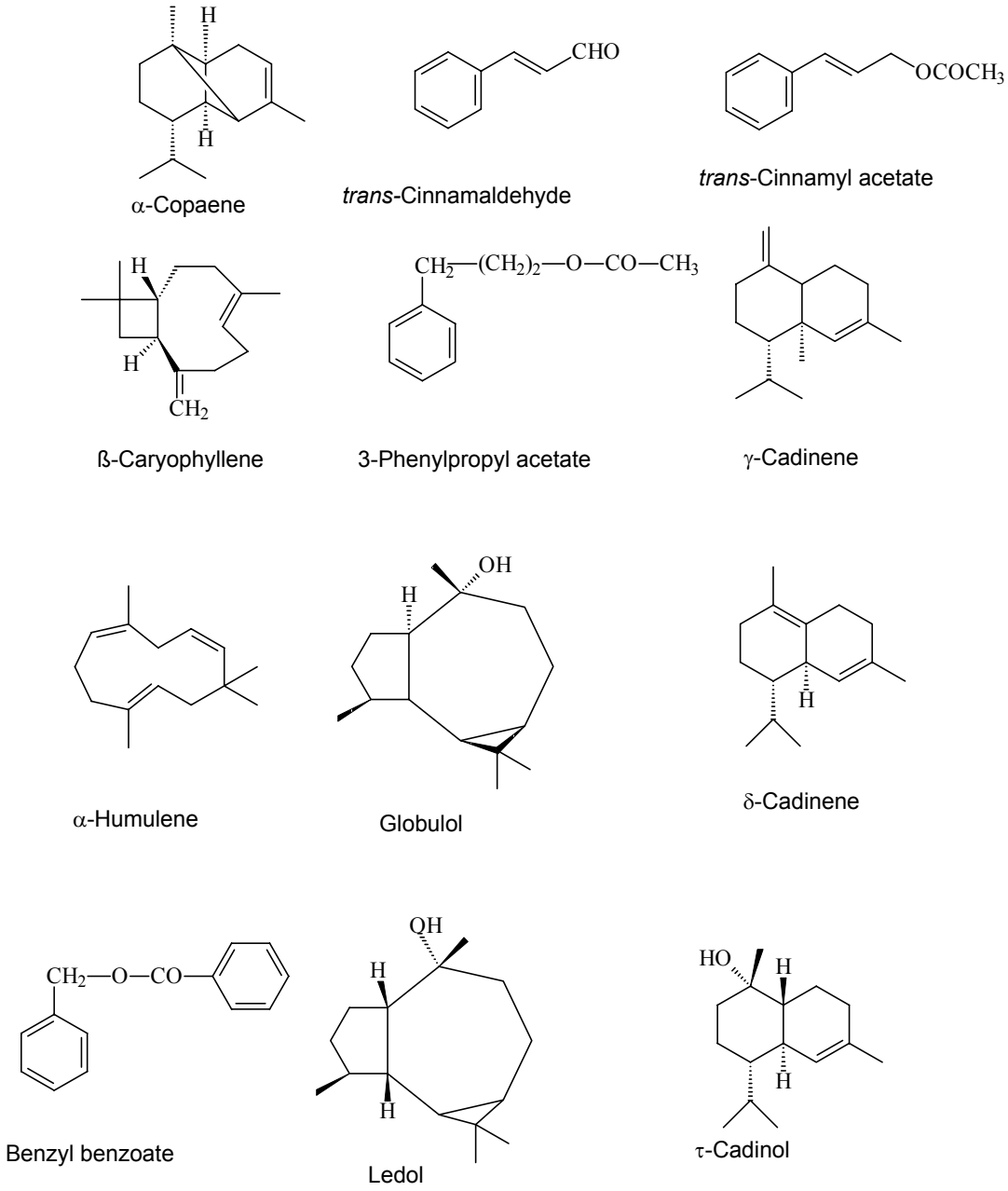


Figure 2.27. Structures of compounds from cinnamon fruit stalks volatile oil.

2.5. BIOGENESIS OF VOLATILE COMPONENTS FROM UNCONVENTIONAL PARTS OF *CINNAMOMUM ZEYLANICUM*

INTRODUCTION

Human beings have confronted with a number of secondary products, since ancient times. While gathering his food he was attracted by certain secondary products and repelled by others. He used many secondary substances to his advantages. He processed cellulose fibres to make cloths and he used wood, a mixture of cellulose and lignin, for building homes and cooking meals. Much has changed since those times and the utilisation of secondary products has increased enormously for various purposes. We enjoy the taste and flavour of beverages. We use medicinal remedies, which in many cases contain secondary products as active principles (Harborne, 1982).

Secondary products dominate taste, flavour and colour of foodstuffs and beverages. These attract and repel human beings in the same way as other vertebrates. Most attractive qualities of foodstuffs are sweetness and aroma; whereas repellent characters are sharpness, bitterness and astringency. But attractiveness or repellence is strongly dose-dependent, as may be seen with the flavouring sulphur compounds of garlic, which are attractive in trace amounts, but repellent if present in higher quantities. Beverages, which are especially attractive, if possess a suitable balance among the attributes of sweet, acidic, and astringent *e.g.,* wines. Usually complex mixtures of secondary products influence on choice of food. However, in some cases the characteristic

flavour is dominated by one main component *viz*., apple (ethyl-2-methylbutyrate), peach (undecalactone), and coconut (nonalactone). Small changes in chemistry may alter these properties completely (Ohlloff, 1978).

Humans are very sensitive to some types of secondary products. The bitter taste of the alkaloid brucine, may be recognized in concentrations of 0.0001% whereas the odour of cucumber in a concentration of 10^{-8}%. The threshold concentrations are highly varied among persons and depend on the physiological state even in the same person. Today, there are only about a dozen major crop plants used in human nutrition: wheat, rice, corn, potato, barley, sweet potato, cassava, grapes, soybeans, sugars, sugar beet and sorghum. In these plants, the concentration of secondary products has been balanced by a plant breeding technique and by selection in comparison with their wild progenitors (Harborne, 1982). In some instances, crop plants were also selected for a higher content off certain types of secondary products. Cultivated apples, pear and other fruits have a better aroma, *i.e.* contain more volatile secondary substances and in addition to a higher sugar content and also possess more attractive pigments than their wild progenitors.

In general, aroma compounds are generated either during normal metabolic processes, or during the processing / cooking of plant parts for edible purposes. The biosynthetic routes lead to three major classes of volatiles (shikimic acid derivatives, terpenoidal derivatives and green leaf volatile derivatives) that are released from plants as shown in Figure 2.28.

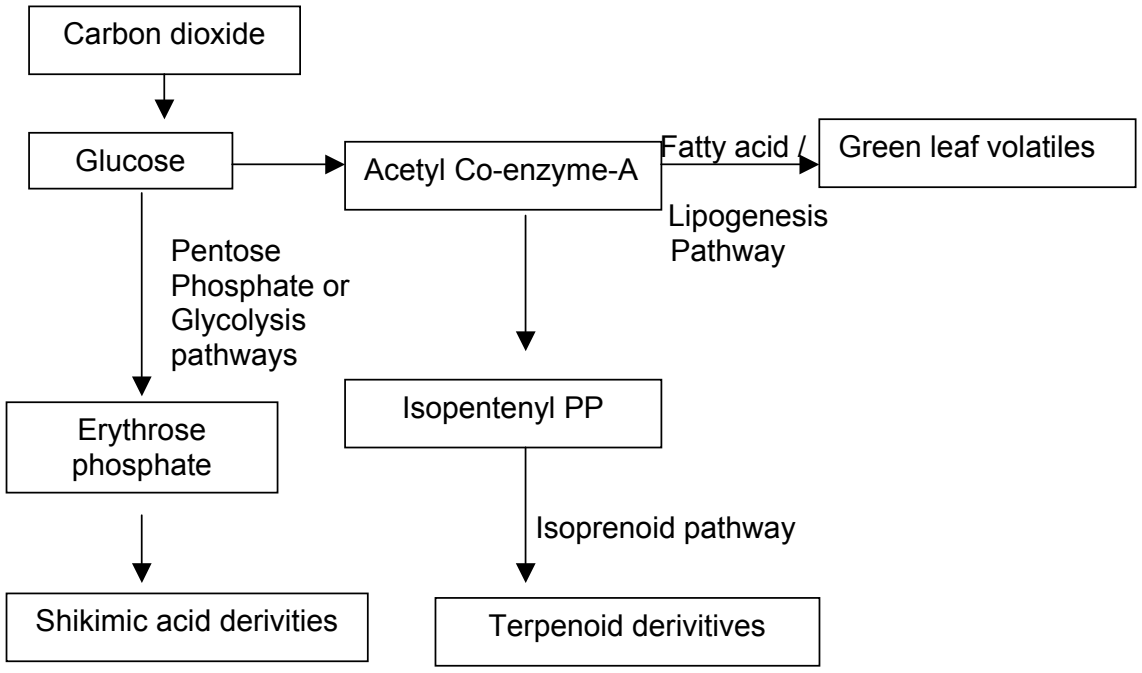


Figure 2.28 Biosynthetic routes for the formation of volatiles from plants.

The general approach to biosynthesis has been to break up the structure into units from which the compound could plausibly be derived (Figure 2.28). Furthermore, this does not mean that the units chosen must necessarily be involved in the building up of the compound. The general principle is that although a particular unit may itself be involved, it is also possible that its equivalent may act as a substitute (*i.e*., any compound that can readily give rise to this unit by means of various reactions) and it may be the actual compound involved in the biosynthesis. Another term used in connection with the study of the biosynthesis of natural products in the living organism is biogenesis (Banthorpe et al., 1972). *This term biogenesis is a collection of hypothesis, which has been proposed to describe the synthesis of natural products in the living organisms* (Banthorpe et al., 1972). Thus, *biogenesis describes the*

hypothesis of transformations, whereas biosynthesis describes the actual pathways for natural products that are synthesized in the living organisms. Often, biogenesis is often an overall picture and does not necessarily state individual steps that are involved in the synthesis. The reactions that are commonly postulated in biogenesis are described as extra skeletal process (Luckner, 1984; Thomson, 1993). In this chapter, biogenesis of volatile compounds from the unconventional parts of cinnamon have been discussed.

Volatile Compounds from the Unconventional parts of Cinnamon

Formation of (E)-Cinnamyl acetate, 3-Phenylpropyl acetate, cinnamyl alcohol, cis and trans-cinnamaldehyde, benzaldehyde, dihydrocinnamaldehyde, benzyl benzoate (shikimic acid metabolites).

The formation and interconversion of phenylpropanoids was explained in Figure 2.29. The exact sequence of the conversions will be a matter for conjecture until experimental evidences becomes available. However, some generalizations appear possible on the basis of existing knowledge (Banthorpe et al., 1972). It has been well documented that shikimic acid is an effective precursor to aromatic natural products such as tannins and lignins and the formation of compounds such as phenylalanine, tyrosine and cinnamic acid (Table 2.5).

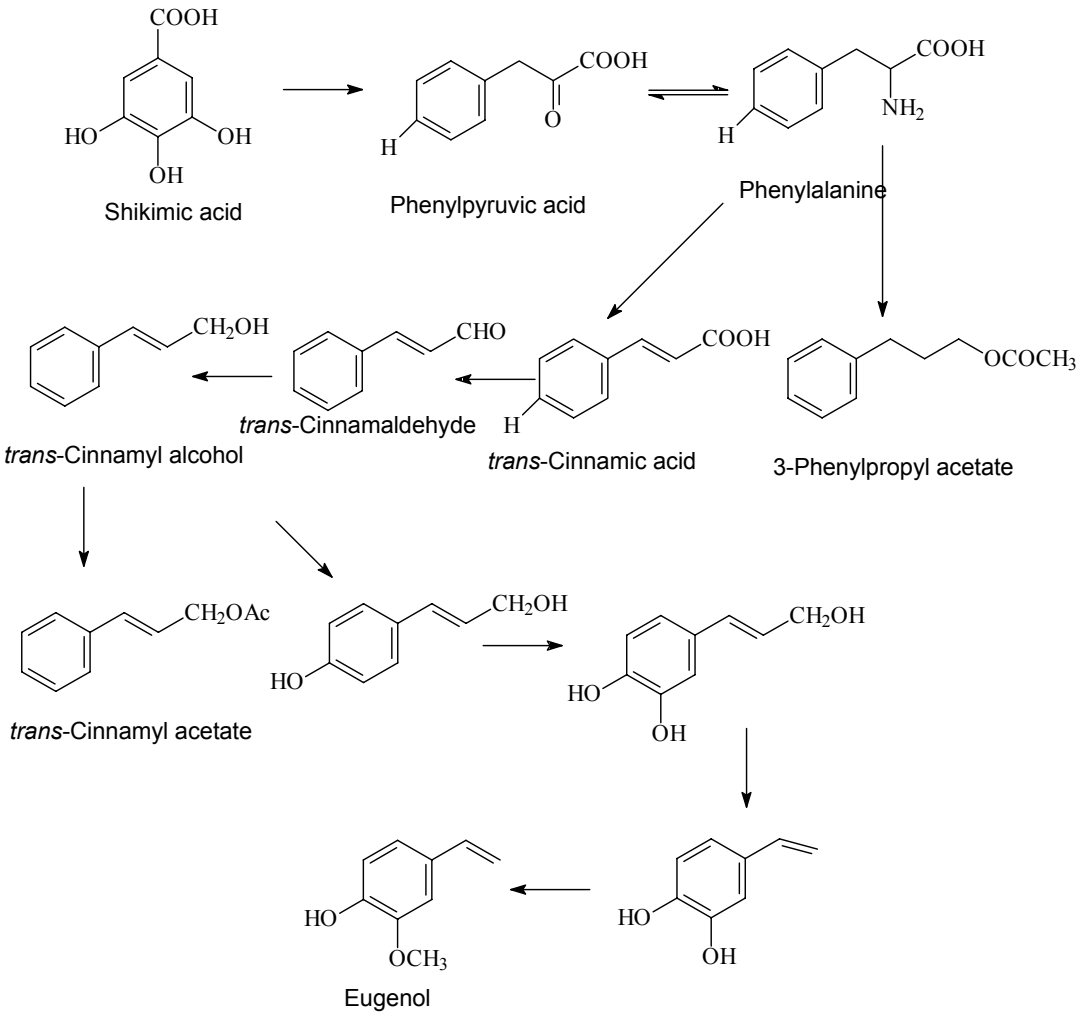


Figure 2.29. Possible routes to the formation of phenylpropanoids in cinnamon

Senanyake et al. (1976) have reported shikimic acid and related compounds are effective precursors to the phenyl propanoids among the volatile constituents of cinnamon. Thus, from a mechanistic viewpoint, the initial hydroxylation of cinnamyl alcohol should be ortho or para to the side chain because of the

Table 2.5. Important precursors of benzoic acid derivatives

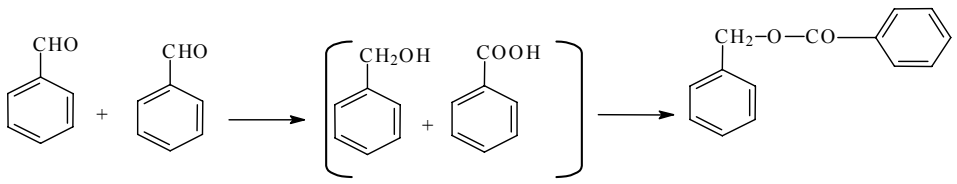
PRECURSOR	BENZOIC ACID DERIVATIVE
Cinnamic acid	Benzoic acid
p-Coumaric acid	p-Hydroxy benzoic acid
Caffeic acid	Protocatecheuic acid
Ferulic acid	Vanillic acid
Sinapic acid	Syringic acid
3,4,5-Trihydroxy cinnamic acid	Gallic acid
o-Coumaric acid	Salicylic acid
2,5-Dihydroxy cinnamic acid	Gentisic acid
2,4-Dihydroxy cinnamic acid	p-Resorcylic acid

extra stabilization achieved by the intermediate due to the pattern of occurrence of mono oxygenated phenylpropanoids in nature. The second hydroxylation of the para oxygenated phenylpropanoid would be expected to occur ortho to the first oxygen due to powerful mesomeric directing influence. However, the cinnamon plant has a unique situation where in the flowers, fruits and fruit stalks have phenyl propanoids *viz., (E)*-cinnamyl acetate, *(E)*-cinnamaldehyde, cinnamyl alcohol and 3-phenylpropyl acetate are predominate.

In plants and animals dihydrocinnamic acids are the important precursors of benzoic acids. The dihydrocinnamic acids are cleaved with the formation of a two-carbon fragment and an aromatic aldehyde, which may be oxidised to the corresponding acids or reduced to the alcohols such as benzyl alcohol and benzoic acid precursors (Figure 2.29). In addition, activated benzoic acid

derivatives (via. CoA ester) can be reduced to benzaldehyde and benzyl alcohol derivatives.

Further, methylation of respective hydroxyl groups may lead to the formation of methoxyl derivatives. Esterification of carboxyl groups with methyl / ethyl alcohol may lead to the respective esters. Finally, benzyl benzoate occurs to a significant extent in cinnamon volatile oils. This compound is neither a phenyl propanoid nor terpenoid. However, its formation in cinnamon could be arising from cinnamldehyde by means of disproportion-type reaction (Figure 2.30).



Benzyl benzoate

Figure 2.30. Possible mechanisms for formation of benzyl benzoate

Formation of Terpenoids

Generally, terpenoids are the primary flavour and fragrance impact molecules found in the essential oil of higher plants. Significant among them are mono- and sesquiterpenoids. Plants produce large amounts of monoterpenoids and acetyl CoA is the starting material for terpenoids. In a branched condensation, the keto function of acetyl CoA reacts with another acetyl CoA molecule to form β-hydroxy-β-methyl glutaryl CoA, which is transformed into the active isoprene unit and ultimately to the terpenoids (Torssell, 1997).

Formation of Monoterpenes

It is also generally accepted that the acyclic monoterpenoids are derived from geranyl pyrophosphate (GPP) and neryl pyrophosphate (NPP) (Figures 2.31 and 2.32).

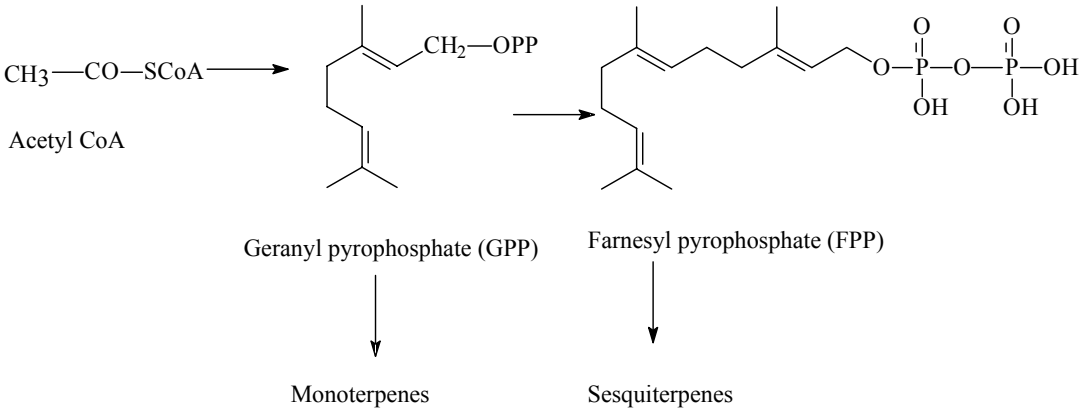


Figure 2. 31. Formation of mono and sesquiterpenes

Cyclic compounds thought to arise from NPP due to the trans cyclic double bond. GPP cannot directly cyclise because of the constrains caused by the *trans* substituted double bond (Figure 2.32) (Valenzuela and Cori, 1967). The terpenoid volatile in cinnamon also might have formed in this fashion. Majority of the minor constituents present in the volatiles from the cinnamon unconventional parts are mono and sesquiterpenoids and their derivatives and these might have formed in the similar way as described (Newman, 1972).

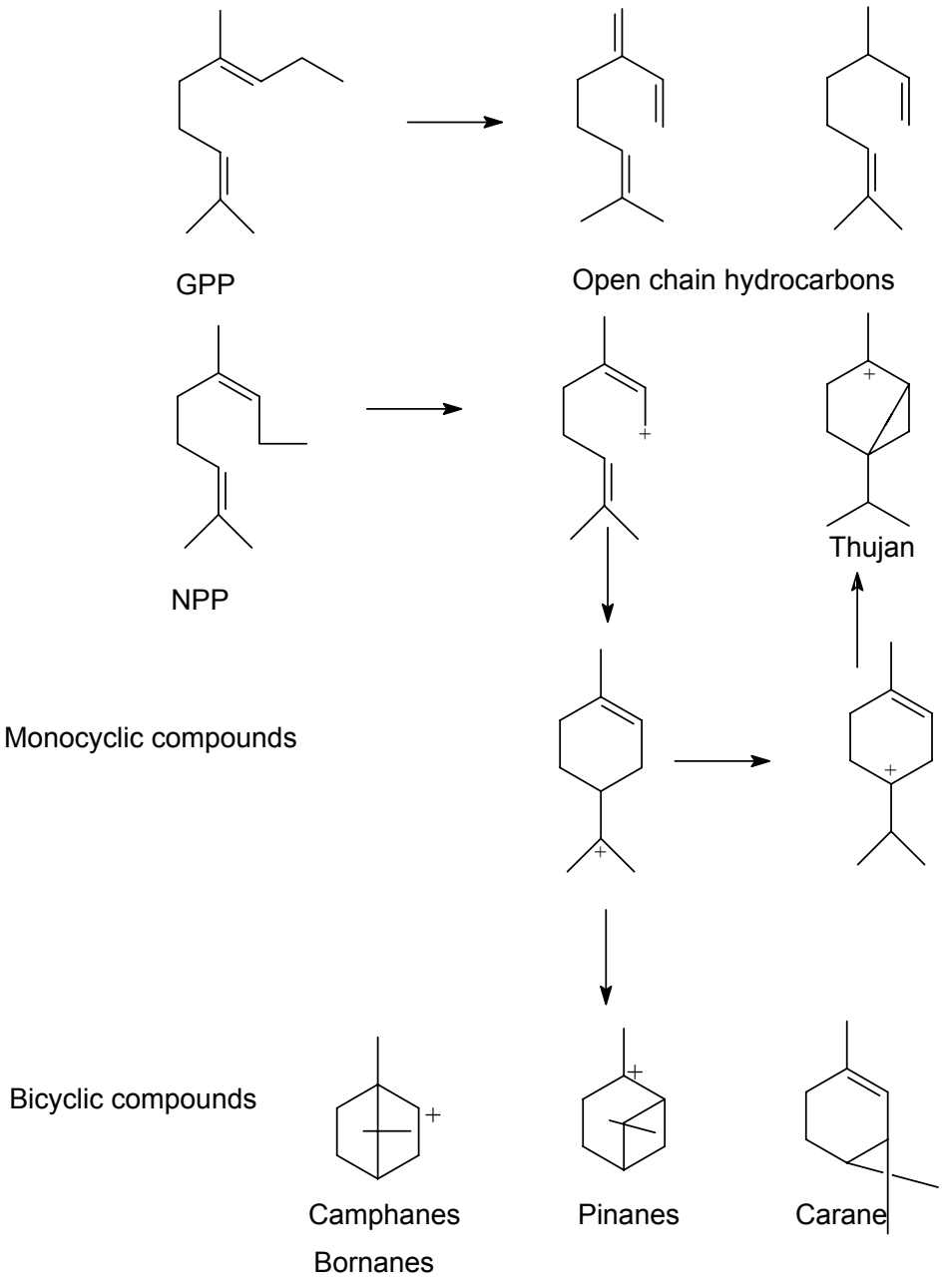


Figure 2.32. Mechanisms for the biosynthesis of monoterpenoids

Formation of Sesquiterpenes

The sesquiterpenes are believed to arise from farnesol (*2-trans-6-trans*-farnesnyl pyrophosphate; Figure 2.31) by transformations analogous to the formation of monoterpenoids from geraniol and nerol. The mode of formation of the variety of terpenoids is well documented and tracer techniques have been successfully used to study the various intermediates (Luckner, 1984). The sesquiterpenic volatiles (*viz.,* β-Caryophyllene, α-humulene, α-copaene, δ- and γ-cadinene, germacrence-B, τ- and α-cadinol, α-bergamotene, α-copaene, α-humulene and δ-cadinene) of unconventional parts of cinnamon might have formed in similar fashion to the above compounds.

Formation of Fatty Acids and their Esters

Acetic acid or its biosynthetic equivalent, acetyl CoA plays an important role in the synthesis of natural compounds. Linear Claisen condensation leads to β-keto esters, which either by reduction and repeated condensation give fatty acids or by further direct condensation, give polyketides. These in turn can cyclise to a vast number of aromatics (Torssell, 1997).

Acetyl CoA is formed in the cell either by degradation on fatty acids decarboxylation of pyruvic acid obtained via glycolysis or degradation of certain amino acids. This can be concluded that the condensation occurs with concomitant exothermic decarboxylation generating the anion. The condensation takes place on a multifunctional enzyme complex, the fatty acid synthetase. Consequently, the condensation must proceed with inversion of

configuration to fit the overall stereo outcome of the process. *e.g.,* the biosynthesis of palmitic acid occurs with one acetyl CoA as a starter, seven malonoyl CoA units and fourteen NADPH are needed. It is rather remarkable that in most organisms the chain elongation practically stops at C_{16}. The enzyme readily accepts a C_{14} CoA ester as starter but reluctantly a C_{16} CoA. The successive addition of two carbon units to acetyl CoA explains why even numbered acids are commonest. Docosanoic acid, tetracosanoic acid and others also might have formed in the similar pathway. However, small amounts of odd numbered acids do occur. These normally originate from propionic acid as starter. Allyl nonanoate is an example that might have originated by the esterification of nonanoic acid (odd numbered acid) by allyl group. Formation of these straight chain saturated fatty acids like tetradecanoic and hexadecanoic acids might have started from the acetyl CoA by polyketide pathway.

2.6. COMPARISON OF CHEMICAL COMPOSITION OF VOLATILE OILS

Volatiles from *Cinnamomum zeylanicum* buds, flowers, fruits and fruit stalks showed many analogies. It can be seen from the earlier chapters 2.1, 2.2, 2.3, and 2.4, the seven volatile components were common in all the four parts of cinnamon, which included the major compound, i.e. *trans*-cinnamyl acetate (Table 2.6). The other six compounds are □-copaene, □-humulene, germacrene-D, □-cadinene, globulol and □-cadinol. Comparison of the chemical composition of volatile oils from, buds, flowers, fruits and fruit stalks revealed that the volatile oil from fruits and fruit stalks contain more relative amounts of shikimic acid derivatives. The character impact compound i.e., *trans*-cinnamyl acetate was found to be the major compound present in the volatile oils of fruits (42% and 54% in sample A and B), flowers (42%) and fruit stalks (36.6%) whereas in the volatile oil from buds present to an extent of 2.4% only. Formation of this compound might have initiated in the buds stage and its further formation could have propagated during the flowering stage and reached a maximum in the fruits stage.

Fourteen compounds represent in the buds volatile oil are found in the fruit stalks oil and constituted 66.34% of the oil (Chapter 2.1 and 2.4). These are α-copaene, (*E*)-cinnamyl acetate, α-humulene, germacrene-D, α-muurolene, γ-cadinene, δ-cadinene, β-guiene, ledol, spathulenol, globulol, α-cadinol, τ-cadinol and benzyl benzoate.

Nine compounds present in the flower volatile oil are found in the fruit stalks oil and constituted 54.5% of the oil (Chapter 2.2 and 2.4). These are (*E*)-cinnamaldehyde, 3-phenylpropyl acetate, α-copaene, (*E*)-cinnamyl acetate, α-humulene, germacrene-D, δ-cadinene, globulol and α-cadinol.

Similarly, seventeen compounds present in the fruits volatile oil are also found in the fruit stalks oil and constituted 87.52% of the oil (Chapter 2.3 and 2.4). The major compounds are *trans*-cinnamyl acetate, β-caryophyllene, α-humulene, τ-cadinol, δ-cadinene and α-copaene.

It may be concluded that mevalonic acid metabolites / terpenoids formed to a maximum extent during the buds stage and increased to some extent during the fruits stage. Further, these are different from oils of other parts of *C. zeylanicum* such as leaf, root bark, and stem bark. Senanayake et al. (1978) reported the chemical compounds of leaf, stem bark and root bark oil by GC. The major compounds was found to be eugenol, cinnamaldehyde and camphor in leaf oil, stem bark oil and root bark oil respectively along with other minor constituents. *trans*-Cinnamyl acetate was found to be 1.7%, 5.0% and traces in leaf, stem bark and root bark oil, respectively. Caryophyllene and α-humulene are also identified in these volatile oils. But there are some similarities as these contain many other compounds that are present in other oils as well. It has been observed that different types of compounds are present at different stages from buds to fruits through flowers, although some of the compounds are similar.

On comparing sesquiterpenoids of cinnamon buds, flowers, fruits and fruit stalks volatile oils, it was found that, one bicyclic hydrocarbon (δ-cadinene) and one oxygenated bicyclic compound (α-cadinol) are found to be common. Tricyclic hydrocarbons are represented by one hydrocarbon (α-copane) and one oxygenated compound (globulol). Three compounds are represented by macrocyclic sesquiterpene hydrocarbons. Macrocyclic oxide is present in three oils except fruit stalk oil.

The content of shikimic acid derivatives are more during the flowering and fruits stage. It may be concluded that mevalonic acid metabolites are formed to a maximum extent during the buds stage and levels increased marginally during the fruits stage. However, the formation of shikimic acid derivatives initiated during buds stage (2.41%) and propagated to the maximum extent during the flowering (49%) and fruits stages (54.78 – 64.03%).

Table 2.6. Comparison of chemical composition of cinnamon buds, flowers, fruits and fruit stalks volatile oils.

Sl. No	Compound	Buds	Flowers	Fruits		Fruit stalks
				A	B	
1	α-Pinene	0.87		1.57	0.12	
2	β-Pinene			0.59	0.09	0.40
3	Borneol		0.17	0.39	0.27	
4	α-Terpineol		0.15	0.32	0.26	
5	*(E)*-Cinnamaldehyde		0.38	4.26	0.58	0.34
6	Cinnamyl alcohol		0.49	2.24	2.04	
7	3-Phenylpropyl acetate		1.99	2.91	4.26	1.45
8	α-Copaene	23.05	3.03	3.10	1.82	3.02
9	α-Bergamotene	27.38	7.97			
10	β-Caryophyllene			13.73	9.21	22.36
11	*(E)*-Cinnamyl acetate	2.41	41.98	42.38	54.18	36.59
12	α-Humulene	6.19	2.40	3.11	2.79	5.49
13	Germacrene-D	2.10	1.31	0.78	0.65	0.53
14	α-Muurolene	1.70				1.29
15	γ-Cadinene	1.57		1.57	1.18	2.27
16	δ-Cadinene	5.97	2.97	3.03	2.28	4.70
17	β-Guaiene	1.91		0.35	0.35	0.73
18	Ledol	1.29				2.55
19	Spathulenol	2.02				0.40
20	Caryophyllene oxide	0.41	7.29	0.91	1.11	
21	Globulol	2.10	3.80	0.50	0.64	0.75
22	10-*epi*-Eudesmol			0.42	0.53	0.65
23	***Cubenol***			0.31	0.29	0.20
24	τ-Cadinol	Tr.		0.81	0.51	4.90
25	α-Cadinol	Tr.	6.35	1.01	1.46	1.66
26	Tetradecanol	4.27	5.05			
27	*epi*-α-Bisabolol	1.90	0.73			
28	n-Heptadecane		2.14			
29	Benzyl benzoate	Tr.		2.65	2.78	1.46

2.7. ISOLATION OF *trans*-CINNAMYL ACETATE

INTRODUCTION

(E)-Cinnamyl acetate is widely used in perfumery because of its excellent sensory and fixative properties. It is used frequently in blossom compositions such as lilac, jasmine, lily of the valley, hyacinth and gardenia to impart balsamic and oriental notes to the fragrance. It is used as a modifier for cinnamic alcohol and as a warm-spicy-floral note in heavy florals, hyacinth etc. It can also introduce warmth to a rose composition at a discrete level of concentration. It is used in flavour composition for imitation apple, apricot, berry, cherry, cinnamon, cassia, grape, peach, pineapple, quince, vanilla etc. (Steffen Archtander, 1969). In addition, it is utilized as modifiers in berry, nut and spice flavour systems (Howe-Grant, 1993). It is used in nonalcoholic beverages (2.7 ppm), ice-creams (6.5 ppm), baked goods (11 ppm), chewing gum (8.7 ppm) and in condiment (2 ppm) (Ash and Ash, 1995). It is also, used in frozen dairy (6.9 ppm), meat products (3.0 ppm), condiment relish (3.66 ppm), soft candy (17.29 ppm), pudina (21.87 ppm), hard candy (73.42 ppm) (Thomas and Nicolo, 1995).

(E)-Cinnamyl acetate was prepared by acetylation of cinnamyl alcohol with acetic acid. (Leonard Levine, 1963). Another method of preparation was pyrolysis of di-esters of 1- phenyl-1, 3-propanediol (1). The assay of the above synthetic product is 98%. Remaining 2% may constitute side products like

substrates, reactants and reagent chemicals. Many times, these chemicals gave undesirable notes to the overall flavour of food systems, which is not acceptable to the consumer. Some times these chemicals are found to be health hazardous and are not metabolised easily and observed to give side effects. Due to these reasons, flavour chemicals from natural sources are more highly priced (10-200 times) than that of corresponding synthetic chemicals. Flavour compounds, isolated from natural sources, also contain minor constituents. These will give characteristic impact to the overall flavour quality in the food system and are considered as harmless, since these are present in the plant material along with flavour compound.

Cinnamon leaf, stem bark and root bark contained *(E)*-cinnamyl acetate to the extent of 1.7%, 5.0% and traces amounts respectively as indicated by GC-MS analysis (Mallavarapu et al., 1995; Senanayake et al., 1978). However, there is no report on the methods for the preparation of *(E)*-cinnamyl acetate from natural sources. Earlier reports on cinnamon revealed that, leaf, stem bark and root bark are used as spices and for the production of volatile oils. Now, unconventional parts such as fruits, flowers, and fruit stalks of *C. zeylanicum* are found to be good sources for *(E)*-cinnamyl acetate. This chapter describes the isolation and identification of *(E)*-cinnamyl acetate from cinnamon volatile oils for the first time.

MATERIALS AND METHODS

Isolation of Volatile Oil

The volatile oils were obtained by hydro-distillation of fruits, flowers and fruit stalks of *C. zeylanicum* as mentioned in the chapters 2.2, 2.3 and 2.4.

Fractionation of Volatile Oils

Ten ml of fruit volatile oil was impregnated on 10 g of silica gel and loaded to 100 g silica gel column chromatography. The column was eluted with 500 ml of non-polar solvent. Then the polarity was increased with 500 ml of halogenated solvent (medium polar). The eluates were concentrated under reduced pressure at 30 °C separately to get fraction I and II with a yield of 3.8 and 4.1 ml. Same procedure was used for fractionation of flowers and a fruit stalks volatile oil of *C. zeylanicum*.

^{1}H NMR Analysis

^{1}H NMR (90 MHz, $CDCl_3$) spectra were recorded on Varian EM-390 instrument. TMS was used as the internal standard.

Determination of Refractive Index

Refractive index was measured using Zeiss butyrorefractometer. The prism was cleaned with alcohol and water circulated at 30 °C. Sample 2-3 drops was placed on the surface of the prism and closed. The mirror was adjusted until the reading is sharp. The instrument was allowed to stand for a few min before the

reading is made so that the sample and the instrument will come to equilibrium. Second reading was noted after a lapse of a few min.

Gas Chromatography Analysis

Fraction I and II were analysed on a Shimadzu GC 15A chromatograph equipped with a FID detector using SE-30 column (10' × 1/8"). Oven temperature was programmed from 75 °C for 5 min, then increased 3°/ min to 230 °C held for 3 min; injector port temperature was 200 °C; detector temperature was 250 °C; nitrogen was used as carrier gas with a flow rate of 40 ml/min. 0.5 μl of samples were injected to GC. Peak areas were computed by a Shimadzu C-R4A chromatopac integrator.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of fraction I and II were carried out using Shimadzu gas chromatograph equipped with QP-5000 Mass Spectrometer (Kyoto, Japan) using SPB-1 capillary column coated with poly dimethylsiloxane (30 m × 0.32 mm i.d.; 0.25 μm film thickness); injector port temperature 200 °C, detector temperature 250 °C and oven temperature was kept initially at 75 °C for 2 min and then increased to 250 °C at the rate of 2 °C /min at which temperature was held for 5 min. Helium was the carrier gas at a flow rate of 1 ml/min. 1.0 μl (1:25 dilution with acetone). Split ratio 1:50; Ionisation Voltage, 70eV.

RESULTS AND DISCUSSION

(E)-cinnamyl acetate was present in the volatile oils of flowers, fruits and fruit stalks in the range of 48, 44-55 and 37% respectively along with many other minor compounds (chapters 2.2, 2.3 and 2.4). A method was developed to obtain >90% *(E)*-cinnamyl acetate from the unconventional parts of cinnamon, which involves the following steps. (i). Hydro-distillation of cinnamon flowers / fruits / fruit stalks and (ii). Fractionation of volatile oil using solid adsorbent and non- polar solvent to obtain > 90% *(E)*-cinnamyl acetate. The isolated fractions I and II were analysed by GC. It was found only one major peak at retention time 27.2 min (Figure 2.33) in fraction II.

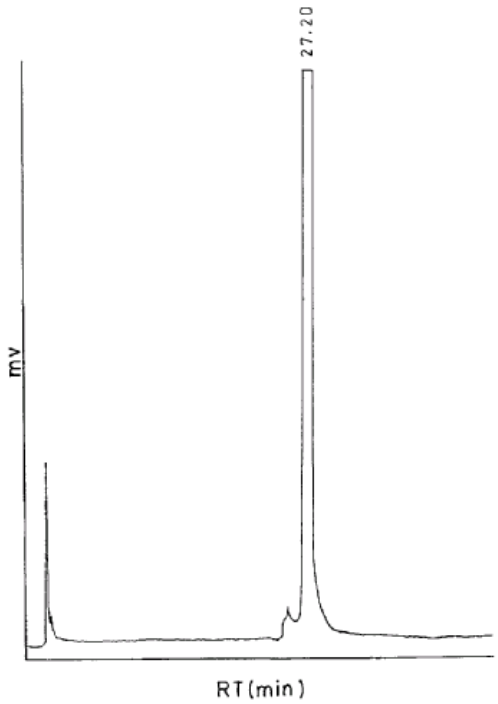


Figure 2.33. GC chromatogram of fraction II.

Retention times of this peak and that of authentic cinnamyl acetate are found to be same. Further, the structure of isolated fraction II was confirmed by GC-

MS. The mass spectrum of fraction II was identified by matching the fragmentation pattern (Figures 2.34 and 2.35) in mass spectra with that of published mass spectra (Adams, 1989). The signals in the ^{1}H NMR spectrum of fraction II at δ 2.1 (s, 3H) indicated the presence of a methyl group adjacent to the ester carbonyl group and δ 7.4 (s, 5H) shows the presence of five aromatic protons. The signals at 6.60 (d, 1H, 17.0 Hz) and 6.2 (dt, 1H, 17.0 and 6.0 Hz) showed the presence of two *trans* protons and 4.5 (d, 6.0 Hz) was assigned to methylene protons. On the basis of above data the isolated fraction II was identified as (*E*)-cinnamyl acetate.

The physical properties of the isolated compound are as follows,

Appearance:	Colourless liquid
Assay:	>94%
BP:	265 °C
Sp. Gravity:	1.047 at 25 °C
Ref. Index:	1.54 at 20 °C
Solubility:	Insoluble in water, soluble in organic solvents.
Organoleptic properties	Characteristic balsamic-floral
Odour:	Sweet taste reminiscent of pineapple.

The remaining (4 - 10%) portion of the fraction II constitutes other flavour compounds, which give desirable overall flavour to the products in food systems. Although these are present only as trace constituents, these compounds can

have a characteristic impact on the over all sensory properties of the natural isolate (Boelens, 1996). These residues are also expected to be metabolisable easily and harmless, since these are also obtained from the same natural source along with the *(E)*-cinnamyl acetate.

CONCLUSION

Volatile oils of flowers, fruits and fruit stalks of cinnamon are found to possess considerable amount of (*E*)-cinnamyl acetate for the first time. These oils were fractionated on silica gel to get cinnamyl acetate (purity > 90%). The structure of the (*E*)-cinnamyl acetate was identified on the basis of mass spectra and ^{1}H NMR spectral data. This compound could be used for the replacement of synthetic (*E*)-cinnamyl acetate in perfumery because of its excellent sensory and fixative properties.

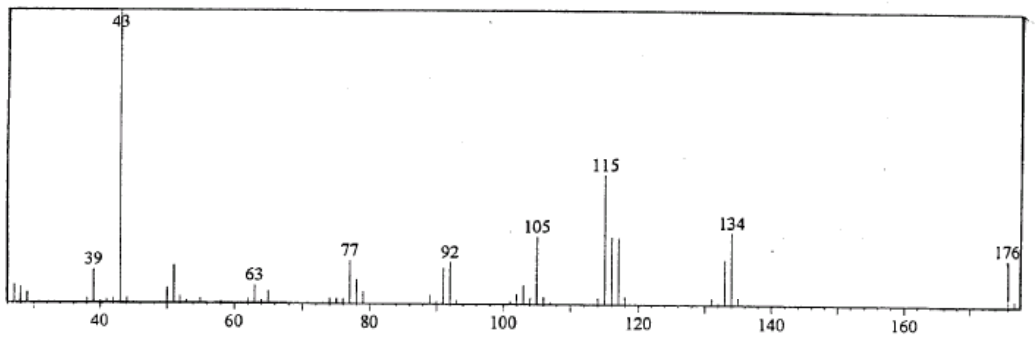


Figure 2.34. Mass spectra of *(E)*-cinnamyl acetate.

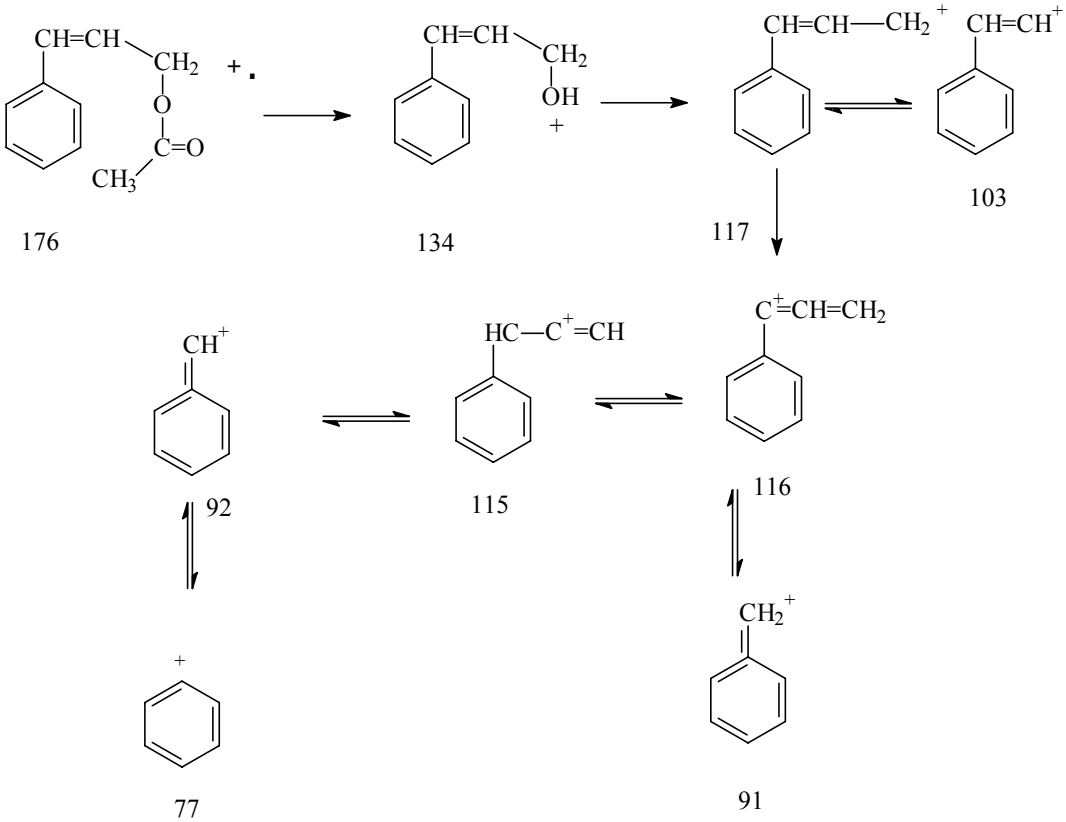


Figure 2.35. Fragmentation pattern of *(E)*-cinnamyl acetate.

PART – A

Chapter - 3

Chemical examination of the solvent extracts from fruits of cinnamon and their bioactivities

3.1. ISOLATION AND IDENTIFICATION OF CHEMICAL CONSTITUENTS

INTRODUCTION

A large variety of complex phenols are widely found in natural products. The interest in these phenolic compounds is because of their known health benefits, which are due to their antioxidant activity as free radical scavengers. In phenolic acids and their esters, this activity depends on the number of hydroxyl groups (Rice Evans et al., 1996). The separation and isolation of these phenolic compounds from their natural sources is actually very complicated, reason for which, many of them are not produced at a large scale and are not commercially available, e.g. proanthocyanidins.

Flavonoids, a group of polyphenolic compounds, are widely distributed and have been reported to act as antioxidants in biological systems (Morel et al., 1993). Flavonoids are considered to have antioxidant activity similar to that of α-tocopherol, vitamin E. It is one of the most common and active naturally occurring antioxidant compounds used in food, because of its activity in both hydrophilic and lipophilic systems (Kühnau, 1976).

Cinnamomum zeylanicum bark contains dimeric, timeric and higher oligomeric proanthocyandins, which are doubly linked bis-flavan-3-ol unit in the molecule (Figure 3.1). This class of proanthocyanidins is not so frequently reported in nature in contrast to the singly linked proanthocyanidins, which occur widely in unripe fruits and in plants of wood habit (Nonaka et al., 1983).

Morimoto et al. (1985) extracted the *Cinnamomum sieboldii* root bark with 80% aqueous acetone and it was fractionated to get (-)-epicatechin, (+)-catechin and twelve proanthocyanidins. Later, Morimoto et al., (1986) reported four procyanidins, one doubly linked procyanidins and two new procyanidins glucosides from the bark of *Cinnamomum cassia*. In this chapter, the isolation of chemical constituents from the extracts of fruits of cinnamon and their identification using spectral data have been reported for the first time.

MATERIALS AND METHODS

Equipment

^{1}H and ^{13}C NMR spectra of compounds 1 - 5 were recorded on Bruker DRX 600 and Bruker Avance 800 instruments (Bruker, Karlsruhe, Germany). IR spectra were recorded on a Bruker-IFS 25 spectrometer using KBr discs.

Extraction

Cinnamon fruits were dried at 30 °C under shade for one week. The dried fruits were powdered (60-80 mesh) and it was extracted (510 g) in a Soxhlet with 1500 ml of hexane for 6 h. The extract was filtered and concentrated *under vacuum* to get 9.60 g of yield. The defatted fruit powder (500 g) was extracted successively in a Soxhlet for 4 h with solvents such as benzene, ethyl acetate, acetone, methanol and water (each 1500 ml). The extracts were concentrated under reduced pressure at 35 °C to remove solvents. The yields of benzene, ethyl acetate, acetone, methanol and water extracts were 3.90, 9.35, 8.70, 24.95, and 19.35 g respectively. Ethyl acetate, acetone, methanol and water

extracts were subjected to HPLC analysis (conditions mentioned in the next paragraph) and the chromatograms are presented in the Figure 3.2.

HPLC Analysis

The high performance liquid chromatographic system consisted of a Hewlett Packard HPLC model HP 1100 Series (Hewlett-Packard, CA, USA) equipped with quaternary pump, fitted with a Zorbax C_{18} (Hewlett Packard, CA, USA) analytical column (25 cm $\times$ 4.6 mm i.d., 5 μm particle size). The injection system (Rheodyne) used was a 20 µl sample loop. Detection was done using a HP 1100 Series variable wavelength detector at wavelength of 280 nm. The gradient mobile phase consists of (A) methanol (B) 1% acetic acid in water, with the flow rate of 0.7 ml/min. The elution program involved a linear gradient from 95 to 50% B in A for 0 - 25 min and 95% B in A by 30 min followed by 5 min of equilibrium with 95% B. The compounds were quantified using HP ChemStation software. The 20 μl of cinnamon extracts and column fractions were injected to HPLC. The column was equilibrated with 95% B and 5% A for 10 min prior to each analysis.

Determination of Total Phenolics

The concentration of phenolics in the extracts was determined as per the method, described by Waterhouse et al., (2000) and results were expressed as catechin equivalents. The cinnamon extracts (10 mg) and catechin (10 mg) were dissolved in a 10 ml of mixture of methanol:water (6:4 v/v). Cinnamon extracts (equivalent to 100 μg) and different concentrations (10-100 μg) of

catechin in 0.2 ml were mixed with 1.0 ml of ten fold diluted Folin-Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate solution. After standing for 30 min at ambient temperature, the absorbance was measured at 765 nm using Genesys-5 UV-visible spectrophotometer. The estimation of phenolics in the fractions was calculated using standard graph (catechin). The experiments were carried out in triplicate and the results were averaged (Table 3.1).

Fractionation

Ten grams of water extract was dissolved in 10 ml of water and transferred to Diaion HP-20SS (80 g) column (60 cm × 30 mm i.d.,) (Scheme -1, Column I). Elution was initiated with 100% water (200 ml) and continued by increasing the proportion of methanol and finally eluted with methanol and acetone (Scheme -1). A total of 20 fractions were collected. All the fractions were monitored by HPLC analysis; fractions of similar composition were combined and concentrated under reduced pressure and lyophilised.

Isolation of Compound 1

Concentrate (2 ml) from fractions 2 and 3 (from Scheme -1; Column I) were loaded to Diaion HP-20 column (15 g). Elution was performed using water, mixtures of water: methanol and methanol to get nine fractions (25 ml) (Scheme -2; Column II). Fraction 1 was concentrated to 2 ml under vacuum and applied to Diaion HP-20 column (10 g; Scheme - 2; Column III) and eluted with water. Ten fractions (15 ml each) were collected and all the fractions were analysed by HPLC. Fractions of the same composition were combined, concentrated under

vacuum and lyophilised. Compound 1 (27.9 mg) of white amorphous powder was obtained from fractions 1 and 2. The purity of compound 1 was analysed by HPLC and it showed a single peak at 9.81 min (Figure 3.3).

Isolation of Compound 2

Concentrate (2 ml) from fractions 8 and 9 (from Scheme - 1; Column I) was loaded to Diaion HP-20 column (20 g; Scheme - 3; Column IV), which was pre-equilibrated with water. First nine fractions (1-9; Scheme 3) of 10 ml each were eluted with water. Elution was continued with methanol and three fractions (10-12) were collected. Finally eluted with acetone (fraction 13). All the fractions were concentrated *under vacuum* and analysed by HPLC. Concentrate from fractions 10 - 12 (1 ml) was re-chromatographed on Sephadex LH-20 (10 g; Scheme - 3; Column V) and twelve fractions of 3 ml each were collected and monitored by HPLC analysis. Compound 2 (16.3 mg) was obtained as a brown amorphous powder from fractions (7-11). HPLC analysis showed single peak at 13.55 min (Figure 3.3).

Isolation of Compound 3

Concentrate (4 ml) from fraction 10 (from Scheme - 1; Column I) was applied to Diaion HP-20 column (20 g gel), which was pre-equilibrated with water (Scheme - 4; Column VI). The column was eluted successively with water, mixtures of water: methanol and methanol. Nine fractions (100 ml each) were collected and concentrated *under vacuum*. All the fractions were monitored by HPLC and fractions containing same composition were combined. Fraction 3 was further chromatographed on Diaion HP-20 column (10 g) and the column

was eluted with mixtures of water: methanol and methanol to get twelve fractions (15 ml each; Scheme - 4; Column VII). The fractions were monitored by HPLC and compound 3 (26 mg) was obtained from fraction 8. HPLC analysis showed single peak at 20.04 min (Figure 3.3).

Isolation of Compounds 4 and 5

Concentrate (5 ml) from the fractions 11 and 12 (from Scheme -1; column I) was applied to a Diaion HP-20 column (40 g; Scheme 5; Column VIII). The column was eluted successively with water, mixtures of water: methanol and methanol. Ten fractions (100 ml each) were collected and concentrated *under vacuum* at 40 ^{o}C. Fraction 7 was further subjected to chromatography on Sephadex LH-20 (7 g) (Scheme - 5; Column IX) and eluted with water, mixture of water: methanol and methanol. A total fourteen fractions (20 ml) were collected and analysed by HPLC. Compound 4 (9.2 mg) and compound 5 (19.2 mg) were obtained from fractions 4 and 10-11, respectively. HPLC analysis of compound 4 and 5 showed peaks at 22.91 min and 24.73 min, respectively (Figure 3.3).

Preparation of reagents for the UV spectral analysis of flavonoids

Sodium methoxide solution. Freshly cut metallic sodium (2.5 g) pieces were cautiously added to dry spectroscopic grade 100 ml methanol. The solution was stored in a glass bottle.

Aluminium chloride solution. Anhydrous aluminium chloride (5 g) was added cautiously to spectroscopic grade 100 ml methanol.

Dilute hydrochloric acid solution. Concentrated hydrochloric acid (50 ml) was added cautiously to 100 ml of distilled water.

Sodium acetate and boric acid. Analytical grade anhydrous sodium acetate and boric acid were used.

Identification

Compound 1, white amorphous powder, Mp 200-201 °C. It gave bluish-green colour with ferric chloride. Analysed for $C_7H_6O_4$ (C 54.41; H 3.95% requires C 54.55; H 3.92%). ^1H and ^{13}C NMR data and spectra are presented in Table 3.2, Figures 3.4-3.6; Mass spectrum showed [M-H^+] 153 (29%), 148 (100%), 146.1 (18%) and 112.9 (5%) (Figure 3.7).

Compound 2, brown amorphous powder, it showed dirty green colour with ferric chloride reaction. It gave a pink colour on heating with dilute hydrochloric acid. $[\alpha]_D^{25}$ + 65.1° (c = 0.5 in acetone), analysed for $C_{45}H_{36}O_{18}$ (C 62.45; H 4.22% requires C 62.50; H 4.20%). ^1H and ^{13}C NMR spectral data are presented (Table 3.3; Figures 3.8 – 3.13). Negative ion mass spectrum showed fragments at 863.1 (M-H^+, 18%), 297.2 (60%), 148.1 (100%) (Figure 3.14), Positive ion mass spectrum showed fragments at 865.2 (M+H^+, 15%), 489 (14%), 177 (72%), 119 (100%) (Figure 3.15).

Compounds 3, amorphous powder, it showed bluish-green colour with ferric chloride reaction. It gave positive for Molish test. $[\alpha]_D^{25}$ – 30.1° (c = 0.5 in

methanol), analysed for $C_{26}H_{35}O_{11}$ (C 59.62; H 6.76% requires C 59.65; H 6.74%). ^{1}H and ^{13}C NMR spectral data are presented (Table 3.4; Figures 3.16 – 3.20). Positive ion mass spectrum showed fragments at m/z 522.3 (M^{+}, 13%), 331.2 (61%), 185.2 (100%), 93 (74%) (Figure 3.21). Negative ion mass spectrum showed fragments at m/z 523 ($[M+1]^{+}$, (100%), 492 (9%), 359 (25%), 329 (28%) (Figure 3.22).

Compounds 4, pale yellow crystals, Mp 214-215 °C, it gave dark dirty-green colour with ferric chloride reaction. It showed positive for Mg+HCl test. Analysed for $C_{27}H_{30}O_{16}$ (C 53.10; H 4.96% requires C 53.12; H 4.95%). UV (MeOH) λ_{max} nm 260, 268 (sh), 298 (sh), 360; $AlC1_3$ 275; 303 (sh), 433; $AlCl_3$ + HCl 271, 300, 364 (sh), 402; NaOMe 272, 327, 410; NaOAc 272; 325; 393; NaOAc + H_3BO_3 262; 298; 388. ^{1}H and ^{13}C NMR spectra are presented in Table 3.5 and Figures 3.23 – 3. 26. Positive ion mass spectrum showed fragments at 611.2 ($M+H^{+}$, 18%), 465.2 (4%), 257.2 (22%), 185.2 (100%), 93 (81%) (Figure 3.27).

Compound 5, pale yellow crystals, Mp 250-252 °C, it showed dark dirty-green colour with ferric chloride reaction and pink colour with Mg+HCl test. Analysed for $C_{21}H_{20}O_{11}$ (C 56.24; H 4.51% requires C 56.25; H 4.50%). UV (MeOH) λ_{max} nm 257, 266 (sh), 301 (sh), 350; $AlC1_3$ 277; 303 (sh), 333, 430; $AlCl_3$ + HCl 272, 303 (sh), 354, 402; NaOMe 272, 329, 394; NaOAc 273; 323 (sh); 372; NaOAc + H_3BO_3 262; 298 (sh); 368. ^{1}H and ^{13}C NMR spectra are presented in Table 3.6 and Figures 3.28 – 3.32. Negative ion mass spectrum showed fragments at 447.1 ($M-H^{+}$, 100%), 297.2 (39%), 148.1 (100%), 146.1 (29%) (Figure 3.33).

Positive ion mass spectra showed fragments at 449.1 (M $+H^+$, 33%), 303.1 (100%), 119 (48%), 85 (41%) (Figure 3.34).

RESULTS AND DISSCUSSION

Cinnamomum zeylanicum fruits were powdered and successively extracted with hexane, benzene, ethyl acetate, acetone, methanol and water. The yields and phenolic content of cinnamon fruit extracts have been presented in Table 3.1. The maximum and minimum yields were obtained with methanol and benzene extracts respectively, with respect to dried fruits (w/w) (Table 3.1). The phenolic content was analysed in all the extracts using Folin-Ciocalteu method and it was found that water extract has maximum phenolic content (44.5 $\pm$ 1.73%) in terms of catechin equivalents, whereas EtOAc extract has the least phenolic content (Table 3.1).

Ethyl acetate, acetone, methanol and water extracts of cinnamon fruits were analysed by HPLC and the chromatograms were presented in Figure 3.2. Water extract showed several peaks indicating the presence of several compounds. Water extract showed maximum phenolic content and highest antioxidant activity as compared to other extracts (Chapter 3.2). The water extract was fractionated on Diaion HP-20SS (Scheme - I) and these fractions were further chromatographed and purified on Diaion HP-20SS, Diaion HP-20 and Sephadex LH-20 (Scheme 2-5). It afforded five compounds *viz.,* 1, 2, 3, 4 and 5. The purity of compounds 1, 2, 3, 4 and 5 have been analysed by HPLC and the chromatograms are presented in Figure 3.3.

Table 3.1 Percentage of various solvents extracts yield and phenolics.

Solvents	**Yield**	**Phenolics ***
Hexane	1.92 ± 0.12	Nil
Benzene	0.78 ± 0.25	Nil
EtOAc	1.87 ± 0.18	14.4 ± 1.4
Acetone	1.74 ± 0.78	15.6 ± 3.9
MeOH	4.99 ± 1.31	40.4 ± 0.7
Water	3.87 ± 0.23	44.5 ± 1.7

*as catechin equivalents present in corresponding extracts

Compound 1 gave positive for ferric chloride reaction indicated the presence of hydroxyl group. It also gave effervescence with dilute sodium bicarbonate, indicated the presence of carboxyl group. UV absorption (λ_{max} at 280 nm indicated the presence of substituted aromatic system. The ^{1}H NMR spectra (Figures 3.4 and 3.5) of compound 1 (Table 3.2) showed ABX pattern for the presence of three aromatic protons at δ (ppm) 7.27 (dd, 1.80; 8.20 Hz), 6.74 (d, 8.2 Hz) and 7.33 (d, 1.80 Hz). ^{13}C NMR spectra (Figure 3.6) showed the presence of seven carbons. The signal at δ (ppm) 167.6 is assigned to carboxyl carbon (COOH). The signals at δ 149.9 and 144.8 were assigned to the C-3 and C-4 carbons linked to hydroxyl groups. The remaining four signals at δ 121.8, 116.6, 115.1 and 122.0 are assigned to C-1 (linked to carboxyl group), C-6, C-2

and C-3 respectively. Also, the ^{13}C NMR chemical shifts of compound 1 have matched with the reported values (Scott, 1970).

Table 3.2. ^{1}H and ^{13}C NMR (DMSO-d_6) chemical shifts of compound (1)***

H/C	^{1}H □.	Multiplicity	*Coupling constants* (J) Hz	^{13}C □.
1				121.8
2	7.33	d	1.80	116.6
3				144.8
4				149.9
5	6.74	d	8.20	115.1
6	7.27	dd	1.80; 8.20	122.0
COOH				167.6

* :600 MHz

** :150.9 MHz

*** :Chemical shifts were compared with Scott, (1970)

Further, the compound 1 was analysed by electrospray fourier-transform ion, ion trap on negative mode. The mass spectrum of compound 1 (Figure 3.7) showed molecular ion peak at 153 [M-H^+], (29%), 148.1 (100%), 146.1 (20%) and 112.9 (5%). On the basis of ^{1}H, ^{13}C NMR and Mass spectral data the compound 1 was identified and characterised as 3, 4-dihydroxybenzoic acid (*viz.,* protocatechuic acid). (Figure 3.35). Finally, the structure of compound 1 was confirmed by HPLC analysis. The retention times of compound 1 and that of authentic protocatechuic acid are found to be similar (Figure 3.36).

Compound 2 was isolated as a brown amorphous powder and developed dirty-green colour with ferric chloride indicating it to be a phenolic compound. It gave a pink colour on heating with dilute methanolic hydrochloric acid; it

demonstrated the presence of anthocyanidin nucleus. It showed UV absorption at (λ_{max}) 223 and 280 nm, confirming it to be an anthocyanidin. High-resolution mass spectral analysis (Figure 3.15) of compound 2 indicated $[M+H]^+$ ions at m/z 865.2, which is consistent with a triflavonoid moiety (Morimoto et al., 1987). The ESI-MS of compound 2 (Figure 3.14) gave an $[M-H]^-$ peak at m/z 863.1, which was consistent with a trimeric procyanidins constituent in which an A type linkage was also present (Foo et al., 2000). The ^1H and ^{13}C NMR spectra (Figure 3.8 - 3.10) confirmed the presence of three anthocyanidin units. Three ^{13}C signals of equal intensities were observed for each carbon of the anthocyanidin unit.

The NMR chemical shifts and their assignments of compound 2 have been presented in Table 3.3. The ^1H NMR spectrum exhibited the presence of an A-type doubly linked unit from the AB coupling system for C-3 and C-4 of anthocyanidin unit at 3.27 and 4.14 (each d, J = 3.5 Hz). This was also supported from the presence of ketal carbon signal at 99.9 for C ring 2nd position (Kamiya et al., 2001). Three carbon signals at δ 67.2, 72.6 and 67.5 correspond to C-3 of the three heterocyclic rings (rings C, F and I) present in three anthocyanidin units (Balde et al., 1991). DQFCOSY, HSQC and HMBC spectra of compound 2 were presented in Figures 3.11 – 3.13. By a combination of DQFCOSY and HMBC, a planar structure was established and the signals of protons and carbons were assigned. On the basis of above spectral data the compound 2 was characterised as epicatechin- (2$\beta\rightarrow$O$\rightarrow$7,4$\beta\rightarrow$8)-epicatechin-(4$\beta\rightarrow$8)-epicatechin (Figure 3.35). The same structure was assigned earlier to

cinnamtannin B-1 (Balde et al., 1991). Further, the NMR (^{1}H and ^{13}C) spectra of compound 2 and cinnamtannin B-1 (Kamiya et al., 2001) were compared and found to be identical (Table 3.3). HPLC analysis of compound 2 and cinnamtannin B-1 (Kind gift from Prof. T. Satake, Kobe Gakuin University, Kobe, Japan) confirmed their identity. Hence the structure of compound 2 is identified as epicatechin- (2β→O→7, 4β→8)-epicatechin- (4β→8)-epicatechin (cinnamtannin B-1). This compound has been reported only three times in nature so far (Nonaka et al., 1983; Balde et al., 1991; Kamiya et al., 2001). However, this is the first report from cinnamon fruits.

Compound 3 gave positive for ferric chloride and Molish test, indicated the presence of hydroxyl groups and sugar moiety. UV spectrum in MeOH showed the (λ_{max}) at 227 and 278 nm, which indicated the presence of aromatic system. IR spectrum of compound 3 showed the peaks at 3345, 1604 and 1517 cm^{-1}, which indicated the presence of hydroxyl and aromatic rings.

^{1}H and ^{13}C NMR spectra of compound 3 were presented in Figures 3.16 and 3.17 respectively. The ^{1}H NMR spectrum showed signals at δ 2.61 (2H, t, 7.5 Hz), 1.81 (2H, m) and 3.56 (2H, t, 6.5 Hz) indicated the presence of tri-methylene protons i.e. a spin system of CH_2-CH_2-CH_2O and signals at 3.85 (s) and 3.83 (s) indicated the presence of two aromatic methoxyl groups. This has been supported by two carbon signals at δ 56.7 and 56.8 in ^{13}C NMR spectrum (Figure 3.17). It showed the presence of three aromatic proton signals at δ 7.12 (J = 8.4 Hz), 6.91 (dd, J = 1.8; 8.40 Hz) and 7.02 (d, J = 1.8 Hz), were

characteristics of ABX system, which indicated *ortho* di-substitution (Table 3.4). It was confirmed by the presence of two signals in its ^{13}C NMR spectrum at δ 150.9 and 147.5 for two *ortho* disubstitued carbons. ^{1}H NMR spectrum also showed the presence of two more aromatic signals at δ 6.71 (1H, s) and 6.72 (1H, Br.s), which indicated the presence of another aromatic system with four carbons substitution. Further, ^{13}C NMR showed the signals for the presence of dihydrobenzofuran skeleton (137.1, 118.0, 145.2, 147.6, 129.6, 114.2, 88.5 and 55.7). The ^{13}C NMR spectrum also showed signals δ 62.4, 77.8, 71.3, 78.1, 74.9 and one signal at δ 102.9 indicating the presence of one anomeric carbon, suggesting that compound 3 contained a glucose unit.

The structure of the compound 3 was further determined by extensive 2D-NMR studies, such as DQFCOSY, HSQC and HMBC (Figures 3.18 – 3.20). Using these spectral data, each proton and carbon signals in the ^{1}H and ^{13}C NMR spectra of compound 3 were assigned (Table 3.4). The correlations between C-6 and C-1, 2, 3, 4, 7 and 8′ were verified by HMBC. Similarly, correlations between C-7’ and C-1′, 2′, 4′, 5′, 6′, 8′, and 9′ were verified by HMBC. The mass spectra of compound 3 were presented in Figures 3.21 – 3.22 and it showed molecular ion peak at 522.3 (18%). On the basis of above spectral data the structure of the compound 3 elucidated as [7′, 8′-dihydro-8′-(hydroxymethyl)-1-(3-hydroxypropyl)-3-(methoxy)-benzofuranyl]-7′-ethoxyphenyl 4′-O-□-D-glucopyranoside (2S-trans). The spectral data of compound (3) was matched to reported values of urolignoside, which confirmed the above structure (Shen et al., 1998).

Compound 4 gave positive ferric chloride reaction and pink colour in Shinoda test, which indicated the presence of a flavonoid with hydroxyl groups. It gave positive Molish test, which suggested the presence of sugar moiety. The UV spectrum of compound 3 in methanol showed (λ_{max}) peaks at 259, 266 (sh), 299 (sh) and 359 nm. The bathochromic shift of about 51 nm without decreasing the intensity of band I by the addition of sodium methoxide indicates the presence of free hydroxyl group at position C-4′ (Mabry et al., 1970) in compound 4. The bathochromic shift of band II (12 nm) with sodium acetate indicates the presence of free 7- hydroxyl group. Further, the presence of B ring ortho dihydroxyl group was confirmed by bathochromic shift of band I (28 nm) in the presence of sodium acetate and boric acid. The presence of 5-hydroxyl group was confirmed by aluminium chloride and hydrochloric acid addition. A stable bathochromic shift of band I and II with aluminium chloride and aluminium chloride and hydrochloric acid shows the presence of hydroxyl group may be at 3rd or 5th position.

IR spectrum of compound 4 showed two strong absorption bands at 3440 and 1685 cm^{-1} corresponding to hydroxyl group and carbonyl group attached to phenyl ring respectively. Mass spectrum of compound 4 have been presented in Figure 3.27. It showed m/z (%) 611.2 (M+H^{+}, 18%), 465.2 (4%), 301 (19%), 257 (22%), 185.2 (100%), 93 (80%).

The ^{1}H NMR spectrum (Figure 3.23) showed the presence of three aromatic proton signals at δ 7.62 (dd, J = 2.2; 8.50 Hz), 7.66 (d, J = 2.2 Hz), and 6.86 (J = 8.4 Hz) were characteristics of H-6′, H-2′ and H-5′ respectively of B-ring of

flavonoid nucleus, which indicated substitution in 3′ and 4′-positions (Table 3.5). It has been confirmed by the presence of two carbon signals in its ^{13}C NMR spectrum (Figure 3.24) at δ 145.8 and 149.9 for C-3′ and C-4′ respectively. Two doublet signals at δ 6.20 (2.2 Hz) and 6.38 (2.2 Hz) were assigned to two protons on C-6 and C-8 in A ring. Hence, the compound 4 is substituted in the remaining positions (*i.e.,* C-3, 5 and 7). The ^{13}C NMR spectrum (Figure 3.24) also supported the substitution. The ^{13}C NMR spectrum also showed nine signals between δ 68 – 80 ppm and two signals at δ 100.0 and 102.4 indicating the presence of two anomeric carbons and one signal at δ 17.9 indicating the presence of one terminal methyl group, suggesting that compound 4 may be flavonoid disaccharide. Signal at δ 1.11 (d, 3H, 6.2 Hz) supported the presence of terminal methyl group and indicated it as rhamnose methyl group. The ^{13}C signals in aliphatic region indicated the sugar unit as rutinose. Connectivities in DQFCOSY (Figure 3.25) and HMBC (Figure 3.26) supported the assignments. The chemical shifts of compound 4 were matched to reported values (Markham et al., 1978). The mass spectrum of compound 4 showed the fragments at m/z 611.16 and 633.18 for $[M+H]^+$ and $[M + Na]^+$ respectively (Figure 3.27). Fragments at m/z 465 and 303 showed the ion peaks for $[M\text{-}Rha]^+$ and $[M\text{-}Rha\text{-}Glu]^+$ suggesting that compound 4 contain two sugar units. Finally, the ^1H and ^{13}C NMR chemical shifts of compound 4 were matched with rutin (Markham et al., 1978), indicating it to be quercetin 3-O-rutinoside. On the basis of above spectral data compound 4 was identified as 3-[[6-O- (6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl] oxy]-2-(3,4-dihydroxy phenyl)-5,7 dihydroxy-4H-1benzopyran-4-one (rutin).

Compound 5 gave pink colour in Shinoda test and positive with ferric chloride, indicating the compound may be flavonoid with phenolic hydroxyl groups. It gave positive for Molish test, which suggested the presence of sugar unit. UV spectral analysis showed the absorption bands at λ_{max}^{MeOH} 257 (band II) and 350 (band I) nm. The bathochromic shift of band I of about 49 nm by the addition of sodium methoxide indicates the presence of free hydroxyl group at position 4' (Mabry et al., 1970). Similarly, bathochromic shift of band II (16 nm) with sodium acetate shows the presence of 7-hydroxyl group. Using the shift reagents $AlCl_3$, $AlCl_3$-HCl, sodium acetate and sodium acetate-boric acid indicated the presence of two free hydroxyl groups at C-5 and C-7. The presence of free hydroxyl at position 5 and ortho dihydroxy system in compound 5 was indicated by the bathochromic shifts 80 nm observed with aluminium chloride and the same is decreased to 52 nm on the addition of hydrochloric acid. Hence, the UV absorption data indicated the presence of the free hydroxyls in compound 4 and also their tentative positions as 5, 7, 3′ and 4′ hydroxyls. The IR spectra gave additional information about the substituents about the compound 5. The presence of hydroxyl group was indicated by the signal at 3400 cm^{-1} (O-H) and 1645 cm^{-1} (α, β-unsaturated C=O) indicating a flavonol like structure such as quercetin (Li et al., 1998).

^{1}H NMR spectrum (Figure 3.28 and Table 3.6) of compound 5 showed evidence of penta-oxygenated flavonoid. Three ABX type aromatic signals at δ 7.33 (d, 2.1 Hz, 1H), 7.30 (dd, 2.1; 8.3 Hz, 1H), 6.91 (d, 8.2 Hz, 1H) were

characteristics of H-2′, H-6′ and H-5′ respectively of B-ring in a flavonoid nucleus, which suggests that the B ring is 3′ and 4′ dihydroxylated. The chemical shifts at δ 6.36 (1H, 2 Hz) and 6.19 (1H, 2 Hz) were assigned to H-6 and H-8 of A-ring. Signal at δ 0.93 showed the presence of methyl group and it may be due the presence of terminal methyl group of rhamnose. The anomeric proton was observed at δ 5.34 (d, 1.5 Hz) indicated the attachment of the anomeric carbon to the flavonoid unit. ^{13}C NMR spectral analysis (Figure 3.29 and Table 3.6) further substantiated these findings. ^{13}C NMR spectrum of compound 5 contained the signals for aglycone unit similar to compound 4 and indicating the presence of quercetin unit. In addition, it showed the presence of signals for one sugar moiety, which includes one methyl signal. The linkage of sugar moiety at C-3 of flavonoid was confirmed from the upfield shift of C-3 signal and the corresponding ortho and para carbon signals were shifted downfield. Assignments were made for all the protons and carbons and connectivities were determined on the basis of DQFCOSY (Figure 3.30), HSQC (Figure 3.31) and HMBC (Figure 3.32) spectra. The chemical shifts of compound 5 were matched to reported values (Jaganmohan Rao et al., 2002). Positive and negative electrospray ionisation mass spectra (ESI-MS) showed the molecular ion $[M+H]^+$ at m/z 449 (33%) corresponding to molecular formula $C_{21}H_{20}O_{11}$ and fragment ions characteristics of attachment of a rhamnosyl residue (Figures 3.33 and 3.34). The fragment ions at 303 $[(M+H)^+ -146]$ showed the loss of rhamnosyl unit. On the basis of above spectral data, compound 5 was identified as quercetin-3-O-α-L-rhamnopyranoside.

CONCLUSION

Cinnamon fruits were extracted with different solvents with increasing polarity. The phenolics were found to be highest in water extract and it was subjected to repeated column chromatography to get five compounds. The compounds 1-5 were identified as protocatechuic acid, cinnamtannin B-1, urolignoside, rutin and quercetin-3-O-α-L-rhamnopyranoside, respectively using extensive NMR and mass spectral studies. This is the first report on non-volatile constituents from cinnamon fruits.

Table 3.3. ^{1}H and ^{13}C NMR chemical shifts and characteristics of HMBC connectivities of compound (2)***

UNIT	H*	Chemical shifts	Multiplicity	J (Hz)	C**	HMBC
1st	2				99.9	
	3	3.27	d, (1H)	3.50	67.2	C-10
	4	*4.14*	d, (1H)	3.50	28.9	C-3
	5				154.2	
	6	6.00	d, (1H)	2.3	96.6	C-5, C-7, C-8, C-10,
	7				157.9	
	8	5.95		2.3	98.3	C-6, C-9, C-10
	9				156.8	
	10				104.9	
	1′				132.5	
	2′	7.02	d, (1H)	2.04	115.7	C-2, C-3′, C-4′, C-6′
	3′				146.3	
	4′				146.6	
	5′	6.81		8.23	115.2	C-4′, C-6′
	6′	6.84	dd, (1H)	2.01, 8.23	119.9	C-2′, C-3′, C-4′
2^{nd}	2	5.69	Br.s, (1H)		78.9	C-1′, C-6′, C-2′
	3	4.11	Br.s, (1H)		72.6	C-2, C-10
	4	4.55	Br.s, (1H)		38.3	C-2, C-3, C-8, C-5, C-9, C-8 (3^{Rd} unit)
	5				155.8	
	6	5.80	Br.s, (1H)		96.1	C-5; C-7, C-8, C-10
	7				151.8	
	8				106.4	
	9				151.1	
	10				106.7	
	1′				131.8	
	2′	7.30	d, (1H)	1.95	116.7	C-2, C-3′, C-4′, C-6′
	3′				145.8	
	4′				145.9	
	5′	6.83	d, (1H)	8.5	116.2	C-1′
	6′	7.18	dd, (1H)	1.95, 8.22	121.4	C-5′
3^{rd}	2	4.38	s, (1H)		80.3	C-3, C-1′, C-2′, C-6′
	3	3.85	Br.s, (1H)		67.5	
	4	2.82	dd, (1H)		29.9	C-10
	5				156.0	
	6	6.08	s, (1H)		96.4	C-5, C-7, C-8, C-10

	7				155.8	
	8				108.9	
	9				155.8	
	10				100.0	
	1′				133.2	
	2′	6.81	d, (1H)	2.1	115.5	C-1′, C-2
	3′				145.5	
	4′				145.3	
	5′	6.74	d, (1H)	8.2	116.0	C-1′, C-3′, C-4′
	6′	6.71	d, (1H)	8.2, 0.79	119.4	C-2, C-4′

* : 800 MHz; ** : 200 MHz

***: chemical shifts were compared with Kamiya et al., (2001)

Table 3.4. ^{1}H and ^{13}C NMR (CD_3OD) chemical shifts and characteristics of HMBC connectivities of compound (3)***

H*	**Chemical Shifts**	**Multiplicity**	**Coupling constants (Hz)**	**C****	***HMBC***
1				137.1	
2	6.71	1H, Br.s		118.0	C-3, C-4, C-6, C-7, C-8′
3				145.2	
4				147.6	
5				129.6	
6	6.72	1H, Br.s		114.2	C-1, C-2, C-3, C-4, C-7, C-8′
7	2.61	2H, t	7.5	32.9	C-1, C-2, C-6
8	1.81	2H, m		35.8	C-1
9	3.56	2H, t	6.5	62.2	C-7, C-8
1′		1H, d	5.9	138.1	
2′	7.02	1H, d	1.8	111.2	C-1′, C-4′, C-6′, C-7′
3′				150.9	
4′				147.5	
5′	7.12	1H, d	8.4	118.1	C-1′, C-2′, C-4′
6′	6.91	1H, dd	2.0, 8.4	119.4	C-2′, C-3′, C-4′, C-7′
7′	5.55	1H, d	6.0	88.5	C-1′, C-2′, C-4′, C-5′, C-6′, C-8′, C-9′
8′	3.43	1H, m		55.7	C-1′, C-4, C-5, C-9′
9′	3.73, 3.83	2H, dd		65.0	C-5, C-7′
3-OCH_3	3.82	3H, s		56.7	C-3′
3’-OCH_3	3.85	3H, s		56.8	C-3
1′′	4.88	1H, d	7.6	102.8	C-4′
2′′	3.49			74.9	C-1′′, C-3′′
3′′	3.38			78.1	C-5′′
4′′	3.39			71.3	C-3′′, C-5′′

5′′	3.46	2H, dd	1.85, 4.8	77.8	C-4′′
6′′	3.68			62.4	C-5′′

* : 800 MHz;

** : 150.9 MHz

*** : Chemical shifts were compared with Shen et al., (1998)

Table 3.5. ^{1}H and ^{13}C NMR (CD_3OD) chemical shifts and characteristics of HMBC connectivities of compound (4)***

H*	Chemical shifts	Multiplicity	J (Hz)	C**	HMBC
2				159.3	
3				135.6	
4				179.4	
5				163.0	
6	6.38	1H, d	2.20	94.9	C-7, C-8, C-9, C-10
7				166.3	
8	6.20	1H, d	2.20	100.0	C-5, C-6, C-7, C-10
9				158.6	
10				105.5	
1′				117.2	
2′	7.66	1H, d	2.20	123.1	C-2, C-3′, C-4′, C-6′
3′				145.8	
4′				149.9	
5′	6.86	1H, d	8.4	116.1	C-3′, C-4′, C-6′
6′	7.62	1H,dd	2.2, 8.50	123.5	C-2, C-4′
1″	5.10	1H, d	7.70	102.4	C-3
2″	3.45	1H, dd	1.35, 7.7	75.7	
3″	3.80	1H, dd	1.5, 11.1	78.2	C-4″
4″	3.27	2H, m		71.4	
5″	3.42	2H, m		77.2	C-3″
6″		2H,		68.5	
1‴	4.49	1H, d	1.46	104.7	C-2‴, C-3‴, C-5″
2‴	3.62	dd	1.63, 3.4	72.1	C-4‴
3‴	3.53	dd	3.47, 9.50	69.7	
4‴	3.38	2H, t		73.9	
5‴	3.27	2H, m		69.7	
6‴	1.11	3H, d	6.20	17.9	C-4‴, C-5‴

* :600 MHz

** :150.9 MHz

*** :Chemical shifts were compared with Markham et al., (1978)

Table 3.6. ^{1}H and ^{13}C NMR (CD_3OD) chemical shifts and characteristics of HMBC connectivities of compound (5)***

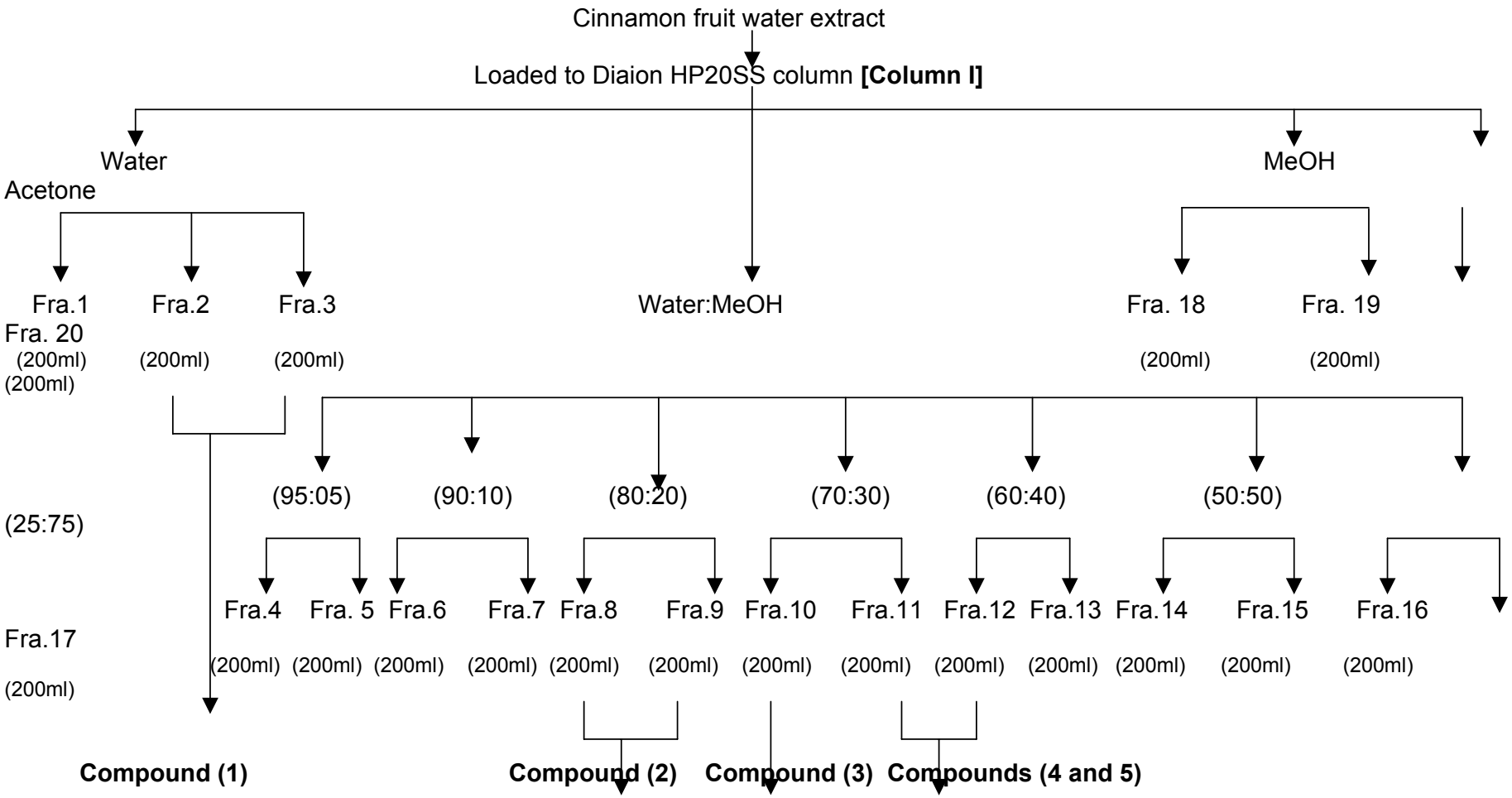
H*	Chemical shifts	Multiplicity	Coupling constants (Hz)	^{13}C **	HMBC
2				159.3	
3				136.2	
4				179.7	
5				163.2	
6	6.19	1H, d	2.0	99.8	C-5, C-7, C-8, C-10
7				165.9	
8	6.36	1H, d	2.1	94.7	C-4, C-6, C-7, C-9, C-10
9				158.5	
10				105.9	
1′				122.9	
2′	7.33	1H, d	2.1	116.9	C-2, C-3′, C-4′, C-6′
3′				146.4	
4′				149.8	
5′	6.91	1H, d	8.2	116.3	C-1′, C-3′, C-4′, C-6′
6′	7.30	1H, dd	2.1, 8.3	122.8	C-2, C-4′, C-5′
1′′	5.34	1H, d	1.5	103.6	C-2′′, C-3′′, C-3
2′′	3.73	1H, dd	1.7, 3.3	72.1	C-3′′, C-4′′
3′′	3.41	1H, dd	3.3, 9.5	72.0	C-2′′, C-4′′
4′′	3.33	1H, d	9.5	73.3	C-3′′, C-5′′
5′′	4.21	1H, m	3.3, 2.9, 9.6	71.9	C-3′′, C-4′′
6′′	0.93	3H, d	6.3	17.6	C-4’’, C-5’’

* : 800 MHz

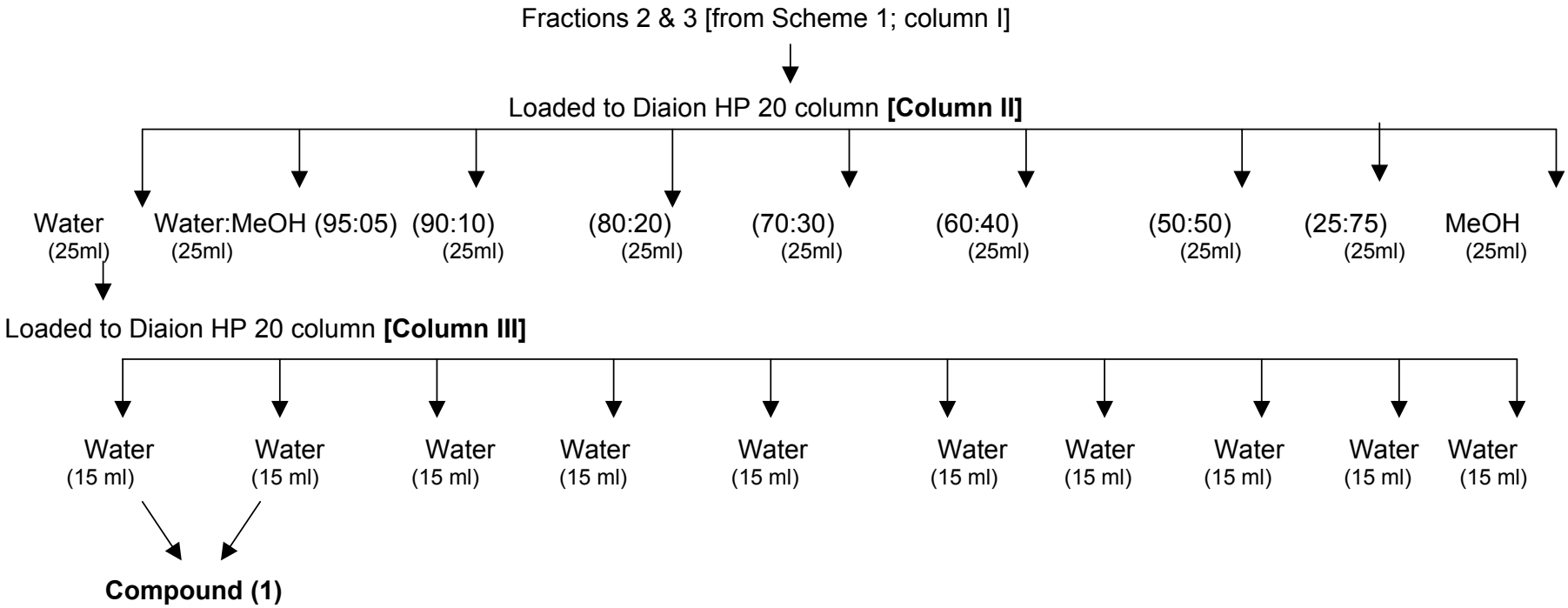
** : 200 MHz

*** : Chemical shifts were compared with Jaganmohan Rao et al., (2002)

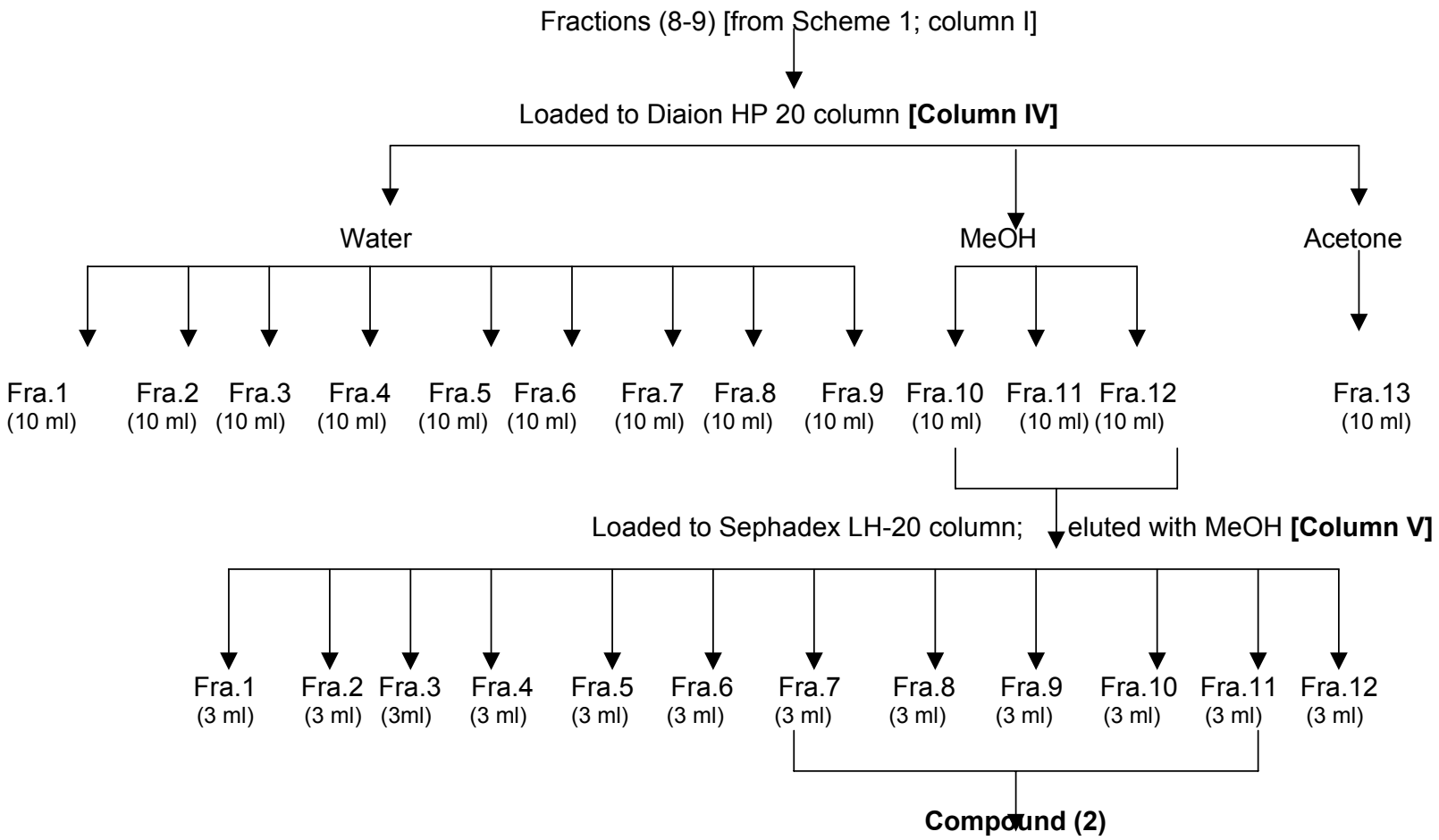
Scheme 1. Fractionation of water extract of cinnamon fruits using HP-20 column



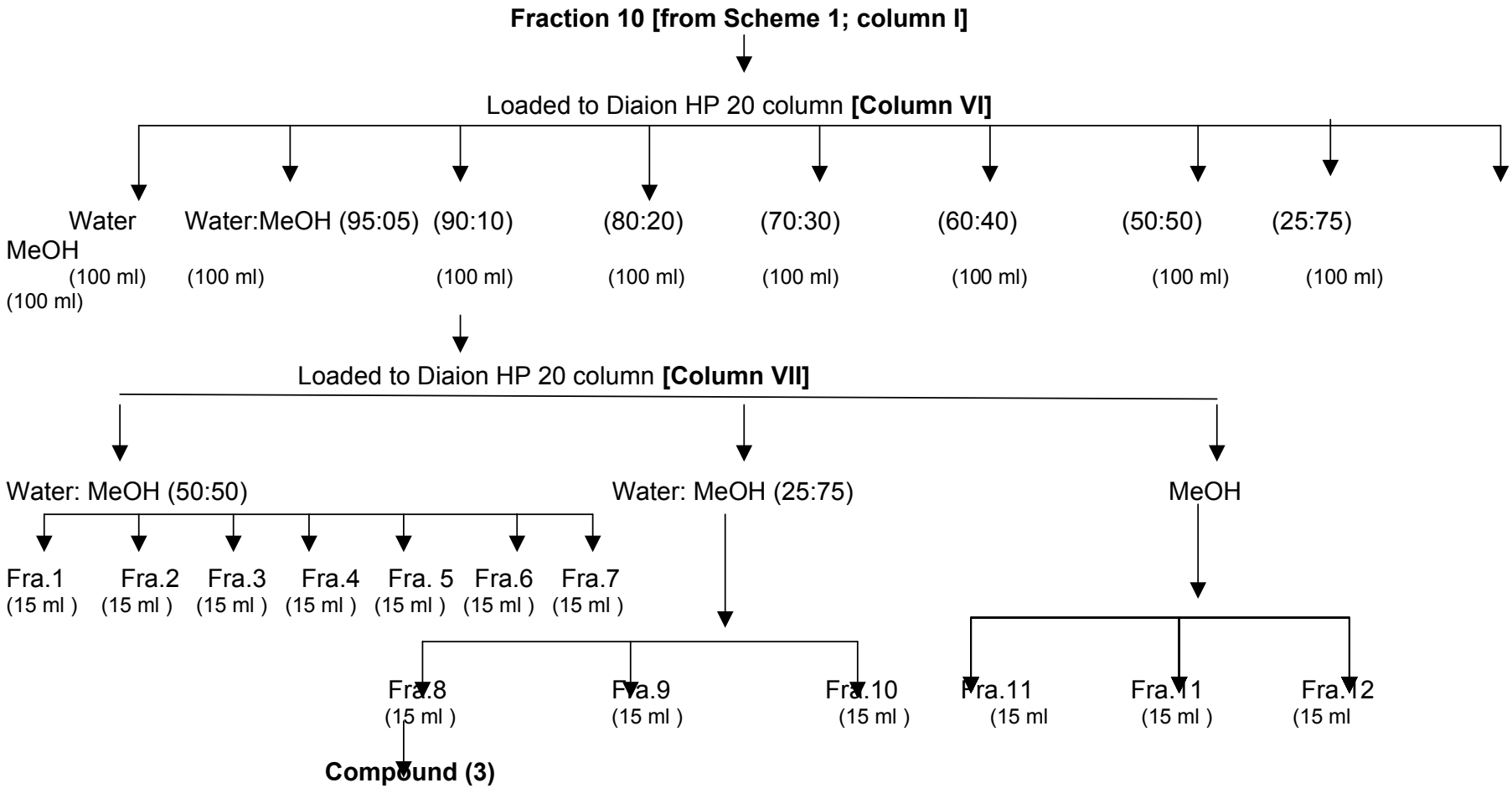
Scheme 2. Purification of compound 1 using HP-20 column.



Scheme 3. Purification of compound 2 using HP-20 and LH-20 columns.



Scheme 4. Purification of compound 3 using HP-20 column.



Scheme 5. Purification of compounds 4 and 5 using HP-20 and LH-20 columns.

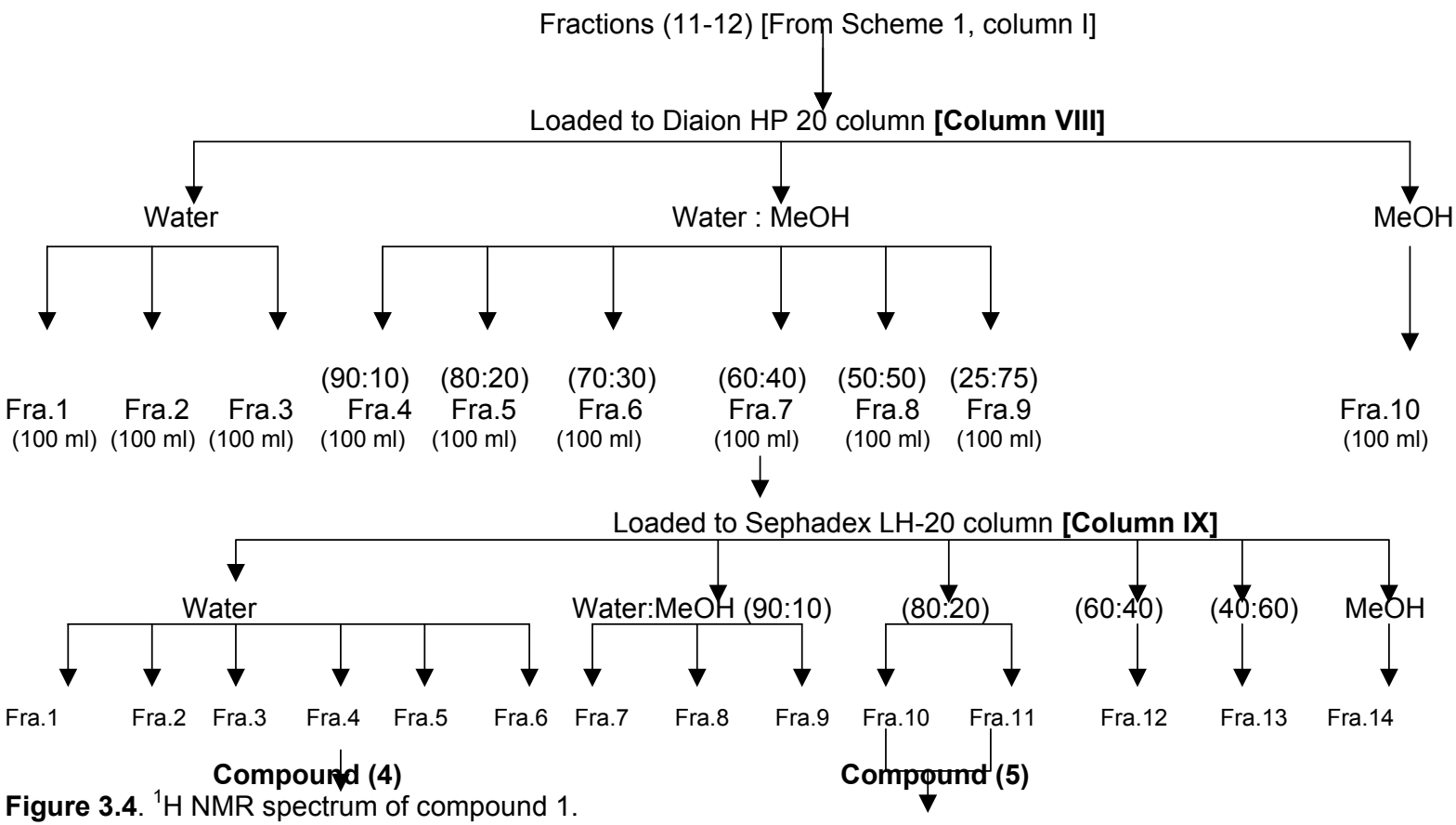


Figure 3.4. ^{1}H NMR spectrum of compound 1.

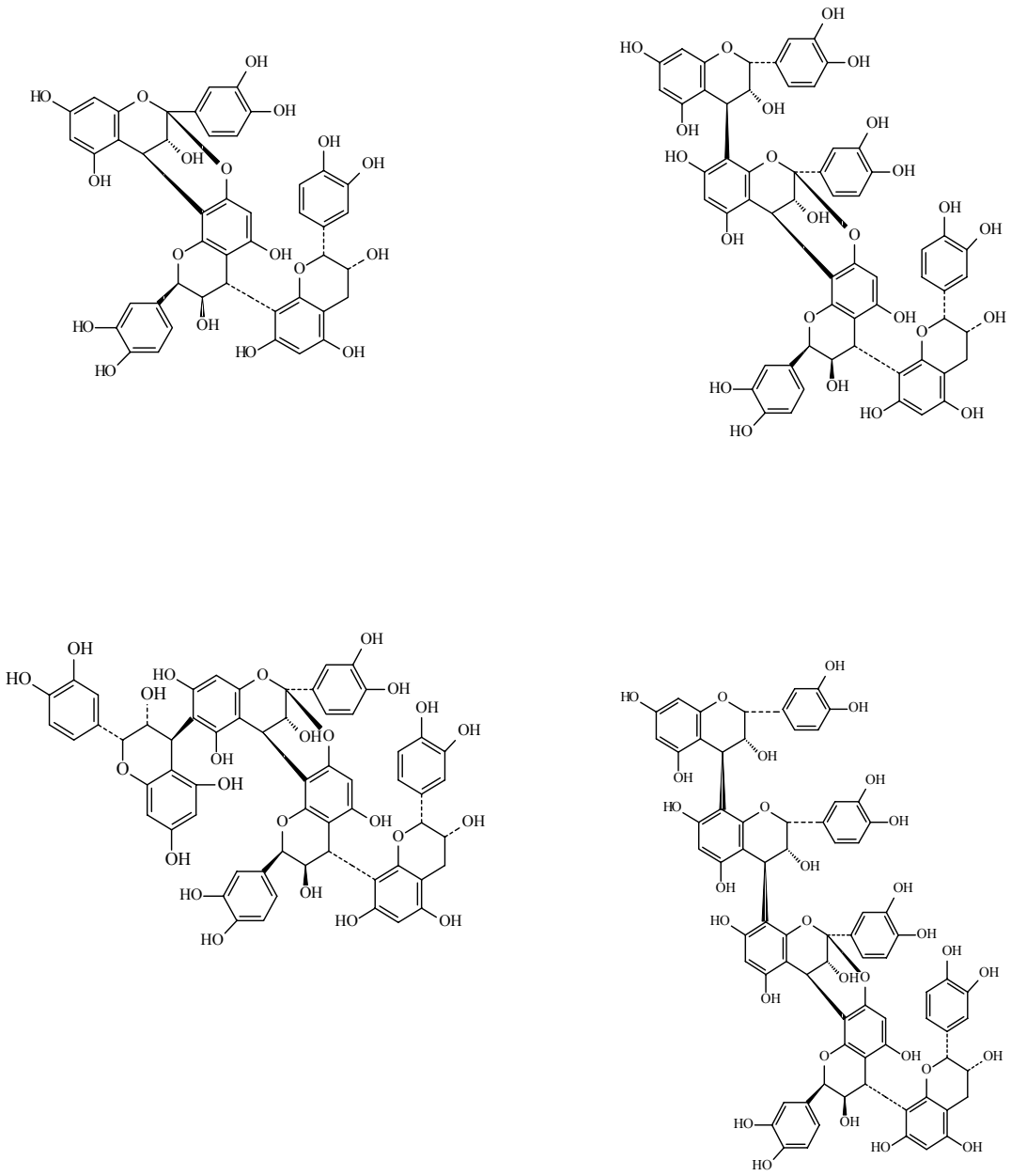


Figure 3.1. Structures of trimeric, tetrameric and pentameric proanthicyanidins from cinnamon bark (Nonaka et al., 1983).

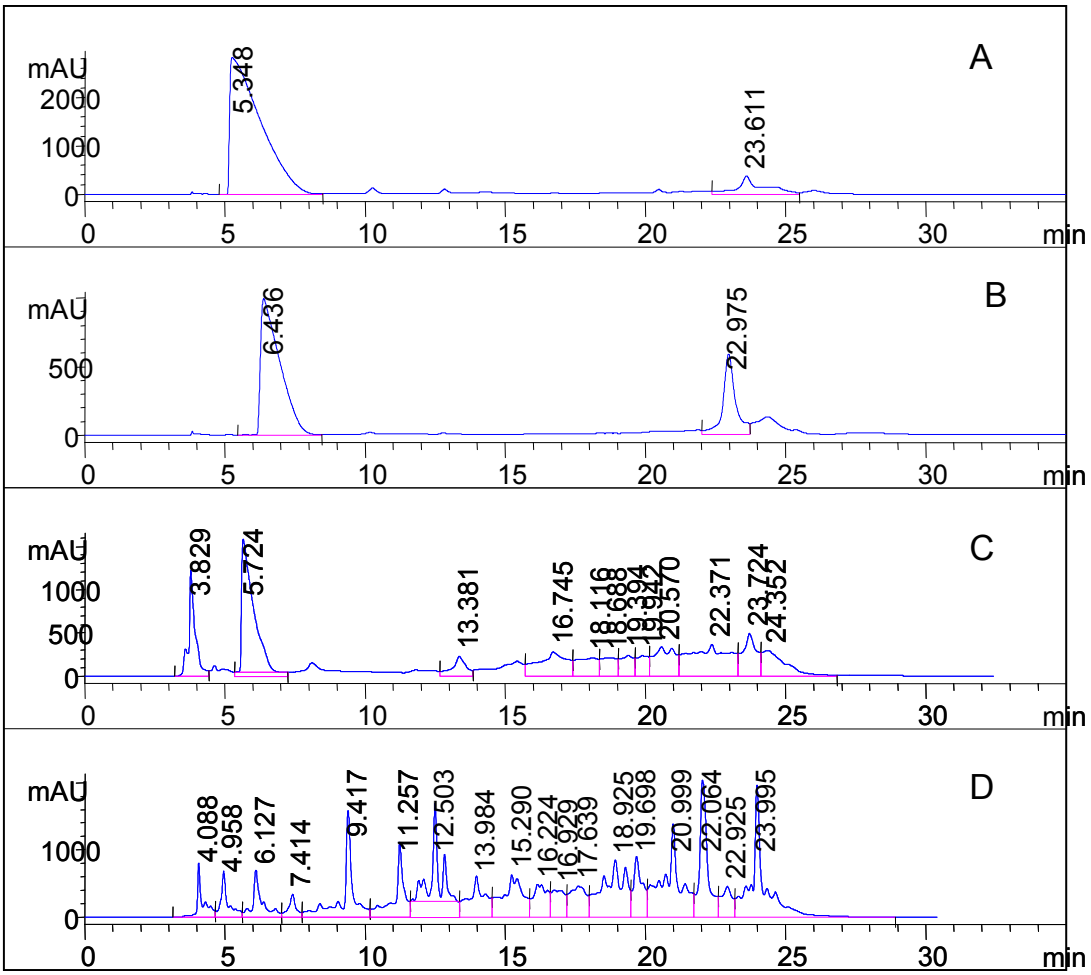


Figure 3.2. HPLC chromatograms of (A). ethyl acetate extract, (B). acetone extract, (C). methanol extract and (D) water extract.

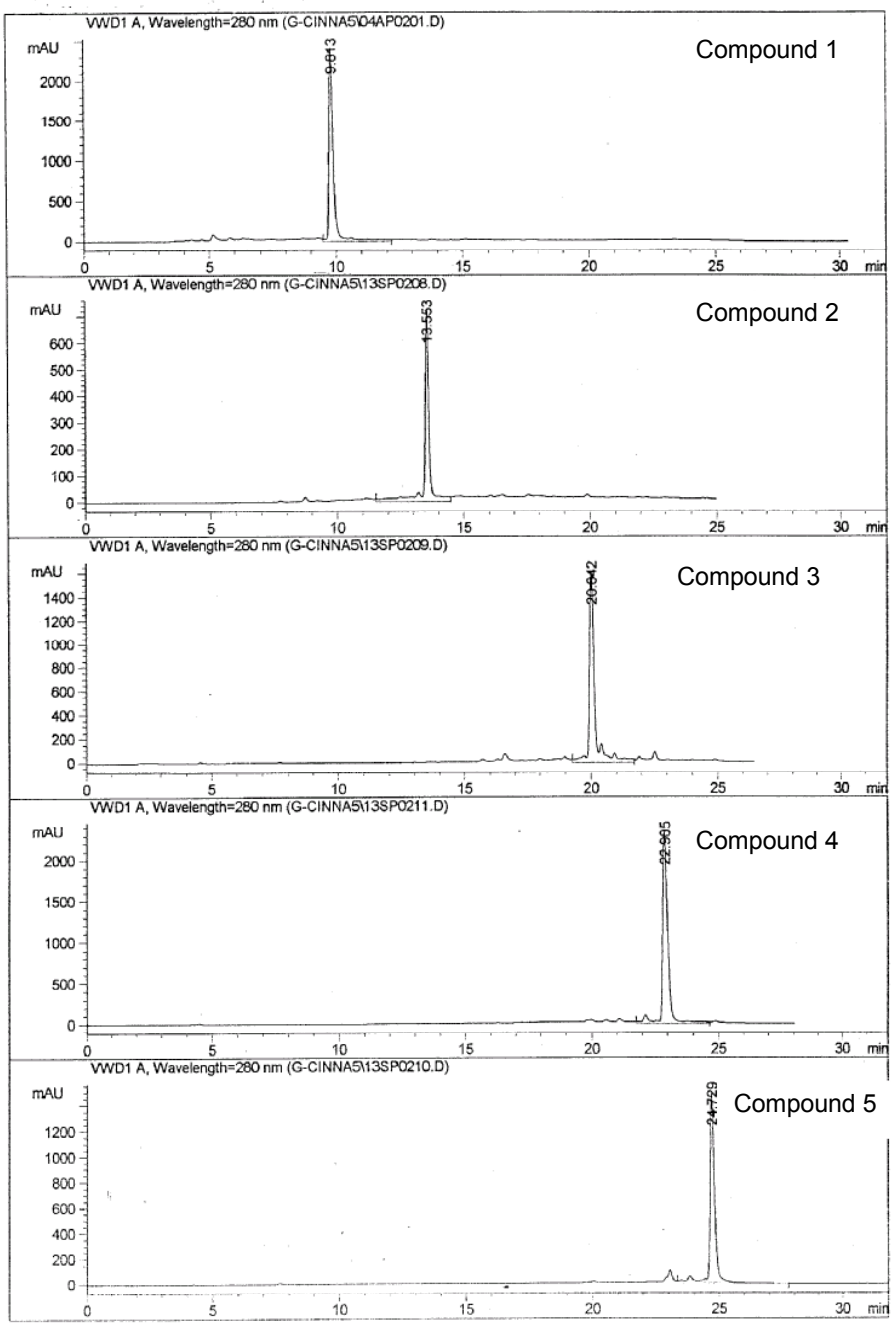


Figure 3.3. HPLC chromatograms of compounds 1-4 using C_{18} column (25 cm × 4.6 mm I.D, 5 μm particle size), detection 280 nm, Mobile phase (A) methanol (B) 1% acetic acid, Flow rate of 0.7 ml/min. Linear gradient from 95 - 50% B in A for 0 - 25 min and 95% B in A by 30 min followed by 5 min of equilibrium with 95% B.

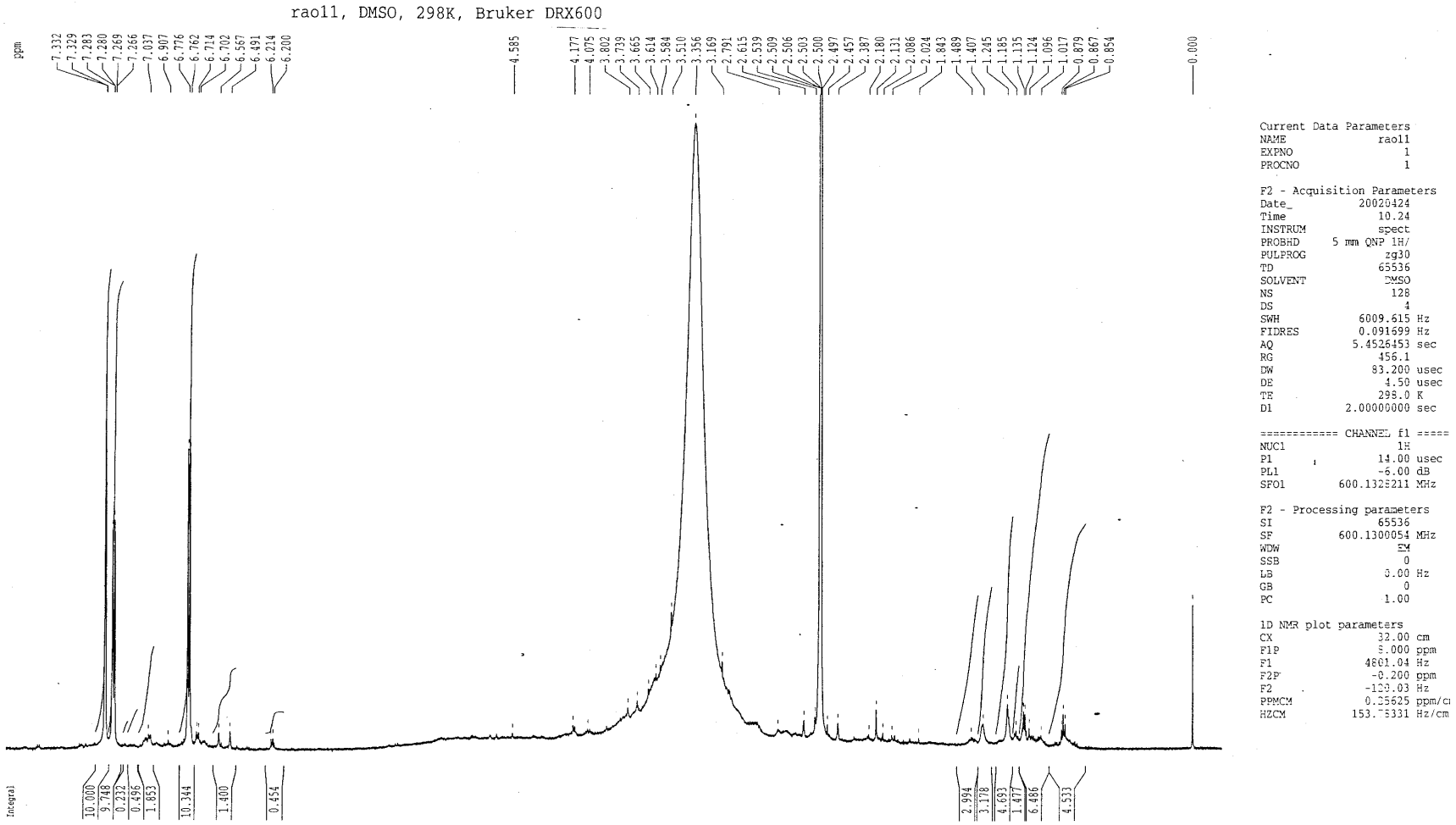


Figure 3.4. ^{1}H NMR spectrum of compound 1.

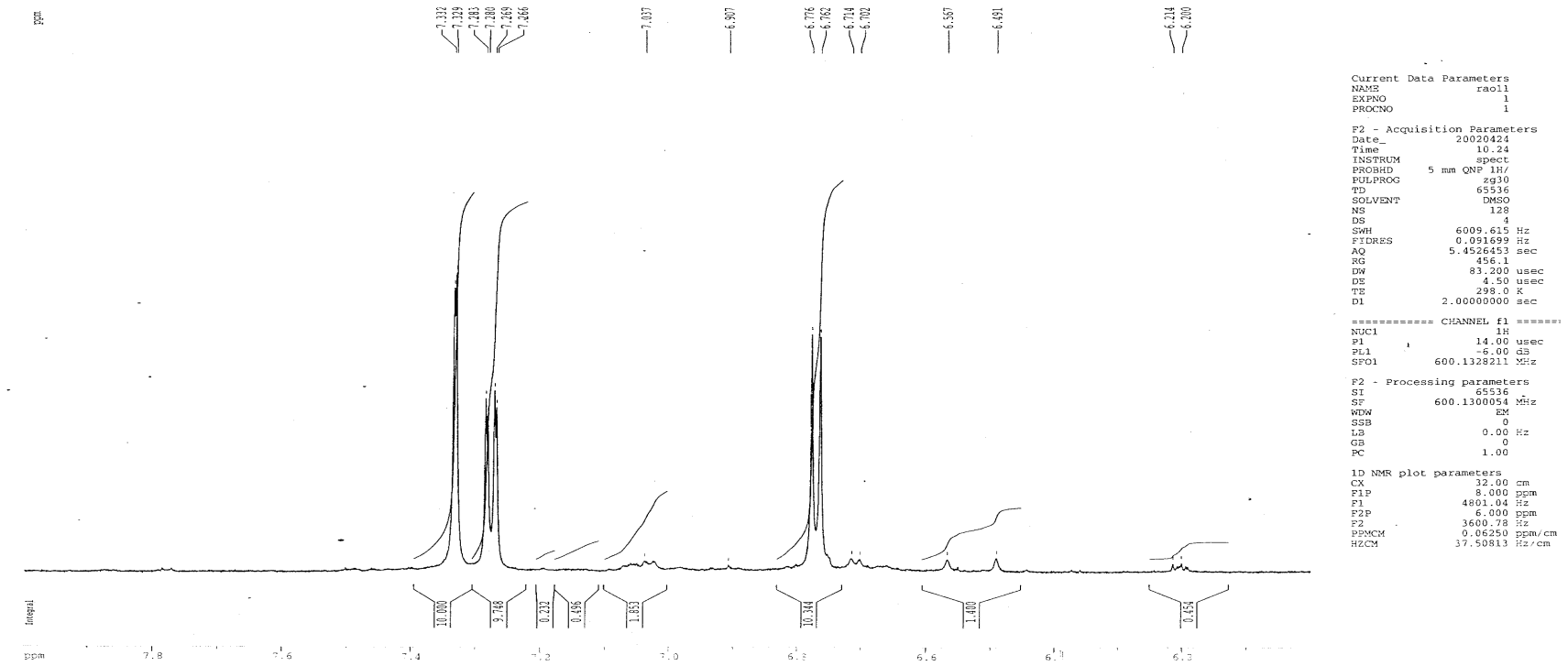


Figure 3.5. ^{1}H NMR spectrum of compound 1 (expanded).

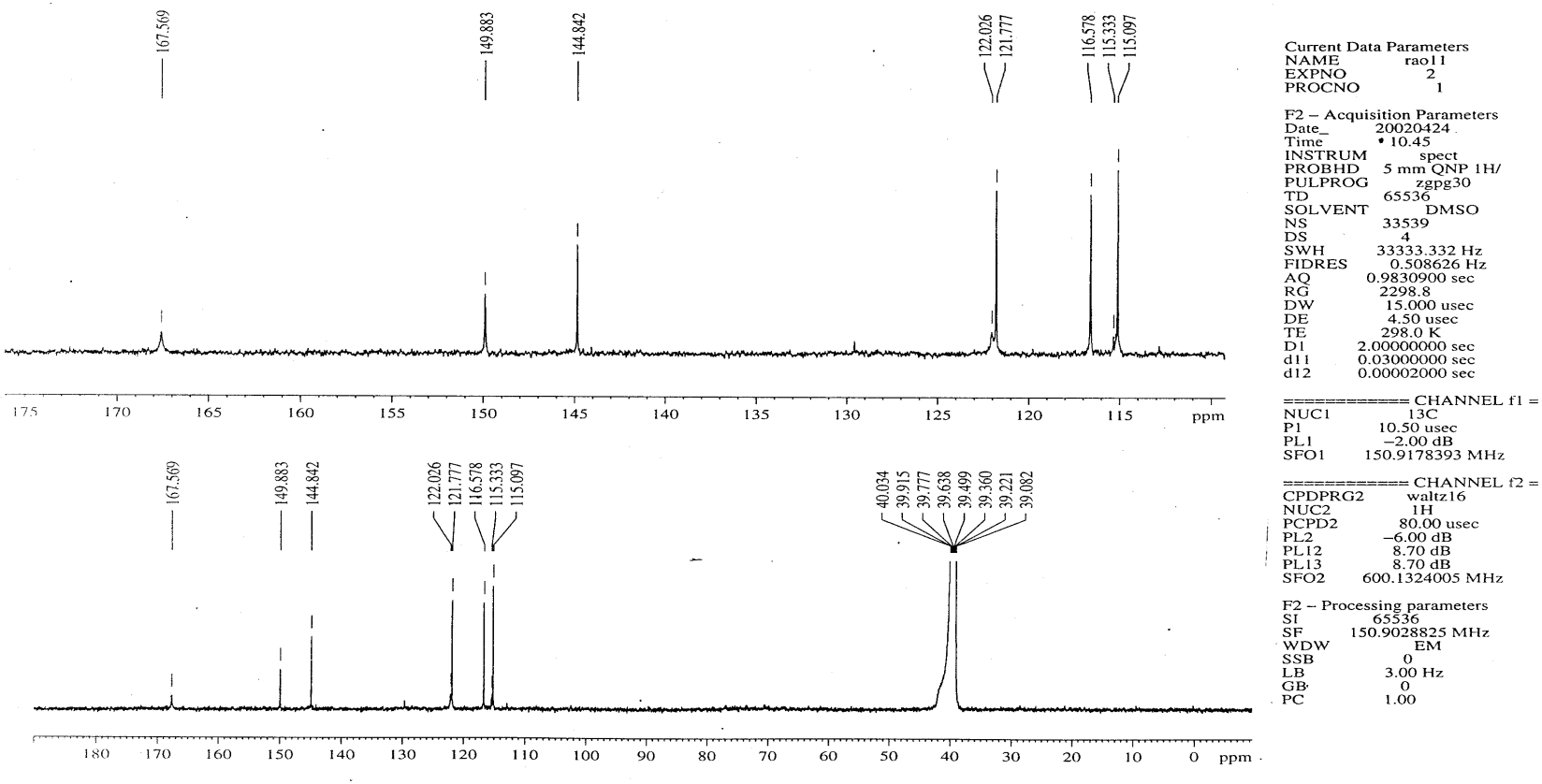


Figure 3.6. ^{13}C NMR spectrum of compound 1.

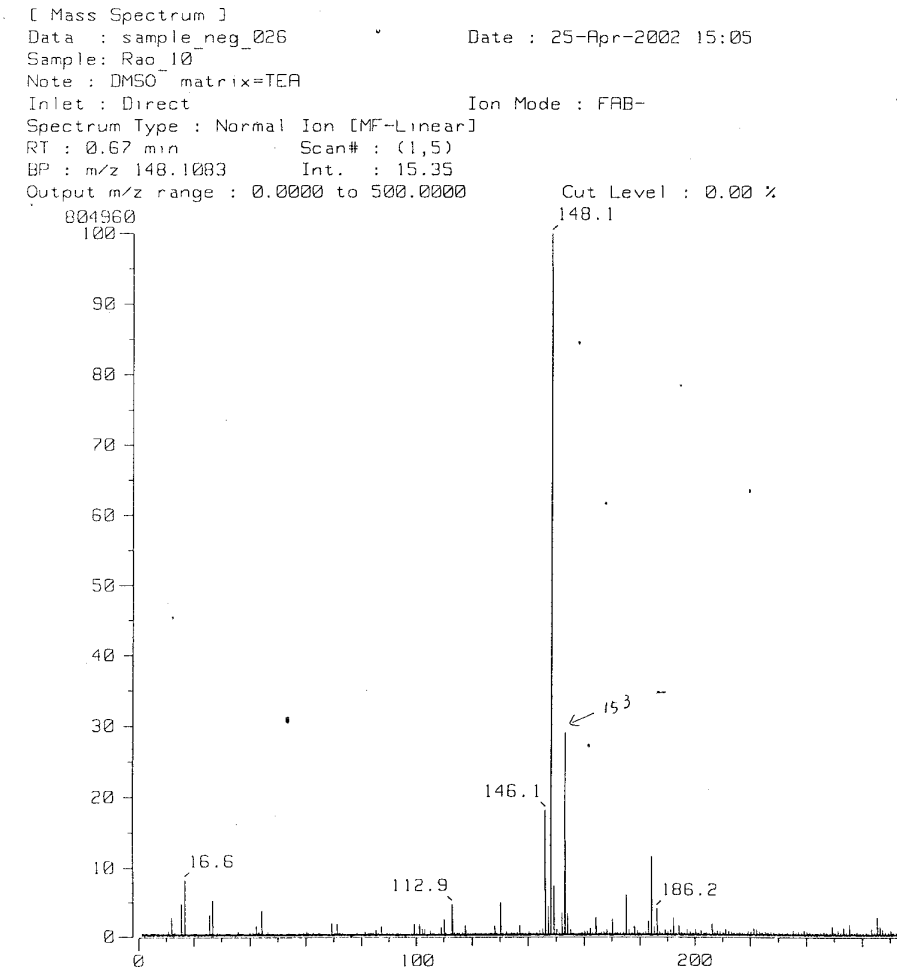


Figure 3.7. Mass spectrum of compound 1 using negative ion mode

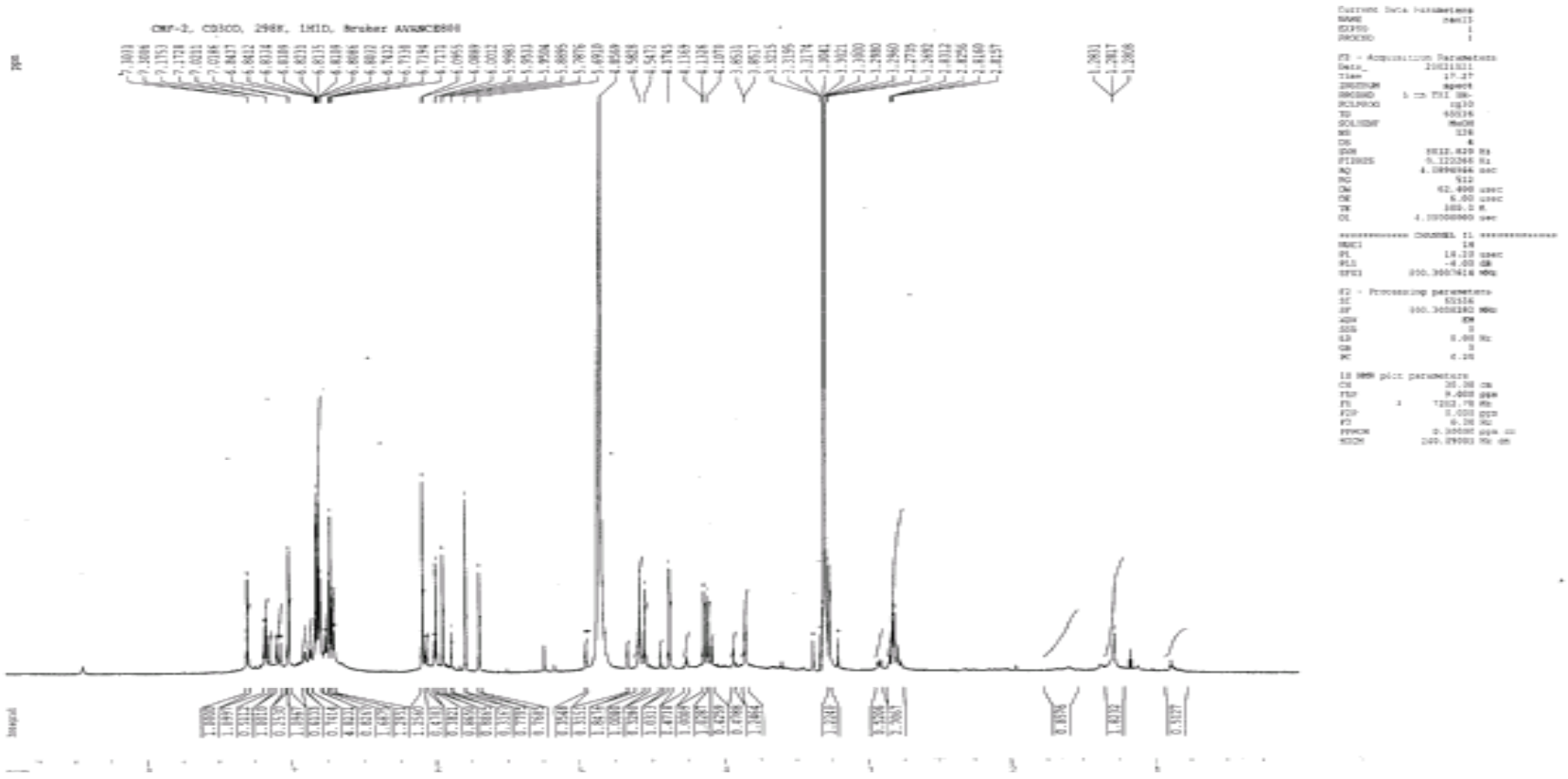
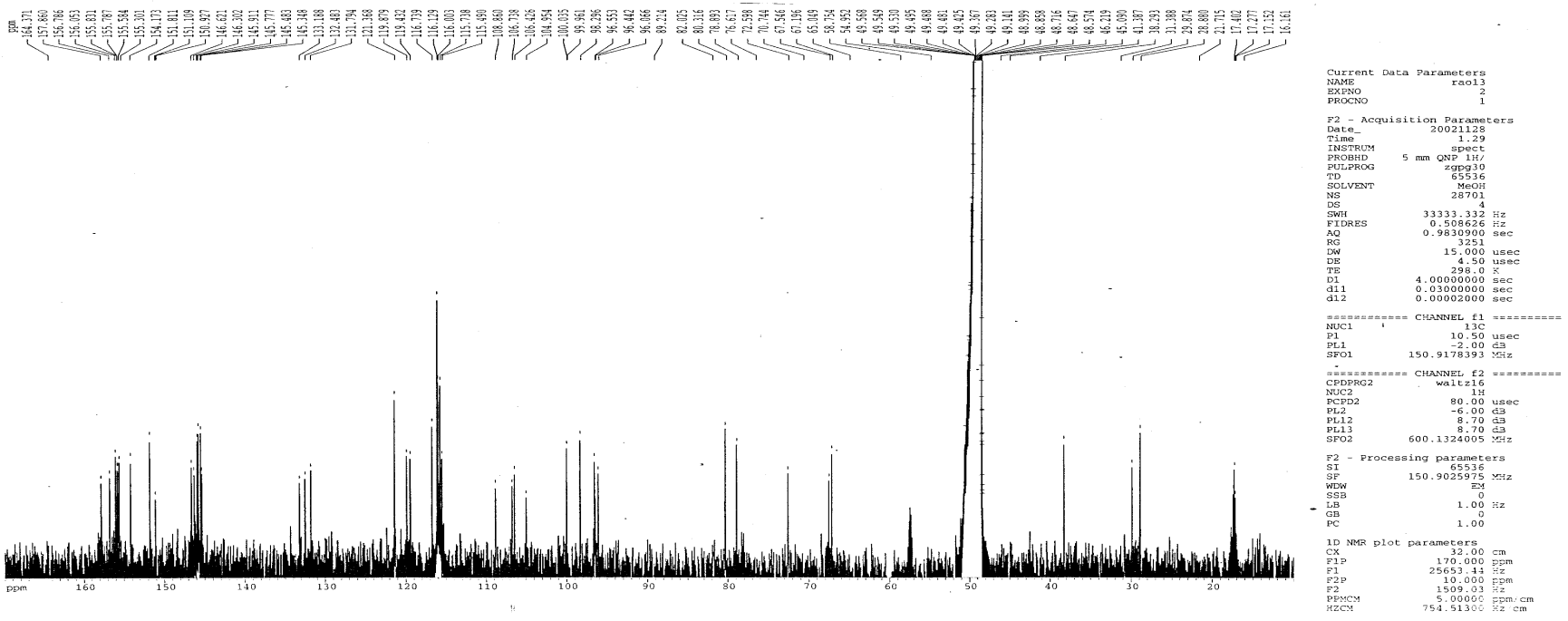


Figure 3.8. ^{1}H NMR spectrum of compound 2.

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Current Data Parameters
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EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
Date_              20021128
Time                  1.29
INSTRUM              spect
PROBHD     5 mm QNP 1H/
PULPROG           zgpg30
TD                 65536
SOLVENT             MeOH
NS                 28701
DS                     4
SWH            33333.332 Hz
FIDRES             0.508626 Hz
AQ                 0.9830900 sec
RG                    3251
DW                   15.000 usec
DE                    4.50 usec
TE                  298.0 K
D1             4.00000000 sec
d11            0.03000000 sec
d12            0.00002000 sec

============ CHANNEL f1 ==========
NUC1                  13C
P1                  10.50 usec
PL1                 -2.00 dB
SFO1          150.9178393 MHz

============ CHANNEL f2 ==========
CPDPRG2           waltz16
NUC2                  1H
PCPD2               80.00 usec
PL2                 -6.00 dB
PL12                 8.70 dB
PL13                 8.70 dB
SFO2          600.1324005 MHz

F2 - Processing parameters
SI                 65536
SF            150.9025975 MHz
WDW                   EM
SSB                    0
LB                  1.00 Hz
GB                     0
PC                  1.00

1D NMR plot parameters
CX                 32.00 cm
F1P               170.000 ppm
F1              25653.11 Hz
F2P                10.000 ppm
F2              1509.03 Hz
PPMCM              5.00000 ppm/cm
HZCM             754.51300 Hz/cm
```

Figure 3.9. ^{13}C NMR spectrum of compound 2.

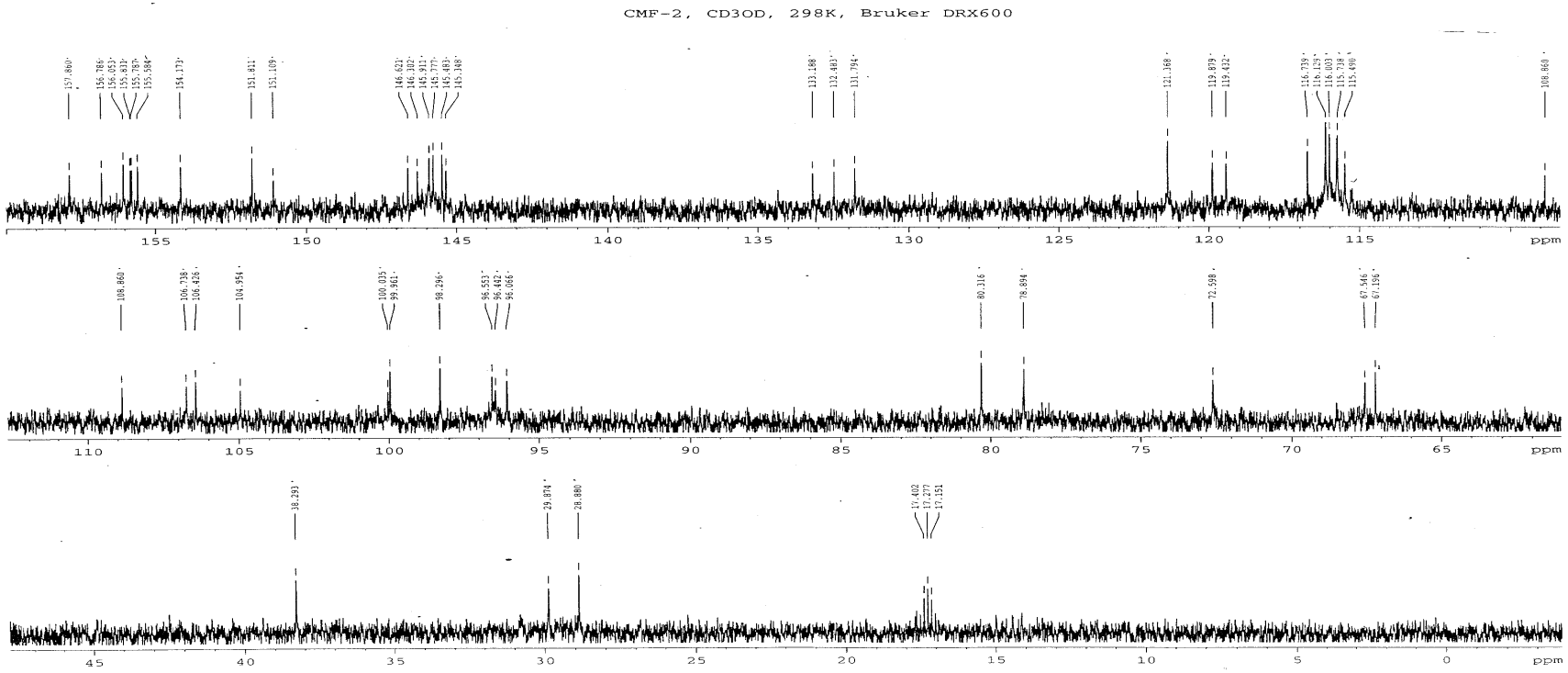


Figure 3.10. ^{13}C NMR spectrum of compound 2 (expanded).

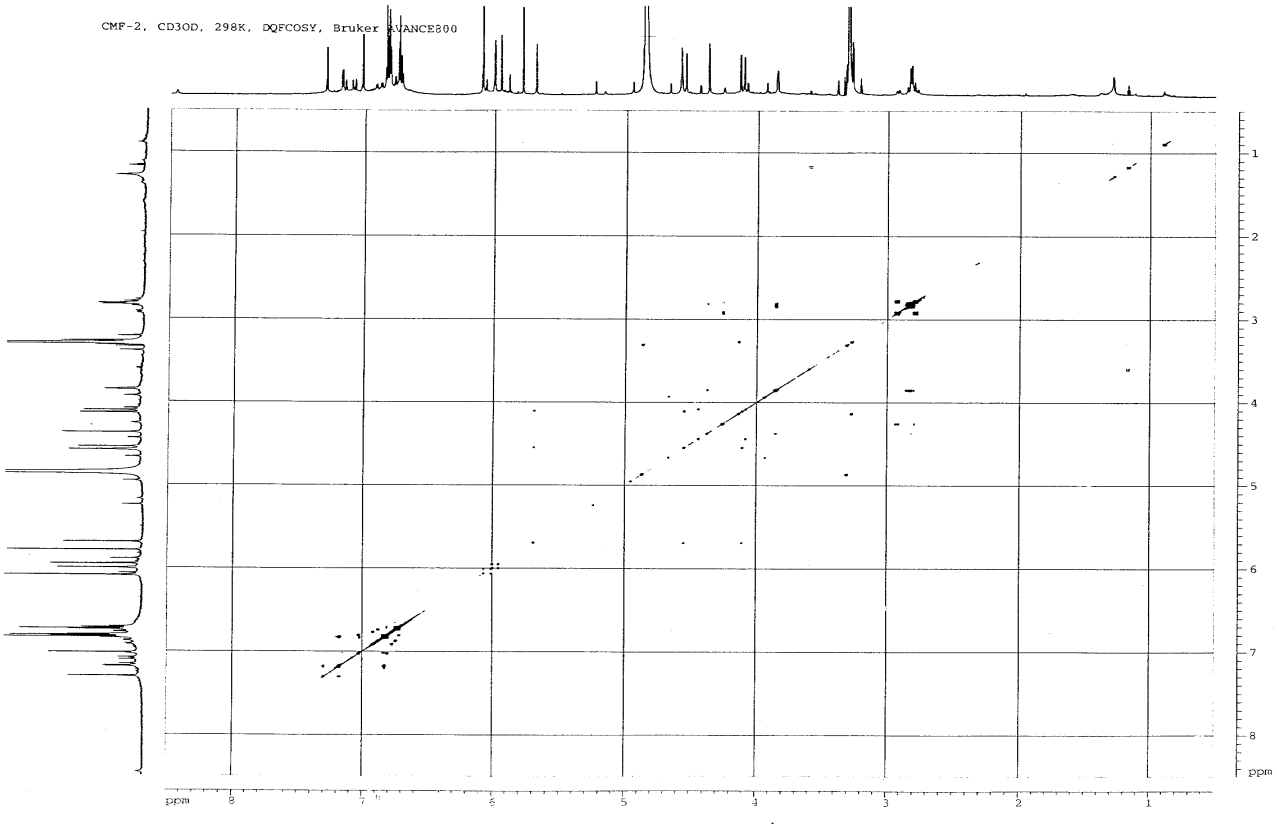


Figure 3.11. Double Quantum Filtered Correlated Spectrum (DQFCOSY) of compound 2.

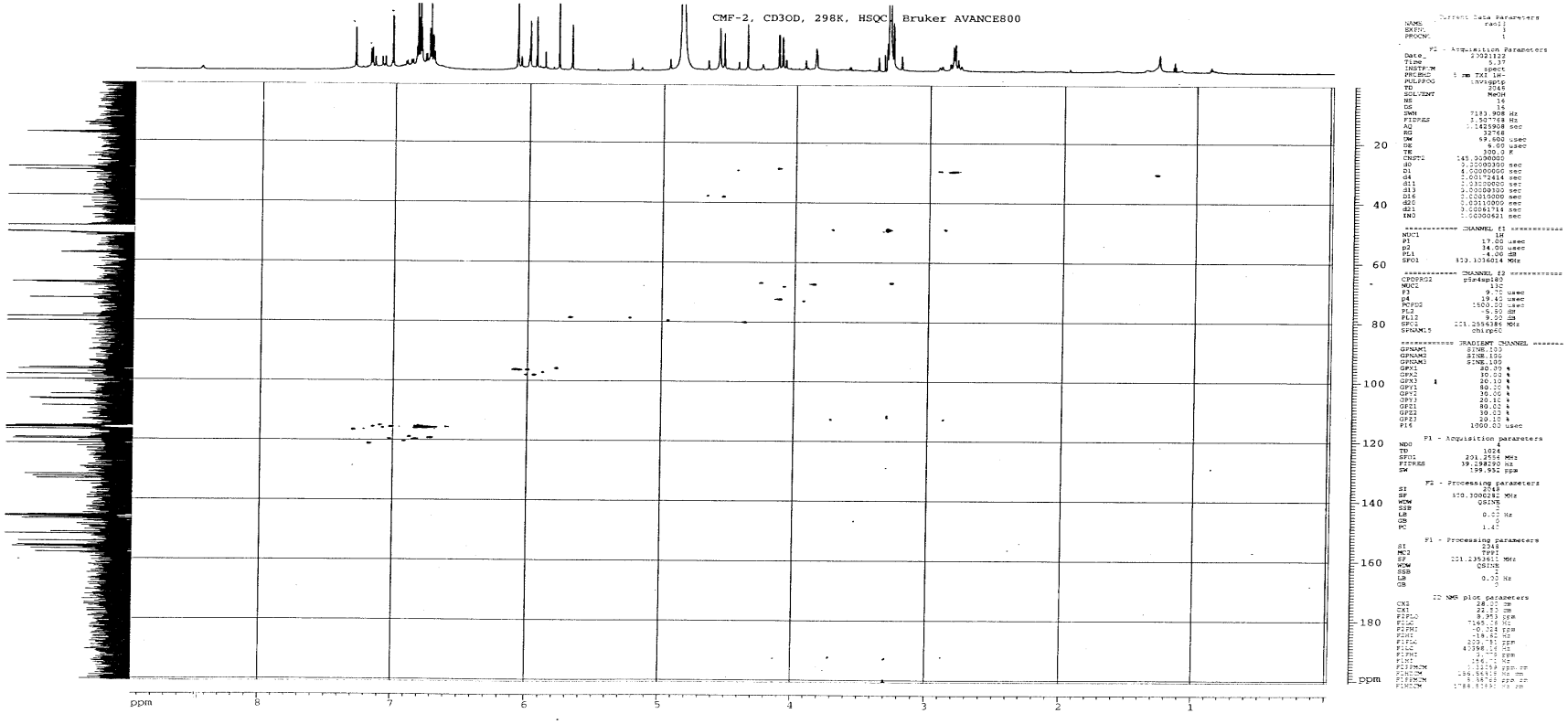


Figure 3.12. 2D ^{1}H-^{13}C Heteronuclear Single-Quantum Correlation Spectrum (HSQC) of compound 2.

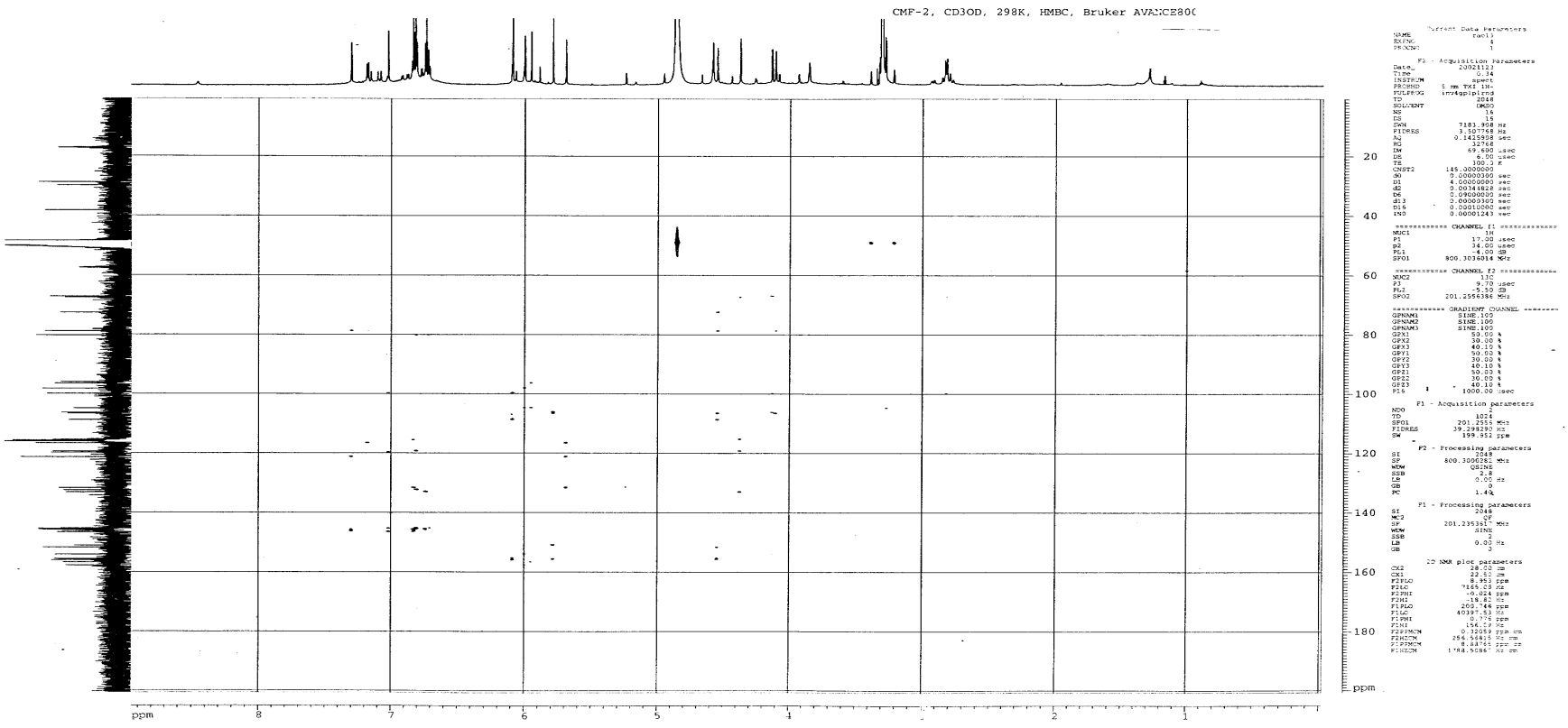


Figure 3.13. 2D ^{1}H-^{13}C Heteronuclear Multiple-Bond Correlation Spectrum (HMBC) of compound 2.

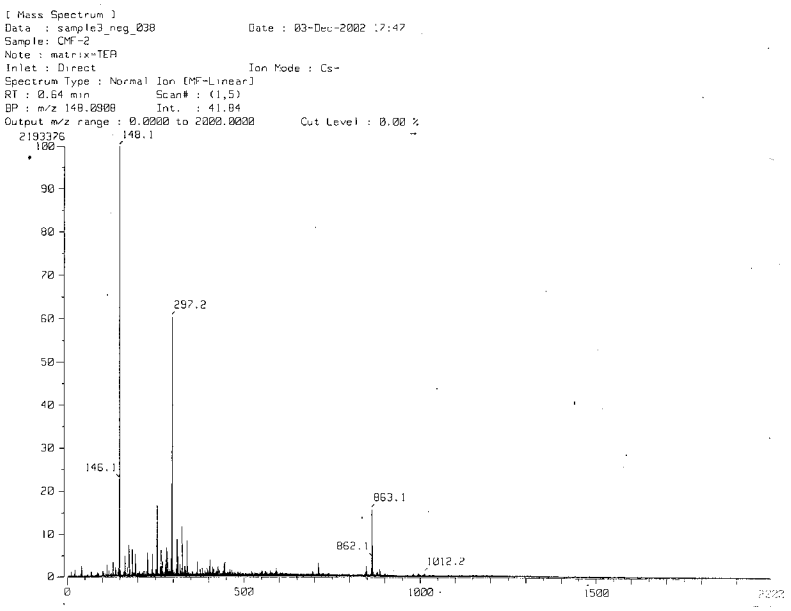


Figure 3.14. Mass spectrum of compound 2 using negative ion mode.

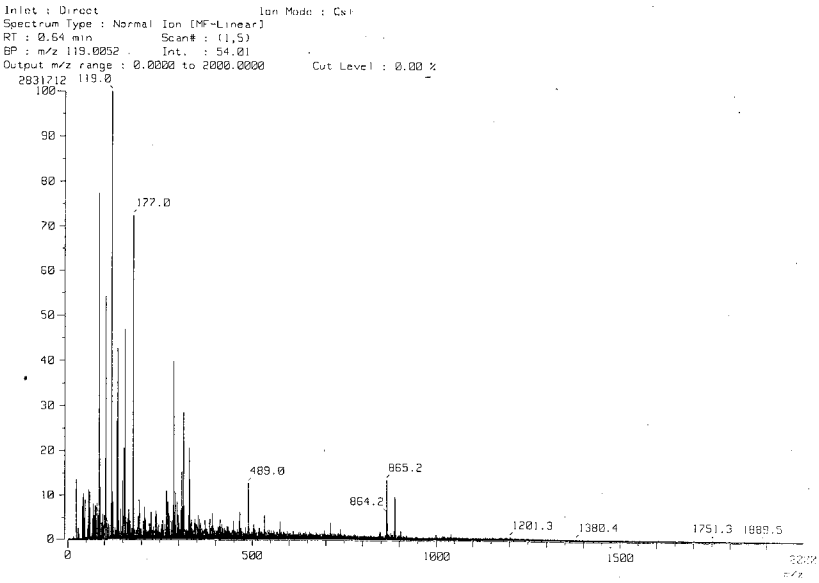
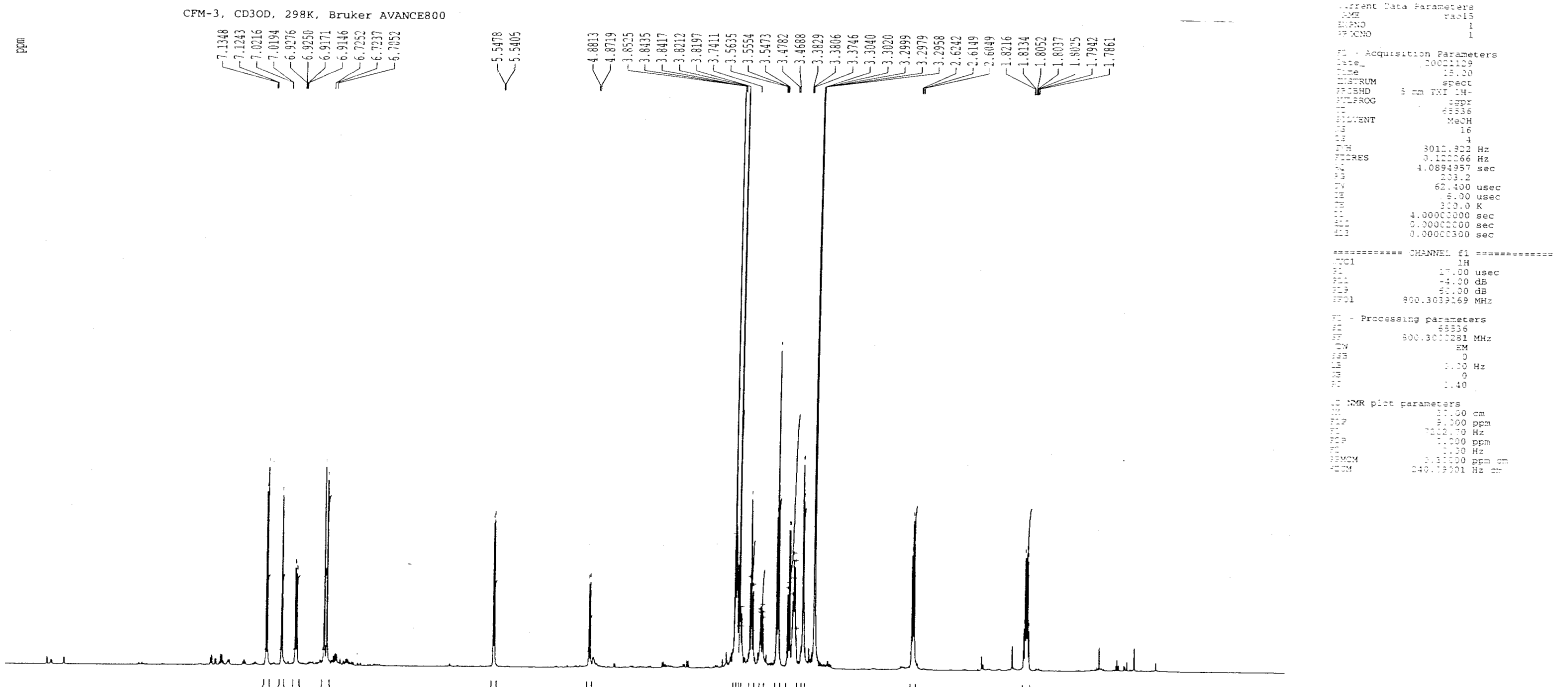


Figure 3.15. Mass spectrum of compound 2 using positive ion mode.



Current Data Parameters
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PROCNO 1

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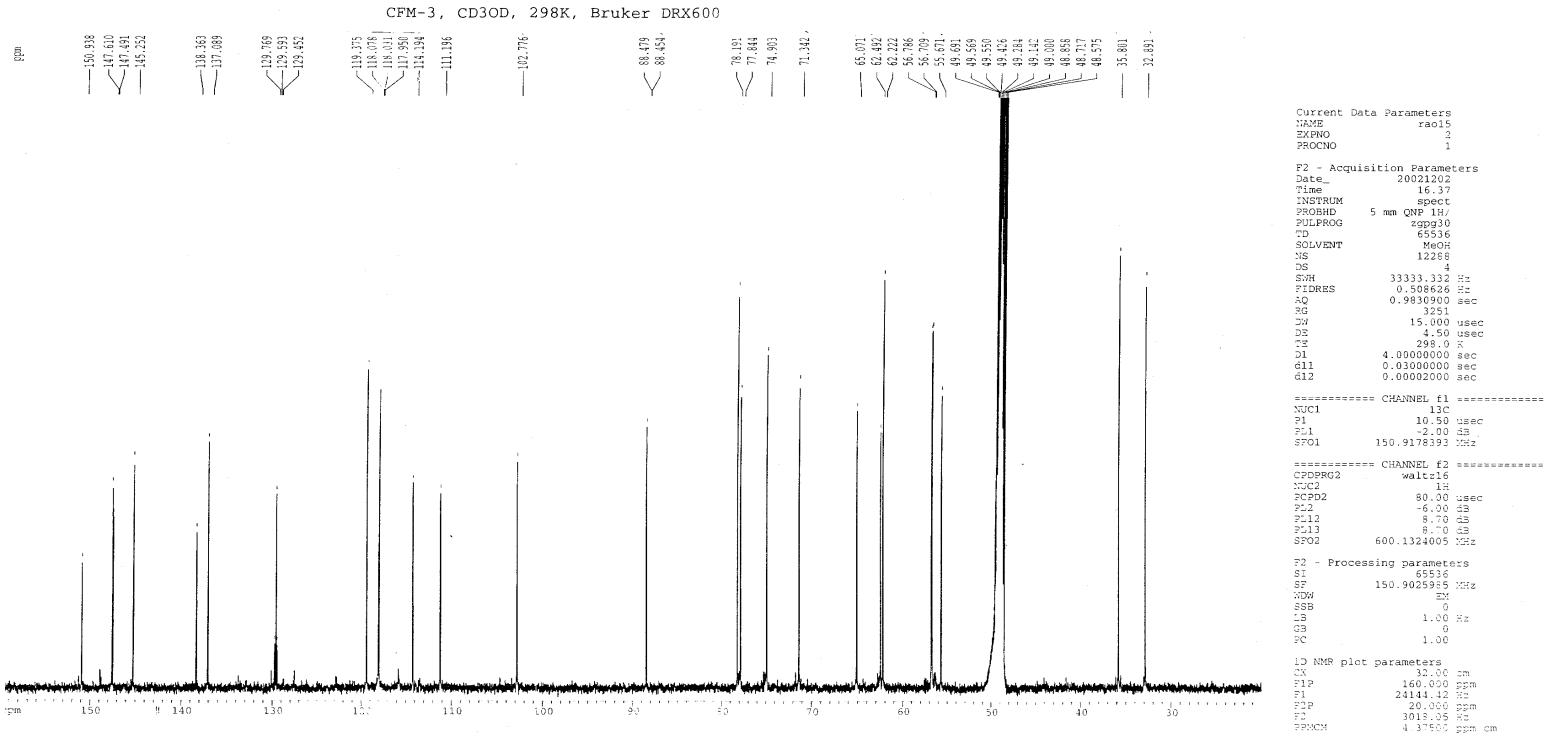
Time 18.20
INSTRUM spect
PROBHD 5 mm TXI 1H/
PULPROG zgpr
TD 65536
SOLVENT MeOH
NS 16
DS 4
SWH 9012.822 Hz
FIDRES 0.137566 Hz
AQ 4.0894957 sec
RG 203.0
DW 62.100 usec
DE 6.00 usec
TE 313.0 K
D1 4.00000000 sec
D11 0.03000000 sec
D12 0.00000300 sec

====== CHANNEL f1 ======
NUC1 1H
P1 17.00 usec
PL1 -4.00 dB
PL9 51.00 dB
SFO1 800.3039169 MHz

F2 - Processing parameters
SI 65536
SF 800.3000281 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.40

1D NMR plot parameters
CX 17.50 cm
F1P 9.000 ppm
F2P 7210.70 Hz
PPP 1.000 ppm
LPP 0.20 Hz
PPMCM 0.51100 ppm/cm
HZCM 240.79301 Hz/cm

Figure 3.16. ^{1}H NMR spectrum of compound 3.




```
Current Data Parameters
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EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
Date_              20021202
Time                  16.37
INSTRUM               spect
PROBHD     5 mm QNP 1H/
PULPROG             zgpg30
TD                   65536
SOLVENT               MeOH
NS                   12288
DS                       4
SWH              33333.332 Hz
FIDRES           0.508626 Hz
AQ               0.9830900 sec
RG                    3251
DW                15.000 usec
DE                 4.50 usec
TE                 298.0 K
D1            4.00000000 sec
d11           0.03000000 sec
d12           0.00002000 sec

============ CHANNEL f1 =============
NUC1                 13C
P1                 10.50 usec
PL1                -2.00 dB
SFO1         150.9178393 MHz

============ CHANNEL f2 =============
CPDPRG2          waltz16
NUC2                 1H
PCPD2              80.00 usec
PL2                -6.00 dB
PL12                8.70 dB
PL13                8.70 dB
SFO2         600.1324005 MHz

F2 - Processing parameters
SI                   65536
SF           150.9025985 MHz
WDW                     EM
SSB                      0
LB                    1.00 Hz
GB                       0
PC                    1.00

1D NMR plot parameters
CX                   32.00 cm
F1P                160.000 ppm
F1                 24141.42 Hz
F2P                 20.000 ppm
F2                  3018.05 Hz
PPMCM              4.37500 ppm/cm
```

Figure 3.17. ^{13}C NMR spectrum of compound 3.

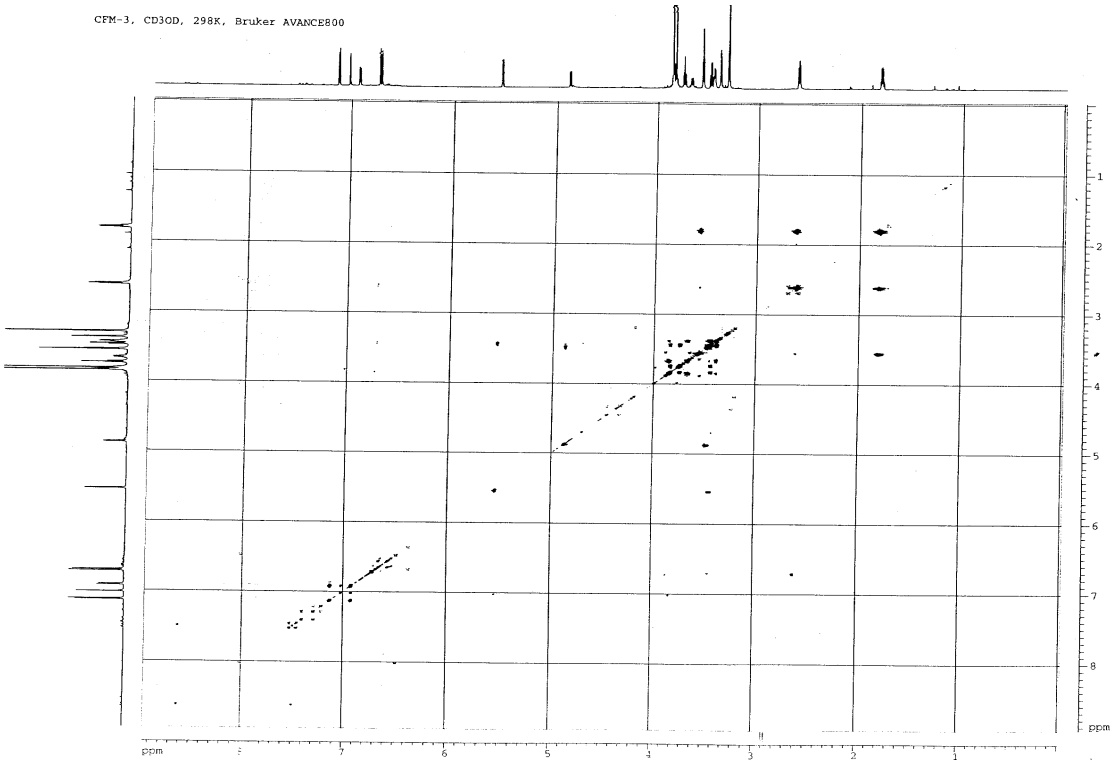


Figure 3.18. Double Quantum Filtered Correlated Spectrum (DQFCOSY) of compound 3.

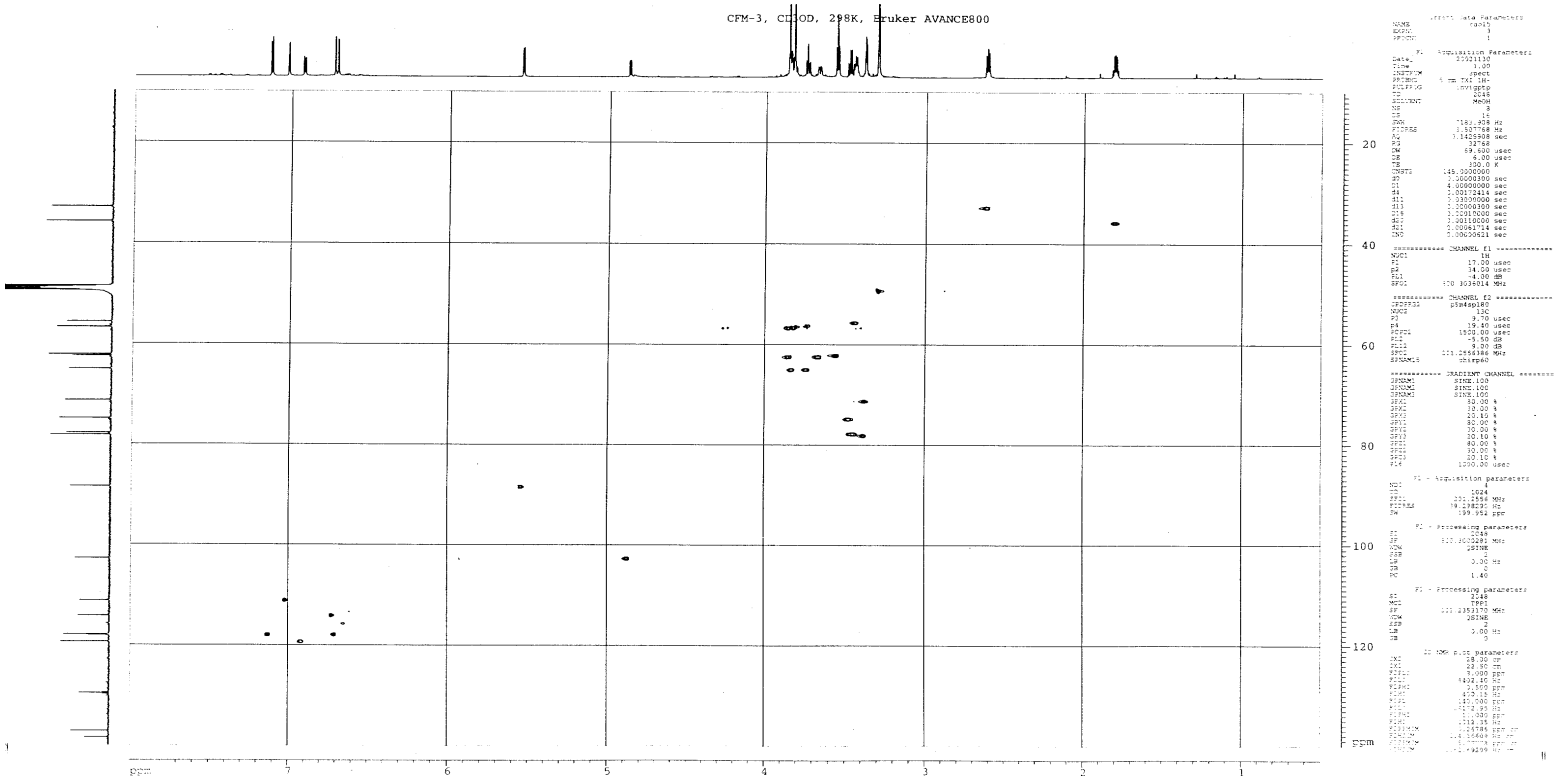


Figure 3. 19. 2D ^{1}H-^{13}C Heteronuclear Single-Quantum Correlation Spectrum (HSQC) of compound 3.

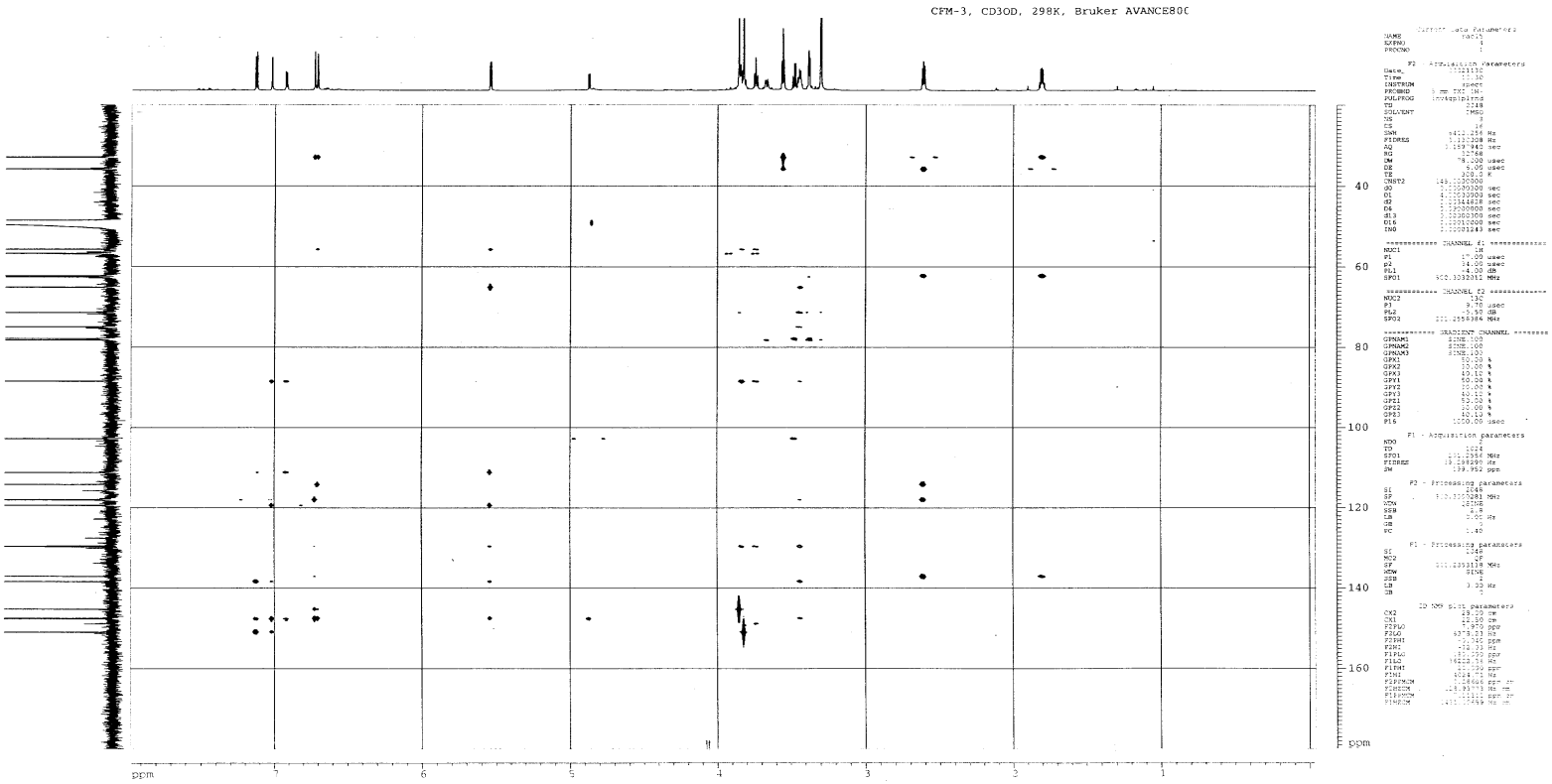


Figure 3. 20. 2D ^{1}H-^{13}C Heteronuclear Multiple-Bond Correlation Spectrum (HMBC) of compound 3.

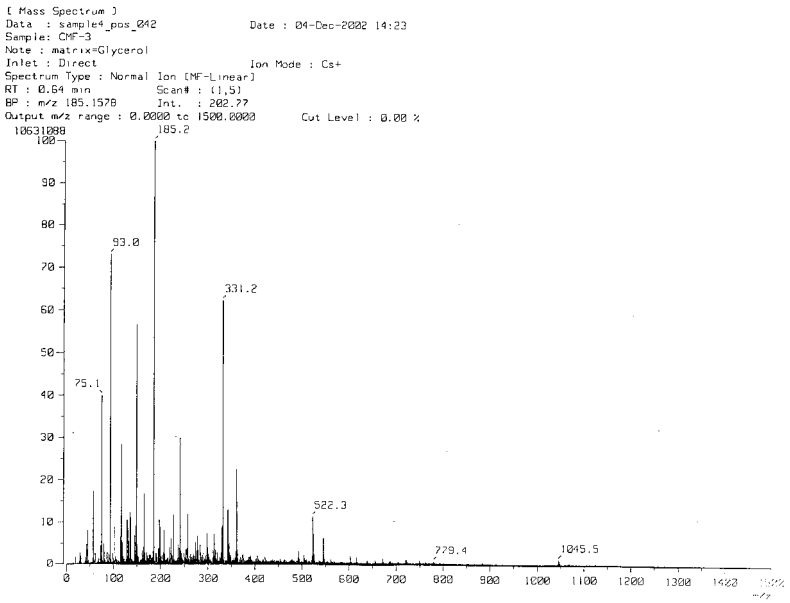


Figure 3.21. Mass spectrum of compound 3 using positive ion mode.

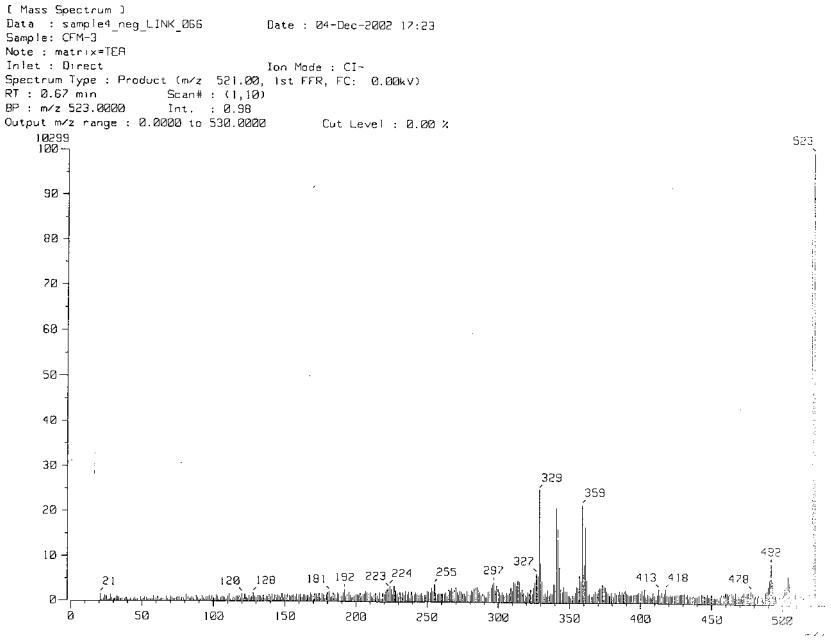


Figure 3.22. Mass spectrum of compound 3 using negative ion mode.

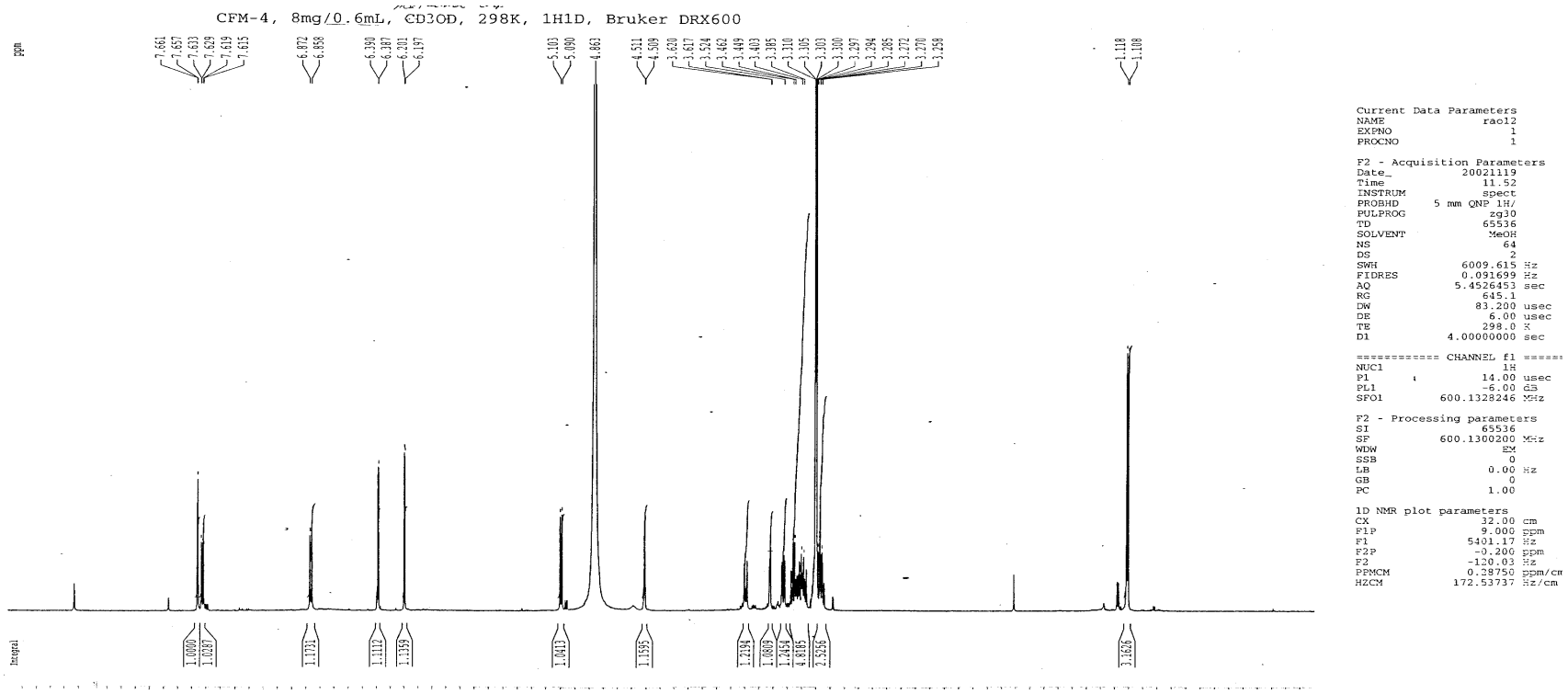


Figure 3. 23. ^{1}H NMR spectrum of compound 4.

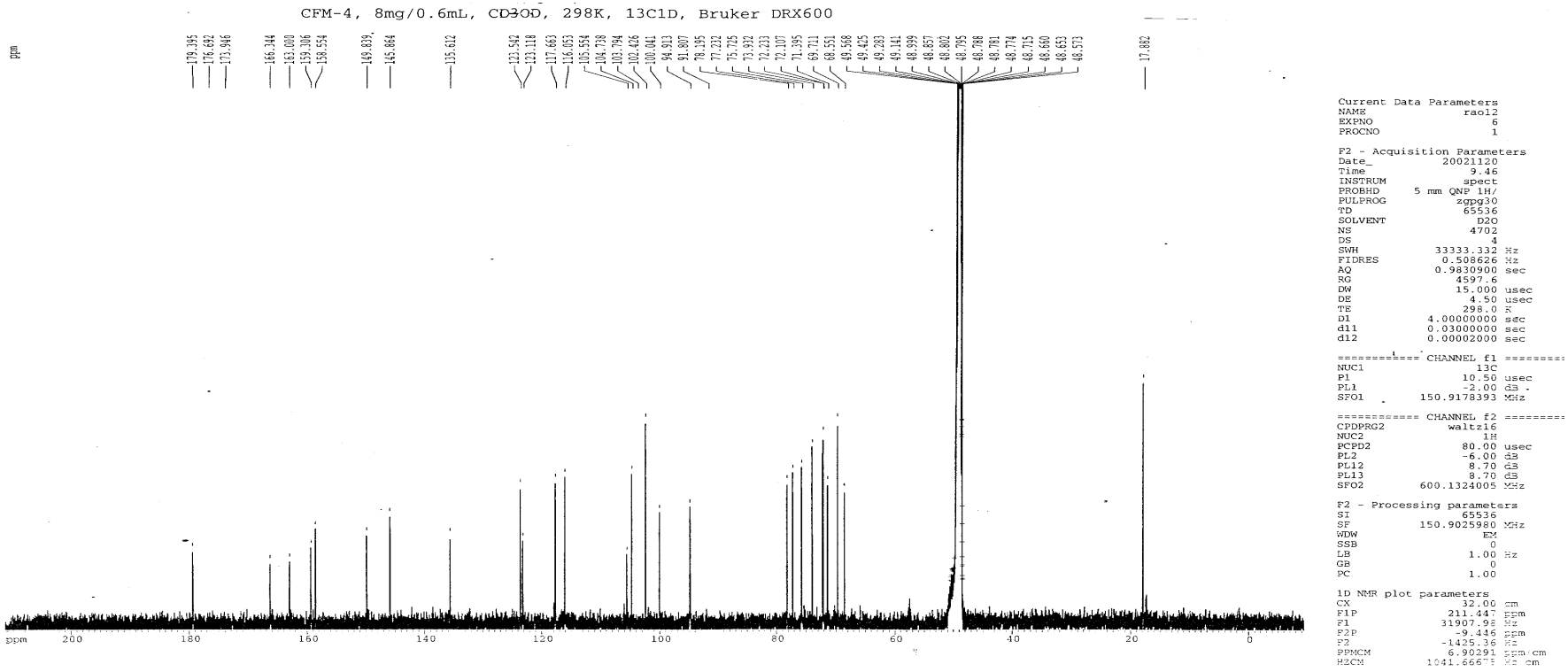
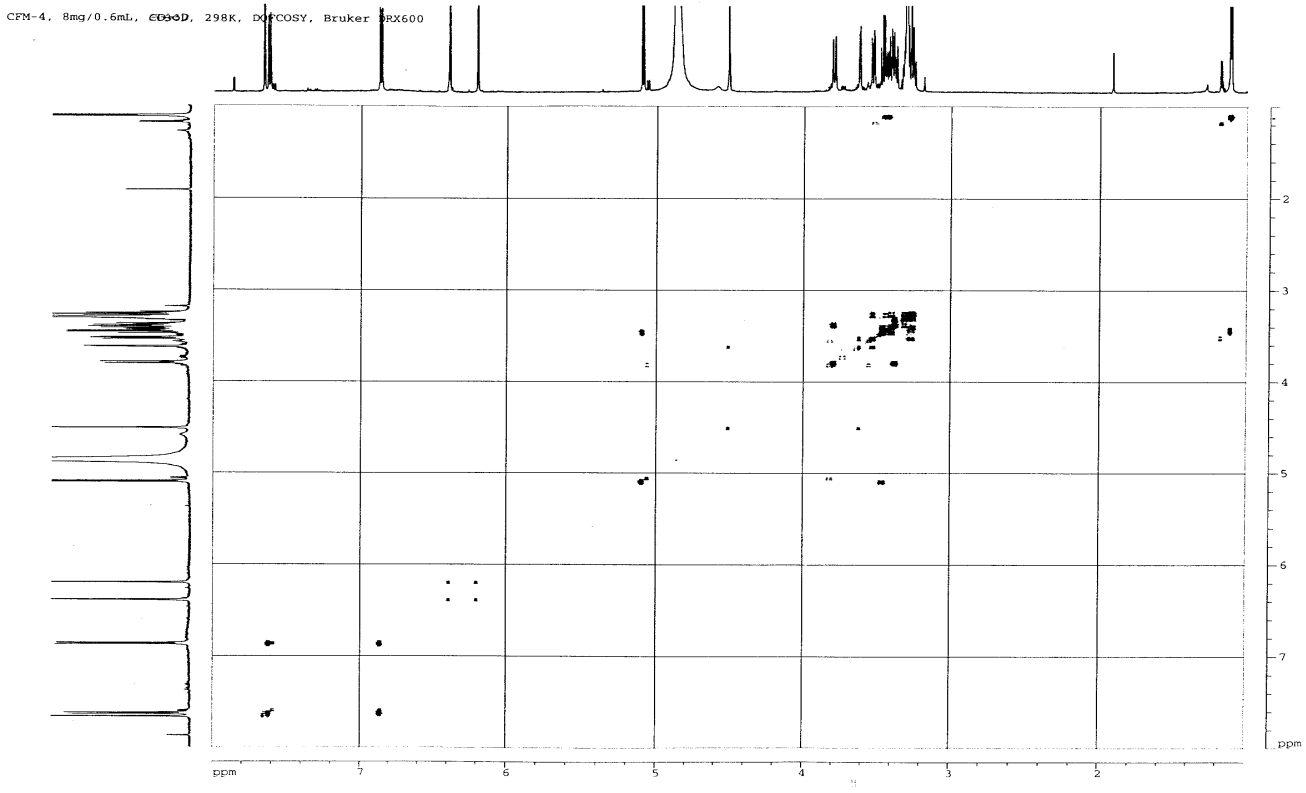


Figure 3. 24. ^{13}C NMR spectrum of compound 4.



Current Data Parameters
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EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
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Time 16.21
INSTRUM spect
PROBHD 5 mm QNP 1H
PULPROG cosygpmfqf
TD 2048
SOLVENT MeOH
NS 4
DS 16
SWH 5387.931 Hz
FIDRES 2.630826 Hz
AQ 0.1901044 sec
RG 325
DW 92.800 usec
DE 4.50 usec
TE 299.0 K
d0 0.00000300 sec
D1 4.000000000 sec
d13 0.00000300 sec
D16 0.00010000 sec
d20 0.00110300 sec
IN0 0.00009280 sec

============ CHANNEL f1 ============
NUC1 1H
P1 15.10 usec
P2 30.20 usec
PL1 -6.00 dB
SFO1 600.1327006 MHz

============ GRADIENT CHANNEL =======
GPNAM1 SINE.100
GPNAM2 SINE.100
GPX1 0.00 %
GPX2 0.00 %
GPY1 0.00 %
GPY2 0.00 %
GPZ1 32.00 %
GPZ2 64.00 %
P16 1000.00 usec

F1 - Acquisition parameters
ND0 2
TD 1024
SFO1 600.1327 MHz
FIDRES 5.261652 Hz
SW 8.978 ppm

F2 - Processing parameters
SI 2048
SF 600.1300209 MHz
WDW QSINE
SSB 2
LB 0.00 Hz
GB 0
PC 1.00

F1 - Processing parameters
SI 2048
MC2 TPPI
SF 600.1300209 MHz
WDW QSINE
SSB 2
LB 0.00 Hz
GB 0

2D NMR plot parameters
CX2 22.50 cm
CY1 22.50 cm
F2PLO 8.000 ppm
F2LO 4801.04 Hz
F2PHI 1.002 ppm
F2HI 600.13 Hz
F1PLO 8.000 ppm
F1LO 4801.04 Hz
F1PHI 1.002 ppm
F1HI 600.13 Hz
F2PPMCM 0.33111 ppm/cm
F2HZCM 198.70711 Hz/cm
F1PPMCM 0.33111 ppm/cm
F1HZCM 198.70711 Hz/cm

Figure 3. 25. Double Quantum Filtered Correlated Spectrum (DQFCOSY) of compound 4.

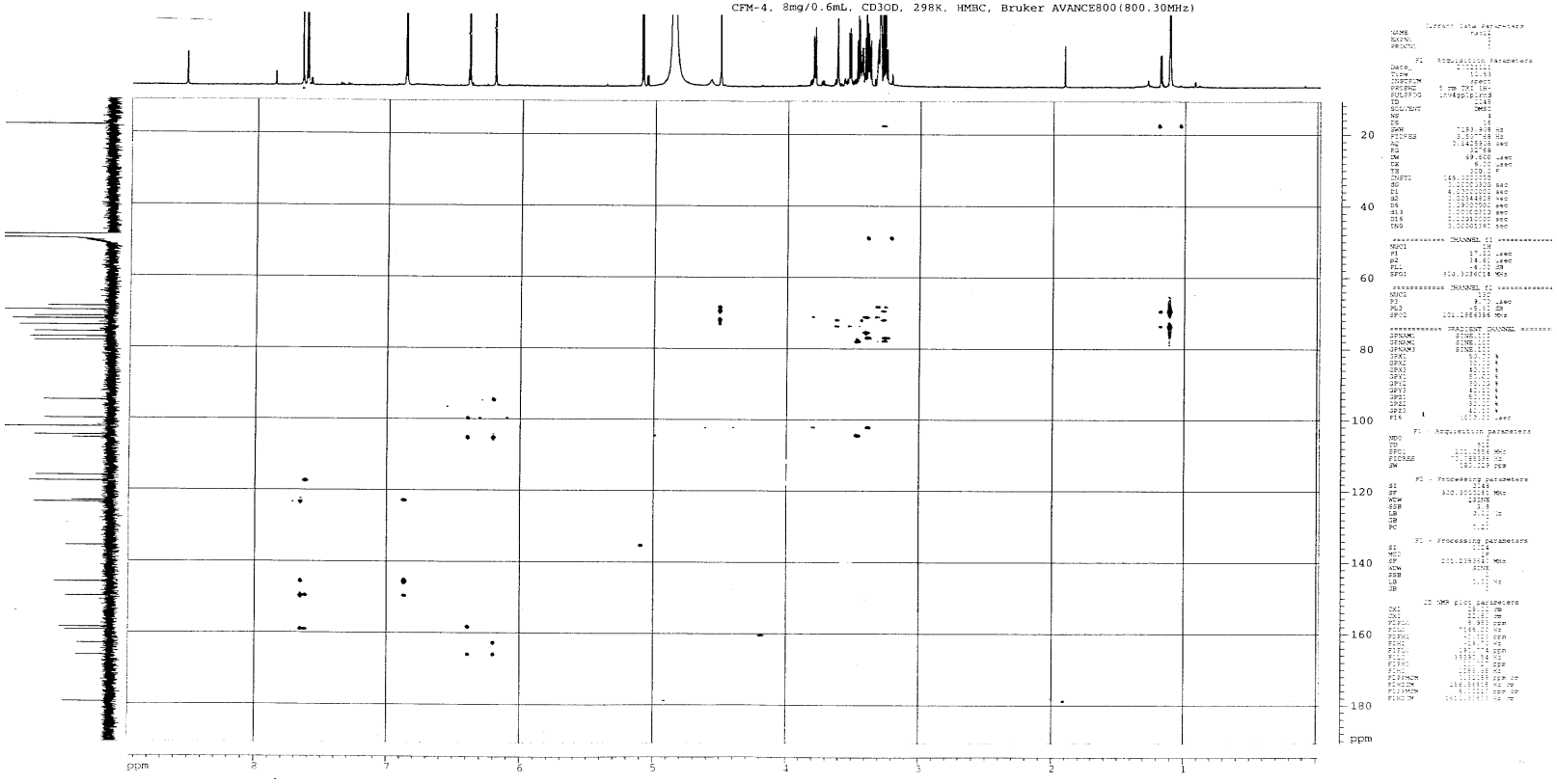
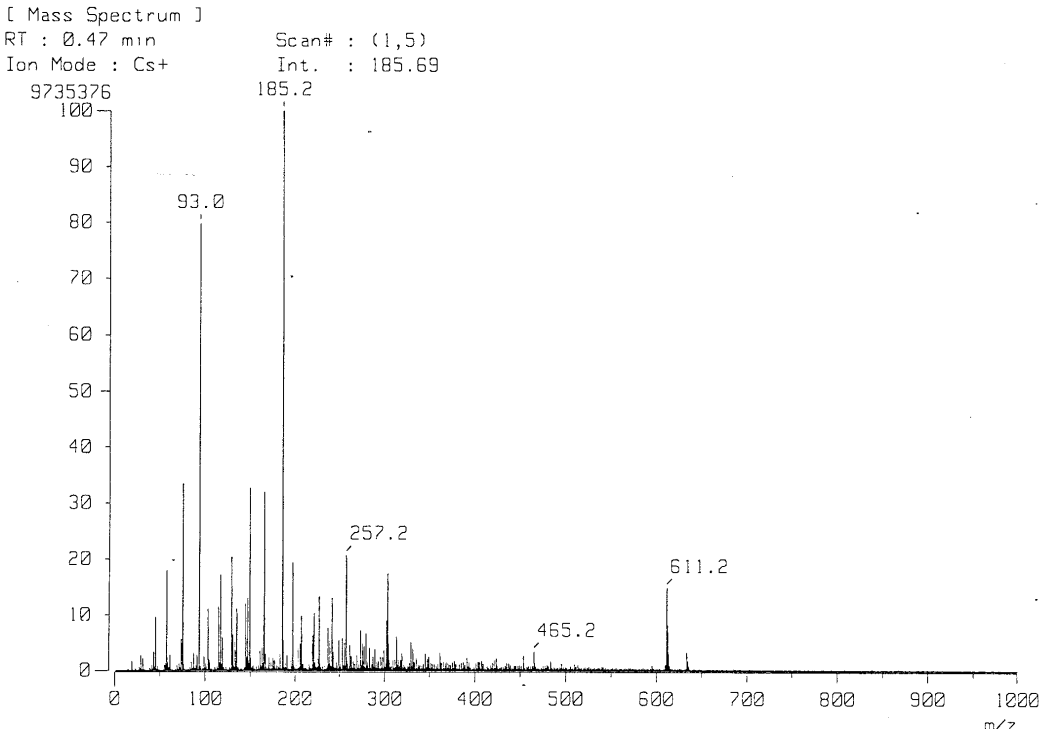
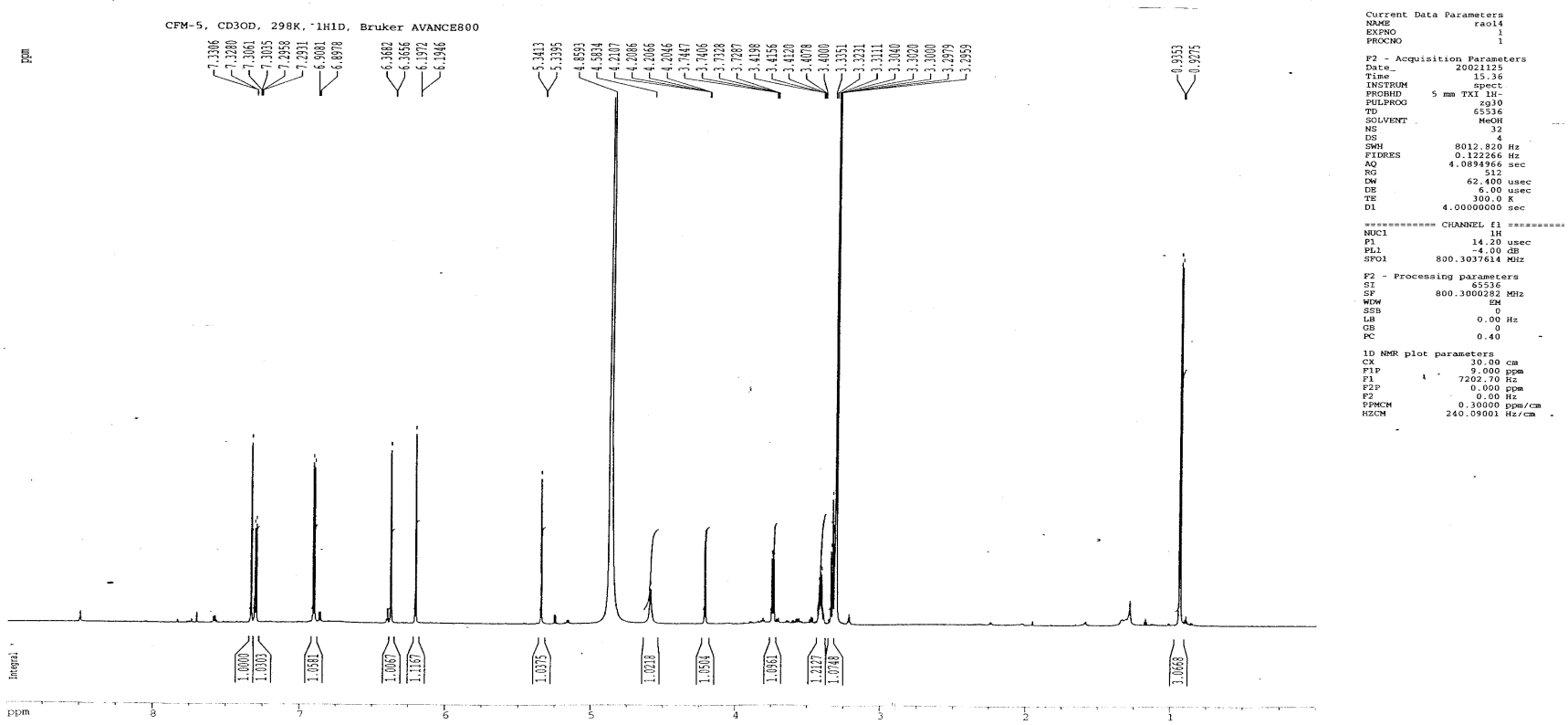


Figure 3. 26. 2D ^{1}H-^{13}C Heteronuclear Multiple-Bond Correlation Spectrum (HMBC) of compound 4.



m/z

Figure 3.27. Mass spectrum of compound 4 using positive ion mode.

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Current Data Parameters
NAME                  raol4
EXPNO                     1
PROCNO                    1

F2 - Acquisition Parameters
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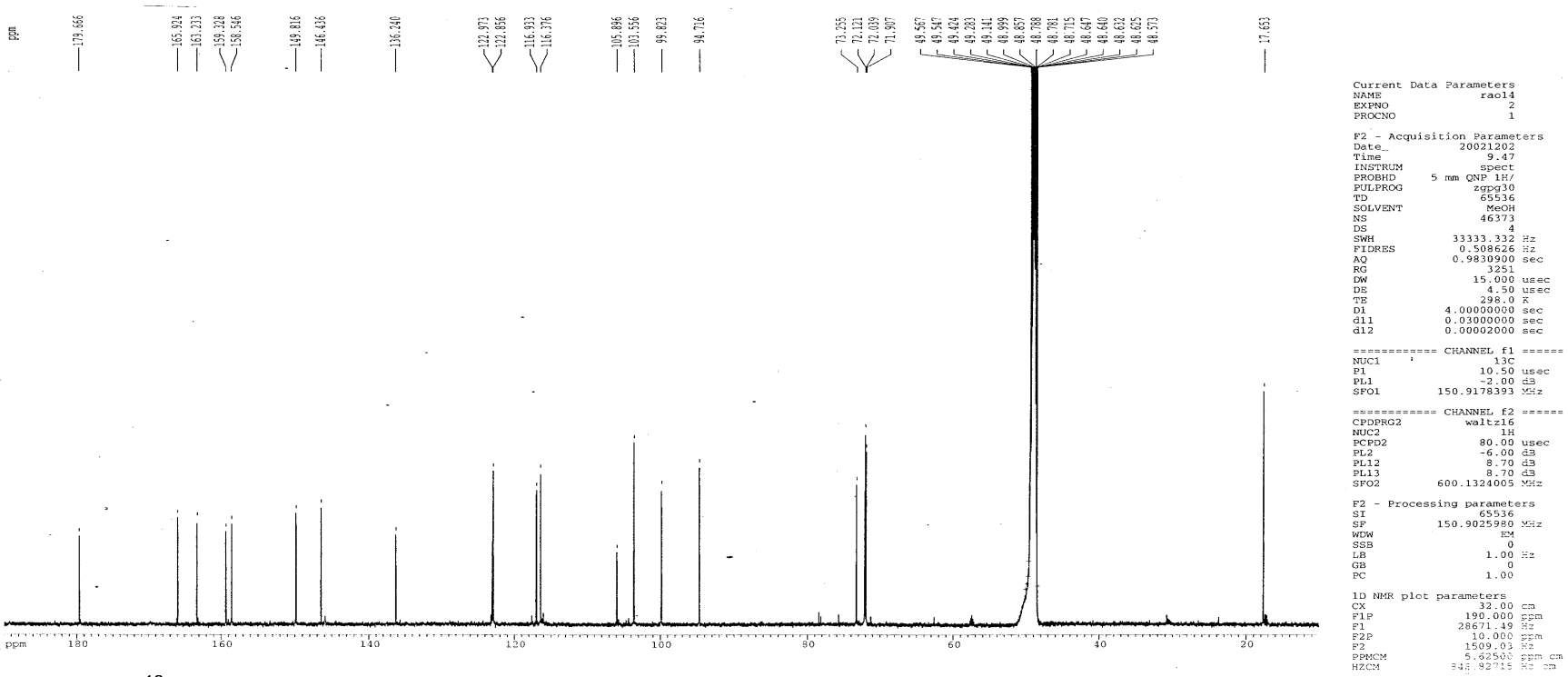
Time                  15.36
INSTRUM               spect
PROBHD     5 mm TXI 1H-
PULPROG               zg30
TD                   65536
SOLVENT               MeOH
NS                      32
DS                       4
SWH                8012.820 Hz
FIDRES             0.122266 Hz
AQ                 4.0894966 sec
RG                     512
DW                  62.400 usec
DE                   6.00 usec
TE                  300.0 K
D1                 4.00000000 sec

========== CHANNEL f1 ==========
NUC1                     1H
P1                   14.20 usec
PL1                  -4.00 dB
SFO1           800.3037614 MHz

F2 - Processing parameters
SI                   65536
SF             800.3000282 MHz
WDW                      EM
SSB                      0
LB                    0.00 Hz
GB                       0
PC                    0.40

1D NMR plot parameters
CX                   30.00 cm
F1P                   9.000 ppm
F1                 7202.70 Hz
F2P                   0.000 ppm
F2                    0.00 Hz
PPMCM             0.30000 ppm/cm
HZCM            240.09001 Hz/cm
```

Figure 3.28. ^{1}H NMR spectrum of compound 5.

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Current Data Parameters
NAME                  raol4
EXPNO                     2
PROCNO                    1

F2 - Acquisition Parameters
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Time                   9.47
INSTRUM               spect
PROBHD     5 mm QNP 1H/
PULPROG             zgpg30
TD                   65536
SOLVENT                MeOH
NS                   46373
DS                       4
SWH              33333.332 Hz
FIDRES           0.508626 Hz
AQ               0.9830900 sec
RG                    3251
DW                15.000 usec
DE                 4.50 usec
TE                 298.0 K
D1             4.00000000 sec
d11            0.03000000 sec
d12            0.00002000 sec

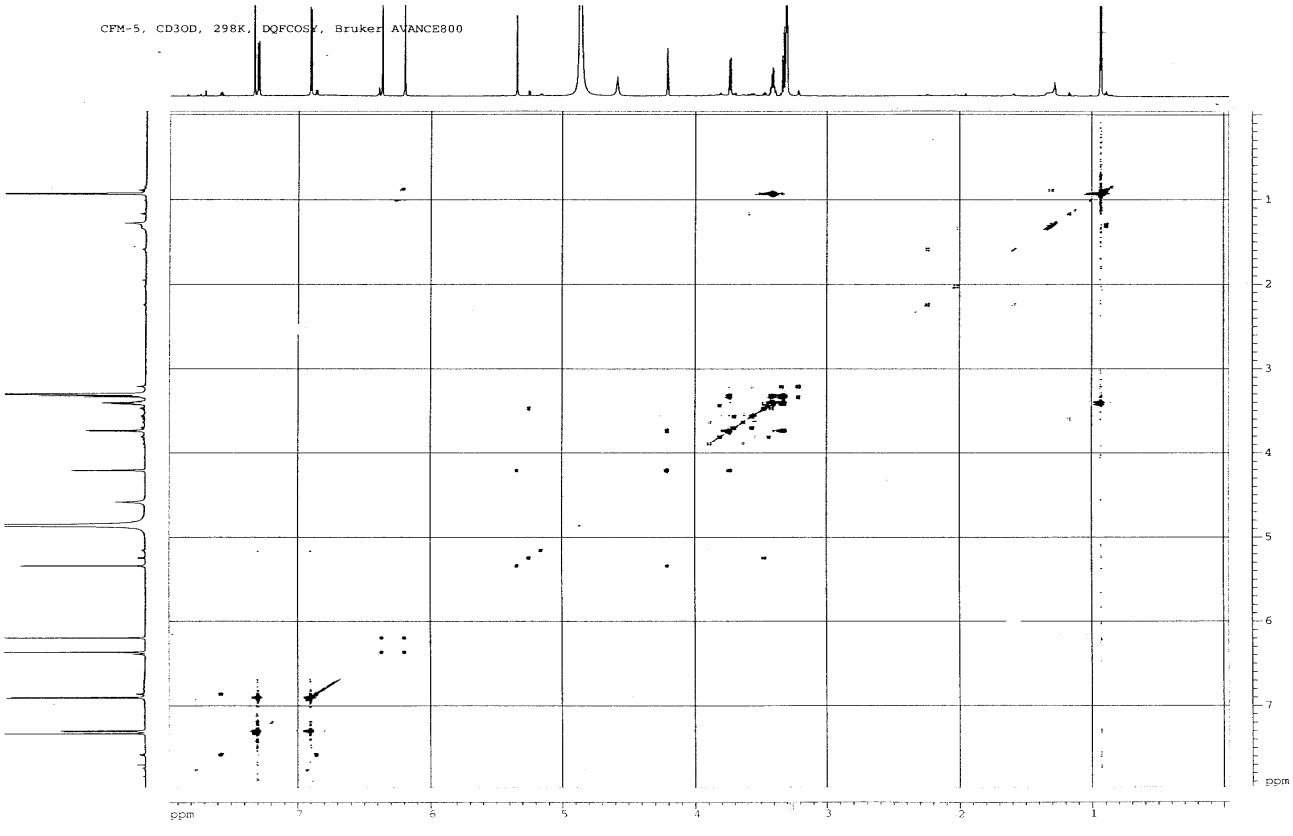
============ CHANNEL f1 ======
NUC1                  13C
P1                 10.50 usec
PL1                -2.00 dB
SFO1          150.9178393 MHz

============ CHANNEL f2 ======
CPDPRG2           waltz16
NUC2                  1H
PCPD2              80.00 usec
PL2                -6.00 dB
PL12                8.70 dB
PL13                8.70 dB
SFO2          600.1324005 MHz

F2 - Processing parameters
SI                   65536
SF            150.9025980 MHz
WDW                     EM
SSB                      0
LB                  1.00 Hz
GB                       0
PC                    1.00

1D NMR plot parameters
CX                  32.00 cm
F1P                190.000 ppm
F1               28671.49 Hz
F2P                 10.000 ppm
F2                1509.03 Hz
PPMCM             5.62500 ppm/cm
HZCM            848.82715 Hz/cm
```

Figure 3.29. ^{13}C NMR spectrum of compound 5.



Current Data Parameters
NAME rao14
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
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Time 15.40
INSTRUM spect
PROBHD 5 mm TXI 1H-
PULPROG cosygpmfqf
TD 2048
SOLVENT MeOH
NS 2
DS 16
SWH 6410.256 Hz
FIDRES 3.128908 Hz
AQ 0.1597940 sec
RG 512
DW 78.000 usec
DE 6.00 usec
TE 300.0 K
d0 0.00009100 sec
D1 1.30000000 sec
d13 0.00003300 sec
D16 0.00010000 sec
d20 0.00110300 sec
IN0 0.00007800 sec

========== CHANNEL f1 ==========
NUC1 1H
P1 17.30 usec
p2 34.90 usec
PL1 -1.30 dB
SFO1 500.3032012 MHz

========== GRADIENT CHANNEL ==========
GPNAM1 SINE.100
GPNAM2 SINE.100
GPX1 45.00 %
GPX2 90.00 %
GPY1 45.00 %
GPY2 90.00 %
GPZ1 31.00 %
GPZ2 64.00 %
P16 1000.00 usec

F1 - Acquisition parameters
ND0 2
TD 1024
SFO1 500.3032 MHz
FIDRES 8.260016 Hz
SW 8.010 ppm

F2 - Processing parameters
SI 1548
SF 500.3000282 MHz
WDW QSINE
SSB 2
LB 0.00 Hz
GB 0
PC 1.40

F1 - Processing parameters
SI 2048
MC2 TPPI
SF 500.3032282 MHz
WDW QSINE
SSB 2
LB 0.00 Hz
GB 0

1D NMR plot parameters
CX2 22.50 cm
CX1 22.50 cm
F2PLO 7.970 ppm
F2LO 6378.11 Hz
F2PHI -0.040 ppm
F2HI -32.15 Hz
F1PLO 7.970 ppm
F1LO 6378.11 Hz
F1PHI -0.040 ppm
F1HI -32.15 Hz
F2PPMCM 0.35599 ppm cm
F2HZCM 284.91027 Hz cm
F1PPMCM 0.35599 ppm cm
F1HZCM 284.91027 Hz cm

Figure 3.30. Double Quantum Filtered Correlated Spectrum (DQFCOSY) of compound 5.

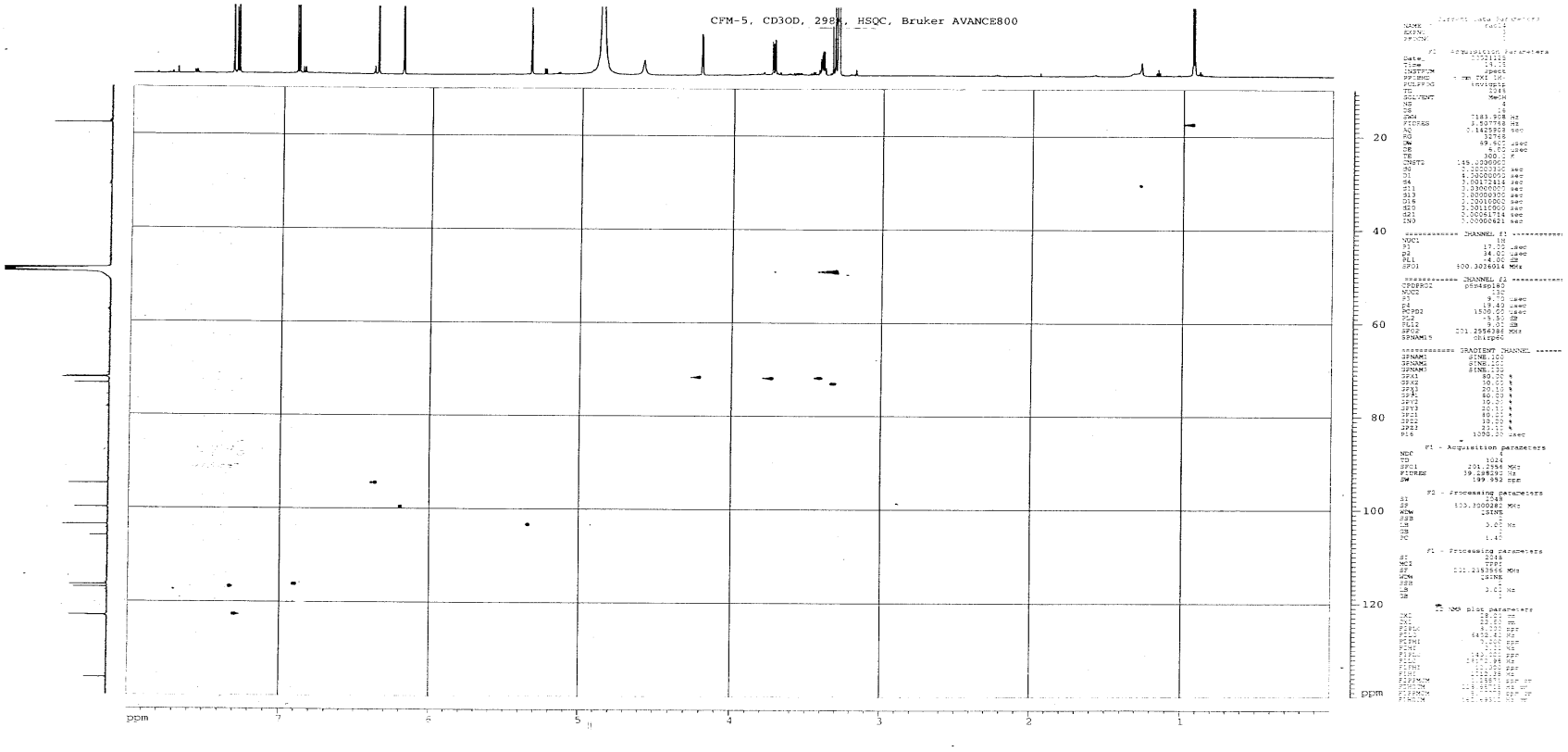


Figure 3. 31. 2D ^{1}H-^{13}C Heteronuclear Single-Quantum Correlation Spectrum (HSQC) of compound 5.

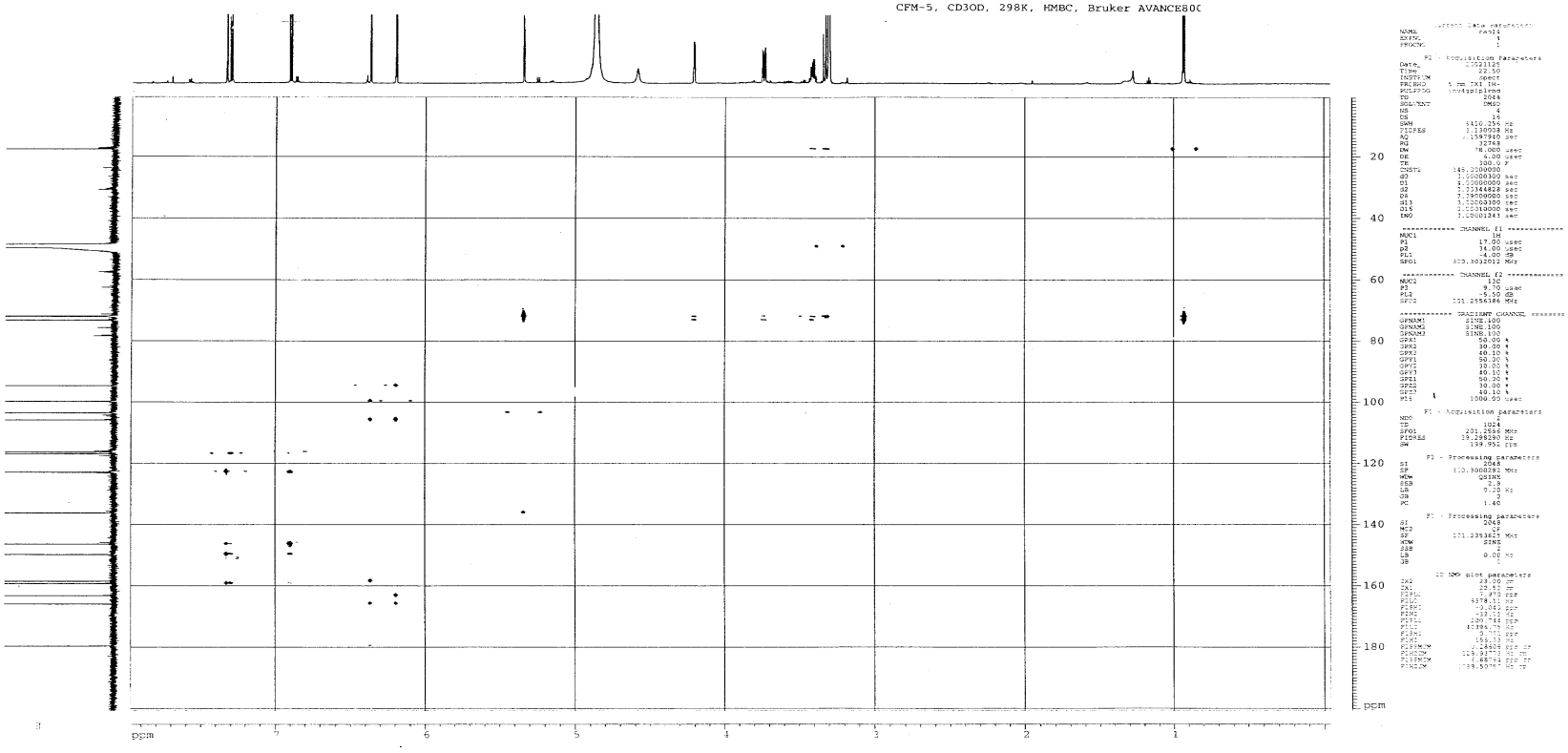


Figure 3. 32. 2D ^{1}H-^{13}C Heteronuclear Multiple-Bond Correlation Spectrum (HMBC) of compound 5.

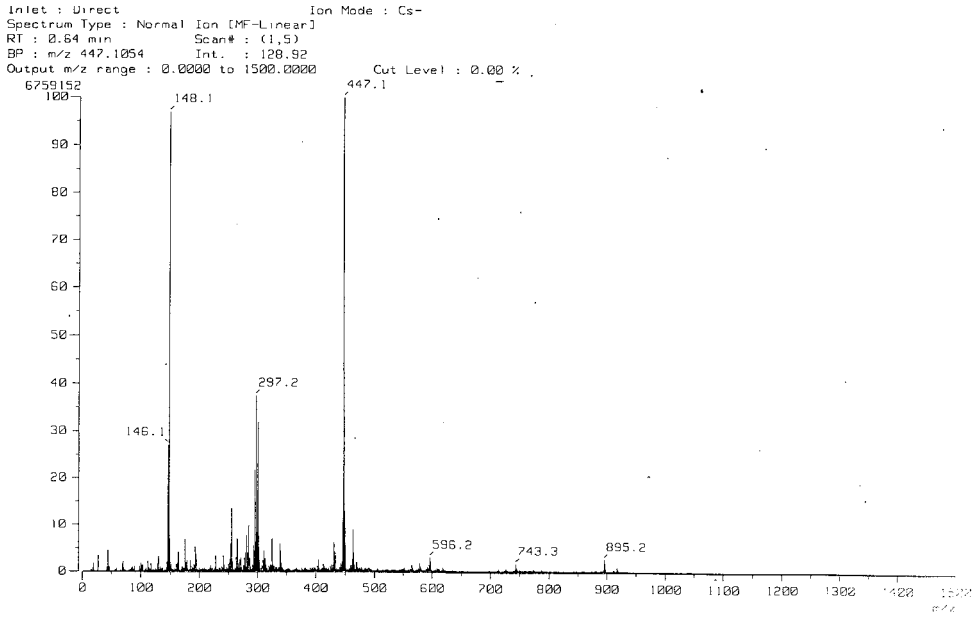


Figure 3. 33. Mass spectrum of compound 5 using negative ion mode.

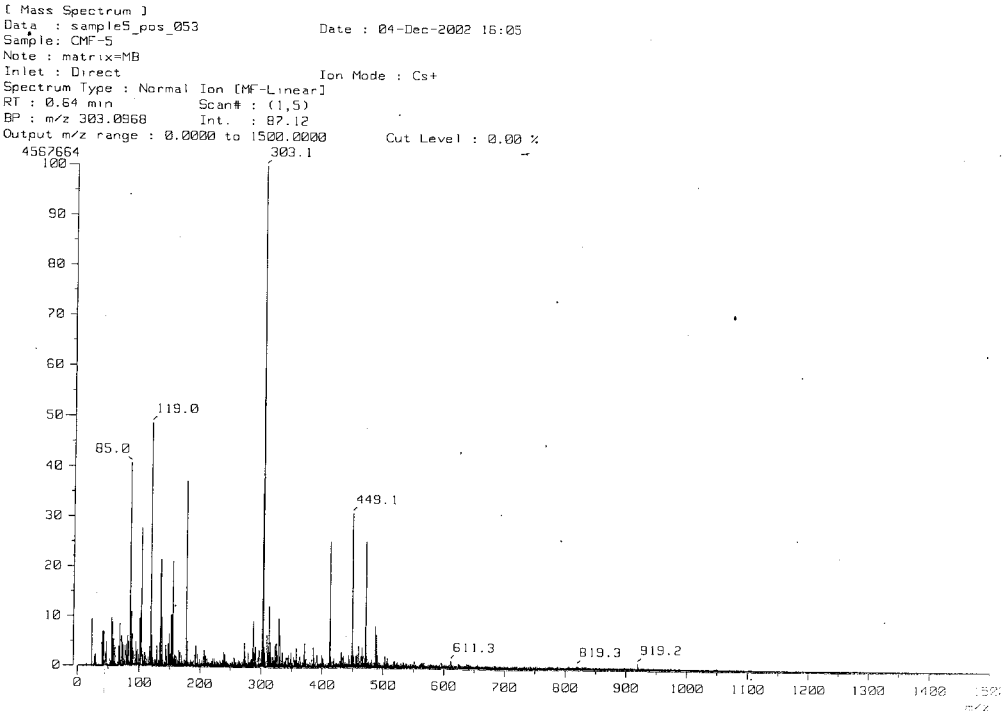
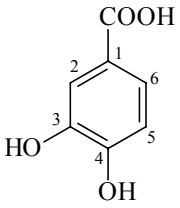
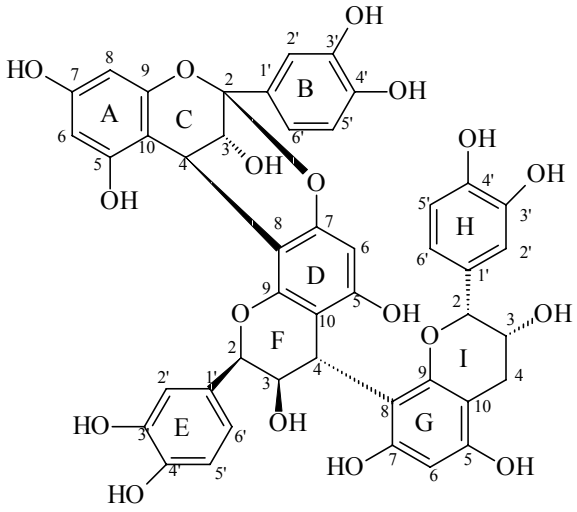


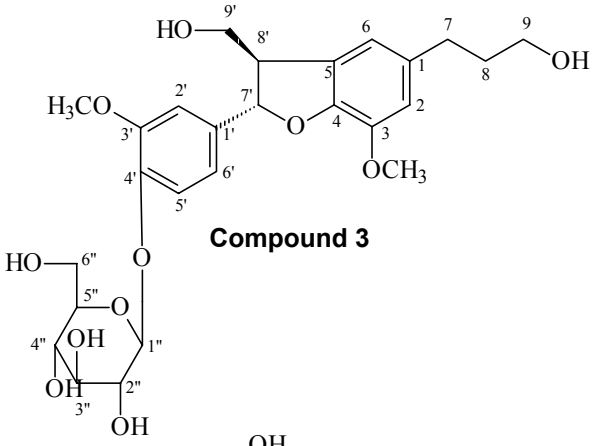
Figure 3. 34. Mass spectrum of compound 5 using positive ion mode.



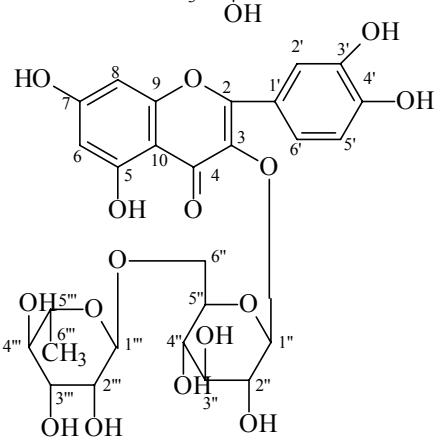
Compound 1



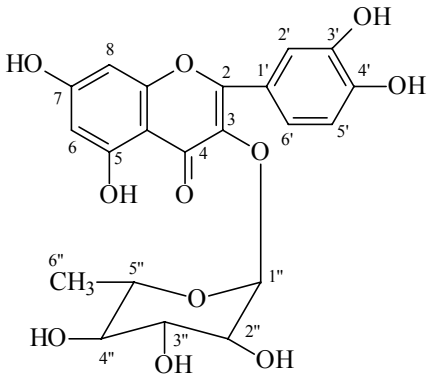
Compound 2



Compound 3



Compound 4



Compound 5

Figure 3.35. Structures of compounds 1-5 isolated from cinnamon fruits.

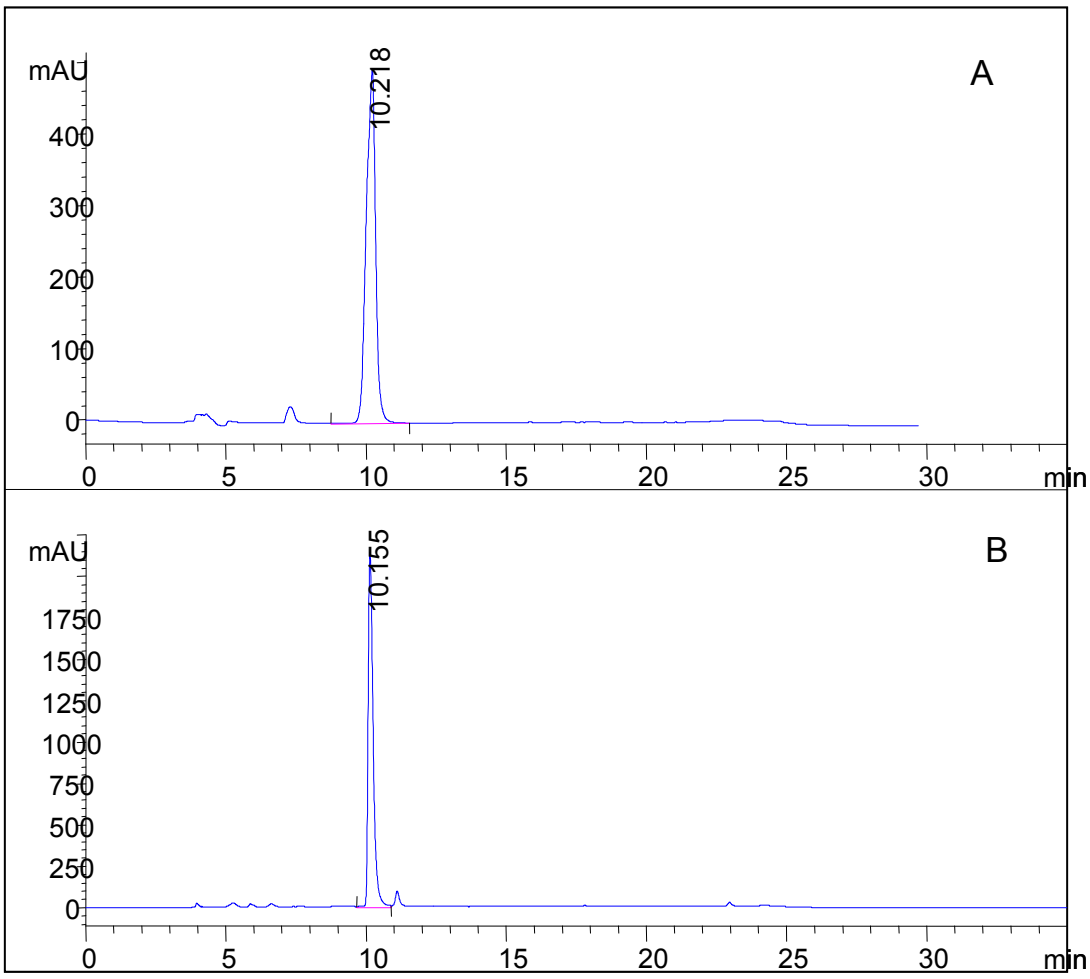


Figure 3.36. HPLC chromatograms of (A). Protocatechuic acid, (B). Compound 1.

3.2. STUDIES ON ANTIOXIDANT ACTIVITY

INTRODUCTION

Antioxidants stabilize polyunsaturated fatty acids in foods by reacting with free radicals, chelating metal ions and interrupting the propagation phase of lipid oxidation. The most widely used synthetic antioxidants are butylated hydroxyanisole (BHA), propyl gallate and tertiary butylhydroquinone (TBHQ) to prevent the oxidation of lipids in foods (Wanasundara and Shahidi, 1998). Even though natural and synthetic antioxidants function similarly, questions have been raised concerning the safety of some of the commercial antioxidants because model studies indicated mutagenesis and carcinogenesis associated with some synthetic antioxidants (Madhavi and Salunkhe, 1995).

Natural antioxidants such as tocopherols, ascorbic acid and flavonoids have gained the interest of consumers, scientists and medical and pharmaceutical industries because of their antitumour, anti-mutagenic and anti-carcinogenic activities. The antioxidant properties of certain plant phenols are well established (Aviram et al., 2000). Plant phenolics constitute one of the major classes of natural antioxidants. These phenolics occur in all parts of the plant including fruits, vegetables, nuts, seeds, leaves, flowers, roots and barks. Many leguminous plants such as soybeans, sesame seeds and rosemary and spices contain significant levels of flavonoids, whose antioxidant activities are comparable to those of many synthetic antioxidants. Therefore, extraction, characterization and utilization of natural antioxidants are of considerable interest (Pokorny, 1991; Aviram et al., 2000).

Phenolic acids (essentially hydroxybenzoic and hydroxycinnamic acid) are widely distributed in plants and can be present in considerable amounts in the human diet. The intake of hydroxybenzoic and hydroxycinnamic acid has been estimated to be around 11 mg/day and 211 mg/day, respectively and the intake of caffeic acid, a hydroxycinnamic acid derivative, has been estimated in the order of 206 mg/day in subjects drinking coffee (Radtke et al., 1998).

Flavonoids are low molecular weight polyphenolic compounds that are widely distributed in vegetables and fruits. Quercetin is the main aglycone found in foods. Many flavonoids have been shown to have antioxidant, antiinflammatory, antiallergic, anticancer, and antihemorragic properties. Recently, flavonoids have attracted increasing attention for their antioxidant properties, which may help to explain the protective effect of vegetable-rich diets on coronary heart disease (Rice Evans et al., 1996).

Proanthocyanidins (condensed tannins) consisting of oligomers and polymers of flavan-3-ol units are the most widely distributed type of tannins in the plant kingdom (Waterman and Mole, 1994). In addition to these widespread 3-hydroxy forms, proanthocyanidins comprising 3-deoxy subunits are also exists, rarely, which have been identified only in sorghum and maize (Stafford, 2000). Human beings consume significant quantities of proanthocyanidins in foods, such as fruits, vegetables, cereals, legumes and grains as well as in beverages, including tea, cocoa and red wine (Deshpande et al., 1986). In addition to the influence on the flavour and appearance of foods (Salunkhe et al., 1990), dietary proanthocyanidins are hypothesized to be beneficial, possibly due to their antioxidant properties and their ability to complex with

macromolecules and metal ions (Haslam, 1996). In nature, proanthocyanidins can influence the behaviour of plant feeding insects and mammals (Waterman and Mole, 1994; Nitao, et al., 2001). Many proanthocyanidins have been reported to possess antimicrobial activity (Bruyne et al., 1999; Jayaprakasha et al., 2003)

In the present study, the antioxidant and radical scavenging activities of various solvent extracts and purified compounds obtained from cinnamon fruits have been assayed using β-carotene-linoleate and DPPH model systems.

MATERIALS AND METHODS

Preparation of solvent extracts and purification

Cinnamon fruits were extracted according to the method described in Part A, Chapter 3.1, using hexane, EtOAc, acetone, MeOH and water. Protocatechuic acid, cinnamtannin B-1, urolignoside, rutin and quercetin-3-O-α-L-rhamnopyranoside were isolated as described in Part A, chapter 3.1, Page No. 89.

Samples Preparation

Ten milligrams of each sample *viz.,* ethyl acetate, acetone, MeOH, water extracts of cinnamon fruits, protocatechuic acid, cinnamtannin B-1, urolignoside, rutin and quercetin-3-O-α-L-rhamnopyranoside were dissolved in methanol and made up to 10 ml with methanol.

Antioxidant Assay using β-Carotene-linoleate Model System

The antioxidant activity of cinnamon fruit extracts and pure compounds were evaluated by the method of Jayaprakasha and Jaganmohan Rao (2000). β-Carotene (0.4 mg) in 0.4 ml of chloroform, 40 mg of linoleic acid and 400 mg of tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed in a 200 ml round bottom flask. Chloroform was removed at 40°C *under vacuum* and the resulting mixture was diluted with 10 ml of distilled water and mixed well for 2 min. To this emulsion, 90 ml of oxygenated water was added and mixed for 1 min. Four millilitres aliquots of the emulsion were pipetted into different test tubes containing 0.2 ml of cinnamon extracts (equivalent to 100 and 200 ppm), pure compounds (equivalent to 50 and 100 ppm) and BHA (equivalent to 50, 100 and 200 ppm) in methanol. BHA was used for comparison purposes. A control containing 0.2 ml of methanol and 4 ml of the above emulsion was prepared. The tubes were placed at 50 °C in a water bath and the optical density at 470 nm was measured at zero time (t = 0). The measurement of optical density was recorded at an intervals of 30 min and untill the colour of β-carotene disappeared in the control tubes (t = 180 min). A mixture prepared as above without β-carotene served as the blank. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula, $AA = 100[1-(A_o-A_t)/(A^\circ_o - A^\circ_t)]$ where A_o and A°_o are the optical density values measured at zero time of the incubation for the test sample and the control, respectively. A_t and A°_t are the optical density measured in the test sample and the control respectively, after incubation for 180 min. All determinations were carried out in triplicate and averaged.

Radical Scavenging Activity using DPPH Method

Different concentrations of cinnamon fruit extracts (25 and 50 ppm), pure compounds (12.5 and 25 ppm) and BHA (12.5, 25 and 50 ppm) were taken in different test tubes. The sample volume was adjusted to 0.1 ml by adding MeOH. Five millilitre aliquots of 0.1 mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27 °C for 20 min (Blois, 1958). The control was prepared as above without any sample or BHA. Methanol was used for the baseline correction. The changes in the optical density (OD) of the samples were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and calculated using the following formula, % Radical scavenging activity = (Control OD – sample OD/Control OD) $\times$ 100.

RESULTS AND DISCUSSION

Dried cinnamon fruits extracts, pure compounds and BHA were dissolved in methanol and used for antioxidant activity. The antioxidant activity of cinnamon fruit extracts and BHA at different concentrations was determined using the bleaching of β-carotene-linoleate model system and the results are presented in Figure 3.37. It was observed that, all the extracts prepared by different solvents exhibited varying degree of antioxidant activity. The EtOAc, acetone, MeOH, water extracts and BHA exhibited 53.5, 71.5, 80.5, 83 and 89% antioxidant activity respectively at 200 ppm, concentration. Water extract was found to possess the maximum antioxidant activity.

Figure 3.38 shows the antioxidant activity of protocatechuic acid, cinnamtannin B-1, urolignoside, rutin, quercetin-3-O-α-L-rhamnopyranoside and BHA at 50 and 100 ppm concentration. Protocatechuic acid showed highest antioxidant activity and urolignoside showed lowest antioxidant activity at 50 and 100 ppm concentrations.

The mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules (Jayaprakasha et al., 2001). As β-carotene molecules loses their double bonds by oxidation, the compound loses its chromophore and characteristic orange colour, which was monitored spectrophotometrically. In the present study, it was observed that, the presence of different extracts of cinnamon fruits / purified compounds could hinder the extent of β-carotene bleaching by neutralising the linoleate free radical and other free radicals formed in the system. The order of antioxidant activity was found to be urolignoside < cinnamtannin B-1 < rutin < quercetin-3-O-α-L-rhamnopyranoside < protocatechuic acid.

Free radical scavenging potentials of cinnamon fruit extracts, purified compounds and BHA at different concentrations were tested by DPPH method and the results are depicted in Figures 3.39 and 3.40. Antioxidants reacts with DPPH, which is a stable free radical and converted to 1, 1-diphenyl-2-picryl hydrazine. The degree of discoloration indicates the scavenging potential of the samples. At 50 ppm, EtOAc, acetone, MeOH and water extracts of cinnamon fruits and BHA exhibited 56, 66, 76, 94 and 96% free radical scavenging activity, respectively. Similarly, protocatechuic acid, cinnamtannin B-1, urolignoside, rutin, quercetin-3-O-α-L-rhamnopyranoside and BHA showed 77.3, 39.3, 30.4, 44.7, 60.3 and 85% free radical scavenging activity respectively, at 12.5 ppm concentration.

It appears that, the radical scavenging activity of the samples (cinnamon extracts or purified compounds) is attributed to their hydrogen donating ability as suggested by Shimada et al. (1992). It is well known that free radicals cause autooxidation of unsaturated lipids in food (Sherwin, 1978). On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable end product, which does not initiate or propagate further oxidation of lipid (Kubow, 1990). The data obtained revealed that the extracts are free radical inhibitors and primary antioxidants that react with free radicals. The degree of antioxidant activity and free radical scavenging activities of the extracts are attributed to their extent of phenolics. The water extract contains maximum phenolics and it showed highest degree of antioxidant and free radical scavenging activities and the same parameter was found to be least with ethyl acetate extract.

Ueda et al. (1996) reported that protocatechuic acid is a strong antioxidative effect 10-fold higher than that of α-tocopherol. In the present study also, protocatechuic acid showed highest antioxidant activity as compared to other tested compounds. It has been observed that, ruitn showed less antioxidant activity as compared to quercetin-3-O-α-L-rhamnopyranoside. In flavonoids, glycosylation of the hydroxyl group at C-3 does not seem to change antioxidant activity notably, as reported by Teissedre et al. (1996). In the present study, the glycosylation of the 3-hydroxyl group in rutin and quercetin-3-O-α-L-rhamnopyranoside showed good antioxidant activity. The low antioxidant activity of rutin as compared to quercetin-3-O-α-L-rhamnopyranoside may be due to the steric hindrance created by the disaccharide moiety. Our results are similar to those of previous studies (Rice-Evans et al., 1996; Woodill and Prior, 2001)

confirming that quercetin and its monoglycosides have the greater antioxidant activity than rutin.

CONCLUSION

The results of the present study indicate the presence of compounds possessing promising antioxidant activity in fruits of *C. zeylanicum*. The different activities of the cinnamon extracts can be ascribed to their difference in their phenolic composition. Water extract showed maximum (83%) antioxidant activity at 200 ppm using β-carotene-linoleate model system. Besides, all the isolated compounds showed good antioxidant activity in β-carotene-linoleate and DPPH model systems. Protocatechuic acid and urolignoside showed maximum (77.3%) and minimum activity (30.4%) respectively at 12.5 ppm using DPPH method. Further, work is required to study the antioxidant activity of above extracts and pure compounds *in vivo* systems. Thus, there will be value addition to the unconventional parts of cinnamon, which has no commercial application at present.

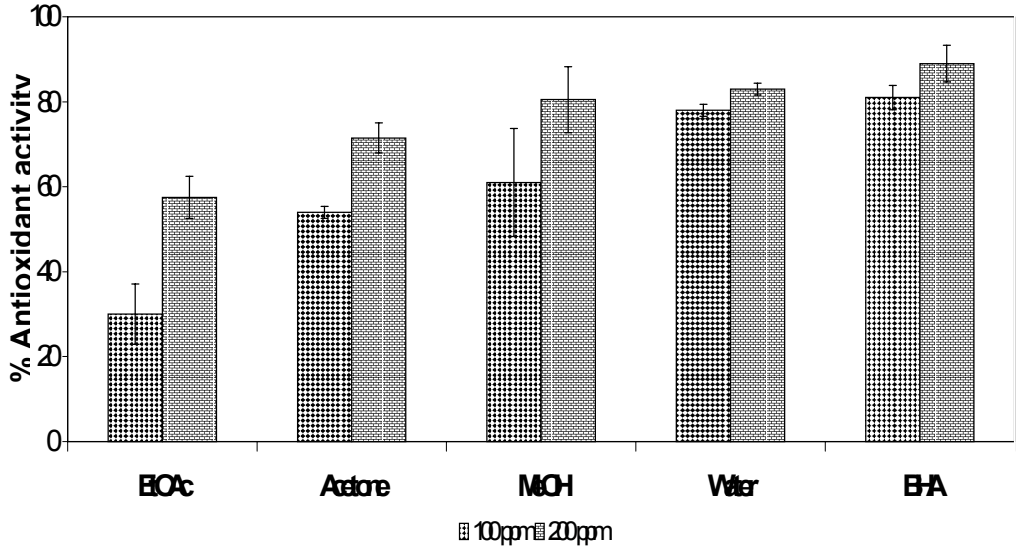


Figure 3.37. Antioxidant activity of cinnamon fruit extracts using β-carotene-linoleate model system.

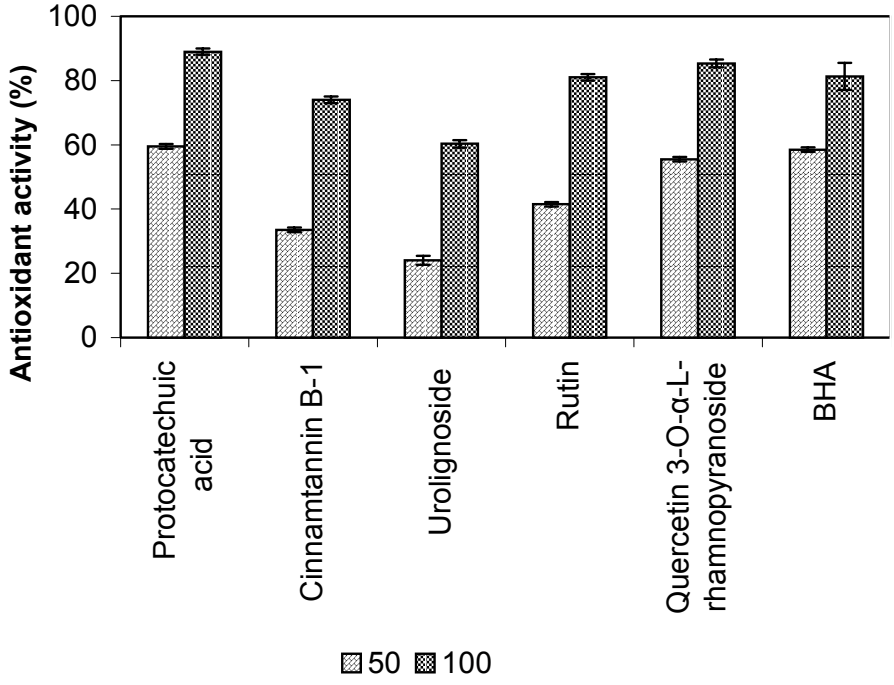


Figure 3.38. Antioxidant activity of purified compounds from cinnamon fruits using β-carotene-lineoleate model system.

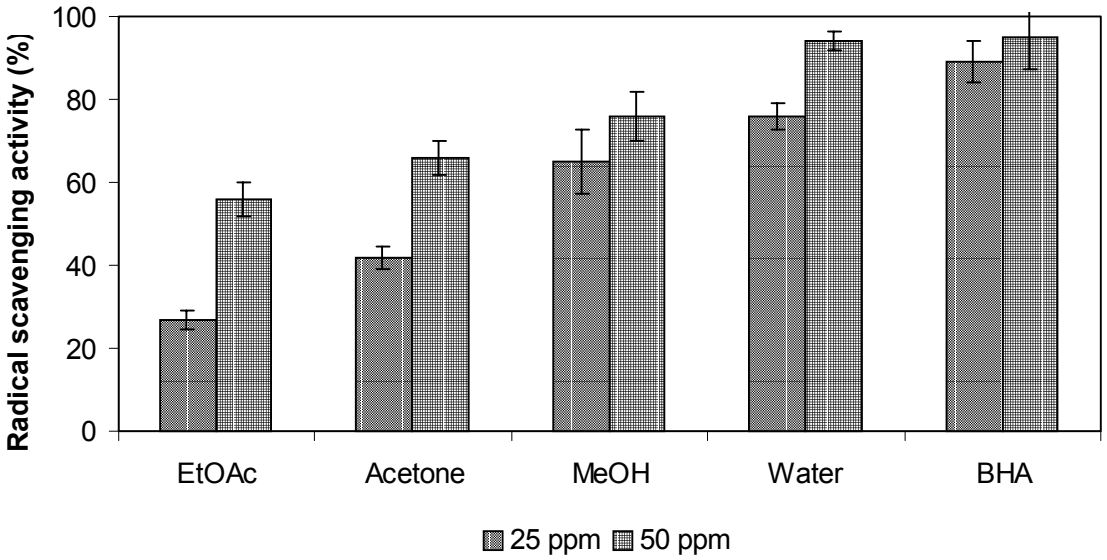


Figure 3.39. Radical scavenging activity of cinnamon fruits extracts using DPPH method at different concentrations.

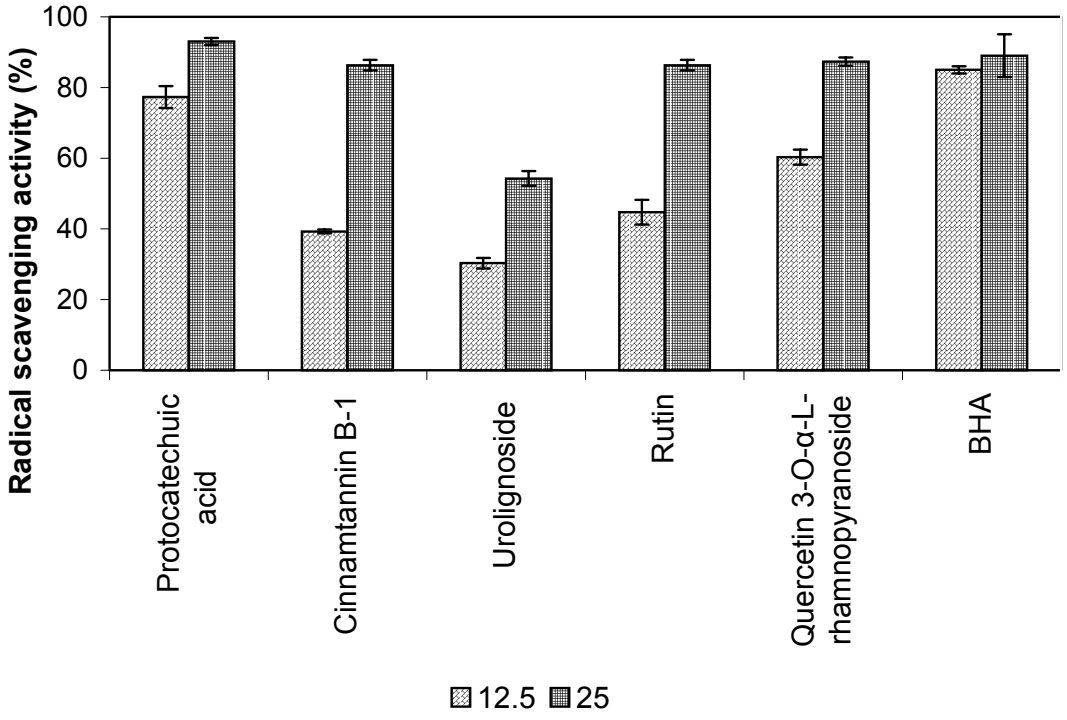


Figure 3.40. Radical scavenging activity of purified compounds from cinnamon fruits using DPPH method at different concentrations.

3.3. STUDIES ON ANTIBACTERIAL ACTIVITY

INTRODUCTION

Volatile oils from aromatic and medicinal plants have been known since antiquity to possess biological activity, notably antibacterial, antifungal and antioxidant properties (Deans and Waterman, 1993). With the growing interest in the use of volatile oils in both the food and the pharmaceutical industries, a systematic examination of plant extracts for these properties has become increasingly important. The use of natural antimicrobial compounds is important not only in the preservation of food, but also in the control of human and plant diseases of microbial origin. Bacterial and fungal infections pose a greater threat to health, most notably in immunocompromised subjects, hence the need to find cheap and effective antimicrobial agents (Baratta et al., 1998). Cinnamon is rich source for essential oils and tannins, which inhibit microbial growth (Bullerman et al., 1977; Chang, 1995).

Although India occupies a foremost position in terms of production of spices and spice products, the standing in the global export market has not been consistent. This has been due to the quality attributes, especially that of microbiological profile. Spices and spice products of Indian origin have shown unacceptable levels of pathogenic bacterial species like *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Staphylococcus aureus,* and others. The occurrence of these pathogens at times is largely due to the practices of handling and the prevailing environmental factors. In this background, an attempt has been made in the present investigation to assess the

antibacterial potential of extracts from cinnamon fruits against those pathogenic and spoilage bacterial species, which tend to occur in the food commodities.

MATERIALS AND METHODS

Extraction

Cinnamon fruit were extracted according to the method described in Part A, Chapter 3.1, Page No 89 using hexane, benzene, ethyl acetate, methanol and water.

Samples Preparation

Hexane, benzene, ethyl acetate, methanol and water extracts (250 mg each) were dissolved in propylene glycol and transferred to 10 ml volumetric flasks separately and made up to the mark.

Preparation of Agar Medium

Nutrient agar (13 g) (HiMedia, Mumbai) was dissolved in water and made up to 1000 ml distilled water and 20 ml aliquots of agar medium were transferred into 100 ml conical flasks and sterilized at 121 °C for 20 min in a autoclave at 15 psi.

Microorganisms and Culture Media

Strains of *Bacillus cereus, B. coagulans*, *B.subtilis, Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from the stock culture collection of Food Microbiology Department, CFTRI, Mysore. The bacterial cultures were maintained at 4 °C on nutrient agar slants and sub-cultured at 15 days intervals. Prior to use, the cultures were grown in nutrient broth at 37 °C for 24 h. A pre-culture was prepared by transferring 1 ml of this culture to 9 ml nutrient broth and cultivated

for 48 h at 37 °C. The cells were harvested by centrifugation at 4000 rpm for 5 min, washed and suspended in saline.

Antibacterial Activity

The cinnamon extracts were tested against different microorganisms as per the method of Chen et al. (1998) with slight modification. To flasks containing 20 ml of melted nutrient agar, different concentrations (250, 500 and 1000 ppm) of test material were added. In case of control, equivalent amount of propylene glycol was added. One hundred μl (about 10^3 cfu/ml) of each bacterium to be tested was inoculated into the flasks under aseptic conditions. The contents were mixed thoroughly and media was then poured into sterilized petri plates in quadruplet and incubated at 37 °C for 20-24 h. The colonies developed after incubation were counted and expressed as colony forming units per ml of culture (cfu/ml). The inhibitory effect was calculated using the following formula: % Inhibition = (1 - T/C) x 100, where T is cfu/ml of test sample and C is cfu/ml of control.

The minimum inhibitory concentration (MIC) was reported as the lowest concentration of the compound capable of inhibiting the complete growth of the bacterium being tested (Naganawa et al., 1996).

RESULTS AND DISCUSSION

The hexane benzene, ethyl acetate, methanol and water extracts of cinnamon fruits tested against a few gram positive and gram negative bacteria and the results are depicted in Figure 3.41 and Table 3.7. Hexane extract was most effective against all the bacteria tested, except *E. coli,* whereas the inhibition occurred at 1500 ppm

(Table 3.7). *B. cereus* and *P. aeruginosa* were inhibited completely at 250 and 200 ppm, respectively. *B. coagulans, S. aureus* and *B. subtilis* were inhibited at 300, 500 and 300 ppm respectively. Ethyl acetate extract was also effective against all the bacteria tested, but its activity was slightly lower than hexane extract. Although both the extracts showed similar pattern of inhibition at 250 and 500 ppm concentrations, inhibition of growth of *B. subtilis* and *P. aeruginosa* was higher than hexane extract. Methanol and water extracts were least effective against all the bacteria tested. Growth of none of the organisms was inhibited completely by these extracts even at $\leq$ 500 ppm except for *B. coagulans* by methanol extract. Benzene extract showed an intermediate activity and it inhibited complete growth of *B. cereus, B. subtilis, B. coagulans* and *P. aeruginosa* at 500 ppm. *E. coli* was most resistant of all the bacteria tested and even at 1000 ppm its growth was not completely inhibited by any of the extract and at this concentration the maximum inhibition was shown by ethyl acetate extract (72.5%) (Figure 3.41).

Table 3.7. Minimum inhibitory concentrations (MIC) of *C. zeylanicum* fruit extracts (ppm).

Microorganisms	**Hexane extract**	**Benzene extract**	**Ethyl acetate extract**	**Methanol extract**	**Water extract**
Bacillus cereus	250	300	250	700	750
B. subtilis	300	500	400	800	1000
B. coagulans	300	400	300	500	1000
Staphylococcus aureus	500	600	500	750	800
Pseudomonas aeruginosa	200	400	250	700	750
Escherichia coli	1500	1500	1400	1500	1500

These observations were confirmed by MIC values (Table 3.7). The MIC values of hexane and ethyl acetate extracts showed highest antibacterial activity. The variable activity observed among different extracts may be due to the differences in active compounds extracted by different solvents. Earlier, Negi and Jayaprakasha (2001) reported variable activity of compounds extracted by different solvents from grapefruit peels. The cinnamon leaf essential oil is reported to have antimicrobial, fungitoxic (Saksena and Saksena, 1984), and nematicidal activity (Tiwari et al., 1994). Recently, the insecticidal constituent of the cinnamon bark volatile oil has been identified as *trans*-cinnamaldehyde. Strong insecticidal activity was also obtained in the commercial eugenol and salicylaldehyde, which are constituents of the cinnamon bark (Mallavarapu et al., 1995). The antibacterial activity of various extracts may be due to variations in their content of active components.

In general, Gram-negative bacteria are reported to be more resistance to external agents as their outer membrane acts as permeability barrier due to the presence of lipopolysaccharides which makes them inherently resistant to antibiotics, detergent and hydrophilic dyes (Nikaido and Vaara, 1985). The reason for higher sensitivity of the Gram-positive bacteria than negative bacteria is due to the differences in cell wall compositions, as the Gram-positive bacteria contain an outer peptidoglycon layer, which is an ineffective permeability barrier (Scherrer and Gerhardt 1971). Although, *E. coli*, a Gram negative bacterium showed similar observation in this study, the other Gram negative bacterium *P. aeruginosa* did not exactly gave similar results when compared to Gram positive bacteria.

CONCLUSION

The results shown above indicate that the extraction of cinnamon fruits with hexane and ethyl acetate yields conserves with high antibacterial activity. The antibacterial properties of cinnamon fruits can add the value to this spice plant, as at present except fruits all parts of this plant are being utilized. This is the first report on the isolation of antibacterial fractions from cinnamon fruits. Further, studies are required to study the antimicrobial activity of cinnamon fruits extracts in food model systems.

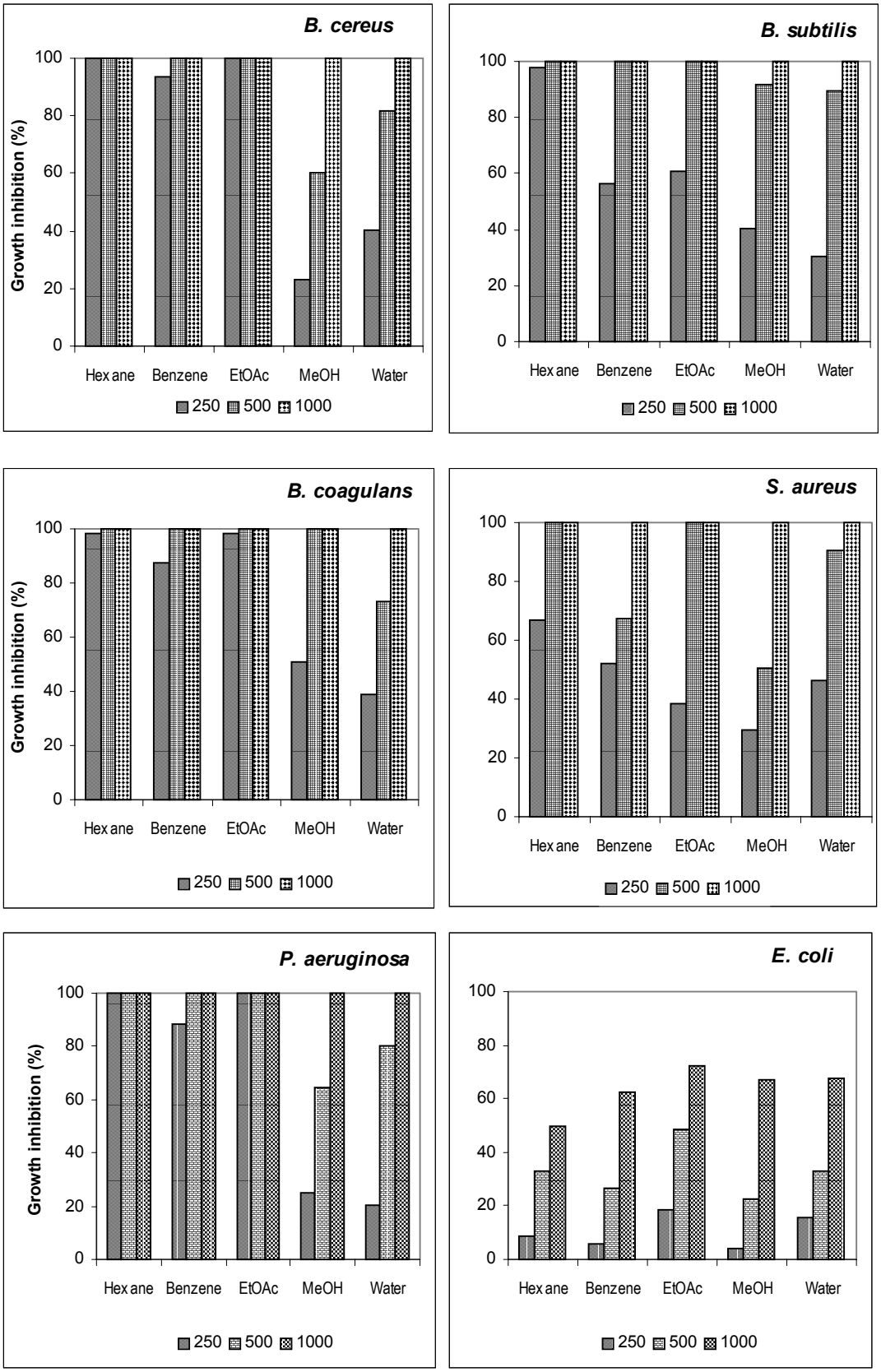


Figure 3.41. Effect of cinnamon fruits different extracts on bacterial growth at different concentrations

3.4. STUDIES ON ANTIMUTAGENIC ACTIVITY

INTRODUCTION

Recently, it has been realized that a large number of naturally occurring compounds have potent anticarcinogenic and antimutagenic activities against environmental carcinogens and mutagens. Mutagenic and carcinogenic agents are omnipresent in the human environment and it seems impossible to eliminate all of them. Moreover, several well-known mutagenic risk factors are closely connected with the modern life style and their entire eradication appears to be very burdensome and even unsustainable. Extensive studies have shown that, dietary habits are important in deciding human health. The human diet contains a great variety of natural antimutagens / anticarcinogens such as fibres, polyphenolic compounds, flavonoids, isoflavones, tocopherols, ascorbic acid etc (Ames, 1983; Stavric, 1994). Therefore, by the regular intake of antimutagenic agents can reduce genotoxic effects of mutagenic and carcinogenic factors (Ikken et al., 1998; 1999).

Many mutagens and carcinogens may act through the generation of reactive oxygen species (ROS). The generation of ROS is associated with environmental pollution, UV radiation and several normal metabolic processes. The role of ROS in various human diseases is increasingly recognised (Halliwell et al., 1992; Ames et al., 1993; Martinez-Cayuela, 1995). ROS may also play a major role as endogenous initiators of degenerative processes, such as DNA damage and mutation (and promotion), that may be related to cancer, heart disease and aging (Ames, 1983). Besides the endogenous defences, the consumption of dietary antioxidants like tocopherol, ascorbic acid, carotenoids, phenolic compounds etc. play vital role in

protecting against ROS (Willet, 1994). Dietary intake of natural antioxidants could be an important aspect of the body's defence mechanism against these agents and also many antioxidants are being identified as anticarcinogens (Ames, 1983). Low dietary intake of fruits and vegetables doubles the risk of most types of cancer as compared to high intake (Ames et al., 1993).

In the earlier chapter 3.2, it was found that the various extracts of cinnamon fruits possess various degree of antioxidant activities. In the present study, aimed to explore whether the antioxidant fractions of the cinnamon fruits are probable antimutagens assayed through Ames test.

MATERIALS AND METHODS

Materials

Sodium ammonium phosphate ($NaHNH_4\ PO_4.\ 4H_2O$) was obtained from Acros Organics, Belgium. Remaining chemicals were obtained from HiMedia, Mumbai.

Preparation of cinnamon extracts

Solvent extracts were prepared as according to the method described in Part A, Chapter 3.1, Page No. 89.

Vogel-Bonner medium E Preparation

Ten grams of Magnesium sulphate ($MgSO_4.\ 7H_2O$), 100 g of citric acid monohydrate, 500 g of potassium phosphate (K_2HPO_4) and 175 g of sodium ammonium phosphate ($NaHNH_4\ PO_4.\ 4H_2O$) were dissolved separately in minimum

quantity of distilled water at 80 °C, mixed and made up to 1 litre with water. The medium was sterilized at 121 °C for 20 min.

Agar (Minimal Agar) Plate Preparation

Antimutagenic activity agar plates were prepared by dissolving Bacto-difco agar (1.5%) and glucose (2%) in Vogel-Bonner medium E. The medium was sterilized and 18 ml media was distributed into sterilized petriplates under aseptic conditions.

Top Agar Preparation

Six grams of agar and 5 g of sodium chloride were dissolved in water. Then add 50 ml of 0.5 mM D-biotin and 50 ml of 0.5 mM L-histidine were added and made up to 1 litre with water. The medium was sterilized at 121 °C for 20 min.

Antimutagenicity assay

The standard plate incorporation test was carried out according to Maron and Ames (1983). In the antimutagenicity test, the inhibitions of mutagenic activity of the sodium azide by the test samples were determined. Two millilitres of top agar was distributed into 13 × 100 mm capped culture tubes held at 45 °C in a heating block. Different concentrations (25, 50, 100 and 200 ppm) of test samples in 0.1 ml of propylene glycol, 0.1 ml of 10 h old culture of *Salmonella typhimurium* TA-100 was added. The bacteria was left at 45 °C for a few min without loss of viability and mixed by vortexing at low speed and then poured onto a minimal glucose agar plate quickly the plate was tilted to form a thin layer. This step is important to achieve a uniform distribution of the top plate. Positive and negative controls were also included in each assay. Sodium azide was used as a diagnostic mutagen (1.5 μg / plate) in positive

control plates. Negative controls were prepared with equivalent amount of propylene glycol instead of sodium azide and test samples, which is required to establish the number of colonies that arise spontaneously for *S. typhimurium* TA-100. The number of histidine^{+} (His^{+}.) revertants colonies were counted after incubation of the plates at 37 °C for 48 h. Each sample was assayed using duplicate plates and the data presented as mean ± SD of three independent assays. The mutagenicity of sodium azide in the absence of test samples was defined as 100% or 0% inhibition. The calculation of % inhibition was done according to the formula given by Ong et al (1986), % inhibition = [1-T/M] x 100 where T is number of revertants per plate in presence of mutagen (sodium azide) and test samples and M is number of revertants per plate in positive control (sodium azide). The number of spontaneous revertants was subtracted from numerator and denominator.

The antimutagenic effect was considered moderate, when the inhibitory effect was 25-40% and strong when more than 40%. Inhibitory effects of less than 25% were considered as weak and was not recognised as positive result (Ikken et al., 1999).

RESULTS AND DISCUSSION

Ames test has been widely used to assess the antimutagenic and anticarcinogenic activity of various compounds (Ikken et al., 1999). The antimutagenic activity of cinnamon extracts against sodium azide was evaluated by means of the Ames test using *S. typhimurium* TA-100 strain. All the cinnamon extracts inhibited the mutagenicity of sodium azide in *Salmonella* strain, which ranged from weak to strong inhibition depending upon the concentration of extract per plate (Figure 3.42).

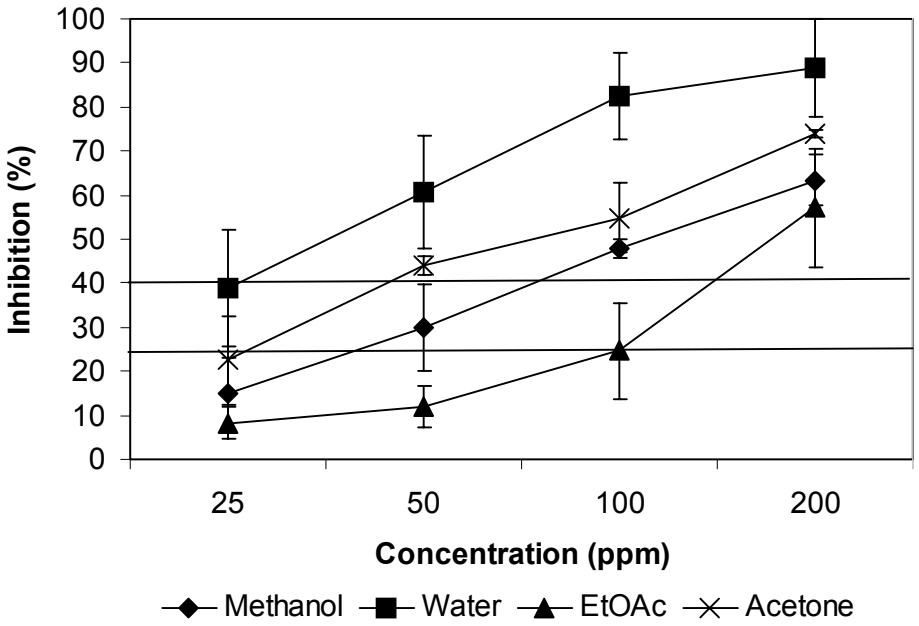


Figure 3.42. Inhibitory effect of cinnamon fruit extracts against the mutagenicity of sodium azide to *Salmonella typhimurium* TA-100.

Strong inhibitory effect was shown by all extracts at 200 ppm concentration. Water extract showed highest inhibition, whereas EtOAc extract showed least inhibition and it showed weak inhibitory effect at all the tested concentration. The antimutagenicity of water extract was followed by extracts with acetone, methanol and ethyl acetate. It has been observed that many plant polyphenols such as ellagic acid, catechins and

chlorogenic, caffeic and ferulic acids act as potent antimutagenic and anticarcinogenic agents (Ayrton et al., 1992; Bu-Abbas et al., 1994). Nasr et al., (1996) have reported that pomegranate peel contains catechin and procyanidins. The presence of similar type of polyphenols (Chapter 3.1) in the cinnamon fruits may be responsible for antimutagenicity of extracts.

Edenbarder and Tang (1997) have reported that the antimutagenic activity of flavonoids is due to the presence of keto group at C-4, whereas the low antimutagenic activity of flavanols and anthocyanidins is due to the lack of keto group at C-4 position. In the flavone, flavanols and flavonol series antimutagenic potencies were collected especially with the presence of polar hydroxyl groups. Besides, antimutagenic activity of glycosides was weaker than those of the corresponding a glycones. The double bond between C-2 and C-3 present in flavones but absent in flavanones was also of importance for antimutagenic activity. In the present study, the antimutagenicity of cinnamon extracts was examined using *Salmonella typhimurium* TA-100 treated with sodium azide. In terms of efficacy these compounds could be ranked in the following increasing order: water extract > acetone extract > methanol extract > EtOAc extract (Figure 3.42). The high activity of water extract may be due to the presence of rutin and quercetin-3-O-α-L-rhamnopyranoside along with other phenolic compounds *viz.,* protocatechuic acid, cinnamatannin-B1, urolignoside**,** (Part A, Chapter 3.1) and this confirms the findings of previous reports (Okuda et al., 1984; Edenharder et al., 1993).

In the earlier chapter 3.2, it was found that the antioxidant activities of cinnamon extracts were in the order of water > methanol > acetone and ethyl acetate. However

in the present study showed that the antimutagenic activity of cinnamon extracts was found to be in the order of water > acetone > methanol > ethyl acetate. Thus, in the present study water and ethyl acetate extracts could find the direct correlation between antioxidant and antimutagenic activities, while acetone and MeOH extracts could not find the direct correlations, even though the antioxidants are probable antimutagens.

A similar trend has been observed in earlier studies (Ikken et al., 1998; 1999) and this is a common situation when complex mixtures are tested. The effect of the modifying chemical can be either enhancing or inhibiting depending on its mechanism of action (Wallum et al., 1990). These results suggests that either a great variety of antimutagenic compounds of different structures exists in spices or that groups of similar compounds with virtually universal distribution in the plant kingdom may be responsible for the direct interaction between the genotoxic reactive intermediates derived from N-nitrosoamines and the antimutagenic compounds.

Active oxygen and free radicals are related to various physiological and pathological events such as inflammation, immunization, aging, mutagenicity and carcinogenicity (Namiki, 1990). Kim et al., (1991) and Ueno et al., (1991) indicated that active oxygen scavengers reduce mutation induced by various mutagens. It has been suggested that compounds, which possess antioxidant activity can inhibit mutation and cancer because they can scavenge a free radical or induce antioxidant enzymes (Hochstein and Atallah, 1988).

CONCLUSION

The present study showed the inhibitory effect of extract of cinnamon fruits extracts against the mutagenicity of sodium azide using the Ames test. Water extract of was found to be most active (≈ 82%). Further, studies are required to study the antimutagenic activity *invivo* experiments.

Summary and conclusions

The increasing consumers preference and health concerns associated with the use of synthetic additives has resulted in an increased demand for natural additives. The agricultural and industrial residues offer untapped source of natural additives. A number of by-products has been previously studied as potential source of food additives. Literature survey revealed that, there are no reports on chemical composition of unconventional parts of cinnamon (a farm by-product) and curcumin removed turmeric oleoresin (industrial by-product i.e. from curcumin production). As a means of easy understanding, the highlights of the present study are presented chapter wise:

Chapter 1. In this chapter, a brief introduction is presented on isolation and identification of natural products. Isolation procedures of volatiles and non-volatiles by different methods such as Conventional techniques, Discontinuous techniques, Continuous techniques, Conventional hybrid (continuous–discontinuous) techniques, Microwave-assisted extraction together with both Supercritical fluid extraction and Continuous subcritical water extraction have been described. Besides, separation methods for individual components and their structure determination techniques using UV, IR, GC, GC-MS, GC-FT-IR, ^{1}H and ^{13}C NMR (one and two dimension) and mass spectral methods were also discussed.

Chapter 2. The literature on chemical composition and bioactivity of *Cinnamomum zeylanicum* (*C. zeylanicum*) and scope of further research and the protocol of the work undertaken have been presented. This chapter has been presented under seven sub-chapters *viz.,* 2.1, 2.2, 2.3, 2.4, 2.5, 2.6 and 2.7 wherein the detailed study on isolation and identification of volatiles from the *C. zeylanicum* buds, flowers, fruits, fruit stalks, biogenesis and comparison of volatile oils, and isolation of *trans*-cinnamyl acetate have been presented.

2.1. The hydro-distilled volatile oil of the *C. zeylanicum* buds was analysed using GC and GC-MS for the first time. Thirty-four compounds representing ≈ 98% of the oil were characterized, consisting of terpene hydrocarbons (78.0%) and oxygenated terpenoids (9.0%). α-Bergamotene (27.4%) and α-copaene (23.1%) were found to be the major compounds.

2.2. The hydro-distilled volatile oil of cinnamon flowers was analysed by GC and GC-MS for the first time. It consists of 23% hydrocarbons and 74% oxygenated compounds. A total of 26 compounds constituting ≈ 97% of the oil were characterized. (*E*)-Cinnamyl acetate (41.9%), *trans*-α-bergamotene (7.9%), and caryophyllene oxide (7.2%) were found to be major compounds.

2.3. The hydro-distilled volatile oil of the *C. zeylanicum* fruits grown in Karnataka and Kerala (South India) were analysed using GC and GC-MS. It consists of hydrocarbons (32.8% and 20.8%) and oxygenated compounds (63.7% and 73.4%). Thirty-four compounds representing more than 94% of the oil were identified. *trans*-Cinnamyl acetate and β-caryophyllene were found to be the major compounds in cinnamon fruit grown in both Karnataka and Kerala. This was the first report on the chemical composition of the volatile oil of fruit *C. zeylanicum.*

2.4. The hydro-distilled volatile oil from cinnamon fruit stalks was analysed using GC and GC-MS. It consists of 44.7% hydrocarbons and 52.6% oxygenated compounds. Twenty-seven compounds constituting ≈ 95.98% of the volatile oil were characterized. *(E)*-Cinnamyl acetate (36.59%) and caryophyllene (22.36%) were found to be major compounds.

2.5. The general pathways of biogenesis leading to the volatile aromatic compounds with reference to mono and sesquiterpenoids and their oxygenated compounds and other selected components present in the cinnamon volatile oils have also been discussed.

2.6. The comparison of the chemical composition of the volatile oils obtained from cinnamon buds, flowers, fruits and fruit stalks have been described. It was found that seven volatile compounds were common in all the four parts of *C. zeylanicum* volatile oils.

2.7. The isolation and identification of *trans*-cinnamyl acetate from *C. zeylanicum* volatile oil was described for the first time. Volatile oils from cinnamon fruits, flowers and fruit stalks were fractionated using column chromatography to obtain the pure compound. The structure of the isolated compound was identified on the basis of ^{1}H NMR and Mass spectra.

Chapter. 3. Solvent extraction, purification and identification of chemical constituents from cinnamon fruit extracts have been presented in this chapter. Besides, antioxidant, antibacterial and antimutagenic activities of cinnamon fruit extracts were included.

3.1. In this part, the extraction of bioactive fraction from cinnamon fruits, separation and identification of individual chemical constituents have been described. The cinnamon fruit powder was successively extracted with hexane, benzene, ethyl acetate, acetone, methanol and water and concentrated *under vacuum* to obtain crude extracts. Water extract was found to possess maximum phenolics and antioxidant activity. Hence, it was used for fractionation using Diaion HP-20SS, Diaion HP-20, Sephadex LH-20 columns. After repeated column chromatography, four pure

compounds were isolated. The purity of the compounds were analysed by HPLC using C_{18} column at 280 nm. The purified compounds (1-5) were identified as 3,4-dihydroxybenzoic acid (protocatechuic acid), epicatechin- (2β→O→7, 4β→8)-epicatechin- (4β→8)-epicatechin (cinnamtannin B-1), [2,3-dihydro-3-(hydroxymethyl)-5-(3-hydroxypropyl)-7-(methoxy) benzofuranyl]-2-methoxyphenyl 4'-O-β-D-glucopyranoside (2S-trans) (Urolignoside)**,** 3-[[6-O-α-L-rhamnopyranosyl)-β-D-glucopyranosyl] oxy]-2-(3,4-dihydroxy phenyl)-5,7 dihydroxy-4H-1benzopyran-4-one (rutin) and quercetin-3-O-α-L-rhamnopyranoside by using spectral data.

3.2. The antioxidant activity of extracts of cinnamon fruits has been described. The ethyl acetate, acetone, methanol, water extracts and BHA were screened for their potential as antioxidant using model systems, like β-carotene-linoleate and 1,1-Diphenyl-2-picrylhydrazyl (DPPH). All the crude extracts showed good antioxidant activity. The water extract showed 83% antioxidant activity at 200 ppm using β-carotene-linoleate system. Similarly, in DPPH system, the activity was 94% at 50 ppm concentration.

3.3. This part describes the antibacterial activity of cinnamon fruits extracts. The extracts *viz.,* hexane, benzene, ethyl acetate, methanol and water were screened for antibacterial activity by pour plate method. All the crude extracts showed a broad spectrum of antibacterial activity. Hexane extract was found to be most effective of all.

3.4. This part describes the antimutagenic activity of cinnamon fruits extracts. Ethyl acetate, methanol and water extracts of cinnamon decreased sodium azide mutagenicity in *Salmonella typhimurium* (TA-100), which ranged from weak to strong inhibition depending upon the concentration of extract per plate.

CONCLUSIONS

The study describes the investigation on the volatile and non-volatile constituents of unconventional parts of cinnamon (farm by-product).

Chemical composition of the volatile oils from cinnamon buds, flowers, fruits and fruit stalks have been reported for the first time. The major flavour compound i.e. *trans*-cinnamyl acetate was isolated (purity >94%) and identified for the first time from cinnamon fruits. The biogenetic pathways on the formation of chemical constituents have been discussed. Besides this, cinnamon fruits were extracted with different solvents with increasing polarity. Antioxidant, antibacterial and antimutagenic activities of these extracts have been determined. The water extract showed maximum antioxidant and antimutagenic activities and contained highest phenolics. Hence, it was used for fractionation to get five pure compounds. The structures of isolated compounds 1-5 have been identified using ^{1}H, ^{13}C NMR (i.e. DQFCOSY, HSQC, HMBC) and high-resolution Mass spectra, as protocatechuic acid, cinnamtannin B-1, urolignoside**,** rutin and quercetin 3-O–α–L-rhamnopyranoside, respectively. Finally, the antioxidant activity of isolated compounds 1-5 was tested for their antioxidant activity. The order of antioxidant activity of the isolated compounds were found to be protocatechuic acid > quercetin-3-O-α-L-rhamnopyranoside > rutin > cinnamtannin B-1 > urolignoside .

Further work is required to study the antioxidant and antibacterial activities of cinnamon fruit extracts and purified compounds in food systems.

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PART- B

Chapter - 1

Turmeric (*Curcuma longa)*: Chemistry, Technology, Biological activities and Uses

INTRODUCTION

Turmeric (*Curcuma longa* L.) belongs to the Zingiberaceae family along with the other noteworthy members like ginger, cardamom and galangal. It belongs to the genus *Curcuma* that consists of hundreds of species of plants that grow to form rhizomes and underground root like stems. Turmeric is grown in warm, rainy regions of the World such as China, India, Indonesia, Jamaica and Peru (Govindarajan, 1980). In India, it is popularly known as *Haldi*. In Malaysia, Indonesia and India, turmeric has been well studied due to its economic importance. Its rhizomes are oblong, ovate, pyriform and often short-branched and these are a household remedy in Nepal (Eigner and Scholz, 1999).

A large number of publications and several reviews have appeared on the chemistry, processing and technology of turmeric (Govindarajan, 1980; Ammon and Wahl 1991, Srimal, 1997, Verghese, 1999; Khanna, 1999). The chemical constituents of turmeric have been studied in relation to the mechanisms of oxygen stress-related processes, including aging. Products isolated from *C. longa* show a strong antioxidant action when tested on the following models systems like oxidation of linoleic acid in air (Toda, 1985; Jitoe et al., 1992; Masuda et al., 1992), and *in vitro* lipid peroxidation of the brain (Sharma, 1976).

CHEMISTRY OF VOLATILE OILS

The aroma of the turmeric is due to its volatile oil, while the phenolic compounds and its analogues account for its bright yellow colour. Due to its lower commercial importance, the chemistry of turmeric oil has not received much attention. Kelkar and Sanjeev Rao (1933) reported that steam-distilled volatile oil is a mixture of

sesquiterpene ketones and alcohols predominantly. Malingre (1975) reported *p*-cymene, β-sesquiphellandrene, turmerone, *ar*-turmerone and sesquiterpene alcohols from *C. longa* (Figure 1.1).

Chen et al. (1983) compared composition of the volatile oils of rhizome and tuber of *C. longa* of Chinese origin and turmerone (24%), *ar*-turmerone (8.4%) and curdione (11.58%) are the major compounds in both the oils (Figure 1.1). However, *ar*-curcumene was found in rhizomes oil to the extent of 12.2%, but it was not reported in tuber oil. Kiso et al. (1983) examined an aqueous ethanolic extract of *C. longa* rhizomes. A new oxygenated sesquiterpene, curlone was isolated. Structural elucidation of curlone was achieved by dehydrogenation to *ar*- turmerone as well as by Mass and NMR (^{1}H and ^{13}C) spectral studies (Figure 1.1). Gopalan and Ratnambal (1987) compared the main constituents of turmeric oils produced from different cultivars. There was considerable quantitative variation in the main components depending upon the cultivars from which the oil was produced. Phan et al. (1987) reported GC-MS analysis of turmeric oil produced by steam distillation of rhizomes of *C. longa* that were grown in Vietnam. Cooray et al. (1988) examined the effect of maturity on the major components of the rhizome oil produced from a single turmeric cultivar grown in Sri Lanka and it was reported that *ar*- turmerone (24.7 - 48.9%) and turmerone (20 - 39%) are the major compounds. Imai et al. (1990) reported the two new sesquiterpene ketones namely turmeronol A and turmeronol B from the dried rhizomes of *C. longa* (Figure 1.1).

Ohshiro et al. (1990) examined sesquiterpenoidal constituents from methanolic extract of *C. longa.* Five sesquiterpenes, *viz.,* germacrone-13-al, 4-hydroxybisabola-

2, 10-diene-9-one, 4-methoxy-5-hydroxybisabola-2, 10-diene-9-one, 2,5-dihydroxybisabola-3, 10-diene and procurcumadiol (Figure 1.2) were isolated and identified by NMR (^{1}H and ^{13}C) spectroscopy It was concluded that, the high content of bisabolene type sesquiterpenes is characteristic for *C. longa* compared with other *Curcuma* species. Nigam and Ahmed (1991) reported the results of their analysis of turmeric oil produced by hydro distillation. The major compound was found to be *ar*-turmerone (59.69%). Zwaving and Bos (1992) compared the composition of the rhizome oils of five different *Curcuma* species using GC-MS. Uehara et al. (1992) analysed hexane extracts of the rhizomes of a number of cultivars of turmeric using GC-MS. It was reported that, the percentages of major components vary *e.g. ar*-turmerone (2.6- 70.3), α-turmerone (trace - 46.2%) and zingiberene (trace - 36.8%) (Figure 1.2).

Konig et al. (1994) reported the presence of (+)-*ar*-curcumene and (-)-β-bisabolene in turmeric oil (Figures 1.1 and 1.2). McCarron et al. (1995) used GC-MS analysis to compare the monoterpene hydrocarbon content of oils produced from the green leaves and fresh rhizome of *C. longa.* It was found that the monoterpene hydrocarbons of the fresh leaf and fresh rhizome oils were 92.9% and 16.3%, respectively. The rhizome oil of *C. longa* of Chinese origin was analysed by GC-MS (Zhu et al., 1995). The oil was reported to contain 17 chemical constituents of which turmerone (24%), *ar*-turmerone (18%) and germacrone (11%) are the major compounds (Figures 1.1 and 1.2).

Vabirua-Lechat et al. (1996) analysed a series of oils produced from plants grown in French Polynesia. The authors examined the composition of oils of dried rhizomes and super critical fluid CO_2 extract of *C. longa*. Twenty-one compounds were

identified in both the oils, without any change in percentage of composition. Hiserodt et al. (1996) examined the volatiles of a number of samples of turmeric powder. Volatile oil was obtained by mixing 20 mg turmeric powder with preconditioned 200 mg of 80-100 mesh Chromosorb W. *ar*- Turmerone, turmerone and curlone (Figure 1.2) were identified as major compounds by Direct Thermal Desorption Gas Chromatography-Mass spectroscopy (DTD-GC-MS). Li et al. (1997) analysed a series of oils produced from several Zingiberaceae plants including the rhizome oil of *C. longa* using GC-MS. Thirty-five components were identified and turmerone (49%), *ar*-curcumene (15%) and *ar*-turmerone (6.4%) are the major compounds. Richmond and Pombo-Villar (1997) determined chemical composition of cyclohexane extract of *C. longa.* The cyclohexane extract was found to contain a series of saturated and unsaturated fatty acids along with sesquiterpenes. Eight fatty acids were reported in *C. longa* namely, tetradecanoic acid, *cis*-9-hexadecenoic acid, hexadecanoic acid, *cis-cis*-9,12-octadecedienoic acid, *cis-trans*-9-octadecenoic acid, octadecanoic acid and eicosanoic acid. Sharma et al. (1997) analysed oil produced from 5-10 month old *C. longa* rhizomes that were grown in Bhutan using GC and GC-MS and the major compounds were found to be *ar*- turmerone (16.7-25.7%), α-turmerone (30.1-32.0%) and β-turmerone (14.7-18.4%) (Figure 1.2). Hu et al. (1997) reported that the oil of *C. longa* contains *ar*-curcumene (34%) using GC-MS analysis.

Kojima et al. (1998) analysed three hydro distilled oils of turmeric using GC and GC-MS. The authors found that the oil contained β-turmerone (11-36%), α-turmerone (19-24%) and *ar*-turmerone (4-14%). More recently, Garg et al. (1999) examined twenty-seven accessions of *C. longa* collected in the Tarai region of Uttar Pradesh in India and the oil content of the fresh rhizomes ranged from 0.16-1.94%. Furthermore,

they determined the content of seven major constituents in these oils. The constituents examined were β-pinene (0.1-23.9%), *ar*-turmerone (0.1 -37.6%) *p*-cymene (0.4-6.0%) α-turmerone (0.1-18.5%) *ar*-curcumene (0.1-4.0%) β-turmerone (0.6-25.2%) and β-curcumene (0.8-5.4%). This study re-emphasises the fact that various studies on turmeric oil have generated very different compositional data. Consumers of turmeric oil need to be quite knowledgeable as to which composition is desirable for their end use. More recently, Gopalan et al. (2000) compared the composition of a supercritical fluid CO_2 extract of turmeric powder with that of a steam distilled oil. The composition of the extract and the oil were very similar.

ISOLATION OF CURCUMINOIDS AND TECHNOLOGICAL ASPECTS

Turmeric has been found to be a rich source of phenolic compounds or curcuminoids. Besides the major compound curcumin, two minor constituents are also isolated (Srinivasan, 1952; Srinivasan, 1953) (Figure 1.3). Janaki and Bose, (1967) reported the isolation of curcuminoids in higher yield (1.1%) involved prior extraction of rhizomes with hexane to remove much of the volatile and fatty components and then extracting with benzene. The concentrate readily crystallised on cooling and was further purified by crystallization from ethanol to yield orange-yellow needles. But, the yield of curcuminoids (1.1%) was poor. Sastry (1970) reported the isolation of curcumin and related demethoxy compounds from the turmeric by extraction with organic solvents. The drawback was low recovery of the curcuminoids (1.5-2.0%). Krishnamurthy et al. (1976) reported the hot and cold percolation extraction methods with good yields with a high recovery of curcumin. Stransky (1979) reported that, the curcumin was isolated from the rhizome by the action of soap solution of about pH 7.0 or slightly higher at 60-90 °C. However, the curcumin obtained by this method was

found to be paste and keeping the solution at alkaline pH at higher temperature may bring structural changes. Tonnesen et al. (1989) reported the isolation of curcumin by insoluble lead salt. Kiuchi et al. (1993) reported a new curcuminoids, *viz.,* cyclocurcumin and it was isolated from the nematocidally active fraction of turmeric with other known curcuminoids.

Considering the potential of curcuminoids, attempts were made by several researchers in the past to isolate curcuminoids from turmeric rhizomes by solvent extraction using organic solvents (Zhang and Yang, 1988; Verghese and Joy, 1989; Xianchum et al., 1993). In the recent years, supercritical fluid-based extraction has gained commercial importance as an efficient method of extraction for natural products. It has been investigated for the extraction of essential oils from *Curcuma longa* (Marsin et al., 1993; Hisashige et al., 1994). Recently, Baumann et al. (2000) have claimed efficient extraction of curcuminoids using supercritical CO_2 modified by 10% ethanol. Although supercritical fluid extraction is known to be a clean technology giving acceptable yields and purity, its major disadvantage lies in its high operating pressures. The scaleup problems could also be severe when the extraction is to be done at large scales.

Numerous methods were available for isolating curcuminoids from *C. longa.* Isolation of pure curcumin from plant material is time consuming and pure curcumin sold on the market is therefore a purified extract containing a mixture of the three curcuminoids *i.e.* curcumin (75-81%), demethoxycurcumin (15-19%) and bisdemethoxycurcumin (2.2-6.6%). Except by the chromatographic routes, all other

methods generally converge to curcuminoids with curcumin as the dominant constituent.

Park and Kim (2002) reported two novel compounds *viz.,* 4□□-(3□□□methoxy-4□□□hydroxyphenyl)-2□□-oxo-3□□-enebutanyl 3-(3□-methoxy-4□hydroxyphenyl) propenoate (calebin-A) and 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 4, 6- heptatrien-3-one and seven known compounds, curcumin, demethoxycurcumin, bisdemethoxycurcumin, 1-hydroxy-1, 7-bis (4-hydroxy-3-methoxyphenyl)-6-heptene-3,5-dione, 1, 7-bis(4-hydroxyphenyl)-1-heptene- 3, 5-dione, 1, 7-bis(4-hydroxyphenyl)-1, 4, 6-heptatrien-3-one and 1, 5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one were isolated from *Curcuma longa* (Figures 1.3 and 1.4).

CHEMISTRY OF CURCUMINOIDS

The colouring principle of turmeric was isolated in the 19th century and was named curcumin. Curcuminoids refers to a group of phenolic compounds present in turmeric, which are chemically related to its principal ingredient curcumin. Three curcuminoids were isolated from turmeric *viz.,* curcumin, demethoxycurcumin and bisdemethoxycurcumin (Figure 1.3). All three impart the hallmark yellow pigmentation to the *Curcuma longa* plant and particularly to its rhizomes. Although the chemical structure of curcumin was determined in the 1970' s and 1980' s, recently the potential uses of curcuminoids in medicine have been studied extensively. The structure of curcumin as diferuloylmethane was confirmed by the degradative work (Majeed et al., 1995). On boiling with alkali, curcumin gave vanillic acid and ferulic acids whose structures were established. Fusion with alkali yielded protocatechuic acid and oxidation with potassium permanganate yielded vanillin. On hydrogenation a mixture

of hexahydro and tetrahydro derivative were obtained. Based on these the structure of curcumin was established as diferuloylmethane.

ANALYSIS OF CURCUMINOIDS

A variety of methods for quantification of the curcuminoids were reported (Tonnesen and Karlsen, 1986). Most of these are spectrophotometric methods, expressing the total colour content of the sample. Commercially obtained *Curcuma* products contain mixtures of curcumin, demethoxycurcumin and bisdemethoxycurcumin. For an exact determination of the curcumin content a pre-separation of the three curcuminoids is essential.

The curcuminoids isolated from *C. longa* exhibit strong absorption between 420 - 430 nm in organic solvents. The official methods for assaying curcumin or *Curcuma* products as food colour additives are based upon direct spectrophotometric absorption measurements (WHO food additives series, 1976; British standard methods of test for spices and condiments, 1983). The evaluation of the total amount of curcuminoids in a sample by use of direct absorption measurements is only valid if the calculations are based on reference values obtained from pure standards. It should however, be noted that the presence of other compounds absorbing in the region of 420-430 nm influence the results strongly.

A direct fluorimetric method for the assay of curcumin in food products was reported (Karasz et al., 1973). The difficulties in obtaining reproducible results could be ascribed to the difference in fluorescence intensity of curcumin and the two demethoxy compounds in organic solvents. At fixed excitation and emission

wavelengths (420 - 470 nm), the relative fluorescence intensities of curcumin, demethoxycurcumin and bisdemethoxycurcumin in ethanol are 1:2.2:10.4 at equimolar concentrations (Tonnessen and Karlsen, 1983). Unless these differences are taken into account small changes in sample composition may lead to large variations in the curcumin content calculated.

To increase the molar absorptivity of curcumin, intensely coloured complexes were developed by reaction with alkalis, strong mineral acids or boric acid (Karasz et al., 1973; Krishnamurthy et al., 1976; Janssen and Gole, 1984). However, the colours formed were found to be very unstable and severe fading was reported after 5-10 minutes with the exception of the boric acid complexes (Dyrssen et al., 1972; Janssen and Gole, 1984).

A pre-separation of the curcuminoids can be accomplished by thin-layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) (Tonnesen and Karlsen, 1983). Separation of the curcuminoids is strongly dependant on the chromatographic conditions. Curcumin and the related 1,3-diketones are shown to adsorb strongly onto the silicic acid used as the solid support in TLC and HPLC. By removing one of the keto groups from a diketone the adsorption to silica gel can be prevented (Tonnesen and Karlsen, 1986). The adsorption is therefore ascribed to intermolecular hydrogen bonding between the keto-enol unit of the 1,3-diketones and the silicic acid. Quantitative analysis of curcumin and related compounds by TLC or HPLC is difficult to carry out unless the chromatographic support is properly deactivated, e.g. the number of free silanol groups is kept at a minimum. HPLC systems based on C_{18} stationary phases did not completely resolve the three

curcuminoids (Asakawa et al., 1981; Smith and Witowska, 1984). A reproducible separation of the coloured compounds was achieved by the use of an amino bonded stationary phase, provided that the water content of the system is kept below 10%. HPLC system based on an amino-bonded stationary phase, however, seems to have a catalytic effect upon curcumin degradation. To obtain reproducible results the experimental conditions must be carefully controlled.

HPLC in combination with fluorescence detection is the most sensitive method for the determination of curcumin, the detection limit lying in the picogram range (Tonnesen and Karlsen, 1983). GC methods provide no alternative to HPLC due to the low volatility and thermally labile nature of the curcuminoids. Spectroscopic methods (IR, NMR, MS) are widely used for identification and characterisation of the curcuminoids (Tonnesen and Karlsen, 1986; Unterhalt, 1980; Roughley and Whiting, 1973; Govindarajan, 1980*).* NMR has also been tried for quantitative determinations (Unterhalt, 1980). Mass spectrometry (MS) is often the method of choice when trace amounts of organic compounds are to be detected. The detection limit for curcumin in biological samples by MS needs to be determined, and then the possibility of using quantitative MS in curcumin analysis could be evaluated. It was reported that the strong interactions observed between curcumin and silanol groups also occur in a glass container. Unless precautions are taken, curcumin in solution will adsorb strongly to the container wall, leading to inaccurate results (Unterhalt, 1980).

BIOLOGICAL ACTIVITIES

Srimal and Dhawan (1973) reported the pharmacological actions of curcumin e.g., the compound was effective in acute as well as chronic models of inflammation. The potency of this drug is approximately equal to phenylbutazone in the carrageenin-induced edema test, but it is only half as active in the chronic experiments. It was observed that curcumin was less toxic than the reference drug (no mortality up to a dose of 2 g/kg). Ammon et al. (1992) demonstrated curcumin as an inhibitor of leucotriene formation in rat peritoneal polymorph nuclear neutrophils (PMNL), with an EC_{50} of 27 x 10^{-7} M, in contrast, to hydrocortisone, which did not show any effect.

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Park and Kim (2002) reported a bioassay-guided fractionation scheme utilizing an assay to detect protection of PC-12 cells from β-amyloid insult. Calebin - A, curcumin, demethoxycurcumin, bisdemethoxycurcumin and 1,7-bis (4-hydroxyphenyl)-1-heptene-3, 5-dione (Figures 1.3 and 1.4) were found to protect PC12 cells from βA insult (ED_{50} 0.5-10 μg/ml) more effectively than Congo red (ED_{50} = 37-39 μg/ml).

Antioxidant Activity

Pulla Reddy and Lokesh (1992) observed that curcumin is capable of scavenging oxygen free radicals such as superoxide anions and hydroxyl radicals, which are the initiators of lipid peroxidation. The effect of curcumin on lipid peroxidation was also studied in various models by several authors.

Curcumin is a good antioxidant and inhibits lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla Reddy and Lokesh, 1994). The lipid peroxidation has a main role in the inflammation, in heart diseases, and in cancer. Unnikrishnan and Rao (1992) studied the antioxidative

properties of curcumin. It was demonstrated that, curcumin protects (52%) hemoglobin from nitrate-induced oxidation to methemoglobin at 400 μM concentration. Sreejayan and Rao (1994) have reported that three curcuminoids were inhibitors of lipid peroxidation in rat brain homogenates and rat liver microsomes. All of these compounds were more active than tocopherol as reference and curcumin showed the better results. In the case of curcumin, the methoxy group seems to play a major role. The phenolic hydroxyl and the methoxyl groups on the phenyl ring and the 1,3-diketone system seem to be important structural features that can contribute to these effects. The diketone system is a potent ligand for metals such as iron, used in these experiments. Another fact proposed is that the antioxidant activity increases when the phenolic hydroxyl group is at the ortho position with respect to methoxy group.

Anti-Protozoal Activity

Araújo et al. (1998, 1999) reported the anti-protozoal activity of curcumin and some semi-synthetic derivatives against tripanosomatids in promastigotes (extracellular) and amastigotes (intracellular) forms of *Leishmania amazonensis*. It was reported that curcumin has an excellent activity (LD_{50} = 24 μM or 9 mg/ml) and the semi-synthetic derivative, methylcurcumin (a non phenolic curcuminoid), has the best action with a LD_{50} < 5 μg/ml and LD_{90} = 35 μM against promastigotes forms. This derivative was tested *in vivo* in mice and showed good activity with 65.5% of inhibition of the lesion size of the footpad of the animals, when compared with the group inoculated with the parasites alone. Another interesting point mentioned is that they did not observe any inflammatory reaction in the area where the drugs were injected, perhaps because curcuminoids are potent inhibitors of inflammation.

Nematocidal Activity

Kiuchi et al. (1993) demonstrated the nematocidal activity of methanolic and chloroform extracts of turmeric against *Toxocara canis*. In this work they isolated a new curcuminoid, the cyclocurcumin. All the substances did not show activity when applied independently, but the activity was observed when they were mixed, suggesting a synergistic action.

Antibacterial Activity

Curcuma oil was tested against cultures of *Staphylococcus albus*, *S. aureus* and *Bacillus typhosus* and the results showed inhibition of the growth of *S. albus* and *S. aureus* at different concentrations (Chopra et al., 1941). Bhavanishankar and Srinivasamurthy (1979) investigated the activity of turmeric fractions against some intestinal bacteria *in vitro*. In this work, total inhibition of growth of *lactobacilli* in the presence of whole turmeric was observed (4.5-90 µl/100 ml). The alcoholic extract was also effective (10-200 mg/ml), but the inhibition was not equal as the whole turmeric. Curcumin (2.5-50 mg/ml) inhibited only *S. aureus.*

Antivenom Activity

Ferreria et al. (1992) reported the activity of turmeric and its constituents against snake venom. The fraction consisting of *ar*-turmerone, isolated from *C. longa* neutralized both the hemorrhagic activity and lethal effect of venom in mice. In this study *ar*-turmerone was capable of abolishing the hemorrhagic activity of *Bothrops* venom and about 70% of the lethal effect of *Crotalus* venom. Immunological studies demonstrated that *ar*-turmerone inhibited the proliferation and the natural killer activity of human lymphocytes.

Anti-HIV

Mazumder et al. (1995) demonstrated that curcumin has an antiviral activity, being a HIV-1 integrase inhibitor (IC_{50} = 40 μM) and suggested that curcumin analogs could be developed as anti-AID's drugs. Data showed that curcumin inhibited the replication of HIV-1 integrase protein. Eigner and Scholz (1999) reported the anti-HIV-1 and anti-HIV-2 activities of curcumin.

Anti-Tumour Activity

Huang et al. (1988), studied the effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumour promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate (TPA) and observed that all these compounds inhibit the epidermal ornithine decarboxylase (ODC) and epidermal DNA synthesis, curcumin being the most effective. Limtrakul et al. (1997) showed an inhibitory effect of curcumin on mouse skin carcinogenesis initiated by 7,12-dimethylbenz (a) anthracene (DMBA) and promoted by TPA. Thus, curcumin administration decreased both the number of tumours per mouse and tumour volume. Huang et al. (1997) reported the effects of a range of doses of curcumin applied topically on TPA-induced tumour promotion. It was reported that application of 100 nmol of curcumin together with 5 nmol TPA twice a week for 18 weeks markedly inhibited TPA-induced tumour promotion. The authors suggested that the effect of curcumin might be linked to its strong inhibitory action on DNA and RNA synthesis. Furthermore, Ozaki et al. (2000) studied the action of curcumin on rabbit osteoclast apoptosis and demonstrated that curcumin drastically inhibits bone resorption and stimulation of apoptosis in the cells. Since cancer and bone inflammation are diseases that increase bone resorption, the authors suggest that curcumin may be useful in the therapy of these diseases. The anti-cancer action

of curcumin has been studied in a standard model of radiation-induced tumour in rat mammary gland (Inano et al., 2000). The authors suggest that curcumin has the potential to be an effective agent for chemoprevention of radiation-induced initiation stage of mammary tumourogenesis.

Anti-Inflammatory Activity

Arora et al. (1971) investigated the anti-inflammatory activity of different fractions of the rhizomes of turmeric in animals. They found that the extracts reduced the granuloma growth and no toxic effects were observed. Chandra and Gupta (1972) demonstrated the anti-inflammatory and anti-arthritic actions of volatile oil of *C. longa.* Ghatak and Basu (1972) showed the action of sodium curcuminate as an anti-inflammatory agent, being better than curcumin and hydrocortisone acetate, in experimental inflammation induced by carrageenin and formalin in albino rats (ED_{50} = 144 µg/kg).

Mukhopadhayaya et al. (1982) demonstrated the activity of curcumin and other semi-synthetic analogues (sodium curcuminate, diacetyl curcumin, triethyl curcumin and tetrahydro curcumin) in carrageenin-induced rat paw edema and cotton pellet granuloma models of inflammation in rats. In these experiments the authors used ferulic acid and phenylbutazone as reference drugs. Curcumin and its analogues showed similar action in carrageenin-induced paw edema in rats; however the sodium curcuminate was the most potent analogue and was more water-soluble than curcumin. Among the curcumin analogues, triethyl curcumin was the most potent anti-inflammatory in the chronic model of inflammation, when compared with the

others as well as with the reference drug. Tetrahydro curcumin showed no activity. In the acute inflammation condition, all the substances were more effective. The authors concluded that the activity of the compounds used in these experiments, would depend on the model of inflammation. Ammon and Wahl (1991) reported that the *Curcuma* extracts showed a high anti-inflammatory effect after parenteral application in standard animal models.

Curcumin can also protect against inflammation-related changes in the liver prostanoids in an animal model of alcohol-caused hepatic injury linked to increased activity in serum enzymes aspartate transaminase and alkaline phosphatase. When the diet of the ethanol-consuming rats was supplemented with curcumin, not only the activity of these serum enzymes was decreased but there was also a reduction in the abnormally raised levels of prostaglandins E_l and E_2 in liver as well as in kidney and brain (Rajakrishnan et al., 2000). More recently, Chuang et al. (2000) have shown that gavage administration of 200 mg of curcumin suppresses diethyl nitrosamine-induced inflammation and hyperplasia in rats, as shown by histopathological examination. Apparently, this effect is due to the fact that curcumin can act as an inhibitor of the inflammation-factor lipoxygenase (Skrzypezac-Jankun et al., 2000).

STRUCTURE-ACTIVITY RELATIONSHIPS

It is known that the structure of curcumin is very similar to diarylheptanoids. Researchers attributed the anti-inflammatory activity of curcumin and its derivatives to the hydroxyl and phenol groups in the molecule, and these groups are also essential for the inhibition of prostaglandins PG synthetase and leucotrienes synthesis (LT) (Kiuchi et al., 1982; Iwakami et al. 1986; Kiuchi et al., 1992). Claeson et al. (1993,

1996) suggested that the anti-inflammatory action is associated with the β-dicarbonylic system, which has the conjugated double bonds (dienes). This system seems to be responsible not only for anti-inflammatory power, but also to antiparasitic activity (Araújo et al., 1998, 1999). The presence of diene ketone system provides a lipophylicity to the compounds, and thus probably better skin penetration.

TRADITIONAL USES

Turmeric is of special importance to man with the discovery that its powdered rhizome, when added to various food preparations, preserve their freshness and impart a characteristic flavour. Turmeric, which belongs to a group of aromatic spices, was originally used as a food additive in curries to improve the storage condition, palatability and preservation of food. In Ayurveda, turmeric has been used internally as a stomachic, tonic and blood purifier and externally in the prevention and treatment of skin diseases (The Wealth of India, 2001; Chopra et al., 1958). The review of literature showed that turmeric is useful in treating a variety of ailments and metabolic disorders. Turmeric roots are known to be antiseptic and aromatic. The bactericidal properties of turmeric have been proved by clinical testing and its use is more than being merely cosmetic (Khanna, 1999). The significance of turmeric in medicine has changed considerably since the discovery of the antioxidant properties of naturally occurring phenolic compounds in turmeric. Traditional Indian medicine claims the use of its powder against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis (Ammon et al., 1992).

THE AIMS AND SCOPE OF THE PRESENT STUDY

Turmeric oleoresin is a value added product produced in large quantities in India (Govindarajan, 1980). Extraction of turmeric is usually accomplished by organic solvents such as ethanol or acetone or methanol and gives turmeric oleoresin, which consists of both flavour and colour principles (Smith and Witoska, 1984). Curcuminoids are produced industrially by treating turmeric oleoresin with isopropyl alcohol. The curcuminoids (20-30%) are collected by centrifugation and the centrifugate is concentrated to recover the solvent. The concentrate (≈70-80%) is known as Curcumin Removed Turmeric Oleoresin (CRTO) or Spent Turmeric Oleoresin. It has a composition of oil, resin and leftover curcuminoids and this has no commercial value at present. This has a small demand in the perfume industry (Saju et al., 1998). Remaining CRTO is used for boiler fuel, as it is waste in the process of curcuminoids extraction (Saju et al., 1998). The above literature survey revealed that, there are no reports on utilization of curcumin removed turmeric oleoresin (industrial by-product i.e. from curcumin production) (Part A, Figure 2.4).

In the background of present knowledgebase, a systematic investigation on the chemical as well as bioactivity studies of the volatiles and non-volatile fractions from curcumin removed turmeric oleoresin were undertaken and the findings of these studies are presented in Part B.

The objectives of the present investigation were (i) Isolation and identification of antibacterial, antifungal, antioxidant and antimutagenic fractions from spent turmeric oleoresin, (ii) Development of a method for the isolation of curcuminoids mixture and individual curcuminoids from spent turmeric oleoresin, (iii) Development of an analytical method for the determination of curcumin, demethoxycurcumin and

bisdemethoxycurcumin from different varieties of turmeric rhizomes and (iv) Antioxidant activity of curcumin, demethoxycurcumin and bisdemethoxycurcumin.

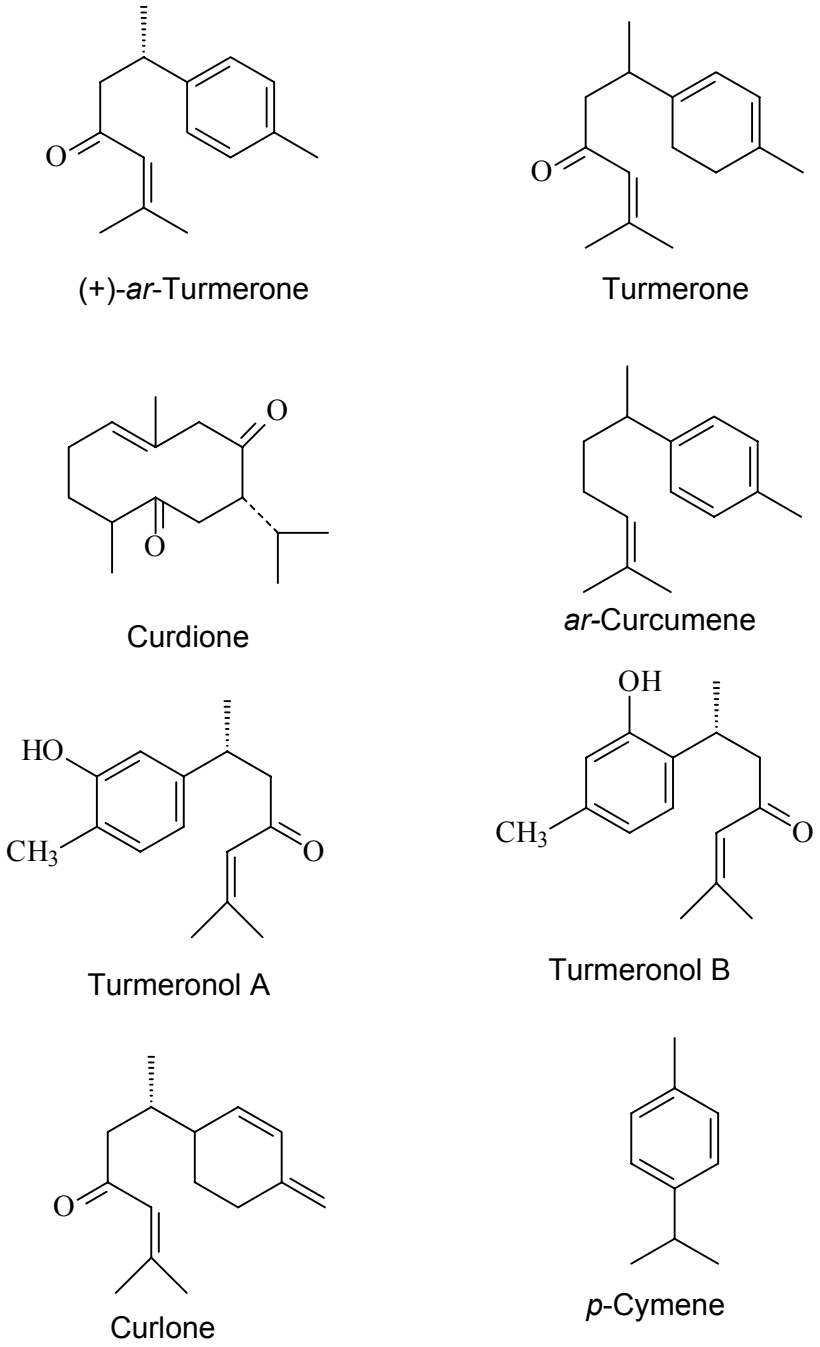
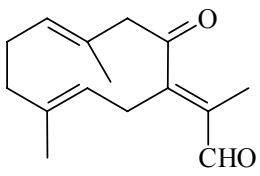
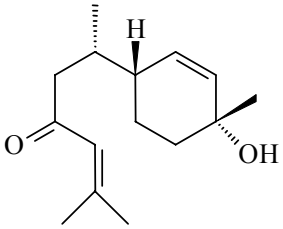


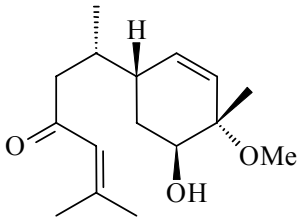
Figure 1.1. Structures of compounds identified in turmeric oil (Malingre, 1975; Chen et al., 1983; Kiso et al., 1983; Imai et al., 1990).



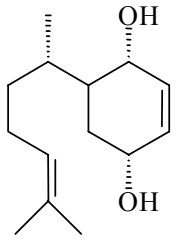
Germacrone-13-al



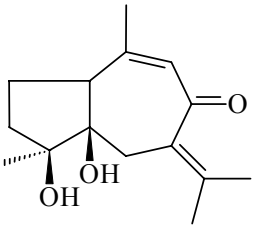
4-Hydroxybisabola--2,10-diene-9-one



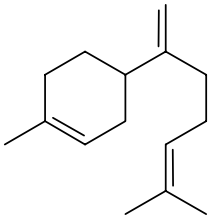
4-Methoxy-5-hydroxy bisabola-2,10-diene-9-one



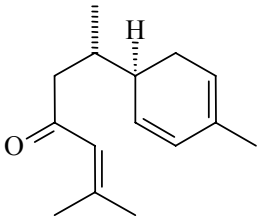
2,5-Dihydroxybisabola--3,10-diene



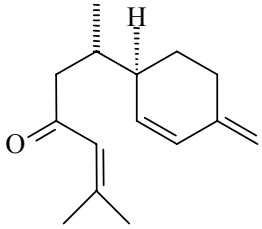
Procurcumadiol



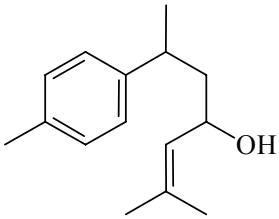
β-Bisabolene



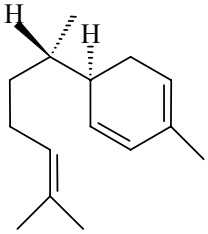
α–Turmerone



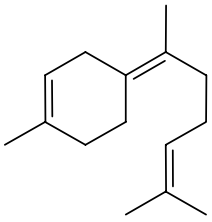
β-Turmerone



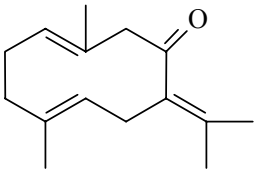
α-Turmerol



α-Zingiberene

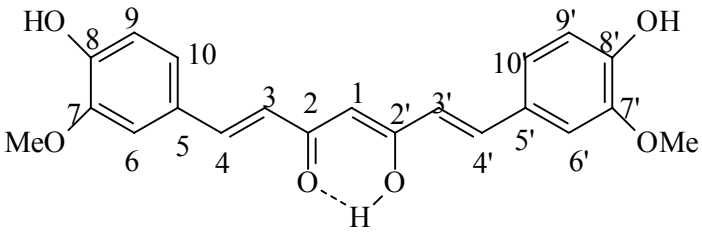


cis–γ-Bisabolene

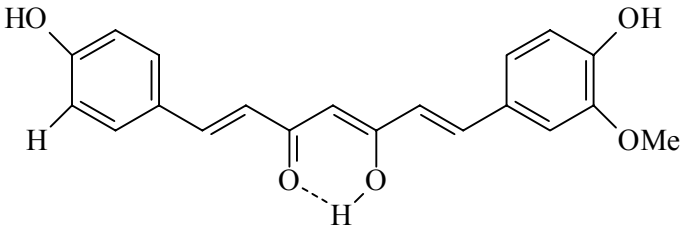


Germacrone

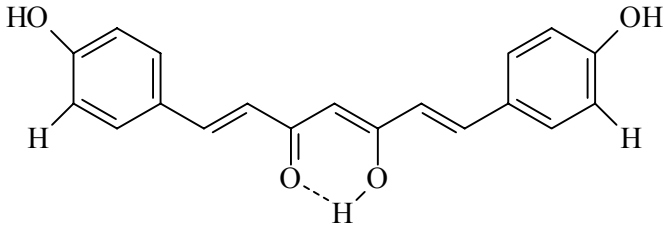
Figure 1.2. Structures of compounds identified in turmeric oil (Ohshiro et al., 1990; Uehara et al., 1992; Konig et al., 1994; Zhu et al., 1995; Sharma et al., 1997).



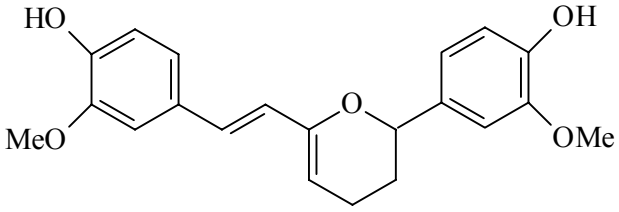
Curcumin (1)



Demethoxycurcumin (2)

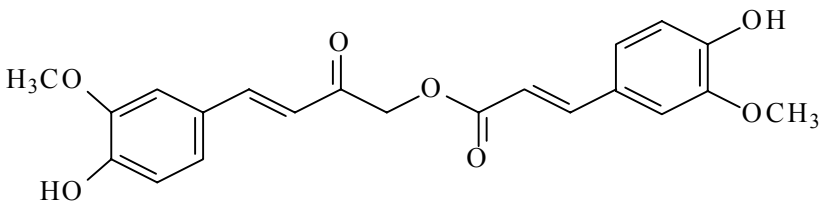


Bisdemethoxycurcumin (3)

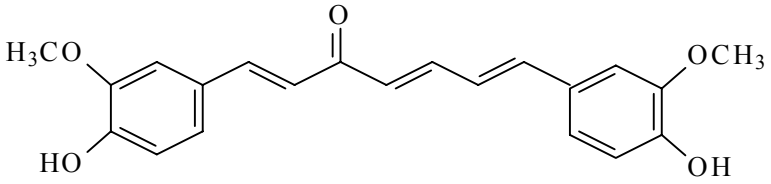


Cyclo curcumin (4)

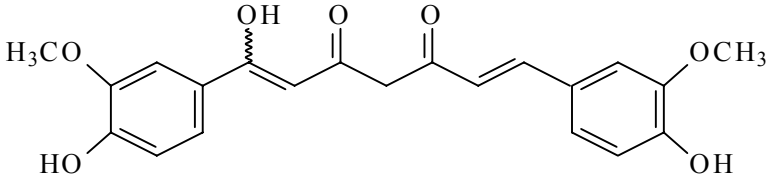
Figure 1.3. Structures of curcuminoids from *C. longa* (Srinivasan, 1953; 1953; Kiuchi et al., 1993).

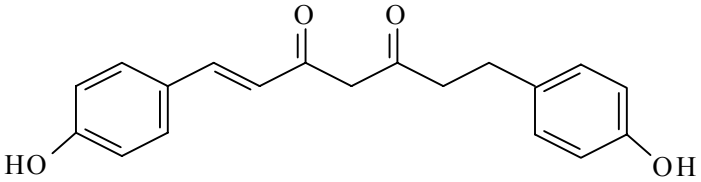


methoxy-4'-hydroxyphenyl)-propenoate (Calebin-A)

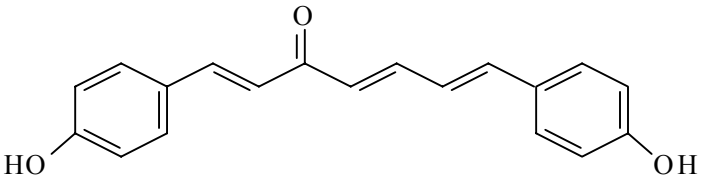


1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatrien-3-one

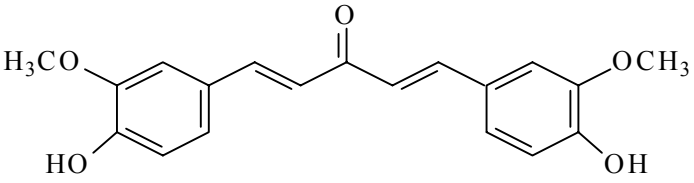




1,7-Bis(4-hydroxyphenyl)-1-heptene-3,5-dione



1,7-Bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one



1,5-Bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one

Figure 1.4. Structures of curcuminoids from *C. longa* (Park and Kim, 2002).

PART – B

Chapter – 2

Studies on volatile oil from spent turmeric oleoresin

2.1. ISOLATION OF ANTIBACTERIAL FRACTIONS AND DETERMINATION OF CHEMICAL COMPOSITION

INTRODUCTION

The spoilage and poisoning of foods by microorganisms are the problems that have not yet been brought under adequate control despite the range of robust preservation techniques available. Consumers are increasingly avoiding foods prepared with preservatives of synthetic chemical origin. Therefore, natural alternatives are needed to achieve sufficient long shelf life for foods and a high degree of safety with respect to foodborn pathogenic microorganisms. In nature, there are a large number of antimicrobial compounds having potential application in the control of microbial spoilage of foods (Hsieh et al., 2001).

There are number of reports available on chemical composition of volatile oil from rhizomes of *Curcuma longa* volatile oil. But, there is no report on the volatile oil from CRTO and its bioactivities. In order to bring out the importance in the context of preference for natural food additives, a systematic investigation was under taken on curcumin removed turmeric oleoresin, a by-product from curcumin manufacturer. This chapter deals with the isolation and identification of antibacterial fraction from CRTO. The active fraction was separated and tested against a few pathogenic microorganisms.

MATERIALS AND METHODS

Materials

First batch (5 Kg) of Mother liquor / curcumin removed turmeric oleoresin (CRTO) was obtained from M/S Flavours and Essences (P) Ltd, Mysore during the year 1998.

Isolation of Oil from CRTO

Fifty grams of CRTO was taken in a 1000 ml round bottom flask, 400 ml of hexane was added and it was subjected to reflux for 1 h at 60 °C. The extract was cooled to ambient temperature (25-30 ^{o}C) and filtered under water suction using Whatman no. 1 filter paper. The filtrate was concentrated *under vacuum* at 30 °C, which gave 25.8 ml (v/w) of oil. Henceforth this oil referred as CRTO oil.

Fractionation of CRTO Oil by Column Chromatography

Five ml of CRTO oil was impregnated on 10 g silica gel and loaded on to a 90 g of silica gel in a glass column (600 × 30 mm), which was pre-equilibrated with hexane. The column was successively eluted with 1000 ml each of hexane, hexane:ethyl acetate (95:5) and ethyl acetate (EtOAc). The solvents from the three elutes were evaporated *under vacuum* and these are designated as fractions I, II and III. The yields of these fractions I, II and III were 1.4, 2.1 and 1.4 ml (v/v), respectively.

Samples Preparation

CRTO oil, fractions I, II and III (100 mg each) were weighed and dissolved in propylene glycol separately and made up to 10 ml with propylene glycol (10 mg/ml).

Preparation of Agar Medium

Nutrient agar (13 g) (HiMedia, Mumbai) was dissolved in water 1000 ml distilled water and 20 ml aliquots of agar medium were transferred into 100 ml conical flasks and sterilized at 121 °C for 20 min.

Organisms and Conditions for Cultivation

Bacterial cultures namely *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were grown in nutrient agar media at 37±1 °C. Each bacterial strain was transferred from stored (4-5 °C) slants to 10 ml nutrient broth and cultivated overnight at 37±1 °C. A preculture was prepared by transferring 1 ml of this culture to 9 ml of nutrient broth and cultivated for 48 h. The cells were harvested by centrifugation (4000 rpm, 5 min), washed and suspended in saline.

Growth Inhibition Assay

The CRTO oil and its column fractions were tested for their effect on the growth of different bacteria as per the method of Chen et al. (1998) with slight modification. Sterilized nutrient agar media was cooled to 45-50 °C. Different quantities of CRTO oil, fraction I, II and III in propylene glycol (equivalent to 25, 50, 100, 200 and 400 ppm) were added to conical flasks under aseptic conditions and mixed well. The 100 µl of each bacterium (10^3 cfu/ml) was inoculated in to the flasks under aseptic conditions. Equivalent amount of propylene glycol was used in controls. Petri plates were incubated at 37±1 °C for 20-24 h. The colonies developed after incubation were counted and expressed as colony forming units per ml of culture (cfu/ml). Studies were performed in triplicate and the inhibitory effect was calculated according to Rico-

Munoz and Davidson (1983) using the following formula, % Inhibition = (1 - T/C) x 100, where T is cfu/ml of test sample and C is cfu/ml of control. The minimum inhibitory concentration (MIC) was reported as the lowest concentration of test compound that demonstrated no visible growth (Naganawa et al., 1996).

Gas Chromatography (GC) Analysis

CRTO oil, fractions I and II were analysed using a Shimadzu GC-15A (Kyoto, Japan) chromatograph equipped with a SE-30 column $(10' \times 1/8'')$. The oven temperature was kept at 75 °C for 2 min, and the programmed to 220 °C at the rate of 2 °C/min and at this temperature the column was maintained for 3 min. The injector port temperature and detector (FID) temperature were 250 °C. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. Peak areas were computed by a Shimadzu C-R4A chromatopak data processor.

Gas Chromatography-Mass Spectral (GC-MS) Analysis

CRTO oil and its fractions were analysed using a Shimadzu GC-17A (Kyoto, Japan) chromatograph equipped with a QP-5000 (Quadrupole) mass spectrometer. The samples were diluted 25 times with acetone and 1 μl was injected. A fused capillary silica column SPB-1 (30 m × 0.32 mm I.D., film thickness 0.25 μm) coated with polydimethylsiloxane was used. Helium was the carrier gas at a flow rate of 1 ml/min. The injector port temperature and detector temperature were 250 °C. The oven temperature was programmed from 65 °C for 2 min, increased to 250 °C at a rate of 2 °C/min and at this temperature the column was maintained for 5 min. The split ratio was 1:25 and the ionisation voltage 70 eV.

RESULTS AND DISCUSSION

Hexane extraction of CRTO at 60 °C gave 51.6% (v/w) of CRTO oil. The oil was broadly fractionated on silica gel column using hexane, 5% EtOAc in hexane and EtOAc as eluents. The yield of Fraction I, II and III were 28, 42 and 28% respectively. CRTO oil and its column fractions exhibited antibacterial effect against all the bacteria tested and the results are presented in Figure 2.1. The number of colonies developed after incubation was taken as index of growth inhibition. Fraction I was more effective against *B. cereus* (54.7%)*, B. subtilis* (52.7%)*, B. coagulans* (62.4%) and *S. aureus* (67.5%), whereas against Gram-negative bacteria it was less effective (*E. coli,* 28.5% and *P. aeruginosa,* 36.8 %) at 50 ppm concentration. Fraction II (obtained by 5% ethyl acetate in hexane) was found to be more effective than fraction I and CRTO oil. In case of Gram-positive bacteria, fraction II brought about complete inhibition of growth at 50 ppm level, except for *B. cereus,* while for Gram- negative bacteria, 200 ppm concentration was required to inhibit complete growth. Fraction III was least effective. CRTO oil was found to be more effective than fraction III against Gram-positive bacteria, but it exhibited around 5% inhibition in *E. coli* and 35% inhibition in *P. aeruginosa* at 50 ppm concentration. Similar sensitivity of Gram- negative and Gram-positive bacteria against different essential oils has been reported (Sivropoulou et al., 1996). The MIC levels of CRTO oil and fraction II are presented in Table 2.1. MIC levels for Gram-positive bacteria were less than or equal to 100 ppm of fraction II, whereas in case of Gram-negative bacteria, 200 ppm of same fraction was required to bring about complete inhibition of growth.

Table 2.1. Minimum Inhibitory Concentration (MIC) of CRTO oil and fraction II

Bacteria	**MIC (ppm)**	
	CRTO oil	**Fraction II**
Gram-positive		
B. cereus	200	100
B.coagulans	50	50
B.subtilis	100	50
S. aureus	100	50
Gram-negative		
E. coli	>200	200
P. aeruginosa	>200	200

CRTO oil, fraction I and II were analysed by GC and GC-MS. Figures 2.2, 2.3 and 2.4 shows the GC-MS total ion chromatograms of CRTO oil, fraction I and II respectively. Retention indices of all compounds were determined using *n*-alkanes as standards (Jennings and Shibamoto, 1980). The compounds were identified by comparison of Kovats indices with reported values (Davies, 1990) and by matching mass spectra with NIST-MS library (NIST62-Lib.) and published mass spectra (Ten Noever Bravw, 1988; Adams, 1989; Hiserodt et al., 1996). Table 2.2 shows Kovats indices (KI) and percentages of chemical constituents of CRTO oil, fraction I and II. The third fraction was mostly non-volatile; hence, it could not be analysed by GC and GC-MS. Thirteen major components major were identified in CRTO oil, fractions I and II and taken as markers. The mass

Table 2.2. Chemical composition of CRTO oil, Fraction I and II

Peak No.	Compound	Peak Area Content (%)			KI	Identification by
		CRTO oil	Fra. I	Fra. II		
1	*ar*- Curcumene	3.49	8.66	0.11	1449	MS, RI
2	α-Zingiberene	2.48	6.48	0.10	1465	MS, RI
3	β-Bisabolene	2.1	5.07	Tr.	1478	MS, RI
4	β-*trans*-Farnesene	6.57	16.45	0.23	1488	MS, RI
5	*ar*-*T*urmerol	0.87	1.00	0.71	1541	MS, RI
6	Compound (1)*	1.90	1.80	1.90	1580	MS, RI
7	Caryophyllen oxide	1.03	2.05	1.00	1588	MS, RI
8	*ar*-Turmerone	62.00	41.36	77.85	1611	MS, RI
9	Turmerone	5.09	4.50	5.17	1622	MS, RI
10	Curlone	3.88	2.55	5.30	1650	MS, RI
11	Compound (2)*	0.43	Tr.	1.11	1685	MS, RI
12	Compound (3)*	Tr.	Tr.	1.00	1702	MS, RI
13	Compound (4)*	Tr.	Tr.	1.84	1717	MS, RI

MS : Mass spectra

KI : Kovats indices

RI : Retention indices

Tr. : Less than 0.09%

* : see Figure 2.9. Structures of compounds (1-4); MS was compared with that of Hiserodt et al. (1996)

spectra of six major compounds and four unidentified oxygenated compounds are presented in Figures 2.5 - 2.7. *ar*-Turmerone (62.0%), *trans*-β-farnesene (6.6%), turmerone (5.1%) and curlone (3.9%) were found to be the major compounds (Figure 2.8). in CRTO oil whereas fraction II contained *ar*-turmerone (77.9%), curlone (5.3%) and turmerone (5.2%) as major components. Fraction I contained *ar*- turmerone (41.4%), β-*trans*-farnesene (16.5%), *ar*-curcumene (8.7%) as the major compounds. Further, oxygenated compounds (1-4) (Figure 2.9) were enriched in fraction II. *ar*-Turmerone and turmerone were found to be the two major constituents of the *Curcuma longa*, which confirms the earlier reports (Govindarajan, 1980).

MIC levels for fraction II were lower due to enrichment of *ar*-turmerone, turmerone, curlone and compounds (1-4) in this fraction and probably the additive /synergistic effect of these compounds may be responsible for high antibacterial activity. Fraction II at 50 ppm was sufficient for complete inhibition of *B. subtilis* and *S. aureus*, whereas CRTO oil inhibited these organisms completely at 100 ppm. In case of *B. coagulans,* 100% growth inhibition was achieved at 50 ppm, but when the inhibitory effect was compared at 25 ppm, it showed 53.6% inhibition for Fraction II and 32.6% for CRTO oil (Figure 2.1). Similarly, growth of *E. coli* and *P. aeruginosa was* inhibited completely at 200 ppm of fraction II, whereas the percentage of inhibition were 35% and 67%, respectively for these organisms, at 200 ppm of the CRTO oil.

CONCLUSION

CRTO oil was found to possess antibacterial activity towards Gram-positive and Gram negative bacteria. Major constituents possessing antibacterial properties were isolated by elution with hexane: ethyl acetate (95: 05) using silica gel column chromatography. The results of the present study are indicative of the utilization of CRTO oil as a preservative agent, which has no commercial application at present.

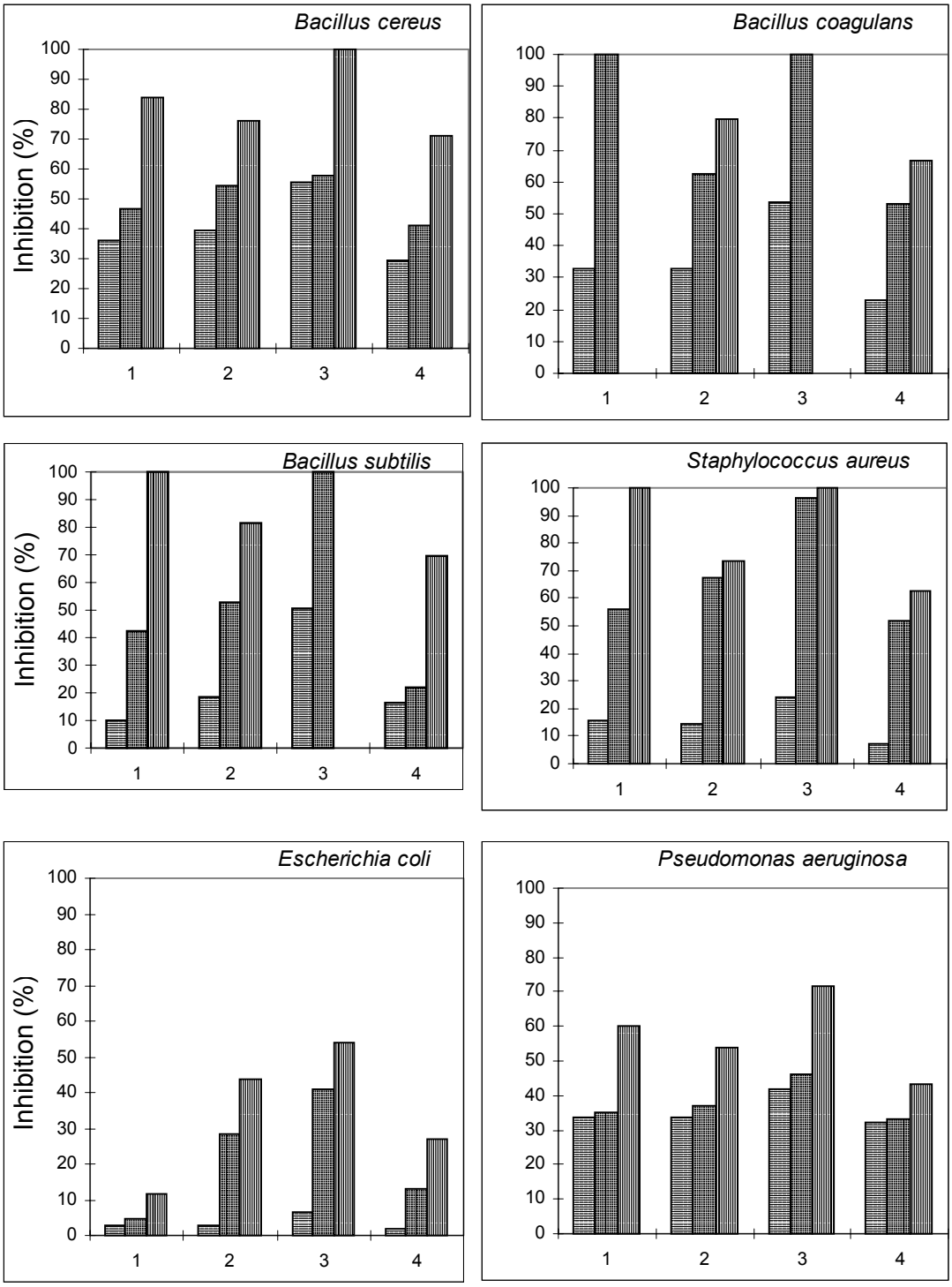
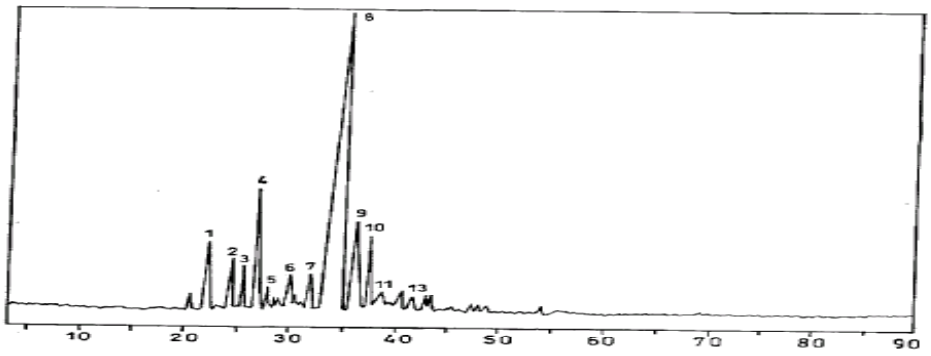


Figure 2.1. Inhibition of bacterial growth by CRTO oil and its fractions: 1-CRTO oil; 2-fraction I; 3-fraction II; 4-fraction III.



RT (min)

Figure 2.2. GC-MS total ion chromatogram of CRTO oil obtained by hexane

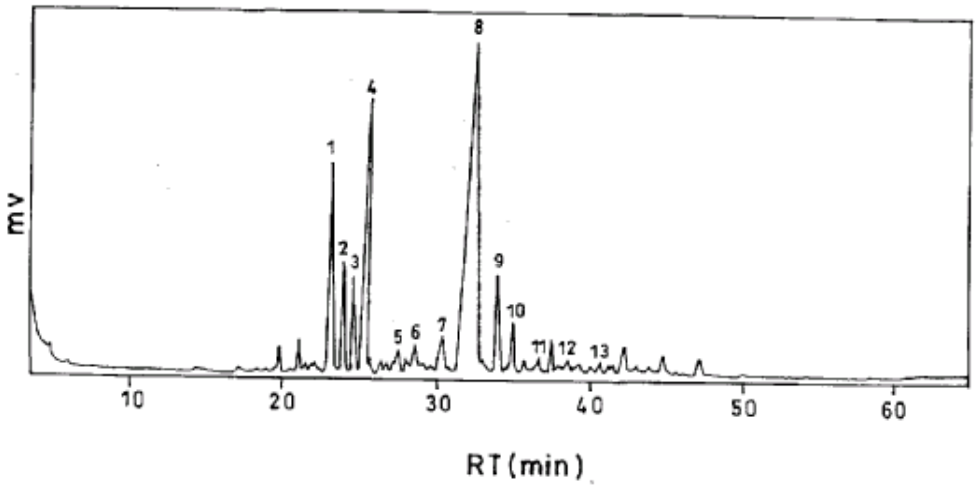


Figure 2.3. GC-MS total ion chromatogram of fraction I obtained by silica gel column chromatography using hexane as an eluent.

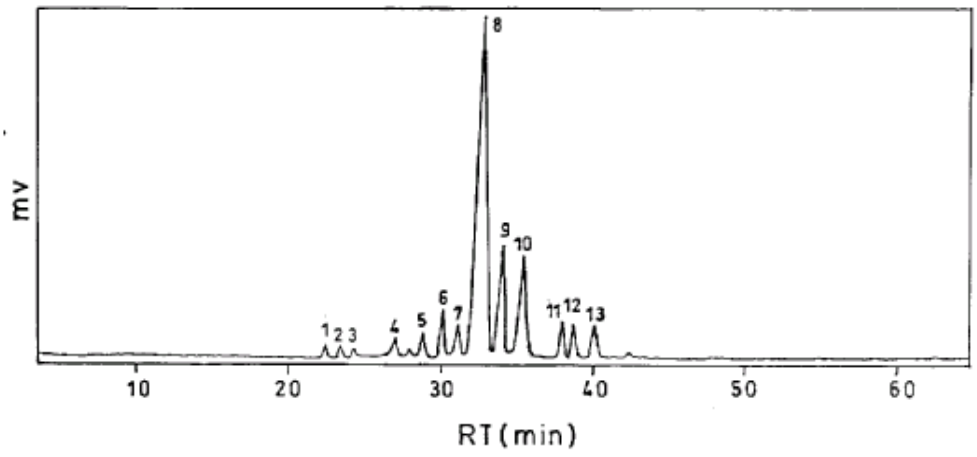


Figure 2.4. GC-MS total ion chromatogram of fraction II obtained by silica gel column chromatography using hexane:ethyl acetate (95:5) as an eluent.

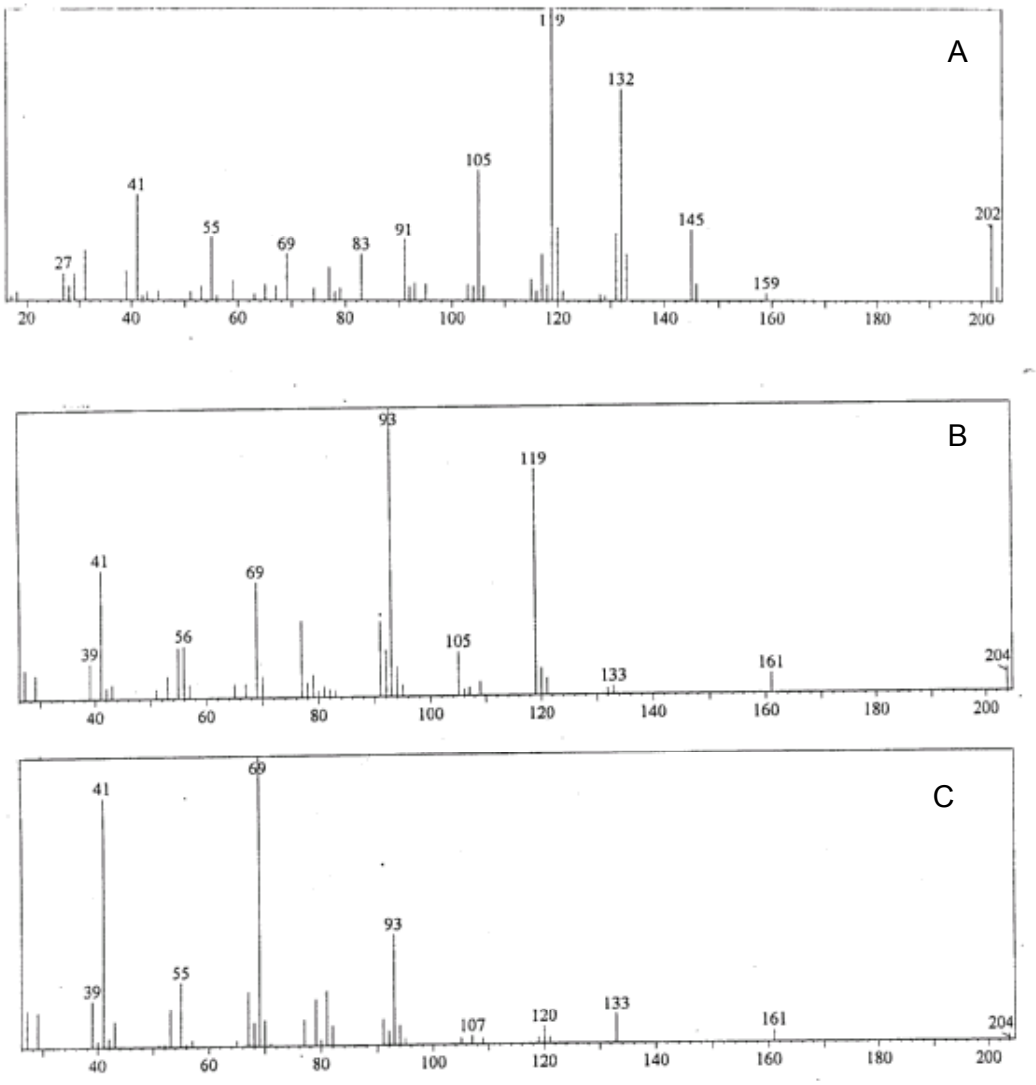


Figure 2.5. Mass spectra of (A). *ar*-Curcumene, (B). α-Zingiberene and (C). β-*trans*-Farnesene, from CRTO oil, fractions I and II;
[X-axis: m/z, Y-axis: Relative abundance (%)].

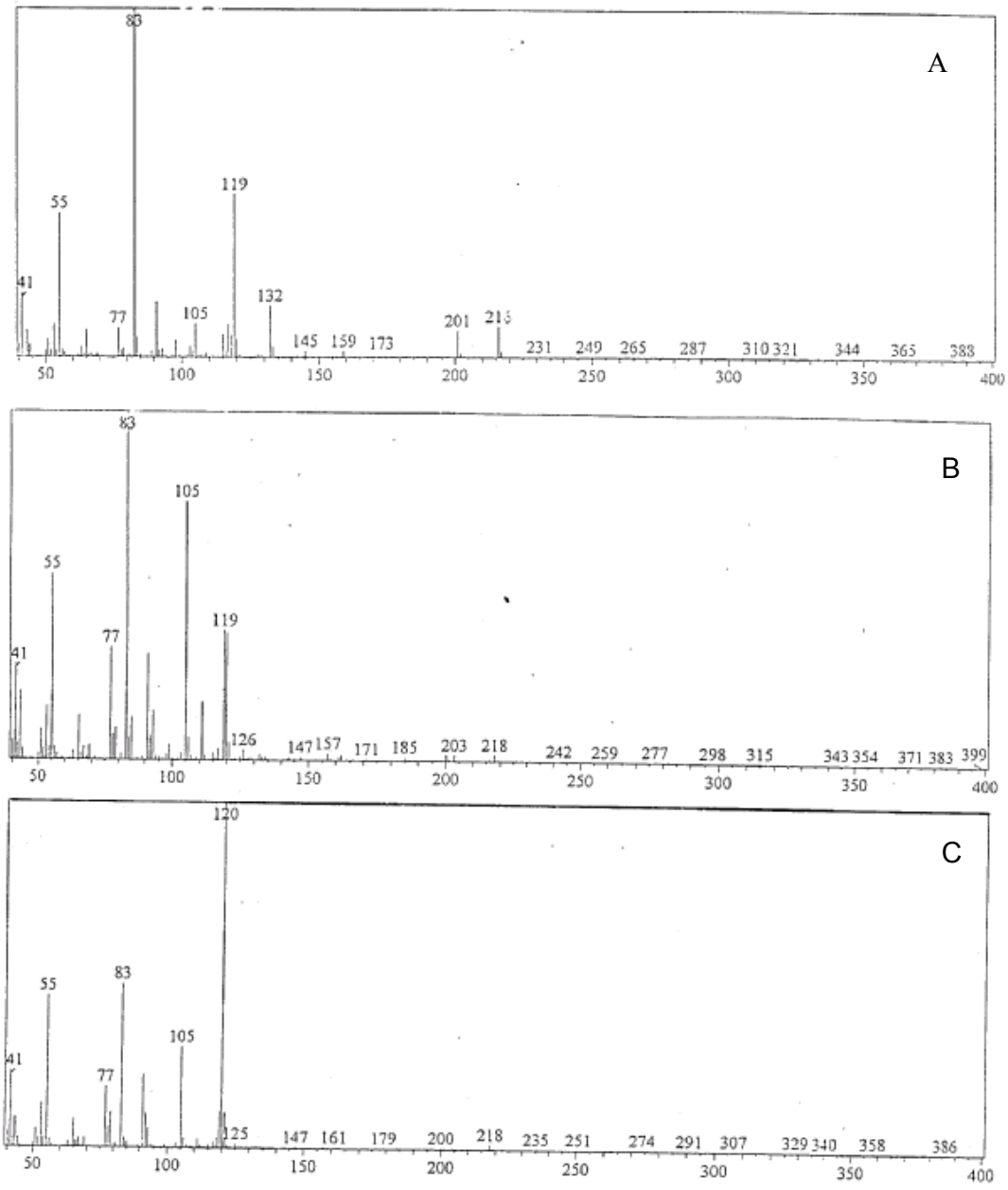


Figure 2.6. Mass spectra of (A). *ar*-Turmerone, (B). Turmerone and (C). Curlone from CRTO oil, fractions I and II;

[X-axis: m/z, Y-axis: Relative abundance (%)].

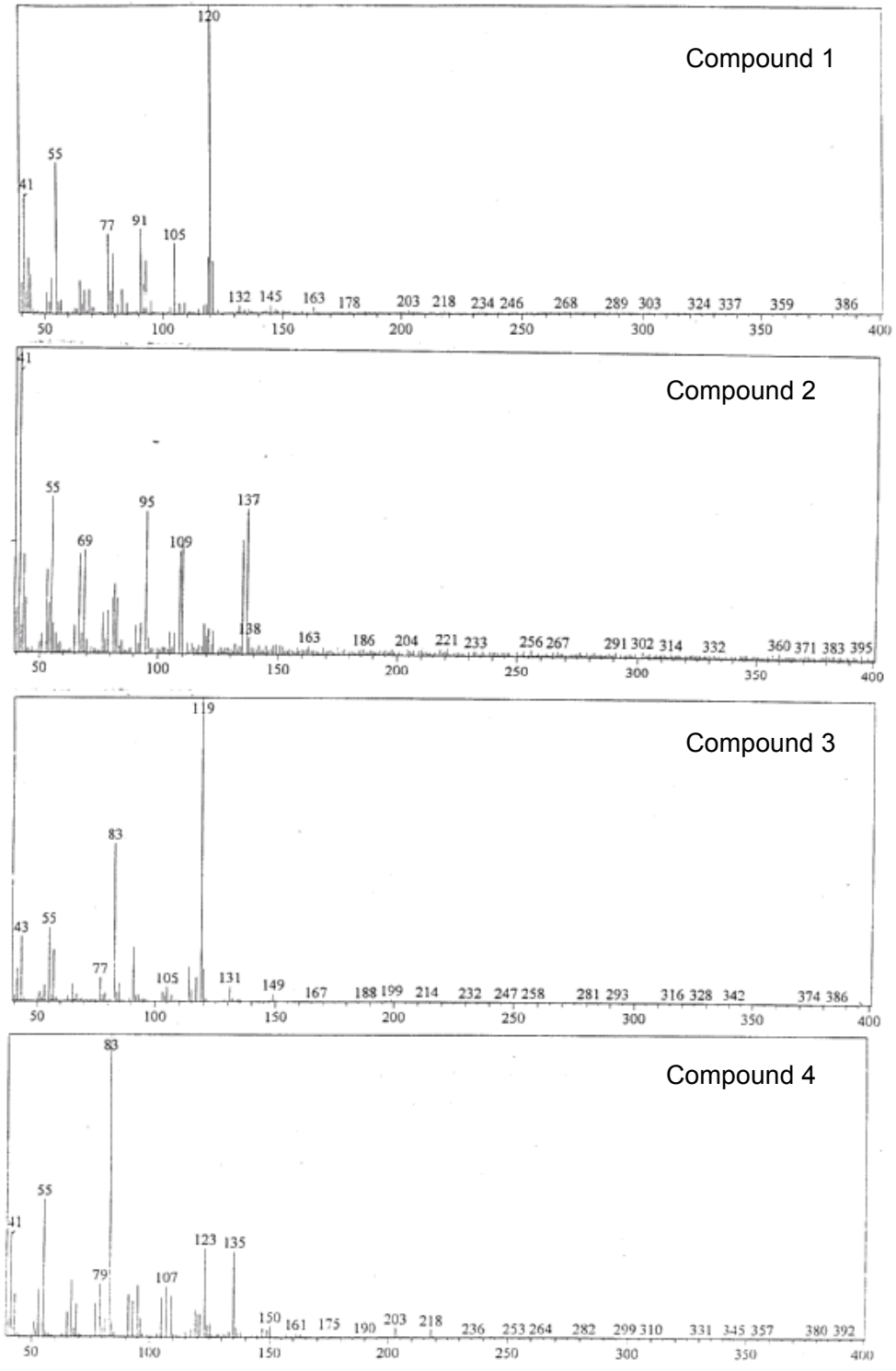
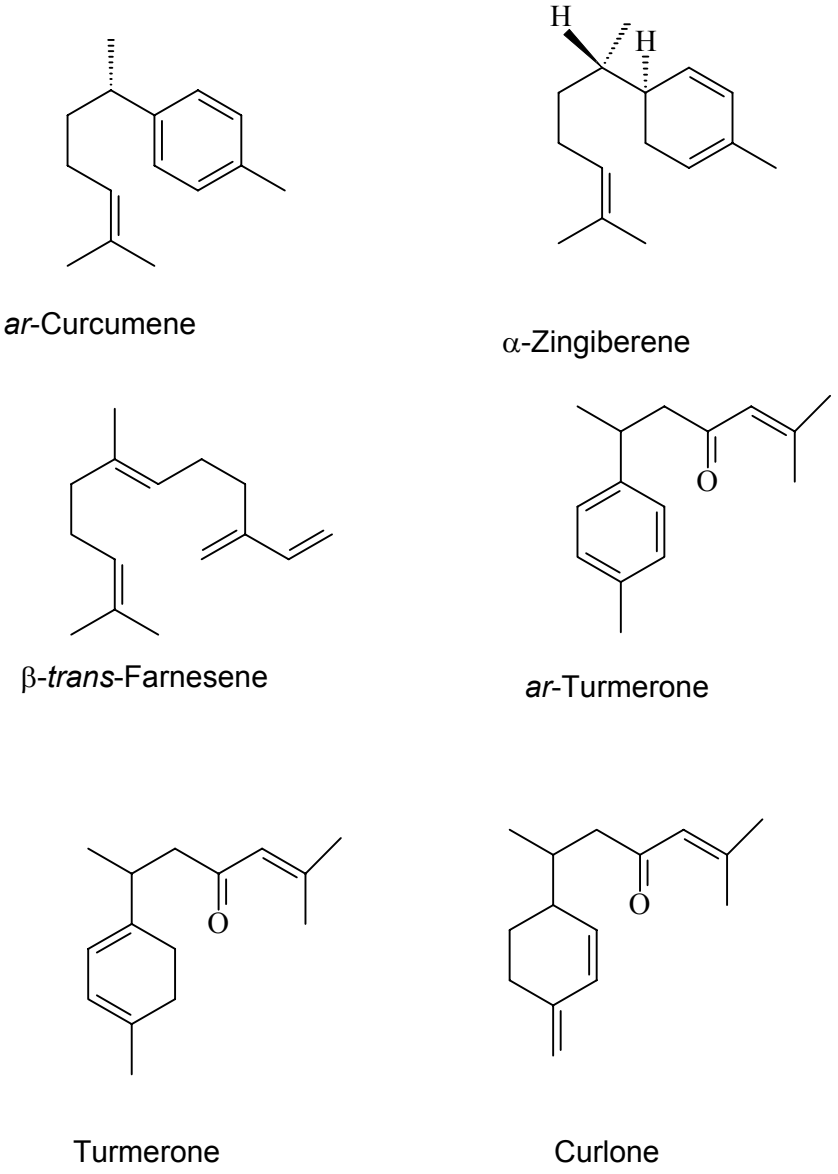


Figure 2.7. Mass spectra of Compounds (1-4) from CRTO oil, fractions I and II; [X-axis: m/z, Y-axis: Relative abundance (%)].



ar-Curcumene

α-Zingiberene

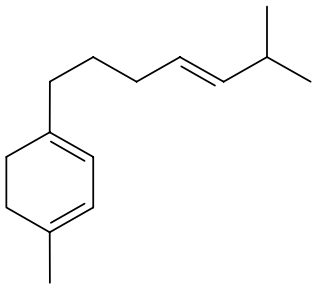
β-*trans*-Farnesene

ar-Turmerone

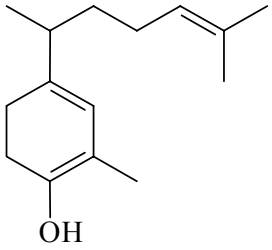
Turmerone

Curlone

Figure 2.8. Structures of major compounds identified in CRTO oil and its fractions (Hiserodt et al., 1996).



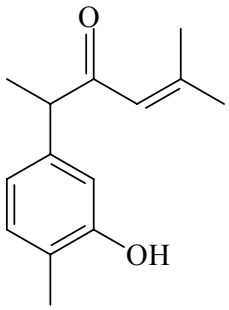
Compound 1 (MW = 204)



Compound 2 (MW = 220)

Unidentified component

Compound 3 (MW = 216)



Compound 4 (MW = 218)

Figure 2.9. Structures of compounds identified in CRTO oil and its fractions (Hiserodt et al., 1996).

2.2. ISOLATION OF ANTIFUNGAL FRACTIONS AND DETERMINATION OF CHEMICAL COMPOSITION

INTRODUCTION

The use of chemicals to enhance the safety of many foods is of great interest to the food industry. Chemical preservatives vary in their ability to kill microorganisms. Effectiveness depends on the types of microorganisms and also on the physical and chemical characteristics of foods (Cherry, 1999). However, the presence of chemical residues in foods and labeling of preservatives on food packages are major concerns to consumers these days. Therefore, the need for naturally derived compounds and other natural products with antimicrobial properties has been explored (Gould, 1996). The stability of some foods against attack by microorganisms is due to the fact that they contain naturally occurring substances with antimicrobial activity. Some spices are known to contain essential oils that possess antimicrobial activity, such as eugenol in cloves, allicin in garlic and cinnamic aldehyde and eugenol in cinnamon bark and leaf respectively (Hsieh et al., 2001).

Safety is a primary consideration for antifungal agents, especially for those in food products, which may be utilized in various quantities on a regular basis. However, the individual activity of antifungal agents characterized in plants is usually not potent enough to be considered for practical use. With the ever increasing demand from consumers for the use of natural compounds as additives / preservatives in food, there is a need to identify new natural sources of commercial significance that can meet consumer demand. It is therefore of interest to investigate the antifungal

properties of CRTO oil, a byproduct from curcumin manufacture. In this chapter, antifungal activity of CRTO oil and its fractions along with their chemical composition was reported.

MATERIALS AND METHODS

Materials

Second batch (5 Kg) of mother liquor/ curcumin removed turmeric oleoresin (CRTO) was obtained from M/S Flavours and Essences (P) Ltd, Mysore during the year 1999.

Isolation of Oil from CRTO

CRTO (100 g) was taken in a 2000 ml round bottom flask, 1000 ml of hexane was added and it was subjected to reflux for 1h at 60 °C. The extract was cooled to ambient temperature and filtered using Whatman No 1 filter. Solvent was removed from filtrate *under vacuum* at 30 °C (Büchi, Switzerland), which gave 56.0 ml of oil and designated as CRTO oil.

Fractional Vacuum Distillation

CRTO oil (20 ml) was taken in a 100 ml round bottom flask. The flask was kept in an oil bath and it was equipped with fractionating column (15 cm) and water condenser. The flask was heated slowly and the system was connected to vacuum pump. The first distillate (2.2 ml v/w) was collected at 80-110 °C under 10 mm vacuum. Similarly, the second distillate (3.4 ml v/w) was collected at 110-120 °C under 10 mm vacuum. The remaining part was not distilled.

Samples Preparation

CRTO oil, distillate I and II (250 mg each) were weighed and dissolved in propylene glycol separately and made up to 10 ml with propylene glycol (25 mg/ml).

Preparation of Agar Medium

Rose bengal chloramphenicol agar (32 g) (HiMedia, Mumbai) was dissolved in 1000 ml distilled water and 20 ml aliquots of agar medium were transferred into 100 ml conical flasks and sterilized at 121 °C for 20 min.

Microbial Culture and Preparation of Spore Suspension

Antifungal activity of CRTO oil and its distilled fractions were tested against *Aspergillus flavus, Aspergillus parasiticus, Fusarium moniliforme* and *Penicillium digitatum.* The fungal strains were maintained at 4 °C in potato dextrose agar (PDA) slants. Strains were grown in potato dextrose agar at 26 °C for 5 days and antifungal activity was evaluated by spore germination method (Paster et al., 1999). The spore suspension was adjusted with sterile saline to a concentration of approximately 1×10^3 spore /ml. The inoculum (spore suspension) was stored at 4 °C for further use.

Antifungal Activity

To the flasks containing 20 ml sterile agar and different concentrations of test material prepared in propylene glycol, 100 μl of spore suspension was inoculated aseptically. In case of control, the equivalent amount of propylene glycol was used in place of test material. The number of spores germinated was counted at regular intervals and germination of spores was expressed as percentage of control.

GC Analysis

GC analyses of CRTO oil, distillate I and II were carried out using a Shimadzu GC-15A (Kyoto, Japan) chromatograph equipped with a SE-30 column $(10' \times 1/8'')$ as per the method described in Part B, Chapter 2.1, page No. 171.

GC-MS Analysis

CRTO oil, distillates I and II were analyzed using a Shimadzu GC-17A (Kyoto, Japan) chromatograph equipped with a QP-5000 (Quadrupole) mass spectrometer as per the method described in Part B, Chapter 2.1, page No. 171.

RESULTS AND DISCUSSION

CRTO was extracted with hexane to separate CRTO oil (56% v/w). The CRTO oil was subjected to vacuum distillation and two fractions were collected. First distillate was collected at 80-110 °C under vacuum (yield 11% v/v). Similarly, the second distillate was collected at 110-120 °C under vacuum (yield 17% v/v). Remaining portion was non-volatile fraction, may be due to the presence of fixed oil in raw turmeric (Dandekar and Gaikar, 2002).

The spore germination assay of *Aspergillus flavus, A. parasiticus, Fusarium moniliforme* and *Penicillium digitatum* against CRTO oil, distillate I and II was carried out and the results (bar graphs) are presented in Figure 2.10. Distillate II was more effective than distillate I in inhibition of germination of spores. In case of *Penicillium digitatum,* distillate II inhibited complete spore germination till 4 days at a concentration of 1.5 mg/ml, whereas 6 mg/ml is required for *Aspergillus flavus*. Similarly, complete spore germination inhibition by distillate II was up to 3 days in case

of *A. parasiticus* and *Fusarium moniliforme* at 6 mg/ml concentration. CRTO oil was less effective than distillate II and it was able to suppress the complete spore germination up to 3 days in case of *Penicillium digitatum* and *A. parasiticus* at 3 and 6 mg/ml concentration respectively. DistIllate I was least effective and was not able to suppress spore germination completely even at 6 mg / ml for *A. parasiticus* and *Fusarium moniliforme,* whereas *Penicillium digitatum* and *Aspergillus flavus* spore germination suppressed completely at 6 mg/ml up to 2 days. Further, it was observed that *Penicillium digitatum,* spore germination was suppressed completely up to 3 days at the same concentration, whereas growth is observed with *Aspergillus flavus* on third day.

The chemical constituents of CRTO oil, distillate I and II were analysed by GC and GC-MS. Retention indices of all the compounds were determined using *n*- alkanes as standards (Jennings and Shibamoto, 1980). The compounds were identified by comparison of Kovats indices with reported values (Davies, 1990) and by matching with NIST-MS library (NIST62-Lib.) and published mass spectra (Adams, 1989; Hiserodt et al., 1996). Figures 2.11, 2.12 and 2.13 show total ion chromatograms of CRTO oil, distillate I and II respectively. Table 2.3 shows chemical constituents of CRTO oil, distillate I and distillate II, and their Kovats indices. The data indicated the presence of twenty-five compounds in CRTO oil, distillate I and II. The mass spectra of major and unidentified compounds are presented in Figures 2.5 - 2.7, 2.14 and 2.15. *ar*-Turmerone (21.4%), α-zingiberene (15%), β-(Z)-farnesene (13.96%), *ar*-curcumene (10.3%), turmerone (6.2%) and curlone (5.1%) were found to be the major compounds in CRTO oil. Distillate I contained α-zingiberene (28%), β- (Z)-farnesene (21%), *ar*-curcumene (18%) whereas distillate II contained *ar*- turmerone (52.6%),

Table 2.3. Chemical composition of CRTO oil, distillate I and II

Peak No	Compound	Peak Areas (%)			KI	Identification by
		CRTO oil	Distillate I	Distillate II		
1	α-Phellandrene	0.42	0.92	Tr.	998	MS, KI
2	*p*-Cymene	0.35	0.40	Tr.	1008	MS, KI
3	1,8-Cineole	1.24	1.30	Tr.	1024	MS, KI
4	*trans*-Ocimene	0.36	0.40	Tr.	1044	MS, KI
5	α-Terpineol	0.22	0.30	Tr.	1149	MS, KI
6	α-*cis*-Bergamotene	2.27	3.10	Tr.	1376	MS, KI
7	Caryophyllene	Tr.	0.25	Tr.	1377	MS, KI
8	*ar*-Curcumene	10.30	18.00	3.02	1447	MS, KI
9	α-Zingiberene	15.03	28.00	4.12	1467	MS, KI
10	β-Bisabolene	3.55	5.50	1.22	1476	MS, KI
11	β-(*Z*)-Farnesene	13.96	21.00	4.71	1493	MS, KI
12	*cis*-γ-Bisabolene	0.99	2.40	0.34	1500	MS, KI
13	Caryophyllene oxide	1.07	1.50	Tr.	1555	MS, KI
14	*ar*-Turmerol	0.52	1.10	0.89	1570	MS, KI
15	Dehydrocurcumene	2.31	3.00	1.00	1576	MS
16	Compound (1)*	0.91	0.60	1.90	1589	MS
17	*ar*-Turmerone	21.40	3.50	52.60	1628	MS, KI
18	Turmerone	6.20	1.00	11.50	1632	MS, KI
19	Curlone	5.10	1.10	8.50	1655	MS, KI
20	Compound (4)*	0.42	---	3.80	1710	MS
21	Compound (5)*	Tr.	---	0.82	1733	MS
22	Compound (6)*	Tr.	---	0.74	1862	MS
23	Compound (7)*	Tr.	---	0.43	1867	MS
24	Hexadecanoic acid	Tr.	---	0.33	1946	MS
25	Heptadeconic acid	0.26	---	0.50	2124	MS

MS : Identification based on mass spectral data.

KI : Kovats indices on SPB column.

Tr. : Less than 0.09%.

RI : Retention indices

* : see Figure 2.16 structures of compounds (1, 4-7); MS was compared with that of Hiserodt et al., (1996).

turmerone (11.5%) and curlone (8.5%) as the major components. Further, oxygenated compounds (1, 4-7; Figure 2.16) were enriched in distillate II. Table 2.2 and 2.3 shows slightly different chemical composition of CRTO oil obtained by hexane

extraction of CRTO in two different batches. This may be due to the different varieties of raw materials used for the extraction of curcuminoids in different batches.

The comparison of chemical composition showed that the distillate II has high concentration of *ar*-turmerone, turmerone, curlone and compounds (4-7; Figure 2.16) as compared to CRTO oil. High antifungal activity of distillate II may be attributed to these compounds. *ar*-Turmerone has been implicated in many biological activities (Baik et al., 1993; Kitahara et al., 1993; Ferreira et al., 1992; Whalon et al., 1998; Helen et al., 1982; Roth et al., 1998). Probably *ar*-turmerone alone or in synergy with turmerone, curlone and compounds (4-7) is responsible for higher antifungal activity of this fraction. Distillate II is more effective antifungal agent as compared to CRTO oil. Distillate I have the least concentration of *ar*-turmerone and it has least antifungal activity. A positive correlation was observed between antifungal activity and concentrations of *ar*-turmerone, turmerone, curlone and compounds (4-7).

CONCLUSION

The study demonstrated antifungal activity of CRTO oil, distillate I and II, against *Aspergillus flavus, Aspergillus parasiticus, Fusarium moniliforme* and *Penicillium digitatum.* Hexane extract of CRTO was subjected to fractional vacuum distillation to get two distillates. The chemical composition of CRTO oil and its distillates was characterized. Further, studies are required to study the antifungal activity in foods and mechanisms of activity for specific microorganisms. Distillate II was found to exhibit a high degree of antifungal activity and has the potential to be used as a preservative.

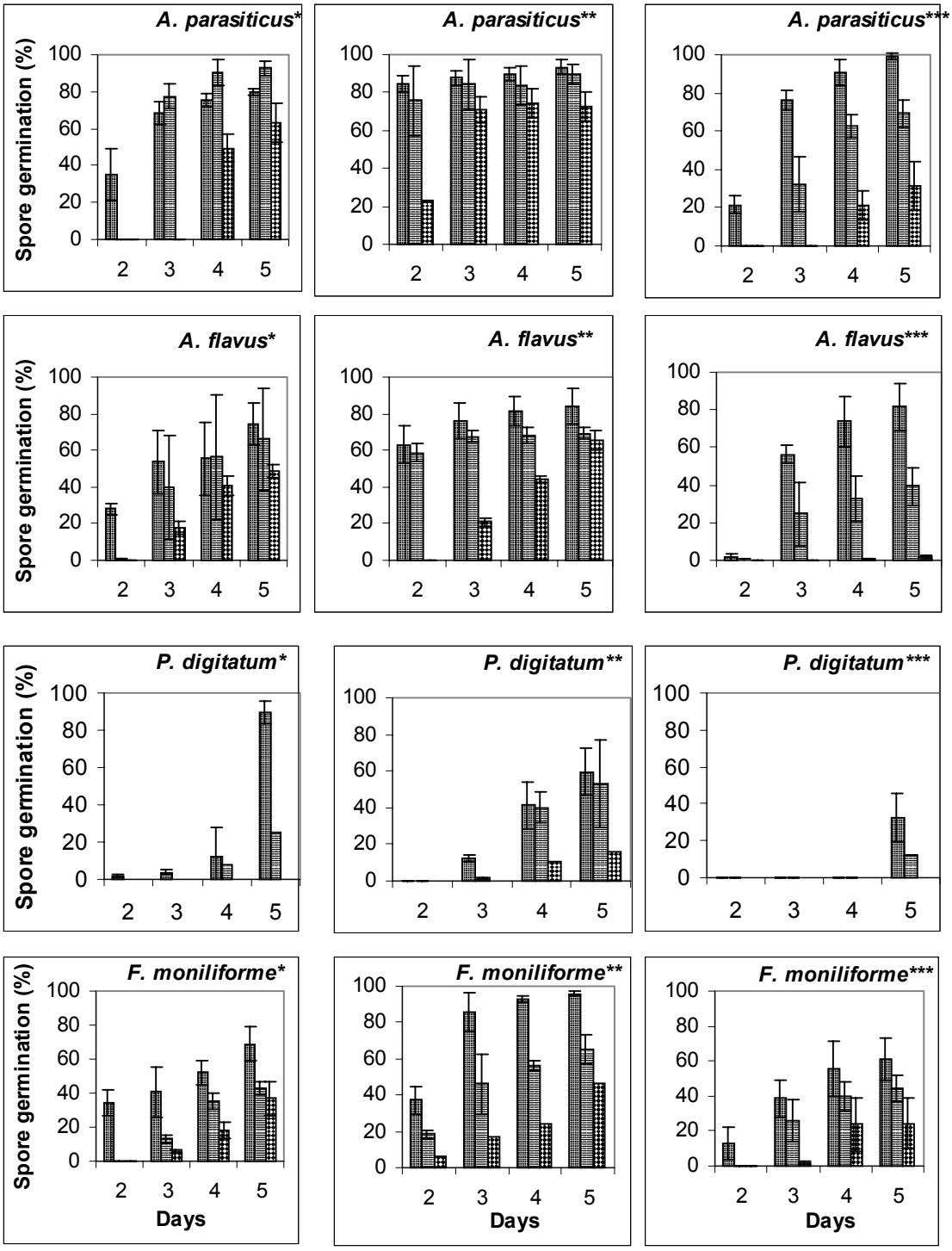


Figure 2.10. Effect of CRTO oil (*), distillate I (**) and II (***) on spore germination of different fungi; X-axis: Number of days; Y-axis: Spore germination (%)

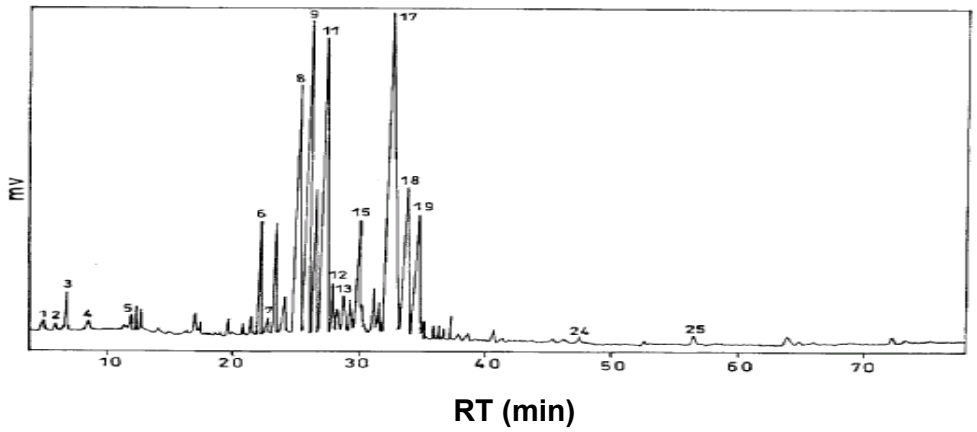


Figure 2.11. GC-MS total ion chromatogram of CRTO oil obtained by hexane extraction of CTRO (2nd batch) at 60 °C.

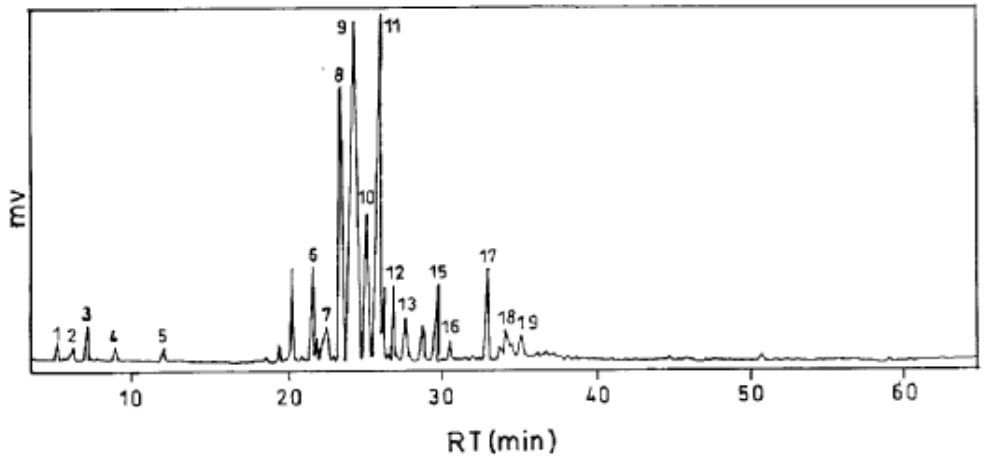


Figure 2.12. GC-MS total ion chromatogram of distillate I obtained by fractional vacuum distillation.

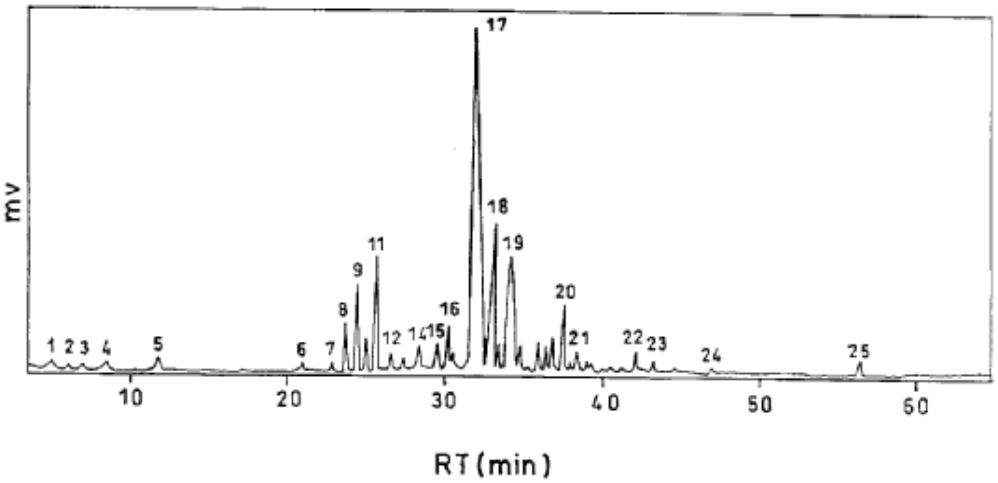


Figure 2.13. GC-MS total ion chromatogram of distillate II obtained by fractional vacuum distillation.

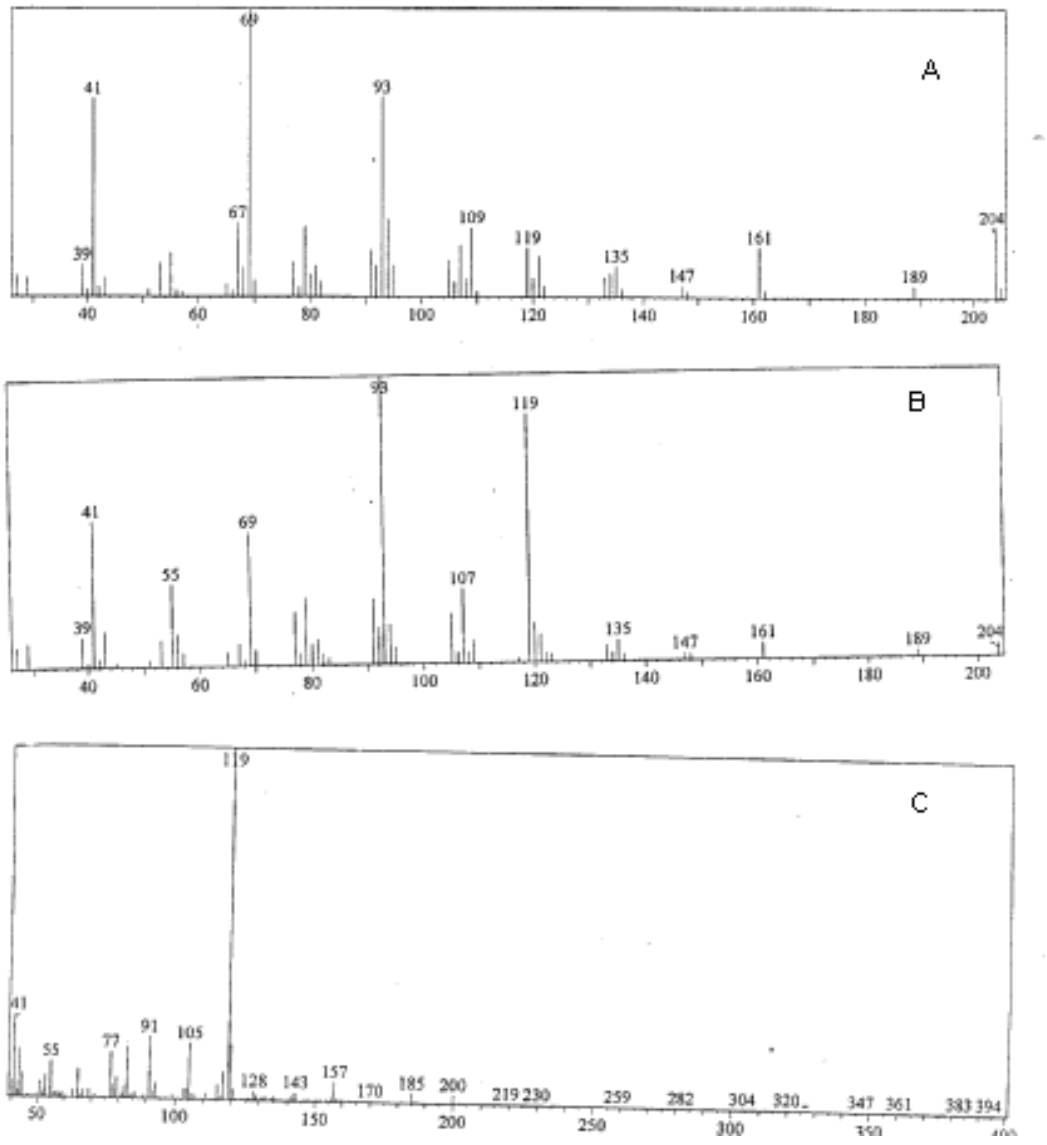


Figure 2.14. Mass spectra of (A). β-Bisabolene (B). *cis*-Bergamotene and (C). Dehydrocurcumene from CRTO oil, distillates I and II;

[X-axis: m/z, Y-axis: Relative abundance (%)].

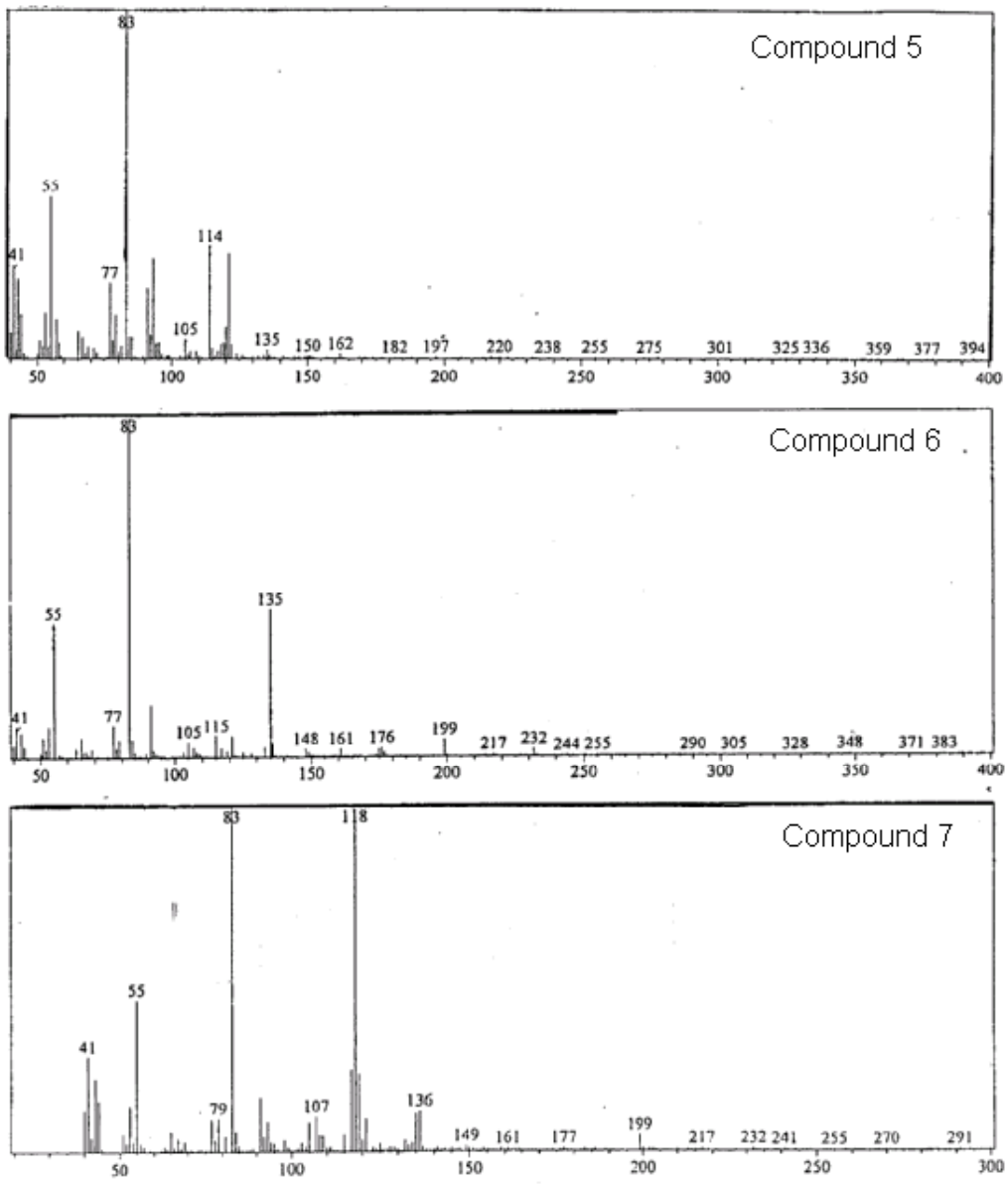
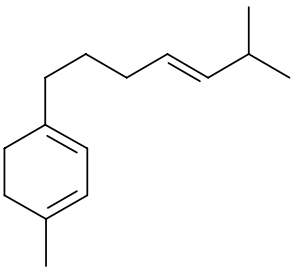
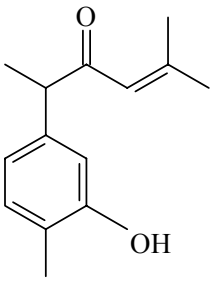


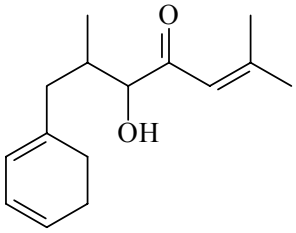
Figure 2.15. Mass spectra of Compounds (5-7) from CRTO oil, distillate I and II; [X-axis: m/z, Y-axis: Relative abundance (%)].



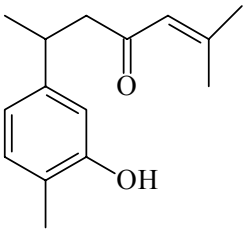
Compound 1
(MW = 204)



Compound 4
(MW = 218)



Compound 5
(MW = 234)



Compound 6
(MW=232)

Unidentified compound = 7
(MW = 216)

Figure 2.16. Structures of compounds identified in CRTO oil, distillate I and II (Hiserodt et al., 1996).

2.3. ISOLATION OF ANTIOXIDANT AND ANTIMUTAGENIC FRACTIONS AND CHARACTERISATION OF CHEMICAL COMPOSITION

INTRODUCTION

Lipid peroxidation is one of the major reasons for deterioration of food products during the processing and storage. The addition of antioxidants is a method of increasing the shelf life, especially of lipids and lipid containing foods. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have restricted use in foods as these synthetic antioxidants are suspected to be carcinogenic (Madhavi and Salunkhe, 1995). Therefore, the importance for the search of natural antioxidants, especially of plant origin has greatly increased in recent years (Loliger, 1991).

In addition to exogenous factors, the generation of reactive oxygen species (ROS) is associated with life under aerobic conditions and produced under normal metabolism and pathophysiological conditions. ROS rapidly interact with biomolecules such as lipids; proteins and DNA to induce membrane damage, oxidation of low-density lipoprotein, oxidation and denaturation of proteins, inactivation of enzymes, breakage of DNA strand and its modification (Cross et al., 1987). The consequences of such extensive damages are involved in the development of various degenerative disease states like atherosclerosis, cardiovascular disease, cataract, tissue damage in rheumatoid arthritis, decline in immune function, dysfunction of brain, and certain types of cancer (Ames et al., 1993; Halliwell, 1994; Basu et al., 1999). It has been

estimated that under normal metabolic conditions, the DNA in each cell of our body is exposed to 10,000 oxidative hits per day leading to the formation of more than twenty different oxidative DNA lesions. Many of these lesions are known to cause mutations, which may contribute to carcinogenesis (Ames et al., 1993). ROS produced by certain chemical carcinogens play a role in carcinogenic process (Cerutti, 1985). Besides the endogenous defenses, consumption of dietary antioxidants could be an important aspect of body's defense mechanism to protect against ROS (Rimm et al., 1993; Willet, 1994; Frei, 1994) and also many antioxidants are being identified as anticarcinogens (Ames, 1983; Frei, 1994).

It is important to note that most of the investigations regarding inhibitory effect of food component on the oxidative damages of biomolecules have been devoted to the foods of plant origin. Diets rich in fruits and vegetables have been associated with lower incidence and mortality rates of cancer (Dragsted et al., 1993; Keys, 1995) and low dietary intake of the same doubles the risk of cancer as compared to high intake (Ames, 1993).

The continuing research indicates that turmeric and its active principle curcumin have unique antioxidant, antimutagenic, antitumerogenic, and anticarcinogenic, anti-inflammatory, antiarthritic, antimicrobial and hypocholesterolemic properties as reviewed elsewhere (Majeed et al., 1995; Miquel et al., 2002). There are number of reports available on turmeric powder and curcumin, but there are no reports on CRTO oil. In this chapter, the antioxidant and antimutagenic activities of CRTO oil and its fractions along with their chemical composition have been reported.

MATERIALS AND METHODS

Materials

Second lot of Mother liquor/ curcumin removed turmeric oleoresin (CRTO) was obtained from M/S Flavours and Essences (P) Ltd, Mysore during the year 1999.

Extraction

CRTO (50 g) and 500 ml of hexane was taken in 1000 ml conical flask. The content was stirred using a magnetic stirrer for 30 min at ambient temperature. The mixture was filtered through a glass filter under water suction using Whatman no 1 filter paper. The residue was re-extracted twice with 100 ml of hexane for 10 min each until no colour was observed in the supernatant. The extracts obtained were pooled and concentrated *under vacuum* at 30 °C, which gave 21.1 ml of CRTO oil.

Fractionation of CRTO Oil

CRTO oil (7 ml) was impregnated with 14 g of silica gel and chromatographed on silica gel (120 g) in a glass column (600 × 30 mm). The column initially equilibrated with hexane and eluted with 1500 ml each of hexane, hexane:benzene (1:1) and benzene. The solvents from elutes were evaporated *under vacuum* to get three fractions I, II and III, the yields of which were 1.4, 2.5 and 2.3 ml (v/w) respectively.

Samples Preparation

CRTO oil and its fractions (20 mg of each) were dissolved in acetone and made up to 10 ml with acetone (2 mg/ml) and used for antioxidant activity. CRTO oil and its fractions (250 mg of each) were dissolved in propylene glycol and made up to 25 ml with propylene glycol (10 mg/ml) and used for antimutagenic activity.

HPLC Analysis of Curcuminoids

The curcuminoids in the CRTO oil and its fractions were analysed by HPLC as described in a separate chapter (Part B, Chapter 3.2).

Antioxidant assay by β-Carotene-linoleate Model System

The antioxidant activity of CRTO oil and its column fractions were evaluated by the β-carotene-linoleate model (Jayaprakasha and Jaganmohan Rao, 2000). 0.2 mg of the β-carotene in 0.5 ml of chloroform, 20 mg of linoleic acid and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed together. The chloroform was removed at 40 °C *under vacuum* using a rotary evaporator. The resulting solution was immediately diluted with 10 ml of triple-distilled water and the emulsion was mixed well for 1 min. The emulsion was further diluted with 40 ml of oxygenated water before use. 4 ml aliquots of this mixture were transferred into different tubes containing 0.2 ml of samples and butylated hydroxyanisole (BHA) at 100 ppm concentrations in ethanol. A control containing 0.2 ml of ethanol and 4 ml of the above mixture was prepared. The tubes were kept at 50 °C in a water bath during the incubation period.

Optical density (OD) at 470 nm was recorded for the assay solutions immediately and at 15 min intervals until the colour of β-carotene disappeared in the control. The antioxidant activity (AA) of the samples were evaluated in terms of bleaching the β-carotene using the following formula. $AA = 100[1-(A_o-A_t)/(A^o_o - A^o_t)]$ where A_o and A^o_o are the OD measured at zero time of the incubation for test samples, BHA and control, respectively. A_t and A^o_t are the OD measured for test samples BHA and control, respectively, after incubation for 90 min. All determinations were performed in triplicate averaged.

Antioxidant Capacity by Phosphomolybdenum Method

The total antioxidant capacity of CRTO oil and its fractions was evaluated by the method of Prieto et al. (1999). An aliquot of 0.1 ml of sample (100 ppm) solution was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to ambient temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in Genesys-5-UV spectrophotometer (Milton Roy, New York, USA). A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and which was incubated under same conditions as rest of the sample. For samples of unknown composition, fat-soluble antioxidant capacity was expressed as equivalents of α-tocopherol (μmol/g of CRTO oil or its fractions).

Media preparation

Media for antimutagenic activity was prepared as described in part A, Chapter 3.4, Page No. 124.

Antimutagenicity assay

The standard plate incorporation test was carried out according to Maron and Ames (1983). In the antimutagenicity test, the inhibitions of mutagenic activity of the sodium azide by the test samples were determined. Two millilitres of top agar was distributed into 13 $\times$ 100 mm capped culture tubes held at 45 °C in a heating block.

Different concentrations of CRTO oil and its column fractions (625, 1250 and 2500 μg/plate) in 0.1 ml of propylene glycol, 0.1 ml of 10 h old culture of *Salmonella typhimurium* TA-100 were added. The bacteria was left at 45 °C for a few min without loss of viability and mixed by vortexing at low speed and then poured onto a minimal glucose agar plate quickly the plate was tilted to form a thin layer. This step is important to achieve a uniform distribution of the top plate. Positive and negative controls were also included in each assay. Sodium azide was used as a diagnostic mutagen (1.5 μg / plate) in positive control plates. Negative controls were prepared with equivalent amount of propylene glycol instead of sodium azide and test samples, which is required to establish the number of colonies that arise spontaneously for *S. typhimurium* TA-100. The number of histidine^{+} (His^{+}.) revertants colonies were counted after incubation of the plates at 37 °C for 48 h. Each sample was assayed using duplicate plates and the data presented as mean ± SD of three independent assays. The mutagenicity of sodium azide in the absence of test samples was defined as 100% or 0% inhibition. The calculation of % inhibition was done according to the formula given by Ong et al (1986), % inhibition = [1-T/M] x 100 where T is number of revertants per plate in presence of mutagen (sodium azide) and test samples and M is number of revertants per plate in positive control (sodium azide). The number of spontaneous revertants was subtracted from numerator and denominator.

The antimutagenic effect was considered moderate when the inhibitory effect was 25-40% and strong when more than 40%. Inhibitory effects of less than 25% were considered as weak and was not recognised as positive result (Ikken et al., 1999).

GC Analysis

GC analysis of CRTO oil and fraction I, II and III were carried out using a Shimadzu GC-15A (Kyoto, Japan) chromatograph equipped with a SE-30 column (10' × 1/8") as per the method described in Part B, Chapter 2.1, Page No. 171.

GC-MS Analysis

CRTO oil and fraction I, II and III were analysed using a Shimadzu GC-17A (Kyoto, Japan) chromatograph equipped with a QP-5000 (Quadrupole) mass spectrometer as per the method described in Part B, Chapter 2.1, Page No. 171.

RESULTS AND DISCUSSION

It was found that CRTO contains substantial amount of CRTO oil (42.2% v/w), which is comparable with earlier reports (Saju et al*.,* 1998). The low yield of CTRO oil as compared to earlier (Chapter 2.2) may be due to ambient extraction, whereas in hot extraction of CRTO yielded 56% oil. Hence, hot extraction may be good for the maximum CRTO oil extraction. Then, the CRTO oil was fractionated using silica gel column chromatography, which yielded 20, 35.7 and 32.9% of fractions I, II and III, respectively.

The presence of curcuminoids was analysed in CRTO oil and its fractions by HPLC method (details of the method development is discussed in part B, Chapter 3.2). The curcuminoids were found in CRTO oil to the extent of 0.15%, but the curcuminoids were absent in column fractions I, II and III (Figure 2.17). This may be due to the fractionation on silica gel using hexane: benzene as eluents. In this study, in the antioxidant and antimutagenic activities of CRTO oil and its fractions (devoid of

curcuminoids) are of interest, therefore the column was not eluted with more polar solvents.

The antioxidant activity of CRTO oil and its fractions was carried out using coupled oxidation of β-carotene and linoleic acid, which was compared with antioxidant activity of BHA (Figure 2. 18). The addition of CRTO oil, column fractions I, II, III and BHA at 100 ppm concentrations prevents the bleaching of β-carotene to different degrees. CRTO oil and its fractions I, II, and III showed 65, 43, 46 and 49% antioxidant activity respectively, at 100 ppm concentration. The marked antioxidant activity of CRTO oil may be due to the presence of curcuminoids (0.15%), which were co-extracted along with CRTO oil from CRTO. However, the decreasing order of antioxidant activity among the fractions was found to be III>II>I.

The quantitative antioxidant capacity of the CRTO oil and its fractions were measured spectrophotometrically by the phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample and the formation of Mo (V) phosphate complex (green colour) with a maximum absorption at 695 nm (Prieto et al., 1999). The antioxidant capacity of CRTO oil and its fractions was found to decrease in the order of fractions III>CRTO oil>II>I (Figure 2.19). The decreasing order of antioxidant capacity of CRTO oil and its fractions is not absolutely correlating with the order of antioxidant activity found in β-carotene-linoleate model system. The active principle in turmeric is a group of phenolic compounds including curcumin, which is very well known for its strong antioxidant activity (Miquel et al., 2002). However, in the present studies, the CRTO oil and its fractions are also found to be

effective antioxidants. The antioxidant activity of CRTO oil and its fractions may be due to their additive or synergetic actions of the compounds present in the oil.

The protective action of CRTO oil and its fractions against the mutagenicity of sodium azide was evaluated by the Ames test using *S. typhimurium* TA-100, as presented in Figure 2.20. It appears that, the antimutagenicity of CRTO oil and its column fractions increases with doses. CRTO oil and its fractions showed an antimutagenicity ranging from weak to strong and even 100%, depending on concentration of the test samples. The order of antimutagenic activity of CRTO oil and its fractions was found to be III>CRTO oil> II>I at the concentration tested.

The chemical composition of CRTO oil, column fractions I, II and III were determined by GC and GC-MS. Figures 2.21, 2.22, 2.23 and 2.24 shows the total ion chromatograms of CRTO oil, fractions I, II and III respectively. Retention indices for all the compounds were determined using *n*-alkanes as standards (Jennings and Shibamato, 1980). The compounds were identified by comparison of retention indices with those reported in the literature (Jennings and Shibamato, 1980; Davies, 1990) and by matching their mass spectral fragmentation patterns with those stored in the spectrometer database, using the NIST62-Lib (Shimadzu Corporation, Japan) MS library or comparison of MS data with those reported in literature (Jennings and Shibamato, 1980; Ten Noever Bravw et al., 1988; Adams, 1989). Table 2.4 shows the Kovats indices and chemical constituents of CRTO oil and its column fractions. The GC-MS data indicated the presence of 15 compounds in CRTO oil and fractions I, II and III. It was found that *ar*-turmerone, turmerone were found to be the major compounds in CRTO oil and its fractions. The mass spectra of major and unidentified

components from CRTO oil and its fractions are presented in Figures 2.5-2.7, 2.14-2.16 and 2.25. *ar*-Turmerone (37.41%), α-zingiberene (3.78%), β-(Z)-farnesene (3.76%), *ar*-curcumene (7.4%), turmerone (18.01%) and curlone (11.58%) were found to be the major compounds in CRTO oil. Fraction I

Table 2.4. Chemical composition of CRTO oil and its column fractions

Peak No.	Compound	Peak areas (%)				KI	Identification method
		CRTO oil	Fra. I	Fra. II	Fra. III		
1	Caryophyllene	3.46	3.70	0.82	Tr.	1428	MS, KI
2	*ar*-Curcumene	7.40	23.54	12.15	0.34	1446	MS, KI
3	α-Zingiberene	3.78	5.17	4.44	0.55	1463	MS, KI
4	β-Bisabolene	0.92	4.52	2.44	0.35	1476	MS, KI
5	β-Farnesene	3.76	14.86	3.40	0.31	1493	MS, KI
6	Compound (8)*	0.84	3.49	2.77	0.21	1510	MS
7	Compound (1)*	1.07	0.84	0.98	0.74	1589	MS
8	*ar*-Turmerone	37.41	23.68	44.75	49.95	1628	MS, KI
9	Turmerone	18.01	2.49	4.80	12.21	1632	MS, KI
10	Curlone	11.59	9.57	13.52	21.57	1655	MS, KI
11	Compound (3)*	1.46	0.81	0.76	0.93	1690	MS
12	Compound (4)*	3.36	0.99	1.59	2.93	1710	MS
13	Compound (6)*	1.22	1.09	1.93	2.30	1733	MS
14	Compound (7)*	0.75	Tr.	0.92	1.87	1867	MS
15	Compound (9)*	0.45	Tr.	Tr.	0.71	1875	MS

MS : Mass spectra

KI : Kovats indices

* : see Figure 2.26 Structures of compounds; MS was compared with that of Hiserodt et al., (1996).

contained α-zingiberene (5.17%), β-(Z)-farnesene (14.86%), *ar*-curcumene (23.54%) whereas fraction II contained *ar*-turmerone (44.75%), turmerone (4.8%) and curlone (13.52%) as the major components. Fraction III contained *ar*-turmerone (49.95%), turmerone (12.21%) and curlone (21.57%) as the major

components. The comparison of chemical composition showed that the fraction III has high concentration of *ar*-turmerone, turmerone, curlone and unidentified compounds (6, 7, 9) (Figure 2.26) as compared to CRTO oil. Tables 2.3 and 2.4 show the chemical composition of CRTO oil in ambient and hot extraction respectively. The difference in composition may be due to partial extraction of CRTO oil during the ambient extraction.

ar-Turmerone, turmerone and curlone were found to be the major compounds present in the CRTO oil and its fractions in the decreasing order of fraction III (71.38%) > CRTO oil (67.01%)> fraction II (63.07%) > fraction I (35.74%). Synthetic turmerone has been reported to act as neoplasm inhibitor and anticarcinogenic (Baik et al., 1993). The extent of the presence of *ar*-turmerone and turmerone in CRTO oil and its fractions may be responsible for their antimutagenic activity. The active principle in turmeric is the curcumin, which has strong antimutagenic activity, both *in vitro* and *in vivo* (Goud et al., 1993). The presence of traces of curcuminoids in CRTO oil may also be responsible for its antimutagenicity.

It has been reported that mutation induced by numerous mutagens was reduced by active oxygen scavengers (Kim et al., 1991; Ueno et al., 1991). It has also been suggested that compounds, which possess antioxidant activity, can inhibit mutation and cancer because they can scavenge free radical or induce antioxidative enzyme (Hochstein and Atallah, 1988). Goud et al. (1993) observed significant increase of xenobiotic metabolizing enzymes such as UDP glucuronyl transferase and glutathione-S-transferase in the liver of turmeric fed rats. Similarly, curcumin an antioxidant, isolated from turmeric was shown to induce glutathione-S-transferase and

glutathione peroxidase, which are involved in detoxification of electrophilic products of lipid peroxidation and contribute to the anti-inflammatory and anticancer activities (Piper et al*.,* 1998). Azuine and Bhide (1992) found the protective effect of dietary turmeric on BP-induced forestomach and DMBA-induced skin tumors with simultaneous increase of glutathione level and activity of glutathione-S-transferase in the liver of mice.

Dietary supplementation of antioxidants present in fruits and vegetables are thought to decrease free radical attack on DNA and hence protect against mutation that causes cancer (Duthie et al*.,* 1996). However, the antioxidant, antimutagenic and other biological activities of curcuminoids have been reported (Majeed et al*.,* 1995; Miquel et al*.,* 2002). In the present study, CRTO oil and its fractions are having both antioxidant and antimutagenic activities. Further, it was observed that the decreasing order of antioxidant activity of CRTO oil and its fractions was almost equivalent to the decreasing order of their antimutagenicity i.e. fraction III > CRTO oil > fraction II > fraction I. The antioxidative properties of CRTO oil and its fractions have therefore played an important role with regard to their antimutagenicity, which seem to explain the action of their antimutagenicity. It has been observed that *ar*-turmerone and turmerone are the major constituents found in CRTO oil and its fractions, and fraction III was found to contain highest percentage of *ar*-turmerone and turmerone. Probably, *ar*-turmerone alone or in synergy with turmerone, curlone and compounds (3,4,6,7,9) (Figure 2.26) could be responsible for the highest antioxidant and antimutagenic activities of fraction III. Further work is required to assess the process of chemoprevention of mutation and carcinogenesis using CRTO oil with special

reference to its fraction III. Thus there will be a value addition to the byproduct of curcumin production, which has no commercial application at present.

CONCLUSION

The results of this study demonstrate that CRTO oil and its column fractions showed antioxidant and antimutagenic properties. Both the activities varied with the extent of fractionation. Moreover, antioxidant and antimutagenic activities of fraction III was greater than those of fraction I, II and CRTO oil. The antioxidative properties of CRTO oil and its fractions seem to explain the activity of antimutagenicity of various fractions. Further, work is required to determine the mechanism involved in the antimutagenic activity of CRTO oil and its fractions, especially for fraction III.

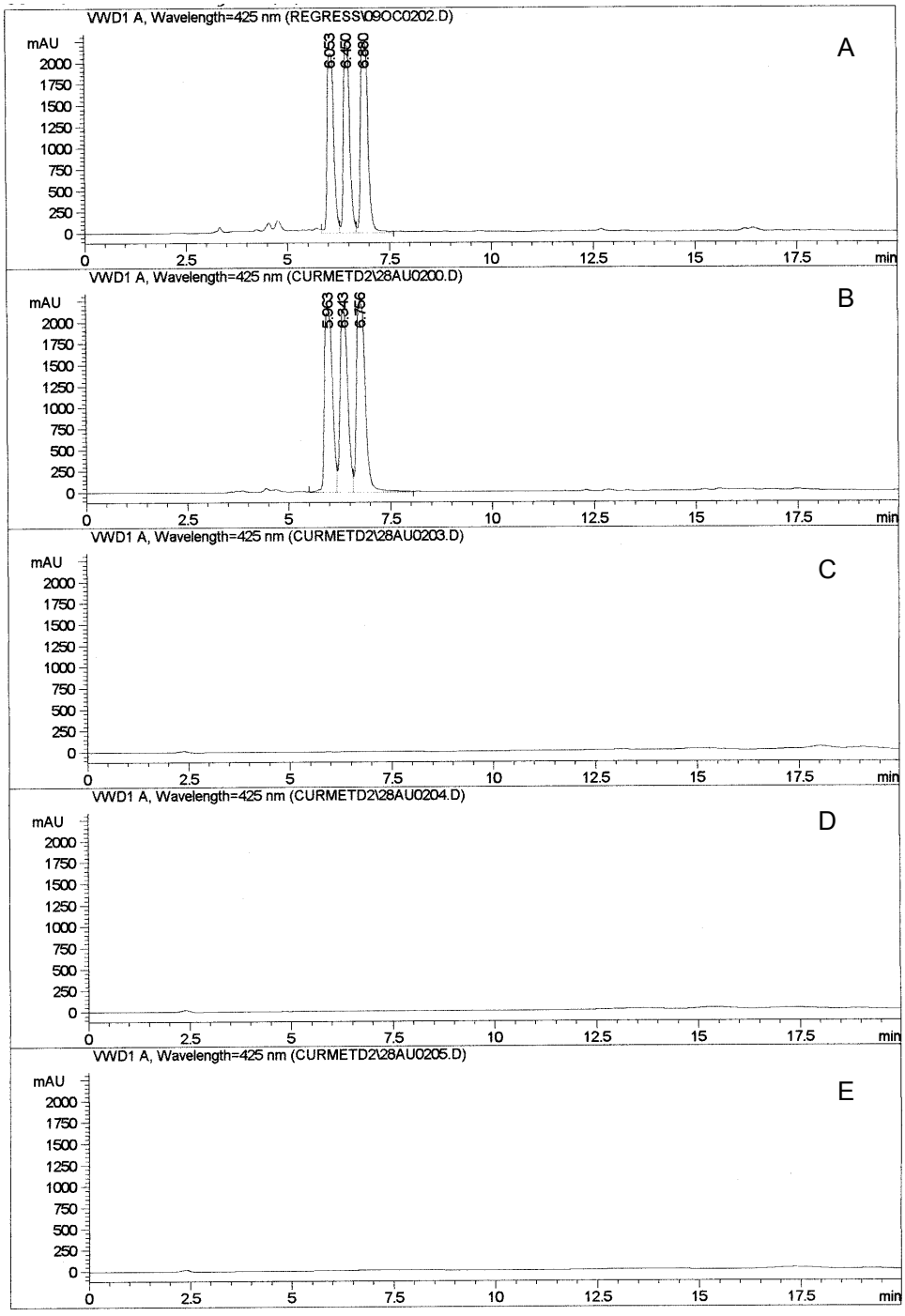


Figure 2.17. HPLC chromatograms of (A). Standard curcuminoids, (B). CRTO oil (C). Fraction I, (D). Fraction II and (E). Fraction III.

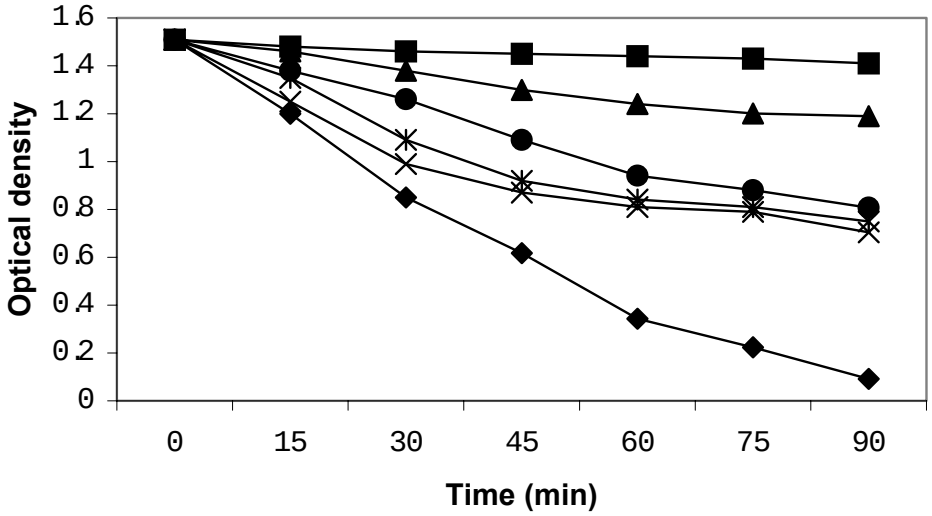
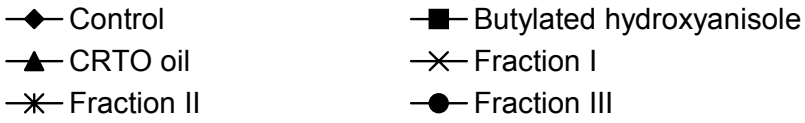


Figure 2.18. Antioxidant activity of CRTO oil and its column fractions by ß-carotene-linoleate model system at 100 ppm



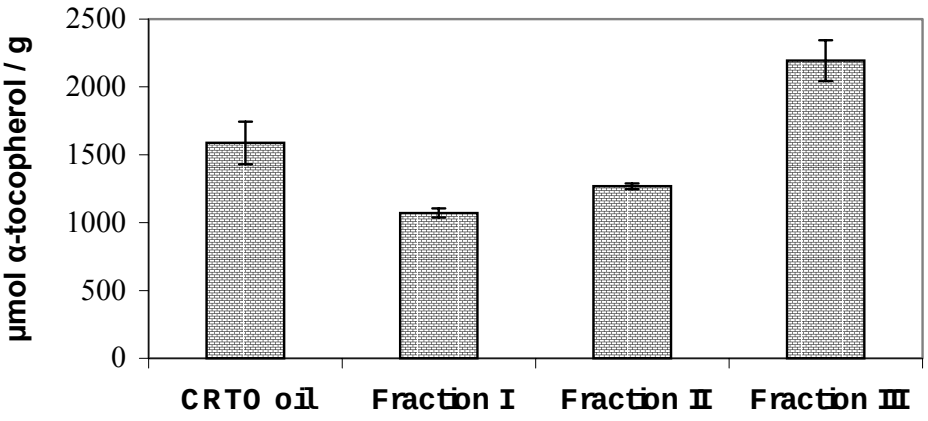


Figure 2.19. Antioxidant capacity of CRTO oil, fractions I, II and III by phosphomolybdenum method

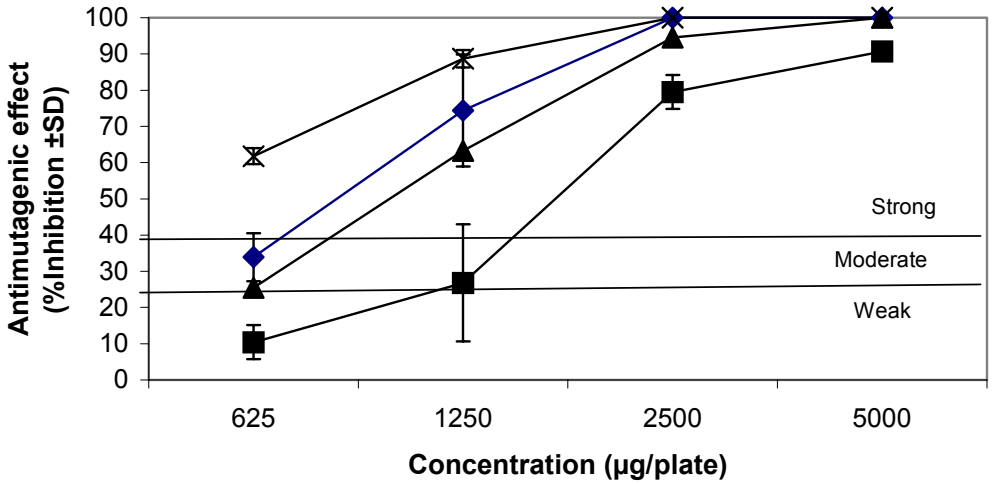


Figure 2.20. Inhibitory effect of CRTO oil and its fractions aganist the mutagenicity of sodium azide to *Salmonella typhimurium* TA-100

CRTO oil — Fraction I — Fraction II — Fraction III

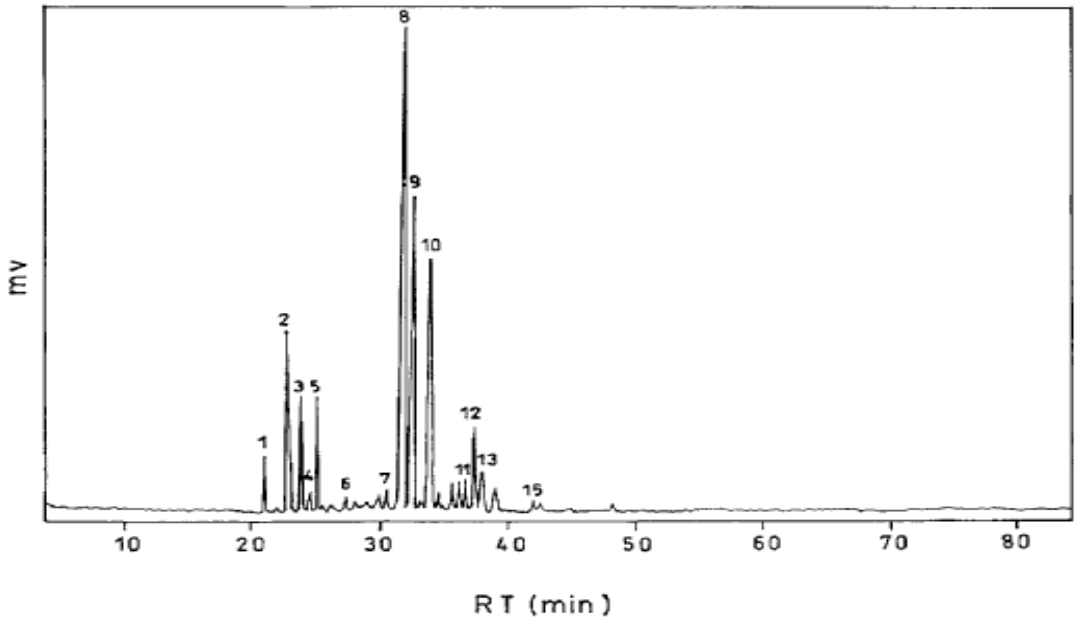


Figure 2.21. GC-MS total ion chromatogram of CRTO oil obtained by hexane extraction at ambient temperature.

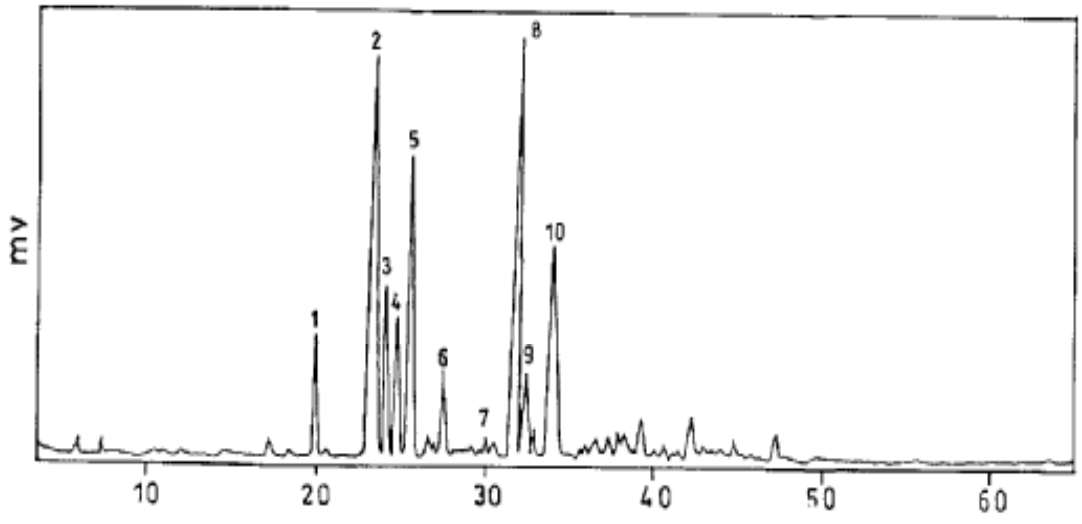


Figure 2.22. GC-MS total ion chromatogram of fraction I obtained by fractionation using silica gel as an adsorbent and hexane as an eluent.

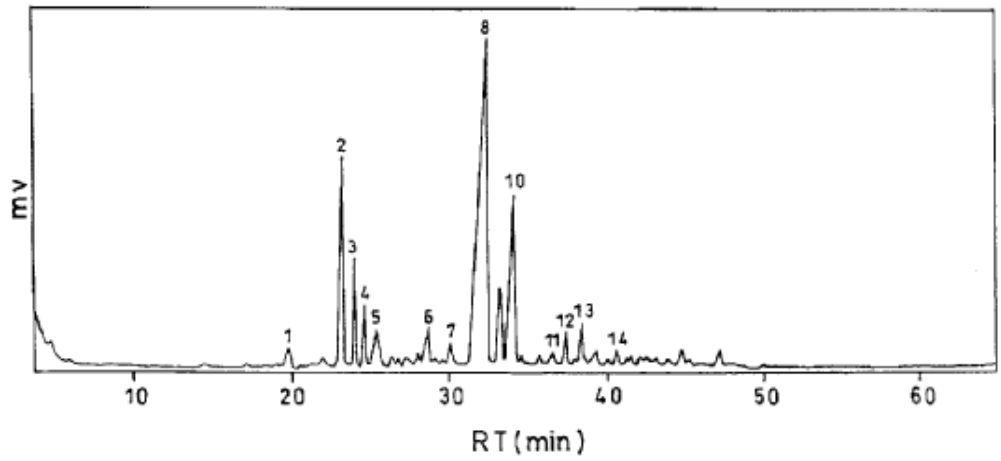


Figure 2.23. GC-MS total ion chromatogram of fraction II obtained by fractionation using silica gel as an adsorbent and hexane:benzene (1:1) as an eluent

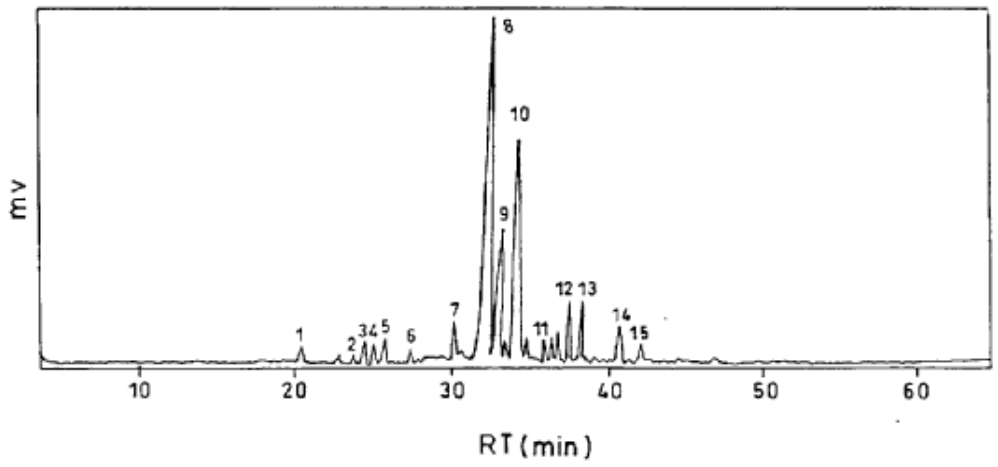


Figure 2.24. GC-MS total ion chromatogram of fraction III obtained by fractionation using silica gel as an adsorbent and benzene as an eluent

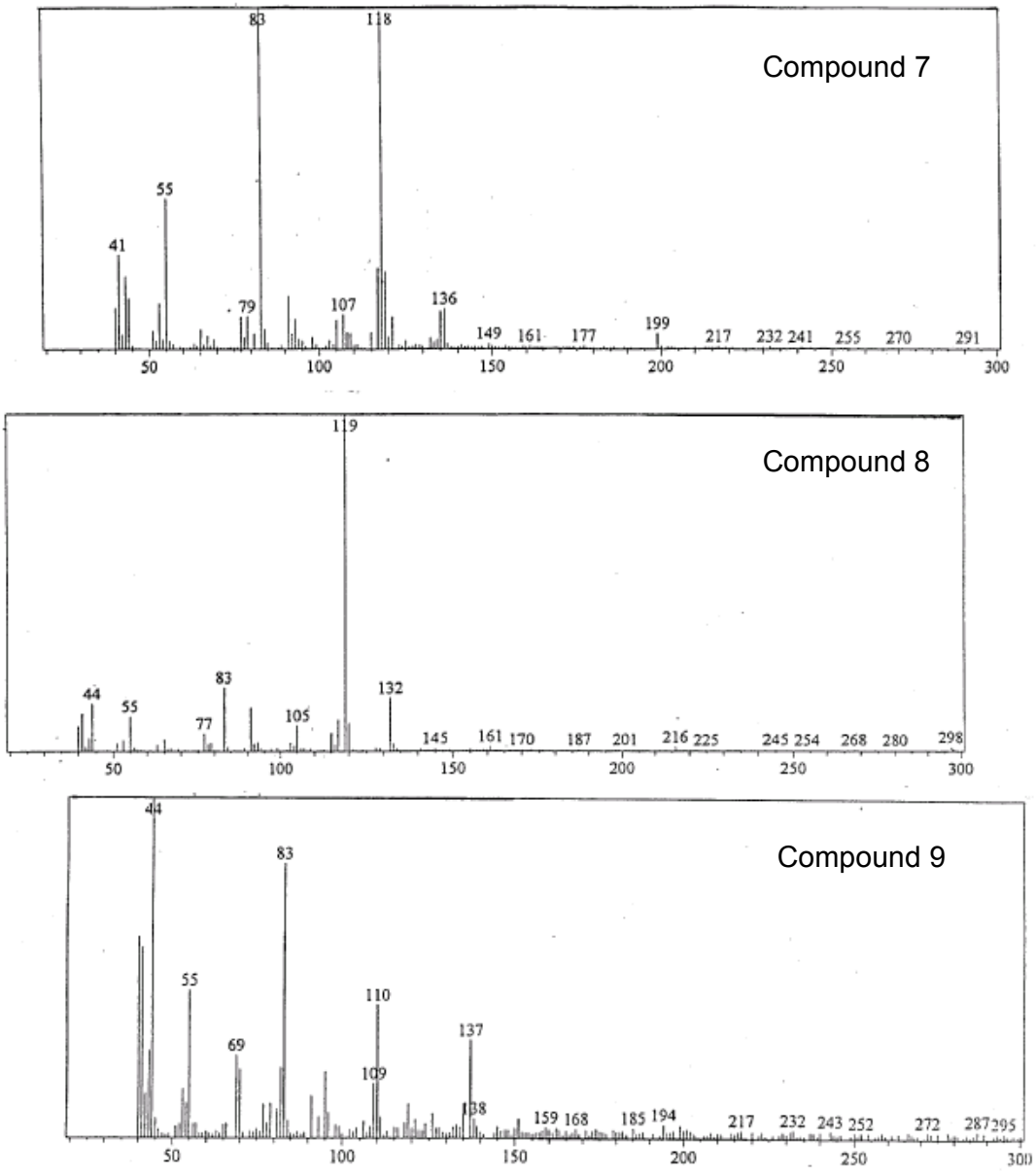
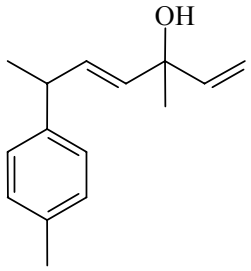
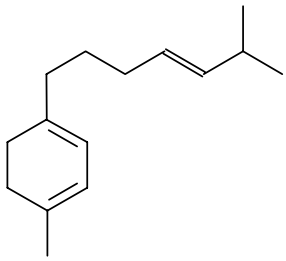


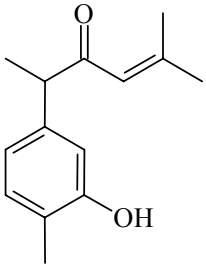
Figure 2.25. Mass spectra of Compounds (7-9) from CRTO oil, fractions I, II and III.



Compound (8)
(MW = 216)



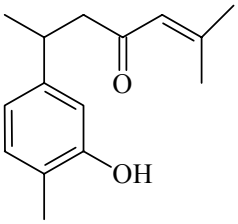
Compound (1)
(MW = 204)



Compound (4)
(MW = 218)

Unidentified component

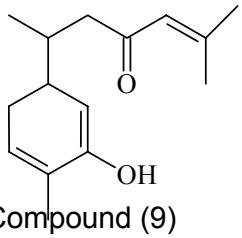
Compound (3)
(MW = 216)



Compound (6)
(MW = 232)

Unidentified component

Compound (7)
(MW = 216)



Compound (9)
(MW = 234)

Figure 2.26. Structures of compounds identified in CRTO oil and its fractions (Hiserodt et al., 1996).

PART -B

Chapter 3.

Studies on curcuminoids from spent turmeric oleoresin

3.1. SEPARATION AND IDENTIFICATION OF CURCUMINOIDS FROM SPENT TURMERIC OLEORESIN

INTRODUCTION

Nature is rich in colour and the majority of plant pigments are not widely exploited for the colouring of food. New sources of plant pigments are needed to be available in sufficient quantities for industrial extraction. There has been much interest in the development of technologies for new natural products as food additives. Natural colourants have become increasingly popular with consumers because synthetic colourants tend to be perceived as undesirable and harmful; some are considered to be responsible for allergenic and intolerance reactions (Blemford, 1995). However, on considering economic, legislative and consumer acceptance aspects of new food colourants, it will be a time consuming and tedious effort. Pigmented by-products from food manufacturing, such as grape, or tomato skins, spices and variety of common food plants enriched in pigments are unaffected by legislative and economic considerations. These can be used to add colour variation to several food preparations without the disadvantages associated with the use of pure colourants (Wissagott and Bortlik, 1996).

Commercially available curcumin consists of a mixture of three curcuminoids *viz.* curcumin (diferuloylmethane), demethoxycurcumin (*p*-hydroxy cinnamoyl, feruloylmethane) and bisdemethoxycurcumin (di-*p*-hydroxy cinnamoylmethane) with curcumin as the main (≈ 77%) constituent (Ahsan et al., 1999) (Part B; Figure 1.3). There is an increasing demand for demethoxycurcumin and bisdemethoxycurcumin due to the discovery of their new biological activities (Simon et al., 1998; Ahsan et al.,

1999; Kim et al., 2001). The pigment curcumin is industrially produced using turmeric oleoresin as the starting material. The mother liquor (approximately 70-80%), after isolation of curcuminoids from oleoresin, has a composition of oil, resin and left over curcuminoids. This material has no commercial value at present (Saju et al., 1998). Hence, an attempt was made to utilize the CRTO for the isolation of left over curcuminoids.

Literature survey revealed that, there is no report on the isolation of left over curcuminoids from spent turmeric oleoresin (Curcumin Removed Turmeric Oleoresin - CRTO). Studies were carried out for the isolation of curcuminoids from CRTO, so that the total yield of curcuminoids from turmeric oleoresin can be improved. Further, the studies were extended for the isolation and characterisation of individual curcuminoids from spent turmeric oleoresin. The identification of these compounds was carried out with the help of spectral studies.

MATERIALS AND METHODS

Isolation of Curcuminoids from CRTO

Slurry of CRTO (25 g) was stirred with 2-4 times (w/v) of petroleum ether at ambient temperature and filtered. The residue (12 g) was extracted with 2-4 times of ethyl acetate at 40-60 °C. Extract was separated by filtration and filtrate was concentrated under vacuum. The concentrate (20 ml) was treated with 2-4 times of petroleum ether to get a precipitate. The precipitated curcuminoids were collected by filtration and dried at 70 °C (2.1 g).

Separation of Individual Curcuminoids

Five grams of CRTO was dissolved in 10 ml of acetone and was impregnated on 10 g of silica gel. Slurry of silica gel (100 g) in hexane was packed into a glass column (600 × 30 mm); prior to sample loading the column was equilibrated with hexane. Then the impregnated sample was loaded onto the column and eluted with 500 ml of hexane. Then the column was eluted sequentially with mixture of benzene and ethyl acetate with increasing polarity. Compound 1 was eluted with benzene:ethyl acetate (82:18 v/v), whereas compounds 2 and 3 were eluted with benzene: ethyl acetate (70:30 v/v) and benzene: ethyl acetate (58:42 v/v) respectively. The elutes were concentrated *under vacuum* and recrystallised to obtain compounds 1 (110 mg), 2 (223 mg) and 3 (170 mg).

HPLC Analysis

The purity of isolated curcuminoids was estimated as described in Part B, Chapter 3.2, Page No. 213.

Identification of Compounds

The melting points of compounds 1, 2 and 3 were recorded as 186-187, 175-76 and 231-232 °C respectively. The purified compounds were subjected to TLC analysis on silica gel (10 × 20 cm; thickness – 0.2 mm) plates, using chloroform: methanol (98:2 v/v) as the developing solvent system. The three curcuminoids are visualised as yellow spots. The R_f values of compounds 1, 2 and 3 were found to be 0.55, 0.50 and 0.43, respectively.

IR and MS Analysis

IR spectra were recorded on a Bruker-IFS 25 spectrometer-using KBr discs (Bruker, Rheinstetten, Germany). Mass spectra were recorded using QP-5000 Quadrapole Mass Spectrometer coupled with Shimadzu GC-17A (Shimadzu, Kyoto, Japan).

NMR Analysis

^{1}H and ^{13}C NMR spectra (DMSO-d_6) were recorded at 400 and 100 MHz respectively on a Bruker AMX 400 FT instrument (Bruker, Rheinstetten, Germany). TMS was used as the internal standard. Chemical shifts (δ ppm) values are presented in Tables 3.1 and 3.2.

RESULTS AND DISCUSSION

The present approach for the isolation of leftover curcuminoids is directed to find a suitable solvent for selective extraction of curcuminoids from CRTO, followed by dilution of the extract to precipitate curcuminoids (Jayaprakasha et al., 2001). CRTO was first treated with non-polar solvent for the removal of oils. The residue was selectively extracted into medium polar solvent. The extract obtained was concentrated to 50%, followed by precipitation of curcuminoids using an apolar solvent (Figure 3.1). The isolated curcuminoids mixture was analyzed by HPLC method as described in Part B, Chapter 3.2. It was found to contain curcumin (30-58.7%), demethoxycurcumin (9 - 14%) and bisdemethoxycurcumin (7.6 - 22.7%). The ratio of three curcuminoids varies based on the source of spent turmeric oleoresin.

HPLC analysis showed that, the percentage of demethoxycurcumin and bisdemethoxycurcumin in the isolated curcuminoids mixture isolated from CRTO, were found to be more when compared to commercially available curcumin (Figure 3.2). Hence, CRTO is a potential source for the isolation of these two curcuminoids. Further, pure curcumin 1, demethoxycurcumin 2 and bisdemethoxycurcumin 3 are not available from commercial sources. Hence, curcuminoids 1, 2 and 3 were purified from the spent turmeric oleoresin by column chromatography using silica gel as adsorbent after initial elution with hexane for the removal of oil part. The separation of the compounds 1, 2 and 3 was achieved on elution with benzene and ethyl acetate mixtures with increasing polarity. The isolated compounds 1, 2 and 3 showed single spots on TLC and well-resolved single peaks in HPLC analysis (Figure 3.3).

The IR spectrum of compound 1 showed absorptions for hydroxyl groups (3400 cm^{-1}) β-diketone (1625 cm^{-1}) and an aromatic ring (1595, 1515 cm^{-1}). ^1H NMR spectrum of compound 1 is presented in Figure 3.4. The singlet at δ 3.85 for six protons showed the presence of two aromatic methoxyl groups. One signal at δ 9.64 (s, 2H) has been assigned to two phenolic hydroxyl groups. A singlet at δ 6.06 (1H) was attributed to the one proton on the central carbon of the β-diketone in its enol form (Vijayalakshmi and Satyanarayana, 1980). Two pairs of doublets at δ 6.75 and 7.57 (2H each, J = 16.0 Hz) were assigned to trans olefinic protons, which are in conjugation with the carbonyl group and phenyl rings. Furthermore, three signals δ 7.32 (d, 2H, J = 2.0 Hz), 7.16 (dd, 2H, J= 2.0 and 8.1 Hz) and 6.85 (d, 2H, J = 8.1 Hz) indicated ABX-pattern for substituted phenyl rings (Table 3.1). These

Table 3.1. ^{1}H NMR ($CDCl_3$) spectral data of compounds 1, 2 and 3.

H	1	2	3
1	6.06 (1H, s)	6.04 (1H, s)	6.03 (1H, s)
2-OH	16.41 (Br.s)	**----**	16.4 (Br.s)
3, 3′	7.57 (2H, d, 16)	7.55 (1H, d, 15.8); 7.54(1H, d, 15.8)	7.56 (2H, d, 15.8)
4, 4′	6.75 (2H, d, 16)	6.76 (1H, 15.8); 6.69 (1H, d, 15.8)	6.68 (2H, d, 15.8)
6, 6′	7.32 (2H, d, 2)	7.32 (1H, Br.s); 7.56 (1H, d, 8.3)	7.56 (2H, d 8.2)
7,7’		6.83 (1H, d, 8.2)	6.83 (2H, d, 8.14)
8,8’-OH	9.64 (2H, s)	9.67 (1H, s); 10.1 (1H, s)	10.03 (s)
9, 9′	6.85 (2H, d, 8.1)	6.83 (1H, d, 8.2); 6.83 (1H, d, 8.2)	6.83 (2H, 8.14)
10, 10′	7.16 (2H, dd, 2; 8.1)	7.56 (1H, d, 8.3); 7.14 (1H, Br. d, 8.2)	7.56 (2H, d, 8.2)
OMe	3.85(6H, s)	3.83 (3H,s)	

Chemical shifts are followed by coupling constants J (in Hz): values in parentheses.

s: singlet

d : doublet

Br: broad

dd: double doublets

assignments suggested the presence of two feruloyl moieties, which was supported by the stable fragment ions at m/z 177 in its mass spectrum. ^{13}C NMR spectra of compound 1 exhibited eleven signals from 21 carbons consistent with symmetry around C-1 carbon. In decoupled and SEFT spectra (Table 3.2; Figure 3.5 and 3.6), signal at 183.2 showed the presence of a carbonyl carbon. The methoxyl groups at C-7 and C-7’ showed a singlet at δ 55.7. Figure 3.7 shows the mass

Table 3. 2. ^{13}C NMR ($CDCl_3$) spectral data of compounds 1, 2 and 3.

C	1	2	3
1	100.8	101.4	100.9
2,2'	183.2	183.3, 183.2	183.2
3,3'	121.1	121.4, 121.6	120.8
4,4'	140.7	140.8, 141.1	140.3
5,5'	126.4	126.4, 126.9	125.8
6,6'	111.5	111.7, 130.8	130.3
7,7'	148.0	148.4, 116.4	115.9
8,8'	149.4	149.7, 160.1	159.8
9,9'	115.8	116.1, 116.2	115.9
10,10'	123.0	123.6, 130.8	130.3
OMe	55.7	56.2	

spectral fragmentation pattern of compound 1. The EI-MS of compound 1 showed a molecular ion peak at m/z 368 compatible with molecular formula $C_{21}H_{20}O_6$ and a characteristic peak at m/z 350, shows the loss of 18 mass units due to a water molecule ($M^+ - H_2O$). Peaks located at m/z 217, 177 and 232 originated as a result of a, b and c cleavages in the central alkene moiety of the compound 1. Fragments at m/z 177 and 137 confirmed the presence of feruloyl and hydoxymethoxy benzyl ion moieties. These data confirmed that the compound 1 to be curcumin.

The IR spectrum of compound 2 showed absorptions for a hydroxyl group (3400 cm^{-1}) and a β-diketone (1625 cm^{-1}). ^1H and ^{13}C NMR spectra of compound 2 are

presented in Figures 3.8 - 3.10. ^{1}H NMR spectrum showed the presence of singlet at δ 3.83 for three protons indicating the presence of one methoxyl group. One proton singlet at δ 6.04 was assigned to the one proton on the central carbon of the β-diketone in its enol form (Vijayalakshmi and Satyanarayana, 1980). Four pairs of doublets at δ 6.69 (1H, 15.8 Hz), 6.76 (1H, 15.8 Hz), 7.55 (1H, 15.8 Hz) and 7.54 (1H, 15.8 Hz) were assigned to *trans* olefinic protons conjugated to the carbonyl group. Further, seven signals due to 1,3,4- and 1,4- substituted phenyl groups were observed at δ 7.32 (1H, Br.s), 6.83 (1H, d, 8.2 Hz), 7.14 (1H, Br.d, 8.2 Hz), 7.56 (2H, d, 8.2 Hz), 6.83 (2H, d, 8.2 Hz). This suggested the presence of a feruloyl moiety, which was supported by the stable fragment ions at m/z 177 and a *p*-hydroxycinnamoyl moiety. The mass spectrum of compound 2 showed a molecular ion peak (M^{+}) at m/z 338 along with another characteristic peak at m/z 320 formed due to loss of one water molecule ($M^{+} - H_2O$). Two groups of peaks located at m/z 177 and 149, 137 and 147, 119 and 107 were characteristic of two differently substituted aromatic moieties, namely feruloyl and *p*-hydroxycinnamoyl respectively. On the basis of these data, compound 2 was identified as demethoxycurcumin.

The IR spectrum of compound 3 exhibited bands at 3200, 1626, 1545 cm^{-1} for O-H, C=O and ring C-O stretching vibrations respectively. Figure 3.11 to 3.13 shows ^{1}H and ^{13} C NMR spectra of compound 3. The ^{1}H NMR spectrum of compound 3 displayed doublets at 7.56 and 6.68 with a coupling constant of 15.8 Hz for the H-4, 4′ and H-3, 3′ signals, a value typical for trans- double bonds. The H-1 signal at δ 6.03 ppm of compound 3 indicates the presence of an enolic proton, since no signal corresponds to CH_2 for the keto form could be found. Among the aromatic protons in compound 3, H-6, 6′ and H-10, 10′ resonated upfield at δ 7.56 (4H, d, 8.2 Hz) while,

H-9, 9′ and H-7, 7′ showed signal at downfield δ 6.83 (4H, d, 8.2). Mass spectrum of compound 3 showed a molecular ion peak (M^+) at m/z 308 and ($M^+ - H_2O$) fragment ion peaks at m/z 219, 202, 187, 160, 147, 119 and 107 characteristic of a *p*-hydroxycinnamoyl moiety were also observed. Thus, the compounds 1, 2 and 3 were characterised and confirmed as curcumin, demethoxycurcumin and bisdemethoxycurcumin respectively, using ^1H and ^{13}C NMR spectra. Chemical shifts of the curcuminoids were in accordance with reported values (Table 3.1 and 3.2) (Roughley and Whiting, 1973; Unterhalt, 1980).

This method for recovery of curcuminoids from CRTO will add to the economics of curcumin manufacture. This method brings in beneficiation to curcumin manufacturing industries. The yield of total curcuminoids was found to be 8-12% with respect to CRTO. Total content of curcuminoids was analysed by HPLC method and it was found to be 60-75% and the composition of curcumin, demethoxycurcumin and bisdemethoxycurcumin was found to be 30-59%, 9-14% and 7.6-22.7%, respectively.

The advantages of the method are

- Isolation of unrecoverable curcuminoids from the CRTO matrix by simple method,
- This method involves only simple and routinely used solvents and these can be recovered for re-use,
- The solubility of curcuminoids mixture in polar solvents obtained in this process is more when compared to the curcuminoids mixture from commercial sources,

as the percentage of the two polar curcuminoids (viz., demethoxycurcumin and bisdemethoxycurcumin) is more.

Recently, demethoxycurcumin and bisdemethoxycurcumin was found to posses a strong antioxidant activity and inhibition of the proliferation of MCF-7 human breast tumour cells has been reported. (Simon et al., 1998; Ahsan et al., 1999; Kim et al., 2001). The concentration of these two components is increased abundantly in spent turmeric oleoresin compared to normal oleoresin. These compounds can be isolated by silica gel column chromatography on large scale for various application purposes. The results of the present study are indicative of the utilization of spent turmeric oleoresin as a natural bioactive component, which at present has no commercial application.

CONCLUSION

Now a days, demethoxycurcumin and bisdemethoxycurcumin was found to posses a strong antioxidant activity and inhibition of the proliferation of MCF-7 human breast tumour cells has been reported. The concentration of these two components is increased abundantly in spent oleoresin compared to commercial turmeric oleoresin. These compounds were isolated by simple precipitation method and the individual curcuminoids were separated on silica gel column chromatography. The results of the present study are indicative of the utilization of spent oleoresin having no commercial application at present, as natural bioactive component or food additive.

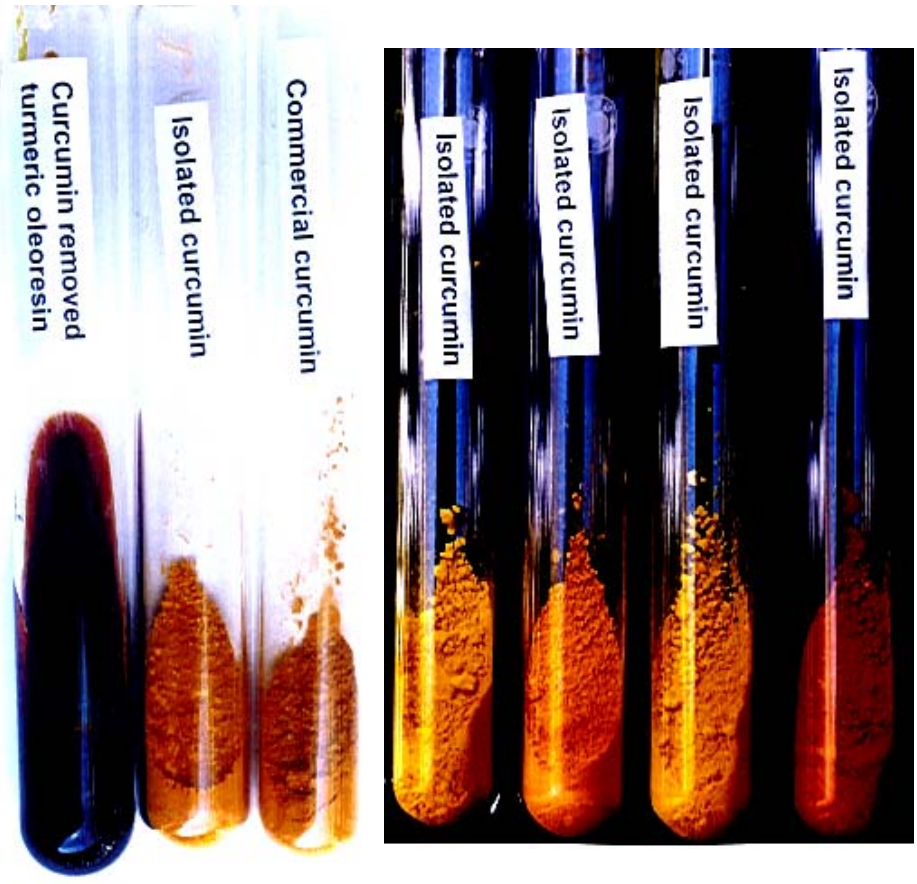


Figure 3.1. Samples of CRTO, commercial curcumin and isolated curcuminoids (different batches).

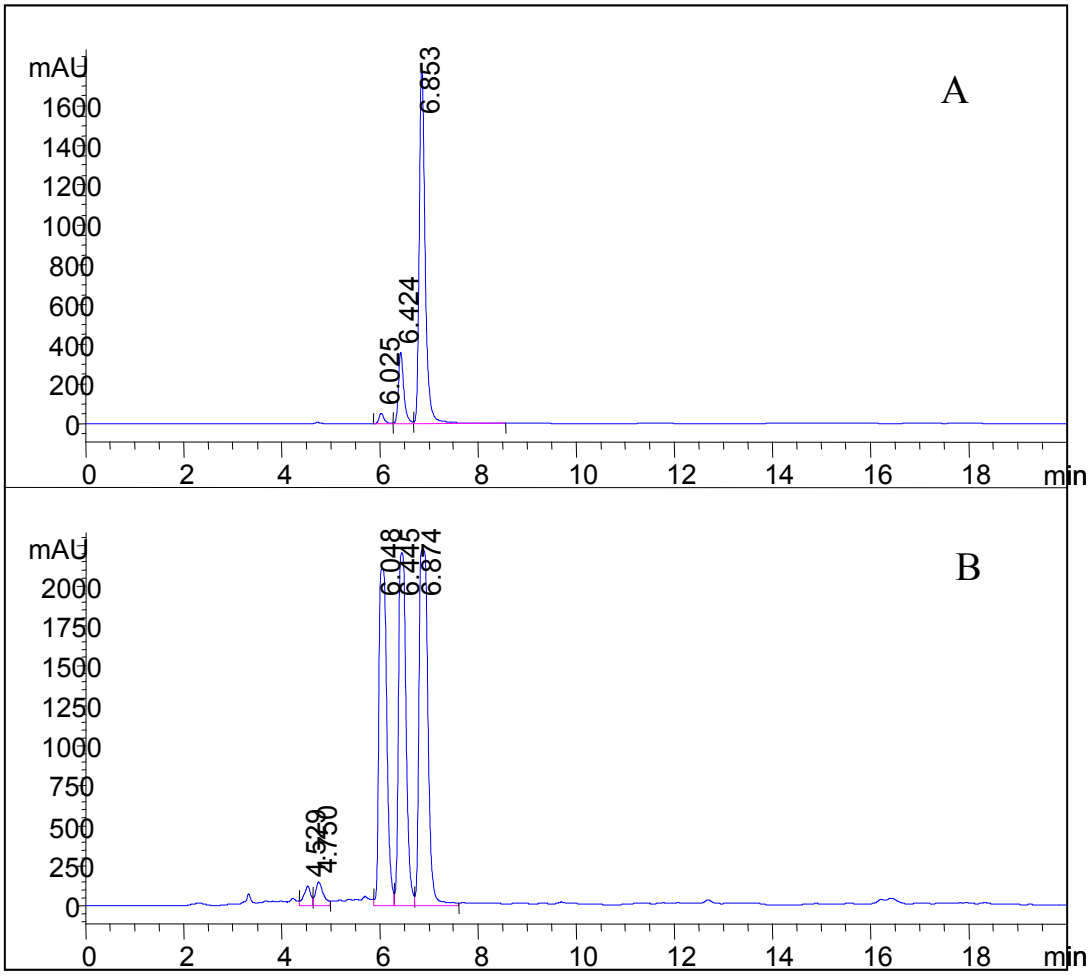


Figure 3.2. HPLC chromatograms of (A). Commercial curcumin (curcuminoids) and (B). Isolated curcuminoids from CRTO.

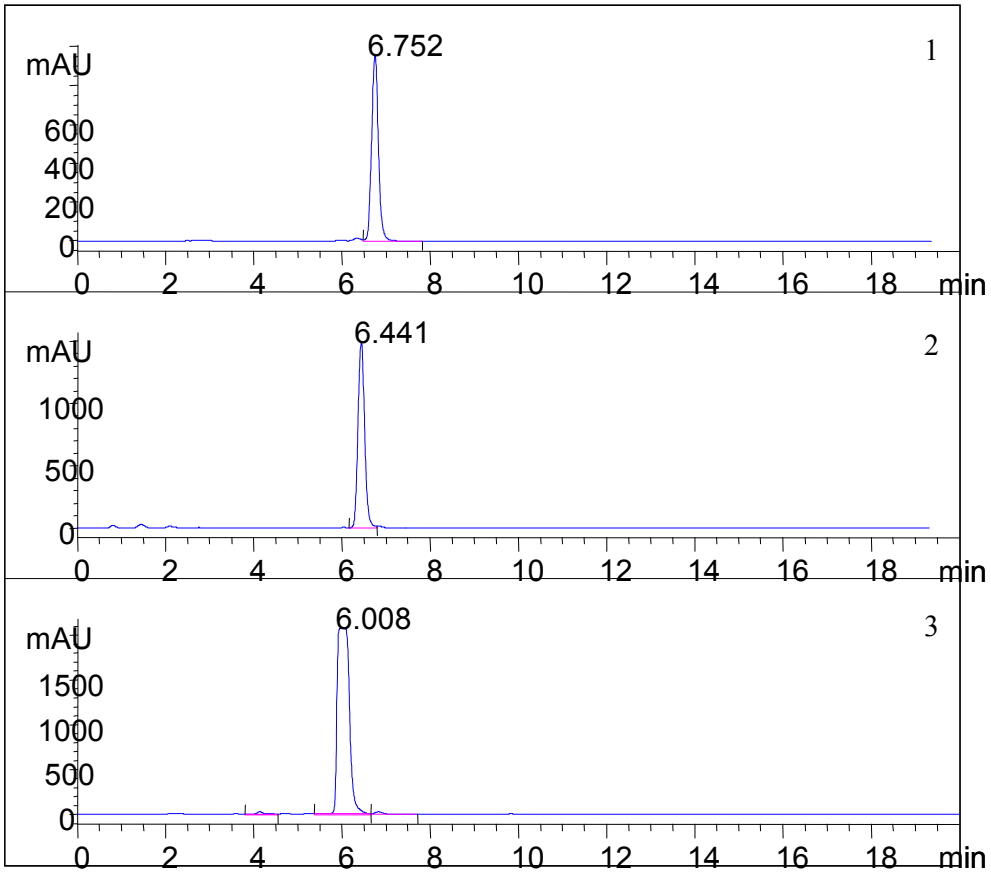
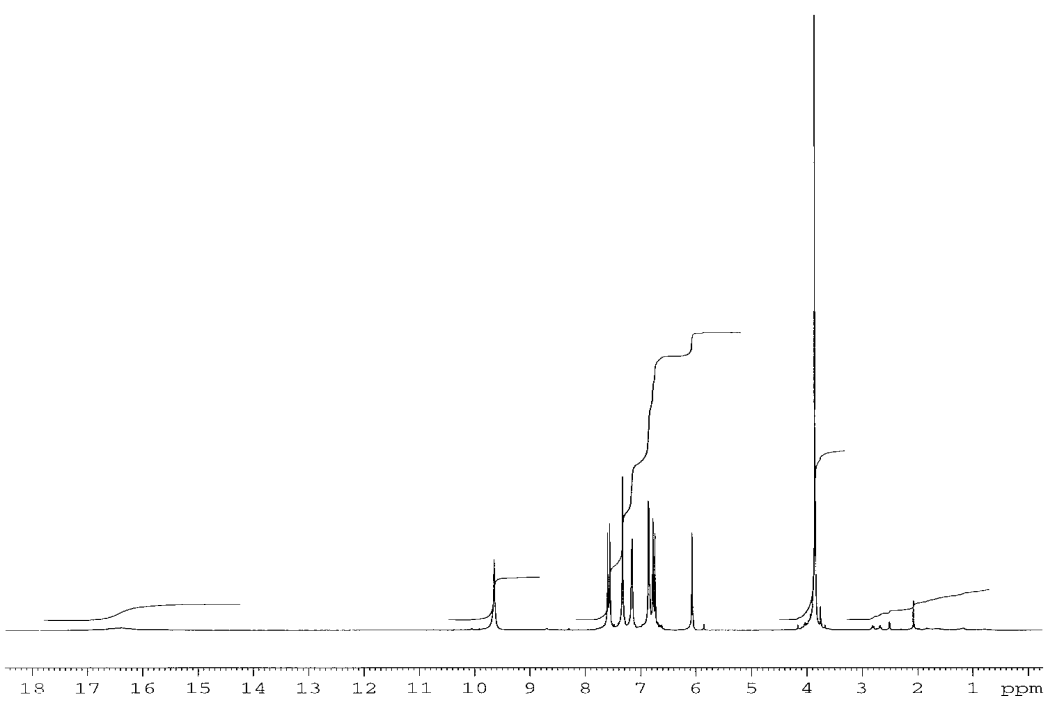


Figure 3.3. HPLC chromatograms of curcumin 1, demethoxycurcumin 2 and bisdemethoxycurcumin 3.

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RG                           64
DW                        61.000 usec
DE                         76.25 usec
TE                         300.0 K
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PC                           1.00
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Figure 3.4. ^{1}H NMR spectrum of compound (1).

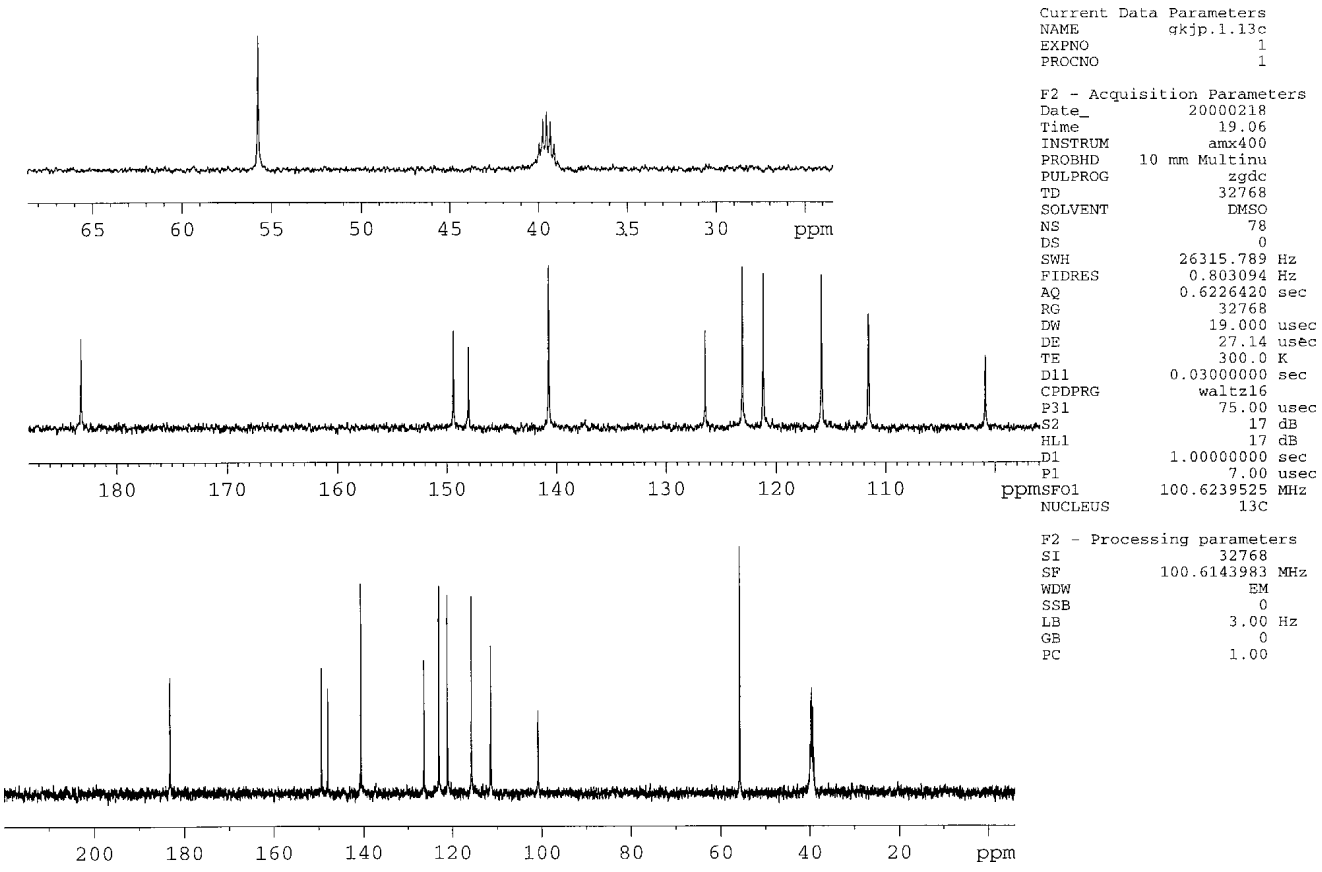


Figure 3.5. ^{13}C NMR spectrum of compound (1).

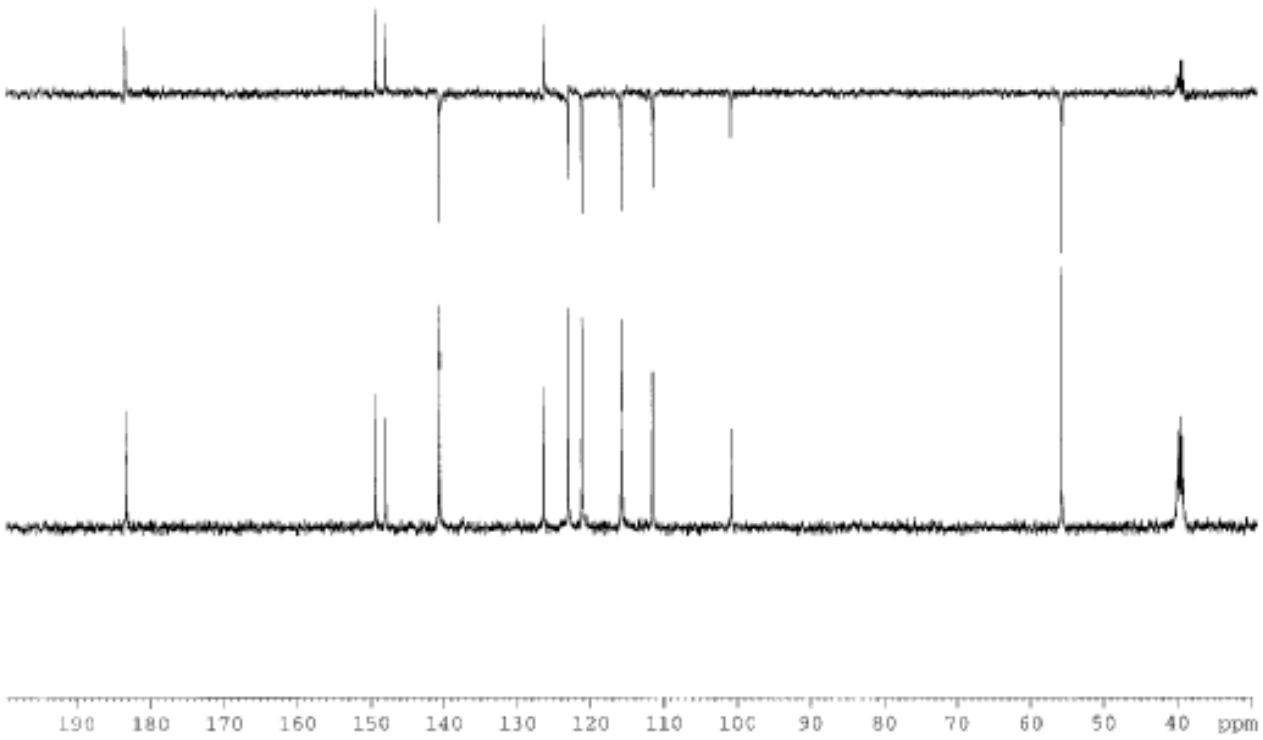


Figure 3.6. ^{13}C NMR SEFT spectrum of compound (1).

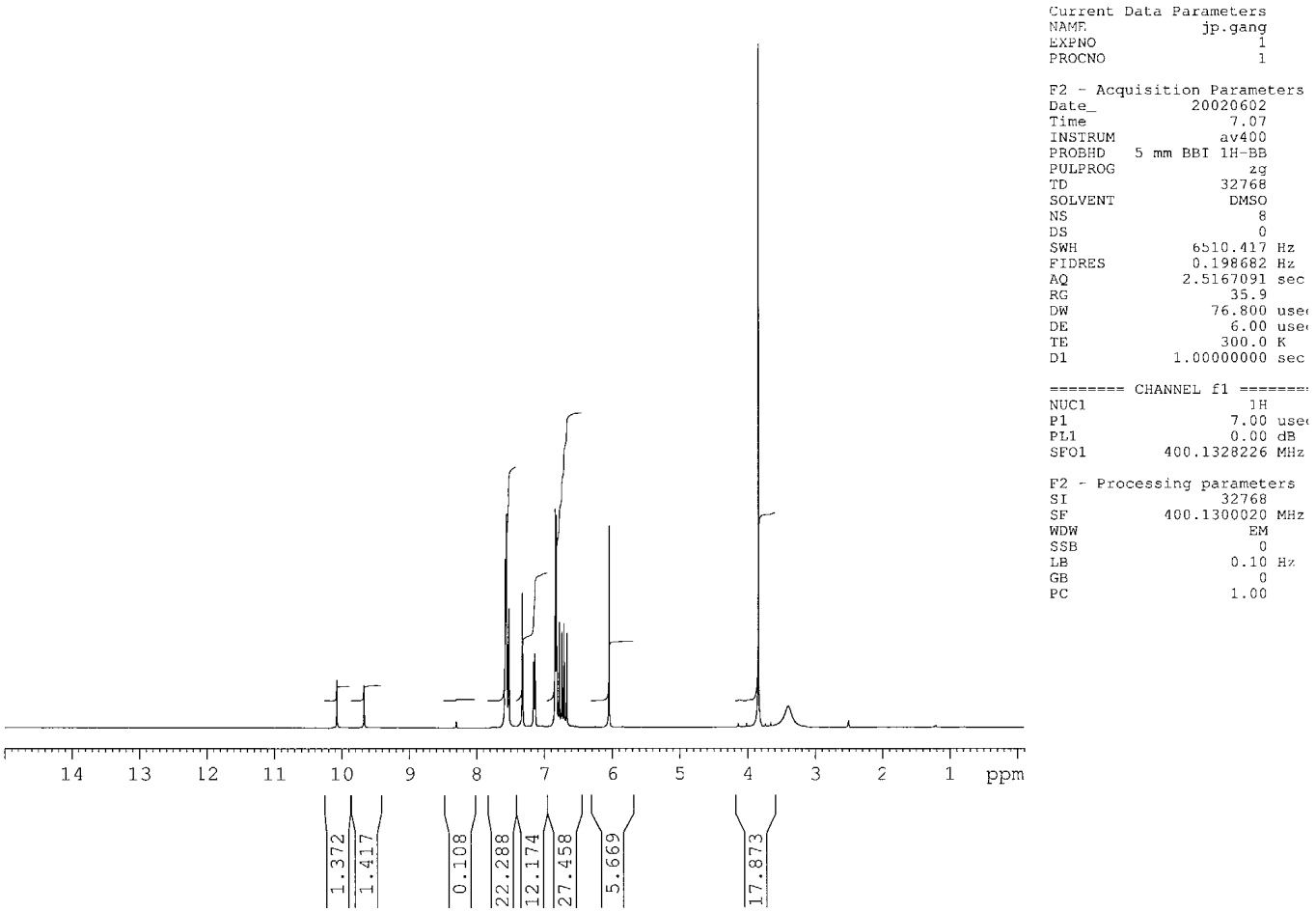
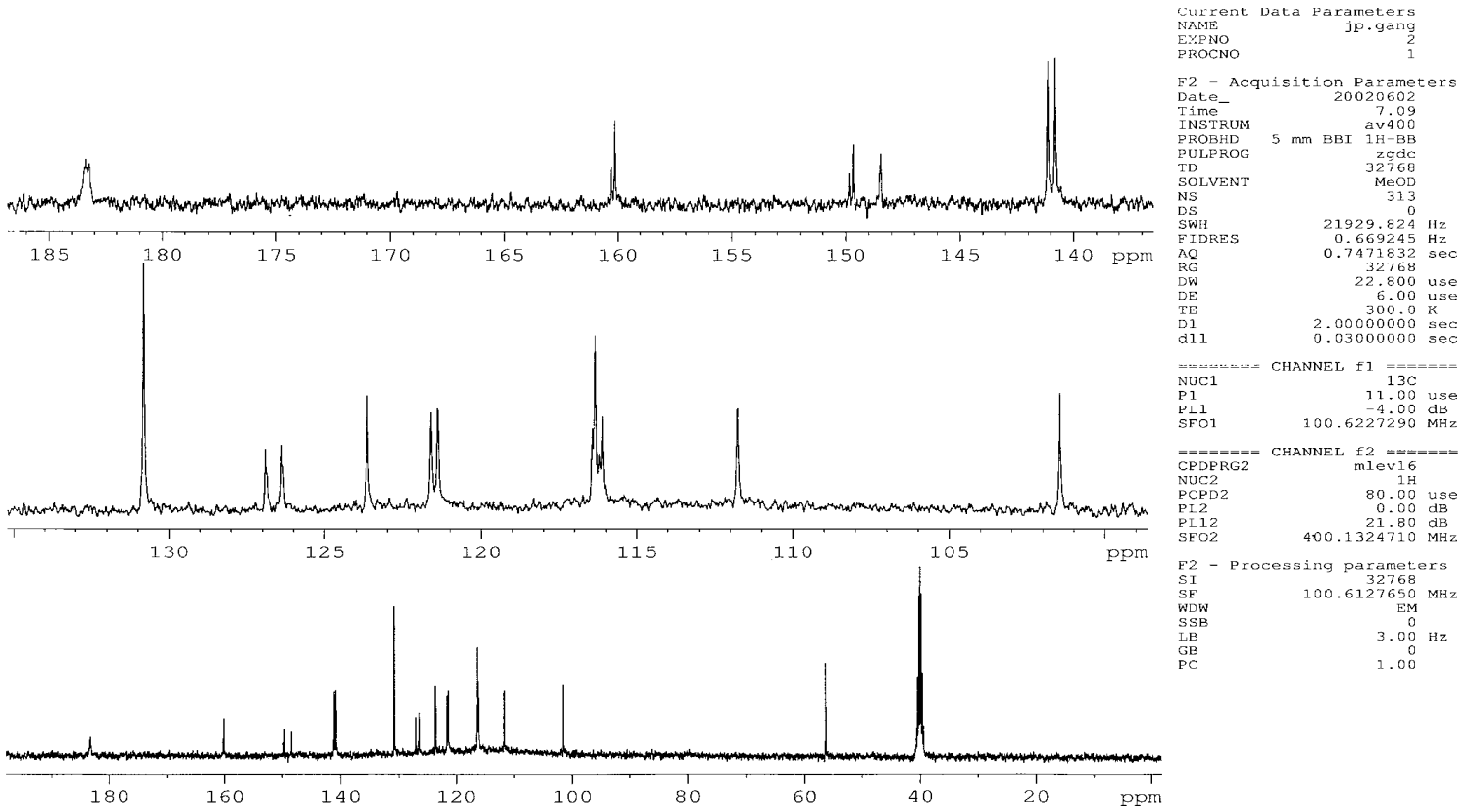


Figure 3.8. ^{1}H NMR spectrum of compound (2).

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PL1                    -4.00 dB
SFO1           100.6227290 MHz

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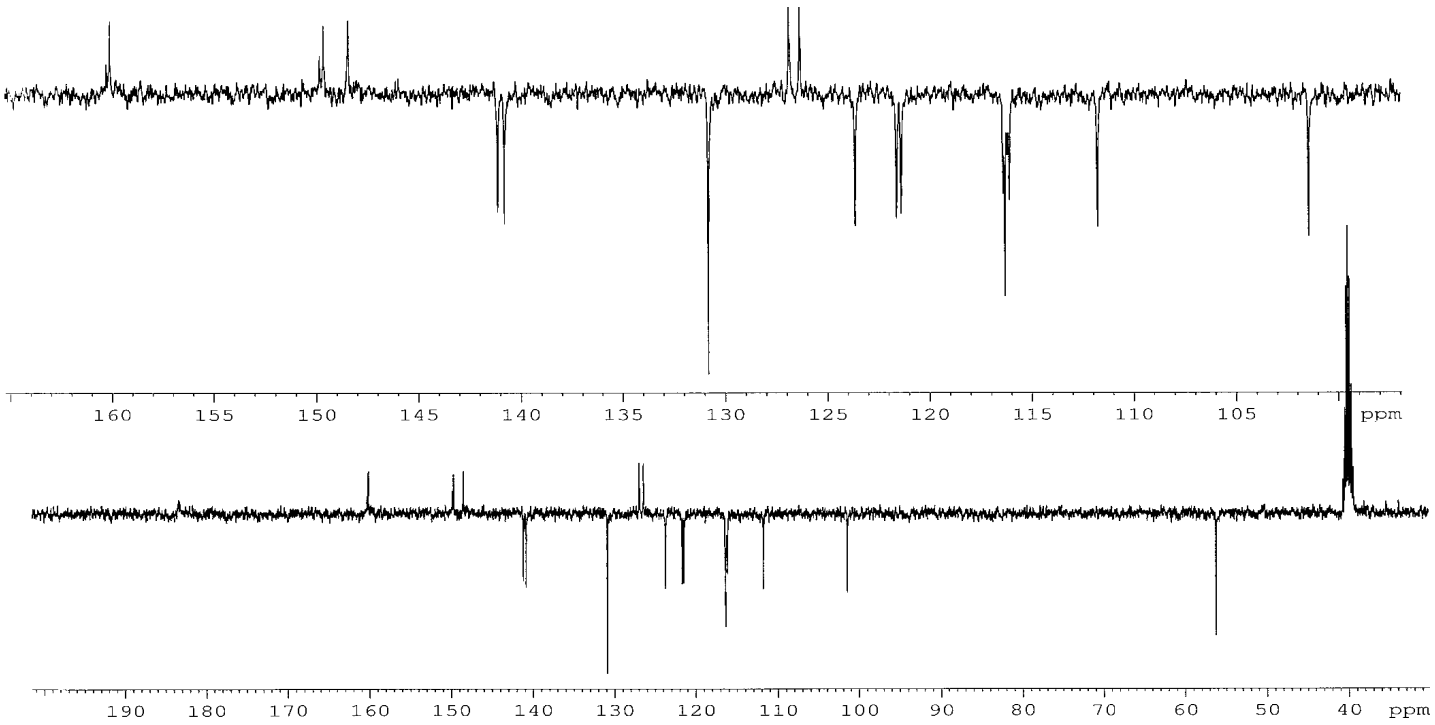
Figure 3.9. ^{13}C NMR spectrum of compound (2).

Figure 3.10. ^{13}C NMR SEFT spectrum of compound (2).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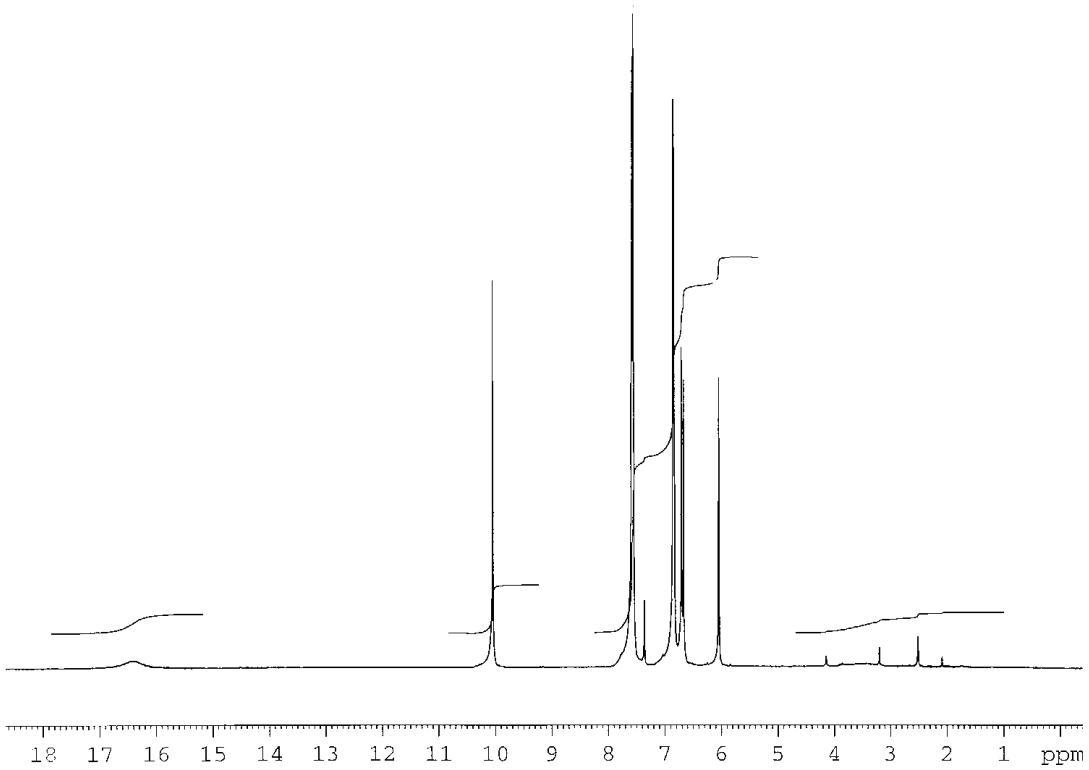


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FIDRES          0.250144 Hz
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RG                   128
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D1             1.00000000 sec
P1                 13.00 usec
SFO1         400.1399354 MHz

F2 - Processing parameters
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Figure 3.11. ^{1}H NMR spectrum of compound (3).

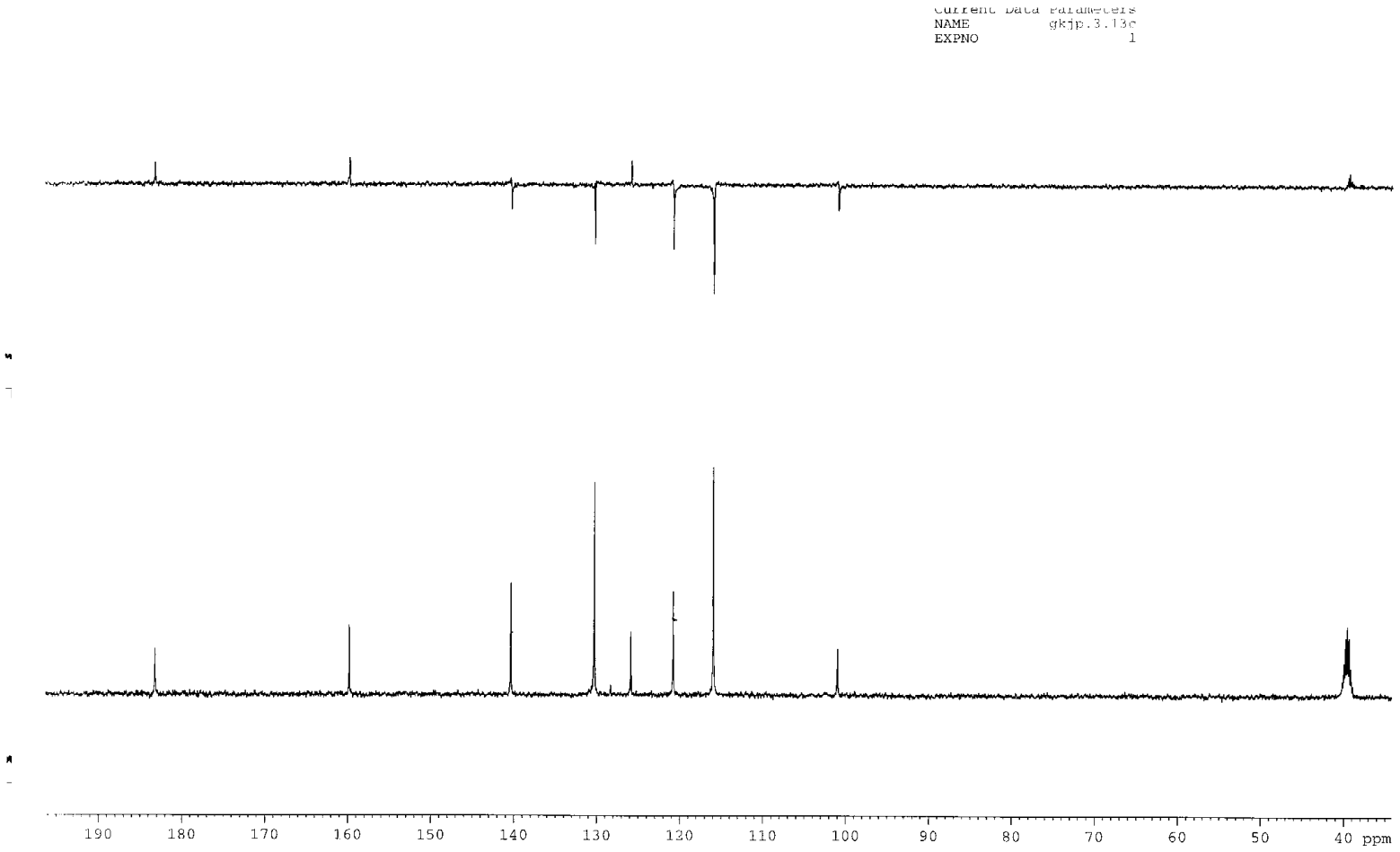

Figure 3.12. ^{13}C NMR spectrum of compound (3).

Figure 3.13. ^{13}C NMR SEFT spectrum of compound (3).

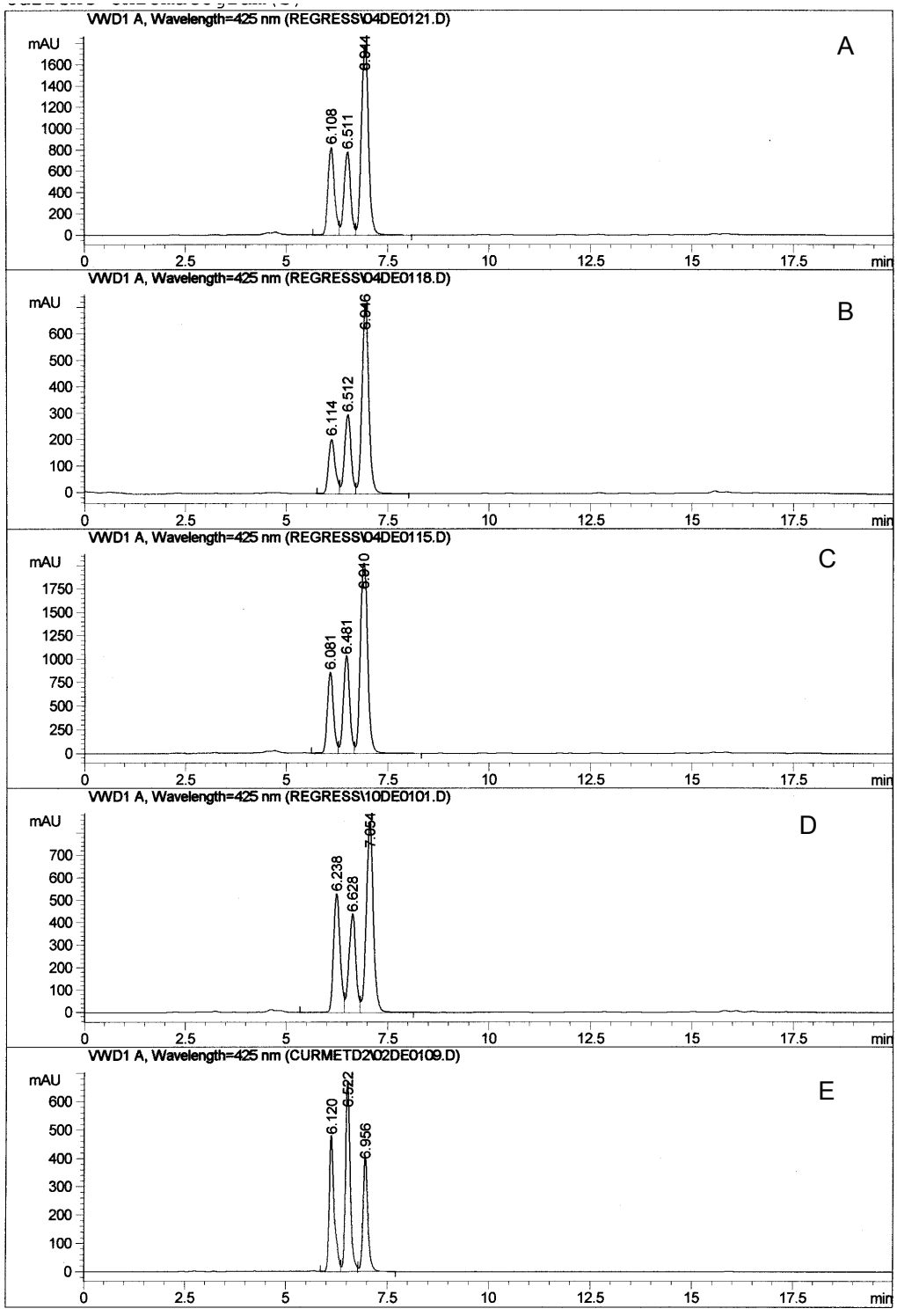

Figure 3.14. HPLC chromatograms of different varieties of *Curcuma longa*, Salem (A), Mysore (B), Erode (C), Balasore (D), Standard mixture of bisdemethoxycurcumin, demethoxycurcumin and curcumin (E).

3.2. DETERMINATION OF INDIVIDUAL CURCUMINOIDS IN DIFFERENT TURMERIC VARIETIES USING IMPROVED HPLC METHOD

INTRODUCTION

Commercially available curcumin / turmeric products consists of a mixture of three curcuminoids *viz.* curcumin, demethoxycurcumin and bisdemethoxycurcumin with curcumin as the main constituent (Ahsan et al., 1999). A variety of methods for quantification of the curcuminoids have been reported. Most of these are spectrophotometric methods, expressing the total color content of the sample (ASTA, 1997). However, it is not possible to quantify the individual curcuminoids with spectrophotometric method. Karasz et al. (1973*)* reported the direct fluorimetric methods for the analysis of curcumin in food materials. Tonnesen and Karlsen (1986) reported the HPLC method for the separation of curcuminoids using a fluorescence detector. Gupta et al. (1999) reported the determination of curcuminoids in turmeric using high-performance thin layer chromatography. However, the linearity was found in the concentration ranged between 1 to 20 μg. Rasmussen et al. (2000) reported the simple and efficient separation of curcuminoids using dihydrogen phosphate impregnated silica gel TLC plates. However, this method can be used for the separation and identification of curcuminoids, but does not enable to quantify individual curcuminoids.

The analysis of individual curcuminoids is possible by HPLC on normal phase or on C_{18} reverse phases. However, the resolution of these compounds to individual peaks was not satisfactory, because of tailing of peaks (Tonnesen and Karlsen, 1983; Smith and Witowska, 1984; Rouseff, 1988). Due to the very labile characteristics of curcuminoids C_{18} columns are preferred for HPLC analysis (Khurana and Ho, 1988).

Of the three commonly used reverse phase solvents, methanol, acetonitrile and tetrahydrofuran (THF), methanol does not provide the necessary resolution/selectivity for the separation of curcuminoids. Using THF instead of acetonitrile as the organic modifier reverses the elution order of the curcuminoids (Smith and Witowska, 1984, Rouseff, 1988). Bailey et al. (1991) reported HPLC method for the separation of curcuminoids. The mobile phase consists of 1% citric acid (pH = 3.0, adjusted with KOH) and THF as organic modifier. Taylor and McDowell (1992) reported separation of three curcuminoids, using a non-silica polymeric column. Hiserodt et al (1996) reported the LC-MS and GC-MS methods for the separation of curcuminoids and volatiles. It involves octadecyl stationary phase using a mobile phase consisting of ammonium acetate with 5% AcOH and acetonitrile. He et al. (1998) reported the on-line-HPLC-UV diode array and electrospray mass spectrometry to analyze curcuminoids and sesquiterpenoids in a fresh turmeric extract. The curcuminoids and sesquiterpenoids were identified at column temperature set at 48 °C. The presence of inorganic salt may contaminate mass spectrometer ion source.

The pigment curcumin is industrially produced using turmeric oleoresin as the starting material. The mother liquor (approximately 70-80%), after isolation of curcuminoids from oleoresin, has a composition of oil, resin and left over curcuminoids. This fraction has no commercial value at present (Saju et al., 1998), but the demand of demethoxycurcumin and bisdemethoxycurcumin is increasing due to the discovery of their new biological activities (Simon et al., 1998; Ahsan et al., 1999; Kim et al., 2001). Hence, a method has been developed for the isolation of curcuminoids from CRTO (Jayaprakasha et al., 2001). This chapter describes the HPLC separation of curcumin, demethoxycurcumin and bisdemethoxycurcumin. The

developed method was satisfactorily applied to determine the content of the individual curcuminoids from commercial turmeric samples.

MATERIALS AND METHODS

Materials

Different turmeric varieties of *Curcuma longa* i.e., were obtained from the cities of Salem (A) and Erode (C) in the state of Tamil Nadu, city of Balasore (D) in the state of Orissa and local market samples from the city of Mysore (B) in the state of Karanataka. Turmeric spent oleoresin (i.e.,Curcumin removed turmeric oleoresin – 2^{nd} lot) was obtained from M/S Flavours and Essences (P) Ltd, Mysore, Karanataka state. Pure curcumin (1), demethoxycurcumin (2) and bisdemethoxycurcumin (3) were isolated as per method described earlier (Part B, Chapter 3.1, Page No. 201).

Equipment

The high-performance liquid chromatographic system consisted of a Hewlett Packard Quaternary pump (Model HP 1100 Series, Hewlett-Packard, CA, USA), fitted with Zorbax analytical (Hewlett-Packard, CA, USA) C_{18} column (250 $\times$ 4.6 mm I.D), 5 μm particle size. The injection system (Rheodyne) used was a 20 µl sample loop. A HP 1100 Series Variable Wavelength Detector was used at a wavelength of 425 nm. A Millipore Swinnex type filter (pore size 0.45 μm, Bangalore, India) was used to remove insoluble impurities from samples before injecting to HPLC.

Preparation of stock solution of curcuminoids for HPLC

Methanolic stock solutions of curcumin, demethoxycurcumin and bisdemethoxycurcumin were prepared separately at a concentration of 0.5 mg/ml.

Sample preparation

Different varieties of turmeric rhizome samples were powdered to get 60 - 80 mesh. The powdered samples (each 5.0 g) were extracted with hexane (100 ml) by using a Soxhlet extractor for 30 min, separately. The hexane extract was discarded and the powder was re-extracted with 150 ml of methanol for 2 h. One ml of this solution was transferred to 10 ml volumetric flask and the volume was made upto the mark with methanol, mixed thoroughly and filtered with Millipore filter (0.45 μm) before subjecting to HPLC analysis.

Chromatographic conditions

Elution was carried out with gradient solvent systems with a flow rate of 1.0 ml/min at ambient temperature. The mobile phase consisted of methanol (A), 2% acetic acid (B) and acetonitrile (C). Quantitative levels of curcuminoids were determined using above solvents programmed linearly from 45% to 65% C in B for 0 to 15 min, then from 65% to 45% C in B for 15-20 min, with a constant of 5% A. The compounds were quantified using the HP CHEMSTATION software.

Validation of HPLC method

Calibration and linearity. The linearity of the method was evaluated by analyzing a series of standard solutions of individual curcuminoids. 10 µl of each of the five working standard solutions containing 0.0625 to 2.0 µg of standard curcumin, demethoxycurcumin and bisdemethoxycurcumin were injected into the HPLC. The elution was carried out as described above and the standard calibration curves were

obtained by plotting concentration of standard curcuminoids versus peak area (average of 3 runs).

Range. The calibration range was chosen due to normal curcuminoids concentrations in turmeric samples. This range included concentrations from lower limit of quantification (LLOQ) to the upper limit of quantification (ULOQ).

Determination of the limit of quantification. The limit of quantification (LOQ) was defined as the lowest standard curcuminoids concentration, which can be determined with an accuracy and precision <20%.

Determination of curcumin, demethoxycurcumin and bisdemethoxy curcumin in samples

The curcuminoids' concentration in the sample volume of 20 μl was calculated based on linear calibration functions and with regard to the dilution factor. The content of curcumin, demethoxycurcumin and bisdemethoxycurcumin was expressed as g /100 g turmeric powder.

RESULTS AND DISCUSSION

Curcuminoids from spent turmeric oleoresin (CRTO) contains more demethoxycurcumin and bisdemethoxycurcumin as compared to commercially available curcumin samples (Figure 3.2). Hence, CRTO is a potential source for the isolation of these two curcuminoids. The major curcuminoid in different commercial varieties of turmeric is curcumin as detected by HPLC (Figure 3.14). Two minor peaks were identified as demethoxycurcumin and bisdemethoxycurcumin. The

percentage compositions of curcuminoids in four different varieties of *Curcuma longa* by HPLC are summarized in Table 3.3.

Table 3.3. Percentage (w/w) of composition of curcuminoids in four different varieties of *Curcuma longa* by HPLC*.

Samples	Curcumin	Demethoxy curcumin	Bisdemethoxy curcumin	Total
Salem (A)	4.14 ± 0.150	2.88 ± 0.025	2.16 ± 0.060	9.18 ± 0.232
Mysore (B)	1.06 ± 0.061	0.86 ± 0.074	0.42 ± 0.036	2.34 ± 0.171
Erode (C)	4.00 ± 0.061	3.36 ± 0.040	1.75 ± 0.050	9.11 ± 0.150
Balasore (D)	5.65 ± 0.040	0.83 ± 0.047	0.62 ± 0.031	7.10 ± 0.119

* : Average of three replications

Curcumin was found to be major compound in all the tested varieties. It was found that, Erode and Salem varieties have more amount of total curcuminoids. Hence, these two varieties may be good sources for the isolation of curcuminoids. Curcumin, demethoxycurcumin and bisdemethoxycurcumin were resolved as individual peaks in all the samples analysed with no interference from other compounds. The identity of

each peak was confirmed by determination of retention times and by spiking with standards.

The purpose of this study was to develop an improved HPLC method for the quantification of curcumin, demethoxycurcumin and bisdemethoxycurcumin. in different turmeric varieties. Asakawa et al. (1981) and Amakawa et al. (1984) reported that the HPLC system based on C_{18} stationary phases does not completely resolve the three curcuminoids. The separation of coloured compounds can be achieved by the use of an amino-bonded stationary phase using the mobile phase with less than 10% water content. Tonnesen and Karlsen (1986) reported the HPLC method for the separation of curcuminoids by using a fluorescence detector. In general, these methods are not stable enough to get reproducible results and problems such as tailing peaks and poor resolution occur. Bailey et al. (1991) have used THF and 1% citric acid (pH 3.0) for the separation of curcuminoids. Citric acid was used in the mobile phase to quench secondary retention mechanisms caused by metals and active silanols on columns where the stationary phase was bonded to silica. Another method (Taylor and McDowell, 1992) involves the separation and identification of curcumin using supelcosil C_{18} column using gradient elution with ammonium acetate-acetic acid and acetonitrile. The presence of inorganic salt in the mobile phases may contaminate the mass spectrometer ion source. Hence, these mobile phases cannot be used when the HPLC is interfaced with a mass spectrometer because of the requirement for volatile mobile phases. He et al. (1998) reported the analysis of curcuminoids and sesquiterpenes using HPLC-UV-ES-MS. The column used was C_{18} and it was maintained at 48 ºC. Regarding the selection of the mobile phase in the present study, a mixture of methanol: 2% acetic acid: acetonitrile gave

optimum chromatographic separation of curcumin, demethoxycurcumin and bisdemethoxycurcumin (Figure 3.3). The resolution was found to be satisfactory. Hence, the same method can be extended to LC-MS. This is an alternative HPLC analysis method to fluorimetric detection, HPTLC and amino bonded silica column for the determination of individual curcuminoids and total content in turmeric samples.

The calibration graphs were prepared to determine the curcuminoids content of different turmeric samples (Figures 3.15, 3.16 and 3.17). Calibration curves were derived from three independent injections of seven concentrations each of curcumin, demethoxycurcumin and bisdemethoxycurcumin verses the peak area. Linearity was found in the concentration range between 0.0625 - 2.0 μg with high reproducibility and accuracy. The regression analysis of experimental data points showed a linear relationship with excellent correlation coefficient (r^2) for curcumin, demethoxycurcumin and bisdemethoxycurcumin and are 0.986, 0.998, and 0.961 respectively. The linear regression equations for the curves for curcumin, demethoxycurcumin and bisdemethoxycurcumin are $y = 13974\ x - 387.62$, $y = 11059\ x - 229.88$ and $y = 8151.7\ x - 114.95$ respectively. The estimated LOQ in this study was found to be 0.05 μg.

CONCLUSION

In the present study, curcuminoids levels in rhizomes of *Curcuma longa* were quantified by rapid and simple analytical procedure requiring minimal sample preparation. The method described is suitable for the routine analysis of a large number of commercial samples of *Curcuma longa* as well as turmeric products with suitable modification of the sample preparation procedure.

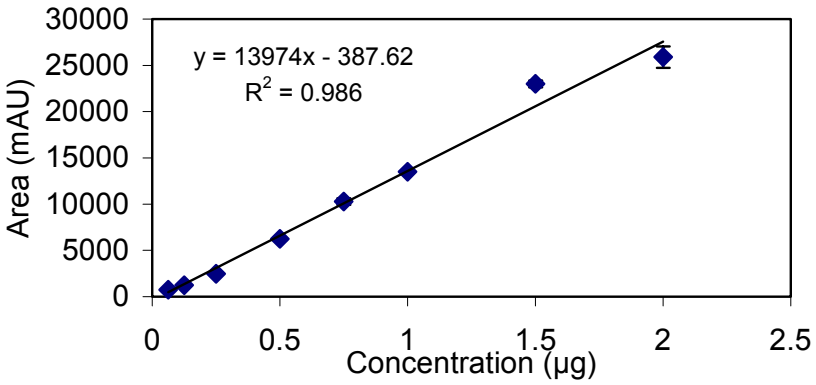

Figure 3.15. Calibration graph for curcumin

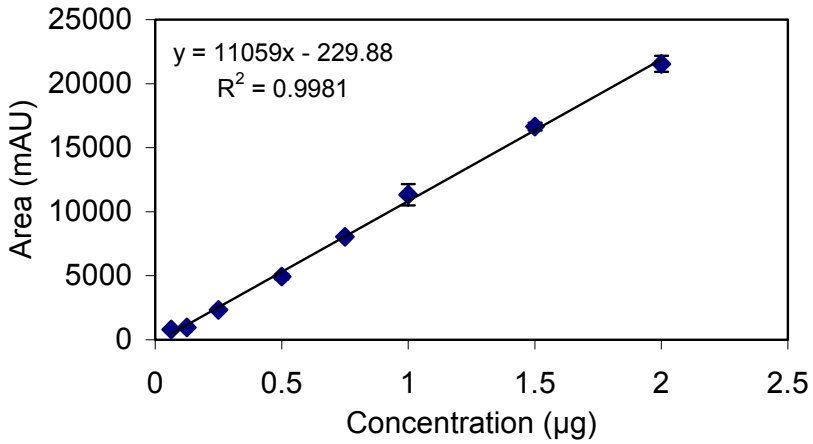

Figure 3.16. Calibration graph for demethoxycurcumin

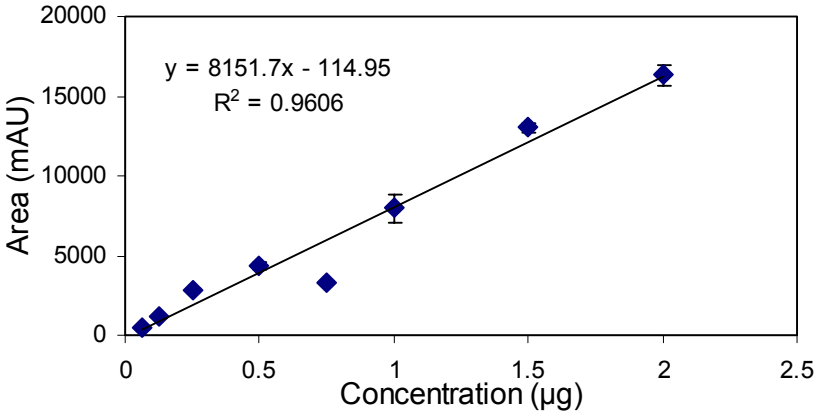

Figure 3.17. Calibration graph for bisdemethoxycurcumin

3.3. ANTIOXIDANT ACTIVITY OF CURCUMIN, DEMETHOXYCURCUMIN AND BISDEMETHOXYCURCUMIN

INTRODUCTION

Reactive intermediates in oxidation processes, particularly free radicals, are receiving increased attention in biology, medicine, food chemistry, and as well as environmental areas (Whitehead et al., 1992; Larson, 1997). Radical species are involved in many oxidative chain reactions. A common example of such a process is lipid peroxidation in foods leading to rancidity. Food additives such as antioxidants can be applied to extend the shelf-life of foods and maintain their safety, nutritional quality, functionality and palatability. Antioxidants must be non-toxic, relatively inexpensive, and effective. These should also possess carry-through effect during processing, and should not alter the quality of the end-product (Reische et al., 1998). Currently, food manufacturers / consumers prefer additives labelled as ‘‘natural’’. Therefore, there is a growing tendency to replace synthetic antioxidants by natural alternatives; rosemary extracts being a prime example. New sources are being screened for potential novel natural antioxidants.

Several studies in recent years have shown that curcumin posses antioxidant, anti-inflammatory, anti-microbial, anti-parasitic, anti-mutagen and anticancer properties (Khanna, 1999). The potential use of curcumin in the prevention of cancer and in the treatment of infection with human immuno-deficiency virus (HIV) is the subject of intensive laboratory and clinical research (Srimal, 1997). Ahsan et al. (1999) reported

the antioxidant and pro-oxidant activity of curcumin and the structure relationship between curcumin, demethoxycurcumin and bisdemethoxycurcumin. Recently, the effect of curcuminoids was examined on the proliferation of MCF-7 human breast tumour cells. It was reported that demethoxycurcumin showed the best inhibition of MCF-7 cells followed by the curcumin and bisdemethoxycurcumin (Simon et al., 1998). Pure curcumin, bisdemethoxycurcumin and demethoxycurcumin are not available from commercial sources. Commercial curcumin contains 77% curcumin, 17% demethoxycurcumin and 3% bisdemethoxycurcumin (Ahsan et al., 1999). In this chapter, the studies on the antioxidant capacity and activity of curcumin, bisdemethoxycurcumin and demethoxycurcumin using phosphomolybdenum and linoleic acid peroxidation methods respectively have been discussed.

MATERIALS AND METHODS

Isolation and purification curcuminoids

Curcumin, demethoxycurcumin and bisdemethoxycurcumin were isolated from CRTO and identified as described in Part B, Chapter 3.1, Page No. 201.

Antioxidant capacity by phosphomolybdenum method

Antioxidant capacity of curcumin, demethoxycurcumin and bisdemethoxycurcumin were evaluated by the method of Prieto et al. (1999). An aliquot of 0.1 ml of curcuminoids (equivalent to 50 and 100 ppm) was combined with 1.0 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Methanol (0.1 ml) was used in place of sample solution for the blank. The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous

solution of each was measured at 695 nm against a blank in a Genesys-5-UV-Visible spectrophotometer (Milton Roy, New York, USA). Antioxidant capacity of curcumin, demethoxycurcumin and bisdemethoxycurcumin was expressed as ascorbic acid equivalents (μmol/g of sample).

Antioxidant activity using linoleic acid peroxidation method

Antioxidant activity of curcumin, demethoxycurcumin and bisdemethoxycurcumin were determined using the thiocyanate method (Yen and Hsieh, 1998). The linoleic acid emulsion was prepared by homogenizing 0.28 g of linoleic acid, 0.28 g of tween-40 as emulsifier and 50 ml of phosphate buffer (0.2 M, pH 7.0). Curcuminoids were dissolved in MeOH and pipetted (0.5 ml) into different test tubes (equivalent to 100 ppm), then were mixed with 2.5 ml of linoleic acid emulsion, 2.5 ml of phosphate buffer (0.2 M, pH 7.0) and incubated at 37 °C for 168 h. The mixture prepared as above without test sample served as control. Aliquots (0.1 ml) were drawn from the incubation mixture at an interval of 24 h and mixed with 5.0 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM in ferrous chloride in 3.5% hydrochloric acid and allowed to stand at room temperature for 3 min. The colour developed was measured at 500 nm. The degree of linoleic acid peroxidation was calculated at 120 h using the following formula (Pin-Der Duh, 1998). Antioxidant activity = [1- (increase in absorbance of sample / increase in absorbance of control)] × 100. BHT was used as standard for comparison. All tests and analysis were carried out in triplicate and averaged.

RESULTS AND DISCUSSION

Pure curcuminoids were isolated from CRTO and identified as described in Part B, Chapter 3.1. Total antioxidant capacities of the individual curcuminoids were quantitatively determined by the formation of phosphomolybdenum complex. This method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V) complex, which has maximal absorption at 695 nm. The results of antioxidant capacity curcuminoids are expressed as water-soluble ascorbic acid equivalents (μmol / g of sample). Curcumin, demethoxycurcumin and bisdemethoxycurcumin exhibited various degrees of antioxidant capacity (Figure 3.18). The antioxidant capacity of curcuminoids was found to decrease in the order curcumin>demethoxycurcumin >bisdemethoxycurcumin. Antioxidant capacity of curcumin, demethoxycurcumin and bisdemethoxycurcumin were found to be 3099 ± 66, 2833 ± 25 and 2677 ± 30 μmol / g ascorbic acid equivalents at 50 ppm concentration respectively. The active principles in turmeric are a group of phenolic compounds including curcumin, which is well known for its strong antioxidant activity (Miquel et al., 2002). However, in the present investigation, it was found that other two curcuminoids are also effective antioxidants.

The antioxidant activity of curcumin, demethoxycurcumin and bisdemethoxycurcumin in preventing the peroxidation of linoleic acid, as measured by thiocyanate method, is shown in Figure 3.19. Absorbance of control has increased up to 2.282 at 120 h, and then it has decreased. This is due to oxidation of linoleic acid, generating linoleic acid hydroperoxides, which decompose to many secondary oxidation products (Hua-Ming et al., 1996). The oxidized products (viz., linoleic acid hydroperoxides) react with ferrous chloride to form ferric chloride, then to ferric thiocyanate of blood-red colour. After the incubation period (120 h), the formation of

peroxides will be stagnated, due to non-availability of linoleic acid. Also, the intermediate products may be converted to stable end products. The non-availability of hydroperoxides, results in the retardation of oxidation of ferrous chloride. Hence, the absorbance will not increase. In the presence of curcumin, bisdemethoxycurcumin, demethoxycurcumin and BHT, oxidation of linoleic acid was very slow. Hence, the colour development will be slow. The antioxidant activities of the curcumin, demethoxycurcumin and bisdemethoxycurcumin were found to be 81.98, 81.77 and 73% respectively at 120 h. The decreasing order of antioxidant activity of curcuminoids is correlating well in the order of antioxidant capacity found in the phosphmolybdenum method.

Ahsan et al. (1999) reported the curcumin, bisdemethoxycurcumin and demethoxycurcumin are able to degrade DNA in the presence of Cu (II), the order of activity being curcumin > demethoxycurcumin > bisdemethoxycurcumin. Curcuminoids are capable of inhibiting damage to super coiled plasmid DNA by hydroxyl radicals. Kim et al. (2001) reported the radical scavenging activity of curcumin, bisdemethoxycurcumin and demethoxycurcumin. It was concluded that, bisdemethoxycurcumin and demethoxycurcumin are good in trapping the DPPH radical as efficiently as well-known strong antioxidant i.e. curcumin. The concentration of these two components is high in spent turmeric oleoresin compared to commercial turmeric oleoresin. The results of the present study indicated that the spent turmeric oleoresin could be used for the isolation of other two curcuminoids, which could be used as potential antioxidants.

CONCLUSION

The results of the present work established that demethoxycurcumin and bisdemethoxycurcumin from CRTO are also good antioxidants along with curcumin. Further, work is required to study mode of their different antioxidant mechanisms *in vitro* and in food model systems.

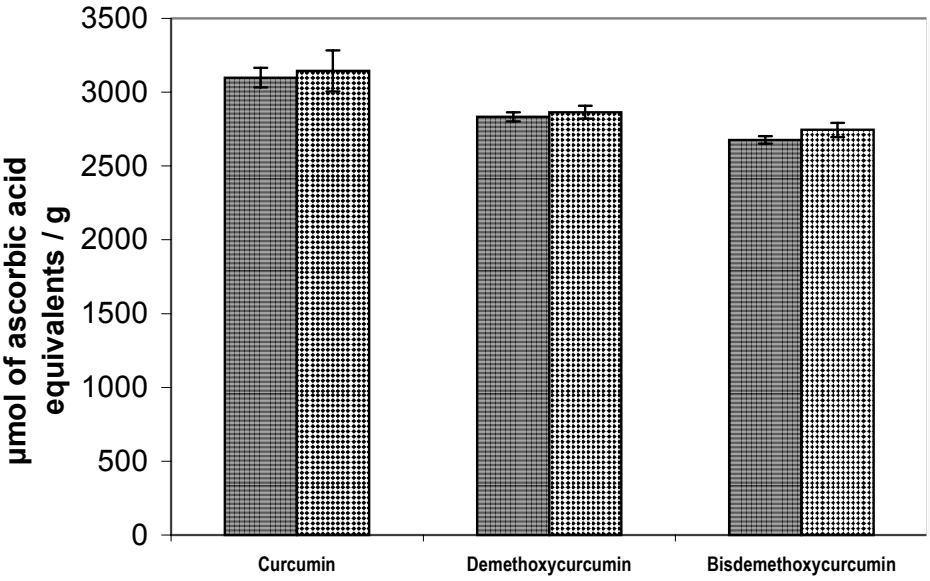

Figure 3.18. Antioxidant capacity of curcuminoids by phosphomolybdenum method

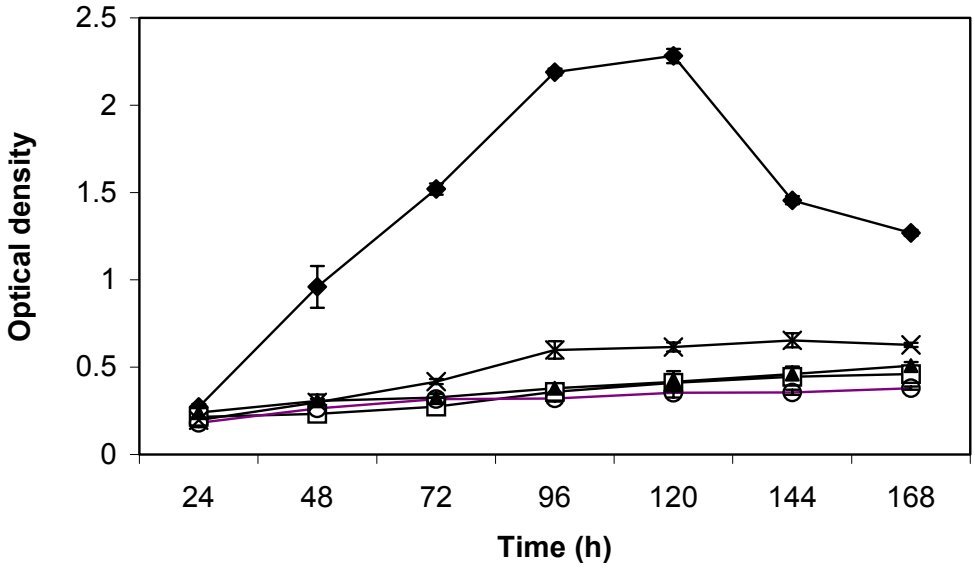

Figure 3.19. Antioxidant activity of curcuminoids and BHT at 100 ppm concentration using thiocyanate method

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Summary and conclusions

Chapter 1. In this chapter, a brief introduction has been presented on recent literature on chemistry, technology and biological activities of *Curcuma longa*.

Chapter 2. Isolation and identification of antibacterial, antifungal and antioxidant and antimutagenic fractions from volatiles of spent turmeric oleoresin have been presented under sub-chapters 2.1, 2.2 and 2.3.

*2.1***.** Curcumin, the yellow colour pigment of turmeric, is produced industrially from turmeric oleoresin. The mother liquor / spent turmeric oleoresin / curcumin removed turmeric oleoresin (CRTO) after isolation of curcumin from turmeric oleoresin, contains approximately 40% oil. The CRTO oil was separated from the mother liquor. This oil was broadly fractionated to get three fractions using column chromatography. These fractions were tested for antibacterial activity by pour plate method against *Bacillus cereus, Bacillus coagulans, Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Fraction II eluted with 5% ethyl acetate in hexane was found to be most active fraction. The CRTO oil, fractions I and II were analysed by GC and GC-MS. Thirteen chemical constituents were identified. *ar*-Turmerone, turmerone and curlone were the major compounds present in these fractions.

2.2. The CRTO oil was subjected to fractional distillation *under vacuum* to get two distillates. These fractions (CRTO oil, distillate I and II) were tested for antifungal activity against *Aspergillus flavus, A. parasiticus, Fusarium moniliforme* and *Penicillium digitatum* by spore germination method. Distillate II was found to be more active. Twenty-five chemical constituents from CRTO oil, distillates I and II were identified by GC and GC-MS. *ar*- Turmerone (52.6%), turmerone (11.5%) and curlone (8.5%) were major compounds present in the distillate II along with other oxygenated compounds.

2.3. CRTO oil was broadly fractionated using silica gel column chromatography to obtain three fractions. Fifteen volatile constituents were

identified by GC and GC-MS. CRTO oil contained *ar*- turmerone (31.32%), turmerone (15.08%) and curlone (9.7%). Also, oxygenated compounds were enriched in fraction III. CRTO oil and its fractions were tested for antioxidant activity using the β-carotene-linoleate model system and the phosphomolybdenum method. The fraction III showed maximum antioxidant capacity. These fractions were also used to determine their protective effect against the mutagenicity of sodium azide by means of the Ames test. CRTO oil, fractions I and II exhibited good antimutagenicity, but fraction III was the most effective. Fraction III contained *ar*-turmerone (44.5%), curlone (19.22%) and turmerone (10.88%) as the major compounds.

Chapter 3. The isolation of curcuminoids from spent turmeric oleoresin / CRTO has been described. Separation of individual curcuminoids and their identification, an improved HPLC method developed for the analysis of curcuminoids from commercial samples of turmeric and antioxidant activity of individual curcuminoids are presented in the sub-chapters.

3.1. Isolation of curcuminoids from CRTO was standardised for the first time. After removal of oil, left over CRTO was extracted with medium polar solvent and the extract concentrated to 50%. Curcuminoids were precipitated by adding non-polar solvent. The composition of isolated curcuminoids mixture was analysed by HPLC. Curcumin, bisdemethoxycurcumin and demethoxycurcumin were separated by column chromatography, identified from their IR, ^{1}H, ^{13}C NMR and Mass spectra.

3.2. An improved HPLC method was developed for the separation of curcumin, bisdemethoxycurcumin and demethoxycurcumin. HPLC separation was performed on a C_{18} column using mixtures of three solvents, *viz.,* methanol, 2% acetic acid, and acetonitrile, with detection at 425 nm. Four different commercially available varieties of turmeric rhizomes were analysed to determine the percentage of these three curcuminoids. The quantities of curcumin, demethoxycurcumin, and bisdemethoxycurcumin as

estimated by HPLC using their calibration curves were found to be 1.06 - 5.65, 0.83 - 3.36 and 0.42 - 2.16%, respectively. The total percentages of curcuminoids varied in the range of 2.34 - 9.18%.

3.3. Antioxidant activity of curcumin, demethoxycurcumin and bisdemethoxycurcumin were determined by phosphomolybdenum and linoleic acid peroxidation methods. Antioxidant capacity of curcumin, bisdemethoxycurcumin and demethoxycurcumin were found to be 3144 ± 140, 2744.8 ± 48 and 2864.1 ± 44 μmol / g ascorbic acid equivalents, respectively at 50 μg/ml concentrations. The antioxidant activities of the curcumin, bisdemethoxycurcumin and demethoxycurcumin were found to be 81.98, 73.0 and 81.77% respectively, using linoleic acid peroxidation method.

The results of the present study are indicative of the utilization of CRTO oil and its fractions can be used as a preservative agent, which has no commercial application at present.

CONCLUSIONS

The study describes the investigation on the volatile and non-volatile constituents from curcumin removed turmeric oleoresin (industrial by-product).

The volatile oil from CRTO was used for the fractionation and characterisation of antibacterial, antifungal, antioxidant and antimutagenic fractions. The chemical composition of these fractions was determined using GC and GC-MS. The study also, helped in devising an analytical method for the determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin using HPLC analysis. After isolating the volatile oil the spent was used for the separation of curcuminoids mixture. The mixture was subjected to column chromatography and three individual curcuminoids *viz.,* curcumin, demethoxycurcumin and bisdemethoxycurcumin were separated and characterised on the basis of IR, ^{1}H and ^{13}C NMR spectra. The purified curcuminoids were used for the HPLC calibration and antioxidant activity. The antioxidant activities of individual curcuminoids were found to be in the decreasing order of curcumin > demethoxycurcumin > bisdemethoxycurcumin.

Further work is required to study the antioxidant activity, antimicrobial and antimutagenic activity of volatile constituents from CRTO in food systems.

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11. Isolation and identification of chemical constituents from *Cinnamomum zeylanicum* fruits and their antioxidant activity (Manuscript under preparation)

PATENTS FILED

1. A process for the isolation of *trans*-cinnamyl acetate from the unconventional parts of cinnamon (*Cinnamomum zeylanicum* Blume), by L. Jagan Mohan Rao, **G. K. Jayaprakasha** and K. K. Sakariah. (India Patent No. 2941/DEL/97, dt. 14/10/97).

2. A process for the recovery of curcuminoids mixture from spent turmeric oleoresin by **G. K. Jayaprakasha,** L. Jagan Mohan Rao and K. K. Sakariah. (Indian Patent No. 168/DEL/02, dated 28/02/2001; CSIR No. 472/NF/2001, dt. 30/10/2001).

3. A one step process for the preparation of antibacterial fraction from the unconventional parts of *Cinnamomum zeylanicum.* By **Jayaprakasha, G.K.,** Negi, P.S. Jagan Mohan Rao, L. and Sakariah, K.K. (Submitted to CFTRI patent cell).

4. A process for the isolation of antioxidants from fruits of *Cinnamomum zeylanicum.* **G. K. Jayaprakasha,** and L. Jagan Mohan Rao (Under preparation)

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