Chemical and biochemical investigations on coffee by-products for value addition

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BY

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CERTIFICATE

I, hereby certify that the thesis entitled "**CHEMICAL AND BIOCHEMICAL INVESTIGATIONS ON COFFEE BY-PRODUCTS FOR VALUE ADDITION"** submitted by Smt. K. Ramalakshmi for the award of the degree of **DOCTOR OF PHILOSOPHY** in **CHEMISTRY** to the **UNIVERSITY OF MYSORE, India** is the result of the research work carried out by her in the Department of Plantation Products, Spices and Flavour Technology, **CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE, Mysore, India** under my direct guidance and supervision during the period 2004-2009.

Dr. L. Jagan Mohan Rao
(Research Guide)

Date:

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DECLARATION

I, hereby declare that the thesis entitled, "**CHEMICAL AND BIOCHEMICAL INVESTIGATIONS ON COFFEE BY-PRODUCTS FOR VALUE ADDITION"** has been submitted to the **UNIVERSITY OF MYSORE, INDIA** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **CHEMISTRY,** is the result of the research work carried out by me , in the Department of Plantation Products, Spices and Flavour Technology, **CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE, Mysore, India** under the guidance of Dr. L. Jagan Mohan Rao during the period 2004-2009.

I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

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Scope & Aim of the Investigation

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Coffee is one of the international products and is the second largest traded commodity in the world next to crude oil. It belongs to Rubiaceae family which contains more than 70 species but two of them are of significant economic importance namely, arabica (Coffea arabica) and robusta (Coffea canephora). According to the latest estimate of International Coffee Organization (ICO) the world coffee production in 2008-2009 is 128.8 million bags (1 bag equals 60 kilograms or 132.276 pounds), nearly 15 million bags over the previous year. Brazil, Columbia and Vietnam are the top three coffee producers accounting for more than 45% of the total world produce while India's contribution is 4.6% (URL-1).

In coffee producing countries, coffee wastes and by-products constitute a source of severe contamination and a serious environmental problem. For this reason, since the middle of the last century, efforts have been made to develop methods for its utilization as a raw material for the production of feeds, beverages, vinegar, biogas, caffeine, pectin, pectic enzymes, protein, and compost. In general, coffee is graded based on the size, colour and percentage of imperfections. In India, apart from size specification, a defect count standard based on the 'percentage of defects by weight' was specified for each of the grades of coffee (Anon, 1982). Defective coffee obtained after grading is termed as low-grade coffee beans (LCB), which includes the imperfections such as blacks, dark brown beans, insect damaged beans, spotted beans, sours, bits and greens (immature beans) etc.

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LCB are obtained as a result of either improper formation within the fruit or by faulty processing. These beans produce undesirable taste in the beverage when mixed with graded beans. LCB represent about 15-20% of coffee production on weight basis and are a problem for disposal. Nearly 1.2-1.5 million tonnes are generated in the coffee industry every year and LCB are rejected in the international market due to the undesirable taste produced in the beverage. Generally LCB are separated from the quality beans prior to selling on external markets and transferred to the internal market. The majority of the roasting industries blend these beans with the healthy ones and over all a low grade roasted and ground coffee are consumed in the respective internal market. In order to eliminate these defective coffee beans from the internal market, ICO urges to find out the ways for alternative uses of them. Therefore, it is worthwhile to find ways of utilization for its value addition.

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Coffee is consumed by the people either as filter coffee or instant coffee. Instant coffee is produced from green coffee after roasting, grinding and extraction of water solubles. After extraction the remaining coffee is called "spent coffee". Almost 50% of the world produce is processed for soluble coffee manufacture. The major problem encountered by the industry is the disposal of spent coffee. Disposal of spent coffee has included sewer discharge, sanitary land fill, incineration, cattle feed, as fillers and adsorbents in thermosetting material (Ligo Eugenie, 1970; Boopathy, 1987; Rizzi & Gutwein, 1994). Waste coffee grounds were used after removing the

oil from the grounds (Sivetz & Desrosier, 1979). Industrial coffee waste was degraded and used as compost (Stahl & Turek, 1991). D- mannose and D-mannitol were prepared by the acid hydrolysis of spent coffee. The spent coffee contains 45% (dry basis) polymeric carbohydrates which needs investigations to make soluble carbohydrates (Stahl & Turek, 1991; Navarini et al., 1999).

Bioactive substances from various plants, animals and microbial sources are gaining importance in recent years and several reports have appeared in identification and isolation of active components. Synthetic antioxidants such as butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) and propyl gallate (PG) are used in food products within certain limits to suppress the development of radicals/peroxides during food storage. Commercial synthetic antioxidants such as BHA are finding disfavour throughout the world and BHT is already banned for food use in many countries including India since they are suspected to be carcinogenic. Therefore, the importance of the search for exploitation of natural antioxidants especially of plant origin has greatly increased in recent years. Antioxidant activity of chlorogenic acid, which is one of the major constituents in coffee beans, and coffee extract, was studied. The activity of coffee brew is not only due to phenolic compounds naturally occurring in green coffee beans but also due to melanoidin and phenylindans which might be produced during roasting.

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Literature survey revealed that the studies on bio-activity of spent and LCB have not been studied. Preliminary work carried out in the laboratory disclosed that the extracts from the above by-products showed antioxidant activity. However, this requires to be validated with conclusive evidences and also systematic basic research work is needed to find out the components responsible for the same. Further, the economic viability of isolation of these compounds needs to be studied. It is in this context this investigation was carried out to study the bio-active compounds of LCB and spent coffee residue.

The main aim of the proposed study is

1. To study the physicochemical characteristics of Low grade coffee beans and spent coffee.
2. To optimize the conditions for obtaining the maximum extraction of conserves using suitable solvents either single or as a mixture.
3. To evaluate the bio activity of the extracts employing *in vitro* and *in vivo* methods.
4. To select the extract having maximum bioactivity and isolation of the components employing chromatographic techniques.
5. Chemical and enzymatic modifications of carbohydrate polymers.
6. To characterise the bio-active components by spectral analysis.

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LIST OF ABBREVIATIONS

8-OHdG	: 8-hydroxydeoxyguanosine
AA	: Antioxidant activity
AAPH	: 2, 2-azinobis (2-amidinopropan) dihydrochloride
AMP	: Adenosine monophophate
AnV	: anisidine value
Ar	: Arabica plantation
AUC	: Area under curve
BHA	: Butylated Hydroxy Anisole
BHT	: Butylated Hydroxy Toulene
Carr	: Cornelli
CGA	: Chlorogenic acids
CQA	: Caffeoylquinic acid
CEH	: Cholesterol esterase
DEFT	: Driven Equilibrium Fourier Transform
d	: doublet
dd	: double doublet
dG	: deoxyguanosine
dl	: deciliter
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl sulphoxide
DNS	: Dinitrosalicylic Acid
DPPH	: α, α - diphenyl β-picrylhydrazyl
EDTA	: Ethylene diamino tetra acetic acid
ELISA	: Enzyme-Linked Immunosorbent Assay
ELSD	: Evaporative Light Scattering Detector
FCS	: Fetal Calf Serum
FRAS	: Free Radical Analytical System
FFA	: Free Fatty Acid
FTIR	: Fourier Transfrom Infrared Spectroscopy
g	: gram
GMP	: Guanosine monophosphate

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h	: hour
HDL	: High Density Lipoprotein
HPLC	: High Performance Liquid Chromatograph
HMBC	: Heteronuclear Multiple Bond Correlation
HRP	: Horseradish Peroxidase
HSQC	: Heteronuclear Single Quantum Correlation
Hz	: Hertz
Ig	: Immunoglobulin
IMP	: Inosine monophosphate
IPA	: Iso Propyl Alcohol
J774A.1	: Mouse macrophage cell line
JaICA	: Japan Institute for the Control of Aging
JCRB	: Japanese Collection of Research Bioresources
LCB	: Low grade coffee beans
LC-MS	: Liquid Chomatography Mass Spectrometry
Me_1	: Methanol extract
ml	: Mililitre
mM	: millimolar
MT buffer	: Modified Tyrode buffer
NAD	: Nicotinamide adenine dinucleotide
NADH	: Nicotinamide adenine dinucleotide reduced
NIHS	: National Institute of Health Sciences
nM	: nanomolar
nm	: nanometer
NMF	: Normal Mice Feed
NMR	: Nuclear Magentic Resonanace
NOESY	: Nuclear Overhauser Effect Spectroscopy
°C	: Degree centigrade
°F	: Degree Fahrenheit
ORAC	: Oxygen Radical Absorbance Capacity
PDA	: Photo diode array
PBS	: Phosphate buffered saline
ppm	: parts per million

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PV	: peroxide value
Rb	: Robusta cherry
RBL	: Rat basophilic leukemia
ROM	: Reactive oxygen metabolites
ROS	: Reactive oxygen species
Rpm	: rotation per minute
RSA	: radical scavenging activity
S	: singlet
SAH	: S-Adenosyl homocysteine
SAM	: S-Adenosyl methionine
SEFT	: Spin Echo Fourier Transform Spectroscopy
SS	: stainless steel
TFA	: tri fluro acetic acid
TMB	: 3, 3’, 5, 5’-tetramethylbenzidine chromatic solution
TNF	: Tumor Necrosis Factor
XMP	: Xanthosine monophosphate
μm	: microns

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MATERIALS & EQUIPMENTS

Plant materials

Two varieties of arabica and robusta processed by dry and wet methods were selected for the studies and were procured from Maruthi coffee curing works, Mysore, India. Spent coffee powder was prepared using a SS column extractor (patented) in the laboratory. Low-grade coffee beans were purchased from Castle Coffee Co, Mysore.

Chemicals and reagents

Reference chemicals such as caffeine, chlorogenic acid, gallic acid, DPPH, BHA, fluorescein sodium salt and Trolox, anti-DNP (dinitrophenyl)-IgE, DNP-HSA, enzymes such as *a- Amylase,* Cellulase, Pullulanase, Pectinase and Folin-Ciocalteu's reagent, were purchased from Sigma Chemical Co., St. Louis, MO. Cell proliferation reagent WST-1 was purchased from Takara Bio Inc., Shiga, Japan. AAPH and Wortmannin were purchased from Wako Pure Chemical Industries, Kyoto, Japan. 8-OHdG ELISA Kit was procured from JaICA, Shizuoka, Japan. Cholesterol assay kits - Wako 439-17501 and Wako 431-52501 were procured from Wako, Japan. Dowex resin was purchased from Bakers Chemicals Company, USA. Diaion HP 20 was purchased from Labion, Pharmaconcept, Mumbai, India. All the other chemicals and solvents used were from Merck, Mumbai, India unless otherwise mentioned in the experiments.

Cell organisms

P388, lymphoid mouse neoplasma cell line (JCRB0017), J774A.1, mouse macrophage cell line (JCRB9108], RBL- 2H3, rat basophilic

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leukaemia [JCRB 0023] were procured from National Institute of Health Sciences, Japanese Collection of Research Bioresource, Tokyo, Japan.

Equipments

All equipments were calibrated before use. All the extracts were concentrated using flash evaporator (Heidolph, RE 120, Germany). Absorbances in the ultraviolet and visible regions were measured using a UV–visible spectrophotometer (Cintra 10, GBC, Australia). Colour measurements were carried out on refelectance meter (Photovolt 575, USA). pH of the solutions was measured using Control Dynamics – APX-175E/C (India) meter. ORAC readings were monitored in multidetection microplate reader (Powerscan, ^{TR} Dainippon Sumitomo Pharma, Osaka, Japan) equipped with Gen 5 software. Absorbance of the cell assay experiments were carried out by ELISA reader (Molecular Devices, $Thermo_{max}$ Microplate Reader, Vista, Canada) and microplate reader (Biorad Model 550, Bio-Rad Laboratories, Hercules, CA).

Separation and analysis of chlorogenic acids and carbohydrates were carried out by HPLC system (Waters 2998) with Empower software equipped with PDA and ELSD detectors respectively. IR spectra were recoded on a Bruker-IFS 25 spectrometer using KBr discs. NMR analysis was carried out by Bruker (Rheinstetten, Germany) DRX 500 NMR instrument operating at 500MHz for ^{1}H and 125 MHz for ^{13}C at room temperature; Scanned region –0 to 20 ppm for ^{1}H and 0–200 ppm for ^{13}C spectra. Tetramethylsilane was used as an internal standard.

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Chemical and biochemical investigations on coffee by-products for value addition

SYNOPSIS
Submitted to the
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Chemical and biochemical investigations on coffee by-products for value addition

Coffee is one of the most important agricultural products traded worldwide. Coffee belongs to Rubiaceae family with more than 70 species but two of them are of significant economic importance namely, arabica (*Coffea arabica*) and robusta (*Coffea canephora*). International Coffee Organization (ICO) estimated the world coffee production in 2008-2009 as 128.8 million bags (1 bag equals 60 kilograms or 132.276 pounds), nearly 15 million bags over the previous year. Coffee is produced in 70 countries of the world. Brazil, Columbia and Vietnam are the top three coffee producers accounting for more than 45% of the total world produce while India's contribution is 4.6%.

In coffee producing countries, coffee wastes and by-products constitute a source of severe contamination and a serious environmental problem. The major by-products of coffee processing industries are coffee pulp, processing effluent, husks, silverskin, low-grade coffee beans and spent coffee residue. The use of coffee by-products and wastes has been the subject of numerous studies and lead to the conclusion that these can be used in a variety of ways.

In general, coffee is graded based on the size, colour and percentage of imperfections. In India, apart from size specification, a defect count standard, based on the 'percentage of defects by weight' was specified for each of the grades of coffee. Defective coffee obtained after grading is termed as ***low-grade coffee beans*** (LCB), which includes the imperfections such as blacks, dark brown beans,

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insect damaged beans, spotted beans, sours, bits and greens (immature beans) etc. LCB are rejected in the international market due to the undesirable taste produced in the beverage. However, majority of the roasting industries blend these beans with the graded ones and overall a low grade roasted and ground coffee are consumed in the respective internal markets. In order to eliminate these defective coffee beans from the internal markets, it is worthwhile to find ways of utilization for its value addition apart from consuming as beverage.

Coffee is consumed by the people either as filter coffee or instant coffee. Instant coffee is produced from green coffee after roasting, grinding and extraction of water solubles. After extraction, the remaining residue is called ***spent coffee***. Almost 50% of the world produce is processed for soluble coffee manufacture. The major problem encountered by the industry is the disposal of spent coffee. Therefore, these two by-products (viz., LCB and spent coffee) were selected for the investigation work to find out the alternate uses.

Chapter 1 entitled ***Coffee: processing, chemistry and by-products*** deals with the general introduction about the statistics of coffee, processing methods and chemical constituents present in the coffee are discussed along with bioactivity including health benefits of coffee.

Chapter 2 entitled ***Physico-chemical characteristics of LCB and spent coffee*** describes in detail about the coffee grading with respect to Indian and world scenario in the 'Introduction' section followed by the description, formation and the nature of LCB. This chapter also deals with physical and chemical attributes of LCB

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and spent coffee residue. Four varieties of graded and LCB were analysed for physical characteristics such as bean density, brightness, titratable acidity, pH, moisture, and total soluble solids and also for chemical composition, viz., caffeine, chlorogenic acids, lipids, sucrose, total polyphenols and proteins. Spent coffee powder from two varieties of coffee namely arabica plantation and robusta cherry, was prepared using a column extractor and analysed for bioactive compounds namely, caffeine, total polyphenols and chlorogenic according to the standard protocols. Marginal differences in the physical and chemical parameters between the graded and defective coffee beans were observed and the results are discussed.

Biochemical studies of the extracts of LCB and spent coffee are explained in Chapter 3 under the title ***Biochemical studies: in vitro and in vivo***. LCB and spent coffee residue were subjected to extraction using various solvents namely hexane, chloroform, acetone and methanol successively using column and soxhlet extractors. The extracts (conserves) were evaluated for antioxidant potential through *in vitro* models such as radical scavenging activity (α,α–diphenyl-β-picrylhydrazyl radical), antioxidant activity (β-carotene-linoleate model system), reducing power (iron reducing activity) and antioxidant capacity (phosphomolybdenum complex). Methanol extract (Me_1) was further analysed for various biological activities such as oxygen radical absorbance capacity (ORAC), anti-tumour (P388 cell assay), anti-inflammatory (J774A.1 cell assay) and anti allergy (RBL- 2H3) *in vitro* and the results are discussed.

Me_1 was also evaluated for oxidative stress in BALB/c mice and explained under the subdivision "Bioactivity *in vivo*" in Chapter 3. Me_1 was mixed with Normal

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Mice Feed and fed to the mice (n=6), upto 14 days of experimental period. Blood and urine samples were collected. Urine samples were quantitatively analysed for oxidative DNA damage in terms of the adduct 8-hydroxydeoxyguanosine (8-OHdG) as a biomarker. Serum samples were analysed for total cholesterol, HDL cholesterol, oxygen radical absorbance capacity and reactive oxygen metabolites. Results suggest that coffee extract showed positive indication in controlling the oxidative stress in mice.

Chapter 4 entitled ***Characterisation of bioactive compounds and value addition*** is divided into two parts. Part I describes the analysis of chlorogenic acid isomers from Me_1 by High Pressure Liquid chromatographic (HPLC) method employing PDA detector. Further, Me1 was subjected to preparative HPLC to separate the constituents. Six fractions were collected and Nuclear Magnetic Resonance spectral analysis was carried out on selected fractions for the identification of compounds. Trigonelline was identified in the first fraction and this is the first report for the presence of this compound in the LCB. Compound present in the second fraction was identified as 3-Caffeoylquinic acid (CQA). The third fraction was found to contain two major compounds viz., 5-CQA and caffeine. Presence of 4-CQA was also observed. Fractions 4, 5 and 6 were identified as 3, 4 diCQA, 3,5, diCQA and 4, 5 diCQA respectively by comparison with the retention times.

Methanol extract was subjected to fractionation by different treatments for the enrichment of phenolics in terms of chlorogenic acids. Me_1 was partitioned in selected solvents individually and successively. The extract was also subjected

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chromatographic separation employing resins. Also, the Me_1 was subjected to lead acetate treatment to precipitate the phenolics. The separated fractions were analysed for the parameters such as chlorogenic acids, total polyphenols and caffeine. Further, the separated fractions were analysed by HPLC for the quantification of isomers of CQAs.

In Part II, utilisation of antioxidant coffee conserve as a natural antioxidant is explained by incorporating Me_1 in sunflower oil. The conserve prepared from low-grade coffee beans, which has the radical scavenging activity of 92.5 % at 100-ppm concentration, was incorporated into sunflower oil at two concentrations (200 & 500 ppm) and stored at 27 and 40 °C in transparent as well in brown coloured glass bottles. The quality parameters such as peroxide value, free fatty acids and p-anisidine value were analysed for a storage period of 120 days. Also, the oil was subjected to FT-IR analysis initially as well at the end of storage period to observe the degraded compounds formed during the storage. Results showed that the conserve was quite effective in restricting the oxidative changes and did not have any adverse effect on the storage quality of the oil.

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Chapter 5 entitled ***Hydrolysis and characterisation of polysaccharides*** describes the methodologies for the solubilisation of polysaccharides from LCB. There is an appreciable amount (~50%) of insoluble polysaccharides present in green coffee beans, it could be possible to modify / degrade them for solubilisation using chemical / enzymatic treatment for further utilization.

Enzymes viz., amylase, cellulase, pullulanase and pectinase were selected for the solubilisation of polysaccharides of Low-grade coffee beans. LCB were treated with enzymes at various concentrations and different incubation times and subjected to extraction. The solubilised sugars were analysed by dintiro salicyclic method for the estimation of simple sugars. Amylase under optimised conditions yielded hydrolysed sugar with 12.5% invert sugars. Sugar profile was analysed by HPLC using Evaporative light scattering detector, which showed three peaks and their retention times were compared with that of the standard sugar. Results are discussed.

References are quoted in alphabetical order of first authors. The list of details about the papers published, symposia papers and patent are included as the ***Outcome of the thesis*** at the end.

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CHAPTER 1

Coffee- Processing & Technology, Chemistry & By-products

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CHAPTER 1

1.1. Facts about coffee

Coffee is one of the international products and is the second largest traded commodity in the world next to crude oil. It belongs to Rubiaceae family which contains more than 70 species but two of them are of significant economic importance namely C*offea arabica* and *Coffea canephora* (Varnam & Sutherland, 1994).

Coffee - the dark, aromatic, non-alcoholic brew, loved by world over for its stimulating and refreshing taste, is a product from the species of *Coffea* genus. The beans from the fruits of these plants are roasted, ground and liquored to produce the fascinating brew. Being the fifth largest producer and a major exporter of coffee in the world, India is a force to reckon within the global coffee industry (Sivetz & Foote, 1963).

The history and development of the coffee beverage is varied and interesting, involving chance occurrences, political intrigue, and pursuit of wealth and power. One of the legend revealed that, the effect of coffee beans on behaviour was noticed by a sheep-herder named Kaldi from Ethiopia, as he tended his sheep. He noticed that the sheep became hyperactive after eating the red cherries from a certain plant when they changed pastures. He tried a few, and was soon as overactive as his herd. The story relates that a monk scolded him for taking of those fruits. However the monks soon

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discovered that this fruit from the shiny green plant could help them stay awake for their prayer (Abraham, 1992; Clifford & Wilson, 1985).

Another legend mentions the name for coffee as “mocha". An Arabian was banished to the desert with his followers to die of starvation. In desperation, he and his friends boiled and ate the fruit from an unknown plant. The brew saved their life and their survival was taken as a religious sign by the residents of the nearest town, Mocha. The plant and its beverage were named Mocha to honour this event. Coffee was believed by some Christians to be the devil's drink. Pope Vincent III heard this and decided to taste it. He enjoyed it so much he baptized it (URL-1).

The word itself is derived from the Arabic word “quahweh”, which is a poetic term for “wine”. The wild coffee plant is indigenous to Ethiopia from which it spread to Arabia and nearby countries due to Arab traders in the beginning of the 18th century. Later, during the 18 and the 19 centuries, there was a steady expansion of coffee drinking throughout the world and Arabica coffee was exported to many countries (Wintgens, 2004).

Coffee was introduced much later to countries beyond Arabia whose inhabitants believed it to be a delicacy and guarded it as top secret. Transportation of the plant out of the Islam nations was forbidden. The actual spread of coffee was started illegally. One Arab named Baba Budan smuggled beans to some mountains near Mysore, India, and started a farm

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there. Early in this century, the descendants of those original plants were found still growing in the region (Sivetz & Foote, 1963).

Coffea belongs to the family Rubiaceae and has almost 500 species. It actually grows like a tree but is pruned to be a shrub. There are about 25 major species within Coffea, but the typical coffee drinker is likely to be familiar with three species: *robusta*, *arabica* and *liberica*. (Varnam & Sutherland, 1994). *Robusta* and *liberica* are used in the mass produced commercial operations because these have a higher yield than the *arabica* (Fig. 1.1) varieties. Beans of the *robusta* and *liberica* species are considered to be flavoured more harshly than the *arabica*. *Arabica* varieties are considered to be the more important type of coffee beans. *Arabica* trees produce less fruit than the other two varieties and require more effort to grow. Specific environmental conditions are required for *Arabica*, whereas the *robusta* and *liberica* grow in a wide range of climatic conditions. *Arabica* tree is more fragile by nature. These require manual harvesting in order to avoid the damage to the plant. The best coffee in the world comes from the *Coffea arabica* plant, which grows at high altitudes throughout the equatorial regions of the world. *Coffea robusta* (Fig. 1.2) is also grown commercially (primarily in Africa), but this plant is used mostly for the lower grades of coffee that are in the market today (URL-2).

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Fig. 1.1. *Coffea arabica*

Fig. 1.2. *Coffea robusta*

Arabica coffee represents approximately 70 percent of the world's coffee production. The arabica plant is typically a large bush that can reach a height of 14 to 20 feet, with dark green, oval-shaped leaves. Its fruits are oval and usually contain two flat seeds. After planting, arabica trees mature in 3 to 4 years and produce their first crop. The arabica plant continues to produce fruits for 20 to 30 years. Arabica trees prefer a seasonal climate of 59-75°F and an annual rainfall of 60 inches (Clifford & Willson, 1985).

1.1.1. World Scenario

International Coffee Organization (ICO) estimated the world coffee production in 2008-2009 is 128.8 million bags (1 bag equals 60 kilograms or 132.276 pounds), nearly 15 million bags over the previous year. Coffee is produced in 70 countries of the world. The top three producing countries account for over 50% of the total production and hence control the world coffee market. Coffee producing countries and quantity of production during 2005-06 is presented in Table 1.1 and Fig. 1.3 respectively. (URL- 3)

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Table 1.1. List of coffee producing countries

No.	Country	Type of coffee	Production (million bags*)
1	Brazil	Arabica & Robusta	35.00
2	Cameroon	Arabica & Robusta	1.00
3	Colombia	Arabica	11.0
4	Costa Rica	Arabica & Robusta	0.60
5	Cuba	Arabica	0.28
6	Dominican Republic	Arabica	0.50
7	Ecuador	Arabica & Robusta	0.70
8	El-Salvador	Arabica	1.30
9	Ethiopia	Arabica	4.50
10	Guatemala	Arabica & Robusta	3.70
11	Guinea	Arabica	4.50
12	Haiti	Arabica	0.37
13	Honduras	Arabica	3.00
14	India	Arabica & Robusta	4.60
15	Indonesia	Arabica & Robusta	6.70
16	Ivory Coast	Robusta	2.50
17	Kenya	Arabica	1.00
18	Madagascar	Arabica & Robusta	0.70
19	Mexico	Arabica	4.20
20	Nicaragua	Arabica	1.40
21	Uganda	Arabica & Robusta	2.70
22	Peru	Arabica	2.70
23	Philippines	Arabica & Robusta	0.50
24	Venezuela	Arabica	0.80
25	Vietnam	Robusta	11.00

*1 bag =60 kg

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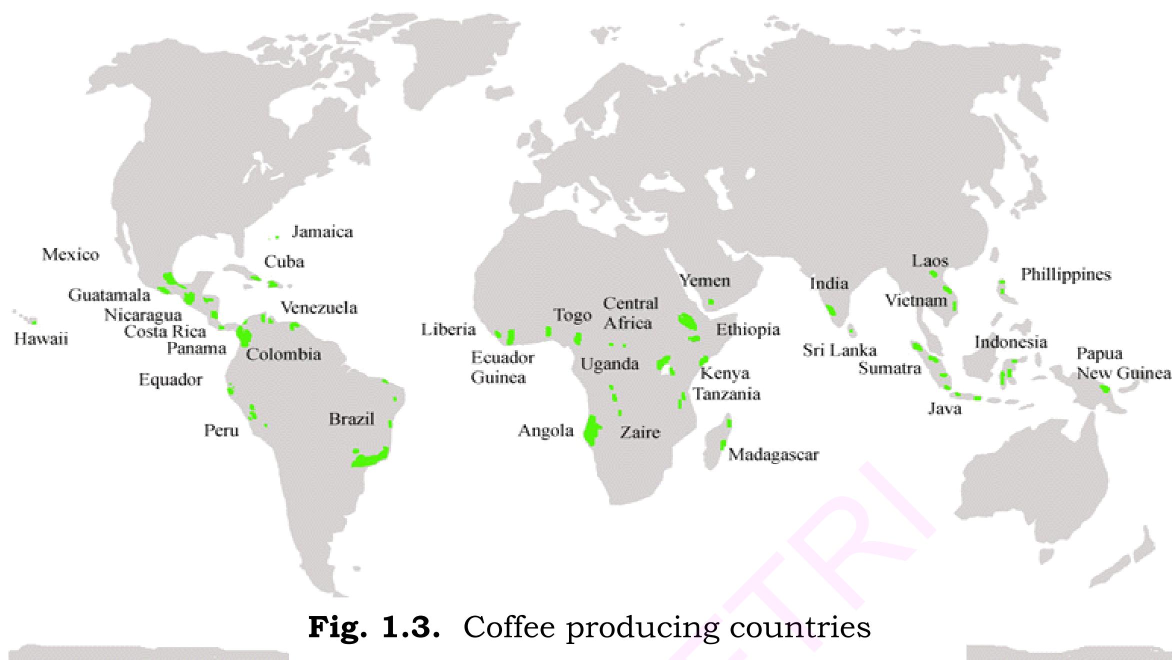

Fig. 1.3. Coffee producing countries

1.1.2. Indian Scenario

India accounts for about 4.5% of world coffee production and most of the coffee cultivated in the southern part of the country. India's domestic coffee market is estimated at around 55,000 tonnes. India exported 2.21 million tonnes of coffee in 2006-07 realising the foreign exchange earnings of $ 395.04 million or Rs.1769 crore. The industry provides employment to 0.6 million people. Most of the coffee are exported to Italy, Germany, Russian federation, Spain, Belgium, Slovenia, US, Japan, Greece, Netherlands and France. Europe accounts for about 70 percent of India's total coffee exports. The three major traditional coffee producing states in southern India are Karnataka (Chikmagalur, Coorg, and Hassan regions), Kerala (Wynad, Travancore and Nelliampathies regions) and Tamil Nadu (Pulneys, Nilgris, Shervroys and Anamalais regions), which contribute 53,

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28 and 11% respectively towards the coffee production. The non- traditional producing regions of coffee in India include Andhra Pradesh, Orissa, Assam, Manipur, Meghalaya, Mizoram, Tripura, Nagaland and Arunachal Pradesh. Robusta coffee production contributes to about 62-65% of the total coffee production whereas Arabica coffee contributes to 35-38% (URL-4).

1.2. Botany

The coffee plant takes approximately three years to develop from seed germination to first flowering and fruit production. The fruit of the coffee plant is known as a cherry, and the beans which develop inside the cherry are used as the basic element for producing roast and ground coffee, soluble coffee powders and coffee liquor. A well-managed coffee tree can be in production for up to 80 years or more, but the economic lifespan of a coffee plantation is rarely more than 30 years. The plant itself is a small tree, maintained at 150 cm in case of *C.arabica* and 170-185 cm in case of *C.robusta* but in the wild the plant can grow up to 7-8 m (Wintgens, 2004).

The taxonomical classification of coffee is as under:

Kingdom : Plantae
Subkingdom : Tracheobionta
Superdivision : Spermatophyta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Asteridae
Order : Rubiales
Family : Rubiaceae

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Various methods of propagation are used, including cuttings, graftings and layering, though cuttings are the normal commercial practice. An altitude of 600-1200m is ideal for coffee but can also be grown at an altitude up to 1800m. The ideal climatic conditions for the plant being 75-80°C and a rain fall of 150-200cm. The yield of coffee is totally dependent on the flowers produced by the plant and more importantly, on the percentage of fruit set from the flowers. The fresh fruit consists of an outer skin over a fleshy pulp in which two seeds are embedded, each flat on one side and convex on the other. Occasionally only one seed, rounded on both sides (pea berry), may be found (Abraham, 1992; Schoenholt, 1993).

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1.3. Processing

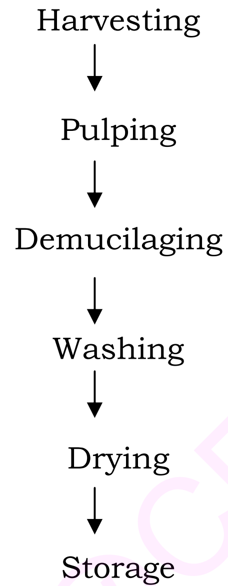
1.3.1. Green bean processing

The processing of green coffee is carried out by two methods namely wet method and dry method.

In the wet method (Fig. 1.4), only ripe fruits having a reddish brown colour are picked, graded and fed to a pulper to remove the outer skin and mucilage. The parchment is washed thoroughly and dried. The coffee prepared by this method is called parchment coffee and this method is practiced in Columbia, Kenya and most of the south and Central American countries. India also processes arabica coffee by this method. In the dry method (Fig. 1.4), ripe, green and under ripe fruits are sorted out and then

dried separately. The coffee obtained by this method is called cherry coffee. Consumers favour parchment coffee from the wet method (Clarke, 1985).

Wet Method



Dry Method

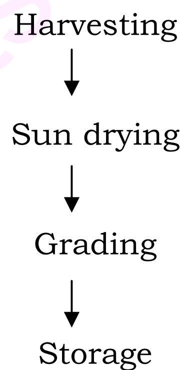


Fig.1.4. Processing methods of coffee

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1.3.2. Roasting of Coffee

Roasting is the first step in the preparation of any consumable product from green coffee. The characteristic aroma is developed during roasting. Coffee received at the roasting plant should be free of extraneous matter, but in practice it is usually contaminated with chaff, strings, wood splinters, stones, pebbles, coins, nails etc., and therefore these must be cleaned well. Large particles are removed using screens and light particles are blown off through air blasting. Nails and other materials are removed using magnetic separators.

1.3.2.1. Roasting Process

Roasting of coffee is a process of exposing the coffee beans to a warming process that is sufficient to drive off the free and bound moisture; dry beans are heated to a temperature of 200-250°C. The time required for roasting is 5-10min in a continuous roaster and more then 20min in non-continuous roaster (Carvalho & Chalfoun, 1989). The degree of roasting is critical to flavour development in the bean and determines many of the flavour characteristics of the brewed coffee. The relationships between different roasting temperatures, weight loss and cup quality are summarized in Table 1.2. The degree of roast is usually assessed from external colour, a final quantitative assessment made using colour reflectance meters (Abraham & Shankaranarayana, 1990; Subramanyan *et al.,* 1960).

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The conventional roasting equipment consists of a metal container in which green coffee is heated while it is continuously rotated. Heat may be supplied by conduction from hot air or more frequently a mixture of both methods of heat transfer together. It is necessary that during the roasting process, heat may be supplied quickly and uniformly and the beans be continuously stirred.

Table 1.2. Roasting parameters and cup quality

Temperature (°C)	Roast	Weight loss %	Cup quality
200	Very light	11-12	Acid taste, less aroma
220	Light	14-15	Better aroma, less astringent, acidic cup
230	Medium	16-20	Optimal quality
240	Dark	21-23	Preferred in Europe
250	Italian	24-25	Dark colour, good taste

Different types of roasters are available. Continuous roasters are used in large scale processing plants because these have greater efficiency and ensure better uniformity than batch type roasters. Continuous roasters consist of either a perforated drum or a cylinder for roasting and subsequent cooling of the beans. The rotating drum principle is used in the commercial roasters. Fluidised bed roasters are used for large scale roasting of the coffee beans. Both heating and cooling are achieved in the same vessel by a fluidised solid contact technique. Fluidised roasters have better control parameters and deliver the product with uniform roasting. The spouted bed roaster is a variant of the fluidised bed roaster that has an

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advantage in large scale roasting and tends to develop unstable fluidisation (Nagaraju & Ramalakshmi, 1995; Nagaraju *et al.,* 1997; Sivetz, 1990). The special feature of the spouted bed roaster is that it has no moving parts or vibratory units, which may damage the final product. The roasting chamber is transparent so that the colour of the roasting beans can be easily controlled visually (Nagaraju & Ramalakshmi, 2002).

1.3.2.2. Changes during roasting

Many types of physical and chemical changes occur during roasting, including changes in colour, size and shape of the bean. The important changes that take place during roasting are, loss of moisture, loss of organic matter and production of CO_2, swelling of bean and consequent changes in density of the bean, decrease in the breaking strength of the bean, caramelisation of sugar and other constituents with consequent changes in colour, formation of typical aroma compounds, decrease in the tannin like constituents and sugars, increase in water soluble matter and formation of niacin and increase in its content during roasting. The chemical composition of green, roasted and brewed solids is presented in Fig.1.5. (Barter, 2004).

Roasting results in loss of weight of the bean. Roasting produces a large amount of CO_2 that under high pressure inside the bean bloats it with the corresponding reduction in the specific gravity from 1.2-1.3 to 0.6-0.8. This results in porous structure and reduction in the breaking strength of

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the roasted bean. The colour of the bean changes from grey green to light brown, dark brown or almost black depending on the type of roast. The pH of the roasted coffee brew falls down to 5.5 – 5.0 from the pH 6.0 of the green beans. This is mainly due to the formation of organic acids (I). In general light roasts give more acid cup then dark roasts. pH of brew cup from medium roast is 5.0, while that of dark roast is upto 5.3.

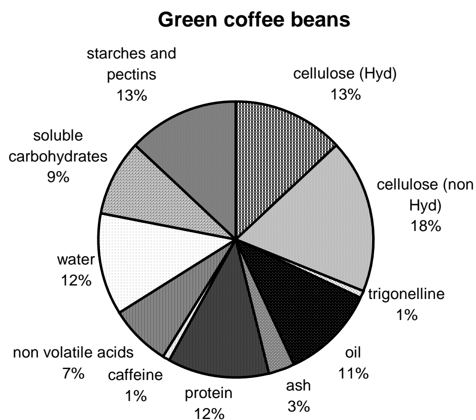

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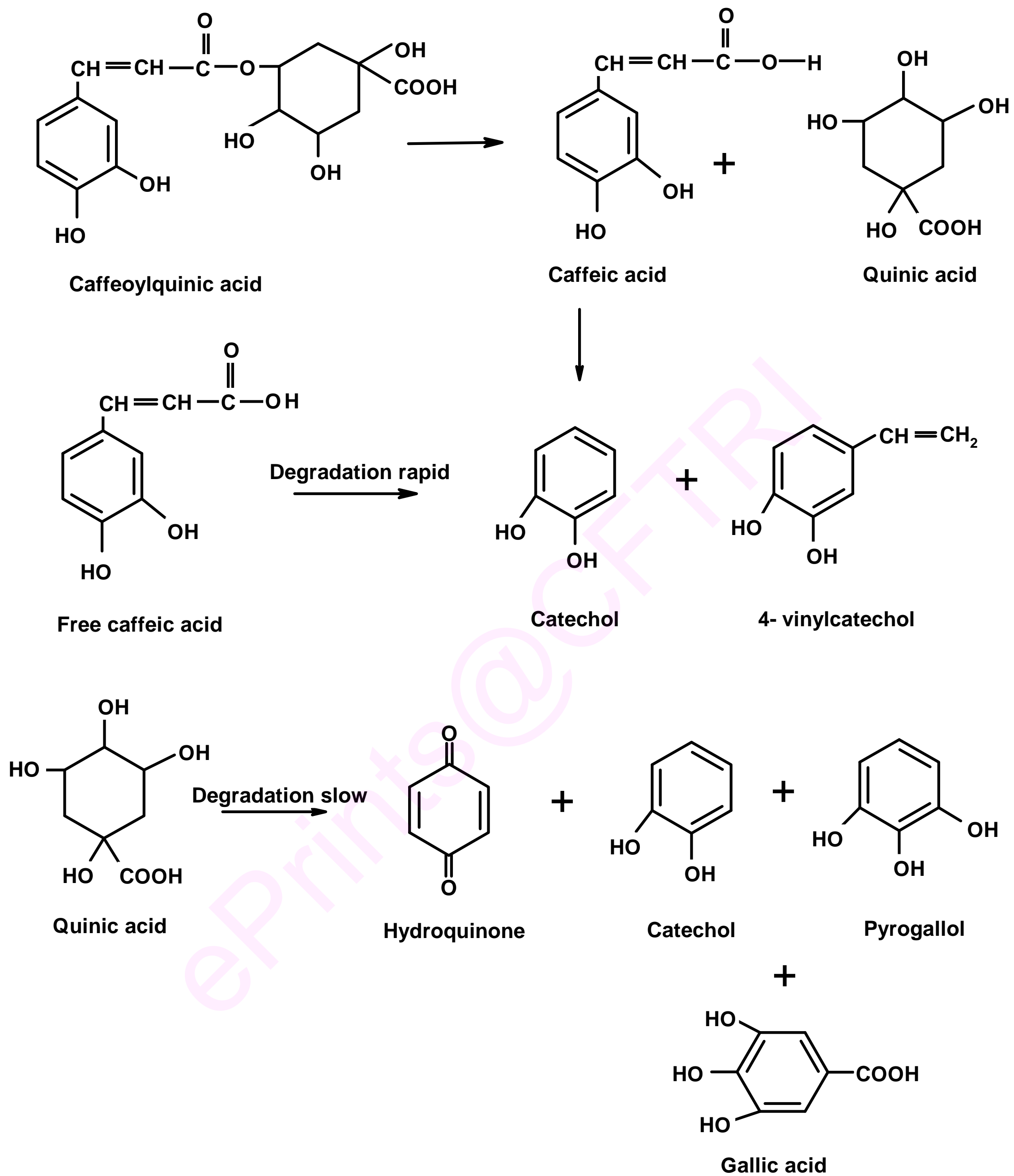
Fig. 1.5. Chemical composition of green, roasted and brewed solids



I. Formation of organic acids during roasting

Sugars and proteins break down to aldehydes, alcohols and acids. Sucrose is the major sugar, which suffers heavy loss during roasting.

Proteins are denatured and are broken down to amino acids. The most significant change occurring during roasting is the formation of aroma compounds. Roasted whole beans retain the characteristic aroma for about a week under normal atmospheric condition. This is mainly due to the carbon dioxide built-up inside the bean providing an inert atmosphere.

The volatile compounds of coffee are largely responsible for the aroma. The green bean does not possess any appealing flavour and its infusion is unpalatable. Roasted coffee contains more than 1000 volatile compounds (Clarke & Vitzthum, 2001). The chemical composition of green and roasted coffee and the important precursors for the formation of volatiles are given in Tables 1.3-1.5 (Ramalakshmi & Raghavan, 2003).

Table 1.3. Chemical composition of green coffee

Constituent	Arabica (%)	Robusta (%)
Caffeine	0.9-1.2	1.6-2.5
Trigonelline	1.0-1.2	0.7-1.0
Ash	3.0-4.2	4.0-4.4
Chlorogenic Acids	5.5-8.0	7.0-10.0
Organic acids	1.5-2.0	1.5-2.0
Sucrose	6.0-8.0	5.0-7.0
Reducing Sugars	0.1-1.0	0.4-1.0
Total polysaccharides	44.0-55.0	37.0-47.0
Lignin	2.0-3.0	2.0-3.0
Protein	11.0-13.0	11.0-13.0
Lipids	14.0-16.0	9.0-13.0

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Table 1.4. Generation of coffee volatiles during roasting

Green coffee	Roast coffee
Lipids	Aliphatic hydrocarbons
Fatty acid	
Higher terpenoids	Monoterpenoids
Lignin	Phenolic compounds
Starch	Acids and aldehydes, Ketones
Sugars	
Peptides , Amino acids	Nitrogenous and Sulfurous compounds
Trigonelline	Nitrogenous compounds

Table 1.5. Chemical composition of roasted coffee

Constituent	Arabica (%)	Robusta (%)
Caffeine	1.0-1.3	1.7-2.4
Trigonelline	0.5-1.0	0.3-0.7
Ash	3.0-4.5	4.0-6.0
Chlorogenic Acids	2.2-4.5	3.8-4.6
Organic acids	1.0-2.4	1.0-2.6
Sucrose	Nil	Nil
Reducing Sugars	0.2-0.3	0.2-0.3
Polysaccharides	24.0-39.0	25.0-37.0
Protein	~ 12	~ 12
Lipids	~ 13	~ 10
Water solubles	26.0-30.0	28.0-32.0

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1.3.2.3. Subsequent operations

After developing the coffee flavour by roasting, efficient extraction of the roasted coffee solubles and volatiles that contribute to the coffee flavour and aroma is essential. The solubles could be extracted from the whole roasted beans, but the yield would be low and flavour would be poor. Extraction may be made to give a higher yield of solubles by breaking down the whole bean to smaller pieces. Grinding is essential to obtain maximum extraction of solubles including aroma and flavour. Various types of grinders based on the principles of cutting, shearing and crushing are available for the purpose of size reduction during large scale grinding of coffee beans. After grinding, the coffee is suitably packed and stored (Sturdivant, 1990).

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1.4. Chemical Composition

The chemical composition of green coffee depends mainly on the variety of coffee, agricultural practices, processing and storage conditions. Green coffee is especially characterized by its content of caffeine, trigonelline and chlorogenic acids; otherwise its composition is similar to other vegetable substances with their protein, carbohydrates, vegetable oil and mineral content. However, the carbohydrate portion consists mainly of polysaccharides and the physical hardness of the same is due to mannan (low degree polymerization). The two main species of coffee, viz., arabica

and robusta differ in composition (Table 1.3) with respect to parameters such as caffeine, chlorogenic acid, and lipids.

1.4.1. Moisture

Moisture is an important parameter, which affects the quality and storage behaviour of coffee. It is generally recognized that green coffee should not be allowed to reach moisture content in excess of 12% corresponding to a relative humidity of 70%. Higher moisture content will result in loss of green colour, favour mould growth, flavour deterioration and possibility of mould toxin formation (Ramalakshmi & Raghavan, 1998). Deterioration is also markedly accelerated by temperature (low temperature is preferred for retaining the colour and flavour quality). Moisture content of 10.5% for plantation coffee and 11% for cherry coffee is generally recommended. The moisture content in green, roast and instant coffees is determined by air oven, vacuum oven or Karl Fischer method. Moisture meters are now available for quick measurement of moisture content in green coffee. The most commonly used Kappa moisture meter works on the principle of dielectric constant. The instrument needs calibration using a reference method, i.e., oven method (Macrae, 1985).

1.4.2. Caffeine

This is perhaps the most important chemical component studied in coffee due to its reported physiological effects. Caffeine (II), 1,3,7-trimethylxanthine, is an alkaloid with a substituted purine ring system.

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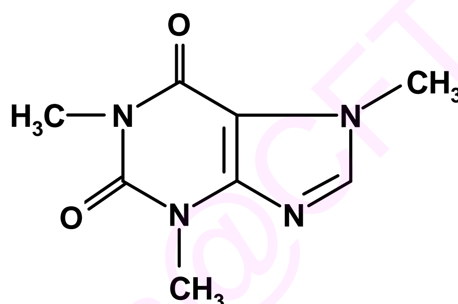
The main physiological effect of caffeine appears to be as a stimulant on the central nervous system. It has an effect on the cardiovascular system with slight increase in blood pressure and heart output. Caffeine also increases the gastric acid secretion. It undergoes bio transformation in the human body to form methylated derivatives of uric acid. Caffeine is definitely not lethal to the system when ingested in the form of beverages unless one consumes 75 cups of coffee, 125 cups of tea or 200 cola beverages within 30 minutes period (Ramalakshmi & Raghavan, 1999).



II. 1,3,7- trimethylxanthine

The caffeine content of green coffee bean varies according to the species; robusta coffee contains about 2.2%, arabica about 1.20% and the hybrid 'arabusta' 1.72%. Environmental and agricultural factors appear to have minimal effect on the caffeine content. During roasting, there is no significant loss in terms of caffeine. A typical cup of regular coffee contains 70 - 140 mg of caffeine depending on preparation, blend and cup size (Ramalakshmi & Raghavan, 1998).

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The structure of caffeine is that of purine derivative xanthine, with methyl substituents attached at positions 1, 3 and 7. Reports on the biosynthesis and degradation of caffeine in coffee are limited. Both processes occur more rapidly in immature than mature fruit. The main biosynthesis route utilises the purine nucleotide for the formation of caffeine (III) is well documented (Waler & Suzuki, 1989). In coffee plants caffeine is synthesised from xanthosine via 7-methylanthosine, 7-methylxanthine and theobromine. S-adenosylmethionine (SAM) is the actual source of the methyl groups. The caffeine is degraded relatively slowly and involving demethylation steps (IV) to yield theobromine and theophylline. Theophylline is catabolised to xanthine via 3-methylxanthine. But it is unclear whether 3-methylxanthine and/ or 7-methylxanthine are intermediates in the conversion of theobromine to xanthine. Xanthine is metabolised to urea (Waler & Suzuki, 1989).

On roasting, caffeine is unchanged though some loss occurs due to sublimation. Caffeine is the most important compound in the analytical parameters of coffee and in the standards and specifications of coffee and coffee products. There are several analytical procedures for the determination of caffeine in coffee such as Baily Andrew, Levin’s and HPLC methods (Ramalakshmi & Raghavan, 2003).

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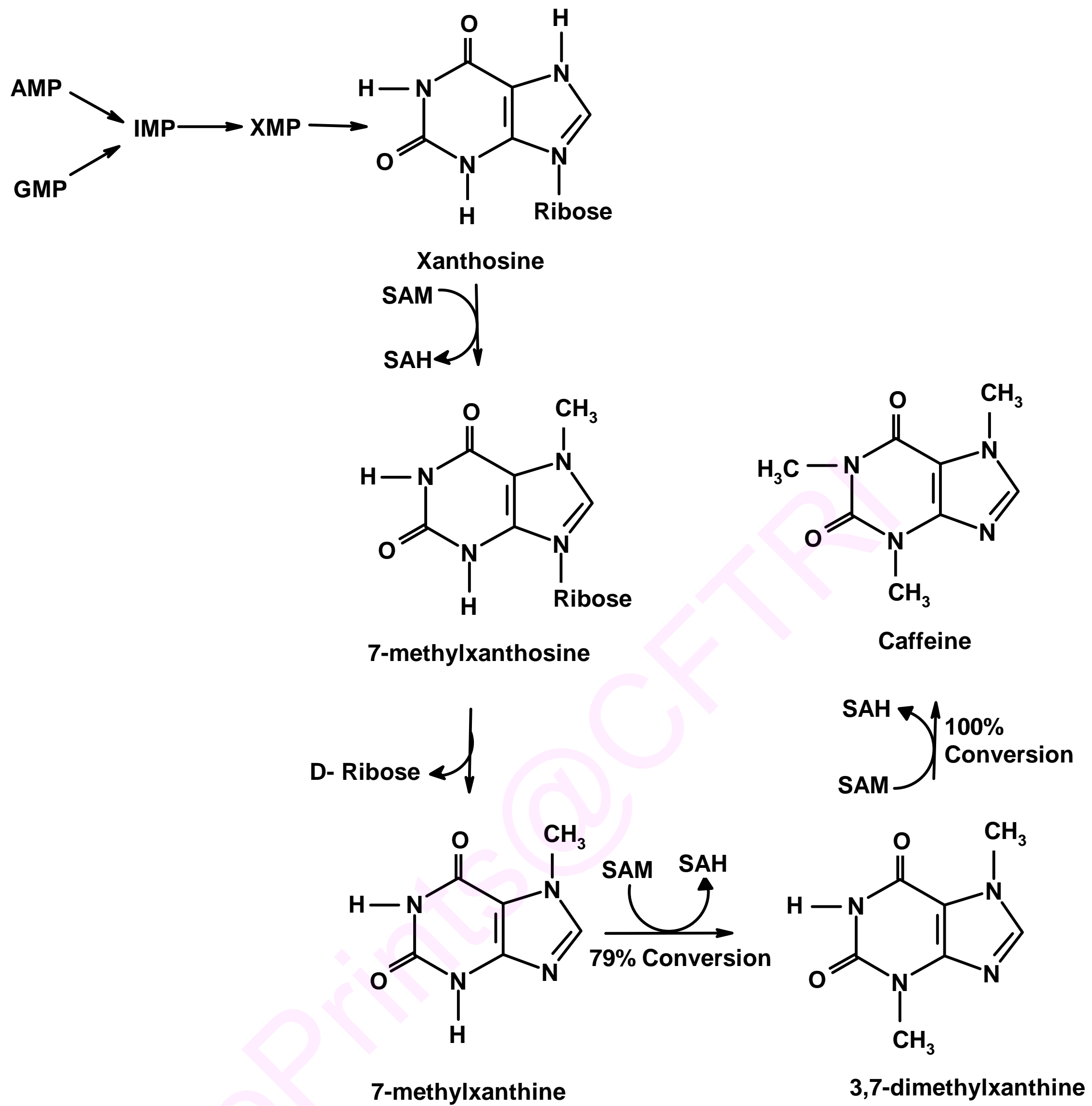

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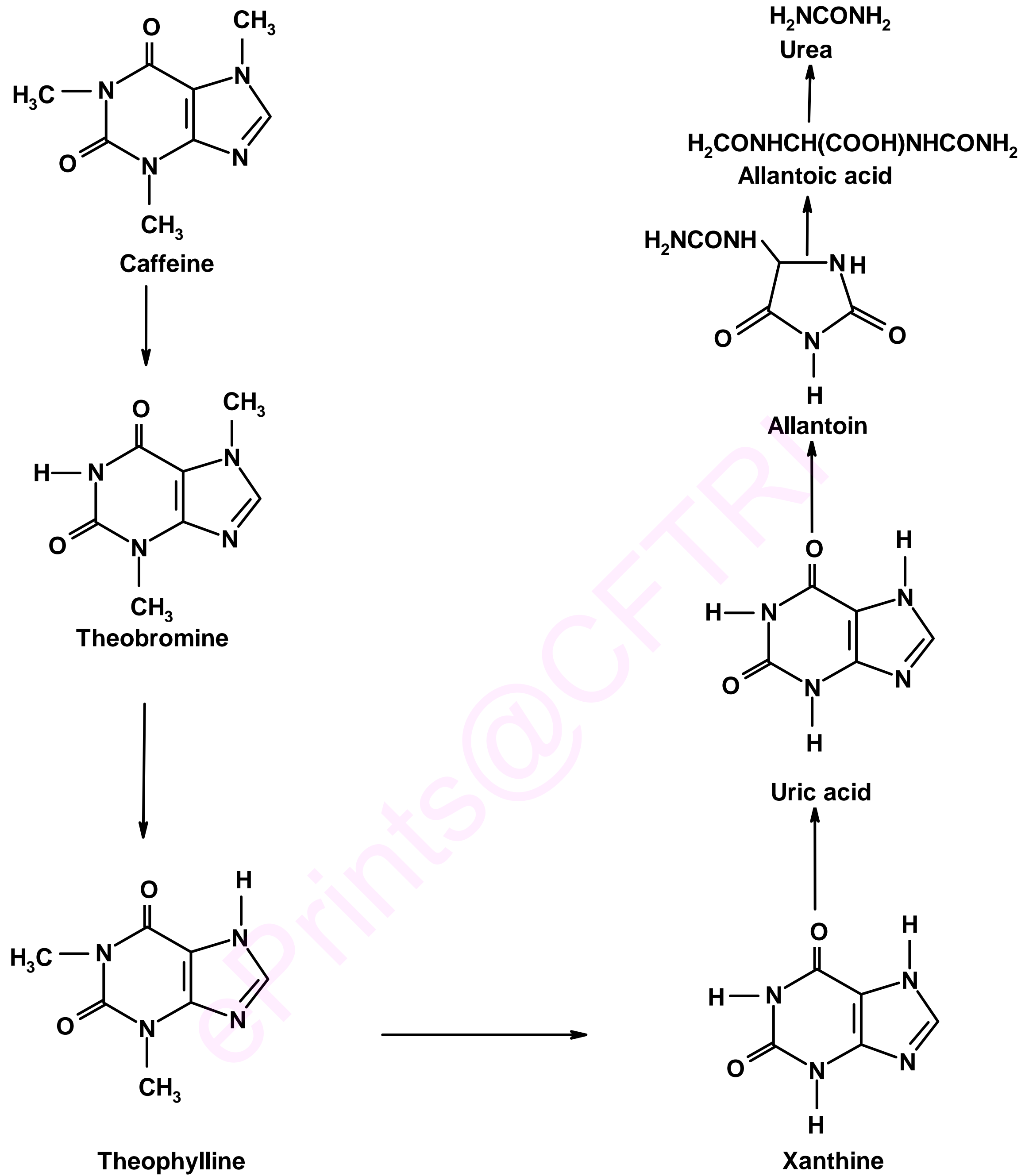
III. Formation of caffeine in *Coffea arabica* fruits



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IV. Degradation of caffeine

1.4.3. Organic acids

Chlorogenic acid is one of the important components along with the other organic acids (V) present in green and roasted coffee beans. Chlorogenic acids contribute to acidic and astringent tastes. Chlorogenic acid comprises of (i) caffeoylquinic acid ester of caffeic and quinic acdis (ii) deiaffeoylquinic acid (iii) feruloylquinic acid (iv) coumaroylquinic acid (v) caffeoylferuloylquinc acid (vi) feruloylcaffeoylquinic acid. These isomers suffer heavy losses during roasting and the degree of loss depends on the type of roasting. Robusta coffee contains high amounts of chlorogenic acid compared to arabica coffee. High astringency of robusta coffee is attributed to dicaffeoylquinic acids and the feruloylquinic acids. Further, the higher content of 4, 5-dicaffeoylquinic acid in robusta appears to contribute to a peculiar lingering metallic taste which is a negative sensory effect (Clifford, 1997). Chlorogenic acid is determined by spectrometric and chromatographic methods, which allow separation and quantification of individual isomers.

During roasting polysaccharides undergo degradation resulting in several organic acids which contribute to the acidity of coffee brew and this is an important sensory quality. The acids reported are citric, malic, lactic, quinic, pyruvic, acetic, oxalic, tartaric, propionic, butyric, valeric etc.

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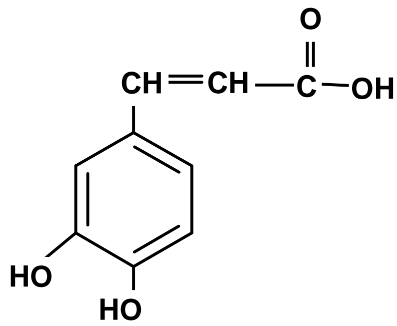
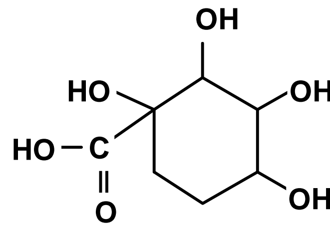
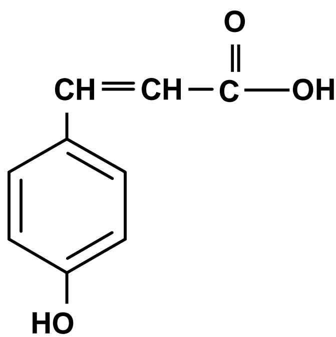


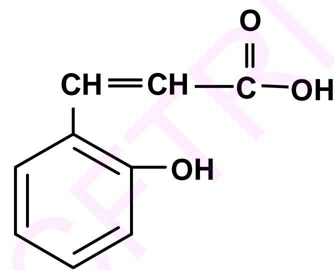
Caffeic acid



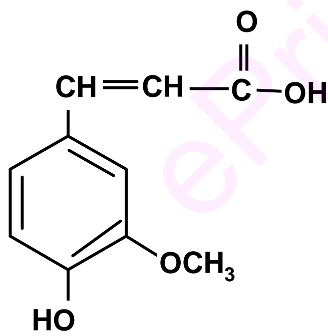
Quinic acid



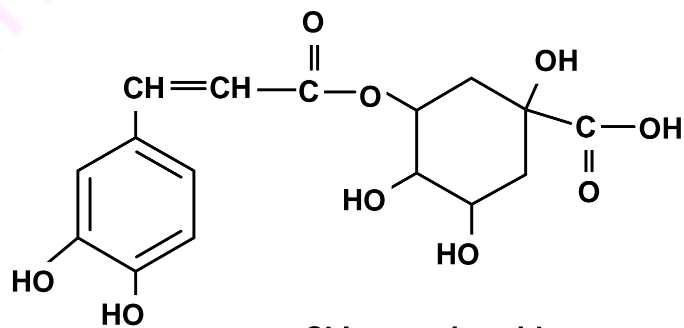
p-coumaric acid



o-coumaric acid



Ferulic acid



Chlorogenic acid

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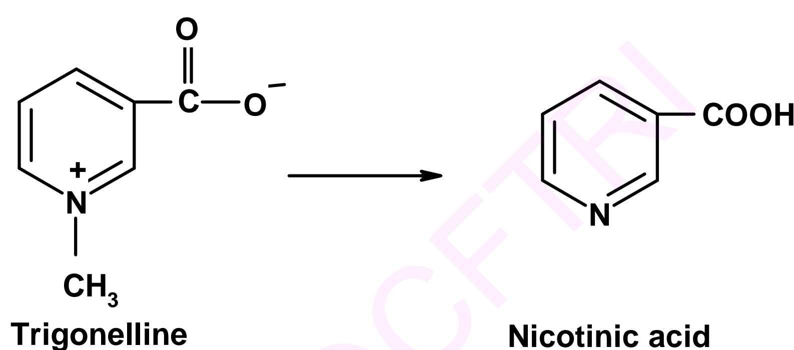
V. Acids present in coffee

1.4.4. Trigonelline and Nicotinic acid

Trigonelline (VI) present in green coffee (about 1%) degrades rapidly on roasting yielding nicotinic acid, nicotinamide and a range of aroma volatiles, which includes pyridines and pyrroles. Roast coffee contains 10 to 40 mg of nicotinic acid per 100g depending on the degree of roasting (Macrae, 1985).



VI. Trigonelline and Nicotinic acid

1.4.5. Proteins

Proteins and amino acids have received relatively little attention. Considerable research (Macrae, 1985) has been carried out to correlate the initial protein content in green coffee to the aroma and mouth feel of the coffee brew obtained from the roasted and ground powder but with limited success. In green coffee, proteins exist in unbound form predominantly in the cytoplasm or bound to cell wall polysaccharides. Proteins are denatured during roasting and broken down to amino acids. The sulphur amino acids like cystein, cysteine and methionine degraded alone or react with maillard

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intermediates. Hydroxyl amino acids like serine and threonine react with sugars during roasting to yield pyrazine and their derivates, pyridines and their derivatives. Free amino acids occur only in traces in roasted coffee (Kusu *et al.,* 1995).

1.4.6. Carbohydrates

Green coffee contains sucrose and a wide range of polysaccharides (arabinogalactan, galactomannan and cellulose). Sucrose is the major sugar which suffers heavy loss during roasting due to caramelisation and other reactions. Because of the severe conditions employed for extraction in instant coffee manufacture, hydrolysis of insoluble polysaccharides takes place yielding soluble oligosaccharides, mainly polymers of mannose and galactose, arabinose and mannose monomers (Fischer *et al.,* 2001). The sugar analysis involves determination of free sugar in green coffee, oligosaccharides in instant coffee and monosaccharides (after hydrolysis of polysaccharides) in all coffee products. Higher levels of total xylose, mannitol, are indicative of the adulteration of instant coffee with coffee husks or skins (MAFF, 1995).

1.4.7. Melanoidins

Proteins and carbohydrates react during roasting forming melanoidins. Melanoidins are derived from Maillard reactions or from carbohydrate caramelization. The melanoidins are the caramelized substances consisting of complicated structures involving fragments of

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phenols, carbocycles, N-hetero-cycles, benzenoids and furanoids. Attempts to isolate browning substance in roasted coffee have remained unsuccessful. Melanoidins appear to have a stimulating effect on the stomach intestinal tract causing irritation in some persons. This irritation is reported to reduce substantially by treating the coffee beans with steam prior to roasting (Steinhart & Luger, 1997).

1.4.8. Minerals

The ash content of green coffee is about 4% (dry matter basis) of which 40% is potassium (dry ashing at 580°C and estimation by flame photometry or atomic absorption spectrometery). In addition to potassium, 30 more elements were quantified in coffee products by atomic absorption or neutron activation analysis and these include magnesium, calcium, rubidium and iron. Manganese content is higher in arabica (25 to 60 ppm) and lower in robusta coffees (10 to 33 ppm). The other trace elements reported in green coffee include zinc, molybdenum, cobalt, copper, strontium and others. There can be considerable contribution of trace elements to the instant coffee from the processing water and this varies with the source of water employed (Sivetz & Desrosier, 1979).

1.4.9. Lipids

Lipids constitute one of the major components of green coffee (arabica 14-16% and robusta 9-13%). Coffee beans are coated with a polar wax (0.2-0.3%), which consists largely of fatty acid esters of 5-hydroxytryptamine,

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which are known to act as mucosal irritants (Clarke & Vitzthum, 2001). The lipids content in boiled coffee, espresso and filter coffee are reported as 2.2%, 0.4% and 0.2% respectively on ground coffee basis. Instant coffee contains very little lipid materials, apart from coffee oil that may be added for aromatization at the end of the process.

1.4.10. Volatile compounds

The green coffee is devoid of any appealing taste or aroma. The pleasant flavour of coffee is formed during roasting involving a wide range of complex chemical reactions like oxidation, reduction, hydrolysis, polymerization and decarboxylation. Roasting alters the colour, size and shape of the bean. The degree of roast is based on flavour preference, which varies from place to place. The most important change occurring in coffee during roasting is the formation of aroma compounds. The important aroma precursors are amino acids, sugars and chlorogenic acids. Some minor compounds are formed from other compounds such as terpenes, trigonelline, sterols and lipids. The most significant reaction in coffee aroma formation is interaction between amino acids and reducing sugars (browning reaction) and also the direct caramelisation (Czerny & Grosch, 1999; Clement & Deatherage, 1957; Abraham & Shankaranarayana, 1990)

The study of flavour compounds includes total volatiles analysis including headspace compounds. Recently, there has been a remarkable progress in the isolation and recovery of the flavour volatiles. Initially, the

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flavour analysis met with several problems such as (a) the number of volatile substances is extremely large (b) the compounds vary in physical and chemical properties and in the levels of concentration (c) isolation and identification procedures may alter the nature of several sensitive compounds resulting in artefacts. In spite of these inherent difficulties, the flavour chemistry of coffee has received maximum attention the world over and about 800 flavour components have so far been identified, but relatively small number of compounds makes a significant contribution to overall flavour. Volatile compounds are being considered desirable at low, but undesirable at higher concentrations. Studies on flavour formation also have received some attention in recent years. The origin of various volatiles (Abraham & Shankaranarayana, 1990; Abraham, 1992) in roast coffee is correlated to aroma precursors such as amino acids, proteins and sugars (Table 1.6). The complete mechanism of flavour formation in coffee from the precursors is not well understood.

The search for key flavour compounds responsible for the characteristic aroma of coffee has not yielded good results. However, some of the important aroma compounds and their contribution to overall flavour are presented in Table 1.7. (Natarajan *et al.,* 1960; Shibamoto, 1991; Abraham & Shankaranarayana, 1987).

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Table 1.6. Formation of aroma volatiles in coffee

Type of volatile compound	Possible precursor	Mechanism of formation
Olefinic hydrocarbons	Saturated hydrocarbons, dicarboxylic amino acids	Pyrolysis
Aromatic hydrocarbons	Glucose, aromatic amino acids	Pyrolysis
Carbonyl compounds	Sugars, fatty acids, amino acids	Pyrolysis, oxidative, decarboxylation
Alcohols	Carbonyl compounds	Reduction
Phenols	Tannins, chlorogenic acid	Pyrolytic degradation
Mercaptans, sulphides, thiophenes	Sulphur amino acids	Pyrolysis
Thiazoles	Cysteine	Pyrolysis
Furan compounds	Carbohydrates	Cyclisation
Pyrazines	Carbohydrates – Amino acid/ammonia	-
Pyrroles	Prolines	-
Pyridines	Trigonelline	-

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Table 1.7. Possible aroma impact compounds

Aroma compound	Flavour effect
Furyl-2-methanethiol	Fresh coffee aroma (10-500 μg/kg/) Stale note (1-10mg/kg)
Kahweofuran	Slight coffee note (10-100mg/kg)
N-Furyl-2-methyl pyrrole	Stale coffee odour
2-Ethyl furan	Burnt, sweet, coffee-like aroma
N-Ethyl-2-formyl pyrrole	Burnt, roast coffee
Thiobutyrolactone	Burnt coffee odour
2-Methyl-2-acetyl thiophene	Coffee –like aroma
2-methyl isoborneol	Earthy, musty

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1.5. Quality of coffee beans

Coffee grading is an essential step to the coffee manufacturers for exporting the coffee. Quality coffee beans are separated according to size and density. The coffee bean has three dimensions: length, width and thickness. A flat bed grader and a drum grader separate the beans according to the size, and pneumatic separators often-called *catadors*, grade the beans by density. Green coffee quality is the result of an interaction of many variables such as varieties, soil, climate, husbandry, latitude, altitude, harvesting, processing, storing etc (Wintgens, 2004).

1.5.1. Major Grades of Indian Coffee beans

Coffee Board, India allows export of coffee of specified types and grades. The major types and grades of coffee (Anon, 1982) are given in Table 1.8.

Table 1.8. Major types and grades of Indian coffee

<table>
<tr><th colspan="2">Arabica</th><th colspan="2">Robusta</th></tr>
<tr><th>Plantation</th><th>Cherry</th><th>Parchment</th><th>Cherry</th></tr>
<tr><td>Plantation PB</td><td>Arabica cherry PB</td><td>Robusta parchment PB</td><td>Robusta cherry PB</td></tr>
<tr><td>Plantation A</td><td>Arabica cherry AB</td><td>Robusta parchment AB</td><td>Robusta cherry AB</td></tr>
<tr><td>Plantation B</td><td>Arabica cherry C</td><td>Robusta parchment C</td><td>Robusta cherry C</td></tr>
<tr><td>Plantation C</td><td>Arabica cherry black / brown</td><td>Robusta parchment blacks/browns</td><td>Robusta cherry blacks/brown</td></tr>
<tr><td>Plantation blacks</td><td>Arabica cherry bits</td><td>Robusta parchment bits</td><td>Robusta cherry bits</td></tr>
<tr><td>Plantation bits</td><td rowspan="2">Arabica cherry bulk</td><td rowspan="2">Robusta parchment bulk</td><td rowspan="2">Robusta cherry bulk</td></tr>
<tr><td>Plantation bulk</td></tr>
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1.6. Coffee by-products

In coffee producing countries, coffee wastes and by-products (Fig. 1.6) constitute a source of severe contamination and a serious environmental problem. The use of coffee by-products and wastes has been the subject of numerous studies that, in general, lead to the conclusion that these can be used in a variety of ways. The by-products of coffee processing are mainly coffee pulp, processing effluent, parchment husks and coffee husks. Since the middle of the last century, efforts have been made to develop methods for their utilisation as a raw material for the production of feeds, beverages,

vinegar, biogas, caffeine, pectin, pectic enzymes, protein, and compost (Mburu & Mwaura, 2006).

Fresh berry gives about 16% of exportable green bean. Coffee pulp represents around 43% of the weight of the coffee fruit on a fresh weight basis, or approximately 28% of the coffee fruit on a dry weight basis. The other by-products of coffee fruit processing are the mucilage, about 5-14% of the dry weight of the fruit, and coffee hulls, representing 10-12% of the weight of the fruit on a dry weight basis (Jan von Enden, 2003).

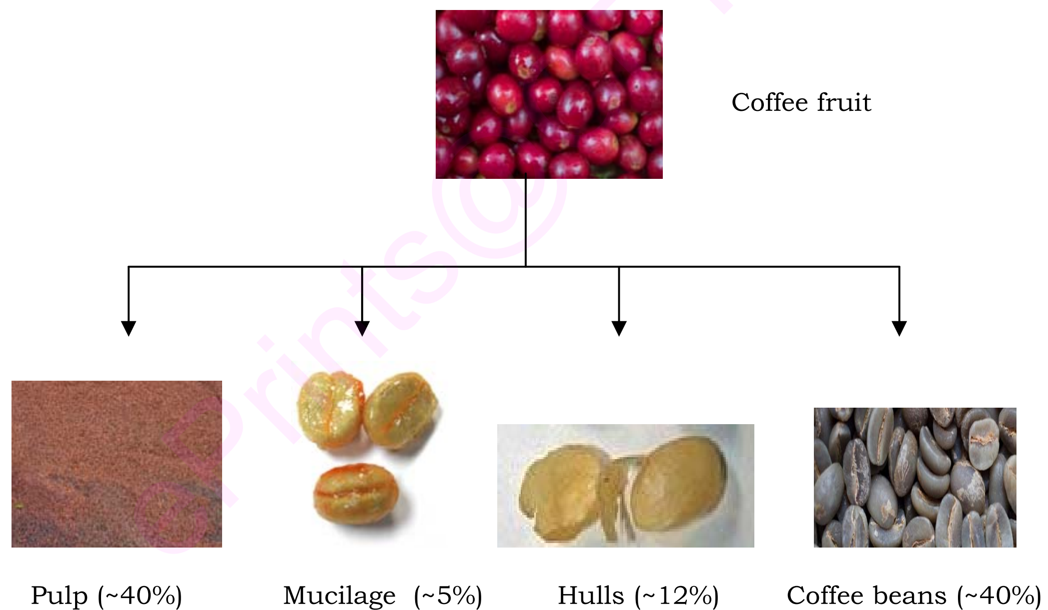

Fig. 1.6. Coffee by-products

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1.6.1. Coffee pulp

Coffee pulp, also identified as coffee fruit without seeds, is an abundant agricultural by-product that causes serious environmental pollution problems. It represents around 43% of the weight of the coffee fruit on a fresh weight basis, or approximately 28% of the coffee fruit on a dry weight basis. The other by-products of coffee fruit processing are the mucilage, about 5% (5-14%) of the dry weight of the fruit, and coffee hulls, representing 12% (10-12%) of the weight of the fruit on a dry weight basis (Braham & Bressani, 1979).

The coffee pulp is obtained either by subjecting the coffee fruit to a depulping operation during wet processing or drying the coffee fruit, followed by a dehulling operation during dry processing. The high water content of the pulp causes problems in handling, transport, stability and processing. The pulp should be dried as quickly as possible to avoid spoilage and should be preserved for further applications. The coffee pulp has been subjected to a drying operation by partial removal of water by pressure, with or without the aid of calcium hydroxide addition. Drying has been accomplished by solar dehydration forced hot air-drying, or both.

In some instances the pulp is thrown into rivers. The fermented pulp when dried is used sometimes as an organic fertilizer applied to the coffee trees. Coffee pulp is a source of nutrients: 0.5% of composted pulp is nitrogen, 0.15% is phosphorus, and 0.5% is potassium. Therefore, pulp can

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be treated and used as organic fertiliser. An alternative use of coffee pulp is ensiling with 4-6% sugar cane molasses. Although fresh coffee pulp can be directly ensilaged, better quality is obtained if moisture content is around 75%. A different and attractive ensiling process is to mix grasses, sorghum or corn, with coffee pulp in layers of about 30 cm with or without sugar cane molasses (4-6%). The silage, whether of coffee pulp alone, or mixed with grasses, is ready to be used in about 3 weeks and if well packed, it can be preserved for upto 18 months. The silage from coffee pulp alone or mixed with other forages can be used as such, or it can be dehydrated (Ramirez-Martinez & Jose, 1998).

In recent years, research has been done on processing coffee pulp by solid state fermentation techniques using *Aspergillus niger*, with some better results in terms of nutritional parameters. Such pulp could be used up to 15% in poultry diets and upto 20% in swine diets (Anon. 1974). Experimental evidence has suggested that a well processed coffee pulp can be fed upto 30% dry weight for dairy and beef cattle, up to 16% for swine, and upto 5% to poultry, in well-balanced diets, particularly with respect to protein (Okai *et al.,* 1985). Coffee pulp can be used as planting soil for mushroom production. After being fermented for two days, the pulp is pasteurised with hot water, drained, dried, and mixed with mushroom spores. Then, they are put in plastic bags. After 3 - 4 weeks, the mushroom grows out of the holes in the bags and is collected. The mushroom can be eaten or dried and sold in the market. Considering the large amount of

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coffee pulp generated every harvesting season, the income from mushroom growing is significant for farmers (Ramirez-Martinez, Jose, 1998).

Although the evidence available is not conclusive, the main nutritional constraints of usage of coffee pulp include the presence of caffeine, tannins and potassium content. Chemical treatment, anaerobic fermentation, solid-state fermentation, and water extraction can reduce caffeine, tannins and potassium significantly.

1.6.2. Coffee Husk

Coffee cherries are generally sun dried during the dry processing of coffee and the outer layer is hulled to get coffee husk. This residue poses a serious environmental concern due to its disposal in rivers, lakes located near coffee processing estates (Roussos *et al.,* 1994). Due to the presence of proteins, fibers, carbohydrates and minerals in coffee husk, it can be used as an animal feed and also organic fertilizer. The presence of physiological factors such as caffeine, polyphenols, tannins, chlorogenic, ferulic and high potassium in coffee husk causes problem in animal feed. (Pandey and Soccol, 2000). Solid state fermentation techniques are adopted to reduce the caffeine level and polyphenol levels since some of the microorganism use the nitrogen and carbon source (Sera *et al.,* 2000).

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Coffee husk is practically pure lignocellulose and has no fertilizer value at all. Coffee husk was used as substrate for production of citric acid by *Aspergillus niger* in a solid-state fermentation system (Brand *et al.,* 2002; Shankaranand & Lonsane, 1994).

1.6.3. Low-grade coffee beans

Defective coffee obtained after grading is termed as low-grade coffee beans (LCB) which contain the imperfections such as blacks, dark brown beans, insect damaged beans, spotted beans, sours, bits and greens (immature beans) etc. LCB is one of the by-products obtained in the coffee producing countries poses a huge problem of disposal. In general, coffee is graded based on the size, colour and percentage of imperfections. In India, apart from size specification, a defect count standard based on the 'percentage of defects by weight' was specified for each of the grades of coffee (Anon, 1982). Both black and sour defects are associated with bean fermentation and are reported to downgrade coffee flavour (Clarke, 1985). Immature beans, those that come from immature fruits, contribute to beverage astringency. LCB are obtained as a result of either improper formation within fruit or by faulty processing. These beans produce undesirable taste in the beverage when mixed with graded beans. It is a difficult task to separate the various imperfections from a sample of coffee on the sole basis of a verbal description. LCB represent about 15-20% of coffee production on weight basis and are a problem for disposal. Generally LCB are separated from the quality beans prior to selling on external

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markets and transferred to the internal market. The majority of the roasting industries blending these beans with the healthy ones and over all a low grade roasted and ground coffee are consumed in the respective internal market. In order to eliminate these defective coffee beans from the internal market, there is a need to find for more attractive alternative uses of them.

1.6.4. Spent coffee grounds

Coffee is consumed by the people either as filter coffee or instant coffee. Instant coffee is produced from green coffee after roasting, grinding and extraction of water solubles. After extraction the remaining coffee is called "spent coffee". Almost 50% of the world produce is processed for soluble coffee manufacture. After extraction of the solubles, the wet spent coffee has to be dried to make it usable as fuel for boilers. Alternatively, the spent has to be cleared from the premises. Since the bulk involved is enormous, it is a tremendous task to get it transported and the whole effort is not worth when viewed in terms of cost involved. The major difficulty encountered by the industry is the disposal of spent coffee. The existing methods of disposal of spent coffee has included sewer discharge, sanitary land fill, incineration, cattle feed, as fillers and adsorbents in thermosetting material (Ligo Eugenie, 1970; Boopathy, 1987; Rizzi and Gutwein, 1994). Waste coffee grounds were used after removing the oil from the grounds (Sivetz and Desrosier, 1979). Industrial coffee waste was degraded and used as compost also (Stahl and Turek, 1991). Further, this has necessitated finding out alternative uses for the spent coffee. D-mannose and D-

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mannitol were prepared by the acid hydrolysis of spent coffee. The spent coffee contains 45% (dry basis) polymeric carbohydrates which needs investigations to make soluble carbohydrates (Stahl and Turek, 1991; Navarini *et al.,* 1999). The spent coffee has been found to be a weedicide, but this has to be validated with conclusive evidences and also systematic basic work is needed to find out the components responsible for this effect. Preliminary work has shown that the spent coffee contains some anti-oxidant compounds and this too requires to be validated. Further, the economic viability of isolation of these active components needs to be taken into consideration. Perhaps the day is not far when the spent coffee will no longer be called 'spent' but will be found more useful in several other ways. It is in this context that investigation is taken up to determine the antioxidant potential of spent coffee.

1.7. Biological activities of coffee and its by-products

Coffee is consumed because of its desirable bitter taste and physiological benefits. The effect of coffee on human physiology varies from person to person and also on the quality and quantity of coffee consumed. Coffee is a complex chemical mixture and composed of over 1000 different chemicals (Clarke & Vitzthum, 2001). Although coffee has a long history of human food use of over 1000 years, until recently most of the studies on its health effects have focused on potential adverse and toxic effects. However, although not yet proven, recently scientific literature suggests the potential

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beneficial health effects of coffee and several of its constituents. For example, positive effects on performance and protection against some types of cancers, liver disease and radiation – induced tissue damage have been documented (Svilaas *et al.,* 2004).

Several diseases have been alleged to be caused or exacerbate by coffee consumption (Leviton *et al.,* 1994). Among others, issues have concerned hypertension, cardiovascular disease, cancer, spontaneous abortion, delayed conception, low birth weight and osteoporosis. Despite a vast amount of research, evidence to support a direct link of coffee with these diseases has been limited and inconsistent. However, although not yet proven, there is an increasing scientific literature suggesting beneficial health effects of coffee and several of its constituents. Coffee contains substantial amount of antioxidants and this may explain some of its potential beneficial activities, although several other important advantageous active components have also been identified. The major pharmacologically active compound in coffee is the caffeine (methylxanthine), which is known to have effects on a number of functions including stimulation of the central nervous system (CNS), stimulation of cardiac muscle and relaxation of smooth muscle especially bronchial muscle and to act on the kidney to produce dieresis. A number of other compounds, such as chlorogenic acid are also present and are also pharmacologically active. The biological and health effects of coffee have

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been extensively investigated in various animal and in vitro model systems as well as in humans.

Coffee contains substantial amounts of antioxidants and this may explain some of its potentially beneficial activities. Whatever effects are attributed specifically to the consumption of coffee, one must assume that these are associated with compounds in coffee beverage. Immediately this focuses attention upon caffeine, the chlorogenic acids, possibly the sparingly water-soluble diterpenes kahweol and cafestol and miscellaneous products of roasting. The biological and health effects of coffee have been extensively investigated in various animals and in vitro model systems as well as in humans. In recent years, due to the increasing interest in finding physiologically functional foodstuffs, the relation between coffee and health has been extensively investigated (George *et al.,* 2008). Coffee exhibits number of bioactivities since it contains many active compounds those exhibit these effects. Few of the significant bioactivities documented are antioxidant (Nicoli *et al.,* 1997; Rosenberg, 1990; Ramalakshmi *et al.,* 2008), anti-carcinogenic (Giovannucci, 1998; Inoue *et al.,* 1998) anti-mutagenic activity (Kim and Levin, 1988) etc.

Coffee by-products possess many medicinal properties. Coffee mucilage and pulp contain pectins, protopectins, sugars and polyphenolic chemicals, anthocyanins, proanthocyanins, and cyanidins, bioflavonoids and tannins and also caffeine and chlorogenic acids. Pectins present in the

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coffee by products can boost the levels of the high density HDLs, which give the real health benefit. There is very little information available for the development of soft or alcoholic drinks from the coffee cherry pulp. Coffee-based spirits are available as Kahlua in Mexico and Caffè Borghetti in Italy (Rajkumar & Giorgio, 2005). Coffee fruit sugars, such as glucose, galactose, rhamnose and arabinose can be revovered from the recycled pulping water.

Coffee fruit contains anthocyanin apart form the antioxidants and flavonoid compounds with other polyphenolics such as chlorogenic acids and caffeine (Rajkumar & Giorgio, 2005). These materials can be extracted and blended into several combinations to make a range of food additives which would be of interest to the food industry. Coffee fruits can be used for the extraction of proanthocyanins, which can be used in food applications. Green coffee beans possess a large amount of polysaccharides, which degrades during roasting. Efforts can be made to isolate these polysaccharides for further use in food applications.

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CHAPTER 2

CHAPTER 2

Physico-chemical characteristics of low grade and spent coffee

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CHAPTER 2

2.1. Introduction

One of the most important aspects of coffee is its quality. Quality is not always appreciated, but it should always be demanded. The quality of green coffee beans is commonly evaluated according to criteria such as bean size, colour, shape, processing method, crop year, flavour or cup quality and presence of defects (Varnam & Sutherland, 1994). Among those, the last two are the most important parameters and are employed worldwide in coffee trading. There are many different approaches to determine the quality of coffee. Some large companies look for a combination of screen size and defect count as a barometer of quality. These types of standards can be useful as a first line of defence against low quality coffee. Apart from screen size, moisture content of the green coffee plays very important role since it reflects during roasting. In general, moisture content of 11-12% in the green bean leads to even coloured roast. These are potentially good quality standards, but do not ensure a great tasting coffee (Clarke & Macrae, 1987).

In general, coffee is graded based on the size of the coffee bean and percentage of imperfections (defect). Bean size plays an important role for roasted whole coffee beans because many consumers associate bean size to quality; however, larger beans do not necessarily taste better than smaller. It has to be pointed out that for roasting, the more uniform the bean size, the better the heat transfer and consequently the roast. It is also advisable

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not to use the blends different species, together e.g. Arabica with Robusta (The Wealth of India, 1950). Apart from size specification, a defect count standard based on the 'percentage of defects by weight' was specified in respect of each of the grades of coffee (Anon, 1982).

Quality determination of green coffee can be separated in three main categories: green coffee grading, sensory evaluation and chemical/analytical measurements. In grading, the main objective is to determine the size distribution of the coffee along with the assessment of the amount of defective beans and their colour. Sensory evaluation focuses on determining the flavour profile. Finally, the analytical and chemical tools will determine moisture content and some of the chemical components, such as caffeine and possible pesticide residues (Abraham, 1992; Sivetz & Desrosier, 1979).

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2.1.1. Grading and sorting

Grading is the separation of beans according to size and density. The coffee bean has three dimensions: length, width and thickness. It is usually oblong, fairly flat and boat shaped. However peaberry has an elongated oval shape. Although round perforations are sufficient to separate most beans, peaberry requires oblong slots. Flat bed grader and drum grader separate the beans according to size and pneumatic separators often called 'catadors' grade the beans by density.

Sieving standards laid down for different types and grades of coffee should also be strictly enforced. Beans size distribution is carried out by means of perforated plates commonly called screens. Screen (sieve) sizes are expressed as numbers e.g., Robusta Grade on Screen 16, or by letters e.g Arabica Grade AA for bold bean, or by description e.g., bold, medium or small. It depends on trade custom in any given country. Depending on the shape of the holes, screens can be grouped in two categories: rounded and slotted. Usually screens with rounded holes measure the bean with and slotted screens separate peaberry beans (ITC, 1992)

The size of the screen hole is usually specified in 1/64 in. For instance, a screen 15 refers to a rounded screen with holes having a diameter of 15/64 in, which is equivalent to 5.95 mm (0.396875 mm X 15). Usually, most green coffee beans will be retained between screens 12 and 19 (Table 2.1.). In the case of slotted screens, there are two widths, also specified in 1/64 in, and the length of the slot usually measures ¾ in (19mm). The most common slotted screens used for green coffee are in the range 8-11 (Wintgens, 2004).

The bean size distribution can be shown in two different ways: cumulative or percentage between screens. It is worthwhile mentioning that the moisture content of the green coffee bean has an effect on bean size: the higher the moisture, the larger the bean.

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Table 2.1. Screen size for screening of coffee beans

Screen No.	Screen diameter (mm)	English description bean size
20	7.94	Very large
19	7.54	Extra large
18	7.14	Large
17	6.75	Bold
16	6.35	Good
15	5.95	Medium
14	5.55	Small
13	5.16	Defect
12	4.76	Defect

However, all coffee for export is graded to exclude the largest and smallest beans and particles (Anon, 1982). The different Indian grade designations for arabica and robusta coffees and then standards are given in Table 2.2.

Sorting of quality beans is usually done in the final stage in the preparation of coffee for export. It is required to remove any defective bean remaining after processing. A certain amount of extraneous material including whole berries, twigs, stones and fragments of husks and parchment may be present and require removal from the beans. Sorting may be carried out physically by blasting air upwards through the beans. The process of air lifting removes the defective beans.

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Table 2.2. Indian grade designations and standards

<table>
<tr><th rowspan="2">Type</th><th rowspan="2">Grade designation</th><th colspan="3">Sieving requirements</th></tr>
<tr><th>Aperture size in mm</th><th>% by wt retained (minimum)</th><th>Garbled standard permissible percentage of Triage/BBB[a]</th></tr>
<tr><td rowspan="9">Arabica plantation (washed)
&
Arabica cherry (unwashed)</td><td>Plantation A</td><td>6.65</td><td>90</td><td>2 of PB
2 of T</td></tr>
<tr><td>B</td><td>6.00</td><td>75</td><td>2 of PB
3 of T</td></tr>
<tr><td>C</td><td>5.50</td><td>75</td><td>-</td></tr>
<tr><td>PB</td><td>-</td><td>-</td><td>3 of T</td></tr>
<tr><td>Bulk</td><td>-</td><td>-</td><td>3 of T</td></tr>
<tr><td>Arabica cherry AB</td><td>6.00</td><td>90</td><td>2 of PB
3 of T</td></tr>
<tr><td>C</td><td>5.50</td><td>75</td><td>2 of BBB</td></tr>
<tr><td>PB</td><td>-</td><td>-</td><td>2 of AB
3 of T</td></tr>
<tr><td>Bulk</td><td>ungraded & ungarbled</td><td>-</td><td>3 of T</td></tr>
<tr><td rowspan="4">Robusta parchment (washed)
&
Robusta cherry (unwashed)</td><td>Robusta parchment AB
& Robusta cherry AB</td><td>6.00</td><td>90</td><td>2 of PB
3 of T</td></tr>
<tr><td>Robusta parchment C
& Robusta cherry C</td><td>5.50</td><td>75</td><td>2 of BBB</td></tr>
<tr><td>Robusta parchment PB
& Robusta cherry PB</td><td>-</td><td>-</td><td>2 of AB
3 of T</td></tr>
<tr><td>Robusta parchment Bulk
& Robusta cherry Bulk</td><td>ungraded & ungarbled</td><td>-</td><td>10 of T</td></tr>
</table>

a Triage: broken withered, spotted, elephant, small, discoloured, malformed beans; pales; and pulper cuts. BBB: blacks, bits, browns. b: Peraberry

In some cases, beans removed by air lifting are considered as a low grade triage coffee. Hand sorting is a traditional process and is highly

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labour-intensive but the cost can be justified by the high quality of hand sorted coffee by expert sorters. Discoloured sour beans are difficult to recognize but complete removal is essential since even a small number adversely affect the flavour of the brew (Ramalakshmi & Raghavan, 2003).

Electronic colour sorting machines of the monochromatic type use the reflection of white light to identify beans whose surface brightness differs from the rest. Bichromatic sorting machines apply a combination of colour and can eliminate a considerable range of offending beans such as black beans, foxy beans, yellow sours, marble beans, water-damaged beans, etc. Ultraviolet sorting machines operate on the same mechanical principles as bichromatic machines but recognize defective beans by exposing them to ultraviolet light (Anon, 1982).

2.1.2. Defective coffee beans

The rejected coffee obtained after grading according to the size and colour is termed as 'Low grade' or 'Substandard' or 'Triage', which contain the defects such as blacks, dark brown beans and damaged beans such as bleached beans (spongy), spotted beans (more than a quarter of a bean surface), insect damaged beans, sours, bits and greens (immature beans). The defective beans (Fig. 2.1.) produce undesirable taste in the beverage, when roasted along with graded beans (Mazzafera, 1999).

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The amount of defective beans has always been associated with quality. It is true that larger amounts of imperfections will increase the probability of finding off-flavours and lesser homogeneity in the cup; however, low amounts of visible defects do not necessarily correlate with higher cup quality.

The assessment of the defective beans count is done by hand picking. The defects of similar category are separated, grouped according to the nature defects and the total amount of defects is declared. Despite improvements made by international organizations and private companies over the past 20 years, the correct identification of physical defects in green coffee remains difficult. Still it is essential to facilitate the identification of defective beans in green coffee and to point out their effect on cup quality. Some of the defective coffee beans are depicted with illustrations, cause of formation and their impact on the coffee beverage (Wintgens, 2004).

Field or process damaged beans

These defects (Fig. 2.1) are caused by stress of coffee beans due to climatic conditions, water or nutrient deficiencies, inadequate cultivation or harvesting practices, unsatisfactory primary processing. Also these defects formed by inadequate primary processing operations like pulping, washing, drying, hulling, cleaning, etc. These beans when roasted become slightly darker in colour than normal beans leads to off-flavors with diminished aroma, flavor and acidity in the cup.

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Amber bean

These coffee beans (Fig. 2.1.) are smooth, yellowish in colour and usually semi-translucid in nature. These are caused by iron deficiency in the soil and/or a high soil pH. These beans lead to produce diminished aroma, flavor and acidity with a grassy or woody character in the cup.

Insect damaged Bean

These kinds of beans (Fig. 2.1.) are with more than three small holes or tunnels inside the bean and damaged by *Coffee Berry Borer*. The holes are circular and clean cut. Their diameter is 0.3-1.5 mm. Sometimes the bean tissue itself has been eaten away by the pest. This gives the bean a ragged aspect. These beans produce significant incidence of off-flavors with a predominantly bitter and tarry flavor which result in total loss of aroma, flavor and acidity.

Elephant and Triangular beans

Elephant beans (Fig. 2.1.) are unusually large in size, spherical in shape and more frequent in robusta beans. These are caused by false polyembryony. Triangular beans (Fig. 2.1) are triangular in shape transversal section and produced by genetic cause resulting from the development of three beans per cherry. During roasting, these beans lead to uneven roasting and however there is no significant effect on the cup quality.

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Immature bean (quaker bean)

These beans (Fig. 2.1.) are small, “boat-shaped” bean often with a wrinkled surface. These have metallic green to dark green colour with black and glossy silverskin, depending on the drying conditions. Cell walls and internal structure are not finally developed. The beans are smaller than mature beans. These are caused by drought, stress, fertilization, pests and diseases. Immature beans leads to slow and irregular roasting and beans remain pale in the roast. These beans produce more bitterness, diminished aroma, flavor and acidity, chemical off-flavor, fermented taste to the coffee beverage and the impact is higher in the beverage.

Black and brown beans

Most of the external and interior (50%) surface of these coffee beans are black in colour. These beans (Fig. 2.1) have dull colour with a granulous external surface and often small-sized beans with adherent silverskin, undesirable appearance, enlarged center-cut, slightly shrunken and often "boat-shaped". These are caused by six reasons.

1) Attacks by pest diseases.
2) Carbohydrate deficiency in the beans due to poor cultural practices and insufficient water during ripening.
3) Over-ripe cherries picked up off the ground.
4) Immature beans affected by faulty drying (i.e. high temperatures).
5) Beans/cherries subjected to over-fermentation by moulds/yeasts and subsequent drying.
6) Poor drying or re-wetting.

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Black beans cause uneven roasting. The beans turn dull and yellowish. They rarely reach the second cracking sound during roasting. These beans produce total loss of aroma, acidity and off-flavours in the beverage. The negative quality produced in the coffee is rated very high.

Waxy and foxy beans

These beans (Fig. 2.1) are with translucent, waxy appearance which has yellowish green to dark reddish brown colour. These are produced, in general, in cherry coffee beans when over-ripe, partly dried. They have tendency to roast quickly, increased roasting loss leads to darker colour. These beans produce low acidity, greenish character and fermented off-flavor to the cup profile. The impact of these beans on the cup profile is rated high. Foxy beans (Fig. 2.1) possess foxy (oxidized reddish to light-brown) silverskin. The reddish visual colour is particularly noticeable in the center-cut. The effect of these beans in the cup quality is almost equivalent to waxy beans and the negative quality produced in the coffee beverage is rated medium on the quality.

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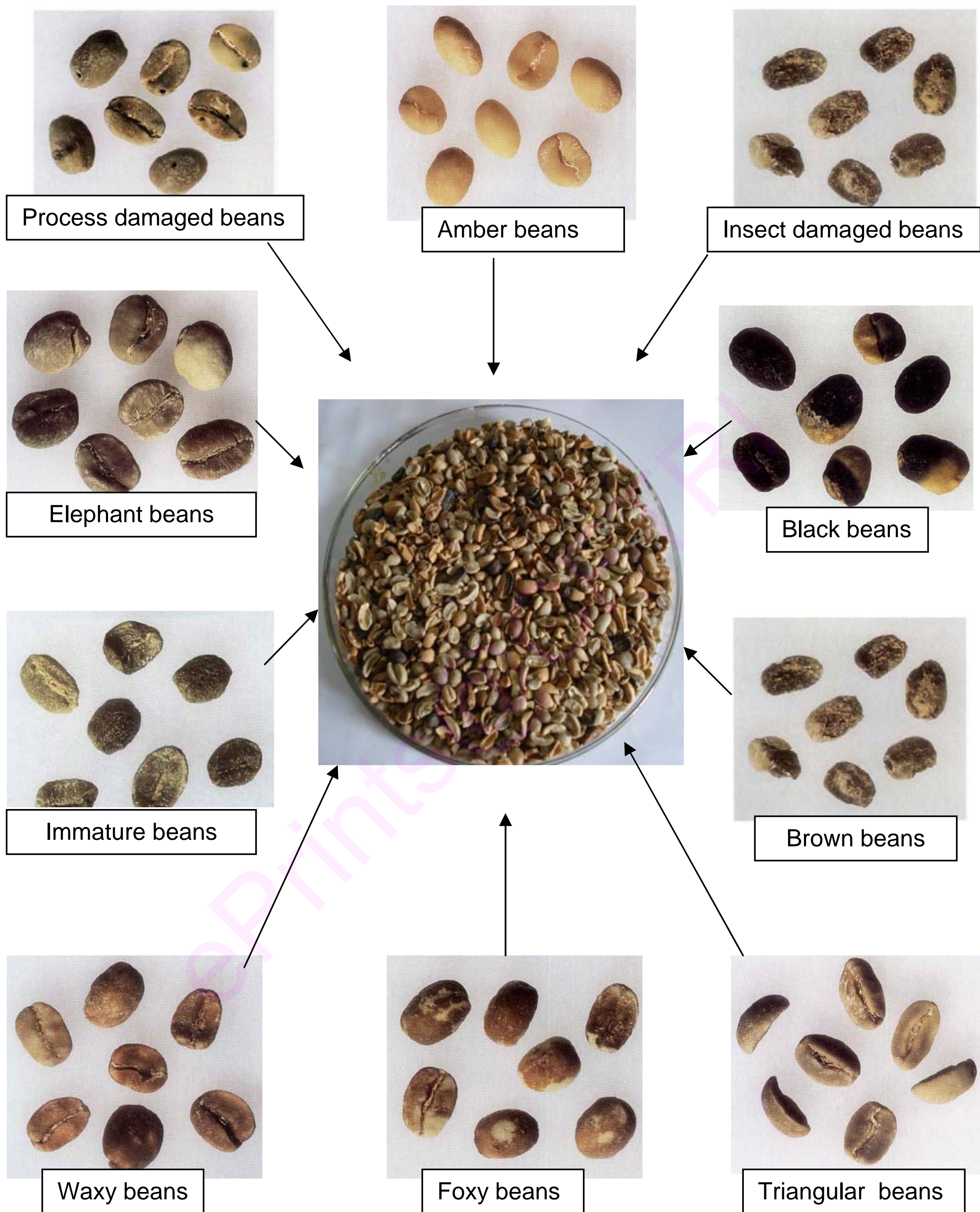

Fig 2.1. Low- grade coffee beans

2.1.3. Impact on the quality

The presence of defects is quite relevant in establishing coffee quality, since they could be associated with specific problems during harvesting and pre-processing operations.

Black beans result from dead beans within the coffee cherries or from beans that fall naturally on the ground by action of rain or over-ripening (Mazzafera, 1999). The presence of sour beans can be associated with 'overfermentation' during wet processing (Clarke & Macrae, 1987), improper drying or picking of overripe cherries (Sivetz & Desrosier, 1979). Immature beans come from immature fruits. Immature-black beans are those that fall on the ground while immature, remaining in contact with the soil and thus subject to fermentation (Mazzafera, 1999). Typical defects, such as black, sour and immature beans, are known to affect beverage quality.

According to Clarke (1987), the black bean is usually associated with a heavy flavour and sour beans contribute to sour and onion tastes. However, specific studies that correlate the presence of such defects with physical and chemical characteristics of the beans are still scarce. Few studies (Clifford & Kazi, 1987) have shown that the reduced contents of chlorogenic acids, and specifically the ratio between monocaffeoylquinic and dicaffeoylquinic acids, were related to the presence of immature beans. Mazzafera (1999) evaluated chemical attributes of immature black and black beans in comparison to non-defective beans. Non-defective beans were

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heavier, more humid leads to higher levels of sucrose, protein and total oil content than either immature-black or black beans.

2.1.4. Spent coffee grounds

Spent coffee grounds are the residues of the soluble coffee processing. With improvements of soluble coffee processing, the daily volume of coffee residues is initially produced from 1.86kg dropped to 0.91 kg for each kilogram of soluble coffee (Silva, 1998). Though the volume of spent coffee grounds produced is decreased, their disposal still is a problem for the coffee industry. Since it is expected that the spent residue contains lesser quantity of the bioactive compounds such as caffeine, tannins and polyphenols, it is difficult for the effective utilization of spent coffee residues thus leading to the problem of environmental pollution.

With the advent of biotechnology, attempts have increasingly been made to make potential use of agro-industrial residues for value addition by production of enzymes, organic acids, single cell protein and bioactive secondary metabolites.

Scope of this chapter

Specific studies that correlate the presence of defects with respect to physical and chemical characteristics of the beans compared to graded coffee in the literature are still limited (Mazzafera, 1999; Franca *et al.,* 2005; Vasconcelos *et al.,* 2006; Oliveira *et al.,* 2006). These studies were

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not made with reference to the Indian coffee beans, which are different from other geographical regions with regard to various physical and chemical characteristics. Also there are no reports on the physico-chemical characteristics of spent coffee residues. Therefore, the present study is focused on the physical properties and chemical composition of the defective coffee beans in comparison to non-defective beans from India.

2.2. Preparation of Low grade green coffee and spent coffee

Two varieties of arabica and robusta processed by dry and wet methods were selected for the studies. Grading was carried out after removing the extraneous matters such as twigs, stones and wood pieces (Ramalakshmi & Raghavan, 2003). Arabica plantation (AP), processed by wet method, were graded in the sieve with the aperture size, 6.65 mm, whereas Arabica cherry (dry method, AC), Robusta parchment (wet method, RP), Robusta cherry (dry method, RC) were graded with the sieve of aperture 5.50mm as per the guidelines of Indian Coffee Board (Anon, 1982). The rejects or defects, (APD, ACD, RPD, RCD) were collected separately from each variety. The electronic colour sorter rejects from AP, AC, RP and RC were added to the respective rejects (APD, ACD, RPD, RCD) and taken for further studies. Also commercially available defective coffee beans (mixture of varieties, CD, commercial defective) were procured from the local market (Mysore, India). These were packed in LDPE (Low Density Poly Ethylene) pouches and preserved at 8-10°C for further analysis.

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Three varieties of coffee beans namely, robusta cherry, arabica plantation and commercial triage coffee beans were procured, roasted and ground to pass through 0.077 inch sieve using the apex mill. Roasted and ground (R & G) coffee was subjected to boiling water extraction using a column (5 kg, Fig. 2.2.) where the temperature of the extraction system was maintained at 92 ± 5 °C. The ratio of material to water ratio was kept as 1:3. Also the domestic type coffee filter was used for preparing spent coffee residue. After extraction the spent coffee was dried and analysed for moisture, total soluble solids, polyphenols, caffeine and chlorogenic acids. A commercial spent coffee sample obtained from M/s Nestle India Ltd. was analysed along with the above samples.

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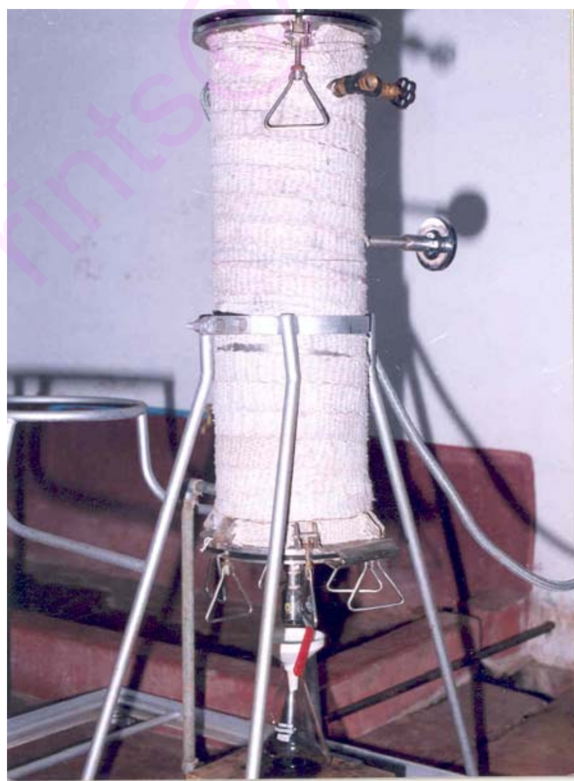

Fig. 2.2. Column used for preparation of spent coffee

2.3. Physical attributes

Graded and defective green coffee beans and spent coffee residue of the above mentioned varieties were analysed for the following physical parameters. All the analyses were carried out in triplicate and statistical analysis was applied.

2.3.1. Experimental methodologies

2.3.1.1. Bean density and brightness

Whole green coffee beans (100 nos.) from each sample were weighed and average bean density was evaluated as the ratio between the weight of the 100 beans and the volume occupied by the beans (Dutra *et al.,* 2001). The colour of the green coffee beans was determined using reflectance meter (Ramalakshmi *et al.,* 2000). Reflectance meter was calibrated to 0 to 100 % reflection with white and black filter plates respectively. Green coffee beans were taken in a Petridish and colour was determined by placing it over the sensor unit and percentage of reflectance was measured.

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2.3.1.2. Titratable acidity and pH

Aqueous ethanol, 80% (75ml) was added to green coffee powder (10g) in a round bottom flask and gently agitated using a magnetic stirrer for 16hs. The mixture was then filtered using Whatman No.1 filter paper. Filtrate (25 ml) was diluted and made up to 100ml using distilled water. Sample solution (25 ml) was titrated against 0.1N NaOH using phenolphthalein as the indicator. Volume of NaOH required for

neutralisation was noted and titratable acidity was calculated. (AOAC, 2000). pH of the green coffee was determined by extracting green coffee powder (3g) in 50ml hot water. The extract was cooled to room temperature and pH of the extractive was measured using a pH meter.

2.3.1.3. Moisture content

Samples of green coffee beans and spent coffee powder (5g) were placed in the hot air oven at a temperature of 105 ± 2°C for 48 h (Mazzafera, 1999). The samples were cooled in a desiccator and the dry weights noted and percentage of moisture calculated.

2.3.1.4. Total soluble solids

Green and spent coffee powder (2 g) was refluxed with hot water (200 ml) over the flame for 1 h. It was cooled and made up to 500 ml. The solution was mixed thoroughly and filtered using Whatmann No.1 filter paper. The filtrate (50 ml) was transferred to a weighed petri dish and evaporated to dryness on a steam bath. The dried sample in the petri dish was heated (105 ± 2°C) for 1h and cooled. The weight of sample was taken to find out the amount of total soluble solids present in the green coffee. (AOAC, 2000).

2.3.2. Results and Discussion

Results of studies on physical attributes of Indian coffee beans are presented in Table 2.3. The weight of 100 seeds of defective coffee beans are

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less compared to the graded beans in all the varieties. The reason could be due to the presence of broken and cut beans. Density of the graded beans (657-710 kg/m^3) was higher than that of the defective coffee beans (520-650 kg/m^3) in all the varieties, which confirms the earlier report (Franca *et al.,* 2005). As expected the density of arabica beans processed by both wet and dry methods (AP, AC) are lesser compared to the robusta variety (RP, RC). However, the densities of both arabica and robusta are lower when compared to Brazilian green coffee. Bulk density of Brazilian coffee beans is reported in the range of 1200-1300 kg/m^3 and 450-800 kg/m^3) for green and roasted beans respectively (Mazzafera, 1999).

Table 2.3. Physical characteristics of graded & defective coffee beans

Samples	**Weight of 100 seeds (g)**	**Average bean density (g/ml)**	**Colour***	**Moisture (%)**
CD	$12.12^{a}\pm0.41$ (10df)	$0.524^{a}\pm0.011$ (10df)	$19.13^{a}\pm0.13$ (10df)	$6.24^{a} \pm 0.20$ (10df)
RC	21.52^{b}	0.706^{b}	22.98^{b}	7.38^{b}
RCD	15.50^{c}	0.644^{c}	19.18^{a}	7.33^{b}
RP	20.86^{b}	0.705^{b}	25.27^{c}	6.51^{a}
RPD	12.26^{a}	0.595^{d}	17.60^{d}	6.89^{ab}
AC	$20.82^{b}\pm0.22$ (8df)	$0.665^{c}\pm0.009$ (8df)	$23.35^{b}\pm0.15$ (8df)	$5.52^{c}\pm0.06$ (8df)
ACD	12.58^{a}	0.573^{d}	20.24^{a}	6.21^{a}
AP	19.32^{bd}	0.657^{c}	29.53^{d}	6.66^{ab}
APD	17.21^{d}	0.623^{cd}	24.36^{c}	6.83^{ab}

* Percentage of reflectance; df: degree of freedom
Means of the same column followed by different letters differ significantly (P<0.05) according to Duncan's New multiple range test.

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Arabica coffee beans have higher colour (percentage of reflectance) value than robusta, which is due to the bright bluish green nature of arabica varieties. However, presence of bleached, black and brown bits in defective coffee beans was found to lower its colour value in every category of samples. Higher titratable acidity in defective coffee could be attributed to fruit fermentation during the drying process. The commercially available defective coffee beans also showed higher titratable acidity like the other category of defective coffee beans. Although significant differences were not found with pH, the lower values were observed for defective coffee beans, which is associated with the highest acidity. This could be attributed to fermentation within the fruit during the drying process or fermentation of the black bean or beans harvested at an inappropriate developmental stage. Titratable acidity and pH values are matching with the literature reported values (Mazzafera, 1999).

Moisture levels (5.52 to 7.38%) of the beans in the present study are marginally lower than the levels reported (Mazzafera, 1999; Clarke, 1985). However, defective coffee beans did not show much variation in moisture levels, when compared to graded beans in the respective category. Total soluble solids content was in the range of 29-34% for graded and 26-32% for defective coffee beans (Table 2.4.). Robusta variety gave more soluble solids than arabica variety processed by both the methods.

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Table 2.4. Physical attributes of graded & defective coffee beans

Samples	TSS (%)	pH	Titrable acidity ml NaOH g^{-1}
CD	$31.02^{a} \pm 0.26$ (10df)	$6.14^{a} \pm 0.01$ (10df)	$2.11^{a} \pm 0.01$ (10df)
RC	32.59^{b}	5.76^{b}	2.02^{b}
RCD	30.18^{a}	5.72^{b}	2.39^{c}
RP	32.10^{b}	6.42^{c}	2.80^{d}
RPD	30.84^{a}	6.01^{d}	2.90^{e}
AC	$31.06^{a} \pm 0.20$ (8df)	$5.81^{b} \pm 0.05$ (8df)	$2.07^{a} \pm 0.02$ (8df)
ACD	27.32^{c}	5.73^{b}	2.16^{a}
AP	31.62^{ab}	6.12^{a}	2.17^{a}
APD	26.57^{c}	5.92^{d}	2.41^{c}

Means of the same column followed by different letters differ significantly ($P<0.05$) according to Duncan's New multiple range test.

The **physical attributes** of R& G coffee powder and **spent coffee residue** of both the varieties along with the commercial spent coffee residue is furnished in table 2.5. Total soluble solids (TSS) of roasted coffee powder obtained from arabica plantation and robusta cherry were found to be 23.8 and 31.9%. (Table 2.5.). The results are in agreement with the values reported in the literature (Sivetz & Desrosier, 1979; Ramalakshmi *et al.,* 2000). TSS content of spent coffee residues are in the range form 3.81 – 11.75% in both the extraction methods in column as well as in coffee filter. The higher TSS content of commercial spent may be due to the presence of amount of chicory which was not disclosed by the supplier.

In general extraction of solubles from R&G coffee is more in column extraction then in filter devices (Gronlund, 1995).

Table 2.5. Physical attributes of R&G and spent coffee residues

Sample	Moisture (%)	TSS (%)
Arabica plantation (Ar)	2.75 ± 0.15	23.80 ± 1.42
Spent Ar (column)	4.72 ± 2.67	10.35± 0.21
Spent Ar (filter)	1.84 ± 0.09	3.81± 0.41
Robusta cherry (Rb)	1.94 ± 0.11	31.92 ± 2.42
Spent Rb (column)	3.87 ± 0.99	7.29± 0.38
Spent Rb (filter)	2.85 ± 0.16	7.76 ± 0.51
Commercial spent coffee	8.78 ± 0.19	11.75 ± 0.81

There was an extraction efficiency of 56.8% in arabica plantation, while it was 77.4% in the case of robusta cherry when the coffee powder was extracted in column extractor. This resulted with the higher TSS content in spent arabica (10.3%) than in spent robusta (7.2 %).

2.4. Chemical attributes

Green and spent coffee were analysed for the following chemical characteristics. All the analyses were carried out in triplicate and the statistical analysis was applied.

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2.4.1. Experimental Methods

2.4.1.1. Caffeine

Green and spent coffee powder sample (5 g) was mixed with magnesium oxide (3 g) and distilled water (100 ml) in a 250ml FB flask. The mixture was refluxed for 45 min on a boiling water bath. After cooling, it was filtered using Whatman No. 1 filter paper. This filtrate was transferred into a separating funnel (250 ml). Sulphuric acid (10 %, 5 ml) was added to the separating funnel and shaken well. Chloroform (15 ml) was added and shaken well. Chloroform extract collected separately and the extraction repeated 4 times. The chloroform extracts were pooled together in another separating funnel (100 ml) and washed with 2% KOH solution (5 ml). Lower chloroform layer was collected and distilled off. Residue in the flask was cooled and dried in an oven at 100-105°C. The weight of the dried residue is noted at room temperature.

The dried caffeine in the FB flask was dissolved in warm water. It was filtered through a fluted Whatman No.1 filter paper into a 250 ml volumetric flask. The flask was rinsed several times with water and carefully made up to volume. From this 250 ml, 10 ml of the aliquot was taken in a 100 ml volumetric flask and made up to the mark. The absorbance was measured at 275 nm using water as blank. The caffeine quantity was calculated using a standard graph prepared from caffeine reference sample (AOAC, 2000).

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2.4.1.2. Chlorogenic acids

Chlorogenic acid was estimated by UV spectrophotometry before and after lead acetate treatment of the coffee extract, followed by measurement of the absorbance at 325nm (AOAC, 2000).

Green and spent coffee powder (1 g) was taken in a 20ml test tube, extracted with petroleum ether (4 x 5ml) and the supernatant was decanted. Residual solvent was removed using a hot water bath. The dried materials were transferred to a conical flask containing (300 ml) boiling water and boiled for 15 min. and cooled. The contents were transferred to a 500 ml volumetric flask and volume made up. The sample was filtered through filter paper (Whatman No. 1).

The filtrate (10 ml) was transferred to a 100ml volumetric flask and made up to volume. This solution was termed A. The absorbance was determined at 325nm using water as blank. Then 100ml of the sample solution was transferred to a 200 ml beaker to which saturated potassium acetate solution (2 ml) and basic lead acetate solution (10 ml) were added with swirling. The beaker was placed on a boiling water bath for 5min while swirling occasionally. It was cooled under tap and placed in ice water bath and stirred mechanically (1 h). The beaker contents were brought down to room temperature and transferred to a 250ml volumetric flask and made up to the volume. The solution was filtered using fluted Whatman No. 50 filter

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paper and this solution was termed as B. Absorbance measured at 325nm immediately.

From standard curve, apparent concentration of chlorogenic acid in solution without lead treatment (Co); apparent concentration in filtrate after lead treatment (C1) were determined. From latter value subtract 0.00045 mg/ml to correct for solubility of lead chlorogenate.

Calculated corrected concentration = Co – [(C1 – 0.00045) / 4] .

2.4.1.3. Proteins

Green coffee powder (0.1 g) was mixed with 5 ml of 0.1N NaOH solution and ground in a mortar using pestle. The solution was filtered using Whatman No 1 filter paper and filtrate was collected. Bradford's reagent, (Commassie blue G-250, Sigma, USA) was prepared by dissolving 20 mg of Bradford Reagent in 10 mL of 95% ethanol. To this 50 ml distilled water and 20 ml of o-phosphoric acid were added. The solution was made up to 200 ml with distilled water.

Filtrate of the sample solution (80 µL) was mixed with Bradford's reagent (3.6 ml) and diluted to 4.0 ml using distilled water. The absorbance of this solution was measured at a wavelength of 595nm and protein content obtained from the standard graph (Bradford, 1976). The experiment was repeated using 100 µL of filtrate and absorbance noted.

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Standard Protein graph was prepared using Bovine's serum albumin (BSA). Standard BSA solution (0.01 % in water) was prepared and known volumes [50 (5 μg), 100 (10 μg), 150 (15 μg), 200 (20 μg), 250 (25 μg) μl] into test tubes containing Bradford reagent (3.6 ml) and volume made up to 4 ml. The absorbance was measured at 595 nm. The concentration of the protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the quantity of protein in unknown samples.

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2.4.1.4. Lipids

Total lipid content was analysed by soxhlet extraction method (Folstar, 1985). Green coffee powder (50 g) was extracted with hexane (1:6) in a soxhlet apparatus for 16 h on a boiling water bath. The solvent extract was desolventised in a rotovapour and residual solvent was removed by keeping the extract in vacuum dessicator. The solvent extract free from traces of solvent was finally weighed and the amount of total lipid content was calculated.

2.4.1.5. Sucrose

Sucrose content was determined using a HPLC method. Powdered green coffee (5 g) was defatted using petroleum ether (30 mL). The defatted coffee powder was transferred to a conical flask and boiling water (30 mL) was and the whole content was boiled for 30 min. The extract was filtered through Whatmann No.1 filter paper. The sample solution was then passed through a 0.45 μm micro filter paper and injected (20 μL) to HPLC having

amino propyl column (250 mm X 4.6 mm, i.d., 5 microns, Macherey Nagel, Germany) and RI detector. The mobile phase used was acetonitrile and water in the proportion of 80:20 at the flow rate of 1 ml/min. Peak areas from the chromatogram of the sample were compared with the standard and the amount of sucrose in the sample was calculated (Mullin *et al.,* 1997).

2.4.1.6. Total polyphenols

Total polyphenol content of the green and spent coffee samples was determined using Folin-Ciocalteu's reagent. Green coffee powder (0.5g) was taken in methanol: water (70:30, 10 mL) solution in a graduated test tube and heated on a water bath (70°C) for 10 min. The sample was subjected to centrifugation for 3500 rpm for 10 min. and the supernatant was separated. Saturated sodium carbonate solution (5 mL) and Folin-Ciocalteu's reagent (0.2 mL) were added to the sample solution and made upto 10 mL with distilled water. The solution was incubated at room temperature for 60 min. and the absorbance of this solution measured at 765 nm. The total polyphenol content of coffee samples is expressed as gallic acid equivalents (Swain & Hillis, 1959). Gallic acid stock solution (0.01 %) was prepared. The working solutions (20-200 μg / 10 ml) were prepared. These solutions were treated as above and the absorbance measured. The standard curve was drawn using gallic acid concentration versus absorbance.

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2.4.2. Results and Discussion

The chemical characteristics of defective coffee in comparison with graded coffee beans are presented in figures 2.3-2.8.

Caffeine content was slightly lower in defective coffee beans. But significant changes were not found, as caffeine content is not much affected by environmental, agricultural and post harvest practices. Caffeine content was in the range of 0.8 –1.8% which is very much matching with the literature value of 0.9-1.4% (Franca *et al.,* 2005; Macrae 1985). Robusta coffee beans contain more caffeine than arabica beans

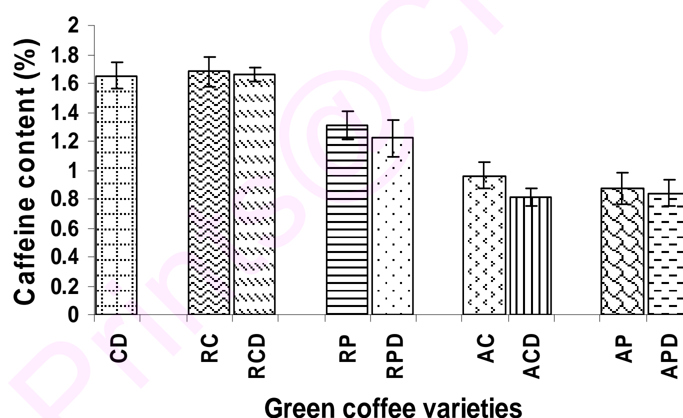

Fig.2.3. Caffeine (%) content in different varieties of green coffee beans

No differences in caffeine contents were encountered among the defective beans. Higher caffeine contents were reported in the endosperm of immature fruits and in the whole immature fruit (Mazzafera *et al.,* 1991). In addition to this, the minor contribution of caffeine to beverage bitterness led

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these authors to conclude that this alkaloid is not responsible for any change in beverage quality.

Chlorogenic acids (CGAs) constituted the second important group of compounds, representing 6 – 10% dry matter of the coffee beans. Chlorogenic acid is one of the key components in coffee responsible for determining the beverage quality as well as its antioxidant activity and in turn for health benefits. Chlorogenic acid content (Fig. 2.4.) was found to be marginally more in defective coffee beans (6.83 - 8.80 %) compared to graded coffee beans (6.35 - 8.32 %). However these values are marginally higher than the values reported by Franca *et al.,* 2005. These could be due to the difference in the extraction procedures or geographical origin. Chlorogenic acids from defective coffee beans perhaps could be utilized as natural antioxidants and may help to add the benefits to the coffee growers / manufacturers.

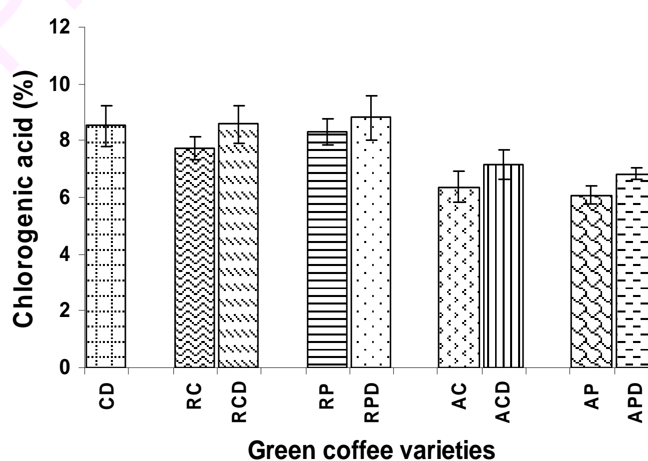

Fig.2.4. Chlorogenic acids (%) in different varieties of green coffee

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The relationship between chemical composition of immature cherries and chlorogenic acid was studied in detail by Clifford & Kazi 1987, who suggested that immature coffee beans might affect the coffee beverage, conferring astringency, which is mainly due to the composition of various constituents of chlorogenic acid. The correlation between the isomers of CGAs and the sensory properties of beverage, though not proven, indicate that these acids have an important role in determining bean and beverage quality (Clifford & Ohiokpehai, 1983; Clifford *et al.,* 1987; Ohiokpehai *et al.,* 1982).

The **protein** content was also less in defective coffee beans (32.81 – 47.15 mg/g) when compared to graded beans (38.75 – 57.16 mg /g; Fig. 2.5.). However, the protein content of the Brazilian immature coffee beans is higher (Mazzafera, 1999) than the defective as well as the graded coffee beans investigated in the present study.

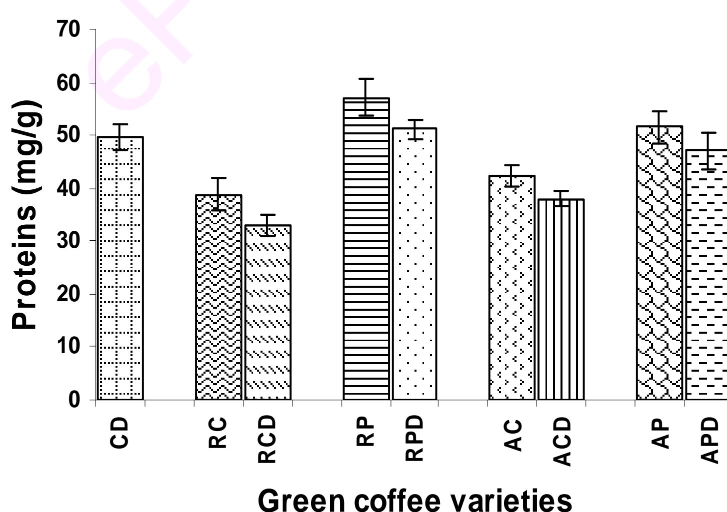

Fig. 2.5. Proteins in different varieties of green coffee beans

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The **lipid** contents of defective coffee beans (7.2-12.7%) were lower than those of corresponding category of graded coffee beans (8.7-16.3%). According to an earlier report, the lipid content of green coffee beans is in the range of 9-16 % (Speer & Kolling, 2001). Non-defective coffee beans found to contain higher lipid contents than defective (black, sour and immature) ones.

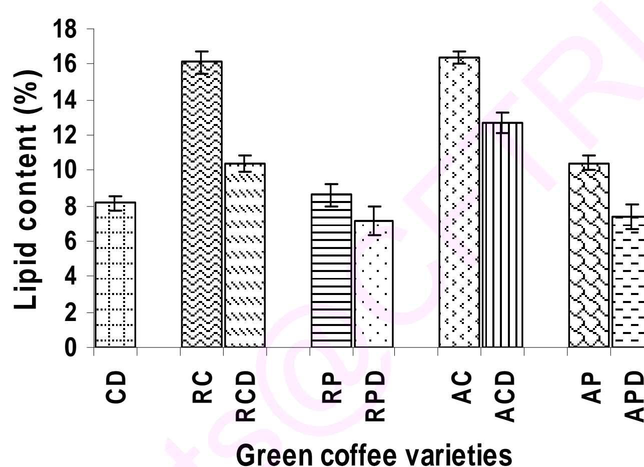

Fig.2.6. Lipid content (%) in different varieties of green coffee beans

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Sucrose was the main carbohydrate, present in the range of 3-5%. Though there was not significant difference in sucrose content between graded and triage [Fig. 2.7] defective coffee beans showed slightly lowersucrose content, which could be attributed to the presence of immature black beans since the degree of ripening is related to the accumulation of sucrose in green coffee beans. It was reported that good beverage was associated with coffee beans of high sucrose content

(Mazzafera 1999). Also it was observed that dry processed coffee showed more sucrose content than the wet processed coffee in both the varieties.

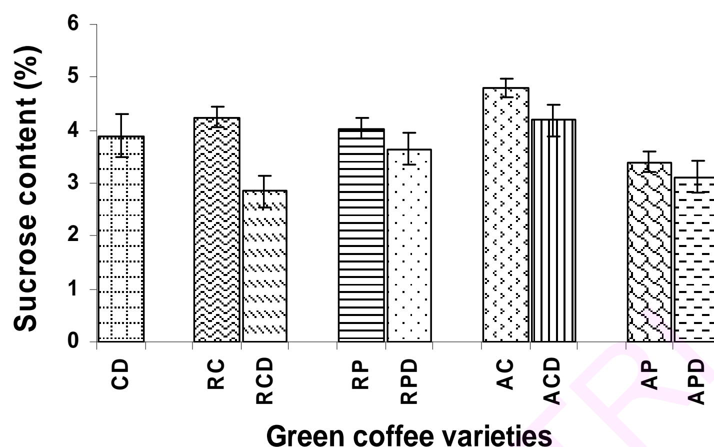

Fig.2.7. Sucrose (%) in different varieties of green coffee beans

Total **polyphenols** was less in defective coffee beans (3.04 - 4.08 % GAE) when compared to graded beans (3.36-4.54 % GAE, Fig. 2.8). Polyphenols can be isolated as enriched fractions and could be used as antioxidants in food products to improve the health benefits as well as to extend shelf life.

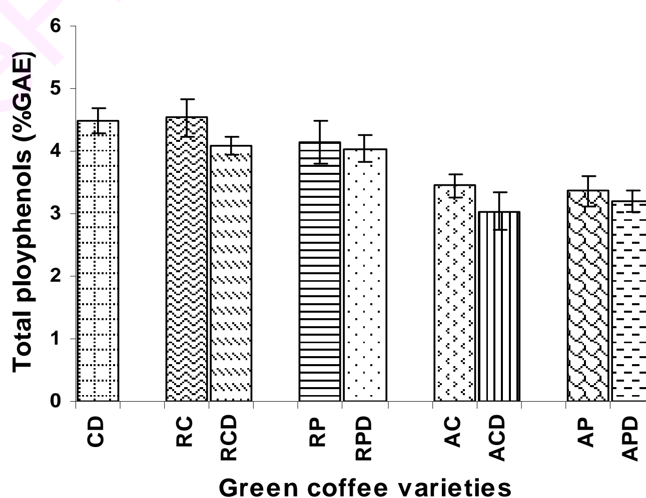

Fig.2.8. Total polyphenol content of different varieties of green coffee beans

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The total polyphenols and chlorogenic acid in the **spent coffee** are in the range of 0.87-2.38% and 0.5-1.45% respectively (Table 2.6).

Table 2.6. Chemical composition (%) of R&G and spent coffee residues

Sample	Total polyphenols (%)	Caffeine (%)	Chlorogenic acids (%)
Arabica plantation (AR)	3.53 ± 0.21	1.60 ± 0.11	2.68 ± 0.16
Ar spent (column)	1.32 ± 0.11	0.53±0.09	1.45±0.05
Ar spent (filter)	0.87 ± 0.04	0.11± 0.01	0.71± 0.01
Robusta cherry (Rb)	4.10 ± 0.23	2.37 ± 0.12	4.00 ± 0.14
Rb spent (column)	1.02 ±0.05	0.22±0.08	1.21±0.09
Rb spent (filter)	1.66 ± 0.09	0.40 ± 0.01	0.85 ± 0.01
Commercial spent coffee	2.38 ± 0.11	0.02± 0.08	0.50 ± 0.01

* as gallic acid equivalents

As expected, the amount of chemical constituents such as phenolics, chlorogenic acids and caffeine content are lesser in spent arabica and robusta than the R&G coffee powder of both the varieties. This is due to the extraction efficiency of 56.8% in arabica plantation and 77.4% in the case of robusta cherry when the coffee powder was extracted in column extractor. Also, the reasons could be (i) the degradation / conversion of these compounds during roasting and (ii) release of the compounds into the water extract during the extraction procedure.

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CHAPTER 3

CHAPTER 3

Biochemical studies: *in vitro* and *in vivo*

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CHAPTER 3

3.1. Introduction

Recent studies carried out by diverse industries have shown that the consumers' demand for health-promoting food products containing the bioactive principles isolated from plant materials is increasing. These types of products are called as "nutraceuticals" a new hybrid term between nutrient and pharmaceuticals. A nutraceutical is a product isolated or purified from foods which is demonstrated to have a physiological benefit or provide protection against chronic diseases.

Besides fruits and vegetables that are recommended as optimal sources of antioxidant components, herbs and spices are reported to contain high amounts of compounds capable of quenching free radicals (Madsen & Bertelsen, 1995), which impart beneficial effects (Capecka *et al.,* 2005) Many herbs and spices, usually added to flavour dishes, are an excellent source of phenolic compounds such as curcumin (VII), lutein (VIII), gingerol (IX), catechins (X, XI) from tea and other compounds have been reported to show good antioxidant activity (Rice-Evans *et al.,* 1996; Zheng & Wang, 2001) and thus they may serve as natural food preservatives.

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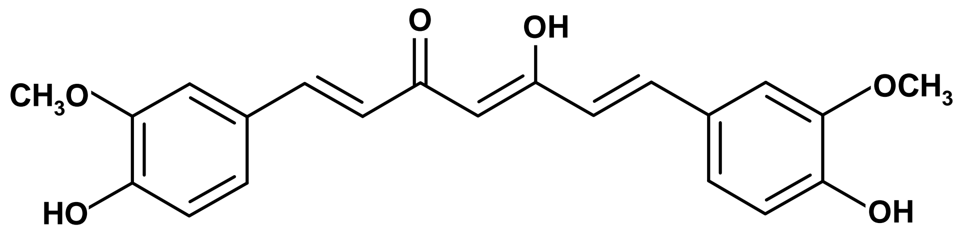
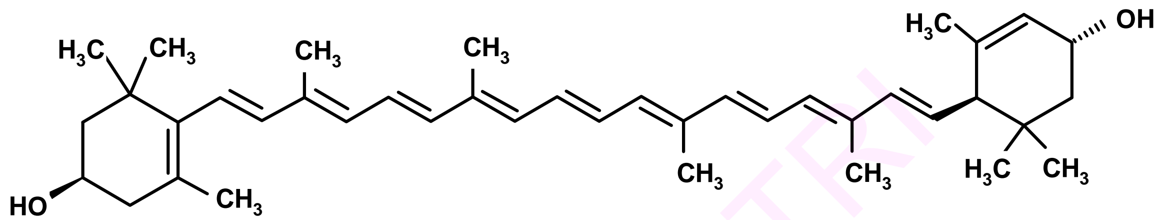
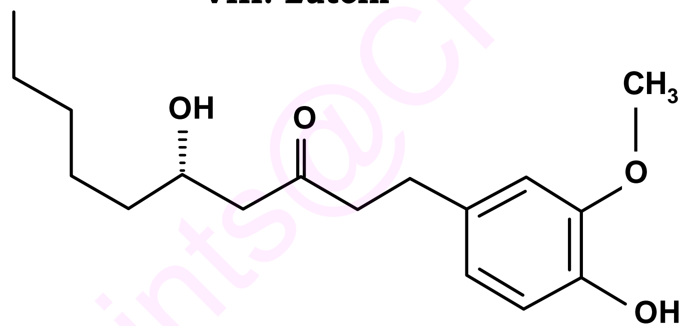


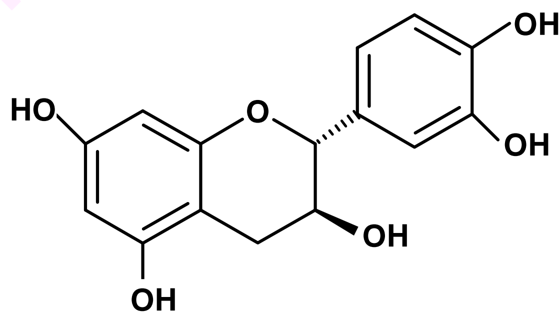
VII. Curcumin



VIII. Lutein



IX. Gingerol



X. Catechin

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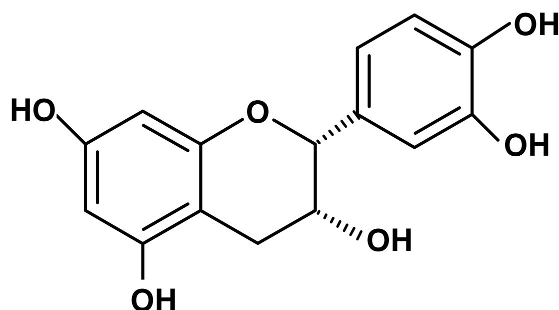

XI. Epicatechin

Coffee is responsible for a number of bioactivities since it contains a number of active compounds that exhibit these effects. Active compounds such as polyphenols including chlorogenic acids (CGA, XII), caffeine (XIII), serotonin (XIV), caffeoyltryptophan (XV), cafestol (XVI), kahweol (XVII) and other phenolics such as caffeic, ferulic and vanillic acids present in the coffee and are reported to be responsible for these bioactivity effects. (Farah, 2006; Clifford, 1999).

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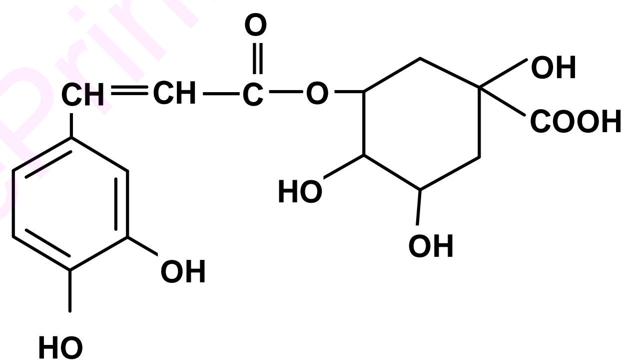
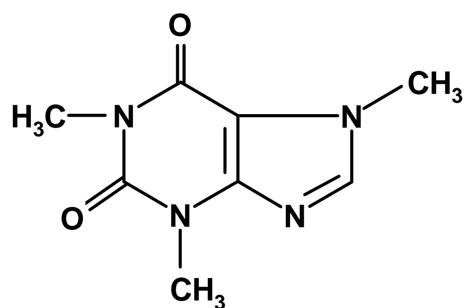
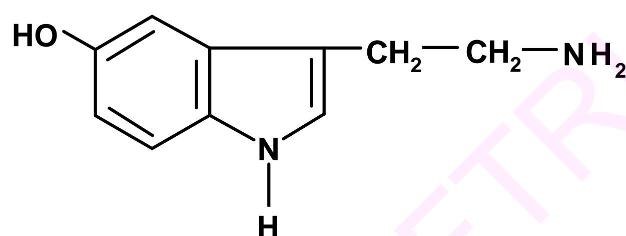


XII. Caffeoylquinic acid



XIII. Caffeine



XIV. Serotonin

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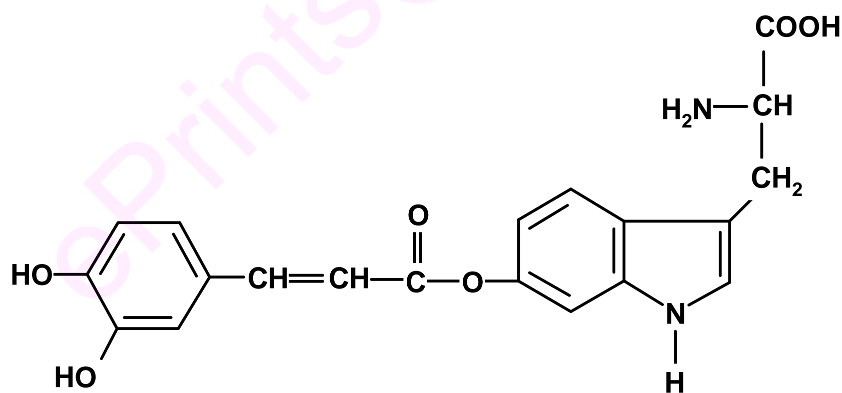
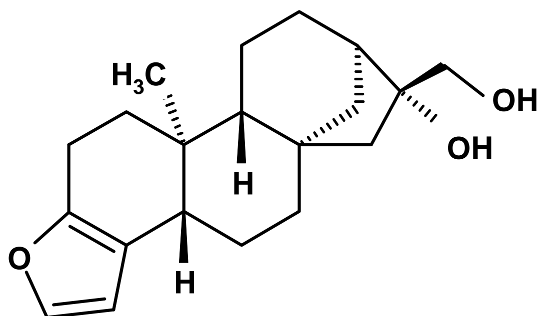
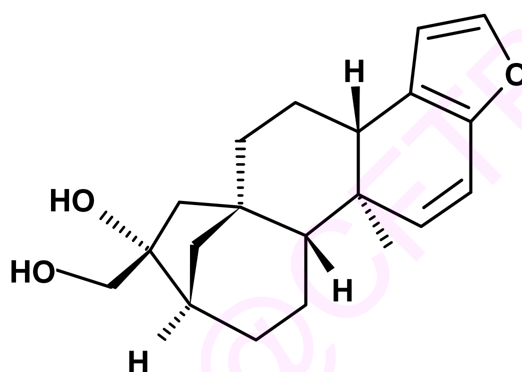


XV. Caffeoyltryptophan



XVI. Cafestol



XVII. Kahweol

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LCB and spent coffee are the coffee by products in the coffee industry. Studies on the physical and chemical characteristics of graded and LCB with reference to Indian region (Ramalakshmi *et al.,* 2007) reveals that there are marginal differences in the composition. In particular, chlorogenic acid, one of the components in coffee responsible for the antioxidant activity was found to be high in defective coffee beans indicating changes in the chemical composition when compared to graded and normal coffee beans.

Scope of the study

Results in chapter 2 suggested that there is an appreciable quantity of polyphenols and chlorogenic acids present in LCB as well as spent coffee residue. It is well known that these two compounds including caffeine are responsible for the bioactivity of coffee. Though there are reports available on the bioactivity of coffee, the information on coffee by-products with reference to the bioactivity is scanty.

Extracts were prepared form LCB and spent coffee residue and evaluated for bio activity with reference to antioxidant, anti-tumour, anti-inflammatory and anti-allergy using in vitro model systems. Also extract of LCB were fed into Balb/c mice and evaluated for the oxidative stress.

3.2. Preparation of extractives

3.2.1. Plant Materials

Commercially available low-grade green coffee beans were ground and sieved using a mesh size-18 (650 μm). Sample in a powder form was packed in low-density polyethylene pouches and preserved at 8-10 ^{0}C for further analysis. Two varieties of graded coffee beans, i.e., arabica plantation (Ar) and robusta cherry (Rb) were medium roasted and ground to obtain a coarse powder (> 650μm).

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Two varieties of roasted and ground coffee powder (1 Kg) were extracted with de-ionized water in column (with SS jacket) where the temperature of the extraction system was maintained at 92 ± 5°C. After extracting 6 h, the spent coffee residue was dried in a hot air oven at 60ºC for 3 h and thus obtained was used for further extraction with suitable organic solvents.

3.2.2. Extraction Methods

Two types of extraction methodologies were followed to prepare the extractives from LCB and spent coffee residues.

3.2.2.1. Soxhlet extraction

LCB and spent coffee powder, 800 g of each were defatted with hexane (for 8h in a soxhlet extraction system (Fig. 3.1). The defatted powder was extracted with series of solvents of increasing polarity viz., chloroform, acetone and methanol until discoloration (8 h) while maintaining a material to solvent ratio of 1:6 to 1:8. The extracts were desolventized in a rotavapour by maintaining the temperature at 50°C and stored in a refrigerator (6-8°C) until further use.

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Fig. 3.1. Soxhlet extraction

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3.2.2.2. Column extraction

LCB powder (100g) was loaded in a glass column (50 cm length with 1.5 cm diameter) fitted with a stopcock. The column (Fig. 3.2) was tapped gently to get a moderate packing. The powder was defatted with hexane (600 ml). The defatted coffee powder was loaded in the column and further extracted with chloroform, acetone and methanol until discoloration (8 h) while maintaining the material to solvent ratio to 1:6 to 1:8. at room temperature. The extract was collected completely and desolventised and kept (6-8°C) for further studies.

Fig. 3.2. Column extraction

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3.3. Bioactivity of extractives – i*n-vitro*

Extracts were evaluated for antioxidant potential through *in vitro* model systems such as β-carotene-linoleate model system (antioxidant activity - AA), α, α - diphenyl β-picrylhydrazyl radical (radical scavenging activity), iron reducing system (reducing power) and phosphomolybdenum complex (antioxidant capacity). The extract of LCB and spent coffee are further evaluated for antioxidant (ORAC assay), anti-tumour (proliferation of P388 cells), anti-inflammatory (cytokine production of J774A.1 cells) and

anti-allergy (degranulation of RBL- 2H3 cells) using in vitro model systems. The extracts were analysed for total polyphenol content, chlorogenic acids and caffeine content according to the procedure explained in chapter 2.

3.3.1. Experimental Methodologies

The extracts were analysed for total polyphenol content, chlorogenic acids and caffeine content according to the procedure described in chapter 2. Also the extracts for antioxidant, anti-tumour and anti-inflammatory activity and antiallergic activities in different in vitro model systems.

3.3.1.1. Antioxidant activity

Antioxidant activity of the extractives was evaluated as described by Jayaprakasha & Jaganmohan Rao (2000). β-Carotene (0.4 mg) in chloroform (0.4ml), 40mg of linoleic acid and 400mg of Tween 40 (polyoxyethylene sorbitan monopalmitate) were mixed in a 250ml round bottom flask. Chloroform was removed at 40°C under vacuum using a rotavapour and the resulting solution was immediately diluted with 10 ml of triple distilled water and the emulsion was mixed well for 2 min. To this emulsion, oxygenated water (90ml) was added and mixed for 1 min. Aliquots of the emulsion (4 ml) were pipetted into different stoppered test tubes containing 1ml of desired amount of extractives (equivalent to 100 - 200ppm) and BHA (equivalent to 200 ppm) in ethanol. BHA was used for comparison purposes. A control consisting of 1 ml of ethanol and 4 ml of the above emulsion was prepared. Optical density of all samples was

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measured immediately (t=0) and second reading after 15 min., followed by readings at 30 min. interval for 3 h (t=180). The tubes were placed in water-bath at 50°C between the readings. All the determinations were performed in duplicate. Measurement of colour was recorded until the colour of β-carotene disappeared. The antioxidant activity (AA) of the extract was evaluated in terms of photo-oxidation of β-carotene using the following expression.

$$AA = 100 \left(1 - \frac{A_0 - A_t}{A_{00} - A_{0t}} \right) \%$$

A_0 and A_{00} are the absorbance values measured at zero time of the incubation for the test sample and the control, respectively. A_t and A_{0t} are the absorbance values measured in the test sample and the control, respectively, after incubation for 180 min. All the determinations were carried out in triplicate and averaged.

3.3.1.2. Radical scavenging activity

Radical scavenging activity of the extracts was evaluated according to the method described by Blois *et al.,* 1958. The extracts and BHA at different concentration (30 - 200 ppm) were taken in different test tubes. Four milliliters of 0.1mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27°C for 20 min. Control was prepared as above without any extract and methanol

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was used for the baseline correction. Optical density (OD) of the samples was measured at 517nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

% Radical scavenging activity = (Control OD – Sample OD/Control OD) X100

3.3.1.3. Oxygen radical antioxidant capacity

Oxygen radical antioxidant capacity (ORAC) of the extracts was evaluated according to the method of Huang *et al.,* 2002. Extracts (0.2 g) of coffee samples were dissolved in boiling water (8 mL) and maintained at 60°C for 10 min. The solutions were centrifuged at 3000 rpm for 5 min. and the supernatant was used for ORAC assay.

Stock solution (8.16 μM) of fluorescein sodium salt was prepared in phosphate buffer solution (pH 7.0). The working solution (81.6 nM) was obtained by subsequent dilution of stock solution with Phosphate buffered saline (PBS pH: 7.1, Sigma, USA). Fresh 2, 2-azinobis (2-amidinopropan) dihydrochloride solution was prepared at a concentration of 200 mM and used for automatic injection. Calibration curve of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was prepared each day by diluting the stock solution (2 mM) with Phosphate buffered saline (PBS). The fluorescence (kexcitation = 485 nm, kemission = 528 nm) was recorded each minute over 40 min. in a multidetection microplate. All samples were

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analysed in triplicate at three dilutions and the mean value was taken for ORAC determination. The quantification of the antioxidant activity was based on the calculation of the area under the curve (AUC), as proposed by Cao & Prior (1999). The antioxidant activity by ORAC was calculated as μmol of trolox equivalents (TE) per gram of sample using the formula:

Relative ORAC value = $[(AUC_{sample} - AUC_{blank}) / (AUC_{Trolox} - AUC_{blank})]$ X molarity of trolox/molarity of sample

The experimental data on triplicate analysis of average value with standard deviation is reported.

3.3.1.4. Reducing power

The method described by Oyaizu *et al.,* 1986 was followed to evaluate the reducing power of the extracts. Different amounts of the extracts (50-200μg) in 1 ml of distilled water was mixed with phosphate buffer (2.5ml, 0.2mol/L., pH6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ml, 1%). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%), 2.5ml was added to the mixture, which was then centrifuged at 3000rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%), and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power.

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3.3.1.5. Antioxidant capacity

The total antioxidant capacity of extracts was evaluated by the method of Prieto *et al.,* 1999. An aliquot of 0.1 ml of sample solution (100 μg/ml) 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 room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. For samples of unknown composition, water-soluble antioxidant capacity was expressed as equivalents of ascorbic acid (μmole/g of extract).

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3.3.1.6. Anti-tumour activity

Anti-tumour activity of the extracts was analysed by P388 cells proliferation assay by the method described by Shinmoto *et al.,* 2001. Extracts (0.1g) were weighed accurately, mixed with 10 ml of dimethyl sulphoxide (DMSO) and stirred in a shaker overnight at room temperature. The solution was centrifuged at 3000 rpm for 5 min. and the supernatant was taken for the assay. Extract was diluted to 2000 ppm concentration with PBS while the concentration of DMSO was kept at 20%.

P388 cell lines were grown in RPMI (Roswell Park Memorial Institute) supplemented with 10% Fetel Calf Serum (FCS), 100U/mL penicillin and 100µg/mL streptomycin, and kept in the incubator (37°C, 5% CO_2, 95% air). Cells were seeded in the culture medium (100 µl) at a concentration of $5x10^4$ cells / well in a microtiter plate (tissue culture grade, 96 wells, flat-bottomed, Falcon BD, USA). Coffee extracts were added into the wells, separately along with DMSO, which acts as a negative control for the cell growth inhibition. After 48 h of incubation, 10 µl of WST-1 reagent was added per well, and the cells were incubated for 4h at 37°C. The absorbance was measured at 490-650 nm in an ELISA reader.

3.3.1.7. Anti-inflammatory activity

Anti-inflammatory activity of the extracts was determined through tumour necrosis factor, (TNF-α/), which is an important cytokine mediated immune response and inflammatory activity. J774A.1 mouse macrophage cell line was selected for the study. The cells were maintained in 10% FCS with Dulbecco's modified Eagle's medium (DMEM) supplemented with 100U/mL penicillin and 100µg/mL streptomycin. The cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. The methodology described by (Herath *et al.,* 2003) was followed.

Sample (0.1 g) solutions were prepared separately from all the extracts, followed by dissolution in 10 mL of DMSO. These extracts were further diluted with Hank's solution to get the final concentration of 1, 3

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and 10μg/ml in the assay medium. Under these conditions, none of the solvents altered the production of TNF- α without the stimulation of Lipopolysaccharide (LPS), procured from E.coli 0111:B4, DIFCO., USA. The cell suspension with the concentration of $2.5X10^5$ cells / well was placed in 96-well cultured plate and incubated for 18 h (37°C, 5% CO_2). After incubation, culture medium was removed, and various concentration (1, 3 and 10μg/ml) of the test compounds and LPS (final concentration, 1.0μg/mL) were added to each well and incubated further for 3 more h. Supernatant was then collected and assayed for TNF-α content using TNF- α enzyme–linked immunosorbent assay kit (e-Bioscience, USA). The inhibition of plant extracts against TNF- α production was calculated comparing the positive control.

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3.3.1.8. Anti-allergic activity

Rat basophilic leukaemia RBL- 2H3, cells are mucosal mast cell type which is a major model for the study of anti allergic activity of food stuffs (Yamashita *et al.,* 2000; Watanabe *et al.,* 2005).

RBL-2H3 cells were grown in Dulbecco's modified Eagle's Medium (Sigma, MD, USA) containing 10% heat in-activated fetel calf serum. The cells were inoculated into a 24 well plate (1 ml of 2.5×10^5 cells) and cultured overnight at 37°C in an atmosphere of 5% CO_2 and 95% air. Mouse monoclonal DNP IgE solution (500 μL, 50ng/ml MT buffer) was

added to each well. The solution containing micro plate was incubated for 2 h; plates were removed and washed with Modified Tyrode buffer (MT). Extracts were diluted to different concentrations (100, 1000, 1000ppm) with MT buffer and were added to in each experimental well (490 μL). Wortmannin solution (5 μM/MT buffer) was used as the positive comparison. After 10 min. of incubation, DNP labelled Human Serum Albumin (final concentration 50 ng/ml) was added to all the wells and the culture incubated for 30 min. The supernatant solution and the cell lysates were collected and analysed antigen-induced de-granulation. The supernatant was collected into the lower wells and the cells were lysed with 500 μL of Triton X 100 (0.1% (w/w MT buffer). The supernatant and the cell lysate (50 μL) was transferred to 96 well ELISA plate and mixed with 100 μL of 0.1 M citrate buffer (pH 4.5) containing 3.3 mM *p*-nitrophenyl-2-acetamide-β-D-glucopyranoside. The mixture was incubated for 25 min. at 37°C. The reaction was stopped by adding 100 μL of 2M glycine buffer (pH 10.0) and the absorbance was measured at 405 nm in a micro plate reader. Degranulation (%) in terms of β-hexasaminidase release was calculated according to Demo *et al.,* (1999).

3.3.1.9. Composition of extractives

The extractives of LCB and spent coffee residues were analysed for polyphenols, chlorogenic acids and caffeine content according to the procedure described in Chapter 2.

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3.3.2. Results and Discussion

Coffee is a complex mixture of several chemicals, containing variable amounts of chlorogenic acid, caffeic acid and other polyphenolic compounds depending on the species. Many of them are found to be potent antioxidants. The **yield of the extractives** using different solvents and the extraction methods of Low grade coffee beans (LCB) are given in Table 3.1. Yield of extraction is more in soxhlet extraction irrespective of the solvents used. Methanol could able to extract more solubles (12.5%) followed by hexane (8.7%) during solvent extraction. The efficiency of the solvents to wards the extraction is in the order of Methanol > Hexane > Chloroform > Acetone.

Table 3.1. Yield of extraction of Low grade coffee beans

Method of extraction	Solvent	Extract (%)
Column	Hexane	7.67
	Chloroform	1.02
	Acetone	0.33
	Methanol	10.90
Soxhlet	Hexane	8.69
	Chloroform	1.56
	Acetone	0.98
	Methanol	12.50

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Since the soxhlet extraction gave better result with respect to extraction over column extraction, soxhlet extraction was followed for the preparation of extractives from spent coffee residue. The results are given in Table 3.2. The efficiency of the solvents towards the extraction is in the order of Hexane > Methanol > Chloroform > Acetone.

Table 3.2. Yield of extraction of spent coffee residue

Method of extraction	Solvent	Extract (%)
Soxhlet	Hexane	14.79
	Chloroform	2.76
	Acetone	0.44
	Methanol	10.95

The **antioxidant activity** of LCB extracts was compared with BHA at different concentrations using β-carotene-linoleate model system and the results are presented in Tables 3.3 & 3.4. The mechanism involved in the bleaching of β-carotene is a free radical mediated phenomenon resulting from hydroperoxides of linoleic acid oxidation which eventually attack the highly unsaturated β-carotene molecules and bring about their rapid discoloration in the absence of an antioxidant (Singh *et al.,* 2002).

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Table 3.3. Antioxidant activity of soxhlet extractives of LCB

Solvents	AA (%) at ppm levels		
	100	**200**	**500**
Hexane	<10	14.00	15.38
Chloroform	23.61	28.26	30.12
Acetone	42.54	45.51	51.21
Methanol	55.33	59.21	65.12
BHA	-	95.09	-

Table 3.4. Antioxidant activity of column extractives of LCB

Solvents	AA(%) at ppm levels		
	100	**200**	**500**
Hexane	< 0.2	0.30	3.81
Chloroform	4.43	20.02	25.28
Acetone	31.73	40.58	45.63
Methanol	35.43	50.07	55.89

The effect of solvent on antioxidant activity of the extracts is in the order of methanol > acetone> chloroform > hexane irrespective of the method of extraction. Soxhlet extractives exhibited more antioxidant activity than column extractives irrespective of the solvents. Methanol extract of LCB showed maximum activity (65%) followed by acetone (51%) and chloroform (30%) on soxhlet extraction. Hexane extract showed very less activity.

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Table 3.5. Antioxidant activity of soxhlet extractives of spent coffee

Solvents	AA (%) at ppm levels	
	200	**500**
Hexane	10.14	22.46
Chloroform	24.78	26.58
Acetone	29.76	44.78
Methanol	57.83	62.19
BHA	95.59	97.23

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Antioxidant activity of the extractives of spent coffee residue was marginally lesser compared to the LCB extracts in respect of the each solvents (Table 3.5). Of all the solvents methanol extract exhibited maximum antioxidant activity (57.83 and 62.19%) at the concentrations of 200 and 500 ppm respectively. The antioxidant activity of the acetone extracts is better than chloroform extracts at both the concentrations. Hexane exhibited very less activity. **Radical scavenging activity** of the extracts was tested using DPPH model system (Jayaprakasha & Jagan Mohan Rao, 2000) and the results are presented in Tables 3.6 & 3.7. The role of antioxidants is their interaction with oxidative free radicals. The essence of DPPH method is that the antioxidants react with the DPPH free radical i.e., (deep violet colour) and convert it to α,α–diphenyl-β-picrylhydrazine with discoloration (XVIII). The degree of discoloration indicates the scavenging potential of the antioxidant sample / conserves

(Abdille *et al.,* 2005). In the present study the extracts were able to decolorize DPPH and it appears that the extracts from the green coffee possess hydrogen donating capabilities to act as antioxidant.

Table 3.6. Radical scavenging activity of soxhlet extractives of LCB

Solvents	RSA (%) at ppm levels			
	30	**50**	**100**	**200**
Hexane	2.60±0.12	5.42±1.02	8.96±1.99	10.26±0.22
Chloroform	18.40±1.11	20.82±1.23	25.52±1.11	27.65±1.23
Acetone	57.90±2.36	80.03±2.14	81.76±1.37	82.50±1.55
Methanol	92.08±1.59	91.00±3.25	92.47±2.11	94.74±1.34
BHA	--	--	95.01±1.89	95.89±2.20

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Table 3.7. Radical scavenging activity of column extractives of LCB

Solvents	RSA (%) at ppm levels			
	30	**50**	**100**	**200**
Hexane	< 1.0	2.23±0.12	8.18±1.21	10.20±1.77
Chloroform	13.3±2.45	19.55±1.12	14.01±2.56	15.88±2.58
Acetone	43.0±1.22	59.64±2.23	57.37±1.99	60.90±2.56
Methanol	54.79±1.98	64.68±2.34	84.17±2.76	85.12±2.99

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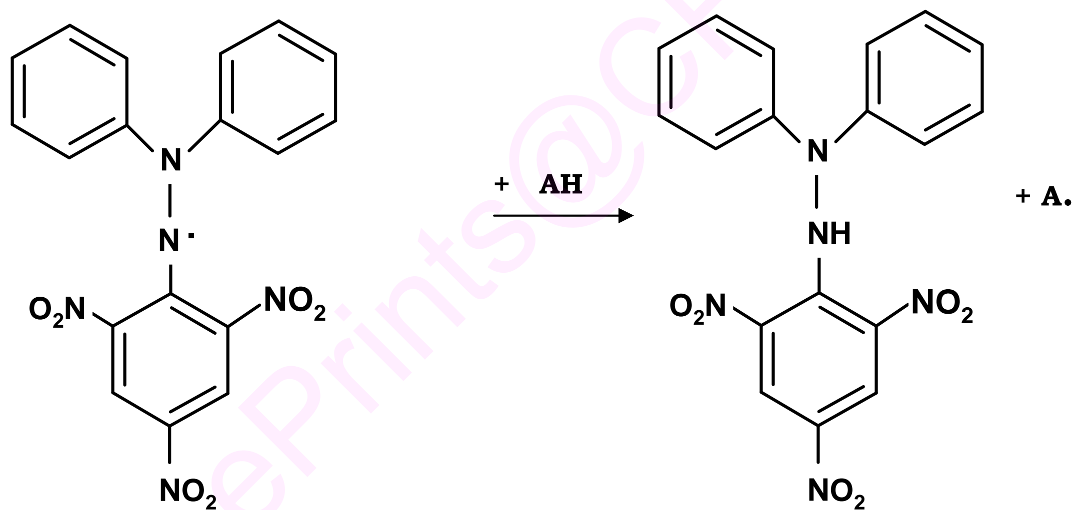

DPPH Radical (Deep violet colour) DPPH (Colourless)

XVIII. Decolourisation of DPPH

The effect of solvents on RSA and AA of the extracts are similar. It is in the order of methanol > acetone> chloroform > hexane irrespective of the method of extraction. In general, soxhlet extractives showed better radical scavenging activity than the column extractives. Also it is observed that methanol extract was found to exhibit maximum radical scavenging activity (92.5%) at 100 ppm concentration, followed by acetone (81%) and chloroform (25%) on soxhlet extraction. Hexane (8%) showed very less or no activity. The synthetic antioxidant namely butylated hydroxy anisole showed activity of 95.01% at the same concentration.

Radical Scavenging activities of methanol extracts of spent arabica and robusta are given in Table 3.8.

Table 3.8. Radical scavenging activity of spent coffee residue

Sample	RSA (%) at ppm levels		
	200	100	50
Spent coffee (Ar)	89.2±3.9	87.9±4.1[ab]	86.9±5.0
Spent coffee (Rb)	84.3±5.9	83.8±5.1[b]	82.0±5.0
BHA	95.5±4.4	95.1±3.1[a]	95.1±4.0

Spent arabica (86.9%) and spent robusta (82.0%) at the concentration of 50 ppm. However, there is no significant ($p<0.05$) difference in radical scavenging activity between the extracts as well as between the

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concentrations (50, 100 and 200 ppm) of each extracts. The slight difference in the activity of the extracts in turn may be due to the presence of polyphenolic compounds mainly the chlorogenic acids. Scavenging activity of water extract obtained from roasted coffee residues was reported as 95.4% at the concentration of 0.2mg/ml by Yen *et al.,* 2005. They have described that apart from polyphenolic compounds, Maillard reaction products formed during roasting are also responsible for scavenging activity of the coffee extracts.

Results of antioxidant potential of acetone and methanol extracts of LCB and spent coffee residue by β-carotene-linoleate and DPPH model systems were promising. Since the methanolic extract of LCB showed highest activity compared to other solvent extracts, further, the yield of methanol extract was higher than the acetone extract, these extract were further evaluated for the other bioactivities.

Accurate measurement of antioxidant capacity requires both inhibition degree and inhibition time since the reaction among free radical, substrate and antioxidant is very complicated which makes it impossible to use a fixed equation to express the kinetic order. **Oxygen radical absorbance capacity** (ORAC) is the only method so far that combines both inhibition time and degree of inhibition into a single quantity. The ORAC assay

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measures the ability of antioxidant compounds to inhibit the loss of fluorescein induced by the peroxyl radical generated from AAPH.

ORAC in terms of Trolox equivalent was given in Table 3.9. Green coffee extract showed higher activity than spent coffee extracts. Over time ROS, generated from the thermal decomposition of AAPH, will quench the signal from the fluorescent probe of fluorescein isothiocyanate sodium salt. The subsequent addition of an antioxidant produces a more stable fluorescence signal, with signal stability depending on the antioxidant's capacity are depicted in the following figure. The data points are summarized over the time by the evaluation software. This is then compared to the standard, Trolox®, and the ORAC values expressed as micromoles of Trolox® equivalents (TE) per gram or per milliliter of sample (µmole of TE/g or µmole of TE/mL).

Table 3.9. ORAC values of extracts from LCB and spent coffee

Sample	**ORAC value (µM TE/g)**
Low grade coffee	4416±215[a]
Spent coffee (Ar)	1821±345[c]
Spent coffee (Rb)	2594±71[b]

Values are mean± SD of triplicate analysis. Values not having similar superscripts in the same column are significantly ($p<0.05$) different.

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It is reported (Wen *et al.,* 2004) that the green coffee extract obtained from *Coffea robusta* showed the value of 40 as µmol Trolox eq/100 g whereas the green coffee extract obtained using LCB in this study showed 100 times more activity (4416 µmol Trolox eq / g). In the present study soxhlet extraction was followed using methanol as an extraction solvent after defatting with hexane, whereas, Wen *et al.,* (2004) refluxed the green coffee powder with hot water and the resultant extract was lyophilized before evaluating for the antioxidant property. Leslie (2007) reported, the whole coffee berry extract exhibited an antioxidant capacity 10 times more than that of green tea by ORAC assay method and further suggested that the antioxidant activity is due to the presence of polyphenols especially chlorogenic acid, proanthocyanidins, quinic and ferulic acids.

There is a significant ($p<0.05$) difference in ORAC value between the extracts of spent robusta and spent arabica. Extract of spent robusta showed more ($p<0.05$) trolox value than spent arabica extract. Earlier report (Castillo *et al.,* 2005) reveals that the brews obtained from C*offea robusta* showed more ORAC value than *Coffea arabica.*

Methanol extract of LCB was evaluated for its reducing power (Iron (III) –Iron (II) reducing system). In this assay, depending on the **reducing power** of antioxidant samples the yellow color of test solution changes into various shades of green and blue colors. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant

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activity. The reduction of the ferricyanide (Fe^{3+}) complex to the ferrous (Fe^{2+}) form can be monitored by measuring the formation of Perl's Prussian blue at 700 nm which occurs in the presence of reductants such as antioxidant substances in the antioxidant samples (Chung *et al.,* 2002). Reducing power of the methanol extract and standards (BHA, ascorbic acid and chlorogenic acid) using the potassium ferricyanide reduction method were depicted in Fig. 3.3. For the measurements of the reductive ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of extract using the method of Oyaizu (1986). The reducing power of methanol extract and standards were increased with increase of sample concentrations. In general, the reducing power observed in the present study was in the following order ascorbic acid > chlorogenic acid >BHA > methanol extract. The data presented here indicate that the marked reducing power of methanol extract seem to be the result of their antioxidant activity. The reducing properties are generally associated with the presence of reductones (Pin-Der-Duh, 1998). It is presumed that the phenolic compounds may act in a similar fashion as reductones by donating electrons and reacting with free radicals to convert them to more stable products and terminating the free radical chain reaction.

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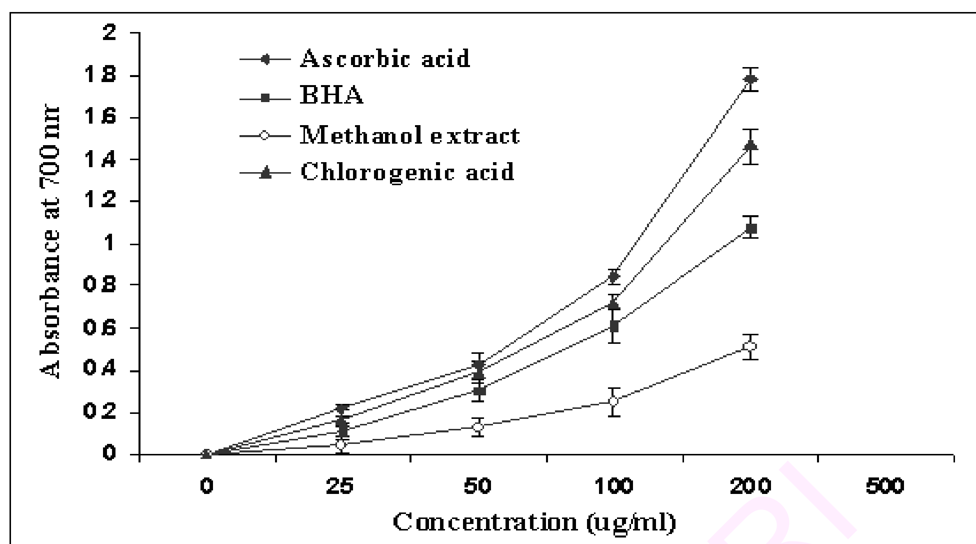

Fig. 3.3. Reducing ability of LCB extract

The **antioxidant capacity** of the extracts was measured spectrophotometrically using phosphomolybdenum method, which is based on the oxidation of Mo (IV) to Mo (V) by the sample analyte and the subsequent formation of green phosphate of Mo (V) compounds with a maximum absorption at 695 nm. The assay was successfully used to quantify vitamin E in soyabean seeds (Prieto *et al.,* 1999) and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant polyphenols. The antioxidant capacity of the methanol extract and propyl gallate showed 1367 ± 54.17 and 5098 ± 34.08 µMole/g as equivalents to ascorbic acid respectively, whereas chlorogenic acid showed 3587.9 ± 43.87 µMole/g (Fig. 3.4).

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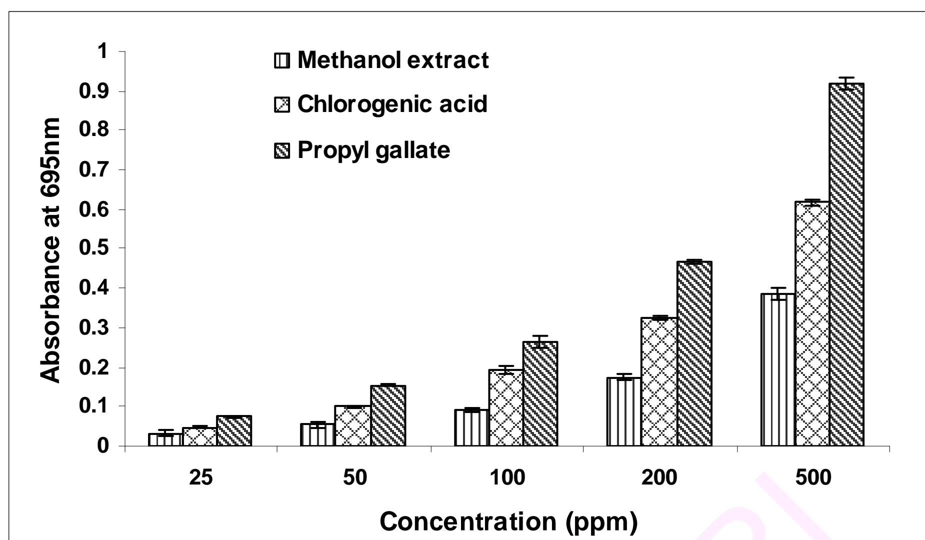

Fig. 3.4. Antioxidant capacity of LCB extract

It was found that all the coffee extracts showed **antitumour activity** of extract by the inhibition of mouse leukemia P388 cells. Several epidemiological studies have investigated to clarify whether coffee consumption induce or promote cancer, still the question remains unclear. Many studies have revealed the protective association between coffee consumption and the risk of certain cancers (Nishi *et al.,* 1996; Schilter *et al.,* 2001). Cell viability was reduced to 50.1±3.6% by LCB extract whereas the extracts obtained from spent arabica plantation and robusta cherry exhibited significantly ($p<0.05$) lower values of 20.6 ± 5.3 and 19.2 ± 4.4% respectively (Fig. 3.5) compared to vehicle (cell line and DMSO) where the cell viability was considered as 100%.

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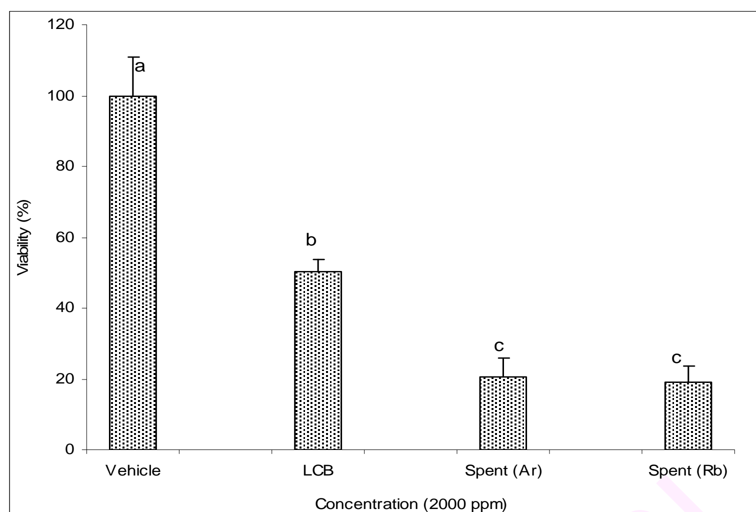

Fig. 3.5. Anti-tumour activity of coffee extracts (P388 proliferation cells)

Values are mean ±SD of triplicate analysis, Values not having similar superscripts are significantly ($p<0.05$) different

There is not much difference between both the varieties of spent coffee extracts on inhibition of cell viability. However, both the spent coffee extracts showed more anti-tumour activity than green coffee extracts though the amount of polyphenols and chlorogenic acids are present more in green coffee extract. This may be due to the possible role of brown pigments (melanoidins and phenolic polymers), formed during the roasting process, which protects the cells from oxidative damage in the biological systems (Wen *et al.,* 2004). Many animal studies also support the anti-carcinogenic property of coffee, which is mainly due to the presence of two diterpenes namely cafestol and kaweol (Cavin *et al.,* 2002).

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There are not many reports on the **anti-inflammatory activity** of coffee. However there are reports relating polyphenols and ant-inflammatory response (Tracy *et al.,* 2003). Researchers are attempting to screen agri / horticultural produce that contain polyphenols, which shows potent anti-inflammatory activity. Inflammation involves a complex web of intra and intercellular cytokine signals. TNF-α, an inflammatory mediator is one of the most important pro-inflammatory cytokines produced by the activated monocytes and macrophages etc., TNF-α is secreted during the early phase of acute chronic diseases such as asthma, rheumatoid, arthritis, septic shock and allergic diseases.

Though all the coffee extracts have substantial quantity of polyphenols, none of the extracts showed anti-inflammatory activity. One of the reports revealed the suppressive effect of red ginsenosides, which is one of the medicinal plant used in Korea and Japan. It is reported that water extract of red ginsenosides possessed anti inflammatory activity and melanoidins present may be responsible for the activity. However, in the present study, extracts obtained from spent coffee residues, though it contains melanoidins apart from polyphenols, they did not show anti-inflammatory activity against the LPS production from J774A.1 mouse monocytes cell line (Fig. 3.6).

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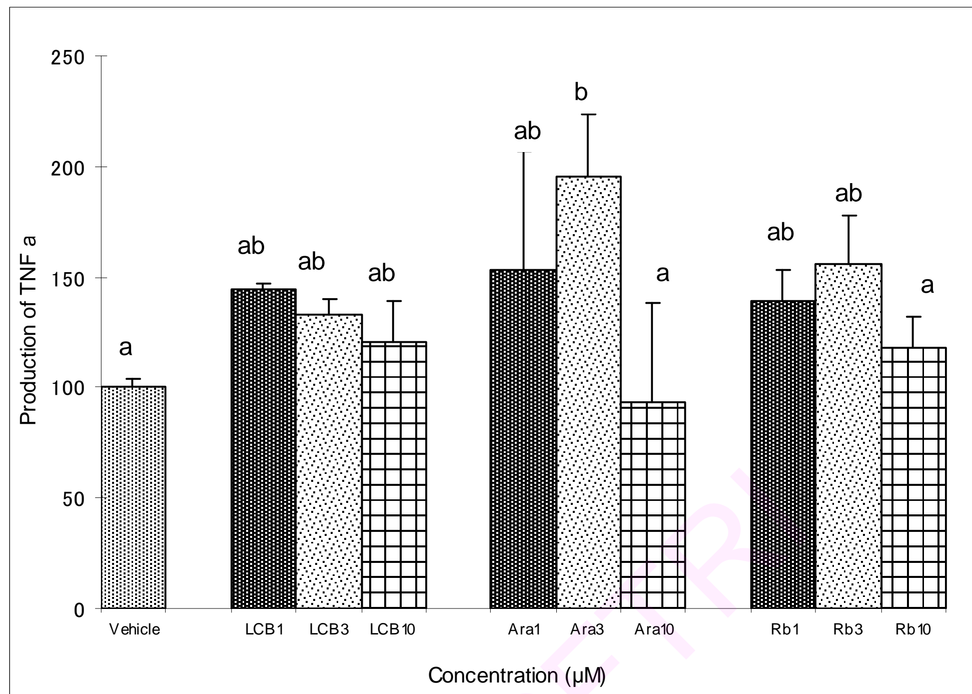

Fig. 3.6. Anti-inflammatory activity of coffee extracts TNF α production from J774A.1 cells

Values are mean ±SD of triplicate analysis, Values not having similar superscripts are significantly ($p<0.05$) different

Food allergy generally showed an immediate hypertensive (Type I) allergic symptoms. Mast cells or basophils play a central role in immediate allergic reactions mediated by immunoglobulin (IgE). Binding multivalent allergens with allergenspecific IgE captured by IgE receptors on the surface of mast cell or basophils leads to the release of inflammatory mediators such as histamine, arachidonic acid metabolites and cytokines. In this study, rat basophilic leukemia (RBL-H3) cell line was selected to estimate the degranulation inhibitors. Earlier reports say that chlorogenic acids are the important allergic constituents of green

coffee bean (Freedman *et al.,* 1964). Recent study (Yamashita *et al.,* 2000) reported that polyphenolic compounds from tea shown to inhibit histamine release from mast cells thereby showing anti-allergic activity. Extracts obtained from spent coffee of both the varieties could inhibit the antigen-specific degranulation as shown in Fig. 3.7. Also increase in concentration of the spent extracts, increases the inhibitory effects significantly ($p<0.05$). There is not much difference in the anti-allergic activity between the varieties of spent coffee. At the concentration of 10000 ppm, extracts of both the varieties spent coffee could inhibit the degranulation to less than 10% compared to the negative control which could inhibit around 56%. However, extract of LCB could not inhibit degranulation even at the higher concentration of 10000 ppm level. When the pure compounds namely chlorogenic acid and caffeine were tested, cholorogenic acids (chl) inhibit degranulation better than caffeine (ca) (Fig. 3.8). At the concentration of 10000 ppm, the inhibition on the effect of degranulation is significantly more ($p<0.05$) for the extracts of spent coffee of both the varieties than the extract of LCB though the amount of chlorogenic acid of spent coffee extracts is significantly ($p<0.05$) less than LCB extract. This result shows that the anti-allergic activity in terms of inhibition of antigen-specific degranluation of mast cell is not only due to chlorogenic acid content, which is the major polyphenol present in coffee. The present result is in good agreement with an earlier report (Layton *et al.,* 1966), where it was reported that

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coffee-specific allergenicity is not entirely due to the chlorogenic acids but due to the protein present as a contaminant in the coffee extracts.

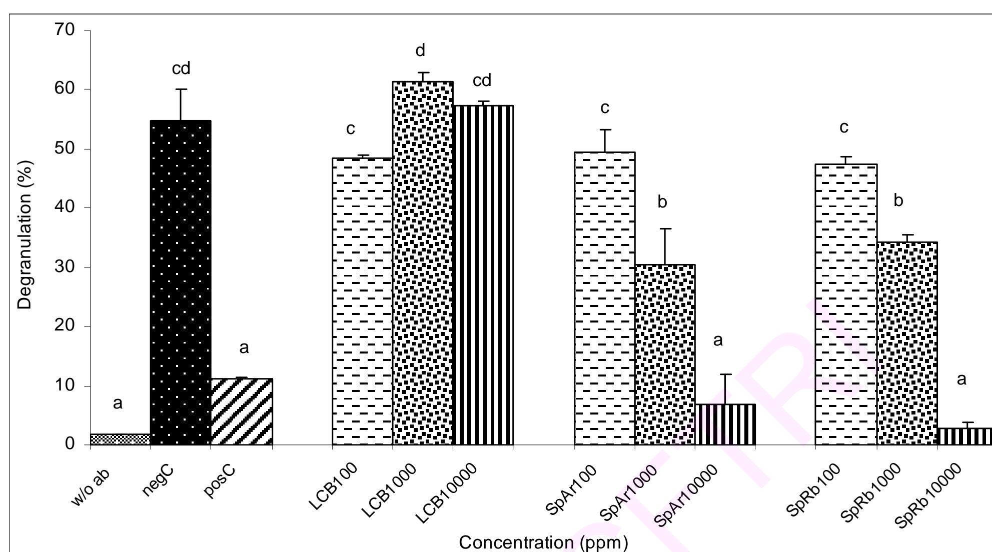

Fig. 3.7. Anti-allergic activity of coffee extracts
Values are mean ±SD of triplicate analysis, Values not having similar superscripts are significantly ($p<0.05$) different

The chemical composition **of extracts of LCB and spent coffee residues are given in the Table 3.10.** As expected the extracts of LCB possess higher phenolics and chlorogenic acids (21.9 and 34.2%) than the spent coffee extracts of both arabica and robusta. The lesser content of these components in spent coffee residues could be (i) the degradation / conversion of these compounds during roasting and (ii) release into the water extract during the preparation of instant coffee.

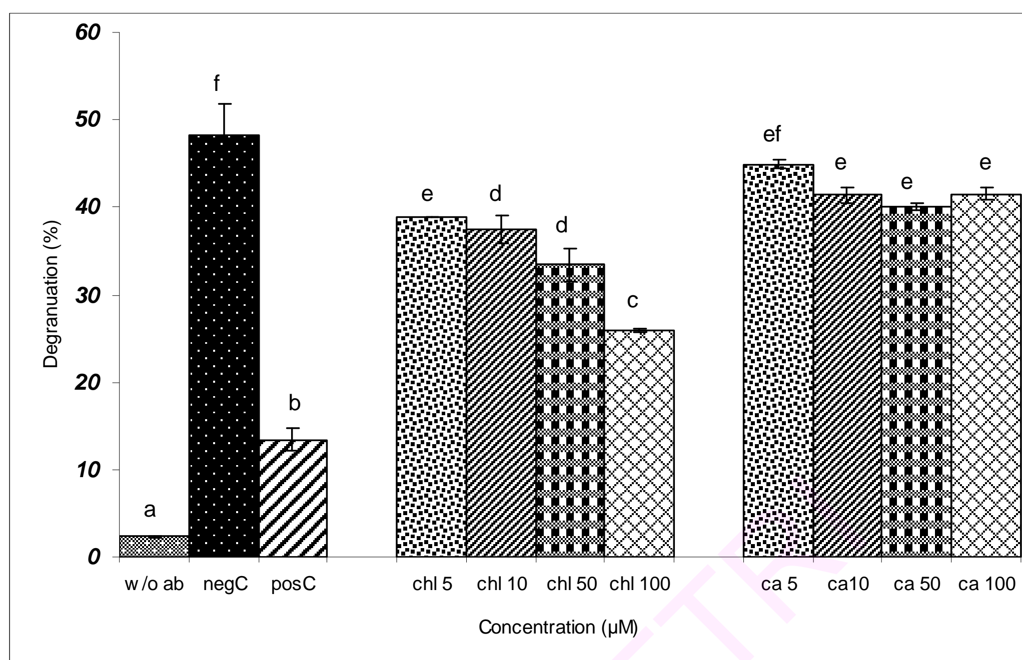

Fig.3.8. Anti-allergic activity of chlorogenic acid & caffeine

Table 3.10. Chemical components of coffee extracts

Sample	Caffeine	Chlorogenic acids	Total Phenolics*
LCB	8.3± 0.4[a]	34.2±0.3[a]	21.9± 0.5[a]
Spent coffee (Ar)	2.5± 0.2[b]	5.62± 0.0[b]	6.32± 0.0[b]
Spent coffee (Rb)	1.0± 0.3[c]	4.87± 0.0[c]	4.81± 0.0[c]

Values are mean ±SD of triplicate analysis, Values not having similar superscripts are significantly ($p<0.05$) different

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3.4. Bioactivity studies (i*n-vivo*)

3.4.1. Oxidative stress

Recently, there is a strong scientific opinion that more than 90 percent of diseases are caused by oxidative stress due to active oxygen, other free radicals and lipid peroxides. Active oxygen, though vital to our body, causes considerable damage to the essential components of human body and disturbs the physiologically important functions of proteins, lipids, enzymes and DNA bearing the genetic code. This leads to disorder in our body, which in turn leads to development of diseases and accelerating aging. It has been demonstrated that oxidative stress causes cancers and many diseases such as arterial sclerosis, high blood pressure, myocardial infarction, cerebral apoplexy, dementia, diabetes, cataract etc.

It has been calculated that about 2 percent of oxygen inhaled by the respiratory system turn to active oxygen in human body and some time reaches at 10 percent. Active oxygen is produced when our body generates energy and also when microphage cells confronts foreign, invading substances which may cause harm to the body and dispose them appropriately in our body system. Thus, active oxygen is essential and useful to our body. However, danger occurs when excess active oxygen is dispersed and which becomes detrimental to organs, body tissues and blood corpuscles.

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Human body is endowed with a counteracting defense system, to suppress excessive active oxygen so that body tissues and metabolic processes are prevented from disruption. This defense system is composed of components such as antioxidant enzymes, trace elements which aid the enzyme activity, vitamins and antioxidants referred to as super vitamins. As long as there is a balance between the oxidative stress and antioxidant system, human body is maintained in an optimal health state. However, once the level of active oxygen released exceeds the protective capacity provided by the antioxidant system, human body suffers from the ill effects of oxidative stress. This leads to illness, death of cells and unstoppable excessive cell division resulting in cancer, and as a consequence acceleration of the aging process. Therefore, the maintenance of the delicate balance between oxidative stress and antioxidant system in proper order becomes very important.

Oxygen free radicals can cause varieties of damage to DNA, including single and double strand breaks, base modifications and abasic sites and they are thought to be involved in the mechanisms of aging and in carcinogenesis and other disorders. Recent evidences (Hwang & Bowen, 2007) have shown that stress can alter hormonal levels, lymphocyte subsets and the production of reactive oxygen species. Oxidative stress is induced by overproduction of reactive oxygen species (ROS) which oxidize lipids, proteins and DNA leads to cell membrane destruction, heart disease,

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diabetics and early ageing. Generally antioxidant compounds are seems to acts against the oxidative stress (Imade *et al.,* 2002). Green coffee extract from low grade green coffee beans showed antioxidant in terms of RSA and ORAC and anti tumour activity. Therefore, green coffee extract from LCB was chosen to determine the oxidative stress in BALB/c mice.

3.4.2. Experimental Methodologies

BALB/c male mice of 6 weeks old were purchased from Charles River Japan Inc, (Yokohama, Japan) and were used for the experiments. All animal experiments were performed in accordance with the guidelines of the National Food Research Institute for Animal Experiments and the Law (no. 105) and Notification (no. 6) of the Japanese Government.

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Two sets of experiments were carried out to determine the effect of green coffee extract on the oxidative stress of mice.

Experiment 1

Mice were separated in two groups (n=6), caged and kept in animal facility of NFRI, Japan. Mice received fresh diet and free access to water in alternate days. Normal Mice Feed (NMF, Oriental Yeast Co Ltd., Tokyo, Japan) and NMF mixed with 1% (w/w) coffee extract diet were fed to the mice upto 14 days of experimental period. Weight of mice and feed were weighed periodically at an interval of 2 days. Blood samples were collected from all the mice at 0, 7th days from tail vein. At the end of 14 days all the

mice were sacrificed, blood and urine were collected. Blood samples were centrifuged at 15000 rpm for 10 min. at 4°C and serum separated. All the samples were kept at -80° till the analyses.

Experiment 2

Mice (n=6) were separated in four groups caged and kept in animal facility of NFRI, Japan. Mice received fresh diet and free access to water in alternate days. NMF was mixed with the coffee extract at 0.1, 0.3 and 1% (w/w) level and fed for the experimental mice along with the control mice for 14 days. Weight of mice and feed were weighed periodically at an interval of 2 days. Blood samples were collected from all the mice at 0, 7th days from tail vein (Tom *et al.,* 2006). At the end of 14 days all the mice were sacrificed, blood and urine were collected. Blood samples were centrifuged at 15000 rpm for 10 min. at 4°C and serum separated. All the samples were kept at -80° till the analyses.

Parameters analyzed

All the blood samples were analysed for total cholesterol, HDL cholesterol using colorimetric assay viz Wako 439-17501 and Wako 431-52501, respectively. Also the serum samples were analysed for Reactive Oxygen Metabolites (ROM's) using Free Radical Analytical System (FRAS). Urine samples were quantitatively analysed for oxidative DNA damage in terms of the adduct 8-hydroxydeoxyguanosine (8-OHdG) as a bio marker by enzyme-linked immunosorbent assay.

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3.4.2.1. 8-hydroxy-2'-deoxyguanosine

The quantitative of determination of oxidative DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) in mice urine samples was determined using 8-OHdG ELISA Kit., (Code: KOG.HSIOE), JaICA, Shizuoka, Japan. This method is high sensitive competitive in vitro enzyme-linked immunosorbent assay (ELISA).

Procedure

Estimation of 8-OHdG in the mice urine samples was determined (Fig. 3.9) according to the method described by Zheng *et al.,* (2007). All the reagents and samples to be examined were brought to room temperature (20-25°C). Primary antibody was reconstituted with the primary antibody solution. Urine samples were diluted with phosphate buffer (10 times). 50 µL of sample or standard 8 OHdG (0-10 ng/ml) was added into each wells of 8-OHdG precoated plate. Reconstituted anti-8-OHdG monoclonal antibody by 1% BSA and 0.05% Tween-20 in Phosphate Bufferd Saline (PBS) (50 µL) was added to wells. The plate was shaken from side to side and mixed fully. The plate was covered with adhesive strip and incubated at 4°C for overnight. After incubation, plate was washed with washing solution (0.05% Tween-20 in PBS, 250 µL) for three times. The plate was inverted and blotted against clean paper. Secondary antibody (HRP-conjugated antibody) was reconstituted with PBS. Constituted Secondary antibody (100 µL) was added to each well and shaken well. The plate was covered with an adhesive

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strip and incubated at room temperature for 1 hour. After incubation, the plate was washed three times with washing solution. Chromatic solution (3, 3’, 5, 5’-tetramethylbenzidine (TMB) as enzyme substrate) was diluted with diluting solution (H_2O_2 in citrate-phosphate buffer, 100 times). Reconstituted enzyme substrate (100 µL) was added per well and incubated at room temperature for 15 min. in the dark. Stop solution (1M phospholic acid, 100 µL) was added to each well and shaken the plate from side to side and mixed fully.

The absorbance was measured at 450 nm using the micro plate reader (Model 550; Bio Rad, CA, USA). Calibration curve was generated and the concentration of 8-OHdG of the samples was calculated.

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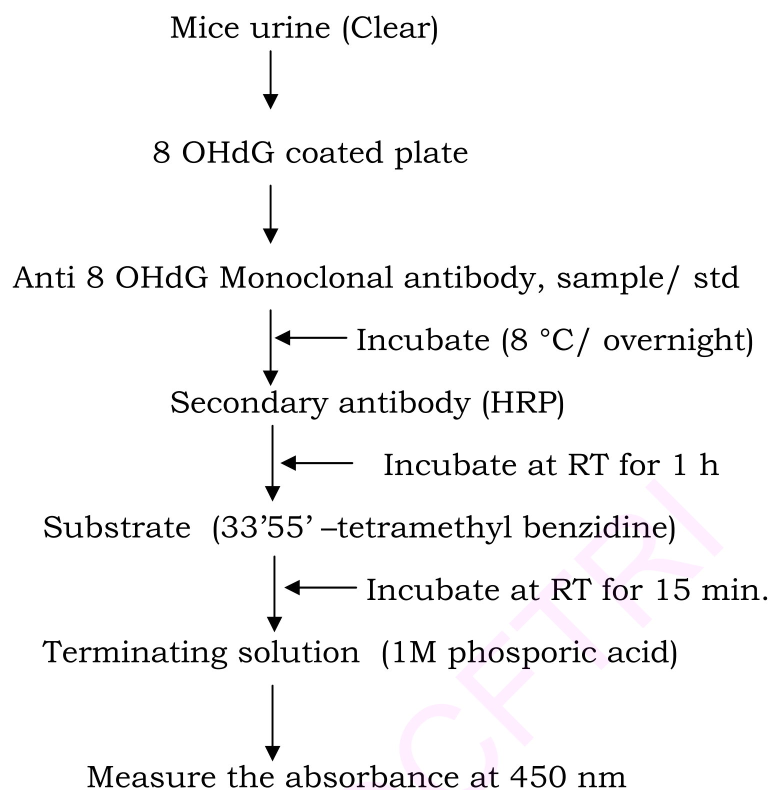

Fig. 3.9. Determination of 8OHdG in mice urine

3.4.2.2. Reactive Oxygen Metabolites (ROM)

The direct measurement reactive species is difficult due to its instability. However reactive oxygen metabolites can be measured by Free Radical Analytical System (Fig. 3.10). The amount of hydro peroxides in serum samples, are proportional to the amount of free radicals. When the serum sample is dissolved in acidic buffer, the hydro peroxides oxidize the an adduct (N,N, diethyl, para- phenylendiamine) to the corresponding cation which absorbance can be measured.

For ROM's value of serum, 20µL of serum was mixed with buffered

solution (1ml, pH 4.8) and chromogenic substrate (10 µL) and mixed. The solution was centrifuged and incubated for 5 min. at 37°C and the absorbance was measured at 505 nm. (Cornelli *et al.,* 2001)

Fig. 3.10. Free Radical Analytical System

3.4.2.3. Total and HDL cholesterol

For total cholesterol, serum (5 µL) was mixed with 750 µL of reaction buffer and incubated at 37°C for 5 min. The absorbance was measured at 595 nm. For HDL cholesterol, serum (100 µL) was mixed with equal amount of precipitation reagent and allowed to stand at room temperature for 10 min. The solution was centrifuged at 3000 rpm for 15 min. at 27 °C. The

supernatant (5 μL) was mixed with reaction buffer (300 μL) and the mixture was incubated for 5 min. at 37 °C and the absorbance measured at 595 nm.

3.4.2.4. Oxygen radical Absorbance Capacity

The serum samples were analysed for antioxidant activity in terms of ORAC according to the method described by Parker *et al.,* (2007) and Prior *et al.,* (2003). Blood sample was centrifuged at 15000 rpm for 10 min. at 4°C. Serum separated the top, transfer in to a vial and store at - 40°C. Serum was combined with equal quantity of perchloric acid, mixed well. The solution was centrifuged at 10000g x10 min. The supernatant solution was anlaysed for ORAC values in terms of Trolox equivalent.

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3.4.3. Results and Discussion

Recently consumers are interested in seeking natural antioxidant compounds in the diet which may help to reduce the oxidative stress. Coffee is one of the most widely consumed beverages throughout the world. Caffeine, one of the main constituent of coffee has a variety of biological and cellular response in the biological system. However, investigations on the biological effects of chlorogenic acids (present in large quantity in coffee) on antioxidation, anti-mutation, anti-inflammatory etc are focused recently.

Oxidative free radicals are by-products of the normal reaction within our body. These reactions include the generation of calories and degradation

of lipids under stress and inflammatory processes. If the balance between oxidative free radical production and eradication maintained, the harmful effects of free radicals would be minimized in our body. If the unwanted free radicals are not eradicated efficiently, oxidative stress occur. Oxidative stress caused by reactive oxygen or free radicals has been shown to associate with the progression of many diseases including cancer, heart diseases, inflammation, depression etc. Oxidative stress plays an important role in the production of reactive oxygen species (ROS). ROS injure protein, lipid and induce cell membrane destruction. But the measurement of ROS is rather difficult in the routine laboratory owing to their biochemical characteristics. However recently a method has been developed for measuring reactive oxygen metabolites (ROM's) using FRAS in terms of hydroperoxide.

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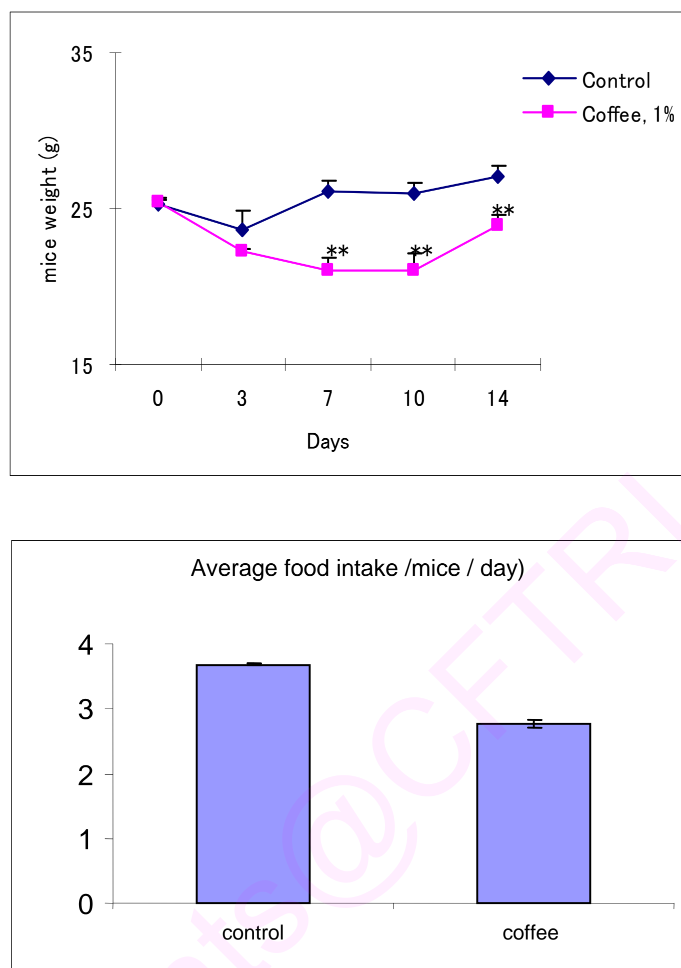
Results of the first experiment suggest that, there is a significant ($p<0.01$) reduction in the ***weight between control and coffee fed mice.***

Though there is a slight reduction of food intake by coffee fed mice in the initial period, (Figs. 3.11 & 3.12) after 10 days of experiment, it is almost equivalent to the control mice. This is due to the astringency and/or bitterness of coffee extract and the mice needs time to adjust with the taste of the feed.



Figs. 3.11 and 3.12. Change in mice weight and food intake (g) during the experimental (1) period

** significantly different ($p<0.01$)

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Results of the **second experiment** with the different level of coffee extract reveal that, there is an appreciable amount of reduction in the weight of mice as well as food intake of mice between control and coffee fed mice in all the levels (Figs 3.13 & 3.14).

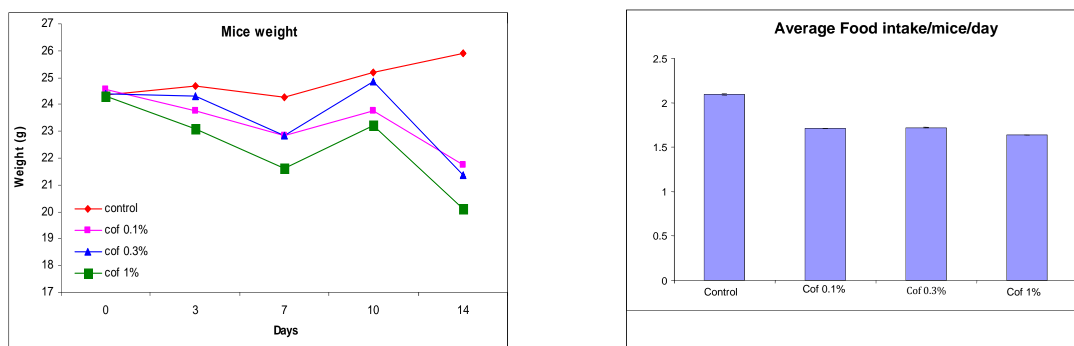

Fig. 3.13 and 3.14. Change in mice weight and food intake (g) during the experimental (2) period

With reference to **Reactive Oxygen metabolites**, there is no significant ($p<0.05$) difference in ROM value (Fig 3.15) and the it is in the safe side of oxidative stress (<300 carr value). Komatsu *et al.,* (2006) experimented the status of Mongolians and Japanese in terms of oxidative stress and the dietary habit to determine the reason for shorter life span of Mongolians which is 16 years shorter than Japanese. They concluded that ROM values Mongolians are 429 Carr U whereas Japanese were found to have 335 Carr U. This shows that oxidative stress is directly proportional to ROM value.

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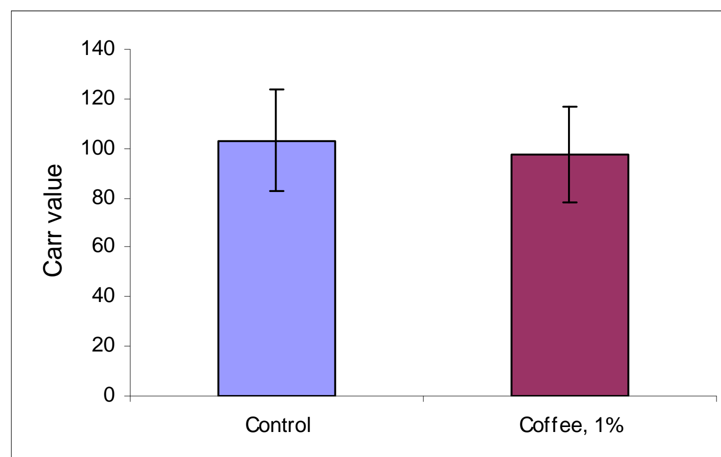

Fig. 3.15. Reactive oxygen metabolites of mice serum (Expt 1)

Many research findings concluded that **8 OHdG**, a DNA base-modified product is one of the good markers to determine the oxidative stress. Deoxyguanosine (dG) is one of the constituents of DNA and when it is oxidized, it is altered into 8-hydroxy-2'-deoxyguanosine (8-OHdG; XIX). First experiment result showed that there is a significant difference ($p<0.05$) in 8 OHdG concentration of the urine between control and coffee fed mice after 14 days of experiment shows the antioxidant effect of coffee extract. There is less amount of 8 OHdG found in coffee fed mice compared to control mice (Fig. 3.16).

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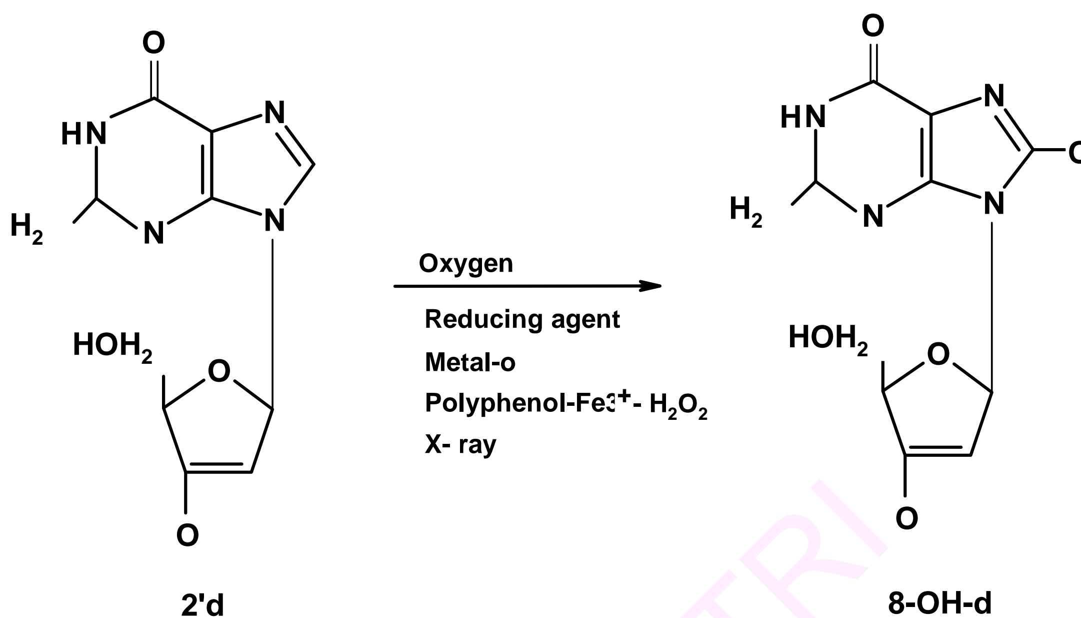
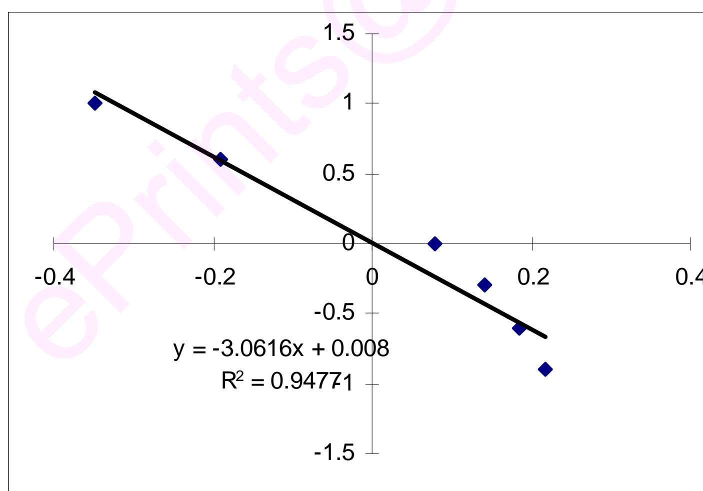


XIX. 8-OHdG formation by oxygen radicals



Standard graph of 8 OHdG (Absorbance Vs log 8-OHdG(ng/ml)

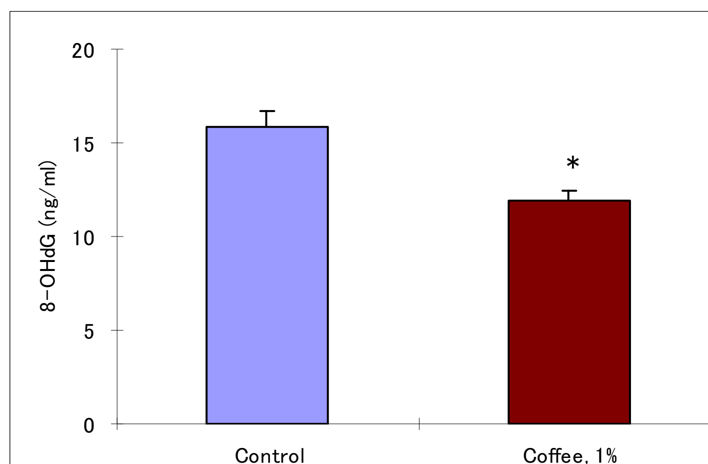


Fig. 3.16. Oxidative stress in terms of 8-OHdG bio marker of mice urine (Expt 1)

* significantly different (p<0.05)

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Second experiment results showed that the level 8-OHdG of coffee fed mice at the level of 1% was slightly low, but not significant (p< 0.05) with the control mice. However there was a significant (p<0.05) increase in the level of 8-OHdG concentration between control and coffee fed mice at the level of 0.1% (Fig. 3.17.). Earlier report (Sakamoto *et al.,* 2005) reveals that there is an increase in 8-OHdG concentration in Wistar rat fed with coffee powder at the level of 1.36%. In another report, (Bouayed *et al.,* 2008) found that anxiety related behaviors were found to decrease in mouse models with the extract of *Prunus dimestica* and further they concluded that chlorogenic acid, one of the poly phenol, is responsible for reducing oxidative stress.

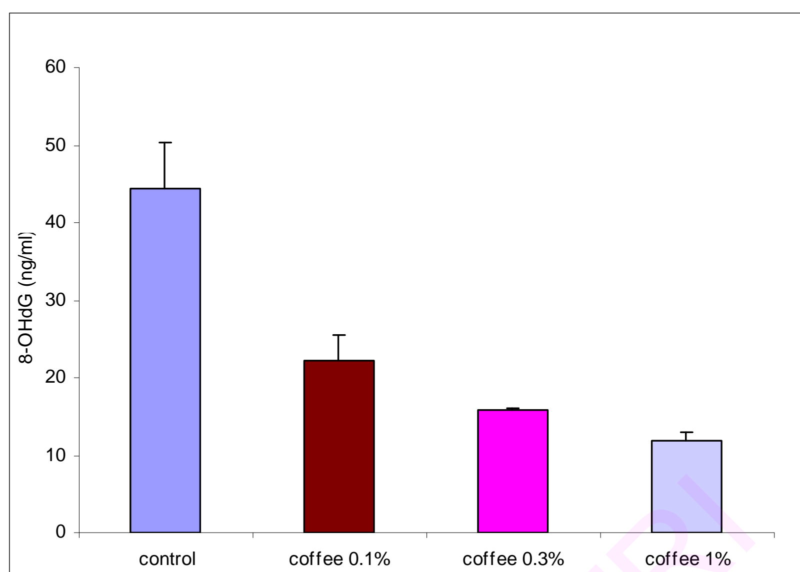

Fig. 3.17. Oxidative stress in terms of 8-HdG bio marker of mice urine (Expt 2)

* significantly different ($p<0.05$)

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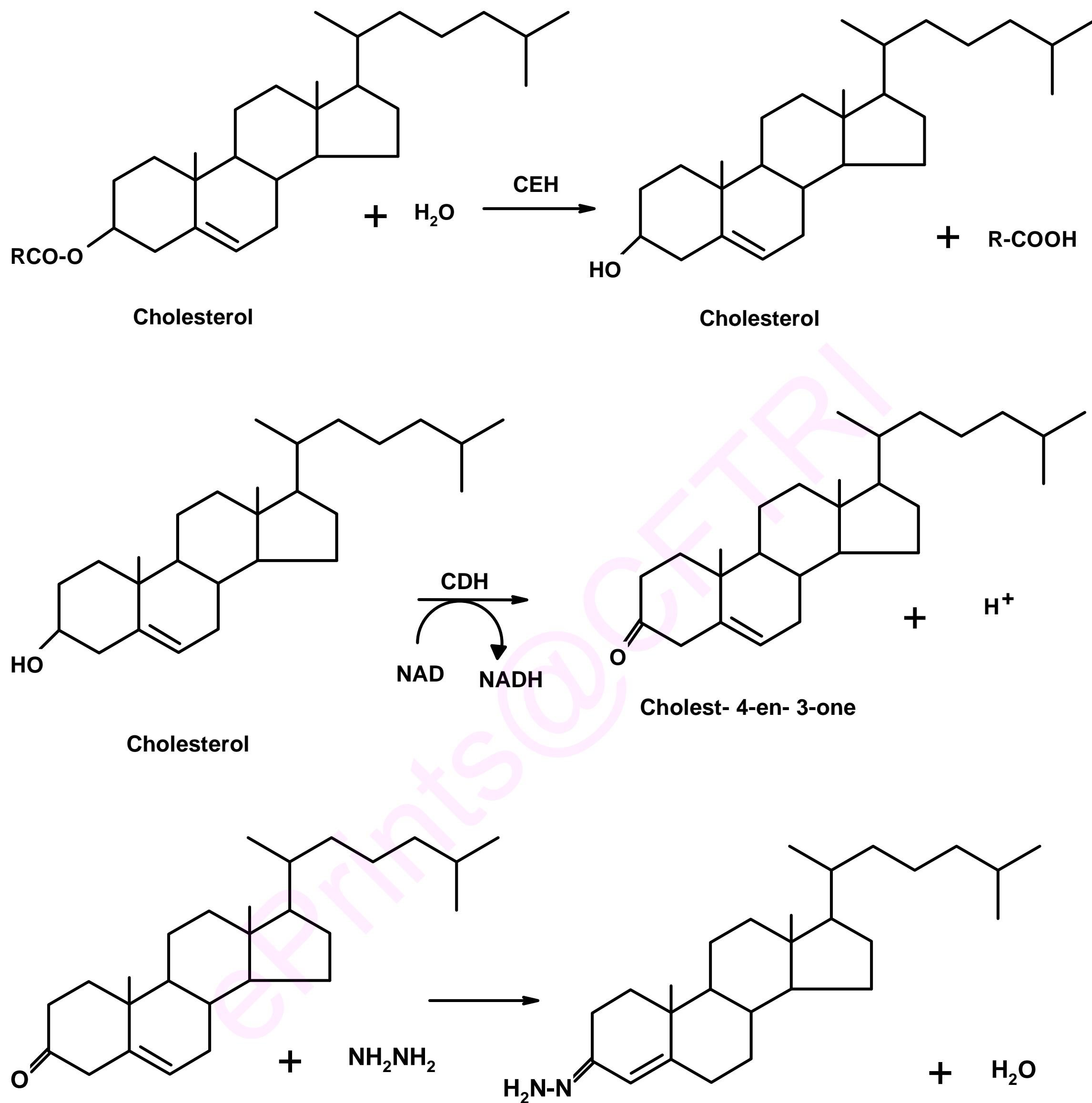
Total cholesterol (TC) and HDL cholesterol (HDLC) of serum samples of control and coffee fed mice were estimated by the enzymatic reaction (XX).

Low density lipoproteins (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of acid phosphotungstic in the presence of magnesium ions. After centrifugation the cholesterol concentration in the HDL (high density lipoprotein) fraction, which remains in the supernatant is determined and expressed as HDLC.



XX. Enzymatic action on cholesterol

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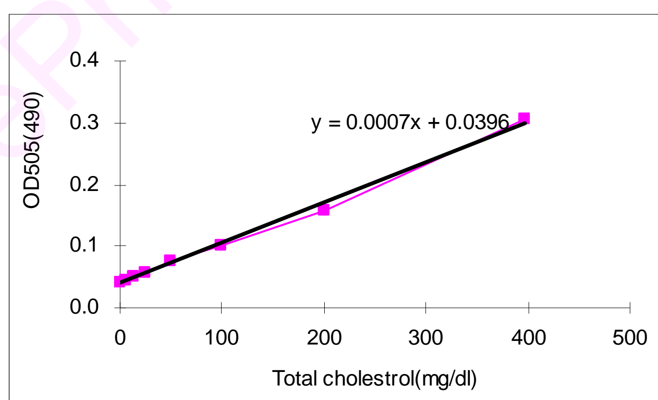
At Oth day there is no difference between TC and HDLC of control and coffee fed mice. After the 14 days of experimental period, there is significant difference ($p<0.05$) in serum total cholesterol level in coffee fed diet mice than the control mice. There is an increase in the cholesterol level in coffee fed diet. Cholesterol level is increased with the concentration of coffee extract in the feed dose dependently. The concentration of the HDL cholesterol also increase in the serum coffee fed diet mice compared to control and there is no significant difference ($p<0.05$) between the concentration of the coffee extract in the diet (Figs. 3.18 to 3.21). Effect of coffee consumption on oxidative susceptibility of lipoproteins was investigated by Yukawa *et al.,* (2004). Decrease in total cholesterol and LDL cholesterol was found in Japanese with coffee diet compared to control group. Also they concluded that coffee ingestion favorably reduce the risk of cardiovascular risk status by reducing and Total and LDL cholesterol.



Standard graph of Total cholesterol

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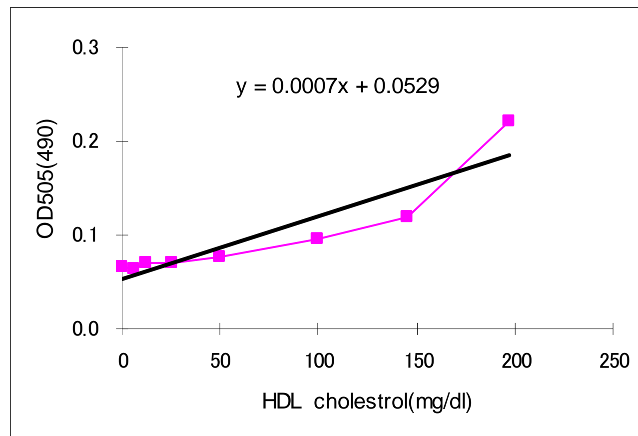

Standard graph of HDL cholesterol

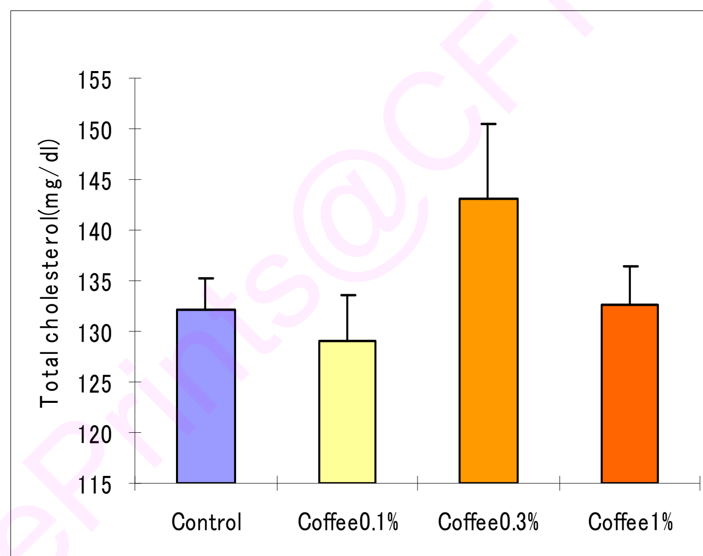

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Fig. 3.18. Total cholesterol levels of mice – 0 day

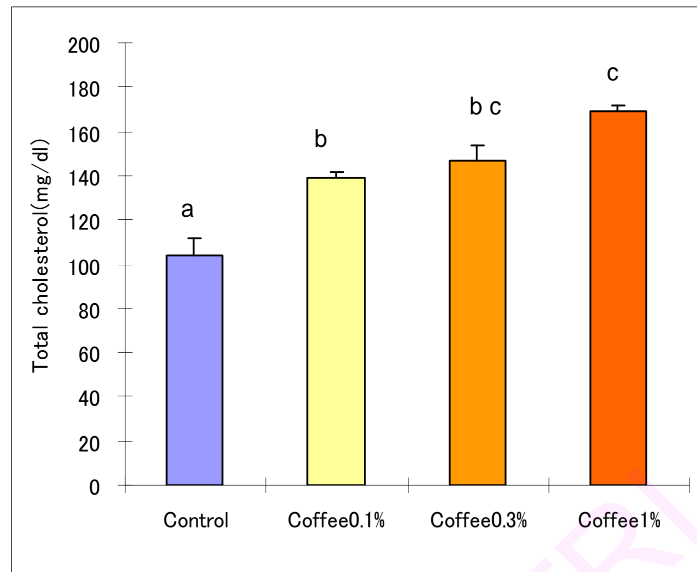

Fig. 3.19. Total cholesterol levels of mice – 14 day

Values not having similar superscripts are significantly ($p<0.05$) different

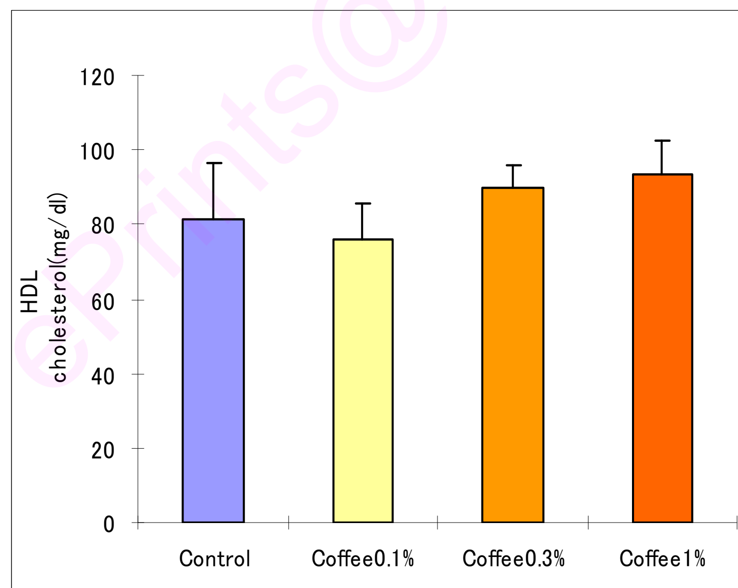

Fig. 3.20. HDL cholesterol levels of mice – 0 day

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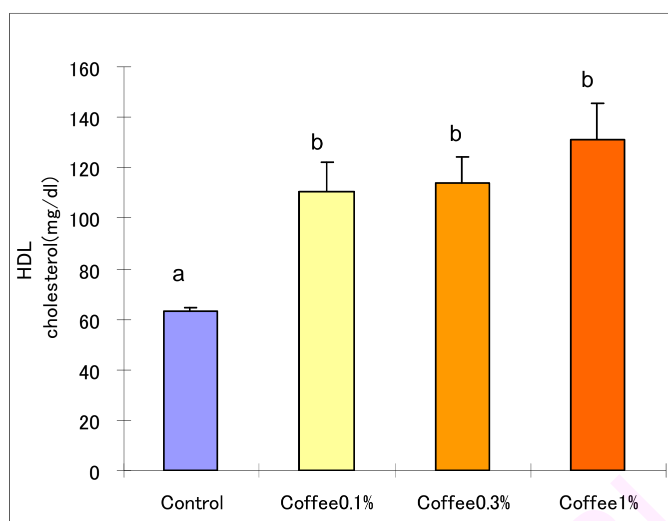

Fig. 3.21. HDL cholesterol levels of mice – 14 day

Values not having similar superscripts are significantly ($p<0.05$) different

The **antioxidant capacity of serum in term of ORAC** values of the coffee fed and control mice are given in Fig. 3.22.

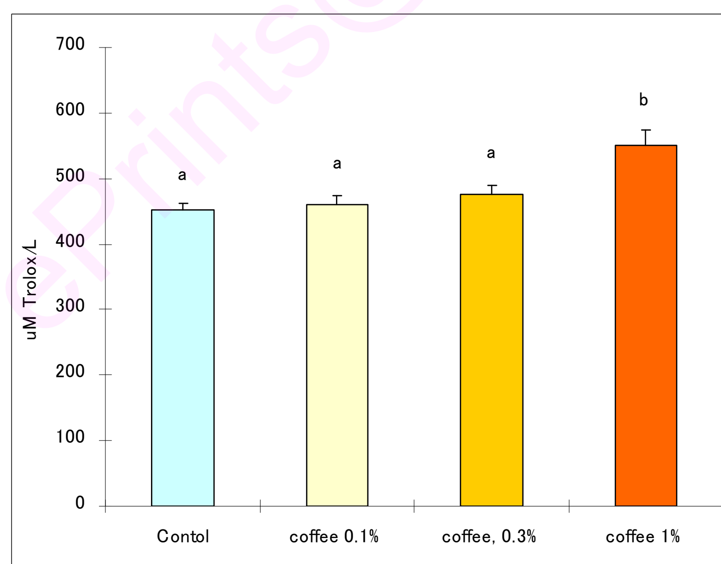

Fig. 3.22. Serum ORAC (μM TE/L) levels of mice – 14 day

Values not having similar superscripts are significantly ($p<0.05$) different

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There is a significant increase in ORAC value in coffee fed mice compared to control mice. This result shows that coffee at the level of 1% has significant effect on increasing the antioxidant capacity of serum. This result expected since green coffee extract showed the higher ORAC value of 4416 ± 215 µM Te/g. Cao *et al.,* (1998) reported that the ORAC values of human plasma increased significantly with the experimental group of subjects with diet supplemented with fruits and vegetables containing antioxidants compared to the control group.

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CHAPTER 4

CHAPTER 4

Characterisation of bioactive compounds and value addition

Part I

Characterisation of isomers of chlorogenic acid from low grade coffee beans and their enrichment in conserves

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CHAPTER 4

Part I

4.1. Introduction

Generally, polyphenolic compounds are found in plants which are reported to have multiple biological effects including antioxidant activity. In recent years, numerous reports have been published on the isolation and identification of novel antioxidants from plants, animals and microbial sources for use in processed food products. Extracts rich in phenolics from plants are of increasing interest in the food industry because these possess many biological properties and can improve the quality and nutraceutical value of food system.

Phenolic compounds are ubiquitous constituents of commonly consumed plant foods such as fruits, vegetables, cereals, legumes, wine, tea and coffee (Cheynier, 2005). These compounds are secondary metabolites of plants and can be grouped into different classes according to their basic chemical structure and into sub classes according to the functional groups in the basic structure (Manach *et al.,* 2004). Most of these compounds have received considerable attention as potentially protective factors against human chronic degenerative diseases viz., cancer and cardiovasucular diseases etc (Rosenberg, 1990; Giovannucci, 1998; Inoue *et al.,* 1998; Kim & Levin, 1988).

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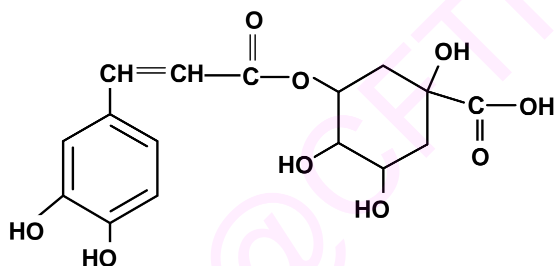
4.1.1. Chlorogenic acids

In coffee beans, phenolic compounds are predominantly present as family of esters formed between *trans*-hydroxy cinnamic acids (caffeic, p-coumaric and ferulic) and quinic acid (1,3,4,5-tetrahydroxycyclohexane carboxylic acid - which has axial hydroxyls on carbons 1 and 3 and equatorial hydroxyls on carbons 4 and 5) and are collectively known as Chlorogenic acid (CGA, XXI), (Clifford, 1985).



XXI. Chlorogenic acid

CGA are present in high concentration in green coffee beans (7-14%) have a marked influence in determining the quality as well as nutraceutical value of coffee beverage (Farah and Donangelo, 2006), as these possess several beneficial health properties such as hypoglycemic, antiviral, hepatoprotective and antispasmodic activities (Basnet *et al.,* 1996; Trute *et al.,* 1997; Moreira *et al.,* 2005).

Scope of the study

Coffee conserve was prepared from LCB using methanol after defatting with hexane. The bioactivity of the extract (Me_1) was evaluated

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using in vitro and in vivo methods and presented in earlier chapters. Methanol extract possess appreciable amounts phenolic content which includes chlorogenic acids which exist as isomers. Since there is no report on the chlorogenic acid isomers from LCB, efforts were made to isolate the isomers and their structure has been elucidated using spectral techniques. It is well known that enrichment of phenolics as well as chlorogenic acids results in increasing the bioactivity of the extract. Efforts were made for the enrichment of extracts for phenolics as well as chlorogenic acids by different methods viz., partitioning in solvents, chromatographic separation using resins and precipitating by lead acetate. Quality of these extracts were evaluated. Though the bioactivity of the extract is proved by model system it is necessary to evaluate the efficacy in food applications. LCB extract was evaluated by incorporating into sunflower oil and oxidative rancidity was monitored.

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4.2. Experimental section

LCB conserve was prepared using methanol as described in the earlier chapters and partitioned in solvents, resin as well as crude chlorogenic acid was isolated by lead acetate precipitation method.

4.2.1 Plant material and chemicals

Dowex resin, Diaion HP 20, BHA, chlorogenic acid, caffeine were purchased from the relevant sources as mentioned earlier.

4.2.2. HPLC analysis of chlorogenic acids

The isomers of chlorogenic acids were analysed by HPLC according to the procedure mentioned by Balyaya & Clifford (1995) with the slight modification.

LCB extract (25 mg) were dissolved in aqueous 70% methanol (10ml). The solution was filtered through membrane filter (0.45μ) and injected. Standard chlorogenic acid and caffeine were individually analysed for retention time. Standard solutions were prepared by dissolving 10mg in distilled water (4ml) and further diluted to 20 times and injected into HPLC and analysed according to the conditions mentioned below:

HPLC system: Waters 2998 Model equipped with Empower software

Column: C18 (Waters; particle size -5 μm, i.d.-4.6 mm, length -250mm)

Mobile Phase : A - 0.5% Trifluoroacetic acid (TFA) in water
B - 45% Acetonitrile in A

Gradient programming :

min.	A (%)	B (%)
0	100	0
56	0	100
66	100	0
76	100	0

Detector: Photo diode array (PDA)

Injection Volume: 20 μl

Flow Rate: 1ml/min.

4.2.3. Separation of constituents by Preparative HPLC

The constituents of chlorogenic acid mixture were separated by preparative HPLC according to the conditions mentioned below. Methanol

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extract (200 mg) was dissolved in aqueous 70% methanol (10ml). The solution was centrifuged (2500 rpm for 5 min.) and filtered through membrane filter (0.45 µ). Total six injections (200 µL each) were made and the separated peaks were collected as three fractions (1, 2 and 3) and desolventised using a rotavapor.

HPLC system: Waters 2998 Model equipped with Empower software

Column : C18 (Waters 10 µm, 19x300 mm)

Mobile Phase: A : 0.5% TFA in water

B : 45% Acetonitrile in A

Programming:

min.	A(%)	B(%)	Flow Rate/min.
0	100	0	10ml
15	50	50	10ml
60	0	100	4ml
70	100	0	10ml
80	100	0	10ml

Detector : PDA (200 to 400 nm)

4.2.4. Characterization by NMR spectroscopy

The methanol extract was subjected to NMR spectral using Bruker NMR instrument operating at 500MHz for ^{1}H and 125 MHz for ^{13}C at room temperature; Scanned region –0 to 20 ppm for ^{1}H and 0–200 ppm for ^{13}C spectra. Tetramethylsilane was used as an internal standard and the values are given below:

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Fraction 1 (Compound 1):

^{1}H NMR (D_2O) δ 9.25 (s), 8.86 (t, J=8Hz), 8.05 (dd, J=6.5, 8 Hz), 4.35 (s); ^{13}C NMR (D_2O) δ 164.5, 147.7, 146.4, 145.3, 131.5, 127.9, 48.4.

Fraction 2 (Compound 2):

^{1}H NMR (D_2O) δ 7.53 (d, J=16Hz), 7.08 (d, J=2 Hz), 7.01 (dd, J= 2,8 Hz), 6.82 (d, J=8Hz), 6.30 (d, J= 16 Hz), 5.28 (d, J=3.5Hz), 4.08 (ddd, J=3,5,10 Hz), 3.65 (dd, J= 3.5, 9.5 Hz), 2.06, (dd, J=3,10Hz), 2.03 (m), 1.95 (dd, J=3,12); ^{13}C NMR (D_2O) δ 178.3, 168.2, 146.8, 145.9, 144.3, 126.9, 122.0, 116.3, 114.9, 114.5, 74.8, 73.2, 72.3, 66.7, 39.9, 35.0.

Fraction 3 (Compound 3a and 3b):

^{1}H NMR (D_2O) δ 7.63 (s), 7.28 (d, J=16Hz), δ 7.17 (d, J=16Hz), 6.71 (d, J= 2 Hz), 6.65 (d, 1.5 Hz), 6.64 (dd, J= 1.5, 8 Hz), 6.55 (d, J=8Hz), 6.1 (d, J= 16 Hz), 5.98 (d, J= 16 Hz), 5.14 (ddd, J= 4, 4, 4.5 Hz), 4.12 (ddd, J=3.5, 6.5, 2.5 Hz), 4.11 (ddd, J=3.5, 8.5, 12 Hz), 3.76 (dd, J= 3.5, 8.5 Hz), 3.75 (ddd, J= 3, 8, 12 Hz), 3.66 (s), 3.20 (s), 3.04(s), 2.11, (dd, J=3.5, 14 Hz), 2.08, (ddd, J=3,5,14 Hz), 2.06 (dd, J=4.5, 13Hz), 2.02 (ddd, J=3,4,14 Hz), 1.98 (dd, J=11, 14), 1.94 (dd, J=13, 4);

^{13}C NMR (D_2O) δ176.8 , 176.6, 168.4,168.2 , 155.5, 151.9, 148.5, 147.5, 146.8, 145.8, 145.6, 143.9, 142.7, 141.6,126.4, 126.1, 121.9, 116.3, 115.4, 114.0, 113.8,107.3, 77.5, 74.8, 74.5, 70.9, 70.4, 68.8, 67.5, 39.5, 36.5, 36.2, 33.1, 29.6, 27.6.

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4.2.5. Enrichment of coffee extracts

4.2.5.1. Partitioning in solvents

1. Partitioning in chloroform

Methanol extract (Me_1, 5g), was dissolved in distilled water (150 ml) and extracted with chloroform (150 ml) for four times in a separating funnel. The chloroform extract as well as aqueous layer were desolventised on a rotavapor at 50°C under reduced pressure of mercury and the obtained solids C(S_1) and C(IS_1) respectively, were preserved in desiccator for further use.

2. Partitioning in Ethyl acetate

Me_1 (5g) is extracted with 600 ml of ethyl acetate in a separating funnel. The ethyl acetate soluble and insoluble extracts were desolventised to obtain the solid portions of EA (S_1) and EA (IS_1).

Successive extraction

In another experiment, extraction was carried out using chloroform and ethyl acetate successively. Me_1 extract (10 g) was dissolved in distilled water (300 ml) and extracted with chloroform (150 ml) for four times. The pooled chloroform extract was desolventised to obtain chloroform soluble extract [C (S_2)]. The aqueous layer was further extracted with ethyl acetate (150ml) for four times. Both the layers of aqueous and ethyl acetate were desolventised to obtain EA (S_2) and EA (IS_2) respectively.

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4.2.5.2. Separation using resins

Dowex 50 Resin

Dowex resin (50g) was prepared for chromatography as per the following procedure: It was percolated in 1N sodium hydroxide and four resin volumes (400ml) passed through for 4 h and the resin was washed to neutral pH. Then, it was percolated in 10% hydrochloric acid and four resin volumes (400ml) passed through for 3 h and the resin was washed to neutral pH. Finally, the resin was rinsed with distilled water to obtain a neutral pH.

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Methanolic extract (5g) was dissolved in 75ml of distilled water. Sample solution was loaded onto the resin bed and 100ml of distilled water was poured slowly. First fraction of elute was collected in a conical flask (100ml). Methanol (100 ml) was passed through slowly and elution was continued. The second fraction (100ml) was collected. Both the fractions were desolventised and the extracts preserved for further analysis.

Diaion HP 20

Diaion HP20 (50g), was taken in a 500ml measuring cylinder. Two bed volumes of methanol was added and kept overnight. Supernatant was discarded and washed 2-3 times with distilled water by stirring for about 3-4 times. LCB extract (5g) was dissolved in distilled water (75ml) and loaded onto the column. Isopropyl alcohol 200ml (IPA) was added and fraction collected. Finally methanol (200ml) was added into the column and the

fraction eluted was collected. Fractions were desolventised and analyzed for phenolics.

4.2.5.3. Precipitation using lead acetate

The chlorogenic acid was isolated from coffee conserve according to the method mentioned by Hulme, 1953, where the isolation of chlorogenic acid from apple fruit was described. Coffee conserve (Me_1, 20g) was dissolved in 500ml of boiling water. Lead acetate solution (100 ml) was added and centrifuged. Supernatant was discarded; precipitate was dissolved in 100ml water and again centrifuged. This was repeated for about four times. Precipitate was then suspended in water (400ml) and pH was adjusted to 1.7 with 10% perchloric acid. Insoluble residue was removed by centrifugation. pH was again adjusted to 4.1 with 10% sodium hydroxide. Precipitate collected was washed with 100ml water, centrifuged and supernatant discarded. Precipitate obtained was dissolved in about 400ml water. Hydrogen sulphide gas passed through the precipitate with vigorous stirring for 20 min. to remove the lead as lead sulphide. Then it was filtered through Whatman No.1 paper in a Buckner funnel. The filtrate was saturated with sodium chloride and extracted with ethyl acetate (8 X 50ml). The extracts were combined and dried over sodium sulphate for 12h. Solvent was filtered and distilled off until liquid just begins to turn cloudy or reaches volume of ~20-30ml. The mixture was cooled on an ice bath and added two volumes of petroleum ether to get a fluffy precipitate. The

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precipitate was collected, washed with petroleum ether and dried in a desiccator.

4.2.6. Quality evaluation of the separated fractions

The isomers of chlorogenic acids of the separated fractions were analysed by HPLC analysis according to the section 4.2.2. Also, the isolated fractions were analysed for total phenolics, chlorogenic acids and caffeine according to the procedure already described in chapter 2.

4.3. Results and Discussion

Coffee is a complex mixture of several compounds. Phenolic compounds are part of the non-volatile extracts. These contain variable amounts of chlorogenic acid, caffeic acid and other polyphenolic compounds depending on the species. Many of them are found to be potent antioxidants. Quinyl esters of *trans*-hydroxycinnamic acids, which are generally known as chlorogenic acids (CGA) in green coffee beans was first reported in 1837 (Sondheimer, 1964). CGA account for 7-10% in *Coffea robusta* and 5 – 8% in *Coffea arabica* on dry mater basis (Clifford, 1985). CGA include three major groups viz., Caffeoylquinic acids (CQAs), feruloylquinic acids (FQAs) and dicaffeoylquinic acids (diCQAs). Each group contains three isomers on the basis of the number and identity of the acylating residues (Clifford, 1985). In this chapter five different methods of separation of CGAs' conserve were attempted and isomers were analysed by HPLC and the results are discussed.

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HPLC analysis

Isomers of chlorogenic acids were separated and eleven peaks were observed in all the fractions, of which seven are major. The pattern of chromatogram is very well matching with the earlier reports (Balyaya and Clifford, 1995; Ky *et al.,* 1997) of graded coffee beans. Therefore the peaks were compared with the literature reported chromatograms and characterised accordingly. The chromatograms of 5-Caffeoylquinic acid and caffeine are given in figs. 4.1.1 and 4.1.2 respectively. HPLC profiles of methanol extract are given in Figs. 4.1.3. and 4.1.4.

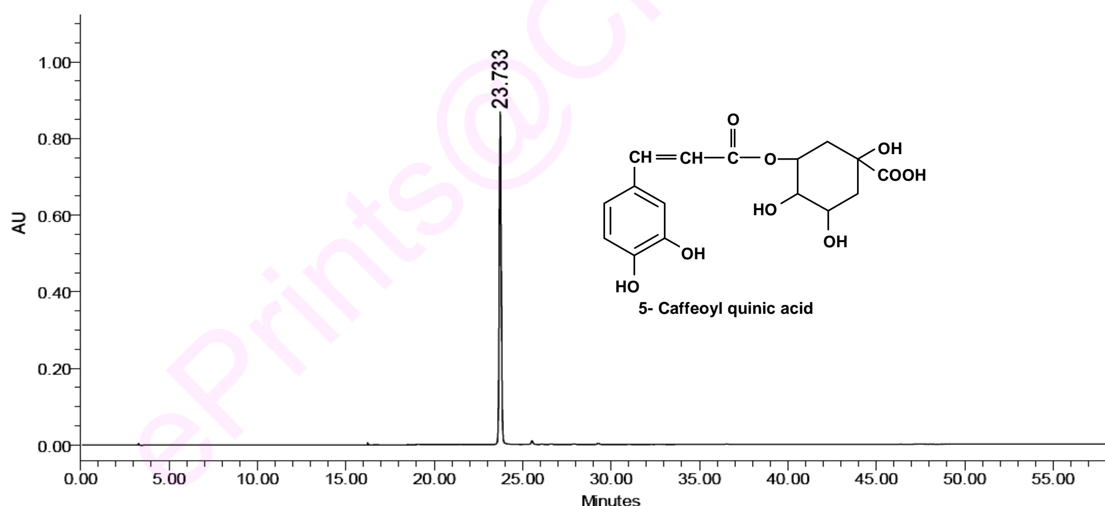

Fig. 4.1.1. HPLC Chromatogram of 5-CQA at 325 nm

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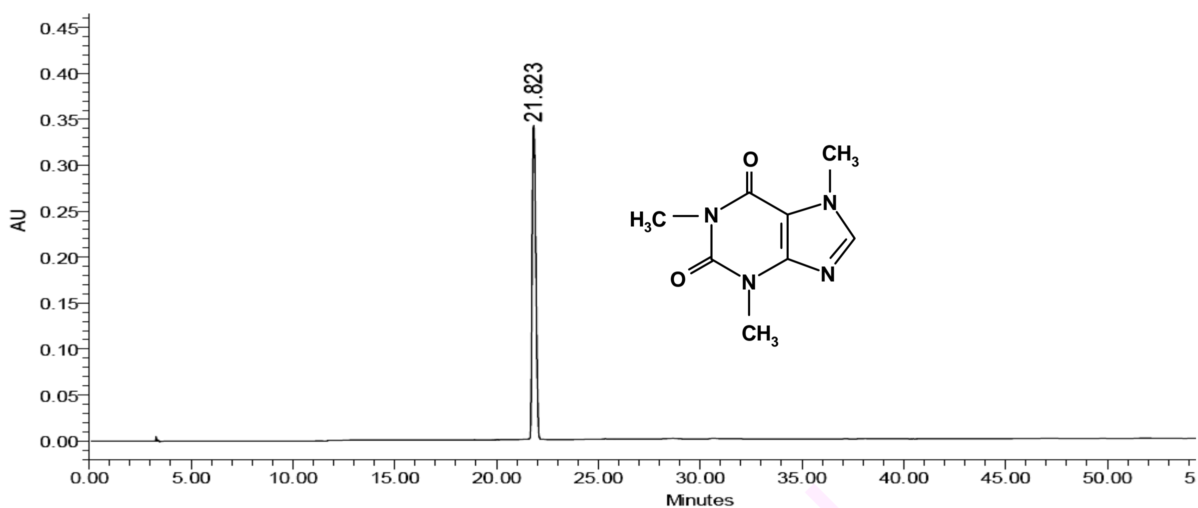

Fig. 4.1.2. Chromatogram of caffeine at 275 nm

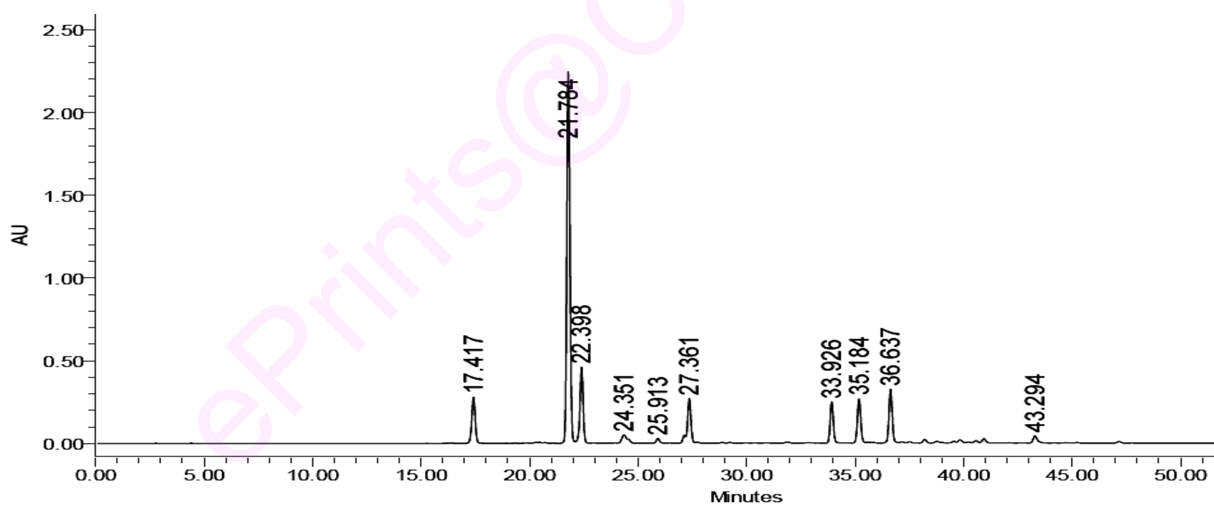

Fig. 4.1.3. Chromatogram of Me_1 at 325 nm

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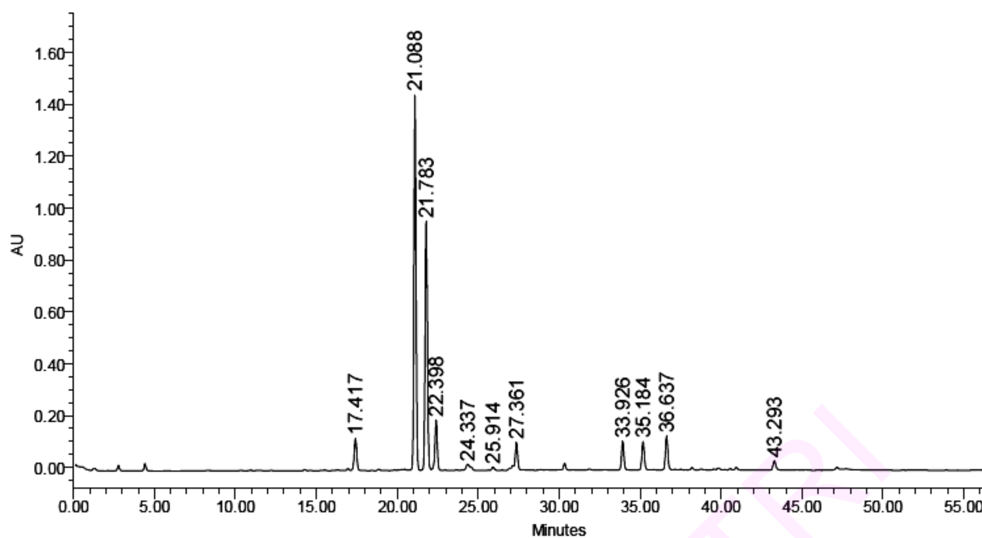

Fig. 4.1.4. Chromatogram of Me_1 at 275 nm

Constituents by preparative HPLC

There is no report on the isolation of chlorogenic acid isomers from Low-grade coffee beans. Therefore an attempt was made to isolate the chlorogenic acid isomers using preparative HPLC. The chromatogram of LCB extract using PDA with the scanning from 200 to 400 nm is given in Fig. 4.1.5.

Though the pattern of the CGA isomers are similar (Balyaya & Clifford, 1995) there are two more predominant peaks were observed. Six fractions were collected consisting of the peaks with retention times (minutes) of 11.026 (fraction 1), 17.122 (fraction 2), 20.335, & 21.003 (fraction 3), 29.272 (fraction 4), 31.312 (fraction 5) and 34.743 (fraction 6). Purity of the isolated fraction was analysed.

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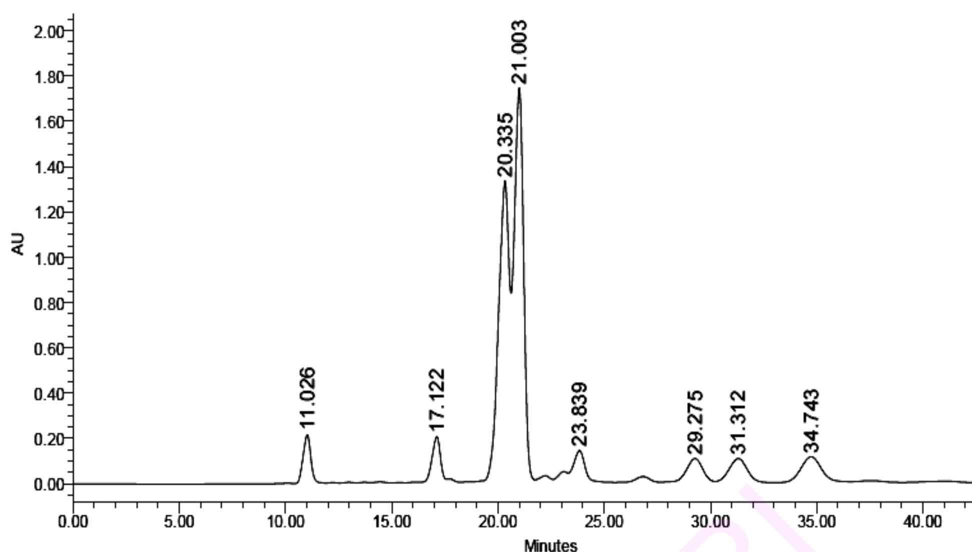

Fig. 4.1.5. Chromatogram of Me_1 using preparatory HPLC column

Fraction 1 and 2 are found to be pure and contained single peak indicating the presence of single compound. Fraction 3 found to contain two close moving peaks, indicating it to be mixture of two components. The fractions 4, 5 and 6 too low in quantity and spectral studies could not be carried out. However, their retention times are comparable with those of the di CQAs. Fractions 1, 2 and 3 were subjected to NMR spectral analysis and results are presented here.

Identification of the isolated compounds by NMR

The NMR spectra obtained for the compound 1 was furnished in the following figures (4.1.6 to 4.1.11a). The chemical shift values were compared with the literature report (Biological Magnetic Resonance Data Bank, 2009) and the compound is characterised as Trigonelline (1-methyl pyridine 3-carboxylic acid; XXII). The slight differences in chemical shift

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values are observed. These are due to the difference in the solvent used. The compound dissolved in pure D_2O in the present study for NMR study, where as earlier reports used buffer also. The spectral assignments are furnished in Table 4.1.1. HSQC and HMBC spectral studies confirmed the assignments.

Table 4.1.1. ^{1}H and ^{13}C NMR chemical shifts and connectivity by HMBC of compound 1

H/C	δ of proton (ppm)	Multiplicity	Coupling constant J (Hz)	δ of carbon (ppm)	HMBC correlations
1				_	
2	9.25	s		146.4	C7, C6, C4, C3, C8
3	-	-		131.5	
4	8.87	d	8	145.3	C2, C6, C5, C7
5	8.05	dd	6, 8	127.9	C3, C6
6	8.86	d	6	147.7	C2, C4, C5, C8
7	-	-		164.5	
8	4.35	s		48.4	C2, C6,

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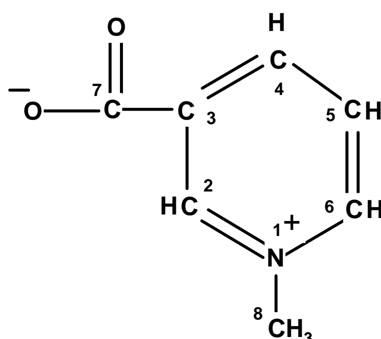

XXII. Compound 1- Trigonelline

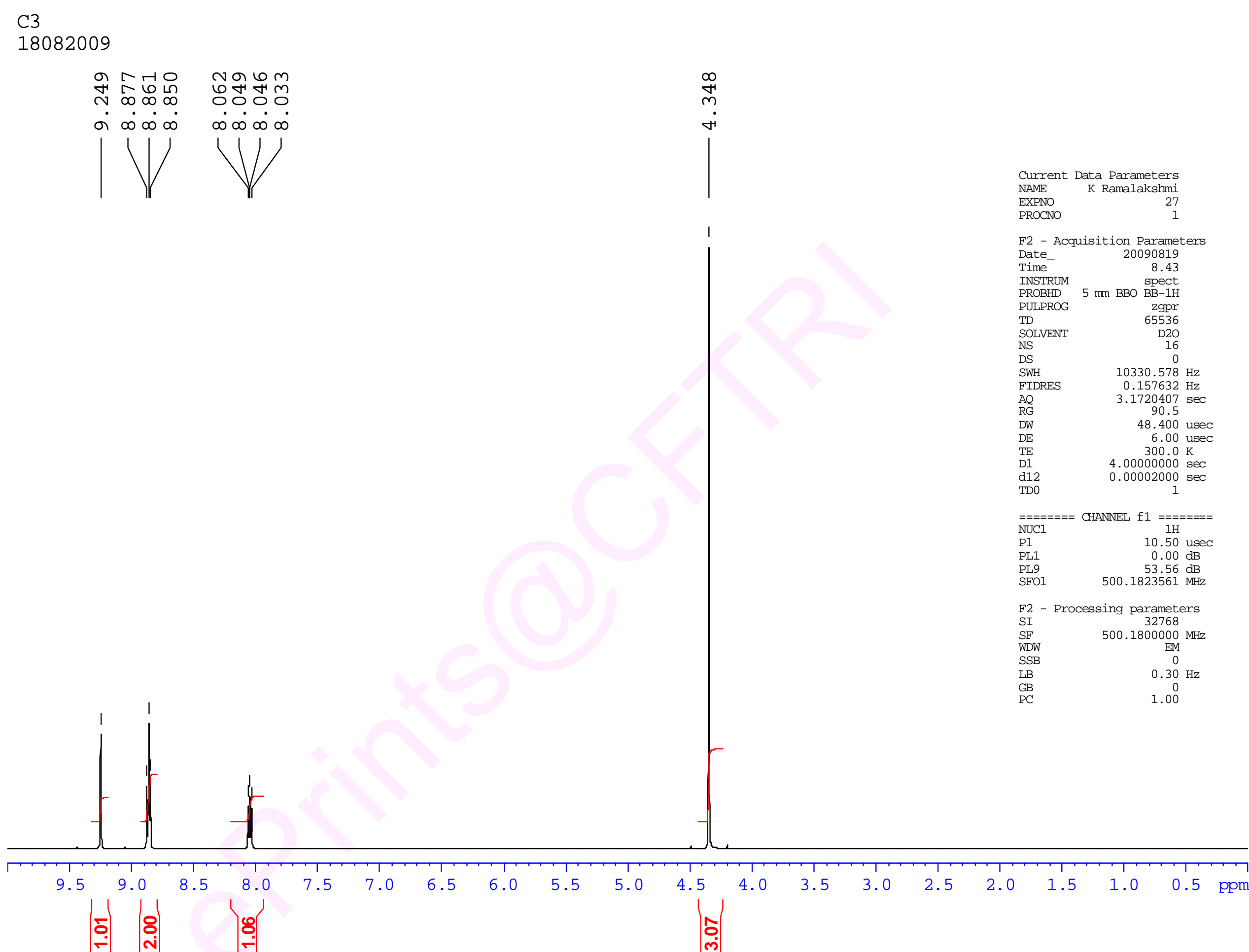

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Fig. 4.1.6. ^{1}H NMR spectrum of compound 1

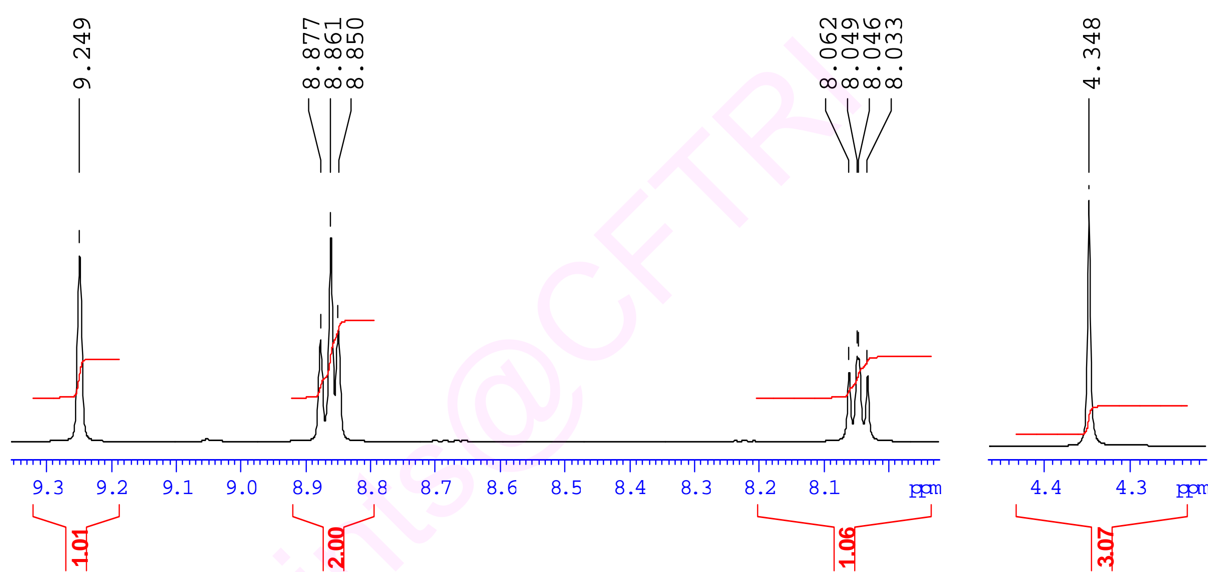

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Fig. 4.1.6a. ^{1}H NMR spectrum of compound 1

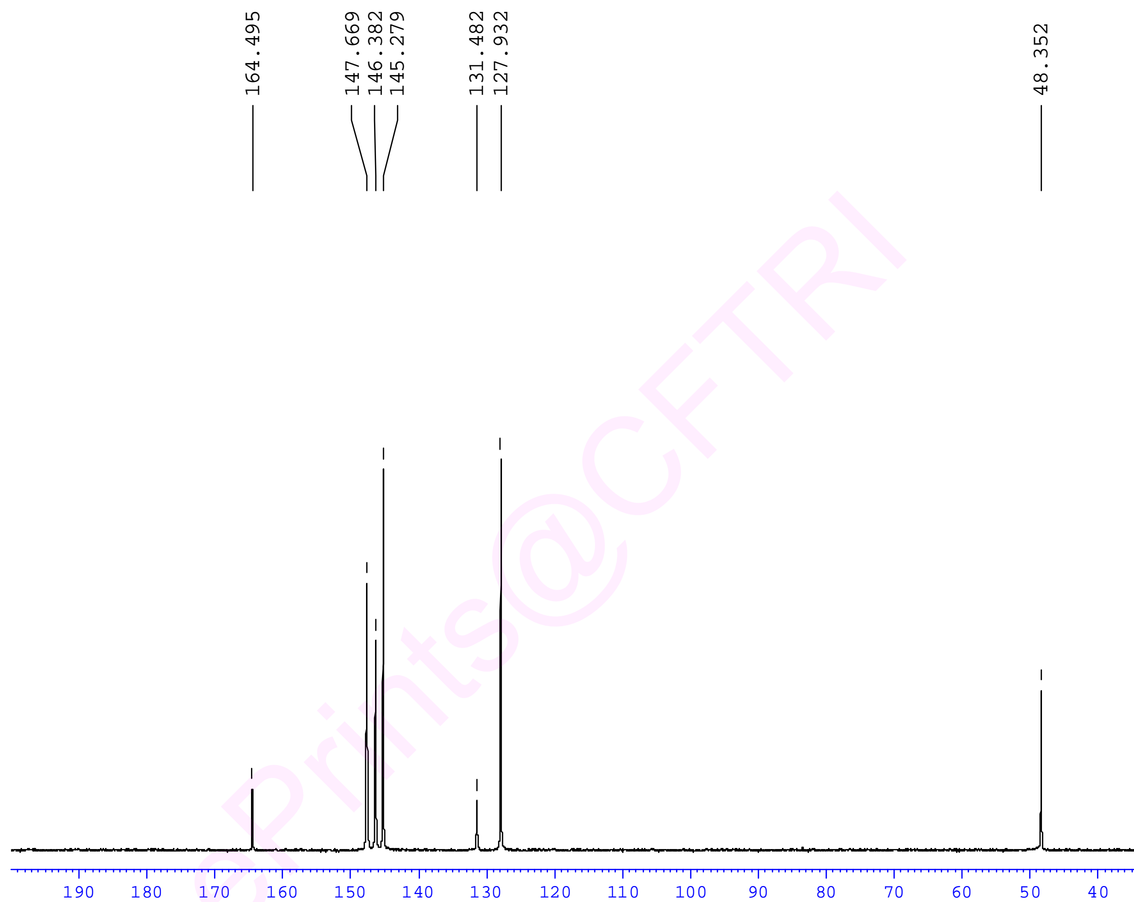

Fig. 4.1.7. ^{13}C NMR spectrum of compound 1

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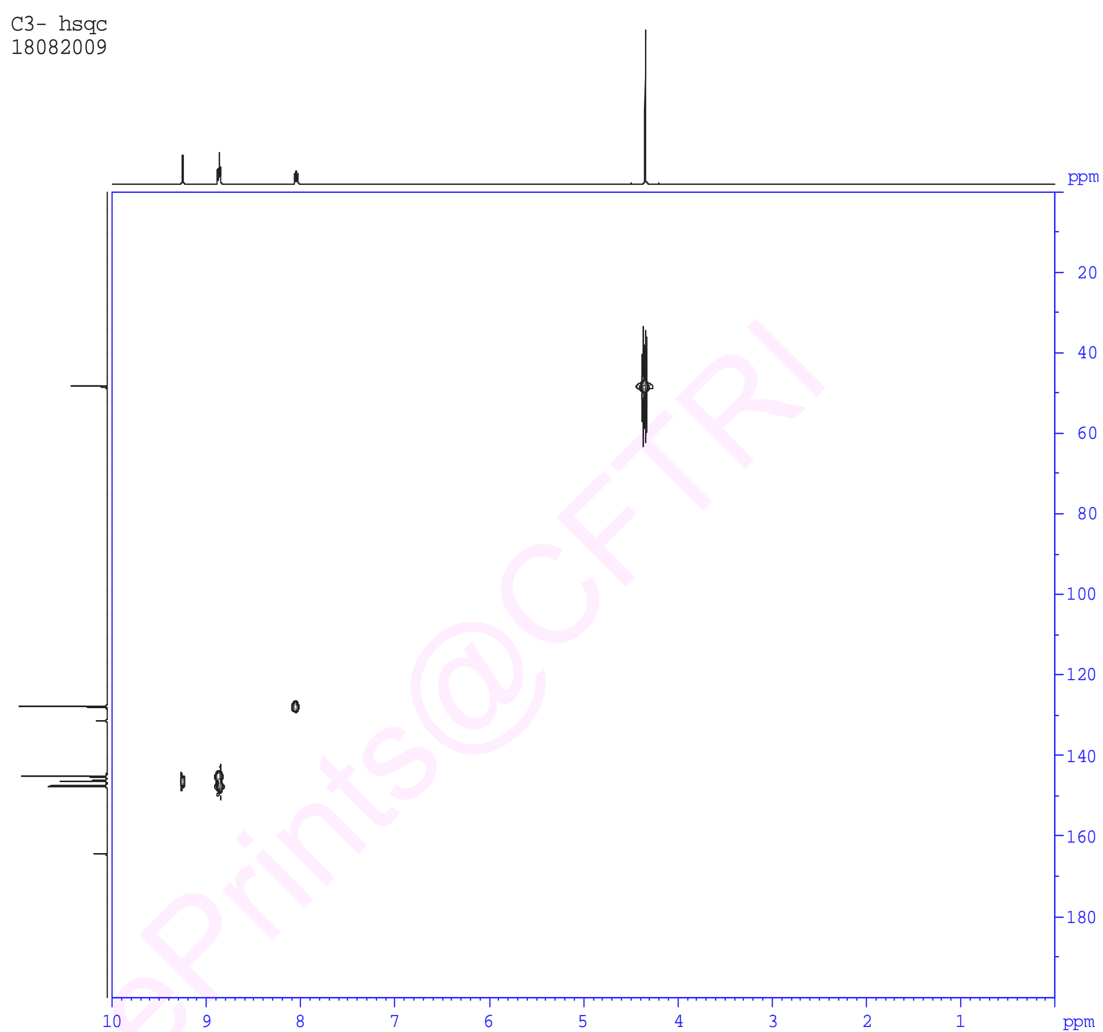

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Fig. 4.1.8. HSQC spectrum of compound 1

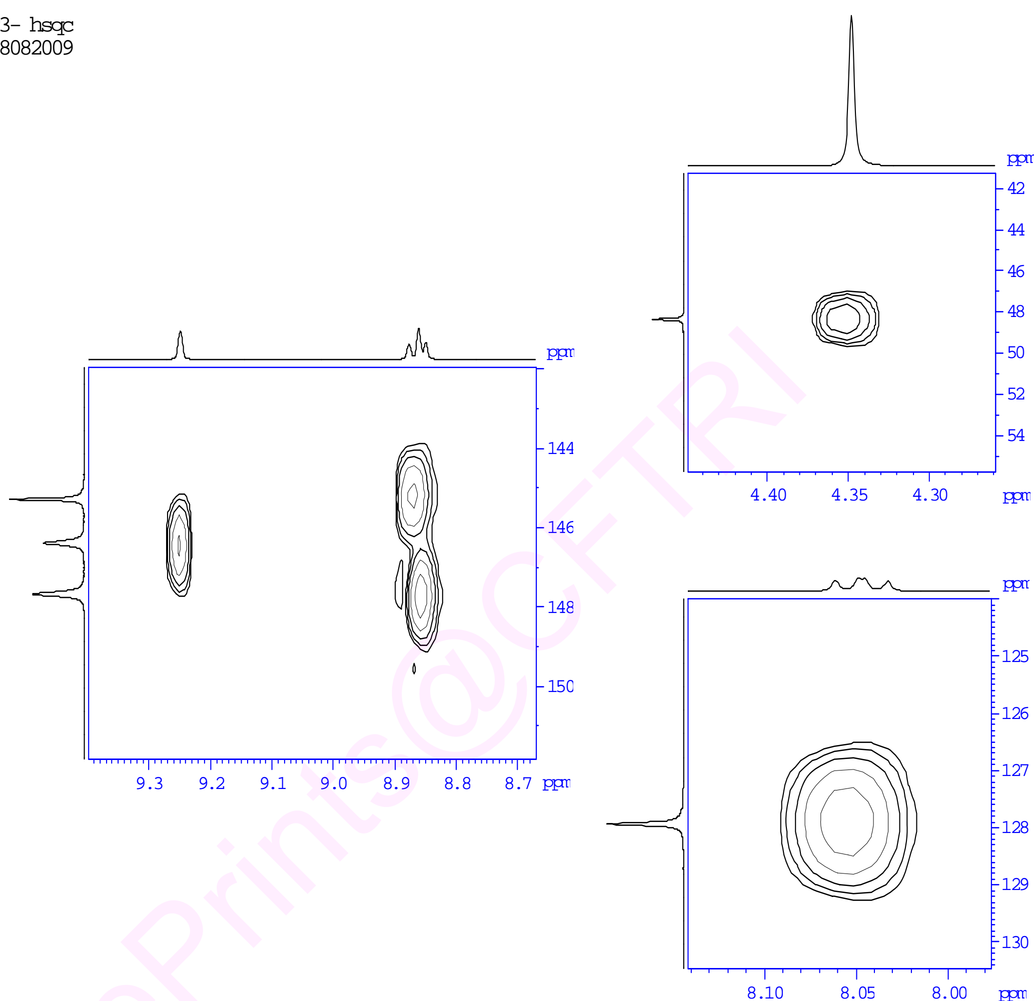

Fig. 4.1.8a. HSQC NMR spectrum of compound 1

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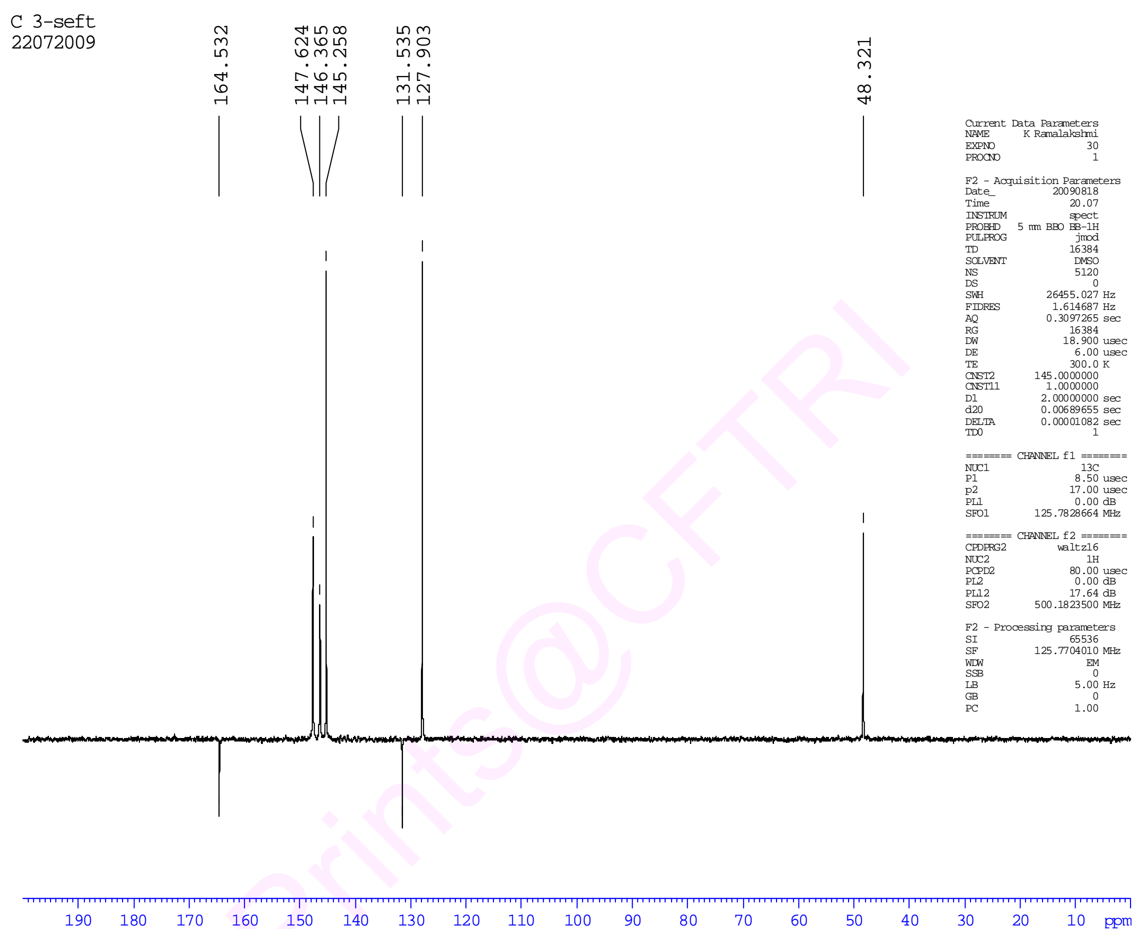

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Fig. 4.1.9. ^{13}C SEFT NMR spectrum of compound 1

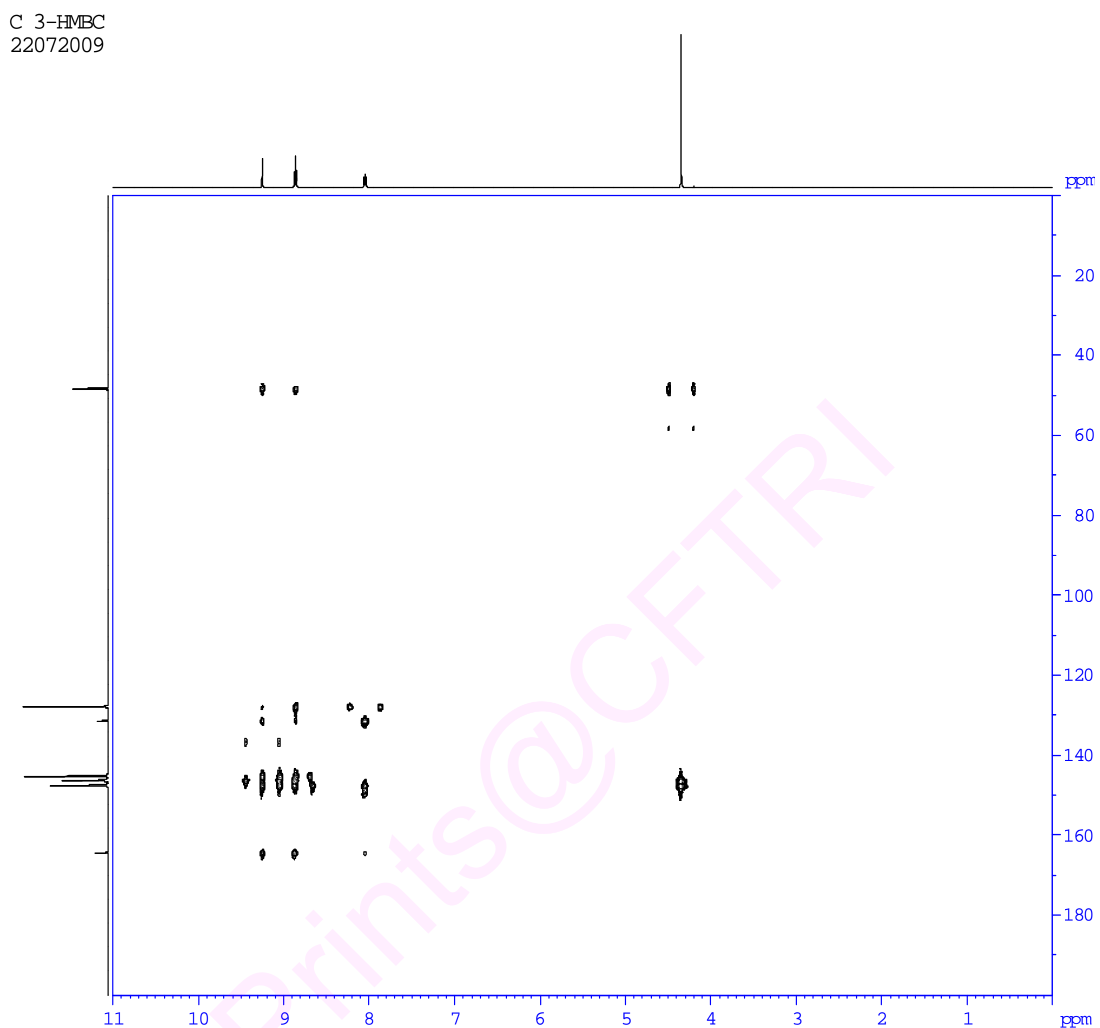

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Fig. 4.1.10. HMBC NMR spectrum of compound 1

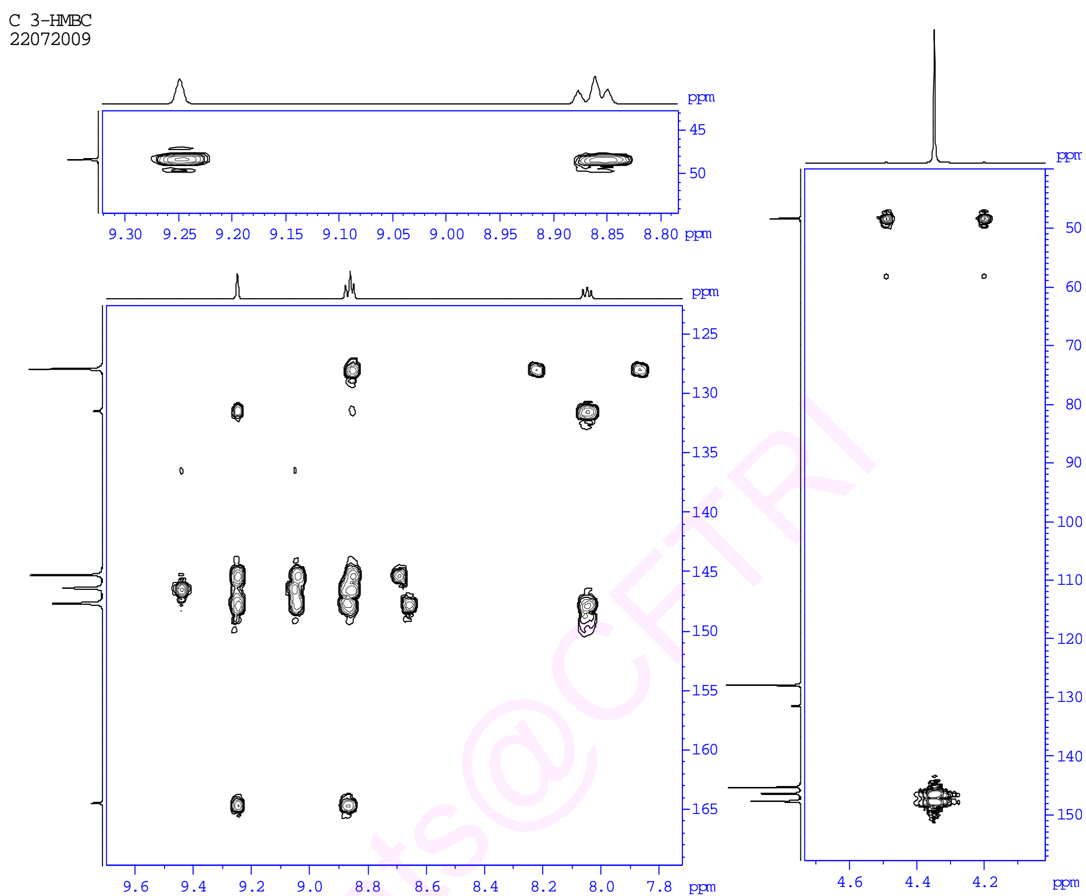

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Fig. 4.1.10a. HMBC NMR spectrum of compound 1

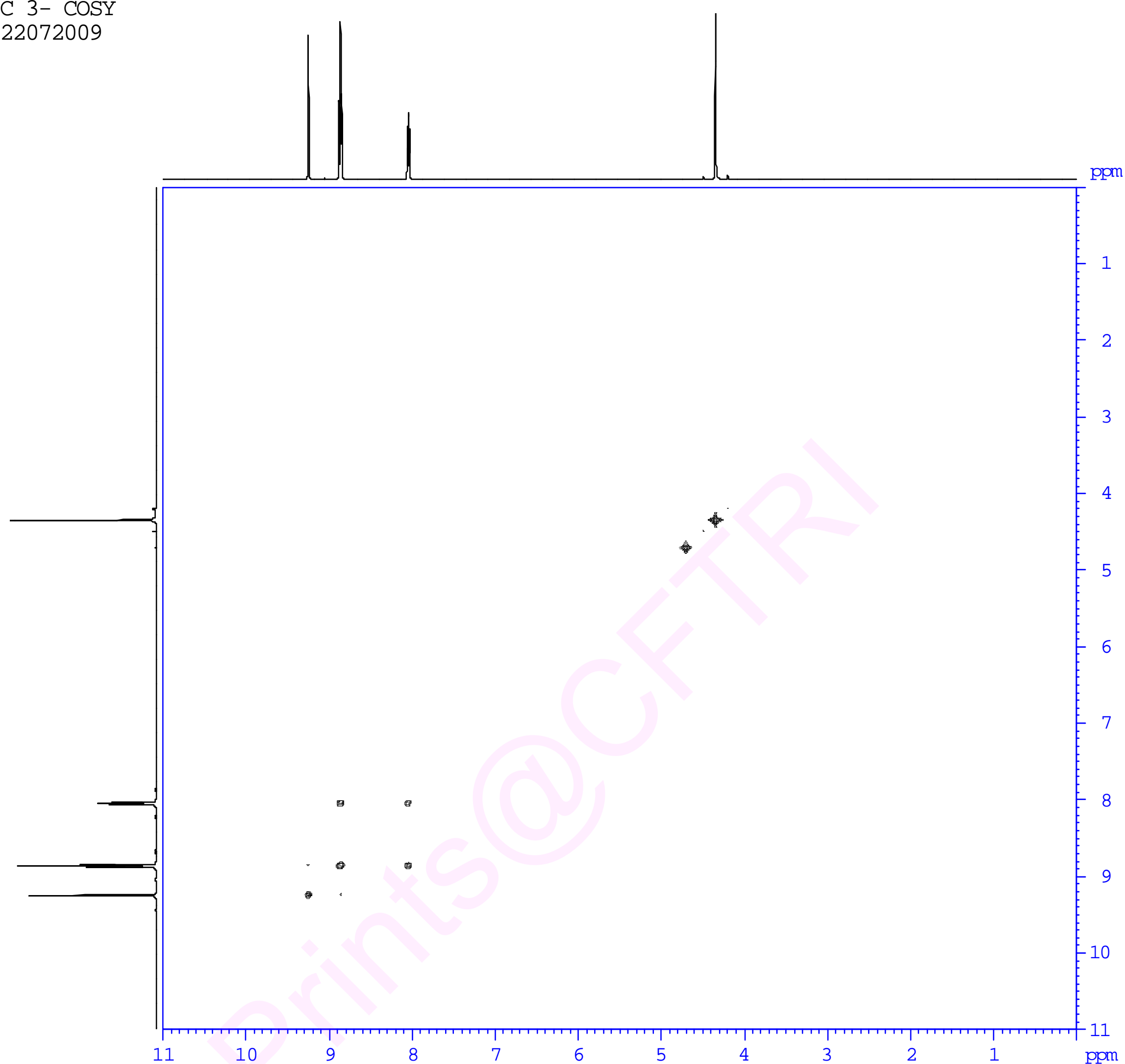

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Fig. 4.1.11. COSY NMR spectrum of compound 1

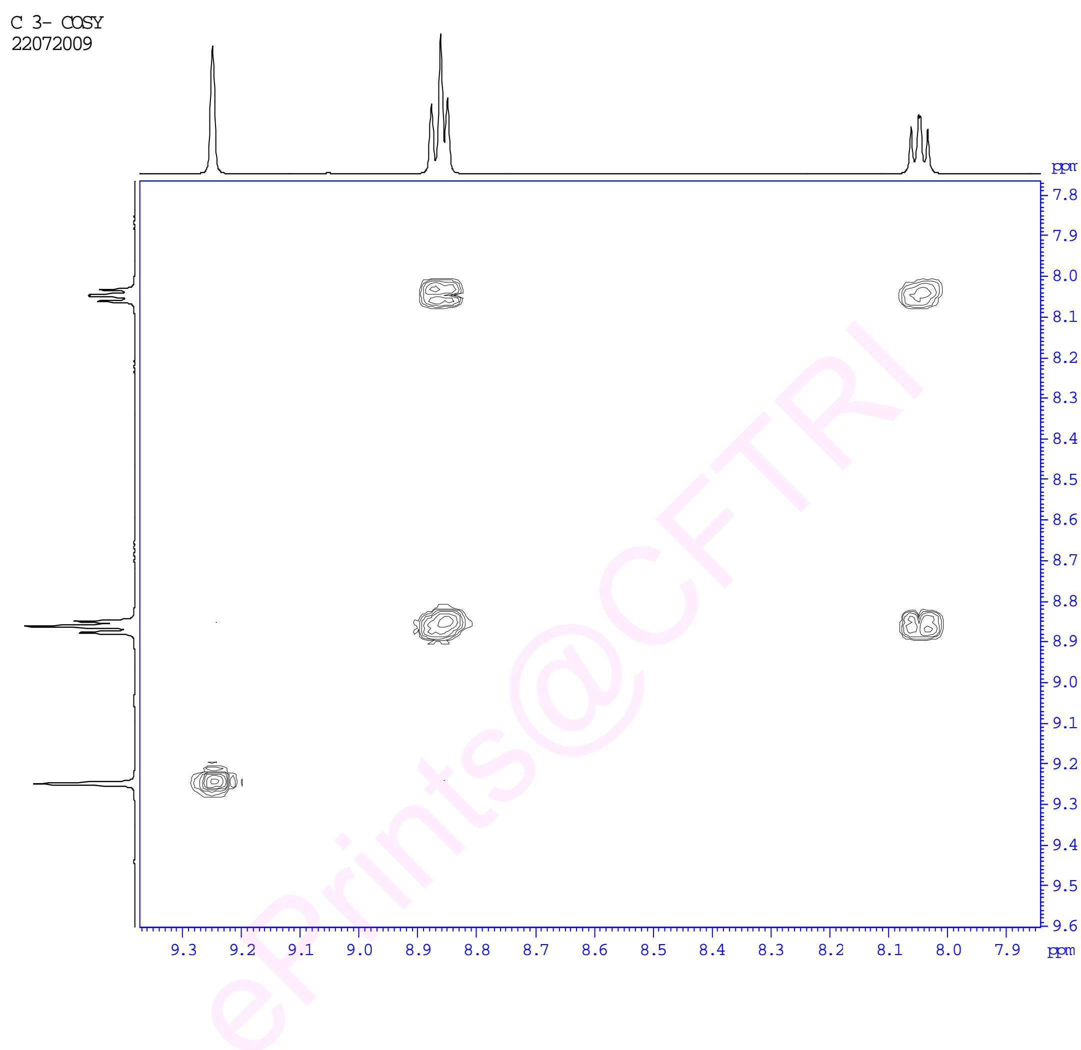

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Fig. 4.1.11a . COSY NMR spectrum of compound 1

Compound 2.

The NMR spectra obtained for the compound 2 were furnished in the following Figs. 4.1.12 to 4.1.16a. The chemical shift values were compared with the literature report (Nakatani *et al.,* 2000, Morishita, 1984, Tatefuji 1996) and the compound is characterised as 3-Caffeoylquinic acid [3-{{(3,4-dihydroxyphenyl)-1-oxy-2-propenyl}oxy}-1, 4, 5-trihydroxy-1{1S-1α, 3β, 4α, 5α cyclohexane carboxylic acid}] (XXIII). The slight differences in chemical shift values are observed. These are due to the difference in the solvent used. The compound dissolved in pure D_2O in the present study for NMR analysis, where as earlier report used CD_3OD. The spectral assignments are furnished in Table 4.1.2. HSQC and HMBC spectral studies confirmed the assignments. C-3 carbon signal shifted to down field by Δδ +5.1 (δ72.3ppm), C-2 carbon signal value moved upfield by Δδ -5.7 (δ 35.0 ppm) and C4 signal moved upfield by Δδ –2.0 (δ 73.2 ppm) when compared to the literature values of quinic acid (Kelley *et al.*, 1976). This confirms the attachment of caffeic acid moiety at the position of C-3 in the quinic acid.

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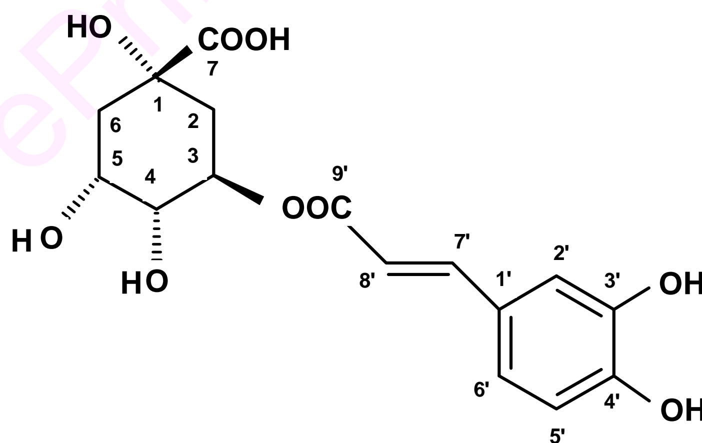
Table 4.1.2. ^{1}H and ^{13}C NMR chemical shifts of compound 2

H/C	δ of protons (ppm)	Multiplicity	Coupling constant J (Hz)	δ of carbons (ppm)	Key HMBC connectivities
1	-	-	-	74.8	
2 ax	2.19	dd	3, 10	35.0	
2eq	2.10	m			
3	5.28	m	3.5	72.3	
4	3.65	dd	3.5, 9.5	73.2	
5	4.08	ddd	3.5, 10	66.7	
6 ax	1.90	dd	3, 12	39.9	
6 eq	2.12	m			
7	-	-	-	176.5	
1′	-	-	-	126.9	
2′	7.08	d	2.0	114.5	C3’, C4’, C6’,C7’
3′	-	-	-	144.3	
47′	-	-	-	146.8	
5′	6.82	d	8	116.3	C1’, C3’
6′	7.01	dd	2, 8	122.0	C2’, C7’
7′	7.53	d	16	145.9	C2’, C6’, C9’
8′	6.30	d	16	114.9	C1’, C9’
9′	-	-	-	168.2	



XXIII. Compound 2: 3-Caffeoylquinic acid

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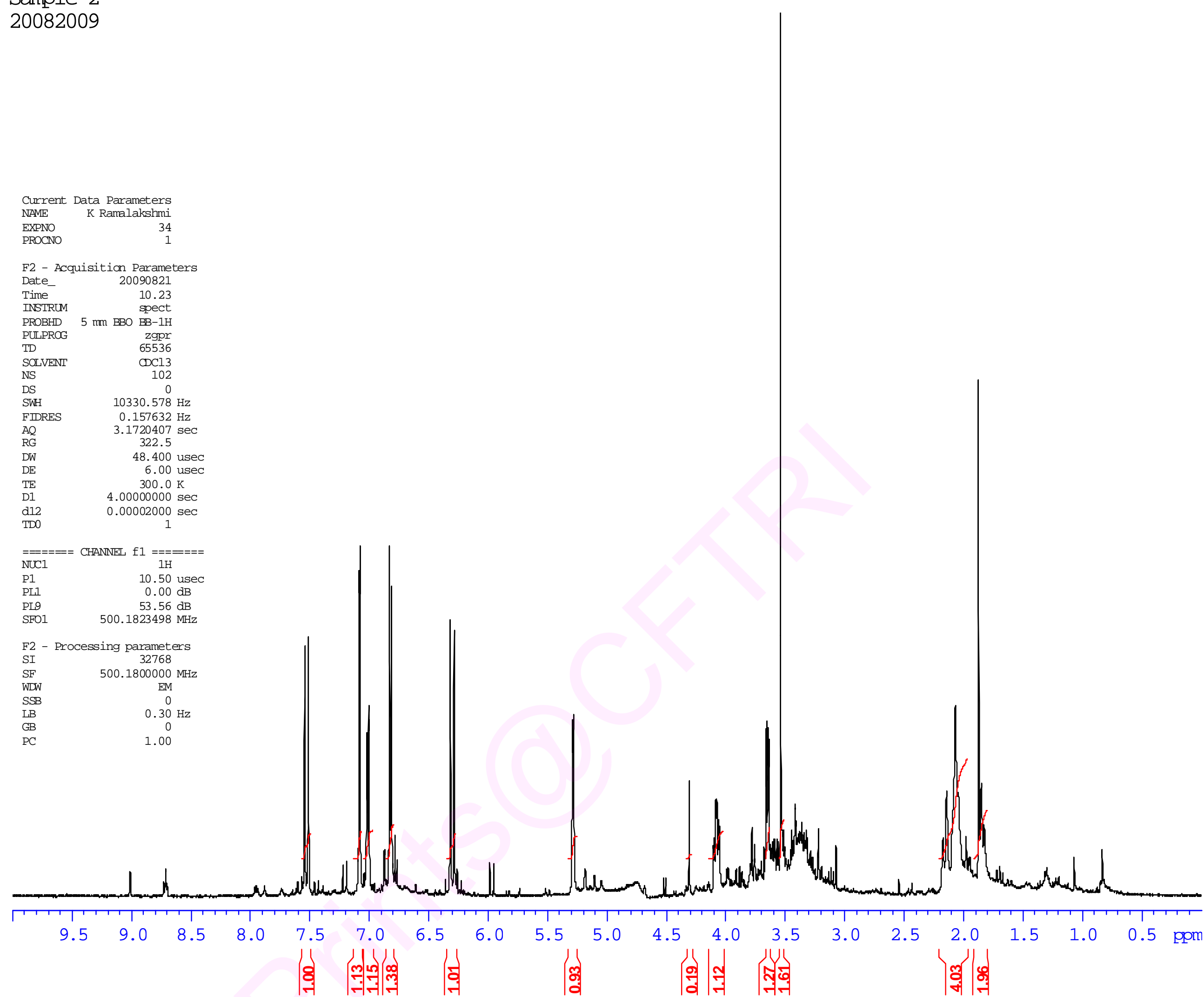

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Fig. 4.1.12. ^{1}H NMR spectrum of compound 2

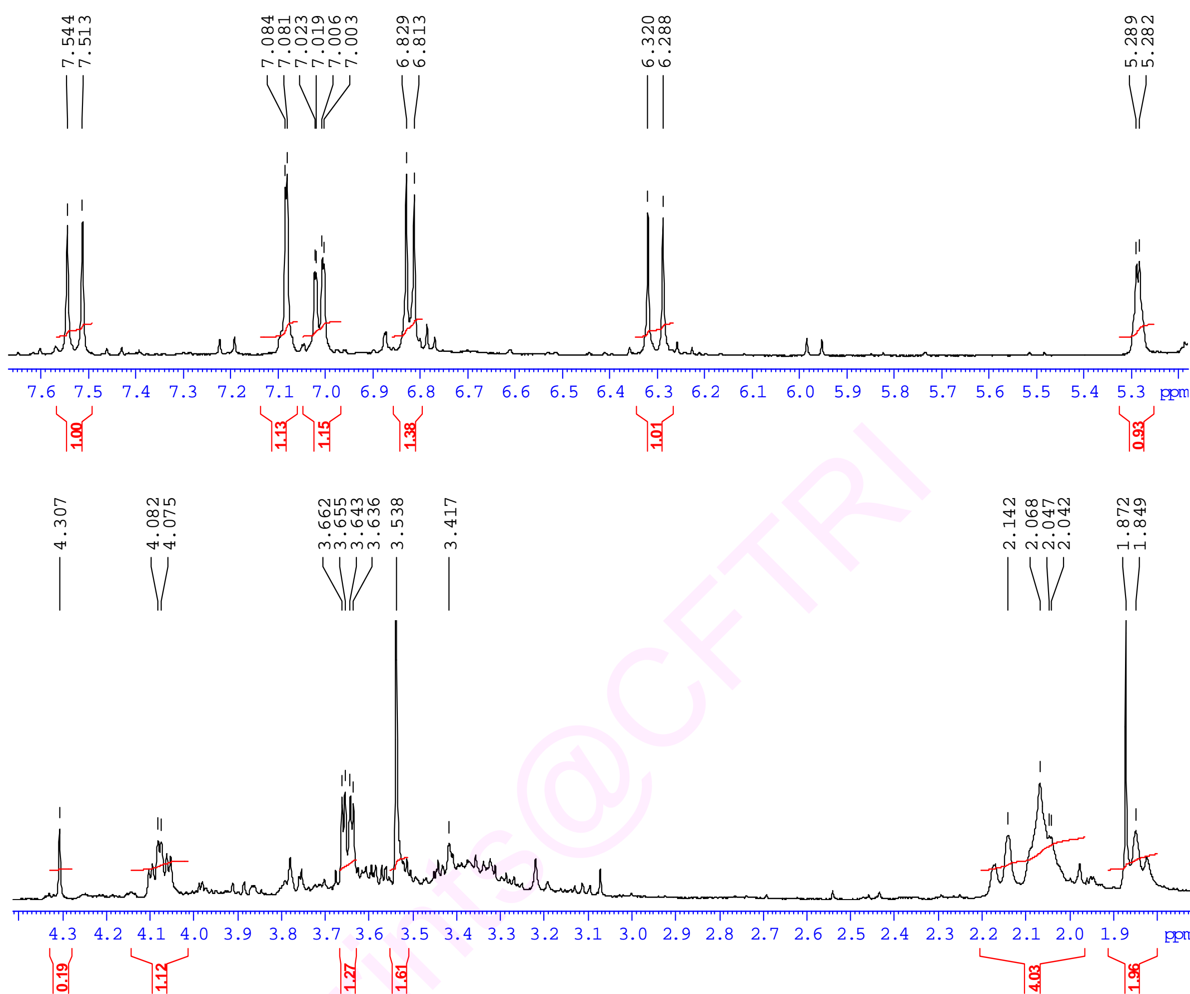

Fig. 4.1.12 a. ^{1}H NMR spectrum of compound 2

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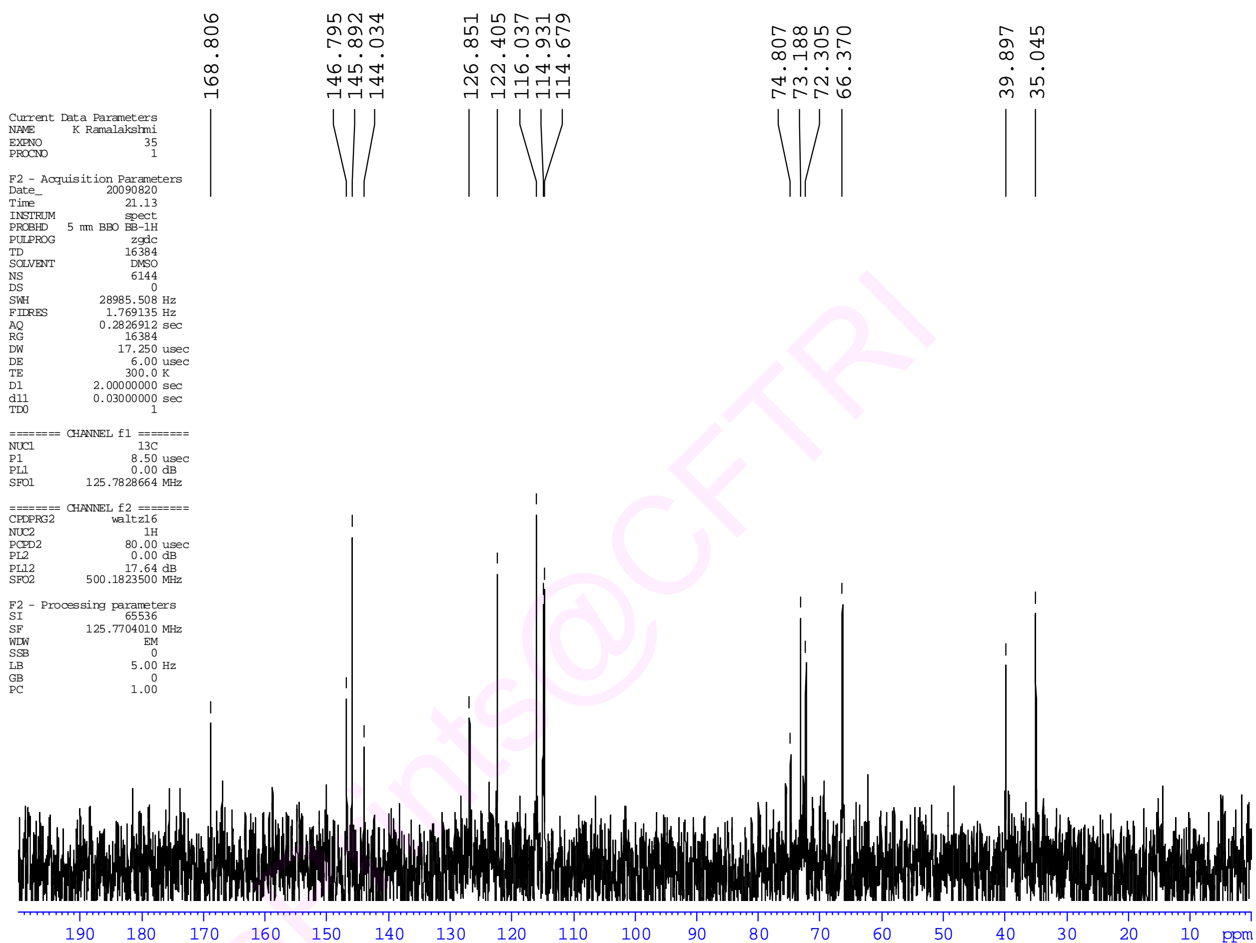

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Fig. 4.1.13. ^{13}C NMR spectrum of compound 2

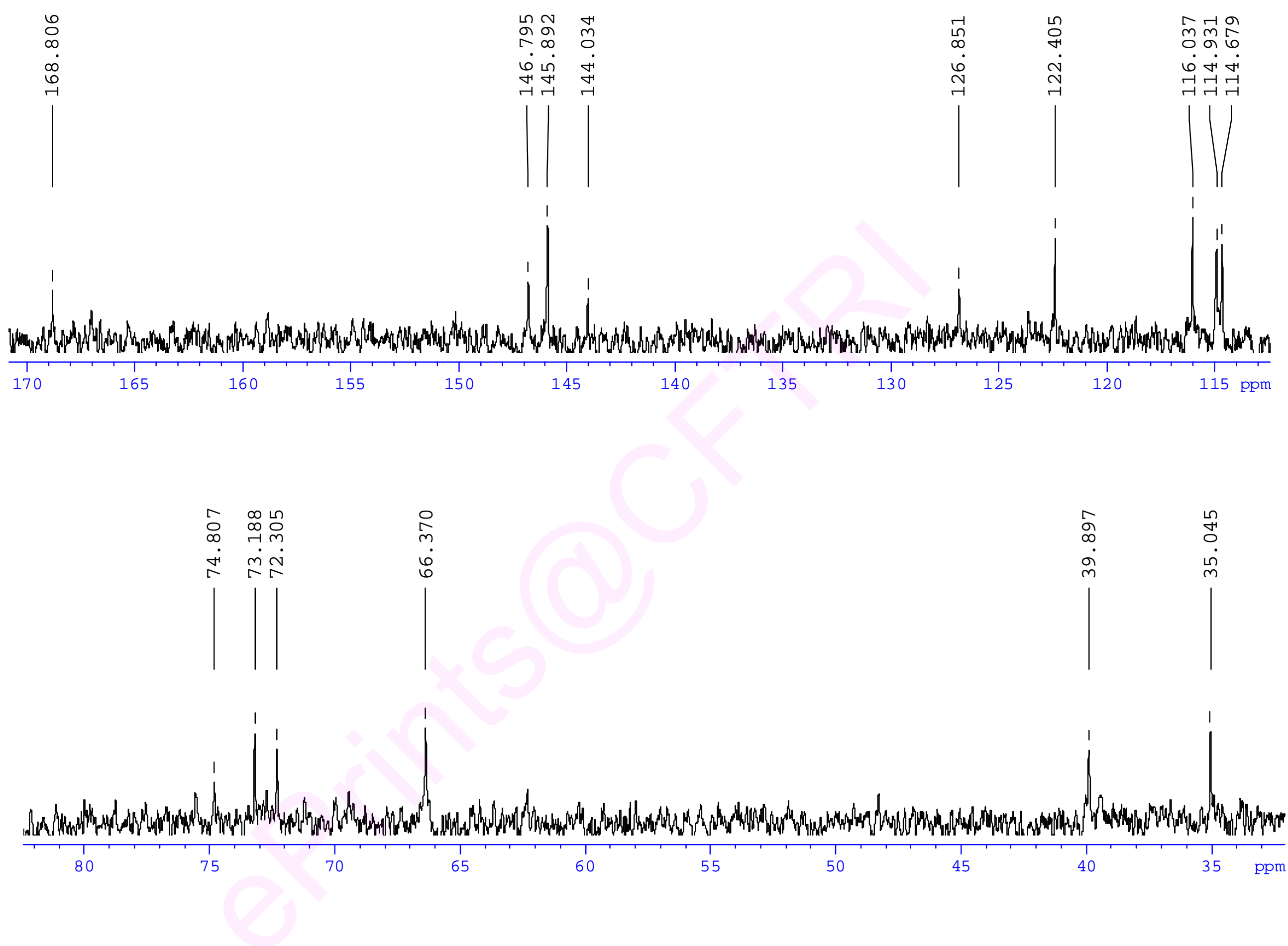

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Fig. 4.1.13a. ^{13}C NMR spectrum of compound 2

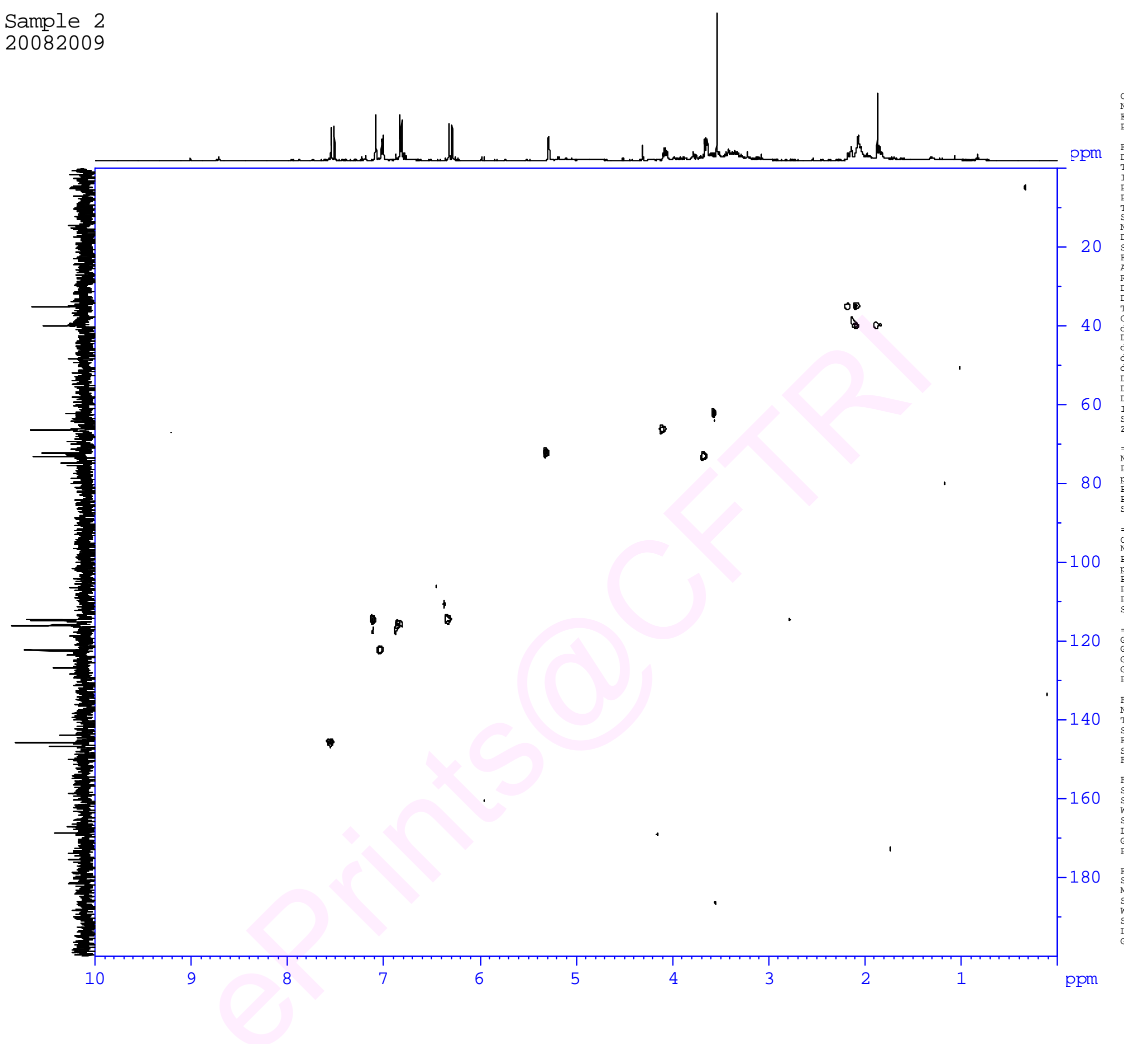

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Fig. 4.1.14. HSQC NMR spectrum of compound 2

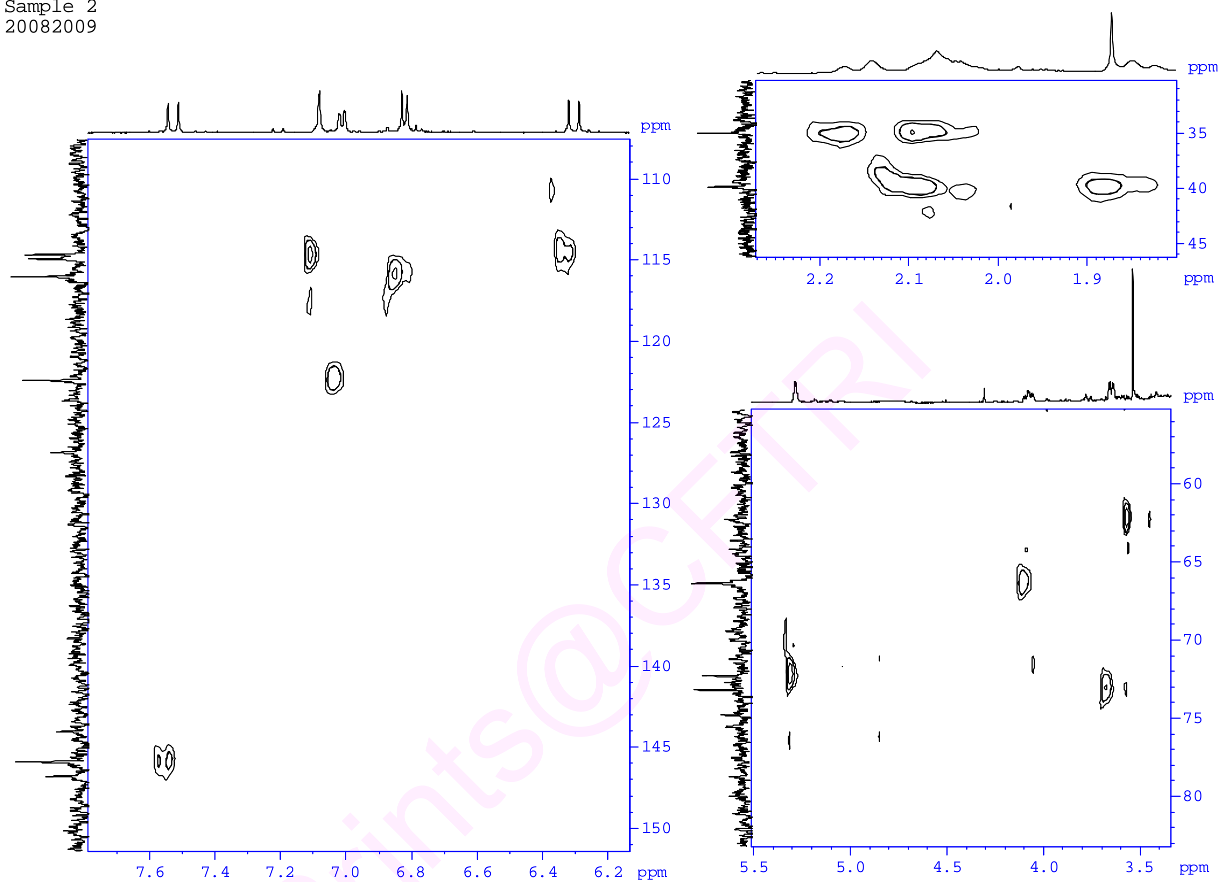

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Fig. 4.1.14a. HSQC NMR spectrum of compound 2

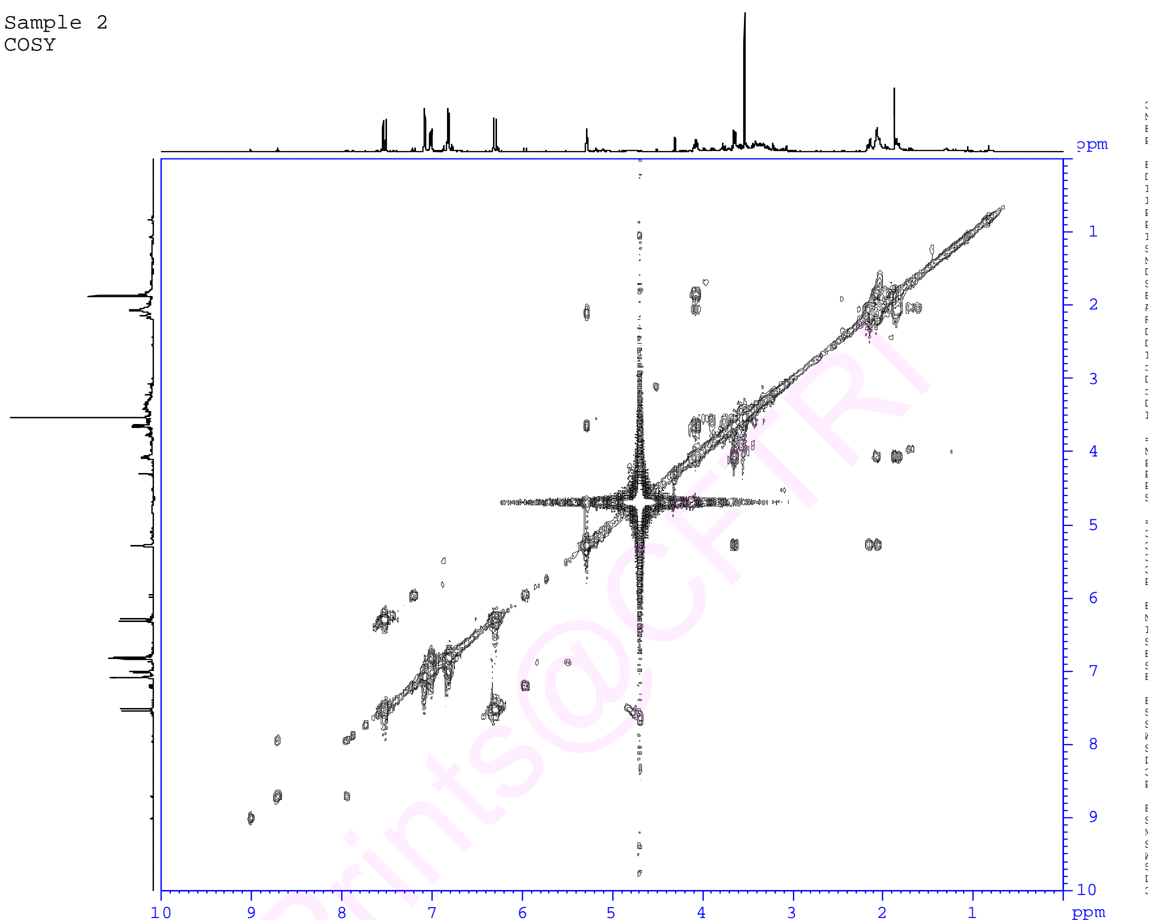

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Fig. 4.1.15. COSY NMR spectrum of compound 2

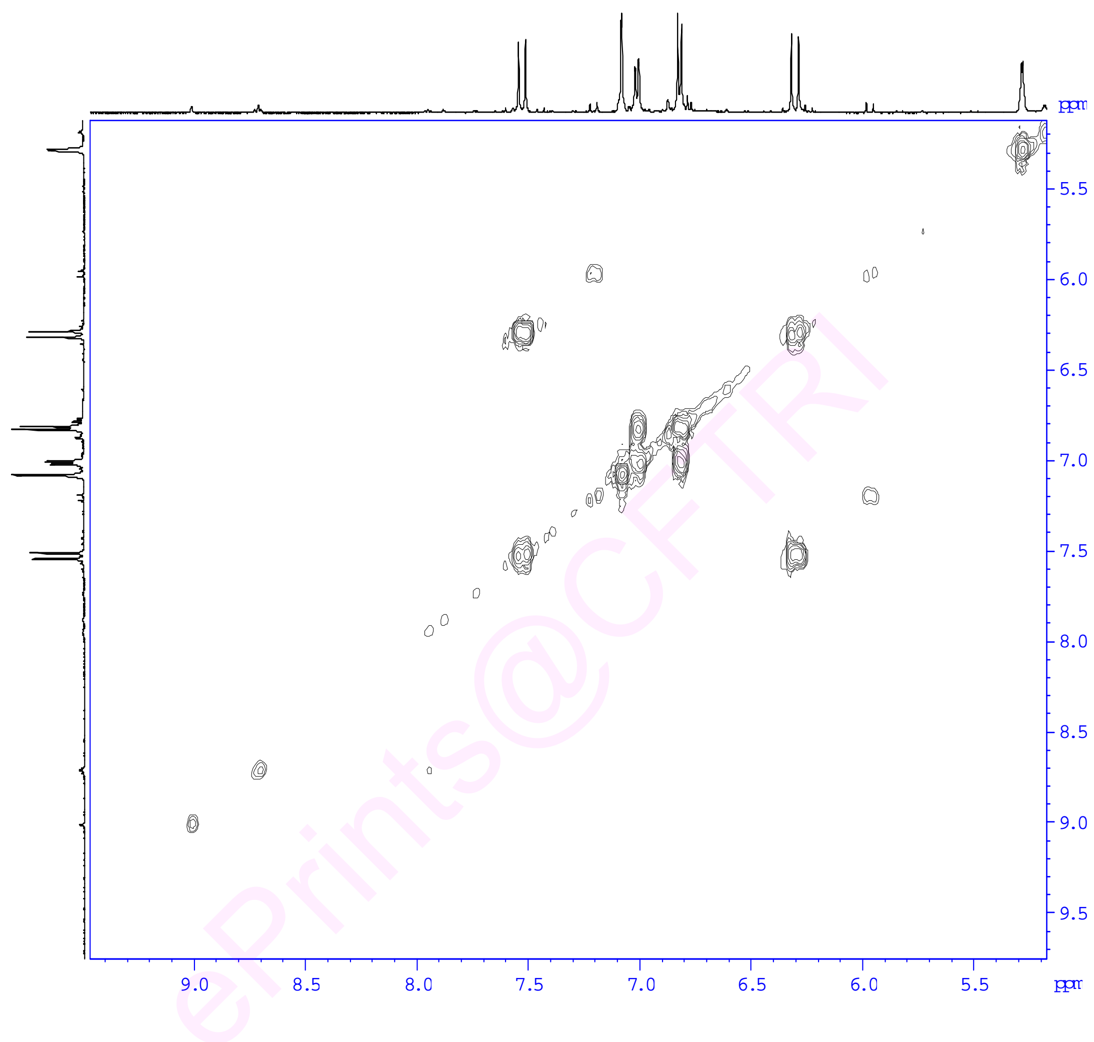

Fig. 4.1.15a. COSY NMR spectrum of compound 2

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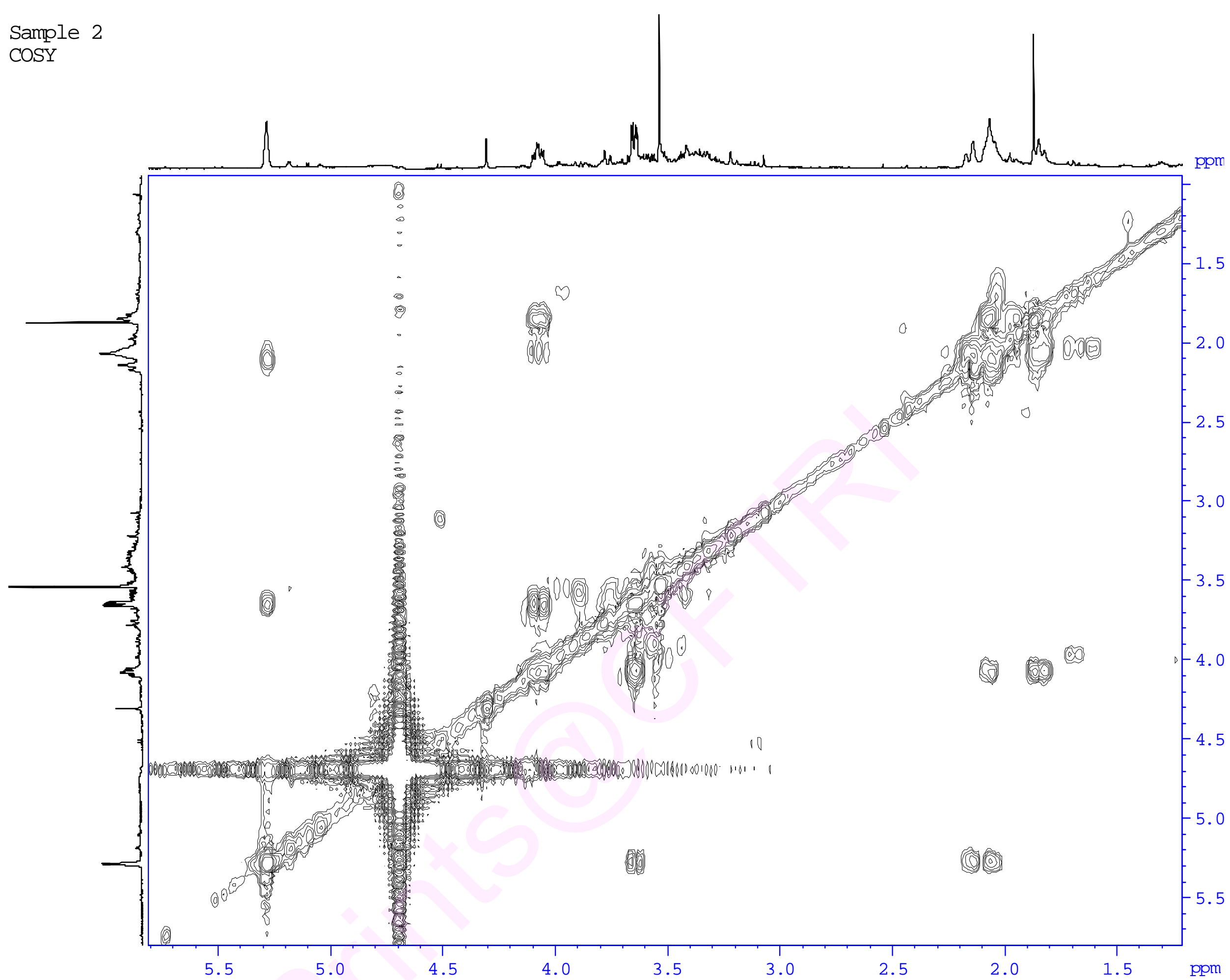

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Fig. 4.1.15b. COSY NMR spectrum of compound 2

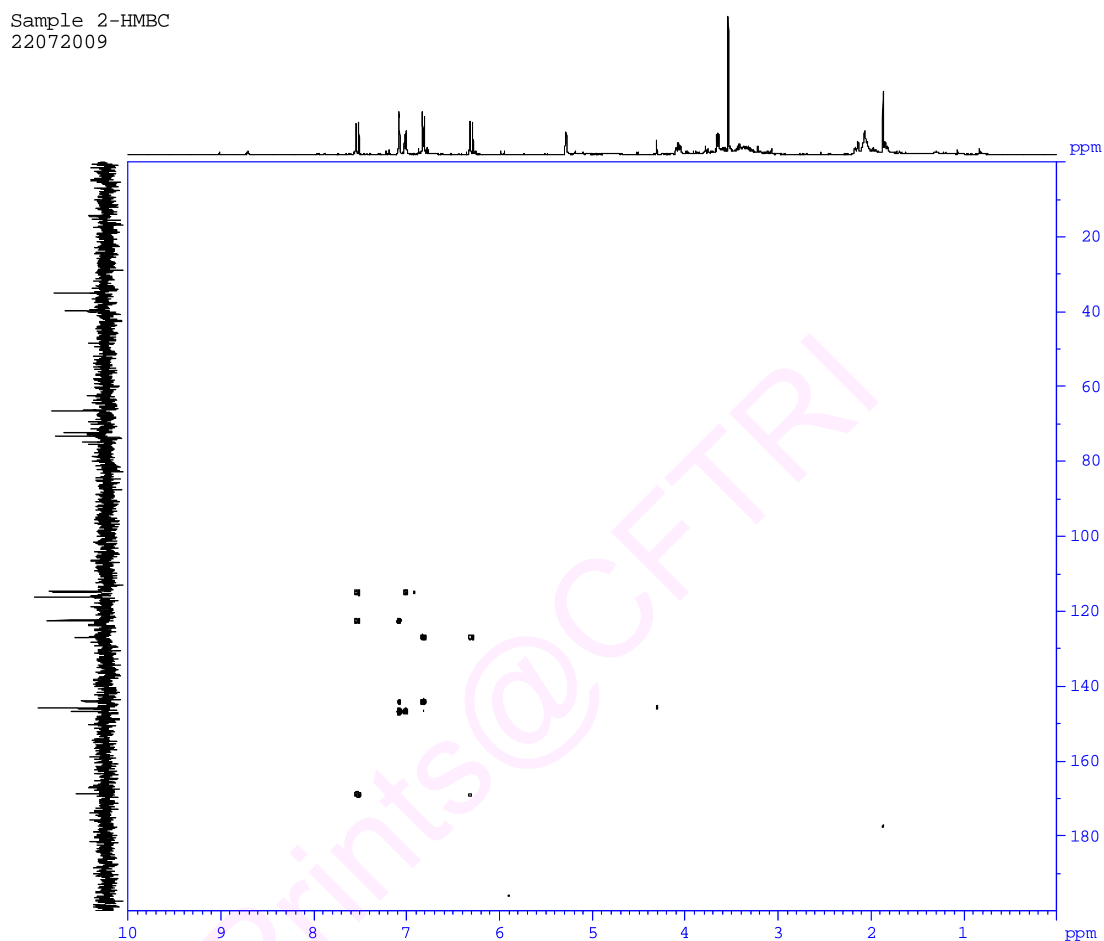

Fig. 4.1.16. HMBC NMR spectrum of compound 2

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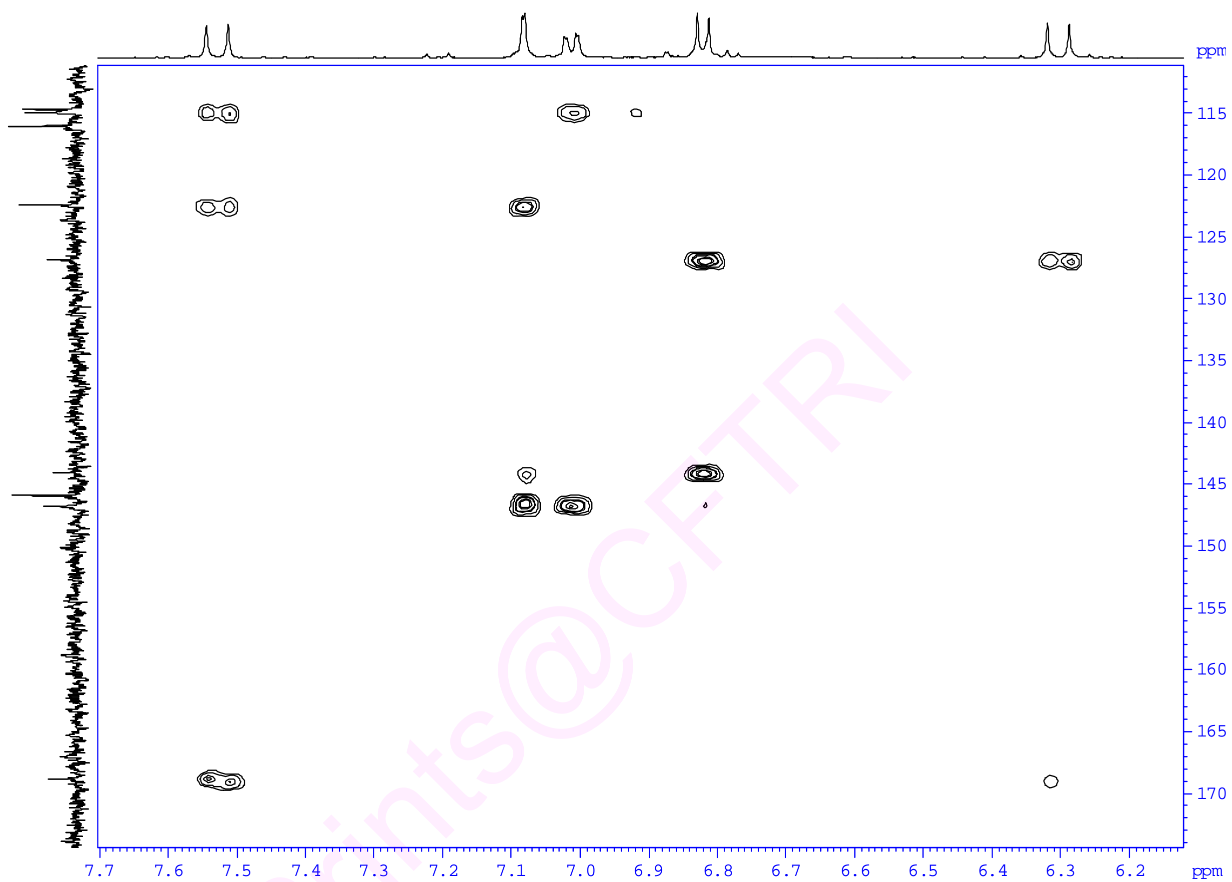

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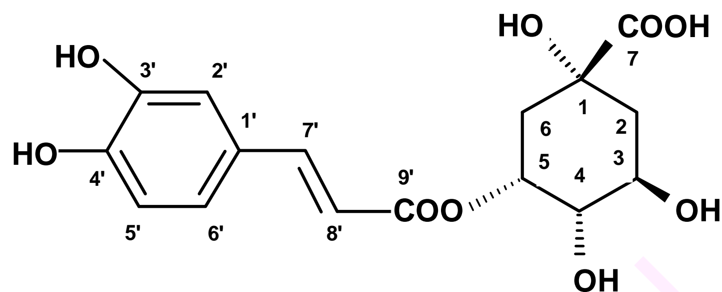
Fig. 4.1.16a. HMBC NMR spectrum of compound 2

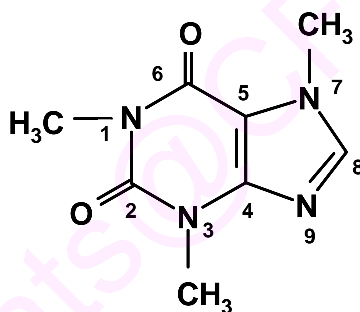
Fraction 3.

The NMR spectra obtained for the fraction 3 were furnished in the following figures (4.1.17 to 4.1.21b). The chemical shift values were compared with the literature report (Parameswaran *et al.,* 2002; Meusinger, 2009; Morishita *et al.,* 1984; Tatefuji *et al.,* 1996; Kelley *et al.,* 1976; Cheminat *et al.,* 1988). It was found to contain two major compounds (3a and 3b) and the compounds are characterised as 5-Caffeoylquinic acid [5-{{(3,4-dihydroxyphenyl)-1-oxy-2-propenyl}oxy}-1,3,4-trihydroxy-1{1S-1α, 3β, 4α, 5α cyclohexane carboxylic acid}] (XXIV) and caffeine (1,3,7-trimethyl xanthine) (XXV). Coupling constants of protons showed that 5H which is equatorial, couples with 4 and 6 axially (J=4, 4.5 Hz) and with 6 equatorially (J =4Hz). Carbon signals clearly shows that the attachment of caffeic acid is at the C5 position of quinic acid. The carbon signal of C4 shifted upfield by Δδ -4.2 (δ 71.0 ppm) and C6 signal also shifted upfield Δδ –1.2 (δ 36.2 ppm) when compared to the literature values of quinic acid (Kelley *et al.*, 1976) . The slight differences in chemical shift values are observed. These are due to the difference in the solvent used. The fraction dissolved in pure D_2O in the present study for NMR analysis, where as earlier reports used CD_3OD. The spectral assignments are furnished in Tables 4.1.3 and 4.1.4. HSQC and HMBC spectral studies confirmed the assignments. Apart from the signals due to caffeine and 5 CQA, some signals with minor intensity were

observed in the ^{13}C and ^{1}H spectra of fraction 3, which may be due to the presence of 4 CQA (XXVI).



XXIV. Compound 3a: 5-Caffeoylquinic acid



XXV. Compound 3b: Caffeine

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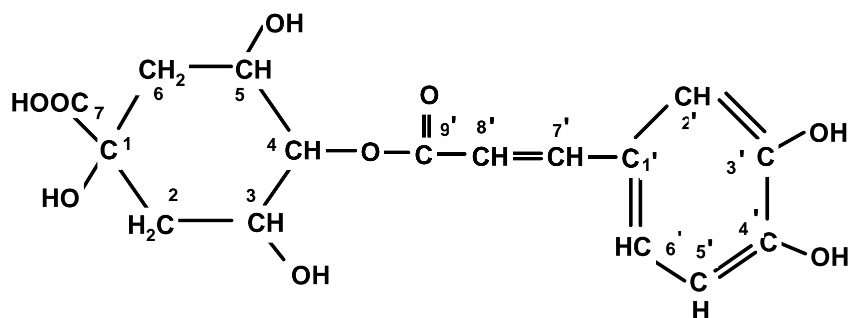

XXVI. 4-Caffeoylquinic acid

Table 4.1.3. ^{1}H and ^{13}C NMR chemical shifts and connectivity by HMBC of compound 3a

H/C	δ of proton (ppm)	Multiplicity	Coupling constant J (Hz)	δ of carbon (ppm)	HMBC
1	-	-	-	74.5	
2 eq	1.94	dd	13,4	36.2	C1, C3, C6, C7
2 ax	2.11	dd	3.5, 14		
3	4.12	ddd	3.5,6.5,2.5	68.8	
4	3.76	dd	3.5, 8.5	71.0	C5, C6
5	5.14	ddd	4, 4, 4.5	70.4	C4, C9’
6 ax	2.06	dd	4.5, 13	36.2	C1, C2, C5
6 eq	2.20	dd	13, 4		
7	-	-	-	176.6	
1′	-	-	-	126.1	
2′	6.65	d	1.5	113.8	C6’, C3’, C4’
3′	-	-	-	142.8	
4$^{7'}$	-	-	-	146.8	
5′	6.55	d	8	115.4	C1’, C3’, C4’
6′	6.64	dd	1.5, 8	121.9	C2’, C4’, C7’
7′	7.17	d	16	143.9	C1’, C6’, C8’, C9’
8′	5.98	d	16	114.1	C1’, C9’
9′	-	-	-	168.2	

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Table 4.1.4. ^{1}H and ^{13}C NMR chemical shifts and connectivity by HMBC of compound 3b

H/C	δ of proton (ppm)	Multiplicity	δ of carbon (ppm)	HMBC
1	-		-	
2	-		151.9	
3	-		-	
4	-		148.5	
5	-		107.3	
6	-		155.5	
7	-		-	
8	7.63	s	141.6	C5, C4, N7- CH_3
N1-CH_3	3.04	s	27.6	C6, C2
N3- CH_3	3.20	s	29.6	C2, C4
N7- CH_3	3.66	s	33.1	C5, C8

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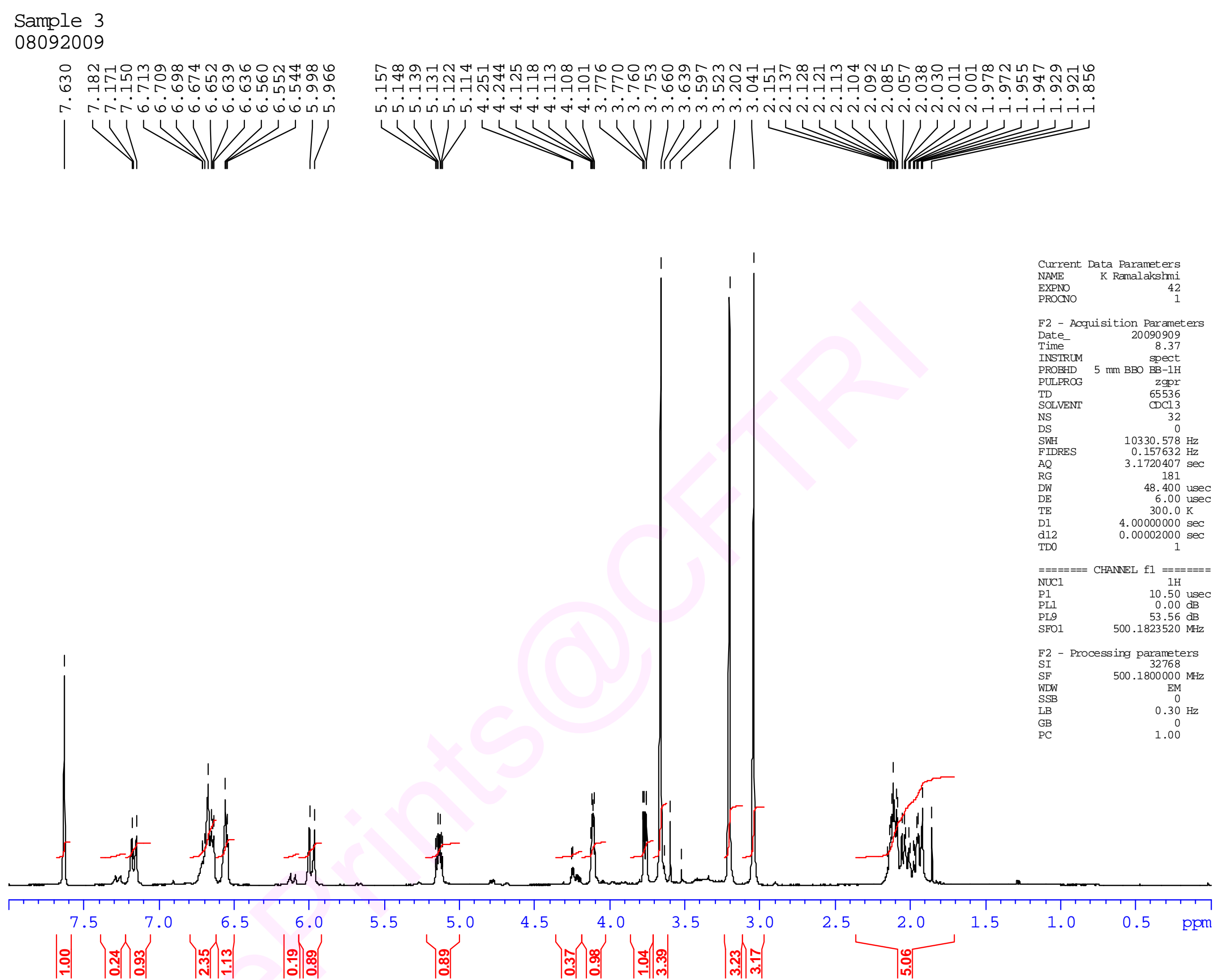

Fig. 4.1.17. ^{1}H NMR spectrum of compound 3

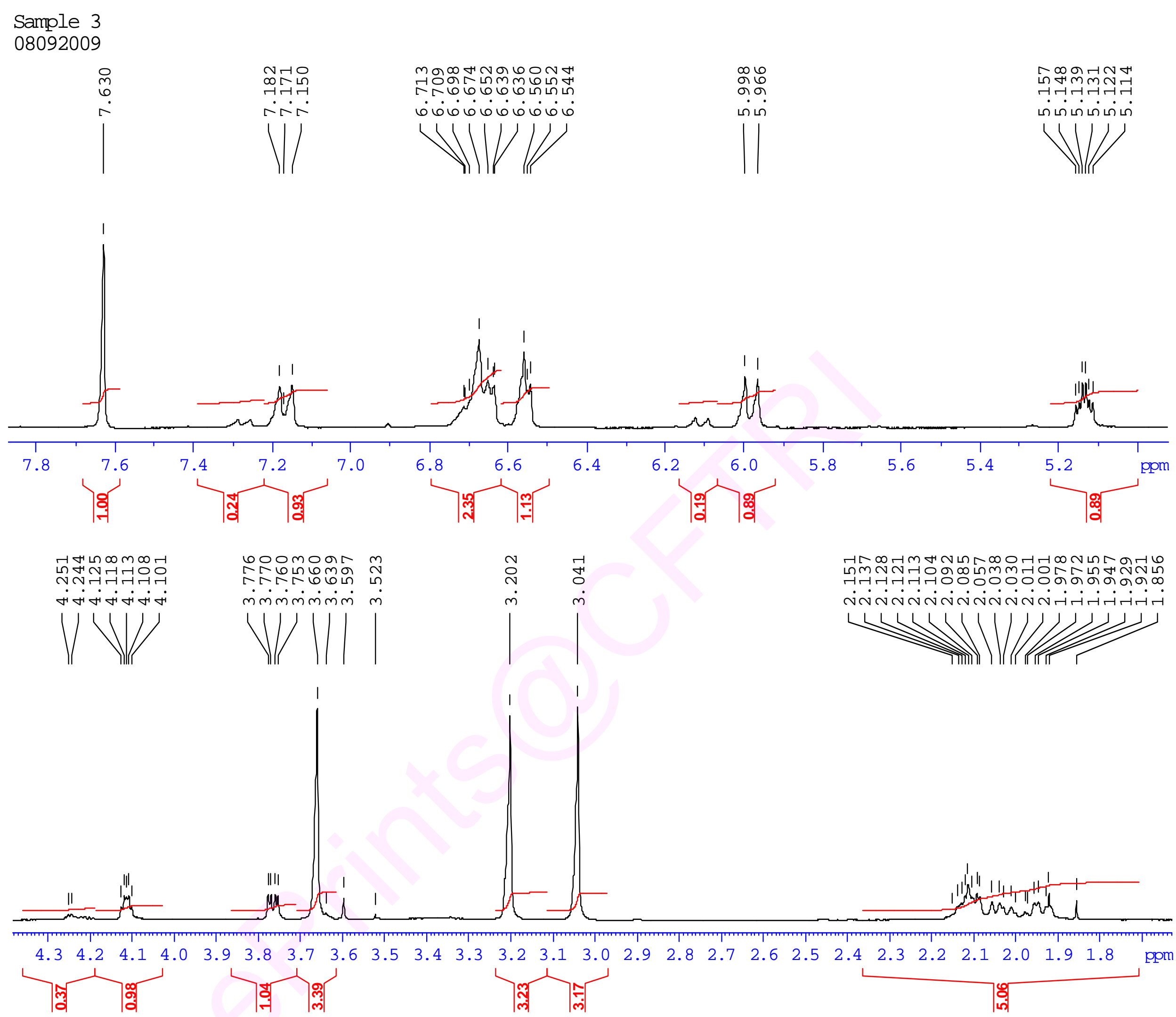

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Fig. 4.1.17a. ^{1}H NMR spectrum of compound 3

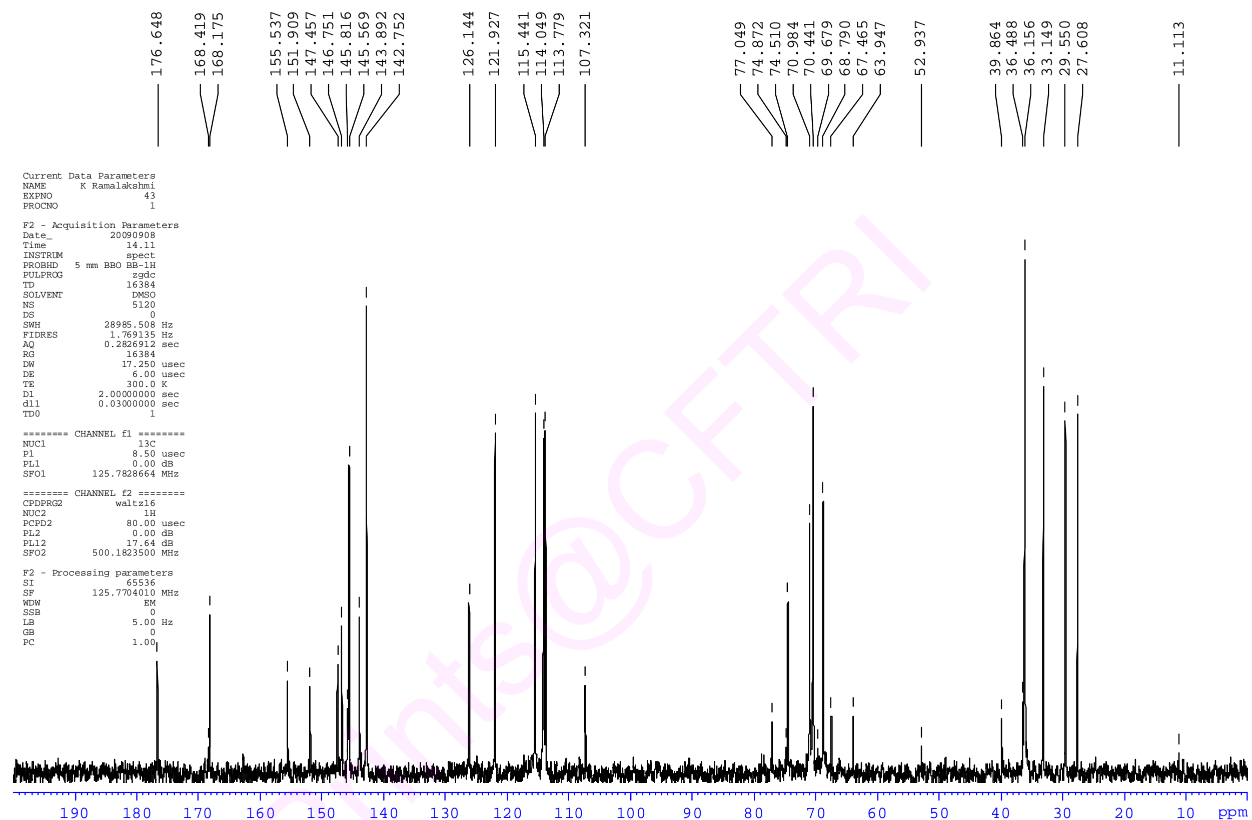

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Fig. 4.1.18. ^{13}C NMR spectrum of compound 3

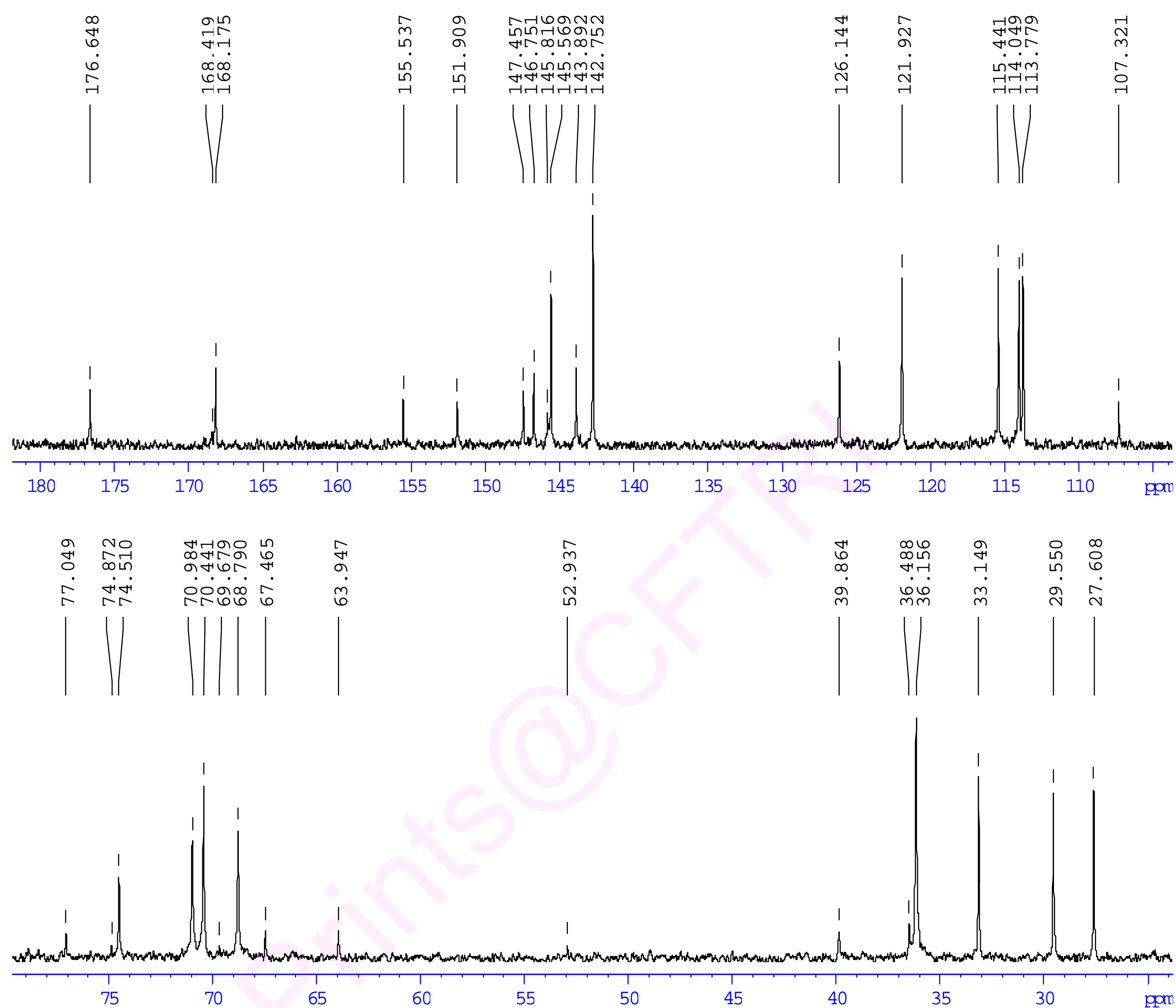

Fig.4.1.18a. ^{13}C NMR spectrum of compound 3

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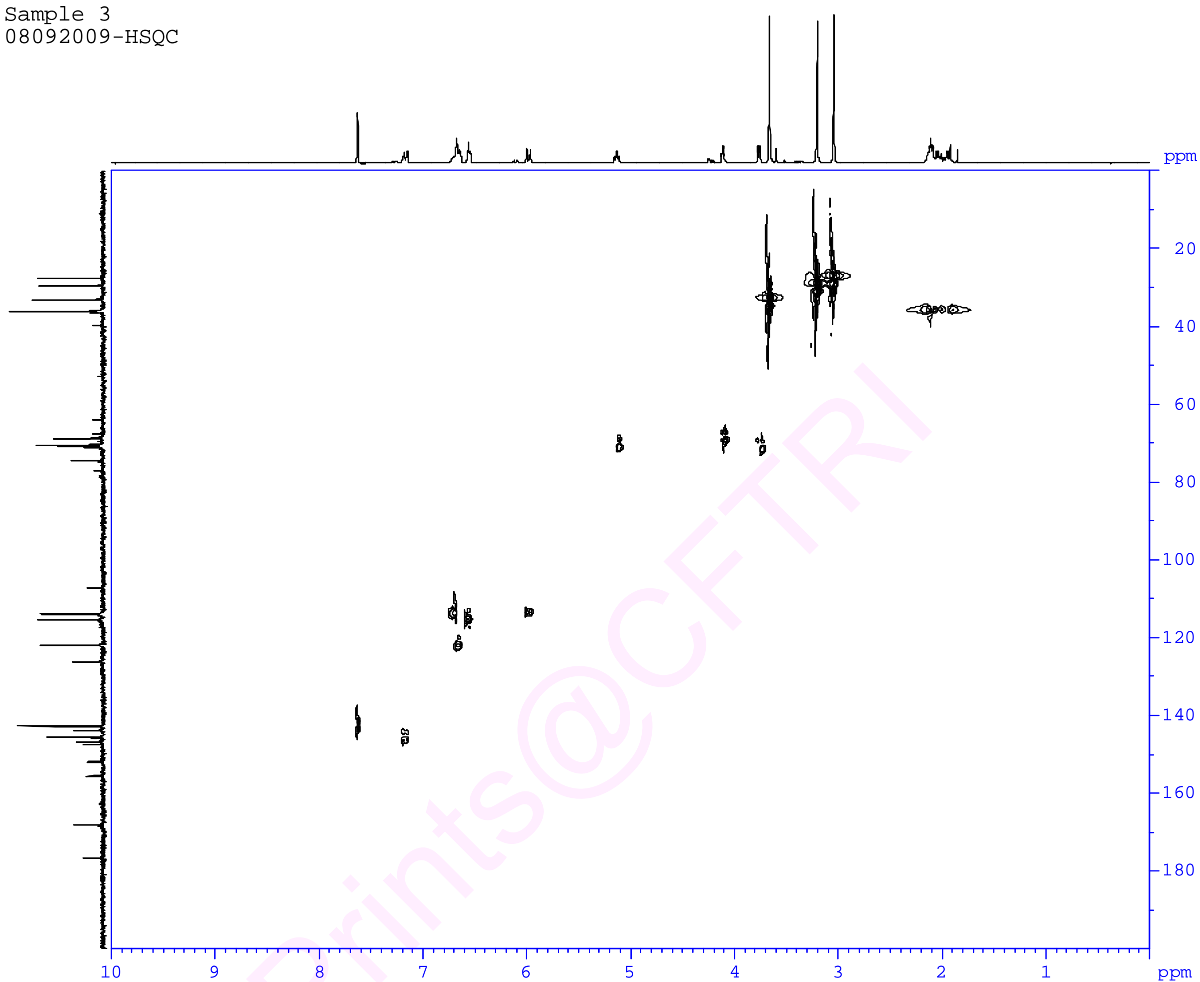

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Fig. 4.1.19. HSQC NMR spectrum of compound 3

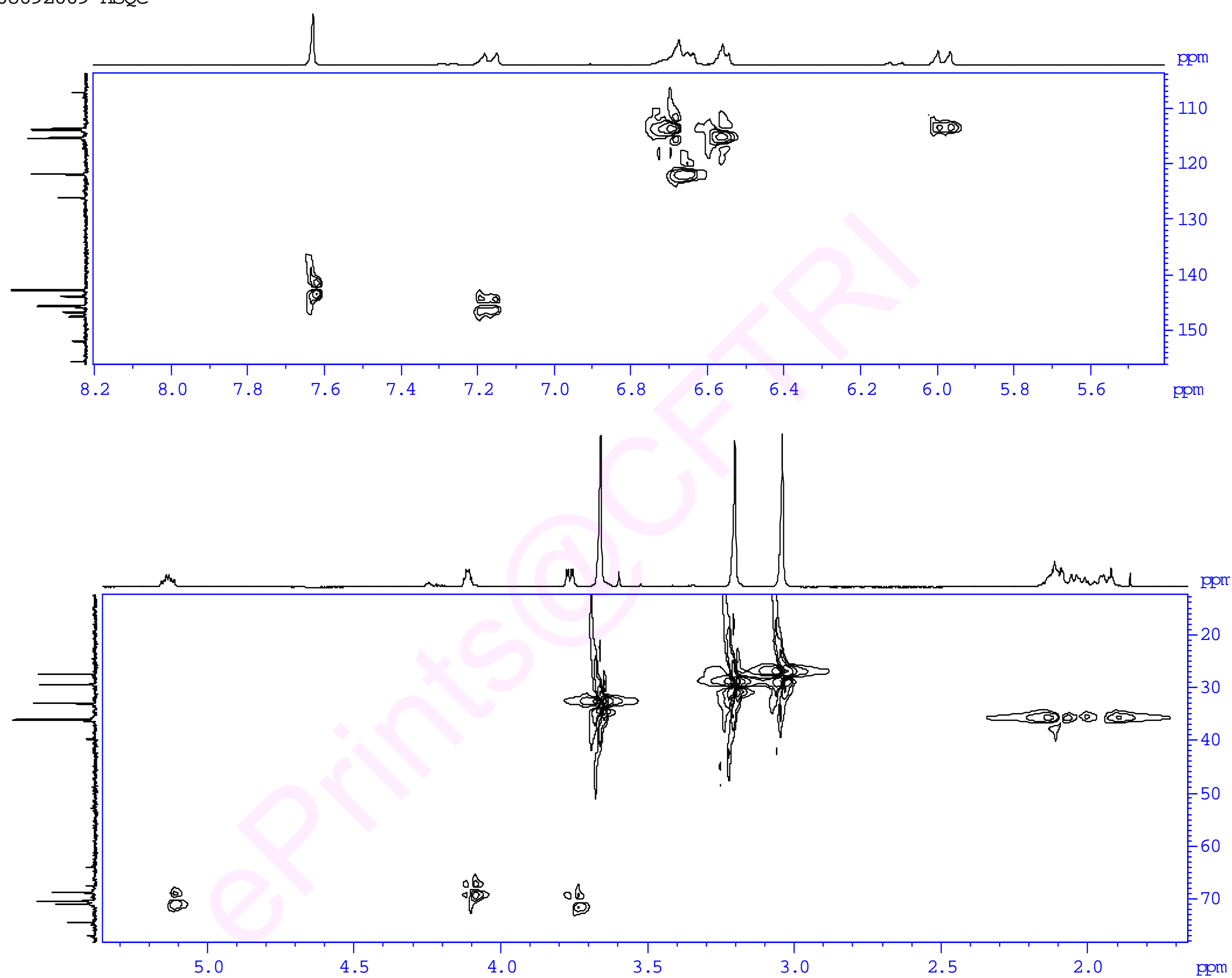

Fig. 4.1.19a. HSQC NMR spectrum of compound 3

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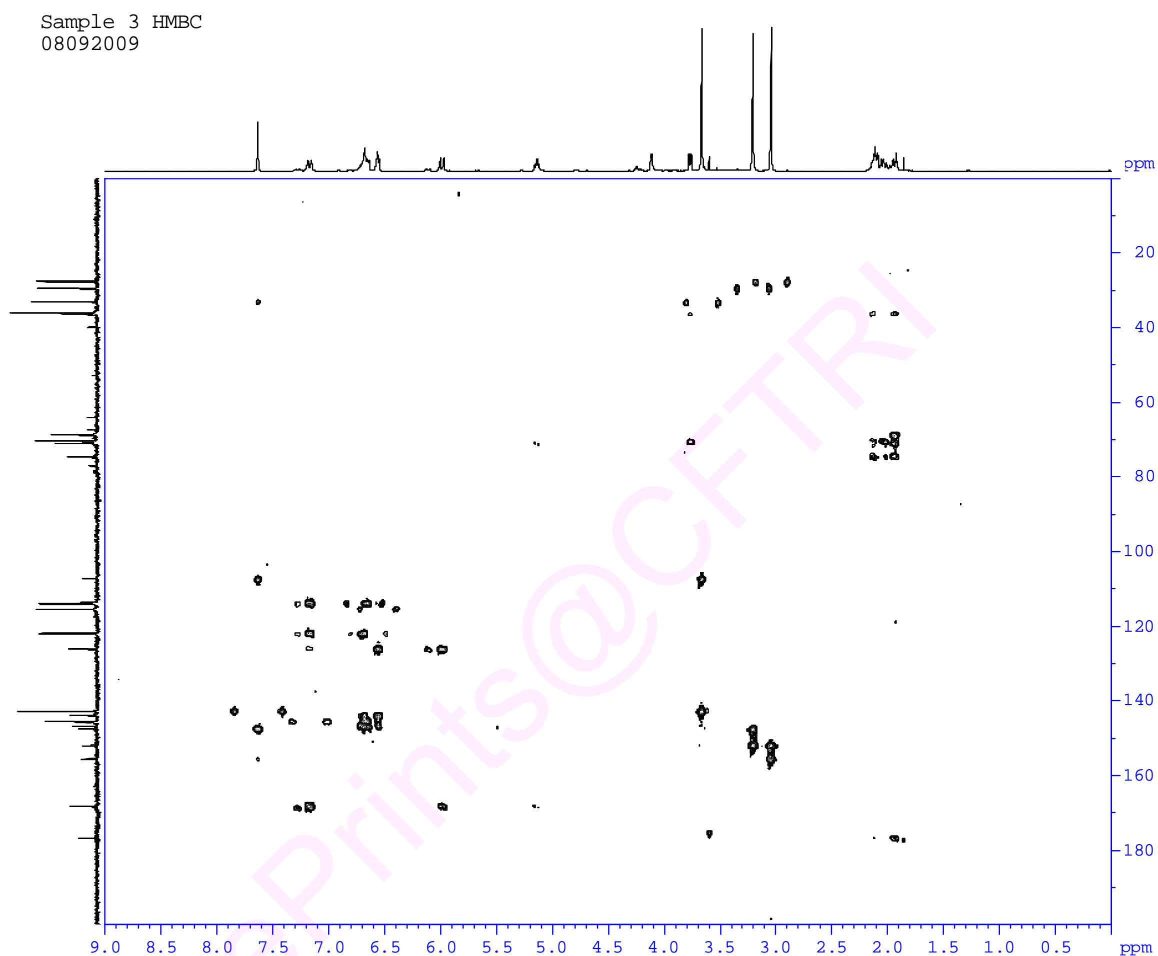

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Fig. 4.1.20. HMBC NMR spectrum of compound 3

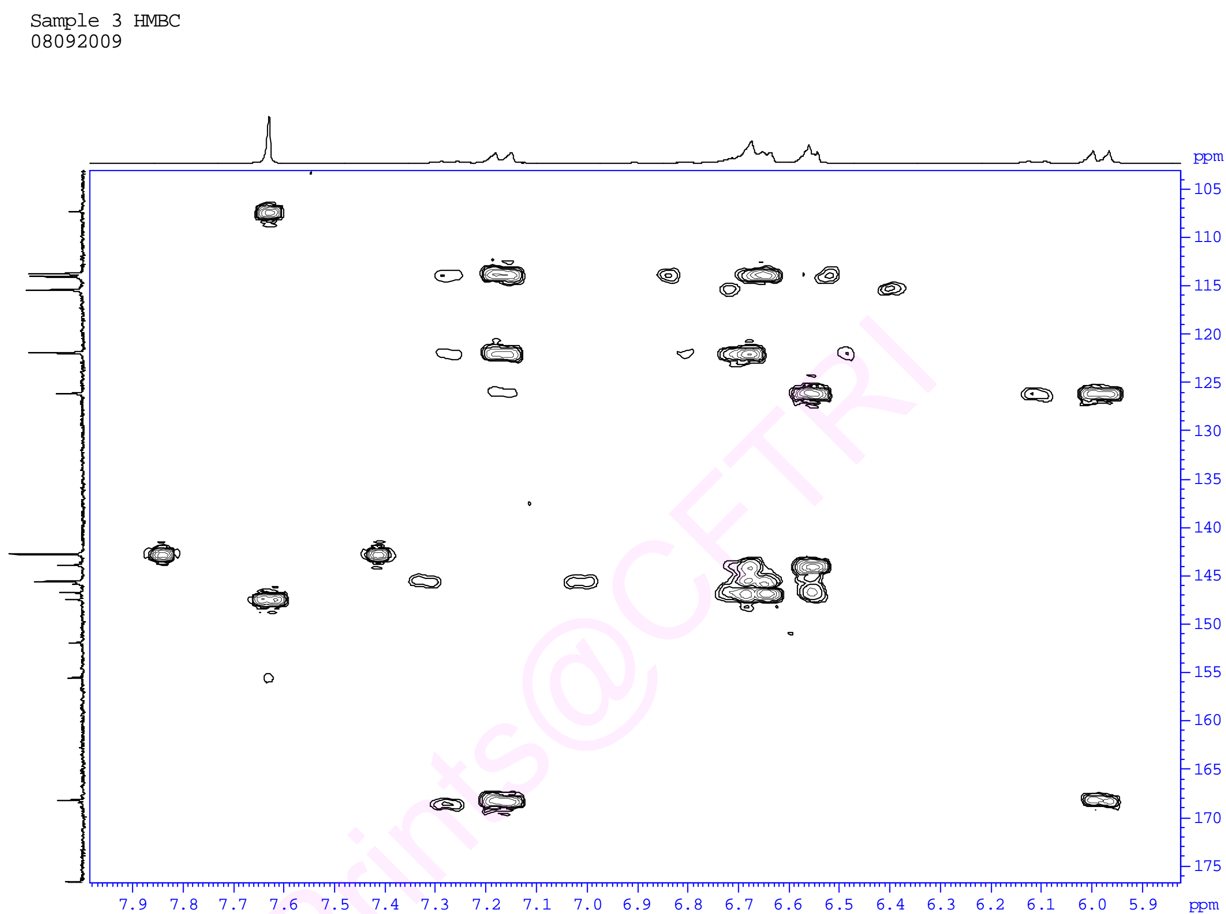

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Fig. 4.1.20a. HMBC NMR spectrum of compound 3

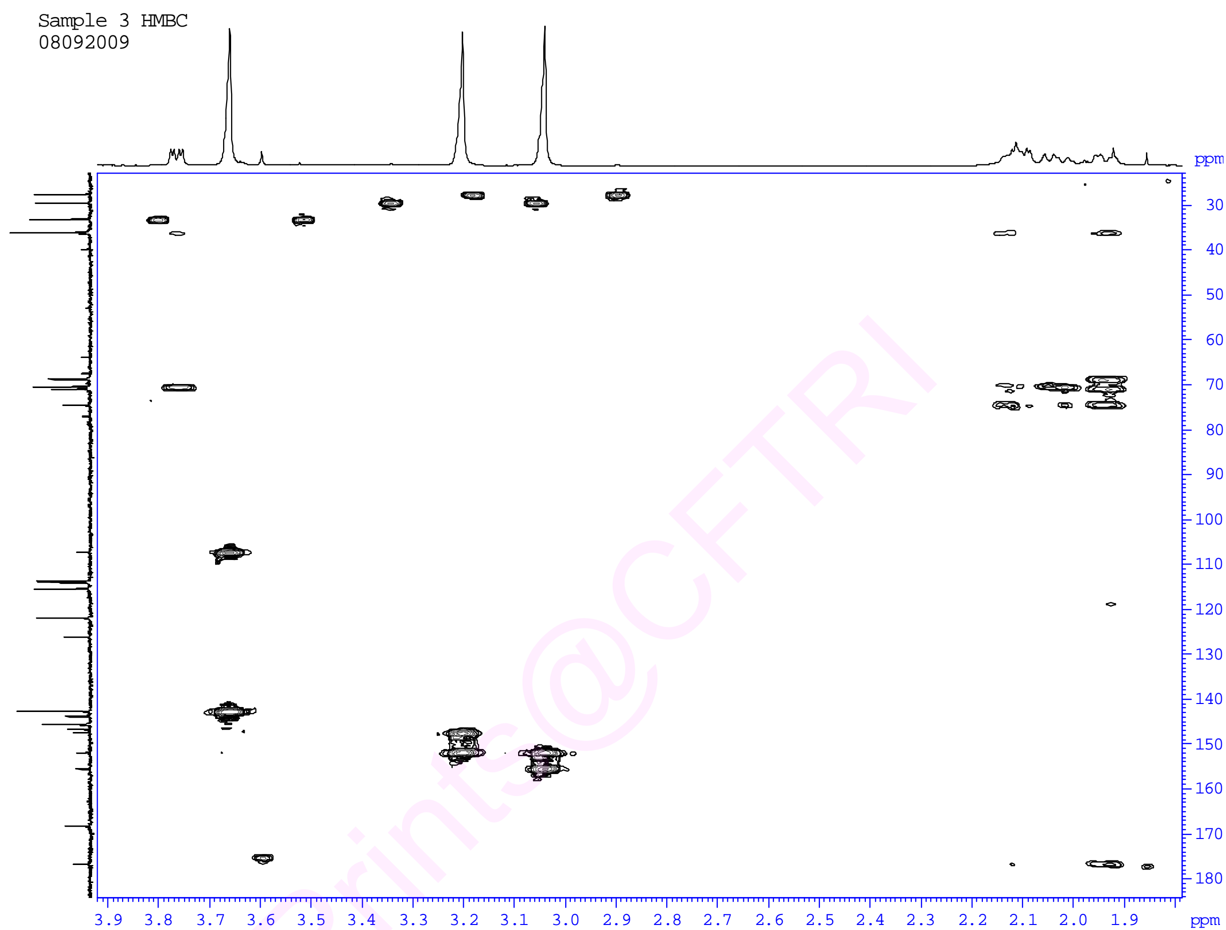

Fig. 4.1.20b. HMBC NMR spectrum of compound 3

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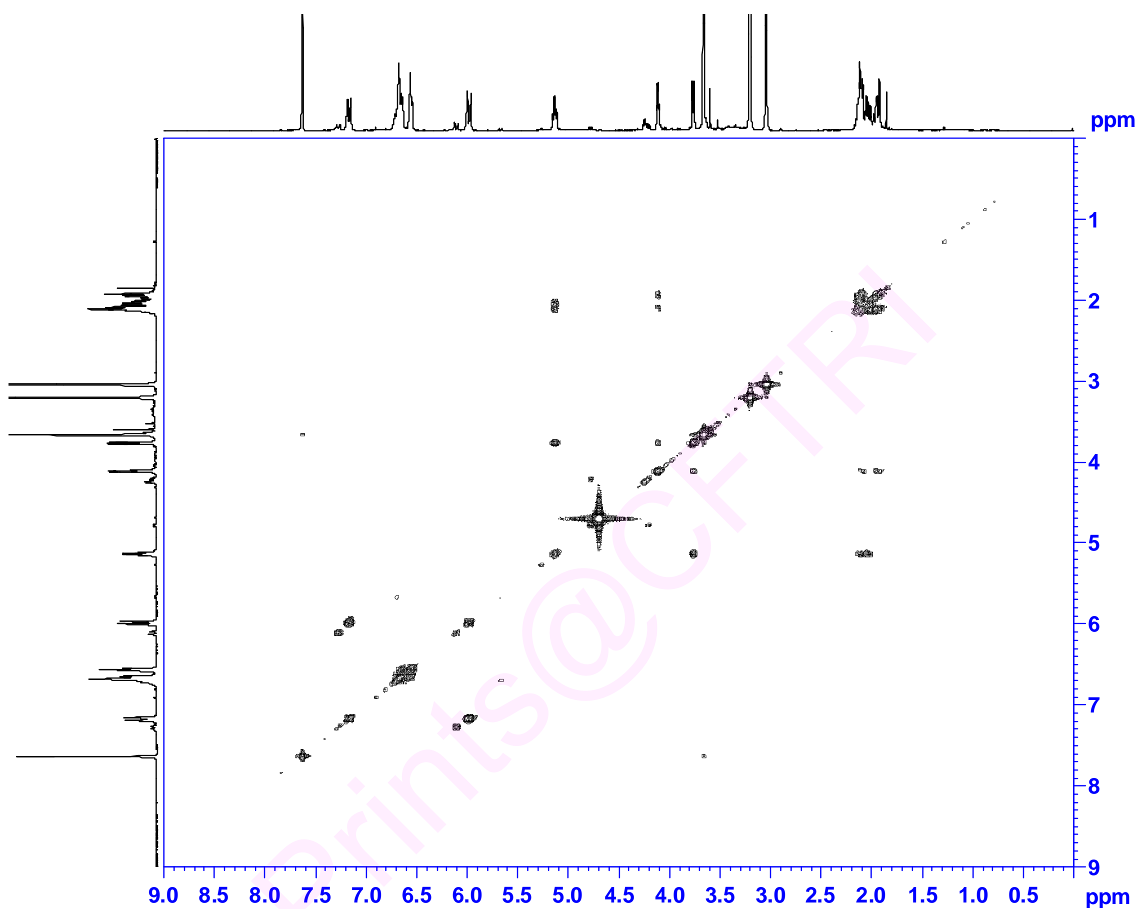

Fig. 4.1.21. COSY NMR spectrum of compound 3

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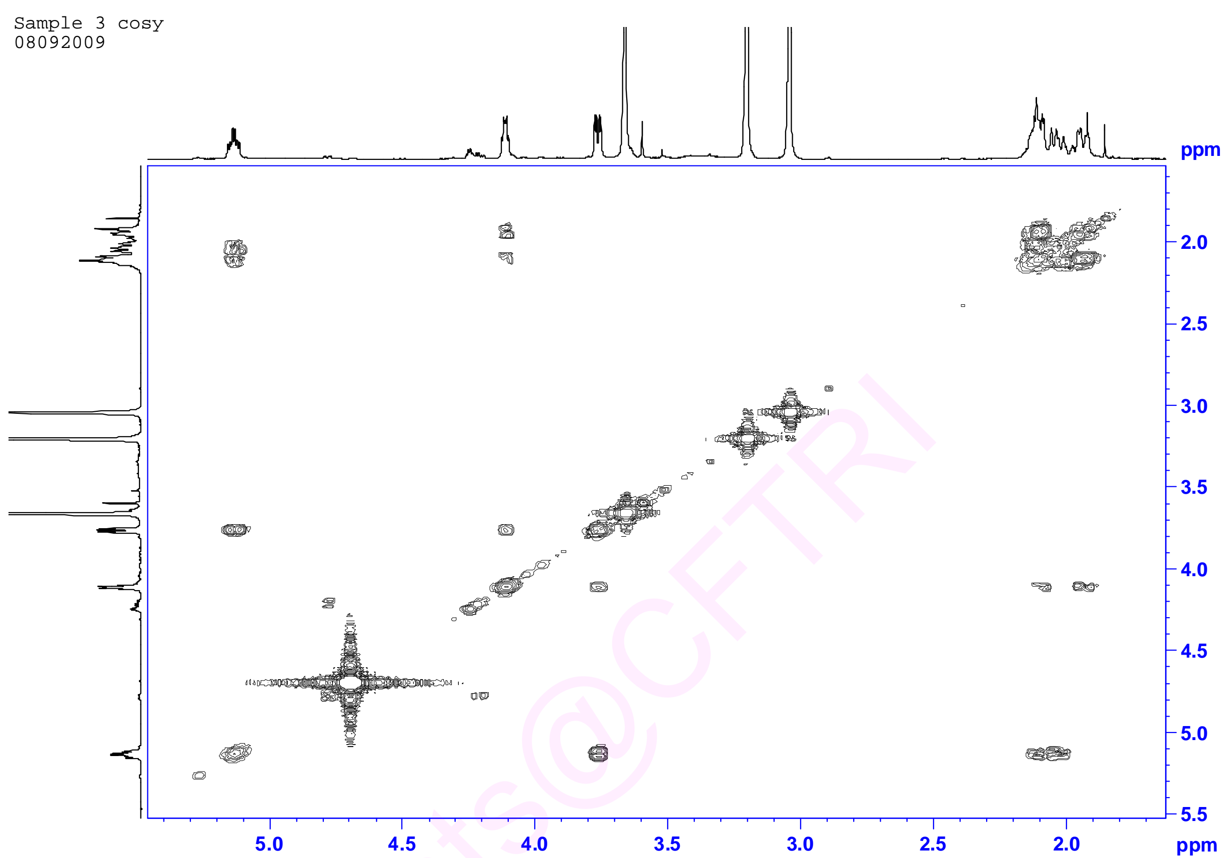

Fig. 4.1.21a. COSY NMR spectrum of compound 3

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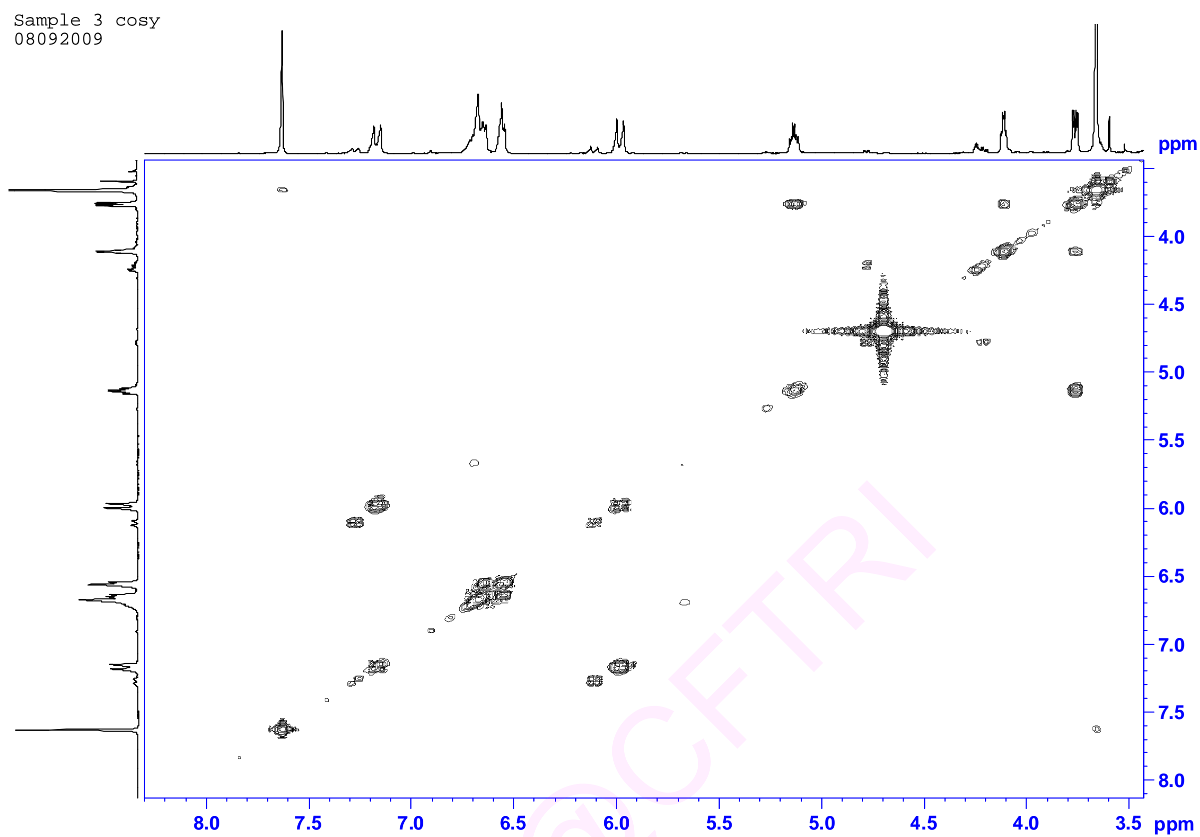

Fig.4.1.21b. COSY NMR spectrum of compound 3

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Chlorogenic acid isomers composition of LCB extract (Me_1)

When the Me_1 of LCB was analysed by preparative HPLC followed by the NMR analysis confimed the presence of trigonelline. It is present 1.16% in the extract. There is no literature report about the presence of trigonelline in the green coffee extract. 3- Caffeoylquinic acid contributes 1.24% in the extract. It could not possible to separate caffeine and 5-caffeoylquinic acids by preparative HPLC since their retention times are very closer. Retention time of 4 and 5-caffeoylquinic acids are also very closer and hence the combined concentration is calculated and presented in the Table (4.1.5.). The concentration of the compounds in fractions (4-6) containing the dimers of caffeoylquinic acids was not sufficient for NMR spectral analysis. However, HPLC profile was similar to the literature reports (Balyaya & Clifford (1995). Hence, the peaks 4, 5 and 6 are tentatively characterised as 3,4 diCQA, 3,5 diCQA and 4,5 diCQA respectively with reference to retention times and their quantities are provided. The chlorogenic acid isomers composition of Me1 along with trigonelline is given in Table 4.1.5.

Table 4.1.5. Composition of Me_1 extract

compound	%
Trigonelline	1.16
3 CQA	1.24
4 CQA & 5 CQA	21.22
unknown	1.22
3,4 diCQA	1.26
3,5 diCQA	1.37
4,5 diCQA	1.67

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Enrichment of antioxidant compounds / chlorogenic acid isomers

Low-grade coffee beans (LCB) were defatted using hexane and extracted with a polar solvent namely methanol in a soxhlet apparatus. Methanol could able to extract 8.96 ± 1.45% of the solids (Table 4.1.6). Methanol extract (Me_1) was partitioned in chloroform and ethyl acetate individually as well as successively to enrich the extract with phenolics. Soluble and insoluble fractions in the respective solvents were desolventised and the yields of the fractions are presented in Table 4.1.6. When the solvents were used for individual fractionation, chloroform yielded 1.83% and 6.76% as soluble and insoluble fractions respectively with respect to green coffee beans (w/w). In the same way, ethyl acetate yielded 1.20% of soluble and 7.31% of insoluble fractions. During the successive extraction of chloroform followed by ethyl acetate the yields of soluble fractions are 1.71 and 0.48 respectively. The amount of insoluble fraction is 6.45%. When the extract (Me_1) was separated on Dowex resin, the yield of the isolated fraction is 4.66%. The isopropyl alcohol elutes from Diaion HP 20 resulted 3.58% of fraction. The yield of precipitated chlorogenic acid using lead salts method is 1.5%

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Table 4.1.6. Yield (%) of separated fractions of LCB extract

Method of fractionation	Nature / medium of extract	Yield (%)	Actual yield (%) from LCB (w/w)
Methanol	Me_1	8.96 ± 1.45	8.96
Individual extraction			
Chloroform	Soluble, $C(S_1)$	20.48 ± 1.21	1.83
	Insoluble, $C(IS_1)$	75.45 ± 2.45	6.76
Ethyl acetate	Soluble, $EA(S_1)$	13.42 ± 1.08	1.20
	Insoluble, $EA(IS_1)$	81.64 ± 2.99	7.31
Successive extraction			
Chloroform	Soluble, $C(S_2)$	19.06 ± 1.08	1.71
Ethyl acetate	Soluble, $EA(S_2$	5.33 ± 1.01	0.48
	Insoluble $EA(IS_2)$	72.05 ± 2.77	6.46
Resin			
Dowex 50	Water (DW)	52.00 ± 1.98	4.66
Diaion HP 20	Isopropyl alcohol (DN)	40.0 ± 2.11	3.58
Precipitation			
Lead acetate	Water (CHL)	1.59 ± 0.99	0.15

Quality characteristic of fractions

All the fractions were solid and slight yellow in colour in nature. However, ethyl acetate soluble fraction was in the paste form when this solvent was used for extraction after chloroform successively. All the fractions were analysed for polyphenols, chlorogenic acid and caffeine and the results are furnished below.

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Polyphenol content

The polyphenol content (Gallic acid equivalent) of the extracts are furnished in Table 4.1.7. The phenolic compounds present in green and roasted coffee are given in XXVII (Clifford *et al.,* 1987). Methanolic extract was found to contain 16.6± 1.43% polyphenols which was slightly lesser than the extract obtained from chapter 3 due to the difference in the variety of LCB which was purchased. Polyphenol was enriched from 4.05% from raw green triage coffee beans. This shows that methanol is a better solvent for extracting polyphenols due to the polar nature. This is very well matching with the literature reports also (Ramalakshmi *et al.,* 2008). As expected chloroform soluble extracts possess lesser polyphenol than the insoluble extracts when this solvent is used as individual as well as successive extraction due to its low polar nature compared to the phenolic compounds present.

Chloroform is selected to remove the alkaloids such as caffeine from the extracts since caffeine has better solubility in chloroform. C (IS1) yielded 21.08± 1.29% of polyphenols. Total Polyphenol content was distributed in ethyl acetate soluble and insoluble portions as 15.30 ± 1.11% and 16.11± 1.49% respectively.

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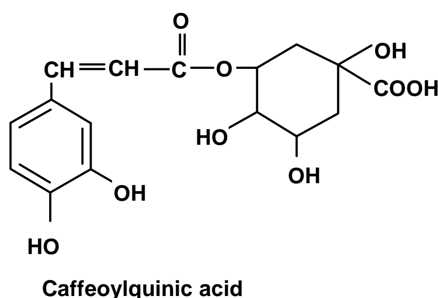
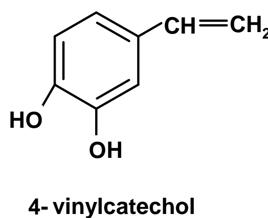
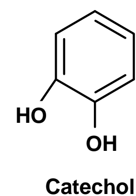


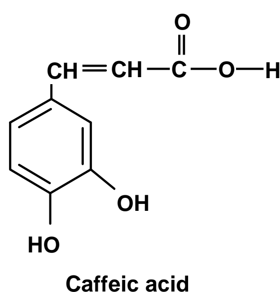
Caffeoylquinic acid



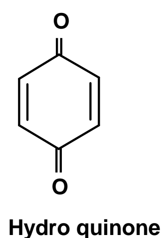
4- vinylcatechol



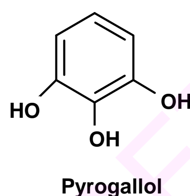
Catechol



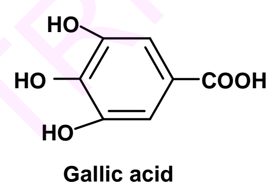
Caffeic acid



Hydro quinone



Pyrogallol



Gallic acid

XXVII. Phenolic compounds present in coffee

Same is the case with successive extraction also, phenols have been found in both the extracts [EA (S_2), EA (IS_2)]. This result brought the conclusion that the quantity of ethyl acetate was not sufficient to isolate the phenols from the extract. Successive extraction of chloroform followed by ethyl acetate was followed (Zhang *et al.,* 2008) for the isolation of phenolics from strawberry fruits. Dowex resin could able to isolate phenolics up to 25.08± 1.83 % from the methanolic extract. Diaion HP 20 was used for the isolation of phenolics compounds by Jayaprakasha *et al.,* (2006), where phenolic constituents of cinnamon fruits were carried out and the compounds have been isolated. However, in our experiments Diaion HP 20 could not give better results with respect to the enrichment phenolics from

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the coffee extract. The isolated chlorgenic acid by precipitation with lead acetate possess maximum phenolic content of 46.33± 1.45%

Table 4.1.7. Polyphenol content of different fractions

Extract	Polyphenol (%)
Me_1	16.6± 1.43
Individual	
$C(S_1)$	2.93± 0.59
$C (IS_1)$	21.08± 1.29
$EA (S_1)$	15.30± 1.11
$EA (IS_1)$	16.11± 1.49
Successive	
$C (S_2)$	1.77± 1.10
$EA (S_2)$	17.80± 1.78
$EA (IS_2)$	18.68± 1.41
Resin	
DW	25.08± 1.83
DN	17.3± 1.19
CHL	46.33± 1.45

Caffeine content

Caffeine is an alkaloid with the substituted purine ring system. The caffeine content of green coffee beans varies according to the species, robusta contains about 2.2%, arabica about 1.2% and the hybrid arabusta about 1.72%. Environmental and agricultural factors appear to have minimal effect on the caffeine content. Caffeine is of major importance with

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respect to the physiological properties of coffee and also in determining the overall cup character of brew.

Methanol extract showed 7.52 ± 1.21% of caffeine from the triage coffee beans (Original caffeine content 1.11%). Chloroform removed most of the caffeine from the methanol extract when chloroform used for isolation of polyphenols. Ethyl acetate also removed caffeine and yielded the insoluble extract with less caffeine. This is expected since caffeine has better solubility in chloroform and ethyl acetate. It is reported that caffeine has a solubility of 15.0 and 4.0 % (w/v) in chloroform and ethyl acetate respectively (Ramalakshmi & Raghavan, 1999). Dowex resin also could separate the caffeine and yielded the extract with less caffeine (3.93± 0.98 %). The caffeine content of DN and CHL fractions are 2.50 ± 0.69 and 0.33 ± 0.11 % respectively.

Radical Scavenging activity of the isolated fractions

Radical scavenging activity of the extracts was tested using DPPH model system (Jayaprakasha & Jagan Mohan Rao, 2000) and the results are presented in Table 4.1.8. The principle involved in this method is that the antioxidants react with the stable free radical i.e., α,α–diphenyl- β-picrylhydrazyl (deep violet colour) and convert it to α,α-diphenyl-β-picrylhydrazine with discoloration. The degree of discoloration indicates the scavenging potential of the antioxidant sample / extracts (Abdille *et al.,* 2005).

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The radical scavenging activity of the extract is increasing with the increase in concentration as expected. C(IS_1) and DW showed more scavenging activity than methanol extract at all the concentration. DW fraction showed maximum activity (>95%) at the concentration of 50 ppm and is comparable to BHA and chlorogenic acids. C(IS_1) showed lesser activity than DW, however it showed more activity than methanol extract. Higher scavenging activities of C(IS_1) and DW is due to the presence of more phenolics as well as chlorogenic acids. CHL showed the highest scavenging activity even at 5 and 10 ppm concentrations, when compared to all other fractions due to the higher amount of phenolics (Table 4.1.8.).

Table 4.1.8. Radical scavenging activities of the separated fractions

Solvents	**Concentration at ppm levels**				
	5	**10**	**20**	**50**	**100**
Me_1	16.94± 3.05	32.79±1.96	84.48±1.08	83.91±0.92 [a]	84.53±0.56
C (IS_1)	35.26 ±2.28	60.12±2078	87.54±0.15	89.34 ±1.37	89.83 ±2.46
DW	22.77±2.78	51.65±1.34	79.45±2.10	95.03 ±0.45	95.60±1.07
CHL	45.67±1.80	76.84±2.06	87.69±1.23	94.78±0.33	95.17 ±0.52
BHA	74.97±2.4	89.03±0.21	92.41± 0.12	95.89± 0.97	95.40±0.35
Chlorogenic acid	54.58 ±3.57	91.73±1.02	93.42±0.98	95.80±0.95	95.95±0.12

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Chlorogenic acids

Chlorogenic acids (CGAs) constituted the second important group of compounds (next to caffeine), representing 6-10% dry matter of the coffee beans. Chlorogenic acids are the key components in coffee and are responsible for determining the beverage quality as well as its antioxidant activity and in turn health benefits. Levels of chlorogenic acid appear to be dependent on species and are unaffected by differences in agronomic practice or method of processing (Clifford and Kazi, 1987)

Five purification methods have been described by Ky *et al.,* 1997, for the isolation of chlorogenic acids from green coffee beans. It is reported that the best method of extraction of chlorogenic acids from green coffee beans is to extract with methanol (70%) using a Tecator Soxtec HT-1043 continuous extraction unit. Our experiments (chapter 3) also resulted with the maximum chlorogenic acid content of 29.6% when methanol was used as the extracting solvent in a soxhlet extraction unit from LCB. During partitioning of Me_1, insoluble extract of chloroform exhibited maximum chlorogenic acid content of 37.3±1.60% in which the chlorogenic acid was enhanced from 29.6%. There is not much enrichment in the chlorogenic acid content when ethyl acetate was used as individual as well as successive extraction. Even Dowex resin could able to isolate the chlorogenic acids rather than the phenolics as total phenolic compounds. The results of the experiments are given in Table 4.1.9. There is slight increase in chlorogenic

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acid content when Diaion HP resin was used for the enrichment. As expected the chlorogenic acid content was highest in CHL from lead salts treatment, compared to all the other extracts. It yielded the extract with 43.5±2.01% of chlorogenic acids. This method of isolation was reported earlier (Hulme, 1953), in which chlorogenic acid was isolated from apple fruits. Crude chlorogenic acids (22.5 g) with the purity of 15.78% were isolated from 70 kg of apple fruit pulp.

Table 4.1.9. Chlorogenic acid content of phenol rich extracts

Sample	Chlorogenic acid (%)
Me_1	29.6±1.01
C (IS_1)	37.3±1.60
EA (IS_2)	30.9±1.231
DW	29.4±1.66
DN	31.9±1.22
CHL	43.5±2.01

Isomers of chlorogenic acids

HPLC profiles of methanol extract and fractions at 325 nm are similar indicating there is no change in the chlorogenic acid composition (Figs. 4.1.21 to 4.1.25). Comparative data for the contents of individual chlorogenic acid isomers of the fractions are given in the Table 4.1.10. The percentages of CQA isomers (XVIII) i.e., 3-Caffeoylquinic acid (3-CQA), 4-

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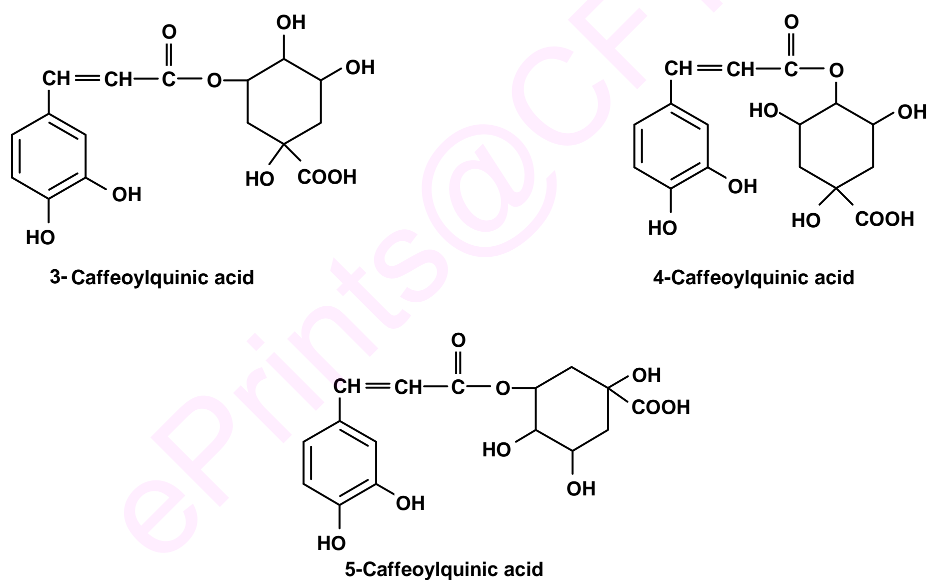
Caffeoylquinic acid (4-CQA) and 5-Caffeoylquinic acid (5-CQA) are increasing in the separated fractions compared to the methanol extract. The levels of FQA isomers (XIX) namely 3-Feruloylquinic acid (3-FQA), 4-Feruloylquinic acid (4-FQA) and 5-Feruloylquinic acid (5-FQA) and diCQA isomers (XXX) namely, 3,4-di Caffeoylquinic acid (3.4 diCQA), 3,5- di Caffeoylquinic acid (3,5 diCQA) and 4,5-di Caffeoylquinic acid (4,5 diCQA) are decreased in the separated fractions. Balyaya and Clifford (1995) reported around 54-58% of CQA isomers, 10-12% of FQA isomers and 18-19% of diCQA isomers in methanol extract of robusta cherry coffee beans.



XXVIII. CQA Isomers

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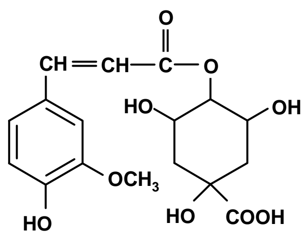
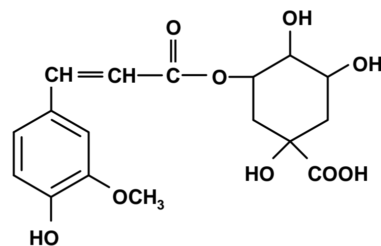
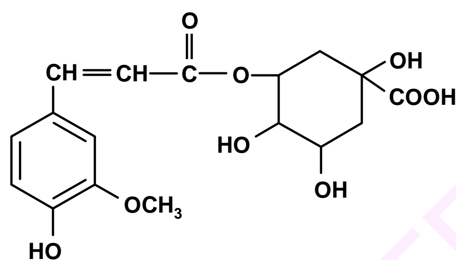


4-Feruloylquinic acid

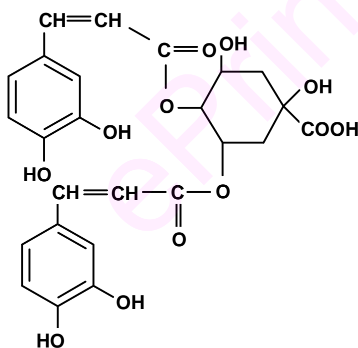


3-Feruloylquinic acid

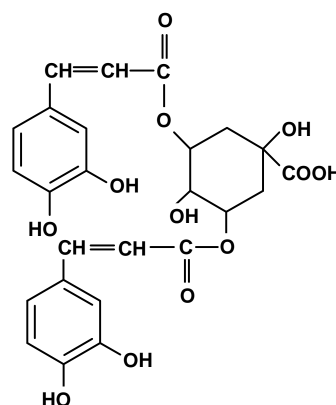


5-Feruloylquinic acid

XXIX. FQA Isomers



3,4-dicaffeoylquinic acid



3,5-dicaffeoylquinic acid

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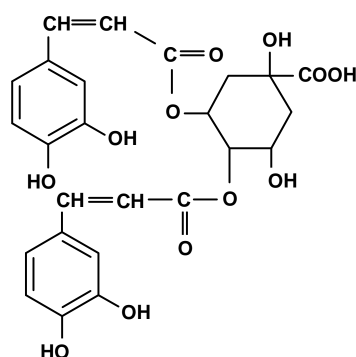

4,5-dicaffeoylquinic acid

XXX. diCQA Isomers

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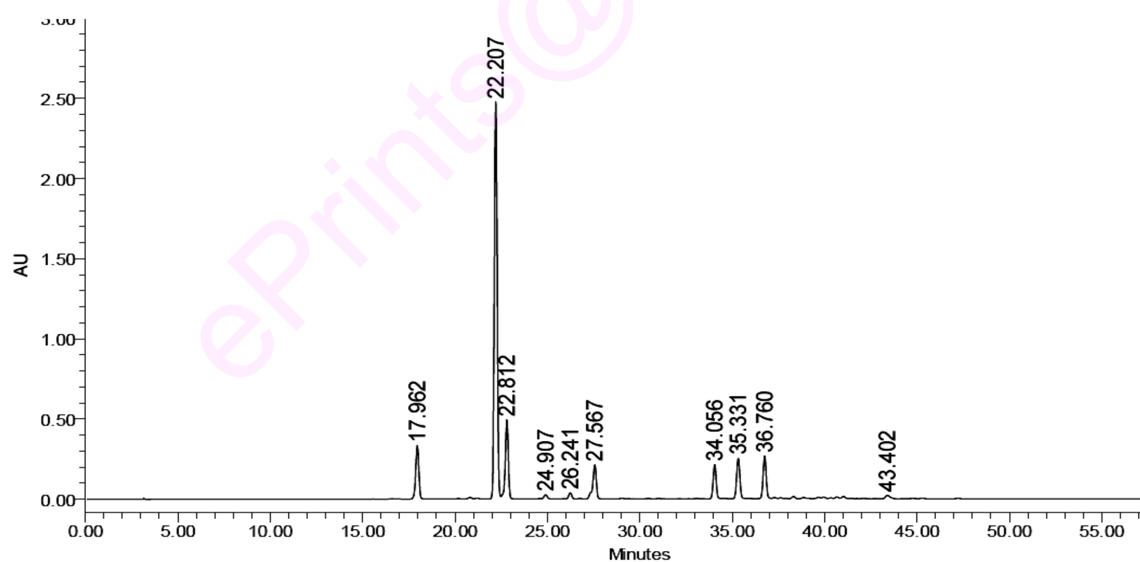

Fig. 4.1.22. HPLC profile of C(IS_1)

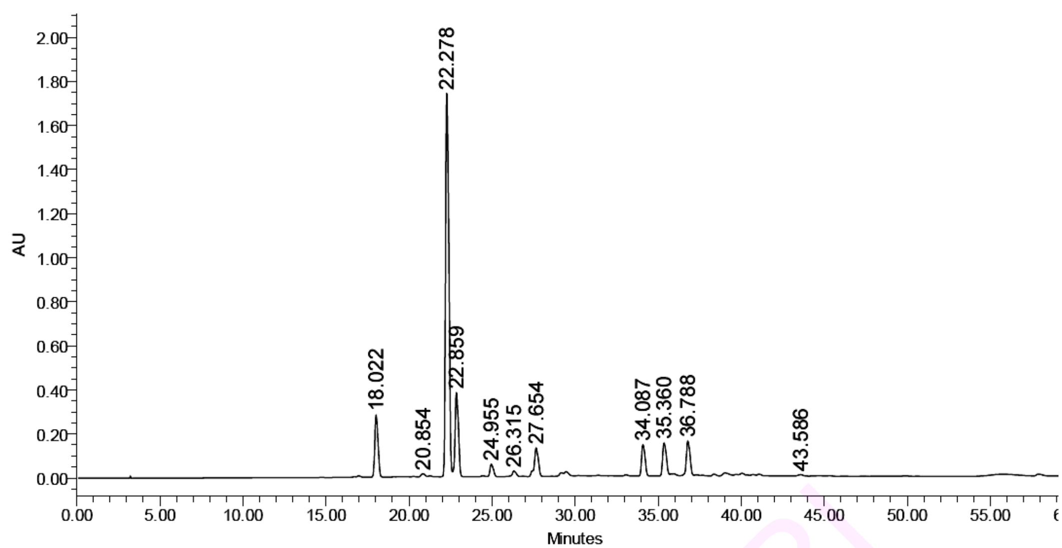

Fig. 4.1.23. HPLC profile of DW

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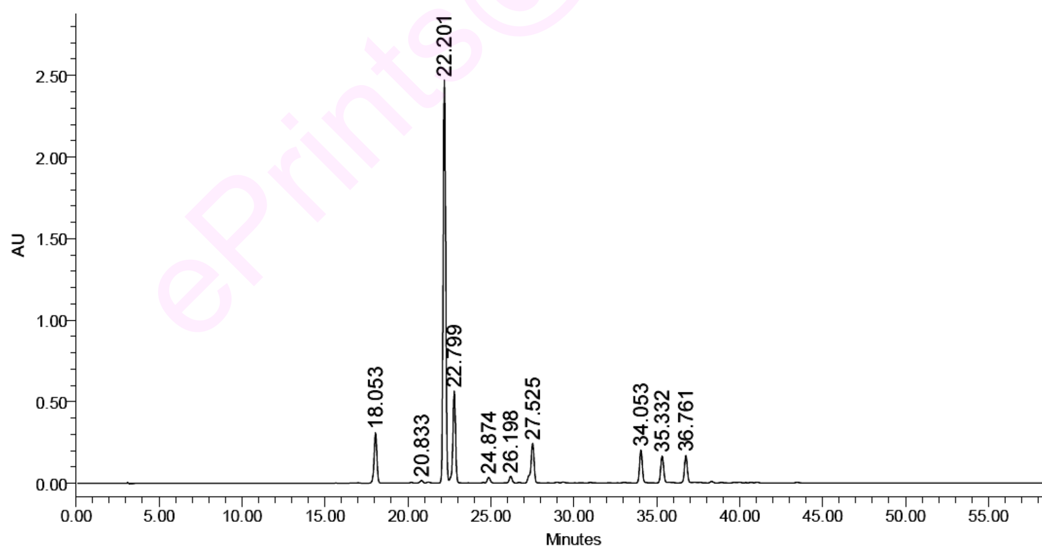

Fig. 4.1.24. HPLC profile of DN

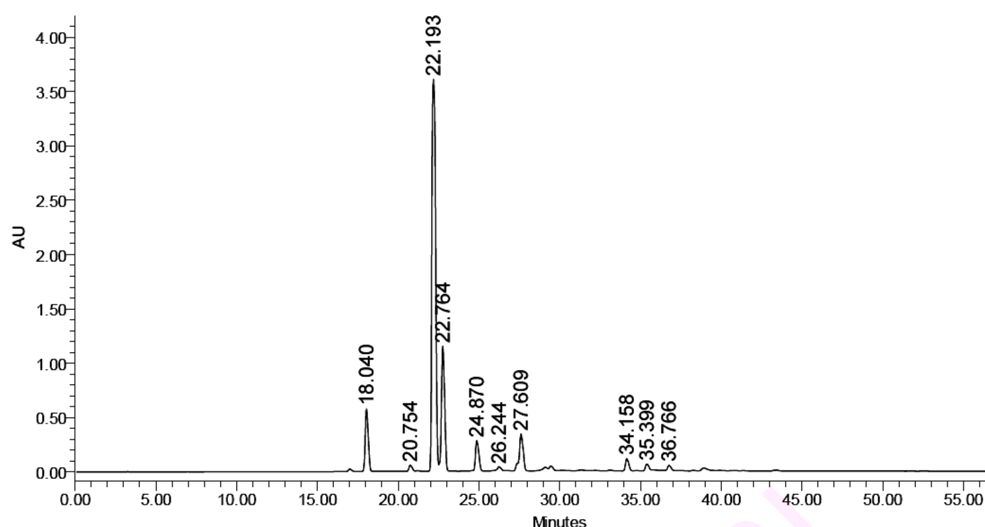

Fig. 4.1.25. HPLC profile of CHL

The relationship between chlorogenic acids with cup quality is still unclear and controversial. Ohiokpehai (1982) reported that the increase in the concentration of diCQA conferred a disagreeable flavour to the coffee beverage. Mazzafera, (1999) observed that the content of phenolic substances and 5-CQA were higher in immature and immature-black beans compared to the quality beans. However Franca *et al.,* (2005) reported lower levels of 5-CQA in black defective beans and further reported that 5-CQA was the main CGA in all defective beans (50-75% of total CGA).

Me_1 extract possess 58.09% of CQA isomers, 7.20% of FQA isomers and 20.38% of diCQA isomers. The concentration of CQA isomers increased in all the isolated fractions irrespective of the solvents and the methodologies used for the fractionation. The concentration of total CQA isomers was highest when phenolics were enriched by precipitation using

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lead salts (Table 4.1.10) though the yield was slightly lesser compared to the other methods of separation (Table 4.1.1).

Table 4.1.10. Chlorogenic acid isomer of isolated fractions

Peak No.	Compound name	RT	Area (%)				
			Me_1	C (IS_1)	DW	DN	CHL
1	3 CQA	17.417	6.99	7.97	8.61	7.58	7.78
2	4 CQA	21.874	51.10	55.07	54.95	56.37	60.51
3	5 CQA						
CQA sub total			**58.09**	**63.04**	**63.56**	**63.95**	**68.35**
4	Caffeic acid	22.398	10.80	11.41	12.04	13.30	16.72
5	5 pCoQA	24.351	2.15	0.65	1.84	0.89	4.22
6	4FQA	25.913	0.70	0.89	0.97	1.04	0.82
7	5FQA	27.361	6.50	5.79	5.03	6.94	5.58
FQA sub total			**7.20**	**6.68**	**6.00**	**7.98**	**6.40**
8	3,4 diCQA	33.926	5.80	4.90	4.68	4.90	1.64
9	3,5 diCQA	35.184	6.68	6.19	5.34	4.28	1.02
10	4,5 diCQA	36.637	7.90	6.41	5.66	4.27	0.88
di CQA total			**20.38**	**17.50**	**15.68**	**13.45**	**3.54**
11	Unknown	43.294	1.38	0.73	0.22	--	---

On the contrary, the concentrations of FQA and diCQA isomers decreased during the enrichment of the extracts. Stalmach *et al.,* (2006) reported that the antioxidant capacity of green coffee beans is due to the presence of chlorogenic acids in particular to 5-CQA and to a lesser degree to 3,4 CQAs and diCQAs. The contribution of FQAs towards antioxidant activity is very less (Stalmach *et al.,* 2006). These results are reflected in the radical scavenging activity of the fractions.

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CHAPTER 4

CHAPTER 4

Characterisation of bioactive compounds and value addition

Part II

Antioxidant efficacy of coffee conserve against oxidative rancidity of sunflower oil

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CHAPTER 4

Part II

4.4. Oxidation of lipids

Oxidation of lipids is a major cause of deterioration in the quality of oils, fats and other fat containing foods. The products of lipid oxidation are known to produce health hazards since they are associated with aging, membrane damage, heart disease and cancer (Cosgrone *et al.,* 1987). Oxidation of lipids are initiated by free radicals leads to the formation of several intermediates including hydro peroxides, peroxides which in turn produce the aldehydes and ketones, as well as other break down products. It is therefore, important to assess the oxidative degradation of fats and oils in the food industry, because free radicals initiated oxidation is one of the main causes of rancidity which lowers quality and nutritional value of foods. Hydro peroxides, which are the primary anti-oxidation products, have no taste and flavour, but their degradation products (aldehydes, ketones etc) are very potent taste and flavour modifiers (Gordon, 1991).

To retard or prevent the oxidative deterioration the antioxidants are added in foods. The antioxidants can be of synthetic or natural origin. Synthetic anti-oxidants BHA, BHT and TBHQ are widely used in the food industry, as these are effective and less expensive (Pinder Duh *et al.,* 1997). However, the use of synthetic antioxidants is restricted in several countries, because of their possible undesirable effects on human health (Branen,

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1975). This necessitates searching antioxidants from the natural sources. These include soyabean, green and black tea, coffee, red wine, spices, herbs, citrus fruits, onions and olives etc. Among the herbs, rosemary is often shown to exhibit the most anti-oxidant activity. Rosmariginone and rosmaridiphenol were identified by Houlihan *et al.,* (1984, 1985 a, b) as anti-oxidants with potency equal / superior to that of BHA and BHT in lord (Che & Tan, 1999).

Recently, due to increasing awareness of health, coffee and its health benefits with reference to antioxidant activity has been investigated by many researchers (George *et al.,* 2008). Coffee is a complex chemical mixture composed of over thousand different chemical components. Active components such as polyphenols including chlorogenic acids (CGA), caffeine, kahweol, cafestol and other phenolics such as caffeic, ferulic and vanillic acids present in the coffee and are reported to be responsible for various beneficial effects (Ramalakshmi *et al.,* 2007). Coffee due to its antioxidant activity is known to show a protective effect on cancer and other cardiovascular diseases. It also protects Low Density Lipoprotein from oxidation, which may be due to the action of several polyphenolic constituents. In addition, these molecules can have a synergistic or antagonistic effect when present in complex mixtures. Robusta exhibits a high antioxidant activity than arabica, which could be due to the higher amount of chlorogenic acid (Richelle, 2001).

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Green coffee was also shown to exhibit in vitro antioxidant activity against lipid peroxidation and antineoplastic activity (Rosenberg, 1990). Phenolic compounds in coffee are known to have antioxidant activity in which the prevalent one is hydroxy cinnamic acid and the major component of this class is caffeic acid, which occurs in food mainly as esters called chlorogenic acid (Rice-evans *et al.,* 1996).

Morishita and Kido (1995) established the radical scavenging activity of coffee using 1,1, diphenyl–2 picryl hydrazyl (DPPH) and superoxide anion mediated linoleic acid peroxidation system in vitro. Ohnishi *et al.,* (1998) showed radical scavenging activity of caffeoyl-tryptophan, a minor constituent in green beans, increased dose-dependently at concentrations ranging from 1 to 50 micromolar. Nakayama (1995) has shown that caffeic acid enhanced hydroxyl radical formation in the presence of transition metal ions such as Fe^{3+}, Cu^{2+}, and Mn^{2+} that causes oxidative damage while caffeic acid esters showed protective effects in the absence of the metal ions.

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Scope of the study

Though the earlier research showed the antioxidant activity of coffee in model system by in vitro methods there is no report about the role of coffee in the food system against antioxidant activity. It is in this context, work was planned to evaluate the efficacy of the green coffee extract from LCB which is one of the by products in coffee industry, in sunflower oil that is devoid of added antioxidants.

4.5. Materials and Methodologies

4.5.1.Incorporation of Coffee conserve into sunflower oil

LCB was ground and sieved using a mesh size-18 (650μm). The green coffee powder was transferred to a soxhlet apparatus and defatted with hexane for 8 h. The defatted powder was extracted with methanol for 8 h while maintaining a material to solvent ratio of 1:8 to 1:12. The extracts were desolventized in a rotavapour by maintaining the temperature at 50°C under reduced pressure and stored in desiccator for further use.

Refined sunflower oil was purchased from Mysore, Karnataka, India, which is devoid of added antioxidants. Coffee conserve was incorporated in sun flower oil at two different concentrations viz. 200 and 500 ppm, to determine the effect of concentration on the activity of conserve as antioxidant. Chlorogenic acid which is the major poly phenol present in coffee conserve, and a synthetic antioxidant namely BHA were incorporated at 100 and 200 ppm levels respectively in order to carry out the relative study between the various antioxidants. All the antioxidants were dissolved in ethanol and methanol mixture at 95±2°C and incorporated into oil and the solvent removed in a rotavapour. The samples were filled in transparent and brown coloured bottles and kept in two conditions viz., ambient (27°C) and accelerated (40°C) temperatures.

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4.5.2. Quality parameters analysed

The sunflower oil samples were analysed for the primary and secondary antioxidative products such as peroxides, carbonyls and acids which are the indicators of the intermediate products formed at different stages of oxidation according to the standard procedures.

4.5.2.1. Peroxide value (AOCS OFFICIAL METHOD Cd 8-53)

The peroxide values of all samples were measured according to AOCS method with the slight modification. Sunflower oil samples (5 g) were dissolved in 30 mL of chloroform:glacial acetic acid (3:2, v/v) and saturated solution of KI (1 ml) was added. The mixture was shaken for 1 min and kept in dark for 5 min. After the addition of 75 mL distilled water, the mixture was titrated against sodium thiosulphate (0.01 N) until yellow colour almost disappears. Then about 0.5 mL of starch indicator solution was added. Titration was continued until blue colour just disappears. Blank was also determined under similar conditions. Peroxide value (meq/kg) was calculated according to the equation

$$PV_{(meq/kg)} = C \times (V_1 - V_2) \times 1000/m$$

Where C is the concentration of sodium thiosulphate (N); V_1, V_2 are the volume of sodium thiosulphate consumed by samples and blank, respectively (mL); m is the mass of sample (g).

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4.5.2.2. Free fatty acids (AOCS OFFICIAL METHOD Ca 5a-40)

Free fatty acids (FFA), as oleic acid (%) in oil samples, were determined using an alkali titration method. Sample (7g) was dissolved in 50 mL ethanol and the mixture was titrated against potassium hydroxide (0.1N) using phenolphthalein solution as an indicator. FFA value (%) was calculated according to the equation:

$$FFA (\%) = (V \times C \times 282 \times 100/m) \times 1000$$

Where V is the volume of potassium hydroxide used for the titration by samples (mL); C is the normality of potassium hydroxide (N); m is the mass of sunflower oil (g) sample and 282 is the equivalent weight of oleic acid.

4.5.2.3. p-Anisidine Value (AOCS OFFICIAL METHOD Cd 18 –19)

p-Anisidine value (AnV) was determined according to AOCS official method Cd 18 –19. Samples (4 g) were dissolved in 25 mL isooctane and absorbance of this fat solution was measured at 350 nm using the spectrophotometer (Ultraviolet–Visible Spectrometer, Cintra 10, GBC, Australia). The above mixture (5 mL) was mixed 0.25% p-anisidine in acetic acid (1 ml) and allowed to stand for 10 min. Absorbance of the solution was read at 350 nm using spectrophotometer. AnV was calculated according to the equation.

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$AnV = 25 \times (1.2 A_1 - A_2)/m$

Where A_1 is the absorbance of the fat solution after reaction with the p-anisidine reagent; A_2 is the absorbance of the fat solution; m is the mass of sample (g) sample.

4.5.2.4. FTIR analysis

Samples were also analysed for FTIR spectral analysis to find out the changes in the chemical composition of the oil samples with different antioxidants. The analyses were carried out initially as well as every fortnight upto 120 days of storage. All experiments were conducted with duplicate sets, and analyses of samples were run in triplicate and averaged.

4.6. Results and Discussion

Auto oxidation is a natural process which occurs between molecular oxygen and unsaturated fatty acids. Auto oxidation takes place via free radical mechanism consisting of three basic steps namely initiation, propagation and termination. Initiation starts with the abstraction of a hydrogen atom adjacent to a double bond in a fatty acid (RH) and this may be catalysed by light, heat or metal ion to form a free radical. The resultant free radical (R.) react with atmospheric oxygen to form an unstable peroxy free radical which may in turn abstract a hydrogen atom from another unsaturated fatty acid to form a hydroperoxide (ROOH) and new alkyl free radical. The new alkyl free radical propogates further oxidation and contributes to chain reaction. The propagation step may be terminated by

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the formation of nonradical products resulting from combination of two radical species (Bolland & Gee, 1946; Fennema, 1987).

The mechanism of lipid auto oxidation has been postulated by Farmer *et al.,* 1942. The rate of oxidation of fatty acids increases in relation to the degree of unsaturation and the availability of oxygen in the system. Oxidative products such as hydroperoxides (primary) and alchohols, aldehydes, ketones, hydrocarbons (secondary) generally possess offensive off flavour. These compounds may also interact with other food components and change the functional and nutritional properties. Various methods are available for the measurement of oxidative rancidity. Coffee conserve was added to the sunflower oil, which contains high amount of linoleic triglycerides, and stored for 120 days. The oxidative stability of sunflower oil in presence of coffee antioxidant conserve was evaluated by measuring the peroxides, carbonyls, free fatty acids and FT-IR spectral analysis. The formation of primary and secondary oxidation products from the selected fatty acid present in sunflower oil (Labuza, 1971; Aruma, 1994) is expressed in the following illustration XXXI.

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RH

Initiation

Abstraction of H atom Initiators(UV light, $^1O_2$ metal catalysts, heat etc)

$R^{\cdot}$ (Lipid free radical) → R-R

Termination

$^3O_2$

Propogation

$ROO^{\cdot}$ → Dimers, polymers, cyclic hydroperoxides hydroperoxy compounds

Cleavage

Aldehydes, ketones, hydrocarbons, furans acids

ROOH (Hydroperoxides)

ROOR, ROR,dimers ← $RO^{\cdot}$ → Keto hydroxy and epoxy comppounds, etc.

Aldehydes | Semialdehydes or oxo esters | Alkyl radicals

O_2 | Condensation | Hydrocarbons | O_2

Hydrocarons, shorter aldehydes, epoxides, acids.

Alkyltrioxanes and dioxolanes

Terminal ROOH

Hydrocarbons, aldehydes, alcohols.

XXXI : Scheme for the autooxidation of unsaturated fatty acids

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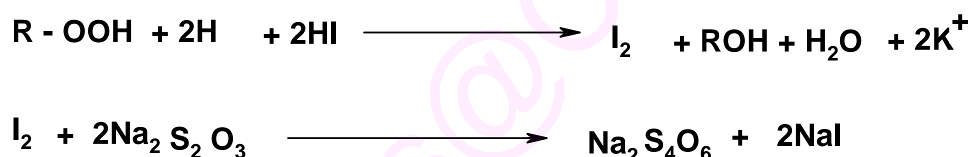
Changes in peroxide value

Hydroperoxides are the resultant products of primary oxidation of fatty acids. It is measured as a peroxide value (PV) and estimated by iodimetric method. The reaction based on the reduction of hydroperoxide group (OOH) with iodide ion (I-). The amount of iodine (I_2) liberated is proportional to the concentration of hydroxides present. Released iodine is titrated against sodium thiosuphate using starch as the indicator. Peroxides value is expressed as milliequivalents of hydroperoxides present in 1000 g of the sample (Bolland & Gee, 1946).



From the Figs. 4.2.1 – 4.2.4., it is observed that there was continuous increase in peroxide values in all the samples as expected. However the rate of increase varied considerably for different samples depending on the storage bottles and conditions.

Conserve @ 200 ppm was effective in arresting the increase in peroxide value up to 50% as compared to the control. Peroxide values obtained for the oil incorporated with 200 ppm was less as compared to the oil having conserve at 500 ppm level, although the oil incorporated with conserve @ 500 ppm also showed reduction in peroxide value as compared

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to control. It indicates that it is quite effective as antioxidant at low concentrations. This result leads to the conclusion that like many other antioxidants, coffee conserve may behave as pro-oxidant at higher concentrations depending on the food system in which it is incorporated (Abdalla & Roozen, 1999, Baurenfield, 1997).

Increase in peroxide value was much lower for the oil stored in brown bottles as compared to transparent bottles. Transparent bottles stored at accelerated conditions showed more peroxide value than oil stored at ambient conditions and the effect was similar for the brown bottles to a great extent (Sherwin *et al.,* 1970, 1976, 1990). This may be attributed to the reason that the formation of peroxides is greatly influenced by the temperature of incubation and varies directly with the temperature. Among all the antioxidants incorporated, coffee conserve at 200 ppm was found most effective in stalling the peroxide formation and was quite comparable to the synthetic grade antioxidant BHA, at the level of 200 ppm. Chlorogenic acid was found effective in controlling the rate of increase of peroxide value in brown bottles irrespective of the condition of storage.

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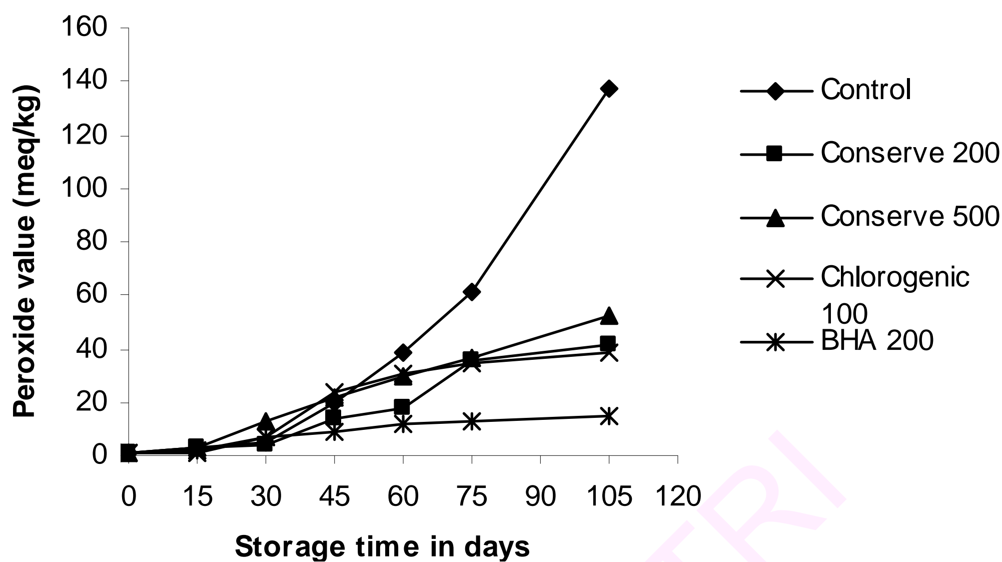

Fig. 4.2.1. Peroxide values of oil in transparent bottles at 27°C

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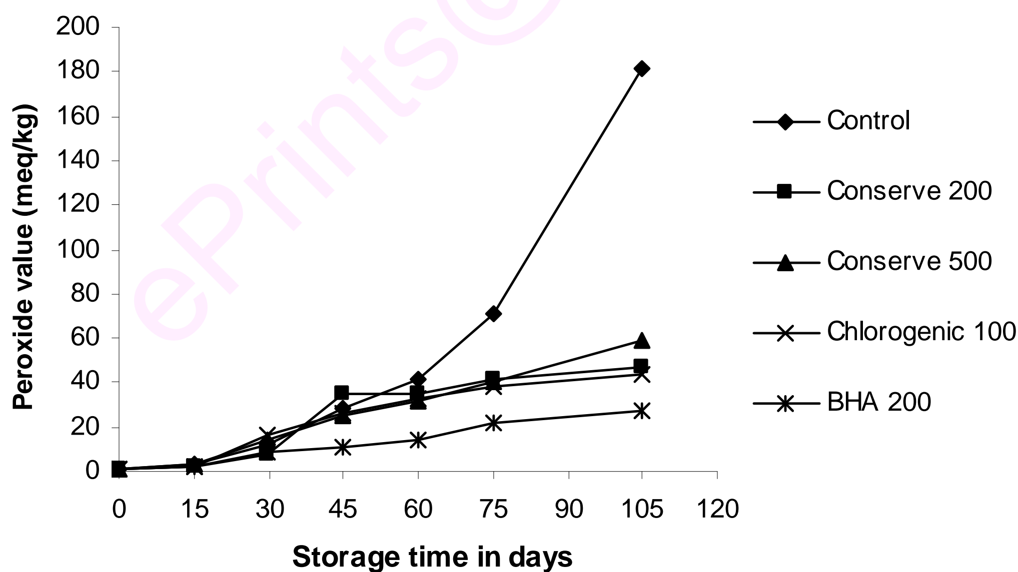

Fig. 4.2.2. Peroxide values of oil in transparent bottles at 40° C

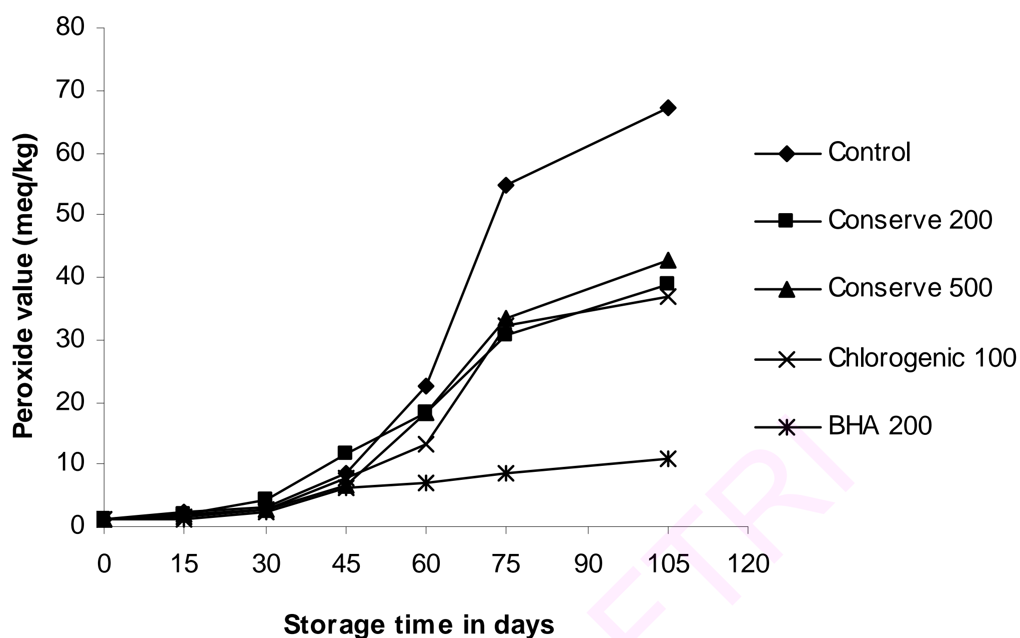

Fig. 4.2.3. Peroxide values of oil stored in brown bottles at 27° C

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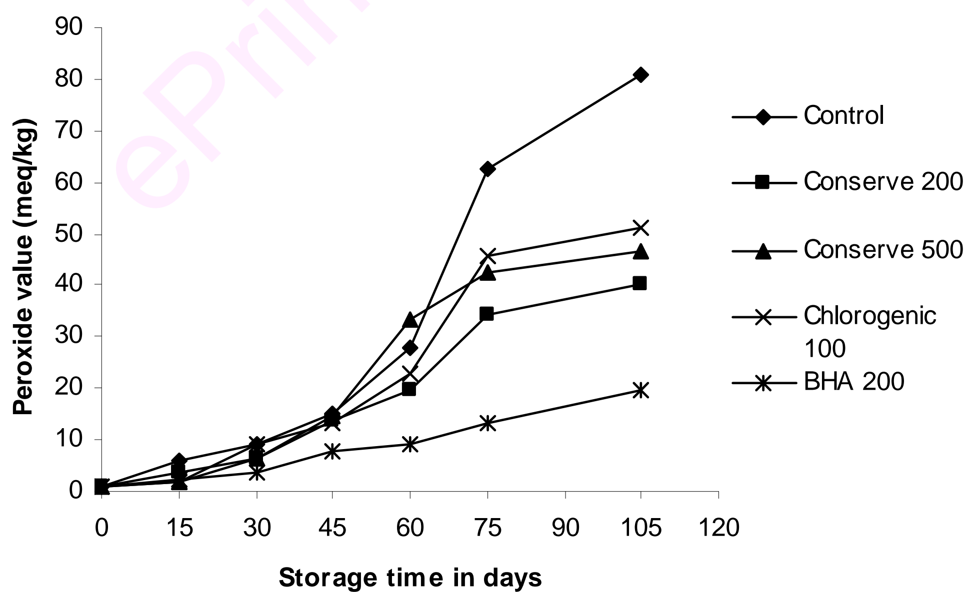

Fig. 4.2.4. Peroxide values of oil stored in brown bottles at 40 °C

Changes in p-anisidine value

p-Anisidine value is defined as 100 times the optical density measured at 350 nm of the solution containing 1.0 g of oil in a 100 ml of mixture containing solvent and reagent (Farmer *et al.,* 1942). *p*-Anisidine value is the indication of the concentration of the aldehydes and ketones especially 2-alkenals in the oil samples which increases as the time of storage increases. This is because of the formation of carbonyl compounds resulting from the oxidation products formed after hydroperoxides. The factors affecting *p*-Anisindine value are extent of aeration and presence of moisture. Carbonyl formed during oxidation forms a complex (XXXII) with anisidine and the absorbance is measured at 350 nm. *p*-Anisindine value of the samples are given in the following Figs. 4.2.5 to 4.2.8. There was incessant increase observed in the p-anisidine value with the period of incubation, which was due to the formation of carbonyl compounds resulting from oxidation of fat molecule (Cort, 1974). p-Anisidine value of control reached a maximum of 25 at the end of 120 days of storage. As compared to the control 40% reduction in the *p*-anisidine value was observed in the samples incorporated at 200 ppm of the conserve which leads to the conclusion that conserve is effective in controlling the formation of aldehydes and ketones. *p*-Anisidine values obtained from the analysis showed that light did not play significant role in the formation of carbonyl carbon compounds since the values obtained for the transparent and brown bottles did not vary significantly.

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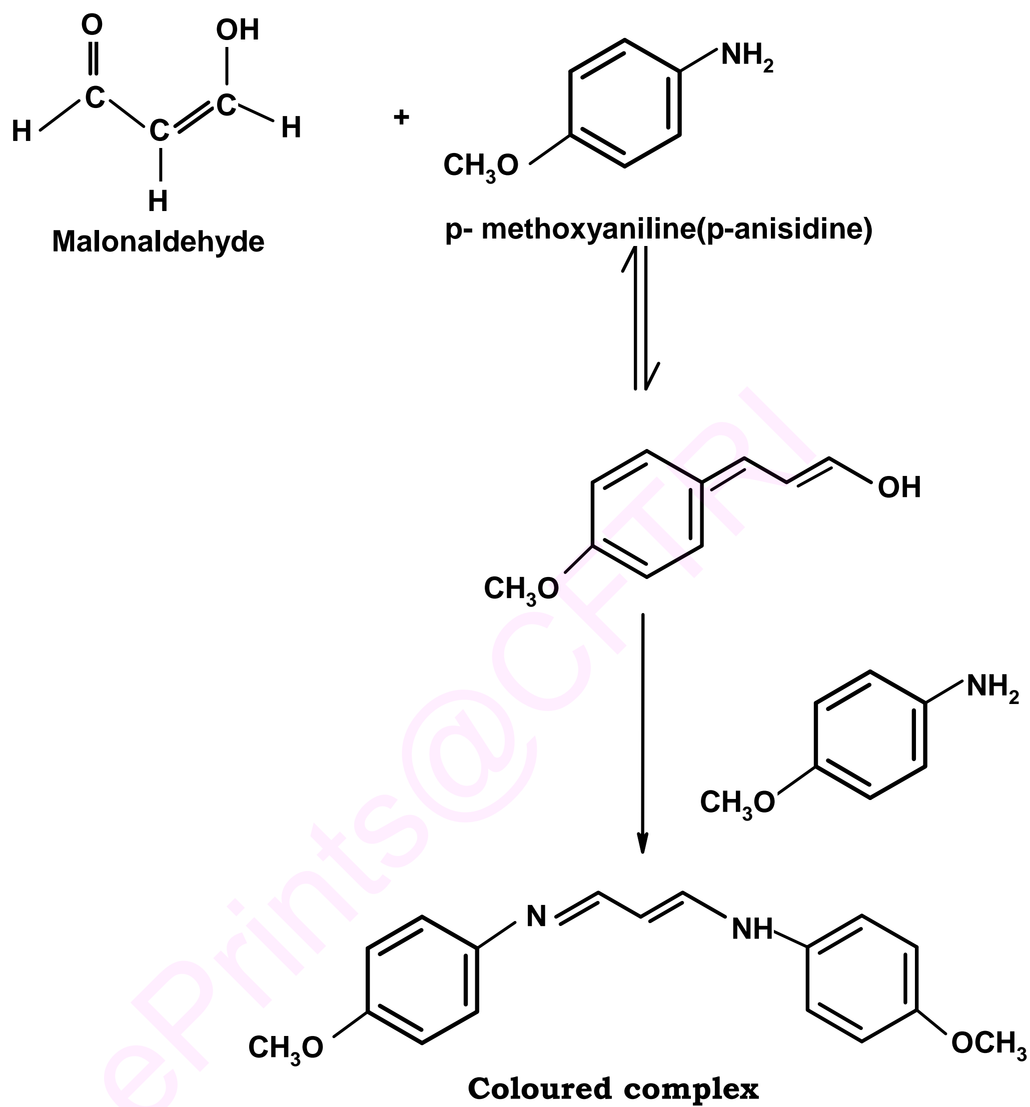

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XXXII: Reaction between p-anisidine and malonaldehyde

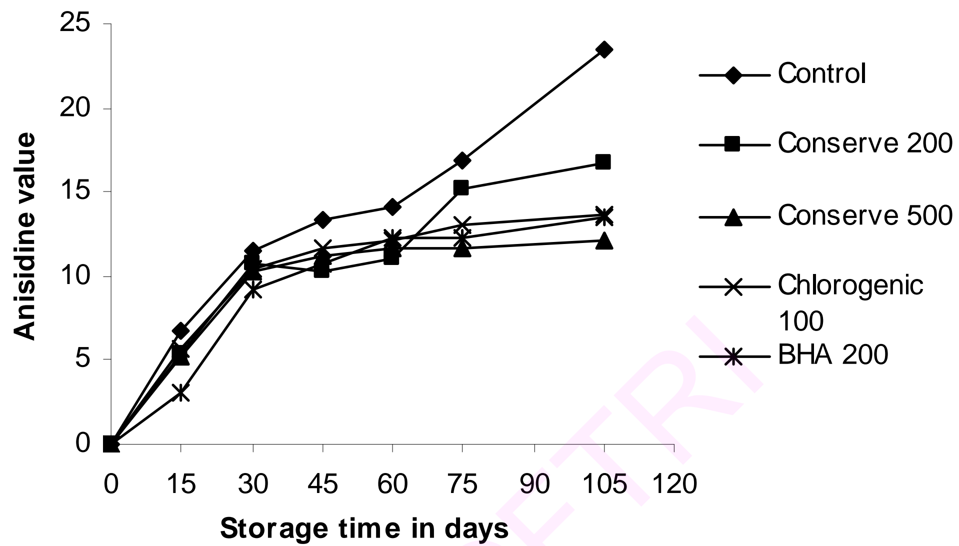

Fig. 4.2.5. p-Anisidine values of oil in transparent bottles at 27°C

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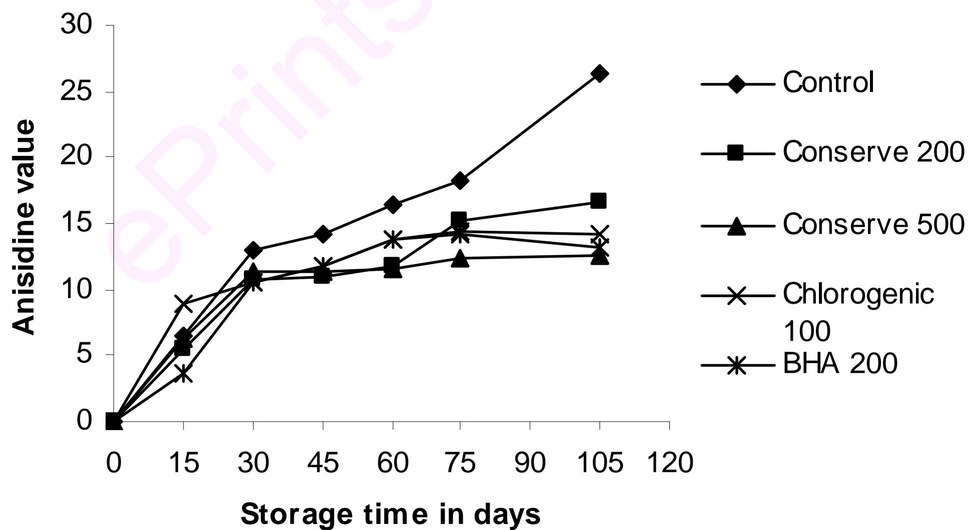

Fig. 4.2.6. p-Anisidine values of oil in transparent bottles at 40°C

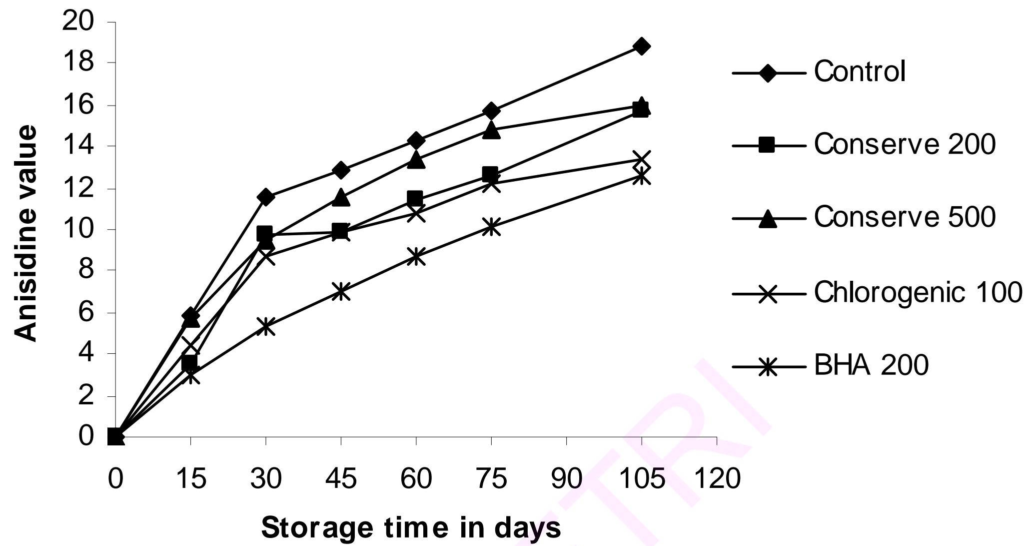

Fig. 4.2.7. p-Anisidine values of oil in brown bottles at 27°C

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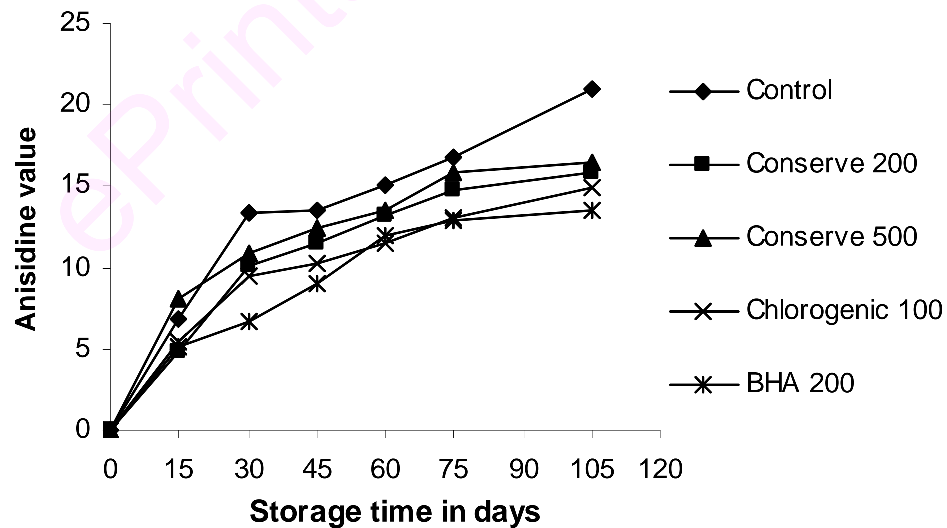

Fig. 4.2.8. p-Anisidine values of oil in brown bottles at 40°C

Changes in free fatty acid content

The free fatty acid percentage in the oil sample indicates the concentration of freely available fatty acids, which are formed due to the cleavage of fat molecule resulting in the formation of glycerol and fatty acids. These fatty acids can be determined by titrating against a standard alkali and the free fatty acid content is calculated. The results of free fatty acid analysis are shown in the following Tables 4.2.1 and 4.2.2.

There was continuous increase observed in the free fatty acid percentage with the period of incubation, which was due to the hydrolysis of fat molecule to glycerol and fatty acid. In general, oil samples in brown bottles with conserve at 200 ppm and stored at 27 ^{0}C showed marginally low free fatty acid percentage as compared to transparent bottles. This shows that light has synergistic effect on free fatty acid formation.

BHA was most effective in arresting the free fatty acid formation compared to the corresponding samples. This is due the fact that it is commercial grade additive, which has been proved as potential antioxidant for oils and fats. However free fatty acid percentage values obtained from the oils with conserve was quite comparable to that of BHA.

The free fatty acid content was lower in oil having 200 ppm of conserve than the oil which had 500 ppm of the conserve which is similar to the trend observed in the peroxide values. Free fatty acids are the ultimate

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products resulting from the fatty acid oxidation which are formed due to the degradation of primary oxidation products.

Table 4.2.1. Free fatty acid (%) content of sunflower oil stored in transparent bottles at different conditions (Initial 0.09)

Temp	Sample	15	30	45	60	75	105	120
	Control		0.09	0.14	0.17	0.22	0.37	0.82
	Conserve, 200 ppm		0.14	0.15	0.17	0.19	0.26	0.52
27°C	Conserve, 500 ppm		0.15	0.17	0.17	0.19	0.27	0.58
	Chlorogenic acid, 100 ppm		0.15	0.17	0.17	0.19	0.24	0.54
	BHA	0.09	0.14	0.14	0.17	0.17	0.17	0.17
	Control		0.17	0.18	0.21	0.25	0.39	0.87
	Conserve, 200 ppm		0.11	0.17	0.17	0.19	0.28	0.57
40°C	Conserve, 500 ppm		0.14	0.15	0.17	0.19	0.29	0.55
	Chlorogenic acid, 100 ppm		0.14	0.17	0.17	0.19	0.25	0.54
	BHA		0.12	0.15	0.17	0.17	0.18	0.20

Table 4.2.2. Free fatty acid (%) content of sunflower oil stored in brown bottles at different conditions

Temp	Sample	15	30	45	60	75	105	120
	Control		0.17	0.19	0.20	0.21	0.35	0.79
	Conserve, 200 ppm		0.12	0.15	0.12	0.17	0.23	0.52
27°C	Conserve, 500 ppm		0.14	0.17	0.18	0.19	0.25	0.58
	Chlorogenic acid, 100 ppm		0.12	0.17	0.17	0.19	0.25	0.48
	BHA	0.09	0.12	0.12	0.17	0.17	0.22	0.17
	Control		0.17	0.20	0.20	0.21	0.39	0.87
	Conserve, 200 ppm		0.12	0.15	0.17	0.17	0.25	0.54
40°C	Conserve, 500 ppm		0.14	0.17	0.17	0.19	0.25	0.54
	Chlorogenic acid, 100 ppm		0.12	0.17	0.17	0.19	0.26	0.58
	BHA		0.14	0.14	0.17	0.17	0.17	0.18

FTIR Analysis

Fourier Transform Infrared Spectroscopy (FTIR) is an analytical technique used to identify organic (and in some cases inorganic) materials. This technique measures the absorption of various infrared light wavelengths by the material of interest. These infrared absorption bands identify specific molecular functional groups and structures. FTIR spectra obtained are presented in the subsequent results.

The peak area observed from the wave number 3000 to 3500 cm^{-1} increased remarkably from 0 to 60 days of the observation and attributed to the considerable increase in the hydroxy groups /compounds, which is also confirmed by the values obtained from peroxide value estimation.

All the samples showed almost similar increase in the peak area near wave number 3000 to 3500 cm^{-1} except for the control samples which showed abrupt rise because of the absence of any antioxidant in the control. Control sample stored in transparent bottles showed noticeable increase in the hydroperoxides (Figs. 4.2.9 to 4.2.11) as indicated by the FTIR analysis, as it is stored at 40°C.

The peak area between the wave number 968 to 1310 cm^{-1} indicate the formation of compounds having *trans* double bond which is due to the formation of some degradation products during storage. All the samples

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exhibited increase in the peak area in this range because of the cleavage of certain fat molecules, which was also evident from fatty acid%.

Double bond near to the carbonyl group indicated the formation of 2-alkenals or other aldehydes. These products are detected by FTIR analysis near wave number 1400 cm^{-1}. The steady increase in all the samples was observed from 0 to 120 days of observation, which is also supported by the results obtained from *p*-anisidine values.

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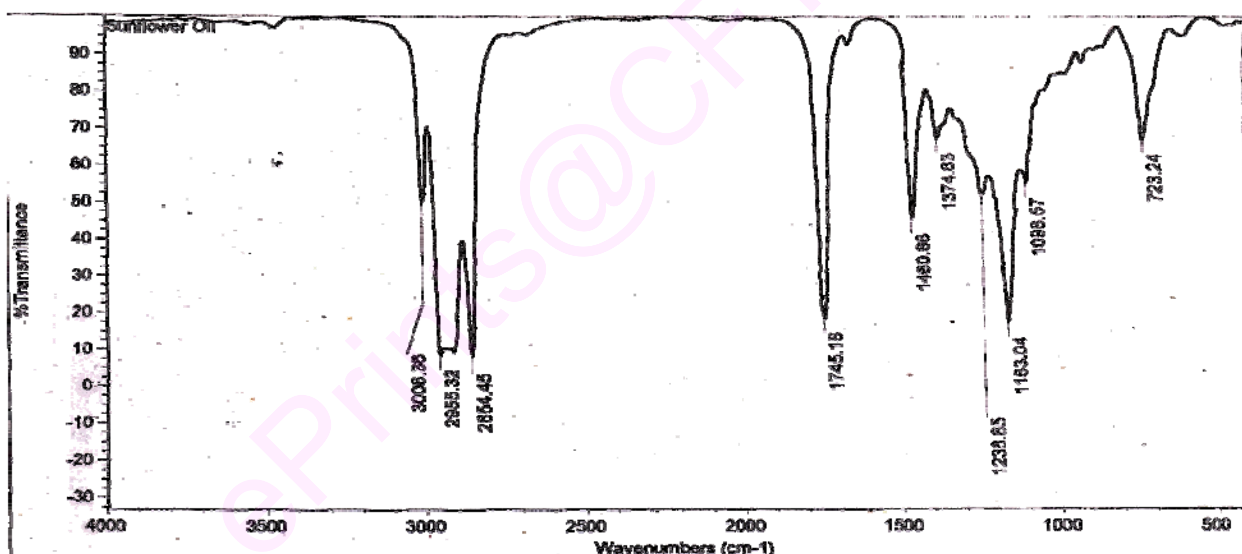

Fig 4.2.9. Sunflower oil (control) - Initial

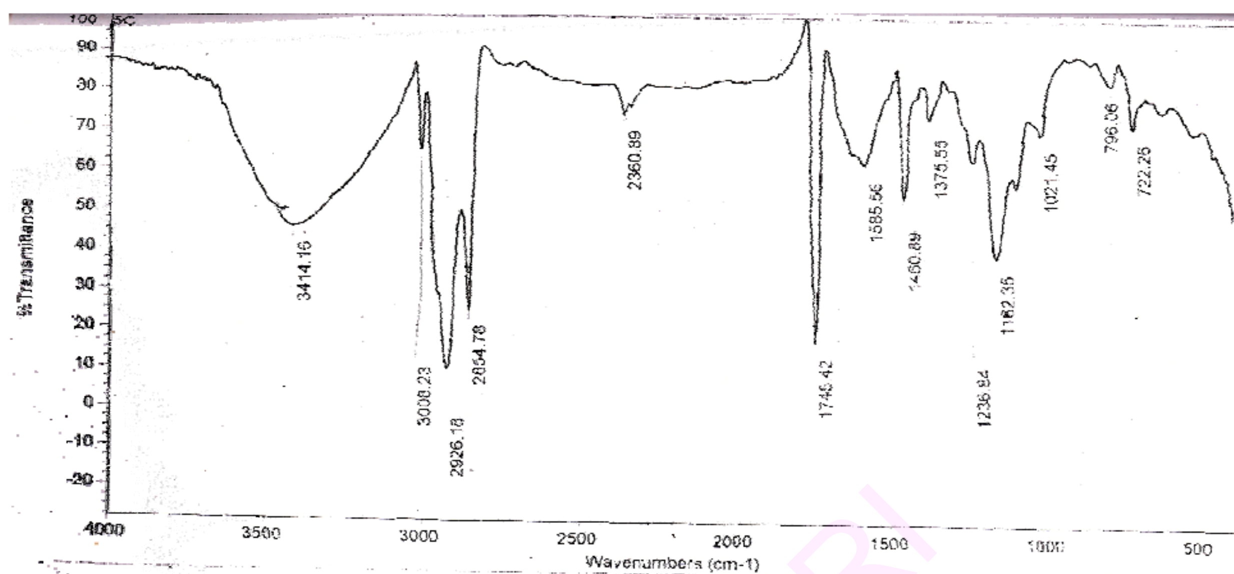

Fig. 4.2.10. Sunflower oil (control) - 120 days storage

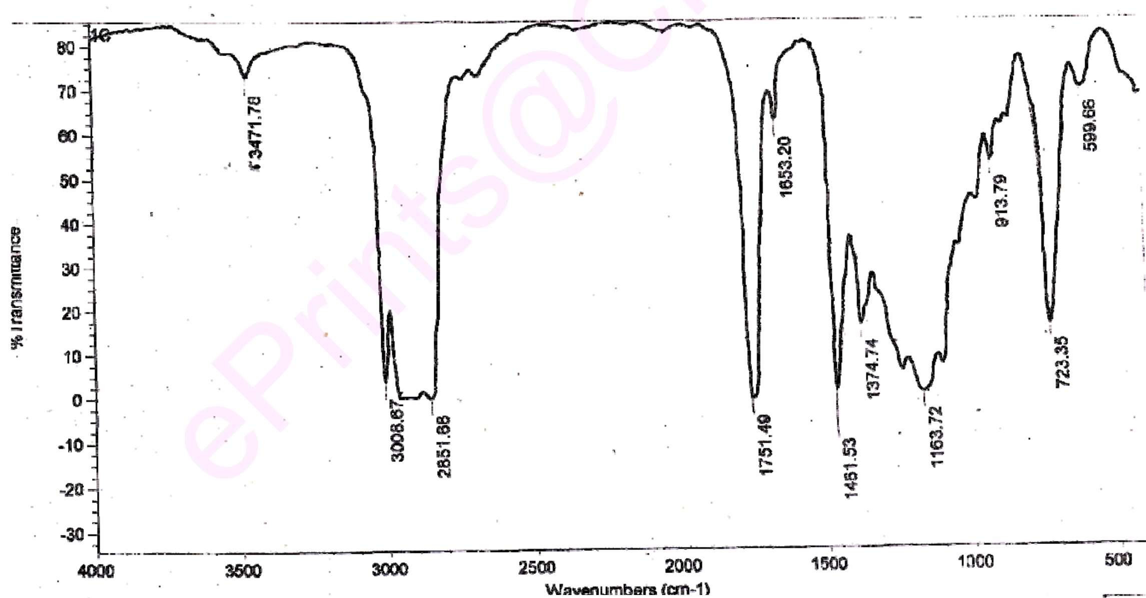

Fig. 4.2.11. Sunflower oil (conserve) - 120 days storage

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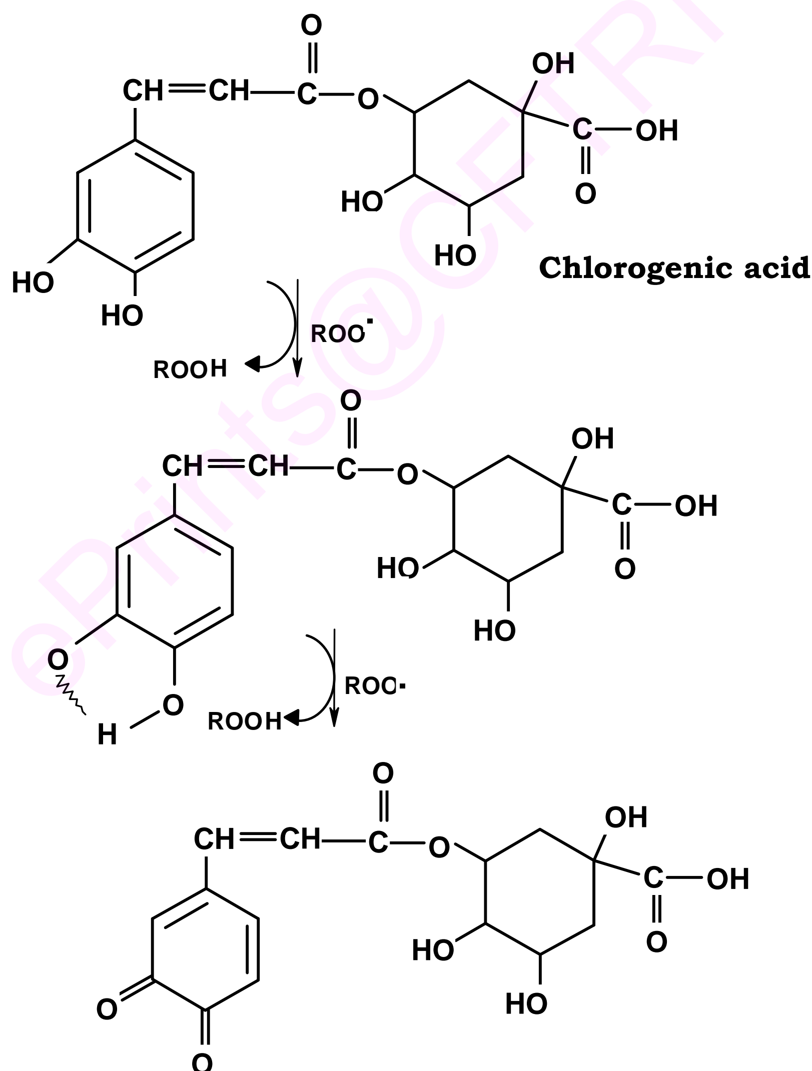
Role of antioxidants on oxidative rancidity

In foods containing lipids, antioxidants delay the onset of oxidation or slow down the rate of auto oxidation. The role of antioxidants is to enhance the food quality to extend the shelf life. The mechanism of the antioxidants on the prevention of auto-oxidation is very broad (Gordon, 1990). In general there are three mechanisms behind the retardation of rancidity by the antioxidants.



XXXIII: Mechanism of chlorogenic acid as a hydrogen donor

They are free radical chain breaking mechanism, metal chelating mechanism and singlet oxygen quenching mechanism (Cuvelier *et al.,* 1992, 1996). Antioxidants containing phenolic groups acts as chain breaking compounds by donating hydrogen to $ROO^{\bullet}$ before its interaction with RH to form $ROO^{\bullet}$ thus preventing the propagation step. Since Chlorogenic acid isomers contain two phenolic hydroxyl groups in the caffeic acid ring, the possible mechanism of its interaction with the fatty acid is given above (XXXIII). In addition the carboxyl group from quinic acid unit also will donate hydrogen to prevent the propagation step.

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CHAPTER 5

CHAPTER 5

Hydrolysis and characterisation of polysaccharides

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CHAPTER 5

5.1. Introduction

The carbohydrates content of the green coffee beans is about 50% of the dry weight. The importance of the carbohydrate in coffee is evident from its presence as a higher quantity compared to other constituents. Carbohydrates (low and high molecular weight) present in coffee undergo extensive chemical changes associated with coffee roasting. The carbohydrate fraction of green coffee is dominated by polysaccharides such as arabinogalactan, galactomannan and cellulose, which contribute to the over all taste of green coffee. In addition, there are small amounts of pectic polysaccharides (Redgwell *et al.,* 2002 a, b) and recently xyloglucan was also shown to be present (Oosterveld *et al.,* 2003) in green coffee beans.

Free monosaccharides are present in mature brown to yellow-green coffee beans. The free part of monosaccharides contains sucrose up to 9000 mg and 4500 mg/100g in arabica and robusta coffees respectively. In arabica green coffee beans the content of free glucose was 30 mg to 38 mg/100 g, free fructose 23 mg to 30 mg/100 g; free galactose 35 mg/ 100g and mannitol 50 mg/100g dried coffee beans, respectively. Mannitol is a powerful scavenger for hydroxyl radicals, which are generated during the peroxidation of lipids in biological membranes. The principal low molecular weight carbohydrate present in green coffee is sucrose and the monosaccharide content is relatively low (Fischer, 1999).

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5.1.1. Galactomannans

There is little information on the biosynthesis of the cell wall polysaccharides. Recently, with the advent of molecular techniques, researchers are interested in finding the mode of biosynthesis of coffee cell wall polymers. Solubilisation of galactomannans determines the yield of coffee extract (Clifford, 1985). Next to cellulose, the most resistant polymers to solubilisation are the galactomannans. One of the principal determinants of galactomannans solubility is the frequency of substitution of galactose residues on the mannan backbone (XXXIV).

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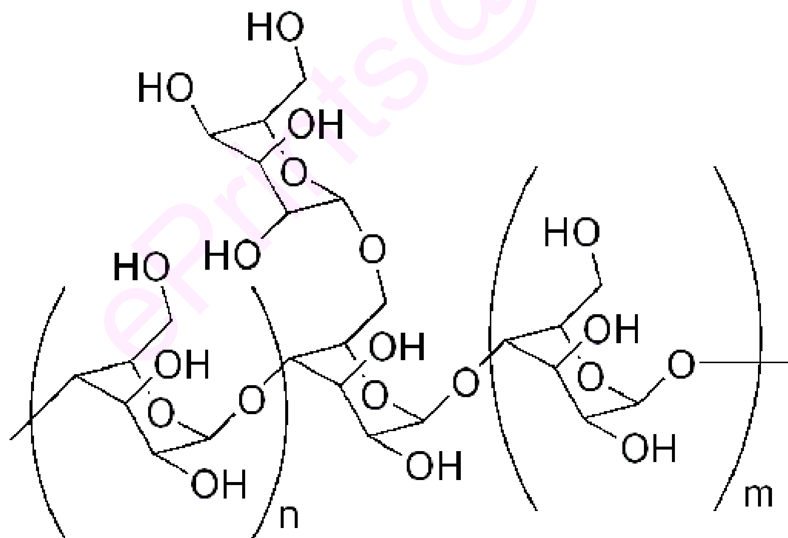

XXXIV. Galactomannan

An increase in the degree of galactosylation on the mannans may increase the degree of solubilisation of the galactomannans. Therefore it is necessary to understand the metabolic steps during the biosynthesis of galactomannans in degree of galactosylation. In some plants, the final degree of galactosylation is determined by the action of α-galactosidase, which regulates the galactosyl residues from the primary metabolic product. Redgwell *et al.,* (2003b) isolated and characterised galactomannans from the endosperm of coffee beans 11, 15, 21, 26, 31 and 37 Weeks after flowering (WAF). At the earliest stage of development of coffee beans, the galactomannans accounted for ~10 % of the polysaccharides which were highly substituted, with Gal/Man ratios between 1:2 and 1:7. At maturity, the galactomannan became the predominant polysaccharide accounting for ~50 % of the total endosperm polysaccharides but their degree of substitution decreased with Gal/Man ratios between 1:7 and 1:40. The decrease in the Gal/Man ratio of the galactomannans commenced between 21 and 26 WAF and was in synchrony with a rise in free galactose.

5.1.2. Arabinogalactan

Arabinogalactan (XXXV) is a natural polysaccharide that is made up of galactose and arabinose sugar units linked together in a ratio of 6:1 respectively. Arabinogalactan occurs in small amounts in many plants and plant-based foods such as fruits, carrots, radishes, wheat flour and coffee

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beans (Bradbury & Halliday, 1990). In most plants, it is found within the sturdy walls that surround each plant cell. It may be bound to proteins and other complex molecules in the cell wall. Extracting and isolating the arabinogalactan from plant cell walls requires chemicals and mechanical treatments. These chemical / mechanical processes may alter the arabinogalactan molecules to a certain degree.

Arabinogalactan contributes to 17% of dry weight of green coffee beans with a molecular weight of 90 kDa to 200 kDa. It is composed of beta-1-3-linked galactan main chain with frequent members of arabinose (pentose) and galactose (hexose) residues at the side chains comprising immuno-modulating properties by stimulating the cellular defence system (Th-1 response) of the body. Mature coffee beans (brown to yellow) contain fewer residues of galactose and arabinose at the side chain of the polysaccharides, making the green coffee bean more resistant to physical breakdown and less soluble in water (Redgwell *et al.,* 2003a). The molecular weight of the arabinogalactan in coffee is higher than in most other plants, improving the cellular defence system of the digestive tract compared to arabinogalactan with lower molecular weight (Gotoda & IwaiI, 2006). A process for extracting arabinogalactans from green as well as roasted coffee by enzymatic hydrolysis was resulted in an aqueous dispersion comprising partially hydrolysed coffee beans and arabinogalactans (US 20070248731). The structure of arabinogalactan was characterised by Wolform and Patin

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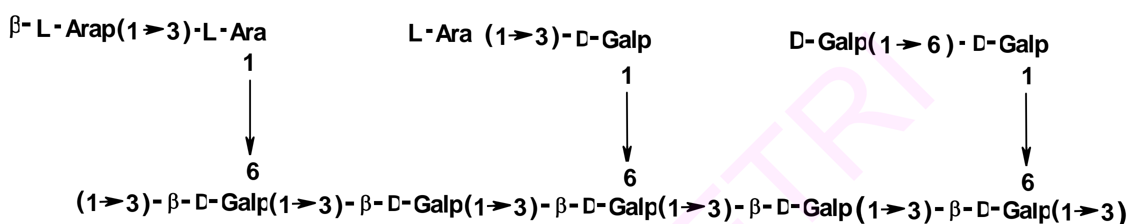
(1965) and suggested that arabinogalactan possesses β 1-3 linked backbone of galactose with frequent single (arabinofuranosyl) and two (galctopyransoyl) linked at C-3 galactopyranosyl units in the main chain. This polymer also contains non terminal arabinose residues (linked at C2 and C5) as well as small propotions of 1, 6 linked galactose residues (Bradbury & Halliday, 1990).



XXXV. Arabinogalactan –core structure

Gal/Ara ratio of the arabinogalactans in the earliest stage of growth was 1-3:1 but this gradually increased during grain growth and reached 2-6:1 at maturity. In addition, at the earliest growth stage the arabinogalactan accounted for ~50 % of the total polysaccharides but this decreased to 34 % in the mature grain.

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5.1.3. Other polysaccharides

Little information is available on the formation of other polysaccharides in the coffee bean cell wall with respect to developmental changes to their structural features. Redgwell *et al.,* (2003 b) reported the composition of monosaccharide of the cell wall material at different stages of development. Arabinose and galactose are derived mostly from the

arabinogalactans and the rhamnose and galacturonic acids are basic structural components of the pectic polysaccharides.

Scope of the study

From the earlier studies, it is concluded that there is a marginal difference in chemical parameters between graded and LCB. There is an appreciable amount (~50%) of polysaccharide present in green coffee beans, which is not soluble in water and degrades during roasting. It may be possible to modify them using chemical/enzymatic treatment for solubilisation and further utilization in food applications. Hence, this chapter deals with the hydrolysis of polysaccharides present in LCB employing different enzymes and characterization of hydrolysed sugars by chromatographic and spectroscopic techniques.

5.2. Materials and Methodologies

The polysaccharides in the Low-grade coffee beans were hydrolysed using acid-base / enzymatic methods and hydrolysed sugars were subjected to HPLC analysis.

5.2.1. Plant material and reagents.

Low grade coffee beans

LCB (500 g each) were weighed, ground and sieved using a mesh size-18 (650μm) and powder was packed in low-density polyethylene pouches and preserved at 8-10° C for further analysis.

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Enzymes

1. *α- Amylase (Aspergillus oryzae).*
2. Cellulase *(Aspergillus niger).*
3. Pullulanase (*Bacillus acidopullulyticus*).
4. Pectinase (*Aspergillus niger).*

3,5 Dinitro Salicylic acid solution

DNS (1g,) was dissolved in NaOH (20 ml of 2N) and slightly warmed for complete dissolution. Distilled water (50 ml) was added along with sodium potassium tartrate (30g). The solution was made upto 100 ml with distilled water. The clear yellow coloured solution was stored in the refrigerator (6-8 ^{o}C) till use.

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Buffers

Acetate Buffer

Acetic acid (0.2 M) was prepared by dissolving 2.85 ml of acetic acid in distilled water (250 ml). Sodium acetate (0.2M) solution was prepared by dissolving 4.1 g of sodium acetate in distilled water (250 ml). pH of the solution was monitored. Sodium acetate solution (70 ml) was added to acetic acid solution (pH 2.0) to bring the pH of the mixture to 4. The normality of the mixture is 0.16 M in terms of acetic acid. This was diluted to get the sodium acetate buffer of 0.05M.

Phosphate buffer

Citric acid solution (0.1 M) was prepared by dissolving 4.80 g of citric acid in distilled water (250 ml). Sodium phosphate solution (0.2M) was prepared by dissolving 7.09 g of anhydrous sodium phosphate in distilled water (250 ml). 30.7ml of citric acid and 23.8 ml of sodium phosphate made up to a volume of 100ml with distilled water to bring the pH of the mixture to 4. Mixture of pH 5, 6, 7 consumed 24.3 ml, 19.6 ml, 12 ml citric acid and 25 ml, 30 ml, 43.5 ml sodium phosphate solution respectively.

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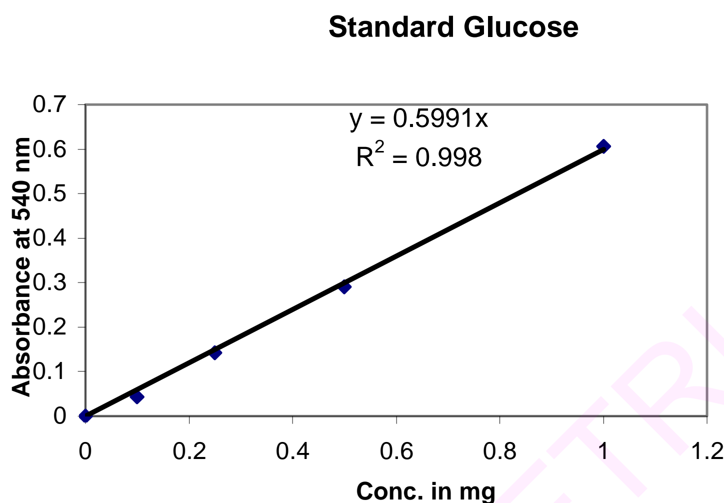
5.2.2. Estimation of Reducing sugars (DNS method)

The sugars in the Low-grade coffee beans were estimated by dinitro salicylic acid method (Fennema, 1996).

An aliquot (0.1 ml) of sample solution in sodium acetate buffer / water was diluted with water to make a reaction mixture (1 ml). DNS solution (1 ml) was added to the reaction mixture and the solution was kept at 70°C in a water bath for 5 min. The solution was cooled to RT and made upto 10 ml with distilled water. The absorbance of the resulting solution was measured at 540 nm. A blank solution was prepared with water and DNS solution treated as above and the absorbance was measured at 540 nm.

Standard glucose was prepared by dissolving 100 mg in 100 ml of distilled water. Aliquots of 0.1, 0.25, 0.5 and 1 ml was made upto 1 ml with water and the glucose was analysed by DNS method according to the

procedure mentioned as above and Calibration curve was prepared using glucose concentration versus absorbance.



5.2.3. Reducing sugars in LCB without hydrolysis

LCB (1g) was mixed with buffer (0.05 M, pH 4, 10 ml) for 10 min by continuous stirring using a magnetic stirrer at room temperature. The solution was filtered using Whatman 1 filter paper and the filtrate was incubated at 37°C for 2 h. After incubation the solution was taken for the analysis of sugars. The solution (0.1 ml) was made up to 1ml with water, 1ml of DNS reagent was added and the resulting solution was maintained at 70°C for 5 min, cooled and diluted to 10 ml, then absorbance was measured at 540 nm and the percentage of sugars was calculated and found that 1.45% of sugars present in LCB without any treatment.

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5.2.4. Hydrolysis using chemicals

LCB (1g) was mixed with distilled water (10 ml) for 10 min by continuous stirring in a magnetic stirrer at room temperature. Diluted hydrochloric acid (1:1, 1 ml) was added to the LCB mixture and maintained at 70°C in a water bath for 10 min. After cooling to room temperature the mixture was neutralized with 4N NaOH. The neutralized mixture was diluted to 50 ml with distilled water. From this solution the maount of invet sugars were analysed according to DNS method

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5.2.5. Enzymatic hydrolysis

Selection and optimisation of dosage of enzymes

LCB (1g) was mixed with 100 ml of buffer and 10 mg of different enzymes namely amylase, cellulase, pullulanase and pectinase individually and incubated at 37°C for 2 h, stirred and filtered. Filtrate (0.1 ml) was made up to 1ml with water, 1ml of DNS reagent was added and the resulting solution was analysed for reducing sugars.

Optimisation of pH

LCB (1g) was mixed with 100 ml of acetate /phosphate buffer of different ranges of pH from 4-8 and amylase (10 mg) and incubated at 37 °C for 2 h and stirred. The filtrate was analysed for reducing sugars.

Optimisation of medium

LCB (1g) was mixed with 100 ml water / buffer and amylase (10 mg) and incubated at 37°C for 2 h and stirred. The filtrate was analysed for

reducing sugars by DNS method. The remaining filtrate was evaporated at 40°C in a rotovapor under vacuum and the powder obtained was dried over P_2O_5 in a desiccator. The yield obtained was noted.

Since there is not much difference in sugar content between the media, further experiments were carried out employing water as the extracting medium.

Enzymatic hydrolysis of LCB (10g) with amylase

LCB (10g) was mixed with water (100 ml) and amylase (100 mg) and incubated at 37°C for 2 h. The solution was filtered through Whatman 1 filter paper. The filtrate was analysed for sugars by DNS method. The remaining filtrate was evaporated using a rotovapor and the powder obtained was dried over P_2O_5 in a desiccator to get the hydrolysed sugars as powder. The sugar composition was analysed using HPLC.

5.2.6. HPLC analysis

The composition of hydrolysed sugars was analysed by HPLC for the identification of individual sugars according to the procedure mentioned below.

High pressure liquid chromatography

The hydrolyzed sugar obtained by treating LCB using enzymes was analysed for the sugar profile by HPLC.

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Sample preparation: Hydrolysed sugars (100 mg) was dissolved in distilled water (1 ml). The solution was filtered through filter paper (4.5μ) and injected into HPLC and analysed under the following conditions.

HPLC system: Waters 2998 Model

Processing software: Empower software

Column : Amino column (Waters Spherisorb 5 μm NH_2, 4.6x250mm)

Mobile Phase : Acetonitrile 80 ml : water 20 ml

Detector : Aquity UPLC ELSD; with the gain 10

Injection Volume : 20 μl

Flow Rate: 1ml/min, Isocratic

Standard sugars namely arabinose, glucose, sucrose and maltose were individually analysed for retention time. Standard solutions were prepared by dissolving 20mg of each in 1ml of distilled water and analysed by HPLC.

5.3. Results and Discussion

Green coffee beans contain storage polysaccharides such as starch and structural support compounds such as cellulose and lignin. The bulk of heteropolysaccharides in the coffee beans are recognized as three fractions namely glycoprotein, water-soluble carbohydrates and holocellulose. Water-soluble and glycoproteins contains mainly galactan with some araban. The holocellulose contains mannan, galactan and cellulose in the ration of 2:1:1

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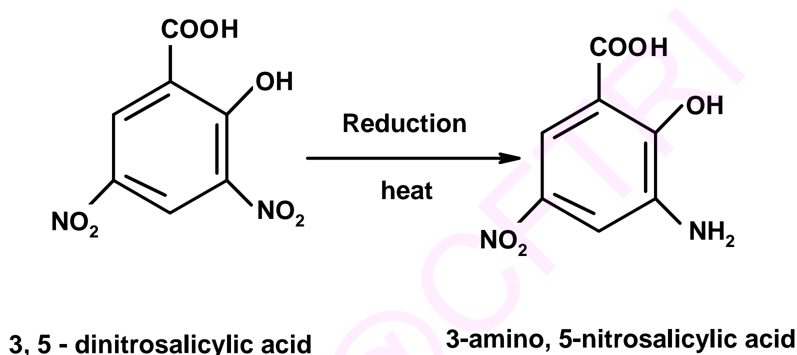
Free reducing sugar is estimated by DNS method. This method tests for the presence of free carbonyl group (C=O). The aldehyde / ketone functional group present in the test sample undergoes oxidation to carboxyl group. Simultaneously, 3,5-dinitrosalicylic acid is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions as mentioned below.



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LCB when subjected to chemical hydrolysis yielded the invert sugar in the mixture with the monosaccharide content of 33.33% using DNS method. The enzymes namely amylase, cellulase, pullulanase and pectinase were selected based on the linkages of the monosaccharide units in the cellulose, galactamannan and arabinogalactan. Results (Table 5.1) suggest that amylase gave better solubilisation of polysachharides than the other enzymes namely cellulase, pectinase and pullulanase.

Table 5.1. Amount of invert sugars in LCB extract

Enzyme (10 mg)	Invert sugars, %
Amylase	17.35 ± 1.32
Cellulase	0.32 ±0.21
Pectinase	7.34±1.01
Pullulanase	2.34± 0.98

Table 5.2. Amount of Sugars in LCB with amylase at different concentration and incubation time

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S.No.	Amylase (mg)	Incub. Time (h)	Reducing sugars, g%
1.	0	0	--
2	0.1	1	3.99 ±0.98
		2	3.75±1.02
		3	4.25±1.11
3	0.5	1	4.91±1.09
		2	5.39±1.09
		3	6.12±1.34
4	1	1	7.02±1.98
		2	8.58±1.01
		3	9.66±1.23
5	10	1	16.16±0.98
		2	18.19±1.01
		3	18.01±1.76
6	100	1	17.47±0.76
		2	18.51±0.29
		3	17.66±1.11

Experiments were conducted to optimise the dosage of the enzyme, incubation time and medium. The results are presented in Table 5.2. Results show that the amount of reducing sugars when 1 g of LCB was

treated with amylase at different concentrations and incubation times. It is seen that 10 mg of amylase is necessary for the hydrolysis of polysaccharides from 1 g of LCB at the incubation time of 2 h and this dosage level is selected for further experiments.

Table 5.3 shows the trend of solubilisation of polysaccharides by amylase at different levels of pH. Results show that there is no positive correlation of pH and solubilisation of polysaccharides by amylase. In general, amylase is most active in the pH levels of 4-6 for the hydrolysis of starch at 35-55°C. However, aqueous medium (pH 5.9) could increase the content of reducing sugar from 15 to 18% and hence water was selected as a medium for hydrolysis of polysaccharides in further experiments.

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Table 5.3. Amount of Sugars in LCB with Amylase at different levels of pH

Enzyme	Buffer/Water	pH	% Reducing sugars
amylase (10mg/1g LCB)	Acetate buffer	4	15.00±1.09
		5	15.02±1.23
	Phosphate buffer	4	15.07±1.35
		5	15.55±1.01
		6	15.19±1.07
		7	15.43±1.23
	Water	5.9	18.77±1.98

For conclusive evidence experiments were repeated with buffer (pH 4, acetate buffer) and water as medium, at a higher scale - amylase

concentration of 100mg for 10g of LCB for 2 h incubation time. The filtrate was dried which yielded the hydrolysed sugar (white powder) of 30.4 and 32.1%, when buffer and water were the medium respectively. The reducing sugars of both the powder were 12.49 and 12.36%. *Oosterveld et al.,* (2003) reported that when green coffee beans were sequentially extracted with EDTA, NaOH, only 7% of the polysaccharides could be hydrolysed and 68% of the polysacchrides remained in the residue.

The sugar composition of hydrolysed sugar was determined employing HPLC analysis equipped with Evaporative Light Scattering Detector (ELSD), which is a universal detector. The chromatograms of hydrolysed sugar obtained are provided below (Fig 5.1)

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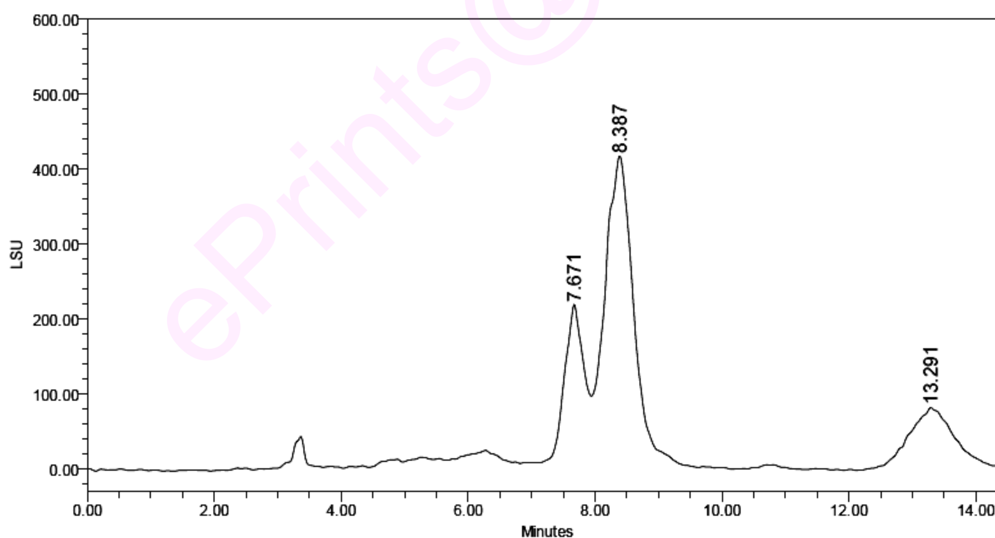

Fig. 5.1. Sugar profile of Hydrolysed sugars from LCB

ELSD makes a light scattering measurement using a photomultiplier of target analytes that have been dried of mobile phase through evaporation. It detects all non-volatile organic compounds after evaporation of medium.

It detects non-volatile organic compounds in sensitivity depending on their mass. The ELSD is particularly suited for detection of sugars, fats and surfactants that have low absorbance and are difficult to detect using UV and other detectors.

The chromatogram of the hydrolysed sugars, using water as medium, showed three major peaks, with the retention time of 7.671, 8.387 and 13.291 min with the corresponding area of 30.55, 57.86 and 11.59 in LSU (light scattering unit) respectively. Authentic samples of standard monosaccharides namely fructose, glucose, (Figs. 5.2 & 5.3) and disaccharides namely sucrose and maltose (Figs. 5.4 & 5.5) were injected individually and the chromatograms are provided below. The chromatogram of the hydrolysed sugar, using buffer as medium, showed similar profile.

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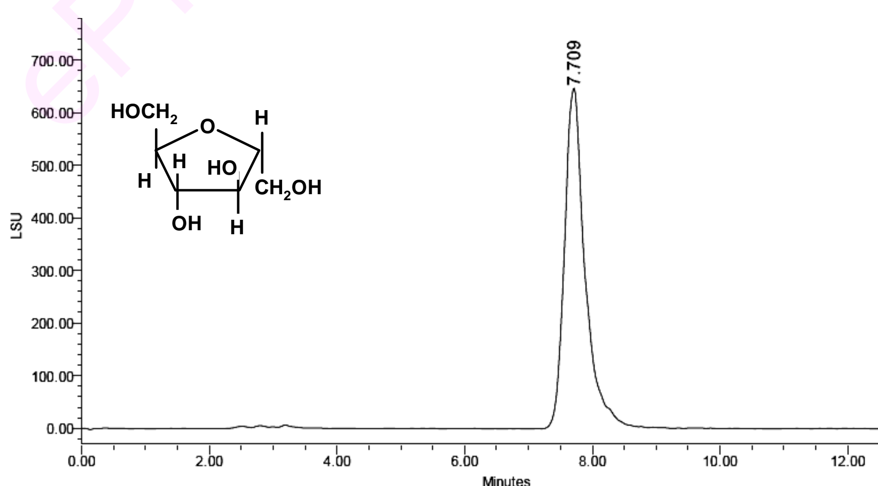

Fig. 5.2. HPLC chromatogram of fructose

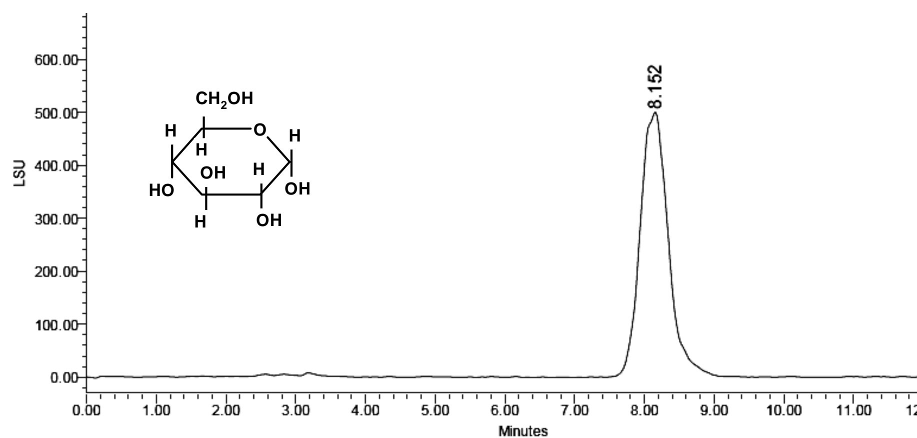

Fig. 5.3. HPLC chromatogram of glucose

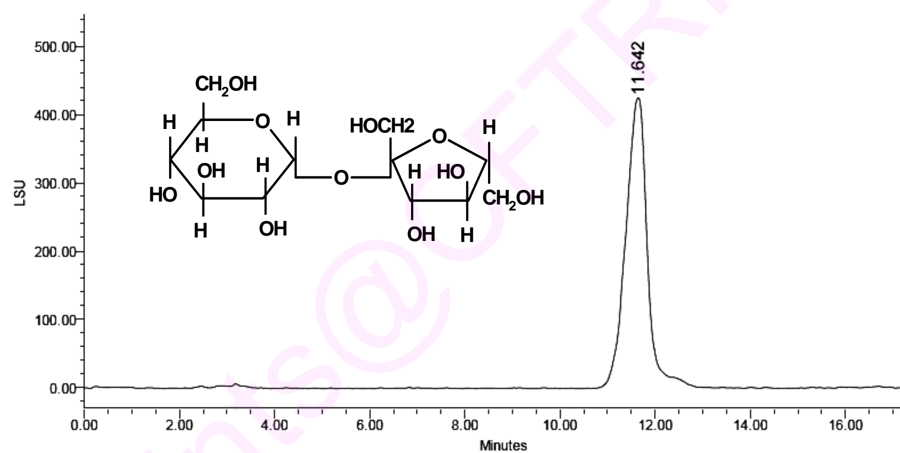

Fig. 5.4. HPLC chromatogram of sucrose

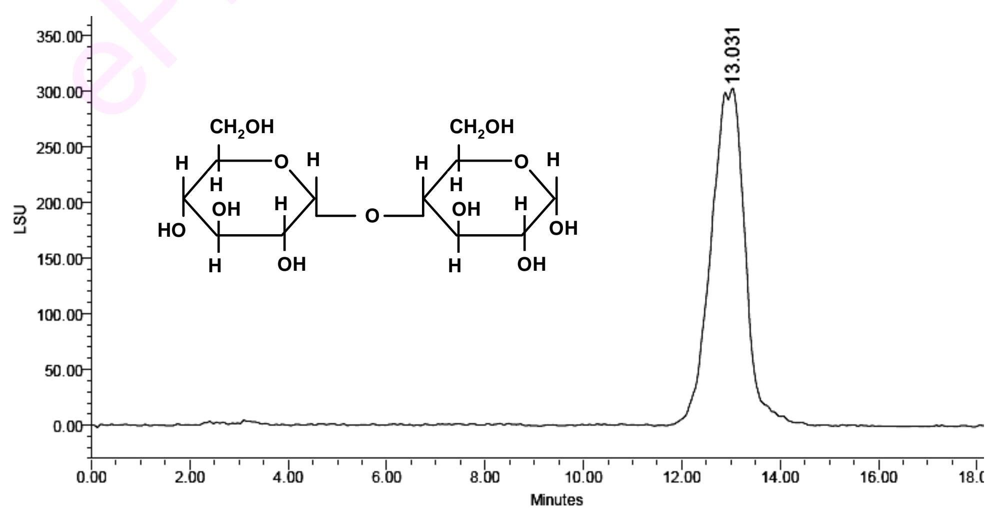

Fig. 5.5. HPLC chromatogram of maltose

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On comparing the retention times of hydrolysed sugar of LCB with the standard sugars, the first peak of LCB (RT: 7.671) matched with fructose (7.709). So, enzyme hydrolysed LCB sugar was spiked with fructose and confirmed that the first peak characterised as fructose (5.6)

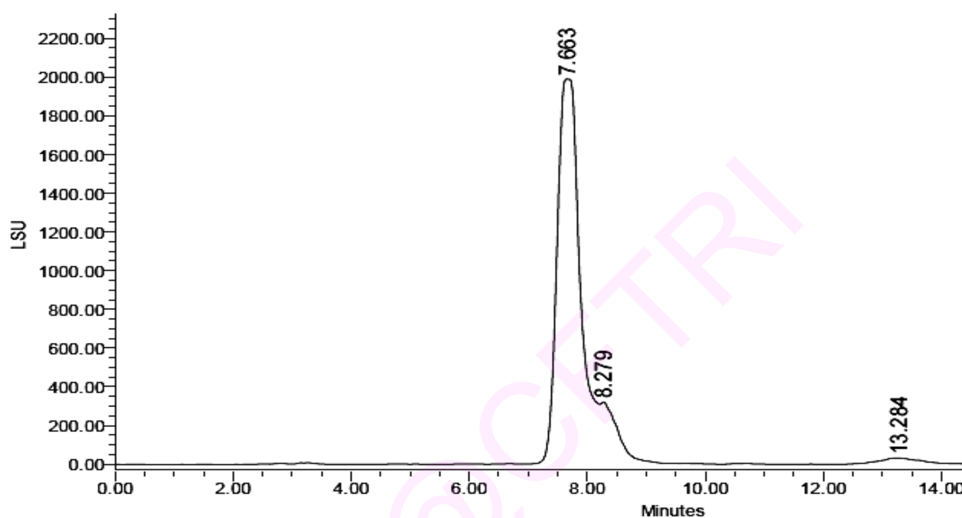

Fig. 5.6. Hydrolysed sugar with fructose spiking

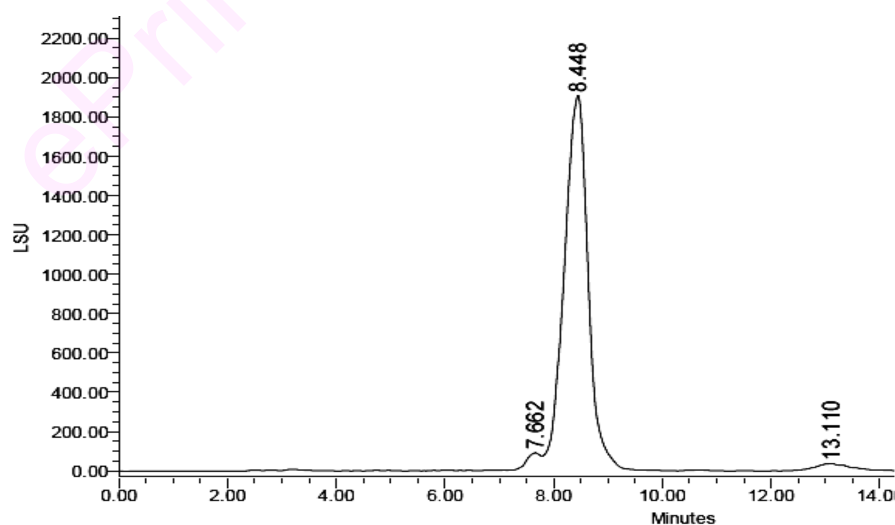

Fig. 5.7. Hydrolysed sugar with glucose spiking

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Second peak with the retention time of 8.387 min matches with glucose (8.152). Enzyme hydrolysed LCB sugar was spiked with glucose and confirmed that the second peak is due to the presence of glucose (Fig. 5.7).

On comparing the retention time of the individual sugars, the third peak (RT: 13.291) matches with retention time of maltose (13.031). This observation is confirmed by spiking enzyme hydrolysed LCB sugar with maltose and the chromatogram is given below (Fig. 5.8.).

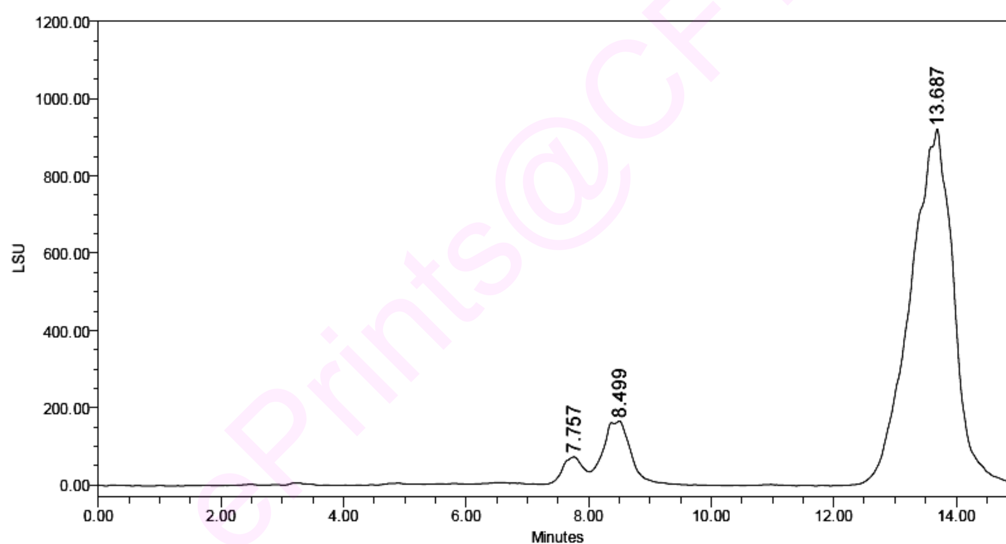

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Fig. 5.8. Hydrolysed sugar with maltose spiking

Green coffee contains 13% of starch and 7-8% of sucrose (Clarke & Vitzthum, 2001). Starch is generally insoluble in water at room temperature. Starch molecules are glucose polymers linked together by the alpha-1,4 and alpha-1,6 glucosidic bonds. Amylases during hydrolysis of starch enter at the 1 -> 4 linkages and break the chain and eventually

produce a mixture of glucose and maltose. Also, the presence of sucrose on inversion lead to the formation of glucose and fructose since the acidic pH was selected for the experiments. However there is no report of the presence of maltose from LCB as well as green coffee beans.

Like starch, cellulose is a polymer of glucose monomer units, linked together at the beta-1,4 locations as opposed to the alpha-1,4 locations for amylose (Meenakshi & Rao, 2007). The difference in the glucose linkage between starch and cellulose makes impossible for the amylase to break down cellulose. Therefore, cellulose degradation is not happening during the hydrolysis of LCB polysaccharides.

There are reports for the presence of low molecular weight sugars in green coffees (*Coffea Arabica*) was reported using LC-MS to processing method and storage conditions (Wintgen, 2004). There is a relationship between post harvesting methods and the amount of low molecular carbohydrates. Though sucrose is the major low molecular carbohydrate, on storage, it cleaves into glucose and fructose.

Oosterveld (2003) reported that part of the galactomannans from green coffee beans could be extracted relatively easy with water. Also the galactomannans were found to have high degree of branching of galactose and mannose. Wolform *et al.,* (1961) isolated galactomannans and suggested that β (1-4) linked mannan with the molecular weight

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approximately 7000 and one galactose unit side chain for every 47 mannose units. In general, arabinogalactan is a member of the carbohydrate family made up of many galactose and arabinose sugar units linked together in a ratio of 6 galactose units to 1 arabinose unit (Fischer, 1999). However, in our experiments, amylase could not hydrolyse either arabinogalactan or galactomannans but could able to hydrolyse starch and sucrose.

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Summary & Conclusion

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SUMMARY AND CONCLUSIONS

Coffee is one of the most popular and widely consumed beverages in the world (Varnam & Sutherland, 1994). In coffee producing countries, by-products/wastes constitute a source of severe contamination and a serious environmental problem due to the availability of enormous quantity. In general, coffee is graded based on the size, colour and percentage of imperfections. Defective coffees obtained after grading is termed as Low-grade green coffee beans (**LCB**), one of the by-products, represent ~15-20% of coffee production and are rejected in the international market due to the undesirable taste produced in the beverage when blended with the graded coffee.

Instant coffee is produced from green coffee after roasting, grinding, extraction and concentration of water solubles. After extraction, the remaining residue, co-product, is referred to as **spent coffee**. Almost 50% of the world's produce is processed for soluble coffee. Therefore, these plant materials were selected for the investigation work to find out the alternate use rather than using for beverage purpose.

Physico-chemical composition

There are no reports available for the physical properties and chemical composition of LCB in comparison to graded coffee beans from Indian region. In the present study, physical characteristics such as bean density, brightness, titratable acidity, pH, moisture, and total soluble solids and also chemical composition, namely, caffeine, chlorogenic acids, lipids, sucrose, total polyphenols (as gallic acid equivalents) , and proteins were evaluated in LCB as

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well as in graded green coffee beans. The physical parameters such as weight, density, and brightness of defective coffee beans were low compared to the graded beans, which is due to the presence of immature, broken, bleached, and black beans. Caffeine content was low in LCB compared to graded beans. Moisture levels (5.52 to 7.38%) of the beans in the present study are marginally lower than the levels reported (Mazzafera, 1999; Clarke, 1985). However, defective coffee beans did not show much variation in moisture levels, when compared to graded beans. Total soluble solids content was in the range of 29-34% for graded and 26-32% for defective coffee beans. Caffeine content was in the range of 0.8 –1.8% which is matching with the literature value of 0.9-1.4% (Franca *et al.*, 2005; Macrae, 1985). Chlorogenic acids in coffee are responsible for antioxidant activity and are found to be marginally more in LCB (6.83-8.80%) compared to graded coffee beans (6.35-8.32%).

Spent coffee powder from two varieties of coffee namely arabica plantation and robusta cherry, was prepared using a column extractor. The total polyphenols and chlorogenic acid in the spent coffee residues are in the range of 0.87-2.38% and 0.5-1.45% respectively.

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Bioactivity *in vitro* and *in vivo* studies

LCB and spent coffee residues were subjected to extraction using various solvents namely hexane, chloroform, acetone and methanol successively in column and soxhet extractors. The extracts (conserve) were evaluated for antioxidant potential through *in vitro* models such as radical scavenging activity (α,α–diphenyl-β-picrylhydrazyl radical), antioxidant activity (β-carotene-linoleate model system), reducing power (iron reducing activity) and antioxidant

capacity (phosphomolybdenum complex). Highest yield of extract (12%) was obtained with methanol followed by hexane (8%) and chloroform (1.5%). Lowest was obtained with acetone (<1%). Also, it was observed that methanol extract was found to exhibit maximum radical scavenging activity (92.5%) followed by extracts obtained with acetone (81%) and chloroform (25%) at 100-ppm concentration.

Further, the methanol extract (Me_1) showed antioxidant activity (58%) at 100 ppm concentration, while the other extracts viz., acetone, chloroform and hexane exhibited 44%, 28%, and 14%, respectively, at the same concentration. The antioxidant capacity of the methanol extract and propyl gallate showed 1367 ± 54.17 and 5098 ± 34.08 µmol/g (as equivalents to ascorbic acid). Reducing power of the extract and standard compounds is in the following order ascorbic acid > chlorogenic acid > BHA > methanol extract. The high antioxidant potential of the methanol extract of low-grade coffee beans is due to the presence of phenolic compounds including chlorogenic acids.

Me_1 was further analysed for oxygen radical absorbance capacity (ORAC), anti-tumour (P388 cell assay), anti-inflammatory (J774A.1 cell assay) and anti allergy (RBL- 2H3) *in vitro*. The antioxidant activity of LCB extract, measured as Trolox equivalents (4416 µM/g) was significantly ($p < 0.05$) higher than that of the spent coffee extracts. However, extracts of spent coffee exhibited significantly ($p < 0.05$) more anti-tumour activity than the LCB extract in terms of cell viability. This could be due to the possible role of brown pigments (melanoidins and phenolic polymers), formed during roasting, which may protect cells from oxidative damage in the biological system. However, both the extracts of LCB and spent coffee showed limited anti-inflammatory

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and anti-allergic activity. The presence of phenolics and chlorogenic acids in appreciable quantities in LCB and brown pigments in spent coffee makes these coffee by-products a source for natural antioxidants.

Me_1 was evaluated for oxidative stress in BALB/c mice *in vivo*. Extract was mixed with normal mice feed and fed to the mice (n=6), up to 14 days. Blood samples were collected from all the mice at 0, 7th days from tail vein. At the end of 14 days all the mice were sacrificed, blood and urine were collected. Urine samples were quantitatively analysed for oxidative DNA damage in terms of the adduct 8-hydroxydeoxyguanosine (8-OHdG) as a bio marker. Serum samples were analysed for total cholesterol, HDL cholesterol oxygen radical absorbance capacity and reactive oxygen metabolites.

At the concentration of 1%, Me_1 reduces the urine oxidative stress in terms of the biomarker as 8-OHdG and increases the serum HDL cholesterol significantly ($p<0.05$) in BALB/c mice at the end of 14 days of experimental period. There is a significant increase in Trolox value in serum of coffee fed mice compared to control mice. There is not significant difference in the reactive oxidative metabolites between control and coffee fed mice. Results suggest that coffee extract showed positive indication in controlling the oxidative stress in BALB/c. Further studies are needed to find out the compounds responsible for the beneficial effect.

Isomers of chlorogenic acid and their enrichment

Methanol extract possess appreciable amounts phenolic content (16.6± 1.43%), which includes chlorogenic acids. Isomers of chlorogenic acids were analysed by HPLC. Me_1 was subjected to preparative HPLC analysis and six

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fractions were collected and analysed by NMR. Results suggest that the compound in first fraction was trigonelline, and this is the first report for the presence of this compound in the extract of LCB. Compound present in the second fraction was identified as 3- CQA. The third fraction found to contain two major compounds viz., 5-CQA and caffeine. Presence of 4-CQA was also observed. Quantities of fractions 4, 5 and 6 were insufficient for NMR spectral analysis and however the compounds present in these fractions were identified as 3, 4 diCQA, 3,5, diCQA and 4, 5 diCQA respectively by comparing the retention times with the literature reported values.

Methanol extract was subjected to different treatment (viz., partitioning in different solvents, separation by chromatography on resins and isolation using lead salts) for the enrichment of phenolics. Among all the methods, precipitation using lead acetate gave more phenolics as well as chlorogenic acids though the yield of the separated fraction was less compared to the others. Isomers of chlorogenic acids was analysed by HPLC and found that the composition of 5-CQA was highest in the isolated fraction by lead acetate precipitation. Partitioning in chloroform and ethyl acetate as individual extraction yielded better yield, phenolics as well as chlorogenic acid contents. In general, the composition of 5-CQA was more in all the isolated fractions than the methanol extract irrespective of the solvents as well as the methodologies attempted. Therefore it is concluded that for food application studies, LCB can be used as raw material for isolating the phenolics.

Me_1 can be fractionated using either chloroform or ethyl acetate since the yield of the fractions as well as phenolic and chlorogenic acid content was relatively higher. However, for pharmaceutical applications, the preferable

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method could be using lead acetate, since the composition of chlorogenic acids is the highest in this fraction compared to all methods. Further purification of chlorogenic acids is necessary for food / pharmaceutical application taken into account of rules and regulations of residual solvents and toxicological experiments.

Efficacy of coffee extract against oxidative rancidity

The conserve prepared from low-grade coffee beans, which has the radical scavenging activity of 92.5 % at 100-ppm concentration, was selected to evaluate its efficacy against oxidative rancidity of sunflower oil. Results showed that the conserve was quite effective in restricting the oxidative changes (viz., Peroxide value, free fatty acids p-anisidine value), which are formed during the storage of refined oil. Also, the conserve was quite soluble in the fat system and did not have any adverse effect on the storage quality of the oil. Results of the study provides scope for the research on the utilization of the low-grade coffee / or its conserve in various food systems. However, further study is required with respect to the other characteristics such as toxicological aspects, carry through effect and standardization of the dosage levels.

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Hydrolysis of polysaccharides

There is an appreciable amount (~50%) of insoluble polysaccharides present in green coffee beans, it could be possible to modify / degrade them for solubilisation using chemical/enzymatic treatment for further utilization.

Enzymes viz., amylase, cellulase, pullulanase and pectinase were selected for the solubilisation of polysachharides of Low grade coffee beans. LCB were

treated with enzymes at various concentrations and different incubation times and subjected to extraction. The solubilised sugars were analysed by dintiro salicyclic method for the estimation of simple sugars. Amylase at the level of 10 mg/1 g of LCB after incubation time of 2h., yielded the extract (30%) with 12.5% sugars. Sugar profile was analysed by HPLC using ELSD, which showed three peaks and their retention times were compared with that of the standard sugar. Peaks were identified as fructose, glucose and maltose with spiking experiments. The ratio of these sugars in hydrolysed sugar mixture is 30.55: 57.86:11.59. The hydrolysed sugar could be partially replaced for chicory which is presently used as an adjunct during the manufacturing of soluble coffee. Further work is necessary for the hydrolysis of arabinogalactan and galactomannans using different enzymes.

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Outcome of the Thesis

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PAPERS PUBLISHED

1. Physicochemical characteristics of green coffee: Comparison of graded and defective beans
   K. Ramalakshmi, I. Rahath Kubra and L. Jagan Mohan Rao
   Journal of Food Science, 2007, 72, S333-337.

2. Antioxidant potential of low-grade coffee beans
   K. Ramalakshmi, I. Rahath Kubra, and L. Jagan Mohan Rao
   Food Research International, 2008, 41, 96-103.

3. Bioactivities of low-grade green coffee and spent coffee in different *in vitro* model systems
   K. Ramalakshmi, L. Jagan Mohan Rao, Yuko Takano-Ishikawa and M. Goto
   Food Chemistry, 2009, 115, 79–85.

4. A Perception on health benefits of coffee
   Sunitha E. George, **K. Ramalakshmi** and L. Jagan Mohan Rao
   CRC Critical Reviews in Food Science and Nutrition, 2008, 48, 464-486.

5. Low grade coffee beans: an alternate use as antioxidants
   K. Ramalakshmi and L. Jagan Mohan Rao
   Food Review Indonesia, 2008, 3, 30-33.

6. Efficacy of green coffee conserve against the oxidative rancidity of sunflower oil. (Manuscript under preparation)

7. Enzymatic hydrolysis and characterization of green coffee polysaccharides. (Manuscript under preparation)

8. Isolation and identification isomers of chlorogenic acids of low grade coffee beans. (Manuscript under preparation)

9. Effect of green coffee conserve on oxidative stress in BALB/c mice. (Manuscript under preparation)

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PAPERS PRESENTED IN CONFERENCES

1. Bio-activities of selected spices and plantation crops in different in vitro model systems.
   K. Ramalakshmi, L. Jagan Mohan Rao, P. Srinivas, Yuko TAKANO-ISHIKAWA and Masao GOTO
   International conference on Polyphenols and Health, Kyoto during 25th – 28th November 2007

2. Effect of green coffee conserve on oxidative stress in BALB/c mice
   K. Ramalakshmi., L. Jagan Mohan Rao., Y. Takano-Ishikawa, Y. and M. Goto
   International Food Convention- 2008 , Mysore during 15-19th December 2008.

HONOURS & AWARDS

1. Awarded ***UNU-Kirin Fellowship*** for the year ***2007-08*** by United Nations University, Tokyo, Japan.

2. Awarded CFTRI Annual award - 2009 in the category of ***Individual Award for Scientific & Technical Contribution in Group III*** (R&D Department)

PATENT FILED

1. A process for the preparation of green coffee conserve from low grade coffee beans -729DEL05
   K. Ramalakshmi, L. Jagan Mohan Rao and B. Raghavan.

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