ANALYSIS OF TRACE ELEMENTS AND ORGANIC CONSTITUENTS IN MEDICINAL HERBS

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

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INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **ANALYSIS OF TRACE ELEMENTS AND ORGANIC CONSTITUENTS IN MEDICINAL HERBS** in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** and submitted in the **Department of Chemistry** of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out by me during a period from **January 2003 to August 2006** under the supervision of **Prof. A.N. GARG**

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Ratnadeep

(RATNADEEP PAUL CHOUDHURY)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

A.N. Garg 30th Aug. 2006

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The Ph.D. Viva-Voce Examination of **RATNADEEP PAUL CHOUDHURY,** Research Scholar, has been held on 26th October, 2006 at 10:30 AM.

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Signature of External Examiner

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(RATNADEEP PAUL CHOUDHURY)

ABSTRACT

Health is a state of physical and mental well being and not merely the absence of a disease. This definition particularly fits well with the concept of *Ayurveda*, based on holistic approach whereas conventional medicines treat the affected part of the body only. Use of medicinal herbs in various civilizations is as old as the mankind itself. WHO estimated herbalism to be the most commonly practiced in all parts of world? In recent years, a global trend is noticed for the revival of interest in the traditional system of medicine. Screening of medicinal herbs has become a potential source of biodynamic compounds of therapeutic value in phytochemical research but little is known on the role of essential trace elements, which play a vital role in health and enzymatic processes.

Popularity of medicinal herbs has also brought concerns and fears over the professionalism, quality, efficacy and safety of herbal products available in the market. Imposing regulatory standards using good agricultural, laboratory, supply and manufacturing practices only can ascertain the public belief that herbal and natural products are safe. Therefore, an extensive investigation of trace element analysis (TEA) and organic constituents are essential. Instrumental neutron activation analysis (INAA) has been used as multielemental technique for the determination of 23-31 elements in a variety of herbs and herbal formulations. AAS was used for the determination of toxic elements Ni, Cd and Pb especially. Also, thin layer (TLC) and column chromatography including preparative TLC were used for the separation of organic constituents, which were identified by ir, NMR spectral and GC-MS methods. Thesis is divided into six chapters, a brief discussion of each follows:

Ch I introduces the ancient Indian medicine system using herbs and their therapeutic or medicinal uses. An overview of traditional medicines and their scientific literature is discussed. General aspects of radioanalytical methods including NAA, its different types and applications in various fields for trace element analysis (TEA) in complex biological samples are emphasized. A general survey of drugs from natural sources is discussed. Literature reports on organic constituents and biocompatible trace elements have been reviewed. Lastly, Aim and Scope of the present work is described.

Ch II describes *Experimental Methodology and Instrumentation* along with sampling methods and sample preparation in NAA and AAS. Major emphasis has been on short

irradiation using pneumatic carrier facility (PCF) in DHRUVA reactor at the BARC, Mumbai resulting in the determination of 20 elements. Also included are details of high-resolution γ spectrometry including associated hardware and software. Modified version of phosphorus determination is described. Also our results on the participation in Intercomparison studies of Corn flour (CF-3) and Soybean flour (SBF-4) are discussed. Separation of organic compounds from the natural products is described along with a brief description of spectral identification and GC-MS methods.

Ch III deals with our results on the analysis of 30 elements in 10 samples of mint (*Mentha Spicata*) leaves collected from four different locations in North-West India. It is enriched in Ca, Mg, K, P, Na and Fe. Variation in elemental contents from different locations is attributed to difference in soil characteristics and environmental factors. Toxic heavy metals Hg (97-983 ng/g), Sb (1.8-315 ng/g), Cd (15-722 ng/g) and As (98-320 ng/g) are all found at sub-ppm level and vary in a wide range. Strong inverse relationship is observed between Na and Mg with Cl (r = -0.95 and –0.97 respectively). An inverse correlation (r = -0.91) was observed between Zn and Cr, essential in enzymatic processes. K/Na in four different locations varies by a factor of 3 while K/P varies in a range of 2-10 with mint leaves from Dehradun showing the lowest ratio. Column and preparative TL chromatography ($CHCl_3$/MeOH/CH_3COOH in 9:2:0.5 v/v) were used to separate menthol and 1,3-dihdrocarveol in methanolic extract. Structure was elucidated by elemental analysis, ir, NMR and GC-MS studies.

DPPH radical scavenging activity of diethyl ether extract was found maximum at ~40 μg/mL and attributed to polyhydroxy compounds. Ten hitherto unknown compounds; 2-(1-methyethylidene) cyclohexanone; 2-hydroxy 3-ethyl 2-cyclopenten-1-one; 4-ethyl 1,3-benzenediol; 4-acetyl 1-methyl cyclohexene; 2-propyl 5-methoxy phenol, carvone, octahydro-1, 4,9,9-tetramethyl methanoazulene; 2-chloro 1-ethyl 5-methoxy 3-methyl benzene; dibutyl phthalate and mono (2-ethyl hexyl ester) hexanedioic acid were identified in diethyl ether extract by GC-MS.

Ch IV: After coming across news reports on Curry leaves (*Murraya Koenigii)* being antidiabetic and anticancerous, we analyzed 28 samples from all over India for 24 elements. Most elements vary in a wide range depending on the origin of their location. It is observed that Br, Cs, Sc, Th and Zn vary by an order of magnitude whereas Fe, Mn, Na, K, Rb, Se and P vary by a factor of 3 to 5 only. Leaves from the southern zone are enriched in K, Mg, Mn, Cl and P but those from the western zone are rich in Na and

Zn. Concentrations of most elements from eastern zone are at par with the mean values. It is known that Cr, Fe, Cu, and Zn play an important role in the maintenance of normoglycemia by activating the β-cells of pancreas. Curry leaves are a rich source of nutrient trace elements such as Fe, Cu and Zn besides Mn, Se and minor constituents (K, Mg, Ca and P). Rb and Cs are linearly correlated (r =0.93) as their salts enhance the absorption of insulin in lower respiratory tract by lowering the breakdown of glucose.

Three new compounds were separated from the ethanolic extract by GC-MS: 3-methylthiopropanenitrile (**I**); 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl ester) (**II**) and 1-penten-3-ol (**III**). **I** is a plant product of biosynthesis of glucatropaelin, **II** an allelopathic compound reducing the need for weed management and **III** a strong antioxidant which can account for the antidiabetic and anticancerous properties.

Ch V describes the analysis of leaves (5), roots (4), fruits (3) and seeds (3) of sixteen anti diabetic herbs including three capsules (Himalaya) and five commercial herbal preparations for 6 minor and 22 trace elements. K (3.20-60.9 mg/g), Ca (4.98-47.8), Mg (0.43-1.92 mg/g), Cl (0.21-11.9 mg/g) and P (0.59-6.11 mg/g) form minor constituents. All these are electrolytic or structural elements and play a role in the fluid balance. Na (0.03-5.67 mg/g) and Fe (0.11-0.27 mg/g) are found in < 0.5% amounts. No single plant part is enriched in all the elements. *Tejpatta*, a leaf used as spice is enriched in Ca (47.8±3.5 mg/g) while roots like *Kutki* and *Naagarmotha* are enriched in Cr (2.15±0.02 μg/g) and Se (1.04±0.07 μg/g) respectively. *Marodphali* a fruit contains elevated concentrations of Fe, Cu and Zn, all correlated with *diabetes*. Seeds of *Jaamun* and *Kaalijeera* are considerably enriched in V (2.97±0.18 μg/g) and Mn (356±10 μg/g) respectively. V and Mn play an important role in controlling *diabetes*. *Bitter gourd* capsule (Himalaya) and powder (Vyas) contain very high amounts of As (1.01±0.07 μg/g and 1.44±0.12 μg/g) and Br (433±27 and 203±6 μg/g) respectively. Hg content also varies in a wide range of 23-143 ng/g but well below the permissible limit (3 μg/g). K/P were found in a range of 6.45-10.7 with a mean of 8.19±1.39. Cu and Zn are well correlated with r = 0.89. Again Rb and Cs are linearly correlated with r = 0.87.

In addition five antidiabetic formulations Madhunashini (Gurukul and Divya), Diabetex (Jagdamba), Jambrushila (UAP) and Diabeticin (BACPO) were analyzed where Cu, P, Fe and Mn contents were 2-3 times higher than raw herbs and capsules while Mg content was higher by a factor of 6. Herbs and capsules have higher concentrations of Na, Ca, Cl, V and Zn. Most elements (K, Cr, Zn, Ba, Rb and Se) are in

comparable range. However, toxic elements (As and Hg) are significantly lower in formulations. Rb and Cs show even better correlation with r = 0.93. Zn and Cr show an inverse correlation with r = -0.81 depicting antagonistic behaviour.

Petroleum ether extracts of *A. indica* (neem) showed four compounds by GC-MS: 1,1,2,3-tetramethylcyclopropane, methyl phenyl sulfone, n-hexanedecanoic acid (palmitic acid) and 9,12,15-octadecatrienal. Octadecatrienal, commonly known as linolenic aldehyde, is reported in the hexane extract of *kewda*, an aromatic plant. Two compounds 7–(but-3-enyl) 1,2-dihydro cyclobutabenzene and 2-hydroxy methyl 1-methoxy 9,10 anthracenedione were separated from the ethyl acetate soluble fraction of petroleum extract and identified by GC-MS. Anthracenediones are a class of anti-cancer agents.

Ch VI deals with the analysis of *trikatu* – used as a stimulant and for treatment of cold is a mixture of three spices of dried ginger (*Z. Officianalis*), black pepper (*P. Nigrum*) and pipali (*P. Longum*). Its five brands from Yogi (Haridwar), Vyas (Indore), Zandu (Mumbai) and Sushrut (Nagpur) Pharmacies and a local sample from Mumbai were analyzed for seven minor (Al, Ca, K, Na, P, Mg and Cl) and 24 trace (As, Au, Ba, Br, Ce, Co, Cr, Cs, Cu, Eu, Fe, Hf, Hg, La, Mn, Rb, Sb, Sc, Se, Sm, Sr, Th, V and Zn) elements. It exhibits higher amounts of Ca (3.83±0.8 mg/g), Fe (0.48±0.20 mg/g), Mn (167±22 μg/g) and Se (0.12±0.4 μg/g), which are all of nutritional importance. Some toxic heavy metals such as Sb, Hg, Th were found below permissible limits. Cu/Zn varies linearly with Zn (r = 0.92) whereas Fe and Mn exhibit inverse relationship (r = -0.89). Ginger is particularly enriched in Ca (12.6±0.1 mg/g), Mg (1.88±0.06 mg/g), Fe (427±34 μg/g) and Mn (266±45 μg/g) whereas black pepper is enriched in P (3.53±0.35 mg/g), Cr (8.65±1.48 μg/g), Se (0.093±0.02 μg/g) and Zn (37.1±6.7 μg/g) contents.

Barbituric and tannic acids were separated from the methanolic extract of pipali and confirmed by elemental analysis, ir spectra and GC-MS. The essential oil obtained by hydro distillation showed 10 compounds; 2,2-dimethyl propanoic acid, decane, 1-decyne, 3,4, 8-trimethyl 1-nonene, undecane, bis- (1-methylpropyl) disulfide, 2-nonynoic acid, 2,4-decadienal, nonanoic acid and tetradecanoic acid, by GC-MS.

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CHAPTER I

ANALYSIS OF HERBS

I.1 TRADITIONAL MEDICINE SYSTEMS

Since the early periods of civilization, human beings have been dependent on plants for their health care and other needs. Plants were the main part of folk medicines practiced in different civilizations including India [1,2], China [3], Middle East [4], Africa [5], South East Asia [6] and South America [7]. Herbs have been called part of *nature's pharmacy*. Today, herbalism is widely practiced in many parts of world, both by the traditional or indigenous healers and also by trained herbalists in the West, who form a part of complementary medicine system. According to a WHO survey, 70-80% of the world population is dependent on plant-based medicines [8]. Medicinal plants were adopted into modern medicine system after these were found effective as drugs through chemical and pharmacological screening. The importance of plants in East European countries especially in the old communist block can be gauged by the fact that ~ 60% of the prescriptions issued contain one or more plant products [9]. In China, more than 80% of the population use the Traditional Chinese Medicine (TCM) derived from plants. In Asia, Africa and South America, folk medicines and traditional system of medicine play a major role in overall health care programme [10]. Although their action can in some ways be similar to modern drugs, herbal remedies are generally gentle and safe. Many of the drugs used as conventional medicine are derived from herbs. Herbalism uses the whole plant or part of the plant such as leaves, flowers, fruits, stems or roots rather than isolating the *active agent*. Plants contain active constituents that work together synergistically with no/low side effects, which may occur if isolated components are used.

(i) Ayurveda; It is the ancient Indian system of medicine originated around 1500 B.C. [11,12]. It had glorious past in India and other parts of the world. The word *Ayurveda* is derived from '*ayur*' meaning 'life' and '*Veda*' meaning 'to know'. Thus, *Ayurveda* means *the science (knowledge) of life*. Ayurvedic Medicine System is now considered as an effective alternative system of medicine. It has a field oriented functional holistic approach, which seeks balance between mind, body, spirit, senses and emotions, a combination of total health. It recognizes the unique constitutional difference between individuals and therefore recommends different regimens for individuals [13]. Ayurvedic concept is interrelated to body, mind, consciousness (or soul) and the *Panchamahabhuta* (or five elements), which blend into three bio-

energetic forces: *Vata* (air and space), *Pitta* (fire and water) and *Kapha* (water and earth) present in all human beings and govern health and physical constitution. This is also called *tridosha*, which should be in equilibrium and any imbalance causes disease. For example, excessive *Pitta* causes irritation and aggressiveness. Similarly excessive *Kapha* results in greed and attachment [14]. Ayurvedic herbal supplements are used to reduce or pacify one or more *doshas* that may be imbalanced or disturbed in our body [15]. *Ayurveda* includes a comprehensive study of anatomy, physiology, pathology, diagnostic systems and treatment strategies. It finds increasing relevance and acceptance in a rapidly increasing global market of competing health care systems [11].

(ii) Other Systems of Medicine; There are many other systems of medicine such as the Chinese, Tibetan and Islamic (Unani Tibb) which have their roots in *Ayurveda*. For example, Buddha (born 550 BC) was a follower of *Ayurveda* and the spread of Buddhism into Tibet during the following centuries was accompanied by increased practice of *Ayurveda*. TCM includes herbal remedies, acupuncture, acupressure, massage and moxibustion [3]. All the ancient civilizations were linked to one another by trade routes, campaigns and wars. Arab traders spread knowledge of Indian plants in their *Material Medica* [4]. This knowledge was passed on to the ancient Greeks and Romans, whose practices eventually form the basis of European medicines including Homoeopathy. In African and South American countries, herbal medicines are highly popular and a major population relies on herbal products.

The world has now started taking a more serious look at herbalism. Only a few plant species of medicinal value have been scientifically evaluated for their possible medical applications. Safety and efficacy data are available for even fewer plants, their extracts, active ingredients and preparations. A number of International organizations such as UNESCO, WHO, UNIDO and Commonwealth Science Council are coming forward to support research programmes in this area. A six volume Compendium of Indian Medicinal Plants [16] has been published by the NISCOM. As a part of the Ministry of Health and Family Welfare, Government of India has established a Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha & Homeopathy (AYUSH). Traditional medicine systems have gained global importance. Hence, a thorough knowledge on their trace element contents and organic constituents attributable to its role in treating ailments is called for in providing safe and effective herbal medicines.

I.2 TRACE ELEMENTS: BIOLOGICAL IMPORTANCE

Various constituents of balanced diet provide energy, growth, replacement of cells and physiological regulation in human body. Our body needs adequate food for growth and development and trace elements are known to play a vital role [17]. An essential element is required to support adequate growth, reproduction and health throughout the life cycle, when all other nutrients are optimal [18]. Essentiality of an element is further proved if it exerts a catalytic or regulatory role in a critical biochemical pathway [19]. A classification of the elements in biological systems is presented in Table I.1.

Table I.1: Classification of elements in biological systems

Structural Elements	C, H, O, P, N, S, Ca
Electrolyte Elements	Ca, Cl, K, Mg, Na
Anions	HCO_3^-, SO_4^{2-}, HPO_4^{2-}
Trace Elements	
Essential	
a. Biologically	Co, Cr, Cu, Fe, I, Mn, Mo, Se, Zn, As, F, Ni, Si, Sn, V
b. Clinically	Cr, Cu, Fe, I, Se, Zn
Toxic	
a. Potentially Toxic	As, Br, Cd, Hg, Pb, Se, Tl
b. Environmental contaminants	Cd, Hg, Pb
c. Industrial Hazards	As, Cr, Hg, Ni, Mn, Pb, Sb, Si, Ba, Sr
d. Radioactive	^{232}Th, ^{235}U, ^{210}Po, ^{222}Rn, ^{234}Pu etc.

Those found at mg/g level are referred to as macronutrients whereas others found at μg/g or ng/g levels are called micro or trace nutrients. They are further subdivided as those for which essentiality has been confirmed by evidences and others whose essentiality has been suggested by impairment of physiological function [20].

These elements have primarily three general physiological roles in biological systems; structural, catalytic and signal transduction. Of the six macronutrients, Ca and P play an important role in the skeletal structure though P is also an important component of phospholipids, phosphoproteins and nucleic acids. Na, K and Cl are responsible in maintaining osmotic pressure, water balance and membrane potential along the cell wall. Mg is primarily an intracellular element exerting regulatory and catalytic role in various biochemical systems [21]. Iron performs both major and

minor functions. It is a component of numerous proteins notably the cytochromes and its deficiency causes anaemia. Deficiency of iodine causes goiter and cretinism and that of Cu gives rise to many distinct aspects of pathology, which can be identified with specific cuproenzymes [18]. Another factor of importance is the bioavailability defined as the proportion of an element in food that can be absorbed by the body. Major functions and deficiency symptoms, which have led to the recognition of essential mineral elements are listed in Table I.2.

As in the case of other essential nutrients, the physiological effects of the essential mineral elements depend on the level of intake/ absorption in the body as illustrated in Fig. I.1.There is a range of concentration, the so-called adequate or optimum range which provides proper function in the body system. If intake is below this range, there is a graded decrease in function until deficiency symptoms appear [22]. Toxic effects start appearing when intake exceeds the safe and adequate range. However, there are some elements, which are toxic even in minute amounts and have no biochemical functions eg. Cd, Pb, Hg and Tl are considered as toxic [23] even though some mercury preparations (especially *bhasmas*) are recommended for effective treatment of many ailments in *Ayurveda* [24].

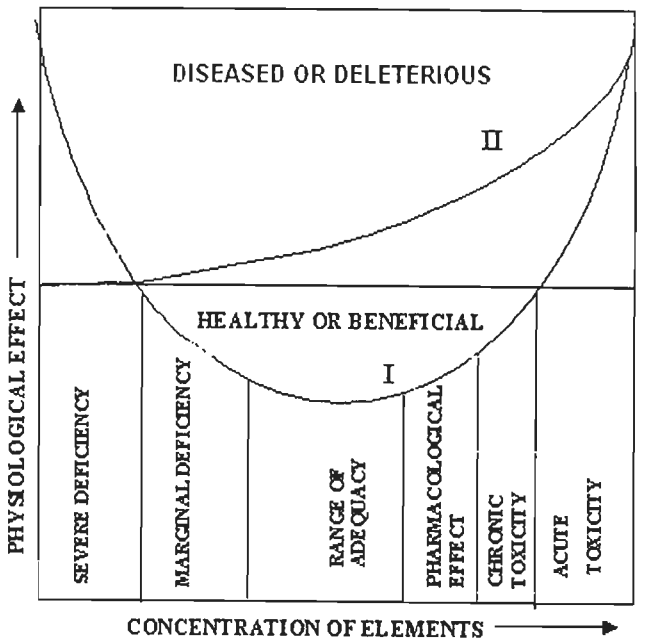


Fig. I.1: Correlation of elemental concentration with its physiological effects

In an industrial environment, many contaminants such as Br, Sb, Ni, Pb and Hg may either be inhaled or be a part of food chain causing deleterious effects [20]. Since we are living in nuclear age, long-term effects of radionuclides such as ^{222}Rn, ^{232}Th, ^{235}U, ^{210}Po and ^{239}Pu resulting from nuclear activities not subject to regulatory safety standards is a major source of concern.

Table I.2 List of essential elements, their metabolic functions and deficiency symptoms

Element	Function	Deficiency/Excess Symptoms
Fe	Oxygen and electron transport, constituent of haemoglobin	Anaemia, Stomatitis, Dysphagia, brittle nails
Cu	Constituent of oxidative enzymes, interaction with iron, cross linking of elastin	Anaemia, Change of ossification, elevated serum cholesterol, diarrhoea, nervous system damage
Mn	Mucopolysaccharide metabolism, constituent of superoxide dismutase	Disturbance in bones and cartilage formation, deafness, dizziness
Zn	Constituent of enzymes involved in energy metabolism and transcription	Growth depression, poor healing of wounds, sexual immaturity, skin lesions, menstrual problems, change of taste, acuity
Co	Constituent of vitamin B_{12}	Loss of appetite, vitamin B_{12} deficiency
Se	Constituent of glutathione peroxidase, interaction with heavy metals	Endemic cardiomyopathy (Keshan disease) premature aging, eye and nerve disorders, infertility, cause of breast cancer
Cr	Potentiation of insulin	Relative insulin resistance, impaired glucose tolerance, elevated serum lipids, fatigue and lack of energy, indigestion
As	DNA repair mechanism	Growth depression
Rb	Stimulate metabolism	Decreases growth and life expectancy
V	Gene regulation in various enzymatic systems	Several physiological malfunctioning including thyroid, glucose and lipid metabolism.
Ni	Interaction with iron absorption, activates enzymes	Anaemia, inconsistent growth
Ca	Essential for formation of healthy bones and teeth, regulates blood clotting, muscle function, nerve transmission	Muscle cramps, brittle bone disease, dental problems
Mg	Helps in absorption of other minerals, stimulates bone growth and promotes the body, use of vitamin B, C, and E	Lack of energy, muscle spasms, weakness asthma, cardiovascular disorders
K	Essential for growth, stimulates nerve impulses, promotes healthy skin, boosts kidney function, combines with Na to regulate heart beats	Irregular heart beats, dry skin, nervous disorders
P	Cell repairs, vital to growth of bones and teeth, helps digest proteins, fats and carbohydrates	Poor growth, arthritis, loss of appetite

I.3 DRUGS FROM NATURAL SOURCES

Plant species have served as sources of medicine for millennia. Many medical practitioners with training in pharmacology and/or pharmacognosy are well aware of a number of modern therapeutic agents that have been derived from plant species. In fact, over 120 pharmaceutical products currently in use are plant-derived and 75% of these were isolated after examining the use of these plants in traditional medicines [25]. Natural products are chemical compounds (or species) derived from living organisms such as plants and animals and these are useful as dietary supplements and for cure of a variety of ailments. Drugs derived from natural products are usually secondary metabolites and their derivatives. Until early 20th century, Organic chemistry was almost exclusively the study and isolation of natural products though its formation was little understood. Modern chemists now employ isolation techniques guided by bioassays to isolate the active compounds in pure form as schematically shown in Fig. I.2.

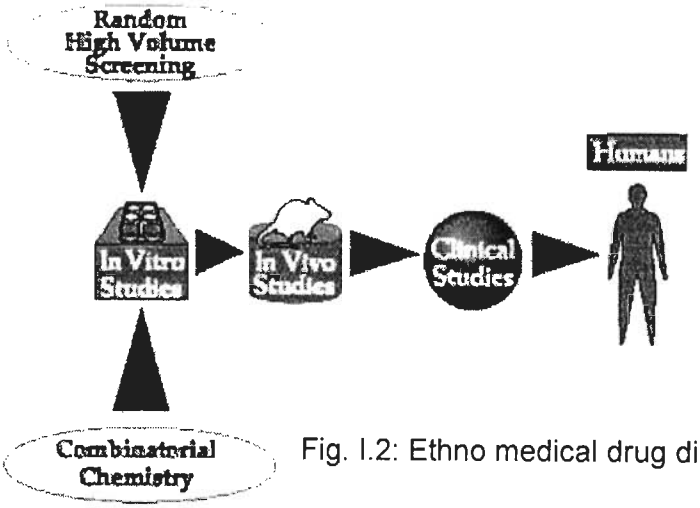
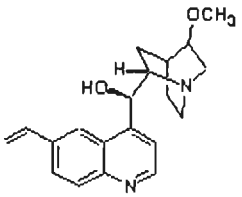


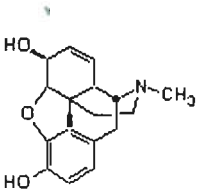
Fig. I.2: Ethno medical drug discovery process

The treatment of diseases with pure pharmaceutical agents is a relatively modern phenomenon. Following folk treatments, chemists and pharmacists began to isolate the compounds responsible for the remedy of an ailment. A large number of drugs are based on plant products viz. terpenes, glycosides, alkaloids, phenols, vitamins etc. Many of the earliest isolated pure compounds with biological activity were alkaloids, which were easy to isolate. Nitrogenous compounds are generally basic and exist in plants as salts. Thus, alkaloids are often extracted with water or mild

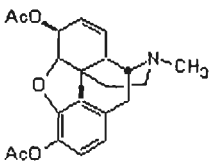
acid and then recovered as crystalline material by treatment with a base. For example, morphine alkaloids, derived from the opium poppy, *Papaver somniferum*, are powerful pain relievers and narcotics [26].



quinine



morphine



heroin

I.4 TRACE ELEMENT ANALYSIS: STATE OF ART

Reliable analytical measurement of nutritional and biomedical samples is an essential ingredient of sound decisions involving many facets of society including safeguarding an individual's health, improving the quality of life and facilitating technological advances. Therefore, an analytical methodology especially for health related problems should meet realistic expectations and the performance of a technique may be judged from the following criteria [27]

(i) Characterization of analytical information

- Accuracy and precision
- Sensitivity
- Multielemental capability

(ii) Sample requirement

- Type of matrix e.g. geological, biological etc.
- Size i.e. amount of sample (mg or even smaller)
- Nondestructive character especially for archaeological, cosmological and forensic samples

(iii) Operational properties

- Turn around time
- Cost effectiveness
- Accessibility and safety of workers
- Routine capability
- Automation

When an analysis is performed for a diagnostically important element, then the most important criterion is the multielemental character of the technique because

many elements interfere in presence of others. Analytical scientists have always been looking for suitable methods, which can meet all the requirements [28]. With technological advancement, automatic instrumental methods of analysis have become the order of the day and an analyst is required to determine analytes at lower and ultra lower concentrations [29]. As a result, limit of detection (LOD) has been decreasing by several orders of magnitude (from nμg/g to pg/g) during the last decade.

Significant developments in the instrumental methods of analysis to meet the ever-increasing demand of the analysts came into reality after the electronic revolution during 60's. The introduction of microcomputer systems and softwares has further changed the face of chemical analysis where one can get the results directly after the sample is introduced into the instrument for analysis. During last few decades, several new analytical techniques especially more sensitive hyphenated techniques have been developed:

- Inductively coupled plasma (ICP)-Atomic Emission Spectroscopy (AES)
- ICP-Mass spectrometry
- X-ray fluorescence (XRF)
- Particle induced X-ray emission (PIXE)
- Isotope dilution - mass spectrometry (IDMS)
- Derivative and Speciation neutron activation analysis involving GC-NAA
- Accelerator Mass Spectrometry (AMS)
- Thermal ionization mass spectrometry (TIMS)

Literature search for 2001-05 has shown approximately 23,000 papers published on the use of various analytical techniques. A pi diagram in Fig. I.3 clearly shows tough competition between AAS, ICP-MS and radioanalytical methods. In recent years AAS and ICP-MS have gained popularity because of long turn around time, radiation hazards and the antinuclear propaganda associated with radioanalytical methods. Still NAA remains

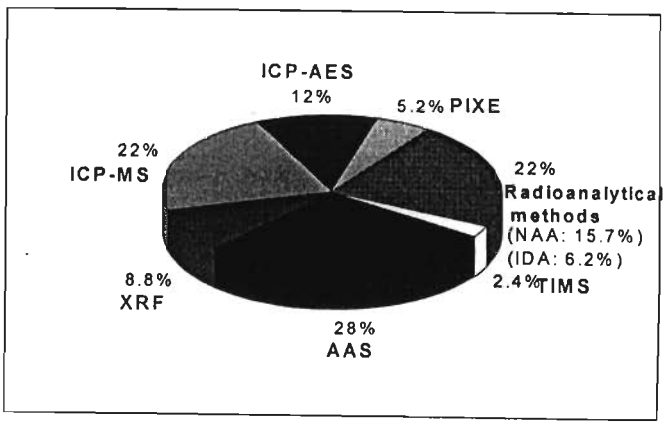


Fig. I.3. Use of Analytical Methods based on No. of Publications during 2001-05

the only technique, which is nondestructive, multielemental and provides accurate and precise data for complex matrices.

No doubt, AAS and ICP-MS are the most widely used techniques for determining metals and metalloids in biological and environmental samples in solution form [30]. AAS is based on the principle of absorption of light to measure the concentration of an element in gas-phase. However, it is primarily unielemental though multielemental hollow cathode lamps have become available. With the development of inductively coupled plasma (ICP) technology in the late 1980's, ICP-MS has the advantage of easy sample introduction and quick analysis with the accurate and low detection limits of a mass spectrometer [31]. It is capable of trace multielement analysis, often below ppb level. It has been used widely over the years, finding applications in a number of different fields including drinking water, wastewater, natural water systems/hydrogeology, geology and soil science, mining/metallurgy, food sciences, and medicine. Thermal ionization mass spectrometry (TIMS) has seen spectacular progress brought about by automation, advances in solid-state electronics, multi-collection techniques and filtering [32]. Consequently, it is possible to measure isotope ratios for elements such as Sr or Nd with a precision better than 10^{-5} on a ng or even lesser sample size.

The method to be employed for the measurement of an analytical signal largely determines the extent and type of sample preparation (including its size) required. Activation methods, XRF and mass spectrometric (MS) methods do not require the sample in solution form. Thus, solid sample analysis can be extremely useful for analysis of biological materials especially for forensic investigations. It is possible to eliminate the time-consuming sample dissolution step, thus reducing the risk of reagent contamination as well as loss of analyte elements. An important consideration, however, is the need of obtaining the sample as a homogenous solid, even though proper statistical treatment of data may enable accurate results to be obtained on samples showing micro-heterogeneity.

I.5 RADIOANALYTICAL TECHNIQUES

The discovery of radioactivity and subsequent use of radioisotopes as radiotracers and application of radiations especially during the 40's and 50's led to the development of many radioanalytical techniques such as activation analysis,

isotope dilution analysis and other radiotracer methods [33-36]. These are more specific, sensitive and accurate than conventional methods of those times. Neutron activation analysis (NAA) using neutron irradiation is one such method, which has evolved as the most widely used technique [37]. Instrumental and methodological developments have further enriched this technique and it is being used in various disciplines for the analysis of a variety of samples.

(i) Historical Developments; Since its discovery by George de von Hevesy and Hilde Levy in 1936, it has traveled a long distance of 70 years. A chronological development in the field of NAA is summarized in Table I.3.

Table I.3 Stages in the development of NAA

1932-1944 (Induction period)	Discovery of Neutrons, Activation Analysis using isotopic neutron sources.
1944-1964 (Nuclear Reactor)	Availability of nuclear research reactors, Exploration of applications
1950-1965 (Scintillation detector)	Fundamental advances in electronics/instrumentation, scintillation detectors and radiochemical neutron activation analysis of complex matrices.
1965-1975 (Solid state detector)	Si(Li), Ge(Li) and HPGe detectors, Multichannel Analyser, High resolution gamma ray spectrometry for multielemental analysis of Lunar rocks, meteorites and cosmic samples
1975-1995 (Methodogical Developments & Applications)	Extensive applications in geological, archeological, biological, environmental, industrial and forensic studies – new era of methodological developments [38-42]; ➢ Epithermal neutron activation analysis (ENAA) ➢ Fast neutron activation analysis (FNAA) ➢ Cyclic neutron activation analysis (CNAA) ➢ Derivative neutron activation analysis (DNAA) ➢ Prompt γ-ray neutron activation analysis (PGNAA) ➢ Monostandard (k_0) NAA method ➢ Fast irradiation and measurement system (FIMS) ➢ In vivo activation analysis (IVAA) ➢ On Line Analysis
1995 - Present	Development of Robotic systems, Specific problem oriented studies in Medicine, Health & Nutrition, Environment, Industry, and National security. Speciation studies (GC-NAA)

In recent years the widespread applications of this technique have been hindered due to lack of funding prompting closure of many reactors. However, its popularity and interest in the scientific community has not diminished. This is evident from the number of publications (Fig. I.3) and the conferences held around the world as listed in Table I.4. The proceedings of MTAA, MARC, NAMLS and APSORC are published in *J. Radioanal. Nucl. Chem.* These conferences provide a forum at

national/international level to present and discuss the emerging trends in methodological, instrumentation and their applications in new areas.

Table I.4. Important Conferences on Nuclear Analytical Techniques

Name of the Conference	Location	Starting (Frequency)
◆ Modern Trends in Activation Analysis (MTAA). MTAA-12, June 20-25, 2007 scheduled in Tokyo, Japan	Around the world (MTAA-1 & 2 were held at TAMU, USA)	1961 (4 years)
◆ Nuclear and Radiochemistry (NRC). NRC-6 held in Aachen, Germany, Aug 29-Sep. 3, 2004	Europe (NRC-1 held at Lindau, Germany)	1984 (4 years)
◆ Methods and Applications of Radioanalytical Chemistry (MARC) Recent MARC-VII held, April 3-7, 2006	Kailua-Kona, Hawaii, USA	1987 (3 years)
◆ Nuclear Analytical Methods in the Life Sciences (NAMLS). NAMLS- 8 held at Rio de Janeiro, April 17-22, 2005	Around the world (NAMLS 1 was held at Amsterdam, The Netherlands)	1967 (Irregular intervals of 4/6 yrs)
◆ Nuclear Analytical Chemistry (NAC) Recent NAC-III held in June 2001	Halifax, Canada	1985 (Irregular intervals)
◆ Asia Pacific Symposium on Radiochemistry (APSORC). APSORC-3 was held in China during Oct 17-21, 2005	Asian-Pacific countries (APSORC-1 & 2 held in Japan)	1997 (4 years)
◆ Nuclear and Radiochemistry Symposia (NUCAR), DAE	India	1992 (Alternate years)

Ever since the first book "Neutron Irradiation and Activation Analysis" by Dennis Taylor was published in 1964, several textbooks [38,39] have appeared, most recent by Loveland et al. [43]. Until 1994, biannual reviews by Ehmann et al. [44] were in much prominence. In recent years several reviews describing present status of NAA and nuclear techniques have appeared [45-54].

***(ii) Principles of NAA*:** The sequence of events occurring during the neutron capture or (n,γ) reaction is illustrated in Fig. I.4. On interaction of neutrons with the target nucleus, a compound nucleus (CN) is formed within $<10^{-15}$ s, which is de-excited through the emission of prompt γ-rays. The CN from the ground state then undergoes β-decay and in the process may populate excited state in the daughter product that deexcites by emitting γ-rays known as delayed γ-rays. Either β or γ

activity is measured to determine the concentration of an element present in the sample.

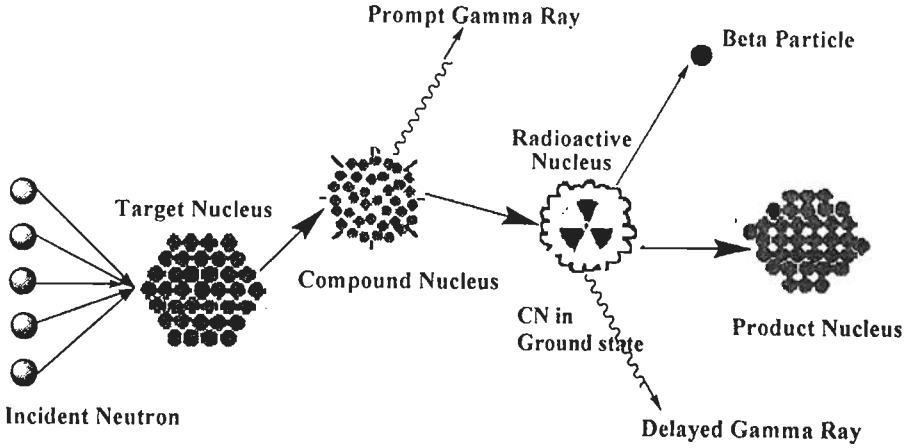


Fig. I.4. Principle of Neutron Activation Analysis

The activity thus produced depends on

- Number of target atoms $\left[\frac{i.w\ N_A}{M}\right]$ where M is the mass of target atom,
- Neutron absorption cross section in cm^2 (σ),
- Neutron flux in $cm^{-2}s^{-1}$ (φ),
- Irradiation time, (t) in s.

If the activity (A) is measured after a delay time (t_d) using a detector having an efficiency ε, then

$$A = \left(\frac{wiN_A}{M}\right)\sigma\phi\left(1-e^{-\lambda t_i}\right)e^{-\lambda t_d}\varepsilon\gamma = \left(\frac{wiN_A}{M}\right)\sigma\phi S D C \varepsilon\gamma \quad \ldots\ldots\ldots(I.1)$$

where $S = \left(1-e^{-\lambda t_i}\right)$ is the saturation factor, $D = \left(e^{-\lambda t_d}\right)$ is delay factor, $C = \left(1-e^{-\lambda t_c}\right)/\lambda$ is the correction factor for decay during counting time t_c and γ is the abundance of γ-ray measured. γ-Activity (A) of an isotope is proportional to the peak area, which is measured using an HPGe detector after subtracting the background. Concentration of an element is represented as

$$w = \frac{A\,M}{i\,N_A\,\sigma\phi\left(i-e^{-\lambda t_i}\right)e^{-\lambda t_d}\gamma\varepsilon} \quad \ldots\ldots\ldots\ldots\ldots\ldots(I.2)$$

This is called the **absolute method** of NAA [55]. Although it is feasible to calculate the concentration of an element by eqn. I.2, it is generally not acceptable and practiced because of significant uncertainties in σ, φ and other parameters. This is because the reactor neutrons have energy spectrum and cross section depends

on neutron energy. In addition, flux density of the reactor can vary during irradiation period. For this reason, a **comparator method** is generally employed, whereby a standard containing known concentration of specific element is irradiated and measured along with the sample(s). Concentration of an element (w') can be represented by an eqn. similar to (I.2). Comparing two such eqns. one gets

$$A' = \left(\frac{w' i N_A}{M}\right)\sigma\,\phi\,\left(1-e^{-\lambda t_i}\right)e^{-\lambda t_d'}\,\varepsilon\,\gamma \quad = \left(\frac{w' i N_A}{M}\right)\sigma\,\phi\,S\,D'\,C\,\varepsilon\,\gamma \qquad \text{.....(I.3)}$$

or

$$\frac{A}{A'} = \frac{w}{w'} \times \frac{e^{-\lambda t_d}}{e^{-\lambda t_d'}} \qquad \text{.....(I.4)}$$

However, if $t_{1/2}$ of the radionuclide is long enough compared to delay (t_d) and counting time (t_c), the delay facor can then be ignored and the eqn. further simplifies to

$$\text{Concn. of element in sample } (S_a) = \text{Concn. of element in standard } (S_t) \times \frac{\text{Activity in sample}}{\text{Activity in standard}} \qquad \text{.....(I.5)}$$

In this regard, either multi-elemental standard or single comparator standard for all elements [56] can be incorporated. In eqn. (I.5), activity is replaced by specific activity to have the uniformity while considering difference in weights of the sample and the comparator standard. Stoichiometrically well-defined standards of different matrices can be obtained from sources that specialize in the preparation of standard reference materials (SRMs) such as National Institute of Standards and Technology (NIST) USA, International Atomic Energy Agency (IAEA) Vienna, National Institute of Environmental Studies (NIES) Japan and Institute of Nuclear Chemistry and Technology (INCT) Poland. Sensitivity of the method depends on atomic mass (M), γ-ray abundance (i), cross section (σ) and half-life ($t_{1/2}$) of the radionuclide including irradiation, delay and counting time.

Apart from the absolute and comparator methods, concentration can also be determined by k_0 standardization [57] where a single element such as Au, Mn, Co, Zr with well defined nuclear characteristics and high thermal neutron absorption cross section (σ) is used as a comparator. The eqn used to calculate (w) is

$$w(\mu g/g) = \frac{A_{sp}}{A_{sp}^{\bullet}}\,\frac{1}{k_0}\,\frac{\left(f+Q_0^{\bullet}(\alpha)\right)\varepsilon^{*}}{\left(f+Q_0(\alpha)\right)\varepsilon} \qquad \text{.....(I.6)}$$

where A_{sp} is specific activity; f is thermal to epithermal flux ratio
Q_o is ratio of the resonance integral to the thermal neutron cross section;
ε is detector efficiency; α is deviation of the epithermal flux from ideality

k_o is $\left(\frac{M^* i \gamma \sigma_{th}}{M i^* \gamma^* \sigma^*_{th}}\right)$ and *corresponds to the comparator element

The k_o factor is an experimentally determined composite factor that contains the values for the nuclear constants needed in the activation analysis. The eqn. I.6 may be written as

$$\rho(\mu g/g) = \frac{A'_p\, p}{A^*_{sp}} \cdot \frac{1}{K_{anal}} \quad \text{where } K_{anal} = k_o \frac{(f+Q_o)}{(f+Q_o)^*} \cdot \frac{\varepsilon}{\varepsilon^*} \quad \ldots\ldots\ldots\ldots\ldots\ldots (I.7)$$

A'_p is the peak area of the i^{th} element corrected for saturation, cooling and decay during counting and normalized for 1 g sample.

Neutron activation can be carried out on-line by measuring prompt γ-rays (PGNAA) and off-line by measuring delayed γ-rays (NAA).

a) Since PGNAA is an on-line technique, measurements are carried out during irradiation itself [58-60]. It is generally performed by using a neutron beam extracted through the reactor beam port. This reduces the flux by an order of 10^3 than samples inside the reactor. PGNAA technique is most applicable to low Z whose product radionuclide decays too quickly (short lived) to be measured off-line.

b) In delayed gamma ray neutron activation analysis (DGNAA) or conventional NAA, counting follows radioactive decay at the end of irradiation. This operational mode is more common. Conventional NAA is useful for the majority of elements in the periodic table.

Selectivity or specificity is the key advantage of NAA because of its dependence on nuclear characteristics over other analytical techniques, which depend on electronic environment.

(iii) Neutron sources: Various types of neutron sources are listed in Table I.5. In addition, plasma gun and plasma pinch neutron sources invented in the early 1960s by J.W. Mather and also independently by N.V. Filippov are now in vogue [65,66]. The plasma gun or focus neutron source, (also called a Farnsworth-Hirsch fusor) produces controlled nuclear fusion by creating a dense plasma within which ionized deuterium and/or tritium gas is heated to sufficiently high temperature for creating

fusion. It is also called a *high-intensity plasma gun device* (HIPGD). The electromagnetic compression of a plasma is called a "pinch". Intense bursts of x-rays and charged particles are emitted as are nuclear fusion neutrons when operated in deuterium. Such a neutron source may find use in medical and security inspection applications and materials modification. Spallation sources also produce neutrons [67,68] by high energy protons on a target and making multiple collisions in a nucleus causing spallation (an intranuclear cascade). The high-energy neutrons emitted in the course of this process then collide with other nuclei, causing similar reactions (an extranuclear cascade). The residual nuclei of the cascade in an excited state give off neutrons. In this process about 10-20 fast neutrons/proton are produced. The quantity of neutrons thus produced is ~ 100 times that of photonuclear reaction and ~ 10 times that produced by fission.

Table I.5 Common Sources of Neutrons for NAA

Reaction	Half life	Average Neutron Energy (MeV)	Neutron yield (ns^{-1} Ci^{-1}, unless otherwise stated)
I. Isotopic Sources [61-63]			
(i) Photo neutron (γ, n)			
^{88}Y with ^{9}Be	106.6 d	0.16	$1x10^5$
^{124}Sb with ^{9}Be	60.2d	0.02	$1.9x10^5$
(ii) Alpha emitter (α, n)			
^{239}Pu with ^{9}Be	$2.4x10^4$ y	3.5	$\approx 10^7$
^{226}Ra with ^{9}Be	1600 y	3.6	$1.1x10^7$
^{241}Am with ^{9}Be	433 y	3.5	$2.2x10^6$
(iii) Spontaneous fission [$^{252}Cf \rightarrow {}^{140}Xe + {}^{108}Ru + 4n$]	2.64 y	2.3	$2.3x10^{12}$ ns^{-1} g^{-1}
II. Machine Type Source [64]			
(i) Cockroft-Walton n-generator $^{3}H(d, n)\ ^{4}He$	-	14.7	$10^8 - 10^{11}$ n/s
(ii) Cyclotron 10 μA of 30 MeV deuterons on Be	-	Broad distribution	$2x10^{11}$ ns^{-1}
[$^{239}Pu(\alpha, n)\ ^{242}Cm$]			
III. Nuclear reactors (Indian)	-	Broad distribution	
APSARA (1956) BARC, Mumbai		Depending on position	$\sim 10^{11}$ n cm^{-2} s^{-1}
CIRUS (1960)			$\sim 10^{12}$
Dhruva (1985)			$\sim 10^{13}$
Kamini (1999)– IGCAR, Kalpakkam			$\sim 10^{10}$

Neutron energy distribution and flux in different reactors and at different positions within a reactor can vary considerably with regard to material used to moderate the primary fission neutrons. In general, neutron energy distribution is quite broad and consist of three principal components (thermal, epithermal and fast) as schematically shown in Fig. I.5.

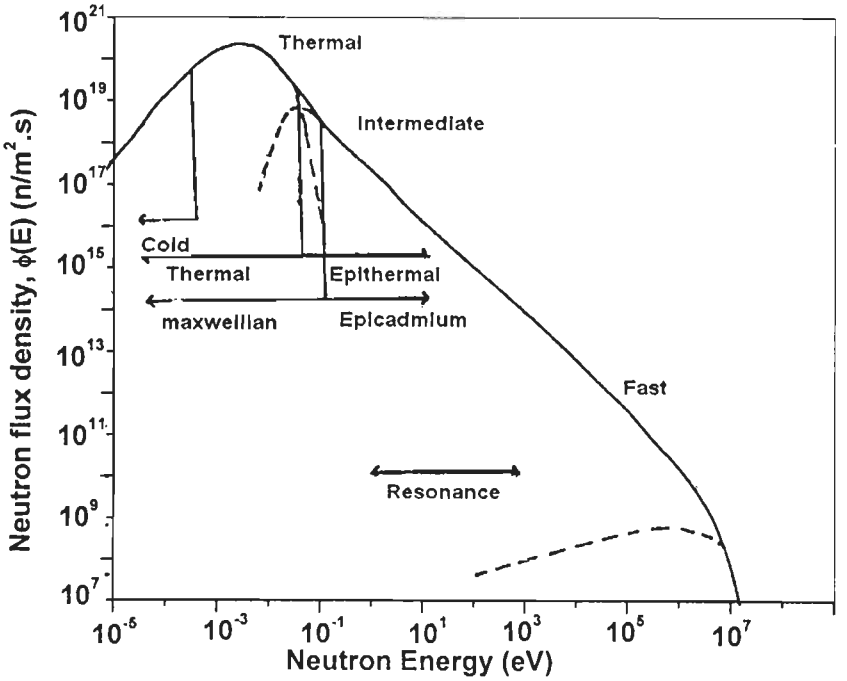


Fig. I.5 Energy Spectrum of Reactor Neutrons

Thermal neutrons with energy 0.025 eV can induce radiative capture (n,γ) reactions in the target nuclei. Delayed γ-rays from products are measured. An advantage of using thermal neutrons is their high cross section. At higher flux of thermal neutrons, Thermal NAA (TNAA) offers potentially higher sensitivity for a large number of elements except lighter elements (such as C, N and O) and potentially toxic elements (e.g Tl, Pb, Ni).

Epithermal neutrons (0.1 to 1.0 eV) have lower end of neutron energy closer to 0.5 eV, which is the Cd cutoff energy (~ 0.55 eV) and the higher end of the range extends up to several keV [69]. Epicadmium neutrons, like thermal neutrons, also induce (n, γ) reactions and delayed gammas are detected. Many elements such as

Ag, As, Au, Ba, Br, Cs, Eu, Ga, Gd, Hf, Ir, La, Ni, Rb, Sm, Sn, Sr, Ta, Tb, U, W and Yb can be determined more accurately and with better detection limits [70].

Higher energy neutrons encompass the widest range of energy, 0.5 – 20 MeV [71] and induce (n, p), (n, α), (n, n') and (n, 2n) reactions in addition to (n, γ) reactions. Mostly FNAA is performed using 14 MeV neutrons produced by Cockroft-Walton neutron generator using the reaction d (t, n) ^{4}He. These are especially useful for the determination of oxygen using the reaction ^{16}O(n, p) ^{16}N [72]

(iv) Interferences and Sources of errors [73-75]: As with any other analytical technique, NAA is also subject to interferences and a number of experimental errors. These may be introduced at all stages of the analytical process; during sample collection, preparation of the sample, irradiation, radioactive assay and finally in the calculation of result. Sampling errors can be more easily avoided in NAA than in any other analytical technique. A common source of error arises while using isotopic neutron sources and small reactors is when samples and standards are not subjected to the same neutron flux as a result of vertical or horizontal flux gradient [76]. For accurate analysis, these have to be compensated or eliminated. Self-shielding is a phenomenon that occurs when the neutron flux experienced in a sample is attenuated, thereby reducing the activation of the nuclides. This effect occurs when a sample contains extremely high concentration of an element with very σ. The principal error in the analysis of materials by NAA is the counting statistical error based on the signal to background ratio at the γ-ray energy region of interest. A 1σ error for a photo peak area determination is approximately equal to the square root of the total counts (background plus net counts) divided by the net counts.

Among the most documented sources of error are the interference reactions [77]. Fast neutrons can induce so called threshold (n.p) and (n,α) reactions yielding positive errors, e.g. determination Na and Al in biological samples is affected in presence of Mg and Al; Si and P respectively if present in the sample.

^{23}Na (n, γ) ^{24}Na : ^{24}Mg (n, p) ^{24}Na and ^{27}Al (n, α) ^{24}Na

^{27}Al (n, γ) ^{28}Al : ^{28}Si (n, p) ^{28}Al and ^{31}P(n,α)^{28}Al

Usually these effects are important only if fast to thermal neutron flux ratio is significant and if the concentration of the element giving rise to interference is a major component. This type of interference can be corrected by irradiating the pure

element which gives rise to interference and it can be estimated quantitatively. Another important interference is spectral in nature and arises specially while using NaI (Tl) detector where two γ-rays with comparable energy cannot be resolved [78]. Pile up peaks due to high activity and sum peaks when overlooked may cause erroneous results [79]. However, with the advent of high-resolution γ ray spectrometry and low noise better electronics, this problem has been solved to a major extent. For example in the determination of selenium using ^{75}Se ($t_{1/2}$ =120 d, E_γ= 264 and 280 keV) in presence of ^{203}Hg (t = 46.6 d, E_γ= 279 keV). Similarly the determination of cobalt via ^{60}Co (1173 & 1332 keV) will cause interference due to sum peaks of ^{82}Br (554, 619 keV and 554.3, 776.5 keV) at 1173.3 and 1330.8 keV respectively.

(v) Applications: Importance of trace element analysis (TEA) in today's context of technological globalization is vital. Some potentialities and capabilities of NAA are illustrated in Fig. I.6. There are several alternative techniques such as XRF, ICP-MS and EDXRF but the only common answer to all the problems is NAA provided a nuclear reactor is available. INAA has the additional advantage that it needs least sample preparation. It has evolved as an established analytical technique for solid samples though liquids could also be analyzed. NAA has a serious limitation that gaseous samples cannot be analyzed. Some important applications in various disciplines with emphasis on bioenvironmental, nutrition and health related studies are described here.

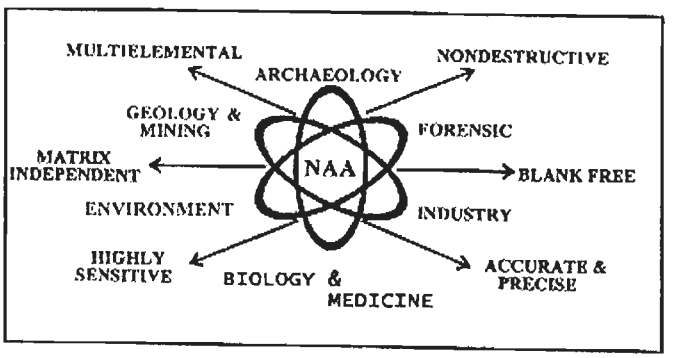


Fig. I.6: Illustration of capabilities and applications of NAA

Applications include **environmental studies** to characterize pollutants, determine their sources and methods of reduction. NAA has made significant contributions in the determination of heavy metals and other inorganic pollutants in a variety of matrices. A number of monographs [80] and reviews [81] have discussed the potential of NAA for environmental studies. INAA has been used for analyzing hair and fish to monitor occupational exposure [82] and aquatic environmental

pollution [83]. A large population exposed to subsoil water contaminated with arsenic showed toxic clinical features called arsenicosis [18,84]. Similarly, airborne particulate matter (PM) from industrial and vehicular emissions has been associated with a wide variety of environmental problems including detrimental health effects [80].

In **forensic studies** non-destructive nature of NAA has proved to be a boon. Some marker elements such as Ba, Sb, Cu, Ag, Fe, Br, Zn and K are analyzed depending on the sample type. Guinn and coworkers [85] carried out pioneering work and solved a typical case of As poisoning. Krishnan and Jervis [86] used NAA to find the muzzle distance of bullet fire and to trace the culprit using Ba and Sb in gun shot residue swapped from hands. Looking to its importance Central Forensic Research Laboratory (CFSL) Kolkata have established NAA laboratory at BARC, Mumbai [87].

Multielemental character of NAA is being extensively used for **health and nutrition studies.** Iyenger [88] has written an excellent monograph on compositional aspects of foods where he advocated the need for mixed total diet reference material. In a special supplement of Food and Nutrition Bulletin, several articles have appeared on the use of nuclear and isotope techniques for addressing nutritional problems [89]. In a recent study, Zaidi et al. [90] have reported trace element evaluation of some medicinal herbs whereby variation in trace element contents of same plant species from different origins were attributed to ecological and geographical variations. Singh and Garg [91] analyzed a large number of cereals, vegetables and spices of Indian origin and used the data for calculating elemental intake. Shi et al. [92] used NAA in combination with HPLC for the determination of low levels of five As species, As(III), As(V), monomethylarsinic acid (MMA), dimethylarsinic acid (DMA), and arsenobetaine (AsB) in H_2O samples. In our laboratory, INAA is being used extensively for multielemental analysis of herbal preparations and its constituents where trace elements play vital role in curing various ailments [93]. The collaboration between activation analysts and medical researchers has resulted in a much deeper understanding of the functions of trace elements in the body [94,95]. Sarmani [96] used NAA to study trace elements in kidney stones. Garg and coworkers [97] used NAA to determine trace element levels in human blood and excision biopsy samples of breast cancer to correlate with clinical stages and with dietary intake. Rajpathak et al. [98] observed that toenail Se

is lower among diabetic men with or without cardiovascular disorder than among healthy controls.

Semiconductor material analysis is another area where NAA has extensive applications. Hamada et al [99] determined ultra trace level of impurities in PbI_2 semiconductor. Continuous increasing performance demands on all types of semiconductors makes it important to measure ultra-trace level metallic impurities in Si wafers. Swanson et al. [100] determined metallic impurities in Si wafers using NAA. Takeuchi et al. [101] used INAA to determine trace impurities of Au, Sb and Ir in a Si single crystal.

NAA finds wide applications in the field of **archaeology**. Prudencio et al. [102] analyzed alkali metals, transition metals, and rare earths in 2 clay reference samples of the National Bureau of Standards, which have been suggested as standards for archaeological pottery studies. It has been concluded that the use of flint clay rather than plastic clay as a standard in NAA for ceramics and clays is preferable since the U content in flint is small enough to avoid correction for U-235 fission products. Carlson and James [103] analyzed REE in lead-glazed earthenware recovered from four Spanish missions in Texas using NAA and concluded that the ceramics characterized by the volcanic ash were manufactured in Mexico while those containing sand were made in Texas. Zao et al. [104] determined 32 elements in raw materials of terracotta warriors and horses of Qin Shihuang's Mausoleum, China and concluded that Lishan clay may be considered as the origin of these raw materials.

Apart from these applications, NAA is also used to produce radioisotopes, widely used in industry and medicine, by bombarding specific elements with neutrons. For example, ^{90}Y used to treat liver cancer [105] is produced by $^{89}Y(n, \gamma)$ reaction. An International Monitoring System (IMS) using NAA is being created to monitor the radionuclide aerosols [106] according to the Comprehensive Nuclear-Test-Ban Treaty (CTBT). It also finds extensive use in nuclear waste management [107]. Today, *in-vivo* NAA [108] is used for diagnosing the presence of diseases or offer effective means for monitoring potentially harmful side effects of therapy. NAA is the most common technique used for the certification of SRMs [109].

Despite limitations of long turn around time, radiation hazards, limited accessibility and limited automation, NAA remains a popular technique for trace and ultra trace element analysis in complex matrices.

I.6 QUALITY ASSURANCE

Quality assessment programme is an overall system of activities whose purpose is to ensure that the overall analysis is being done systematically and one has full control over the whole process [110]. It involves a continuing evaluation of the products and of the performances of the production system. Two concepts are involved in quality assessment (QA); quality control (QC) and quality assurance. Quality control is the overall system of activities whose aim is to control the quality of a product or service so that it meets the needs of users. The purpose is to provide a satisfactory, adequately dependable, and economic quality. Quality assurance refers to systems of external requirements placed upon laboratories by governmental agencies or private accreditation organizations. It provides the producer or user of a product or a service the assurance that it meets defined standards of quality with a stated level of confidence say 90% or 99%. QC procedures may or may not be required by quality assurance systems. Accredited laboratories develop an analytical methodology ensuring quality assurance through years of experience.

Quality assurance for sampling implies controlling the analytical blank. This **blank** not only contains the contamination within the analyte, but also the background-to-noise signal during the measurement. An empirical rule in the case of trace element analysis is that the blank should never exceed one-third of the concentration level expected in the sample. One should have full knowledge of contamination, for discovering ways to minimize the run-to-run variation in analytical practice, and for improving the signal-to-noise and sample-to-blank ratios [111]. QC must cover this specific phase of the procedure. Two very important operational terms for describing the quality of the data are precision and accuracy. Precision describes the variability of the individual results of replicate measurements and accuracy denotes the closeness of a measured value to the true value. The actual error of the analysis result is usually unknown as true value is hypothetical whereas the error of the determination is inferred from its precision. Nearly all measurements carry a certain degree of bias due to some systematic error inherent in the method. An important aid in this endeavor is the use of standard reference materials (SRMs), certified on the authority of International organizations [112]. The need for RMs certified for trace element species is evident. They are the best tool for harmonizing the results and providing quality assurance. They have the distinct advantage of

wide acceptance, and they form the basis for inter-comparison of measurement systems and for testing data produced under diverse conditions and by various laboratories. When an analyst obtains a result, which is close enough to the certified value, accuracy and tracability are established. However, it is also very important that it should be reproducible [113].

Z- scores are a special application of the transformation rules [114]. The Z score for an item indicates how far and in what direction the result deviates from the mean of its distribution, expressed in units of its standard deviation. Thus *Z-value* or *Z score* expresses the divergence of the experimental result x from the most probable result μ as a number of standard deviation σ. The larger the value of Z, the less reliable is the experimental result.

I.7 COMPARISON OF NAA WITH OTHER TECHNIQUES

The development of analytical instrumentation over the past few decades has undergone a sea change and allowed to detect trace metals at sub-ppb or even lower levels. As recently as early 1960s, trace element determinations were predominantly carried out by traditional wet chemical methods such as volumetric, gravimetric, or colorimetric assays. It wasn't until the development of atomic absorption spectroscopy (AAS) technique in late1960s that the clinical community realized about a highly sensitive and diverse trace analysis technique that could be automated [30]. With the advent of ICP technology ICP-AES and ICP-MS were developed after 1980s. As a result NAA, AAS, and ICP-AES or ICP-MS are in tough competition with each other as evident from Fig. I.3. Today NAA has emerged as a front-runner between various analytical techniques for trace element determination.

NAA is often nondestructive in that the integrity of the sample is not changed in any manner by prechemistry or the addition of any foreign material before irradiation [33, 39]. Thus the problem of reagent introduced contaminants unlike AAS or ICP is completely avoided. The analytical approach for NAA for most of the elements of interest is primarily an instrumental technique. In some cases though, post irradiation chemistry may be required. Thus the technician time per sample analysis is low, producing an efficient, low cost analytical approach. NAA is a multi-element technique in that many elements can be analyzed simultaneously in a given sample by recording γ spectrum without changing or altering the apparatus as is necessary in AAS. NAA is fast in that for many elements, several samples can be

irradiated at a given time and counted later on following a given decay schedule. A significant advantage of NAA is its sensitivity to trace elements. The sensitivity obtained by activation analysis is a function of the neutron absorption cross section (σ in cm^2) of the element in question, available neutron flux (φ in n cm^{-2} s^{-1}), length of irradiation, resolution and efficiency of the detector, matrix composition, and the total sample size. Hence, increasing neutron flux and irradiation time, and the major advances in detector technology especially with regard to increased efficiency and resolution have pushed the detection limits of most elements of interest to the very low levels (10^{-12} g). Comparison of detection limits of commonly used analytical methods is listed in Table I.6.

Table I.6. Comparison of detection limits of commonly used analytical methods*

Elements	Approximate Detection limit				Elements	Approximate Detection limit			
	NAA (ng/g)	AAS (μg/g)	XRF (μg/g)	ICP-MS (pg/g)		NAA (ng/g)	AAS (μg/g)	XRF (μg/g)	ICP-MS (pg/g)
Al	1	10	100	300	Mg	10	4	100	100
Sb	0.1	0.8	0.3	0.5	Mn	0.01	1	1	30
As	0.01	0.2	0.5	25	Hg	1	2	1	50
Ba	1	0.01	2	2	P	1,000	40	50	500
Br	0.1	-	0.5	2,000	K	1	0.1	10	200
Ca	100	0.01	5	500	Rb	1	0.5	0.5	1
Ce	1	-	5	1	Sm	0.01	1	5	0.5
Cs	0.1	4	5	0.1	Sc	0.01	0.5	2	0.5
Cl	1	-	10	--	Se	0.1	0.25	0.5	10
Cr	1	1	2	3	Na	0.1	0.2	500	500
Co	0.1	2	1	3	Sr	10	0.1	0.5	5
Cu	1	1	1	20	Th	0.1	-	1	0.01
Eu	0.1	0.02	2	0.01	V	0.1	0.2	1	1
Fe	10	2	1	50	Zn	1	1	1	100
La	0.01	2	3	0.1					

*All these values have been taken from the Internet (www. me.utexas.edu)

Detection limit is a statistical concept, based on the ability of a measurement method to determine an analyte in a sample matrix, regardless of its source of origin. Without a precise statistical definition, it is impossible to determine a numerical value for the limit of detection (LOD) [113]. In general, all LODs are defined in terms of 3σ where σ is the *standard deviation* of the measurement on blank specimens. It is observed that NAA shows much lower detection limits for most of the elements compared to AAS and XRF. Being in computer age, Faltejsek and Obrusnik [115]

developed a PC-based computer program QAS, designed for quality assessment (QAS) of results obtained by NAA.

I.8 LITERATURE SURVEY

Most of the published literatures on herbs from India are not scientifically authentic as there are very few epidemiological studies. Such literature is mostly available in *Sanskrit* and popular books. Earlier writings recommend large number of herbs for treatment based on hearsay, folklore and traditions being followed through generations [1,2,10]. Some are so comprehensive and indiscriminate that some plants are recommended for the treatment of several chronic diseases e.g. Neem (*Azadirachta indica*) and Amalaki (*Embelica officianilis*) to name a few [2, 118]. The criterion that seems lacking is scientific evidence, dosage and efficacy of a particular herb for a particular ailment. In *Ayurveda*, oldest treatises are *Charak Samhita* [116] and *Sushruta Samhita* [117] written by legendary sages *Charak* and *Susruta*, well known physician and surgeon respectively of ancient times. A book on *Dravyaguna Vigyan* (properties of vegetable drugs) describes properties of various herbs, its characteristics and uses [118]. Two recent books by Sivarajan and Balachandran [1] and by Paranjpe [2] describe majority of herbs in a comprehensive and authentic way. During past few decades, many researchers from various countries and particularly from China, Brazil and Africa have analyzed herbs particularly for the isolation of organic constituents [119]. However, very few attempts have been made to determine elemental contents in medicinal herbs and correlate these with various biochemical and enzymatic processes. Ever since, importance of trace elements in human nutrition has been realized [21], a variety of analytical techniques such as NAA [39], XRF [27], PIXE, AAS [30], ICP-AES and ICP-MS [27] including various electro analytical methods have been employed to determine trace elements, mostly in later part of 20^{th} century.

(i) Inorganic mineral elements: First reports on the elemental analysis of herbs appeared from China [120] and Nigeria [121] around 1984. During 1990's, many workers around the world started analyzing local medicinal plants extensively. An extensive literature search has shown immense interest among Chinese, Brazilian and South African workers [122-135]. Chen [122] determined As, Br, Sb and I in eight Chinese herbs using epithermal NAA. Wang et al [123] determined 10 microelements in Chinese medicinal herbs for curing hypertension using AAS.

Hamzah et al. [124] analyzed 10 popular Chinese herbs used as an alternative medicine in Malaysia for 16 trace and major elements using NAA. *Angelica Keiskei*, a Taiwanese herb was analyzed for 17 elements using NAA [122]. Amin et al [125] used slurry sampling electro thermal AAS for determining Cu content in Japanese herbal medicines. Sarmani et al [126] used k_0 NAA for determining 29 elements in Malaysian medicinal herbs.

There has been an immense interest in the analysis of medicinal herbs from South American countries, especially Brazil. Saiki et al [127] have published several papers on the multielement NAA in Brazilian traditional medicines and herbal extracts. Caldas and Machado [128] reported toxic metals (Cd, Hg and Pb) in Brazilian medicinal herbs by AAS. Vega-Carrillo et al [129] reported elemental distribution in folk medicines from Mexico. Gomez et al. [130] determined 9 essential elements in the extract of *Hypericum perforatum*, a medicinal plant from Argentina using flame AAS.

Because of geographical reasons medicinal herbs from South African countries (Ghana, Nigeria) have attracted much attention, not only within the country but in the western world as well. Serfor-Armah [131] determined 17 elements from five Ghanian medicinal plants. Obiajunwa et al. [132] determined 14 elements in Nigerian medicinal plants using EDXRF. Shabana et al. [133] determined Pb, Cd and Hg contents in Egyptian herbal tea. Jawad et al. [134] analyzed Pb, Fe, Cu, Cd, Cr and Mn contents in Iraqi medicinal plants. Abu-Irmaileh and Afifi [135] reviewed trace elements and organic constituents to assess the current situation of sales and uses of herbal medicines in Jordan

Comparatively speaking, traditional herbal remedies have received little attention in Western Europe and North America. However, there is increasing interest in the scientific community to analyze herbs from South African and other countries. Thomson and Ward [136] used FAAS and ICP-MS for determining 21 elements in 11 herbal remedies. Razic et al [137] used AAS for determining 9 elements in *Echinacea purpurea*, a medicinal herb from Yugoslavia. Chizzola et al. [138] determined Cd, Cu, Fe, Mn, Pb and Zn contents in Austrian medicinal and aromatic plants. Kanias et al. [139] reported Co, Fe, Eu, Rb, Sc, Sr, Th and Zn in Greek *Eucalyptus* leaves by NAA. Mukhammedov et al [140] used NAA for determining 22 elements in Russian herbs (now Uzbekistan). Kist et al. [141] further used proton activation along with NAA for 32 elements in five medicinal herbs from

Uzbekistan. Szakova and Mader [142] used dry ashing and wet decomposition technique for the determination of 11 essential trace elements in Czech medicinal herbs. Lozak et al [143] determined several macro and microelements in Polish medicinal herbs by ICP-MS and AAS. Looking to its importance, an SRM of Mixed Polish Herbs (INCT-MPH-2) has been developed by Dybczynski et al. [144]

On Indian scenario, our group first reported (1996) elemental contents in several plant parts often used as medicines in the Indian *Ayurvedic* system [145]. Since then, our interest has been growing and so also other workers have taken keen interest (146-148). Rajurkar et al. [146] from Pune determined trace elements in medicinal plants used for cardiovascular, diabetic and urinary tract disorders. Naidu et al. [147] from Tirupati (A.P.) have analyzed leaves and other plant parts. Recently, Mohanta et al. [148] have used NAA to determine 14 elements in medicinal plants of North Eastern India. Several reports from Pakistan have appeared on the elemental analysis of medicinally important herbs. Sahito et al. [149] determined mineral contents in medicinally important plants of Pakistan using AAS. Zaidi et al [90] reported trace element evaluation of Pakistani medicinal herbs by NAA.

In recent years, many Western scholars have been opposing the use of herbal medicines on the plea of their toxic metal contents especially Cd, Pb, Hg etc. As a result, use of many herbal medicines even by native residents in these countries has been banned. In this regard some workers have taken special efforts to determine toxic metal contents in the medicinal herbs of their respective countries [128,133]. It has been suggested that use of natural herbs grown on industrially polluted soil or environment are harmful and their use may damage health. Chan [150] has specially emphasized the importance of good agricultural, laboratory, manufacturing and supply practices for such medicines. In a way quality control of herbal medicines has become integral part of the production and use of such medicines on a large scale especially in Western countries.

(ii) Organic constituents: Several prominent groups in different parts of India have taken keen interest in isolating active ingredients such as alkaloids, flavonoids, glycosides, tannins etc. from a large number of plant parts of medicinal importance. Central Drug Research Institute Lucknow (CDRI) is a premier institution where plant based drug development research is being carried out since about half a century. A six-volume Compendium on Indian Medicinal Herbs [16] has been published.

Compounds of natural origin play a major role as drugs and as lead structures for the development of synthetic molecules. Fabricant and Farnsworth [151] reviewed 122 compounds of defined structures from 94 plant species used globally as drugs. It has been observed that 80% of these have had an ethno medical use identical or related to the current use of the active constituent of the plant. Yamamura et al. [152] determined chemical structures of 4 antihistaminic flavonoids, three new and five known glucosides of lower alcohols and rosmarinic acid in mint by using column chromatography and HPLC. Fletcher et al.[153] showed that rosmarinic acid is the major contributor to the antioxidant capacity as observed by DPPH assay. Oke and Hamburger [154] have screened 22 Nigerian medicinal plants for their antioxidant activity using DPPH method. Palaniswamy et al. [155] determined antioxidant vitamins, α-tocopherol, β-carotene and lutein in fresh curry leaves using reversed phase gradient HPLC. Srivastava and Singh [156] studied the essential oil from curry leaves for its anti fungal activity and attributed it to β-caryophyllene (29.0%) and β-gurjunene (21.0%). Daulatabad et al. [157] reported lauric (2.8%), myristic (31.7%), palmitic (4.7%), stearic (6.5%), oleic (32.2%), linoleic (16.1%), malvalic (1.2%), sterculic (1.8%), and vernolic acids (3.0%) in *jaamun* seed oil. Naik et al [158] have found *amalaki* to be rich in phytochemicals having high antiradical properties and a very good antioxidant.

Thus it is amply clear that analysis of herbs for inorganic and organic constituents is very essential to understand their pharmacological action in the human body, for quality control and to understand its toxic effects, if any.

I.9 AIM AND SCOPE OF THE PRESENT WORK

Since the beginning of civilization, various plant parts such as root, stem, leaves, flowers, fruits and seed or their extracts have been used for healing purposes and maintaining health. *Ayurveda*, the ancient Indian medicine system forms the basis of usage of herbs and in many modern medicines, these form an integral part [11-15]. Since plants are a rich source of essential trace elements and because of the importance of trace elements in various metabolic processes, there is a need to know the elemental contents in herbs and herbal formulations [21-23]. For the past one decade, our group has been actively working on the analysis of medicinal herbs and herbal formulations. Present work was undertaken to further

consolidate our efforts in this direction in a more comprehensive manner. To be pharmacologically effective or essential, these trace elements are likely to be combined or chelated with some natural organic ligands so that resultant molecule could be physiologically absorbed in the body and prevent or cure impairment caused by the deficiency of the element. Hence an attempt has been made to identify some organic molecules, which may possibly act as an antioxidant and/or exhibit other therapeutic properties.

Though NAA is now a well-established and developed technique, we have optimized experimental parameters for the analysis of trace elements by short-term reactor irradiation using pneumatic carrier facility (PCF) of the Dhruva reactor. It has been shown that by 1 min irradiation at 10^{13} n cm^{-2} s^{-1} followed by counting at different intervals, up to 20 elements could be determined. However, by long irradiation, additional elements could be determined. Besides, AAS has been used for the determination of environmentally toxic elements Ni, Cd and Pb because of inherent problems in NAA determination.

Overall, the thesis is divided into six chapters. ***Ch I*** introduces the importance of analysis of herbs, a brief literature survey and a description of the technique of NAA. ***Ch II*** deals with the instrumentation and methodology adopted for elemental analysis and identification and structure determination of the organic constituents. Also described are our results on the participation of elemental analysis of proposed reference materials of Corn (CF-3) and Soybean (SBF-4) flour samples from the Institute of Nuclear Chemistry and Technology (INCT) Poland. Following are the salient features of the work described in chapters III to VI.

- Determination of 30 elements in *Mentha Spicata* (Mint) collected from four different locations separated by 320 km in Northwest India by NAA and AAS.
- A new compound 1,3-dihydro carveol along with menthol were isolated from the methanolic extract of mint and identified by elemental analysis, IR, NMR and GC-MS studies.
- DPPH free radical scavenging activity of mint in dichloromethane, diethyl ether and methanol was carried out. Furthermore 10 compounds from diethyl ether extract were identified by GC-MS.
- 28 samples of curry (*Murraya Koenigii*) leaves collected from 19 Indian states have been analyzed for 28 elements by NAA and AAS.

- Three new compounds, 3-methylthiopropanenitrile, 1,2-benzenedicarboxylic acid mono-(2-ethylhexyl ester) and 1-penten-3-ol were isolated from the ethanolic extract of curry leaves and identified by spectral techniques and GC-MS.
- Various plant parts such as leaves, roots, fruits and seeds (16 raw samples and 3 capsule preparations from Himalaya Drugs, Bangalore) commonly used for treatment of diabetes were analyzed for 28 elements by NAA and AAS.
- Five antidiabetic herbal formulations from Gurukul, Divya, Jagdamba, UAP and BACPO pharmaceuticals were analyzed for 6 minor and 22 trace elements by NAA and AAS.
- 3 new compounds 1,1,2,3-tetramethylcyclopropane, methyl phenyl sulfone and 9,12,15-octadecatrienal along with n-hexanedecanoic acid (palmitic acid) were isolated from the petroleum ether extract of Neem leaves and identified by GC-MS
- 5 brands of *Trikatu* – an Ayurvedic formulation commonly used as a stimulant and for treatment of cold, procured from Yogi (Haridwar), Vyas (Indore), Zandu (Mumbai) and Sushrut (Nagpur) Pharmacies and a sample from Mumbai were analyzed for 7 minor and 24 trace elements by NAA and AAS.
- Individual constituents dried ginger (*Zingiber Officianalis*), black pepper (*Piper Nigrum*) and pipali (*Piper Longum*) were also analyzed for their elemental contents.
- Methanolic extract of pipali was used for the isolation of barbituric and tannic acids, which were identified by elemental analysis and spectral methods. GC-MS was used for further confirmation of structures.
- Hydrodistillation of pipali yielded an essential oil whose GC-MS studies showed 10 compounds; 2,2-dimethyl propanoic acid, decane, 1-decyne, 3,4, 8-trimethyl 1-nonene, undecane, bis- (1-methylpropyl) disulfide, 2-nonynoic acid, 2,4-decadienal, nonanoic acid and tetradecanoic acid

Attempts have been made to correlate elements such as Na vs Mg, Na vs Cl, Rb vs Cs, Cu vs Zn, Fe vs Mn in various medicinal herbs and formulations. K/P in various plant parts has been found to be of special significance. It has been observed that many herbs are enriched in one or more essential trace elements, which remain bound with organic molecules so as to make them bioavailable and compatible. Some herbal preparations, which represent combination of two or

more herbs, make them especially useful for enhancing vitality and resistance to the immune system. The thesis is an attempt to correlate the elemental contents and organic constituents with the therapeutic uses of medicinal herbs.

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CHAPTER II

METHODOLOGY & INSTRUMENTATION

A part of the work was presented at the Eleventh International Conference on Modern Trends in Activation Analysis (MTAA-11) held at the University of Surrey, Guildford, UK, 20-25 June 2004 and will appear in *J. Radioanal. Nucl. Chem.*, **271,** (2007) In Press

Minor, trace and toxic element analysis of medicinal herbs is a challenging task before analytical chemists because of its complex matrix character. Since these are grown in natural environment, these are likely to be contaminated by pollutants depending on where a particular plant is grown. Besides soil characteristics and ecological factors may also affect its elemental composition. Also, sampling errors, interferences, data validation and inappropriate data analysis may lead to serious errors. Since we have adopted two methodologies, NAA and AAS for trace element analysis (TEA), only these two techniques will be described here. The different stages in the NAA and AAS methodologies with emphasis on instrumentation and data analysis are discussed here.

II.1 SAMPLING

It is the process of extracting an aliquot from a large quantity of material, which is a true representative of the whole material. It refers particularly to the choice and collection of sample and its storage. For medicinal herbs, identity and validity of the sample with respect to location and other specific characteristics such as soil conditions and surrounding environment must be known. Plant materials derived from the same species can show significant differences in quality when cultivated in different environments owing to the influence of soil, climate and other factors such as the age and section of the plant. Careful sectioning is necessary as various segments like root, stem, leaves, fruits, seeds have different medicinal properties. Similarly fresh or old leaves may also differ in composition.

In recent years, many Western countries have banned the use of Indian herbal products owing to the presence of toxic elements and other carcinogens. Poor quality of herbal medicines is a direct consequence of the use of pesticides and chemical treatment during storage. Environmental pollution due to industrial and vehicular emissions and other anthropogenic activities cannot be ignored either. Hence, sampling location should be carefully chosen from pollution free atmosphere away from the highways. Also, care must be taken such that samples are not collected from a place where excessive fertilizers, insecticides and fungicides have been used. If sampling is not done properly, all the labour and time spent during analysis may be completely wasted. Therefore, it is essential to follow appropriate sampling methodology and protocol [1-3].

Curren and King [4] reviewed modern methods and developed techniques for sampling. Woittiez and Sloot [5] reviewed the aspects of trace element sampling and sample preparation, mainly for biomedical and environmental studies. Park and Pohland [6] described the factors affecting the sampling, which include nature of the analyte, physical characteristics of the product and sample size. World Health Organization (WHO) has developed sampling guidelines based on good agricultural and collection practices (GACP) of medicinal herbs [7]. Heydorn [8] discussed the problems of sampling and sample handling for clinical studies. IAEA technical reports deal with the precautions in sampling for NAA in details [1, 9].

In the present study, herbal preparations and formulations were of brand names and procured from the chemist shops locally or from other cities. Raw samples were collected in a few cases only and their details are given in individual chapters.

II.2 SAMPLE PREPARATION

Sample preparation procedures and equipment may differ depending on the type of sample (leaves, stem, root, seed or blood, tissue, bone) to be analyzed. This aspect of preparing the sample for analysis requires adequate space, equipment and knowledge of preparing the sample properly. The aim is to reduce the sample size to allow a representative sample for analysis. Since NAA is virtually a blank free technique, special care is taken to avoid contamination before irradiation. In fact, the only steps where contamination can influence the final result are sampling, sample handling and storage. In case of raw samples or leaves plucked from a tree, **removal of surface contamination** is the primary step. It is a prerequisite for all types of biological samples including medicinal herbs. Even while collecting the sample, care must be taken to wear disposable gloves. All the unwanted dust particles and extraneous material should be removed while processing. Surface contamination is removed by wiping the individual plant parts with a tissue paper and then by repeated washing with double distilled water [10]. Drying is essential to remove moisture content. Samples are air-dried in sunlight and then in an oven at 80°C for 24 h. Alternatively samples may also be dried under infrafil lamp where temperature does not go beyond 80°C. For biological samples, this is the most appropriate method, which ensures that volatile elements such as As, Se, Hg etc. are not lost [11].

Certain types of samples absorb water from the air on standing. Hence, after withdrawing the samples from oven, it should be kept in a desiccator so that moisture does not get reabsorbed. To be on safe side, use of talc free gloves, laminar hood and a dust free chamber (shown in Fig. II.1) is a prerequisite.

Fig. II.1 Dust free chamber for sample preparation

Sample homogenization was done by crushing, grinding and pulverizing the dried sample in an agate mortar. Samples were then sieved to uniform particle size by passing through 100-mesh sieve. Literature reports exist on sampling and sample size necessary for analysis [12,13].

The goal of **sample preservation and storage** is to prevent any undesirable change between the time sample is collected and analysis is done. The matrix properties, humidity, pH, temperature, duration of storage and the container material are linked to the contamination and to maintain originality of the sample. For biological samples, preservation by freezing at 0°C and even at refrigerator temperature is a must [14]. To avoid biodegradation due to microorganisms, samples may be irradiated with ^{60}Co γ-rays at 3-5 Mrads. Sample container should preferably be made of polyethylene or PTFE, which should be perfectly cleaned and dried. Zeisler et al. [15] reported that under these conditions of storage, there was no loss in elemental concentration over a long period of time. A careful choice of valid sample and its proper preservation can claim good quality analytical data.

II.3 PREPARATION OF STANDARDS

Standardization of a result is the basis for accuracy of the analytical facility and methodology. The approach to standardization varies with the technique and

depends on the background of the analyst and the type of matrix to be analyzed. In the present study, NAA has been used in the comparator mode and the standards used were multielemental synthetic (primary) and secondary SRM/CRMs preferably of similar matrix.

(i) Primary (Multielemental Synthetic) Standard: For the ultimate accuracy, there is no substitute for such in-house comparator standards [16,17]. These are prepared by weighing known amount of the respective element or its compound in high purity or purest form (assay 99.9% or better) and with known stoichiometry, deposited on specific and inert substrate such as Whatman filter paper, alumina, SiO_2, magnesia, cellulose, resin etc. [18]. Potential sources of error including evaporation loss, pipette calibration, contamination of the standard from the reagent, laboratory environment, purity and stoichiometry of the salts and cross contamination of one element by the addition of a second should be taken into account while preparing such standards. In Table II.1 are listed the constituents of a typical synthetic standard prepared and used in this study. These were prepared in a clean glove box by depositing aq. acidic solutions (in dil. HCl) of 1 to 5 μg of AnalaR/GR/HP grade salts on a Whatman filter paper No. 42 strip. It was handled with a plastic tweezers, dried in a glove box under infrafil lamp and packed in an aluminum foil. In order to check the accuracy of our standards and thereby results, we irradiated two primary standard at a time and calculated results using the other as comparator. Data are given in Table II.2, where amount taken and relative error are also listed.

Table II.1 Constituents of a typical synthetic multielemental standard

Element	Compound/ Reagent	Firm and Purity	Amt. of Element deposited
As	As_2O_3	BDH, England, 99.8%	0.5-0.7 μg
Co	$Co(OOC.CH_3)_2.4H_2O$	E. Merck, India, 99%	5-8 μg
Cr	$Cr(NO_3)_3.9H_2O$	Fluka, Germany, 98%	1-2 μg
Fe	$(NH_4)_2Fe(SO_4)_2.6H_2O$	E. Merck, India, 99%	10-15 μg
Hg	$(CH_3COO)_2Hg$	E. Merck, India, 99%	1-2 μg
Mn	$KMnO_4$	E. Merck, India, 99.5%	5-10 μg
Se	SeO_2 in HNO_3, 1.0 μg /mL	Merck, Germany, 998±5 mg/L	0.2-0.5 μg
Zn	$Zn(OOC.CH_3)_2.2H_2O$	Merck, Germany, 99.5%	2-3 μg

Table II.2 Data validity using synthetic primary standards

Element	Standard-I		Standard-II	
	Concentration (ppm)	Error (%)	Concentration (ppm)	Error (%)
As	0.68±0.05 (0.7)	2.9	0.57±0.01 (0.5)	14.0
Co	7.98±0.6 (7.5)	6.4	5.51±0.04 (5)	10.2
Cr	0.93±0.02 (1)	7.0	1.53±0.05 (1.5)	2.0
Fe	14.7±0.03 (15)	2.0	11.7 ±0.02 (12)	2.5
Hg	1.69±0.02 (1.5)	12.7	1.87±0.02 (2.0)	6.5
Mn	5.23±0.06 (5.0)	4.6	7.67±0.11 (8.0)	4.1
Se	0.38±0.01 (0.4)	5.0	0.23±0.02 (0.25)	8.0
Zn	2.37±0.04 (2.5)	5.2	1.85±0.04 (2)	7.5

Values in parenthesis are the amount taken using E. Merck 5L79657 transferpette

(ii) Standard Reference Materials (SRMs): In view of the many potential errors in preparing multielemental standards and poor stability, the simplest quality assurance procedure in NAA is to include 2-4 Standard/Certified Reference Material (RM) in each batch of irradiation. If the results obtained for the RM agree with the certified values within ±5-10% uncertainty, then corresponding concentration for the analyte can be considered with a fair degree of confidence.

RMs available through the international agencies viz. NIST (USA), IAEA (Vienna), INCT (Poland) and NIES (Japan) have been widely used as primary standards in many INAA studies [19-21]. In present studies, at least two RMs in short irradiation (1-5 min.) and 3-4 in long irradiation batches were used. Efforts were made to choose RMs of similar matrix of plant origin but from different agencies. In Table II.3 are listed RMs analyzed in present studies.

Table II.3 Standard Reference Materials of botanical origin analyzed in this study

Standard	Identification No.	Source
Apple Leaves	SRM-1515	NIST, USA
Cabbage	IAEA-359	IAEA, Vienna
Oriental Tobacco Leaves	CTA-OTL-1	INCT, Poland
Peach leaves	SRM-1547	NIST, USA
Pine Needles	SRM-1575	NIST, USA
Virginia Tobacco Leaves	CTA-VTL-2	INCT, Poland
Mixed Polish Herbs	MPH-2	INCT, Poland
Wheat Flour	SRM-1567	NIST, USA
Rice Flour	SRM-1568a	NIST, USA
Whey Powder	IAEA-155	IAEA, Vienna
Rice Flour	No.10a	NIES, Japan

Analysis of RMs and INAA has become complementary to each other because of its inherent characteristics. NAA is now referred to as a reference method to have good quality data for candidate RMs for trace elemental analysis [22-24]. These RMs are quite costly and difficult to procure and store.

II.4 SAMPLE PACKING

A variety of packing materials have been in use for NAA depending on the neutron flux, duration of irradiation and the nature of the matrix. The packing material has to sustain the conditions during irradiation in the reactor. It should be able to withstand the effects of radiation, heat and mechanical impact [25]. The material has also to be of high purity so as not to contaminate the sample.

The most commonly used materials for sample packing are polyethylene (or Alkathene), high purity quartz ampoule and aluminum foil. In the present study Alkathene (pure form of polypropylene) was used for short irradiation of 1-5 min. Alkathene bag stock is available in the form of flattened tubing in width (half the circumference) from 2.5 cm up and in-wall thickness from 25-150 microns. All the samples and standards each of 30-50 mg were accurately weighed and packed in Alkathene bags and heat sealed twice. For prolonged reactor irradiation, 40-80 mg each of samples and standards were packed in high purity aluminum foil (Superwrap). The sealed packets were then numbered using waterproof black marker pen. All the packets were sealed in a dust free and clean chamber. These were then collectively packed in a bundle to be fitted in the irradiation container as shown in Fig. II.2 A, B & C.

Fig. II.2 Irradiation containers for (A) CIRUS, (B) Dhruva and (C) Rabbit for Dhruva PCF

The samples packed in Alkathene or aluminum foil are inserted in a larger irradiation container for insertion into the irradiation position. The size depends on the size of the irradiation position, which is usually made to fit. For irradiation in APSARA

reactor, we have used polyethylene container while aluminum container is used for 1-3 d irradiation in Dhruva/CIRUS reactor. High-density polypropylene (PP) rabbits having high purity and resistance to strong mechanical impact are used while using the pneumatic carrier facility (PCF) of CIRUS/Dhruva reactor at the Bhabha Atomic Research Centre (BARC), Mumbai.

II.5 IRRADIATION

The samples along with comparator standards are irradiated at high Cd ratio position (high thermal to fast neutron ratio) in a reactor. The primary factors to be considered are the isotope of interest, the anticipated concentration level, neutron capture cross section and half-life, all of which are taken into consideration to fix the irradiation time. Sample size and interfering activities should also be taken into consideration before irradiation. Besides, flux variation is another important parameter in comparative NAA. Since all the samples along with RMs were irradiated in a small packet (3 cm long x 1.5 cm dia), vertical and horizontal flux variations are likely to be minimal or insignificant. In this study, we were fortunate to have been able to use pneumatic carrier facility of Dhruva and CIRUS reactors at BARC, Mumbai where irradiation times were 1 and 2 min respectively. This was found to be extremely useful for the determination of short-lived nuclides such as ^{52}V, ^{28}Al, ^{27}Mg, ^{38}Cl, ^{49}Ca and at least 10 more nuclides. The use of PCF allows short transfer time (~6s) and convenient access to the high flux irradiation position enabling minimal loss of activity due to delay thereby ensuring better reproducibility [26,27]. Sample packed in *rabbit* (Fig. II.2 C) made from high density PP (26 cm diax30 cm length) is transported through a pneumatic tube (dia 30 mm) with pressurized air though N_2/He is also used to reduce ^{41}Ar background. Schematics of the PCF system of Dhruva reactor and the rabbit used in CIRUS reactor are shown in Fig II.3. The rabbit is thermally and mechanically stable and can withstand

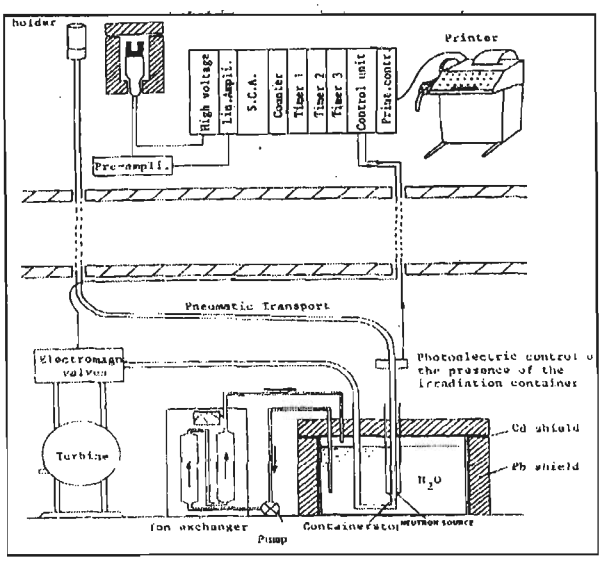


Fig.II.3 Schematic of PCF in Dhruva reactor

radiation field in the reactor. Besides PCF of Dhruva and CIRUS reactors, 5 min irradiation in APSARA was also availed followed by counting at the reactor site and later at the Radiochemistry Division of BARC.

Long irradiations of 1 to 3 d were carried out in Dhruva or CIRUS reactors respectively and after suitable cooling, samples were air lifted to Delhi airport and then brought to our laboratories at Roorkee. Irradiation and counting schedule including radionuclides identified are listed in Table II.4.

Table II.4 Irradiation, delay and counting schedule

Reactor (Irradiation time)	Delay	Counting	Radionuclides Identified
Dhruva/ CIRUS (1 min / 2 min)	3 min.	60 s	^{28}Al, ^{49}Ca, ^{27}Mg, ^{52}V.
	10 min.	100 s	^{28}Al, ^{49}Ca, ^{27}Mg, ^{52}V
	20 min.	300 s	^{38}Cl, ^{56}Mn
	2 h	100 s	^{56}Mn, ^{24}Na, ^{42}K, ^{198}Au
	1 d	4000 s	^{24}Na, ^{42}K, ^{82}Br, ^{76}As, ^{140}La, ^{82}Br.
	20 d	2000 s	^{32}P(β^-)
APSARA (5 min.)	5 min	50 s	^{28}Al, ^{49}Ca, ^{27}Mg,
		100 s	^{28}Al, ^{49}Ca, ^{27}Mg,
		300 s	^{38}Cl, ^{56}Mn, ^{24}Na, ^{42}K
Dhruva/ CIRUS (1 d/ 3 d)	10 d	1h	^{140}La, ^{233}Th(^{233}Pa), ^{86}Rb, ^{82}Br.
	12d	2h	^{75}Se, ^{124}Sb, ^{51}Cr, ^{131}Ba, ^{141}Ce,
	20 d	6h	^{203}Hg, ^{181}Hf, ^{59}Fe, ^{60}Co, ^{46}Sc, ^{152}Eu.
	25 d	1 h	^{32}P(β^-).
	40 d	12 h	^{65}Zn, ^{134}Cs, ^{60}Co, ^{152}Eu.

II.6 POST IRRADIATION TREATMENT

Soon after irradiation and suitable cooling, the container is cut open and samples are unpacked. Surface of the Alkathene/aluminum packed samples was decontaminated by swiping with acetone soaked cotton/ tissue paper. These are then mounted on Perspex sheets with suitable and reproducible geometry. . For long irradiations of a few hours or days, Al-foil wrapper is first carefully cleaned, recognized by weighing and then counted. After initial counting these were unwrapped and the samples were transferred to pre-weighed and precleaned polythene container. Alternatively tracing/ butter paper packets (1 cm x 1 cm) were also used. This is to avoid impurities in Al-foil, which may get activated (long lived radionuclides) and interfere with the sample activity. Extreme care was taken during transfer of the irradiated samples for repacking. Normally 80-95 % of the sample

could be recovered. All the operations with activated sample were carried out inside a glove box.

II.7 ASSAY OF RADIOACTIVITY

One of the steps of INAA procedure is the measurement of induced γ activity with an exception of ^{32}P (β^- emitter) and is done by high-resolution γ-ray spectrometry [1, 28]. The basic set up for γ-ray spectrometry consists of

- Semiconductor detector with preamplifier
- High voltage power supply
- Spectroscopy amplifier
- Analog-to-digital (ADC) converter
- Multichannel pulse height analyzer (MCA), 8 k
- Computer system with input-output facility and
- Printer

Sometimes, two or more of these functions are combined. e.g. ADC and MCA or MCA and computer system. Following are the instrumental details.

(i) Detection System: We have used coaxial HPGe detector with large volume, which can be used for detecting high-energy γ rays. Schematics of HPGe detection system with all the accessories is shown in Fig.II.4.Such semiconductor detectors are operated at liquid N_2 temperature of 77 K. The crystal is mounted on a vacuum cryostat, thermally connected to Cu rod called *cold finger*, which dissipates heat from the crystal to the cooling medium. One of the basic advantages of HPGe is that it can be stored at room temperature without any damage to the crystal as long as HV bias is removed.

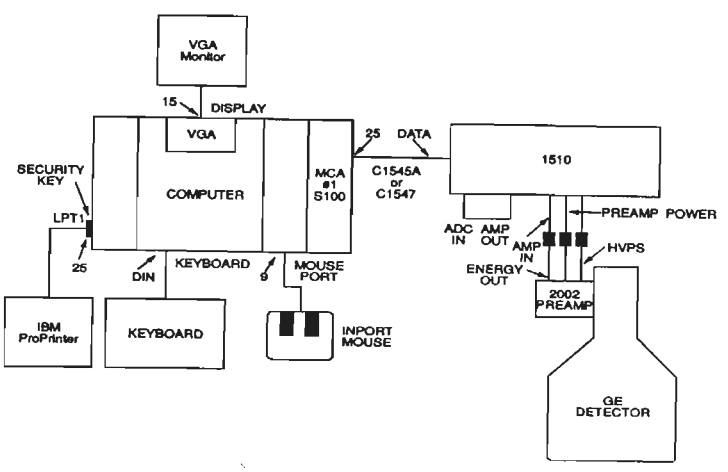


Fig. II.4 A Schematic Diagram of the Counting Setup

A two-pronged approach was adopted to measure the activity; activity due to short-lived radionuclides were counted using 80 cm^3 coaxial detector (EG & G, ORTEC) and 4 K MCA with a DOS based pcaiii software at BARC, long lived activity was counted at Roorkee by using a coaxial HPGe detector with 8 K MCA and window based Genie 2000 software (Canberra, USA). A photographic illustration of the counting set up with lead shielding and computer is shown in Fig. II.5.

Detector Model: GC2018
Cryostat Model: 7600 SL
Serial Number: 05017312
Diameter = 60.5 mm
Length = 29.5 mm
Preamplifier Model: 2002 CSL
Bias voltage: (+) 4000 V dc
Relative Efficiency: 20%
Peak/Compton Ratio: 50:1
Resolution: 1.8 keV (FWHM) at 1332 keV of ^{60}Co and 0.9 keV at 122 keV of ^{57}Co

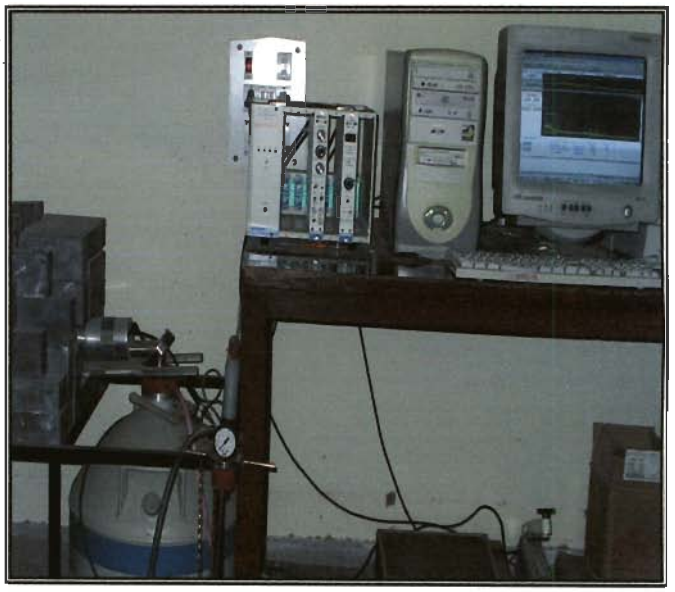


Fig. II.5 Photographic illustration of HPGe setup

An indication of the quality of the detector to produce peaks with (semi) Gaussian shapes is obtained by the ratio of the full width at tenth maxima (FWTM) and full width at fiftieth maxima (FWFM) to the FWHM value. The values for Gaussian peaks in the detector are FWTM/FWHM =1.91 and FWFM/FWHM=2.43. Typical shape and energy calibration using ^{152}Eu source are shown in Fig. II.6 and 7 respectively.

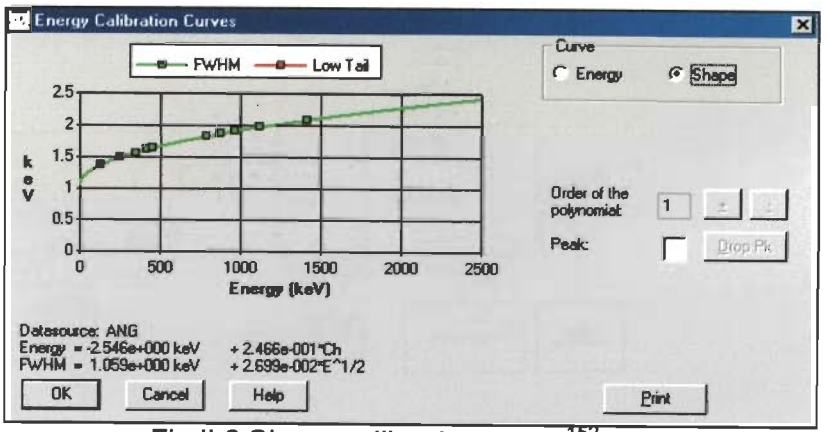


Fig II.6 Shape calibration using ^{152}Eu

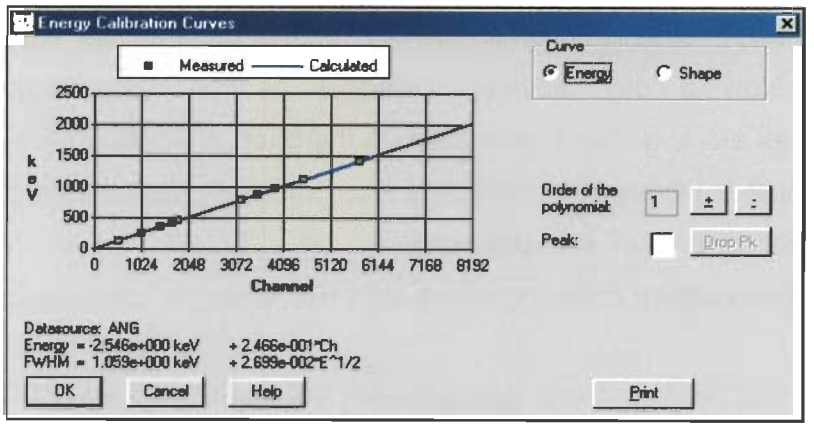


Fig II.7 Energy calibration using ^{152}Eu

Radionuclides are then identified from their characteristic photo peaks. Nuclear characteristics of the radionuclides identified/determined by (n, γ) reaction in this study are listed in Table II.5.

Table II.5 Nuclear Characteristics of the Radionuclides Identified/Determined

Target Element (Nuclide)	Isotopic Abundance (%)	Product Nuclide	Half Life ($t_{1/2}$)	Cross Section σ (b)	Energy E_γ (keV)
Al (^{27}Al)	100	^{28}Al	2.241m	0.23	1779
As (^{75}As)	100	^{76}As	26.3h	4.3	559, 657
Au (^{197}Au)	100	^{198}Au	2.7d	98.8	412
Ba (^{130}Ba)	0.1	^{131}Ba	12d	8.8	373, 496
Br (^{81}Br)	49.5	^{82}Br	35.4h	3.31	554, 776
Ca (^{48}Ca)	0.187	^{49}Ca	8.718m	1.1	3084
Ce (^{140}Ce)	88.5	^{141}Ce	33d	0.6	145
Cl (^{37}Cl)	24.4	^{38}Cl	37.2m	0.43	1643, 2167
Co (^{59}Co)	100	^{60}Co	5.27y	37.2	1173, 1332
Cr (^{50}Cr)	4.35	^{51}Cr	27.8d	15.9	320
Cs (^{133}Cs)	100	^{134}Cs	2.06y	30	605,796
Cu (^{63}Cu)	69.1	^{64}Cu	12.8 h	45	511
Eu (^{151}Eu)	47.8	^{152}Eu	13.5y	5900	244, 1408
Fe (^{58}Fe)	0.33	^{59}Fe	44.6d	1.2	1099, 1291
Hf (^{180}Hf)	35.1	^{181}Hf	42.4d	10	482
Hg (^{202}Hg)	29.7	^{203}Hg	46.6d	3.8	279
K (^{41}K)	6.88	^{42}K	12.5h	1.30	1524
La (^{139}La)	99.9	^{140}La	40h	8.93	487, 1596
Mg (^{26}Mg)	11.17	^{27}Mg	9.46m	0.038	1014
Na (^{23}Na)	100	^{24}Na	15h	0.5	1368
Mn (^{55}Mn)	100	^{56}Mn	2.58h	13.3	846
P (^{31}P)	100	^{32}P	14.3d	0.19	1708(β^-)
Rb (^{85}Rb)	72.1	^{86}Rb	18.7d	0.91	1077
Sb (^{123}Sb)	42.8	^{124}Sb	60d	3.3	603, 1691
Sc (^{45}Sc)	100	^{46}Sc	83.8d	27.2	889, 1120
Se (^{74}Se)	0.89	^{75}Se	120d	52	264
Sm (^{152}Sm)	0.21	^{153}Sm	47 h	5800	103
Sr (^{84}Sr)	0.56	^{84}Sr	65.2d	1.4	514
Th (^{232}Th)	100	$^{233}Th(^{233}Pa)$	27d	7.4	312
V (^{51}V)	100	^{52}V	3.74m	4.9	1434
Zn (^{64}Zn)	48.6	^{65}Zn	244d	0.76	1115

In some cases, however, half-life of the radionuclide e.g. ^{27}Mg was also followed. Now a day, several updatable neutron cross-section and energy programmes are available constructed on the basis of RNAL [29]. Since our system also had built-in Library of Nuclides it got automatically calibrated though a difference of maximum up to 1 keV was observed.

(ii) Data Processing: Following steps are involved in the computerized analysis of a peak.

- Smoothening of experimental data
- Peak searching
- Selection of fitting intervals
- Peak energy calculation
- Peak area calculation

For NAA, the most important of all these factors is peak area calculation represented by: $A = \sum_{-n}^{+n} \alpha_i - \frac{n+1}{2}(b_{+n} + b_{-n})$ where, n = number of channels on right (+) and left (-) of the i^{th} peak channel, α_i = total counts in i channels, $b_{\pm n}$ = background counts in 2n channels as determined from a straight line drawn between left to right of the peak channel as shown in Fig.II.8.

However, the superimposition of photo peaks on each other or the high Compton background makes it difficult to precisely determine the net counts in the region of interest (ROI). The base area is mostly taken as trapezoidal summation of the average counts in each channel giving the background over which the photo peak is situated. Several workers [30] have reported the validation and loss free counting for NAA where the use of peaks in the corrected spectrum has been emphasized for the concentration calculation while the data from an uncorrected spectrum determines the counting statistics. In recent

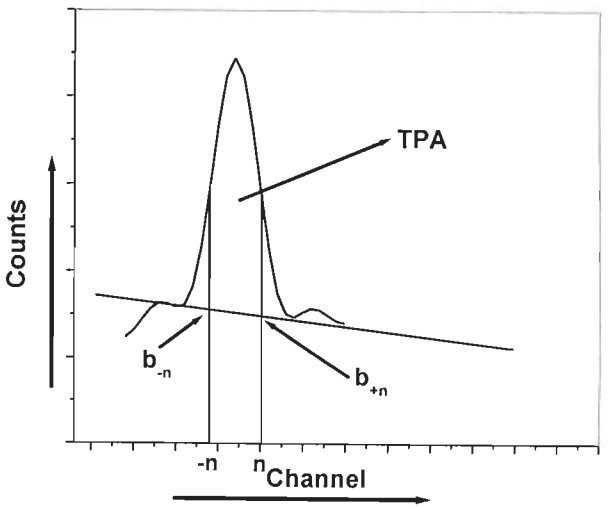


Fig. II.8 An Illustration of the Measurement of Total Peak Area (TPA)

years Compton suppression technique has become a preferred technique for short-lived nuclides where background noise is much less resulting in enhanced sensitivity [31]. Landsberger et al. and several other groups [31] have used it most extensively to reduce the spectral interferences and thereby lower the detection limits for many elements. In recent years several softwares have been developed whereby closely lying multiplets can be resolved into individual photo peaks. These include SAMPO 90 [32], DECHAOS [33], (SPAAC) [34], HYPERMET [35], UNISAMPO and SHAMAN [36].

Concentrations were then calculated using the comparator method using eqn. (I.5) Recently, Landsberger et al. [37] developed Microsoft Excel as the analysis engine for the input data based on report files created by Canberra's Genie-2000 spectroscopy system.

II.8 DETERMINATION OF PHOSPHORUS

Phosphorus is an important and essential structural element providing strength to bones in animal kingdom and as phosphate in the form of nucleotides serving as a source of a high free energy bond performing an important function in conserving and providing bursts of energy [38]. It is involved in many metabolic pathways as a part of adenosine triphosphate (ATP) and found everywhere in the earth's crust, environment plants and animal tissues [39]. Phosphorus is one of the macro ingredients in fertilizers and detergents, which find their way into the water bodies leading to increase in phosphorus levels causing eutrophication of system. Oxo acids and salts of phosphorus are of interest in biological, agricultural and food industry. Therefore, determination of phosphorus is important in several fields like environment, biology and geology. Its determination especially in biological samples is difficult though many instrumental methods are reported in literature [40-43]. Weginwar et al. [40] first developed a NAA procedure for determining mg amounts of P in biological tissues and plant materials whereby β-activity due to ^{32}P formed by (n,γ) reaction in a reactor was determined by using a gas flow proportional counter and 27 mg cm^{-2} Al filter after a delay period of ~ 3 weeks. Scindia et al. [41] reported its trace determination using preconcentration chemical procedure followed by derivative NAA by extracting molybdovanadophosphoric acid in methyl isobutyl ketone (MIBK) and determining the activity of ^{52}V ($t_{1/2}$ = 3.4 min). Porte et al. [42] determined P in bones by 2 min thermal neutron irradiation in a reactor followed by

the measurement of the bremsstrahlung produced by the β-emission of ^{32}P in a Ge-detector surrounded by an anti-Compton shield. During recently held MARC-VII conference Goerner et al. [43] reported a method for P in organic materials by using instrumental photon activation analysis.

During past one decade the method followed earlier in our laboratory [40] has been modified as in the new method β activity is measured by using an end window G.M. counter (Model GC602A Nucleonix, Hyderabad) and Al absorber of 27 mg cm^{-2} to cut off low energy β particles. We have been using this method for determining P content in a variety of biological RMs and samples with a detection limit of 0.1 mg/g [44]. It has been our experience during our participation in the Intercomparison studies from NIST (USA), IAEA (Vienna) and INCT (Poland) that our data have been found acceptable within <±5% of the certified values. In order to be sure if no activity due to any other β emitting radionuclide is being counted, Feathers analyzer method was employed to determine β_{max} and half-life was followed for the samples and RMs and the results compared with that for ^{32}P tracer solution. β_{max} was found to be 1725 keV and typical half-life plots are shown in Fig. II.9.

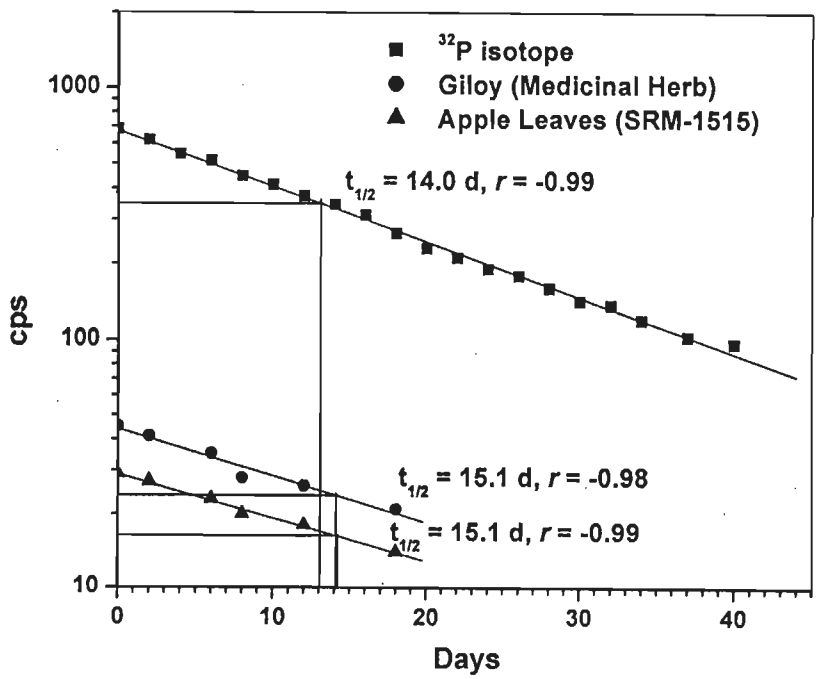


Fig II.9 Decay plots of ^{32}P in tracer solution, a Herb and biological RM

The $t_{1/2}$ of ^{32}P tracer solution obtained from BRIT, Mumbai is found to be 14.33 ± 0.19 d compared to 14.23 d reported in literature [45]. However, for biological RMs and samples it was found to be 15.3±0.2 d. Thus the error for tracer solution was just + 0.77 % whereas for samples it is much higher (+7.21%). Probably longer half-life observed in the biological samples and RMs may be attributed to trace amounts of some long-lived β emitters such as ^{35}S ($t_{1/2}$=87 d) and ^{45}Ca ($t_{1/2}$=164 d). Further, it seems all the low energy β particles are not being cutoff by the Al absorber. However, our data seem to be acceptable within ±10 % limits because of the difficulty in the NAA determination of phosphorus.

Recently, we participated in an intercomparison study of two candidate RMs; Corn flour (CF-3) and Soybean flour (SBF-4) from INCT, Poland where P content was found to be 3.54±0.43 and 6.11±0.29 mg/g compared to the certified values of 2.83±0.10 and 6.56±0.34 mg/g [46] respectively. Further, Z-score values for P in CF-3 and SBF-4 were calculated as 1.65 (p=0.834) and 1.32 (p=0.892) respectively suggesting that our data should be acceptable within 95% confidence limit. The details of the intercomparison study are given in Section II.10. Concentration of P in some biological SRMs and medicinal herbs analyzed in this study are listed in Table II.6. SD values obtained by replicate analyses in RMs are always small with RSD<5% in most cases. However, while analyzing samples if the same specie is collected from geographically different regions, SD and RSD values become large. This is particularly evident from P concentration in curry (0.97±0.40) and mint (3.88±0.94) leaves where 28 and 10 samples were derived from far and wide. It may be due to differential uptake of phosphorus from the soil. A comparison of P concentration data in most RMs is within ±5% of the certified values with relative error in the range –7.74 to +7.91%. In view of these observations, same degree of accuracy and precision is expected for various samples as well. Large SD values in some cases may however be attributed to different geo-ecological conditions wherefrom samples were derived.

The proposed method has a limitation that it is good only if P is present at mg/g level, which is the case in all kinds of biological samples, be it plant material or a tissue. Another limitation is that the sample should be in dried and powdered form, which of course is the limitation of NAA itself.

Table II.6 Phosphorus concentration in biological SRMs, medicinal herbs and formulations

Sample	Phosphorus content (mg/g)		Relative Error (%)
	Present work	Certified value	
Reference Material; Pine Needles (NIST, SRM-1575a)	1.25±0.09	1.20±0.20	+4.1
Rice Flour (NIST, SRM-1568)	1.47±0.04	1.58±0.08	-6.96
Peach Leaves (NIST, SRM-1547)	1.43±0.11	1.55± 0.05	-7.74
Apple Leaves (NIST, SRM- 1515)	1.60+0.07	1.59±0.11	+0.63
Cabbage (IAEA, CRM-359)	5.12±0.11	[5.27] Information Value	-2.85
Cabbage Leaves (INCT, CL-1)	3.29±0.23	[3.55] Information Value	-7.32
Tobacco Leaves (INCT, CTA-OTL-1)	2.71±0.17	2.89±0.13	-6.23
Tobacco Leaves (INCT, CTA-VTL-2)	2.59±0.13	2.40±0.08	+7.91
Mixed Polish Herbs (INCT-MPH-2)	2.34±0.06	[2.50] Information Value	-6.40
Biological Sample; Mint (n=10)		3.88±0.94	*Ch. III*
Curry leaves (n=28)		0.97±0.40	*Ch. IV*
Neem leaves (n =11)		2.16±0.74	*Ch. V*
Arjuna bark (n = 5)		0.37±0.16	Ref. 47
Neem bark (n = 3)		0.58±0.04	*Ch. V*
Antidiabetic Herbs: Leaves (n = 3)		2.15±0.48	*Ch. V*
Roots (n = 5)		1.65±0.87	*Ch. V*
Seeds (n = 3)		3.19±1.03	*Ch. V*
Fruits (n = 3)		1.04±0.42	*Ch. V*
Bark (n = 1)		0.61±0.09	*Ch. V*
Herbal Formulations (n = 5)		4.13±1.59	*Ch. V*
Trikatu (n=5)		3.35± 0.37	*Ch. VI*
Trifala (n= 8 brands)		3.58±0.01	Ref. 48
Pragya-peya (n=3)		1.20±0.04	Ref. 49
Chewing Tobacco (20)		2.70±0.54	Ref. 50

II.9 SAMPLE DISSOLUTION FOR AAS

AAS is now a well-established technique for the determination of trace elements covering a wide range of analyte types including metals and a few of the nonmetals. Unlike in INAA where no sample dissolution is required, in case of Atomic Absorption Spectrophotometry (AAS), it is the liquid sample, which is aspirated into the flame, and hence it is essential to have the sample in solution form. AAS makes use of light wavelengths being specifically absorbed by the analyte element present as gaseous atom in the aerosol. The characteristic wavelength corresponds to the energy needed to promote electrons from the ground to excited energy level [2, 51]. AAS has many uses for the determination of trace elements in different areas of chemistry and especially in biological specimens. However, it has a great disadvantage of being destructive and mostly unielemental. It is the most sought for analytical technique for a variety of sample matrices. Bings et al. [52] have reviewed some recent developments in AAS methodology. AAS was specially used for the determination of Ni, Cd and Pb, which are difficult to be determined by thermal NAA but are environmentally important. It may be mentioned that while Ni is considered as essential element [53], Cd and Pb are highly toxic and environmental contaminants [54].

The inorganic content of most medicinal herbs is only a minor constituent and interfered by major constituents. Therefore, first task in inorganic analysis is to remove the organic matter. Many decomposition methods are available for mineralisation of plant material, before the determination of trace elements including dry ashing and wet digestion. Besides, microwave digestion procedure is also frequently used in AAS as described by Soylak et al. [55] for trace heavy metal contents in Turkish cereals, pulses and spices.

Procedure; 2 g dried powder sample was accurately weighed and digested in a mixture of nitric acid and perchloric acid (5:1) as suggested in literature [56]. After digestion, dilution and filtration on a Whatman filter paper no 42, 2-3 drops of HCl were added and the solution was made up to 25 mL. All the solutions were stored in tight capped polythene bottles. These were appropriately diluted and used for the determination of various elements. Details of the instrument are given in Section II.12.

II.10 PARTICIPATION IN INTERCOMPARISON STUDY

Based on the quality of our INAA data during past two and half decades our group is being invited to participate in Intercomparison studies of candidate reference materials developed by NIST (USA), IAEA (Vienna) and INCT (Poland). Prof. R. Dybczynski (INCT, Poland) has been pioneer in developing RMs of biological origin. In continuation of our earlier participation on RMs Tobacco leaves (OTL-1 and VTL-2), Tea Leaves (TL-1) and Mixed Polish Herbs (INCT-MPH-2), we were invited (2003) to participate in the Intercomparison study of two candidate RMs Corn flour (CF3) and Soybean flour (SBF-4). Moisture contents for CF-3 and SBF-4 were found to be 8.77±0.22% and 5.44±0.07% respectively. Elemental concentrations were corrected for the moisture content and the final data on dry wt basis were submitted in May 2004.Final reports are still awaited though preliminary results [46] are available.

INCT-CF-3: The material was prepared from corn grown in Poland according to Polish standard PN-A-74205: 1997. The material was sieved through 250 μm nylon sieves. Approximately 50 kg of sieved corn flour was collected and stored in a polyethylene (PE) bags. Optical microscopy examination revealed Martin's diameter to be below 25 μm for over 98% of particles.

INCT-SBF-4: It was prepared from Soya bean grown in India, not genetically modified. After milling, the material was sieved through 150 μm nylon sieves. Approximately 50 kg of sieved Soya bean flour was collected and stored in polyethylene (PE) bags. Optical microscopy examination revealed Martin's diameter to be below 50 μm for over 90% of particles.

In both cases whole lot of flours were then homogenized by mixing for 20 h in a 110 dm^3 PE drum rotated in three directions. Preliminary homogeneity testing by XRF method and final checking of homogeneity by NAA after distribution of the material into containers revealed, that it is sufficiently homogeneous at least for a sample size ≥ 100 mg. In order to assure the long-term stability, all containers of both flour samples (CF-3 and SBF-4) were sterilized by electron beam radiation. Long-term stability was checked by analyzing concentrations of selected elements in the material stored in the air-conditioned room at 20°C. Short-term stability was examined by the determination of concentrations of the selected elements in the

bottle stored in the CO_2 incubator at 37°C. The shelf life of CF-3 and SBF-4 was established to be up to 31 December 2015.

Analytical Procedure: For determining short lived nuclides 20-30 mg samples along with standards were packed in Alkathene and irradiated for 5 min. in APSARA at ~10^{11} n cm^{-2} s^{-1}. For long-lived nuclides, however, ~50 mg samples along with standards were packed in Aluminum foil (Suprafine) and irradiated for 1 d in Dhruva at ~10^{13} n cm^{-2} s^{-1}. A synthetic primary standard containing As, Fe, Zn, Co, Cr and Se along with four RMs of Rice flour (1568a) [57] and Wheat Flour (1567) [58] from NIST (USA), Whey Powder (IAEA-155) [59] and Rice Flour no. 10a [60] from NIES, Japan were used as comparator standards. Final data as obtained on dry wt basis along with certified values [46], including RSD, error and Z-score are listed in Table II.7. Also Z-score plot is shown in Fig. II.10.

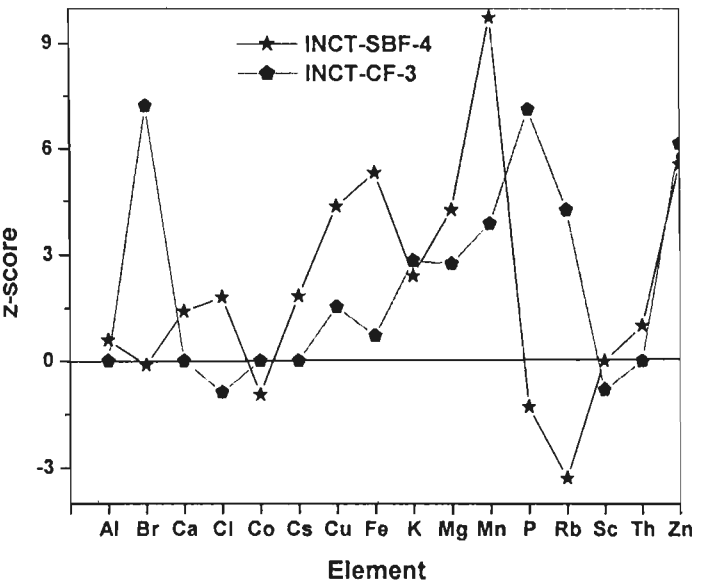


Fig II.10 Z-score plots for CF-3 and SBF-4

Results: The material was certified on the basis of a worldwide interlaboratory comparison, in which 92 laboratories from 19 countries participated. Analytical uncertainties and stability uncertainties were quantified to arrive at combined uncertainties. A close look at Fig. 11.10 shows that Z-score for most elements besides Cu, Fe, Mg and Mn for SBF-4 and Br, Mn, P, Rb and Zn lies between +3 to –3 suggesting that our data to be within 95% confidence limit. A perusal of the data in Table II.7 shows that %RSD for all elements in both the RMs is < 10%. Our data for Al, Br, Ca, Cl, Co, Cs, K and P in SBF-4 are within ± 10%. Concentration data in SBF-4 for Cl, Cr, Na and Sc are higher by ~25% in SBF-4. This could be due to the fact that only informative values are available at the moment. For Corn Flour data for Cl, Cs, Cu, K, Sb and Sc are within +15%.

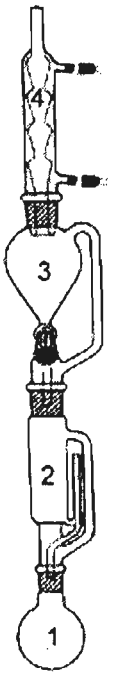
Table II.7 Comparison of our data with the certified/informative values [465]

Element	INCT-SBF-4				INCT-CF-3			
	This work	% RSD	% Error	Z-score (p)	This work	% RSD	Error	Z-score (p)
Al (µg/g)	47.7±1.7 (45.5±3.7)	3.56	+4.84	+0.60 (0.73)	17.1±1.0 [12]	5.85	+42.5	-
Br (µg/g)	2.38±0.21 (2.40±0.17)	8.82	-0.83	-0.12 (0.55)	0.75±0.06 (0.39±0.05)	8.0	+92.3	+7.2 (1)
Ca (mg/g)	2.71±0.21 (2.47±0.17)	7.7	+9.72	+1.41 (0.92)	ND (40)	-	-	-
Cl (µg/g)	73±6 (64.5±4.7)	8.21	+13.2	+1.81 (0.96)	368±15 (397±33)	4.08	-7.30	-0.88 (0.81)
Co (ng/g)	90.1±2.1 (95.6±5.8)	2.33	-5.75	-0.95 (0.83)	13±1 [16]	7.70	-18.8	-
Cr (µg/g)	0.29±0.02 [0.23]	6.90	+23.1	-	0.27±0.02 [0.14]	7.41	+92.9	-
Cs (ng/g)	137±5 (129.1±4.3)	3.65	+6.11	+1.84 (0.97)	3.58±0.12 [4]	3.35	-10.5	-
Cu (µg/g)	12.3±0.06 (14.3±0.46)	0.49	-14.0	-4.35 (1.0)	1.43±0.09 (1.63±0.13)	6.29	-12.3	-1.54 (0.94)
Eu (ng/g)	1.72±0.10 -	5.81	-	-	2.51±0.04 -	1.59	-	-
Fe (µg/g)	112±3 (90.8±4.0)	2.68	+23.3	+5.3 (1.0)	33.0±1.4 (32.0±1.4)	4.24	+3.13	+0.71 (0.76)
K (mg/g)	26.6±2.2 (24.2±0.83)	8.27	+9.91	+2.41 (0.99)	3.50±0.30 (3.16±0.12)	8.57	+10.8	+2.83 (0.99)
La (µg/g)	0.37±0.03 (0.02±0.002)	8.11	+1750	+175 (1)	0.38±0.01 (0.007±0.001)	2.63	+5329	+373 (1)
Mg (mg/g)	3.34±0.17 (3.00±0.08)	5.09	+11.3	+4.25 (1)	1.18±0.06 (1.07±0.04)	5.08	+10.3	+2.75 (0.99)
Mn (µg/g)	43±3 (32.3±1.1)	6.98	+30.0	+9.73 (1)	5.83±0.13 (4.98±0.22)	2.23	+17.1	+3.86 (1)
Na (µ g/g)	6.73±0.32 [5.5]	4.75	+22.3	-	6.9±0.6 [4.4]	8.70	+56.8	-
P (mg/g)	6.11±0.29 (6.56±0.35)	4.75	-6.86	-1.29 (0.90)	3.54±0.23 (2.83±0.10)	6.50	+25.1	+7.1 (1)
Rb (µg/g)	26.1±1.4 (31.7±1.7)	5.36	-17.7	-3.29 (1)	1.08±0.09 (0.91±0.04)	8.33	+18.9	+4.25 (1)
Sb (ng/g)	11.7±0.3 -	2.56	-	-	9.7±0.4 [11]	4.12	-11.8	-
Sc (ng/g)	8.74±0.53 [7]	6.06	+24.9	-	1.87±0.07 (2.13±0.32)	3.74	-10.8	-0.81 (0.79)
Sn (ng/g)	2.19±0.15 -	6.85	-	-	3.41±0.15 -	4.40	-	-
Th (ng/g)	7.9±0.4 (7.08±0.82)	5.06	+11.6	+1.0 (0.84)	7.71±0.24 -	3.11	-	-
Zn (µg/g)	45.1±4.1 (52.3±1.3)	9.09	-13.8	-5.54 (1.0)	25.0±1.7 (20.1±0.8)	6.80	+25.4	+6.13 (1)

In general our data are on the higher side of the reported values. We have also reported data for Eu, Sb and Sn for which no certified/information values are available and these may be taken as information values

II.11 SEPARATION OF ORGANIC CONSTITUENTS

Soxhlet is the most commonly used laboratory glassware employed for the extraction of natural products by repeated washing (percolation) with predistilled organic solvent (methanol/ ethanol/ dichloromethane/ diethyl ether/ petroleum ether) under reflux. 50-100 g dried herb powder was taken in a cellulose thimble in the extraction chamber (2) suspended on a 5 L RB flask (1) containing 2 L solvent. It is designed such that when the solvent surrounding the sample exceeds a certain level it overflows and trickles back down into the boiling flask. A refluxing condenser (4) with a separating funnel (3) helps in recondensation of the excess solvent. The flask is heated and the solvent starts evaporating which then moves up into the condenser where it is condensed and trickles into the extraction chamber (2). At the end of the extraction, the flask containing the solvent and the extract is removed wherefrom the solvent is distilled off. The residual extract is weighed and percentage of the organic extract is calculated. This is the crude separated fraction, which may contain several compounds.



In order to check the number of constituents, ***thin layer chromatography*** (TLC) is carried out on a glass plate (5cm X 20 cm) coated with 0.5 mm thick Silica Gel containing 13% $CaSO_4$ as binder (SRL, Mumbai). A spot of organic extract in a solvent was put and after drying the plate was developed in several chambers containing solvent(s) with increasing polarity where the components of the samples have different solubilities. A typical solvent set with increasing polarity is Heptane<Hexane<Pentane<Cyclohexane<Petroleum.Ether<CCl_4<Benzene< Diethyl Ether<Dichloromethane<Tetrahydrofuran (THF)<$CHCl_3$<Ethylacetate (EtOAc) <Acetone<Methanol<Ethanol< Acetic Acid< Dimethylformamide (DMF)< Dimethylsulfoxide (DMSO)<Water.

As a result, the constituent(s) move up and separate out depending on its nature and the interaction of the constituent with the solvent mixture. The plate after drying in an oven at ~80 °C is then kept in an iodine chamber for development of spots. A circular spot with no tailing is indicative of a pure compound with a definite R_f value. In case of

non-separable constituents, however, a non-symmetrical wide spot with tailing is observed and may be rejected.

The dried extract is dissolved in a non-polar solvent (e.g. petroleum ether) and poured slowly from the side of a 1.5 cm X 45 cm ***glass column*** fitted with sintered frit or glass wool and filled with 60-120 mesh Silica Gel-G (Merck, Mumbai). Afterwards, elution is done using different solvent mixtures in varying ratios depending on the increasing order of their polarities as mentioned above. The solvent elutes the sample through the column allowing the components to separate on the basis of adsorption. Care was taken to control the flow rate. Various fractions were collected (~5 mL) in small test tubes and the solvent was distilled off. Again, TLC check was made and the plate was developed. Pure fractions were kept aside for further analysis while the impure fractions were mixed together and distilled to ~5 mL. The mixture or impure fractions having close R_f values was subjected to ***preparative TLC*** on a glass plate (20 cm x 20 cm) with 1 mm thick Silica Gel coated on it. The bands are developed in a solvent system and then in an iodine chamber. Bands were cut and dissolved in a suitable solvent, which was filtered off using glass wool. Again, the solvent was distilled off and the pure constituents after TLC check were recrystallized. C, H, N and S analysis was done and the structures were elucidated by infrared and NMR spectral and GC-MS studies.

II.12 INSTRUMENTATION

(i) AAS: Atomic absorption spectrophotometer (GBC Avanta, Australia) was used at the Institute Instrumentation Centre (IIC). It uses acetylene-air/acetylene-nitrous oxide mixture to ignite the flame. Only Ni, Cd and Pb were determined by AAS. For each element the instrument was first calibrated using at least four standard solutions (prepared from accurately weighed AR grade salts and dissolving in doubly distilled water with a few drops of HCl) of specific concentration range prepared from high purity grade salts of respective elements. After calibration, stock sample solutions were suitably diluted to get absorbance in the calibration range. Calibration curves were drawn after setting various parameters including wavelength, concentration range and sensitivity for the elements given in Table II.8. Flow rate of air and acetylene was maintained at 10.1 and 2.6 L/ min respectively.

Table II.8 Experimental parameters for AAS determination

Element	Wavelength (nm)	Concn Range (µg/mL)	Slit width (nm)	Sensitivity (ng/mL)
Cd	228.8	0.2-1.8	0.9	9
Ni	232.0	1.8-8.0	0.4	40
Pb	217	2.5-20	0.9	60

(ii) Elemental Analyzer: Elementar Vario-EL III (Germany) connected to a thermal conductivity detector (TCD) was used to carry out C, H, N and S analysis. Supply gas was He (99.996% pure) and O_2 (99.995% pure) with a flow rate of 250 mL min^{-1}. The instrument was preloaded with Vario EL software. It was calibrated with Sulphanilic acid (GR, E Merck) in the beginning and at the end of the day.

(iii) Infrared Spectra: These were recorded in KBr (AR) using Thermo Nicolet (Nexus, USA) FT-IR Spectrophotometer in the range 400-4000 cm^{-1}. A pinch of sample and ~50 mg KBr were thoroughly grounded in an agate mortar and the mixture was put on the pellet holder and pressure was applied through the hydraulic machine to make a thin film. Identity of the compound was confirmed by matching the spectra with those reported in Aldrich library [61].

(iv) NMR: ^{1}H and ^{13}C NMR spectra were recorded on 200 and 300 MHz av Bruker spectrometer using $CDCl_3$ and D_2O solvents and TMS as an internal standard. These were obtained through the courtesy of Prof. A.S. Brar (IIT, Delhi) and Dr. Alok Srivastava (Punjab University, Chandigarh). Spectra were matched with those reported in Aldrich Library [62].

(v) GC-MS: A Perkin-Elmer Clarus-500 single unit gas chromatograph (66 cm X 40 cm X 72 cm) coupled with a mass spectrometer was used. The compound mixture was separated on a fused silica capillary column 30 mm x 0.32 mm x 0.25 µm film thickness, in a temperature program from 50 (2 min hold) to 250 °C (10 min hold) at a heating rate of 8 °C min^{-1}. The injector temperature was also maintained at 250 °C, and the carrier gas was He at a flow rate of 1mL min^{-1}. The interface, which kept the capillary column end into the ion source block, was maintained at 280 °C. The mass spectrometer is fitted with a quadrupole prefilter assembly. The detector consists of a common dynode, phosphor plate and PMT. The Turbo Mass software v.4.4 is preloaded into the system. Compounds were further confirmed by their mass fragmentation pattern and comparing the individual spectra with those of built-in NIST 2.0 mass spectral database [63].

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CHAPTER III

MINT LEAVES

A part of the work presented here has appeared in *J. Pharm. Biomed. Anal.*, **41** (2006) 825-832

III.1 GENERAL CHARACTERISTICS

Mint is a common name for members of the *Labiatae* family, Genus *Mentha* of chiefly annual or perennial herbs [1]. Members of this family are found throughout the world, but mainly in the Mediterranean region, where these plants form a dominant part of the vegetation. The *Labiatae* typically have square stems, paired opposite leaves and tubular flowers with two lips, the upper divided into two lobes and the lower into three [2]. The family is well known for the aromatic volatile or essential oils in the foliage, which are used in perfumes, flavourings and medicines. Species of the *Labiatae* are often grown as ornamentals as well as in herbal gardens and have become naturalized as wildflowers.

The most common and popular varieties of mint are Spearmint *(Mentha spicata),* Peppermint *(M. piperita),* Apple Mint *(M. suaveolens),* Curly Mint *(M. spicata variety crispii),* Pennyroyal *(M. pulegium)*, Pineapple Mint *(M. suaveolens 'Variegata')* and Water or Bog Mint *(M. aquatica)*. Commercially the most important species is peppermint (*M. piperita*).

Spearmint (*M. Spicata*) is the most common mint grown commercially in home gardens. Other common names are mint, brown mint, garden mint, lamb mint, mackerel mint, our Lady's mint, sage of Bethlehem, *pudina* (Hindi, Bengali and Marathi), *putiha* and *Rochin* (Sanskrit), *fudino* (Gujarati), *fujnaj* (Assamese) and pudin in French. Its leaves are smooth and bright green with elongated pointed end. The flowers are pink to lilac in colour and grow in clusters on the ends of the stem. Spearmint grows in a wide range of climatic conditions though ideally it requires plenty of Sun and grows best in summers at higher latitudes in deep, rich soils of friable texture high in organic matter. The preferred pH range is from 6.0 to 7.5 [3]. Spearmint is an ancient herb used since antiquity for its culinary, medicinal and aromatic properties. Its characteristic smell has made it as the most popular perfuming herb throughout history [4].

Around the globe from Europe to India to the Middle East, spearmint has been used as a strewing herb to clear the air in temples and houses. Mint has also come

to symbolize hospitality in many cultural civilizations. In ancient Greece, spearmint leaves were rubbed on dining table to welcome the guests while in Middle East, the guests traditionally were offered mint tea [5]. Beginning with 14^{th} century, spearmint was used for whitening teeth and its distilled oil is still used to flavor toothpaste and chewing gum. It is well described in literature as a part of Indian recipes [6,7]. Mint is an excellent recipe in hot summer days though it can be used round the year. During winters when fresh mint leaves are not available, dried leaves powder in curd is used. A cup of fresh mint tea soothes the stomach and nerves. A combination of mint, fennel, onions and oranges makes a quick and easy salad [8]. Plain yogurt with chopped fresh mint leaves or dried powder and garlic makes an excellent recipe for summers. Mint leaves added to fruit salad give it a unique perk. Chopped mint leaves added to tomato soup complements the sweet acidity of the tomatoes nicely. Mint leaves added to sugarcane juice enhance its flavour.

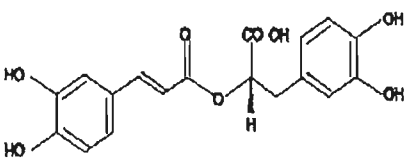
III.2 MEDICINAL IMPORTANCE

Spearmint has served as an important medicinal herb for millennia. It is widely used in every Indian household, both as a medicine and a flavouring agent. Essential oil of Mint is used as a component of many drugs. *Pudin Hara,* which relieves stomachache and cures flatulence is marketed by Dabur India Ltd. as extract and capsules. Our food ranking system shows mint to deliver a wide range of traditional nutrients. Mint is an excellent source of Vitamins A and C, the former notably through its concentration of carotenoids, including β-carotene. Both Vitamin C and β-carotene play an important role in decreasing colorectal cancer risk. Vitamin C is the main water-soluble antioxidant in the body, which decreases levels of free radicals that can cause damage to cells. Some studies have shown that Vitamin C intake helps decrease the incidence of colon tumours [9]. Carotenoids have also been shown to increase cell differentiation and protect cells against carcinogenic chemicals that could damage DNA. Vitamin A, structurally similar to β-carotene, may help to decrease risk by preventing excessive colon cell proliferation and tumour formation [10]. In addition to all the healing properties, mint emerges as an excellent source of dietary fiber, Vitamin B_2 and foliates. Their high nutrient density and low calorie status qualifies mint as a good source of vitamins B_3 and B_6 as well. Its organic and inorganic constituents reported in literature [7, 11] are listed in Table III.1.

Table III. 1 Organic and Inorganic constituents of Mint leaves

Organic	mg/100 g of edible portion	Element	mg/ 100 g of edible portion
Fats	600	Calcium	200
Fibres	2000	Phosphorus	62
Carbohydrates	5800	Iron	15.6
Carotene	1.62	Magnesium	60
Thiamine	0.05	Copper	0.18
Riboflavin	0.26	Manganese	0.57
Miamin	50	Zinc	4.4
Niacin	1.0	Chromium	0.08
Nicotinic acid	0.4	Sulphur	84
Folic acid	0.114	Chlorine	34
Vitamin C	27	Total Minerals	16 mg
Essential Oil	0.25-0.5 %	Moisture	84.9%
Oxalic acid	33		
Energy (kCal)	48		

Besides being used as a spice to impart aroma and flavour to dishes, mint has several health benefits. Randomized control trials have shown the ability of mint oil to relieve symptoms of irritable bowel syndrome including indigestion, dyspepsia, colonic muscle spasms and cold hyperalgesia [12]. Spearmint contains a monoterpene called Perillyl alcohol, which stops the growth of pancreatic, mammary and liver tumours and protects against cancer formation in the colon, skin and lungs [13]. Essential oil of spearmint also stops the growth of different bacteria including *H. pylori*, *S. enteritidis* and *E. coli* [14]. Spearmint contains *rosmarinic acid*, which is beneficial in asthma. In addition to its antioxidant ability to neutralize free radicals, rosmarinic acid has been shown to block the production of pro-inflammatory chemicals like leukotrienes. It acts as insect phagostimulant and have antifungal properties [15].



Rosmarinic acid

III.3 LITERATURE SURVEY

Most work on *Mentha spicata* pertains to the organic constituents though scanty efforts have been made to determine its elemental composition. Elmastas et al. [16] isolated S-Carvone from *Mentha spicata* using chromatographic methods and characterized with GS-MS, FT-IR and NMR studies. De Carvalho and Da Fonseca

[17] reviewed several applications of carvone as fragrance, flavour and antimicrobial agent along with its relevance in medicine. Yamamura et al. [18] identified four known flavonoids, three new and five known glucosides of lower alcohols and rosmarinic acid using column chromatography and HPLC. Lin et al [19] developed a sensitive liquid chromatographic method for the quantitative analysis of enantiomeric (+)-menthol and (-)-menthol based on their derivatization with a fluorescent reagent, naproxen acyl chloride in toluene.

(i) Essential oil: Steam distillation and analysis of essential oil of mint leaves from Sudan by GC and GC/MS revealed twenty-two compounds, the major ones being carvone (78.9%), limonene (8.8%), 1,8-cineole (2.6%), menthone (1.6%), linalool (3.2%) and isomenthone (0.6%) [20]. Kofidis et al. [21] observed that the essential oil obtained from the Greek leaves was characterized by a very high content in linalool (85.0-93.9%). However, Kokkini et al. [22] did not observe any linalool in the GC-MS of essential oil in mint from the island of Crete (Greece). More than 95% of the components were identified in the essential oil from Algiers, carvone being the major component [23]. Mestri [24] reviewed chemical composition of different varieties of mint oil along with physicochemical properties such as specific gravity, optical rotation, refractive index, solubility in alcohol-water mixes, boiling temperature, odour, colour etc. Park et al. [25] tested nematicidal activity of the essential oils from spearmint and other plant species against the pine wood nematode, *Bursaphelenchus xylophilus* and observed that responses varied with plant material and concentration. Khanuja et al. [26] invented a cream based preparation of mint oil and garlic extract having a potent anti-dermatophytic activity. Shan [27] patented a medicine containing mint leaves along with other auxillary materials, which relieve inflammation, arrest bleeding, stop pain and eliminate watery distension and ulcer.

(ii) Antioxidant activity: Proestos et al. [28] employed RP-HPLC with UV detection for the identification and quantification of the phenolic antioxidants ferulic acid (1.1-280 mg/100 g) and caffeic acid (1.2-60 mg/100 g) present in methanolic extract. Kosar et al. [29] used online HPLC-DPPH method for the detection of polar and nonpolar radical scavenging compounds in complex plant extracts. Voirin et al. [30] extracted external lipophilic methylated flavonoids from the diethyl ether extract of dried leaves. Arumugam et al. [31] analyzed four solvent fractions in hexane,

chloroform, ethyl acetate and water of ethanolic extracts of dried leaves for total antioxidant activity (TAA) and relative antioxidant activity. Tognolini et al. [32] studied its essential oil for antiplatelet activity and observed a significant correlation between antiplatelet potency and phenylpropanoid content (54-86%) suggesting a key role for this moiety in the prevention of clot formation. Fletcher et al. [33] carried out DPPH radical assay of heat-stressed and non-stressed plants and observed *rosmarinic acid* to be the major contributor to the antioxidant activity. Akdogan et al.[34] studied the effect of mint tea from Turkey containing different phenolic compounds on iron metabolism in Wistar rats. It was observed that herbal tea inhibited iron absorption, which was dose dependent. Yu et al. [35] studied its antimutagenic activity in Salmonella assay and observed inhibition of carcinogen activation. Elmastas et al. [36] observed that the ethanolic extract has effective reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities. Kulkarni et al. [37] evaluated antioxidant activity of wheatgrass under different growth conditions. It has been shown that ethanolic extracts had higher phenolic and flavonoid content than the aqueous extracts. Kanatt et al. [38] have studied antioxidant potential of mint in radiation processed lamb meat. Mint extract has been found to exhibit retarded lipid oxidation and better storage capacity after 4 weeks chilling. Asrar et al [39] observed that accumulation of Mn in roots was greater than shoots, indicating significant metal immobilization by the roots.

(iii) Trace Elements: Only few references exist on the elemental contents in *Mentha spicata*. Lozak et al. [40] determined 20 elements in *mint* and their infusions by ICP-MS and AAS. Zeinali et al. [41] studied the diversity amongst twelve accessions of Iranian *mint* in relation to yield and mineral contents. In an ICMR compilation [7] concentrations of some selected elements are mentioned as listed in Table III.1. Rajput et al. [42] carried out a field experiment in subtropical climate of northern India to study the response of *mint* to application of six micronutrients (Fe, Mn, Zn, Cu, B and Mo). From neighbouring Pakistan, some workers have analysed organic and inorganic constituents in field mint [43, 44]. Balaji et al. [45] have analyzed a few essential elements in *mint* from Tirupati in southern parts of India.

III.4 PRESENT STUDY

Ten samples of spearmint collected from four different locations in North-West India separated by over 300 km were analyzed for 7 minor (Al, Mg, Ca, K, Na, P and Cl) and 20 trace (As, Au, Ba, Br, Co, Cr, Cs, Eu, Fe, Hf, Hg, La, Mn, Rb, Sb, Sc, Se, Sn, Th and Zn) elements by INAA and Cd, Ni & Pb by AAS.

Column chromatography and preparative TLC were used for the separation of chlorophyll b, menthol and 1,3-dihydro carveol in methanol extract, which were confirmed by elemental analysis, IR, NMR spectral and GC-MS studies.

From the diethyl ether extract following 10 compounds were identified by GC-MS;

- 2-(1-Methyethylidene) cyclohexanone;
- 2-Hydroxy 3-ethyl 2-cyclopenten-1-one;
- 4-Ethyl 1,3-benzenediol;
- 4-Acetyl 1-methyl cyclohexene;
- 2-Propyl 5-methoxy phenol,
- Carvone,
- Octahydro-1, 4,9,9-tetramethyl methanoazulene;
- 2-Chloro 1-ethyl 5-methoxy 3-methyl benzene;
- Dibutyl phthalate and
- Mono (2-ethyl hexyl ester) hexanedioic acid.

Antioxidant behaviour was studied for diethyl ether, dichloromethane and methanol extracts by DPPH free radical scavenging activity.

III.5 EXPERIMENTAL

(i) Sample collection and preparation: Ten samples of fresh *mint* leaves were collected from home gardens or purchased from the local vegetable shops in following cities within a time interval of 6 months during early 2004:

- Roorkee (n=4),
- Dehradun (n=2),
- Uttar Kashi (n=2) and
- Baghpat (n=2).

All the sampling locations shown in Fig. III.1 were at least 100 km away from each other. Dehradun, the capital city of Uttaranchal and Uttar Kashi are hilly areas while Roorkee and Baghpat are in the plains. Leaves were separated from the stems

and soaked in water to remove any dirt. Further, its surface contamination was wiped with tissue paper and left for air drying and then in an oven at < 80 ^{0}C. The samples were powdered in agate mortar and passed through 100-mesh sieve. Various RMs such as Apple Leaves (SRM-1515) from the NIST (USA) [46], Mixed Polished Herbs (MPH-2) from the INCT (Poland) [47] and Cabbage leaves (IAEA-359) from IAEA (Vienna) [48] were used as comparator standards and dried as per recommended procedure before use.

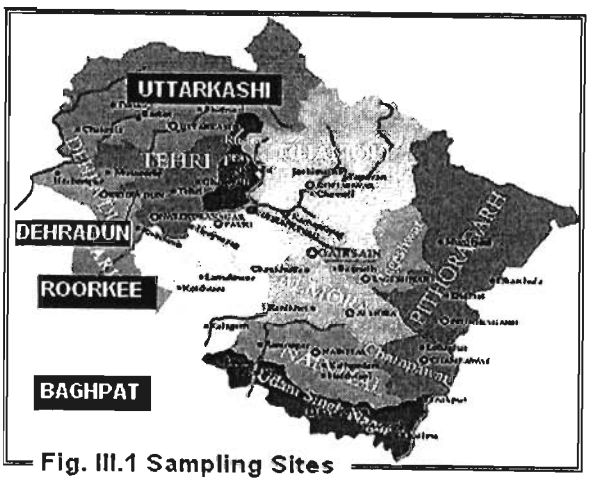


Fig. III.1 Sampling Sites

(ii) Irradiation and Counting: About 50 mg each of powdered samples and RMs were weighed accurately and packed in Alkathene or aluminum foil (Superwrap) for short (1 min) and long (3 d) irradiations respectively in CIRUS reactor at the BARC, Trombay, Mumbai, India, at a thermal neutron flux of ~10^{13} n cm^{-2} s^{-1}. Irradiation details are described in *Ch. II.5.* Details of counting set up are the same as mentioned in *Ch. II.7.* Irradiation and counting schedule followed and elements determined are given in Table II.2. Cd, Ni and Pb were determined by AAS as described in *Ch. II.9.* Elemental contents were calculated by comparator method using RMs as comparators.

(iii) Organic constituents. 50 g dried mint leaves were taken in Soxhlet extraction system in petroleum ether (40-60 ^{0}C) and extraction was carried out for 48 hrs. The extract was filtered where soluble part was colourless and insoluble part was greenish oily liquid. From the soluble portion, the solvent was distilled off and preparative TLC (20 cm×20 cm) was used to separate individual components using chloroform/ethyl acetate (3:2) solvent mixture. Two spots were observed, one of which had R_f = 0.83. It was scrapped from the plate and dissolved in chloroform. After filtration the solvent was distilled off whence a white crystalline compound was

obtained with melting pt. =187 ^{0}C. It was later confirmed as chlorophyll b. The insoluble portion was shaken thoroughly with methanol where it was found to be soluble. Then it was distilled on water bath. A white crystalline substance was collected in the distillation flask leaving behind the yellow volatile oil. The crude white crystalline compound thus obtained was subjected to column chromatography using petroleum ether/diethyl ether (50:1), petroleum ether/chloroform (10:1), chloroform/ethyl acetate (20:1), chloroform/methanol (10:1) and methanol/water (9:1) as eluting solvents. From the last fraction of methanol/water (9:1), a pure compound was obtained whose purity was checked by TLC using chloroform/ ethyl acetate/ water (9:9:1) whence R_f = 0.55 was found and m. pt. = 39 ^{0}C. It was later confirmed as menthol. The crude yellowish oil was subjected to preparative TLC (20 cm×20 cm) using chloroform/methanol (1:1) whence three well-defined bands corresponding to R_f = 0.79, 0.63 and 0.31 were observed. The first band at 0.79 was scrapped, dissolved in acetone, filtered and finally the acetone was distilled off. A pale yellow liquid was obtained which was later confirmed as 1,3-dihydro carveol. Other two fractions with R_f =0.63 and 0.61 could not be identified. Separation scheme of organic constituents is shown in Fig. III.2.

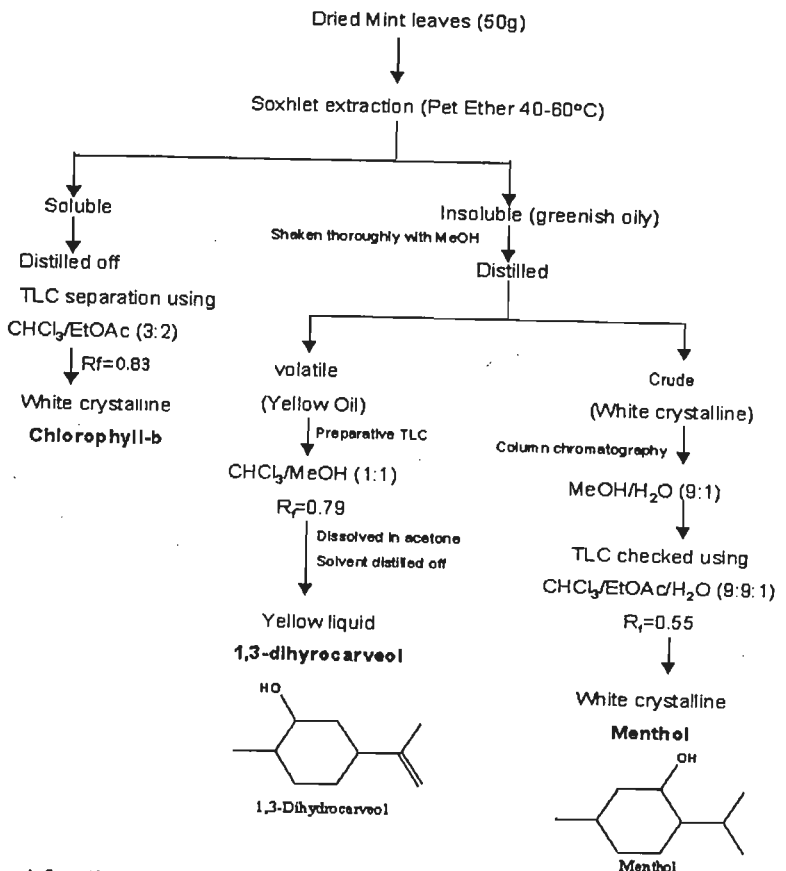


Fig. III.2 Flow sheet for the separation of menthol and 1,3-dihdrocarveol

Further more, GC-MS analysis of the diethyl ether extract was carried out whereby ten compounds: 2-(1-methyethylidene) cyclohexanone; 2-hydroxy 3-ethyl 2-cyclopenten-1-one; 4-ethyl 1,3-benzenediol; 4-acetyl 1-methyl cyclohexene; 2-propyl 5-methoxy phenol, carvone, octahydro-1, 4,9,9- tetramethyl methanoazulene; 2-chloro 1-ethyl 5-methoxy 3-methyl benzene; dibutyl phthalate and mono (2-ethyl hexyl ester) hexanedioic acid were identified.

(iv) DPPH Assay. The spectrophotometric assay of diethyl extract, ethanol and dichloromethane extract were carried out using purple coloured ethanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, which got bleached by the H atom or electron donation ability of the extract. Various concentrations of the extracts were added to 100μM ethanol solution of DPPH. After 30 min incubation period, absorbance was recorded at $\lambda_{max.}$ = 517 nm. These measurements were carried out at the Bio Organic Division (Dr. S. Chattopadhyay) of BARC, Mumbai. Inhibition of free radical in percent was calculated using the relationship:

$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction containing all reagents except the test compound, and A_{sample} is the absorbance of the test compound.

The concentration or inhibition capacity ($IC_{0.2000}$) of the test compound that induced a decrease of 0.20 in absorbance during the 30 min observation was taken as the free radical scavenging potency [49].

III.6 RESULTS

Mean Elemental concentrations in RM, Apple leaves (SRM-1515) along with its certified values, RSD, error (%) and Z-score are given in Table III.2. A comparison of the ranges and mean elemental concentrations of 30 elements including those determined by AAS in ten *mint* leaves samples collected from four different locations in North-West India is given in Table III.3. Range, median and mean elemental concentrations along with SD in *Mint* leaves (n=10) are given in Table III.4. A comparison of our data with those of others in literature is listed in Table III.5.

Table III.2 Elemental concentrations in Reference Materials for data validation

Element	Apple Leaves (NIST, SRM-1515)	Error (%)	RSD (%)	Z-score
Al (mg/g)	0.26 ±0.02 (0.29±0.01)	-10.3	7.69	-1.5
As (μg/g)	0.41± 0.05 (0.38±0.006)	7.89	7.89	0.6
Ba (μg/g)	55.0±5.0 (49.0±2.0)	12.2	9.09	1.2
Br (μg/g)	1.66± 0.05 [1.8]	-7.77	3.01	-
Ca (mg/g)	15.5± 1.0 (15.3±0.2)	1.31	6.45	0.2
Cl (mg/g)	0.58±0.04 (0.579±0.023)	1.73	6.90	0.03
Co (ng/g)	85.5± 7.5 [90]	-5.0	8.74	-
Cr (μg/g)	0.35± 0.02 (0.30)	16.7	5.71	2.5
Cs (ng/g)	201 ± 16 [187]	7.49	7.96	-
Cu (μg/g)	5.58± 0.28 (5.64±0.24)	-1.06	5.02	-0.21
Fe (μg/g)	77±7 (83±5)	-7.22	9.09	-0.86
Hg (ng/g)	47.7± 2.7 (44±4)	8.41	5.66	1.37
K (mg/g)	15.8± 1.3 (16.1±0.2)	-1.86	8.22	-0.23
La (μg/g)	18.2± 0.05 [20]	9.0	0.27	-
Mg(mg/g)	2.75±0.25 (2.71±0.08)	1.48	9.09	0.16
Mn (μg/g)	49.2 ± 2.2 (54 ± 3)	-8.88	4.47	-2.18
Na (mg/g)	26.0± 2.4 (24.4±1.2)	6.55	9.23	0.67
P (mg/g)	1.60± 0.07 (1.59±0.11)	0.63	4.38	0.14
Rb (μg/g)	10.1± 0.2 (10.2±1.5)	-0.98	1.98	-0.50
Sb (ng/g)	14.1±2.0 [13]	8.46	14.2	-
Sc (ng/g)	35.0±3.1 [30]	16.6	8.86	-
Th (ng/g)	33.0±3.0 [30]	10.0	9.09	-
Zn (μg/g)	12.8± 0.1 (12.5±0.3)	2.4	0.78	3.0

Table III. 3 Range and mean elemental concentrations in *Mint* leaves from different locations.

Element	Roorkee (n=4)		Dehradun (n=2)		Baghpat (n=2)		Uttarkashi (n=2)	
	Range	Mean± SD	Range	Mean± SD	Range	Mean± SD	Range	Mean± SD
Al (mg/g)	0.80-0.94	0.65±0.22	0.22-0.24	0.23±0.01	0.98-1.12	1.05±0.07	0.25-0.29	0.27±0.02
As (ng/g)	306-320	313±7	98-250	174±76	-	<20	144-167	156 ±12
Au (ng/g)	13.9-15.7	14.8±0.9	1.5-3.7	2.6±1.1	-	<0.5	1.7-1.9	1.8±0.1
Ba (µg/g)	30.2-42.9	37.3±4.6	25.9-30.5	28.2±2.3	50.7-55.3	53.0±2.3	18.4-20.2	19.3±0.9
Br (µg/g)	3.26-3.50	3.38±0.12	4.74-5.24	4.99±0.25	-	<0.10	1.41-1.43	1.42±0.01
Ca (mg/g)	11.0-15.3	12.8±1.7	15.5-16.8	16.2±0.7	5.82-8.95	7.39±1.57	11.1-14.7	12.9±1.8
Cd (ng/g)	199	199	15-275	213±63	-	<15	450-772	611±11
Cl (mg/g)	4.77-8.14	6.10±1.36	6.67-10.0	8.34±1.67	7.55-7.91	7.73±0.18	9.88-10.5	10.2±0.31
Co (ng/g)	69-151	114±32	85-99	92±7	86-93	90±4	70-92	81±11
Cr (µg/g)	1.27-1.60	1.42±0.15	1.10-1.30	1.20±0.10	1.70-1.71	1.71±0.05	0.99-1.21	1.10±0.11
Cs (ng/g)	131-215	168±37	129-152	141±12	252-282	267±15	44.7-44.8	44.8±0.1
Cu (µg/g)	15.7-18.0	16.5±1.1	17.2-20.2	18.7±1.5	16.8-18.5	17.7±0.9	13.8-16.7	15.3±1.5
Eu (ng/g)	14.7-48.5	28.6±12.3	40.8-64.8	52.8±12.0	25.2-27.9	26.6±1.4	44.1-45.5	4.8±0.1
Fe (µg/g)	86.9-138	103±21	120-139	130±10	88.9-100	94.4±5.5	101-129	115±14
Hf (ng/g)	125-192	167±32	143-183	163±20	110-210	160±50	173-201	187±14
Hg (ng/g)	131-983	410±338	93-115	104±11	101-186	144±43	97-114	106±9
K (mg/g)	16.1-53.3	28.5±14.6	12.4-14.0	13.2±0.8	25.9-29.1	27.5±1.6	13.2-25.5	19.4±6.2
La (µg/g)	1.32-1.71	1.51±0.14	1.38-1.84	1.61±0.23	1.47-1.65	1.56±0.10	1.13-1.41	1.27±0.14
Mg (mg/g)	4.63-6.10	5.51±0.58	3.90-5.15	4.53±0.63	4.53-5.43	4.98±0.45	3.49-3.75	3.62±0.11
Mn (µg/g)	46.1-69.6	57.5±8.7	37.0-57.4	47.2±10.2	48.0-63.7	55.1±7.9	48.0-51.2	49.6±1.6
Na (mg/g)	0.41-0.86	0.64±0.17	0.36-0.43	0.40±0.04	0.51-0.53	0.52±0.01	0.21-0.25	0.23±0.02
Ni (µg/g)	1.17	1.17	3.22	3.22	<0.2	<0.2	0.37-2.82	1.90±1.17
P (mg/g)	3.20-3.42	3.31±0.11	4.46-4.61	4.54±0.04	2.48-2.88	2.68±0.20	4.86-5.12	4.99±0.13
Rb (µg/g)	11.1-30.4	23.1±7.4	21.9-29.3	25.6±3.7	16.0-16.4	30.7±1.15	29.5-31.8	16.2±0.2
Sb (ng/g)	23.5-315	138±110	15.8-27.2	21.5±5.7	178-282	240±52	12.1-19.6	15.9±3.8
Sc (ng/g)	42-139	69.3±40.4	34-42	38±4	55-62	59±4	29-36	33±4
Se (ng/g)	127-197	162±35	-	<100	187-195	191±4	-	<100
Sn (ng/g)	153-184	168±15	172-191	182±10	-	<30	147-172	160±13
Th (ng/g)	44-74	58.3±11.1	39-50	44.5±5.5	110-193	152±42	31-37	34±3
Zn (µg/g)	16.0-24.3	19.9±3.5	22.5-28.4	25.5±3.0	14.8-15.8	15.3±0.5	23.2-25.4	24.3±1.1

NOTE: Pb could not be detected as it was below detection limit of 60 ng/g

Table III. 4 Range, median and mean elemental concentrations in *Mint* leaves (n=10)

Elements	***Range***	***Median±SD***	***Mean±SD***
Al (mg/g)	0.22-1.12	0.49±0.26	0.57±0.35
As (ng/g)	98-320	186±65	196±89
Au (ng/g)	1.5-15.7	6.7±4.2	4.9±6.1
Ba (μg/g)	18.4-55.3	39.6±10.8	35.0±12.3
Br (μg/g)	1.41-5.24	2.98±1.12	3.26±1.80
Ca (mg/g)	5.82-16.8	11.7±3.21	12.4±3.49
Cd (ng/g)	15-772	297±222	369±252
Cl (mg/g)	4.77-10.5	6.98±1.68	7.69±2.02
Co (ng/g)	69-151	86.3±4.0	97.9±26.2
Cr (μg/g)	0.99-1.71	1.20±0.21	1.37±0.25
Cs (ng/g)	44.7-282	1132±69	157±78
Cu (μg/g)	13.8-20.2	15.2±1.87	16.9±1.80
Eu (ng/g)	14.7-64.8	30.5±14.7	36.3±14.9
Fe (μg/g)	86.9-139	103±15	108±22
Hf (ng/g)	110-210	142±29	178±22
Hg (ng/g)	93-983	210±261	235±272
K (mg/g)	12.4-53.3	21.6±12.0	23.4±12.1
La (μg/g)	1.32-1.84	1.47±0.15	1.49±0.20
Mg (mg/g)	3.90-6.10	4.70±0.64	4.83±0.92
Mn (μg/g)	37.0-69.6	49.2±9.54	53.5±9.6
Na (mg/g)	0.21-0.86	0.43±0.19	0.48±0.20
Ni (μg/g)	0.37-3.22	1.38±0.83	1.90±1.35
P (mg/g)	2.48-5.12	3.02±0.77	3.88±0.94
Rb (μg/g)	11.1-31.8	21.8±6.06	23.7±7.18
Sb (ng/g)	12.1-315	95.3±91.6	109±115
Sc (ng/g)	29-139	51.7±32.2	54.0±32.0
Se (ng/g)	127-197	169.3±20.5	177±33.2
Sn (ng/g)	147-191	186±12.9	173±17
Th (ng/g)	31-193	81.0±47.4	69.3±49.1
Zn (μg/g)	14.8-25.4	18.2±3.10	21.0±4.70

Table III.5 Comparison of data with literature

Elements	Present Work	Zaidi et al. (2004) [44]	Gopalan et al. (1999) [7]	Balaji et al. (2000) [45]
Al (mg/g)	0.57±0.35	-	-	0.40±0.03
Ba (μg/g)	35.0±12.3	61.4±5.2	-	-
Br(μg/g)	3.26±1.80	6.20±0.41	-	6.5±0.4
Ca (mg/g)	12.4±3.5	-	2.0	16.5±0.3
Cl (mg/g)	7.69±2.02	2.18±0.10	0.34	14.2±0.1
Co(ng/g)	97.9±26.2	110±70	-	-
Cr(μg/g)	1.37±0.25	1.68±0.08	0.8	-
Cs(ng/g)	157.1±77.9	130±10	-	-
Eu(ng/g)	36.3±14.9	52±4	-	-
Fe(μg/g)	108±22	861±44	160	-
Hf(ng/g)	178±22	62±3	-	-
Hg(ng/g)	235±272	12.0±2.0	-	-
K(mg/g)	23.4±12.1	1.97±0.07	57.0	24.9±3.2
Mg (mg/g)	4.83±0.92	-	0.60	8.5±0.6
Mn(μg/g)	53.5±9.6	51.1±2.6	5.7	50.2±4.4
Na(mg/g)	0.48±0.20	0.67±0.03	-	1.11±0.08
Rb(μg/g)	23.7±7.18	3.15±0.24	-	-
Sb(ng/g)	109±115	60.0±4.0	-	-
Sc(ng/g)	54.0±32.0	280±30	-	-
Se(ng/g)	177±33.2	4580±300	-	-
Th(ng/g)	69.3±49.1	260±30	-	-
Zn(μg/g)	21.0±4.70	169±8.6	44.0	-

III.7 ELEMENTAL CONTENTS

Data in Table III.2 match well within ±10% of the certified values for most elements with exceptions of Cr, Cs, Fe, Sc and Th and standard deviations were <10% suggesting a high order of precision. Therefore, it is presumed that elemental concentrations in *mint* leaves reported in this study should be accurate and precise within ±10%. Z-score plot for Apple leaves (SRM-1515) is shown in Fig. III.3.For most elements Z-score lie between ±3 further emphasizing that our values are correct within 99% confidence limit.

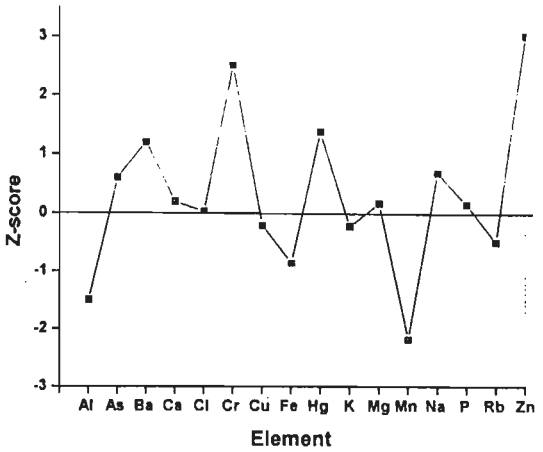


Fig. III.3 Z-score plot of elements in Apple leaves (SRM-1515)

A perusal of elemental data in Table III.3 shows that mean elemental contents in mint leaves from one location to another vary in a small range for most elements (Ca, Cl, Cr, Cu, Fe, K, Mg, Mn, Na, P, K, Se and Zn) whereas for others these are in a wider range. It is observed that mean values of four different locations do not vary significantly and their corresponding SD values are very small suggesting not much variation within a small area where soil characteristics do not change significantly. However, SD values for n=10 (Table III.4) are large suggesting significant variation due to difference in soil characteristics from 4 different regions which are > 100 km away from each other.

It is observed that *mint* is enriched in several essential elements such as Ca (12.4±3.49 mg/g), Mg (4.83±0.92 mg/g), K (23.4±12.1 mg/g), P (3.88±0.94 mg/g) and to a lesser extent in Na (0.48±0.20 mg/g) and Fe (108±22 μg/g). Mint leaves from Roorkee are especially enriched in K (28.5±14.6 mg/g), Mg (5.51 ±0.58 mg/g), and Se (162±35 ng/g) whereas those from Dehradun are enriched in Ca (16.2±0.7 mg/g), Fe (130±10 μg/g) and Zn (25.5±3.0 μg/g). Other micronutrients viz. Mn (53.5±9.6 μg/g), Zn (21.0±4.70 μg/g), Cu (16.9±1.80 μg/g) and Cr (1.37±0.25 μg/g) from

various locations vary in a small range as evident from SD values suggesting independence from the soil characteristics and geo-environmental conditions within a small region. Another structural element phosphorus was also found in a comparable range, 2.48-5.12 mg/g in all the samples. Elemental profiles showing variation of essential and trace element concentrations in *mint* leaves from different locations are illustrated in Figs. III.4 and 5 respectively. It is observed that most elemental contents in *mint* leaves exhibit small variations with very little effect of geo-environmental factors though Mn, Fe, Cu, and Zn are found at μg/g level and Co at ng/g level only. Incidentally samples from Dehradun and Uttarkashi, two hilly areas, exhibit depleted amounts of K, Mg, Na, Cr and Se. In view of the antioxidant properties of Mn (II), Fe (II) and Zn (II) [50, 51], their elemental contents are of special importance.

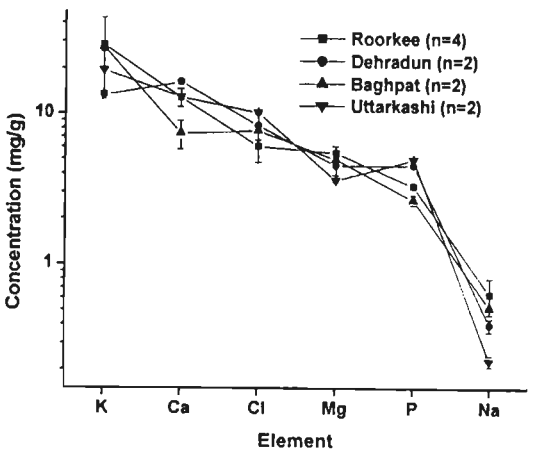


Fig III.4 Concentration profile of minor essential elements in Mint leaves

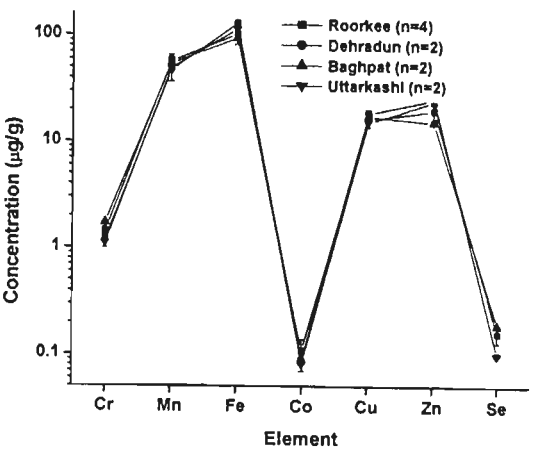


Fig III.5 Concentration profile of essential trace elements in Mint leaves

The toxic heavy metals like Hg (93-983 ng/g), Sb (12.1-315 ng/g), Ni (0.37-3.22 ng/g), Cd (15-772 ng/g) and As (98-320 ng/g) are all found at ng/g level only but vary by more than an order of magnitude. This is essentially due to variation in environmental factors. Leaves from Roorkee show much higher amounts of As (313±7 ng/g), Hg (410±338 ng/g) and Cd (199 ng/g) whereas Ni content (3.22 ng/g) is higher in Dehradun. This may possibly be attributed to the fact that both Roorkee and Dehradun (capital city of Uttaranchal state) are relatively urbanized townships where these pollutant elements could originate from industrial emissions and anthropogenic activities [52]. Not surprisingly, Uttarkashi, a hilly town having no industrial activity shows much lower concentrations of Br (1.42±0.01 μg/g) and Ni

(1.90±1.17 ng/g). On the other hand leaves from Baghpat, a town near Delhi the capital and a mega city of India show higher amounts of Cs (267±15 ng/g), Hf (160±50 ng/g) and Th (152±42 ng/g). However, Pb was found below the detection limit of 60 ng/g possibly because the samples were collected from home gardens where least vehicular emissions exist. All these toxic elements are below the permissible limits specified by the US FDA and hence *mint* leaves are safe to eat. Variation of toxic elemental contents in Mint leaves is shown in Fig. III. 6.

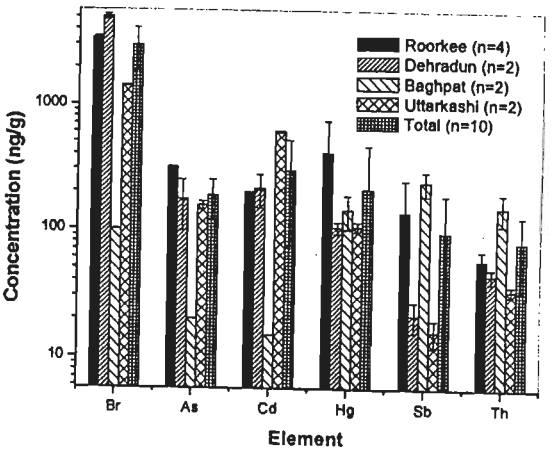


Fig III.6 Concentration of toxic elements in Mint leaves

A comparison of mean and median values in Table III.4 shows striking similarity for most minor and trace elements. However for toxic elements such as Cd, Hg, Sb, Th etc., which are environmental contaminants, these are widely different.

III.8 ELEMENTAL CORRELATIONS

There exists a strong inverse correlation between Na & Mg with Cl as shown in Figs. III.7 and 8 with r = -0.95 and -0.97 respectively. Similarly Cr and Zn, the two essential elements well known for their role in biochemical processes, are also inversely correlated (Fig. III.9), with r = -0.91.This is interesting because the

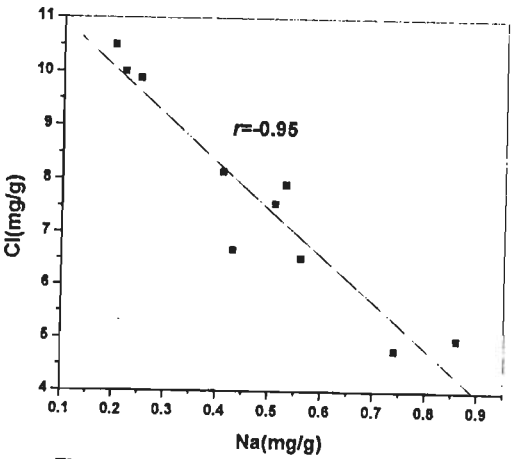


Fig. III.7 Correlation of Cl with Na in *Mint leaves*

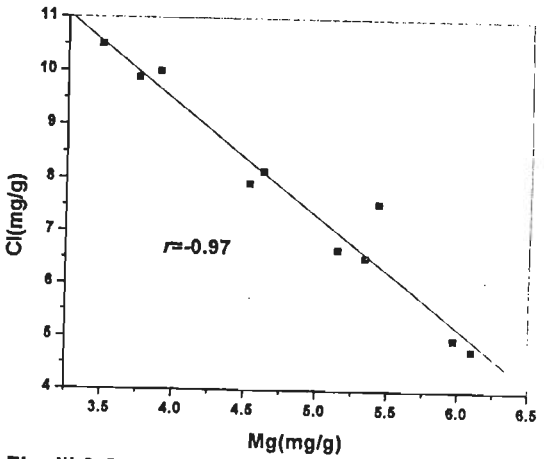


Fig. III.8 Correlation of Cl with Mg in Mint leaves

availability of Zn in the range of 14.8-28.4 μg/g may be beneficial for diabetic patients as its deficiency has been correlated with acute and chronic mal absorption states [53, 54]. Cr (III) may be bound with glycine, cysteine and glutamic acids to form complex molecules called glucose tolerance factor [55].

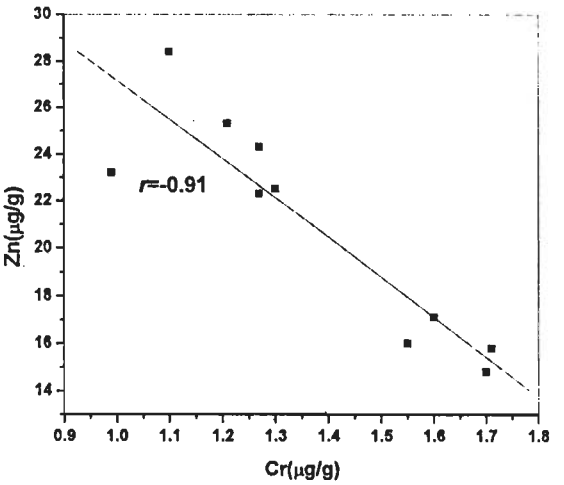


Fig.III.9 Correlation of Zn with Cr in Mint leaves

Similarly, small amounts of Se (177±33.2 ng/g) may be responsible for its anticancer properties because Se as glutathione peroxidase inhibits the replication of tumour virus and prevents the malignant transformation of cells [56]. Promising antimutagenic and anticarcinogenic potential of this herb may possibly be due to potential bioavailability of these elements. K/Na and K/P ratios in *mint* leaves collected from 4 different locations are bar plotted in Figs. III. 10 and 11 respectively. K/Na in four different locations varies by a factor of 3 while K/P varies in a much wider range of 2 to 10. In both cases, *mint* leaves from Dehradun show the lowest ratio. This is essentially due to change in geo-environmental factors at these four places.

Mint leaves have been analyzed by several workers from different parts of the country as well as from neighbouring Pakistan [7, 43,44]. Therefore, we compared

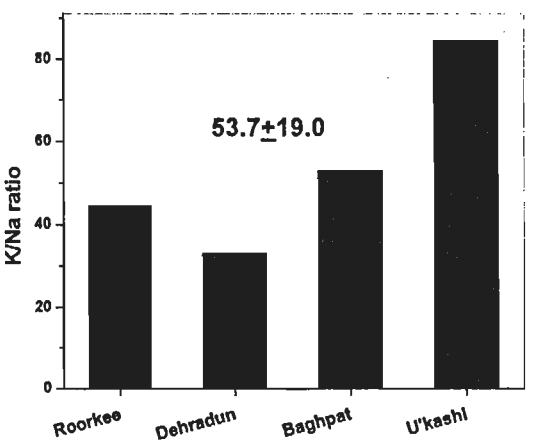


Fig III.10 K/Na ratio in Mint leaves from different locations

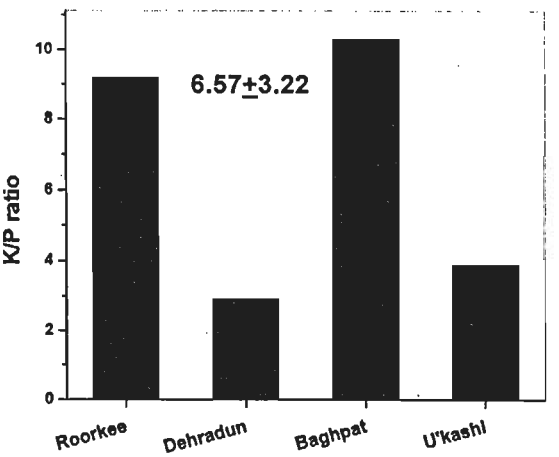


Fig. III.11 K/P ratio in Mint leaves from different locations

our data with that in literature (Table III.5). Our data for Na, Co, Cr, Cs and Eu are in excellent agreement with those reported by Zaidi et al. [44] whereas Al, K and Mn match with those of Balaji et al [43]. Concentration of Fe (108±22 μg/g) matches well with that reported in the compilation by Gopalan et al. (156 μg/g) [7] though Zaidi et al. [44] reported a substantially higher value (861± 44 μg/g). It should be noted that most other analyses are on the basis of one or two samples whereas we have analyzed 10 samples collected from 4 different locations. On the basis of this comparison, it can be inferred that our data for other elements should be reliable though others have not reported it.

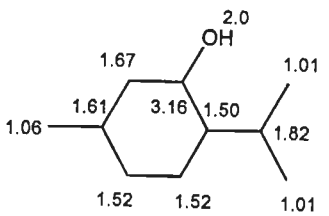
III. 9 ORGANIC CONSTITUENTS

Besides methanolic extract where three compounds were elucidated, diethyl ether extract was also analyzed by GC-MS

(i) Methanolic extract: In Fig. III.2 is shown column chromatographic and preparative TLC separations of chlorophyll b, menthol and 1,3-dihydro carveol. The structures were elucidated on basis of elemental analysis, IR, NMR [57,58] and GC-MS spectral studies [59].

A. Chlorophyll b: It was obtained as a white crystalline compound and characterized by CHN analysis C (%): 62.10, H (%): 14.14, N (%): 9.21, ir spectra in KBr (cm^{-1}): 3435 (ν_{N-H}), 2925 (ν_{C-H}), 2134 (ν_{C-N}), 1735 ($\nu_{C=O}$); visible (methanol) in nm: 412, 660 and functional group analysis for unsaturation and aldehyde.

B. Menthol (*l*): White crystalline, CH analysis, C (%): 76.18 (76.9), H (%): 13.2 (12.8), IR in KBr (cm^{-1}): 3265 (ν_{O-H}), 2928 and 2870(ν_{C-H}), 1630 ($\nu_{C=C}$), 1458 (δ_{O-H}), 1040 (ν_{C-O-C}); ^1H NMR in DMSO (δ): 1.06(d, 3H), 1.01(m, 3H), 1.50(m, 1H), 1.61(m, 1H), 1.82(m, 1H), 2.0(s, 1H), 3.16(q, 2H), 1.52(m, 2H), 1.67(q, 2H); GC-MS (m/z): 138 ($C_{10}H_{18}^+$), 123 ($C_9H_{15}^+$), 109 ($C_8H_{13}^+$), 95 ($C_7H_{11}^+$), 81 ($C_6H_9^+$), 55 ($C_4H_7^+$). Mass spectrum and possible mechanism of fragmentation pattern are shown in Fig. III.12



Menthol (*l*)

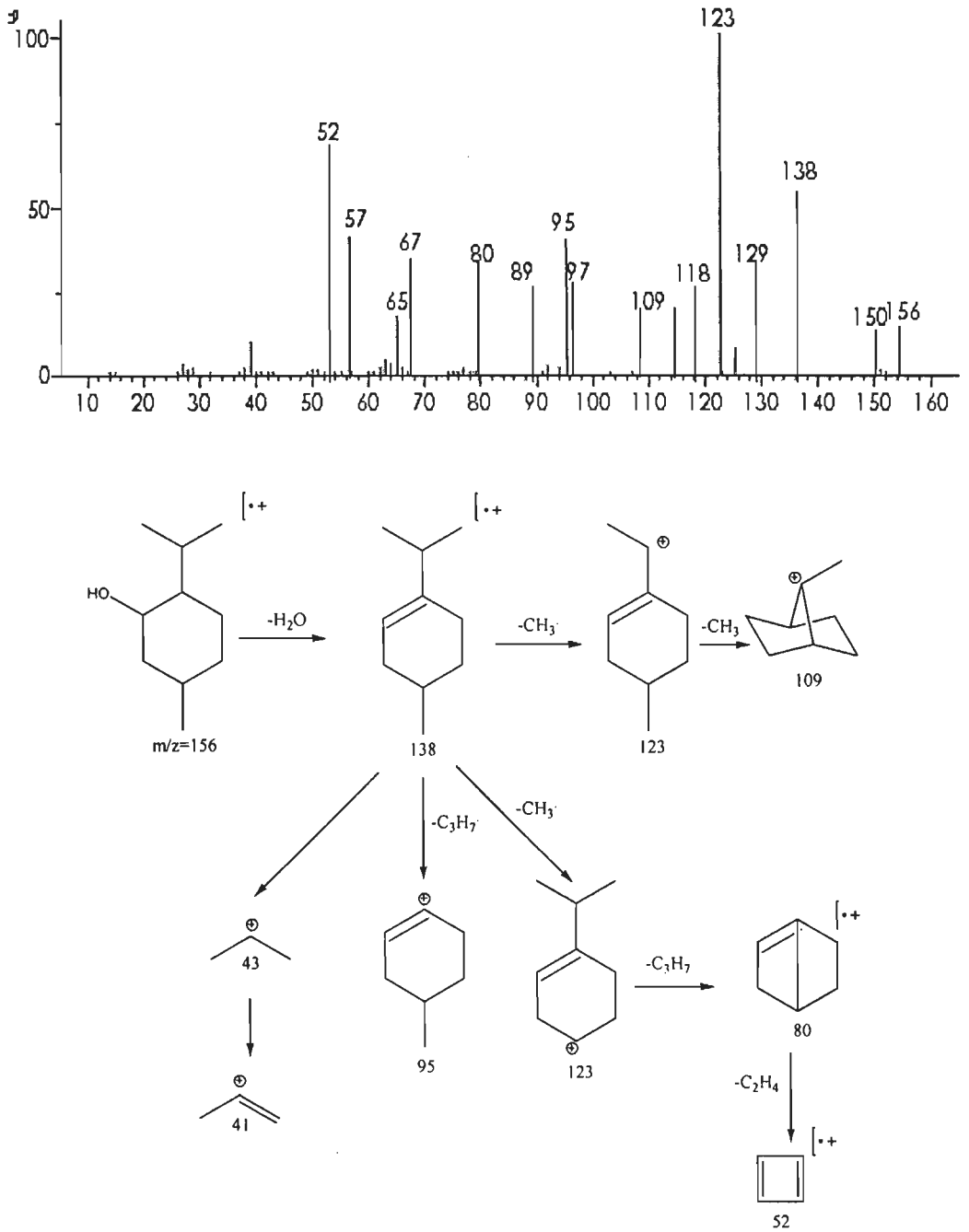


Fig. III.12 Mass spectrum and fragmentation pattern of menthol (*I*)

C. 1,3-dihydrocarveol (*II*): Yellowish oil, CH analysis; C (%): 78.10(78.9), H (%): 11.1(10.5); uv (methanol) in nm: 216, 258, 312; IR in KBr (cm^{-1}): 3444 (ν_{O-H}), 1638 ($\nu_{C=C}$), 1397 (δ_{O-H}), 1021(ν_{C-O-C}); ^1H NMR in D_2O (δ): 1.71(s, 3H), 1.06 (d, 3H), 1.69 (m, 1H), 2.12 (s, 1H), 3.16 (m, IH), 2.0 (S, 1H), 4.66 (D, 1H), 1.72 (Q, 2H), 1.57 (M, 2H), 1.52 (M, 2H). ^1H NMR spectrum matched with Aldrich library

[58]; GC-MS (m/z): 152 ($C_{10}H_{16}O^+$), 134($C_{10}H_{14}^+$), 119($C_9H_{11}^+$), 92($C_7H_8^+$). Mass spectrum matched well with that of NIST 2.0 mass spectral database [59]. Mass fragmentation and the proposed mechanism explaining all the fragmentation peaks is shown in Fig. III.13.

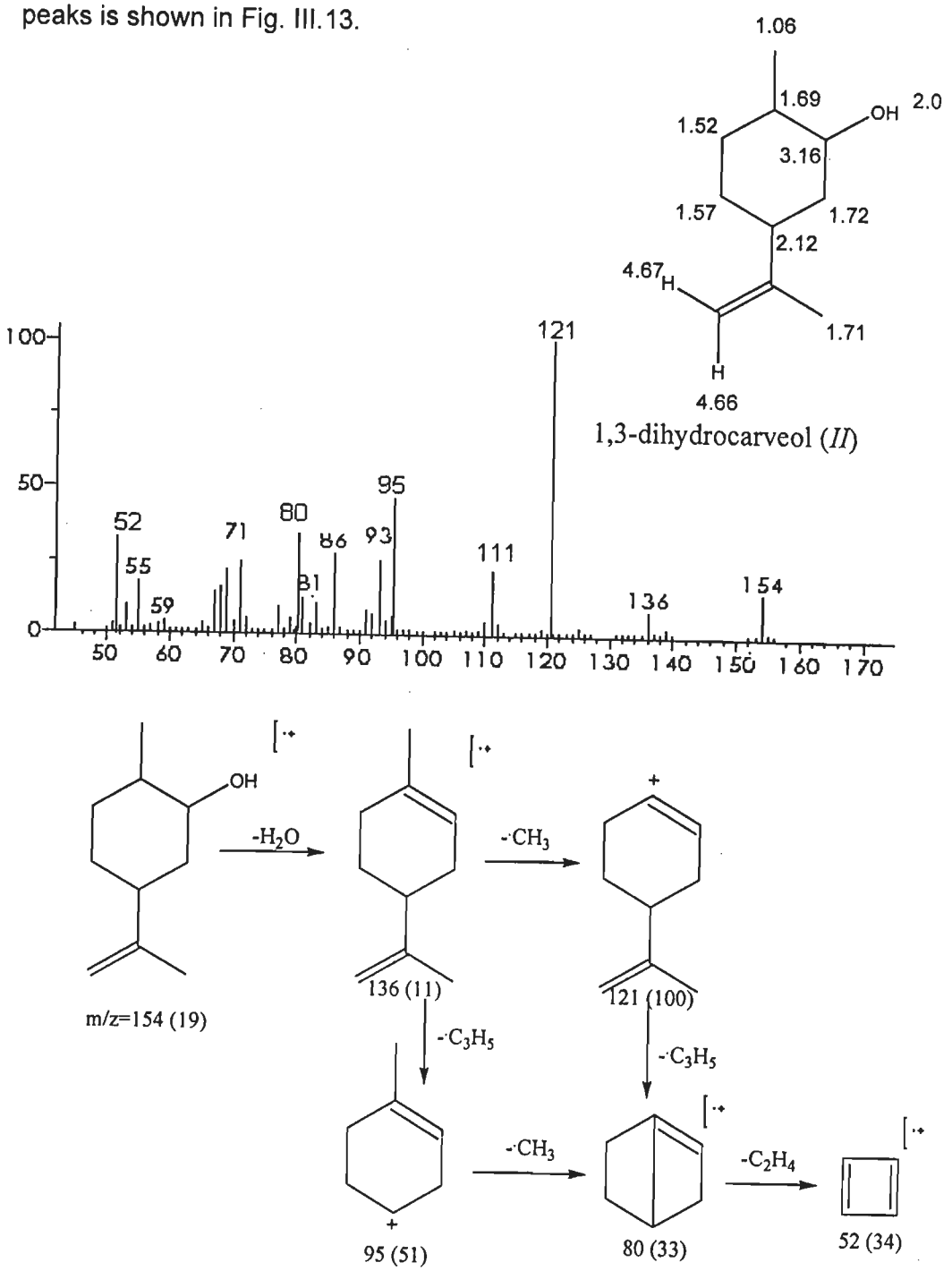


Fig.III.13 Mass spectrum and fragmentation pattern of 1,3-dihydrocarveol (*II*)

(ii) Diethyl ether extract: In order to identify the organic constituents responsible for the antioxidant property of the diethyl ether extract, it was subjected to GC-MS

studies whereby following 10 compounds were identified by comparing their individual spectra with those of NIST 2.0 mass spectral database [59]; 2-(1-methyethylidene) cyclohexanone (*III*); 2-hydroxy 3-ethyl 2-cyclopenten-1-one (*IV*); 4-ethyl 1,3-benzenediol (*V*); 4-acetyl 1-methyl cyclohexene (*VI*); 2-propyl 5-methoxy phenol (*VII*), carvone (*VIII*), octahydro-1, 4,9,9-tetramethyl methanoazulene (*IX*); 2-chloro 1-ethyl 5-methoxy 3-methyl benzene (*X*); dibutyl phthalate (*XI*) and mono (2-ethyl hexyl ester) hexanedioic acid (*XII*). The compounds along with R_t, base peak, molecular ion peak and other prominent peaks are listed in Table III. 6. Typical fragmentation pattern of a few are shown in Figs. III. 14-21 respectively.

Table III.6 Organic constituents identified from the diethyl extract by GC-MS

S/No.	Compound	R_t (min)	Base Peak	Molecular wt.	Characteristic m/z
(III)	Cyclohexanone, 2-(1-methylethylidene)	8.69	123	138	95,67
(IV)	2-hydroxy 3-ethyl 2-cyclopenten-1-one	9.99	126	126	111,84,82,97,70,69
(v)	4-ethyl 1,3-benzenediol	11.12	123	138	109,91,67
(vi)	4-acetyl 1-methyl cyclohexene	12.22	123	138	108,95
(vii)	2-propyl 5-methoxy phenol	12.73	166	166	151,137,123,105,77
(viii)	Carvone	13.83	135	150	109,94,54
(ix)	Octahydro 1,4,9,9 methanoazulene	14.29	149	278	121, 104, 76, 65
(x)	2-chloro 1-ethyl 5-methoxy 3-methyl benzene	14.69	184	184	186,169, 155, 153, 149
(xi)	Dibutyl phthalate	18.79	148	278	177, 130, 77
(xii)	Mono (2-ethyl hexyl ester)	19.17	129	258	147,112,71,70,57

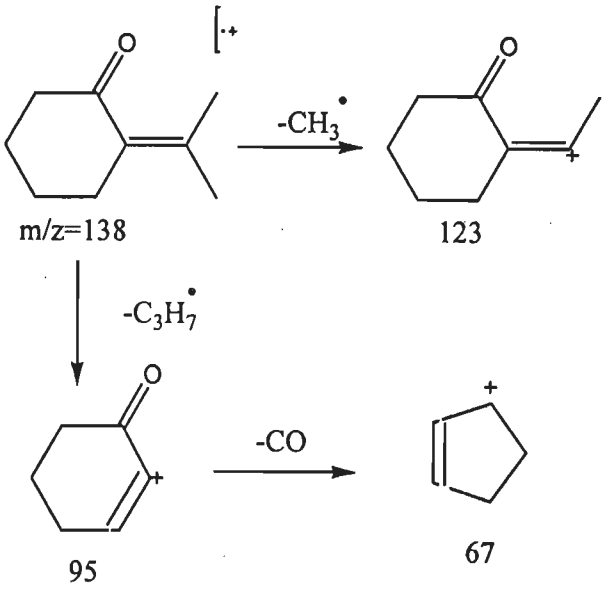


Fig.III.14 Mass fragmentation pattern of 2-(1-methyethylidene) cyclohexanone (*III*)

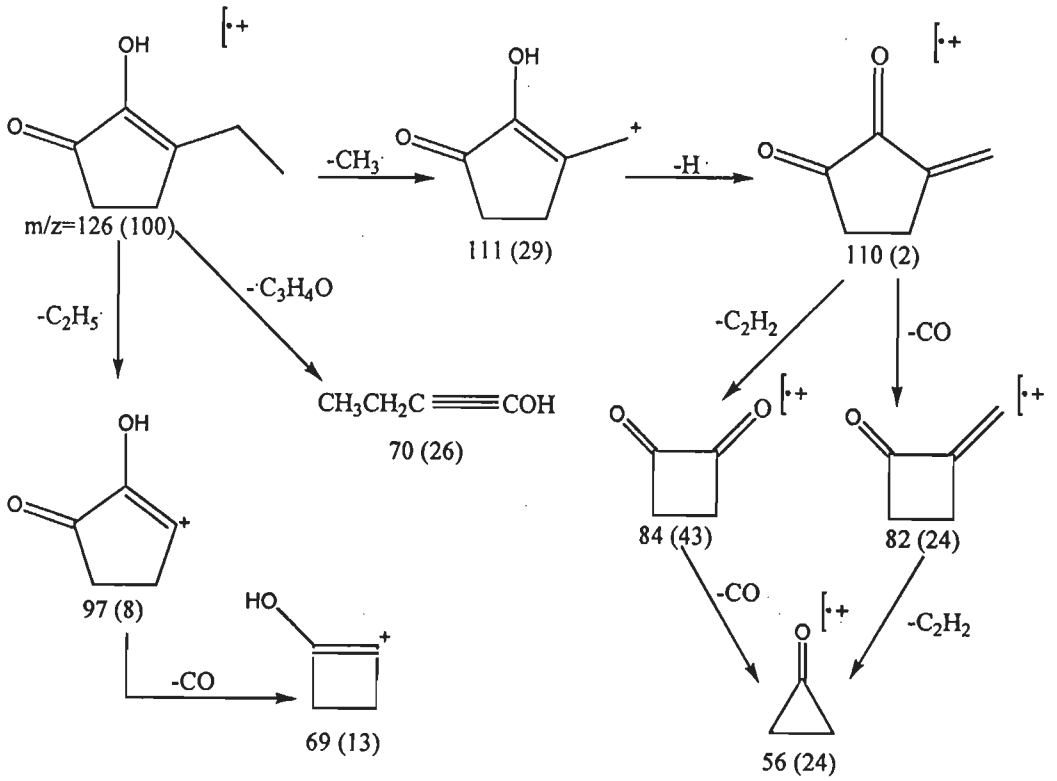


Fig.III.15 Mass fragmentation pattern of 2-hydroxy 3-ethyl 2-cyclopenten-1-one (*IV*)

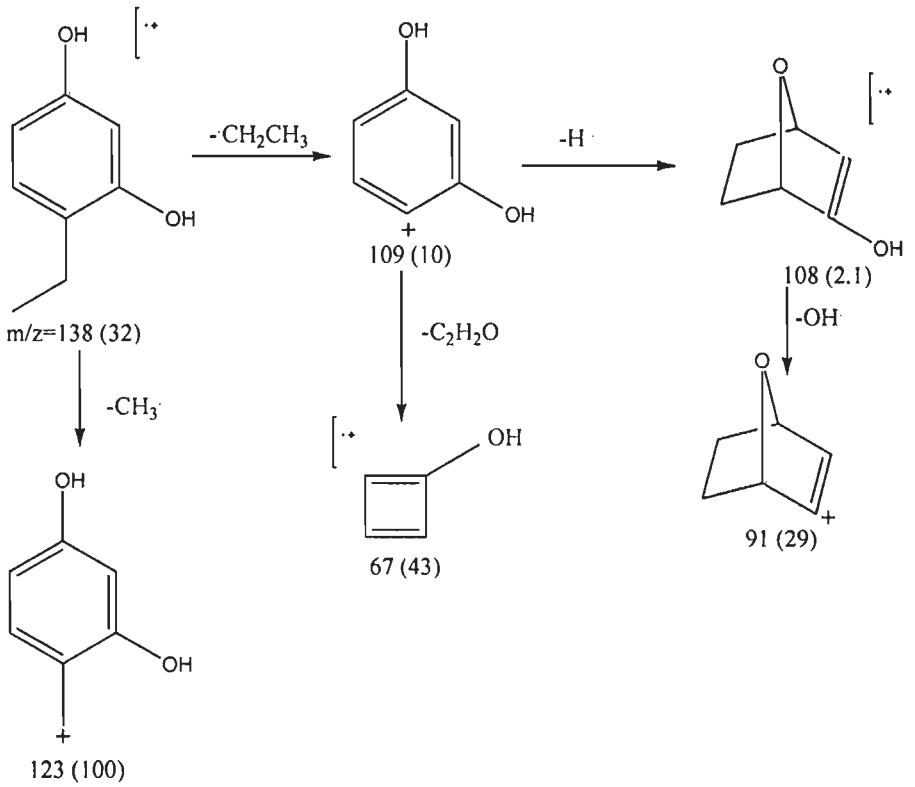


Fig.III.16 Mass fragmentation pattern of 4-ethyl 1,3-benzenediol (*V*)

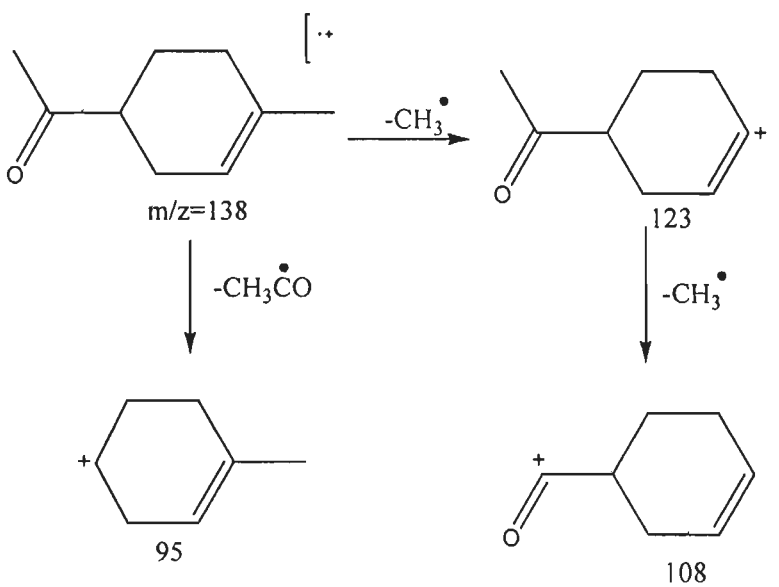


Fig.III.17 Mass fragmentation pattern of 4-acetyl 1-methyl cyclohexene (*VI*)

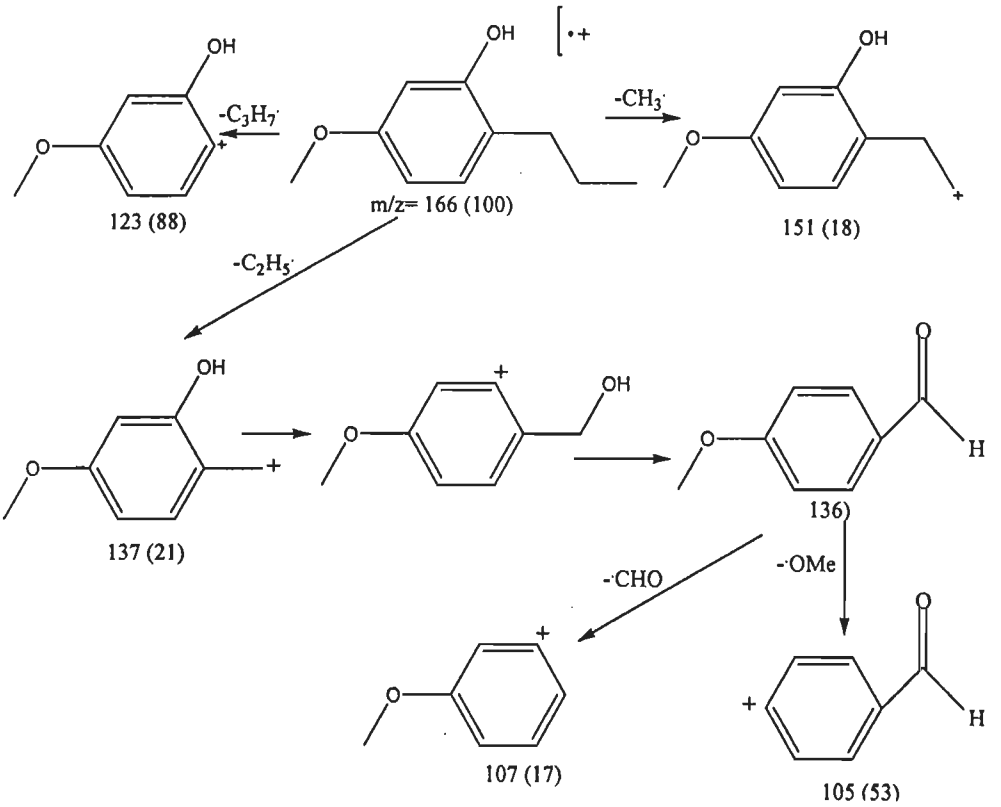


Fig.III.18 Mass fragmentation pattern of 2-propyl 5-methoxy phenol (***VII***)

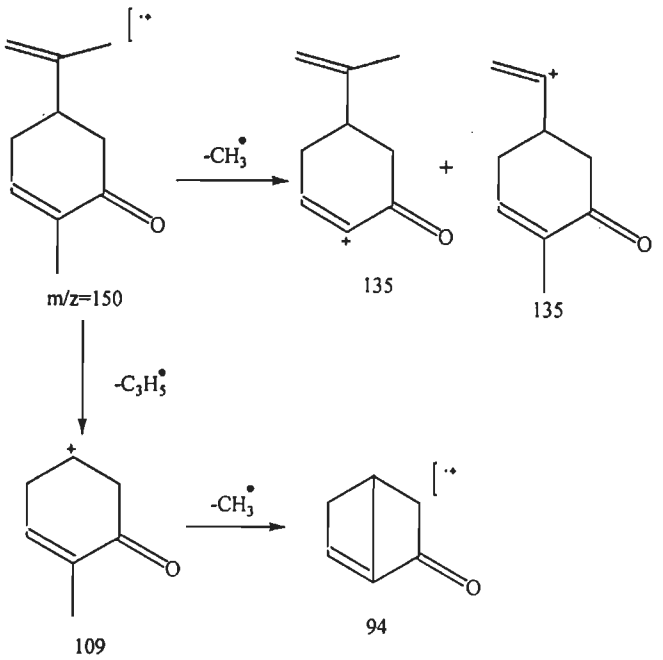


Fig.III.19 Mass fragmentation pattern of carvone (***VIII***)

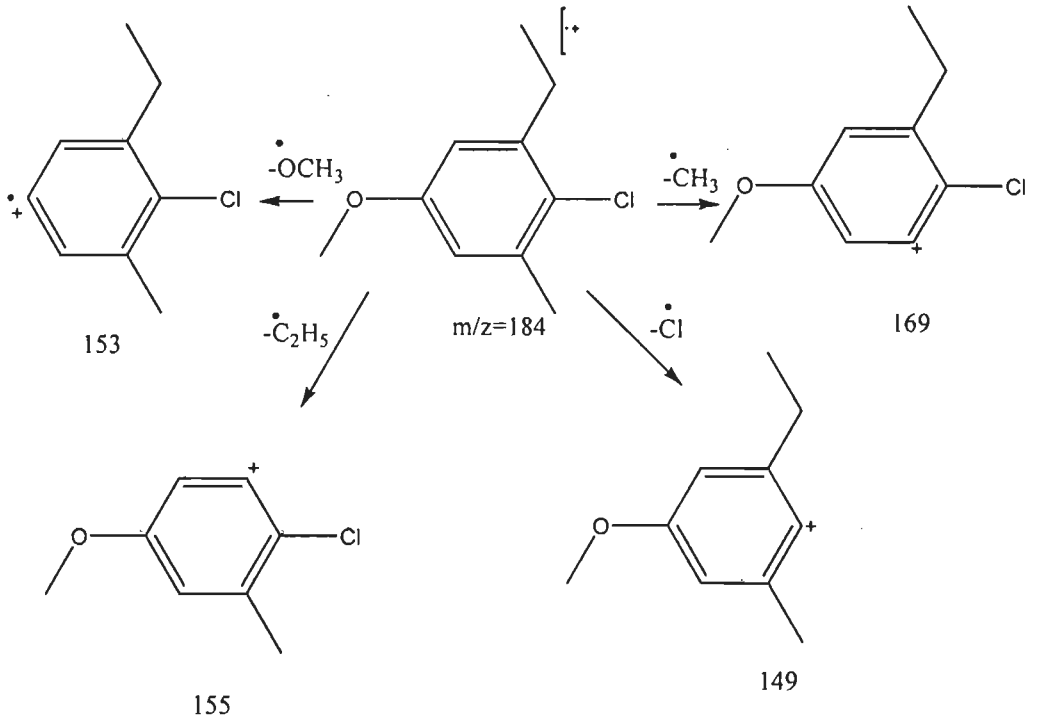


Fig.III.20 Mass fragmentation pattern of 2-chloro 1-ethyl 5-methoxy 3-methyl benzene (***X***)

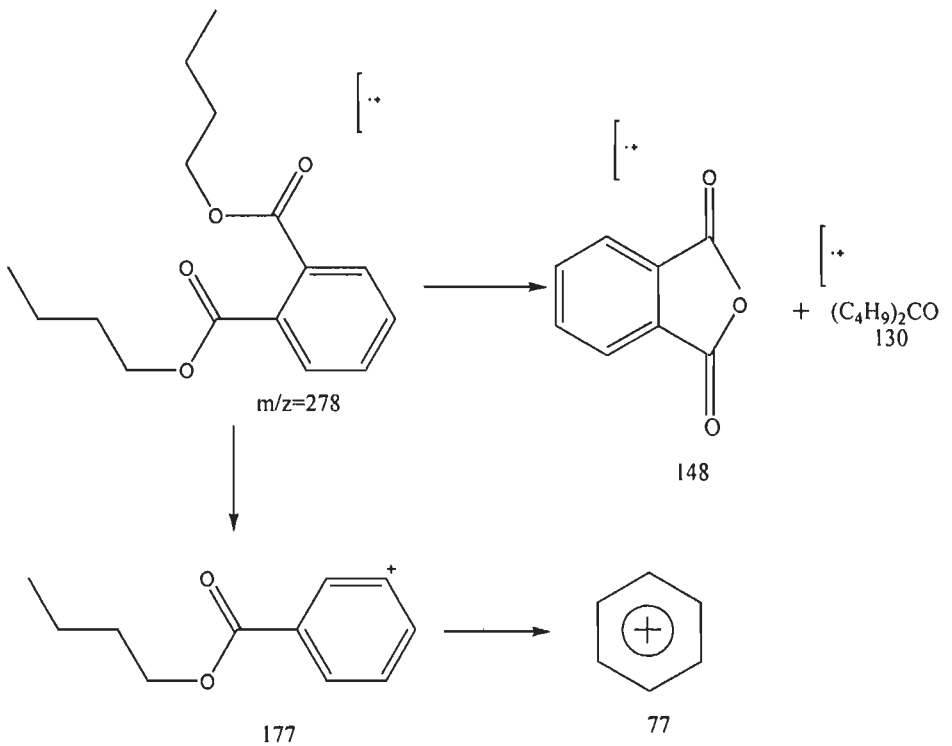


Fig.III.21 Mass fragmentation pattern of dibutyl phthalate (***XI***)

Thus alcoholic and phenolic groups present in all these compounds are likely to be responsible for the free radical scavenging/antioxidant activity of the mint leaves [60]. Stephens and Tores [61] filed an US patent regarding the use of 2-hydroxy-3-ethyl-2-cyclopenten-1-one (***IV***) as a flavouring agent in edible food compositions. Luo et al. [62] reported 4-ethyl 1,3 benzenediol (***V***) isolated from *B. Japanensis yunnanensis* and evaluated its antibacterial activity. Carvone (***VIII***) has been reported to be the chief constituent in mint leaves [20, 23]. Nath et al. [63] reported the effectiveness of dibutyl phthalate as an insect repeller. Both carvone (***VIII***) and dibutyl phthalate (***XI***) could explain the role of mint leaves as a potent anti-insect agent for grain protection [64].

III.10 ANTIOXIDANT BEHAVIOUR

Reactive oxygen species (ROS) including superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) and non-free radical species such as H_2O_2 are likely to induce oxidative damage to biomolecules such as lipids, nucleicacids, proteins and carbohydrates. Their damage causes malaria, immunodeficiency syndrome, heart disease, stroke, diabetes and cancer [65]. Natural antioxidants found in plants commonly consumed as a part of diet may play an important role in minimizing oxidative deterioration of lipids [66] and to maximize food quality. It is observed from Fig. III.22 that diethyl ether extract shows almost 100 % activity at ~40 µg/L whereas the other two extracts in dichloromethane and methanol show very little activity (maximum <10%). Natural antioxidative substances usually have a phenolic moiety in their structure [67] and occur widely in plants. Plant phenolics are multifunctional and act as free

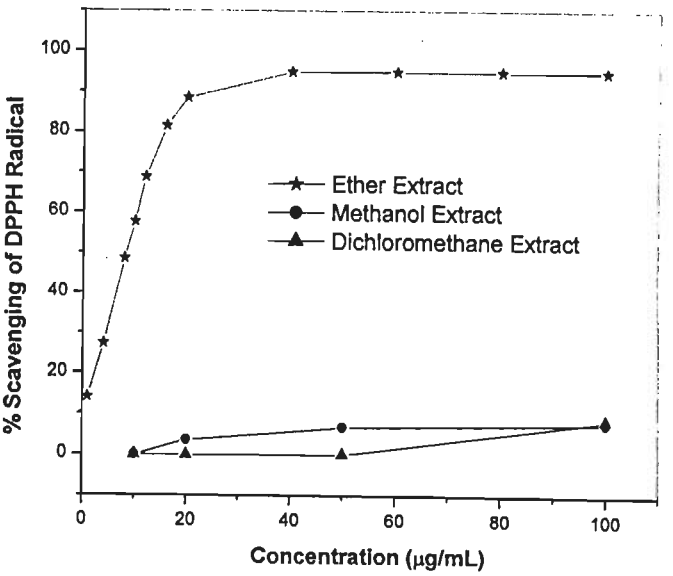


Fig III.22 DPPH radical scavenging activity of *Mint* leaves

radical terminator, metal chelators and singlet oxygen quenchers. Strong free radical scavenging activity of the diethyl ether extract indicates the presence of natural phenolics in the extract. This is confirmed by the presence of phenolic compounds such as V and VII as confirmed by GC-MS.

III.11 TRACE ELEMENTS vs. ORGANIC CONSTITUENTS

Inorganic elements such as Mn, Fe, Cu and Zn, which are found at significant concentrations of approximately 50, 100, 15 and 20 μg/g respectively may remain associated with the organic constituents like menthol and carveol making them bioavailable. Lu et al [68] filed a patent reporting the formation of synthetic prostaglandin (high energy fatty acids) via complex formation of Cu with cyclopentenone. Narde and Purohit [69] developed a qualitative and quantitative method for the analysis of -dihydric phenols based on formation of metal-arene complex with Cu (II). Kolosov et al. [70] carried out ESR measurements of Cu (II) complexes of carvone and its derivatives. Nurullaev [71] studied the IR absorption spectra of the complexes of dibutyl phthalate with Mo (VI). Coassin et al. [49] and Zago and Oteiza [51] have shown antioxidant behaviour of Mn^{2+} and Zn^{2+} respectively. Thus various metal ions in complex form with phenolic/alcoholic compounds may act as antioxidants and exhibit beneficial properties.

CONCLUSION

On the basis of analytical data on minor, essential trace and toxic elements in mint leaves (n=10) from four different locations and the confirmation of a new carveol compound including 10 organic compounds, following generalizations can be made:

- Mint is enriched in Ca (12.4±3.49 mg/g), Mg (4.83±0.92 mg/g), K (23.4±12.1 mg/g), P (3.88±0.94 mg/g) and to a lesser extent in Na (0.64±0.20 mg/g) and Fe (108±22 μg/g).
- Ranges of essential and trace elemental concentrations in four different locations do not vary significantly. This is primarily because soil characteristics within a small area from where the samples were derived, do not vary significantly.
- Mint leaves from Roorkee are especially enriched in K (28.5±14.6 mg/g), Mg

(5.51 ±0.58 mg/g), and Se (162±35 ng/g) whereas those from Dehradun are enriched in Ca (16.2±0.7 mg/g), Fe (130±10 μg/g) and Zn (25.5±3.0 μg/g).

- Trace amounts of Se (177±33.2 ng/g) may be responsible for its anticancer properties Se as glutathione peroxidase inhibits the replication of tumor virus and prevents the malignant transformation of cells
- The toxic heavy metals like Hg (97-983 ng/g), Sb (1.8-315 ng/g), Cd (15-772 ng/g) and As (98-320 ng/g) are all found at ng/g level and Pb was found below detection limit of 60 ng/g. Thus mint does not seem to have accumulating properties for toxic elements and its leaves are safe to consume.
- Leaves from Roorkee show somewhat higher amounts of As (313±7 ng/g), Hg (410±338 ng/g) and Cd (199 ng/g). This may possibly be attributed to the fact that Roorkee is developing into an urbanized township where these pollutant elements could originate from the spray of pesticides, industrial emissions and anthropogenic activities.
- Uttarkashi, a hilly town having no industrial activity shows much lower concentrations of Br (1.42±0.01 μg/g) and Ni (1.90±1.17 ng/g). On the other hand leaves from Baghpat, a town near Delhi the capital and a mega city of India show higher amounts of Cs (267±15 ng/g) and Th (152±42 ng/g). All these toxic elements are below the permissible limits specified by the US FDA
- Na and Mg are inversely correlated with Cl (r = -0.95 and -0.97) respectively. Similarly, Cr and Zn, the two essential elements well known for their role in biochemical processes, are also inversely correlated with r = -0.91.
- K/Na in leaves from four different locations varies in a range of 31.1 to 83.2 while K/P varies in a much wider range of 2 to 10.
- Extracts of *mint* were assayed for DPPH free radical scavenging activity. It is observed that diethyl ether extract shows ~100 % activity at ~40 μg/L whereas dichloromethane and methanol extracts show very little activity.
- A new compound 1,3 dihydrocarveol, possibly responsible for its antioxidant activity, was isolated from the methanolic extract of leaves by column and thin layer chromatography (TLC) and identified by spectral methods and GC-MS.
- GC-MS of diethyl ether extract showed three ketones, two phenols, two esters, an alcohol, azulene and ether. Phenols, alcohols, esters, ketones and ether may act as antioxidants.

- Many of the organic constituents have strong binding sites where inorganic elements such as V, Cr, Mn, Fe, Co, Cu and Zn, may remain complexed thus making them easily bioavailable.
- Since mint is used both as a flavouring agent as an spice-cum-medicine, it is essential to have strict quality control with regard to good agricultural practices, additives and stabilizers while preparing medicines.
- The base line data on essential trace elements and organic constituents may help to develop an understanding for its pharmacological action.

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CHAPTER IV

CURRY LEAVES

A part of the work has appeared in J. Radioanal. Nucl. Chem.**270** (2006)187-195

IV.1 ORIGIN AND ETYMOLOGY

Curry-leaf plant, a native of India and Sri Lanka is a small deciduous tree with pungent aromatic leaves. It is found almost everywhere in the Indian subcontinent excluding the higher levels of the Himalayas. Curry leaves grow throughout mainland and is commonly found in forests as gregarious undergrowth [1]. In the East, its range extends into Burma. The botanical name *Murraya koenigii* refers to two 18th century botanists: the Swede Johann Andreas Murray (1740–1791) and the German Johann Gerhard König (1728–1785) [2]. The English term *curry* is of Indian origin encompassing spicy preparation of vegetables, lentils and other food. The botanical name of curry leaves is *Murraya Koenigii* and is known by different names in various Indian languages and other countries as listed in Table IV.1. Most Indian cuisines use the subtle flavouring of this highly aromatic leafy spice. The plant grows best in tropical and sub-tropical climate in sunny to semi-shaded locations, though they can be sustained in other climates by moving to warm protected areas in winter and maintaining humid conditions during hot and dry summers. It grows to about 2.5 m high the main stem has dark green to brownish colour with numerous dots on it. Its bark can be peeled off longitudinally, exposing the white wood underneath, the girth of the main stem being 16 cm. The long slender leaves are dark green on top and pale inside. The leaves 5 cm long, 2 cm broad and 0.5 cm long petiole with reticulate venation have a strong, warm aroma when bruised or rubbed. Flowers are bisexual, white, funnel shaped, sweetly scented and stalked together [3] .It adorns every house yard in southern parts of India where it is widely used as flavouring agent and as a spice. Though it is considered as a vegetable but it is more like a spice for flavouring curry preparations and salties and is also used as a medicinal plant [4].

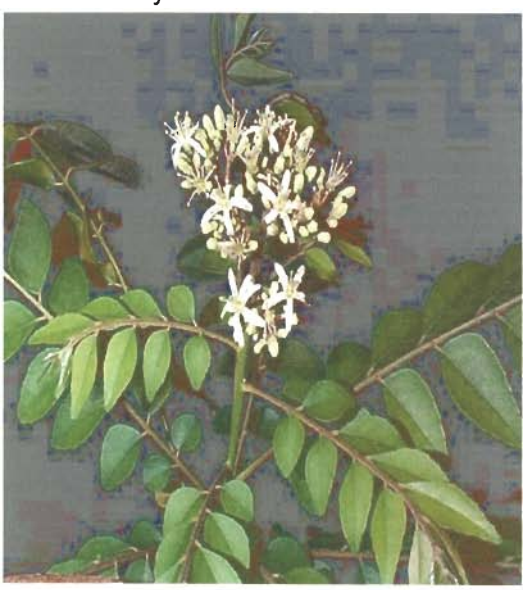


Table IV.1 Nomenclature of Curry leaves in Indian languages and other countries [5]

Indian Languages		Foreign Languages	
Assamese	Bisharhari	Arabic	Waraq al-kari
Bengali	Barsunga	Burmese	Pindosin, Pyim daw thein
Gujarati	Mitho limdo	Chinese	Ga lei yihp
Hindi	Meetha neem, Kari patta,	Dutch	Kerriebladeren
Kannada	Karibevu	English	Curry leaves
Malayalam	Kareapela	French	Feuilles de Cari
Marathi	Kadhilimb, Karhilimb	German	Curryblätter
Oriya	Basango	Hungarian	Curry levelek
Punjabi	Karipata, Karipatta, Bowala	Indonesian	Daun kari
Sanskrit	Girinimba, Suravi	Italian	Fogli di Cari
Tamil	Kariveppilai	Japanese	Kare-rihu, Nanyōzansiyō,
Telugu	Karivepaku	Portuguese	Folhas de Caril
		Russian	Listya karri
		Singhalese	Karapincha
		Spanish	Hoja, Hojas de Curry

The western world is fast taking enthusiastically to Indian curry leaves as a spice as well as medicinal herb. The leaves are used to flavour a range of dishes and typically they are fried in oil until crisp to impart flavour to vegetable preparations cooked in sesame oil. Fresh leaves release strong aroma while cooking. Drying the leaves is not very successful, as they tend to lose flavour. However, their dry powder is also used in cities where the leaves may not be available. Recently agriculturists from USA have developed a variety in curry leaves under the name *Suwasini* which has a strong aroma, dry matter content, essential oil and resistant to leaf spot disease [6]. Gopalan et al. [7] have compiled organic and inorganic constituents in curry leaves as listed in Table IV.2

Table IV. 2 Organic and Inorganic constituents of Curry leaves [7]

Organic	mg/100 g of Edible portion	Element	mg/ 100 g of Edible portion
Fats	1000	Calcium	830
Fibres	6400	Phosphorus	57
Carbohydrates	18700	Iron	0.93
Proteins	6100	Magnesium	44
Carotene	7.56	Copper	0.10
Thiamine	0.08	Manganese	0.15
Riboflavin	0.21	Zinc	0.20
Niacin	2.3	Chromium	0.006
Folic acid	0.09	Sulphur	81
Vitamin C	4	Chlorine	198
Oxalic acid	132	Moisture	63.8%
Energy (kCal)	108		

IV.2 HEALTH BENEFITS

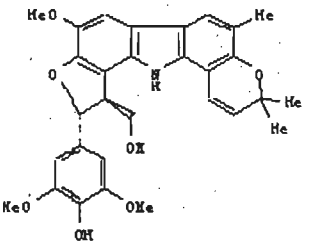
The plant has been used extensively in traditional Indian medicine system for a variety of ailments [4, 8]. British pharmacist Peter Houghton [9] showed curry leaves to be potential antidiabetic as it inhibits a digestive enzyme (pancreatic α - amylase) involved in the breakdown of starch to glucose. The oil derived from the leaves is used in perfumes and soap industry. Leaves are packed with minerals, vitamins A, B and are rich sources of carbohydrates, proteins, amino acids and alkaloids [10-12]. Besides its medicinal use as an anti-diabetic plant [13-15], curry leaves have several other uses as described in literature [4, 8]. Following are some of the common uses [16];

- Fresh juice of curry leaves mixed with limejuice and sugar cures morning sickness, nausea and vomiting due to indigestion.
- A glass of buttermilk with a pinch of salt and a spoonful of grounded curry leaf paste taken on an empty stomach relieves stomachache.
- Chewing the tender leaves helps control loose motions whereas fully-grown curry leaves are beneficial in controlling *diabetes* and in weight loss.
- Leaves cooked in milk and ground to a paste, when applied to poisonous insect bites and other wounds, relieves pain and removes swelling.
- Leaves ground with turmeric and taken daily are an effective remedy for allergic reactions. Also its paste applied on the foot prevents cracking.
- Curry leaves and black pepper beaten with sour curd is beneficial for gas formation due to indigestion.
- Leaves boiled in coconut oil act as an excellent hair tonic. It also stimulates hair growth and retains its natural pigmentation.

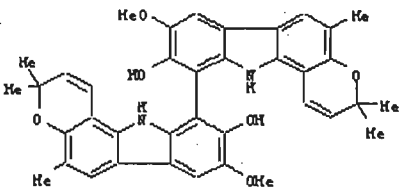
IV.3 ORGANIC CONSTITUENTS

Fresh leaves are rich in essential oil, but the exact amount depends on the extraction technique. Typically, the content ranges from 0.5 to 2.7%. MacLeod and Pieres [17] identified several aroma components β-caryophyllene, β-gurjunene, β-elemene, β-phellandrene, β-thujene, α-selinene, β-bisabolene, limonene, β-trans-ocimene and β-cadinene from the Sri Lankan leaves. Raina et al. [18] observed a large variability in the composition of the essential oil of curry leaves. In North Indian plants, monoterpenes prevail (β-phellandrene, α-pinene, β-pinene), whereas those

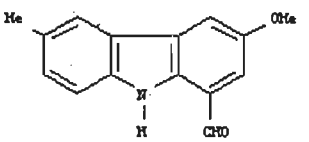
from South India yield sesquiterpenes: β-caryophyllene, aromadendrene, α-selinene. Besides, several new carbazole alkaloids like Murrayanine , 8,8" biskoenigine [19], 1-formyl 3-methoxy 6-methyl carbazole, 6,7-dimethoxy 1-hydroxy 3- methyl carbazole [20], bismurrayafoline E [21], Girinimbine and bicyclomahanimbiline [22] having antioxidative properties were isolated.



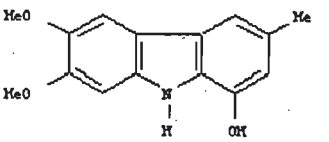
Murrayanine



8,8"-biskoenigine



1-formyl 3-methoxy 6-methyl carbazole



6,7-dimethoxy 1-hydroxy 3-methyl carbazole

IV.4 LITERATURE SURVEY

Most studies on the curry leaves reported in literature pertain to the organic contents viz. essential oils, alkaloids in various parts such as leaves, roots and fruits and their antioxidant properties. Wang et al. [19] isolated two carbazole alkaloids murrayanine and 8,8"-biskoenigine through oxidative coupling using a solid-state reaction. Chowdhury et al. [20] isolated two alkaloids, 1-formyl-3-methoxy-6-methylcarbazole and 6, 7-dimethoxy-1-hydroxy-3-methylcarbazole from the leaves. Nutan et al. [21] reported bismurrayafoline E, another carbazole alkaloid from the ethanolic extract of the leaves. Sukari et al. [23] extracted murrayazoline and murrayacine from the $CHCl_3$ extract and elucidated their structure using high-resolution NMR, IR and mass spectrometry. Bringmann et al. [24] carried out the total synthesis of the Murraya alkaloid murrastifoline F, an unsymmetrical N,C-bonded heterobiarylic biscarbazole. Adebajo and Reisch [25] reported 9 minor furocoumarins; Xanthotoxin, isobyakangelicol, phellopterin, gosferol, neobyakangelicol, byakangelicol, byakangelicin and isogosferol from *Murraya koenigii* seeds. Ramsewak et al. [26] isolated three bioactive carbazole alkaloids, mahanimbine, murrayanol, and mahanine having antimicrobial and mosquitocidal activities. Chakrabarty et al. [27] identified two alkaloids, 9-carbethoxy-3-

methylcarbazole and 9-formyl-3-methylcarbazole which displayed cytotoxicity against mouse leukemia cell lines. Reisch et al. [28] isolated isoheraclenin, isoimperatorin, oxypeucedanin, isopimpinellin, and bergapten from Sri Lankan seeds. Bhattacharya et al. [29] isolated Murrayazolinol from the stem bark and elucidated its structure by spectral means. Palaniswamy et al. [30] determined antioxidant vitamins, α-tocopherol, β-carotene and lutein in fresh leaves using reversed phase gradient HPLC. Dasgupta et al. [31] studied the anticarcinogenic potential of curry leaf extract and found it to be useful for the prevention of stomach and skin cancer. Tachibana et al. [32] evaluated the antioxidative properties of 12 carbazole alkaloids against 1,1- DPPH radical and suggested that an aryl hydroxyl substituent on the carbazole rings plays a role in stabilizing the thermal oxidation and rate of reaction against DPPH radical. Srivastava and Singh [33] found β-caryophyllene (29.0%) and β-gurjunene (21.0%) to be responsible for the antifungal activity of essential oil. Akerele and Ayinde [34] observed that the volatile oil and aqueous extracts of *Murraya koenigii* were active against gram-positive bacteria.

Only scanty reports are available on the minor and trace element composition of curry leaves. Kariyanna [35] has suggested dark coloured leaves as an indicator for Mn and light green due to Fe. Gopalan et al. [7] has compiled 8 essential elements along with organic compounds, which play a vital role in human metabolism. Narendhirakannnan et al. [36] studied the elemental composition in the curry leaves and other traditional medicinal plants widely used in the treatment of diabetes and related metabolic disorders using AAS. Ray et al. [37] determined K, Ca, Fe, Cr, Mn, Cu, Zn, Rb, Sr, and Pb content in the leaves using EDXRF. Balaji et al. [38] employed k_0 NAA for determining 11 essential elements in curry leaves from Tirupati. Singh and Garg [39] also reported 20 elements in curry leaves (also called *meetha neem*) from Nagpur city.

IV.5 PRESENT STUDY

In the present study, 28 samples of fresh curry leaves (*Murraya Koenigii)* collected from 19 states all over India were analysed for 6 minor (Ca, Cl, K, Mg, Na and P) and 23 trace (As, Ba, Br, Cd, Ce, Co, Cr, Cs, Cu, Fe, Hg, La, Mn, Ni, Pb, Rb, Sb, Sc, Se, Sr, Th, V and Zn) elements by NAA and AAS. Further, column and thin layer chromatography were used for separating three organic constituents

- 3-methylthiopropanenitrile;

- 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl ester) and
- 1-penten-3-ol

from the ethanolic extract and characterized by ir and GC-MS.

IV.6 EXPERIMENTAL

(i) Sample collection and preparation: Tender leaves were collected from 28 cities/towns spread over 19 Indian states as shown in Fig. IV.1 and listed in Table IV.3. On an average, ~100 g leaves were collected from the very bottom to the top of the tree mostly from residential areas. Volunteers were requested to follow the protocol in the collection of samples as described in ***Ch. II***. These were thoroughly washed with distilled water to remove any dirt and other surface contamination. The leaves were air dried and stored in polyethylene bags.

Fig. IV.1 Sample collection sites in Indian map

Table IV.3 Sample collection sites

S.No	Place	State	S.No	Place	State
1	Churachandpur	Manipur	15	Indore	Madhya Pradesh
2	Silchar	Assam	16	Kangra	Himachal Pradesh
3	Kolkata	West Bengal	17	Hissar	Haryana
4	Cuttack	Orissa	18	Pathankot	Punjab
5	Bokaro	Jharkhand	19	Roorkee	Uttaranchal
6	Jamshedpur	Jharkhand	20	Bijnaur	Uttar Pradesh
7	Chapra	Bihar	21	Lucknow	Uttar Pradesh
8	Bhilwara	Rajasthan	22	Hyderabad-1	Andhra Pradesh
9	Jaipur	Rajasthan	23	Hyderabad-2	Andhra Pradesh
10	Baroda	Gujarat	24	Vizag	Andhra Pradesh
11	Mumbai-1	Maharashtra	25	Vijaywada	Andhra Pradesh
12	Mumbai-2	Maharashtra	26	Pallakad	Kerala
13	Pune	Maharashtra	27	Pondicherry	Pondicherry
14	Nagpur	Maharashtra	28	Madurai	Tamil Nadu

Finally, these were dried at 80°C for overnight in an oven and crushed to homogenous fine powder (100 mesh) in an agate mortar. The powdered samples were stored in pre cleaned polyethylene vials and handled with extreme care in a glove box to avoid contamination. A primary standard for As, Co, Fe, Hg, Se and Zn along with two reference materials (RMs); Peach Leaves (SRM-1547) from the NIST (USA) [40] and Mixed Polished Herbs (MPH-2) [41] from INCT (Poland) were used as comparators.

(ii) Irradiation and Counting: About 50 mg each of powdered samples and RMs were weighed accurately and packed in alkathene/ aluminum foil (Superwrap) for short (2 min)/ long (1d) irradiation in CIRUS/Dhruva reactor respectively at the BARC, Trombay, Mumbai, India, at thermal neutron flux of ~10^{13} n cm^{-2} s^{-1}. Care was taken to obtain maximum elemental information from more than one counting and the reproducibility of data was checked. Cd, Ni and Pb were determined by AAS as described in *Ch. II.9*. Elemental contents were calculated by comparator method using synthetic multielemental standard and RMs as comparators.

(iii) Organic constituents: 65 *g* dried leaves were extracted with petroleum ether (60-80°C) in a Soxhlet for 36 h so as to remove fatty acids and resins. The solid plant material was then air dried and re-extracted with 95% ethanol for 6 h. The solvent was then removed from the extract in a rotavapor. The residue was passed through a column (45 cm x 2.5 cm) and eluted with several solvent systems such as petroleum ether (PE), PE-CH_2Cl_2 (2:1), PE-CH_2Cl_2 (1:1), PE-EtOAc (9:1), $CHCl_3$-MeOH (99:1) and finally with EtOAc- MeOH (19:1). Only PE-EtOAc (9:1) elute showed distinct spots on TLC plate whereas other eluates showed a few spots in meager amounts, which were discarded. The PE-EtOAc (9:1) fraction (120 mg) was rechromatographed on a similar column whence various solvent mixtures of PE-EtOAc (50:1, 25:1, 10:1) were attempted. It was observed that only 25:1 eluate furnished positive TLC positive test with iodine. The residue (80 mg) so obtained was recrystallised from PE-CH_2Cl_2, which appeared to be homogenous on PE-benzene (1:1). A TLC examination in PE-$CHCl_3$ (1:1), however, demonstrated three components, which were separated by preparative TLC (20 cm x 20 cm) plate with 1mm thick layer using PE-$CHCl_3$ (1:1) as developing solvent system. Three bands corresponding to R_f=0.53 (3-methylthiopropanenitrile, 14mg), 0.41(1,2-

benzenedicaroxylic acid mono 2-ethyl hexyl ester, 22mg) and 0.38(1-penten-3-ol, 18mg) were separated. Schematic of flowsheet is shown in Fig. IV.2

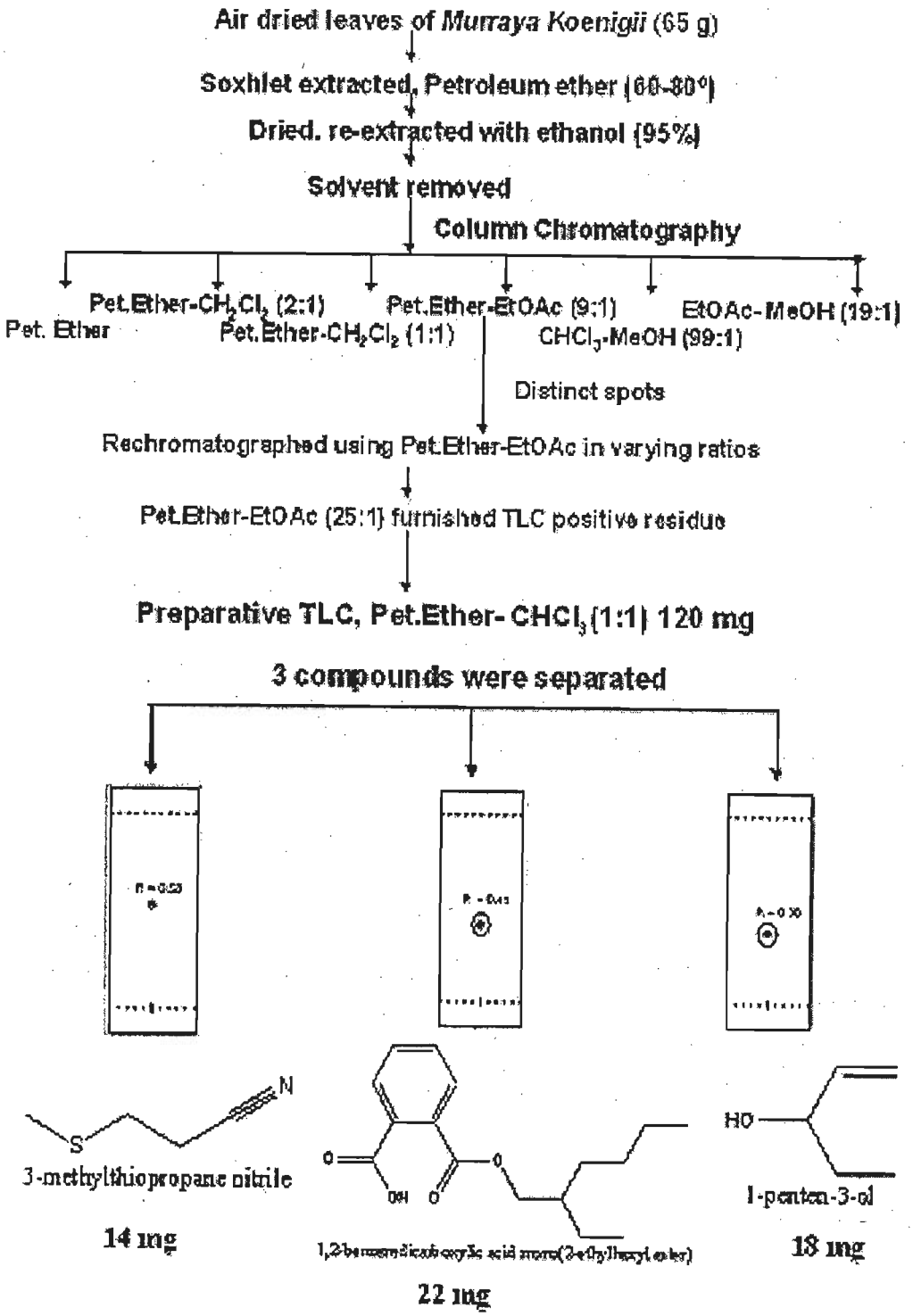


Fig. IV.2 Flow sheet for the separation of organic constituents

IV.7 RESULTS

Elemental concentrations were calculated using synthetic multielemental standard and two RMs as comparators. Mean ± SD data for Peach leaves (SRM-1547) along with certified/ information values; error %, RSD and Z-score values are listed in Table IV.4. It is observed that our data match well within ± 5-10% of the certified values with exceptions of As, La, Sb and Se. Also, RSDs were <10% in all cases suggesting high order of precision in our measurements. Further Z-score values in Fig. IV.3 are all below 3 except Ca and Mg suggesting that the data should be within 99% confidence limits or better. It may be noted that these elements were determined from its short-lived nuclides with $t_{1/2} \approx 10$ min.

Table IV.4 Elemental concentrations in Reference Materials used for data validation

Element	Peach Leaves (SRM-1547)		% Error	% RSD	Z-score
	This work	*Certified*			
As (ng/g)	51.6±0.7	(60±18)	-14	1.4	-0.47
Ba (μg/g)	136±7	(124±4)	9.7	5.1	3.0
Br (μg/g)	10.3±0.6	[11]	-6.4	5.8	-
Ca (mg/g)	16.3±0.5	(15.6±0.2)	4.5	3.1	3.5
Ce (μg/g)	10.1±1.1	[10]	1.0	11.0	-
Cl (mg/g)	0.39±0.03	(0.36±0.02)	8.3	7.7	1.5
Co (ng/g)	64.6±0.4	[70]	-7.7	0.6	-
Cr (μg/g)	1.10±0.10	[1]	10	9.0	-
Cs (ng/g)	85.1±0.4	{-}	-	0.5	-
Fe (μg/g)	221±18	(218±14)	1.3	8.1	0.21
Hg (ng/g)	28.0±0.6	(31.0±7.0)	-9.7	2.1	-0.73
K (mg/g)	24.7±2.0	(24.3±1.0)	1.2	8.1	0.40
La (μg/g)	7.98±0.05	[9]	-11.3	0.6	-
Mg (mg/g)	4.72±0.28	(4.32±0.08)	9.3	5.9	5.0
Mn (μg/g)	101±5	(98.0±3.0)	3.0	5.0	1.0
Na (μg/g)	22.2±1.0	(24.0±2.0)	-7.5	4.5	-0.90
P (mg/g)	1.26±0.10	(1.37±0.07)	-8.0	7.9	-1.57
Rb (μg/g)	21.6±2.1	19.7±1.2	9.6	9.7	1.58
Sb(ng/g)	17.9±1.1	[20]	-10.5	6.1	-
Sc (ng/g)	42.1±0.3	[40]	5.3	0.7	-
Se (ng/g)	101±2	(120±9)	-15.8	1.9	-2.11
Sr (μg/g)	49.2±1.1	(53±4)	-9.1	2.2	-0.95
Th (ng/g)	53.5±2.0	[50]	7.0	3.7	-
Zn(μg/g)	18.9±0.1	(17.9±0.4)	5.6	0.5	2.5

In parentheses ( ) are certified values, in [ ] are information values, In {-} no data available;

Therefore, it is assumed that our data for curry leaves should be reliable within ± 10%. Elemental concentrations in individual samples of curry leaves from East (n=7), West (n=8), North (n=6) and South (n=7) zones of 19 Indian states are listed in Tables IV.5, 6, 7 and 8 respectively. Ranges and mean elemental concentrations in 4 different zones are listed in Table IV.9. Further ranges, mean± SD and median ± SD for all the elements in curry leaves along with a comparison with literature reports [7, 38, 39] are listed in Table IV.10. *SD* of median was calculated using the equation $\left[SD = \frac{1.25\ R}{1.35\ \sqrt{n}} \right]$ where R is the range and n the number of samples analyzed.

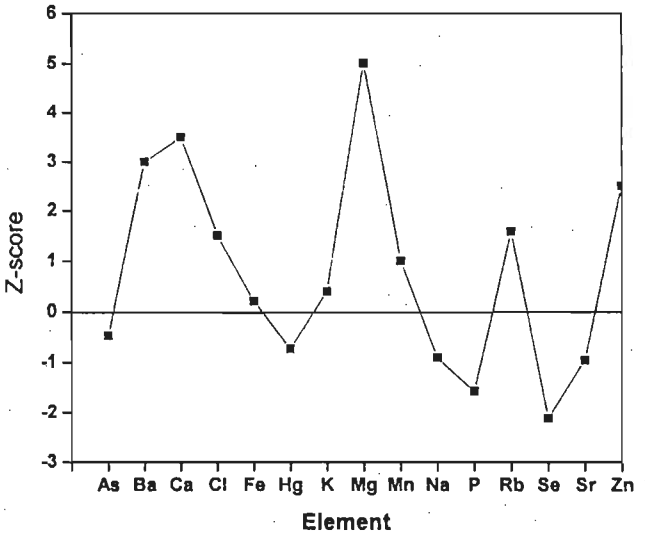


Fig. IV.3 Z-score plot for elements in Peach leaves (SRM-1547)

IV.8 ELEMENTAL CONTENTS

(i) Elemental contents in four zones: A perusal of elemental contents in Table IV.5 shows that most elements from the **East zone** vary in a narrow range by a factor of 3 except Cd, Co, Cu, Sb and Sc. Th and Pb contents vary by an order of magnitude. It is observed that leaves from Kolkata, a mega city show highest Ca (18.4±0.5 mg/g), K (27.9±1.8 mg/g) and P (1.48±0.11 mg/g) contents. Leaves from Bokaro have the highest Na (313±23 μg/g) and Cl (1.90±0.21 mg/g) contents. Fe, Co, Cs and Rb contents are more in leaves from Jamshedpur. Amongst toxic elements, Hg, Sb, Cd and Ni contents are higher in Jamshedpur sample while Bokaro sample has the highest As (81.4±3.9 ng/g) and Pb (35.5μg/g) contents. Both these two cities (Bokaro and Jamshedpur) are known for steel plants in the state of Jharkhand and high concentrations of toxic elements in leaves from these cities indicate the pollution effects of industrial emissions and other anthropogenic activities.

Table IV.5 Mean elemental concentration of curry leaves (n=7) collected from East zone

Element	C'chanpur	Silchar	Kolkata	Cuttack	Bokaro	Chapra	J'shedpur
	Manipur	Assam	W. B'gal	Orissa	J'Khand	Bihar	J'Khand
NAA							
As (ng/g)	31.3±1.4	43.2±1.9	69.8±3.2	58.7±2.6	81.4±3.9	64.7±2.9	36.8±1.7
Ba (μg/g)	57.7±3.1	19.7±1.1	36.2±2.0	41.2±1.7	41.1±2.5	35.1±1.9	32.1±1.8
Br (μg/g)	7.10±0.22	6.70±0.11	6.66±0.16	10.4±0.35	5.33±0.20	6.06±0.19	4.18±0.16
Ca (mg/g)	15.1±0.8	15.8±0.4	18.4±0.5	12.3±0.5	12.9±0.4	16.2±0.9	14.1±0.7
Ce (μg/g)	3.24±0.09	3.19±0.21	5.79±0.33	2.92±0.08	1.81±0.52	3.20±0.14	3.59±0.10
Cl (mg/g)	1.31±0.04	1.29±0.07	0.76±0.02	1.01±0.03	1.90±0.21	1.63+0.16	1.86±0.19
Co (ng/g)	35.1±0.2	58.8±0.4	56.9±0.4	90.5±3.4	102±1	137±2	168±6
Cr (μg/g)	0.83±0.01	0.68±0.03	0.71±0.02	0.86±0.03	0.53±0.01	0.60±0.01	0.67±0.01
Cs (ng/g)	21.3±0.3	31.3±0.6	40.5±0.3	17.3±0.2	7.70±0.15	19.2±0.3	48.3±1.2
Cu (μg/g)	14.6±0.9	71.7±3.6	14.0±0.8	32.5±2.1	11.2±0.7	25.6±1.7	41.1±3.1
Fe (μg/g)	137±1	154±1	159±1	116±6	127±1	196±5	283±9
Hg (ng/g)	12.7±0.91	29.2±1.0	27.7±1.1	42.3±2.0	15.6±1.2	24.8±1.5	45.0±2.9
K (mg/g)	10.8±0.5	23.2±1.1	27.9±1.8	20.6±0.9	10.3±1.6	11.2±0.5	14.5±0.7
La (μg/g)	4.74±0.20	4.75±0.21	6.11±0.24	4.19±0.19	3.82±0.16	6.49±0.28	5.07±0.23
Mg (mg/g)	3.36±0.16	2.61±0.29	2.76±0.22	3.65±0.27	4.58±0.38	5.16±0.69	4.27±0.31
Mn (μg/g)	32.8±0.7	28.8±0.5	24.8±0.5	30.2±0.8	61.6±2.7	30.6±0.6	38.9±0.9
Na (μg/g)	107±5	158±15	119±8	146±9	313±23	230±9	346±13
P (mg/g)	0.80±0.03	0.80±0.05	1.48±0.11	1.03±0.04	1.04±0.06	1.03±0.04	1.32±0.05
Rb (μg/g)	10.6±0.1	11.2±1.0	15.5±2.0	6.00±0.12	5.15±1.13	5.89±0.14	19.2±0.3
Sb(ng/g)	21.1±0.4	28.6±1.1	31.5±2.2	33.1±0.6	77.9±0.9	68.9±1.1	83.9±1.4
Sc (ng/g)	13.6±0.4	20.5±0.2	28.6±0.2	47.4±2.9	45.0±0.7	49.1±1.4	25.2±0.7
Se (ng/g)	56.3±1.8	94.1±0.8	92.4±0.8	108±4	107±1	60.8±2.0	73.5±2.4
Sr (μg/g)	43.1±0.9	24.5±0.6	87.2±2.1	64.0±1.3	52.3±1.1	60.3±1.3	42.1±0.9
Th (ng/g)	49.1±3.3	21.5±0.8	31.1±0.4	37.1±1.2	9.33±0.14	59.8±2.8	80.1±3.8
V (μg/g)	1.25±0.06	2.31±0.16	<0.15	1.96±0.13	<0.15	0.69±0.05	1.51±0.09
Zn (μg/g)	30.2±0.4	24.8±0.2	21.0±0.2	22.8±1.1	17.0±0.3	21.7±1.0	19.4±0.8
AAS							
Cd (μg/g)	0.98	2.44	1.58	3.56	1.85	1.09	5.17
Ni (μg/g)	2.25	3.58	3.08	1.89	3.26	1.24	4.17
Pb (μg/g)	4.87	27.6	37.3	8.51	35.5	3.42	9.37

Table IV.6 Mean elemental concentration of curry leaves (n=8) collected from West zone

Element	Bhilwara	Jaipur	Baroda	Mumbai-1 (BARC)	Mumbai-2 (Andheri)	Pune	Nagpur	Indore
	Rajasthan		Gujarat	Maharashtra				M. P'desh
NAA								
As (ng/g)	40.7±1.7	105±4	63.8±2.1	112±6	86.3±5.1	90.4±5.3	71.2±2.8	50.7±2.1
Ba (µg/g)	36.5±0.9	45.3±1.3	53.4±1.8	80.4±3.4	69.7±2.6	22.7±0.7	60.3±2.4	57.8±1.9
Br (µg/g)	1.69±0.06	7.49±0.58	2.43±0.09	13.9±0.29	15.3±0.34	8.19±0.08	14.6±0.30	5.24±0.14
Ca (mg/g)	18.1±0.4	15.6±0.7	19.3±0.8	17.2±0.4	13.1±0.3	9.44±0.2	12.6±0.4	19.7±0.4
Ce (µg/g)	1.43±0.41	2.31±0.05	0.90±0.23	1.60±0.06	88.8±3.8	4.75±0.75	1.47±0.11	7.18±0.41
Cl (mg/g)	1.39±0.06	1.78±0.11	2.43±0.16	1.85±0.13	2.03±0.15	1.48±0.20	1.44±0.10	1.21±0.09
Co (ng/g)	48.6±0.3	148±7	49.8±2.5	141±6	147±6	116±5	96.6±0.8	77.2±0.7
Cr (µg/g)	0.99±0.02	0.64±0.01	0.52±0.01	0.89±0.03	2.05±0.07	0.93±0.04	1.02±0.04	1.13±0.02
Cs (ng/g)	35.8±0.2	29.6±0.3	12.2±0.1	35.4±0.5	28.7±0.4	40.2±0.7	55.4±0.6	30.1±0.2
Cu (µg/g)	11.1±0.7	7.52±0.5	16.0±1.0	12.3±0.9	8.96±0.8	8.50±0.8	21.7±1.8	10.7±0.9
Fe (µg/g)	72.5±0.2	209±7	145±1	174±8	228±11	169±2	173±1	195±2
Hg (ng/g)	76.5±1.4	33.4±1.9	14.7±1.0	68.6±3.3	12.3±0.5	64.3±0.9	57.8±1.2	16.0±0.7
K (mg/g)	10.9±1.1	20.0±0.8	13.7±1.7	25.9±2.1	28.3±2.3	12.9±1.0	13.7±0.8	30.3±1.6
La (µg/g)	2.06±0.09	3.37±0.15	4.15±0.17	2.11±0.13	2.87±0.16	4.98±0.21	3.83±0.17	2.26±0.12
Mg (mg/g)	4.86±0.55	4.15±0.16	6.90±0.30	4.63±0.51	3.89±0.24	3.83±0.15	4.09±0.14	1.14±0.11
Mn (µg/g)	56.6±0.5	40.8±2.1	38.6±1.9	38.9±2.0	41.3±2.3	40.9±2.2	40.5±2.4	27.8±1.5
Na (µg/g)	120±4	274±11	244±19	256±20	302±24	455±22	201±14	133±13
P (mg/g)	0.43±0.04	1.26±0.05	1.01±0.08	1.40±0.07	1.62±0.08	0.89±0.08	0.61±0.05	0.46±0.03
Rb (µg/g)	9.47±0.41	14.3±0.1	7.45±0.24	15.0±0.3	10.9±0.2	13.9±0.7	20.9±1.2	10.1±1.3
Sb(ng/g)	80.7±3.5	54.4±1.0	50.9±0.7	45.6±0.9	64.7±3.9	65.3±1.9	49.6±1.3	64.4±2.6
Sc (ng/g)	5.43±0.16	35.2±0.9	4.98±0.14	41.0±2.5	57.7±2.1	42.3±0.3	40.1±0.2	42.5±0.2
Se (ng/g)	131±3	91.0±3.0	69.3±0.4	60.1±2.2	162±6	59.9±1.2	50.9±0.5	61.2±1.6
Sr (µg/g)	30.6±0.9	25.9±0.6	22.4±0.5	35.4±0.7	37.8±0.7	47.9±0.8	50.2±1.0	40.2±0.8
Th (ng/g)	8.69±0.37	97.5±4.5	6.60±0.18	97.0±3.3	94.3±4.5	64.4±2.1	44.0±1.0	41.9±0.5
V (µg/g)	0.67±0.05	1.38±0.05	<0.15	0.92±0.06	1.21±0.07	<0.15	1.03±0.06	<0.15
Zn(µg/g)	10.0±0.2	30.2±1.5	70.5±0.5	30.1±1.4	49.9±2.3	51.9±1.4	24.8±0.2	11.9±0.1
AAS								
Cd (ng/g)	3.04	2.12	3.01	5.07	2.64	1.89	3.41	2.43
Ni (µg/g)	3.40	1.68	6.65	4.08	2.16	2.78	6.09	3.06
Pb (µg/g)	36.6	7.96	36.2	5.74	5.17	17.4	79.1	63.4

Table IV.7 Mean elemental concentration of curry leaves (n=6) collected from North zone

Element	Kangra	Hissar	Pathankot	Roorkee	Bijnaur	Lucknow
	H. P'desh	Haryana	Punjab	Uttaranchal	Uttar Pradesh	
NAA						
As (ng/g)	ND	48.3±2.1	ND	ND	56.3±2.5	69.8±3.1
Ba (μg/g)	34.8±2.0	19.9±1.1	26.2±1.5	19.3±0.9	24.7±1.3	56.6±3.1
Br (μg/g)	2.24±0.04	5.03±0.39	3.76±0.13	2.27±0.14	4.46±0.35	7.49±0.65
Ca (mg/g)	28.3±0.6	25.4±0.2	22.4±0.2	21.6±0.9	33.2±0.8	23.1±0.3
Ce (μg/g)	4.26±0.31	1.61±0.07	1.16±0.48	3.32±0.70	1.11±0.05	1.51±0.06
Cl (mg/g)	1.67±0.02	2.01±0.06	1.76±0.03	3.13±0.02	3.64±0.22	2.79±0.03
Co (ng/g)	34.9±0.2	89.2±1.2	72.2±0.9	50.6±2.9	85.3±3.2	102±4
Cr (μg/g)	0.66±0.01	1.05±0.01	0.40±0.01	0.23±0.01	0.94±0.01	0.80±0.01
Cs (ng/g)	86.0±0.3	59.3±0.6	21.5±0.2	16.4±0.2	84.2±0.8	98.7±0.9
Cu (μg/g)	1.39±0.11	4.25±0.34	9.12±0.81	9.39±0.86	10.9±0.9	6.18±0.43
Fe (μg/g)	159±1	112±1	135±1	140±1	158±2	215±4
Hg (ng/g)	25.3±1.0	18.1±0.9	29.9±0.9	20.4±1.2	22.0±1.2	42.9±2.5
K (mg/g)	15.8±1.2	18.4±0.8	13.6±1.9	18.0±1.9	17.5±0.8	23.9±1.1
La (μg/g)	2.57±0.12	4.01±0.17	4.97±0.24	4.18±0.19	10.6±0.45	10.7±0.55
Mg (mg/g)	2.56±0.19	2.81±0.07	4.51±0.20	2.58±0.18	2.61±0.05	2.14±0.16
Mn (μg/g)	45.6±0.5	49.1±2.4	43.1±0.4	44.1±2.3	53.1±0.6	56.3±0.08
Na (μg/g)	104±9	177±7	189±11	214±3	81.5±3.8	70.9±3.3
P (mg/g)	0.58±0.04	2.16±0.08	1.69±0.11	1.17±0.07	1.48±0.06	1.61±0.06
Rb (μg/g)	27.2±2.3	21.1±0.4	12.4±2.3	6.99±1.23	33.7±0.5	20.4±0.4
Sb (ng/g)	48.8±2.5	37.9±0.6	57.4±3.1	14.1±0.4	25.3±0.4	21.9±0.4
Sc (ng/g)	23.9±0.2	43.8±1.3	21.4±0.6	13.6±0.3	60.6±1.7	50.2±1.8
Se (ng/g)	49.6±0.5	58.5±1.9	116±2	40.1±0.3	53.0±1.7	52.5±1.4
Sr (μg/g)	52.4±1.3	31.5±0.7	33.9±0.9	20.6±0.4	27.1±0.6	25.9±0.6
Th (ng/g)	23.7±0.4	64.6±3.0	41.2±0.9	20.0±0.2	64.3±3.0	115±5
V (μg/g)	2.73±0.17	2.15±0.15	<0.15	1.99±0.11	1.30±0.10	1.02±0.08
Zn(μg/g)	13.3±0 .1	25.6±0.3	12.2±0.3	7.90±0.20	28.7±0.8	30.1±1.1
AAS						
Cd (ng/g)	2.12	3.14	2.81	1.39	2.09	3.74
Ni (μg/g)	4.52	2.08	3.92	3.00	4.15	1.57
Pb (μg/g)	28.0	7.12	38.4	10.4	6.98	10.2

Table IV.8 Mean elemental concentration of curry leaves (n=7) collected from South zone

Element	H'dabad-1	H'dabad-2	Vizag	V'wada	Pallakad	P'cherry	Madurai
	Andhra Pradesh				Kerala		T. Nadu
NAA							
As (ng/g)	97.2±4.3	131±6	271±12	104±4.6	73.4±3.7	57.7±2.1	39.6±2.0
Ba (μg/g)	42.1±1.6	67.8±3.7	48.1±1.8	38.6±1.5	53.6±2.1	26.0±1.0	35.9±1.3
Br (μg/g)	9.60±0.17	9.50±0.81	18.2±1.4	13.3±1.1	3.55±0.15	14.8±1.2	8.24±0.06
Ca (mg/g)	15.3±0.1	18.6±0.9	14.9±0.7	20.5±1.1	23.5±1.0	16.3±0.8	21.2±0.6
Ce (μg/g)	2.94±0.41	2.95±0.14	2.76±0.11	2.38±0.12	3.24±0.72	1.80±0.11	4.82±0.21
Cl (mg/g)	2.60±0.07	2.11±0.06	2.13±0.06	1.84±0.04	4.02±0.14	3.56±0.11	2.38±0.08
Co (ng/g)	69.5±2.9	47.0±1.8	78.6±2.4	38.1±1.2	20.4±0.6	27.9±0.9	63.6±0.2
Cr (μg/g)	0.67±0.03	1.02±0.01	0.92±0.01	0.89±0.01	0.79±0.01	0.67±0.01	0.75±0.03
Cs (ng/g)	38.7±0.5	41.3±0.8	50.2±1.1	42.7±1.7	43.4±0.1	36.2±0.1	43.9±0.5
Cu (μg/g)	10.3±0.9	6.15±0.46	3.66±0.27	7.23±0.64	63.3±4.1	10.4±0.9	11.7±1.0
Fe (μg/g)	108±2	139±3	211±5	175±4	101±1	125±2	120±1
Hg (ng/g)	34.7±0.6	35.5±2.8	41.0±2.4	26.3±1.4	49.1±1.1	15.7±0.8	21.6±0.8
K (mg/g)	17.1±1.0	15.6±0.7	20.6±0.9	18.4±0.8	16.4±1.4	22.0±1.1	19.1±1.0
La (μg/g)	2.29±0.09	3.71±0.16	1.13±0.05	2.95±0.13	4.12±0.19	5.58±0.25	3.56±0.14
Mg (mg/g)	4.78±0.15	4.03±0.11	4.39±0.14	6.05±0.19	7.19±0.30	5.26±0.17	6.88±0.20
Mn (μg/g)	62.3±3.7	57.1±3.1	41.7±2.4	53.1±2.8	61.2±4.0	46.9±2.7	63.0±1.9
Na (μg/g)	179±16	194±8	154±6	198±8	277±24	153±12	187±14
P (mg/g)	0.83±0.07	0.86±0.35	1.02±0.04	0.77±0.03	1.59±0.11	0.72±0.02	0.99±0.08
Rb (μg/g)	18.8±08	13.2±0.2	24.3±0.5	15.2±0.3	8.44±1.93	7.34±0.16	20.3±1.2
Sb (ng/g)	46.1±1.5	40.0±1.1	20.9±0.6	12.4±0.4	2.61±0.12	27.2±0.9	51.5±0.3
Sc (ng/g)	46.1±1.5	63.9±1.1	73.9±1.2	75.7±1.4	22.4±1.9	34.5±0.5	37.8±1.2
Se (ng/g)	27.9±0.6	24.1±0.7	39.1±1.3	34.3±1.1	45.8±1.4	44.1±1.4	26.1±0.4
Sr (μg/g)	25.5±0.6	38.0±0.8	35.2±0.8	48.0±1.0	38.9±0.9	30.5±0.7	57.1±1.2
Th (ng/g)	36.9±1.5	64.0±3.0	86.5±4.0	32.5±1.5	16.9±0.5	39.8±1.9	41.9±1.1
V (μg/g)	1.23±0.09	1.75±0.13	<0.15	0.83±0.06	2.89±0.21	2.04±0.16	<0.15
Zn(μg/g)	17.4±0.5	21.2±0.8	30.2±1.2	23.5±1.0	10.9±0.3	24.7±1.1	15.8±0.1
AAS							
Cd (ng/g)	2.94	1.92	3.78	4.02	2.14	2.56	2.47
Ni (μg/g)	4.36	5.21	2.13	3.42	6.73	1.93	3.80
Pb (μg/g)	37.4	13.3	7.14	11.8	20.9	5.31	28.0

Table IV.9 Ranges and mean elemental concentration of curry leaves (n=28) collected from different zones of India

Element	Eastern Zone (n=7)		Western Zone (n=8)		Northern Zone (n=6)		Southern Zone (n=7)	
	Range	Mean ±SD	Range	Mean ±SD	Range	Mean±SD	Range	Mean ±SD
As (ng/g)	31.3-81.4	55.1±18.5	40.7-105	77.5±25.3	48.3-69.8	58.1±10.9	39.6-271	111±77
Ba (µg/g)	19.7-57.7	37.6±11.5	22.7-80.4	53.3±18.3	19.3-56.6	30.3±14.1	26.0-67.8	44.6±13.5
Br (µg/g)	4.18-10.4	6.63±1.93	1.69-15.3	8.61±5.44	2.24-7.49	4.21±1.97	3.55-13.3	11.0±4.8
Ca (mg/g)	12.3-18.4	15.0±2.1	9.44-19.7	15.6±3.64	21.6-33.2	25.7±4.4	14.9-23.5	18.6±3.3
Cd (µg/g)	0.98-5.17	2.38±1.51	1.89-5.07	2.95±0.99	1.39-3.74	2.55±0.85	1.92-4.02	2.83±0.80
Ce (µg/g)	1.81-5.79	3.39±1.20	0.90-88.8	13.6±30.5	1.16-4.26	2.16±1.31	1.80-4.82	2.98±0.94
Cl (mg/g)	0.76-1.86	1.39±0.43	1.21-2.43	1.70±0.40	1.67-3.64	2.50±0.81	1.84-4.02	2.66±0.82
Co (ng/g)	35.1-168	92.6±47.4	48.6-148	103±42	34.9-102	72.4±25.3	20.4-78.6	49.3±22.0
Cr (µg/g)	0.53-0.86	0.70±0.12	0.52-2.05	1.02±0.46	0.23-1.05	0.68±0.32	0.67-1.02	0.82±0.13
Cs (ng/g)	17.3-48.3	26.5±14.2	12.2-55.4	33.4±12.2	16.4-98.7	61.0±35.0	36.2-50.2	42.3±4.4
Cu (µg/g)	11.2-41.1	30.1±21.3	7.52-21.7	12.1±4.7	1.39-10.9	6.87±3.61	3.66-63.3	16.1±21.0
Fe (µg/g)	116-283	167±57	72.5-228	171±47.3	112-215	153±34.9	101-211	140±40
Hg (ng/g)	12.7-45.0	38.2±12.2	1.23-76.5	41.6±28.8	18.1-42.9	26.4±9.1	15.7-49.1	32.0±11.5
K (mg/g)	10.3-27.9	16.9±7.0	10.9-30.3	19.5±7.75	13.6-23.9	17.9±3.7	15.6-22.0	18.5±2.30
La (µg/g)	3.82-6.49	5.02±0.97	2.06-4.98	3.20±1.07	2.57-10.7	6.17±3.55	1.13-5.58	3.33±1.41
Mg (mg/g)	2.61-5.16	3.77±0.95	1.14-6.90	4.19±1.58	2.14-4.51	2.87±0.83	4.03-7.19	5.51±1.22
Mn (µg/g)	24.8-61.6	35.4±12.3	27.8-56.6	40.7±7.81	43.1-56.3	48.6±5.28	41.7-63.0	55.0±8.2
Na (µg/g)	107-346	203±96	120-455	248±106	70.9-214	139±61	153-277	192±42
Ni (µg/g)	1.24-4.17	2.78±1.03	1.68-6.65	3.73±1.79	1.57-4.52	3.21±1.19	1.93-6.73	3.94±1.69
P (mg/g)	0.80-1.48	1.07±0.25	0.43-1.62	0.96±0.44	0.58-2.16	1.45±0.53	0.72-1.59	0.97±0.29
Pb (µg/g)	3.42-37.3	18.1±14.8	5.17-79.1	31.4±27.9	7.12-38.4	16.9±13.2	5.31-37.4	17.7±11.7
Rb (µg/g)	5.15-15.5	10.5±5.4	7.45-20.9	12.8±4.22	6.99-27.2	20.3±9.7	7.34-24.3	15.4±6.2
Sb (ng/g)	21.1-83.9	49.3±26.5	45.6-80.7	59.5±11.5	14.1-57.4	34.2±16.7	2.61-51.5	28.1±18.1
Sc (ng/g)	13.6-49.1	32.8±14.3	4.98-57.7	33.7±18.7	21.4-60.6	35.6±18.6	22.4-75.7	50.6±20.8
Se (ng/g)	56.3-108	84.6±21.2	50.9-131	85.7±40.2	40.1-116	61.6±27.3	24.1-45.8	34.5±8.8
Sr (µg/g)	24.5-87.2	53.4±19.9	22.4-50.2	36.3±9.9	20.6-52.4	31.9±11.1	25.5-57.1	39.0±10.6
Th (ng/g)	9.33-80.1	41.1±24.0	6.60-97.5	56.8±37.7	20.0-115	54.8±35.1	16.9-86.5	45.5±22.8
V (µg/g)	0.69-2.31	1.54±0.63	0.67-1.21	1.04±0.27	1.02-2.73	1.94±0.68	0.83-2.89	1.75±0.79
Zn(µg/g)	17.0-30.2	22.4±4.2	10.0-70.5	34.9±21.0	7.90-30.1	19.6±9.6	10.9-30.2	20.5±6.4

Table IV.10 Range and Mean elemental contents in curry leaves (n=28) and its comparison with literature studies

Element	Curry Leaves (n=28)			Gopalan et al [7]	Balaji et al [38]	Garg &Singh [39]
	Range	Mean ±SD	Median±SD			
As (ng/g)	31.3-271	78.2±47.7	69.8±44.4	ND	ND	ND
Ba (μg/g)	19.3-80.4	42.2±16.4	38.6±10.7	ND	ND	ND
Br (μg/g)	1.69-15.3	7.74±4.44	6.70±2.38	ND	2.96±0.35	64.1
Ca (mg/g)	9.44-33.2	18.4±5.24	17.2±4.16	8.30	22.9±1.05	ND
Cd (μg/g)	0.98-5.17	2.69±1.05	2.47±0.55	ND	ND	ND
Ce (μg/g)	0.90-88.8	5.93±16.3	2.92±15.4	1.98	5.5±0.2	5.43
Cl (mg/g)	0.76-4.02	2.04±0.80	1.84±0.57	ND	ND	740
Co (ng/g)	20.4-168	80.4±40.3	72.2±25.8	0.6	ND	2.27
Cr (μg/g)	0.52-2.05	0.82±0.32	0.79±0.27	ND	ND	ND
Cs (ng/g)	12.2-98.7	39.8±21.8	36.2±15.1	ND	ND	ND
Cu (μg/g)	1.39-63.3	16.5±16.8	10.7±10.8	ND	ND	ND
Fe (μg/g)	72.5-283	158±45	154±36.9	93	ND	1920
Hg (ng/g)	1.23-76.5	03±17.6	27.7±13.2	ND	ND	133
K (mg/g)	10.3-30.3	18.2±5.5	17.5±3.50	ND	17.8±0.6	22.6
La (μg/g)	1.13-10.7	4.33±2.19	4.01±1.68	ND	ND	ND
Mg (mg/g)	1.14-7.19	4.13±1.48	4.09±1.06	4.4	8.3±0.4	ND
Mn (μg/g)	24.8-63.0	44.6±11.3	44.1±6.69	15	67.15±3.31	86.9
Na (μg/g)	70.9-455	199±87	187±67	ND	1710±70	1020
Ni (μg/g)	1.24-6.73	3.44±1.48	3.26±0.96	ND	ND	ND
P (mg/g)	0.43-2.16	1.09±0.42	1.02±0.30	0.57	ND	8.1
Pb (μg/g)	3.42-79.1	21.5±18.8	11.8±13.2	ND	ND	ND
Rb (μg/g)	5.15-27.2	14.5±7.0	14.3±3.86	ND	ND	15.6
Sb (ng/g)	2.61-83.9	43.8±21.8	45.6±14.1	ND	ND	ND
Sc (ng/g)	4.98-75.7	38.1±18.7	40.1±12.4	ND	ND	730
Se (ng/g)	24.1-131	67.5±33.7	58.5±18.7	ND	ND	670
Sr (μg/g)	20.6-87.2	40.3±15.0	37.8±11.7	ND	ND	ND
Th (ng/g)	6.60-115	49.6±29.8	41.9±19.0	ND	ND	710
V (μg/g)	0.67-2.89	1.54±0.65	1.02±0.23	ND	ND	ND
Zn(μg/g)	7.90-70.5	24.9±13.7	22.8±11.0	20	ND	89.4

Incidentally, leaves from Churachandpur, a sleepy town without any industrial base, from the state of Manipur in North-East shows the lowest concentrations of As, Hg, Sb and Cd. However, leaves from Cuttack contain higher concentrations of Cr (0.86±0.03 μg/g) and Se (108±4 ng/g), In general, elemental concentrations vary in a small range. Br content, however, from this seashore city of Orissa is highest.

It is observed from Table IV.6 that elemental contents in leaves from the **West zone** vary in a much wider range probably because of large variation in soil characteristics. Br, Ce, Hg, Pb, Sc and Th vary by an order of magnitude and more. Leaves from Baroda contain higher concentrations of Cl, Mg and Zn while those from Indore show highest Ca and K contents. Samples from Mumbai-2 (BARC, Mumbai) have higher Co, Cr, Fe, P and Se contents whereas those from Pune are rich in Na. Among toxic elements, Bhilwara, the industrial city of Rajasthan has higher Hg and Sb contents. Higher Cd and Pb contents are observed in leaves from Mumbai-1 and Nagpur, mega cities of this zone. Yet again, sample collected from near sea-shore (Mumbai-2) has higher Br content.

Most of the samples collected from the **North zone** are from small suburban towns except Lucknow-capital city of Uttar Pradesh. Hence, elemental contents do not vary much. Not surprisingly, a look at Table IV.7 shows that leaves from Lucknow contain the highest As, Br, Hg and Cd contents. However, the samples from this city are enriched in Co, Cs, Fe, K, Mn and Zn. Bijnaur leaves have highest Ca, Cl, Cu and Rb contents while Roorkee sample is rich in Na. Higher Sb and Pb contents are observed in leaves from Pathankot, an industrial town.

Elemental contents in leaves from **South zone** vary in a relatively wider range compared to those from North zone. Cu and Sb contents vary by 20-fold. A cursory look into Table IV.8 shows that leaves from Pallakad, least polluted city are enriched in Ca, Cl, Cu, Mg, Na, P and Se. However, leaves from Vizag (near Bay of Bengal) known for Navy and other heavy industries are rich in Co, Cs, Fe, Rb and Zn. Pb content is highest in leaves from Hyderabad, a mega city. Similar to our observation from other zones, Vizag, sample has highest Br content. Thus Br content is high in samples from cities near sea. Further, mean content is highest for samples from South zone (11.0 ± 4.8 μg/g) and least in North zone (4.21± 1.97μg/g). It is reported that higher Br concentration in plant species from Atlantic forests could be attributed to air pollution due to sea and could be used for biomonitoring [42].

(ii) Zonal variations: Bar plots showing variation in minor, essential and toxic trace elemental contents in curry leaves from four zones are shown in Fig. IV.4, 5 and 6 respectively. A comparison of elemental contents of curry leaves from 4 different zones (Table IV.9) shows that leaves from Western zone are most enriched in minor (Na, K and Fe) and trace (Cr, Zn, Co and Se) elements. Leaves from the North zone are especially enriched in Na while those from South zone show the highest Mg and Cl contents. West zone leaves contain higher contents of As, Hg, Pb, Sb and Th. This is understandable as leaves were collected from most populous cities. Br and Ni contents are higher in leaves from the South zone. Most elemental contents in leaves from the East zone are at par with the total mean contents as listed in Table IV.10 except Cs, which is lowest. Ca content in North zone is highest (25.7±4.4 mg/g) whereas Se is lowest in South Zone (34.5±8.8 ng/g). A comparison of means and medians (Table IV. 10) shows that medians in all cases are lower compared to the means by ~5%, though in cases of Ba, Cd, Cl, Cs and Zn, medians are still lower by 8-9%. It may be concluded that most variations in elemental contents are uniform all over India irrespective of wide differences in soil characteristics and the variation seem to be real rather than artificial. In other words, it is due to the inherent nature of the plant specie. Elemental uptake by a plant is its characteristic property and may depend on the use of fertilizers, irrigation water and different

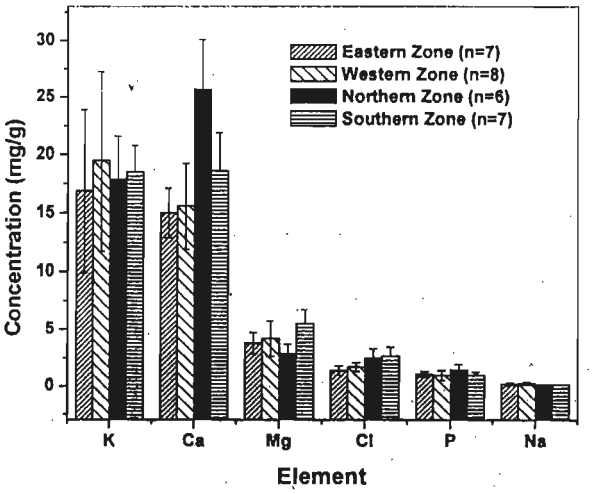


Fig. IV.4 Concentration of minor elements in Curry leaves from different zones in India

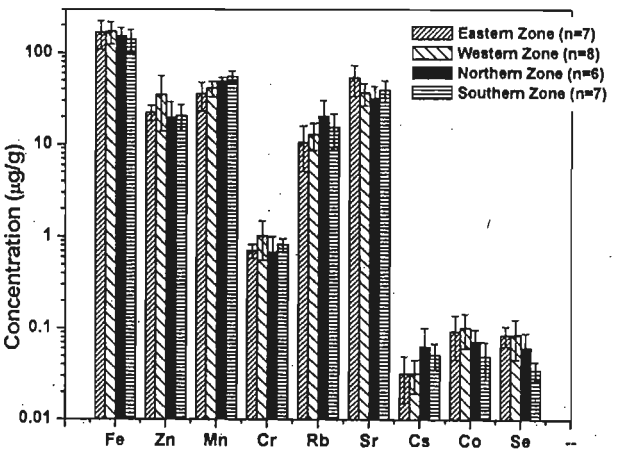


Fig. IV.5 Concentration of trace elements in Curry leaves from different zones in India

climatological conditions. *Murraya Koenegii* leaves are enriched in K, Mg, Ca and P as minor constituents (> 1 mg/g) and trace amounts of Fe, Mn, Cu, Rb and Zn (>20 μg/g). However, V and Cr whose compounds are considered insulin like are found at ~1μg/g only. It seems bioavailability rather than the total amount of an element is more important. Curry leaves are able to supply V, Cr in a suitable form so as to enhance its importance in curing diabetes [9]

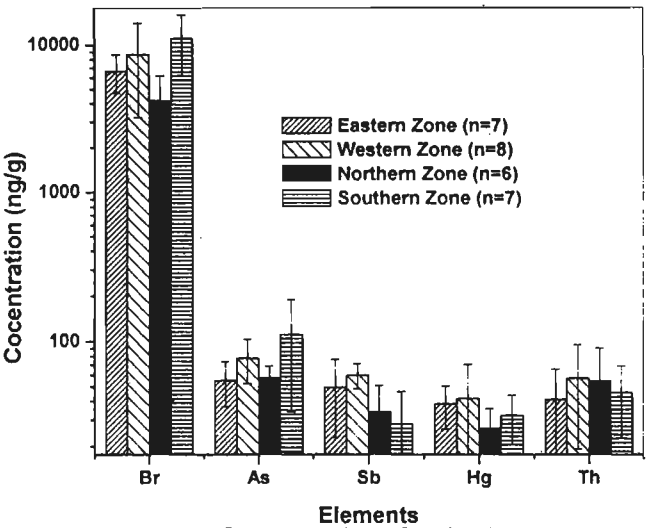


Fig. IV.6 Concentration of toxic elements in Curry leaves from different zones in India

(iii) Interelement Correlations: Alkali and alkaline earth elements (sodium, potassium, magnesium and calcium) together with chloride ions form the minor constituents and must be in balance in extra cellular fluid responsible for muscular irritability. Magnesium has been particularly shown to play a significant role as a regulatory cation in direct and indirect traumatic brain injury [43]. Recently it has been reported that trace amounts of rubidium and cesium help in the breakdown of starch to glucose ratio [44]. Rb and Cs contents in East, West, North and South zones vary linearly with r = 0.96, 0.85, 0.82 and 0.66 respectively. However, regression coefficient for all 28 samples is found to be 0.82 as shown in Fig. IV.7. Thus correlation is excellent in leaves from the East zone but poor in South zone. K/P ratio is considered as an indicator of diagnostic importance. It is found to vary in a large range of 6.39 to

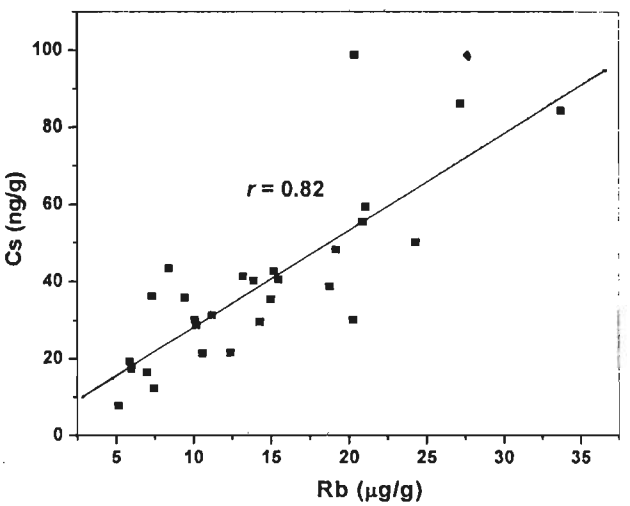


Fig. IV.7 Correlation of Rb vs Cs in Curry leaves

65.9 in four zones with a mean value of 18.6±11.2. However, Ca/P ratio lies in a comparatively smaller range of 8.09-48.8 with a mean value of 19.2±9.9. Thus, both the ratios are comparable supporting the view that K, Ca and P concentrations are in comparable range irrespective of the zone from where curry leaves are derived.

A comparison of our elemental concentration data with those reported in the literature in Table IV.10 shows that Zn content is comparable with those reported by Gopalan et al [7] while Ca, Ce, K and Mn contents are at par with those reported by Balaji et al. [38]. Rb value is in perfect agreement with that of Singh and Garg [39]. Thus, other concentrations should also be real.

***(iv) Use of Curry Leaves/Extract in diabetes*:** The aqueous extract of *Murraya koenigii* leaves have been extensively evaluated for its hypoglycemic activity without any side effects and toxicity [45-47] where it has been shown to considerably reduce blood sugar levels. It is now well known that Cr, V, Mn, Zn, Cu and Se play an important role in biochemical processes and especially in *diabetes*. Haratake et al. [48] showed that when V (IV, V) hydroxamic acid complex is injected into the streptozotocin induced diabetic mice glucose levels were effectively lowered. Thus, vanadium complexes show insulin like activity. Chromium also plays an important role in diabetes treatment, as it is required for maintenance of normal glucose metabolism. It is directly related to the function of insulin by way of glucose tolerance factor (GTF), which contains glutamic acid, cysteine and niacin. Cr (III) is absorbed in GTF to the extent of 10-85% as GTF is essential for the efficient use of insulin and enhances the removal of glucose from the blood. Yang et al. [49] reported that Cr (III) complexes play a key role in carbohydrate and lipid metabolism. Cr(phenylalanine)$_3$ complex has been shown to be insulin sensitive.

An earlier report from our group has indicated lowering of zinc level in the blood of diabetic patients [50]. About 85% of zinc combines with protein for transport after its absorption and its turnover is rapid in the pancreas. Hasse and Maret [51] showed that insulin/zinc interactions, insulinomimetic effects of zinc and controlling the cellular redox state indicate specific role of zinc in pathobiochemistry of *diabetes*. Deficiency of zinc causes diabetic hyposmia, hypogeusia or coma. In a Russian patent, Akbarov and Aripkhodzhaeba [52] have shown that coordination compound of Mn with glutamic acid and Vitamin C intensively lowers the blood glucose level in *diabetes* and shows diuretic action. Mueller et al. [53] have shown

that glutathione peroxidase and thioredoxin reductases selenium contribute to the maintenance of cellular antioxidative balance when taken up at the recommended dietary level of 150 μg/d. Thus, high doses of selenate (Selenium VI) have been shown to normalize hyperglycemia. Also Cu deficiency has been correlated with type II *diabetes* [54]. Thus all these elements as coordination compounds show strong correlation with *diabetes* and their supplementation in the form of natural herbs may be responsible for the antidiabetic action of *Murraya Koenigii* leaves.

(v) Toxic Elements; Toxicity of medicinal herbs is of much greater concern today then ever before [55]. In recent years much emphasis is being laid on toxic element contents as several western countries have banned *Ayurvedic* drugs. A cursory look at Table 8 shows that mean As (78.2±47.7 ng/g), Cd (2.69 ± 1.05 μg/g), Pb (21.5±18.8 μg/g) and Hg (23.2±17.6 ng/g) are present in significant amounts. Though As and Hg contents are below permissible limits of 10 and 1 ppm respectively slated by USFDA but Cd and Pb contents are somewhat higher, more than permissible limits of 0.3 and 10 ppm respectively. High Pb content could be due to vehicular and industrial emissions in cities. Similarly Th, a radioactive element is also present in significant amounts (49.6±29.8 ng/g) Curry leaves are used more as a spice and flavouring agent and its total consumption may be just about a few grams per day. Therefore, total dietary intake of toxic elements is insignificant to be a cause of concern.

IV.9 ORGANIC CONSTITUENTS

Three new compounds with R_f values of 0.53, 0.41 and 0.38 respectively were separated from the ethanolic extract. These were identified and confirmed by spectral methods and GC-MS fragmentation patterns.

(i) 3-methylthiopropanenitrile; Yellowish oil, ^{1}H NMR in $CDCl_3$ (δ): 2.81 (t, 2H, H-3), 2.78 (t, 2H, H-2), 2.09 (s, 3H, H-6) ^{13}C NMR in $CDCl_3$: 117.7 (C-1), 30.5 (C-3), 19.4 (C-2), ^{1}H & ^{13}C NMR spectra matched with that reported in Aldrich library [56] database. IR spectral assignments and mass fragments are listed in Table IV.11.

Table IV.11. IR and Mass spectral assignments of 3-methylthiopropanenitrile

IR Spectral bands		GC-MS Fragments	
Wavenumber (cm^{-1})	Assignment	(m/z)	Assignment
3442	ν_{N-H}	101	$C_4H_7NS^{+\cdot}$
3123	ν_{C-H}	73	$C_3H_5S^+$
2187	$\nu_{C\equiv N}$	61	$C_2H_5S^+$
1626	ν_{C-C}	60	$C_2H_4S^{+\cdot}$
1593	δ_{C-H}	54	$C_3H_4N^+$
643	ν_{C-S}	53	$C_3H_3N^{+\cdot}$

Mass spectrum of 3-methylthiopropanenitrile and proposed mechanism explaining all the fragmentation peaks is shown in. IV.8.

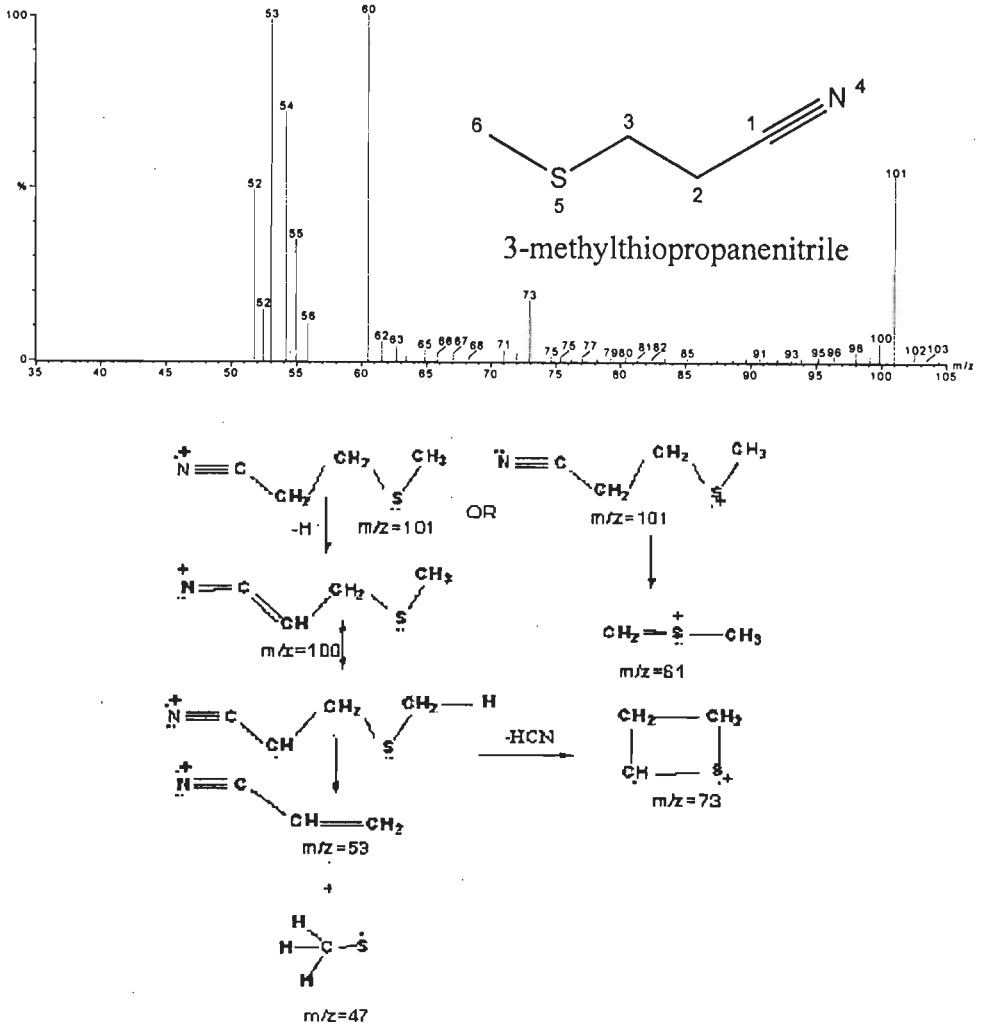


Fig. IV.8 Mass spectrum and fragmentation pattern of 3-methylthiopropanenitrile

This compound is a plant product of biosynthesis of glucatropaelin– a glucosinolate whose breakdown products may be responsible for the characteristic flavour of curry leaves. The GC-MS spectrum reported by Salamon and Davies [57] is in accordance

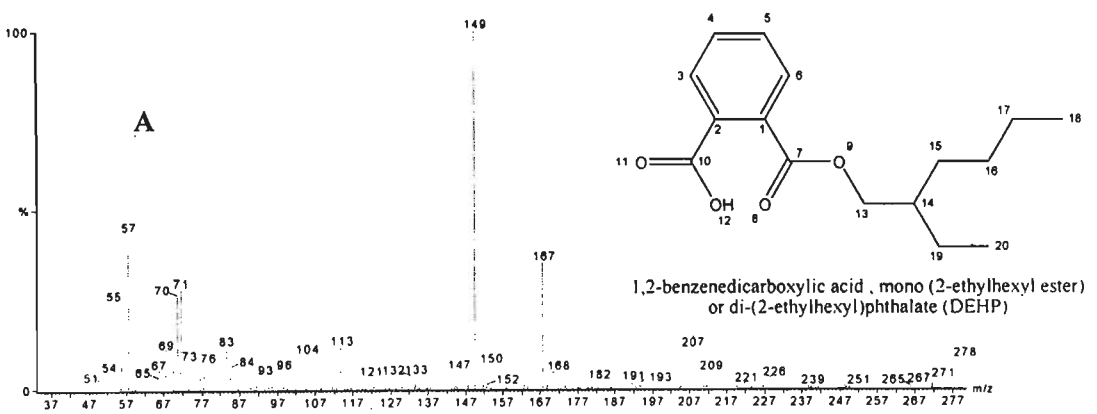
with our observations. It has also been detected as aroma component of cantaloupe Cucumis where it was identified by GC-MS [58]. The biological role of glucatropaelin and their degradation products is not well understood but it may act as a sink for nutrients like nitrogen and sulfur while the products of hydrolysis may play a role in the plant defense system against insects, fungi and microorganism infections.

(ii) 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl ester): White crystalline solid, m.pt = 221°C, ^{1}H NMR in $CDCl_3$ (δ) 8.24, 8.18, 7.68, 7.71 (m, benzylic protons), 11.0 (s, H-12), 2.07 (m, 1H, H-14), 1.25, 1.29, 1.33 (m, 2H, H-17), 0.96 (t, H-20): ^{13}C NMR in $CDCl_3$ (δ): 130-133 (C, benzylic carbons), 166.0 (C-10), 169 (C-7), 69.7 C-13), 39.6 (C-14), 30.8 (C-15), 29.4, (C-16), ^{1}H & ^{13}C NMR spectrum matched with Aldrich library [56]. IR spectral assignments and mass fragments are listed in Table IV.12.

Table IV.12 IR and Mass spectral assignments of 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl ester)

IR Spectral bands		GC-MS Fragments	
Wavenumber (cm^{-1})	**Assignment**	**(m/z)**	**Assignment**
3438	ν_{O-H}	278	$C_{16}H_{22}O_4^{+\cdot}$
2921	ν_{C-H}	167	$C_8H_7O_4^{+}$
1704	$\nu_{C=O}$ Carboxylate	149	$C_8H_5O_3^{+}$
1631	ν_{C-C}	113	$C_8H_{17}^{+}$
1465	δ_{O-H}	57	$C_4H_9^{+}$
1384	ν_{C-O}		

Mass spectrum and proposed fragmentation peaks of 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl ester) are shown in Fig. IV.9 (A & B).



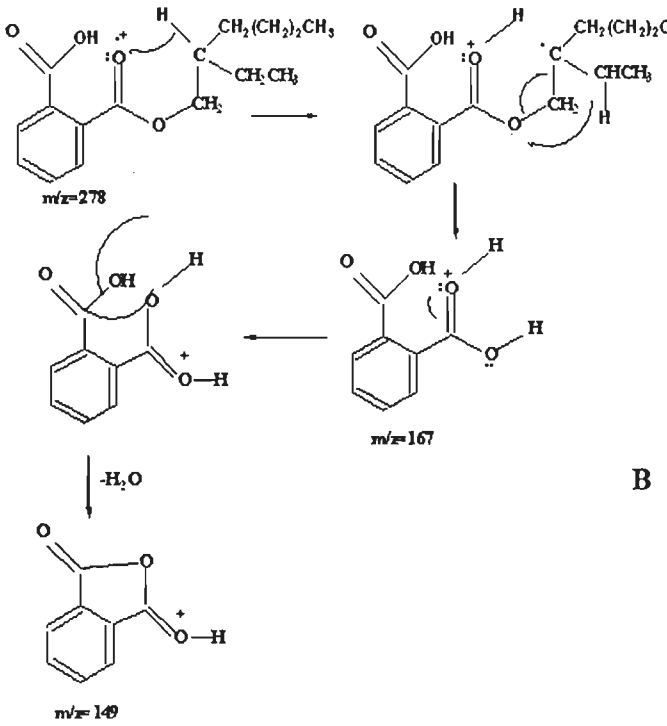


Fig. IV.9 Mass spectrum (**A**) and fragmentation pattern (**B**) of 1,2-benzenedicaroxylic acid mono (2-ethyl hexyl) ester

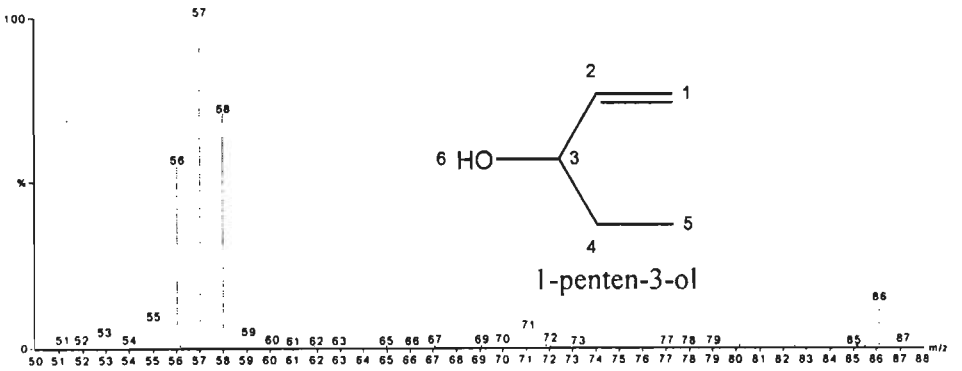
Colon et al. [59] have reported m/z = 149 as one of the characteristic ions for the detection of phthalate ester. Bell et al. [60] have suggested another name for the ester as di-(2-ethylhexyl) phthalate (DEHP) and reported that it reduces serum cholesterol and lipid biosynthesis in rats and rabbits. In a recent study Xie et al [61] have suggested that curry leaves may prove to be of clinical importance in improving the management of high cholesterol level and type 2 *diabetes*. Labrada [62] has suggested the ester as an allelopathic compound, which reduces the need for weed management in other crops. Many esters have distinctive odour, which has led to their widespread use as artificial flavouring and fragrances. This compound may act as the most potent ligand, which can coordinate with the essential micronutrients such as Cr, Mn, Fe, Co and Zn making them bioavailable to our body system. It not only contributes to the aroma of curry leaves but also emphasizes its importance as a medicinal herb.

(iii) 1-penten-3-ol: Colourless liquid, ^{13}C NMR in D_2O (δ): 136.3 (C-2), 115.8 (C-1), 75.6 (C-3), 30.0 (C-4) ^{1}H NMR & ^{13}C NMR spectrum matched with Aldrich library [56]. IR spectral assignments and mass fragments are listed in Table IV.13.Mass

spectrum and the proposed mechanism explaining all the fragmentation peaks of 1-penten-3-ol are shown in Fig. IV.10.

Table IV.13 IR and Mass spectral assignments of 1-penten-3-ol

IR Spectral bands		GC-MS Fragments	
Wavenumber (cm^{-1})	Assignment	m/z	Assignment
3404	ν_{O-H}	86	$C_5H_{10}O^{+\cdot}$
2956	ν_{C-H}	71	$C_4H_7O^{+}$
1633	$\nu_{C=C}$	58	$C_3H_6O^{+\cdot}$
1465 & 1381	δ_{O-H}	57	$C_3H_5O^{+}$
1060	ν_{C-O}	56	$C_3H_4O^{+\cdot}$



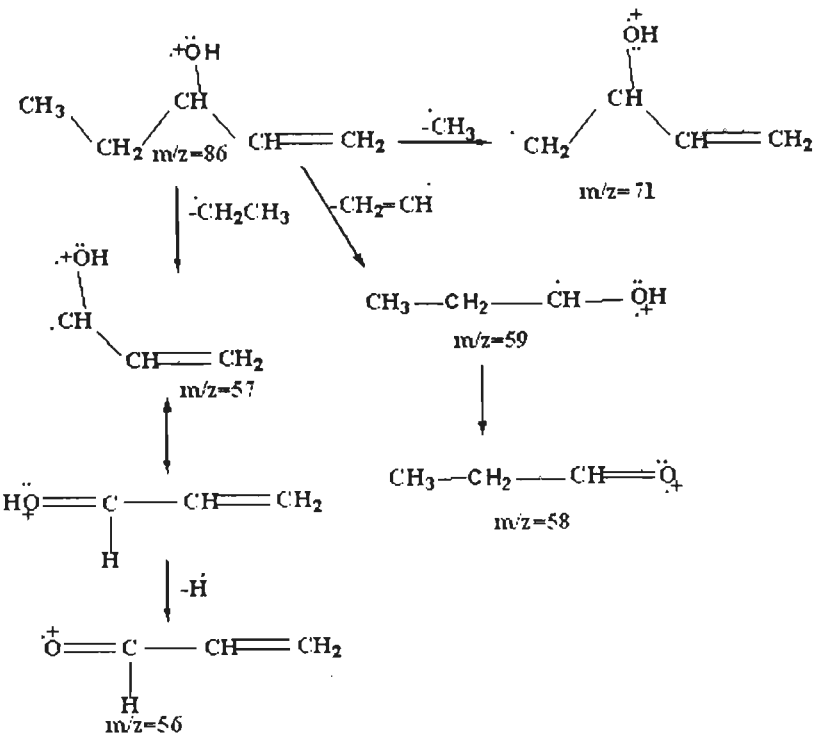


Fig. IV.10 Mass spectrum and fragmentation pattern of 1-penten-3-ol

This compound has been already identified in lettuce leaves [63] and turnip [64] but not in curry leaves so far. Fischer et al. [65] observed large release of pentenol following mechanical wounding of leaves. Enzymatic formation of this compound can be explained by known O_2-dependent lipoxygenase (LOX) reaction, which depends on α-linoleic acid or its hydroperoxide. Being alcoholic in nature, it acts as an antioxidant, which may account for the antidiabetic and anticancerous properties of *Murraya Koenigii.*

CONCLUSION

On the basis of INAA and AAS data on essential minor, trace and toxic elements in curry leaves (n=28) from various Indian states and the three organic constituents identified in ethanolic extract, following generalizations can be made:

- Curry leaves are enriched in K (18.2±5.5 mg/g), Mg (4.13±1.48 mg/g), P (1.09±0.42 mg/g) and Fe (158±45 μg/g).
- High contents of V (1.54±0.65 μg/g), Cr (0.82±0.32 μg/g) and Zn (24.9±13.7 μg/g) could be responsible for its anti-diabetic properties.
- Most elements in leaves from East zone vary in a narrow range and these are at par with the total all India mean contents. Two industrial cities Jamshedpur and Bokaro from the East zone show high Hg, Cd As, Ni and Sb contents.
- Leaves from West zone are enriched in most minor (Na, K and Fe) and trace (Cr, Zn, Co and Se) elements
- Leaves from the North zone are enriched in Na while those from South zone have highest Mg and Cl contents.
- Toxic element (As, Hg, Pb, Sb) contents in West zone samples are higher whereas Th. Br and Ni contents are higher in South zone samples. However, these are well below the USFDA permissible limits.
- Medians for most elements are lower compared to the means by ~ 5%, though in cases of Ba, Cd, Cl, Cs and Zn, these are still lower by ~9%.
- Rb and Cs contents are linearly correlated (r = 0.96). As per US patent Rb and Cs help in the breakdown of starch to glucose ratio.
- A novel compound 3-methylthiopropanenitrile, a plant product of biosynthesis of glucatropaelin– a glucosinolates, has been identified and its breakdown

products may be responsible for the characteristic flavour of curry leaves.

- 1,2-benzenedicaroxylic acid mono (2-ethyl hexyl) ester, reported to reduce serum cholesterol and lipid biosynthesis in rats and rabbits could be responsible for antidiabetic propetry of curry leaves. Besides, the ester is used extensively as a flavouring agent and is an allelopathic compound, which reduces the need for weed management in other crops. Carboxylate group in this compound may possibly act as a ligand coordinating with the essential micronutrients (Cr, Mn, Fe, Co and Zn).
- 1-penten-3-ol, reported in lettuce leaves and turnip has been identified in curry leaves. Being alcoholic in nature, it may act as an antioxidant and possibly account for its antidiabetic and anticancerous properties.

Thus, antidiabetic, antioxidant and cholesterol reducing medicinal properties of curry leaves can be attributed to the presence of various organic constituents as well as associated trace metals.

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CHAPTER V

ANTIDIABETIC HERBS & FORMULATIONS

A part of the work was presented at the Seventh International Conference on Methods and Applications in Radioanalytical Chemistry (MARC VII), Kailua-Kona, Hawaii, April 3-7, 2006 and the paper will appear in *J. Radioanal. Nucl. Chem.* (2007)

V.1 DIABETES MELLITUS

Diabetes Mellitus is a chronic disease in which the body fails to produce any insulin (Type 1, also called insulin-dependent or juvenile-onset), or the insulin that it produces is unable to adequately trigger the conversion of food into energy (Type 2, also called non-insulin-dependent or adult-onset). This raises the blood glucose level. Under healthy conditions, insulin produced by the pancreas controls blood-glucose levels. It flows through our blood to various tissues where it binds to cell-surface receptors initiating a complex biochemical cascade culminating in glucose uptake into cells to fuel metabolic processes. As blood-glucose level declines, the pancreas shuts down insulin production to prevent hypoglycemia (low blood sugar) and, in turn, the liver, the body's nutrient-processing organ, starts releasing glucose back into the blood [1].

Generally, *diabetes mellitus* has no symptoms and in type 2 diabetes in particular, symptoms develop slowly but often vary. Two symptoms that occur with the disease are increased thirst and frequent urination [2]. Excess glucose circulating in the body draws water from tissues leading to dehydration. Other symptoms are weight loss, blurred vision, increased hunger, frequent skin, bladder or gum infections, irritability, tingling or numbness in hands or feet, slow to heal wounds, and extreme unexplained fatigue [3]. Some frequent complications of long-term diabetes are diabetic neuropathy [4], proliferative retinopathy [5], cardiovascular- renal disease and atherosclerosis [6, 7]. If there is too much insulin in the body compared to the amount of blood sugar and the blood sugar falls below normal level, a condition known as hypoglycaemia occurs. The first sign is mild hunger, quickly followed by dizziness, sweating, palpitation, mental confusion and eventual loss of consciousness [8].

Diabetes mellitus has affected more than hundred fifty million people worldwide and is on continuous rise. In India, the prevalence rate of diabetes is estimated to be ~8%, especially in urban population [9]. In Chinese medicine system, it is referred to as thirst disease and is caused due to excessive heat in the stomach. The Chinese pharmacopoeia recommends many herbs for this disease [2]. Providing modern medical healthcare in developing countries such as India is still a far-reaching goal due to economic constraints. Therefore, it is prudent to look for options in herbal medicine for diabetes.

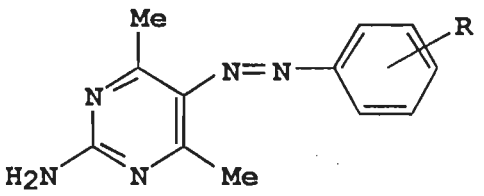
V.2 HERBAL TREATMENT-MECHANISMS

Since antiquity, diabetes has been treated with plant medicines. Recent scientific investigations have confirmed the efficacy of many herbs and herbal preparations some of which are remarkably effective [10]. Ayurvedic herbs herbs have proven antioxidant, anti-inflammatory and immunomodulatory effects [11]. The World Health Organization (WHO) has recognized the importance of antidiabetic plants in the development of economic and effective treatment for diabetes. According to a recent survey of 2003, over 150 million people worldwide are estimated to be affected by *diabetes* [12]. Oral antidiabetic agents exert their effects by various mechanisms

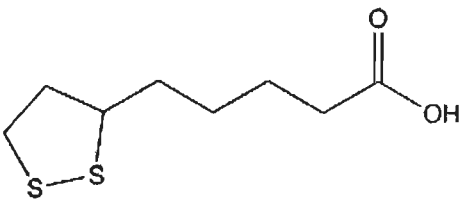
- Stimulating β cells in the pancreas to produce more insulin
- Increases the sensitivity of muscles and other tissues to insulin
- Decreases gluconeogenesis by the liver
- Delaying the absorption of carbohydrates from the gastrointestinal tract.

As early as 600 B.C., *Sushruta* advised the affluent diabetes to indulge in vigorous exercise but exhorted the thin diabetic not to exert too much. It has been demonstrated that exercise enhances glucose uptake and induce a fall in blood sugar [11]. Natural compounds with antidiabetic activity include complex carbohydrates, alkaloids, glycopeptides, terpenoids, peptides and amines, steroids, flavonoids, lipids, coumarins, sulfur compounds, metal ions and others [13].

Plants are considered as a rich source of essential and trace elements along with several organic compounds like α-lipoic acid, phenylazopyrimidine, and nicotinamide. These are prescribed because of their bioavailability and least side effects accompanying low cost. Hence a search for herbal antidiabetic agents has been initiated.



Phenylazopyrimidine derivative



α-lipoic acid

V.3 TRACE ELEMENTS AND DIABETES

Chromium, vanadium, manganese, zinc, copper, iron and selenium play an important role in biochemical processes and especially so in *diabetes*. Trace amounts of Cr and V are essential for proper carbohydrate metabolism. Chromium is required for maintenance of normal glucose metabolism and directly related to the function of insulin by way of glucose tolerance factor (GTF) containing glutamic acid, cysteine and niacin. Cr(III) is absorbed in GTF to the extent of 10-85% as GTF is essential for the efficient use of insulin and enhances the removal of glucose from the blood [14]. Yang et al. [15] reported that Cr(III) complexes play a key role in carbohydrate and lipid metabolism. Cr (phenylalanine)$_3$ complex has been shown to be insulin sensitive. Sekar et al [16] have shown that protein tyrosine kinase activation has a major role in the therapeutic efficacy of V in treating *diabetes mellitus*. Haratake et al. [17] showed that when V (IV, V) hydroxamic acid complex is injected into the streptozotocin induced diabetic mice glucose levels were effectively lowered. Preet et al. [18] observed that lower doses of Na_3VO_4 act as an efficient antidiabetic agent to effectively control the long-term complications of diabetes in tissues like peripheral nerve. Tas [19] observed that vanadyl sulfate treatment improved glycemic control and prevented hyperphagia and polydipsia in diabetic rats. Thus, vanadium complexes showed insulin like activity.

Se is a dietary antioxidant and its supplement could also be a useful therapeutic measure to delay the onset of diabetic nephropathy [20]. High doses of selenate (selenium VI) have been shown to normalize hyperglycemia [21]. Heikkila and Cabbat [22] observed that DETAPAC, an iron-chelating agent given to swiss-webstar mice prior to alloxan protects them from diabetogenic action of alloxan. The complication of diabetes may be mediated through oxidative stress and though indirectly, zinc also plays a key role in cellular antioxidative defence [23]. Garg et al [24] observed a lowering of Zn level in the blood of diabetic patients. In a Russian patent, Akbarov and Aripkhodzhaeba [25] have shown that manganese coordination compound with glutamic acid and Vitamin C intensively lowers the blood glucose level in diabetes and shows diuretic action. Frank et al. [26] correlated Cu deficiency with Type 2 diabetes. Diabetics often have low levels of Mg, a mineral required for many physiological functions. Individuals with the least amount of Mg in their blood have twice the diabetes incidence compared to those with highest levels. It has been

shown that when Mg deficient patients were given supplements, their responsiveness to insulin and glucose metabolism improved [27].

V.4 HERBAL CONCERN

Antidiabetic plants have been used since long by herbal medicine practitioners in India and China for treating individuals with non-insulin-dependent (type 2) diabetes [2, 28]. In such cases patient's response must be carefully monitored. It has been observed that significant benefits can be gained from such therapies. However, the use of such herbs by type 1 (insulin-dependent) diabetics can be hazardous and require that such patients carefully monitor their blood sugar to prevent hypoglycemic and hyperglycemic episodes. Consultation with the prescribing physician is necessary and an integrative management of the case by conventional and herbal practitioners working together should be preferred. The shared goal would be to regulate the dosage of both types of medication and enable a smooth transition to lower dependence on insulin in cases where such is desirable and attainable. While hypoglycemic herbs may offer promise in the treatment of diabetes in their combined effect with insulin, treatment is inherently disruptive and extreme caution must be exercised in order to promote a smooth transition, maintain suitable blood sugar levels and avoid insulin shock.

V.5 DESCRIPTION OF HERBS

The term *diabetes mellitus* was recognized as *Madhumeha* in primeval times. Our ancient physicians had mastered the science of managing this disorder with effective balance of *Aushada* - some herbs or plant food sources as medicine. Those indigenous plant sources may not be as effective as insulin in lowering the blood sugar but the combination therapy seems to equate with the modern methods of drug, diet and exercise. However, down the line in the quest of advancement, the indigenous therapy is extinguishing. Scientific investigations have confirmed the efficacy of many herbs, some of which are remarkably effective. Only those herbs that appear most effective, are relatively non-toxic and have substantial documentation of efficacy are covered here [29-44]

(i) Neem/Azadirachta Indica (leaves): This tree is found in the Western Himalayas of India and is cultivated in the tropical regions of the world such as Indonesia, Australia and West Africa. Its tender leaves are considered to be antidiabetic in

Ayurvedic medicine and for a variety of folk medicinal applications. Recent investigations on the leaves have identified its anticancerous properties also [29].

(ii) Gurmaar/Gymnema sylvestre (leaves): Gymnema sylvestre is known as *gurmaar* (which kills jaggery or sweet) due to the unique property of the plant to antagonize the sweet taste. It has several ethnomedicinal values as various tribals/traditional communities and rural people of India find diverse medicinal uses viz. antidiabetic, diuretic and is useful in cough and throat trouble. Besides, it has strong effect on reducing blood sugar [30]. Sometimes called *madhu-nashini,* it has a unique property of abolishing sweet taste when applied to the tongue [11].

(iii) Palas/Butea monosperma (leaves): Butea monosperma or Butea frondosa, known as Dhak, flame of the forest, Pâlāsh or Bastard Teak, is native to India and Southeast Asia, where it is used for timber, resin, fodder, medicine and as dye. The decoction of flowers is useful in diabetes and also for cough, whooping cough, stomach gas, gastrointestinal colic and insomnia. It serves as a stimulant to appetite and it sometimes made into a salve for treatment of gout [31].

(iv) Tejpatta/Cinnamomum Tamala (leaves): These leaves are used as a spice in Indian cooking for flavouring food especially curries rice etc. It is also used in Indian system of traditional medicines. It is widely used in pharmaceutical preparations because of its hypoglycemic, stimulant and carminative properties [32]. It has digestive properties and form an ingredient of many formulations prescribed for gastrointestinal disorders.

(v) Tejpan/Laurus nobilis (leaves): The leaves are used extensively in French, Italian, Spanish and Creole cooking to flavour soups, stews, sauces, marinades and poultry and fish dishes. Infusion of the leaves is reputed to soothe the stomach and relieve flatulence. Oil pressed from the berries was once a popular liniment for arthritis and sore muscles and is still used in perfumes, candles and soaps [33].

(vi) Vijaysar/Pterocarpus Marsupium (bark): The plant is commonly known as Bijasal or Bija in Hindi. The heartwood is astringent, bitter acrid, anti inflammatory, and anthelmintic. It is considered magical for diabetes. It is good for elephantiasis, leucoderma, diarrhoea, dysentery, rectalgia, cough and grayness of hair [34].

(vii) Kutki/Picrorhiza Kurroa (roots): Picrorhiza kurroa is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers and to treat dyspepsia, chronic

diarrhea, and scorpion sting. Decoction of the roots in small doses act as laxative and in larger dose, it is an abortifacient [35].

(viii) Giloy (Guduchi) /Tinospora cordifolia (roots): The root of this herb is used in seminal weakness and urinary affections. It is also a valuable tonic. Other applications of this herb include fever, gout, jaundice, torpidity of the liver, skin diseases, secondary syphilis, rheumatism, constipation, tuberculosis and leprosy. It is a blood purifier and may be useful in AIDS and other immune diseases. It is also being proposed for cancer patients before and after chemotherapy [36].

(ix) Naagarmotha/Cyperus rotundus (roots): This grass-like herb is found in South India. It has tuberous roots or rhizomes that are fragrant. Medical applications include anthelmintic, anti-fungal, anti-parasitic, anti-rheumatic, antispasmodic, aphrodisiac, astringent, carminative, demulcent, diaphoretic, diuretic, emmenagogue, galactagogue, refrigerant, stimulant and tonic [37].

(x) Garlic/Allium sativum (bulb): *Garlic* as a spice has been used for thousands of years by millions of people all over the world. *Garlic* bulb is beneficial in atherosclerosis, blood and lymph cleanser, cold and flu, colic, congestive heart failure, convulsions, cough, edema, heart disease, haemorrhoids, high cholesterol, hypertension, hypertriglyceridemia, hysteria, immune function, impotence, indigestion, nerve and bone tissue rejuvenative, paralysis, recurrent ear infection, rheumatism, skin diseases, tuberculosis, tumors, round worms and yeast infection [38]. It is widely used in the preparation of vegetable curries and sprinkling the powder on eatables

(xi) Marodphali/Helicteres isora (fruits): This East Indian shrub is often cultivated for its hairy leaves and orange-red flowers. According to the traditional healers, the spirally coiled fruit of this herb is used in griping of bowels related trouble. In Hindi, griping is named as *Marod*. Patients suffering from diarrhoea are advised to take the leachate empty stomach next morning [39].

(xii) Bitter Gourd (Karela) /Momordica charantia (fruits): It is primarily a vegetable. In tropical regions, the fruit is used for a dazzling array of medicaments. It is believed to improve numerous infections and severe conditions of cancer, leukemia, and diabetes. The leaves and fruit both have been used occasionally to make tea and beer or to season soups in the Western world [40].

(xiii) Amalaki/Emblica officinalis (fruits): It has been regarded as a sacred tree in India. The tree was worshipped as Mother Earth and is believed to nurture

humankind because the fruits are very nourishing. Fresh fruit is refrigerant, diuretic, laxative and carminative. Dried fruit is sour and astringent. It is also aphrodisiac, hemostatic, nutritive tonic (memory enhancer), rejuvenative (for *Pitta*). It increases red blood cell count. It is one of the highest natural sources of Vitamin C (3,000 mg per fruit) [41]

(xiv) Kaali Jeera/Cuminum cyminum (seeds): This variety of spice has long been recognized as an ingredient for stimulating the appetite as well as an aid in the relief of nausea. In India it had been used as a medicine for variety of ailments from toothache to paralysis. They have been used as carminatives, reducing stomach and intestinal gas and they are found to stimulate the activity of the heart and kidneys [42]. It is also an effective insecticide against houseflies. Gardeners use pepper sprays against several kinds of pests.

(xv) Fenugreek/Trigonella foenum (seeds): Fenugreek leaves are a common vegetable but its seeds are more commonly used as a spice. Dried powdered leaves called *kasturi methi* is also a spice used for providing flavour/aroma to curries. In ancient times, fenugreek seeds found a wide range of medicinal uses from the treatment of wounds to cure abscesses, arthritis, bronchitis, and digestive problems. Traditional Chinese herbalists used it for kidney problems and conditions affecting the male reproductive tract. *Fenugreek* was and remains a food and a spice commonly eaten in many parts of the world. *Fenugreek* is useful for atherosclerosis, constipation and diabetes [43].

(xvi) Jamun/Syzygium cuminii (seeds): According to *Ayurveda*, bark is sweet, acrid, hot, astringent to bowels, improves voice, and useful in treatment of asthma, thirst, fatigue, dysentery, heavy speech, bronchitis etc. Fruits are sweet and tasty and used as an astringent to bowels. According to Unani system of medicine, fruit is useful in treatment of liver complaints whereas seeds are useful in treatment of syphilis. Many traditional healers recommend its use as a preventive measure to diabetes [44].

V.6 LITERATURE SURVEY

Ethnobotanical information reports about 800 plants that may possess antidiabetic potential [45]. However, most work reported in the literature pertains to the isolation of organic constituents exhibiting hypoglycemic activity of their extracts [46-71] though some workers have also determined trace element contents and

correlated these with the cure of *diabetes*. In the following lines is reviewed literature survey on various species and the work related with *diabetes* studies.

Halim [46] observed that water extract of dried *neem* leaves administered orally to alloxan diabetic rats once a day for 8 weeks caused significant lowering of blood sugar. Kar et al. [47] evaluated the hypoglycaemic activity of the *neem* and observed significant blood glucose lowering activity. Wang et al. [48] isolated 16β,28-dihydroxy-olean-12-ene-29-acid30-β-D-glucuronide (gymnemic acid A) from *gurmaar*. It has been suggested that 13.4 mg/kg of gymnemic acid may prove to be potentially effective in the amelioration of corticosteroid-induced diabetes mellitus/hyperglycemia [49]. Trigggiani et al. [50] reviewed the current concepts dealing with the usefulness of *palas* in treating diabetic patients. *Palas* leaves have been found to possess antioxidant properties due to its ability to reduce lipid peroxidation [51]. Rana and Blazquez [52] reported eugenol (81.69%) along with appreciable amounts of β-phellandrene (4.08 %) and cymol (1.37 %) in *tejpatta*. Singh et al. [53] isolated five flavonoids kaempferol, quercetin, myricetin, kaempferol-3-O-rhamnoside and quercitrin from *tejpatta*. Tanaka et al. [54] isolated four flavonoids and six sesquiterpenes, eremanthine, dehydrocostus lactone, costunolide, zaluzanin C, zaluzanin D and reynosin from *Tejpan* leaves. Aqueous extract of *vijaysar* bark have been shown to have statistically significant hypoglycaemic activity on alloxan-induced diabetic rats [55]. Zhang et al. [56] carried out a bioassay guided phytochemical study of the ethyl acetate extract of *kutki* roots and isolated five triterpenoids. A new HPTLC method has been developed for the simultaneous quantification of picroside-I and picroside-II in *kutki* [57].

Jagetia and Rao [58] observed cytotoxic effect of *giloy* root extract on tumour cells. Oral administration of an alcoholic extract of *giloy* roots at a dose of 100 mg/kg body weight to diabetic rats orally for six weeks normalize the antioxidant status of liver and kidney [59]. A number of terpenes such as α-copaene (1.97%), cyperene (15.73%), α-bisabolene (2.14%), β-gurjunene (1.29%), 2-methoxy-8-methyl-1,4-naphthalenedione (4.01%), β-selinene (17.99%), oxo-β-ylangene (3.00%), 4,4β,5,6,7,8-hexahydro-4β,5-dimethyl-3-(1-Me ethylidene)-2(3H)-naphthalenone (8.11%), β-cyperone (26.15%), longipinocarvone (1.11%) have been found the main constituents of *Naagarmotha* roots [60]. Lanzotti [61] reviewed the major volatile and non-volatile phytoconstituents of *garlic*. Recent studies suggest that *garlic* extact

contains S-Allylcysteine, which inhibits the formation of glycation-derived free radicals [62].

Kamiya et al. [63] isolated five flavonoid glucuronides from *Marodphali*. It has found tom possess hypoglycaemic and hepatoprotective activity and is able to ameliorate biochemical damage in STZ induced diabetic rats [64]. Shetty et al. [65] observed beneficial effects of dried *bitter gourd* powder in the diet. Tannin and flavonoid contents in the various extracts of *Amalaki* have been found to have strong relation (r= 0.88) [66]. Dhandapani et al. [67] observed that supplementation of *Kaalijeera* can reduce free radical mediated oxidative stress to the cells in diabetes mellitus. Kim and Lee [68] invented a hyperglycemic and diabetic complication-preventing compound, which inhibits the activity of α-glucosidase to prevent diabetic complication.

Siddiqui et al. [69] demonstrated that combined therapy of vanadate and *fenugreek* seeds is found to be the most effective remedy in normalization of altered membrane linked functions and of glucose transport distribution without any harmful side effect. Jirovetz et al. [70] observed monoterpenes and sesquiterpenes as the major constituents of the essential oil from *Jamun* leaves. Daulatabad et al. [71] reported a number of fatty acids such as lauric (2.8%), myristic (31.7%), palmitic (4.7%), stearic (6.5%), oleic (32.2%), linoleic (16.1%), malvalic (1.2%), sterculic (1.8%), and vernolic acid (3.0%) in *Jamun* seed oil.

Compared to organic constituents, very little has been reported on the role of micronutrients, which also play an important role in diabetes [72-87]. Rajurkar and Pardeshi [72] reported 15 elements in 25 antidiabetic herbs including *neem*, fenugreek, *bitter gourd* and *Naagarmotha* by INAA and AAS. Ray et al. [73] carried out elemental analysis of anti-diabetic medicinal plants using EDXRF. Sahito et al. [74] identified 15 essential, trace and toxic elements in the water extract of various parts of *neem*. Ajasa et al. [75] determined selected toxic trace metals and macronutrients along with P in Nigerian medicinal herbs. Fatima et al. [76] investigated a large number of indigenous medicinal plants including *gurmaar* for metal contents using AAS. Khan et al. [77] determined Cr in some hypoglycemic plants including *gurmaar*. Rajasekaran et al [78] have shown several inorganic elements (V, Mn, Cr, Cu and Zn) in Aloe vera leaf gel and their role in diabetes related biochemical alterations in experimental rats. Samudralwar and Garg [79]

determined 13 elements in the Indian herbal and other medicinal preparations including *giloy*. Singh and Garg [80] determined 6 minor and 16 trace elements in cereals, vegetables and 20 spices including *tejpatta*. Chowdhury et al. [81] studied fatty acid and mineral compositions of the seeds of 20 plant species including *vijaysar*. Haider et al. [82] determined heavy metals such as Cu, Cr, Mn, Ni, Zn, Cd and Pb in *Naagarmotha* and some other medicinal plants collected from different ecological zones of India. Several scattered reports have described mineral contents in a variety of herbs from their respective countries by ICP-AES, INAA, AAS and total reflection XRF [83-88] and emphasized the importance of essential nutrients.

V.7 PRESENT STUDY

Twenty samples of 16 antidiabetic herbs including 3 commercially available capsules were analyzed for 6 minor (Na, K, Ca, Cl, Mg, and P) and 23 trace (As, Ba, Br, Cd, Ce, Co, Cr, Cs, Cu, Fe, Hg, La, Mn, Ni, Pb, Rb, Sb, Sc, Se, Sm, Th, V and Zn) elements by using NAA and AAS. The herbs so chosen are commonly used in Indian household as some of these are spices, vegetables and fruits and are easily available. In addition, 5 antidiabetic herbal formulations were also analyzed for 29 elements. Following four compounds were identified in the petroleum extract of *neem* leaves.

- 1,1,2,3-tetramethylcyclopropane
- Methyl phenyl sulfone
- n-hexanedecanoic acid and
- 9,12,15-octadecatrienal

Further, following two compounds were separated from the ethyl acetate soluble fraction of the petroleum extract of *neem* leaves.

- 7–(but-3-enyl) 1,2-dihydro cyclobutabenzene and
- 9,10 anthracenedione (2-hydroxy methyl) 1-methoxy

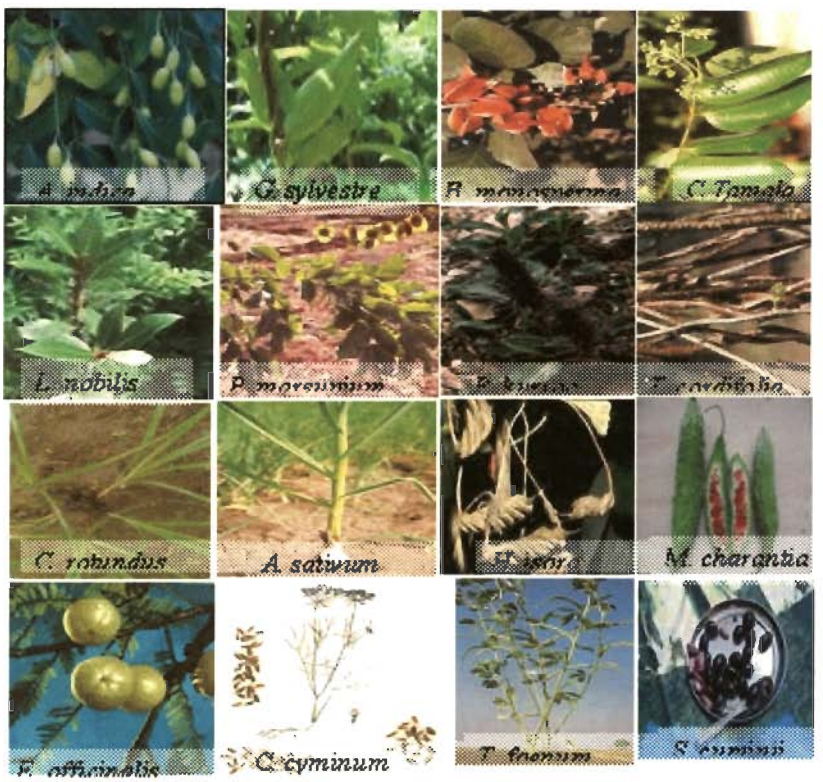
V.8 EXPERIMENTAL

(i) ***Sample Preparation:*** Four types of plant parts viz. roots, bark, seeds and fruits were selected and procured as follows

- ***Vyas Pharmacy, Indore (Powder form)*** **[V]*:*** Leaves of *Neem (A. indica), Tejpatta (C. tamala) and Tejpan (L. nobilis);* root of *Kutki (P. kurrooa);* bark of *Vijaysar (P. marsupium),* seeds of *Kaali Jeera (C. cyminun), Fenugreek (T.*

foenum) and Jaamun (S. cuminii), fruits *of Bitter Gourd (M. charantia), Amalakii (E. officinalis)*

- ***Yogi Pharmacy, Haridwar (Raw form)*** **[Y]*:*** Leaves of *Gurmaar (G. sylvestre)* and *Palas (B. monosperma)*; roots of *Giloy (T. cordifolia),* and *Naagarmotha (C. rotundeus)* and *Morarphali (H. isora),* a fruit.
- ***Local Market (Whole)*** **[L]*:*** *Jaamun* and *Fenugreek* seeds
- ***Himalaya Drugs, Bangalore (Capsules)*** **[H]*:*** Capsules of *Neem, Garlic (A. sativum)* and *Bitter Gourd.*



Details of 5 antidiabetic herbal formulations are given in Table V.1. All the samples were so chosen as that consumed by middle income group. These were cut opened and the powders were used as such. Two RMs of Peach Leaves (SRM-1547) from NIST, USA and Mixed Polish Herbs (MPH-2) from INCT, Poland were used as comparator standards.

Table V.1. Details of antidiabetic herbal formulations

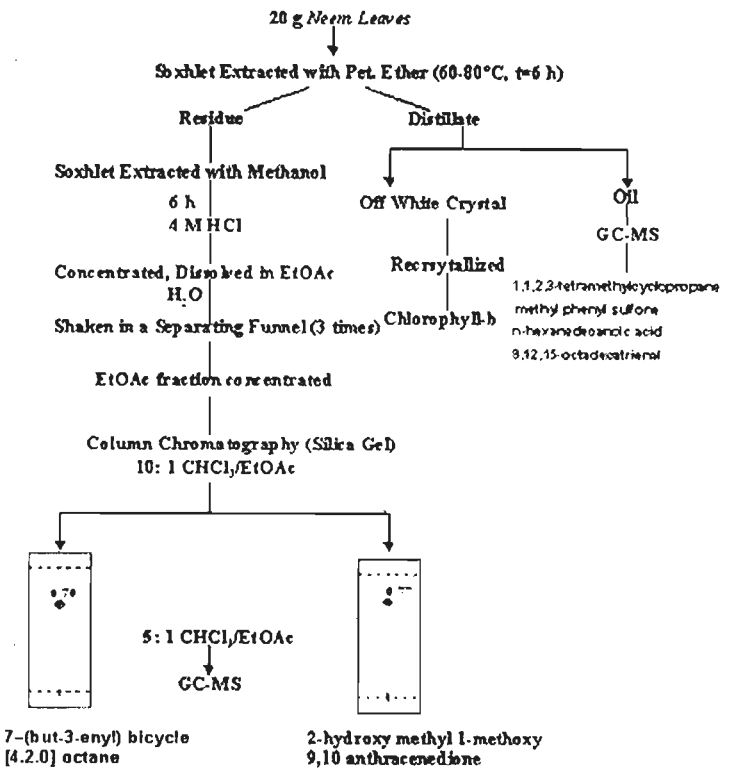
FORMULATION (Pharmaceutical Co.)	Constituents	Dosage
Diabetex Sri Jagdamba Ayurvedic Pharmacy, Haridwar,	Agar, Tagar, Sweet Chandan, Sal Sar, Khail Sar, Beet root, Krish-Sar, Seeres, Sunn, Dhava, Arjuna, Amaltas, Bhojpatra, Madhuvinashi, Tar, supari, Gurmaar, Rasol	2 tab twice a day with milk/water
Divya (Divya Yog Mandir Trust, Kankhal, Haridwar)	Giloy, Baheda, Chirayata, Amalaki, Barangi, Neem, Gurmaar, Bel, Kutki, Haldi, Ashwagandha, Hadjora, Jamun, Ajwayan, Gokhru, Anar, Haritaki, Babul	2 tab twice a day with water
Jambrushila (UAP Pharmaceuticals Pvt. Ltd., Moraiya, Ahmedabad),	Jamunbij, Mamejara, Billipatra, Shuddha shilajit, Tribang bhasmas, Gurmaar, Neem patra, Karela	2 tab thrice a day with water
Diabeticin (BACPO Pharmaceuticals Ind. Ltd, Noida)	Gurmaar, Billipatra, Shilajit, Jamun, Nyagrodha, Karela, Neem, Vijaysar, Trifala	1-2 tab twice a day with water after meals
Madhunashini (Gurukul Kangri Pharmacy, Haridwar)	Not available	2 tab twice a day with water

(ii) Irradiation and Counting: 30-40 mg aliquots of each of the samples and RMs in dried form were packed in polythene/aluminum in batches of 5 and 10. These were irradiated at a thermal neutron flux of ~10^{13} n $cm^{-2}s^{-1}$ in the CIRUS reactor at the BARC Mumbai for 2 min and 3 d respectively. Irradiation and counting details are same as mentioned in *Ch. II.7*. Cd, Ni and Pb were determined by AAS as described in *Ch. II.9*. Elemental contents were calculated by comparator method using RMs as comparators.

V.9 ORGANIC CONSTITUENTS IN NEEM LEAVES

(i) Separation: Air-dried *neem* leaves (20 g) were successively extracted with petroleum ether (60-80°C) in a Soxhlet for 6 hr. On cooling, off white crystals separate out. These were filtered and recrystallized with dichloromethane. TLC examination in $CHCl_3$/MeOH (10:1) shows a single compound (Chlorophyll-b) at R_f=0.81 with m.pt. of 192° C. Oily distillate was subjected to GC-MS wherein four compounds at R_t of 6.70, 15.3, 20.1 and 20.5, which were identified as 1,1,2,3-tetramethylcyclopropane, methyl phenyl sulfone, n-hexanedecanoic acid and 9,12,15-octadecatrienal min respectively. The residue was further extracted in Soxhlet for 6 h in MeOH. The extract was then filtered and 4M HCl was added to precipitate out the

resins. After filtration, the extract was dissolved in 200 mL ethyl acetate. To 50 mL of the extract, 25 mL distilled water was added in a separating funnel and shaken thoroughly. The water-soluble portion was discarded and 25 mL fresh distilled water was added again. The process was repeated 4 times for complete extraction. The ethyl acetate soluble was then distilled and the extract was chromatographed over silica gel G (200 g), eluting with petroleum ether (60-80°C), petroleum ether-CH_2Cl_2 (2:1), $CHCl_3$, $CHCl_3$- EtOAc (10:1) and finally with EtOAc. TLC check with $CHCl_3$-EtOAc (10:1) fraction showed two distinct spots, which were separated by preparative TLC (20 cmx 20 cm) on 0.5 mm thick layers using $CHCl_3$- EtOAc (5:1) as developing system. Two bands at R_f=0.77 and 0.70 were allowed to develop in an iodine chamber, scrapped out and dissolved in EtOAc. It was then filtered using cotton wad and the solvent was distilled off. Individual compounds 7–(but-3-enyl) 1,2-dihydro cyclobutabenzene (R_f=0.70) and 9,10 anthracenedione (2-hydroxy methyl) 1-methoxy (R_f=0.77) were identified using GC-MS. Schematic of the separation procedure is shown in Fig. V. 1.



V.1 Flow sheet showing separation of organic constituents in *Neem* leaves

(ii) GC-MS identification: Gas chromatogram of petroleum ether extract showed four compounds with retention times (R_t) of. 6.70, 15.3, 20.1 and 20.5min. (Fig. V. 2).

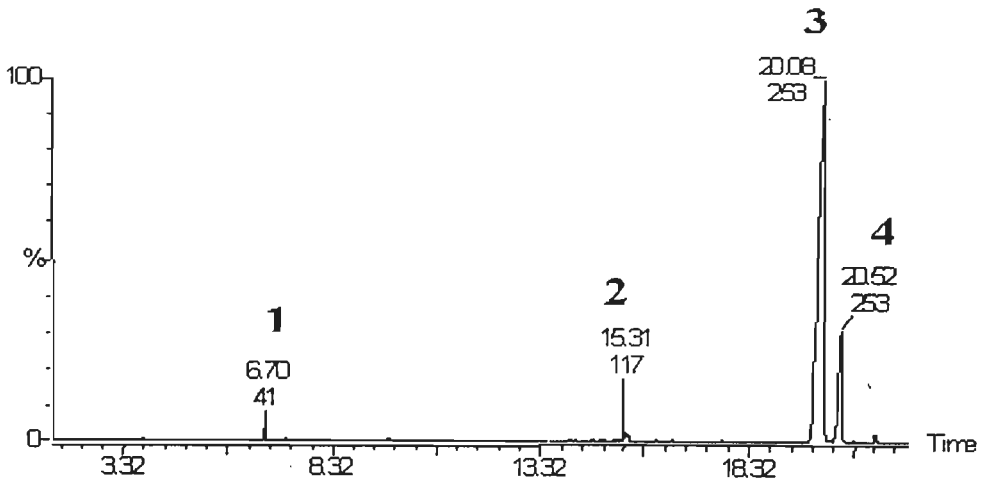


Fig.V.2 Mass chromatogram of the petroleum extract in Neem leaves

These were identified as 1,1,2,3-tetramethylcyclopropane, methyl phenyl sulfone, n-hexanedecanoic acid and 9,12,15-octadecatrienal respectively by studying their mass fragmentation pattern and comparing the individual spectra with those of NIST 2.0 mass spectral database [89]. Mass spectra along with fragmentation pattern of the first three compounds are shown in Fig. V. 3 to 5.

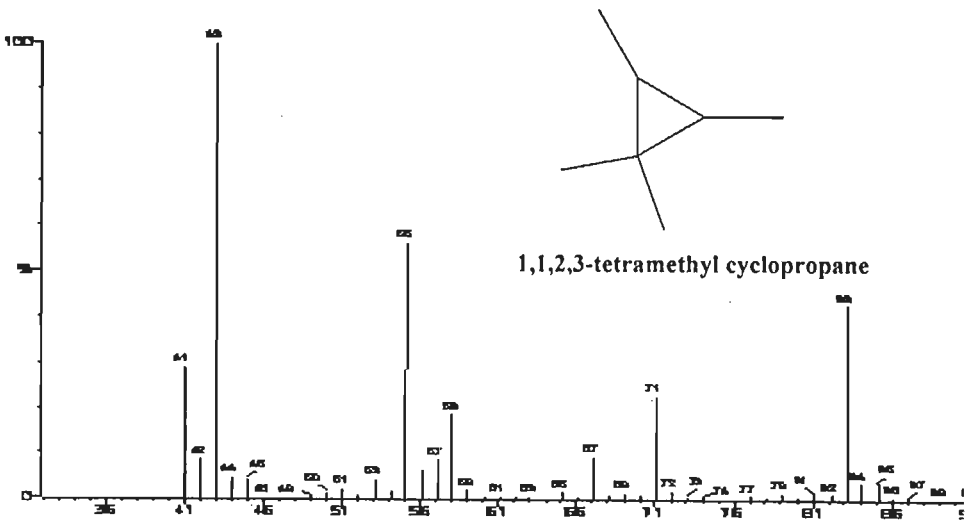


Fig.V.3 A Mass spectrum of 1, 1,2,3-tetramethyl cyclopropane

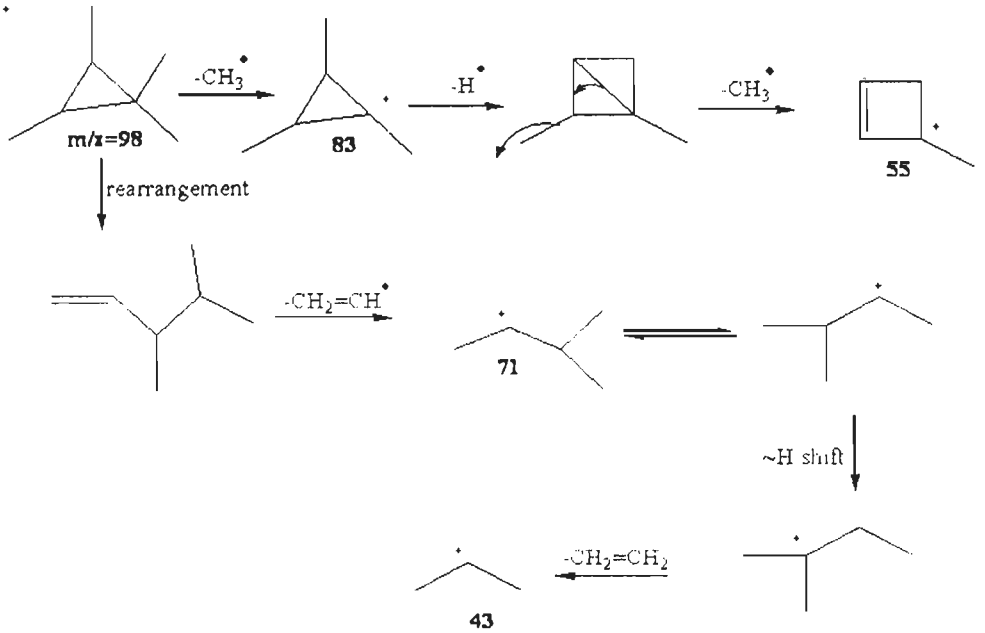


Fig.V.3B Mass fragmentation pattern of 1, 1,2,3-tetramethyl cyclopropane

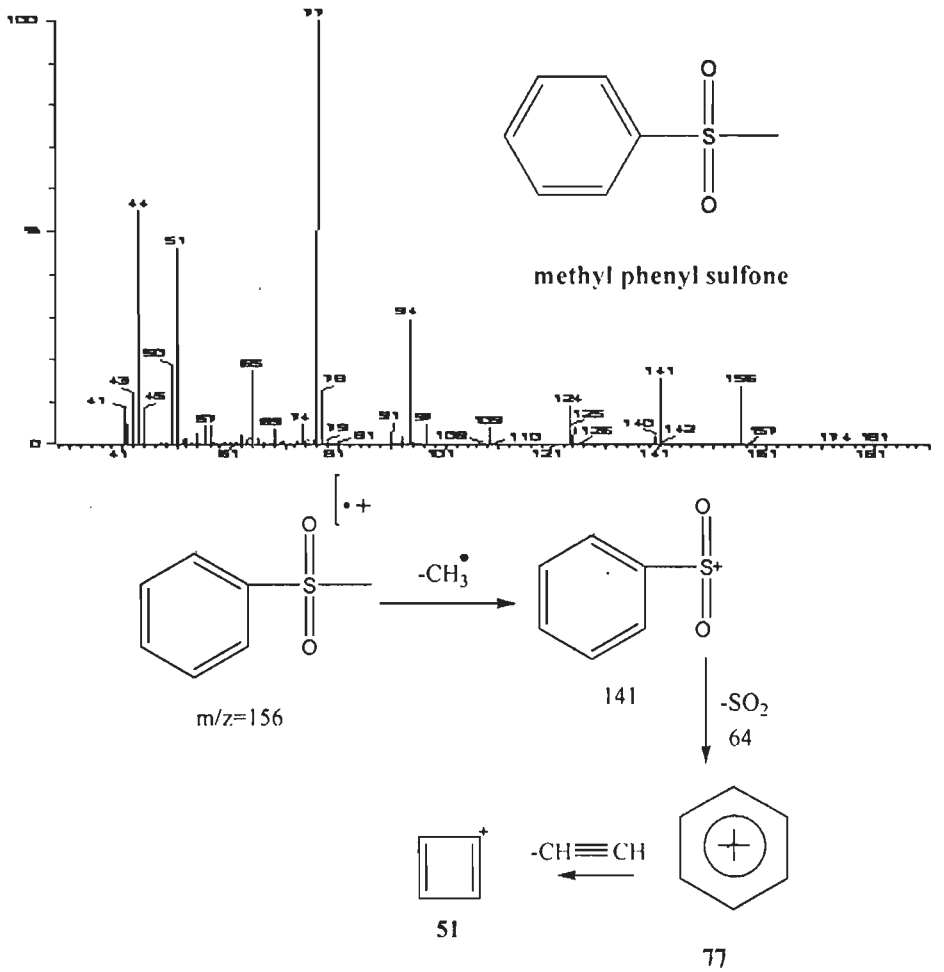


Fig.V.4 Mass spectrum and fragmentation pattern of methyl phenyl sulfone

Methyl sulfone derivative has great promise as anti-malarial agent [90] and it has been reported that various concentrations of *neem* oil when exposed to human body provides ~90% protection from anopheline mosquitoes bite [91].

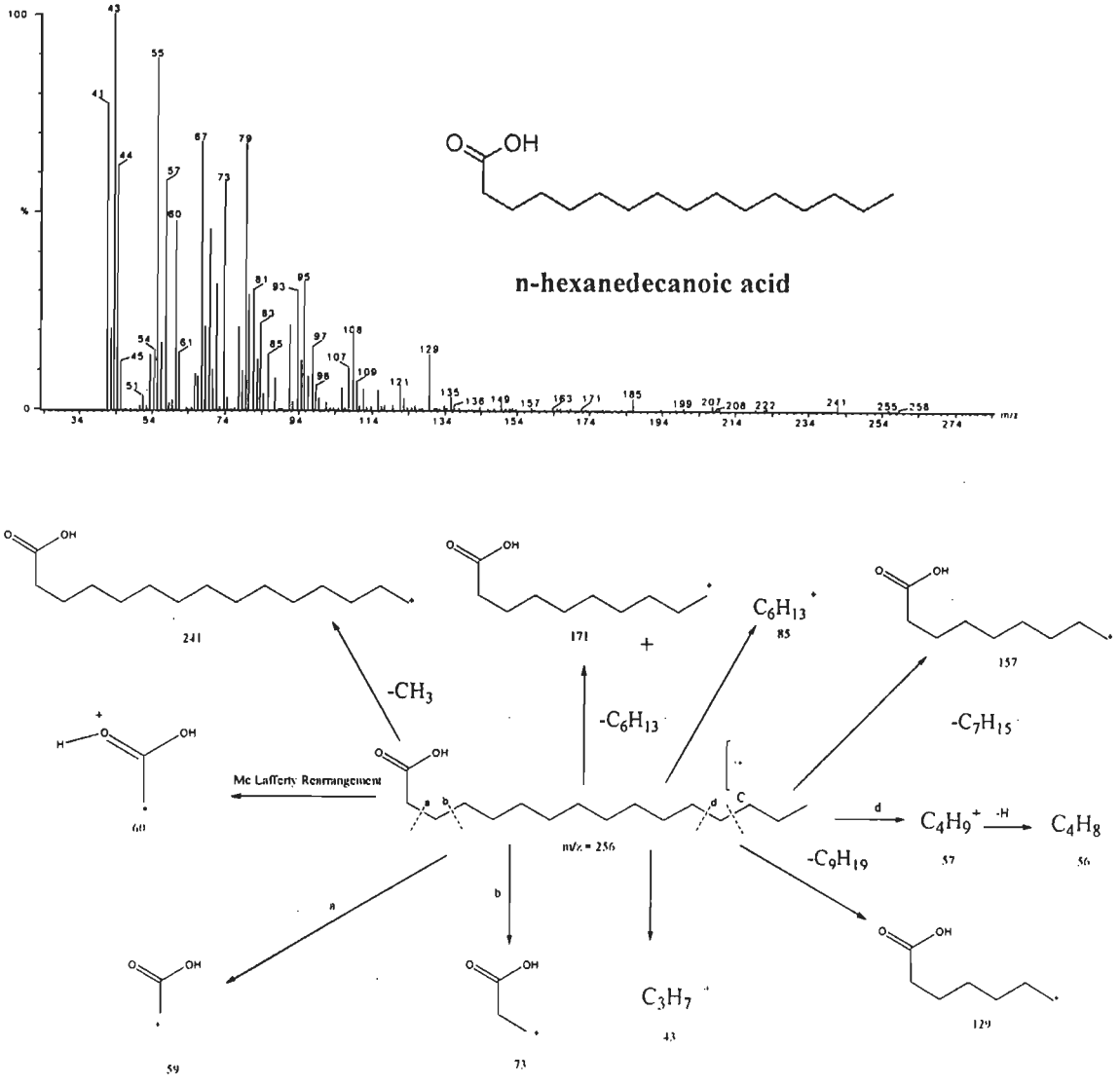


Fig.V.5 Mass spectrum and fragmentation pattern of n-hexane decanoic acid

Of these, only hexane decanoic acid, commonly known as palmitic acid has been reported in neem kernel [92]. Fatty acids are used world wide in cosmetics and anti aging formula. Sodium palmitate is used in bar soap, baby soap, exfoliant/scrub and body wash/cleanser. The aldehyde 9, 12, 15 octadecatrienal was identified by comparing mass spectra with NIST library [89]. This compound commonly known as linolenic aldehyde is reported in the hexane extract of *kewda*, an aromatic plant [93].

Fatty polyunsaturated aldehyde augments the flavour and aroma of foodstuffs, chewing gum and toothpaste. Over the years, *neem* based toothpastes have been very popular. Infact, thin tender stem of neem is widely used as chewing stick in the morning in rural parts of India.

Furthermore two compounds 7–(but-3-enyl) 1,2-dihydro cyclobutabenzene and 9,10 anthracenedione (2-hydroxy methyl) 1-methoxy were separated from the ethyl acetate soluble fraction of the petroleum ether extract of *neem* leaves. Mass spectra of 7–(but-3-enyl) 1,2-dihydro cyclobutabenzene and 9,10 anthracenedione (2-hydroxy methyl) 1-methoxy are shown in Figs. V.6 and 7 respectively. However, the fragmentation pattern of the first compound is explained here but not for the second.

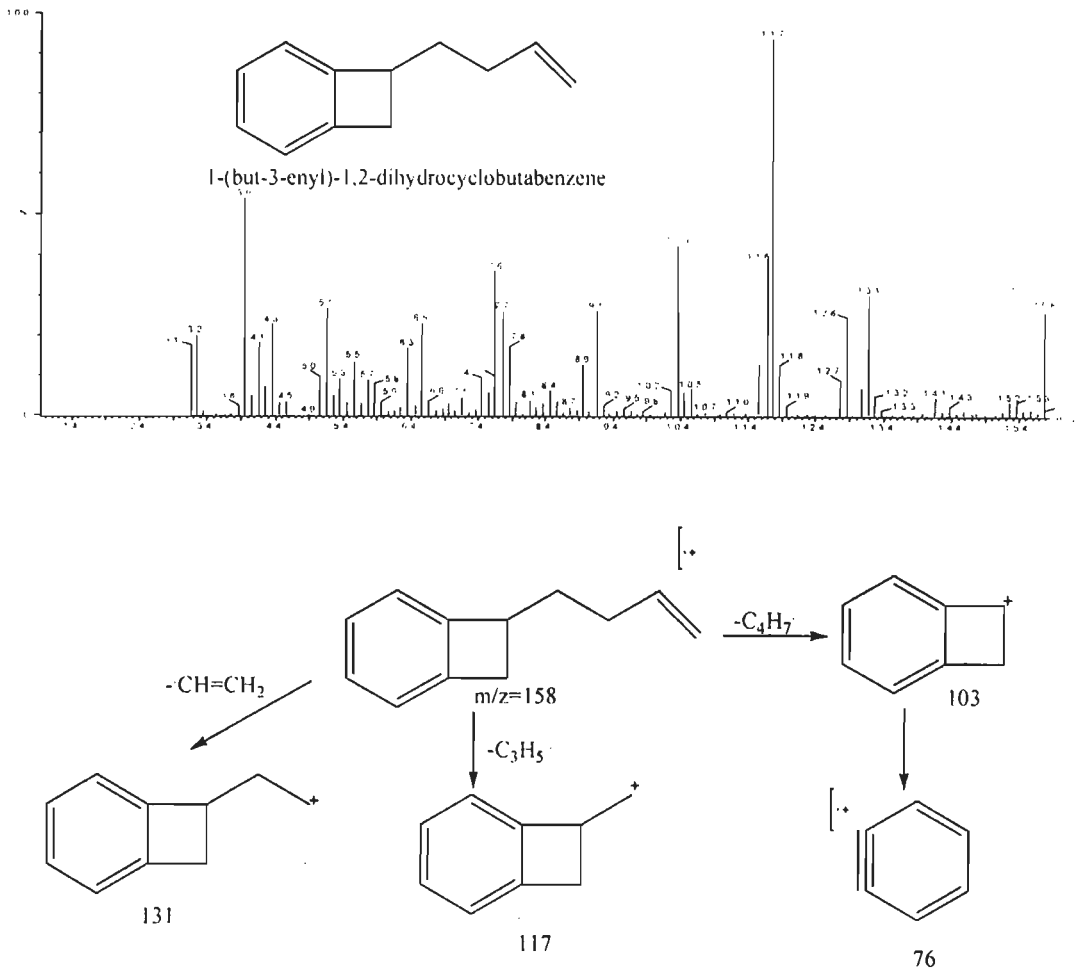


Fig. V. 6 Mass spectrum and fragmentation pattern of 7–(but-3-enyl) 1,2-dihydro cyclobutabenzene

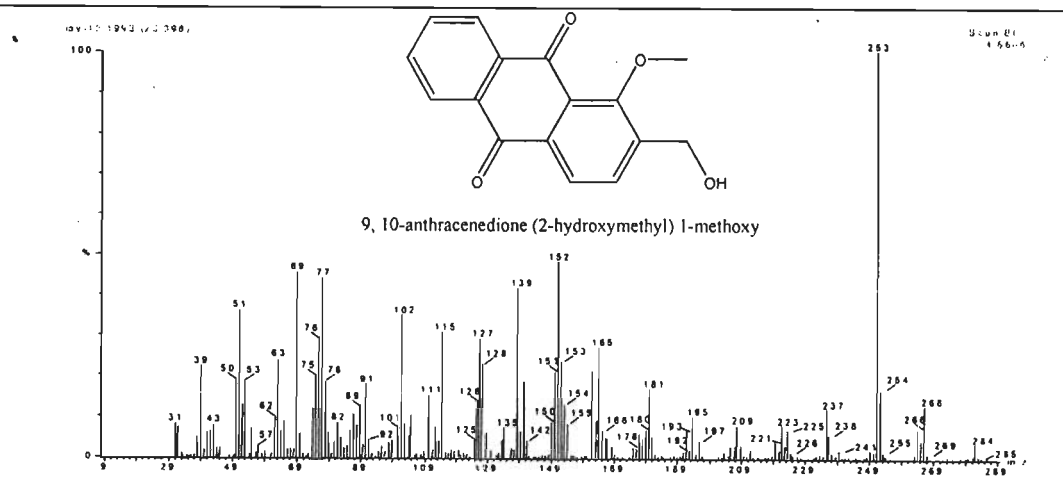


Fig. V. 7 Mass spectrum of 9, 10-anthracenedione (2-hydroxymethyl) 1-methoxy

Rauwald [94] observed that anthraquinones, known as active metabolites of emodin-type O- and C-glycosyl compdounds influence the ion transport across colon cells and act as laxatives.

V.10 RESULTS

Elemental concentrations in MPH-2 along with certified/ information values, error % and RSD are listed in Table V.2. It is observed that our data match well within ± 5-10% of the certified values with the exception of a few elements such as Ce, Cl, Co, Cu, Hf, Hg and Zn but Z-score values in Fig. V. 8 for all elements are between +3 and –3 suggesting our data are within 99% confidence limit. Also, RSDs were <10% in all cases suggesting high order of precision. Therefore, it is assumed that our data for antidiabetic herbs and herbal formulations should be reliable within ± 10%. Concentrations of essential, trace and toxic elements along with mean ± SD and median in antidiabetic herbs are listed in Table V.3A and B. Concentrations of Ni, Cd and Pb as determined by AAS are listed

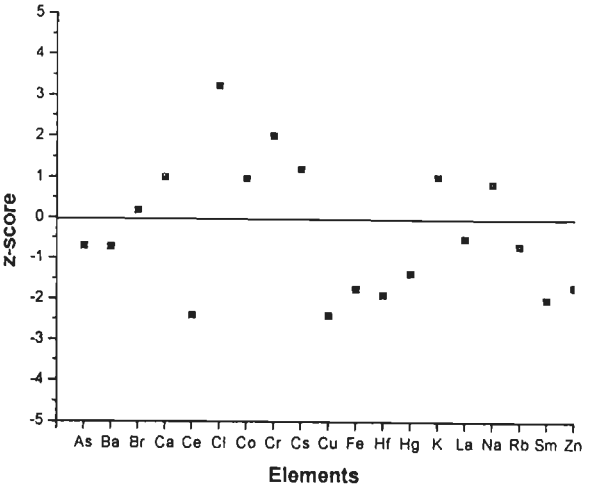


Fig. V.8 Z-score plot for elements in MPH-2

in Table V.4: Concentration of 29 elements along with mean ± SD in five antidiabetic formulations as determined by NAA and AAS is listed in Table V.5.

Table V.2: Elemental concentrations in Mixed Polish Herbs (INCT-MPH-2) used for data validation

Element	This Work	Certified	Error%	RSD%	Z-score
As (ng/g)	207±7	191±23	+8.38	3.38	0.70
Ba (μg/g)	34.3±1.8	32.5±2.5	+5.54	5.24	0.72
Br (μg/g)	7.60±0.5	7.71±0.61	-1.43	6.58	0.18
Ca (mg/g)	10.1±0.9	10.8±0.7	-6.48	8.91	1.0
Ce (μg/g)	1.36±0.2	1.12±0.10	+21.4	14.7	2.4
Cl (mg/g)	2.16±0.11	2.84±0.20	-23.9	5.09	3.4
Co (ng/g)	186±15	210±25	-11.4	8.06	0.98
Cr (μg/g)	1.73±0.02	1.69±0.13	+2.37	1.16	0.31
Cs (ng/g)	82±5	76±7	+7.89	6.10	0.86
Cu (μg/g)	6.78±0.51	7.77±0.53	-12.7	7.52	1.87
Eu (ng/g)	17.3±0.7	15.7±1.8	+10.2	4.05	0.89
Fe (μg/g)	481±22	460	+4.56	4.57	-
Hf (ng/g)	274±17	236±20	+13.8	6.20	1.90
Hg (ng/g)	19.8±1.6	17.6±1.6	+11.1	8.08	1.38
K (mg/g)	17.9±1.4	19.1±1.2	-6.28	7.82	1.0
La (μg/g)	595±34	571±46	+4.20	5.71	0.52
Mg (mg/g)	6.27±0.36	-	-	5.74	-
Mn (μg/g)	178±14	191±12	-6.81	7.87	1.08
Na (μg/g)	365±18	350	+4.29	4.93	-
P (mg/g)	2.38±0.08	2.5	-4.80	3.36	-
Rb (μg/g)	11.2±0.4	10. 7±0.7	+4.67	3.57	0.71
Sb (ng/g)	61.6±4.2	65.5±9.1	-5.95	6.81	0.43
Sc (ng/g)	115±4	123±9	-6.50	3.48	0.89
Se (ng/g)	136±11	-	-	8.09	-
Sm (ng/g)	111±7	94.4±8.2	+17.2	6.31	2.02
Th (ng/g)	173±4	154±13	+12.3	2.31	0.68
V (μg/g)	1.01±0.02	0.95±0.16	+6.32	1.98	0.38
Zn (μg/g)	37.1±1.9	33.5±2.1	+10.7	5.12	1.71

V.11 ELEMENTAL CONTENTS IN HERBS

(i) Minor constituents: A perusal of data in Tables V.3 A shows that K (3.20-60.9 mg/g), Ca (4.98-47.8), and Cl (0.21-11.9 mg/g) form minor constituents as these are all found in found at~1%. It is observed that Na (0.77±1.28 mg/g) Mg (0.94±0.41 mg/g) and P (2.09±1.27 mg/g) are found at ≈ 0.1% amounts. However, antidiabetic herbs are most enriched in K (17.9±13.2 mg/g) and Ca (19.5±10.5 mg/g). All these are electrolytic or structural elements and play an important role in fluid balance [84].

Table V.3A Concentrations of minor and trace elements in herbs and capsules used as anti-diabetic drugs (n=20 samples)

Sample	Na (mg/g)	K (mg/g)	Ca (mg/g)	Mg (mg/g)	Cl (mg/g)	P (mg/g)	V (µg/g)	Cr (µg/g)	Mn (µg/g)	Fe (µg/g)	Cu (µg/g)	Zn (µg/g)
Gurmaar (Y)	0.36±0.01	24.8±0.7	17.3±1.2	0.49±0.02	1.25±0.07	2.32±0.01	2.36±0.16	1.94±0.10	53.2±1.4	261±4	13.4±0.8	44.8±1.9
Giloy (Y)	0.08±0.01	19.1±0.6	8.30±0.42	0.83±0.04	2.33±0.16	2.34±0.01	1.69±0.11	1.04±0.04	73.8±2.6	175±3	2.46±0.21	32.7±1.3
Jamun (L)	0.04±0.01	8.90±0.3	35.3±2.80	1.01±0.08	1.49±0.08	1.20±0.03	2.97±0.18	1.96±0.07	42.7±3.1	186±3	7.31±0.44	35.2±1.1
Jamun (V)	0.10±0.01	13.1±0.3	31.6±1.2	1.92±0.08	0.98±0.03	0.99±0.03	2.01±0.11	2.00±0.02	22.8±0.4	147±4	4.25±0.39	28.3±1.5
Palas (Y)	0.10±0.01	24.0±0.7	12.6±1.2	0.72±0.04	3.25±0.22	2.75±0.08	0.96±0.05	1.54±0.06	58.6±4.9	231±4	15.9±1.1	45.4±2.1
Marodphali (Y)	1.36±0.13	7.10±0.2	15.8±1.3	1.24±0.07	1.68±0.09	0.93±0.02	0.79±0.04	1.79±0.07	22.2±1.4	276±5	39.9±2.7	58.9±2.2
Naagarmotha (Y)	1.40±0.04	8.70±0.3	19.2±1.6	0.88±0.05	2.53±0.17	1.28±0.03	1.02±0.06	1.42±0.10	34.3±2.6	214±3	8.69±0.53	38.3±1.5
Fenugreek (L)	1.66±0.04	12.2±0.4	18.6±2.3	1.41±0.11	8.63±0.12	1.87±0.05	2.15±0.14	1.47±0.20	24.3±0.8	266±5	31.5±2.9	50.6±2.6
Fenugreek (V)	0.08±0.01	16.1±0.2	19.9±0.4	1.22±0.07	6.36±0.31	2.13±0.10	2.47±0.13	1.16±0.14	37.8±2.6	257±24	7.17±0.57	30.2±1.8
Neem (H)	0.68±0.02	20.2±0.6	28.3±1.6	0.58±0.04	9.21±0.33	2.89±0.03	1.77±0.12	1.84±0.07	29.2±1.2	173±3	7.32±0.33	35.1±1.4
Neem (V)	0.21±0.01	15.7±0.2	24.8±1.9	0.49±0.03	11.9±0.9	1.86±0.06	1.82±0.07	1.58±0.19	32.9±2.1	131±12	4.24±0.34	28.6±2.2
Garlic (H)	0.29±0.01	5.30±0.2	7.56±0.27	0.43±0.02	7.03±0.62	0.59±0.01	1.29±0.07	1.01±0.04	28.3±1.5	159±3	3.72±0.26	31.5±1.3
B. Gourd (H)	1.55±0.04	60.9±1.8	11.3±1.0	0.49±0.03	10.6±0.4	6.11±0.11	2.26±0.18	1.28±0.05	55.6±3.7	180±3	10.5±0.6	35.2±1.3
B. Gourd (V)	5.67±0.17	43.7±1.47	8.63±0.42	1.45±0.13	8.27±0.43	4.36±0.15	2.51±0.14	1.58±0.02	58.8±2.1	242±6	3.57±0.22	32.9±2.1
Vijaysar (V)	0.03±0.01	13.2±0.6	4.98±0.12	0.52±0.03	0.21±0.01	2.31±0.06	1.93±0.13	0.99±0.01	13.7±0.4	216±10	5.63±0.47	30.6±1.6
Tejpatta (V)	0.22±0.01	9.76±0.2	47.8±3.5	1.36±0.12	6.00±0.01	1.36±0.04	0.88±0.05	0.59±0.01	54.0±3.3	247±8	5.13±0.44	49.7±1.8
Kaalijeera (V)	1.04±0.04	14.9±0.9	17.5±0.1	0.97±0.04	6.11±0.16	1.87±0.05	0.91±0.06	1.16±0.01	356±10	260±6	6.24±0.28	33.2±1.3
Tejpan (V)	0.19±0.04	14.3±0.8	21.6±0.6	0.69±0.04	3.63±0.20	1.55±0.04	0.64±0.03	0.88±0.01	68.9±4.8	111±3	3.72±0.31	23.4±1.2
Amalaki (V)	0.27±0.05	9.03±0.30	13.9±0.8	0.88±0.05	8.32±0.21	1.12±0.07	1.36±0.09	0.27±0.02	29.6±1.9	112±3	3.14±0.28	26.5±1.4
Kutki (V)	0.13±0.03	17.2±0.12	24.6±1.6	1.18±0.09	1.96±0.07	2.06±0.08	1.01±0.05	2.15±0.02	64.8±4.2	268±12	8.04±0.65	36.1±2.0
Mean±SD	*0.77±1.28*	*17.9±13.2*	*19.5±10.5*	*0.94±0.41*	*5.09±3.58*	*2.09±1.27*	*1.64±0.69*	*1.38±0.50*	*58.1±72.3*	*206±55*	*9.59±9.67*	*36.4±9.1*
Median	*0.52±0.29*	*15.7±3.5*	*16.6±0.8*	*0.78±0.06*	*2.89±0.36*	*2.10±0.23*	*1.25±0.24*	*1.45±0.06*	*38.5±4.2*	*200±13*	*8.91±7.83*	*36.8±4.2*

Table V.3B Concentrations of essential trace and toxic elements in herbs and capsules used as anti-diabetic drugs (n=20 samples)

Sample	Ba (μg/g)	Rb (μg/g)	Se (ng/g)	Co (ng/g)	Cs (ng/g)	As (ng/g)	Br (μg/g)	Ce (μg/g)	Hg (ng/g)	Sb (ng/g)	La (μg/g)	Sc (ng/g)	Th (ng/g)
Gurmaar (Y)	40.3±2.3	25.3±1.6	126±7	94±8	152±11	635±40	25.1±1.5	4.69±0.74	67±3	22.1±1.5	3.07±0.14	103±5	56±4
Giloy (Y)	46.5±2.7	11.1±0.5	121±8	55±5	84±4	279±18	1.29±0.1	1.81±0.22	69±4	15.7± 1.3	1.03±0.05	33±2	21±2
Jamun (L)	81.6±4.3	13.8±0.8	73±5	58±5	107±9	489±36	1.30±0.1	1.60±0.20	51±5	42.7± 0.6	0.68±0.03	29±1	17±1
Jamun (V)	71.8±1.9	17.2±0.4	98±6	42±3	124±6	277±8	1.50±0.1	1.99±0.20	43±3	38.9± 4.1	0.90±0.06	182±6	146±7
Palas (Y)	33.7±2.5	28.8±1.7	126±6	73±6	143±11	796±49	103±6.4	1.94±0.24	88±6	27.0± 1.7	1.07±0.05	36±2	24±7
Marodphali (Y)	67.9±3.9	22.5±1.3	169±12	115±10	148±17	827±53	17.5±1.1	11.0±1.3	92±7	27.4± 9.7	8.35±0.38	93±5	474±36
Naagarmotha (Y)	58.2±3.4	22.0±1.3	1044±70	92±8	113±15	930±60	16.5±1.0	15.8±1.9	47±3	18.0±1.1	10.3±0.5	80±5	291±17
Fenugreek (L)	99.3±5.7	11.0±0.6	291±19	91±8	106±8	569±41	89.9±5.5	2.54±0.31	143±9	24.6±1.4	1.83±0.08	55±3	36±3
Fenugreek (V)	81.9±5.6	23.8±2.0	72±4	58±7	159±7	156±12	6.86±0.3	3.58±0.59	32±2	25.0±1.6	3.59±0.26	229±16	365±27
Neem (H)	204±12	14.6±0.9	100±7	59±5	88±7	358±29	398±48	3.31±0.41	67±4	20.2±0.9	1.12±0.05	32±2	173±11
Neem (V)	201±14	27.5±2.3	80±5	46±6	165±8	211±15	36.8±1.6	3.18±0.53	35±2	15.6±1.0	3.65±0.26	291±24	314±21
Garlic (H)	23.6±1.4	4.15±0.2	267±13	55±5	57±3	868±55	72.4±4.4	1.42±0.18	63±4	43.3±3.6	0.73±0.03	29±2	162±9
B. Gourd (H)	33.8±2.0	39.1±2.8	91±6	72±7	158±13	1013±65	433±27	1.56±0.19	33±2	53.4±3.7	0.99±0.05	29±1	162±9
B. Gourd (V)	37.3±1.0	44.7±1.1	135±9	65±4	185±12	1439±123	203±6	2.05±0.20	54±3	55.1± 8.1	2.18±0.10	138±5	119±6
Vijaysar (V)	51.2±1.4	19.4±0.5	116±7	84±5	128±7	277±7	18.5±1.3	2.11±0.21	30±2	34.7±3.5	6.59±0.10	62±2	397±23
Tejpatta (V)	31.2±0.8	15.9±0.4	93±5	41±3	81±4	803±22	74.1±2.1	2.59±0.25	27±2	21.9±1.6	3.51±0.10	27±1	129±10
Kaalijeera (V)	68.7±1.8	51.5±1.2	250±18	62±4	279±18	441±21	18.7±0.6	5.90±0.54	68±4	34.6±4.4	1.04±0.08	14±1	83±6
Tejpan (V)	71.9±1.9	27.8±0.6	112±5	52±3	114±12	125±5	70.8±0.5	3.47±0.34	38±2	23.6± 3.0	0.99±0.08	56±2	87±5
Amalaki (V)	54.0±1.5	28.6±0.7	153±9	37±3	97±8	129±4	4.07±0.1	2.08±0.20	42±3	21.8±2.8	0.33±0.06	63±2	168±8
Kutki (V)	42.4±1.1	42.9±1.1	227±13	54±3	278±19	99±4	2.62±0.2	8.39±0.82	23±2	26.1±1.6	0.51±0.01	115±4	236±18
Mean±SD	*70.0±49.5*	*24.6±12.3*	*187±212*	*65±21*	*138±58*	*536±367*	*79.7±125*	*4.05±3.70*	*55.6±28.5*	*29.6±11.7*	*2.62±2.78*	*85±74*	*173±134*
Median	*52.4±36.1*	*17.9±4.1*	*244±103*	*73±5*	*110±3*	*868±10*	*48.8±94.7*	*2.24±2.96*	*65±19*	*24.8±1.6*	*1.09±2.11*	*35±12*	*232±98*

Table V.4 Concentrations of essential trace and toxic elements in antidiabetic herbs using AAS*

Sample	Pb (μg/g)	Cd (μg/g)	Ni (μg/g)
Gurmaar (Y)	11.4	6.73	0.59
Giloy (Y)	14.2	4.75	1.03
Jamun (L)	5.33	4.95	0.90
Jamun (V)	10.2	6.03	1.86
Palas (Y)	8.95	3.15	2.29
Marodphali (Y)	9.15	5.63	0.79
Naagarmotha (Y)	14.8	4.88	0.67
Fenugreek (L)	2.70	4.95	1.38
Fenugreek (V)	7.48	3.20	0.42
Neem (H)	4.80	2.78	1.70
Neem (V)	4.20	4.08	1.05
Garlic (H)	5.15	2.95	1.04
B. Gourd (H)	11.5	3.78	1.57
B. Gourd (V)	6.65	4.25	0.54
Vijaysar (V)	9.23	3.33	0.30
Tejpatta (V)	4.75	4.90	1.41
Kaalijeera (V)	6.80	2.90	1.78
Tejpan (V)	9.55	4.28	2.06
Amalaki (V)	2.93	5.88	0.89
Kutki (V)	6.30	4.88	1.28
Mean±SD	7.80±3.39	4.41±1.12	1.18±0.55
Range	2.70-14.8	2.78 -6.73	0.30-2.29

* These are based on single sample analysis though in each case solution was aspirated three times and mean absorbances were noted

Table V.5 Concentration of essential, trace and toxic elements in antidiabetic formulations

Elements	Madhunashini	Jambrushila	Divya	Diabetex	Diabeticin	Mean±SD
As (ng/g)	490±18	97.3±3.7	73.6±2.8	32.0±1.2	56.1±2.1	150±192
Ba (μg/g)	98.3±6.2	123±9	87.1±5.7	41.9±2.4	88.2±5.7	87.7±29.4
Br (μg/g)	9.78±0.28	79.6±2.3	16.4±0.5	8.78±0.31	25.0±0.8	27.9±29.6
Ca (mg/g)	9.85±0.16	24.1±0.6	11.2±0.1	6.58±0.01	7.63±0.01	11.0±6.1
Cd (μg/g)	5.85	12.8	3.63	2.90	10.4	7.11±3.86
Ce (μg/g)	16.9±1.2	29.7±2.1	11.9±0.8	7.63±0.51	14.6±1.3	16.1±8.3
Cl (mg/g)	1.63±0.02	3.32±0.15	2.53±0.03	2.32±0.07	2.14±0.07	2.39±0.62
Co (ng/g)	112±9	87±7	53±3	148±12	117±9	103±36
Cr (μg/g)	2.02±0.16	1.85±0.12	3.11±0.23	1.27±0.09	2.28±0.19	2.11±0.67
Cs (ng/g)	114±9	171±14	187±11	93±7	153±13	144±39
Cu (μg/g)	16.7±0.3	22.9±0.4	14.1±0.3	20.1±0.4	4.55±0.08	15.7±7.1
Fe (μg/g)	415±25	698±41	318±7	569±34	297±14	459±171
Hg (ng/g)	44.5±2.3	37.0±1.4	31.6±1.2	17.7±0.7	13.7±0.5	28.9±13.0
K (mg/g)	20.7±0.2	14.5±0.2	29.3±0.4	15.7±0.2	11.7±0.1	18.4±6.9
La (μg/g)	8.14±0.62	3.15±0.16	0.97±0.06	6.26±0.32	0.52±0.03	3.81±3.32
Mg (mg/g)	7.04±0.02	8.42±0.04	6.57±0.08	3.45±0.03	4.25±0.05	6.57±1.73
Mn (μg/g)	250±8	147±5	231±7	140±5	26.7±0.6	143±23
Na (μg/g)	307±15	686±22	318±10	263±9	48.4±1.5	324±230
Ni (μg/g)	6.05	4.35	2.43	2.55	7.40	4.56±1.94
P (mg/g)	3.12±0.18	4.97±0.34	1.89±0.09	4.79±0.30	5.86±0.41	4.13±1.59
Pb (μg/g)	7.43	17.1	3.48	5.38	14.6	9.60±5.31
Rb (μg/g)	19.6±0.5	28.9±0.9	28.5±1.2	15.1±0.8	20.3±1.2	22.5±6.0
Sb (ng/g)	117±9	201±16	89.1±4.3	142±11	51.2±3.2	120±56
Sc (ng/g)	29±2	83±6	62±4	56±3	18±1	50±26
Se (ng/g)	124±6	415±26	213±18	267±13	169±11	238±112
Sm (ng/g)	141±7	201±10	141±8	126±7	130±7	148±30
Th (ng/g)	241±15	108±9	83±5	114±9	78±4	125±67
V (μg/g)	2.50±0.08	0.78±0.01	1.18±0.04	0.23±0.01	0.26±0.01	0.99±0.93
Zn (μg/g)	21.5±1.5	31.8±1.9	18.9±1.2	39.2±2.4	16.6±1.1	25.6±9.57

It means that diabetic herbs may be considered as vital to provide body strength. Almost all elements vary by an order of magnitude except Co (65.0±21.0 ng/g), Cr (1.38±0.50 μg/g), Fe (232±97 μg/g), V (1.64±0.69μg/g) and Zn (35.6±11.0 μg/g), which vary in a narrow range. This variation in elemental concentration is essentially due to the differential uptake by the plant from the soil or due to inherent nature of the plant species. *Vijaysar* (P. marsupium), one of the most potent flora against *diabetes* is deficient in essential elements viz. Na (0.03±0.01 mg/g), Ca (4.98±0.12 mg/g), Cl (0.21 ±0.01 mg/g) and Mn (13.7 ±0.4 μg/g) but enriched in V (1.93±0.13 μg/g), Cr (5.63±0.47 μg/g), Zn (30.6±1.6 μg/g), and Rb (19.4±0.5 μg/g). It is observed that both brands (Himalaya and Vyas Pharmacy) of *bitter gourd* (Karela) capsule contain elevated amounts of K, Cl and P. Similarly both brands of *Jamun* and *tejpatta* are enriched in Ca and Mg. Our values for K and Cl in *neem, fenugreek, bitter gourd* and *Naagarmotha* are in excellent agreement with those reported by Rajurkar and Pardeshi [67]. Bar plots showing variation in concentration of minor elements are shown in Fig. V. 9.

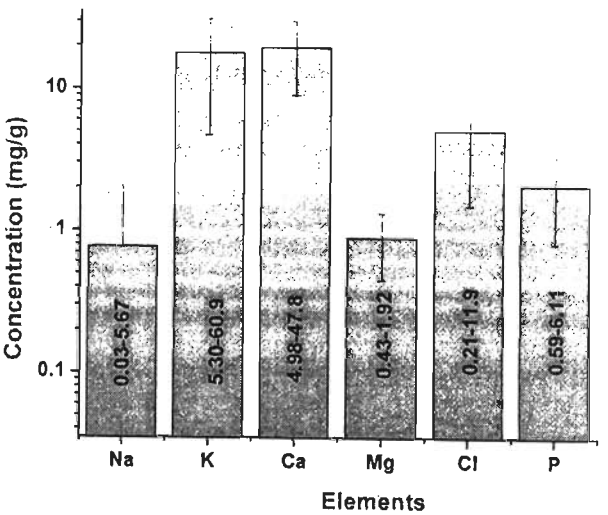


Fig. V.9 Concentration of minor elements in 20 antidiabetic herbs

(ii) Essential trace elements: In general, 20 samples analyzed in this study can be broadly divided into three groups; various plant parts viz. roots, leaves, fruits and seeds, commercially available powders and capsule powders marketed by Himalaya Drugs. Some of these are vegetables (*bitter gourd*) and others are spices (*fenugreek seeds, garlic* and *tejpatta*). A comparison of mean contents of essential trace elements in antidiabetic herbs is shown in Fig. V.10. Fe, Mn, Rb and Zn contents are ~ 100

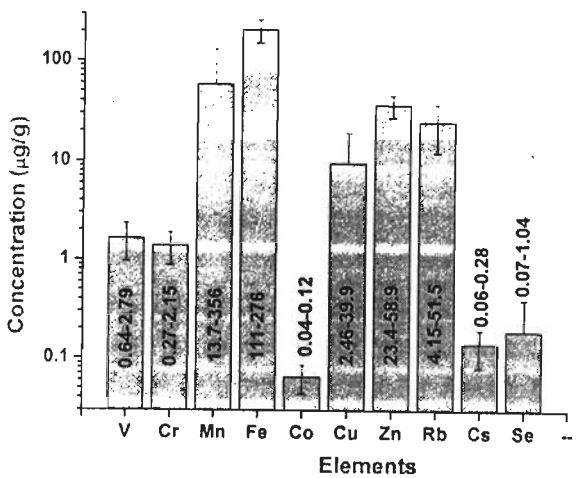


Fig V.10 Concentration of essential trace elements in 20 antidiabetic herbs

μg/g whereas V and Cr are found at ~1 μg/g level. It is observed that no single plant part or herb is rich in all the constituents. However, most commonly used herbs such as *gurmaar, jamun, fenugreek, neem* and *bitter gourd* are all, in general, enriched in V, Cr, Fe, Cu and Zn which play an important role in *diabetes*. *kutki* and *naagarmotha*, both roots are enriched in Cr (2.15±0.02 μg/g) and Se (1044±70 ng/g) respectively. Mn varies in a large range of 13.7-356 μg/g with highest content in *Kaalijeera*. The differing Mn levels can be attributed to the selectivity of the plant and not to any parameter connected with essentiality. In *jamun*, it is observed that the powdered variety from Vyas Pharmacy contains higher amounts of Na, K, Mg, Rb and Cs while locally prepared sample is enriched in Cl, P, V, Mn, Fe, Cu and Zn. In *bitter gourd*, the powdered variety is rich in Na, Mg and Se whereas the commercially marketed capsules contain significantly higher amounts of K, Ca, Cl, P and Cu. This suggests addition of preservatives to capsules (possibly common salt). In *neem*, the capsule is much enriched in Na while the rest are in comparable amounts.

(iii) Rare Earth Elements: Also, contents of 5 REE (La, Ce, Sm, Eu and Th) have been determined. While La and Ce are in μg/g amounts, Sm, Eu and Th are in ng/g amounts. It is observed that whole plant parts contain much higher contents of La, Ce and Sm while the powdered one contain elevated amounts of Th and Eu. Possibly REE uptake may be governed by the plant species, growing season and various geo environmental factors and properties of the host soil. Some acute toxicity and sub chronic toxicity tests have shown that low doses of REE had no significant teratogeny a mutagenicity [95] though no precise evaluations for the long-term biological effects of rare earths on the human health are known.

(iv) Toxic Elements: In recent years much emphasis is being laid on toxic element contents as several western countries have banned *Ayurvedic* drugs because of above permissible quantities of As, Cd and Pb [96, 97]. Health Canada, the government department, issued a health warning to consumers and made public a list of "unapproved Indian *Ayurvedic* products" on its website on July 14, 2005 [98], which also includes *bitter gourd* (Karela) capsule by Himalaya Drugs and Pharmaceuticals. Infact, karela in both capsule and powder forms contains very high amounts of As (1.01±0.07 μg/g and 1.44±0.12 μg/g) and Br (433±27 and 203±6 μg/g) respectively. This level of As, though high, is still below the permissible limits slated as 10 μg/g specified by the US FDA [99] for herbal products. A comparison of toxic element contents in antidiabetic herbs is shown in Fig. V.11. It seems, high As content may be

the inherent nature of the *bitter gourd* plant itself. Both, As (99-1013 ng/g) and Br (1.29-433 μg/g) contents vary in a wide range. However, *kutki* and *giloy* contain the lowest amount of As (99±4 ng/g) and Br (1.29±0.1μg/g) respectively. Hg content also varies in a wide range of 23-143 ng/g but well below the permissible limit of 3 μg/g. Besides, Cd (4.41±1.12 μg/g) and Pb (7.80±3.39 μg/g) were determined by AAS. Cd contents vary in a narrow range of 2.78-6.73 μg/g but Pb contents vary by a factor of 5. It is observed that samples collected from Yogi Pharmacy, Haridwar in general have a higher Pb content. Not much variation in Pb content is observed in *neem* capsules and powder but *bitter gourd* powder from Himalaya Drugs, Bangalore shows a 2-fold higher Pb content than the powder. In general, toxic constituents are below the permissible limits and hence safe to consume.

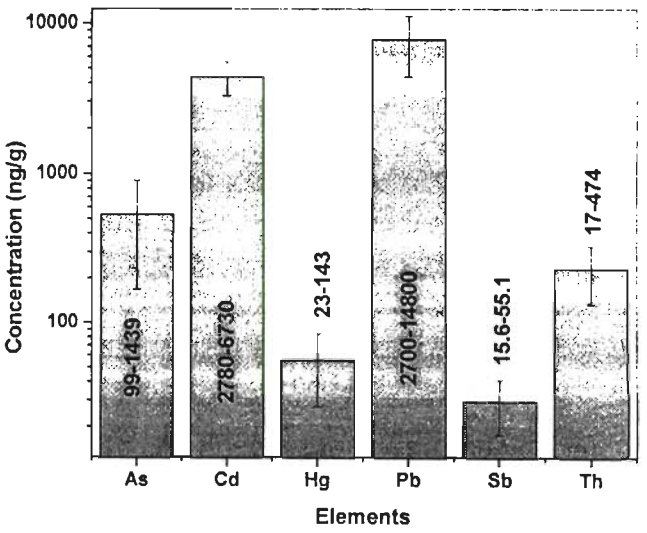


Fig. V.11 Concentration of toxic elements in 20 antidiabetic herbs

Since a large number of samples were analyzed and each one contains varying amounts of elements, mean± *SD* as well as median ± *SD* were calculated. Comparison of mean and median values is a measure of uniform spread in different values. In general, it is observed that mean values are higher than medians except for P, Fe, Cu and Zn, where both are comparable. Mean values of K, Ca, Hg and Sb are higher than the median values by 15-20% while Na, Mg, V, Cr, Ba, Rb and Cs values are higher by 20-50%. For Cl, Mn, Br, Ce, La and Sc, means are higher by more than 50%. Only for a few elements, median values are higher than means. Medians of Cr, Th, Se and Co are higher by 5-20% while arsenic shows the highest variation of ~40%. Large differences in mean and medians causing wide spread may be due to different herbs, each of which has its own characteristics.

(v) Interelemental Correlations: Several literature reports suggest inter relationships in plant species [100, 101]. K/P ratio lies in a very close range of 6.45-10.7 with a mean of 8.19±1.39 but Ca/ P ratio varies by an order of magnitude (3.55-35.1) with a mean of

12.6±9.55. Cu and Zn, both essential elements for biochemical processes, are well correlated in our study of antidiabetic herbs. An excellent linear relationship (r = 0.89) is observed between Cu and Zn in plant parts (Fig. V.12). The other strong linear correlationship exists between Rb and Cs with r = 0.87 as shown in Fig. V.13. According to an US patent, Rb and Cs help in the breakdown of starch to glucose ratio [102]

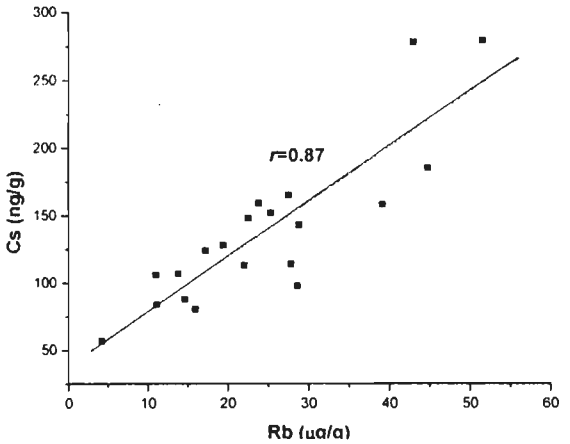


Fig. V.12 Correlation of Rb vs Cs in antidiabetic plants

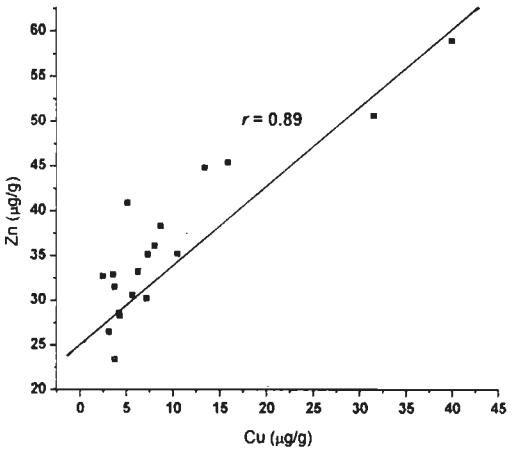


Fig. V.13 Correlation of Zn vs Cu in antidiabetic herbs

(vi) Variation amongst plant parts: Wide variations with large SD values are observed in elemental contents amongst individual plant parts. Fruits contain high Na (243±2.86 µg/g), K (19.9±20.6 mg/g), Cl (6.09±3.82 mg/g), Cu (15.5±21.1µg/g) and Zn (39.4±17.2 µg/g). *Bitter gourd* powder from the Vyas Pharmacy has higher Na (5.67± 0.17 mg/g) and K (43.7±, 1.47 mg/g) contents whereas *Marodphali* has exceedingly high Cu (39.9±2.7µg/g) and Zn (58.9±2.2µg/g) contents. In general leaves contain higher Ca content (24.8±12.2 mg/g), as *tejpatta* has elevated level (47.8±3.5 mg/g). Vijaysar, a bark has highest P (2.31±0.06 mg/g) content. Roots (*giloy*, *naagarmotha* and *kutki*) have higher Mn (57.6±20.7µg/g) and Se (464±505 ng/g) contents. *Giloy* and *naagarmotha* contain highest Mn and Se contents respectively. In three seeds of *fenugreek*, jamun and *kaalijeera* much

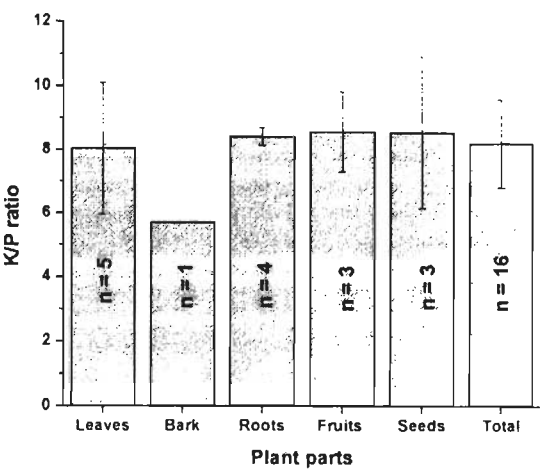


Fig. V.14 Variation in K/P ratio

higher contents of V (2.03±0.69 μg/g), Cr (1.86±0.59 μg/g) and Fe (223±48 μg/g) are observed. All these elements are important in diabetes.

A plot of K/P ratio in individual plant parts (Fig. V.14) shows comparable values in leaves (8.03±2.07), roots (8.41±0.29), fruits (8.56±1.26) and seeds (8.53±2.38) whereas in bark it is the least (5.71). In view of these observations, it is concluded that all plant parts are not enriched in essential minor and trace elements. Hence a combination of various antidiabetic plant parts made into a formulation could perhaps be a better alternative to cure diabetes.

V.12 ELEMENTAL CONTENTS IN FORMULATIONS

As mentioned earlier, formulations are mixtures of several different plant parts to enhance its efficacy for the treatment of a particular disease. However, it is not known as to in what ratio these different plant parts are mixed. All the five formulations analyzed here (Table V.1) are different but some of its contents are common viz *gurmaar*, *neem*, *karela* and *jamun*. It has been observed that none of these herbs are enriched in all the elements individually. Therefore, a combination could be more effective in the treatment of diabetes. Probably this is what must have been happening in these formulations marketed by different pharmaceutical companies.A perusal of elemental data in five antidiabetic formulations in Table V.5 shows that *Jambrushila* is most enriched in Ca, Mg, Na, Cl, Fe, Cu, Rb, and Se. All others are enriched in one or two elements e.g. *Madhunashini* in Mn and V and *Divya* in K and Cr. Several minor (K, Mg, Ca and P as shown in Fig. V.15) and trace (Cu, Cr, Rb, Fe, Mn, Se and Zn as shown in Fig. V.16) elements vary in a close range by a factor of 2-3. However, V content varies by an order of magnitude, being lowest in

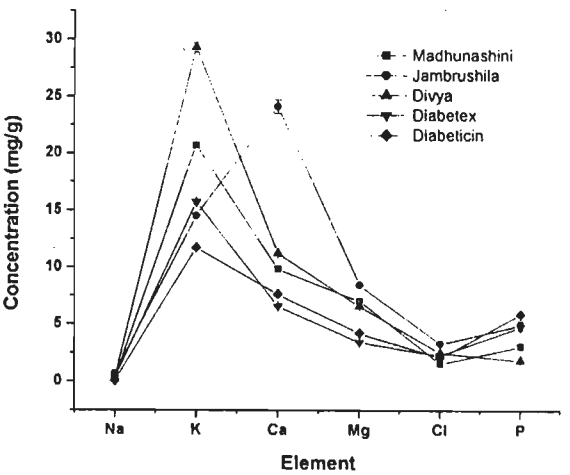


Fig V.15 Concentration of minor elements in antidiabetic formulations

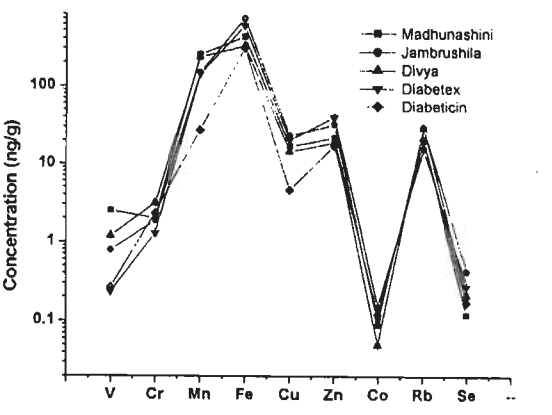


Fig V.16 Concentration of essential trace elements in antidiabetic formulations

Diabetex (0.23 μg/g) and highest in *Madhunashini* (2.50 μg/g). In general, all herbal formulations are enriched in macronutrients (K, Ca, Mg, Cl and P) and micronutrients (Cr, Mn, Fe, Cu, Zn, Rb V, and Se). Though Na, K, Ca, Cl, and P are not directly associated with diabetes, but their deficiency may prove to be fatal. Thus all the formulations have been made such that each one is enriched in macro and micronutrients to avoid nutritional deficiency.

Many Ayurvedic formulations have been found to contain high amounts of toxic elements such as Cd, Pb and Hg. Toxic element profile in Fig. V.17 shows that Br, Cd and Pb are all found at ~10 μg/g level whereas As, Sb and Th are at ~0.1 μg/g level. This has brought herbo-mineral medicines into the realm of debate [96,97]. As per USFDA maximum permissible limits of these toxic elements are: Pb (10 ppm), As (5 ppm), Cd (2 ppm) and Hg (3 ppm) [98]. *Jambrushila* has somewhat higher amounts of Pb and Cd and so is *Madhunashini* with highest amounts of As (0.49±0.02 μg/g) and Hg (44.5±2.3 ng/g) contents. Mean content of Th, a radioactive element, is found to be 0.12±0.07 μg/g. Overall, mean toxic element contents are below permissible limits with some exceptions (Pb and Cd contents in *Jambrushila* are higher and a cause of concern) and hence these medicines should be safe to consume.

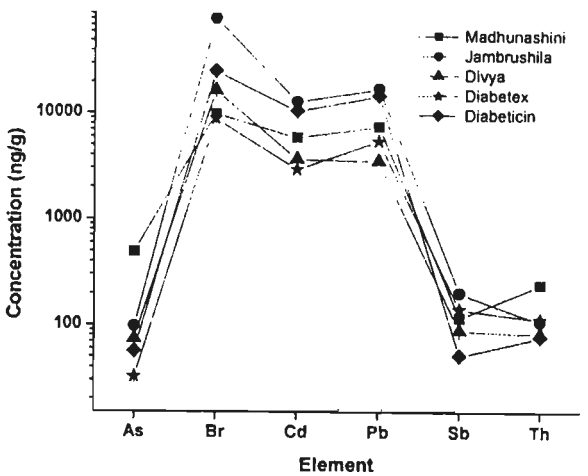


Fig V.17 Concentration of toxic elements in antidiabetic formulations

There also exists strong interrelationship between various elements important in diabetes. K/P ratio, a diagnostic factor, lies in a broad range of 1.99-15.5 with a mean of 6.06±5.59. However, Ca/P ratio lies in a closer range of 1.30-5.93 with a mean of 3.32±2.06. An inverse correlation is observed between Zn and Cr with r = -0.81 as shown in Fig. V.18. Similar to antidiabetic herbs (Fig. V.12), strong positive relationship is observed between Rb and Cs (r = 0.93) as shown in Fig. V.19.

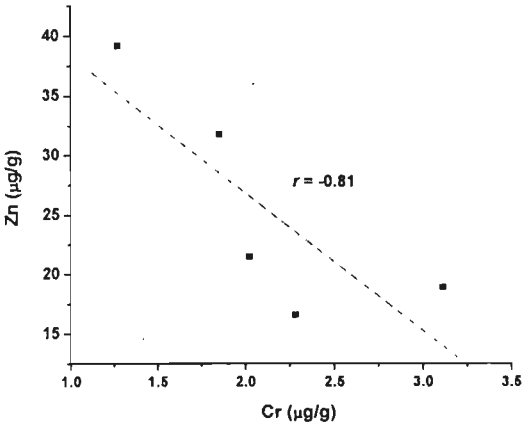


Fig. V.18 Correlation of Zn vs Cr in herbal formulations

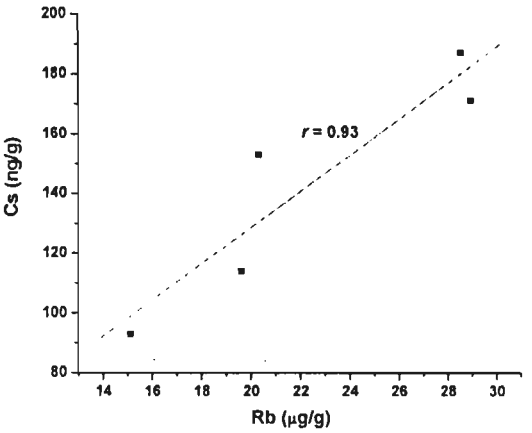


Fig. V.19 Correlation of Rb vs Cs in herbal formulations

V.13 HERBS vs. FORMULATIONS

A comparison of elemental data in Tables V.3, 4 (antidiabetic herbs) and 5 (herbal formulations) shows that herbs are richer in Na, Ca, Cl contents then formulations but diabetically important elements such as Cr (2.11±0.67 µg/g), Cu (15.7±7.11 µg/g), Fe (459±171 µg/g), Mg (6.57±1.73 µg/g), Mn (143±23 µg/g) and Se (238±112 µg/g) are much higher in formulations. A bar plots comparison of minor and essential trace element concentrations between herbs and formulations are shown in Fig. V.20 and 21 respectively. It is observed that Na, K, Mn, Cu, Zn and Rb contents are comparable in both cases. It seems that the formulations have been prepared such that all the essential macro and micronutrients are supplied in proper amounts to the patient.

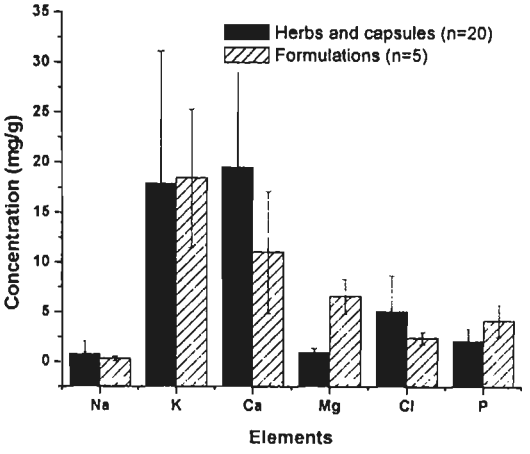


Fig. V.20 Comparison of minor elements in herbs and formulations

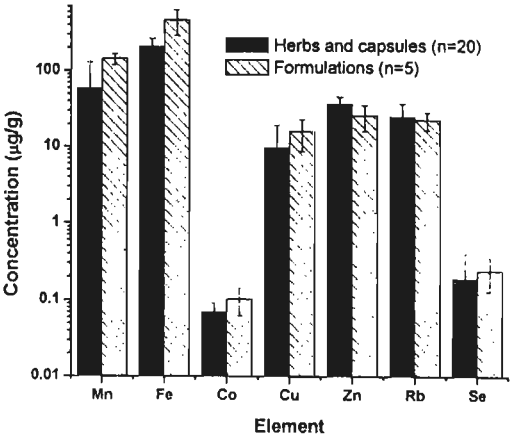


Fig. V.21 Comparison of essential trace elements in herbs and formulations

However, comparison of toxic elements in herbs and formulations indicates almost comparable amounts though formulations contain a bit higher Cd, Pb and Sb contents. This may probably be due to improper way of manufacturing practices like grinding and mixing where contamination must have taken place. Thus, there is a strong need of following good manufacturing practices (GMP) and thereby maintain quality control by the pharmaceutical companies.

CONCLUSION

On the basis of the analytical data for 30 elements in 20 antidiabetic herbs including 3 commercially branded capsules and 5 herbal formulations of different brands and compositions, following generalizations can be made;

- No single herb is enriched in all the elements. Structural elements K, Ca, Mg, Cl and P each found at ~1% are minor constituents.
- Approximate contents of essential trace elements are: V & Cr ~1μg/g, Mn ~ 60 μg/g, Fe~ 200 μg/g, Cu ~10 μg/g, Zn ~ 40 μg/g, Rb ~25 μg/g and Se ~0.2 μg/g. Of these Mn, Cu and Se vary in a wide range and all others in a narrow range. It is due to the differential uptake by the plant from the soil or due to inherent nature of the plant species.
- It is observed that fruits contain high Na, K, Cl, Cu and Zn contents. However, roots have higher Mn and Se, while V, Cr and Fe contents are higher in seeds
- *Bitter Gourd* capsules contain elevated amounts of K (60.9±1.8 mg/g) and P (6.11±0.11 mg/g), which could possibly be due to addition of preservatives.
- Higher amounts of Na, K, Mg, Rb and Cs were observed in *jamun*, Vyas Pharmacy while locally procured sample is enriched in Cl, P, V, Mn, Fe, Cu and Zn. Similarly *Bitter gourd* powder from Vyas Pharmacy is enriched in Na, Mg and Se whereas capsules from Himalaya Drugs contain significantly higher amounts of K, Ca, Cl, P. However, Cu, Cr, Mn, Fe, Zn, Rb, Cs and Se contents are comparable in both cases.
- Raw herbs have higher contents of La, Ce and Sm while the powdered one contain elevated amounts of Th and Eu. The REE uptake is governed by the plant species, growing season and various environmental factors as well as the properties of the host soil.

- *Bitter Gourd* in both capsule and powder form contains very high amount of As (1.01±0.07 μg/g and 1.44±0.12 μg/g) and Br (433±27 and 203±6 μg/g) respectively. This level of As, though high, is below the permissible limits.
- K/P ratio in herbs and capsules lies in a very close range of 6.45-10.7 with a mean of 8.19±1.39. Cu & Zn (r = 0.89) and Rb & Cs (r = 0.87) are linearly correlated in antidiabetic herbs.
- Mean contents of Ca, K, Mg and P in five antidiabetic herbal formulations are in the range 0.5 – 2.0%. The minor constituents vary by a factor of 2-4 but Na content in five brands varies by an order of magnitude.
- *Jambrushila* is enriched in Mg, Ca, Zn, Fe, Rb, Cu and Se contents, which are all diabetically important elements. Incidentally, its constituents *neem, gurmaar, jamun* and *bitter gourd* are also enriched in most of these elements.
- Much wider variation in toxic elements are observed for different brands of formulations. Cd, Pb, Sb and Th contents vary by a factor of ~5 while As and Br contents vary by an order of magnitude. These contents are well below the permissible limit specified by the USFDA.
- In general, antidiabetic herbs are richer in Na, Ca, Cl contents compared to formulations, which have higher Cr, Cu, Fe, and Mn contents. V, Rb and Se contents are, however, comparable but Zn content is halved. These elements are directly related to *diabetes* and other related complications
- K/P ratio in formulations lies in a broad range of 1.99-15.5 with a mean of 6.06±5.59 (somewhat lower than for herbs). Ca/P ratio lies in a narrow range of 1.30-5.93 with a mean of 3.32±2.06.
- An inverse correlation is observed between Zn and Cr (r = -0.81) and a strong linear relationship is observed between Rb and Cs (r = 0.93).
- Two new compounds 7–(but-3-enyl) 1,2-dihydro cyclobutabenzene and 9,10 anthracenedione (2-hydroxy methyl) 1-methoxy were separated from the ethyl acetate fraction of the petroleum ether extract of *neem* leaves. Anthraquinones, known as active metabolites of emodin-type O- and C-glycosyl compdounds influence the ion transport across colon cells and act as laxatives.
- Four new compounds: 1,1,2,3-tetramethylcyclopropane, methyl phenyl sulfone, n-hexanedecanoic (palmitic) acid and 9,12,15-octadecatrienal have been identified in petroleum ether extract of neem leaves. Palmitic acid may be useful in the manufacture of neem-based soap. Methyl sulfone derivative has a promise

as anti-malarial agent. 9, 12, 15 octadecatrienal commonly known as linolenic aldehyde is used to augment the flavour and aroma of foodstuffs including chewing gum and toothpaste.

- It is proposed that the essential trace elements (Mn, Fe, Cu, Zn etc) might be complexed with the organic compounds (anthraquinone derivative) acting as ligands thus making them available to the body system in the cure of diabetes.

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CHAPTER VI

TRIKATU: A HERBAL FORMULATION

A part of the work was presented at the International Conference on Application of Radiotracers in Chemical, Environmental and Biological Sciences (ARCEBS 06) held at the Saha Institute of Nuclear Physics (SINP), Kolkata, Jan 23-27, 2006. It has been accepted for publication in *J. Radioanal. Nucl. Chem.* (2007)

VI.1 HERBAL FORMULATION

The science of herbal formulation is one of the *Ayurveda's* most significant contributions to healthcare. In earlier times many of the traditional physicians called *Vaidyas* enjoyed a high level of local acceptance and respect, and thus had considerable influence on health beliefs and practices. According to them, some Ayurvedic formulations containing more than one plant species enhance the potency and support the primary plant species. Sometimes secondary plant species are added to the formulation to counteract any possible adverse side effects from the actions of the primary plant. *Vaidyas* use the whole herb or plant part in the preparation of medicine, whereas in the pharmaceutical industry, active ingredient is extracted to make plant-derived drugs. A herbal formulation may be considered as a herbal drug derived from a single or several herbs prepared/processed in a specified manner as described in old texts. The notion of using the whole herb or plant part may also contribute to a balanced formula rather than an isolated chemical constituent that is less likely to have adverse side effects.

Unfortunately, there has been a rapid decline in the traditional practice of individual healers identifying plants and preparing a formulation for direct distribution to patients. Because of rapid socio-economic changes, widespread urbanization and globalization, most of the *Vaidyas* have grown increasingly dependent on the products supplied by the Ayurvedic pharmaceutical industry. With the increased availability and acceptance of conventional Western medicine, many of those who are familiar with the tenets and benefits of *Ayurveda* are concerned that this traditional healing system is not receiving the respect it deserves. By documenting the herbal formulations prepared by traditional *Vaidyas*, it may be possible to prevent unfortunate scenario like the recent attempt by commercial interests in the United States to patent the traditional Ayurvedic medicinal plant and culinary spice turmeric (*Curcuma domestica*) [1].

A number of studies by various researchers have documented the use of traditional medicinal plants in India [2-5]. About 3500 Ayurvedic formulations

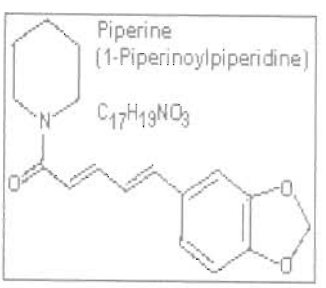
have been documented, as well as additional formulations based on the Siddha and Unani traditions, including details about their combinations have been proposed [6]. For centuries, the Ayurvedic herbal formulation *Chyavanprash*, a super-concentrated mixture of vitamin-rich herbs and minerals has been hailed as the ultimate anti-aging and anti-stress tonic [7]. *Trifala*, a mixture of *Amalaki*, *Haritaki* and *Bibhitaki* is considered the most effective and safest laxative and colon tonic by most health care practitioners [8]. A herbal drink called *Pragya-peya,* a mixture of 12 herbs has been developed by Shanti kunj, Haridwar and its analysis has been reported by our group [9]. The ancient ayurvedic texts maintain that how a formulation is prepared and it is as crucial to its efficacy as what goes into that formula. Ayurvedic herb processing, or sanskar, is therefore a meticulous and highly developed science that ranges from instructions on when to harvest a specific herb to the sequence in which ingredients are to be added to a formulation and how it is administered. However, much of the knowledge held by various traditional herbal healers regarding their use of medicinal plants has not been scientifically documented so as to be acceptable to modern medical practitioners.

VI.2 *TRIKATU*: A MIXTURE OF THREE SPICES

Trikatu is a Sanskrit word meaning 'three spices' in powder form. This ancient formulation is made up of three herbal stimulants; black pepper (*Piper nigrum*), Indian long pepper (*Piper longum*) and the rhizomes of ginger (*Zingiber officianilis*) in equal amounts. It is prescribed as an essential part of many multi-herb preparations, as it has been shown to increase the bioavailability of nutrients, foods, and medicines [10]. The *Trikatu* group of drugs increase bioavailability either by promoting rapid absorption from the gastrointestinal tract, or by protecting the drug from being metabolised/oxidised in its first passage through the liver after being absorbed, or by a combination of these two mechanisms. It is highly effective in dyspepsia accompanied with clinical symptoms of achlorhydria and hypochlorhydria and provides a balanced heat to warm digestion and circulation and prevents the formation of gastric mucosa [11].

Its thermogenic effect, metabolic enhancement, and nutrient bioavailability enhancing properties render *Trikatu* particularly beneficial in the support of respiratory health. This is done by reducing *Kapha* and increasing *Pitta* through the rejuvenation of low *Agni* and the burning away of *Ama*. It is strongly heating and thus removes cold, congestion, reviving weak organic functions. It is also an aphrodisiac and strengthens reproductive functions, warming, and energizing.It is useful in cases of obesity, weak digestion, high cholesterol, high triglycerides, hypothyroid, slow metabolism, congestion, cough, and edema. It is antiallergenic, carminative, antiflatulent, and acts as a natural antihistamine. *Trikatu* is ideally taken one hour before or after each meal in a dose of ½ to 1 teaspoon with honey in a thick paste or luke warm water as prescribed by the physician. A brief discussion on the individual constituents follows

(i) Black Pepper (Piper Nigrum): Its common name in India is *Kali Mirch*. The plant is a thick glabrous climber, the fruit is glabrous, 6 mm in diameter, which is initially green but turns black on drying. Black pepper contains volatile oil, the crystalline alkaloids-piperine, piperidine, piperetine and a resin [12]. The minor alkaloids present are piperetine, piperolein A, piperolein B, piperanine, trichostachine. The volatile oil contains large amounts of terpenes and α-pinene, phellandrene, dipentene and sesquiterpenes [13]. It is a commonly used spice all over India and other parts of world as well. Piperine enhances the pesticidal properties while the benzene extract is markedly fungitoxic. When given orally to rats at 100 mg/kg it shows slight febrifugal activity [14]. It is used commonly as a culinary seasoning, but is also prescribed for dyspepsia, flatulence, diarrhoea and used as gargle for sore throat [15]. A poultice made from pepper, salt and vinegar is used to soften corns. Unani system uses black pepper as a carminative, aphrodisiac and antispasmodic.



(ii) Long Pepper (Piper Longum): Indian Long Pepper or *Pipali*, indigenous to Northeastern and Southern India and Sri Lanka, is a powerful stimulant for both the digestive and the respiratory systems. It has been shown to have a rejuvenating effect on the lungs. Pipali plays an important role in aiding the thermogenic response, i.e. the release of metabolic heat energy. This effect is the result of increased thyroid hormone level in the body and makes it a typical Ayurvedic complementary component whose benefit is to increase the bioavailability and enhance absorption of the other active ingredients [10]. Long pepper is mainly used in pickles (achar). The fruit, which is very small, is sunk inside the fleshy spike is blackish green and is used in medicines. The root, which is thick and branched, is also medicinally important. The fruit contains 1% volatile oil, resin, alkaloids piperin and piperlonguminine, a waxy alkaloid (N-isobutyl deca-trans-2-trans-4-dienamide) and a terpenoid substance [16]. The roots have piperine, periongumine and dihydro-stigmasterol, which are responsible to promote respiratory health [17]. It also contains 1% essential oil, which exhibits antibacterial activity. A common use of the fruit is in the prevention of recurrent attacks of bronchial asthma and in malaria [18]. *Piper longum* differs little in its medicinal values from *P. nigrum,* as it is less aromatic and more acrid. It is widely used in Siddha, Ayurveda and Unani systems of medicine, particularly for diseases of the respiratory tract. The root is used for bronchitis, stomachache, and diseases of spleen, gout, lumbago and tumours. It improves appetite as well. In *Ayurveda*, the infusion is used as a stimulant, carminative and alterative and is more powerful than black pepper. Powdered long pepper administered with honey relieves cough, cold, asthma hoarseness and hiccup.

(iii) Ginger (Zingiber Officianalis): Rhizomes of *ginger* is the most widely used and available herbal remedy on the planet, with billions of people using it every day both as food and medicine. Ginger is used either fresh or dried powder in nearly two thirds of all traditional Indian and Chinese herbal formulations. Fresh

ginger is used to relieve dryness and heat, while dried ginger called *saunth* is used to relieve dampness and chill. Ginger warms the energy channels and stops bleeding, especially uterine bleeding. Typical preparations include tea, tincture, encapsulation, in herbal formulations, and in cooking. Ginger relieves the pain of rheumatoid arthritis by stopping the immune system production of inflammatory leukotrienes. Ginger is especially useful in small doses during pregnancy. Ginger is also an age-old remedy for morning sickness [19]. When used in herbal formulation, ginger protects the body from carcinogenic effects of valproic acid (Depakote), a medication commonly prescribed for migraine and seizure disorders but excessive use may cause gastro-intestinal upset [20]. Organic constituents present in it are 1,8-cineole, 6-gingerol, 6-shogaol, 8-shogaol, acetic acid, α-linolenic acid, α-phellandrene, α-pinene, α-terpinene, α-terpineol, arginine, ascorbic acid, β-bisolene, β-carotene, β-pinene, β-sitosterol, caffeic acid, camphor, capsaicin, chlorogenic acid, curcumene, gingerols, sesquiphellandrene, zingiberene, resins, starches, fats and proteins.

VI.3 LITERATURE SURVEY

Most work reported in literature on *trikatu* deals with its pharmacological aspects. Nothing has been reported on the essential and trace element contents in *Trikatu* though its constituents have been widely analyzed. Atal et al. [10] evaluated the scientific basis of the use of *trikatu* group of acrids in a large number of Ayurvedic prescriptions using ^{3}H-labelled vascine and sparteine as model drugs. Johri and Zutsi [9] reviewed its uses due to the bioavailability enhancing action on other medicaments. Sivakumar and Sivakumar [21] ascertained its efficacy as a hypolipidaemic agent. Karan et al. [22] observed significant lowering of peak plasma concentration of rifampicin and isoniazid in rabbits treated with a single dose of *trikatu*. Lala et al. [23] studied the effect of *trikatu* on the pharmokinetics and pharmodynamics of diclofenac sodium, a non-steroidal anti-inflammatory drug.

Wei et al. [24] isolated 15 novel dimeric amide alkaloids possessing a cyclohexene ring along with 4 others possessing a cyclobutane ring from *black pepper*. Al-Bataina et al. [25] analyzed Mg, Al, Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Cu, and Zn in black pepper and other spices using XRF. Nalini et al. [26] observed that *black pepper* suppresses colon carcinogenesis in 1,2-dimethylhydazione (DMH) induced cancer in rats. Anuradha et al. [27] isolated a new alkamide, isodihydropiperlonguminine from the hexane extract of dried fruits of *long pepper*. Singh and Garg [28a] determined 20 elements (As, Ba, Br, Ca, Cl, Co, Cr, Cu, Fe, K, Mn, Mo, Na, P, Rb, Sb, Sc, Se, Sr and Zn) from *long pepper* and other plant parts using INAA. These authors have further analyzed *black pepper* for 20 elements [28b]. Agrawal et al. [29] studied the antiulcer effect of *long pepper* in rats and concluded that the antiulcerogenic is due to the augmentation of mucin secretion and decreased cell shedding. Yang [30] extracted flavonoids from 80% aq. ethanolic extract of dried ginger and studied the stability and antioxidative activity of the extract under different conditions. Alam and Rehman [31] determined the elemental composition of *ginger* and other spices commonly consumed in Bangladesh and India using GF-AAS. Akoachere et al. [32] investigated the antibacterial activity of the ethanolic extract of *ginger* on four respiratory tract pathogens and observed positive results.

VI.4 PRESENT STUDY

Five different brands of *trikatu*, procured from Yogi (Haridwar), Vyas (Indore), Zandu (Mumbai) and Sushrut (Nagpur) Pharmacies and a local sample from Mumbai were analyzed for seven minor (Al, Ca, K, Na, P, Mg and Cl) and 24 trace (As, Au, Ba, Br, Ce, Co, Cr, Cs, Cu, Eu, Fe, Hf, Hg, La, Mn, Rb, Sb, Sc, Se, Sm, Sr, Th, V and Zn) elements by NAA. Further Ni, Cd and Pb were determined by AAS.

Column separation was carried out for *pipali* for the separation and isolation of barbituric and tannic acids as confirmed by elemental analysis, ir spectra and GC-MS. Hydrodistillation of *pipali* yielded an essential oil whose GC-MS studies showed 10 compounds; 2,2-dimethyl propanoic acid, decane, 1-

decyne, 3,4, 8-trimethyl 1-nonene, undecane, bis- (1-methylpropyl) disulfide, 2-nonynoic acid, 2,4-decadienal, nonanoic acid and tetradecanoic acid.

VI.5 EXPERIMENTAL

(i) Sample collection and preparation; Four different brands of trikatu were obtained from the Vyas Pharmacy (Indore, M.P.), Yogi Pharmacy (Haridwar, UA), Zandu (Mumbai) and Sushrut Pharmacies (Nagpur) both from Mahrashtra. Also a locally prepared powdered sample from Mumbai was procured. The sample from Sushrut was analyzed in capsule as well as raw powdered form through the courtesy of Dr. J. Deopujari, Nagpur. Its three constituents in powder form in duplicates were procured locally at different intervals. These were passed through a sieve of 100 mesh and oven dried at 80 °C for 2 h before use. The samples were stored in precleaned polyethylene capped bottles and handled with extreme care in a glove box (Fig. II.1). Three RMs of biological origin, Apple leaves (SRM-1515) [33], Peach leaves (SRM-1547) [34] procured from the NIST, USA and Mixed Polish Herbs (MPH-2) [35] from INCT, Poland were used as comparator standards and for data validation.

(ii) Irradiation and Counting: 30-50 mg aliquots each of samples and RMs in dried form were packed in alkathene and irradiated together in a batch of 5/10 for 5 min/7h respectively at a thermal flux of 10^{12} n $cm^{-2}s^{-1}$ in the APSARA reactor (BARC, Mumbai). Irradiation details are described in *Ch. II.5*. Details of counting set up are same as mentioned in *Ch. II.7*. Irradiation and counting schedule followed and elements determined are given in Table II.2. Cd, Ni and Pb were determined by AAS as described in *Ch. II.9*. Elemental contents were calculated by comparator method using RMs as comparators. Data were considered only if the values for RMs matched with the certified values within ±10%.

VI. 6 ORGANIC CONSTITUENTS IN *PIPALI*

Indian long pepper has been relatively less investigated specie for its organic constituents.

(i) Methanolic extract; 100 g dried powder of *pipali* was extracted successively with 1:1 aqueous methanol by using a Soxhlet extractor for 6 h at a temperature not exceeding 65 °C. The extract was filtered using Whatman filter paper and concentrated. Column separation was carried out using a set of solvents with increasing polarity in the order; petroleum-ether < chloroform < ethyl acetate < ethanol < methanol < water. Methanol - water fraction (25:2) yielded two distinct spots at R_f =0.74 and 0.59 which were later developed on a preparative TLC plate (20x20 cm^2) in a mixture of methanol/water (50:1). The two compounds were scrapped out, dissolved, filtered and distilled and finally recrystallized in acetone. Their C, H and N contents were determined and IR spectra were recorded in KBr. Separation scheme is shown in Fig. VI.1

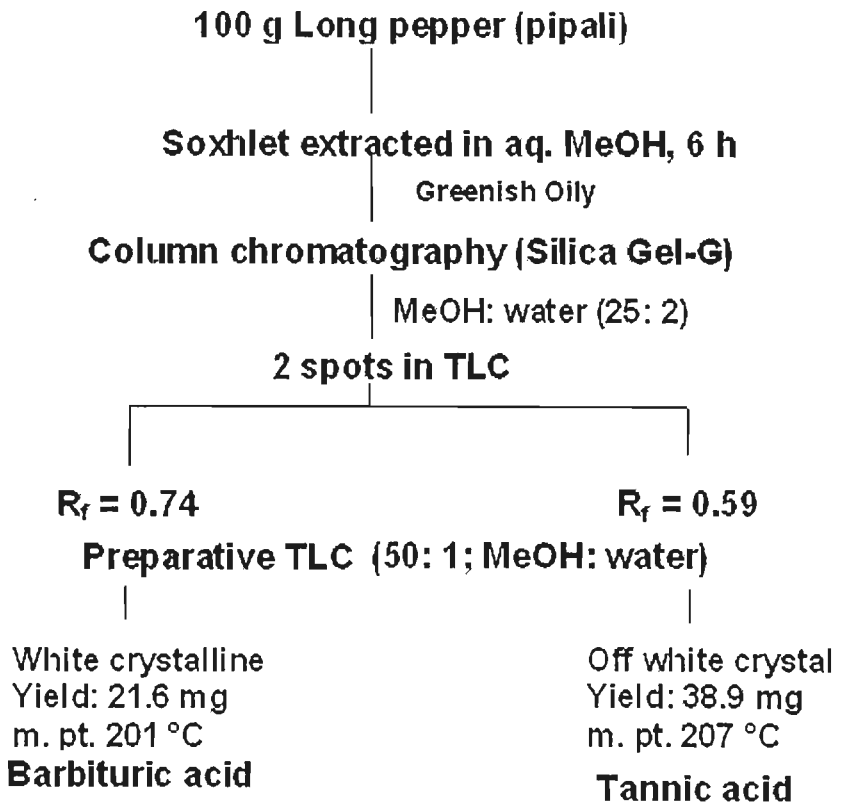


Fig. VI.1 Flowsheet showing the separation scheme of barbituric and tannic acid

Barbituric acid: White crystalline, Yield: 21.6 mg, m.pt. 201 °C (Lit.198 °C); CHN analysis; C (calc.%): 38.16 (37.51), H (%): 3.07 (3.15), N (%): 22.04 (21.87). IR spectrum KBr (cm^{-1}): 3437 (ν_{N-H}), 2966(ν_{C-H}), 1653 ($\nu_{C=O}$), 1277 (ν_{C-N}); Its

retention time (R_t) in methanol was 15.3 min and the mass spectrum could be explained in terms of various fragments as shown in Fig. VI.2.

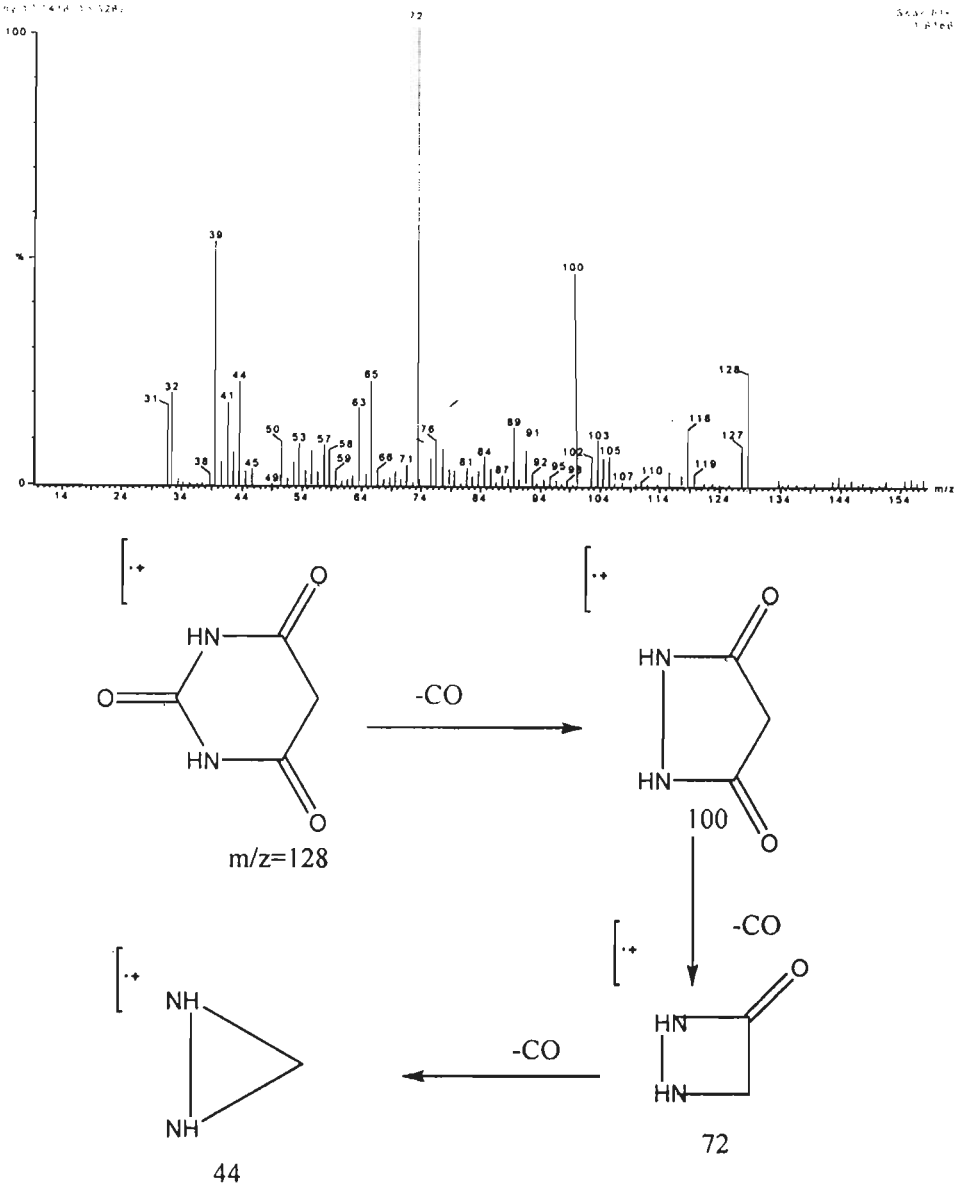


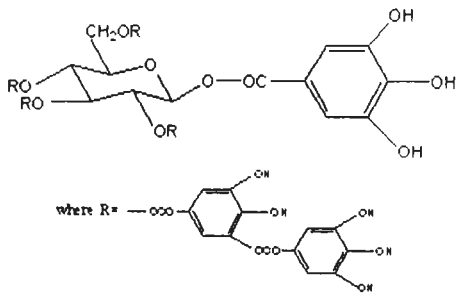
Fig. VI.2 Mass spectrum and fragmentation pattern of barbituric acid

Barbiturate drugs act as central nervous system (CNS) depressants and produce wide spectrum of effects such as mild sedation to anesthesia [36]. Some barbiturates are also used as anticonvulsants. Singh et al. [37] observed that *pipali* extracts had a central stimulant action in frogs, mice, rats, and dogs.

Though this compound is not reported previously, the sedative action of pipali could be attributed to barbituric acid.

Tannic acid: Off white crystalline, Yield: 38.9 mg, m.pt. 207 °C (Lit. 210 °C); CHN analysis (calcd. %); C: 26.18 (25.76), H: 1.61 (1.47) IR spectrum in KBr (cm^{-1}): 3425 (ν_{O-H}), 2852(ν_{C-H}), 1712 ($\nu_{C=O}$), 1205 (ν_{C-O});

Tannic acid is present in all foods derived from plants. It has been widely investigated as a chemopreventive agent because of many health-promoting properties [38-40]. Sehrawat et al. [41] observed that oral treatment of rats with tannic acid resulted in significant recovery of hepatic glutathione content, antioxidant and phase-II metabolizing enzymes thereby suppressing the tumour promotion stage. Sunila and Kuttan [42] observed immunomodulatory and antitumour activity in alcoholic extract of *pipali* and it might be attributed to tannic acid.



Tannic acid

(ii) Essential Oil; 50 g air-dried finely powdered sample was water-distilled for 4 h using a Clevenger apparatus when pale yellow and odourless oil (yield 2.31% v/w) was obtained. It was dried over anhydrous sodium sulphate, filtered and was analyzed by GC-MS. The components were identified based on the comparison of their relative retention time and mass spectra with those from the NIST spectral database [43]. Mass chromatogram of the essential oil extracted from *pipali* is shown in Fig. VI. 3.

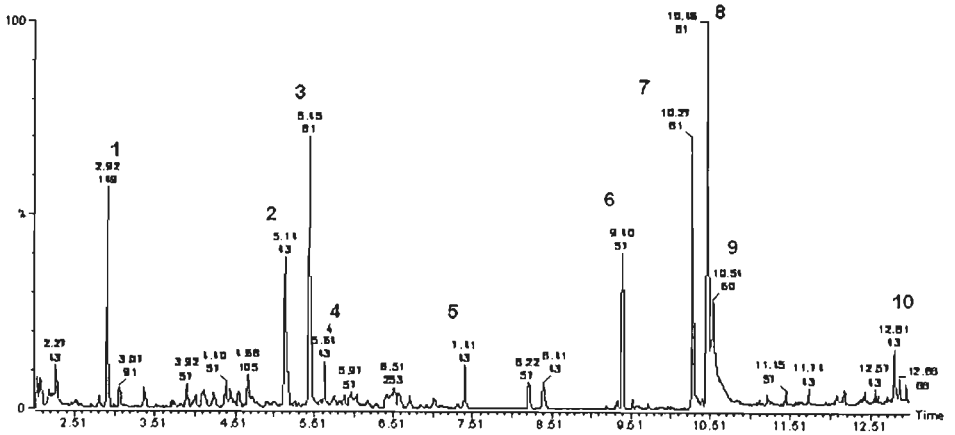


Fig. VI.3 Mass chromatogram of the essential oil in *pipali*

Mass spectra along with fragmentation of 10 compounds are shown in Fig. VI. 4-13.

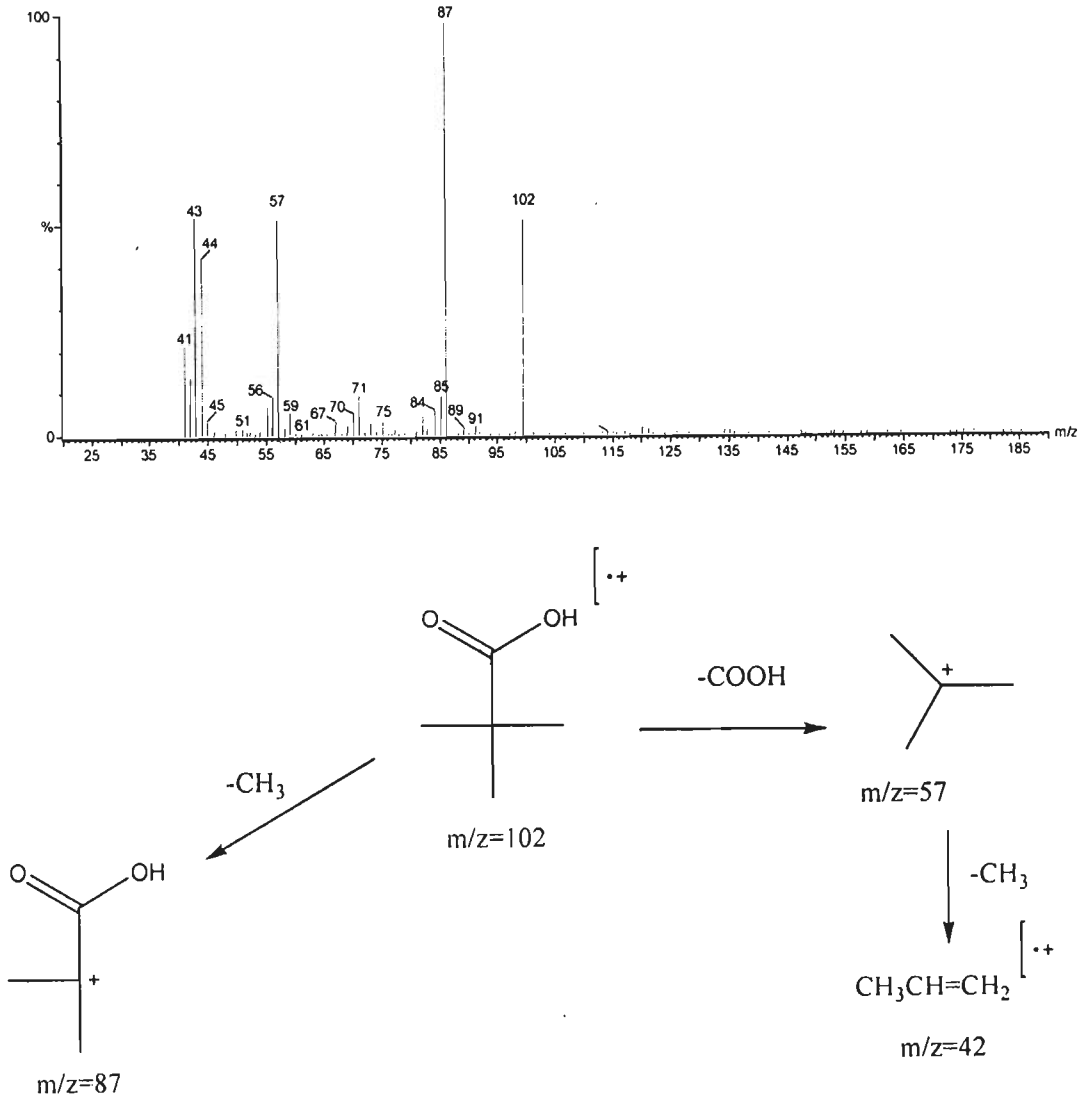


Fig. VI.4 Mass spectrum and fragmentation pattern of 2,2-dimethyl propanoic acid (R_t = 2.90)

2,2-dimethyl propanoic acid also known as pivalic acid was detected in the essential oil of fennel seeds [44]. Pivalic acid is produced in large quantities by the pharmaceutical industry, and is thought to have minimal toxicity. However,

the compound has been found to reduce the fertility of male hamsters [45]. This compound could be responsible for the antifertility activity of piper longum [46].

Fig. VI.5 Mass spectrum and fragmentation pattern of decane (R_t =5.14)

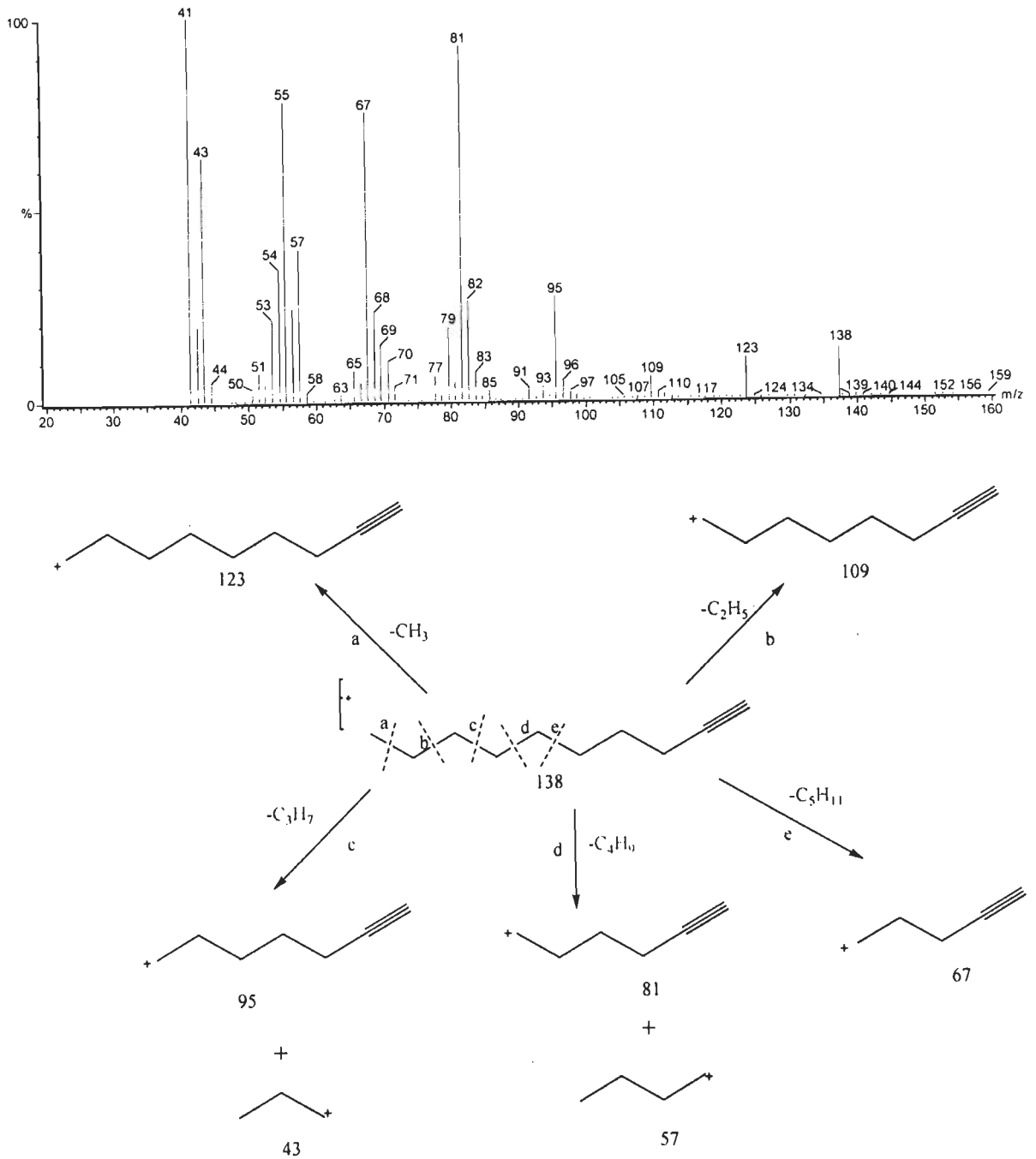


Fig. VI.6 Mass spectrum and fragmentation pattern of 1-decyne (R_t =5.46)

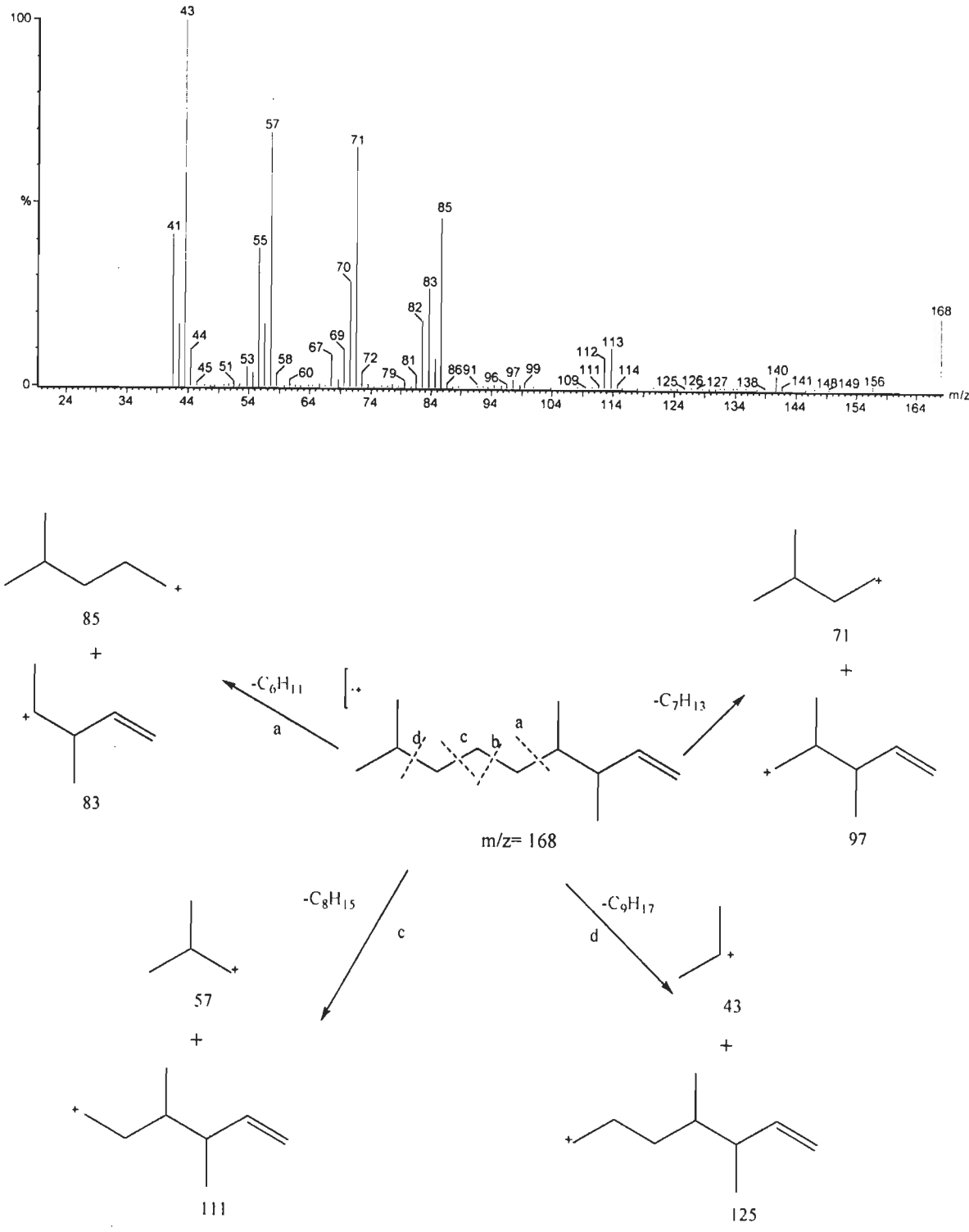


Fig. VI.7 Mass spectrum and Fragmentation pattern of 3,4, 8-trimethyl 1-nonene (R_t =5.64)

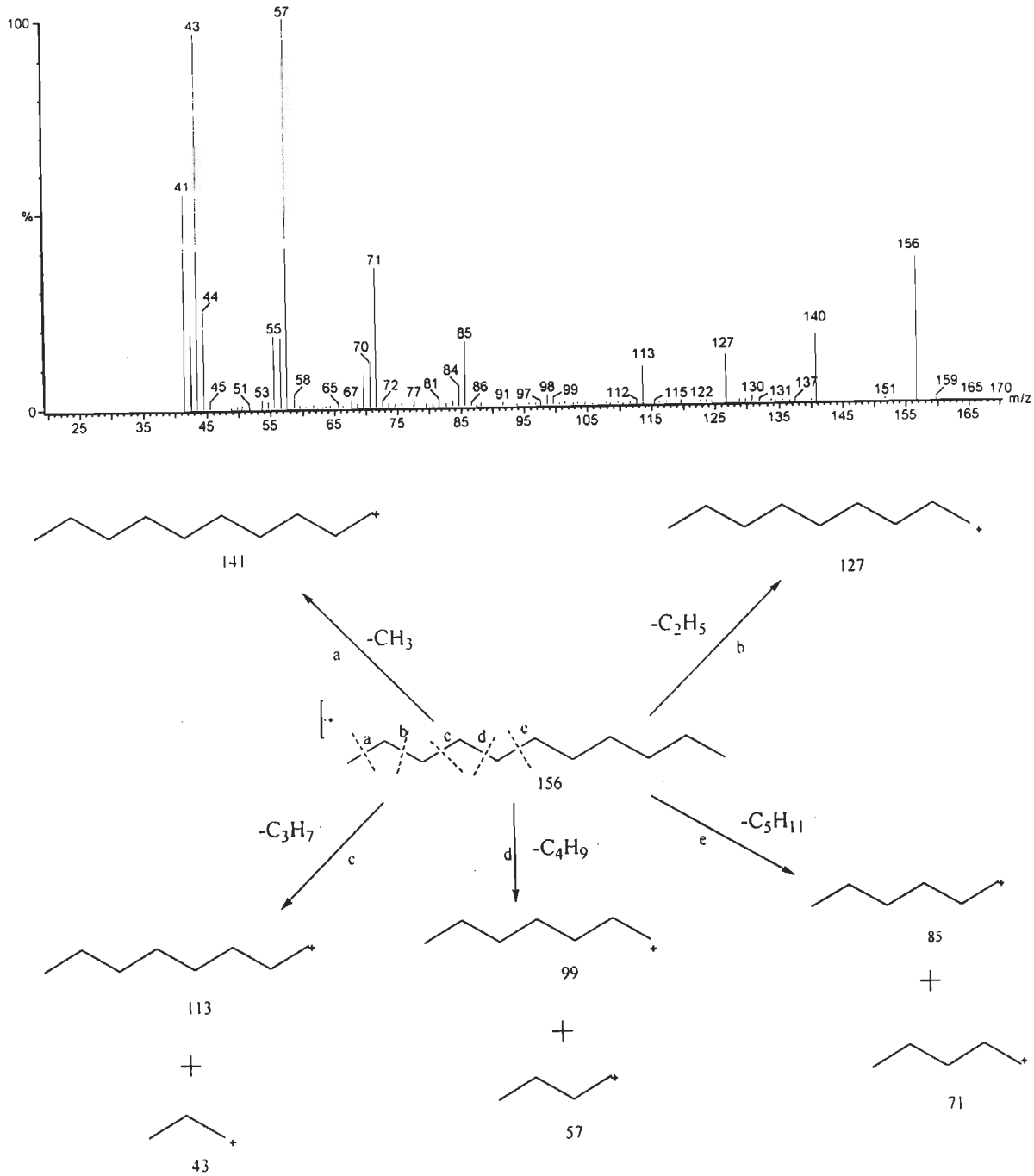


Fig VI.8 Mass spectrum and Fragmentation pattern of undecane (R_t =7.43)

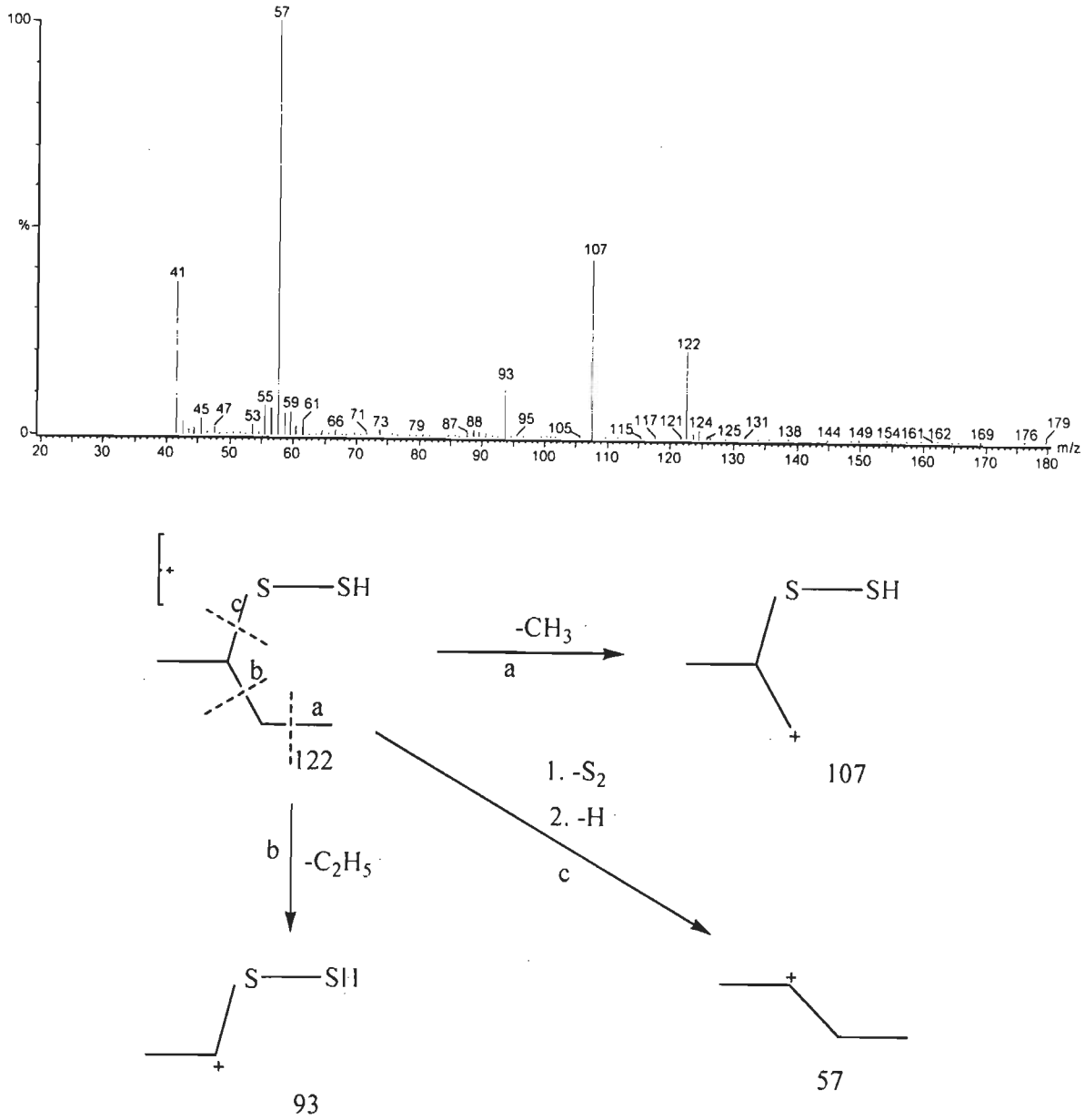


Fig. VI.9 Mass spectrum and fragmentation pattern of bis- (1-methylpropyl) disulfide (R_t =9.41)

Sefidkon et al. [47] observed bis- (1-methylpropyl) disulfide to be the main constituent in gum of *Ferula assafoetida* collected from Iran.

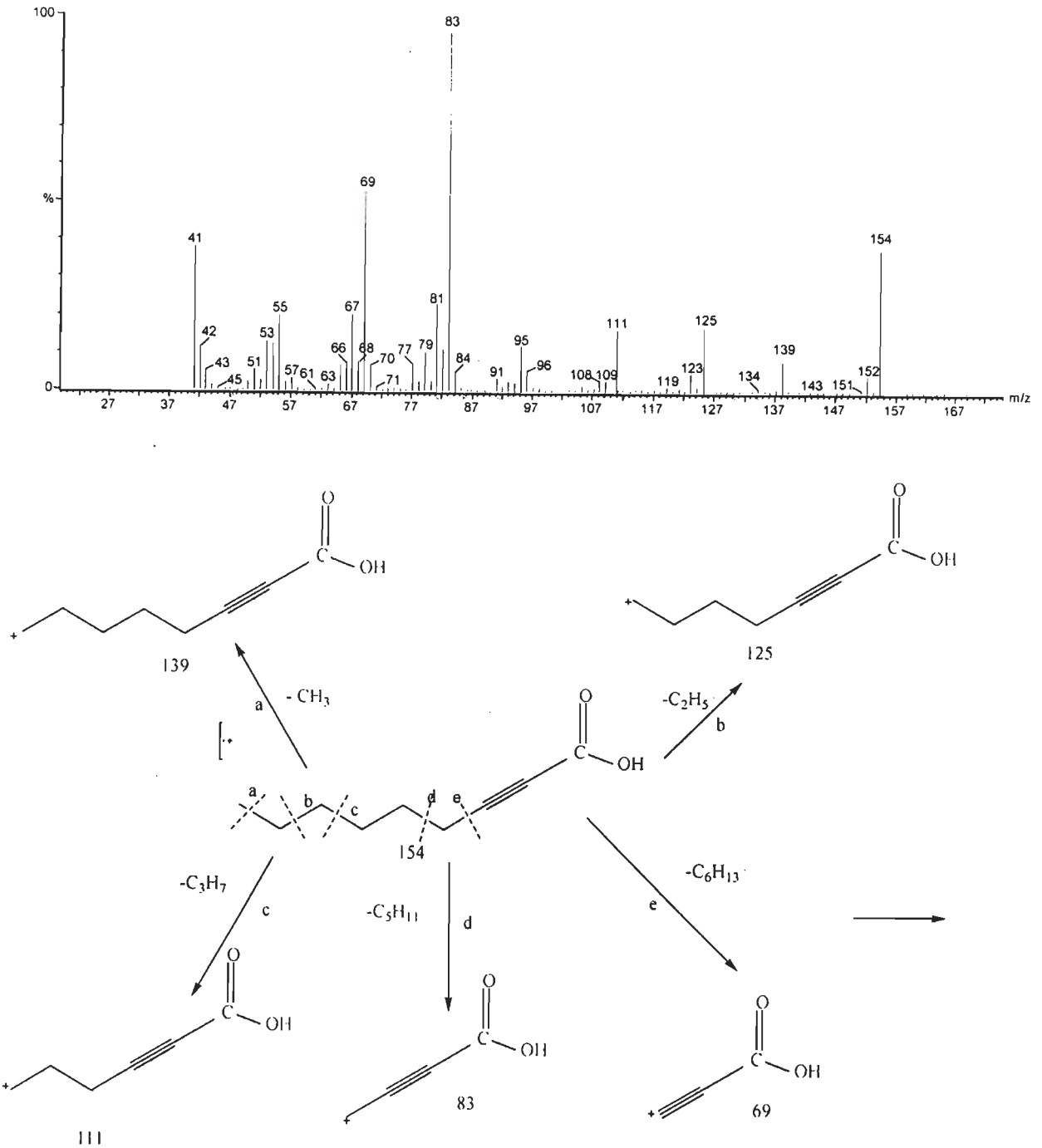


Fig. VI.10 Mass spectrum and fragmentation pattern of 2, 4-nonynoic acid (R_t =10.27)

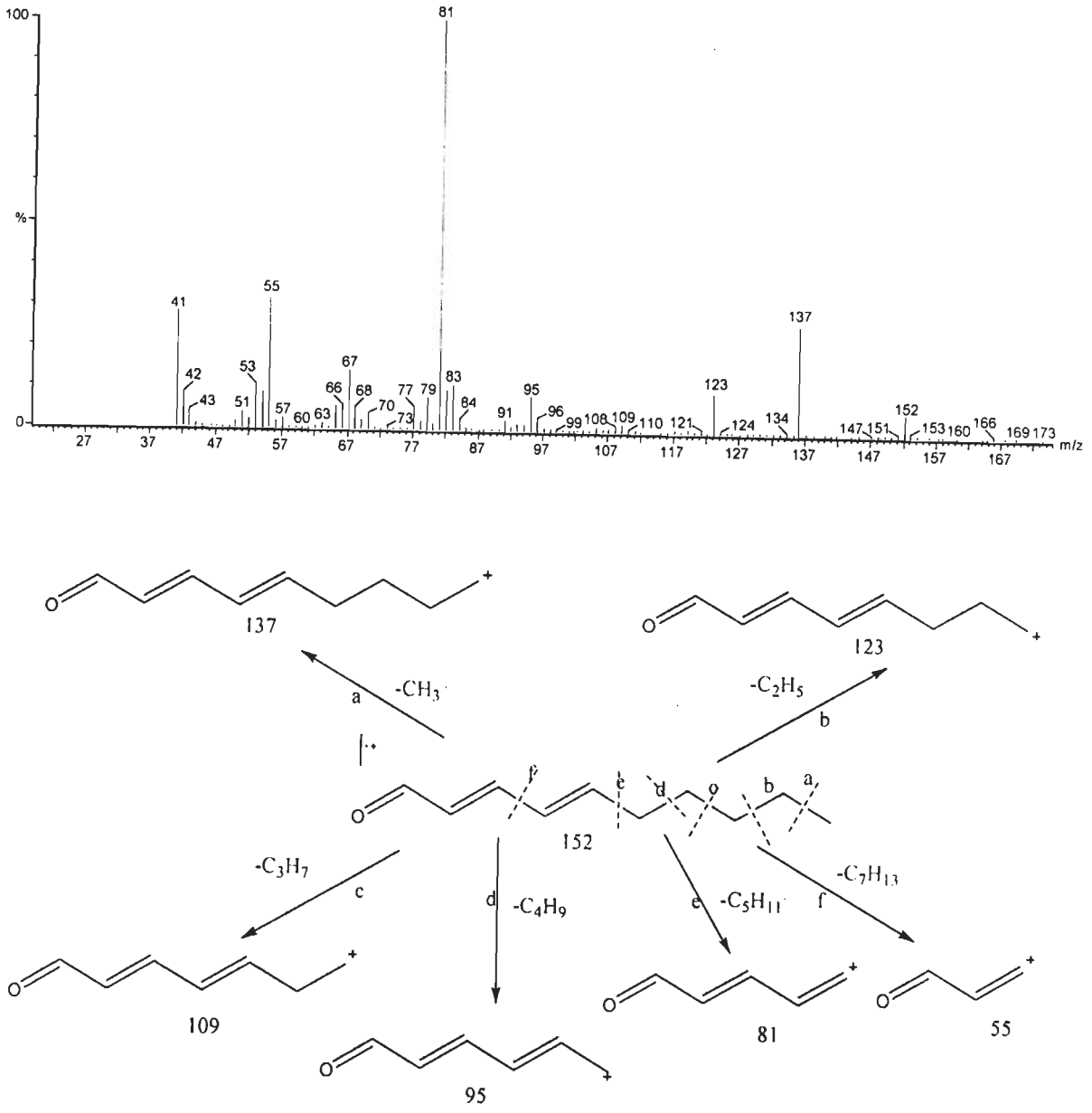


Fig. VI.11 Mass spectrum and fragmentation pattern of 2-decadienal (R_t =10.45)

2-decadienal has been reported in the volatile compounds of red pepper [48]. It is used to synthesize flavour and as an ingredient in soaps and cosmetics.

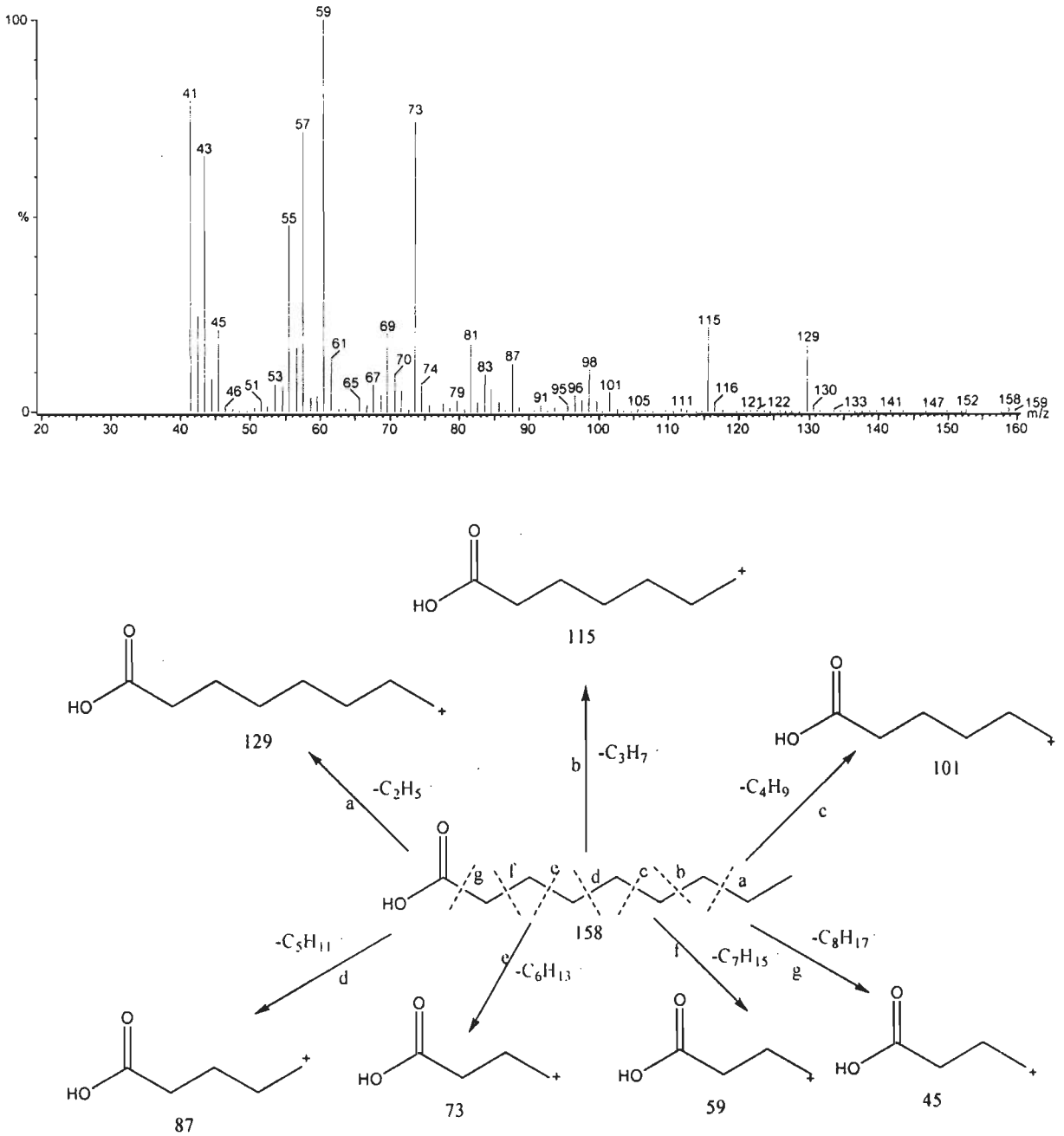


Fig. VI.12 Mass spectrum and fragmentation pattern of nonanoic acid (R_t =10.55)

Chadeganipour and Haims [49] studied the antifungal activitiy of nonanoic acid, also called pelargonic acid on *Microsporum gypseum*.

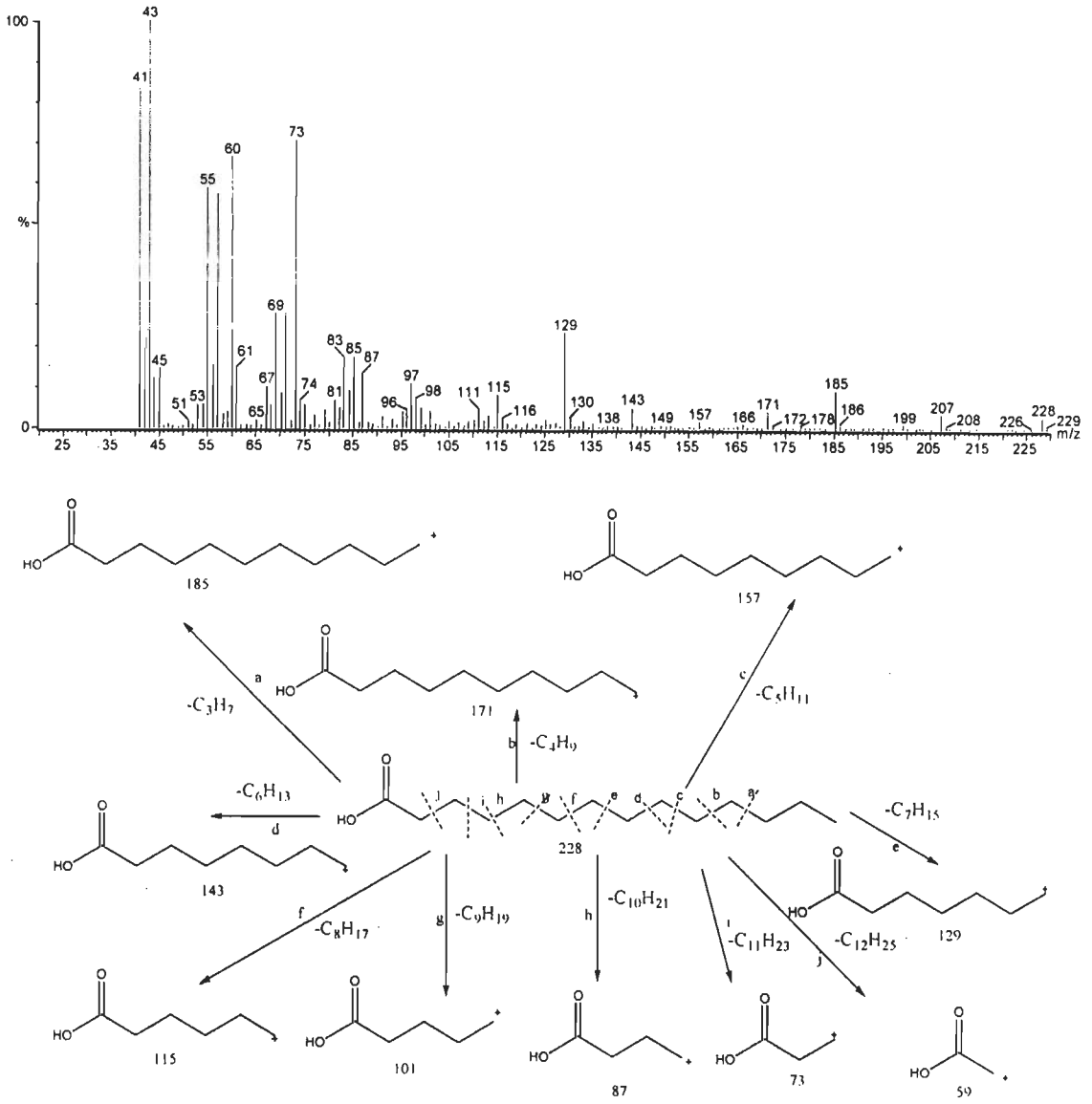


Fig. VI.13 Mass spectrum and fragmentation pattern of tetradecanoic acid (R_t =12.81)

Tetradecanoic acid is commonly known as Myristic acid. This saturated 14-carbon fatty acid occurs in most animal and vegetable fats, particularly butterfat and coconut, palm, and nutmeg oils.

Decane, 1-decyne, undecane, 2-nonynoic acid, nonanoic acid and tetradecanoic acid are all reported earlier in the essential oil of *pipali* [16,50]. Tannic, 2-nonynoic, nonanoic and tetradecanoic acids along with decane, 1-decyne, undecane are strong antioxidants [51-53] and are likely to be responsible for the antioxidant property of *pipali* [54].

VI.7 ELEMENTAL CONTENTS IN *TRIKATU*

Elemental concentrations in various *trikatu* brands and its 3 constituents (in duplicate) are listed in Table VI.1. Also included in the table are ranges of concentrations in 6 brands along with their mean ±SD. Elemental concentrations for Ni, Cd and Pb as obtained by AAS are also included in the same table. A typical γ ray spectrum for short irradiated (5 min) sample is shown in Fig. VI.14 and photo peaks corresponding to short-lived nuclides such as ^{27}Al, ^{52}V, ^{24}Na, ^{42}K, ^{27}Mg, ^{49}Ca ^{38}Cl and ^{56}Mn are marked in the figure. Significance of elemental contents in different brands and constituents are discussed.

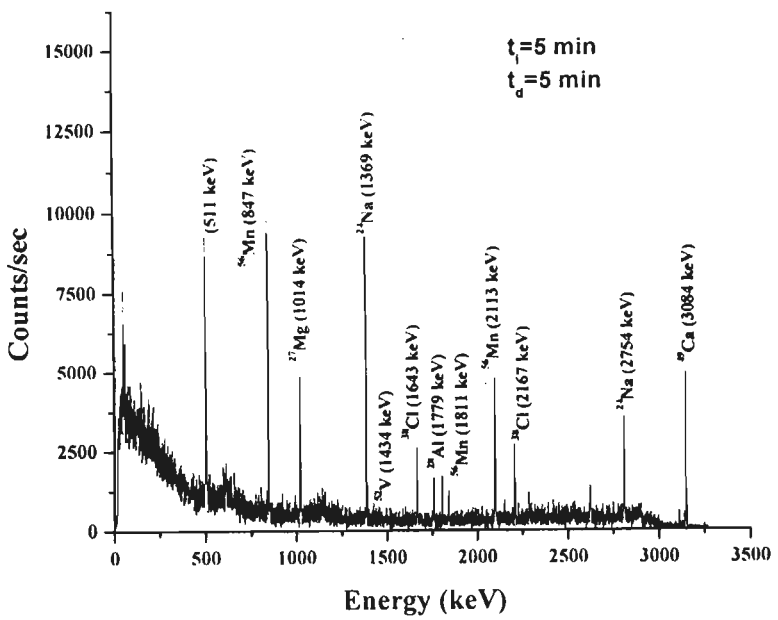


Fig.VI.14 Typical γ-ray spectrum for short-lived nuclides in *trikatu*

Table VI.1 Concentration of minor, trace and toxic element in *Trikatu* and its three constituents

Element	Yogi Pharmacy (Haridwar)	Vyas Pharmacy (Indore) n=2	Local (Mumbai)	Zandu Pharm. (Mumbai)	Sushruta Pharm. (Nagpur)		Range	Mean±SD	Black Pepper, n=2	Pipali, n=2	Ginger, n=2
					Capsule	Powder					
Al (ng/g)	416±23	386±32	325±27	240±14	183±9	224±13	183-416	296± 94	347± 14	106± 5	640±25
As (µg/g)	0.50±0.03	0.20±0.06	0.19±0.01	0.14±0.06	0.13±0.06	0.32±0.08	0.13-0.50	0.25± 0.14	0.21± 0.01	0.47± 0.11	0.18±0.04
Au (ng/g)	11.5±0.3	4.77±0.42	2.80±0.21	5.98±0.41	2.15±0.09	2.34±0.06	2.15-11.5	4.92± 3.56	2.94± 0.64	1.85± 0.36	2.74±0.04
Ba (µg/g)	21.9±1.6	25.0±1.6	26.1±1.6	19.3±0.8	21.8±2.8	27.2±1.6	19.3-27.2	23.6± 3.0	55.1± 8.1	18.9± 5.2	34.6±4.4
Br (µg/g)	11.6±0.5	9.08±0.52	14.6±0.7	7.90±0.35	11.9±1.9	14.3±1.3	7.90-14.6	11.6±2.7	17.1± 1.6	10.2± 1.4	13.6±5.5
Ca (mg/g)	3.02±0.21	4.80±0.38	3.65±0.35	4.29±0.18	3.83±0.07	3.81±0.06	3.02-4.80	3.90± 0.60	5.10± 0.03	6.30± 0.04	12.6± 0.1
Cd (µg/g)	ND	0.2	ND	0.24	0.12	0.16	0.12-0.24	0.18± 0.05	0.09	0.41	0.06
Ce (µg/g)	0.81±0.04	0.57±0.05	0.72±0.03	0.59±0.04	0.39±0.04	0.69±0.07	0.39-0.81	0.63± 0.15	0.73± 0.32	0.56± 0.03	1.35± 0.09
Cl (mg/g)	3.96±0.04	2.27±0.19	3.30±0.04	2.70±0.17	3.77±0.05	2.39±0.04	2.27-3.96	3.07± 0.72	2.28± 0.19	2.57± 0.21	1.41± 0.11
Co (µg/g)	0.25±0.01	0.21±0.04	0.61±0.02	0.30±0.01	0.28±0.01	0.22±0.01	0.21-0.61	0.30± 0.15	0.15± 0.02	0.10± 0.01	0.13± 0.02
Cr (µg/g)	1.08±0.12	1.07±0.07	2.36±0.36	0.73±0.02	0.89±0.09	0.64±0.04	0.64-2.36	1.13± 0.63	8.65± 0.48	3.03± 0.18	1.85± 0.31
Cs (ng/g)	85.6±5.5	54.3±6.8	86.3±2.8	2.83±0.13	56.8±1.5	41.2±0.9	41.2-86.3	67.1± 18.9	41.5± 1.4	105± 12	38.8± 3.5
Cu(µg/g)	16.5±0.4	12.6±0.1	14.3±0.4	15.6±1.0	23.9±2.6	18.9±1.3	12.6-23.9	17.0± 4.0	13.0± 1.3	28.1± 2.6	17.5± 1.0
Eu (ng/g)	15.2±1.2	101±13	28.6±1.8	153±3	32.5±2.2	67.7±5.1	15.2-153	66.3± 52.7	191± 15	153± 23	152± 18
Fe(µg/g)	535±27	493±41	901±51	686±19	312±18	518±28	312-901	574± 200	393± 37	172± 16	427± 34
Hf (ng/g)	90.0±2.0	13.1±0.2	118±12	167±13	95.0±2.1	74.2±3.8	13.1-167	92.9± 50.7	25.1± 1.5	19.8± 3.1	25.3±5.5
Hg (ng/g)	34.3±3.7	67.7±3.6	60.3±5.5	31.0±2.0	55.6±4.2	92.4±6.8	31.0-92.4	56.9± 22.7	60.1± 5.1	34.2± 11.1	72± 40
K (mg/g)	18.9±0.2	20.1±2.0	16.2±0.2	15.3±0.6	21.7±0.7	26.1±1.1	15.3-26.1	19.7± 3.9	17.8± 1.6	16.0± 2.1	21.4± 1.4
La(µg/g)	2.26±0.18	3.58±0.18	1.21±0.15	3.16±0.14	<0.42	3.53±0.21	0.42-3.58	2.36± 1.31	5.17± 3.63	3.47± 0.36	2.92± 0.51
Mg mg/g)	<0.2	0.47±0.04	<0.2	1.65±0.05	0.82±0.02	0.84±0.05	0.2-1.65	0.70± 0.55	<0.2	0.90± 0.07	1.88± 0.06
Mn(µg/g)	105±3	162±15	87.8±3.7	117±4	199±18	155±13	88-199	138± 42	128± 31	50.3± 8.1	266± 45
Na (µg/g)	1110±70	276±12	200±13	172±5	1587±60	1327±49	172-1587	779± 636	190± 7	123± 8	310± 44
Ni (µg/g)	ND	3.65	ND	0.75	1.37	1.89	0.75-3.65	1.92± 1.25	2.78	1.54	0.68
P(mg/g)	3.36±0.22	3.58±0.26	3.18±0.16	2.71±0.21	3.58±0.21	3.71±0.29	2.71-3.71	3.35± 0.37	3.53± 0.35	2.93± 0.69	2.65± 0.35
Pb (µg/g)	ND	2.15	ND	3.86	1.71	2.59	1.71-3.86	2.58± 0.93	0.98	1.52	1.86
Rb (µg/g)	19.7±0.8	20.1±2.8	28.9±1.9	26.5±0.5	23.9±2.6	14.9±1.1	14.9-28.9	22.3± 5.1	18.7± 2.0	33.7± 3.9	14.5± 1.6
Sb (µg/g)	34.7±3.5	23.4±2.1	43.3±3.6	20.2±0.9	24.6±1.4	18.0±1.1	18.0-43.3	27.4± 9.7	27.0± 1.7	42.7± 0.6	15.7± 1.3
Sc (ng/g)	138±3	58.6±6.8	185±4	42.6±0.9	76.0±2.0	31.2±2.1	31.2-185	88.6± 60.4	51.6± 2.7	16.8± 4.7	62.5± 48.6
Se (µg/g)	0.11±0.01	0.10±0.01	0.12±0.01	0.21±0.01	0.09±0.01	0.08±0.01	0.08-0.21	0.12± 0.05	0.09± 0.02	0.06± 0.01	0.08± 0.02
Sm (ng/g)	218±16	125±12	112±18	155±15	326±23	341±24	112-341	213± 100	95.0± 2.0	121± 6	126± 2
Sr(µg/g)	17.5±1.0	19.3±1.8	23.3±2.6	14.4±0.6	21.8±2.0	15.4±1.2	14.4-23.3	18.6± 3.5	38.9± 4.1	16.9± 0.7	17.8± 0.2
Th(ng/g)	205±12	137±14	167±7	157±6	153±13	125±10	125-205	157± 12	129± 3	110± 12	123± 32
V (µg/g)	1.07±0.02	2.39±0.19	2.37±0.06	0.85±0.03	1.69±0.06	1.85±0.08	0.85-2.39	1.70± 0.64	0.89± 0.02	1.12± 0.05	0.98± 0.04
Zn(µg/g)	17.8±1.9	21.0±0.17	27.5±3.2	23.2±0.8	19.7±2.1	17.9±1.6	17.8-27.5	21.2± 1.7	37.1± 6.7	14.5± 1.1	35.3± 1.6

The minor constituents of *trikatu* are Na, K, Ca, Cl, P and to some extent Mg and Fe whereas V, Mn, Cr, Cu, Co, Zn, Rb, and Se are in traces. Despite the fact that 5 brands of *trikatu* were obtained from different parts of India where 3 constituents are likely to have been grown under different soil conditions, most elemental contents vary in a close range by a factor of 2-3; Ca (3.02-4.80 mg/g), Cl (2.27-3.96 mg/g), Fe (0.31-0.90 mg/g), K (15.3-26.1 mg/g), P (2.71-3.71 mg/g), Zn (17.8-27.5 μg/g), Se (0.08-0.21 μg/g), Co (0.21-0.61 μg/g) and V (0.85-2.39 μg/g). However, Na (172-1587 μg/g) content varies by an order of magnitude. Variations of minor and trace elements in various brands of *trikatu* are shown in Fig. VI.15 and 16 respectively. Such variations may be attributed to differential elemental uptake by the respective plants from the soil and other geo-environmental factors as suggested by Zaidi et al [55]. Higher Na content (>1 mg/g) in 3 brands (Yogi Pharmacy, Sushruta capsule and its powder) may be due to deliberate addition of common salt to suppress its pungent taste. In general, *trikatu* exhibits higher concentrations of several nutritionally important elements such as Ca (3.90 ±0.60 mg/g), Fe (0.57±0.20 mg/g), Mn (138±42 μg/g), Se (0.12±0.05 μg/g) and Zn (21.2 ±1.7μg/g). High Fe content could explain the role of *trikatu* as a digestive healer [9]. Two samples of Vyas Pharmacy collected at an interval of a

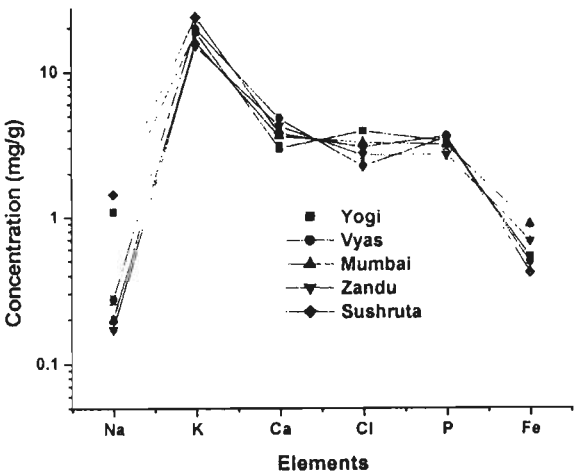
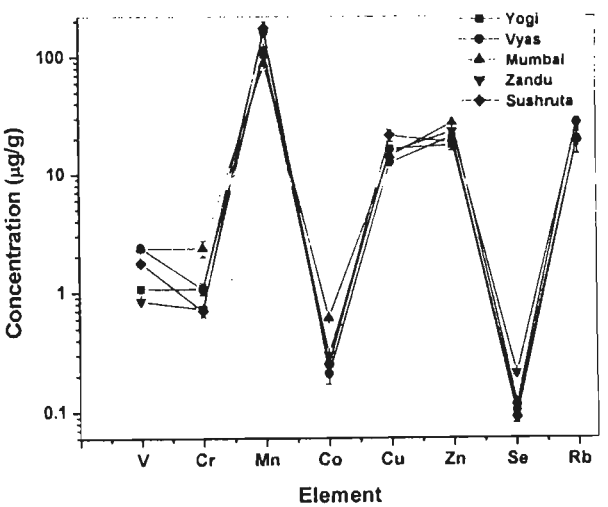


Fig. VI.15 Variation in concentration of minor elements in different *trikatu* brands



Fug. VI.16 Variation in trace element concentration in different *trikatu* brands

year do not show widely different elemental contents while exhibiting highest Ca and V. Similarly the 2 samples from Sushrut Pharmacy, Nagpur (capsule and powder) do not differ significantly in their elemental contents. Out of the 5 brands analyzed in this study, a sample from Mumbai seems most enriched in Ba, Cr, Co, Fe, Zn, Rb and Cs whereas the sample from Zandu exhibits highest Se content. Se as glutathione peroxidase inhibits the replication of tumor virus and prevents the malignant transformation of cells [56]. It is observed that contents of toxic elements such as As, Hg, Cd and Pb are well below permissible level [57]. Hence the product seems safe for human consumption as a drug.

VI.8 ELEMENTAL CONTENTS IN CONSTITUENTS

Out of the three constituents of *trikatu*, ginger also called *saunth* in dried form, is a household spice-cum-medicine. It is particularly enriched in Ca (12.6±0.1 mg/g), Mg (1.88±0.06 mg/g), Fe (427±34 μg/g) and Mn (266±45 μg/g) whereas black pepper another widely used spice is enriched in P (3.53±0.35 mg/g), Cr (8.65±1.48 μg/g), Se (0.093±0.02 μg/g) and Zn (37.1±6.7 μg/g) contents. *Pipali*, on the other hand has higher Cu (28.1±2.6 μg/g) and V (1.12±0.05 μg/g) contents. A histographic comparison of minor, trace and toxic elements in *trikatu* and its 3 constituents are shown in Figs. VI.17, 18 and 19 respectively.

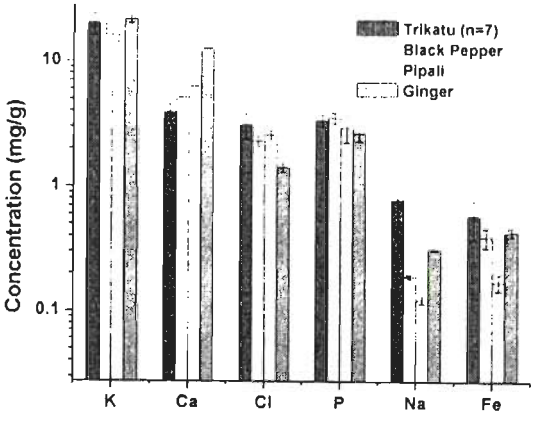


Fig.VI.17 Concentration of minor elements in *trikatu* and its constituents)

A perusal of the figures shows that all the elemental contents in the three constituents are comparable with the mean values in *trikatu* suggesting that it is an intimate mixture of three constituents.

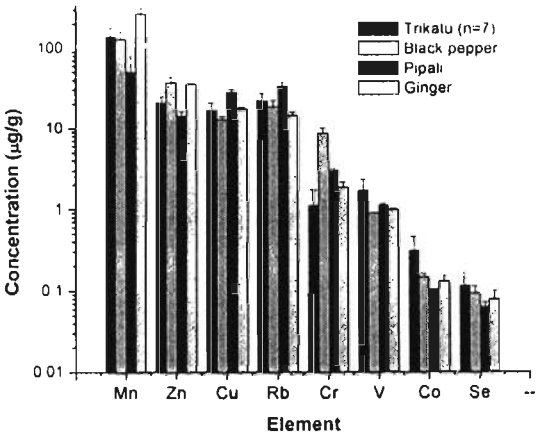


Fig. VI.18 Concentration of essential trace elements in *trikatu* and its constituents

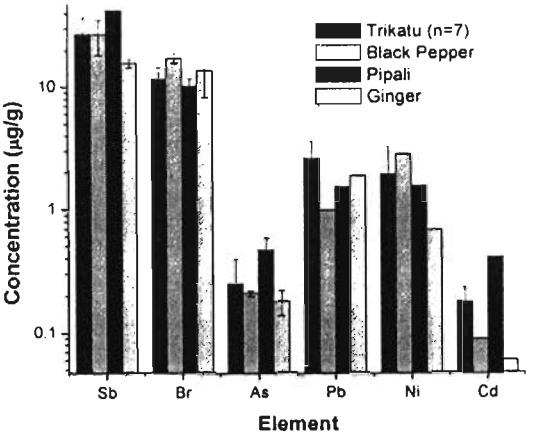


Fig.VI.19 Concentration of toxic elements in *trikatu* and its constituents

VI.9 ELEMENTAL CORRELATIONS

In accordance with the observation of several elemental correlations in biological tissues and plant species, we have observed an inverse correlation between Fe and Mn in *trikatu* (r =-0.89) and its constituents (Fig. VI.20). However, Cu/Zn ratio varies linearly (r = 0.92) with Zn (Fig.VI.21).

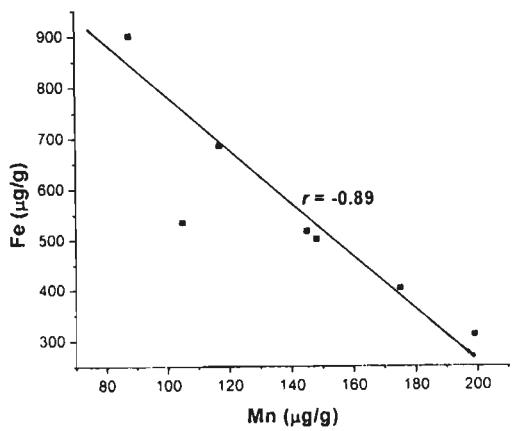


Fig VI.20 Correlation of Fe vs Mn in *Trikatu*

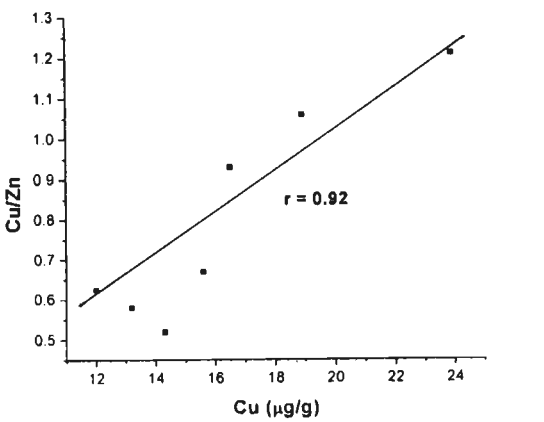


Fig. VI.21 Correlation of Cu/Zn to Cu in *Trikatu*

It is possible that essential elements might be present in the form of complexes with organic compounds such as barbituric and tannic acids, making them bioavailable. Many complex species of barbiturates are reported in literature [58].

CONCLUSION

Five brands of *Trikatu,* a herbal formulation of three spices has been analyzed for 7 minor (Al, Ca, K, Na, P, Mg and Cl) and 27 trace (As, Au, Ba, Br, Cd, Ce, Co, Cr, Cs, Cu, Eu, Fe, Hf, Hg, La, Mn, Ni, Pb, Rb, Sb, Sc, Se, Sm, Sr, Th, V and Zn) elements by INAA and AAS. Also *pipali* has been analyzed for organic constituents. Following generalizations can be made;

- *Trikatu* exhibits higher concentrations of several nutritionally important elements such as K, Ca, Fe, Mn, Se and Zn. High Fe content could explain the role of *trikatu* as providing strength to the body system.
- Most elemental contents vary in a close range by a factor of 2-3 despite the fact that 5 brands were obtained from different parts of India where 3 constituents are likely to have been grown under different soil conditions.
- Na (172-1587 μg/g) content varies by an order of magnitude. Higher Na content (>1 mg/g) in 3 brands (Yogi Pharmacy, Sushruta capsule and its powder) may be due to deliberate addition of common salt to suppress its bitter taste.
- Two samples from Sushrut Pharmacy, Nagpur (capsule and powder) do not differ significantly in their elemental contents. Sample from Mumbai are most enriched in Ba, Cr, Co, Fe, Zn, Rb and Cs whereas the sample from Zandu Pharmacy exhibits highest Se content.
- Toxic elements such as As, Hg, Cd and Pb are well below permissible limits.
- *Ginger* is particularly enriched in Ca, Mg, Fe and Mn whereas black pepper is enriched in P, Cr, Se and Zn contents. *Pipali*, on the other hand has a higher Cu and V contents.
- All the elemental contents in the three constituents are comparable with the mean values in *trikatu* suggesting that it is an intimate mixture of three constituents.
- Fe and Mn are inversely correlated with r = -0.89 in *trikatu* and its constituents but Cu/Zn ratio varies linearly (r = 0.92) with Zn.
- Barbituric acid and tannic acid were separated from the methanolic extract of *pipali.* Barbituric acid may be responsible for its sedative action on the Central Nervous System (CNS). Tannic acid, a chemopreventive agent may be responsible for its immunomodulatory and antitumour activity.

- Ten compounds including 4 hydrocarbons, 4 fatty acids, an aldehyde and a disulfide were identified from the GC-MS of the essential oil of *pipali*.
- 2, 2-dimethyl propanoic acid could be responsible for the antifertility activity of *pipali*.
- Nonanoic acid, also called pelargonic acid is an antifungal agent. Tetradecanoic acid (Myristic acid), occurs in most animal and vegetable fats, particularly butterfat and coconut, palm, and nutmeg oils. It is used as an ingredient in soaps and cosmetics.
- Tannic, 2-nonynoic, nonanoic and tetradecanoic acids along with decane, 1-decyne, undecane are strong antioxidants and are likely to be responsible for the antioxidant property of *pipali*.

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CONCLUSIONS

Trace elements at μg/g or ng/g level in our body activate vital functions and biochemical processes. The study of trace element metabolism and the nature of disorders arising from its deficiency is a field of scientific endeavour cutting across the conventionally accepted boundaries of biochemistry, physiology, nutrition and medicine. Deficiency or imbalance, whether occurring naturally or from human activities, has been shown to cause health problems. The existing knowledge of trace element nutrition to problems of human health depends on a clear understanding of events that link to the clinical manifestation of deficiencies. Therefore, it is essential to use a more sensitive, multielemental analytical technique applicable to all kinds of biological matrices.

In recent years, many researchers have found medicinal herbs as a panacea for the treatment of chronic ailments including diabetes. As a result many pharmaceutical firms have sprung up with branded products without following set guidelines for their standardization. In order to know the availability of essential trace and toxic elements, leaves of *Mentha Spicata* (Mint) and *Murraya Koenigii* (Curry), antidiabetic herbs and herbal formulations and *trikatu* - an herbal formulation including its three constituents were analyzed for 26-34 elements by instrumental neutron activation analysis (INAA) and atomic absorption spectrophotometry (AAS). In addition, some new organic constituents have been identified in organic extracts of specie in each case. Antioxidant behaviour of *M. spicata* has been studied by DPPH radical scavenging activity. On the basis of analytical data and identification of organic constituents, following generalizations can be made.

- INAA using short and long irradiation with high flux of thermal neutrons in a nuclear reactor followed by high-resolution γ ray spectrometry is ideal for the multielemental determination of up to 30 elements. However, AAS has been used as a complementary technique for the determination of toxic elements, Ni, Cd and Pb, which were otherwise difficult to be determined by INAA.
- Short irradiation (1-5 min) using Pneumatic Carrier Facility (PCF) in a high flux reactor is most ideal for the determination of ~20 elements. It has the advantage of low turn around time and producing least radiation hazards.
- Synthetic multielemental standards and RMs from IAEA (Vienna), NIST (USA) and INCT (Poland) were used as comparator standards and for the validation of analytical data. In each case Z-score plot was drawn.

- Intercomparison study of two candidate RMs Corn flour (CF-3) and Soybean flour (SBF-4) from INCT, Poland shows reasonable agreement with the certified values.
- A simple, fast and nondestructive NAA method has been developed for the determination of P in biological samples based on the reaction $^{31}P\ (n,\gamma)\ ^{32}P$ ($t_{1/2}$=14.2 d) where β-activity of ^{32}P is measured by using an end window G.M counter and an Al filter of 27 mg cm^{-2}. The method has LOD of 0.1 mg/g.
- Each herb or plant specie is enriched in some essential macro and micro nutrient element which remain associated with the organic compounds acting as ligands to make them bioavailable/biocompatible and hence easily assimilable.
- No single herb is enriched in all the nutrient elements though some herbs may be enriched in several elements. In general herbs have least side effects.
- A study of the elemental profiles for macro and micronutrients in different samples of the same specie (especially mint and curry leaves) and different brands of antidiabetic herbal formulations and *trikatu* show similar trends.
- In general, medicinal herbs are enriched in Na, K, Ca, Mg, P and Cl which are otherwise required for the general well being. K content is always found in higher amount than Na. Structural elements (Ca, Mg and P) are found at 1-10 mg/g levels while Fe content is at < 1 mg/g level. Mn, Cu, Zn and Rb contents are found in the range 20-50 μg/g, V & Cr in the range 1-2 μg/g and Co & Se at <1μg/g.
- Concentrations of toxic elements (As, Cd, Hg, Pb, Sb and Th) in medicinal herbs are below the permissible limits specified by the WHO and USFDA with few exceptions.
- *M. spicata* (mint) is particularly enriched in Mn (53.5±9.6 μg/g) and Zn (21.0±4.7 μg/g) as lowering of Mn and Zn are related with gastric and indigestion related disorders. Also, higher Se (177±33 ng/g) content may be responsible for its anticancer properties.
- *M. koenigii* (curry) leaves are especially enriched in V (1.54±0.65 μg/g), Cr (0.82±0.32 μg/g) and Zn (24.9±13.7 μg/g), which play an important role in the treatment of diabetes. High Mn content (44.6±11.3 μg/g) could be responsible for curing indigestion.
- Antidiabetic herbs are enriched in Na, Ca, Cl contents but some elements of importance in diabetes (Cr, Cu, Fe, Mg, Mn and Se) are higher in diabetic formulations. K and P contents are also higher.

- *Trikatu*-a digestive healer has higher Fe (574± 200 μg/g) an integral part of lactoferrin, an enzyme responsible for ameliorating dyspepsia and gastric mucosa. Elemental contents in three constituents are comparable with the mean values. Ginger is particularly enriched in Ca, Mg, Fe and Mn, black pepper in P, Cr, Se and Zn contents whereas *Pipali* has a higher Cu and V contents.
- Rb and Cs help in slowing the break down of starch to glucose (US patent). Rb vs Cs are linearly correlated in all diabetic herbs and so are Cu vs Zn in antidiabetic herbs and *Trikatu*. Cr vs Zn, Fe vs Mn, Na vs Mg and Na vs Cl show inverse relationships.
- K, Ca and P are all macro elements (~0.1%). K/P and Ca/P in various plant parts are of special significance.
- DPPH free radical scavenging activity of diethyl ether extract of *mint* leaves shows ~100 % activity at ~40 μg/L. This extract showed 10 compounds including 3 ketones, 2 each of phenols and esters, an alcohol and ether contributing to the antioxidant behaviour.
- Three new organic constituents; 3-methylthiopropanenitrile; 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl ester) and 1-penten-3-ol were separated from the ethanolic extract of *curry* leaves. The ester and the alcohol may act as an antioxidant.
- Petroleum ether extract of *neem* leaves showed four compounds 1,1,2,3-tetramethylcyclopropane, methyl phenyl sulfone, n-hexanedecanoic acid and 9,12,15-octadecatrienal. Last three act as anti-malarial agent, as ingredient in soaps and detergents and to augment the flavour and aroma of foodstuffs.
- Barbituric and tannic acids were separated from the methanolic extract of *pipali*. The former may be responsible for its sedative action and the later is a chemopreventive and anti-tumour agent. Further, 10 more compounds including 4 hydrocarbons, 4 fatty acids, an aldehyde and a disulfide were identified from its essential oil.
- Inorganic elements such as Mn, Fe, Co, Cu and Zn, may remain complexed with the organic constituents making them easily bioavailable.

Present study attempts to provide base line data on essential and toxic elements including those of the organic constituents, which may help to develop an understanding for its pharmacological action. It may also help to have strict quality control with regard to good agricultural practices, additives and stabilizers to enhance their efficacy.

FUTURE SCOPE OF WORK

Many Ayurvedic drugs have proven antioxidant, anti-inflammatory and immunomodulatory effects and can be used for chemoprevention of chronic diseases. Validation of the concept necessitates long-term prospective clinical studies, first on experimental animals and then humans. It is essential to validate the Ayurvedic claims based on experimental wisdom of several centuries. In order to do this, each of the 700 herbal drugs described in *Charak Samhita* should be thoroughly analyzed for its inorganic and organic constituents including speciation studies as to how and which metal ions are bound with organic molecules and investigate their role in the cure of diseases. These so called novel molecules should then be subjected to mechanism based screening. There is a tremendous opportunity to interpret action of these drugs in term of current knowledge of molecular pharmacology. An imaginative thinking needs to be coupled to cutting edge technology to fully exploit the potential benefits of *Ayurveda*'s rich ancient wisdom.

It is essential that each drug be investigated for its pharmacological action in a systematic manner based on modern principles of medicine. This can be achieved only if an interdisciplinary approach is followed by experts from chemistry, biology, pharmacology and medicine along with a group of volunteers who are ready to offer themselves for clinical trials. This seems to be the only way to popularize medicinal herbs at the International level.

LIST OF PAPERS PUBLISHED/ACCEPTED/COMMUNICATED

1. Analysis of *Indian Mint* (*Mentha Spicata*) for essential, trace and toxic elements, its characterization and antioxidant behaviour by **R. Paul Choudhury**, A. Kumar and A.N. Garg, *J. Pharm. Biomed. Anal.* **41** (2006) 825-832

2. Short irradiation instrumental neutron activation analysis of essential and trace elements in Curry leaves (*Murraya Koenigii)* and its organic constituents by GC-MS by **R. P. Choudhury**, G. Jain and A.N. Garg, *J. Radioanal. Nucl. Chem.* **270** (2006) 187-195.

3. Phosphorus Content in Biological Standards and Samples by Thermal Neutron Irradiation and β^- Counting by A.N. Garg, A. Kumar and **R. Paul Choudhury,** *J. Radioanal. Nucl. Chem.* **271** (2007), In Press

4. Elemental characterization of Trifala powders and tablets by INAA, Thermal analysis and spectral studies of Gallic acid by **R. Paul Choudhury** and A.N. Garg, *J. Herbal Pharm.* **6** (2007) In Press

5. Thermal Neutron Activation Analysis of Essential and Trace Elements and Organic Constituents in *Trikatu:* An Ayurvedic Formulation by **R. Paul Choudhury**, A. Kumar, A. V. R. Reddy and A.N. Garg, *J. Radioanal. Nucl. Chem.,* Accepted

6. Availability of essential trace elements in medicinal herbs used for *Diabetes Mellitus* and their possible correlations by **R. Paul Choudhury**, R.N. Acharya, A.G.C. Nair, A.V.R. Reddy and A.N. Garg, *J. Radioanal. Nucl. Chem.,* Accepted

7. Chromatographic separation of Organic constituents from *Indian Mint* (*Mentha Spicata*) and their identification using GC-MS, **R. Paul Choudhury** and A.N. Garg, *Food Res. Intl.*, Communicated.

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LIST OF PAPERS PRESENTED AT NATIONAL / INTERNATIONAL CONFERENCES

1. TLC Separation, Spectral Identification of Organic Components and Availability of Essential Elements in *Trifala* by **R. Paul Choudhury**, A. Kumar and A.N. Garg, 91st Indian Science Congress, Punjab University, Chandigarh, 3-7Jan 2004, Abstract # 162.

2. Analysis of Trace Elements and Organic Constituents in Brahmi Leaves by A. Kumar, **R. Paul Choudhury** and A.N. Garg, Sixth National Symposium in Chemistry, IIT Kanpur, 6-8 Feb 2004, Abstract # P-146.

3. Phosphorus Content in Biological Standards and Samples by Thermal Neutron Irradiation and β^- Counting by A.N. Garg, A. Kumar and **R. Paul Choudhury,** Eleventh International Conference on Modern Trends in Activation Analysis (MTAA-11), Guildford, UK, Jun 20-25, 2004 Abstract # M-173.

4. Analysis of Chewing Tobacco for Essential and toxic Elements by NAA and AAS by A.N. Garg, A. Kumar, **R. Paul Choudhury** and S. Kar, ICOB-4 & ISCNP-24 IUPAC International Conference on Biodiversity and Natural Products: Chemistry and Medical Applications, New Delhi, 26-31 Jan, 2004 Abstract # P-99.

5. Synthesis, Characterization and Ion-Exchange behaviour of Zirconium Tungstate by A.N. Garg, K.D. Singh and **R. Paul Choudhury,** Nuclear and Radiochemistry Symposium (NUCAR05), Guru Nanak Dev University, Amritsar, 15-18 Mar 2005. Abstract # CA-31

6. Comparative study of Essential and toxic elements in Chewing tobacco and Pan Masalas by INAA and AAS by **R. Paul Choudhury**, A.N. Garg, A.G.C. Nair and A.V. R. Reddy, Nuclear and Radiochemistry Symposium (NUCAR05), Guru Nanak Dev University, Amritsar, 15-18 Mar 2005. Abstract # RA-2

7. Short Irradiation Instrumental Neutron Activation Analysis of Essential and trace elements in *Murraya Koenigii* (Curry leaves) and its organic constituents by GC-MS by A.N. Garg and **R. Paul Choudhury**, Eight International Conference on Nuclear Analytical Conference in Life Sciences (NAMLS8) Rio de Janeiro, Brazil, 17-22 Apr 2005, Log #328.

8. How safe are Medicinal Herbs? Elemental Characterization of Medicinal Herbs and Herbal Formulations by INAA, A.N. Garg and **R. Paul Choudhury,** *Trans. Am. Nucl. Soc.*, **93** (2005)

9. Thermal Neutron Activation Analysis of Essential and Trace Elements and Organic Constituents in *Trikatu:* An Ayurvedic Formulation by **R. Paul Choudhury**, A. Kumar, A. V. R. Reddy and A.N. Garg, 6th International Conference on Application of Radiotracers in Chemical, Environmental and Biological Sciences (ARCEBS 06), Kolkata, 23-27 Jan 2006, Log #229

10. Availability of essential trace elements in medicinal herbs used for *Diabetes Mellitus* and their possible correlations by **R. Paul Choudhury**, R.N. Acharya, A.G.C. Nair, A.V.R. Reddy, A.N. Garg, Seventh International Conference on Methods and Applications of Radioanalytical Chemistry (MARC VII) Hawaii, USA, 3-7 Apr 2006, Log #144