NUTRITIONAL VALUE OF *MORINGA OLEIFERA* AS A DIETARY SUPPLEMENT

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Certificate

This is to certify that Ms. Soma Majhi bearing the Exam Roll No: M4PHA 13-27 and Regd No: 117196 of 2011-2012, has successfully completed the research work on the subject entitled ***"Nutritional value of Moringa oleifera as a Dietary supplement"*** under the supervision and guidance of Dr. Pulok k. Mukherjee, Director, School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University Kolkata. She has carried out her research work independently and with proper attention to our entire satisfaction which is being presented through this thesis submitted in partial fulfillment of the requirements for the degree of Master of Pharmacy of Jadavpur University, Kolkata- 700032.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of my "Nutritional value of *Moringa oleifera* as a Dietary supplement" studies.

All information in this document have been obtained and presented in accordance with accordance rule and ethical conduct.

I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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ABBREVIATION

AAS	Atomic Absorption Spectroscopy
CGA	Chlorogenic Acid
CQA	Caffeoyl-quinic Acid
DS	Dietary Supplement
EBV-EA	Epstein-Barr-virus-early antigen
FICCI	Food safety and Standard Authority of India
FIM	Foundation of Innovation Medicine
GLA	Gamma Linoleic Acid
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HSV	Herpes simplex virus
LDL	Low Density Lipoprotein
LOD	Limit of Detection
LOQ	Limit of Quantification
M. oleifera	*Moringa oleifera*
R^2	Correlation Coefficient
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
R_t	Retention time
SD	Standard Deviation
TLC	Thin Layer Chromatography
UV-VIS	Ultra violet visible spectrophotometer
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

DEDICATED
TO
MY PARENTS

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PREFACE

Dietary supplements (DS) is a preparation intended to supplement the diet. Sometimes it is not possible to achieve micro and macronutrients essential for human health in regular diet. Herbal supplements obtained from plant origin are helpful in these regards. The functional component of a functional food can be an essential macronutrient or micronutrient, a nutrient that is not considered essential or a non-nutritive component. They are preferable compared to other due to their safety and low cost.

Moringa oleifera is an unbelievable source of all macro, micro elements amino acids and dietary fibre. No single food contains all of the vitamins and minerals. So it is unbelievable to get all the nutrients in a single plant. That's why it is called as 'miracle tree'. It is a good source of tocopherols, β-carotene (pro-vitamin A), vitamin C, calcium, protein, potassium. The leaf is highly nutritious and contains significant quantities of crude protein, vitamins and minerals. The pod is an excellent source of amino acid. Therefore the present investigation has been carried out to explore this plant as a nutritional supplement by estimating the food value.

The investigational work carried out has been represented in the thesis through the following section:

- Review of literature related to Dietary supplement.
- Review of literature related to the plant profile, phytopharmacology, pharmacological activity, uses of *Moringa oleifera*.
- Standardization of different parts of *M. olefera* (leaf, flower) and formulation prepared from it through High Performance Thin Layer Chromatography.
- Evaluation of total phenolic and total flavonoid content of different parts of *M. olefera* (leaf, pod, flower) and formulation prepared from it.
- Preparation of dietary supplement from *Moringa oleifera* and evaluation of its nutritional value.
- Analysis of Heavy metals.

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CONTENT

1.1. Introduction

Nutraceuticals was defined by Stephen DeFellce in 1979, founder and chairman of the Foundation of Innovation Medicine (FIM), New Jersey, USA. A nutraceutical is considered as a non-toxic food or its part which having basic nutritional value provides health benefits including the prevention of disease or promotion of health (Bieselskl, 2001). The nutritional value of the food should be standardized in the nutraceutical product and production must follow the standard guideline. As per Dietary Supplement Health and Education act of 1994, the definition of nutraceuticals has been extended to include herbs, other botanicals, vitamins, minerals, amino acids and any dietary substances for use by humans to supplement the diet (Srividya et al., 2010). Nutraceuticals are derived from food sources that having extra nutritional value in addition. They are broadly classified as Dietary supplement and Functional foods.

Dietary supplement is one type of preparation (other than tobacco) containing one or more valuable nutrients mainly vitamins, minerals, amino acid, fatty acids, fibre, botanicals, metabolites, concentrate or combination form which intended to supplement the daily diet. It can be consumed as solid dosages form like powder, tablet and capsule or as liquid dosage forms. It is not used as a functional food or essential items. Some of the vital nutrients may not be consumed in sufficient quantity or not be present in normal diet. Dietary supplement plays an important role in these regards. They supplement the diet and improve our health. They contribute to our health maintenance and well-being (McDonald and Nicholson, 2006).

Historically, people have used herbal medicines to prevent illness, cure infection, reduce fever, and heal wounds. Herbal medicines can also treat constipation, weight management, cardiovascular health, immune-modulators and memory loss. Research on some herbs and plant products has shown that they may have some of the same effects that conventional medicines do, while others may have no effect or may be harmful. People have used the active ingredients in dietary supplements for thousands of years for the maintenance of health and treatment. Commonly used dietary supplements include vitamins and minerals (such as vitamin C or a multivitamin), botanicals (herbs and plant products, such as St. John's wort), and substances that come from a natural source (such as omega-3 fatty acids). Consumer interest in the relationship between the diet and health has increased the demand on nutraceuticals. Hence it is challenging to utilize natural resources in the direction of health care system. Dietary supplements will play a key role in that system in nearby future.

1.2. Difference between Dietary Supplements, foods and drugs

Pharmaceutical and nutrients both can cure and prevent diseases. But marketing of drugs need authorized permission from regulatory body set by government. Licensing procedure ensure, a drug must have safety and efficacy profile prior to incorporate in health care. Nutraceutical are not labelled as drugs because they have not gone through a strict approval process. “Nutraceuticals” can restore, correcting or modifying physiological function that may help to prevent diseases in human being. Nutraceutical mostly obtained from natural sources exception is synthetic vitamins (Rajasekaran et al., 2008).

Functional food is slightly differing from the conventional food because it having certain physiological action that may prevents the risk of chronic disease. All functional foods are not nutraceutical. Preparation of food by using "scientific intelligence" with or without knowledge of how or why it is being used, the food is called "functional food". Thus, functional food provides the body with the required amount of vitamins, fats, proteins, carbohydrates, etc., needed for its healthy survival (FAO, 2007).

The nutraceuticals differ from dietary supplements in the two following aspect:

a) Nutraceutical must supplement the diet but should also assist in the prevention and/or treatment of diseases and/or disorder.

b) Nutraceuticals are presented as a conventional food or as the sole item of meal or diet.

Use of nutraceuticals has been increasing day to day globally due to their false perception that they have potent therapeutic value and safe for use but the high cost of prescription pharmaceuticals helps nutraceuticals to solidify their presence in the global market of therapies and therapeutic agents.

1.3. Types of dietary supplement

Dietary supplements widely used for maintenance of human health, some important uses are Vital organ specific care (brain, heart, lungs, liver, kidney); Disease specific care (diabetes, obesity, hyperlipidemia, hypertension, asthma, osteoarthritis, osteoporosis, mood disorder); General care (rejuvenators, immunity enhancers and stress busters); Reproductive health (aphrodisiac, pregnancy condition, lactated mother); Gender specific (Paediatric and geriatric nutritional supplement: Vitamins and minerals); Sport nutrition and Energy drinks (protein

supplements, malted beverages, fruit based products and glucose); Cosmetics (skin whitening, anti-ageing, anti-wrinkle, repair hair-damage).

Dietary supplements are also categories based on their composition.

1.3.1. Vitamins supplements

Dietary supplements add large amounts of micronutrients to the diet. For example, vitamins include vitamin A, vitamin C, vitamin D, vitamin E and vitamin K, the family of B vitamins, which includes thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (H), folic acid (B_9), cyanocobalamin (B_{12}) etc. Significant adult population intakes an inadequate amount of dietary micronutrients. Sometimes, overdose of the dietary supplement may cause adverse effect. The amount of a vitamin is usually expressed on a supplement label as a percentage of the daily value, which is the term used for the amount recommended by the regulatory body (Bailey et al., 2012). A vitamin serves a specific benefit to the body. For example, vitamin A supports vision and bone growth, whereas vitamin E strengthens the immune system and helps to repair DNA. Vitamin D is important for keeping bones strong and reducing bone loss. Vitamin deficiency can impair the body's ability to heal and protect itself.

1.3.2. Mineral supplements

Mineral supplements are often chelated, or bound, with bioavailable compounds that may improve absorption. There are twenty two well known minerals essential to human health, they are divided into "major" minerals and "trace" minerals present in the body. Minerals include calcium, chromium, copper, iodine, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc. Deficiency of either a major or trace mineral can produce equally harmful effects. Minerals play remarkable role in different physiological function e.g. participate in muscle contraction, blood formation, building protein, energy production and numerous other body functions. Sodium, potassium and chloride are electrically charges "Electrolytes" that helps for conduction of electrical impulses along nerves which transport substances in and out of the cells. In addition minerals regulate pH balance of blood and other fluids as well as fluid pressure between cells and the blood.

1.3.3. Protein and amino acid supplements

Protein and amino acids are fundamental substances present in the human diet. Main source of proteins are animal or vegetable origin. Animal sources provide all essential amino acids, whereas vegetable sources generally lack one or more of the essential amino acids. Animal

sources of dietary protein not only contain abundant vitamins and minerals, but it also contains some amount of saturated fat compared to vegetable sources. The advanced processing techniques have taken attention in this regards and has produced some derivative products such as whey, casein and soy (Hoffman and Michael, 2004). Single amino acids also may be used for treating health disorders. Amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.

1.3.4. Herbal supplements

The use of herbal supplements has expanded rapidly during the first decade. Herbal supplement contains botanicals/herbs. It may contain single herb or mixtures of herbs. An herb is a plant or plant part (such as leaves, flowers, fruit, seeds, bark and stem) that may used for flavour, smell, and/or potential health-related properties. They differ from vitamin and mineral supplements in that they are considered to have medicinal value. These products are believed to be useful for a range of ailments including skin disease, sexual problems, and mental problems. Examples of these types of supplements include turmeric, ginkgo biloba, and St. John's wort. Botanicals are one of humanity's oldest health care tools, and the basis of many modern medicines (Shahrokh et al., 2005).

1.3.5. Fibre supplements

Dietary fibre preparation from defatted rice bran has laxative and cholesterol-lowering ability with attendant benefits towards prevention or alleviation of cardiovascular disease, diabetes, obesity and colon cancer. It has been suggested that rice bran is a good fibre source that can be added to various food products (Hamid and Luan, 2000). Psyllium is a dietary fibre is valuable in the management of irritable bowel syndrome, inflammatory bowel disease-ulcerative colitis, colon cancer, constipation (Baljit, 2007).

1.3.6. Probiotic supplements

Usage of probiotics (live viable microbial organisms) in the treatment of specific diseases has evolved into an extremely valuable option. The ability to reduce antibiotic use, the apparently very high index of safety, and the public's positive perception about "natural" or "alternative" therapies has increased their uses. These products manipulated the intestinal microflora to maintain the normal balance between pathogenic and non-pathogenic bacteria. Certain probiotics will be effective in the treatment or prevention of certain conditions (Vanderhoof et al., 1999). Lactobacillus GG has been shown to be effective in the treatment or prevention

of a number of problems including acute diarrhoea in children, travellers' Diarrhoea in adults, Crohn's disease, and reduction of the incidence of antibiotic-associated diarrhoea in infants. Most probiotic preparations are comprised of one or more lactic acid bacteria (LAB). Within this group, strains of *Lactobacillus*, *Bifidobacterium* sp. and occasionally *Streptococcus* are most commonly used. A supplementary use of oral digestive enzymes and probiotics are decreasing the incidence of breast, colon-rectal, prostate and bronchogenic cancer (Gibson and Fuller, 2000; Divisi et al., 2006).

1.4. Role of dietary supplement on human health

1.4.1. Cancer

Cancer has emerged as a major public health problem in developing and developed countries. A healthy lifestyle and diet can help in preventing cancer. People who consume large amount of lutein-rich foods such as chicken eggs, spinach, tomatoes, oranges and leafy greens experienced the lowest incidence of colon cancer (Nkondjock and Ghadirian, 2004). Recently, attention has been on phytochemicals that possess cancer-preventive properties. Besides chemopreventive components in vegetables and fruits, some phytochemicals derived from herbs and spices also have potential anticarcinogenic and antimutagenic activities, among other beneficial health effect. A broad range of phyto-pharmaceuticals with a claimed hormonal activity, called "phyto-estrogens", is recommended for prevention of prostate/breast cancer (Limer and Speirs, 2004).

Flavonoids found in citrus fruit appear to protect against cancer by acting as antioxidants. Soy foods are a unique dietary source of isoflavones, the polyphenolic phytochemicals exemplified by epigallocatechin gallate from tea, curcumin from curry and soya isoflavones possess cancer chemopreventive properties. Ellagic acid is a proven anti-carcinogen is used in alternative medicine and to prevent cancer. It is present in strawberries, cranberries, walnuts, pecans, pomegranates and the best source, red raspberry seeds. Majority of the studies indicate a preventive role of nutraceuticals in cancer (Rajasekaran et al., 2008).

1.4.2. Cardiovascular diseases

Cardiovascular diseases (CVD) is the name for the group of disorders of the heart and blood vessels and include hypertension (high blood pressure), coronary heart disease (heart attack), cerebrovascular disease (stroke), heart failure, peripheral vascular disease, etc. In 1999 CVD alone contributed to a third of global deaths and by 2010 it would be the leading cause of death in developing countries. Majority of the CVD are preventable and controllable. It was

reported that low intake of fruits and vegetables is associated with a high mortality in cardiovascular disease. Many research studies have identified a protective role for a diet rich in fruits and vegetables against CVD (Rissanen et al., 2003; Temple and Gladwin, 2003).

Nutraceuticals in the form of antioxidants, dietary fibres, omega-3 polyunsaturated fatty acids, vitamins, and minerals are recommended together with physical exercise for prevention and treatment of CVD. It has been demonstrated that the molecules like polyphenols present in grapes and wine alter cellular metabolism and signalling, which may help reducing arterial disease (German and Walzem, 2000). Nutrients and nutraceuticals possesses the antihypertensive activity through calcium channel blocking activity include α-Lipoic acid, magnesium, vitamin B6 (pyridoxine), vitamin C, N-acetyl cysteine, hawthorne, Celery, ω-3 fatty acids etc (Houston, 2005).

1.4.3. Obesity

Obesity, defined as an unhealthy amount of body fat, is a well-established risk factor for many disorders like angina pectoris, congestive heart failure, hypertension, hyperlipidemia, respiratory disorders, renal vein thrombosis, osteoarthritis, cancer, reduced fertility etc. One of the primary causes this rapid rise in obesity rates is the increased availability of high-fat, energy dense foods. Excessive consumption of energy-rich foods (snacks, processed foods and drinks) can encourage weight gain, which calls for a limit in the consumption of saturated and trans fats apart from sugars and salt in the diet (Mermel, 2004).

Caloric restriction and increased physical activity has been shown to be only more or less successful in managing obesity. Nutraceuticals can also help to treat obesity. A tolerable and effective nutraceutical that can increase energy expenditure and/or decrease caloric intake is desirable for body weight reduction. Herbal stimulants, such as green tea, ephedrine, caffeine, ma huang-guarana and chitosan have proved effective in facilitating weight loss. However, their use is controversial due to their ability to cause undesired effects. Buckwheat seed proteins have beneficial role in obesity and constipation acting similar to natural fibres present in food. 5-hydroxytryptophan and green tea extract may promote weight loss, while the former decreases appetite, the later increases the energy expenditure (Daly et al., 1993; Boozer et al., 2001; Dulloo et al., 1999).

1.4.4. Diabetes

Diabetes mellitus is characterized by abnormally high levels of blood glucose, either due to insufficient insulin production, or due to its ineffectiveness. The most common forms of

diabetes are type 1 diabetes (5%), an autoimmune disorder, and type 2 diabetes (95%), which is associated with obesity with other factors (Wild et al., 2004). Widespread use of herbal dietary supplements that are believed to benefit type II diabetes mellitus, few have been proven to do so in properly designed randomized trials. Isoflavones are phytoestrogens have a structural/functional similarity to human estrogen and have been consumed by humans world-wide. Among all the phytoestrogens, soy isoflavones have been studied most. A high isoflavone intake (20–100 mg/day) is associated with lower incidence and mortality rate of type II diabetes. Good magnesium status reduces diabetes risk and improves insulin sensitivity; chromium picolinate, calcium and vitamin D appear to promote insulin sensitivity and improve glycemic control in some diabetics; extracts of bitter melon and of cinnamon have the potential to treat and possibly prevent diabetes. However it has been suggested that nutraceuticals with meaningful doses of combinations may substantially prevent type II diabetes (McCarty, 2005).

1.4.5. Immune boosters and anti-inflammatory agents

Various nutrients in the diet play a crucial role in maintaining an "optimal" immune response, on the organism's immune status and susceptibility to a variety of disease conditions. Nutraceuticals that belong to the category of immune boosters and/or anti-viral agents are useful to improve immune function and accelerate wound-healing. They include extracts from the coneflowers, or herbs of the genus Echinacea, such as *Echinacea purpurea*, *Echinacea angustfolia*, *Echinacea pillida*. Goldenseal is an immune booster withantibiotic activity. *Astragalus membranaceous* and *Astragalus mongolicus* are also effective immune boosters in either their natural or processed forms. *Astragalus* stimulates development and transformation of stem cells in the marrow and lymph tissue to active immune cells (Rajasekaran et al., 2008).

Nutraceuticals are used for treating problems with inflammation and auto-immune diseases. Numerous nutraceuticals vitamin C and vitamin D that may influence osteoarthritis pathophysiology, including glucosamine, chondroitin, Sadenosylmethionine, ginger and avocado/soybean unsaponifiables, have been tested in clinical trials (Jang et al., 1997). Gamma linolenic acids (GLA) also have immunomodulator potential which is present in green vegetable in very less quantity. Long known for its myriad health benefits, vitamin C is one of the most popular nutrients taken in supplement form and for good reason. This potent antioxidant has been shown to protect against immune system deficiencies, cardiovascular disease, prenatal health problems, eye disease and even wrinkly skin.

1.4.6. Miscellaneous

Good quality nutritional supplements (combinations rather than isolated single nutrients) can play a valuable role in the health of human being; therefore, emphasis must always be on eating a good diet. Naturally occurring bioactive compounds such compounds include catechins, curcumin, isoflavones, resveratrol, proanthocyanidins, flavonoids, Saponins, terpenes, chitin, chitosan, vitamins B_3, vitamin D_3, fatty acids, peptides and amino acids (alpha 2-macroglobulin, arginine, phenylalanine) etc are used for treatment of cardiac and circulatory disorder. They are also possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities. With the rapidly increasing interest in the nutraceutical revolution, we need to establish a vibrant nutraceutical research community which is absolutely necessary to convert the majority of potential nutraceuticals to establish ones thereby truly delivering their enormous benefits to all of us.

1.5. Consumption of dietary supplement worldwide and India

As per the Food Safety Standard Act, 2006 (Chapter 4, Section 22) it has been recommended that Food should be classified as follows:

- Novel foods
- Genetically modified food
- Irradiated food
- Organic foods
- Foods for special dietary use
- Functional foods
- Nutraceuticals
- Health Supplements

Global nutraceutical market was estimated to be US$ 140.1 billion in 2010. Of this USA and Europe formed the largest markets accounting to 36 % and 25 % respectively.

Indian nutraceutical industry was estimated at US $ 2 Billion, roughly 1.5 percent of the global nutraceutical industry. India gives a promising market for traditional herbal ingredients (usually ayurvedic) into the nutraceutical portfolio Chyawanprash supplements (market size US $74.5 Million in 2010). Indian nutraceutical industry has produced 40% dietary supplement and 60% functional foods and beverages. Increased consumption of functional

foods and beverages are expected to increase in next five years resulting 67% share in the market in which dietary supplements will comprise 33%.

India is major cultivator of food product among the developed countries but still malnutrition is serious problem in India. Food safety and Standard authority of India (FICCI) reported that 40% death in India and other developing country due to nutrition related risk factor. Modern lifestyle cost of leaving, burden of diseases increases day by day life which affects health care system directly. The research of nutraceuticals products development should be goal oriented therefore we get much helpful information to be used in preventive measures of disorders and clinical practices. The health promoting foods are contributing promising economical growth of the country (Devla et al., 2011).

Chapter -1

A review on dietary supplements

1.1 Introduction

1.2 Difference between dietary supplements, foods and drugs

1.3 Types of dietary supplement

1.4 Role of dietary supplement on human health

1.5 Consumption of dietary supplement worldwide and India

1. Rationale behind choosing *Moringa oleifera* as a nutritional Supplement

Moringa oleifera is a highly valued plant and provide outstanding source of nutrition. The leaves, fruit, flowers and immature pods of this tree are used as a highly nutritive vegetable in many countries, particularly in India, Pakistan, Philippines, Hawaii and many parts of Africa (D'souza and Kulkarni, 1993; Anwar and Bhanger, 2003; Anwar et al., 2005). Different tissues of Moringa are good sources of tocopherols (Sanchez-Machado et al., 2006). The leaves of this plant are rich source of β-carotene (pro-vitamin A) that's why it is used to improve human health in vitamin A-deficient diets (Babu, 2000). Various macro and micro minerals have been analysed previously in the leaves of this plant (Barminas et al., 1998; Freiberger et al., 1998; Sena et al., 1998). Through literature review it has been observed that leaf powder of *Moringa oleifera* is a good nutritional supplement for children. The leaves contain seven times the vitamin C in oranges, four times the calcium in milk, four times the β-carotene in carrots, twice the protein in milk and three times the potassium in bananas (Ramachandran et al., 1980).

Sometimes it is not possible to achieve micro and macronutrients essential for human health in regular diet. Herbal supplements obtained from plant origin are helpful in these regards. But it is required to maintain and analyse their nutritional value because essential nutrient become harmful or toxic when they exceed a certain level (Giacomino et al., 2011). Botanicals (single herb or extract, powder, capsule, or tablet forms) are a significant potential source of metal contamination (Liva, 2007; Amster et al., 2007). Thus, it is important to quantify nutritional content in herbal supplements that's why the analysis of food and dietary supplements is constantly increasing.

2. The major highlights of these studies are

- Collection and authentication of leaf, pod, and flower of *Moringa oleifera.*
- Standardization of different parts of *Moringa oleifera* (leaf, pod & flower) and the formulation prepared from it by High performance thin layer chromatography (HPTLC)
- Estimation of total phenolic and total flavonoid content in different parts of *Moringa oleifera* (leaf, pod & flower) and the formulation prepared from it.
- Preparation of dietary supplement from *Moringa oleifera* and estimating the nutritional value.
- Heavy metal analysis by Atomic absorption spectrophotometry.

Chapter – 2

Aim and objective of the study

3.1. Introduction

Fig.3.1. *Moringa oleifera* plant

Moringa oleifera Lam (syn. M. ptreygosperma Gaertner.) is one of the best known and most widely distributed naturalized species of Moringaceae family. It is commonly referred to as "The Miracle Tree" (Palada, 1996), 'drumstick tree' or 'horseradish tree'. Its several medicinal, industrial and nutritional uses make it a significantly valuable plant in world wide. It is distributed in many countries of the tropics and subtropics and cultivated all over the world due to its multiple utilities. It is known as different names in different region: Assamese: Sojina; Bengali: Sojne; Chinese: La mu; English: Moringa, Drumstick tree, Horseradish tree; Hindi: Sahjan. It is a highly valued plant and provide outstanding source of nutrition. In The leaves, fruit, flowers and immature pods of this tree are used as a highly nutritive vegetable in many countries, particularly in India, Pakistan, Philippines, Hawaii and many parts of Africa (D'souza and Kulkarni, 1993; Anwar and Bhanger, 2003; Anwar et al., 2005). Different parts of this plant are used for various purposes. The leaf of this plant increases woman's milk production and is sometimes prescribed for anaemia; that's why it is also known as 'mother's best friend' (Estrella et al., 2000; Siddhuraju and Becker, 2003).

Moringa has several traditional and therapeutic uses. It is being used for more than twenty years in Ghana and other parts of the world as a nutritional supplement. However, it is become popular in the entire society. Despite the nutraceutical importance, different parts of the plant have different pharmacological activity. Moringa tree has an enormous approach in treating malnutrition, especially among infants and mothers. Almost all the parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, haematological and hepatorenal disorders (The Wealth of India., 1962; Singh and Kumar, 1999; Morimitsu et al., 2000; Siddhuraju and Becker, 2003). Various types of compounds such as ascorbic acid, flavonoids, phenolics and carotenoids found in leaves acts as a good source of natural antioxidants (Dillard and German, 2000; Siddhuraju and Becker, 2003). The leaf is highly nutritious and contains significant quantities of crude protein (20-29%), vitamins and minerals (Olugbemi et al., 2010; Abou-Elezz et al., 2011) and juice of leaves are being

applied in eye infections (The Wealth of India., 1962). Moringa seeds are reported to show antimicrobial activity (The Wealth of India., 1962). The roots and seed extract have shown antimicrobial activity (Eilert et al., 1981). The ethanolic extract of the leaves of Moringa was reported for its antimicrobial activities (Pal et al., 1995a). The plant is also well known for its various medicinal properties such as reducing blood pressure, tumour healing properties, anti-fertility activity, antibacterial activity (Biswas et al., 1950; Shukla et al., 1981; Eilert et al., 1981). The aqueous extract and alcoholic extract of Moringa root-wood reported to reduce and prevent the growth of urinary stones (Karadi et al., 2006). A significant number of primary and secondary metabolites and pharmacological activities have been reported for this plant. Hence this review contains necessary and useful information on botany, pharmacognosy, traditional use, phytochemistry, chemistry and pharmacology of this useful plant.

3.2. Growth, cultivation and distribution

M. oleifera plant is a perennial, evergreen tree that grows up to 20 ft (6.1 m) tall, with a straight trunk with corky, whitish bark. It grows well in hot, semi-arid and humid regions and in well-drained sandy or loamy soils. The tree is grown mainly in semiarid, tropical, and subtropical areas. It is also widely cultivated in Africa, Cambodia, Nepal, Indonesia, Malaysia, Mexico, Central and South America, and Sri Lanka. Optimum leaf and pod production requires high average daily temperatures of 25-30° C (77-86° F), well-distributed annual rainfall of 1000-2000 mm (40-80 in), high solar radiation and well-drained soils. Growth slows significantly under temperatures below 20°C (68° F). Ideal elevation is less than 600 m (1, 970 f). The plant is relatively tolerant of drought and poor soils and responds well to irrigation and fertilization. It can tolerate a wide range of soil types and pH (4.5-9) but prefers well-drained soils in the neutral pH range. It grows best in dry, sandy soil, including coastal areas. It is a fast-growing, drought-resistant tree that is native to the southern foothills of the Himalayas in north-western India. India is the largest producer of Moringa, with an annual production of 1.1 to 1.3 million tonnes of tender fruits from an area of 380 km² (Odee, 1998).

3.3. Taxonomy

Binomial name	*Moringa oleifera*.
Kingdom	Plantae – Plants
Subkingdom	Tracheobionta - Vascular plants

Superdivision	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	Magnoliopsida - Dicotyledons
Subclass	Dilleniidae
Order	Capparales
Family	Moringaceae – Horse-radish tree family
Genus	Moringa Adans. – moringa
Species	*Moringa oleifera* Lam. – horseradishtree

3.4. Pharmacognosy and morphology

It is a small tree (7±12 m high) with thick grey bark, fragrant white flowers and long green pods which give the tree its name. The tree has tuberous taproot and brittle stem is with corky bark.

Stem: Moringa has a short normally straight stem. The stem is poorly formed. The stem has a height of 1.5-2 m before it begins branching but can reach up to 3.0 m (Foidl et al., 2001).

Branch: The extended branches grow in a disorganized manner and umbrella shaped.

Leaves: The leaves are pale green to deep green in colour, compound, and 30-60 cm in length. The leaves are bipinnate or more commonly tripinnate, up to 45 cm long, and are alternate and spirally arranged on the twigs. Pinnae and pinnules are opposite. It has many small leaflets which are 1.2 to 2.0 cm long and 0.6 to 1.0 cm wide. The lateral leaflets are elliptic and the terminal ones obovate. The leaflets are finely hairy, green and almost hairless on the upper surface, paler and hairless beneath, with red-tinged mid veins, with entire (not toothed) margins, and are rounded or blunt-pointed at the apex and short-pointed at the base. The twigs are finely hairy and green, becoming brown.

Flowers: The flowers are yellowish white or cream coloured, bisexual and 10- 25 cm long and 2.5 cm wide. It has pleasant fragrance and produced profusely in axillary, drooping panicles 10-25 cm long (Sachan et al., 2010). The five-reflexed sepals are linear-lanceolate. The five petals are slender-spatulate. They surround the five stamens and five staminodes and are reflexed except for the lowest.

Fruit: The pods/fruits are 1 to 4 ft (30-120 cm) long and 1.8 cm (0.7 in) wide and tapering at both ends. It is green to brown in colour. The pod contains about 10 to 20 seeds embedded in the fleshy pith. The seeds are dark brown and the kernel is surrounded by a lightly wooded shell with three papery wings. The fruits are pendulous, linear, three-sided pods with

nine longitudinal ridges, usually 20 to 50 cm long, but occasionally up to 1 m or longer, and 2.0 to 2.5 cm broad. When matured, the fruit becomes brown and has 10–50 seeds inside (Vlahof et al., 2002).

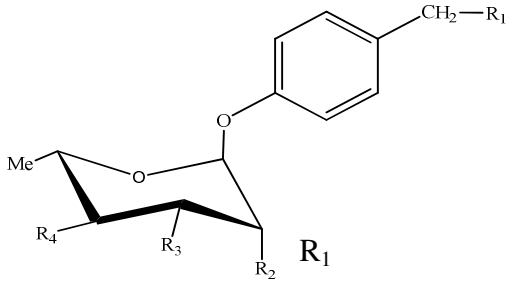
3.5. Phytoconstituents

Different parts of Moringa containing the simple sugar, rhamnose, vitamins, minerals and carotenoid (β- carotene). Moringa species are rich sources of various phyto-chemicals including rare sugar derivative called glucosinolates and isothiocyanates. The predominant glucosinolate is 4-O-(α-L-rhamnopyranosyloxy)-benzylglucosinolate (glucomoringin) and its three mono-acetyl-rhamnose isomers of this glucosinolate (Fig.3.1.a) (Fahey et al., 2001; Bennett et al., 2003; Kjaer et al., 1979). The root of this plant contains high concentration of 4- (α-L-rhamnopyranosyloxy)-benzylglucosinolate and benzylglucosinolate (Fig.3.1.b) (Foidl et al., 2001). Leaves and the flowers of Moringa also contain 4-hydroxybenzyl-glucosinolate (Fig.3.1.c). The leaves of plant also have low amount of various nitriles, thiocarbamates and carbamates that having strong hypotensive and spasmolytic effects (Leuck and Kunz, 1998). Naturally occurring iso-thiocyanates, 4-[(4′-O-acetyl-α-i-rhamnosyloxy) benzyl] (Fig.3.1. g), significantly inhibition on Epstein–Barr virus that may cause tumor (Murakami et al., 1998). Niazirin and niazirinin are the nitrile glycoside and three mustard oil glycoside found in the ethanolic extract of leaves. (Bennett et al., 2003) (Fig.3.1.i).

Presence of high concentrations of oestrogenic substances, iron, calcium, phosphorus, copper, vitamins A and C, α-tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β-carotene, protein, and essential amino acids such as methionine, cystine, tryptophan and lysine in Moringa leaves and pods make it a virtually ideal dietary supplement (Makkar and Becker, 1996). The plant is also a good source of α, γ and δ tocopherols (Anwar and Bhanger, 2003; Tsaknis et al., 1999).

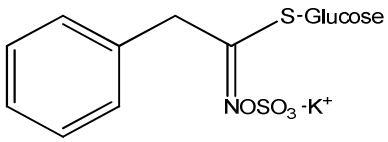
Leaves of this plant act as a natural antioxidant due to the presence of various compounds such as ascorbic acid, flavonoids, phenolics and carotenoids (Anwar et al., 2005; Makkar and Becker, 1996). Leaves, roots, pods and seeds of the plant contain 3- caffeoylquinic acid and 5- caffeoylquinic acid (Fig. 3.1.d and Fig.3.1.e) and flavonols have been reported in different tissues of *M. oleifera*. The plant flavonoid are flavonol glycosides and it present in the form of glucosides, rutinosides and malonylglucosides of quercetin > kaempferol > isorhamnetin. Some of them quercetin-3-o-glucoside, quercetin-3-o-(6- malonyl glucoside) and lower amounts of kaempferol- 3-o glucoside and kaempferol- 3-o-(6- malonyl glucoside) (Bennett et al., 2003; Manguro & Lemmen, 2007) (Fig.3.1.f).

Fig. 3.1. Phyto-constituents of *Moringa oleifera*



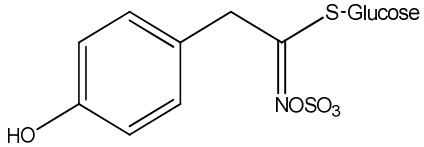
	R_1	R_2	R_3	R_4
4-(α- L-Rhamnopyranosyloxy)-benzylglucosinolate	-CH(=$NOSO_3K^+$)-S-Glc	OH	OH	OH
4"-Acetyl-4-(α-L-Rhamnopyranosyloxy)-benzylglucosinolate	-CH(=$NOSO_3K^+$)-S-Glc	OH	OH	O-AC
4-(α-L-Rhamnopyranosyloxy)-benzylisothiocyanate	-NCS	OH	OH	OH
4-(α-L-Rhamnopyranosyloxy)-benzylcyanide	-CN	OH	OH	OH

(a)



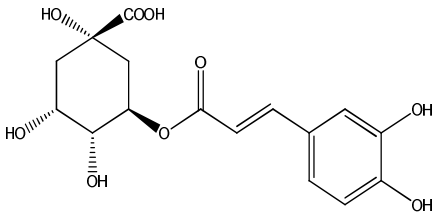
Benzylglucosinolate (Glucotropaeolin)

(b)



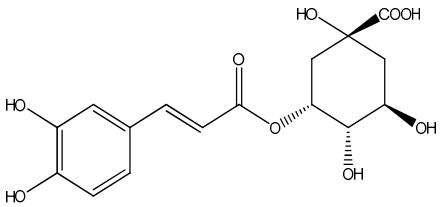
4-hydroxybenzylglucosinolate

(c)



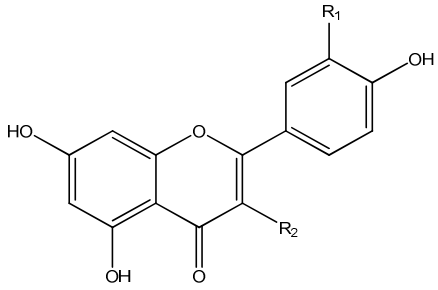
Neochlorogenic Acid (3- caffeoylquinic acid)

(d)



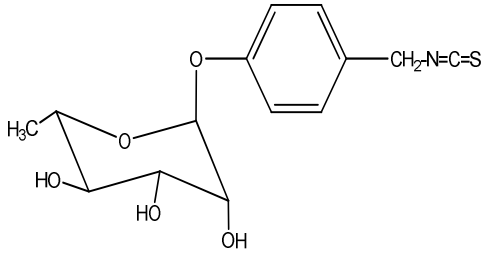
Chlorogenic Acid (5- caffeoylquinic acid)

(e)

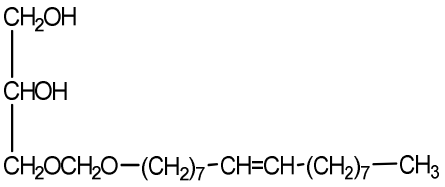


	R_1	R_2
Kaemferole 3-O-Glucoside	H	Glc
Quercetin 3-O-Glucoside	OH	Glc
Rutin	OH	Glc-Rham

(f)

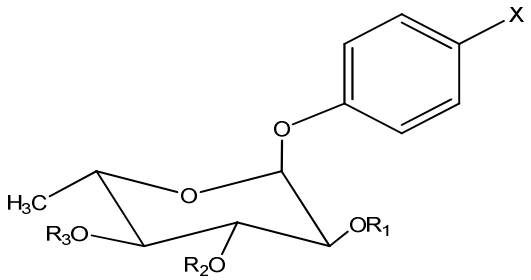


4-(α-L-rhamnosyloxy) benzyl isothiocyanate



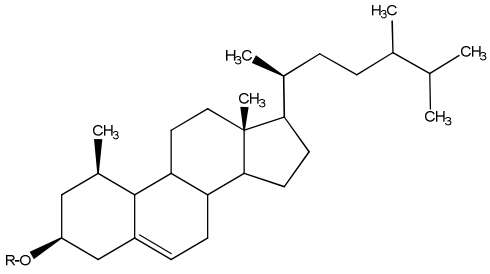
Glycerol-1-(9-octadecanoate)

(g) (h)



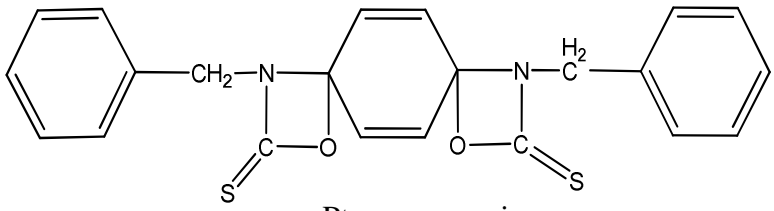
NiaZirin ($R_1 = R_2 = R_3 = H$, X = CN)
Niazirinin ($R_1 = R_2 = H$, R_3 = Ac, X = CN
Niazimin A/B ($R_1 = R_2 = H$, R_3 = Ac, X = CH_2-NH-CO-OEt)
Niazinin A/B ($R_1 = R_2$ =R3 = H, X = CH_2-NH-(C=S)-OMe)
Niazicin A/B ($R_1 = R_2 = H$, R_3 = Ac, X = CH_2-NH-(C=S)-OMe)
Niazimicin ($R_1 = R_2 = R_3 = H$, X = CH_2-NH-(C=S)-OEt)
Niaziminin A/B ($R_1 = R_2 = H$, R_3 = Ac, X = CH_2-NH-(C=S)-OEt)

(i)



R = H β-sitosterol
R = 6' -O-oleoyl-β-D-glucopyranosyl
R = β-D-glucopyranosyl

(j)



Pterygospermin

(k)

Vanillin, β-sitosterol, β-sitostenone, 4-hydroxymellin and octacosanoic acid, 4- (α-L-rhamnopyranosyloxy)-benzylglucosinolate have been isolated from the stem of Moringa(Faizi et al., 1994a). Purified gum exudate obtained from the stem of *Moringa oleifera* reported to contain L-arabinose, D-galactose, D-glucuronic acid, L-rhamnose, D-mannose and D-xylose while a homogeneous, degraded-gum polysaccharide consisting of L-galactose, -glucuronic acid and L-mannose has been obtained on mild hydrolysis of the whole gum with acid (Bhattacharya et al., 1982).

Flowers of Moringa plant is full of various amino acids; sugar such as sucrose & D-glucose, and potassium and calcium. It also contains flavonoids, alkaloids, kaempherol, rhamnetin, isoquercitrin and kaempferitrin (Ruckmani et al., 1998; Faizi et al., 1994a; Siddhuraju and Becker, 2003). It has been reported that Moringa roots have the antibacterial activity and the active antibiotic principle, pterygospermin (Fig.3.1.k) is responsible for antibacterial and fungicidal effects (Ruckmani et al., 1998). The cytokinins have been shown to be present in the fruit (Nagar et al., 1982). A new O-ethyl-4-(α-L-rhamnosyloxy)benzyl carbamate together with seven known bioactive compounds, 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, niazimicin, β-sitosterol-3-O-β-D glucopyranoside, niazirin ,β-sitosterol (Fig 3.1. j) and glycerol-1-(9-octadecanoate) (Fig.3.1.h) have been isolated from the ethanol extract of the Moringa seed (Guevara et al., 1999).

Moringa seed oil contain only mono-unsaturated fatty acids, two ω-9 mono-unsaturated acids, (cis-9-octadecenoic and cis-11-eicosenoic acids) and one ω-7 mono-unsaturated acid (cis-11-octadecenoic acid). The composition of the sterols of seed oil mainly consists of campesterol, stigmasterol, β-sitosterol, Δ5-avenasterol and clerosterol accompanied by minute amounts of 24-methylenecholesterol, Δ7-campestanol, stigmastanol and 28-isoavenasterol (Tsaknis et al., 1999; Anwar and Bhanger, 2003; Anwar et al., 2005). The sterol composition of Moringa seed oil differs greatly from most of the conventional edible oils (Rossell, 1991).

3.6. Traditional uses

Moringa oleifera is tropical trees with a multiple uses. It is traditionaly used for the circulatory system disorder because Moringa leaves have potential hypotensive action (Abe and Ohtani, 2013). It is also used for treatment of enteric-infection due to its anti-microbial properties. The leaves are used as hypocholesterolemic and hypoglycemic agents. Additionally, the leaves have been reported for its antitumour, antioxidant, anti-inflammatory/diuretic properties, anti-hepatotoxic, anti-fertility, anti-urolithiatic and

analgesic activities (Asare et al., 2012). The medicinal value of Moringa oil has been documented by ancient cultures. Moringa oil has tremendous cosmetic value and is used in body and hair care as a moisturizer and skin conditioner (Paliwal et al., 2011). It has been used for different industrial and medicinal uses (CSIR, 1962). The seed flower and leaves of this plant are eaten in many countries as a vegetable. The different parts of the plant are consumed and used for different purposes. The seed are used in curry powder. The seed can be used as less expensive biosorbent and can remove cadmium from aqueous media (Sharma et al., 2006). The seeds act as a natural coagulant, flocculant, absorbent for the treatment of ground water. It reduces the total hardness, acidity, Turbidity, alkalinity, chloride (Mangale et al., 2012). The combined dose of *Moringa oleifera* seed powder and chlorine can give best results and ground water can be used for drinking purpose. The seed is not giving toxic effect. Water treatment by the seed is a cheaper method. Moringa seeds are used in the rural areas for drinking water treatment. The sludge settled down in bottom of the tank after treatment can be used as bio-fertilizer. Moringa seeds could remove up to 97% of the algae present (Shehata et al., 2002). The aqueous solution of seed composed of heterogeneous complex mixture of amino acid can bind metal ions and increase the sorption of metal ions. (Brostlap and Schuurmans, 1988). Oil is extracted from the seed which is called Ben-oil or Behen oil has been used for human consumption. They are utilized for also utilized in wastewater treatment and water purification purpose (Ndabigengeser and Narasiah, 1998). This oil has been used for illumination and as a lubricant for fine machinery (The Wealth of India 1962, Qaiser, 1973.). It is also used extensively in enfleurage process (The Wealth of India, 1962). The seeds are used traditionally in rural areas of Sudan and Malawi for the clarification of drinking water (Muyibi and Evison 1995; Anwar et al. 2007).The leaves can increase breast milk production when mixed with chicken or shellfish soup. 80% ethanolic extract of leaves was reported to increase nodulation of black-gram (Bose, 1980). The water extracted kernels of the plant used for the purification of water (Holmes et al., 1994). The water extract is used in many developing countries as a coagulant in place of aluminium sulphate. In southern India the villagers uses the leaves to prepare cow and buffalo ghee from butter fat and the fruits are also added in curries and sold in stores (Siddhuraju and Becker 2003). Dehusked Moringa press cake can remove hydrophobic organic pollutants from water (Boucher et al., 2007), and extracts might remove other pollutants, such as heavy metals and surfactants (Beltran-Heredia and Sanchez-Martin, 2008; 2009). The leaves contain seven times the vitamin C in oranges, four times the calcium in milk, four times the β-carotene in carrots, twice the protein in milk and three times the potassium in bananas. In India and other parts

of the world *Moringa oleifera* was used as a nutritional supplement and now it is beginning to gain popularity in the entire society due to its medicinal and food value (Asare et al., 2012).

3.7.Pharmacological activity

3.7.1. Antihypertensive, diuretic and cholesterol lowering activities

Moringa leaf juice has blood pressure lowering activity. If the plant has diuretic as well lipid and blood pressure lowering constituents then it is highly useful in cardiovascular disorders. (The Wealth of India, 1962). Nitrile, mustard oil glycosides and thiocarbamate glycosides of Moringa are responsible for the blood pressure lowering effect (Faizi et al., 1994a; 1994b; 1995). Four pure isolated compounds of Moringa leaves niazinin A, niazinin B, niazimicin and niazinin (A and B) show hypotensive effect in rats model possible mechanism may be a calcium antagonist effect (Gilani et al., 1994a).

Ethanolic and aqueous extracts of whole pods and its parts, i.e. coat, pulp and seed have also blood pressure lowering effect, among them seed shows more prominent activity (Faizi et al., 1998). Bio assay guided isolation revel thiocarbamate and isothiocyanate glycosides from pods of Moringa that have hypotensive potential (Faizi et al., 1995).

Aqueous infusion of Moringa roots, leaves, flowers, gum and seeds possess diuretic activity and diuretic components are played a complementary role in the overall blood pressure lowering effect of this plant (Morton, 1991; Caceres et al., 1992). The crude extract of Moringa leaves has a significant cholesterol lowering action in the serum of high fat diet fed rats which might be attributed to the presence of a bioactive phytoconstituent, i.e. β-sitosterol (Ghasi et al., 2000). Mehta et al., 2003 was reported that Moringa fruit has been significantly reduced the atherogenic principle in blood including serum cholesterol, phospholipids, triglycerides, low density lipoprotein (LDL), very low density lipoprotein (VLDL) and reduced the lipid profile of liver and augmented the excretion of faecal cholesterol.

3.7.2. Antispasmodic, antiulcer and hepatoprotective activities

Moringa roots have been reported to possess antispasmodic activity (Caceres et al., 1992). The leaves have been extensively studied pharmacologically and it has been found that the ethanol extract and its constituents exhibit antispasmodic effects possibly through calcium channel blockade (Gilani et al., 1992; 1994a; Dangi et al., 2002). The antispasmodic activity of the ethanol extract of Moringa leaves is due to active compound 4-[α-(L-rhamnosyloxy) benzyl]-O-methyl thiocarbamate (trans), The compound is also liable for its use in diarrhea

(Gilani et al.,1992). Moreover, gastrointestinal motility disorder is treated by spasmolytic activity of different constituent in the plant (Gilani et al., 1994a).

The methanol and aqueous extract of Moringa leaf showed antiulcerogenic and hepatoprotective effects in rats (Pal et al., 1995a). The roots have also been reported to possess hepatoprotective activity. The aqueous and alcohol extracts from Moringa flowers were also found to have a significant hepatoprotective effect (Ruckmani et al., 1998). This activity may be due to the presence of quercetin, a well known flavonoid with hepatoprotective activity (Gilani et al., 1997).

3.7.3. Antibacterial and antifungal activities

Moringa roots have antibacterial activity (Padmarao et al., 1996) and are reported to be rich in antimicrobial agents. The active antibiotic principle is pterygospermin, which has powerful anti-bacterial and fungicidal effects (Ruckmani et al., 1998). A similar compound is found to be responsible for the antibacterial and fungicidal effects of its flowers (Das et al., 1957). The root extract also have antimicrobial activity due to the presence of 4-α-L-rhamnosyloxy benzyl isothiocyanate (Eilert et al., 1981). The aglycone of deoxy-niazimicine (N-benzyl, S-ethyl thioformate) isolated from ethanol extract of the root bark was found to be responsible for the antibacterial and antifungal activities (Nikkon et al., 2003). The bark extract has been shown to possess antifungal activity (Bhatnagar et al., 1961), while the juice from the stem bark showed antibacterial effect against *Staphylococcus aureus* (Mehta et al., 2003). The fresh leaf juice was found to inhibit the growth of pathogenic microorganisms e.g. *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Caceres et al., 1992).

3.7.4. Antitumor and anticancer activities

Moringa leaves are potential source of antitumor compound. O-Ethyl-4-(α-L-rhamnosyloxy) benzyl carbamate and 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, niazimicin, niazirin, 3-O-(6′-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol, β-sitosterol , glycerol-1-9-octadecanoate and β-sitosterol-3-O-β-D-glucopyranoside have been tested on *in-vitro* assay Epstein–Barr virus-early antigen (EBV-EA) activation in Raji cells for their potential antitumor activity. Niazimicin has been proposed to be a potent chemopreventive agent in chemical carcinogenesis (Guevara et al., 1999). The seed extracts have also been found to be effective on hepatic carcinoma, antioxidant parameters and skin papillomagenesis in mice (Bharali et al., 2003). A seed ointment had a similar effect to neomycin against *Staphylococcus aureus* pyodermia in mice (Caceres and Lopez, 1991). It has been found that niaziminin, a thiocarbamate from the leaves of Moringa, exhibits inhibition of tumor-promoter-induced

Epstein–Barr virus activation. On the other hand, among the isothiocyanates, naturally occurring 4-[(4′-O-acetyl-α-i-rhamnosyloxy) benzyl], significantly inhibited tumor-promoter induced Epstein–Barr virus activation, suggesting that the isothiocyano group is a critical structural factor for activity (Murakami et al., 1998).

3.7.5. Other diverse activities

Moringa oleifera has also been reported to exhibit other diverse activities. Aqueous leaf extracts regulate thyroid hormone and can be used to treat hyperthyroidism and exhibit an antioxidant effect (Pal et al., 1995a; 1995b; Tahiliani and Kar, 2000). A methanol extract of Moringa leaves conferred significant radiation protection to the bone marrow chromosomes in mice (Rao et al., 2001). Moringa leaves are effective for the regulation of thyroid hormone status (Tahiliani and Kar, 2000). A recent report showed that Moringa leaf may be applicable as a prophylactic or therapeutic anti-HSV(Herpes simplex virus type 1) medicine and may be effective against the acyclovir-resistant variant (Lipipun et al., 2003). The flowers and leaves also are considered to be of high medicinal value with anthelmintic activity (Bhattacharya et al., 1982). An infusion of leaf juice was shown to reduce glucose levels in rabbits (Makonnen et al., 1997). *Moringa oleifera* is coming to the forefront as a result of scientific evidence that Moringa is an important source of naturally occurring phytochemicals and this provides a basis for future viable developments. Different parts of Moringa are also incorporated in various marketed health formulations, such as Rumalaya and Septilin (the Himalaya Drug Company, Bangalore, India), Orthoherb (Walter Bushnell Ltd, Mumbai, India), Kupid Fort (Pharma Products Pvt. Ltd, Thayavur, India) and Livospin (Herbals APS Pvt. Ltd, Patna, India), which are reputed as remedies available for a variety of human health disorders (Mehta et al., 2003). Moringa seeds have specific protein fractions for skin and hair care. Two new active components for the cosmetic industry have been extracted from oil cake. “Purisoft” consists of peptides of the Moringa seed. It protects the human skin from environmental influences and combats premature skin aging. With dual activity, antipollution and conditioning/strengthening of hair, the Moringa seed extract is a globally acceptable innovative solution for hair care.

A different part of Moringa contains new compounds which have possible antitumor properties. As well as the antispasmodic, antiinflammatory, antihypertensive and diuretic activities of Moringa seed give new dimension of its use as edible plant. Moringa roots and leaves have been used traditionally to treat constipation. Scientific studies verify these claims; therefore, mechanism of antispasmodic action is come out (Gilani et al., 2000). The

available information on α-, β- and γ - tocopherol content in samples of various parts of this edible plant is very limited. β-carotene and vitamins A and C present in Moringa, serve as an explanation for their mode of action in the induction of antioxidant profiles, however, the exact mechanism is not yet elucidated. Niazimicin, a potent antitumor molecule is present in the seed of the plant; its inhibitory mechanism on tumor proliferation should be investigated by isolating more pure samples (Anwar et al., 2007). In view of its multiple uses, its cultivation should be promoted to get more useable parts of the plant that have to be use for treatment of human beings.

Chapter -3

Moringa oleifera: A review

3.1. Introduction

3.2. Growth, cultivation and distribution

3.3. Taxonomy

3.4. Pharmacognosy and morphology

3.5. Phytoconstituents

3.6. Traditional uses

3.7. Pharmacological activity

4.1. Introduction

In herbal medicine, plant material has been processed in a repeatable operation so that a discrete marker constituent is at a verified concentration is standardized. Active constituent concentrations may be misleading measures of potency if cofactors are not present. A further problem is that the important constituent is often unknown. For instance St John's wort is often standardized to the antiviral constituent hypericin which is now known to be the active ingredient for antidepressant use. Standardization has not been standardized yet: different companies use different markers, or different levels of the same markers, or different methods of testing for marker compounds. The plant material is extracted and the obtained extract is used for development of the standardization. In modern times processes like HPLC (High performance liquid chromatography), GC (Gas chromatography), UV/VIS (Ultraviolet/Visible spectrophotometry) or AA (Atomic absorption spectroscopy) are used to identify species, measure bacteriological contamination, assess potency and eventually creating certificates of analysis for the material. High performance thin layer chromatography (HPTLC) is another tool for standardization.

Moringa oleifera is a rich source of phytochemicals and important nutrients that can have positive effects on health. It was found to contain various phytochemicals including uncommon sugar-modified glucosinolates, although there are only details on quantity and profiles for *M. oleifera, M. peregrine* and *M. stenopetala* (Bennett et al., 2003; Fahey et al., 2001). The predominant glucosinolate is 4-O-(a-L-rhamnopyranosyloxy)-benzylglucosinolate (glucomoringin) and depending on the tissues three mono-acetyl-rhamnose isomers of this glucosinolate have also been detected (Bennett et al., 2003; Kjaer et al., 1979). There are also reports of various nitriles, thiocarbamates and carbamates, with strong hypotensive and spasmolytic effects, present in leaves of *M. oleifera* (Leuck & Kunz, 1998). It is reported to contain various phenolic compounds like 5-caffeoylquinic acid (5-CQA), 3-caffeoylquinic acid (3-CQA) compound which has health promoting effect. Caffeoylquinic acids (5- and 3-isomers) were detected in all tissues with the exception of roots, pods and seeds. It was also reported to contain flavonoids like derivatives of quercetin and kaempferol (Amaglo et al., 2010).

4.2. Collection and authentication of plant material

The leaf, pod and flower of *Moringa oleifera* was collected from local market area of Jadavpur, Kolkata and authenticated by Dr. S. Rajan, Field botanist, the medicinal plant

collection unit, Ooty, Tamilnadu, Govt. of India. A voucher specimen (SNPS- 1075) of the plant has been reserved in our laboratory (fig. 4.1.).

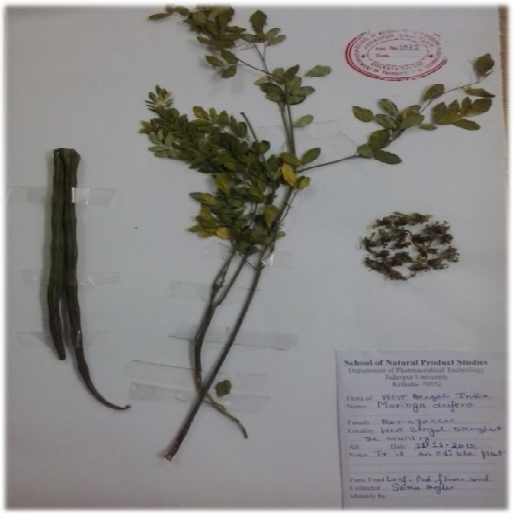


Fig. 4.1. Voucher specimen of *Moringa oleifera*

4.3. Extraction procedure

The collected samples were shade dried and were grind to make fine powder. 100 gms of each of the powdered samples were extracted with hydroalcoholic solvent (water: methanol 30:70) by cold maceration process for 7 days. The process was repeated for 3 times. Then it was filtered and the filtrates of each sample were collected. They are concentrated under vacuum by a rotary vacuum evaporator. The excess methanol may present will be evaporated by this process. Now the samples were freeze dried to remove the excess water present in the sample completely to get a constant weight. The percentage yield of the each extract was found to be 11.54% (leaf), 17.32% (pod), and 21.54% (flower). Each of the extract was then subjected to standardization through High Performance Thin Layer Chromatography (HPTLC) by selecting a suitable marker.

4.4. Rationale behind choosing Chlorogenic acid (CGA) as a marker for standarization

Literature survey reveals that the leaf extracts of *Moringa oleifera* have been reported to exhibit antioxidant activity both in vitro and in vivo due to abundant phenolic acids and flavonoids (Chumark et al., 2008; Verma et al., 2009). Some researchers claimed that Moringa leaves were rich in Chlorogenic acid, Gallic acid, Kaempferol and Quercetin glycosides (Bennett et al., 2003). The flowering part of the plant was also reported to contain CGA. Presence of highest concentration of total phenolics, total flavonoids in the plant

among other major active compounds, and the highest antioxidant activity promoted us in choosing CGA as a marker for standardization.

4.5. HPTLC chromatographic analysis

4.5.1. Equipment

A Camag HPTLC system equipped with a sample applicator Linomat III, twin through plate development chamber, TLC Scanner III and Wincats integration software 4.02 (Switzerland) was used for the experiment. Solvents and reagents Analytical-grade ethyl acetate, methanol, and formic acid were obtained from SD Fine Chemicals Ltd. (Mumbai, India). TLC aluminium plates pre-coated with silica gel $60F_{254}$ (10×10 cm, 0.2mm thick) were obtained from E. Merck Ltd. (Mumbai, India).

4.5.2. Preparation of Standard and Test Sample

For preparing the standard Chlorogenic acid (CGA) solution 1mg of standard CGA was accurately weighed and dissolved in 1ml of methanol to prepare 1mg/ml of solution. The three samples (leaf, flower and formulation) were prepared in a concentration of 10 mg/ml in methanol. All the samples were sonicated and filtered through Whatman No. 1 filter paper pore size 11 mm (Maidstone, UK)

4.5.3. Sample application

Prior to the study, plates were washed with methanol and dried at room temperature for 10 min. Standard CGA and the extracts were applied on the 10 ×10 cm aluminium backed silica gel 60 F_{254} HPTLC plates. Samples were applied to the plates, as 5mm bands, using a Camag Linomat III automated spray-on band applicator equipped with a 100 µL syringe (Hamilton, USA).

4.5.4. Development of TLC plate

The final composition of mobile phase was developed experimentally after several TLC experiments. In order to obtain the best separation, ethyl acetate: water: formic acid (16: 2: 3 v/v/v) was found to be the most suitable mobile phase for extraction to resolve CGA. Ascending development of the plate was performed at 25 ±2° C with the same solvent system in a Camag twin-tough chamber, previously saturated with mobile phase for 30min. The average development time was 15min. The solvent was allowed to run 80 mm from the application spot. The plates were taken out for air drying for 10 min.

4.5.5. Densitometry

Densitometric scanning was performed at 366 nm with a Camag TLC scanner III equipped with Wincats software, using a deuterium light source; the slit dimension was 6.00×0.45 mm. The image of the developed plate is presented in Fig. 4.2. To estimate the limit of detection (LOD) five different levels (2, 4, 6, 8 and 10.0 µg/mL) of the standard stock solution (1mg/mL) of CGA were prepared and used accordingly. Blank methanol was also spotted three times following the same method and the signal-to-noise ratio was determined. The LOD and LOQ were experimentally verified by diluting the known concentration of CGA until the average responses were approximately 3 or 10 times the standard deviation of the response for three replicate determinations for LOD and LOQ respectively.

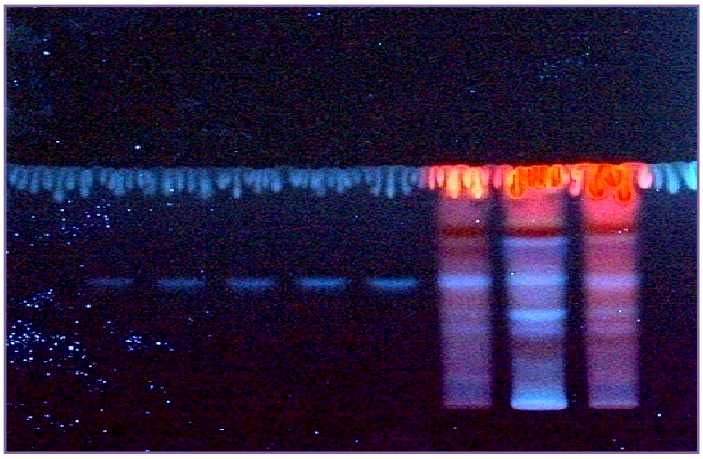


Fig. 4. 2. The developed HPTLC plate under 366 nm

4.5.6. Calibration curve of CGA and its estimation in Moringa oleifera

The calibration curve was plotted based on the application of different concentrations of CGA ranging from 2 to 10 µg. The correlation coefficient (*r*) value was found to be 0.99400, which indicated a good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation Y = 1131.61 + 763.697x, where Y represents the response as area and x represents the concentration (Fig. 4. 3).

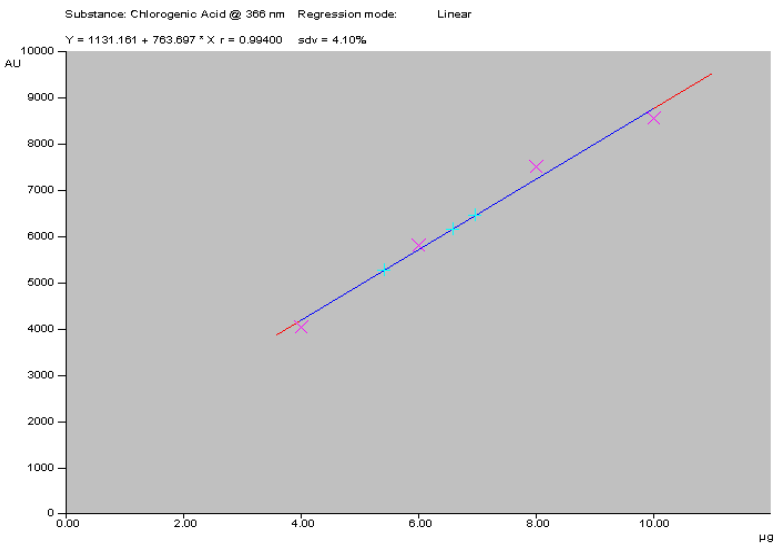


Fig. 4. 3. Calibration curve of Chlorogenic acid

4.6. Validation of HPTLC method

4.6.1. Linearity

The linearity was obtained by analysing five standard CGA solutions (as mentioned earlier) and a representative calibration curve of CGA was obtained. The result indicated a good linear relationship between the concentrations and peak areas (Fig. 4.3).

4.6.2. Accuracy

Standard addition method was used to perform the recovery study. The recovery of added standard (CGA) was studied at three different levels. The extract used for recovery studies were pre-analysed by the developed method as mentioned earlier. The percentage recovery of CGA in the extracts and the formulation is presented in (Table 4. 5 - 4. 7).

4.6.3. Specificity

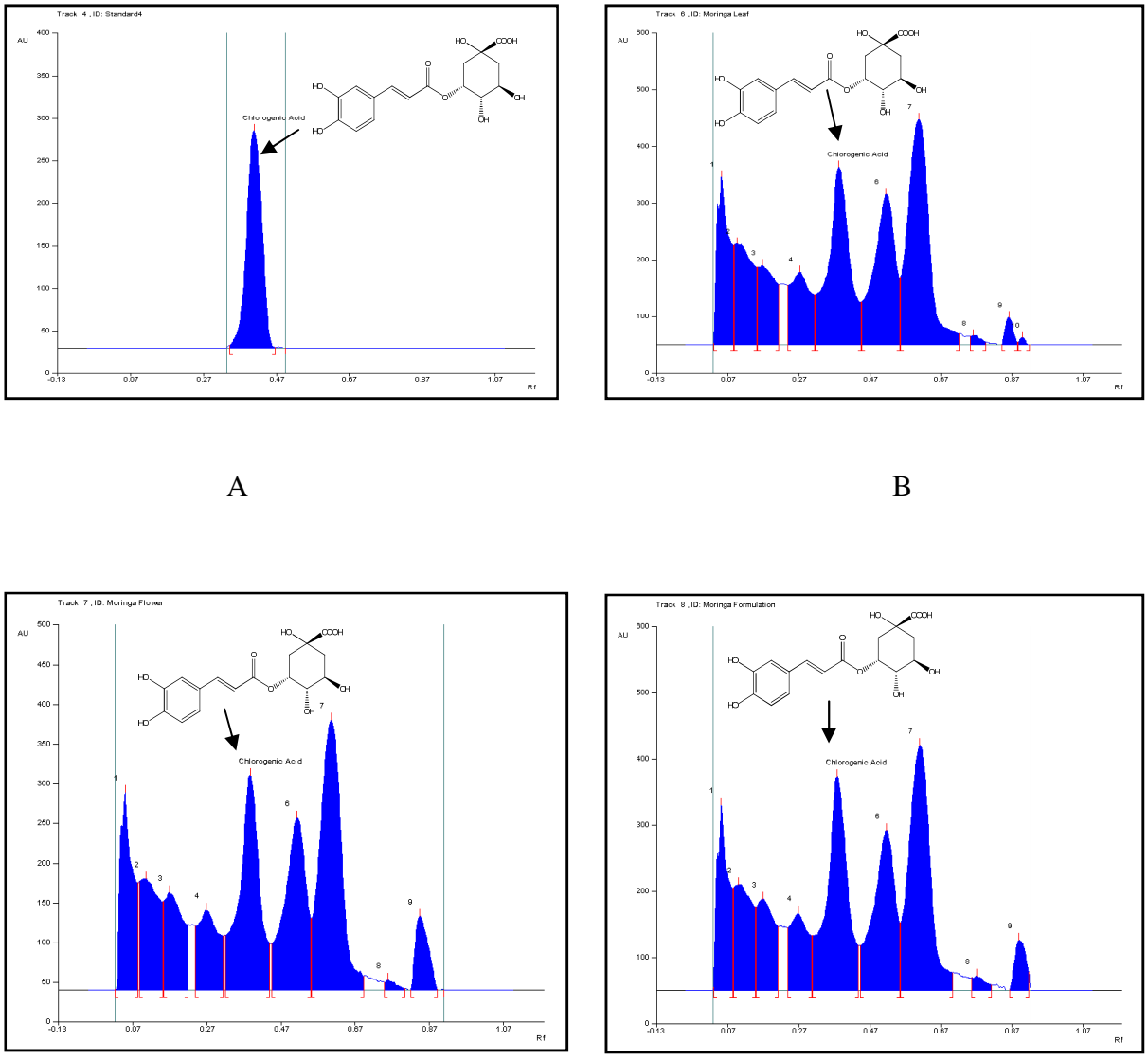
It was observed that the peak of standard did not interfere with the peak of CGA in the extract and therefore the method can be considered as specific. The chromatogram of standard CGA and CGA from the extract was matched in a similar fashion. The LOQ was determined based on the standard deviation of the response of blank and slope estimated from the calibration curve of standard solution of CGA.

4.6.4. Intra- and inter-day precision

The reproducibility of the method was determined by different analysts using the sample from the same homogeneous batch and repeatability was determined by intra-day and inter-day precision, expressed in terms of relative standard deviation (%RSD) and standard error (SE). The intra-day precision was determined at three different concentration levels of CGA of 4, 8 and 10 µg/spot three times on the same day. Inter-day precision was determined at three different concentrations of CGA (4, 8 and 10 µg/spot) three times on three different days over a period of one week (Table 4. 4).

4.7. Result and discussion

The method was performed by following the method of Amaglo et al. 2010. Best fit calibration curve (Fig. 4.3.) was described by the linear regression (Y= 1131.61 + 763.697x) with a correlation coefficient of 0.9940. During mobile phase optimization it was observed that the CGA cannot be detected in pod at 366nm. CGA can only be detected in leaf, flower and formulation of *M. oleifera*.



A B

C D

Fig. 4.4. (A) HPTLC chromatogram of Standard Chlorogenic acid. (B) HPTLC chromatogram of *M. oleifera* leaf. (C) HPTLC chromatogram of *M. oleifera* flower. (D) HPTLC chromatogram of *M. oleifera* formulation.

Hence Chromatogram of all the three samples has been demonstrated in Fig. 4.4. (A-D) The intra-day precision was determined at three different concentration levels of CGA (4, 8, and 10 µg/spot) three times on the same day. Inter-day precision was determined at three different concentrations of CGA (4, 8, and 10 µg/spot) three times on three different days over a period of one week gives an effective result (Table 4. 4).

Table 4.4
Intra and inter-day precision study (n=3)

Amount (µg/spot)	Intra-day precision				Inter-day precision			
	Mean Area	S.D.	% RSD	S.E.	Mean Area	S.D.	% RSD	S.E.
4	4049.96	36.18	0.89	21.08	4185.6	39.23	0.93	32.05
8	7431.28	41.26	0.80	34.82	7601.31	36.13	0.68	29.19
10	8508.55	47.94	0.57	38.78	8676.12	35.41	0.63	25.11

Table 4. 5
Recovery study of CGA from *M. oleifera* leaf extract

Sample no.	Amount of sample (µl)	Excess CGA added (µg)	Expected CGA (µg)	CGA found (µg)	Average CGA found (µg)	Average recovery (%)	RSD (%)
1.	10	0	2.96	2.912 2.947 2.932 2.981 2.916 2.949	2. 94	99.33	0.139
2.	10	2	4.96	4.91 4.982 4.95 4.921 4.895 4.923	4.93	99.21	0.851
3.	10	4	6.96	6.912 6.961 6.955 6.901 6.923 6.954	6.951	99.82	0.505

Table 4.6
Recovery study of CGA from *M. oleifera* flower extract

Sample no.	Amount of sample (µl)	Excess CGA added (µg)	Expected CGA (µg)	CGA found (µg)	Average CGA found (µg)	Average recovery (%)	RSD (%)
1.	10	0	2.46	2.431 2.452 2.455 2.471 2.450 2.459	2.441	99.23	0.90
2.	10	2	4.46	4.48 4.399 4.45 4.42 4.425 4.64	4.437	99.48	0.96
3.	10	4	6.46	6.451 6.459 6.431 6.429 6.449 6.355	6.429	99.52	0.59

Table 4.7
Recovery study of CGA from *M. oleifera* formulation

Sample no.	Amount of sample (µl)	Excess CGA added (µg)	Expected CGA (µg)	CGA found (µg)	Average CGA found (µg)	Average recovery (%)	RSD (%)
1.	10	0	3.16	3.151 3.149 3.159 3.141 3.095 3.156	3.17	98.10	0.76
2.	10	2	5.16	5.133 5.152 5.161 5.189 5.126 5.118	5.146	99.72	0.51
3.	10	4	7.16	7.101 7.131 7.156 7.119 7.126 7.159	7.132	99.60	0.311

The recovery study was performed for each sample of *M. oleifera* (leaf, flower, & formulation) individually. Average recovery during recovery studies was found in the range of 98-100% and the % RSD value obtained were in the range of 0.1-1 (Table 4.5-4.7). The linearity range was found in the range of 4µg- 10µg/spot. From the each chromatogram of the samples and the calibration curve it was found that the formulation contain highest concentration of CGA (3.16 % w/w) whereas the CGA was not found in pod. The leaf and the flowering part contain 2.99 % w/w and 2.46 % w/w of CGA per weight of extract respectively. The developed method is very precise, accurate and reproducible with a narrow linearity range. Thus it can be used at an industrial level to commercialize the method so as to get a fast and reliable analysis of the chlorogenic acid in sample with a controlled amount of Chlorogenic acid.

Chapter – 4

Standardization of *Moringa oleifera* by High Performance Thin Layer Chromatography (HPTLC)

4.1. Introduction

4.2. Collection and authentication of plant material

4.3. Extraction procedure

4.4. Rationale behind choosing Chlorogenic acid as a marker for standardization

4.5. HPTLC chromatographic analysis

4.6. Validation of HPTLC method

4.7. Result and discussion

5.1. Introduction

The discovery of medicinal plants is growing day to day. People are using herbal medicines due to increasing faith in herbal medicine. Wide range of diseases can be cured by allopathic medicine but its high prices and side-effects diminishing people interest and to return to herbal medicines which having fewer side effects with effective cost (Kala, 2005). Free radicals are generated in the body due to oxidative stress. Researchers are working on finding natural antioxidants from plant origin. The use of herbal extracts and nutritional supplements either as alternative or complimentary medicine for treatment of inflammatory diseases is well documented in Ayurveda. Estimation of total phenolic content has been carried out for 5000 years (Dahanukar et al., 2000). The main issue of public health is the acute need for basic health care. The World Health Organization (WHO) indicates that the rural people do not have access to adequate health care services or they cannot afford this costly region. Therefore, innovative alternative approaches are being taken to combat this problem. Medicinal plants offer alternative remedies with tremendous opportunities. The WHO estimates that more than 80% of the world's population rely either solely on traditional health care system (Gias, 1998). Medicinal and aromatic plants has been exploited greatly in recent years as pharmaceuticals, herbal remedies, flavourings, perfumes and cosmetics, and other natural products has (Kumar et al. 2000).

Antioxidants are the compounds prevent oxidative damage caused by free radical. It can interfere with the oxidation process by reacting with free radicals, chelating, and catalytic metals and also by acting as oxygen scavengers (Shahidi and Wanasundara 1992). The potentially reactive derivatives of oxygen, ascribed as ROS (reactive oxygen molecules) such as O_2^-, H_2O_2 and OH, are continuously generated inside the human body as a consequence of exposure to a of exogenous chemicals and/or a number of endogenous metabolic processes involving redox enzymes and bioenergetic electron transfer. The ROS generated in the body are detoxified by the antioxidants present in-vivo under normal circumstances and equilibrium is maintained between the ROS generation and the antioxidants present. However ROS overproduction and/or inadequate antioxidant defense, make this equilibrium hampered and there is increased accumulation of ROS as a result it produces oxidative stress (Kohen and Gati, 2000). Therefore antioxidants from natural origin have been increased in now days.

Moringa oleifera is the most widely cultivated species of a moringaceae family. Medicinal properties of plants have been investigated throughout the world, due to their potent antioxidant activities. The successive aqueous extract of *Moringa oleifera* is reported to exhibit strong scavenging effect on 2, 2-diphenyl-2-picryl hydrazyl (DPPH) free radical,

superoxide, nitric oxide radical and inhibition of lipid per oxidation (Sreelatha & Padma, 2009). Therapeutic uses of medicinal plants as antioxidants in reducing such free radical have been raised in now a day. Besides well identified and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices, some natural antioxidant (e.g. rosemary and sage) are already exploited commercially also as antioxidant additives or a nutritional supplements (Schuler, 1990). It has been mentioned the antioxidant activity of plants might be due to their phenolic compounds (Cook and Samman, 1996). Flavonoids are a group of polyphenolic compounds associated with the free radical scavenging activity, hydrolytic and oxidative enzymes inhibiting activity and have anti-inflammatory action (Frankel, 1995). Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity (Gryglewski et al., 1987). Flavonoids are constituents of fruits, vegetables, nuts, plant-derived beverages such as tea and wine and traditional Eastern medicines such as Ginkgo biloba, as well as components present in a plethora of herbal-containing dietary supplements.

They have been known as plant pigments for over a century and belong to a vast group of phenolic compounds that are widely distributed in all foods of plant origin. North American diet, flavonoid glycosides are unavoidably consumed daily, with an estimated total consumption of 1 g/d (Formica and Regelson, 1995), which could be much higher if dietary supplements are also consumed. As an example, dietary supplements of quercetin have been suggested to contain doses which are up to 20 times higher than those which would be obtained in a typical vegetarian diet (Skibola and Smith, 2000). Flavonoids are known to be powerful antioxidants in vitro (Pannala et al., 1998; Kerry and Rice-Evans, 1999). Derived polyphenols from plants are of great importance because of their potential antioxidant and antimicrobial properties. Phenolic compounds exhibit a considerable free radical scavenging activity, which is determined by their reactivity as hydrogen or electron-donating agents, the stability of the resulting antioxidant derived radical, their reactivity with other antioxidants and finally their metal chelating properties (Amo-Lee et al., 2001; Tuladhar and Rao, 2010; Wojdylo, 2007).

5.2. Method of estimation

5.2.1. Chemicals and equipments

Gallic acid, Folin-Ciocalteu reagent, sodium bicarbonate, aluminum chloride were purchased from Merck Mumbai, deionized water, SpectraMax M5 Multi-Mode Microplate Readers, microtest plate was purchased from Tarson Mumbai.

5.2.2. Determination of total phenolic content

The content of total phenolic compounds was determined by using Folin-Ciocalteu procedure (Pothitirat et al., 2009). A standard sample of Gallic acid (1000 µg/mL) was prepared. From this 200 µL solution was picked up and mixed with 500 µL of the Folin-Ciocalteu reagent (diluted 1:10 with deionized water) and 800 µL of sodium bicarbonate solution (7.5%, w/v) was added. The mixture was allowed to stand at room temperature for 30 min with intermittent shaking. The absorbance was measured at 765 nm using a SpectraMax M5 Multi-Mode Microplate Readers. The test samples of *Moringa oleifera* (leaf, pod, flower and their formulation) were also prepared same manner. The content of total phenolic compounds was calculated as mean ± SD (n = 3) and calculated as grams of Gallic acid equivalents (GAE) in 100 g of the extract.

5.2.3. Determination of total flavonoid content

Total flavonoids were analyzed using aluminum chloride colorimetric method (Pothitirat et al., 2009). Standard quercetin (1000 µg/mL) of 500 µL was mixed with 500 µL of 2% aluminum chloride solution. The mixture was allowed to stand at room temperature for 10 min with intermittent shaking. The absorbance of the mixture was measured at 415 nm against a blank sample without aluminium chloride using SpectraMax M5 Multi-Mode Microplate Readers. The content of total flavonoids was calculated as Mean ± SD (n = 3) and calculated as grams of quercetin equivalents in 100 g of the extract and dried powder.

5.3. Result and discussion

Moringa oleifera is a rich source of phytochemicals and important nutrients that can have positive effects on health. The total phenolic content and and total flavonoid content in

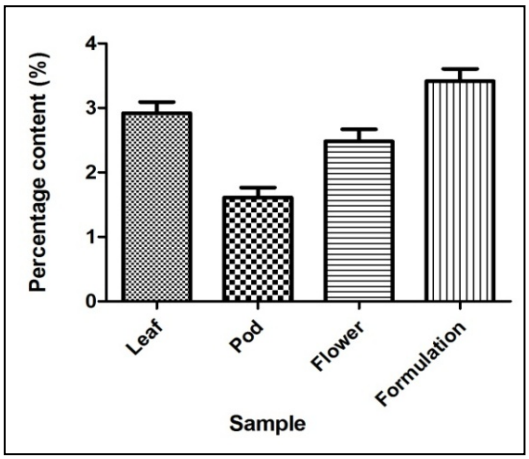


Fig. 5. 1. Total phenolic content. Data expressed as mean±SD (n=3)

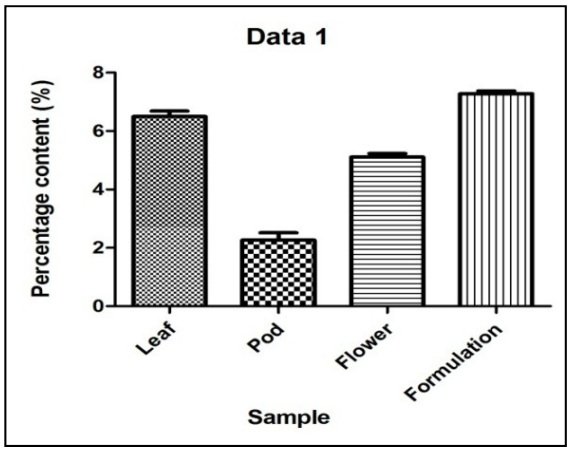


Fig. 5. 2. Total Flavonoid content, Data expressed as mean±SD (n=3)

Moringa oleifera test samples (leaf, flower, pod, extract and formulation) was obtained from this experiment. The results are expressed and presented in Table 5.1. The content of the total phenolic is greater than the total flavonoid content found in this plant. The results have been statistically evaluated by using Graph pad prism 5.0 versions which are presented in Fig. 5.1-5.2. From this estimation study the phenolic content in *Moringa oleifera* was found in the order of formulation > leaf > flower > pod and the total flavonoid content was found to be formulation>leaf>flower> and pod.

Table 5.1
Total Phenolic and Total Flavonoid content in *Moringa oleifera* leaf, pod, flower and its formulation (n=3)

Content	Samples				
	Leaf	Pod	Flower	Formulation	Units
Total phenolic content	6.5±0.31	2.267±0.43	5.11±0.21	7.27±0.18	g GAE/100 g extract
Total flavonoid content	2.917±0.18	1.610±0.15	2.48±0.18	3.41±0.19	g QE/100 g extract

Data are expressed as Mean ± SD.

Antioxidants are the compounds that can inhibit and scavenge free radicals, thus providing protection to human against infections and degenerative diseases. A number of scientific reports indicate that certain terpenoids, steroids and phenolic compounds such as tannins, coumarins and flavonoids have protective effects due to their antioxidant properties (Chandrasekhar et al., 2006). Polyphenols, flavonoids and phenolic compounds are the major antioxidants present in herbs. From this study it has been observed that the plant is a rich source of phenolics and flavonoids which may contribute to the stronger antioxidant profile.

Chapter – 5

Estimation of total phenolic and total flavonoid content

5.1. Introduction

5.2. Method of estimation

5.3. Result and discussion

6.1. Introduction

Macronutrients are the chemical substances required in relatively large amounts (> 0.005% body weight). They are required for energy supply and for growth and maintenance of the body. The macronutrients includes proteins, carbohydrates, fats and some of the minerals like Na, Mg, K, Ca, P, S and Cl.

Micronutrients are the substances required in very small amounts (mg or µg) mainly function as a co-factor of enzymes (<0.005% body weight). The micronutrients includes trace minerals like Fe, Cu, F, Zn, I, Se, Mn, Mo, Cr, Co and B and vitamins A, B, C, D, E, K. Minerals and most vitamins (except vitamin D) are not synthesized by the body and must be taken in the diet. They are essential for the plants growth and only needed in small amounts. That's why they are called as the "minor elements". The seven micronutrients are boron, chlorine, copper, iron, manganese, molybdenum and zinc. Micronutrients add more mineral content to the plants. They play an important role in detoxifying the body and warding off harmful diseases. Due to the high amounts of antioxidants that they contain, micronutrients can essentially help to protect the body against many age-related diseases. They are essential in helping to neutralize free radicals and eliminate toxins from the body, which in turn promotes longevity. In addition, micronutrients help the body to create enzymes and other important components that are essential for healthy body functions. Both of these types of nutrients can be obtained from the diet and supplements. Typically, all whole foods, including meat, dairy, and eggs, as well as fruits and vegetables, nuts, and seeds, contain good amounts of macro and micronutrients. Unlike micronutrients, which do not contain calories, macronutrients are loaded with them in order to provide energy to every organ and cell within the body. The energy helps the organs, such as the brain, kidneys, and the heart, to operate at optimum levels. A deficiency can cause the organs to lose their vital functions and result in fatigue. As example deficiency of iron, decreases the production of haemoglobin which causes anaemia. Deficiency of zinc causes short stature, anaemia, poor gonadal function, impaired healing of wounds and cognitive and motor function. It can also lead to appetite disorders. Deficiency of vitamin A causes night blindness. A lower consumption of protein may lead to malnutrition.

Dietary fibre is another compound required for health maintenance. They are found mainly in fruits, vegetables, whole grains and legumes. It can prevent or relieve constipation. It includes all parts of plant foods that body can't digest or absorb. A high-fiber diet can normalizes bowel movements, Lowers cholesterol levels, control blood sugar levels.

Moringa oleifera is an unbelievable source of all macro, micro elements, and dietary fibre. No single food contains all of the vitamins and minerals. So it is unbelievable to get all the nutrients in a single plant. That's why it is called as 'miracle tree' (Palada, 1996; Fuglie, 1999), It is a good source of tocopherols (Sanchez-Machado et al., 2006). β-carotene (pro-vitamin A), vitamin C, calcium, protein, potassium (Ramachandran et al., 1980). The leaf is highly nutritious and contains significant quantities of crude protein (20 to 29%), vitamins and minerals (Elkhalifa et al., 2007; Kakengi et al., 2007; Olugbemi et al., 2010; Abou-Elezz, et al., 2011). Therefore investigation of these micro and macronutrients has been performed through this study.

6.2. Preparation of dietary supplement

The *Moringa oleifera* plant is an outstanding source of nutrition. The leaf of this plant contains vitamin A, vitamin B, vitamin C and minerals (Jules and Paull, 2008). The pod of this plant contains high amount of minerals. The leaf, pod, and flower are consumed as vegetables in most of the regions. 1kg of leaf, pod and flower of *Moringa oleifera* were collected from local market of Jadavpur, Kolkata. The collected samples were cleaned by water and shade dried. The dried samples were ground and passed through a sieve (20 mesh). To prepare the dietary supplement 50 gm of each sample powder (leaf pod and flower) were accurately weighed and mixed in 1:1:1 ratio. This will be called as dietary supplement formulation. The powdered samples were kept in sealed containers and protected from light until used. All these three powder samples and the dietary supplement formulation will be used in the experiment for the evaluation of nutritional value.

6.3. Evaluation of Nutritional Value

6.3.1. Estimation of Macronutrients

The macronutrients includes proteins, carbohydrates, fats and some of the minerals like Na, Mg, K, Ca, P, S and Cl. The macronutrients analysed for the experimental samples and dietary supplement has been described below.

6.3.1.1. Protein

Proteins are large biological molecules consisting of one or more amino acids chain. They can perform wide array of functions including catalyzing metabolic reactions, DNA replication, and can transport molecules from one location to another. Most microorganisms and plants can biosynthesize amino acids, while animals must obtain some of the amino acids from the diet (Voet and Voet, 2004). In animals, amino acids are obtained through the

consumption of foods containing protein. Amino acids are also an important dietary source of nitrogen.

6.3.1.1.1. Chemicals and equipments

Biuret reagent, Standard Bovine Serum Albumin (BSA) was purchased from Merck Mumbai. SpectraMax M5 Multi-Mode Microplate Readers, 96 well micro test plate was purchased from Tarson Mumbai.

6.3.1.1.2. Procedure

The protein estimation was performed by the Biuret method**.** 1 mg of Standard BSA was prepared in water to prepare 1mg/ml of standard solution. For the calibration curve five dilutions were prepared from the stock solution in 20-60 µg/ml concentration range. 0.5 ml from each of the sample was used for further analysis. The test samples of were also dissolved in water from where 0.5 ml from each was collected for further analysis. To 0.5 ml of each standard and sample solution 2.5 ml Biuret reagent was added and allowed to stand for 30 mins. The absorbance was measured at 550 nm using SpectraMax M5 Multi-Mode Microplate Readers.

6.3.1.1.3. Results and discussion

The calibration curve of Bovine Serum Albumin is presented below in Fig 6.1.

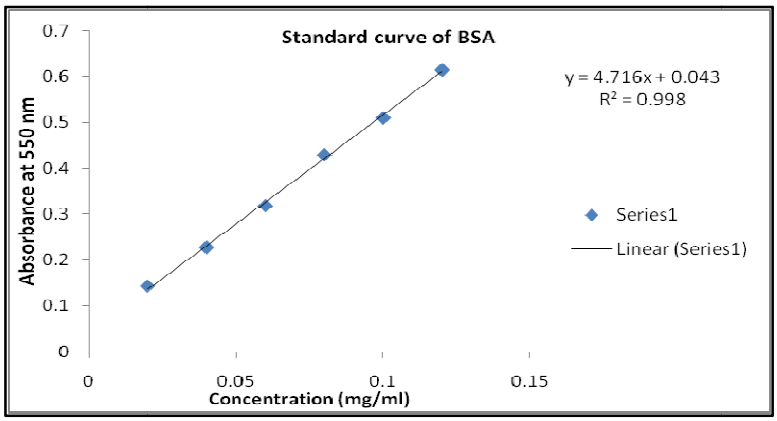


Fig.6.1. Calibration curve of Bovine Serum Albumin

From the calibration curve the unknown concentration of total protein in the test samples and the formulation was determined. The calibration curve shows a good linearity in the concentration range of 20-60 µg/ml. The concentration protein in the test samples has been statistically evaluated by using Graph pad prism version 5. The results are shown in Fig 6. 2. It was observed that the protein content is highest in the dietary formulation compared to other

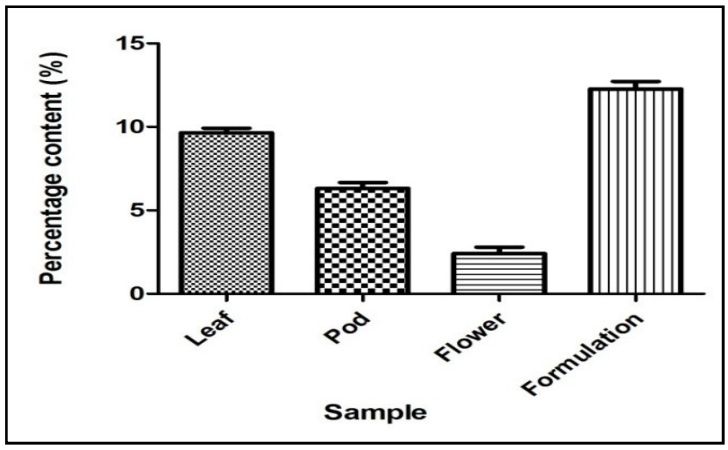


Fig.6.2. Percentage content of protein in *Moringa oleifera* test samples and dietary formulation (Data expressed as Mean ± SD) (n=3)

test samples (leaf, flower, & pod). The leaf also contains larger amounts of protein. The protein content is represented in Table 6.1.

Table 6. 1
Protein content in *Moringa oleifera* test samples and formulation (n=3).

Sample	% w/w
Leaf	9.643 ± 0.28
Pod	6.315 ± 0.35
Flower	2.420 ± 0.37
Formulation	12.26 ± 0.45

Data are expressed as Mean± SD.

6.3.1.2. Carbohydrate

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. They are the common source of energy in living organisms; however, no carbohydrate is an essential nutrient in humans (Westman, 2002).The basic units of carbohydrates are the monosaccharides which cannot be split by hydrolysis into simpler sugars. The carbohydrate content can be measured by hydrolyzing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides. In this experiment carbohydrate content in test sample of *Moringa oleifera* and its prepared dietary formulation has been estimated by anthrone method. In this experiment total carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural.

This compound forms with anthrone a green colored product with an absorption maximum at 630 nm.

6.3.1.2.1. Chemicals and equipments

Anthrone, sulphuric acid, glucose were purchased from Merck Mumbai. 96 well micro test plate were purchased from Tarson Mumbai. SpectraMax M5 Multi-Mode Microplate readers.

6.3.1.2.2. Procedure

Anthrone reagent was prepared by dissolving 200mg anthrone in 100 mL of ice cold 95% H_2SO_4. The standard solution was prepared by dissolving 10 mg glucose in 100 ml water to prepare 0.1mg/ml stock. 100 mg of the each test sample were hydrolysed by keeping it in boiling water bath for 3 hours with 5mL of 2.5 N-HCl and cool to room temperature. After that the solutions were neutralized. The volume was made up to 100 ml with water and the supernatant were collected for analysis. Five dilutions of the standards were prepared (0.02-0.1mg/ml). 4 ml of anthone reagent was added to each of the standard and sample solution. The solutions were heated for eight minutes in a boiling water bath. After some time green colour was developed which on rapid cooling converted to deep green colour. The absorbance was taken at 630 nm.

6.3.1.2.3. Results and Discussion

The calibration curve of glucose is given in Fig 6. 3. From the calibration curve the total carbohydrate in the test samples were measured. The amounts of the unknown concentration

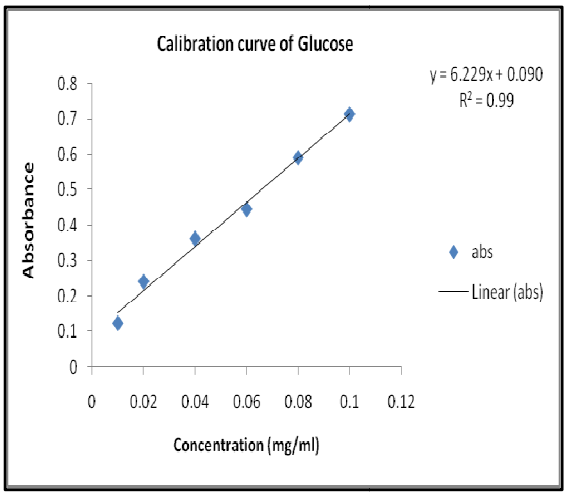


Fig. 6. 3. Calibration curve of Glucose

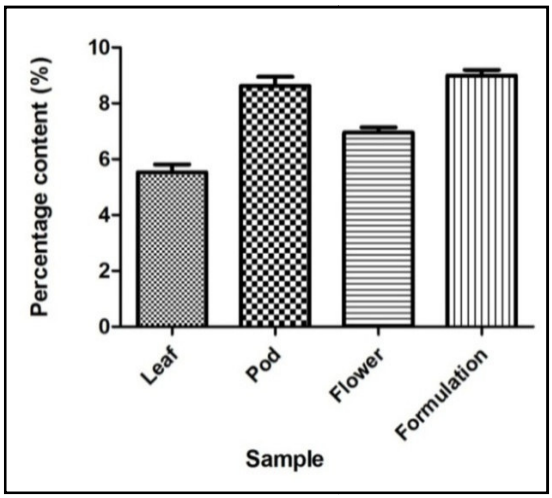


Fig. 6.4. Percentage content of Carbohydrate in test samples and dietary formulation (Data expressed as Mean ± SD) (n = 3)

carbohydrate present in the test samples has been statistically evaluated by using Graph pad prism version 5. The results are shown in Fig. 6.4. It was observed that the carbohydrate content is highest in the formulation compared to other test sample (leaf, flower, & pod) has been presented in Table 6. 2.

Table 6. 2
Carbohydrate content in *Moringa oleifera* test samples and formulation (n=3).

Sample	% Content
Leaf	5.527 ± 0.28
Pod	8.617 ± 0.34
Flower	6.953 ± 0.18
Formulation	8.997 ± 0.19

Data are expressed as Mean ± SD

6.3.1.3. Total fat, total dietary fibre and Moisture content

Fats play an important role in maintaining health. They can maintain healthy skin and hair, insulating body organs against shock, maintaining body temperature, and promoting healthy cell function. They also serve as energy stores for the body. They are broken down in the body to release glycerol and free fatty acids. The glycerol can be converted to glucose by the liver and thus used as a source of energy. Dietary fiber is a compound required for health maintenance. They are found mainly in fruits, vegetables, whole grains and legumes. It can prevent or relieve constipation. It includes all parts of plant foods that body can't digest or absorb. A high-fiber diet can normalizes bowel movements, lowers cholesterol levels, control blood sugar levels. The estimation of total fat, dietary fibre and moisture present in

Table 6. 3
Total dietary fibre, crude fat and moisture content in *Moringa oleifera* test samples and formulation.

Analysis	Leaf	Pod	Flower	Formulation	Unit
Total dietary fibre	32	69.24	25.68	48.44	g/100g
Moisture	11.11	8.95	13.11	10.16	g/100g
Fat	1.17	0.71	1.51	2.11	g/100g

Moringa oleifera and its formulation were done by SGS Chemical, Gurgaon. The dietary fibre was estimated by the method of AOAC 985.29. The moisture content and total fat was estimated by the method of IS: 7874 (part- I): 1975. The data are presented in g/100g unit in Table 6. 3. It has been observed from the report that the total dietary fibre content is highest in the pod of *Moringa oleifera* where as formulation and the flower contains highest amount of fat and moisture respectively.

6.3.1.4. Phosphorus

Phosphorus is essential for life. Phosphate is a component of DNA, RNA, ATP, and phospholipids form all cell membranes. It is the correlating link between phosphorus and life. Hence it is important to take a certain amount of phosphorus in diet.

6.3.1.4.1. Chemicals and equipment

KH_2PO_4, Ammonium molybdate, Hydrazine sulphate were purchased from Merck Mumbai. Spectra Max M5 Multi-Mode Microplate Readers, Micro Test Plate was purchased from Tarson Mumbai, double distilled water.

6.3.1.4.2. Preparation of phosphorus standard solution

0.4393 m of KH_2PO_4 was dissolved in water and diluted to 1 litre to produce 100 ppm solution. Working standard was prepared by diluting 25 ml of stock solution to 500 ml to make 5ppm solution. Aliquots of standard solution was treated to prepare 1-25 ppm concentration and to construct calibration curve. The method was performed according to the method described in AOAC, 1990. Aliquots of standard solution were transferred to 100 ml volumetric flask. 5 ml of ammonium molybdate solution was added and mixed. Then 5 ml of hydrazine sulphate solution was added and the solutions were diluted to 70 ml with water. The solution was mixed properly and placed in boiling water bath for 9 mins. Then the solutions were cooled rapidly and diluted to volume. The experimental samples were treated in the same manner as the standard.

6.3.1.4.3. Result and discussion

The estimation was performed according to the method present in (AOAC, 1990). The results are integrated by the Software: SoftMax®. Pro. Calibration curve prepared with standard KH_2PO_4. Correlation coefficient was found to be 0.995. A good linearity was observed in the concentration range of 1-25 µg/ml Fig. 6.5. The phosphorus present in the test samples was also determined from the equation of the calibration curve Y= 27.45x+0.081 (Table 6.4). The

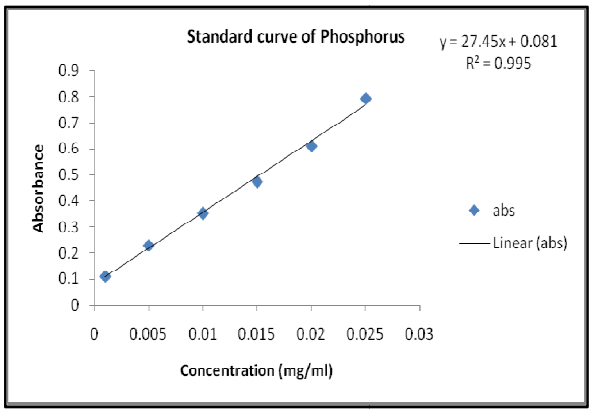


Fig. 6. 5. Calibration curve of Standard Phosphorus

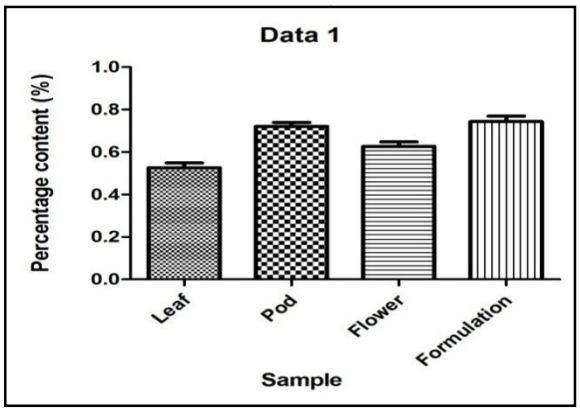


Fig. 6.6. Percentage content of Phosphorus in the experimental samples. Values as expressed as Mean ± SD (n = 3)

Table 6.4.
Phosphorus content *Moringa oleifera* test samples and formulation (n=3).

Sample	% Content
Leaf	0.527 ± 0.02
Pod	0.72 ± 0.01
Flower	0.627 ± 0.02
Formulation	0.744 ± 0.02

Data expressed as Mean ± SD

data are also statistically evaluated by using graph prism 5.0 versions. The datas are represented as Mean ± SD (n=3) in Fig. 6.6.

6.3.1.5. Magnesium

Magnesium is a vital component of a healthy human diet. Low levels of magnesium in the body have been associated with asthma, diabetes, and osteoporosis. Proper amount magnesium plays an important role in preventing stroke and heart attack. Magnesium ions are essential to the basic nucleic acid chemistry, and thus are essential to all cells of living organisms.

6.3.1.5.1. Chemicals and equipment

Magnesium sulphate, Titan-yellow, NaOH, Trichloroacetic. All these reagents were obtained from Merck Mumbai. Spectra Max M5 Multi-Mode Microplate Readers, Micro Test Plate was obtained from Tarson Mumbai, Double distilled water.

6.3.1.5.2. Preparation of the standard solution

10.131 g. $MgSO_4.7H_20$ were dissolved in distilled water, 0.5 ml. chloroform was added and the volume was made upto 1 1itre with distilled water (1mg/ml). Working standard was prepared by diluting 2 ml. of the stock diluted to 100 ml so that 1 ml. working standard contains 0.02 mg/ml. Trichloroacetic acid. 10% (w/v) solution was also prepared. To prepare the Titan yellow solution, 100 mg of Titan yellow was dissolved in water and made to 200 ml to prepare 0.05% Titan yellow solution. Sodium hydroxide 4 (N) was also prepared for the analysis.

6.3.1.5.3. Procedure

To 4 ml of sample 8 ml. water and 4 ml, 10% trichloroacetic acid was added. The solutions were well mixed and was allowed to stand for 5 min. and then filtered through a Whatman no. 42 paper. To 8 ml. of the water-clear filtrate 1.5 ml. Titan yellow and 2 ml 4 (N) NaOH were added. A water-blank was prepared by taking 6 ml distilled water, adding 2 ml 10% trichloroacetic acid, titan yellow and 2 ml NaOH.

6.3.1.5.4. Preparation of calibration curve

For the calibration curve different concentration of the standard was prepared from the working standard ranging from 0.02-0.1mg/ml. The experimental test samples and formulation were also treated in the same way. The absorbance of the solution was measured at 525 nm in Spectra Max M5 Multi-Mode Microplate Readers.

6.3.1.5.5. Results and discussion

The estimation was performed according to the method of (Garner, 1946). The results are integrated by the Software : SoftMax®. Pro. Calibration curve prepared with standard

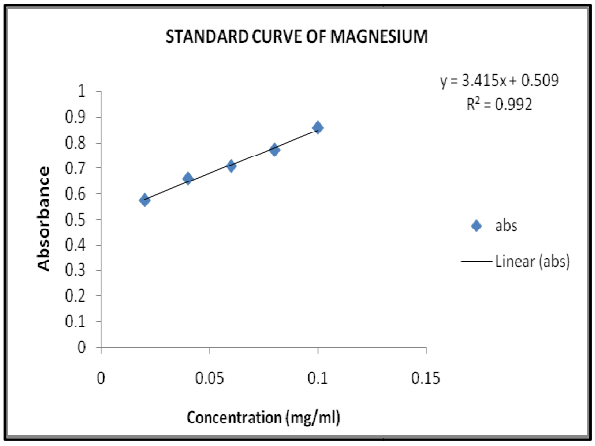


Fig. 6.7. Calibration curve of Magnesium

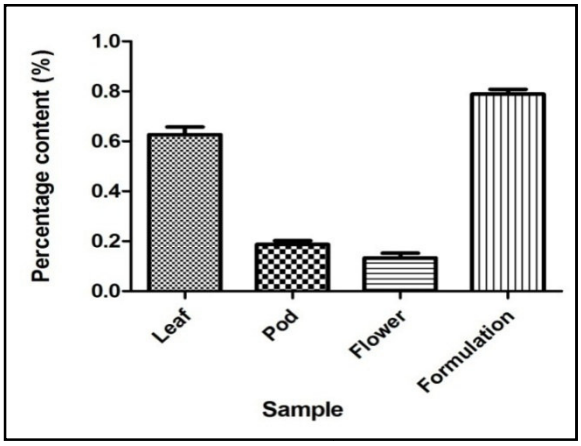


Fig. 6.8. Percentage content of Phosphorus in the experimental samples. Values as expressed as Mean ± SD (n = 3)

magnesium sulphate. Correlation coefficient was found to be 0.992. A good linearity was observed in the concentration range of 0.02-0.1 mg/ml Fig. 6.7. The phosphorus present in the test samples was also determined from the equation of the calibration curve (Table 6.5.). The data are also statistically evaluated by using prism 5.0 versions. The data are represented as Mean ± SD (n = 3) in Fig. 6.8.

Table 6.5.
Magnesium content *Moringa oleifera* test samples and formulation (n = 3).

Sample	% Content
Leaf	0.63 ± 0.03
Pod	0.197 ± 0.015
Flower	0.13 ± 0.018
Formulation	0.744 ± 0.019

Data expressed as Mean ± SD

6.3.1.6. Sodium, Potassium, and Calcium

Sodium controls blood pressure and blood volume in human body. They are needed for muscles and nerves to work properly. Sodium occurs naturally in most foods. The most common form of sodium is sodium chloride.

Potassium is an essential macro mineral and main intracellular ion for all types of cells. It is important in maintaining fluid and electrolyte balance in the bodies of humans and animals. Potassium ion is necessary for the function of all living cells, and is thus present in all plant and animal tissues. Potassium depletion in animals, including humans, results in various neurological dysfunctions.

Calcium is an important component of a healthy diet and for life. As a major mineral it is used in mineralization of bone, teeth and shells. Its supplement used to prevent and to treat calcium deficiencies

The estimation of sodium potassium and calcium in *Moringa oleifera* test samples and its formulation was carried out by SGS chemical, Gurgaon by the method of AOAC 2011.14.

6.3.1.6.1. Results and discussion

The results are given below in tabular form in Table 6.6. It was observed that the formulation contains highest concentration of sodium, potassium, and calcium. Whereas the flower contains

Table 6.6.
Mineral content in *Moringa oleifera* test samples and its formulation.

Minerals	Leaf	Pod	Flower	Formulation	Unit
Sodium	54.73	63.08	49.06	83.64	mg/100g
Potassium	2081.21	1577.22	1233.76	2243.92	mg/100g
Calcium	993.52	347.83	541.08	2187.97	mg/100g

lowest amount of sodium, and potassium compared to others. The calcium content is lowest in the pod.

6.3.2. *Estimation of Micronutrients*

6.3.2.1. Vitamin A

It is a fat soluble vitamin (Fig 6.9.) and essential nutrient needed in small amounts by humans for the normal functioning of the visual system; growth and developm ent; and maintenance of epithelial cellular integrity, immune function, and reproduction. These dietary needs for vitamin A are normally provided for as preformed retinol (mainly as retinyl ester) and provitamin A carotenoids. Vitamin A deficiency is not easily defined, WHO defines it as tissue concentrations of vitamin A low enough to have adverse health consequences even if there is no evidence of clinical xerophthalmia (WHO, 1996).

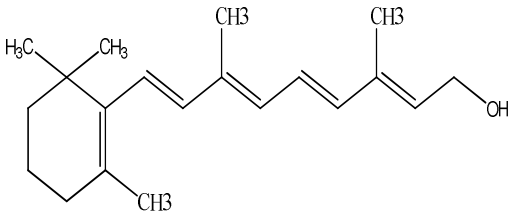


Fig. 6.9. Structure of Vitamin A

6.3.2.1.1. Chemicals and Equipments

Tri-chloro aceteic acid solution and chloroform was obtained from Merck (Mumbai, India). The standard vitamin A acetate was purchased from CDL (No.CDL/REF.STD.2013/402), SpectraMax M5 Multi-Mode Microplate Readers, 96 well micro test plate was purchased from Tarson Mumbai.

6.3.2.1.2 Procedure

The method was performed according to the method of Carr and Price, 1926. Serial dilutions of all-trans vitamin A acetate ranging from 2 to10 µg/ml were prepared from stock solution. To 1 ml of known concentration of vitamin A acetate in a 1 cm quartz cell, 1 ml of TCA in chloroform was added. A blue-colored complex was appeared. The test samples of M. *Moringa oleifera* were accurately weighed and dissolved in methanol to prepare a concentration of 10mg/ml and treated as same manner as the standard. Absorbance was

recorded at 620 nm using SpectraMax M5 Multi-Mode Microplate Readers. By using SoftMax® Pro softwar the results are integrated.

6.3.2.1.3 Results

The calibration curve of vitamin A acetate is given in Fig. 6.10. From the calibration curve the unknown concentration of vitamin A present in the test samples and the formulation was determined. The amounts of the unknown concentration of vitamin A present in the test

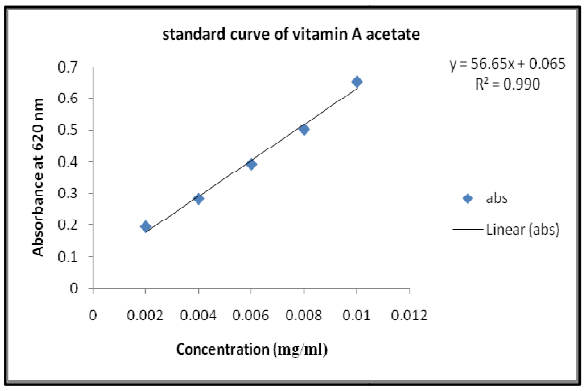


Fig.6.10. Calibration curve standard vitamin A

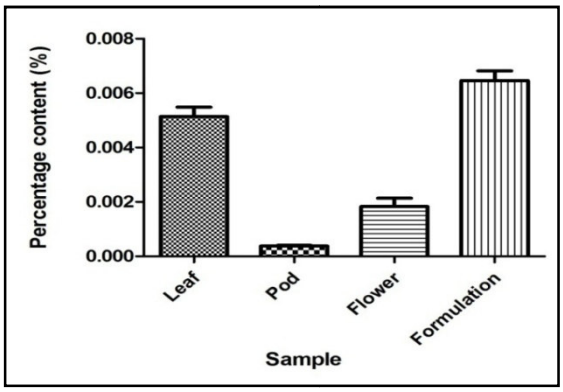


Fig.6.11. Percentage content of Vitamin A in the experimental samples. Values as expressed as Mean ± SD (n=3)

Table 6.7

Vitamin A content in *Moringa oleifera* test samples and its formulation (n=3).

Sample	% Content
Leaf	0.005 ± 0.00035
Pod	0.0003 ± 0.0001
Flower	0.0018 ± 0.00030
Formulation	0.0064 ± 0.00035

Values expressed as Mean ± SD.

samples has been statistically evaluated by using Graph pad prism version 5. The results are shown in Fig. 6.11. It was observed that the vitamin A content is highest in the formulation compared to other test samples (leaf, flower, & pod). The vitamin A content in is represented in Table 6.7.

6.3.3.2. Vitamin E

It is a group of eight lipid-soluble compounds synthesised by plants (Fig. 6.12). These compounds fall into two classes, tocopherols and tocotrienols, which exhibit the biological antioxidant activity of vitamin E. Vitamins in both classes, are designated by the Greek letters α, ß, and λ. The most biologically active antioxidant is d-α-tocopherol. All higher plants

appear to contain α-tocopherol in leaves and other green parts, while λ-tocopherol is generally present in lower concentration. A daily intakes of 4 mg and 3 mg of α-tocopherol equivalents could be adequate for men and women respectively.

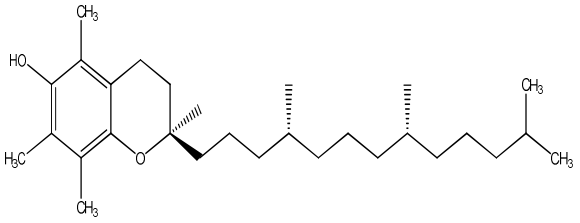


Fig. 6.12. Structure of Vitamin E

6.3.3.2.1. Method of estimation

The vitamin content in *Moringa oleifera* test samples and its formulation was determined by SGS chemical, Gurgaon by following the SGS SOP.

6.3.3.2.2. Results and discussion

The tocopherol content in the pod was found to be lower than other samples. The formulation contains 0.024% of tocopherol. The values are expressed as % content. The values are presented in Table 6.8.

Table 6. 8
Vitamin E content in *Moringa oleifera* test samples and its formulation (n=3).

Sample	% Content
Leaf	0.0096%
Flower	0.0051%
Pod	0.00109%
Formulation	0.0247%

6.3.3. 3. Vitamin C

Vitamin C or L-ascorbic acid, or simply ascorbate (the anion of ascorbic acid), is an essential nutrient for humans and certain other animal species. Vitamin C refers to ascorbic acid and its salts, and some oxidized forms of the molecule like dehydroascorbic acid. Ascorbate and ascorbic acid are both naturally present in the body. Ascorbate may also act as an antioxidant against oxidative stress (Padayatty et al., 2003). The Fig.6.13.represent the structure of vitamin C. Various methods has been reported for the estimation of ascorbic acid. The most widely used are the colorimetric or spectrophotometric methods depending on the reactions with 2, 6-dichloroindophenol (Pepkowitx, 1943), diazotized 4-methoxy-2-nitroaniline

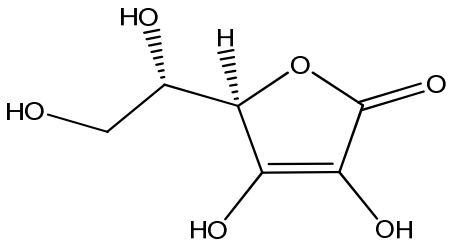


Fig.6.13. Structure of Ascorbic acid

(Schmall et al., 1953), 2, 4-dinitrophenylhydrazine (Roe and Kuether, 1943). Ascorbic acid has also been determined as its molybdophosphate complex. The procedure was done by according to the method of (Elnenasy and Soliman, 1979) where a reaction between ascorbic acid and the ammonium molybdate occurs to produce a blue molybdate complex.

6.3.3.3.1. Chemicals and equipment

Standard Ascorbic acid, Ammonium molybdate (10% aqueous), 10% w/v sulphuric acid; trichloroacetic acid (10%) was obtained from Merck (Mumbai, India), SpectraMax M5 Multi-Mode Microplate Readers, 96 well micro test plate was purchased from Tarson Mumbai.

6.3.3.3.2. Preparation of standard and sample solution

Stock standard ascorbic acid solution was freshly prepared in pure water at a concentration of 1mg/ml. working standard was prepared by diluting, the stock in the concentration range of 0.1-0.5 mg of ascorbic acid/ml. 10 mg of sample was transferred to a 10-ml volumetric flask, and 2 ml of 10% sulphuric acid and 4 ml of ammonium molybdate solution added; mix and leave for 1 hr. A blue colour will be appeared. Then it was dilute to the mark with water and mixed. The absorbance was measured at 730 nm colorimetrically by using SpectraMax M5 Multi-Mode Microplate Readers against a reagent blank prepared in the same manner but without the ascorbic acid. A calibration curve was constructed by using different concentrations (2-10 µg/ml) of ascorbic acid.

6.3.3.3.3. Results & discussion

The estimation was performed according to the method of (Elnenasy and Soliman, 1979). The results are integrated by Software : SoftMax®. Pro Calibration curve prepared with

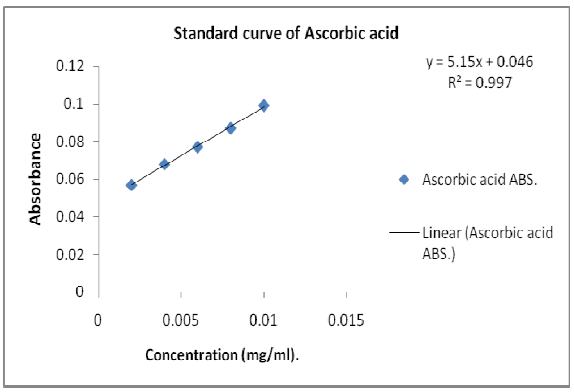


Fig. 6.14. Calibration curve of Ascorbic acid

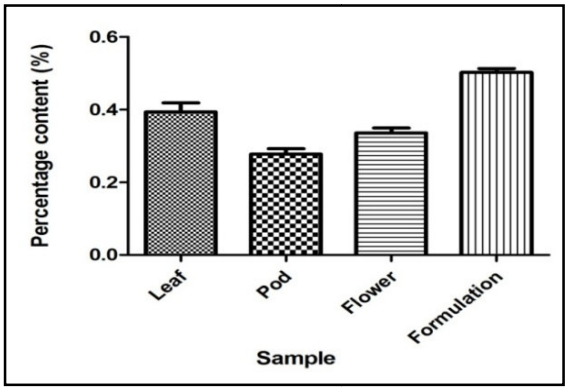


Fig. 6.15. Percentage content of Ascorbic acid in the experimental samples. Values as expressed as Mean ± SD (n=3)

standard ascorbic acid. Correlation coefficient was found to be >0.99. A good linearity was observed in the concentration range of 2-10 µg/ml Fig. 6.14. The ascorbic acid present in the

test samples was also determined from the equation of the calibration curve Y= 5.15x+0.046 (Table 6.9.). The data are also statistically evaluated by using Graph pad prism 5.0 versions. The data are represented as Mean ± SD (n=3) in Figure 6.15.

Table 6.9
Vitamin C content in *Moringa oleifera* test samples and its formulation (n=3).

Sample	% w/w
Leaf	0.3933 ± 0.02
Pod	0.2773 ± 0.01
Flower	0.3360 ± 0.01
Formulation	0.5023 ± 0.01

Data expressed as Mean ± SD.

6.3.3.4. Vitamin B_1

It is the "sulfur-containing vitamin" water-soluble vitamin of the B complex. If not present in the diet produces detrimental neurological effects. Its phosphate derivatives are involved in many cellular processes. The best-characterized form is thiamine pyrophosphate (TPP), a coenzyme in the catabolism of sugars and amino acids. All living organisms use thiamine, but it is synthesized only in bacteria, fungi, and plants. Animals must obtain it from their diet, and thus, for them, it is an essential nutrient (Fig. 6.16.).

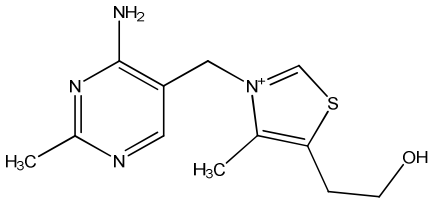


Fig. 6.16. Structure of Vitamin B_1

6.3.3.4.1. Procedure

The vitamin B_1 content in the experimental samples was determined by SGS chemical, Gurgaon by following the SGS SOP.

6.3.3.4.2. Result

The thiamine content in the experimental formulation was found to be 3.12mg/100 gram. The lowest value 1.02 mg/100g was found in the flower of *Moringa oleifera*. The data are represented a tabular form in table no 6.10. as % content value.

Table 6.10
Vitamin B_1 content *Moringa oleifera* test samples and its formulation (n=3).

Sample	% w/w
Leaf	0.001%
Flower	0.0008%
Pod	0.0011%
Formulation	0.0031%

Data expressed as Mean ± SD.

6.3.3.5. Vitamin B_2

Vitamin B_2 is a yellow to orange-yellow crystalline compound. It is also known as Riboflavin (Fig. 6.17). It is an easily absorbed colored micronutrient with a key role in maintaining health in humans and animals. It is the central component of the cofactors FAD and FMN, and is therefore required by all flavoproteins. The estimation of this vitamin in the test samples was performed according to the method of Shah et al., 2012.

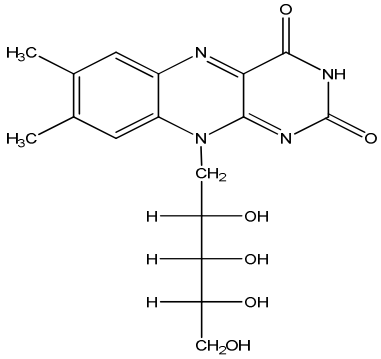


Fig. 6.17. Structure of Vitamin B2

6.3.3.5.1. Reagents and equipments

Standard Riboflavin purchased from CDL (No.CDL/REF.STD.2013/402), sodium hydroxide obtained from Merck Mumbai, SpectraMax M5 Multi-Mode Microplate Readers, 96 well micro test plate.

6.3.3.5.2. Preparation of Stock Solution

Standard stock solution of Riboflavin was prepared by dissolving 100mg of Riboflavin in 100ml of 0.1N NaOH to prepare 1000 µg/ml concentration. The 1000ppm solution was further diluted with 0.1N NaOH to prepare 100 ppm concentration.

6.3.3.5.3. Preparation of Calibration Curve

For preparing the calibration curve the 100 ppm stock solution was diluted in different concentration in a working range of 5-25 µg/ml. Calibration curve was constructed using 0.1 N NaOH as blank. The absorbance was recorded at 415 nm using SpectraMax M5 Multi-Mode Microplate Readers.

6.3.3.5.4. Preparation of sample solution

1mg of each sample was dissolved in 0.1 N NaOH to produce 1mg/ml solution. The amounts of riboflavin present in the samples were determined from the calibration curve.

6.3.3.5.5. Results & discussion

The estimation was performed according to the method of (Shah et al., 2012.). The results are integrated by Software : SoftMax®. Pro. Calibration curve prepared with standard riboflavin. Correlation coefficient was found to be >0.99. A good linearity was observed in the concentration range of 5-25 µg/ml Fig. 6.18. The riboflavin present in the test samples was also determined from the equation of the calibration curve Y= 24.84x+0.02 (Table 6.11). The data are also statistically evaluated by using prism 5.0 versions. The data are represented as Mean ± SD (n=3) in Fig. 6.19.

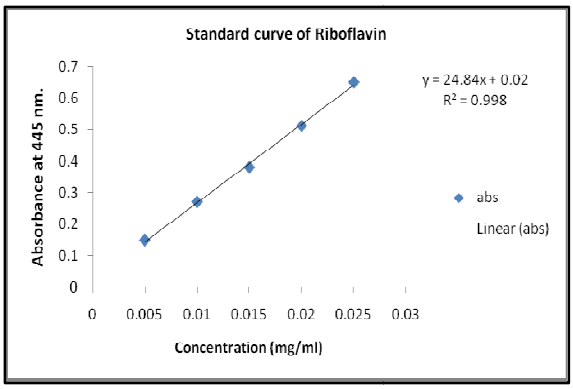


Fig. 6.18. Calibration curve of Vitamin B_2

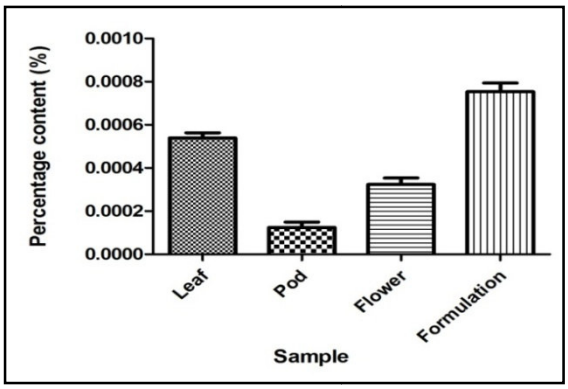


Fig. 6.19. Percentage content of Riboflavin in the experimental samples. Values as expressed as Mean ± SD (n=3)

Table 6.11

Vitamin B_2 content *Moringa oleifera* test samples and its formulation (n=3). Data are expressed as Mean ± SD

Sample	% Content
Leaf	0.0005383 ± 0.0002
Pod	0.000125 ± 0.0001
Flower	0.000323 ± 0.0001
Formulation	0.000753 ± 0.0001

6.3.3.6. Vitamin B_6

Vitamin B_6 is one of the compounds that can be called as Pyridoxine. Pyridoxine assists in the balancing of sodium and potassium as well as promoting red blood cell production. It is linked to cardiovascular health by decreasing the formation of homocysteine. Pyridoxine may help balance hormonal changes in women and aid the immune system (Kashanian et al., 2007). The Fig. 6.20 represents the structure of Vitamin B_6.

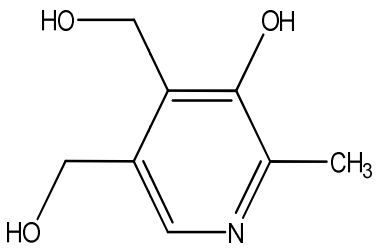


Fig. 6.20. Structure of Vitamin B6

6.3.3.6.1. Chemicals and equipments

Pyridoxine Hydrochloride was obtained from CDL (No.CDL/REF.STD.2013/402), hydrochloric acid was obtained from Merck Mumbai; SpectraMax M5 Multi-Mode Microplate Readers, 96 well micro test plate was purchased from Tarson Mumbai.

6.3.3.6.2. Preparation of Standard Stock Solution and sample solution

10.0 mg of Pyridoxine hydrochloride was accurately weighed and transferred to 100 ml volumetric flask. Add 40 ml of distilled water to dissolve and volume was made up-to 100 ml with distilled water to obtain stock solution of drug concentration of 100µg/ml. The samples were accurately weighed 100 mg and dissolved in 10 ml water to produce 10 mg/ml of solution.

6.3.3.6.3. Preparation of calibration curve for Pyridoxine hydrochloride

From standard stock solution of Pyridoxine hydrochloride 2, 4, 6, 8, 10 µg/ml solutions were prepared by using 0.1N hydrochloric acid. In the similar way the samples are also prepared 1mg/ml using 0.1(N) Hydrochloric acid. The absorbance was measured at 302 nm using. SpectraMax M5 Multi-Mode Microplate Readers. Calibration curve was constructed from absorbance measure at 302 nm against 0.1N hydrochloric acid as blank.

6.3.3.6.4. Results and discussion

The estimation was performed according to the method of (Gowekar et al. 2012). Calibration curve prepared with standard pyridoxine hydrochloride. Correlation coefficient was found to

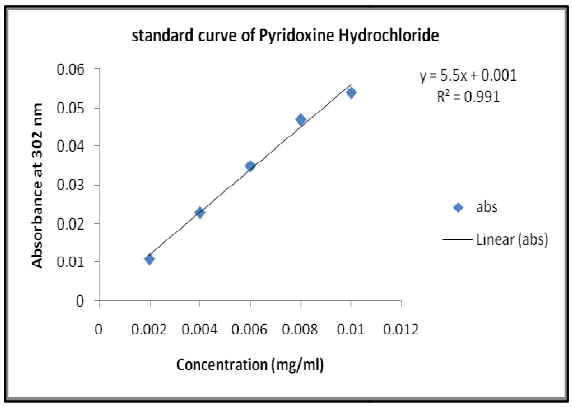


Fig. 6.21. Calibration curve of Vitamin B_6

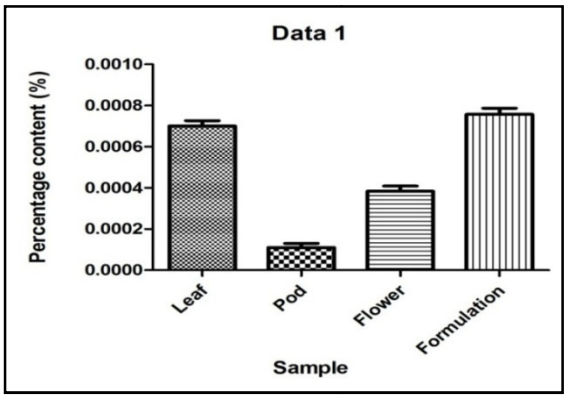


Fig. 6.22. Percentage content of pyridoxine in experimental samples. Values are expressed as Mean ± SD (n=3)

Table 6.12.
Vitamin B_6 content *Moringa oleifera* test samples and its formulation (n=3).

Sample	% Content
Leaf	0.0007±0.003
Pod	0.00011±0.005
Flower	0.0003833±0.007
Formulation	0.00076±0.004

Data expressed as Mean ± SD.

be >0.99. A good linearity was observed in the concentration range of 2-10 µg/ml Fig. 6.21. The pyridoxine content present in the test samples was determined from the equation of the calibration curve Y= 5.5x+0.001 (Table 6.12.). The data are also statistically evaluated by using Graph pad prism 5.0 Versions. The data are represented as Mean ± SD (n=3) Fig. 6.22.

6.3.3.7. Simultaneous estimation of vitamin Folic acid (B_9) and Niacin (B_3) by RP- HPLC method

Niacin is also known as vitamin B_3, nicotinamide and vitamin PP (Fig. 6.23.). Niacin is one of five vitamins (when lacking in human diet) associated with a pandemic deficiency disease: niacin deficiency (pellagra). Niacin or nicotinamide is a component of cozymase, and possesses a curative effect on pellagra. It has been used for over 50 years to increase HDL levels in the blood and has been found to modestly decrease the risk of cardiovascular disease in a number of controlled human trials (Bruckert et al., 2010).

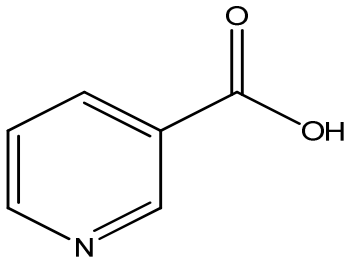


Fig. 6.23. Structure of Vitamin B_3

Folic acid is also known as folate, vitamin M or vitamin B_9 (Fig. 6.24.). Itself it is not biologically active, but its biological importance is due to tetrahydrofolate and other derivatives after its conversion to dihydrofolic acid in the liver (Bailey and Ayling, 2009).

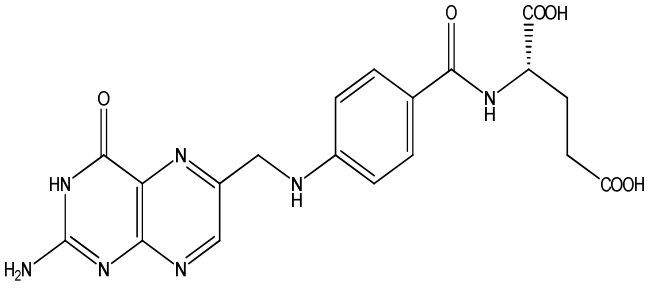


Fig.6.24. Structure of Vitamin B_9

Folic acid and folate is essential for numerous bodily functions. Humans cannot synthesize folate de novo; therefore, folate has to be supplied through the diet to meet their daily requirements. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA as well as to act as a cofactor in certain biological reactions (Weinstein et al., 2003). It is especially important in aiding rapid cell division and growth, such as in infancy and pregnancy. Children and adults both require folic acid to produce healthy red blood cells and prevent anaemia (Carmel, 2005).

Different methods of simultaneous estimation of vitamins have been found through literature survey. A simultaneous method was developed according to the method of Perveen et al., 2009. The method validation was performed. The estimation method and method validations are given below.

6.3.3.7.1. Chemicals and equipments

Methanol of HPLC grade was procured from Merck (Mumbai, India). All other solvents used were of analytical grade, procured from Merck. Vitamin B_9 standard (Folic acid) and Vitamin B_3 (Niacin) standard were purchased from Central Drug Laboratory (Kolkata). Membrane filters of 0.45 mm pore size (Millipore) were used for filtration of the mobile phase and Whatman's syringe filters (NYL 0.45 mm) were used for the filtration of samples.

The HPLC system (Waters, Milford, MA, USA) used for the analysis consisted of a 600 controller pump, a dual-wavelength ultraviolet–visible (UV-Vis) detector equipped with an on-line degasser AF 2489 and a rheodyne 7725i injector with a 20 µL loop. Separation was achieved using Waters Spherisorb ODS2, 5 µm, 250 × 4.6mm (Irelend) columns. Quantitative estimation was performed with Empower2 software program using the external calibration method. A Milli-Q Academic water purification system (Bedford, MA, USA) equipped with 0.22 mm Millipak Express filter and Eyela (Tokyo, Japan) rotary vacuum evaporate were used.

6.3.3.7.2. Preparation of standard and sample solution

An accurately weighed 1mg of vitamin B_9 and B_3 standard were dissolved in 1ml of HPLC grade methanol respectively to obtain a standard stock solution of 1mg/ml. The vitamin B_9 standard was then subsequently diluted with methanol to prepare the standard solution of 1, 5, 10, 20, 40, 60, 80, 100, and 150µg/ml. Similarly the vitamin B_3 standard was also diluted with methanol to prepare the standard solution of 1, 10, 20, 40, 60, 80 µg/ml. An accurately weighed 100mg of test samples were dissolved in methanol to prepare the sample solution of 1mg/ml concentration. Then the sample and the standard solutions were sonicated and passed through 0.45 µm syringe filter prior to inject into the HPLC column.

6.3.3.7.3. Chromatographic condition

HPLC assays were performed using isocratic conditions by the external standard method. Mobile phase composition was optimized to (water: methanol:: 85:15 v/v) which was degassed and filtered through membrane prior to run in the column. The pH of the mobile phase was optimized to 3.8. Temperature of the column was kept at 25°C, and each injection volume was 20 µL. Flow rate was set at 1.0 mL/min. The quantitative estimation of each vitamin compounds present in the plant was determined using a calibration curve of standard folic acid (B_9) and niacin (B_3). Detection of the compounds was performed at 254 nm. Peak identification was achieved by comparison of the retention time of individual standards with the sample.

6.3.3.7.4. Preparation of calibration curve

The linear calibration plot of the prepared standards was constructed by Means of linear regression analysis between peak areas and concentration. Calibration curve established with dilutions of each vitamin standard, at concentrations ranging from 1 to 150µg/ml for Folic acid and 1-80 µg/ml for Niacin respectively. A calibration curve was drawn using the different concentration range for both vitamins (Fig. 6.25 A-B). Peak areas of the obtained from the chromatography were plotted against the concentration of the .

6.3.3.7.5. Method validation

Validation of the HPLC method was done as recommended by the International Conference on Harmonization (ICH) guidelines (ICH, 1996, 2005), defining the linearity, specificity, peak purity, limits of quantification and detection, precision, accuracy and robustness.

6.3.3.7.6. Specificity

The results of the test samples were checked in terms of specificity according to the ICH recommendations to minimize errors due to the contamination of the sample. The specificity of the method was determined by analyzing the standards and test samples. The purity of the peaks was checked using multivariate analysis by comparison of retention times and peak areas of standard compounds and .

6.3.3.7.7. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were calculated based on the ICH guidelines by determining the SD of the response and the slope of the linear equation. The following equations were used to calculate the LOD and LOQ (ICH, 1996, 2005):

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

Where σ = standard deviation of the response from a number of blank run and S = slope of the calibration plot.

6.3.3.7.8. Accuracy

Accuracy of the method was determined by percentage recovery of the markers in the plant . The method was studied by performing standard addition technique and is expressed in terms of percentage relative standard deviations (%RSD) from Mean recovery of the theoretical concentration. Prior to the injection, the tests were spiked with three different known amounts of standard compounds in triplicates. Analyses were done under the set ambient conditions to calculate the overall average recovery. The Mean amounts of the markers achieved were taken as 'real values' to calculate the spike recoveries.

6.3.3.7.9. Precision

The precision of the method was assessed by injecting six replicates at three different concentrations for both of the reference compounds and the values were represented as %RSD of intraday and inter-day runs. The Mean amount and RSD values were calculated.

The intra-day precision of the assay was determined by analyzing three concentrations in 1 day. Also, the inter-day precision was resolute over three successive days by analyzing the same concentrations. Injections were done in six replicates to determine the repeatability of the process.

6.3.3.7.10. Robustness

The robustness of the proposed method was investigated by an analysis of samples under different experimental conditions. Test solutions were analyzed with the variation of flow rate, mobile phase composition, detection wavelength and column temperature and using different columns of same configuration to determine their influence effect on the retention time.

6.3.3.7.11. Statistical analysis

Statistical analysis was performed using the Graph Pad Prism Version 5.0. The results are represented as the Mean ± SD.

6.3.3.7.12. Results & discussion

The content of vitamin B_9 and B_3 in *Moringa oleifera* and formulation was determined using calibration curve plotted between Mean peak area (Y- axis) and concentration (X- axis) (Fig. 6.25. A-B).

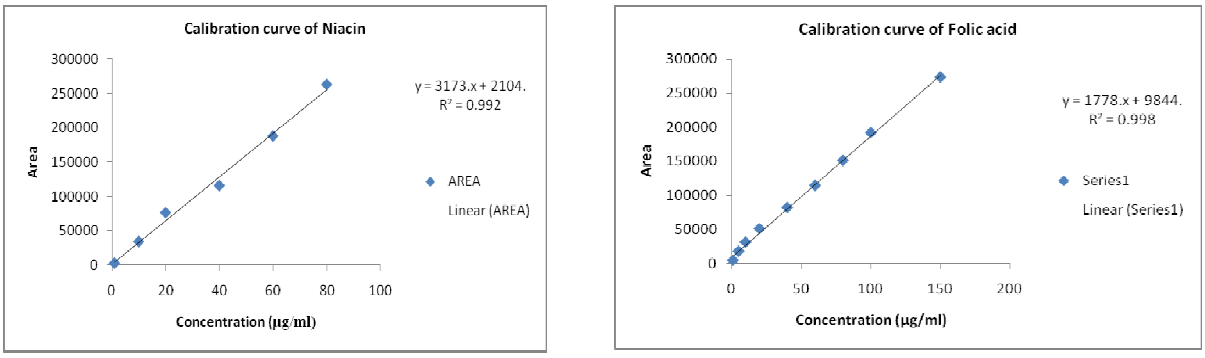


Fig. 6.25. (A-B) The Calibration Curve of Folic Acid and Niacin respectively

The chromatograms of each sample are presented in Fig. 6.26. (A-E) Linearity was evaluated by regression analysis using different concentration of the standard B_9 (1-150 µg/ml) and standard B_3 (1-80 µg/ml). The coefficient of determinants (r^2) was greater than 0.99, which represents that the data is closest to the line of best fit. Chromatograms were found to be directly proportional to concentrations of the calibration solutions. Retention time of the

standard vitamin B_9 and B_3 was found to be 2.82 and 7.35 mints respectively (Fig.6.26. A.). The vitamin content in each sample was calculated from the areas of each of individual from their respective chromatogram. The content of vitamin B_9 was found to be 0.074 % w/w, 0.097 % w/w, and 0.118 w/w % in pod, flower and formulation respectively. The content of vitamin B_3 was found to be 0.0163% w/w, 0. 0354% w/w and 0.0379% w/w in pod, flower and formulation respectively. The B_9 content in the leaf was detected but cannot be quantified from the calibration curve and the vitamin B_3 content was not found in leaf. From the RP-HPLC data it was observed that the formulation contains highest amount of vitamin B_3 and vitamin B_9.

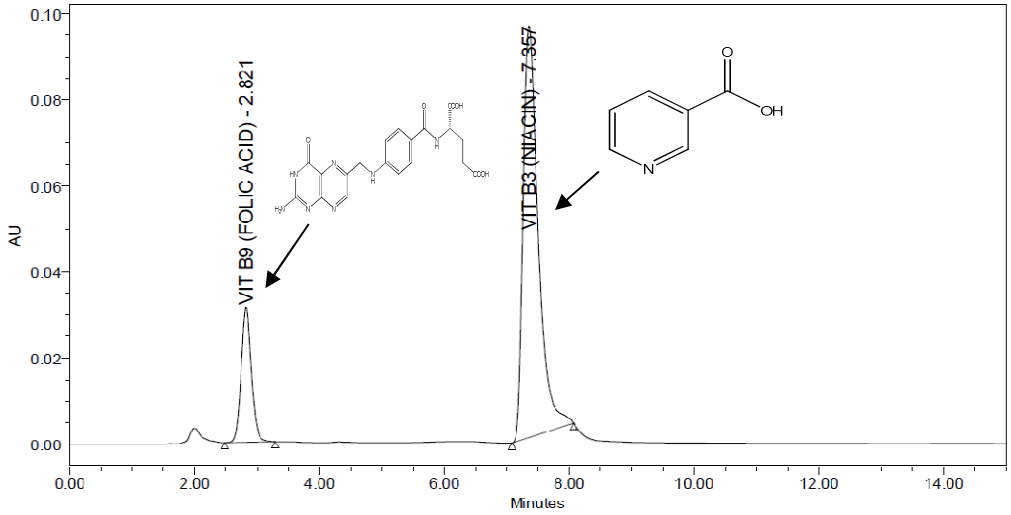


Fig. 6.26. A.

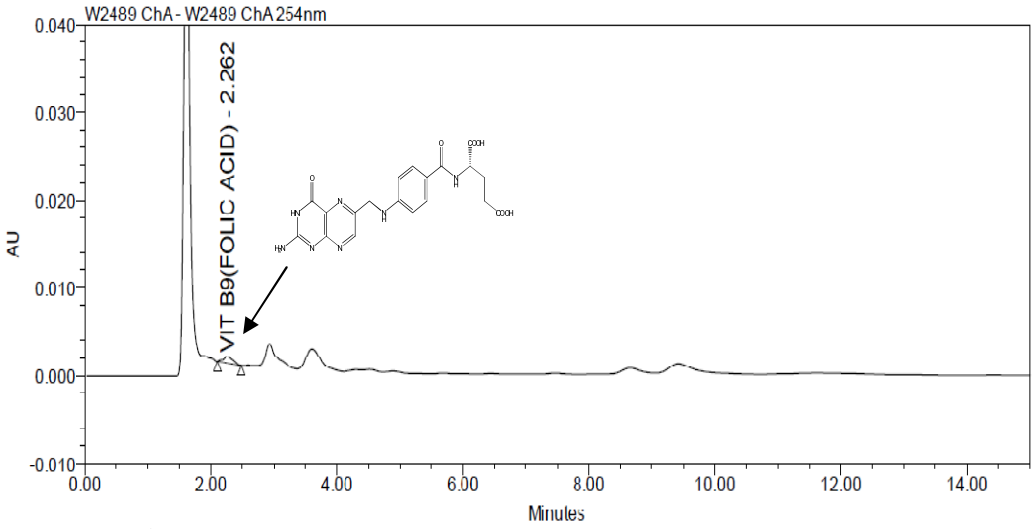


Fig. 6.26. B

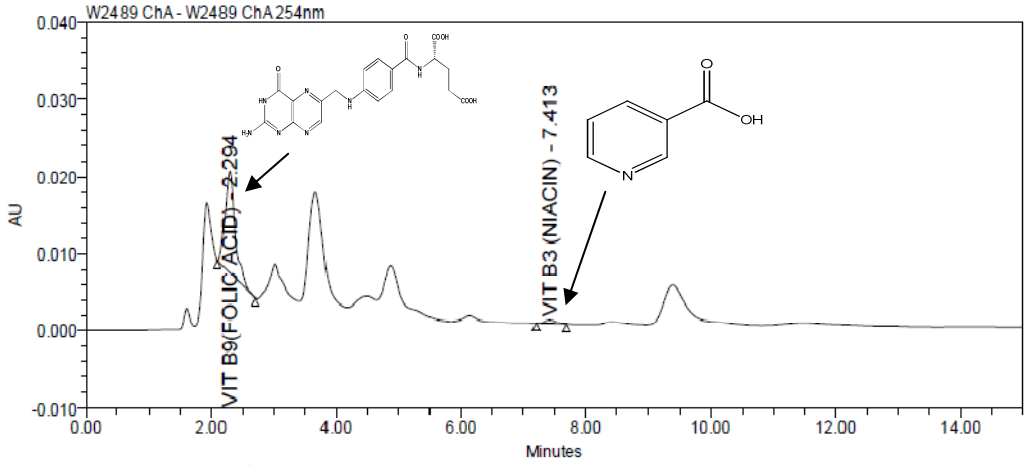


Fig. 6.26. C

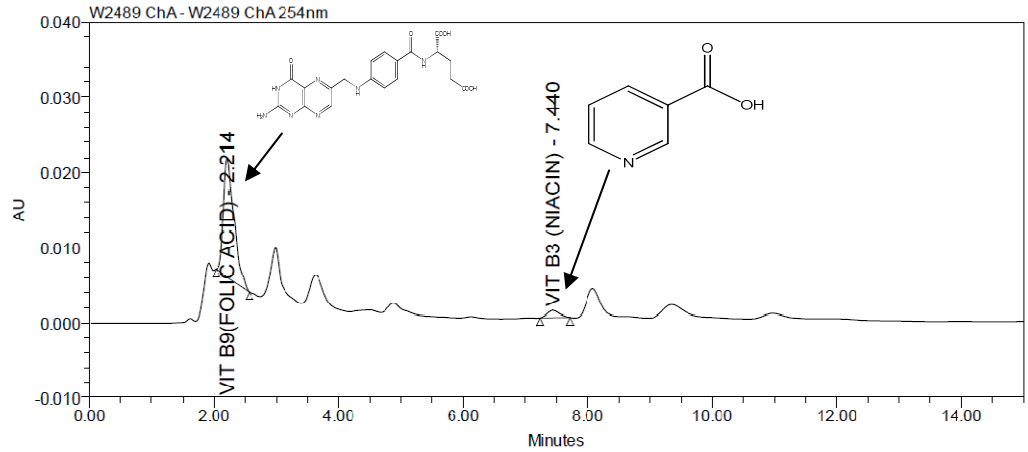


Fig. 6.26. D

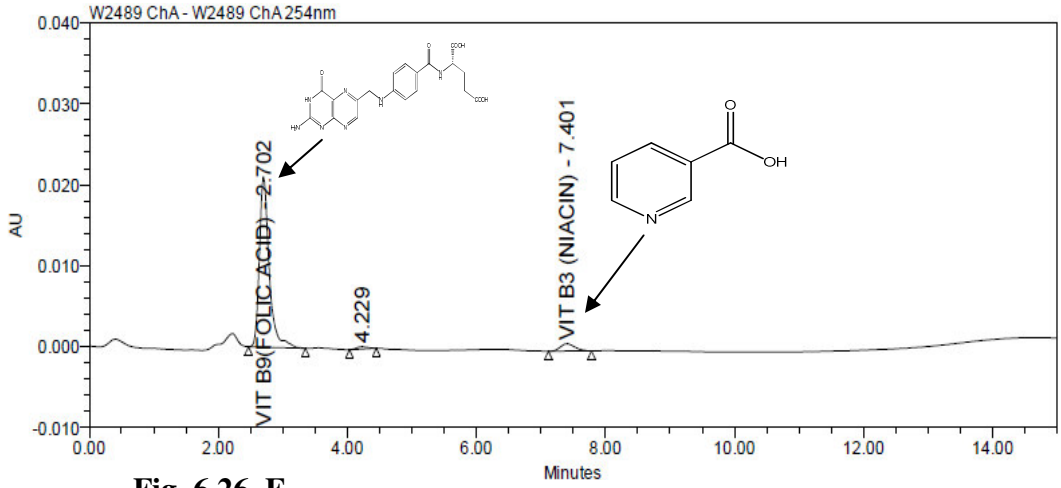


Fig. 6.26. E

Fig. 6.26. (A) RP-HPLC chromatogram of Standard Folic acid (B_9) and Standard Niacin. (B_3). (B) RP-HPLC chromatogram of *Moringa oleifera* leaf. (C) RP-HPLC chromatogram of *M. Moringa oleifera* pod. (D) RP-HPLC chromatogram of *M. Moringa oleifera* flower. (E) RP-HPLC chromatogram of *M. Moringa oleifera* formulation.

Table 6.13
Limit of detection (LOD) and limit of quantification (LOQ) of Folic acid (B_9) and Niacin (B_3)

Vitamins	Linearity range (µg/ml)	LOD	LOQ	Correlation coefficient	Regression equation
Folic acid (B9)	10-150	1.98	5.29	>0.99	Y = 1778x + 3844
Niacin (B3)	10-80	1.57	4.76	>0.99	Y = 3173x + 2104

Table 6.14
Recovery study of vitamins folic acid (B_9) and Niacin (B_3) from *M. oleifera* flower extract

Vitamin B_9						Vitamin B_3					
Amount of sample (µg)	Excess vitamin B9 added (µg)	Expected amount (µg)	Amount found (µg)	Percentage Recovery (%)	Mean % recovery	Amount of sample (µg)	Excess vitamin B3 added (µg)	Expected amount (µg)	Amount found (µg)	Percentage Recovery (%)	Mean percentage Recovery (%)
97.85	10	107.85	106.28	98.54	98.67	3.54	10	13.54	12.91	95.34	96.7
	40	137.85	135.91	98.59			40	43.54	41.77	95.79	
	80	177.85	175.79	98.84			80	83.54	82.58	98.85	

Table 6. 15
Recovery study of vitamins folic acid (B_9) and Niacin (B_3) from *M. oleifera* pod extract

Vitamin B_9						Vitamin B_3					
Amount of sample (µg)	Excess vitamin B9 added (µg)	Expected amount (µg)	Amount found (µg)	Percentage Recovery (%)	Mean % recovery	Amount of sample (µg)	Excess vitamin B3 added (µg)	Expected amount (µg)	Amount found (µg)	Percentage Recovery (%)	Mean %recovery
73.90	10	83.90	81.26	96.85	97.18	1.63	10	11.63	10.89	93.63	95.49
	40	113.90	110.91	97.37			40	41.63	39.92	95.89	
	80	153.90	149.78	97.32			80	81.63	79.13	96.94	

Table 6.16
Recovery study of vitamins folic acid (B_9) and Niacin (B_3) from *M. oleifera* formulation

M. Oleifera formulation											
Vitamin B_9						Vitamin B_3					
Amount of sample (µg)	Excess vitamin B9 added (µg)	Expected amount (µg)	Amount found (µg)	Percentage Recovery (%)	Mean percentage recovery (%)	Amount of sample (µg)	Excess vitamin B3 added (µg)	Expected amount (µg)	Amount found (µg)	Percentage Recovery (%)	Mean percentage recovery (%)
118.93	10	128.93	125.79	97.56	98.09	3.79	10	13.79	12.99	94.20	95.25
93	40	158.93	156.21	98.29			40	43.79	41.56	94.91	
	80	198.93	195.78	98.42			80	83.79	80.97	96.63	

Table 6.17
Intra-day precision and Inter-day precision of vitamin B_9 (Folic acid) and vitamin B_3 (Niacin)

Vitamin B_9								Vitamin B_3							
Intra-day precision (n=6)				Inter-day precision (n=6)				Intra-day precision (n=6)				Inter-day precision (n=6)			
Retention time		Response		Retention time		Response		Retention time		Response		Retention time		Response	
Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD	Mean	% RSD
2.82	0.56	31211.17	0.67	2.89	0.43	33195.45	0.87	7.34	0.81	35154.5	0.87	7.47	0.92	36271.23	0.91
2.85	0.72	82051.33	0.65	2.91	0.51	84101.61	0.69	7.39	0.73	116826.5	0.88	7.52	0.89	131231.41	0.91
2.81	0.88	283019	0.70	2.87	0.59	291709.91	0.81	7.31	0.89	260114.2	0.75	7.49	0.77	278110.09	0.86

This describes a simple, rapid, economic and accurate quantitative simultaneous estimation of water soluble vitamins folic acid and niacin in neutraceutical products by RP-HPLC. A validation of this method was carried out and showed that specificity, robustness and precision are guaranteed.

6.3.3.8. Zinc

Zinc is an essential micro mineral for health maintenance. Zinc deficiency causes growth retardation in children, delayed sexual maturation, infection susceptibility, and diarrhea. Consumption of excess zinc can cause ataxia, lethargy and copper deficiency.

In this Micronutrient estimation the Zinc has been quantified by Colorimetric procedure which has been described here. The other micro nutrients minerals like iron, Manganese, and copper has been analysed by atomic absorption spectrophotometric method described in chapter no. 7.

6.3.3.8.1. Reagents and equipments required

Hydrochloric acid, tri-chloroacetic acid solution, alkaline ammonium citrate, Dithiozone, Chloroform was purchased from Merck Mumbai, Spectra Max M5 Multi-Mode Microplate Readers, double distilled water; Micro Test Plate was purchased from Tarson Mumbai.

6.3.3.8.2. Preparation of standard solution

0.440 g of zinc sulphate was dissolved in water containing 1 ml of 5M acetic acid and sufficient water was added to produce 100.0 ml (100 ppm). 1 volume of zinc standard solution (100 ppm Zn) was diluted to 10 volumes with water immediately before use. The final concentration was 10 ppm zinc.

6.3.3.8.3. Procedure

1 to 5 ml of the test sample solution was Pipetted into a centrifuge tube. 5 ml of trichloroacetic acid solution and sufficient water was added to produce 40.0 ml. Mix. It was centrifuged for few minutes. A few volume of the supernatant liquid was transferred to a separating funnel and water was added to produce 20 ml. 1.5 ml of alkaline ammonium citrate solution and 35 ml of dithizone standard solution was then added. Shake vigorously several times and the chloroform layer was allowed to separate. The absorbance of the chloroform layer was measured at 530 nm using a blank sample obtained by repeating the determination omitting the preparation under examination. The calibration curve was constructed by using 1-5 ppm zinc from the stock solution (10 ppm Zn) and repeating the determination. The content of zinc in the test samples was determined.

6.3.3.8.4. Results & discussion

The estimation was performed according to the method of (IP, 2007). The results are integrated by the Software: SoftMax®. Pro. Calibration curve prepared with standard Zinc sulphate. Correlation coefficient was found to be 0.994. A good linearity was observed in the concentration range of 1-5 ppm zinc Fig. 6.27. The zinc present in the test samples was

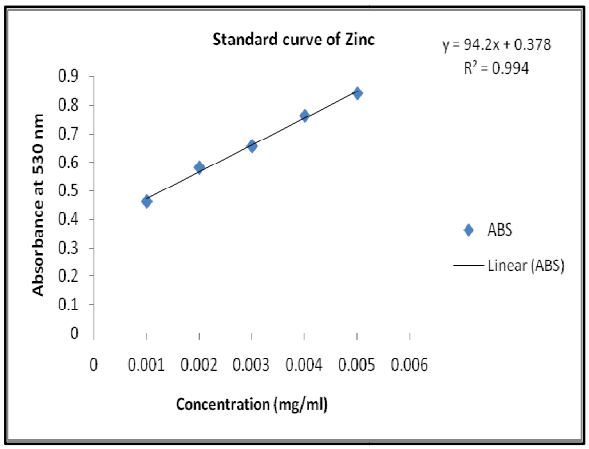


Fig. 6.27. Calibration curve of zinc

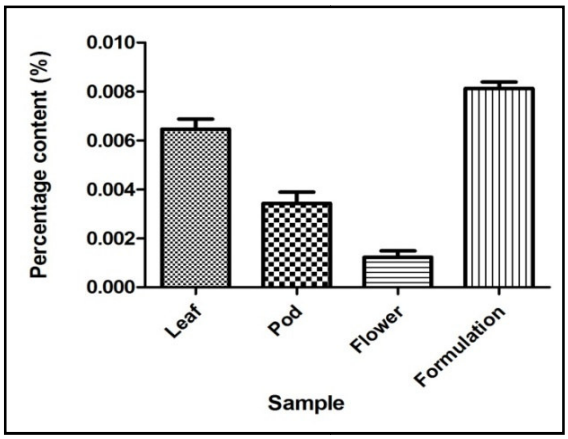


Fig. 6.28. Percentage content of zinc in the experimental samples. Values are expressed as Mean ± SD (n=3)

determined from the equation of the calibration curve (Table 6.18.). The data are also statistically evaluated by using prism 5.0 versions. The data are represented as Mean ± SD of triplicate in Fig. 6. 28.

Table 6.18

Percentage content of zinc *Moringa oleifera* test samples and its formulation (n=3).

Sample	% Content
Leaf	0.0064 ± 0.0004
Pod	0.0034 ± 0.00045
Flower	0.0012 ± 0.00025
Formulation	0.0081 ± 0.0002

Values expressed as Mean ± SD

6.4. A brief note about food value of *Moringa oleifera*

Moringa oleifera is a potent super food with a uniquely balanced combination of vitamins, minerals, complete protein, and powerful antioxidants. According to some studies that have been conducted on the nutritional content of *Moringa oleifera*, it is the most nutritious plant. It is called as the miracle tree. It provides an outstanding source of nutrition. The nutritional profile of its different parts and its prepared formulation has been studied in this experiment.

Table 6.19.

Nutritional profile of *Moringa oleifera* leaf, pod, flower and its formulation (% w/w)

Nutritional profile	Leaf	Pod	Flower	Formulation
Energy (kcal)	71.01	66.03	51.07	103.99
Protein	9.643±0.28	6.315±0.35	2.420±0.37	12.26±0.45
Crude Fat	1.17	0.71	1.51	2.11
Carbohydrate	5.527±0.28	8.617±0.34	6.953±0.18	8.997±0.19
Total Dietary fibre	32	69.24	25.68	48.44
Sodium	0.054	0.063	0.0490	0.0836
Potassium	2.08	1.57	1.23	2.24
Calcium	0.993	0.347	0.541	2.187
Magnesium	0.63±0.03	0.197±0.015	0.13±0.018	0.744±0.019
Phosphorus	0.527±0.02	0.72±0.01	0.627±0.02	0.744±0.02
Zinc	0.0064±0.0004	0.0034±0.00045	0.0012±0.00025	0.0081±0.0002
Copper	0.119±0.41	0.30±0.39	0.10±0.61	0.341±0.52
Iron	0.761±0.12	0.52±0.33	0.50±0.43	0.77±0.51
Manganese	0.57±0.71	0.49±0.69	0.50±0.32	0.61±0.5
Vitamin A	0.005±0.00035	0.0003±0.00001	0.0018±0.00030	0.0064±0.00035
Vitamin E	0.0096	0.0010	0.0051	0.0247
Vitamin C	0.3933±0.02	0.2773±0.01	0.3360±0.01	0.5023±0.01
Vitamin B_1	0.001	0.0011	0.0008	0.0031
Vitamin B_2	0.0005383±0.0002	0.000125 ± 0.0001	0.000323±0.0001	0.000753±0.0001
Vitamin B_3	ND	0.016	0.035	0.037
Vitamin B_6	0.0007±0.003	0.00011±0.005	0.0003833±0.007	0.00076±0.004
Vitamin B_9	ND	0.074	0.097	0.118

ND= Not detected.

It was observed that the prepared dietary formulation contains a larger amount of macro and micronutrients compared to its dietary ingredients. The data's obtained were statistically evaluated. The heavy metal content was also analysed by atomic absorption spectrophotometry (AAS), because heavy metals are a potential source of contamination in herbs. The heavy metal content has been given in chapter 7. Iron, copper and manganese were also estimated by AAS. The food value is depicted in Table 6.19. In addition to

vitamins and minerals, this plant is known to provide every essential amino acid required to synthesize protein. The human body requires several specific amino acids, called essential amino acids, to form other non-essential amino acids, which together create protein, the substance that powers our bodies and enables us to stay healthy and strong. Plant foods do not generally provide every essential amino acid, and so a plant-based diet must incorporate several different plants, each with different essential amino acids, in order to create a complete protein source. There are, however, a few exceptions like *Moringa oleifera* which provide every essential amino acid and give the body everything to synthesize protein. Hence the excellent macro and micro nutrient source statistically verified through this study is exploring its importance as a neutraceutical.

Chapter – 6

Nutritional evaluation of dietary supplement from *Moringa oleifera*

6.1. Introduction

6.2. Preparation of dietary supplement

6.3. Evaluation of nutritional value

6.4. A brief note about food value of *Moringa oleifera*

7.1. Introduction

Heavy metals are one of the main sources of pollution in the environment. Metals are classified into two categories essential metals (Fe, Cu and Zn) and toxic metals (Pb and Cd). It has been observed that many therapeutic effects of medicinal herbs used in the phytotherapy are due to the presence of very minute quantities of trace elements. These elements are iron (Fe), copper (Cu), cobalt, nickel (Ni), zinc (Zn), magnesium, manganese (Mn), molybdenum, chromium (Cr), vanadium, lithium, selenium, fluorine (F) and iodine (I) (Shirin et al., 2010). Plants readily assimilate such elements through roots, which are dissolved in water and remains in ionic forms. Some metal elements are essential for consumers in trace amounts. For example iron (Fe) is responsible for blood formation, myoglobin, and formation of many of enzymes involved in RBC formation. Non essential metals like (Pb), cadmium (Cd) and mercury (Hg) are toxic even in a very low concentrations for human health and environment (LLobet et al., 2003). Essential metals also produce harmful effect when they are taken in high concentration. For example cadmium is absorbed by many plants but it presents a major problem for food stuffs. It has adverse effects on brain, metabolism and also has severe effects such as prostate cancer, kidney, liver, lungs, bone damage. Excess iron concentration causes vomiting, diarrhoea, and intestinal damage. A high concentration of copper causes liver damage. Zn reduces the immune function and levels of high-density lipoproteins (Food and Drug Administration, 2001). Hg causes neurological disorders and has toxic effect on the kidney (Haider et al., 2004). Pb induces renal tumors, reduce cognitive development and increase blood pressure and cardiovascular disease in adults. Cr is required in trace amounts in humans for sugar and lipid metabolism, whereas its hexavalent form is extremely toxic and carcinogenic (Kota's and Stasicka, 2000). It can easily permeate into the human cell and transferred into more stable form, which can damage DNA (Pellerin and Booker, 2000). For normal synthesis and secretion of insulin manganese is required. It acts as a cofactor for a number of enzymatic systems (Korc, 1983). Ni is mostly present in the pancreas, where it plays an important role in the production of insulin. It's minute quantity is required and its deficiency causes liver disorder. Whereas at higher concentration it shows allergic dermatitis known as Ni itch (Pendias and Pendias, 1992). Arsenic is associated with the cancers of skin and internal organs. Thus, quantification of metals in plants, especially medicinal herbs, is part of quality control, which has been established by their purity, safety and efficacy (Ajasa et al., 2004; Mukherjee, 2002). World Health organization (WHO, 1989) declares the maximum permissible levels in food and drug

materials for arsenic (As), Cd and Pb as amount to 1.0, 0.3 and 10 mg/kg, respectively, (Basgel and Erdemoglu, 2006).

Moringa (*Moringa oleifera*) is a plant that is commonly consumed as a nutritional supplement by some communities in South Africa. Contamination of Moringa with toxic heavy metals could be deadly for consumers. The plant is used mainly by disadvantaged communities as a nutritional supplement in their daily diets, and so it is important to know the nutritional benefits of Moringa. As well as nutritional benefits, it is also important to determine any detrimental effects of this plant consumption, such as heavy metal toxicity.

Determination of heavy metals was performed using Thermofisher AA 303 atomic absorption spectrophotometer and the light source was a hollow cathode lamp. For analysis of all the metals oxy- acetylene flame was used. For the determination of arsenic and mercury hydride generator was used and Hg was analysed by the way of cold vapour analysis. The standard instrumental condition and experimental condition was maintained. Arsenic and mercury were converted into their volatile hydride forms using sodium borohydride and concentrated HCl. The vapour of hydride generated in the system was sent to the optical cell using peristaltic pump to produce metallic hydride. The generated gas liquid mixture was separated by the separator 12 into gas and liquids. The separated specimen gas was introduced into the heating section 30. Electricity was supplied from the power source 28 to the specimen heating section 30 where the specimen gas introduced is heated and separated into hydrogen and a specimen metal vapour to be measured. The metal vapour was the introduced into the measuring section 34 arranged between magnetic poles of the magnet 32 where the metal vapour was subjected to the atomic absorptiometric analysis based on the Zeeman effect. (Yashusi et al., 1999)

7.2. Reagent and chemicals

Milli-Q Water (Millipore, USA) was used throughout AQ 2 the analysis; nitric acid (HNO_3), perchloric acid ($HClO_4$), hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) were of analytical grade. Stock solution of 1000 ppm concentration for all the metals was procured from Merck (Darmstadt, Germany). All the working concentrations were prepared freshly on the day of analysis.

7.3. Instrumentation

The atomic absorption measurements were performed using Thermofisher AA 303 atomic absorption spectrometer with hollow cathode lamp light source. For the analysis of all the

metals, oxy-acetylene flame was used. Arsenic and Hg was determined using hydride generator, where these metals were converted into their volatile hydride forms using sodium borohydride and concentrated HCl. The vapour of hydride generated in the system was sent to the optical cell using peristaltic pump. Determination of Hg was carried out by the way of cold vapour analysis. The standard instrumental configuration and experimental condition maintained for the analysis of Cu, Cr, Mn, Fe, Ni, As, Pb, and Hg has been given in Table 7.1.

Table 7. 1

AAS specification for heavy metal analysis

Elements	Cu	Cr	Mn	Fe	Ni	As	Pb	Hg
Wavelength	324.7	357.9	279.5	248.3	232.0	193.7	217.0	253.7
Current (mA)	5.0	5.0	5.0	9.0	9.0	12.0	9.0	3.0
Flame	AA	AA	AA	AA	AA	AA	AA	AA
Fuel (L/min)	3.05	2.90	2.95	2.99	2.94	2.40	2.90	7.66
Slit width (nm)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Working range (ppm)	1–5	2–8	1–5	2–10	3–10	0.04–0.1	2–10	1–0.2
Read time (sec)	3	3	3	3	3	3	3	3
Wash time (sec)	10	10	10	10	10	10	10	10

AA: Air:Acetylene; Cu: Copper; Cr: Chromium; Mn: Manganese; Fe: Iron; Ni: Nickel; AS: Arsenic; Pb: Lead; Hg: Mercury; AAS: Atomic absorption spectrometer.

7.4. Sample preparation

For the analysis, samples (leaf, flower, pod and formulation of *M. oleifera*) were ground to a fine powder and dried at 55–70ºC for 6–8 h in a controlled environment, to remove moisture. Immediately after drying, accurately weighed sample of 3.0 g was placed in a flask and treated with 3 ml of concentrated HNO_3 for 4–5 h. A mixture of HNO_3 and $HClO_4$ in a ratio of 2:1 (3 ml/g of sample) was added. The mixture was heated at 120–130ºC for 5–6 h, until fumes stop and until the resulting solution is clear. Then, 10 ml of milli-Q water was added and boiled again for 10–15 min and the volume was reduced to half, cooled to room temperature and filtered using Whatman filter paper no. 42. The entire filtrate was mixed and

made the volume upto 50 ml with milli-Q water. A blank was also prepared for every sample in the same way. Each sample was aspirated twice and the experiment was repeated for five times.

7.5. Statistical analysis

The data were represented as the Mean ± SD using the Graph pad prism Version 5.0. on the basis of the number of samples analyzed.

7.6. Result and discussion

Metals are natural components in soil. To grow and complete the life cycle, plants must acquire not only macronutrients but also essential micronutrient. Plant needs to detoxify the metals once it takes them up, and the metals need to be transported to the stems and leaves of the plant, and stored there because it may lead to metal contamination which may affect human health or the environment. In the present work, concentration of eight metals, including heavy metals, were determined for *M.oleifera* (leaf, pod, flower) and the formulation. The quantitative determinations were carried out using standard calibration curve obtained by the standard solution of metals having optimal detectable concentration ranges. The concentration of the metals obtained in plant material was expressed in terms of parts per million (Table 7.2). The levels of heavy metals quantified in all the plant samples were well in prescribed limits (WHO, 1999). Arsenic and Mercury was present in a very negligible amount in all the samples. Copper, Manganese and Iron is present in all the samples in an appreciable amount. The dietary formulation contains highest amount of trace metals compared to its dietary ingredients. The concentrations of Cu determined were found to be present in a considerable amount with highest concentration in *M. oleifera* formulation and lowest in the flower. Fe is one of the most important elements required in the human body for the circulation of oxygen in the blood. The concentration of Fe present in the plant was in appreciable quantity to justify its use in human circulation system, with a highest value in formulation and lowest in the flower. Mn is one of the most important elements required for many enzymatic activities. The concentration quantified in the analyzed samples varies from highest concentration in the formulation with a lowest value in pod. Mercury and arsenic was not detected in the leaf and flower respectively. In the formulation and the pod of *M. oleifera* arsenic and mercury present in a very negligible amount. The concentration of Ni in the plant sample was found to vary with a minimum in flower to maximum in formulation. The trace metal Cr was quantified and found to be minimum in the flower of *M. oleifera* and maximum in formulation.

Table 7.2
Metal content in different parts of *M. oleifera* and its formulation by AAS (in ppm).Values are Mean ± SD, n= 5, ND= not detected

M. oleifera parts	Trace metals					Heavy metals		
	Cu	Fe	Cr	Ni	Mn	Pb	As	Hg
Leaf	1192.92 ± 0.41	7610.09 ± 0.12	1.312 ± 0.33	3.168 ± 0.43	5712.46 ±0.71	1.478 ± 0.56	ND	ND
Pod	3095.71 ± 0.39	5230.18 ± 0.33	1.001 ± 0.41	3.341 ± 0.23	4971.12 ±0.69	1.198 ± 0.76	0.021± 0.31	0.003 ± 0.61
Flower	1016.23 ± 0.61	5052.52 ± 0.43	0.891 ± 0.15	2.981 ± 0.55	5015.16±0.32	1.341±0.51	ND	0.001 ± 0.64
Formulation	3418.76 ± 0.52	7709.34 ± 0.51	1.781 ± 0.71	5.117± 0.45	6113.14 ±0.51	1.926 ± 0.34	0.017± 0.43	0.005 ± 0.48

It was observed that the heavy metals present in the different parts of the *M. oleifera* plant (pod, flower, leaf) and in the formulation were within the prescribed limits and other trace metals were also found to be present in a considerable amount. So the plant materials collected were safe and may not produce any toxic effect on health and environment. As a result the level of heavy metals quantified in the plant sample was significant and within the prescribed limits (WHO, 1999).

Chapter – 7

Determination of trace and heavy metals in *Moringa oleifera*

7.1. Introduction

7.2. Reagent and chemicals

7.3. Instrumentation

7.4. Sample preparation

7.5. Statistical analysis

7.6. Result and discussion

Summary and Conclusion

About 2000 years ago, Hippocrates correctly emphasized “Let food be your medicine and medicine be your food". Currently there is an increased global interest due to the recognition that “nutraceuticals” play a major role in health enhancement. Hence a “nutraceutical” is any substance that may be considered as a food or part of a food that provides medical or health benefits, encompassing, prevention and treatment of diseases. Dietary supplement is a preparation intended to supplement the diet. However, supplements should not replace variety of foods that are important to a healthy diet, so it is necessary to take variety of foods as well. We are currently eating foods that are out of balance even though the requirements for these nutrients for optimal health have remained constant (Eaton and Konner, 1985). The difference between the two is termed as “nutrient gap”. This can have adverse influences on the energy processes of the body that are necessary for all vital functions. Currently neutraceuticals are recognised as being beneficial in coronary heart disease, obesity, diabetes, cancer, osteoporosis and other chronic and degenerative diseases such as Parkinson's and Alzheimer's diseases. Evidences indicate that the mechanistic actions of natural compounds involve a wide array of biological processes, including activation of antioxidant defences, signal transduction pathways, cell survival-associated gene expression, cell proliferation and differentiation and preservation of mitochondrial integrity. It appears that these properties play a crucial role in the protection against the pathologies of numerous age-related or chronic diseases (Mandel et al., 2005). In India, nearly 20% of the total population and 44% of young children (below 5 years of age) are undernourished, numbers which are significantly higher than even the poorer sub-Saharan African countries. Iron deficiency anaemia during pregnancy accounts for one-fifth of maternal deaths in India and the prevalence of this deficiency in women has alarming. Iodine and vitamin A deficiencies in India are still above the WHO specified desired levels. Annually as many as 0.3 million children succumb to vitamin A deficiency related diseases. The impact of these deficiencies is a productivity loss of around one percentage point of India’s GDP (Dzanis, 1998).

Moringa oleifera Lam (Moringaceae) has an impressive range of medicinal uses with high nutritional value. Different parts of this plant contain a profile of important minerals, and are a good source of protein, vitamins, beta-carotene, amino acids and various phenolics. Apart from that this plant also a rich source of vitamins (mainly water soluble) and also some essential elements like zinc, magnesium, manganese, phosphorus, calcium in trace amount.

In the present study the nutritional value of *Moringa oleifera* as a neutraceutical has been explored. Evaluations of the neutritional properties of this plant have a great importance in this work. The extracts were standardized with the help of HPTLC using Chlorogenic acid as marker. The CGA was found to be (3.16 % w/w) in the formulation of *M. oleifera,* whereas the CGA was not found in pod. The leaf and the flowering part contain 2.99 % w/w and 2.46 % w/w of CGA per weight of extract respectively. CGA cannot be detected in the pod of this plant. During the total phenolic and flavonoid content determination it was observed that total phenolic (7.27%) and flavonoid (3.41%) content in the formulation was greater than its dietary ingredients. The total phenolic and flavonoid content will attribute to their antioxidant profile.

In the nutritional evaluation study it has been observed that the formulation and the leaf were found to be enriched with an excellent protein source (12.26%, 9.64% respectively). The vitamin content was better achieved in the formulation and in the flower of this plant. The presence of Vitamin C, Folic acid (Vitamin B_9) and Niacin (Vitamin B_3) are maximum in the formulation with compared to its ingredients. Among the minerals, potassium (2.24%), calcium (2.18%) and iron (0.77%) content is significant. The total dietary fiber was present in higher amount in the pod compared to formulation, leaf and flower of *M.oleifera.* The heavy meals were also analyzed by Atomic absorption spectrophotometer and it was found within the WHO prescribed limit. The nutritional values were statistically evaluated. From the nutritional evaluation it can be concluded that this formulation can be used as a neutraceuticals and it is very safe for human consumption. This plant is also economical to all regions and was used by many people due to its multiple utilities. Now a day, there is a growing demand for plant based medicines, health products, pharmaceuticals, food supplement, cosmetics etc. in the national and international market (Dureza et al., 2003). But most of the food supplements are not having proper quality evaluation. Also there are some foods used traditionally, having some ethnopharmacological relevance but no scientific evidence. The major challenge is to develop those scientific rationales behind their use. This work highlights on the exploration of therapeutic benefits of a food plant having medicinal value to a high extent. The result shows that the plant provides an excellent source of macro nutrients and micronutrients. Hence it can also be further explored for development of nutritional supplement in future which may be useful in various disease conditions and thus promote the quality of life.

Chapter - 8

Summary and conclusion

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Chapter – 9

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