CHAPTER VII
DISCUSSION

Current trend observed in the healthcare of human society is shift towards Nutraceuticals for the maintenance of health. As existing synthetic Nutraceutical products, are expensive and are also prone to produce side effects, the need of the hour is to develop effective, safe human friendly herbal Nutraceutical. Recent researchers have demonstrated that natural products, including crude extracts and isolated compounds from plants, can balance nutritional deficiencies and can also prevent unwanted side effects and contribute towards the maintenance of good health.

Hence, researchers have started focusing their research towards natural products such as plants for developing Nutraceutical. So that in the present study, attempts were made to screen the Nutraceutical potentials of some common herbal drugs available in and around Thanjavur, Tamil Nadu, and attempts were also made to develop a formulation using the plants selected with a view to develop a human friendly, biocompatible, cost effective herbal Nutraceutical.

Literature review carried out in the present work revealed that plants rich in lignins, phenols, tannins, flavonoids and alkaloids are used as a potent herbal Nutraceutical sources. This prompted us to select and evaluate the Nutraceutical potentials of the common herbs rich in these phytoconstituents.

Commonly used herbal products rich in alkaloids, flavonoids, phenols and proteins were selected for the present study. Formulations were made from the aqueous extracts of flower buds of *Capparis spinosa* L., seeds of *Caesalpinia bonducella* L., fruits of *Luffa acutangula* L. and aerial parts of *Cassia occidentalis* L. A formulation was also prepared using these plant aqueous extracts. The selected plant drug sources were collected from in and around Thanjavur and Mannargudi and were identified with the help of Flora of Presidency of Madras and authenticated by comparing with voucher specimens deposited at RAPINAT Herbarium, Department of Botany, St.Joseph’s College, Tiruchirappalli.

After proper identification and authentication the test drugs were standardized chemically, which is necessary to maintain the uniformity and reproducibility in the efficacy. This in turn will contribute towards the global acceptance of the developed
formulation as potential herbal Nutraceutical. To ensure reproducible quality of herbal products, proper control of starting material is very much essential. In recent years, there has been an emphasis to determine quality standards for medicinal plants. Keeping these views WHO has suggested quality standards for herbal drugs.

According to WHO, passing the test of identity, purity and strength is the first step towards establishing the quality. In the present dissertation as a first step these standards were determined as per WHO approved protocols for the selected plant sources before evaluating their Nutraceutical potential.

7.1. Pharmacognostical standardization

7.1.1. Botanical standardization studies

7.1.1.1. Organoleptic evaluation

Organoleptic evaluation is a technique of qualitative evaluation based on the study of sensory profiles of whole drugs (Kokate et al., 2007). The Organoleptic studies show the important characteristics of the drugs, the typical tongue sensation and the odour may screen the preliminary phytochemical constituents.

Flower buds of *C. spinosa* L. are light green in colour and bitter in taste. Seeds of *C. bonducella* L. are light brown in colour and possessed characteristic odour and astringent taste. Fruits of *L. acutangula* L. are brown in colour and astringent in taste. Aerial parts of *C. occidentalis* L. are brown in colour and revealed a pleasant odour with bitter taste. Selected polyherbal formulation (Nutraceutical) is greenish brown in colour and odour nothing characteristic.

7.1.1.2. Macroscopic features

Flower buds of *C. spinosa* L. are white, solitary, axillary. Sepals are subequal, petals are white, and anther filaments are purple and are longer than the petals. Seed coat of *C. bonducella* L. is hard, glossy, and greenish to light brown in colour. Seed is traversed by circular and vertical faint markings of the cracks, forming uniform rectangular to squarish rectulations all over the surface. Seeds lead colored, 1.3cm. long. A raised hilum with remains of the stalk lies in the centre of the dark spot, at the narrow edge of the seed. Adjacent to the hilum, lies a faint coloured circular to oval elevated micropyle. In dry seed, kernel gets detached from the testa, is composed of three distinct layers, the outermost thin and brittle, the middle one broad, fibrous
and dark brown and the innermost white and papery. Stem of *C. occidentalis* L. is hard woody. Green colour lanceolate or ovate lanceolate type of compound leaves present. Flower is yellow, inflorescence few flowered, axillary, and also forming terminal panicle; bracts caducous. Fruit is a flat pod 10-12cm. long with 10-30 seeds. Areolate seeds are pointed at end and blunt at the other end. Fruits of *L. occutangula* L. are obovate, pale brown, outer surface covered with 8-10 prominent longitudinal ribs. Single fruit is divided into 3 chambers. The inner part is fibrous and easily detachable and has ten acute lines on it, which is from base to apex of the fruit. Fruits are narrow towards bottom and broader towards apex of the fruit.

### 7.1.1.3. Microscopic studies

According to World Health Organization (WHO) the microscopic description of a medicinal plant is the important step towards establishing its identity and should be carried out before initiating any scientific analysis.

### 7.1.1.3.1. Powder microscopic studies

Powder Microscopic studies were carried out in the selected plant drugs for developed formulation and the results were presented. Powder microscopy of flower buds of *C. spinosa* L. revealed the presence of lignified parenchyma cells, long curved uniseriate trichome, short trichome, sclerenchyma cells, striated sclereid, prismatic calcium oxalate crystal, xylem vessels with spiral thickening and simple starch grain. Powder microscopy of seeds of *C. bonducella* L. revealed the presence of prismatic calcium oxalate crystal, parenchyma cell with starch grains, simple and compound starch grain, linea lucida, oil globules and sclereids.

Powder microscopy of fruits of *L. acutangula* L. revealed the presence of epidermis with striated cuticle, epidermis with stomata, uniseriate trichome, fiber, prismatic calcium oxalate crystal, lignified parenchyma, xylem vessels with spiral thickening, simple starch grain and brown contents. Powder microscopy of aerial parts of *C. occidentalis* L. revealed the presence of prismatic calcium oxalate crystal, druses calcium oxalate crystal, oil globules, fiber, compound starch grain, lignified fiber, linea lucida, sclereids and brown contents. These microscopic features observed could be useful as microscopic standard for the authentication of the selected
CHAPTER VII
DISCUSSION

Biochemical Evaluation and Validation Studies on Herbal Nutraceutical

plant drugs. Presence of these features in the end product developed confirms usage of all selected plant drugs in the final formulation.

Powder microscopy of selected polyherbal (Nutraceutical) formulation revealed the presence of compound starch grains, simple starch grains, sclereids, lignified fiber, prismatic calcium oxalate crystals and xylem vessels.

7.1.1.4. Physicochemical analysis

7.1.1.4. 1. Test for identity, purity and strength

Physicochemical constants were determined for the selected plants and developed formulation.

Loss on drying

The percentage of active chemical constituents in crude drugs is mentioned on air dried basis. Therefore, the loss on drying of plant materials should be determined and the water content should also be controlled. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water. Moisture content directly affects the appearance, texture and its storage (Salunkhe, 1974). The test for loss on drying determines both water and volatile matter and also prevents contamination and deterioration (Anonymous, 2002).

The result obtained for the test drugs (C. spinosa, C. bonducella, L. acutangula, C. occidentalis and formulations) on loss on drying revealed low level of moisture content which implies that the shelf life for this plant material could be longer.

Ash value

Total ash and acid insoluble ash contents are important indices to illustrate the quality as well as purity of crude drugs. Total ash includes physiological ash, which is derived from the plant tissue itself, and non physiological ash, which is often from environmental contaminations such as sand and soil. The acid insoluble ash content is another index to illustrate the quality. Water soluble ash is the water soluble portion of the total ash.

Total ash, acid insoluble ash, water soluble ash and sulphated ash percentage were determined for the selected drug sources (C. spinosa L., C. bonducella L., L. acutangula L., C. occidentalis L. and formulations) as per WHO protocols. The data
of the ash values obtained in the present work revealed that the selected plant drugs are rich in mineral contents. Loss on drying and total ash content also suggested that the selected plant drugs are free from impurities.

**Extractive values**

The extractive values were determined to assess the amount of soluble compounds. This is obtained by exhausting plant sample powder with solvents of increasing polarity and is indicative of approximate measures of their chemical constituents extracted with those solvents from a specific amount of air dried plant material. In the selected plant drugs, it is noted that the water extractive value was found to be higher than other extractives values (Table 24, 42, 60 and 78) suggesting high polar compounds in the test drugs. High extractive value gives an idea of the amount of the phytoconstituents and less extractive value indicates adulteration and substitution of drug (Ramakrishnan et al., 2015).

Successful prediction of natural compounds from plant material largely depends on the type of solvent used in the extraction process. The traditional healers use primarily water as the solvent, hence in the present work also aqueous extract were used to evaluate the Nutraceutical potentials of selected plants and developed formulation.

7.1.2. **Chemical standardization studies**

7.1.2. 1. **Fluorescence features**

Fluorescence is an important phenomenon exhibited by various chemical constituents present in plant material. The samples as such and after treatment with various solvents were subjected to fluorescence analysis. Observations were made under visible light and UV light of short wave length and long wave length separately. The fluorescence colour is specific for each compound. A non fluorescent compound may fluoresce if mixed with impurities that are fluorescent. Hence, it is useful in detecting the adulterants and substitutes. Some constituents show fluorescence in the visible range in many natural products (e.g., alkaloids like berberine), which do not visibly fluoresce in day light. If the substances themselves are not fluorescent, they may often be converted into fluorescent derivatives by applying different reagents.
hence some crude drugs are often assessed qualitatively in this way and it is an important parameter of pharmacognostic evaluation (Ali, 2008). Yellow chromophore normally indicates the presence of flavones and green chromophore indicates the presence of sterols in the selected plant drugs.

7.1.2. 2. Preliminary phytochemical screening

The dried and coarsely powdered samples were extracted in various organic solvents in the order of increasing polarity (hexane, chloroform, ethyl acetate, ethanol and water) using successive solvent extraction method. Extracts thus obtained were subjected to qualitative detection of various chemical constituents present in different extracts using standard textual methods.

Preliminary phytochemical screening of flower buds of *C. spinosa* L. revealed the presence of alkaloids, reducing sugars, carbohydrates, saponins, phenolic compounds, tannins, anthraquinones and lignins in aqueous extract. Preliminary phytochemical screening of seeds of *C. bonducella* L. revealed the presence of alkaloids, saponins, flavonoids, phenolic compounds and tannins in aqueous extract.

The preliminary phytochemical screening data suggested the presence of reducing sugars, glycosides, flavonoids and phenolic compounds in the aqueous extract of fruits of *L. acutangula* L. The preliminary phytochemical screening of aqueous extract of aerial parts of *C. occidentalis* L. results depicted the presence of reducing sugars, carbohydrates, steroids, flavonoids, quinone, anthroquinone, proteins and amino acids.

7.1.2. 3. Estimation of important organic constituents

Major organic constituents (secondary metabolites) present in *C. spinosa* L., *C. bonducella* L., *L. acutangula* L., *C. occidentalis* L. and formulations were estimated. Today food industries are very much interested in using the plant extracts having good amount of secondary metabolites such as flavonoids, phenolic acids and tannins as they are known anti-oxidants. Diverse biological activities, such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities are also possessed by these anti-oxidant compounds (Chung *et al*., 1998).

Flavonoids present in the Nutraceutical might help in inflammatory conditions (Kunle and Egharevba, 2009) alkaloids in microbial infections and in decreasing
blood pressure, balancing the nervous system (Ronan et al., 2009). Phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites (Singh and Singh, 2007). Natural anti-oxidants mainly come from plants in the form of phenolic compounds such as flavonoids, phenolic acids and tannins (Ali et al., 2008). Plant tannins are well known anti-oxidants in medicinal plants, foods and edible fruits (Yoshida et al., 2010). They have been considered to be cardio-protective, anti-inflammatory, anti-carcinogenic and anti-mutagenic, among others. Tannins enhance glucose uptake and inhibit adipogenesis, thus being potential drugs for the treatment of NIDDM (Mamta Kumari and Shashi Jain, 2013). Lignins are protecting against various ailments like hypercholesterolemia, intermittent claudication, benign prostatic hyperplasia and cardiovascular diseases and also having enormous Nutraceutical properties like anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-oxidant, anti-thrombotic, cardioprotective and vasodilatory effects, besides being anti-viral, and anti-cancer (Pasha et al., 2013).

Active constituents present such as alkaloids, flavonoids, phenols, tannins, and lignins in the selected plant drugs in the aqueous extract were estimated qualitatively. Lignins was in higher percentage as compared to tannins, phenols, flavonoids and alkaloids. It was found that seeds of C. bonducella L. have more amount of lignins (74.70 mg/g) followed by L. acutangula L. (58.70 mg/g), C. spinosa L. (47.00 mg/g) and C. occidentalis L. (0.34 mg/g). Flavonoids were found to be more in C. occidentalis L. (02.45 mg/g), followed by C.spinosa L. (00.52 mg/g) and L. acutangula L. (00.45 mg/g) than C. bonducella L. (00.33 mg/g). Tannin content was found to be higher in C.spinosa L. 09.74 mg/g, in C. bonducella L., it is 04.90 mg/g and in L. acutangula L. it is (01.84 mg/g), in C. occidentalis L., it is 0.21 mg/g. Phenol content was higher in L. acutangula L. (0.62 mg/g) and C. bonducella L. (0.60 mg/g) as compared to C.spinosa L. (0.51 mg/g) and C. occidentalis L. (0.16 mg/g). Alkaloid content was found to be higher in C. occidentalis L. (01.56mg/g) as compared to C.spinosa L. (00.22 mg/g), L. acutangula L. (00.19 mg/g) and C. bonducella L. (00.12 mg/g).

Lignin content was rich in selected plant sources, but flavonoid content was found to be high in formulation. Because, lignin synthesis repression leads to the
redirection of the metabolic flux into flavonoids through nonenzymic reaction (Basseau et al., 2007). Flavonoid content (0.3 mg/g) was found to be higher in formulation (3) followed by formulation (1) and formulation (2) (0.2 mg/g). Moderate amount of phenol was present in formulation (3) (0.08 mg/g). Alkaloids, tannins and lignins were also present in the formulations.

7.1.2. 4. HPTLC fingerprinting profile

HPTLC and GC-MS analysis were carried out in the hexane and chloroform extracts of the samples for the identification of volatile and nonvolatile compounds present in the selected plant sources and to determine chemical standards for assessing the genuineness and quality of the herbal drugs under study.

In the last two decades HPTLC method has emerged as an important tool for the qualitative and quantitative phytochemical analysis of herbal drugs and formulation. HPTLC fingerprinting of the selected plants were determined with Rf values, observed under 254 and 366 nm.

HPTLC fingerprinting profiles of flower buds of C. spinosa L. showed eleven spots, two at Rf 0.13 and 0.77 (blue) and nine spots at Rf 0.07, 0.20, 0.43, 0.52, 0.66, 0.72, 0.82, 0.86 and 0.99 (pink) in chloroform extract while only three spots in hexane extract at Rf 0.84 (blue), and at Rf 0.79 and 0.99 (pink).

HPTLC fingerprinting profiles of seeds of C. bonduc L. revealed four spots at Rf 0.41, 0.52, 0.87 and 0.96 (all pink) in chloroform extract while five spots at Rf 0.66, 0.74 and 0.81 (all pink), at Rf 0.93 and 1.00 (blue) in hexane extract.

HPTLC fingerprinting of chloroform and hexane extracts of fruits of L. acutangula L. revealed eleven spots at Rf 0.12, 0.41, 0.73 and 0.85 (all blue), at Rf 0.08, 0.26, 0.31, 0.54, 0.59, 0.77 and 0.99 (all pink) in chloroform extract while three spots at Rf 0.14, 0.57 and 0.80 (all blue), and eight spots at Rf 0.05, 0.24, 0.50, 0.71, 0.76, 0.85, 0.92 and 0.99 (all pink) in hexane extract.

HPTLC fingerprinting of chloroform and hexane extracts of aerial parts of C. occidentalis L. showed ten spots at Rf 0.06 (yellow), Rf 0.91 (blue), Rf 0.13 and 0.75 (green), Rf 0.32, 0.56 and 0.98 (red), Rf 0.38, 0.68 and 0.81 (brown) in chloroform extract while ten bands at Rf 0.07 (yellow), Rf 0.76 (blue), Rf 0.25 and 0.67 (green), Rf 0.39 and 0.98 (red), Rf 0.41, 0.47, 0.56 and 0.76 (all brown) in hexane extract.
HPTLC fingerprinting profiles of selected formulation (polyherbal Nutraceutical) revealed the presences of twelve spots with one at Rf 0.06(yellow), one at Rf 0.20(pink), one at Rf 0.33(red), two at Rf 0.27 and 0.40 (brown), two at Rf 0.16 and 0.55(green) and five at Rf 0.43, 0.67, 0.76, 0.83 and 0.99 (blue) colors were noted at 10µl formulation. Eleven spots at Rf 0.05, 0.19, 0.27, 0.32, 0.42, 0.53, 0.67, 0.75, 0.82, 0.95 and 0.99 with different colors were noted at 5µl formulation. HPTLC fingerprinting of test drug suggested the presence of different types of phytochemical compounds.

7.1.2.5. GC-MS analysis

In the present work, GC-MS analysis of chloroform and hexane extracts of selected plant parts were carried out.

Table 117: GC-MS analysis of chloroform and hexane extracts of selected plant parts

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of the selected plant drugs</th>
<th>No. of compounds identified</th>
<th>Chloroform extract</th>
<th>Hexane extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flower buds of <em>C. spinosa</em> L.</td>
<td></td>
<td>29</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>Seeds of <em>C. bonducella</em> L.</td>
<td></td>
<td>38</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>Fruits of <em>L. acutangula</em> L.</td>
<td></td>
<td>35</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>Aerial parts of <em>C. occidentalis</em> L.</td>
<td></td>
<td>52</td>
<td>45</td>
</tr>
</tbody>
</table>

The GC-MS profiles with n-Hexadecanoic acid as magic compound could be used as chemical marker for deciding the genuine of the selected plants.

7.1.3. Estimation of inorganic constituents

Estimation of metals in medicinal plants is a part of quality control studies to establish their purity, safety and efficacy. Considering the importance of trace elements in various human metabolic processes and also considering their Nutraceutical properties, in the present investigation, we have applied the analytical techniques such as flame photometry, atomic absorption spectroscopy and X-Ray fluorescence spectroscopy to detect various essential elements present in different parts of selected plants. Mineral and heavy metal contents are also determined in the formulations.
Medicinal plants contain both organic and inorganic constituents. The human body needs a number of minerals to maintain good health (Ajasa *et al.*, 2004). Macro and micro elements influence biochemical processes in the human organism. Active constituents of plants i.e. metabolic products of plant cells and a number of mineral elements play an important role in the metabolism (Kolasani *et al.*, 2011). Some mineral elements remain chelated with organic ligands and make them bioavailable to the body system (Choudhury and Garg, 2007). They are intimately involved in the physiological functions and are important cofactors in the production of enzymes. They are also necessary for the maintenance and regulation of cell, genes and membrane functions. Elemental deficiencies result in the reduced activity of the concerned enzymes. However, since each element is related to so many enzymes, deficiency of a single element is often not associated with any specific clinical manifestations, but rather manifests as a combination of various symptoms (Osamu Wada, 2004).

Determination of mineral elements in plants is very important since the quality of many foods and medicines depends upon the content and type of minerals (Bahadur *et al.*, 2011). Malnutrition is of major concern for many tropical developing countries. Deficiency or excess of elements may cause a number of disorders. For example, Iron deficiency anemia affects one third of the world population (Kumari *et al.*, 2004). Iron is needed for the production of red blood cell and enzymes help in fighting against anemia.

Low levels of Zn can induce the pathogenesis of lung cancer (Cobanoglu *et al.*, 2010). The positive impact of zinc supplementation on the growth of some stunted children and on the prevalence of selected childhood diseases suggests that zinc deficiency is likely to be a significant public health problem, especially in developing countries (Osendarp *et al.*, 2003; Hussain *et al.*, 2009). According to FAO’s food balance data, it has been calculated that about 20% of the world’s population could be at risk of zinc deficiency with the average daily intake less than 70 µg per day (Holt and Brown, 2004). Deficiency of calcium and phosphorous leads to the classical bone symptoms associated with rickets, such as bowlegs, knock knees, curvature of the spine, pelvic and thoracic deformities and osteoporosis (Reid *et al.*, 2009). Breast
cancer patients had low levels of Ca, Mg, Fe, Cu, Mn and Zn in their hair (Joo et al., 2009). Magnesium is needed for enzyme action, balanced hormones, a healthy nervous system and cardiovascular system. Recent studies confirm that magnesium plays a key role in the preventing cardiovascular diseases (Liana C Del Gobbo et al., 2013).

Iron, zinc, copper and manganese are playing an important role in the improvement of anti-oxidant system. The mineral elements zinc, molybdate, manganese and chromium are involved in glucose homeostasis and are therefore critical in the management of diabetes. Zinc and chromium are cofactors for insulin synthesis. The activity of a copper-zinc dependent enzyme, superoxide dismutase a powerful anti-oxidant protecting cells against free radical injury just like the manganese is dependent on superoxide dismutase (Hathcock, 1997).

Table 118: Quantity of major organic constituents

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zn – Zinc</td>
<td>0.09±0.18</td>
<td>0.15±0.02</td>
<td>0.06±0.01</td>
<td>0.14±0.04</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>2</td>
<td>Ca – Calcium</td>
<td>24.31±0.29</td>
<td>13.33±0.07</td>
<td>10.79±0.06</td>
<td>0.26±0.08</td>
<td>8.19±0.01</td>
</tr>
<tr>
<td>3</td>
<td>Fe – Iron</td>
<td>0.75±0.83</td>
<td>0.38±0.05</td>
<td>0.85±0.04</td>
<td>11.03±0.01</td>
<td>2.34±0.04</td>
</tr>
<tr>
<td>4</td>
<td>P-phosphorus</td>
<td>2.93±0.21</td>
<td>0.31±0.02</td>
<td>4.86±0.02</td>
<td>0.54±0.08</td>
<td>0.06±0.02</td>
</tr>
<tr>
<td>5</td>
<td>Mg-Magnesium</td>
<td>3.44±0.07</td>
<td>0.96±0.01</td>
<td>2.62±0.05</td>
<td>1.54±0.01</td>
<td>1.28±0.02</td>
</tr>
<tr>
<td>6</td>
<td>Cu – Copper</td>
<td>0.09±0.15</td>
<td>0.16±0.04</td>
<td>0.10±0.03</td>
<td>0.74±0.05</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>7</td>
<td>Mn - Manganese</td>
<td>-</td>
<td>-</td>
<td>0.07±0.05</td>
<td>0.21±0.09</td>
<td>1.56±0.03</td>
</tr>
</tbody>
</table>

In the selected plant sources and developed formulation these important elements (Zn, Ca, Fe, P, Mg, Cu and Mn) were presented in good amount. Heavy metal analysis revealed that all selected plants as well as developed formulation were free from heavy metals. They were present with in the limits.

7.2. Analysis of Nutraceutical values

Data obtained on the Nutraceutical values of selected plant sources and developed formulation revealed the presence of good amount of carbohydrates,
proteins and fats that play major roles in metabolism and are required in large amount. Deficiencies or excess of nutrients especially proteins, carbohydrates and vitamins lead to various complications and metabolic disorders in human beings. The need of nutrients can be fulfilled by additional food stuffs or supplements. The presence of proteins will serve as building block of cells, muscles, cartilage, skin, hormones, enzymes and vitamins.

Intake of crude fibre present in the selected plants can lower the serum cholesterol level, risk of coronary heart disease, hypertension, constipation, diabetes, and colon and breast cancer. The recommended dietary allowances of fibers essential for children, adults, and pregnant and lactating mothers are 19-25 g/d, 21-38 g/d, and 28-29 g/d, respectively (Mark Hanson et al., 2009). The selected plant drugs and formulations possess recommended fibre content.

Flavonoids present in the test drugs will enhance the effects of vitamin C and functions as anti-oxidants. Ascorbic acid protects low density lipoproteins *ex vivo* against oxidation. The presence of high quantities of vitamin E confirms the capacity of providing protection from free radicals and products of oxygenation. Vitamin E works in conjunction with other anti-oxidant nutrients present in these plant materials to quench free radicals. Vitamin E also inhibits lipoxigenase, an enzyme responsible for the formation of pro inflammatory leukotrienes (Belinda S O’Connell, 2001).

Catalase converts the reactive oxygen species hydrogen peroxide to water and oxygen and mitigates the toxic effects of hydrogen peroxide thereby it prevents oxidative stress. Oxidative stress is hypothesized to play a role in the development of many chronic or late onset diseases such as diabetes, asthma, Alzheimer's disease, systemic lupus erythematosus, rheumatoid arthritis, and cancers, which have been associated with decreases in catalase activity. A study from the Institute of Cytology and Genetics suggested that oxidative stress, accumulation of protein and DNA damage could be reduced in the presence of anti-oxidant enzymes catalase in the cytosol and mitochondrial extracts from liver cells of rats (Sinitsyna et al., 2006).

One of the most familiar functions of lipase in the body is the digestion of dietary fat. Another important function of lipase is to help the body package cholesterol for transport and also helps in the biosynthesis of the vitamins A, D, E
and K. Alkaline phosphatase hydrolyzes the phosphate moiety and makes tyrosine available for conversion to catecholamine that are then used to cross link proteins during sclerotization (Lunan and Mitchell, 1969).

From Nutraceutical point of view, flower buds of *C. spinosa* L. possess total carbohydrates content (15.3 mg/g), free amino acids (1.93 mg/g), proteins (26.1 mg/g), free fatty acids (79.1 mg/g), fats (5.5 mg/g), cholesterol (0.05 mg/g), energy value (215.1 kcal) and crude fibre (7.2 mg/g). Carbohydrate value agrees with USDA Nutrient database (1999), but protein, fat, crude fiber and energy values differ from the same database values. Flower buds of *C. spinosa* also possess vitamin B1 (0.40 µg/g), vitamin B2 (0.20 µg/g), vitamin B3 (0.80 µg/g), vitamin C (1.50 µg/g) and vitamin E (0.02 µg/g) and also good measured enzyme activity such as catalase (11.5 µMoles of H₂O₂ utilized /min/ mg protein), amylase (11.7 amount of starch hydrolyzed /S /g), lipase (12.2 micro equivalent of oleic acid liberated per hr/g ), acid phosphatase (0.29U/L = µmol/L in min) and alkaline phosphatase (0.49 U/L = µmol/L in min).

Seeds of *C. bonducella* has total carbohydrates (18.4 mg/g), free amino acids (1.82 mg/g), proteins (17.6 mg/g), free fatty acids (0.03 mg/g), fats (3.6 mg/g), cholesterol (0.02 mg/g), energy value (176.4 kcal) and crude fibre content (3.3 mg/g). Carbohydrate, protein, and fat value agree with Ravikanth (2014), but crude fibre and energy values differ. Seeds of *C. bonducella* has vitamin B1 (10.60 µg/g), vitamin B2 (89.60 µg/g), vitamin B3 (22.60 µg/g), vitamin C (4.20 µg/g) and vitamin E (6.09 µg/g) and also good measured enzyme activity such as catalase (9.6 µMoles of H₂O₂ utilized /min/ mg protein), amylase (12.3 amount of starch hydrolyzed /S /g), lipase (12.9 micro equivalent of oleic acid liberated per hr/g ), acid phosphatase (0.25 U/L = µmol/L in min) and alkaline phosphatase (0.56 U/L = µmol/L in min).

Fruits of *L. acutangula* contains total carbohydrates (5.5 mg/g), free amino acids (1.52 mg/g), proteins (9.6 mg/g), free fatty acids(43.9 mg/g), fats (2.5 mg/g), cholesterol (0.02 mg/g), energy value (82.9 kcal) and crude fibre content (2.8 mg/g). Carbohydrate, protein, fat and energy values are higher, but Crude fibre value lower when compared to Jaysingrao and Sunil (2014) report. Fruits of *L. acutangula* also contain vitamin B1 (0.70 µg/g), vitamin B2 (0.20 µg/g), vitamin B3 (3.80 µg/g),
vitamin C (2.05 µg/g) and vitamin E (0.01 µg/g) and also good measured enzyme activity such as catalase (10.4 µMoles of H₂O₂ utilized /min/ mg protein), amylase (12.4 amount of starch hydrolyzed /S /g), lipase(10.7 micro equivalent of oleic acid liberated per hr/g ), acid phosphatase (0.24 U/L = µmol/L in min) and alkaline phosphatase (0.39 U/L = µmol/L in min).

Aerial parts of *C. occidentalis* possess total carbohydrates content (1.38 mg/g), free amino acids (1.52 mg/g), proteins (0.49 mg/g), free fatty acids (37.12 mg/g), fats (0.03 mg/g), cholesterol (0.03 mg/g), energy value (7.77 kcal) and crude fibre (5.69 mg/g). Fat content agree with Gwarzo (2014) study, but the values of carbohydrate, protein and crude fibre are higher and energy value is lower. Aerial parts of *C. occidentalis* also possess vitamin B1 (06.90 µg/g), vitamin B2 (71.50 µg/g), vitamin B3 (12.60 µg/g), vitamin C (1.36 µg/g) and vitamin E (0.47 µg/g) and also good measured enzyme activity such as catalase (09.81 µMoles of H₂O₂ utilized /min/ mg protein), amylase (10.80 amount of starch hydrolyzed /S /g), lipase(13.60 micro equivalent of oleic acid liberated per hr/g), acid phosphatase (0.21 U/L = µmol/L in min) and alkaline phosphatase (0.41U/L = µmol/L in min).

Formulation possess total carbohydrates content (8-9 mg/g), free amino acids(0.1-0.2 mg/g), proteins (2 mg/g), free fatty acids (0.1 mg/g), fats (10-12 mg/g) , cholesterol (0.01- 0.02 mg/g), crude fibre (4-5 mg/g), vitamin B1 (2 µg/g), vitamin B2 (1 µg/g), vitamin B3 (2-4 µg/g), vitamin C (1-2 µg/g) and vitamin E (1.0- 1.2 µg/g) and also good measured enzyme activity such as catalase (0.03-0.06 µMoles of H₂O₂ utilized /min/ mg protein), amylase (0.01-0.02 amount of starch hydrolyzed /S /g), lipase (0.01- 0.03 micro equivalent of oleic acid liberated per hr/g ), acid phosphatase (0.01 U/L = µmol/L in min) and alkaline phosphatase ( 0.01-0.05 U/L = µmol/L in min).

**7.3. Safety profiles**

**7.3.1. Pesticides residue analysis**

Pesticides residue like Gamma BHC and BHC Tech were found to be absent. PP DDE, 2,4 DDT, aldrin, dieldrin and endosulfan were found to be present within the permissible limits (Table 99).
7.3.2. Total microbial load determination

Lyophilized aqueous extract of flower buds of *C.spinosa* L., seeds of *C.bonducella* L., fruits of *L.acutangula* L., aerial part of *C.occidentalis* L. and selected formulation were screened for the presence of total viable aerobic bacterial and fungal counts and the obtained results were shown in table 33, 51, 69, 87 and 100. Bacterial and fungal counts of the samples were found to be within the permissible limits as prescribed in Indian pharmacopoeia.

Due to the presence of flavonoids and tannins, the selected plant drugs and developed formulation may reveal anti-oxidant and anti-inflammatory activity hence with a view to develop Nutraceutical for patients suffering from inflammations, the test drugs were evaluated for their anti-oxidant and anti-inflammatory efficacy through *In vitro* analysis.

7.4. *In vitro* studies

7.4.1. Anti-oxidant activity

A large and growing volume of scientific investigation supports a comprehensive food and supplement based strategy for controlling free radical formation and thus reducing the incidence and severity of many chronic illnesses. An anti-oxidant rich Nutraceutical is very much beneficial to health in conjunction with good nutrition and regular exercise. There is a belief that anti-oxidants and anti-oxidant rich nutraceuticals provide an anti-aging mechanism within the body. Anti-oxidants can certainly slow down the progression of aging. Provitamin A, vitamin C, vitamin E, zinc, copper, manganese and selenium is act as anti-oxidants, and together they create a great boost. In all the test drugs these anti-oxidants are present in sufficient quantity hence all the test drugs were evaluated for their anti-oxidant potentials employing *In vitro* assays.

Maximum anti-oxidant activity was exhibited by selected plants and developed formulation at a dose level of 1000 µg/ml, which is comparable to that of standard BHT. The activity was dose dependent, which has been found to be increased with an increase in the concentration of the extract. Such radical scavengers may protect tissues from ROS and thereby prevent oxidative damage related diseases.
7.4.2. Anti-inflammatory activity

A large body of epidemiological and experimental data has demonstrated a direct link between inflammation and complex diseases such as obesity, asthma and allergies, cardiovascular disease, type 2 diabetes, rheumatoid arthritis, bowel disease and several types of cancer (http://www.nutraceuticalsworld.com/issues/2015-01/view_features/nutritions-growing-role-in-fighting-inflammation/). Anti-inflammatory agents, including non-steroidal anti-inflammatory drugs (NSAID) and disease modifying anti-rheumatic drugs (DMARDs), are widely used in treating various disorders. However, many of them have dose dependent side effects, and none of them are suitable for primary prevention, which significantly limits their use. On the other hand, it has been recognized that lifestyle and environment play an important role in inflammatory responses.

In this respect, Nutraceutical can be key elements in managing the inflammatory process by

- Inactivating inflammatory molecules through direct binding
- By inhibiting the enzyme lipooxygenase and arresting numerous biochemical pathways responsible for inflammation with both enzymatic and non-enzymatic targets such as prostaglandin E2, cyclooxygenase 1+2, interleukin-1 and isoprostanes (Dilip Ghosh, 2015).

Denaturation of proteins is a well documented cause of inflammation. Since during inflammation condition, protein of the cell gets denatured, albumin protein is used as a model. This model is used to evaluate the protective effect of plant extracts during heat induced denaturation. The neutrophils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further protein denaturation and subsequent tissue inflammation and damage (Chou, 1997). Proteases have been implicated in arthritic reactions. Neutrophils contain neutral serine protease in their liposomal granules. Leukocyte protease plays an important role in the development of tissue damage during inflammatory reactions and significant level of protection is provided by protease inhibitors (Das and Chatterjee, 1995; Sakat et al., 2010).
Stabilization of liposomal membrane is important in limiting the inflammatory response by inhibiting the release of liposomal constituents of activated neutrophil such as bactericidal enzymes and protease, which cause further tissue inflammation and damage upon extracellular release (Da Silveira E Sa et al., 2013). Human red blood cell membrane (HRBC) is analogous to the liposomal membrane and its stabilization implies that the extract may stabilize liposomal membranes. Hypotonicity induced HRBC membrane damage can be taken as an In vitro measure of anti-inflammatory activity of the selected plant extracts (Ballabeni et al., 2010).

In the present study, 10 different concentrations of the lyophilized aqueous extract of flower buds of *C.spinosa* L., seeds of *C.bonducella* L., fruits of *L.acutangula* L., aerial part of *C.occidentalis* L. and selected formulation has been evaluated for anti-inflammatory activity and the data of the results obtained were shown in table 35, 53, 71, 89 and 102.

High concentration (1000 µg/ml) of flower buds of *C.spinosa* L., seeds of *C.bonducella* L., fruits of *L.acutangula* L., aerial part of *C.occidentalis* L. and selected formulation were found to possess maximum anti-inflammatory efficacy, which is comparable to the activity of the standard aspirin. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the anti-inflammatory activity of many plants (Govindappa et al., 2011). This result confirms the test drugs exhibited anti-inflammatory efficacy in a dose dependent manner due to the presence of bioactive compounds such as flavonoids and polyphenols.

### 7.4.3. Anti-bacterial activity

In the present work, four different concentrations of the lyophilized aqueous extract of flower buds of *C.spinosa* L., seeds of *C.bonducella* L., fruits of *L.acutangula* L., aerial part of *C.occidentalis* L. and selected formulation has been evaluated for anti-bacterial activity against food poison causing bacterial pathogens employing disc diffusion method and the data of the results obtained were shown in table 36, 54, 72, 90 and 103.

Maximum anti-bacterial activity exhibited by selected plants and developed Nutraceutical formulation was at a dose level of 1000 µg/ml, which is comparable to that of standard gentamicin. The activity is dose dependent, which has been found to
be increased with an increase in the concentration of the extract. The result of this study indicated that the selected plants and Nutraceutical formulation contain some major compounds that inhibit bacterial growth.

7.5. Toxicity studies

The assumption that medicinal plants are natural and their formulations are safe and must have influenced their indiscriminate use, especially by the local population. Their origin however should not guarantee their safety, since there are no sufficient preclinical, safety and animal toxicity report to support the nutritional or beneficial claims made for many of these herbal products. Several researchers have reported the safety and potential toxicity, as well as risks associated with the use of some vegetable species and herbal products (Agbaire et al., 2013; Withawaskul et al., 2003; Whiting et al., 2002).

Toxicity study is essential for Nutraceutical development process. The preclinical toxicity study on various biological systems reveals the organ and dose specific toxic effects of an investigational product. The toxicity of substances can be observed by (a) In vitro studies (b) In vivo exposure on experimental animals. The preclinical toxicity study is needed to initiate the clinical evaluation of investigational product.

7.5.1. In vitro study

Based on activity of the enzymes (AST, ALT and LDH) in both tissue homogenate and medium, the cytotoxicity of the developed formulation was found to be null. The lipid peroxidation observed in liver tissue based on MDA content is comparable to that of control and standard. Hence all concentrations were noted as safe and they could be used for further study (Table 104, 105 and 106).

7.5.2. In vivo studies

7.5.2.1. Acute oral toxicity study

The investigation of acute oral toxicity is an initial step in the evaluation of the biological effect of any substance. The route of administration depends on the dosage. Based on historical research, the oral route administration is the most convenient and commonly used one for studying acute toxicity. The absorption might be slow, but this method costs less and is painless to the animals (Patricia V. Turner et al., 2011).
Since the extract is administered orally, the animals should be fasted prior to taking the dose because food and other chemicals in the digestive tracts may affect the reaction(s) of the extract.

In acute toxicity study there was no mortality observed at the maximum dose level 2000mg/kg body weight administered orally, which is the single high dose recommended by OECD guidelines 423 for testing acute toxicity. The body weights of the animals (Table 37, 55, 73, 91 and 107) and feed intake (Table 38, 56, 74, 92 and 108) were calculated and are recorded. There were no significant changes in body weight. However, all animals exhibited a normal increment in body weight without drastic difference. Although, the body weights of the entire rats were increased after the oral administration of selected plants and formulation, the growth is not affected.

The general behavioral changes of the rats were observed following oral administration of test samples at 2000 mg/kg doses and were graded through time. The extract did not produce any abnormality in catalepsy, chromodacryorrhea, salivation, polyuria, nasal discharge, dullness, tremors, diarrhea, respiratory distress and coma in all the treated rats during the study period. The internal organs of treated rats did not show any unusual signs and were found to be normal in both size and colour. The results indicated that the test substances treatment by an acute oral route at dose up to 2000 mg/kg did not produce death in 50% of rats during 24 h or 14 days of observation.

According to OECD guidelines (1981) and Kennedy et al., (1986), substances that present LD$_{50}$ higher than 5.00 mg/kg by the oral route can be considered practically non toxic. Therefore, it can be suggested that the acute toxicity of the selected plant extracts and developed formulation is practically null by the oral route. The absence of signs of morbidity and mortality in the oral dose study is probably an indication of the relative safety of the short term administration of the selected formulation.

No toxic symptom or mortality was observed in any animal. All treated animals lived up to 14 days after the administration of test substances. Our test suggested that acute oral administration of selected plants and developed formulation does not cause
any apparent acute toxicity, since there was no sign of toxicity or change in general behavior or other activities of the animals.

7.5.2.2. Sub-acute oral toxicity study

The safety of plant and plants products for human use is essential and can be determined by evaluating their toxicity profile, which is usually carried out in various experimental animal models to predict toxicity and to provide guidelines for selecting a safe dose for humans. Analysis of blood parameters is relevant in risk evaluation as changes in the hematological system have higher predictive value for studies (Olson et al., 2000).

In acute toxicity test, the test samples were found to be nontoxic at the dose level of 2000 mg/kg body weight. The dose selected for the sub-acute toxicity study was 250 mg, 500 mg and 1000 mg/kg b.w. All the animals were free of intoxicating signs throughout the dosing period of 28 days.

In the present study, after sub-acute exposure, the animals were active and responsive to stimuli, with no clinical signs that could be associated with local or systemic toxic effects. There were no deaths and the behavior of animals remained normal for the species. The body weight and food intakes were not altered during the treatment period. This suggests no serious toxic effects as the selected herbal Nutraceutical formulation. However, scientific evidence confirmed that increases or decreases in the body weights are accompanied with accumulation of fats and physiological adaptation responses to the test substances rather than to the toxic effects of chemicals or drugs that lead to decrease appetite and, hence, lower caloric intake by the animal (Arsad et al., 2013). The present results suggest that the oral doses of formulation is non toxic in rats.

The haematopoietic system is one of the most sensitive targets for toxic chemicals and an important index of physiological and pathological status in humans and animals. In this study, the data of the haematological parameter shows that there was statically slight significant increase of neutrophil, HCT and PCT in male rats. There were no changes in any other hematological measures of both male and female groups. These variations were within the normal laboratory range. Increases in these values were not considered as toxicologically relevant. This indicating that the
Biochemical Evaluation and Validation Studies on Herbal Nutraceutical formulation had no toxic effects on the circulating blood cells as well as their production. Blood is the main medium of transport for many nutrients and foreign bodies in the body. Due to this reason, components of the blood such as red blood cells, white blood counts, platelets and haemoglobin are first exposed to significant concentrations of toxic compounds. An analysis of blood parameters is relevant to risk evaluation as the changes in the haematological system have a higher predictive value for human toxicity, when the data are extrapolated from animal studies (Olson et al., 2000). Damage to the blood cells has an adverse effect on the normal functioning of the body, since the administration of selected Nutraceutical formulation do not cause any significant change in the haematological parameters as compared to the controls, the developed formulation can be suggested as non toxic one.

Transaminases (ALT and AST) are good indices of liver damage. Many studies have confirmed that elevated serum levels of hepatic transaminases are not directly linked for liver injury but increased levels are responsible to cause cellular leakage and damage of cell membrane to cells in the liver (Kausar et al., 2010). ALP and bilirubin are most often measured to indicate bileduct obstruction. In this study, there were no deleterious changes found in the levels of transaminases in the plasma of treated animals. The observed significant differences in the plasma levels of total glucose, total protein, albumin, AST, ALT, ALP, total bilirubin, urea and uric acid might be an indication that developed Nutraceutical formulation may have some hepato renal protective properties. Creatinine, uric acid and urea are known to be effective indicators of renal function and any rise in the levels of these parameters indicates a marked renal damage (Gowda et al., 2010). The results from this study however suggest that formulation does not have a negative effect but rather seems to have a protective effect on the kidney. This may probably be an indication that the developed nutraceutical formulation did not interfere with the capacity of the kidney to excrete these metabolites. It is therefore evident that the formulation at doses tested did not cause renal impairment or kidney damage. Nevertheless, further studies are needed to scientifically establish this speculated hepatic and renal protective effect of the formulation. The possible safety of developed nutraceutical formulation was also revealed by the lipid profile. The observed significant changes in the level of total
cholesterol, HDL and triglyceride level could suggest that the developed formulation is non toxic. These observations were further confirmed by the histological assessment of the organs as shown in Figures 44, 45 and 46.

The assessment of pathological changes in the organs such as liver, kidney and brain of treated animals, both macro and microscopically, is the basis for a safety assessment. In this study, there were no pathological features observed in the developed Nutraceutical formulation administered groups. These results proved to be consistent with haematological and biochemical studies, confirming that the developed formulation is safe and non toxic and could be well used for Nutraceutical purposes.