CHAPTER 5

DISCUSSION

Medicinal plants are the richest bio-resource to produce drugs of traditional medicines, modern medicines, nutraceuticals, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube et al., 2008). Medicinal plants maintain the health and vitality of individuals and cure various diseases, including diabetes mellitus without causing toxicity. The mechanism of most of the herbals used to treat diabetes has not been defined (Bailey and Day, 1989).

Diabetes is one of the most prevalent chronic disease in the world affecting nearly 25% of the population. It is one of the oldest disease known to mankind and yet with the tremendous scientific advances witnessed in this century, medical science cannot claim that it knows all that needs to be known about this disease, including its management. Insulin, sulphonylureas and few biguanides are drugs used in the treatment of hyperglycemia in diabetes mellitus. Even insulin therapy does not reinstate a permanent normal pattern of glucose homoestasis and carries an increased risk of atherogenesis and hypoglycemia. World Health Organization has recommended the medicinal plant research warrant attention (Dhar et al., 1968; Hussain, 2002). This is the main reason for the persistent interest all over the world to explore alternative remedies from so-called ‘Alternative systems’ of medicine (Satyavathi et al., 1989).

In recent times many traditionally used medicinally important plants were tested for their antidiabetic potential by various investigators in the experimental animals (Ravi et al., 2000). It has been attributed that the antihyperglycemic effect of these plants is due to their ability to restore the function of pancreatic tissue by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to facilitate metabolites in insulin dependent processes. Hence treatment with herbal drugs has an effect on protecting β-cells and smoothing out fluctuation in glucose levels (Jia et al., 2003 and Elder, 2004).

The results of the present investigation “Phytochemical profile and antidiabetic activity of ethanolic extracts of leaf and fruit of Trichosanthes dioica Roxb. and leaf of Clitoria ternatea L. in streptozotocin induced diabetic rats” are discussed in this chapter.
5.1 PHASE I - Pharmacocochemical characterization of plant materials

5.1.1 Physico-chemical properties

The percentage of physico-chemical properties in crude drugs is mentioned on air dried basis. Moisture facilitates the enzyme hydrolysis or growth of microbes which lead to deterioration of the drugs. Therefore, the loss on drying (LOD) 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. The test for loss on drying determines both water content and volatile matter present in the plant materials (Quality Control Methods for Medicinal Plant Materials, 2002; Kokate et al., 2007).

5.1.1.1 Moisture content

Loss on drying at 110°C is one of the major factors responsible for the deterioration of the drugs and formulations. In the present study, the percentage loss on drying for leaf and fruit of T.dioica and leaf of C.ternatea is below 8% implying that the plants parts can be stored for a long period and will not easily be attacked by microbes. Low moisture content is always desirable for higher stability of drugs.

5.1.1.2 Ash values

The ash value determination is an important parameter to standardize the herbal drugs. The residue remaining after incineration of plant material is the ash content or ash value, which simply represents the amount of inorganic salts. The ash values obtained from the plant tissue is called ‘physiological ash’ as well as from extraneous matter is called ‘non-physiological ash’, which is the residue of the extraneous matter adhering to the plant surface. The determination of the physiological ash and non-physiological ash together is called the total ash determination. The total ash determination is particularly important in the evaluation of purity of drugs, i.e., the presence or absence of foreign organic matters such as metallic salts and/or silica (Ali, 1994).

Total ash may vary within wide limits for specimen of genuine drugs due to the variable natural ash. In such cases, the ash obtained is treated with acid in which most of the natural ash is soluble leaving the silica as acid insoluble ash which represents most of the ash from the contaminating soil. Since the ash values are constant for a given drug,
these values are also one of the diagnostic parameters of the drug (Lalitharani et al., 2013).

Acid insoluble ash is a part of total ash and measures the amount of silica present, especially as sand and siliceous earth. Water soluble ash is the water soluble portion of the total ash. These ash values are important pharmacognostic tool to standardize the crude drugs (Musa et al., 2006 and Kokate et al., 2007).

In the present study, appreciable amount of ash values implies that the plants have a higher organic content and fairly low inorganic content. All the investigated samples have more water soluble ash than acid insoluble ash. It shows that a very small amount of the inorganic component is acid insoluble. These ash values are generally considered as the index of the purity as well as identity of the drug.

5.1.1.3 Extractive values

A successive extractive value reveals the solubility and polarity particulars of the metabolites in the crude drugs. Extractive values are useful for the evaluation of nature of the active phytoconstituents present in the drug especially when the constituents of a drug cannot be readily estimated by any other means (Kokate, 2001).

The water soluble extractive value plays an important role in evaluation of crude drugs. Less extractive value indicates addition of exhausted material to the crude drug, adulteration or incorrect processing during drying or storage. The alcohol soluble extractive value also indicates the same purpose as the water soluble extractive value. The ether soluble extractive value signifies the presence of amounts of fats, lipids and some steroids present in the drug (Ruchi Tripathi et al., 2013).

In the present study, the higher percentage of extractive values of crude drugs in water and ethanol extracts implies that water and ethanol are better solvents for extraction than petroleum ether and chloroform. The extractive percentage clearly indicates that the leaf and fruit of T.dioica and leaf of C.ternatea are best for drug action and effects. The variation in the extractive values may be possible due to the presence of specific compound according to the solubility, soil condition, atmospheric condition and water content of the sample.
5.1.1.4 Crude fiber content

Crude fiber is the fraction of carbohydrate that remains after treatment with acid and alkali. In the present study, substantial amount of the crude fiber content were found in leaf and fruit of *T. dioica* and leaf of *C. ternatea*. A high fiber diet has been proved to work better in controlling diabetes than the diet recommended by the ADA and may control blood sugar levels like oral diabetes drugs (Chandalia *et al*., 2000). Administration of insulin levels were 12% lower in the group eating the high fiber diet compared to the group eating the ADA diet, indicating a beneficial increase in the body’s sensitivity to insulin. High fiber supplements have improved glucose tolerance (Landin *et al*., 1992; Hallfrisch *et al*., 1995). This reveals that one of the factors for antihyperglycemic activity of the leaf and fruit powder of *T. dioica* and leaf powder of *C. ternatea* might be due the fiber content.

Similar studies were made in leaves of *Zanthoxylum armatum* (Kamalesh Upreti *et al*., 2013), roots of *Anogeissus latifolia* (Bhargava *et al*., 2013) and leaves of *Andrographis paniculata* (Burm. F.) Nees. (Meenu Sharma *et al*., 2013).

5.1.2 Fluorescence analysis

Fluorescence analysis is the phenomenon exhibited by various chemical constituents present in the plant material under UV light which can be used to characterize the crude drugs. This analysis is adequately sensitive and enables the precise and accurate determination of the components over a satisfactory concentration range without several time consuming dilution steps prior to other analyses of pharmaceutical samples (Chakravarthy *et al*., 1980; Pimenta *et al*., 2006).

The fluorescent colour is specific for each compound. Different plant materials give different coloration when treated with various chemicals. It acts as a pharmacognostic parameter for identification and standardization of a particular drug from its adulterants. Different crude drugs when viewed under UV light showed different fluorescence at different wavelengths. This is due to the presence of different chemical constituents in the drug (Ansari *et al*., 2006; Reddy and Chaturvedi, 2010).

A correlation exists between a compound present in the drugs and their fluorescent behaviour under different conditions. Coumarin especially hydroxyl amino
acid derivatives like O-coumaric acid appears yellowish green in alkaline condition under short UV radiation. Flavonones which are light yellow in aqueous condition, under UV light, turns to bright yellow under alkaline conditions. Similarly the phytosterols, when treated with 50% H$_2$SO$_4$ shows green fluorescence under UV light. Terpenoids, especially sapogenins, exhibits yellow green fluorescence under short UV light (Harborne, 1976). Quinine, aconitin, berberin and emetin show specific colours of fluorescence (Aconitin - light blue; berberin - light yellow; emetin - orange). Fixed oils and fats show the least fluorescence (Evans, 1996).

In the present study, the fluorescent analysis clearly showed that the powdered leaf and fruit of *T.dioica* and leaf of *C.ternatea* exhibited clear fluorescence behaviours at different radiations. The major bioactive compounds present in the crude drugs were found to be coumarins, flavonoids, tannins, alkaloids, steroids and saponins.

Many researchers have worked out fluorescence analysis of different medicinal plants like *Hygrophila auriculata* (Hussain *et al.*, 2011); *Amorphophallus paeonifolius* Nicols.*var paeonifolius* (Manju madhavan and Regi Raphael, 2012); *Cissus quadrangularis* (Teware Kalpana, 2013) and *Sageretia thea* (Sumaira Shah *et al.*, 2013).

### 5.1.3 Preliminary phytochemical characterization

The phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemo profiling and marker compound analysis (Zade *et al.*, 2013). Presence or absence of certain important bioactive compounds in an extract is determined by colour reactions of the compounds with specific chemicals which act as dyes. This procedure is a simple preliminary pre-requisite before going for detailed phytochemical investigation (Daffodil *et al.*, 2013).

In India traditional communities like tribal and rural populations are frequently using the crude extracts of local plants for medicinal and other purposes. Crude extracts and medicines manufactured on the principles of natural compounds even by pharmaceutical companies, may lead to large scale exposure of humans to natural products. The first step towards this goal is the biological and phytochemical screening of plant extracts from traditional preparations used in popular medicine (Alonso *et al.*, 1995 and Sohni *et al.*, 1995). The phytocompounds are well known to have curative activity
against several human problems such as diuretics, skin diseases (Kirtikar and Basu, 1980), hypercholesterolemia (Govind Sharma and Pant, 1992) and hyperglycemic disorders (Sharmila et al., 2007 and Prashant Kumar Rai et al., 2008) and therefore could suggest the folk use of the medicinal plants.

In the present study, occurrence of wide range of active phytocompounds such as alkaloids, anthraquinone, catechins, coumarins, flavonoids, glycosides, phenols, quinones, saponins, steroids, sugars, tannins and xanthoproteins were seen in methonal and ethanol extracts when compared to other solvents indicated that these compounds were not disturbed during the preparation and storage in the former solvents.

Methanol and ethanol seems to possess high extraction capacity when compared to other solvents. Compared to methanol, ethanol showed stronger extraction capacity. It may be due to the nature of active constituents that are heat labile, but stable in the ethanol (OECD, 2002). Ethanol is one of the good solvents in plant extractions which include low toxicity, easy evaporation at low heat, preservative action, inability to cause the extract to complex or dissociate. Hence the ethanol extracts of leaf and fruit of T. dioica and leaf of C. ternatea were used for further investigation which includes identification of pharmacologically active chemical compounds and quantitative estimation of the above mentioned plants.

Some phytochemists carried out phytochemical analysis of various parts in various plants. A wide range of active compounds like flavonoids, steroids, terpenoids, saponins, tannins, proteins and essential oil were present in ethanolic extract of Artemisia Nilagirica (Devmurari et al., 2010). Whole plant of ethanolic extracts of Vernonia cinerea contained glycosides, esters, flavonoids, steroids, tannins and terpanoids (Sheetal Choudhary et al., 2013).

5.1.4 Quantification of phytochemicals and nutrients

Plants possess potent biochemicals and have been components of phytomedicine. Plant based natural constituents can be derived from any part of the plant. Systemic screening and quantification of these compounds with a purpose of discovering new bioactive compounds is a routine activity in many laboratories (Prashant Tiwari et al., 2011).
5.1.4.1 Phytochemicals or secondary metabolites

In the present examination, flavonoids, total phenolics and tannins were present in considerable quantity in the plant extracts. These secondary metabolites act as major contributors to the antioxidant activity of fruits, vegetables and medicinal plants (Shahidi and Wanasundara, 1992).

Flavonoids act as insulin secretagogues (mimetics) (Geetha et al., 1994). They were known to regenerate the damaged β-cells in the alloxan induced diabetic rats (Chakravarthy et al., 1980). In plants, flavonoids serve as protectors against a wide variety of environmental stresses and are responsible for the radical scavenging effect while, in humans, flavonoids appear to function as “biological response modifiers”. It has been demonstrated to have anti-inflammatory, anti-allergenic, anti-viral, anti-ageing and anti-carcinogenic activity (Hunt et al., 1990; Ceriello et al., 1992; Hunt and Wolff, 1991; Dominguez et al., 1989) and protection against heart diseases through the inhibition of cyclooxygenase activities in platelets and macrophages (Hunt and Wolff, 1991).

The broad therapeutic effects of flavonoids can be largely attributed to their antioxidant properties. It inhibits lipid peroxidation by scavenging free radicals or by other mechanisms such as singlet oxygen quenching, metal ion chelation and lipoxygenase inhibition (Yanishlieva-Maslarova, 2001). Epidemiological studies suggest that the consumption of flavonoid rich foods protects against human diseases associated with oxidative stress like neurodegenerative diseases, diabetes mellitus (Scalbert et al., 2005), platelet aggregation (Daniel et al., 1999), cardiovascular diseases, cancer and osteoporosis (Morton et al., 2000; Shahidi, 2000a; Shui and Leong, 2006).

Phenolic compounds have strong in vitro and in vivo antioxidant activities associated with their ability to scavenge free radicals, break radical chain reactions, chelate metals ions (Shahidi, 2000a) and prevent oxidative damage of lipids and lipoproteins (Arts and Hollman, 2005; Lambert et al., 2005; Joseph et al., 2005). It acts as free radical terminators (Kessler et al., 2003). These compounds are very important plant constituents because their hydroxyl groups confer scavenging ability (Cook and Samman, 1996). In diabetes it suppresses post prandial hyperglycemia and glucose transport across the small intestine (Yoshikawa et al., 1997). The antioxidant activity of phenolics mainly depends on the number and the position of hydrogen donating hydroxyl
groups on the aromatic cycles of the phenolic molecules (Dziedzic and Hudson, 1983; Lien et al., 1999; Rice-Evans et al., 1996).

Many tannin containing drugs are used in medicine as astringent. They are used in the treatment of burns as they precipitate the proteins of exposed tissues to form a protective covering (Handa and Kapoor, 1992). They are also medically used as healing agents in inflammation, leucorrhoea, gonorrhoea, burns and piles and as antidote (Ali, 1994). Tannic acid content of the water extract of Ficus bengalensis has significant hypoglycemic effect on alloxan-induced mild and severe diabetes in rabbits (Gupta et al., 2002). Tannins and flavonoids have antilipoperoxidant activity in ethanolic extracts of Vitis vinifera leaves (Nilufer Sendogdu et al., 2006).

Saponins, a group of natural products, reduces the uptake of certain nutrients including glucose and cholesterol at the gut through intra-luminal physico-chemical interactions and delay glucose transfer from the stomach to the small intestine (Yuan et al., 1998). Hence, it has been reported to have hypocholesterolemic effect and thus may aid lessening metabolic burden that would have been placed in the liver (Price et al., 1987). Alkaloid from stem of Tinospora cordifolia (Patel and Mishra, 2011) and saponin from Anemarrhena asphodeloides (Nakashima et al., 1993) possess hypoglycemic activity.

Hence the presence of the above mentioned phytochemicals in the plant extracts might serve in the prevention of diabetes mellitus along with protection from free radicals produced in the body systems due to various metabolic activities.

5.1.4.2 Nutrients (Total carbohydrates, total proteins and vitamin C)

Diabetes disrupts the mechanism by which the body controls blood sugar. Health professionals have recommended sugar restriction to people with diabetes, even though short term high sugar diets have been shown in some studies, not to cause blood sugar problems in people with diabeties (Abraira and Derler 1988; Colagiuri et al., 1989; Loghmani et al., 1991).

The ADA guidelines do not prohibit the use of moderate amount of sugar, as long as the goal of normalizing blood levels of glucose, triglycerides and cholesterol are being
achieved (American Diabetes Association, 1999). Hence, the carbohydrate present in the ethanolic extracts of leaf and fruit of *T. dioica* and leaf of *C. ternatea* might not cause blood sugar problems in people with diabetes.

The study revealed that the ethanolic extracts of test drugs possess good amount of proteins and also vitamin C. Diabetic patients show reduction in protein content in the body, hence consuming the above mentioned extracts will have additional beneficiary effect and vitamin C also exhibits antioxidant activity. Phytochemicals working together with nutrients and fibers may help to slow the ageing process and reduce the risk of many diseases, including cancer, heart disease, stroke, diabetes mellitus, high blood pressure, cataracts, osteoporosis and urinary tract infections (American Diabetes Association, 1999)

### 5.1.5 HPTLC analysis

HPTLC studies have shown that it is more versatile than ordinary TLC methods, as the spots were well resolved. HPTLC fingerprint analysis can be used as a diagnostic tool for the correct identification of the plant. It is a good estimator of genetic variability in plant populations. The presence or absence of chemical constituent has been found useful in the placement of the plant in taxonomic categories. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. HPTLC fingerprinting is proved to be a linear, precise and accurate method for herbal identification and can be used further in authentication and characterization of the medicinally important plants (Sushma *et al*., 2013).

HPTLC has emerged as an important tool in a wide range of fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis. It is one of the analytical tools to check the presence of adulterants in ayurvedic drugs (Yamuna Devi *et al*., 2012).

In the present study, the HPTLC investigation confirmed the presence of flavonoids, glycosides, saponins, steroids and alkaloids in the ethanolic extracts of leaf and fruit of *T. dioica* and leaf of *C. ternatea*. This could make the investigated plants useful for treating different ailments and a potential for providing useful drugs of human use.
Similar studies were made which revealed that hydroalcoholic extracts of Berberis aristata contained steroidal, phenolic and flavonoidal compounds (Patel et al., 2012) and ethanolic extract of leaf of Pisonea aculeate reported 11 polyvalent phytoconstituents (Mamoon Hussain Syed et al., 2013).

5.1.6 FT-IR spectroscopy analysis

FT-IR spectroscopy is a simple and high resolution method used for identification of different bonds and secondary metabolites functional groups based on the peak values in the region of infrared radiation (Griffiths and de Haseth, 1986; Egwaikidi et al., 2009; Janakiraman et al., 2011).

In this study, the ethanolic extracts of leaf and fruit of T.dioica and leaf of C.ternatea were passed into FT-IR and functional groups of the components were separated based on its peak. The spectra showed substantial overlap of each absorption spectrum of various components, each band represents an overall overlap of some characteristic absorption peaks of functional groups in the samples.

In the current study, the FT-IR analysis of ethanolic extracts of leaf of T.dioica showed different peaks. It confirmed the presence of functional groups such as alcohols, phenolics, alkanes, alkynes, primary alcohols, halogen compounds and sulfonic acids in the ethanol extract of leaf of T.dioica; amines, alkanes, primary alcohols, phenolics, halogen compounds, alkynes, benzene ring, primary amines/ carbon-nitrogen compounds and sulfonic acids in the ethanolic extract of fruit of T.dioica and alcohols, phenolics, primary alcohols, alkanes, alkynes and ketones in the ethanolic extract of leaf of C.ternatea. The above infrared functional group characteristics were cited in literature (John Coates, 2000; Naira Nayeen and Karvekar, 2010).

Many other researchers also worked on FT-IR analysis like Mohammad Nisar et al. (2011) in Rhododendron arboretum and Babu et al. (2013) in Ulva lactuca Linn. Different extracts of Vitex altissima leaf was analysed by FT-IR and confirmed the presence of alcohols, phenols, alkanes, alkynes, alkyl halides, aldehydes, carboxylic acids, aromatics, nitro compounds and amines (Sahaya Sathish et al., 2012).
This current result confirmed the presence of different functional groups in all the three samples, which might be a perfect product for any kind of pharmaceutical applications.

### 5.1.7 GC-MS analysis

GC-MS analysis is one of the initial steps towards understanding the nature of active principles in medicinal plants and to determine whether the plant species contains any individual compound or group of compounds.

The spectrum profile of GC-MS confirmed the presence of major components with their retention time. The heights of the peak indicate the relative concentrations of the components present in the extracts. On comparison of the mass spectra of the constituent with the NIST library, the phytoconstituents were characterized and identified.

In the present study, among the identified phytochemicals in the investigated samples, tetradecanoic acid and n-Hexadecanoic acid, hexadecanoic acid, methyl ester (CAS) and hexa decanoic acid, ethyl ester (CAS) have the antioxidant property and hypocholesterolemic activity. 9, 12-Octadecadienoic acid (Z,Z)- has hypocholesterolemic, hepatoprotective and cancer preventive activity. 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)- (Omega -3-fatty acids) were found to be essential for normal growth and development and may play an important role in the prevention and treatment of diabetes, coronary artery disease, hypertension and cancer. Using Dr. Duke’s phytochemical and ethnobotanical database (online), the biological activity of the identified phytocomponents were ascertained.

Similar observations were reported in methanolic extract of leaf of *Cassia italica* which revealed the presence of 17 compounds (Sermakkani and Thangapandian, 2012), forty nine different phytochemical compounds identified in ethanolic extract of *Maranta arundinacea* L. (Nishaa et al., 2013), fourteen major phytochemical compounds with different therapeutic activities from ethanolic extracts of *Calotropis gigantean* flower (Dhivya and Manimegalai, 2013) and thirteen different phytochemical compounds identified in leaves of *Andrographis paniculata* subjected to GC-MS analysis (Kalaivani et al., 2012).
PHARMACOLOGICAL STUDIES

5.2 PHASE II - *In vitro* antioxidant potential

Several methods have been developed to estimate the antioxidant capacity of different plant materials (Guo et al., 2003). A single assay is not sufficient to evaluate the total antioxidant activity (Frankel and Meyer, 2000; Silva et al., 2006). The *in vitro* antioxidant activity (free radical scavenging activity) of ethanolic extracts of leaf and fruit of *T. dioica* and leaf of *C. ternatea* was evaluated by DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, assay of phosphomolybdenum and reducing power.

5.2.1 DPPH radical scavenging activity

DPPH assay is the most widely reported method for screening antioxidant activity of many plant drugs, based on the reduction of methanolic solution of coloured free radical DPPH by free radical scavenger.

DPPH radical scavenging is considered to be good *in vitro* model widely used to assess antioxidant efficacy of single compound as well as of different plant extracts within a very short period of time (Katalinic et al., 2006). Unlike laboratory generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelating and enzyme inhibition (Yen and Hung, 2000; Mau et al., 2002; Cheung et al., 2003; Amarowicz et al., 2004). In radical form, DPPH disappears on reduction by an antioxidant compound or a radical species to become a stable diamagnetic molecule resulting in the colour change from purple to yellow, due to the formation of diphenyl picryl hydrazine. It could be taken as an indication of the hydrogen donating ability of the extracts (Oktay et al., 2003; Shon et al., 2003; Lee et al., 2007).

In the present study, the percentage of DPPH scavenging effect increases with the concentration of samples in 60 µg to 300 µg in all the samples and the results were in agreement with findings of Khalil et al. (2007). As compared to 60 µg concentrations, 300 µg concentration of leaf (76.71%) and fruit (78.38%) of *T. dioica* and leaf of *C. ternatea* (60.13%) increased DPPH radical scavenging activity. Among the extracts, the leaf extract of *C. ternatea* appeared to have the highest potential for DPPH radical
scavenging activity indicated by the lowest IC$_{50}$ value. The results indicated that the extracts with their proton donating ability, could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants (Marxen et al., 2007).

Similar DPPH radical scavenging activity was reported in water and ethanol extracts of roots of *Cassine transvaalensis* (Mothanka et al., 2008) and methanol extract of *Mitragyna* leaf (Parthasarathy et al., 2009).

**5.2.2 Hydroxyl radical scavenging activity**

Hydroxyl radicals are one of the quick indicators of the lipid peroxidation process by abstracting hydrogen atom from unsaturated fatty acids or simply autooxidation of polyunsaturated fatty acids found primarily in membranes (Kappus, 1991). Scavenging of hydroxyl radicals is an important antioxidant activity, because of its very high reactivity, which can easily cross the cell membranes at specific sites and reacts with most of the biomolecules and furthermore cause tissue damage and cell death. Thus, removing hydroxyl radical is very important task for the protection of living systems (Yang et al., 2012).

In the present study, hydroxyl radical scavenging activity of ethanolic extracts of leaf and fruit of *T.dioica* and leaf of *C.ternatea* showed a dose dependent hydroxyl radical scavenging activity. Out of the five concentrations tested for hydroxyl radical scavenging activity, higher concentrations (240 and 300 µg/ml) have demonstrated good hydroxyl radical scavenging activity. 120 and 180 µg/ml concentrations showed slightly better inhibition than 60 µg concentration. As compared to the values of *T.dioica* leaf and fruit, *C.ternatea* leaf has recorded 23.46% and 15.79% respectively increased hydroxyl radical scavenging activity at 300 µg/ml concentration along with increased inhibition capacity. IC$_{50}$ values of the extracts were more than the standard mannitol.

The values of DPPH radical scavenging activity was more or less equal to the values of hydroxyl radical scavenging activity.

The results of the present study were in close agreement with hydroxyl radical scavenging activity of *Sauropus bacciformis* (Jenecius et al., 2012), *Begonia malabarica*,...
5.2.3 Superoxide radical scavenging activity

Superoxide anions are the most common free radicals in *in vivo*. It is also very harmful to cellular components and produced from molecular oxygen due to oxidative enzymes of body as well as via non-enzymatic reaction such as autooxidation by catecholamines (Naskar et al., 2010).

Superoxide anion is a reduced form of molecular oxygen and is generated in a variety of biological systems. Mitochondria generate energy using a 4-electron transport chain reaction, reducing oxygen to water. Some of the electrons escapes from the chain reaction of mitochondria and directly react with oxygen and form superoxide anion. It plays an important role in the formation of more dangerous reactive oxygen species, including hydrogen peroxide, hydroxyl radical and singlet oxygen that have potential for reacting with biological macromolecules including lipids, proteins and DNA and thereby, inducing tissue damage (Halliwell and Gutteridge, 1984, Halliwell and Chirico, 1993; Lee et al., 2004). The concentration of superoxide anions increase under conditions of oxidative stress (Lee et al., 2002). Overproduction of superoxide anion radical contributes to redox imbalance and associated with harmful physiological consequences (Pervaiz and Clement, 2007).

In the current study, the ethanolic extracts of leaf and fruit of *T.dioica* and leaf of *C.ternatea* were shown to have significant superoxide radical scavenging activity and it was dose dependent. As compared to the values of leaf and fruit of *T.dioica*, *C.ternatea* leaf recorded 30.08% and 23.44% respectively increased superoxide radical scavenging activity at 300 µg/ml concentration. The plant extracts scavenge superoxide radicals by combining with superoxide radical ions to form stable radicals, thus terminating the radical chain reaction (Wang et al., 2009).

The highest antioxidant activity was noted in the extract of *C.ternatea* leaf followed by *T.dioica* fruit and leaf respectively. It might be due to the presence of good amount of phenolics and flavonoids in *C.ternatea* leaf when compared to other two samples. Many researchers have reported close relationship between phenolic content and
antioxidant activity of the plant extracts (Velioglu et al., 1998; Deighton et al., 2000 and Sasikumar et al., 2009). Superoxide radical scavenging activity is increased with increasing flavonoid content (Choudhary and Swarnkar, 2011). The result was in accordance with that of Baccharis grisebachii (Tapia et al., 2004), Calendula officinalis (Preethi et al., 2006) and Cassia siamea and Cassia javanica (Kaur and Arora, 2011).

This study showed correlation between antioxidant activity and flavonoids and phenolic content of the plant extracts. But there are some reports that showed no correlation between antioxidant activity and phenolic content of certain medicinal plants (Saikia and Sristisri Upadhyaya, 2011).

5.2.4 Reducing power assay

In reducing power assay, the presence of antioxidants in the sample reduced Fe\textsuperscript{3+}/ferricyanide complex to the ferrous form. This assay measures the electron-donating capacity of an antioxidant (Yen and Chen, 1995; Hinnenburg et al., 2006). This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and increase in absorbance could indicate an increase in reducing power (Umamaheswari and Chatterjee., 2008; Aderegun et al., 2009).

In the present investigation, a higher absorbance was seen in the samples which indicated a higher reducing power. All the studied plant samples exhibited good reducing activity. But, the ethanolic extract of leaf of C. ternatea was shown to have significant reducing power. The finding was in conformity with the flower of Hypericum perforatum (Fathi and Ebrahimzadeh, 2013) and Hugonia mystax leaf (Rajeswari et al., 2014). It may be suggested that the ethanolic extracts of test plants have high redox potentials and can act as reducing agents.

Higher amounts of reductone, could react with free radicals to stabilise and block radical chain reactions. (Isabel Ferreira et al., 2007). Reductants may serve as a significant indicator of the antioxidant capacity (Yildirim et al., 2000 and Amarowicz et al., 2004).
5.2.5 Total antioxidant capacity

Phosphomolybdenum assay is based on the reduction of Mo (VI) to Mo (V) in the presence of antioxidant compounds and the subsequent formation of a green phosphate Mo (V) complex at acidic pH (Prieto et al., 1999).

In the present analysis, when compared to leaf and fruit of T.dioica, leaf of C.ternatea has 40.75% and 38.84% respectively higher ascorbic acid equivalents. The good antioxidant activity might be attributed to the presence of phytochemicals present in the ethanolic extracts of leaf of C.ternatea followed by fruit and leaf of of T.dioica. The results were in agreement with findings in ethanolic extract of different Selaginella species (Sivaraman et al., 2013) and crude extracts of fresh and dry leaves of Lactuca sativa L. (Rahma Said Salim Al Nomaani et al., 2013).

Phytochemicals have long been recognized to possess many properties including antioxidant, anti-allergic, anti-inflammatory, antiviral, anti-proliferative and anticarcinogenic effects (Eastwood, 1999). They can have complementary and overlapping mechanisms of action in the body including, modulation of detoxification enzymes, delay of the oxidation process, inhibition of the polymerization chain initiated by free radicals and other subsequent oxidizing reactions, stimulation of the immune system and modulation of hormone mechanism (Halliwell and Aruoma, 1991). As plants produce significant amount of antioxidants to prevent the oxidative stress caused by protons and oxygen, they represent a potential source of new compounds with antioxidant activity.

Among the extracts, the leaf extract of C.ternatea appeared to have the highest potential for free radical scavenging activity, hence it may be considered as more potent in vitro antioxidant than other two test samples.

5.3 PHASE III - Acute oral toxicity studies (LD$_{50}$)

Natural products have been used for thousands of years as folk medicine and are promising sources for novel therapeutic agents. They have been used and investigated as promising agents to prevent various diseases. Even though the plants are less toxic and producing very least side effects, it is mandatory to evaluate the toxicity before using as a
medicine. The lethal dosage not only designates the toxic level of a particular extract, it also helps in determining the effective dosage that can be used for the experiment.

In the present study, there was no lethality or any toxic reactions found in the animals at any of the doses selected till the end of investigation period. Hence it may be suggested that the ethanolic extracts of leaf and fruit of *T.dioica* and leaf of *C.ternatea* were non-toxic or safe upto 2000 mg/kg bw.

### 5.4 PHASE IV - Antidiabetic activity

#### 5.4.1 Body weight

Body weight is considered as an indirect index of health status and recovery from disease (Morton and Griffiths, 1985; Calvino *et al.*, 1987). Hyperglycemia is the most critical problems in the diabetes with generally, decrease of body weight as progress of diabetes (Anitha Devi *et al.*, 2003). It is also associated with polyurea, polydypsia, polyphagia and glycosuria (Bastaki, 2005). Weight loss in diabetes mellitus results from a combination of dehydration (caused by frequent urination), increased degradation/breakdown of muscle and adipose tissue proteins for provision of gluconeogenic aminoacids due to unavailability of carbohydrate as energy source (Pepato *et al.*, 1996) and enhanced mobilization of fat stores (provision of FFAs to be used as fuel) (Bastaki, 2005; Smith *et al.*, 2005).

Glycosuria is known to cause a significant loss of calories for every gram of glucose excreted and presumably this loss results in severe weight loss in spite of increased appetite. These events are directly or indirectly related to insulin deficiency or lack of insulin actions (Smith *et al.*, 2005). For this reason, weight reduction is being used as a marker of diabetes mellitus induced by STZ (Ghosh *et al.*, 1994). Therefore, the hypoglycemic as well as body weight maintaining effects have been considered as the essential characteristics of an antidiabetic agent and the efficacy of the herbal extracts has been screened primarily based on these effects.

In the present investigation, the animals treated with individual and combined extracts and the known drug treated groups tend to gain weight. This weight gain was seen from 14th day onwards till end of the treatment period. The progressive weight gain
in the diabetic treated groups showed that severe weight loss was prevented probably due to interaction of several bioactives. This appreciation in weight indicated that the treatment allowed the tissues to access the glucose both to supply energy and spared some to build tissue materials required for growth by decreasing both metabolic rate and glycosuria (Makimattila et al., 1999).

Similar observations were seen in aqueous extracts of bark of *Helicteres isora* L. (Ganesan Kumar et al., 2007) and *Hericium erinaceus* (Bin Liang, 2013) in STZ-induced diabetic rats.

5.4.2 Oral glucose tolerance test (OGTT)

In the present OGTT study, at 3 hrs the blood glucose level was increased to a peak of 67.29% in STZ-induced diabetic rats when compared to normal control rats. The study also indicated that the ethanolic extracts of leaf and fruit of *T.dioica* and leaf of *C.ternatea* treated groups produced a fall in the blood glucose level to 65.33%, 67.41% and 66.6% respectively at 3 hrs when compared to diabetic rats. The extracts might be enhancing glucose utilization, so OGTT was significantly decreased in glucose-loaded rats.

Similar observations were seen in ethanol extract of *Helianthus annuus* L. seeds (Shivani Saini and Sunil Sharma, 2013) and methanol and aqueous extracts of aerial parts of *Sida cordifolia* (Gagandeep Kaur et al., 2013).

5.4.3 Blood glucose level

In the present study, the blood glucose level of the diabetic rats treated with individual and combined ethanolic extracts of leaf and fruit of *T.dioica* and leaf of *C.ternatea* produced a significant reduction when compared with diabetic control rats. *C.ternatea* leaf extract indicated a maximum reduction in blood glucose level nearly to the level of normal control rats at 28th day of treatment. This might be due to the antihyperglycemic activity of the plant extracts.

The antidiabetic effect of ethanolic extracts of leaf and fruit of *T.dioica* and leaf of *C.ternatea* could be due to the possible presence of the aforementioned constituents in the above said part of the plants used in this particular study, which could act
independently or synergistically to enhance the activity of glycolytic and gluconeogenic enzymes.

The different classes of phytochemical compounds isolated from plants including alkaloids (vindoline), flavonoids (epicatechin), tannin (catechin), etc., are documented to have the potential to decrease the blood glucose level (Oubre et al., 1997; Miura et al., 2005; Ragavan and Krishnakumari, 2006). Several authors have reported that flavonoids, sterols/terpenoids and phenolic acids are known to be bioactive antidiabetic principles (Oliver-Bever, 1986 and Rhemann and Zaman, 1989).

Similar study was made by Virendra Singh et al. (2011) in ethanolic extract of *Flacourtia indica* leaf.

5.4.4 Blood glucose, serum insulin, glycosylated hemoglobin, urea and creatinine levels

5.4.4.1 Blood glucose and serum insulin

Insulin is a natural hormone which controls the level of glucose in the blood. In addition to its role of regulating glucose metabolism, insulin also stimulates lipogenesis, diminishes lipolysis, increases aminoacid transport into cells, modulates transcription, altering the cell content of numerous mRNAs, stimulates growth, DNA synthesis and cell replication.

In the present study, the STZ-induced diabetic rats (Group II) elicited significant rise in blood glucose to a level of 62.92% and decreased serum insulin to 68.21% compared to normal control rats (Group I). On the contrary, diabetic rats treated with ethanolic extracts of individual and combined extracts of leaf and fruit of *T. dioica* and leaf of *C. ternatea* and standard drug glibenclamide for 28 days, exhibited decrease in blood glucose and increase in serum insulin levels. It was observed that ethanolic extracts reversed these effects in diabetic animals.

STZ-induced diabetes exhibit most of the diabetic complications mediated through oxidative stress involving in pancreatic cell destruction (Ozturia et al., 1996; Tomlinson et al., 1992). The ethanolic extracts contain phytochemicals such as flavonoids, total phenolics, tannins, glycosides and alkaloids which might have played an
important role in reduction of oxidative stress of pancreatic β-cells. This might have lead to increased glucose metabolism. Another possible mechanism may be by potentiation of the insulin from β-cells of islets of Langerhans or its responsiveness (Padmini and Chakrabati, 1982).

In this context, a number of other plants have also been reported to have antihyperglycemic and insulin-release stimulatory effects of ethanolic extract of fruit of *Terminalia chebula* (Senthilkumar *et al*., 2006), whole plant of *Kyllinga triceps* (Lal *et al*., 2012), hydroalcoholic extract of *Cestrum nocturnum* leaves (Anil Kamboj *et al*., 2013).

**5.4.4.2 Glycosylated hemoglobin**

Glycosylated hemoglobin or HbA$_{1C}$ is produced by abnormal attachment of sugar to hemoglobin. It has been found to be increased over a long period of time in diabetes. The amount of this increase is directly proportional to the fasting blood glucose level (Peters *et al*., 1996). It is formed progressively and irreversibly over a period of time and is stable over the life span of the red blood cells. It is unaffected by diet, insulin or exercise, even on the day of test. Therefore, glycosylated hemoglobin can be used as an excellent marker of overall glycemic control (Bunn *et al*., 1978; Bunn, 1981).

In present study, STZ-induced diabetic rats (Group II) showed significantly increased glycosylated hemoglobin (HbA$_{1C}$) level when compared with normal control rats (Group I). The percentage of HbA$_{1C}$ in normal control groups has shown 3.68 ± 0.11 and the same is elevated nearly three times in STZ-induced diabetic control animals. The significant decrease in the level of glycosylated hemoglobin in STZ-induced diabetic rats following leaf and fruit of *T.dioica*, leaf of *C.ternatea* and standard drug therapy indicated that the overall blood glucose level was controlled, probably due to improvement in insulin secretion. It is noteworthy that the serum insulin level in diabetic animals treated with leaf and fruit of *T.dioica* and leaf of *C.ternatea* also increased when compared to the diabetic control animals.

This result is in agreement with the finding of Alagammal *et al.* (2012c) who have reported that treatment with *Polygala rosmarinifolia* normalized the increased glycosylated hemoglobin level in STZ-induced diabetic rats. In this respect, similar
reports were seen for extracts of Gymnema sylvestre (Shanmugasundaram et al., 1981), Momordica charantia (Cakici et al., 1994) and Enicostemma littorale (Maroo et al., 2002).

5.4.4.3 Urea and Creatinine

Urea is the main end product of protein metabolism. Aminoacid deamination takes place in the liver, which is also the site of urea cycle, where ammonia is converted into urea and excreted through urine. Creatinine is a protein produced by muscle and released into the blood. It is also a waste product formed in muscle by creatinine metabolism. It is synthesized in the liver, passes into the circulation and is taken up almost entirely by skeletal muscle. The creatinine level in the blood is determined by the rate it is being removed, which is roughly a measure of kidney function. Its retention in the blood is evidence of kidney impairment.

In the present study, a significant elevation in markers of renal dysfunction such as urea and creatinine were observed in STZ-induced diabetic rats (Group II) when compared to normal control rats (Group I). After treatment of STZ-diabetic rats with test drugs and glibenclamide reversed urea and creatinine level to near normal.

STZ-induced diabetes in rats have increased the activities of the mitochondrial urea metabolizing enzymes which results in formation of urea in hepatocytes and increase the urea excretion in diabetes (Jorda et al., 1982). STZ can also cause an abnormal glomerular function, marked by reduction in the glomerular filtration rates, which was accompanied by an increase in the serum creatinine level, indicating the induction of acute renal failure. This further confirms the utility of these plants in diabetes associated with renal complications and also authenticate the folk medicinal usage of these plants for kidney disorders. The results were in agreement with finding of methanolic extract of seeds of Cuminum cyminum (Jagtap and Patil, 2010).

Considering above all the parameters, combined extract of T.dioica fruit and C.ternatea leaf and was found to record beneficial effect than other test groups as antidiabetic agent.
5.4.5 Liver marker enzymes

Streptozotocin has a profound effect on the activity of hepatic marker enzymes. The animals treated with STZ developed hepatic damage which is evident from the increase in the enzyme activities (Hwang et al., 2005).

In the present investigation, when rats were exposed to STZ, the activities of the liver marker enzymes were significantly increased when compared to the control group. Also it revealed that the treatment of diabetic animals with individual and combined ethanolic extracts of leaf and fruit of *T. dioica*, leaf of *C. ternatea* and glibenclamide treated groups resulted in reduced the level of SGOT, SGPT, LDH and ALP in the serum of STZ-induced diabetic rats. However combined extracts were found to be better than individual extracts.

The elevated levels of these marker enzymes in diabetic rats may be to increase the concentration of glucose precursors. In other words, the gluconeogenic action of SGOT and SGPT plays the role of providing new supplies of glucose from other sources such as amino acids. Absence of insulin and increased availability of aminoacids were responsible for the increased gluconeogenesis and ketogenesis observed in diabetes (Sivajothi et al., 2007).

The elevated marker enzymes also indicated that diabetes may induce hepatic dysfunction. It has been found that liver was necrotized in diabetic patients (Larcan et al., 1979). Therefore, the increment in the activities of SGOT, SGPT, LDH and ALP in serum may also be due to the leakage of these enzymes from the liver cytosol into the blood stream (Navarro et al., 1993), which gives an indication on the hepatotoxic effect of STZ (Onaolapo and Onaolapo, 2011). On the other hand, treatment of the diabetic rats with ethanolic extracts of plants significantly restored liver marker enzymes level.

The results suggested that test samples possess hepatoprotective capacity due to flavonoids and other components such as saponins, tannins, triterpenes and alkaloids etc. This was in agreement with results of *Brassica oleracea* (Asadujjaman et al., 2011).This also leads to improvement in the carbohydrate, fat and protein metabolism in drug treated groups (Ahmed et al., 2000).
These results were supported by investigations in plants like *Brassica oleracea* (Tawfeq Al-Howiriny, 2008) alcoholic extract of *Momordica charantia*, *Aegle marmelos* and *Eugenia jambolana* (Sundaram *et al.*, 2009) and *Wattakaka volubilis* (Maruthupandian *et al.*, 2010).

### 5.4.6 Total protein, albumin and globulin levels in serum

The levels of serum protein, albumin and globulin were significantly decreased in STZ-induced diabetic rats when compared to control group. This was in agreement with hypoalbuminemia observed in diabetes (Porte and Hatler, 1981). On the other hand, oral administration of ethanolic extracts of leaf and fruit of *T.dioica*, leaf of *C.ternatea* and combined extracts treated diabetic rats never deviated protein metabolism from normal range.

Hypoalbuminemia is a common problem in diabetic animals and is generally attributed in the presence of nephropathy. An overall reduction in serum total protein in diabetic animals and consequent albumin fall were observed in the present study. The reversal of these changes by ethanolic extracts of leaf and fruit of *T.dioica*, leaf of *C.ternatea* therapy proved that insulin deficiency has been grossly corrected. Animals, which received standard drug glibenclamide, also showed the similar result.

These results were in accordance with the effect of *Caseria esculenta* root (Prakasam *et al.*, 2004), *Eugenia singampatiana* bedd leaves (Kala *et al.*, 2012a) and *Polygala rosmarinifolia* (Alagammal *et al.*, 2012c) in diabetic rats.

### 5.4.7 Carbohydrate metabolizing enzymes in liver

Liver is an insulin dependent organ that plays a pivotal role in homeostasis of blood glucose (Arathi and Sachdanandam, 2003). It is the main site for glycolysis, a process where glucose is degraded and gluconeogenesis, where glucose is synthesized from lactate, amino acids and glycerol. These are the two important complementary events that balance the glucose load in our body (Prakasam *et al.*, 2002). Insulin influences the intracellular utilization of glucose in a number of ways. It increases hepatic glycolysis by increasing the activity and amount of several key enzymes including glucokinase, phosphofructokinase and pyruvate kinase (Murray *et al.*, 2000). It may be
important both in the binding of hexokinase to cell membranes, in particular to mitochondria and in the stability of hexokinase Type II (Walters and McLean, 1968).

5.4.7.1 Glucokinase (Hexokinase D)

Hexokinase is universally present in cells of all types. Hepatocytes contain a form of hexokinase called hexokinase D or glucokinase that is more specific for glucose and differ from other forms of hexokinase in kinetic and regulatory properties (Khanna et al., 1981). Glucokinase is the prime and rate limiting enzyme catalyses the phosphorylation of glucose to glucose-6-phosphate in glycolysis and plays a central role in regulation of hepatic glucose storage and disposal (O’Doherty et al., 1999). Following a carbohydrate rich meal, hepatic glucokinase clears a significant amount of glucose from the blood circulation and facilitates its conversion into glycogen and fatty acids (Smith et al., 2005; Agius, 2007). Thus, hepatic glucokinase play a significant role in the prevention of postprandial hyperglycemia.

In the present study, the decreased glucokinase activity in diabetic rats revealed the impairment of the enzyme. As reported by Ragavan and Krishnakumari (2006), insulin stimulates and activates hexokinase activity. Being an insulin-dependent enzyme, the hepatic glucokinase activity of diabetic rats is almost entirely inhibited or inactivated due to the absence of insulin (Gupta et al., 1999). Impairment of glucokinase activity suggests the impaired oxidation of glucose via glycolysis, weakens peripheral glucose utilization and augmented hepatic glucose production, leading to its accumulation, resulting in hyperglycemia (Vestergaard, 1999).

The increased activity of glucokinase in plant extracts and known drug treated groups suggested a shift towards carbohydrate metabolism and it enhanced the utilization of glucose at peripheral sites.

5.4.7.2 Pyruvate kinase

Pyruvate kinase is regulated at the mRNA levels in insulin dependent diabetes (Noguchi et al., 1985). The leaf and fruit of T.dioica and leaf of C.ternatea and glibenclamide treated diabetic rats showed increased activity of pyruvate kinase that may increase the utilization of glucose. The finding suggested that the leaf and fruit of T.dioica
and leaf of *C.ternatea* were improvable to the glucose metabolism by increased utilization of glucose.

### 5.4.7.3 Glucose-6-phosphatase and Fructose-1,6-bisphosphatase

Glucose-6-phosphatase, a crucial gluconeogenic enzyme, is mainly found as an integral protein in the lumen of the endoplasmic reticulum of hepatocytes that catalyzes the dephosphorylation of glucose-6-phosphate to glucose in the liver (Roden and Bernroider, 2003). Fructose-1,6-bisphosphatase is another gluconeogenic enzyme that catalyzes one of the irreversible steps in gluconeogenesis and serves as a site for the regulation of gluconeogenesis (Pilkis and Claus, 1991).

In the present study, activities of these enzymes were increased significantly in diabetic rats which might be due to the activation or increased synthesis of these enzymes contributing to the increased glucose production during diabetes (Baquer *et al.*, 1998). It could also be due to insulin deficiency.

Oral administration of ethanolic extracts of leaf and fruit of *T.dioica*, leaf of *C.ternatea* and combined extracts of *T.dioica* leaf + *C.ternatea* leaf and *T.dioica* fruit + *C.ternatea* leaf reversed the glucose-6-phosphatase and fructose-1,6-bisphosphatase activities in STZ-induced diabetic rats which illustrate improved glycemic control.

As per the study conducted by Ragavan and Krishnakumari (2006), it was revealed that *Terminalia arjuna* bark extract decreased the activity of gluconeogenic enzymes. The same results were also obtained in the present study.

### 5.4.7.4 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase is a key enzyme which catalyses the first and rate limiting step of the hexose monophosphate (HMP) shunt. A decrease in the activity of glucose-6-phosphate dehydrogenase has been observed in diabetic rats. This finding is in conformity with the study of Panneerselvam and Govindaswamy (2002). Treatment with ethanolic extracts of plants increased the activity of the enzyme, via., increased secretion of insulin which increases the influxes of glucose into pentose monophosphate shunt in an attempt to reduce high blood glucose levels. This results in an increased
production of the reducing agent, NADPH, with concomitant decrease in oxidative stress (Ugochukwu and Babady, 2002).

As per the study conducted by Karuna Rasineni (2010), it was revealed that leaf of *Catharanthus roseus* increased the activity of glucose-6-phosphate dehydrogenase. Similar finding was also observed in the present study.

### 5.4.8 Hepatic glycogen metabolism

#### 5.4.8.1 Glycogen, glycogen synthase and glycogen phosphorylase

Glycogen is the primary intracellular storable form of glucose. Diabetes mellitus is known to impair the normal capacity of the liver to synthesize glycogen. The liver glycogen is markedly decreased in diabetic animals (Bollen *et al.*, 1998) which is in proportion to insulin deficiency (Stalmans *et al.*, 1997). The regulation of glycogen metabolism *in vivo* occurs by the multifunctional enzyme glycogen synthase and glycogen phosphorylase (Carabaa *et al.*, 1990).

In the present study, the reduced glycogen store in diabetic rats can be attributed to reduced activity of glycogen synthase and increased activity of glycogen phosphorylase. Treatment of the diabetic rats with experimental plant extracts and glibenclamide elevated the glycogen content and the activity of glycogen synthesizing enzyme in liver.

The glycogen phosphorylase and glycogen synthase are reciprocal in nature. This was well documented in the present study. Treatment of the diabetic rats with experimental plant extracts and glibenclamide decreased the glycogen phosphorylase activity. It revealed that the diabetic condition was alleviated due to treatment with the plant extracts.

The study was supported by Akatsuka *et al.* (1983) and Roesler and Khanderwal, (1986). Similar studies were done in the methanolic root extract of *Salacia chinensis* (Sellamuthu *et al.*, 2009) and aqueous leaf extract of *Talinum triangulare* (Sudha Rani, 2013).
5.5 PHASE V- Antihyperlipidemic activity

5.5.1 Lipid profile

Diabetes mellitus is one of the most common metabolic diseases which lead to derangements in glucose and lipid metabolism (Fumelli et al., 1996). In the post prandial state elevated serum insulin increases lipoprotein lipase activity in adipose tissue and promotes fuel storage as triglycerides in normal metabolism (Bhagavan, 2002). The insulin deficiency in diabetes condition decreases the activity of lipoprotein lipase, thus leading to deranged lipoprotein metabolism (Ranganathan et al., 2000). During diabetes, lipogenesis is decreased while lipolysis is increased in the hepatic tissue, which is the outcome of underutilization of glucose resulting in increased lipolysis and stimulation in the activities of gluconeogenic enzymes (Gupta et al., 1999).

In the present study, serum total cholesterol of STZ-induced diabetic control rats was significantly elevated 48.22% than the normal control rats. The continuous treatment of the different ethanolic extracts of plants for a period of 28 days produced a noteworthy depletion in the serum total cholesterol level when compared to STZ–induced untreated diabetic control rats. The high dose (400 mg/kg bw) of single extract and combined extract treated groups were found to be more effective in lowering the total cholesterol concentration than the low dose of single drug treated groups. The known drug treated group also found to be more effective in the reduction of total cholesterol concentration.

The same trend was seen in the levels of TG, LDL, VLDL and PL whereas HDL level in the untreated diabetic control group have shown significant depletion. On the other hand, both high and low dose of C.ternatea and combined plant extract treated groups were found to be good in restoring back to normalcy and this effect was similar to the known drug treated group.

Excess of fatty acids in serum produced by the STZ-induced diabetes promotes conversion of excess fatty acids into phospholipids and cholesterol in liver. These two substances along with excess triglyceride formed at the same time in liver may be discharge into blood in the form of lipoproteins (Bopanna et al., 1997). The abnormally high concentration of serum lipids in the diabetic subject is mainly due to increase in the mobilization of free fatty acids from the peripheral fat depots, since insulin inhibits the
hormone sensitive lipase (Sharma et al., 1996; Pushparaj et al., 2000). The marked hyperlipidemia that characterizes the diabetic state may therefore, be regarded as a consequence of the uninhibited actions of lipolytic hormones on the fat depots (Goodman and Gilman, 1985).

Hypercholesterolemia and hypertriglyceridemia are independent risk factors that alone or together can accelerate the development of coronary artery disease and progression of atherosclerotic lesions (Mc Kenney, 2001). The hypercholesterolemia is a consequence of accelerated fatty acid oxidation to acetyl CoA which is the primary substrate for cholesterol synthesis (West et al., 1966). Increased plasma cholesterol in diabetic rats may also due to diminishing its clearance from blood. Plasma LDL can undergo reuptake in the liver via specific receptors and get cleared from the circulation. This increase in plasma LDL concentration may be due to defective receptors for LDL. Oxidized LDL is thought to promote atherogenesis by increased lipid peroxidation (Nishigake et al., 1981).

HDL can be protective by reversing cholesterol transport, inhibiting the oxidation of LDL and by neutralizing the atherogeneic effects of oxidized LDL. HDL helps to scavenge cholesterol from extra hepatic tissues (Brewer, 2004). Decreased HDL can contribute to the increased cholesterol levels. A greater increase of LDL may cause a greater decrease of HDL as there is a reciprocal relation between the concentration of LDL and HDL. There is evidence linking increased serum cholesterol to a higher risk for developing coronary heart disease (Glueck et al., 1976). LDL is a major risk factor, whereas HDL is a protective factor for heart diseases. Moreover, HDL is involved in the degradation of cholesterol (Castelli et al., 1986).

Insulin has a potent inhibitory effect on lipolysis in adipocytes, insulin deficiency is associated with excess lipolysis and increased influx of free fatty acids to the liver. The increased levels of low-density lipoprotein (LDL) and very low density lipoprotein (VLDL) in the diabetic animals might be due to stimulation of hepatic triglyceride synthesis as a result of free fatty acid influx (Coppack, 1994). Phospholipids are present in cell membrane and make up vast majority of the surface lipoprotein forming a lipid bilayer that acts as an interface with both polar plasma environment and non-polar lipoprotein of lipoprotein core (Cohn and Roth, 1996).
Previous studies have reported that some of the phytocomponents like flavonoids, saponins, alkaloids and tannins elicit a wide range of biological activities which include hypoglycemic, hypolipidemia, hypoazotemia among others. Specifically, saponin is known to elicit serum cholesterol lowering activity by causing resin-like action, thereby reducing the enterohepatic circulation of bile acids (Topping et al., 1980). In the process, the conversion of cholesterol to bile acid is enhanced in the liver resulting in concomitant hypocholesterolemia (Kritchevsky, 1977; Potter et al., 1979). Also saponin reduces the uptake of certain nutrients including glucose and cholesterol at the gut through intraluminal physicochemical reaction. Hence, it has been reported to have hypocholesterotemic effect and thus may aid lessening metabolic burden that would have been placed in the liver (Rajan et al., 2012).

The presence of these phytocomponents in the ethanolic extracts of leaf and fruit of T.dioica and leaf of C.ternatea in high concentrations could account for these observed biological effects, particularly hypoglycemic and hypolipidemic effects. Moreover its antihyperlipidemic effect could represent a protective mechanism against the development of atherosclerosis.

Similar observations were seen in Phyllantus niruri extract (Nwanjo et al., 2007) and Ficus bengalensis leaf extract (Manikandaselvi et al., 2012).

5.6 PHASE VI - *In vivo* antioxidant activity

5.6.1 Lipid peroxidation

Lipid peroxidation is a highly destructive process and induces alteration in structure and functions of cellular membranes (Kale and Sitaswad, 1990). It is measured by thiobarbituric acid reactive substance (TBARS), which is an index of MDA production.

The results of the present study, showed increased lipid peroxidation (LPO) on serum, liver and kidney of STZ-induced diabetic rats. Earlier studies have confirmed that there is an increased lipid peroxidation in liver, kidney and brain of diabetic rats (Latha and Pari, 2003a; Ananthan et al., 2004). This may be due to relatively high concentration
of early peroxidizable fatty acids contained by the tissues. It has been proved that hyperglycemia generates oxidative stress leading to the development of diabetic complications (Baynes, 1991). The increased lipid peroxidation in the diabetic animals may be due to the observed remarkable increase in the concentration of TBARS and hydroperoxides in the liver and kidney of diabetic rats (Stanely Mainzen Prince and Menon, 2001).

Hyperglycemia results in free radical formation through various biochemical reactions. The tremendous increase in LPO was observed in diabetic rats is attributed to chronic hyperglycemia. It causes increased production of ROS due to autooxidation of monosaccharides, which leads to the production of superoxide and hydroxyl radicals. This, in turn, causes tissue damage by reacting with PUFAs in membrane (Wolff and Dean, 1987; Das et al., 2000). In diabetes, hypoinsulinaemia increases the activity of the enzyme, fatty acyl coenzyme A oxidase, which initiates β-oxidation of fatty acids, also results in lipid peroxidation (Oberley, 1988; Baynes, 1995).

Increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors (Baynes, 1995). Peroxidation of membrane lipids associated with increased membrane rigidity and reduced cells survival has been implicated in diabetes mellitus (Selvam and Anuradha, 1988).

In the present study, the levels of LPO was found significantly reduced after the supplementation with the ethanolic extracts of *T. dioica* (leaf and fruit) and *C. ternatea* (leaf) and glibenclamide. This indicated that plants extracts inhibited oxidative damage due to the antiperoxidative effect of ingredients present in them.

This could be correlated with the previous studies of Prince and Menon (1998) and Prince *et al.* (2004) on *Syzigium cuminii*, Pari and Latha (2002) on *Cassia auriculanta* flower and Latha and Pari (2003b) on *Scoparia dulcis* indicating anti-peroxidative and anti-hyperlipidemic effects in diabetic animals.
5.6.2 Enzymatic antioxidant activities

The antioxidant systems were composed of enzymatic and non-enzymatic antioxidants. There are three main antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

5.6.2.1 Superoxide dismutase

Superoxide dismutase has been postulated as one of the most important enzymes in the enzymatic antioxidant defense system which catalyses the dismutation of superoxide radicals to produce $\text{H}_2\text{O}_2$ and molecular oxygen which can be rapidly converted to water by CAT and GPx, thereby diminishing the toxic effects caused by the radicals (Winterbourn, 1995).

In the present study, a significant decline in the activity of SOD in serum and tissues of STZ-induced diabetic rats was found when compared with control rats.

STZ-induced hyperglycemia provoked the generation of superoxide and hydroxyl radicals, this induced various injuries in surrounding organs and plays an important role in several clinical disorders. The decrease in SOD activity in diabetic control rats could result from inactivation by $\text{H}_2\text{O}_2$ or by glycation of the enzyme or by induced oxidative stress which in turn causes lipid peroxidation (Sozmen et al., 2001; Vincent et al., 2004; Kaleem et al., 2006).

5.6.2.2 Catalase

Catalase is an enzymatic antioxidant widely distributed in all animal tissues and its highest activity is found in the red cells and in liver (Chance et al., 1992). It catalyzes the decomposition of $\text{H}_2\text{O}_2$ generated by the activity of SOD into less reactive gaseous oxygen and water molecule (Gaetani et al., 1996). Therefore, the reduction in the activity of this enzyme may result in a number of deleterious effects.

In the present study, a significant reduction in the activity of CAT in serum and tissues of diabetic control rats suggested that there is an increased endogenous production of superoxide radicals and hydrogen peroxides which could inactivate and reduce the enzyme level (Wohaieb and Godin, 1987). It could also be due to glycation of enzyme (Yan and Harding, 1999). $\text{H}_2\text{O}_2$ is toxic by itself and can be a precursor of other toxic
species. It can react with \( \text{O}_2^- \) to form ‘OH and results in increased lipid peroxidation and hence higher TBARS level (Kono and Fridovich, 1982). The ‘OH radical is also thought to be the primary reactive molecule in the redox activation of enzymes (Salahudeen, 1995).

5.6.2.3 Glutathione peroxidase

Glutathione peroxidase is a general name of enzyme family with peroxidase activity whose main biological role is to protect the organisms from oxidative damage. It plays a primary role in the reduction of \( \text{H}_2\text{O}_2 \) in the presence of GSH-forming oxidized glutathione, (GSSG) and other hydroperoxides including lipid peroxides, thereby protecting cell protein and membrane from oxidative stress (Jacob, 1995).

GPx activity is also reduced in diabetic condition. \( \text{O}_2^- \) reacts with selenium at the active site of GPx and thereby inactivating the enzyme (Blum and Fridorich, 1985). It might be also due to insufficient availability of reduced glutathione (Illing et al., 1991).

The plant extracts and glibenclamide treated groups (Group III – Group XI) demonstrated a significant increase in the serum, hepatic and kidney SOD, CAT and GPx activities. Any compound with rich antioxidant properties, might contribute towards the partial or total alleviation of organ damage. Therefore, removal of superoxide and hydroxyl radicals were probably one of the effective defenses of a living body against diseases (Pushparaj et al., 2000).

The antioxidant property was evidently exhibited in the ethanolic extracts of leaf and fruit of \( T.dioica \) and leaf of \( C.ternatea \) against oxygen free radicals. This means that the flavonoids, glycosides and phenolic compounds present in the plant extracts might have reduced the glycation of enzymes, reduced the oxidative stress (free radicals scavenging activity), detoxified the endogenous metabolic peroxides generated by STZ, or they may reduce free radical related diseases and improve the activities of the antioxidant enzymes. Glibenclamide also showed increase in antioxidant enzymes which was due to its hypoglycemic effects.
In the present study, increased activities of SOD, CAT and GPx in the serum, liver and kidney of diabetic rats confirm that the investigated drugs do extend a clear protection against LPO in STZ-induced diabetic rats.

The observation of the present study were supported by the studies in methanolic extracts of *Allium sativum*, *Allium ascalonicum* and *Salvia officinalis* (Mohammad Fehresti Sani *et al.*, 2012), whole plant of *Polygala rosmarinifolia* (Nishanthini and Mohan, 2012), fruit of *Calamus erectus* and whole plant of *Polygala rosmarinifolia* (Mitali Ghosal and Palash Mandal, 2013) and aqueous extract of *Cassia sophera* dried seeds (Rambir Singh *et al.*, 2013).

5.6.3 Non-enzymic antioxidants

Non-enzymic antioxidants such as reduced glutathione (GSH), vitamin C, E and A play an excellent role in protecting the cells from oxidative damage (Anuradha and Selvam, 1993).

5.6.3.1 Reduced glutathione

Reduced glutathione acts as primary line of defence to cope with the deleterious effects of oxygen free radical species (Bradley and Nathan, 1984). It is a major endogenous non-enzymic antioxidant which counter balance free radicals mediated damage. It has a multifaceted role in antioxidant defence system. It is a direct scavenger of free radicals as well as a co-substrate for peroxide detoxification (Winterbourn, 1995). It is well established that GSH is involved in the protection of normal cells and tissue structure and function by maintaining the redox homeostasis, quenching of free radicals, participation in detoxification of xenobiotic reactions, regulation of immune function and keeps up the cellular levels of the active forms of vitamin C and E by neutralizing the free radicals (Robak and Marcinkiewicz, 1995).

The decreased levels of GSH in the serum, liver and kidney of STZ-induced diabetic rats were due to chronic oxidative stress seen in diabetic condition. GSH protects the cells against oxidative stress by reacting with peroxides and hydroperoxides. Decreased activity of GSH is due to decrease GSH formation which requires NADPH and glutathione reductase (Garg *et al.*, 1996). Maintenance of NADPH/NADP⁺ ratio plays a crucial role in the regeneration of GSH from GSSG (Jain, 1998). GSH is also used by
aldose reductase for the reduction of glucose to sorbitol through the polyol pathway. The competition for NADPH could be responsible for the decreased glutathione levels found in diabetes mellitus (De Mattia et al., 1994).

A significant elevation of serum, liver and kidney GSH level were observed in the extracts treated diabetic rats (Group III – Group X). This indicated that the extracts can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH or have both effects.

Administration of ethanolic extracts of leaf and fruit of *T. dioica* and leaf of *C. ternatea*, combined leaf extracts of *T. dioica* + *C. ternatea* and fruit extract of *T. dioica* + leaf extract of *C. ternatea* to the diabetic rats, maintained the levels of non-enzymic antioxidants to near normal by improving the GSH status in serum, liver and kidney. The results of the present study were in parallel with the finding in *Emblica officinalis* fruit (Khandelwal et al., 2002) and *Casearia esculenta* root (Prakasam et al., 2005). They suggested that dietary antioxidants in general, act by removing ROS before they have a chance to cause damage to biological molecules.

### 5.6.3.2 Vitamin E

Non-enzymic antioxidant like reduced glutathione, ascorbate and α-tocopherol play an excellent role in protecting the cells from oxidative damage (Anuradha and Selvam, 1993). All these act synergistically as cellular antioxidants. The most important antioxidant in the cell membrane is α-tocopherol (Santhakumari et al., 2003). This molecule is known as chain breaking antioxidant because its function is to intercept lipid peroxyl radicals (LOO•) and so terminate lipid peroxidation chain reactions.

The resultant radical is relatively stable and in normal circumstances, insufficiently reactive to initiate lipid peroxidation itself. This is an essential criterion of a good antioxidant thus protecting cell structures against damage. It helps to build normal and red blood cells as well as working as an antioxidant.

In the present study, the level of Vitamin E is lower in diabetic rats which represented a increased utilization of the vitamin due to oxidative stress in diabetes. This vitamin exists in interconvertible (reduction and oxidized) form. Thus reduction in the
level of antioxidant vitamin E can be attributed to reduced regeneration from their oxidised form. People with diabetes may also have greater antioxidant requirements because of increased free radical production with hyperglycemia (Konig et al., 1998; Jouad et al., 2000).

Elevated levels of vitamin E in experimental groups documented that it prevented the destructive damage that may occur in diabetes. It also may be effective in reducing glycosylation (Ceriello et al., 1992; Duntas et al., 1996; Jain et al., 1996; Jain et al., 2000). Vitamin E supplementation may protect against diabetic retinopathy, nephropathy and stroke (Leppala et al., 2000). It decreases the requirement of insulin by diabetic people (Manning et al., 2004).

5.6.3.3 Vitamin C

Ascorbic acid is known to act as an antioxidant both \textit{in vivo} and \textit{in vitro}. It functions as a free radical scavenger and successfully prevents detectable oxidative damage under all types of oxidative stress. It plays an important role in detoxification of reactive intermediates produced by cytochrome \text{P}_{450}, which detoxify xenobiotics.

Reduction in tissue ascorbic acid was observed in STZ-induced diabetic rats. The decrease could have been due to increased utilization of ascorbic acid as an antioxidant defense against increased reactive oxygen species or to a decrease in the GSH level, since GSH is required for the recycling of ascorbic acid (Hunt, 1996).

The significant increase of vitamin C in ethanolic extracts and glibenclamide treated groups when compared to diabetic control might be due to the potent antioxidant effect of the plant extracts. It can protect cell membranes and lipoprotein particles from the oxidative damage by regenerating the antioxidant form of vitamin E. Vitamin C and E act synergistically in scavenging a wide variety of ROS (Senthilkumar and Jeyaprakash, 2012).

5.6.3.4 Vitamin A

The total carotenoids (Vitamin A) have been shown to inhibit tissue lipid peroxidation (Kartha and Krishnamurthy, 1977; Kartha and Krishnamurthy, 1978). Vitamin A supplementation, increases the activity of antioxidant enzymes catalase and
SOD (Helen and Vijayammal, 1997). Beta-carotene and other carotenoids are also believed to provide antioxidant protection to lipid rich tissues. Research suggests beta carotene may work synergistically with vitamin E (Suzuki *et al*., 2002).

In the present study, the decreased level of vitamin A observed in untreated diabetic groups might be due to the liberation of lipid peroxide. It is known to be an important natural antioxidant capable of counteracting oxygen free radicals and exerts a protective effect of antioxidant (Wang *et al*., 2008).

Increased levels of vitamin A in plant extracts treated rats might be due to decreased levels of lipid peroxides. This can be attributed to the free radical scavenging potential of the plant extracts shown by the *in vitro* analysis of the study. The reference drug glibenclamide treated diabetic group also showed increased levels of non-enzymic antioxidants. The results are in accordance with the study in aqueous extract of root of *Casearia esculenta* (Prakasam *et al*., 2005).

### 5.7 PHASE VII - Histopathological studies

#### 5.7.1 Histopathological studies in pancreatic tissues

One of the major findings of this study is that the histopathological investigation along with the biochemical evaluations demonstrated the possibility of the pancreatic tissue regeneration upon combined extract treatment. The regeneration of the pancreas of the STZ destructed islets is probably due to the fact that pancreas contains few stable cells which have the capacity of regeneration. In this regard, both the plant extracts may contain progenitors cell which may be mobilize into injured pancreatic tissue. On the other hand, progenitor cells may be participating in this repair mechanism. However, the source and nature of these progenitor cells was not determined in this study.

The findings of histopathological investigations in liver and kidney revealed a normal cellular architecture in normal control group. Cellular necrosis in liver and congestion of convoluted tubules along with dearranged glomeruli in kidney were noticed in the diabetic control group. Combined plant extracts treated diabetic groups presented a pattern similar to normal control group however individual extracts revealed the same pattern with some modifications.
In the present findings, taken together with other biochemical results may support the hypothesis that the tissues are made by the combined plant extract. Hence it may concluded that combined plant extract more effectively stimulate tissue repair than the other individual plant extract treatment and may be clinically beneficial as an agent to restore or maintain tissues after injury. The results of this study may encourage clinical studies to evaluate the potential benefit of combined plant extract administration.

From the above results it can be said that the combined extract of fruit of *T.dioica* and leaf of *C.ternatea* possess good quality of phytochemicals and significant antidiabetic, antihyperlipidemic and antioxidant activities.