5.1 PHYTOCHEMICAL SCREENING AND STANDARDIZATION

Standardization has been defined as the best technical application consensual wisdom inclusive of processes for selection in making appropriate choices for ratification coupled with consistent decisions for maintaining obtained standards (Pandey and Tripathi, 2014). Plant quality is an important determinant of the phytochemical constituents and invariably the biological activities of an extract. These factors depend on plant part used, genetic variation, geographical location, climatic conditions, collection period, drying methods, and storage conditions. In the present work, the total ash value of plant material is an indicator of the amount of minerals and earthy material attached to the plant; the presence of sugar, acids, and inorganic compounds is indicated by water soluble ash value; and the acid insoluble ash values indicate the presence of polar constituents. Determination of loss on drying is for getting an idea about the moisture content in the plant material; less value of moisture content could prevent bacterial, fungal, and yeast growth.

Secondary metabolites are the biologically active compounds that are non-nutritive to the plants, but provide protective or disease deterrent properties to them. These chemicals are produced by the plants to protect their selves from various diseases (Mazid et al., 2011; Murugesan et al., 2016). The recent research has demonstrated that many of these secondary metabolites show promising action against human diseases. The secondary metabolites include alkaloids, tannins, saponins, terpenoids, flavonoids, phenolics, cardiac glycosides etc. (Erum et al., 2015; Mudasar et al., 2016). These secondary metabolites, also known as phytochemicals have been shown to produce potent antioxidant, anti-microbial, anti-inflammatory, antihepatotoxic, anticancer, antihyperlipidemic, anti-diabetic effects which might be achieved through the regulation of enzymatic reactions and hormone action, gene expression, immune response, cell division, cellular transport and a series of other physiological processes (Katalinic et al., 2004; Mulabagal and Tsay, 2004; Karsha and Borneo, 2008; Lakshmi, 2010).

The preliminary phytochemical screening showed that the plant Eremurus himalaicus contained alkaloids, tannins, saponins, terpenoids, flavonoids, phenolics and
cardiac glycosides. The presence of these biologically active phytochemicals may be responsible for imparting various pharmacological properties to the plant (Singh et al., 2002; Agarwal and Rangari, 2003; Mbagwu et al., 2007; Narendhirakannan et al., 2007).

### 5.2 ANTIOXIDANT ACTIVITY

Total phenolics and flavonoids content, and *in vitro* antioxidant activity of ethyl acetate, methanol and aqueous extracts of *Eremurus himalaicus* were determined. Various solvents were used for extraction with increasing polarity which has been proven to be effective in earlier studies (Stankovic et al., 2010). The phytocompounds like flavonoids and phenolics exhibit various biological activities, one of the most important activities being the antioxidant activity (Adil et al., 2015). They directly or indirectly scavenge the free radicals through a series of coupled reactions (Lewis et al., 1993). The antioxidant activity exhibited by flavonoid and phenolic compounds may be due to the presence of hydroxyl groups which confers the free radical scavenging capacity to them (Naczk et al., 2004). In our study, the extracts of *Eremurus himalaicus* showed the highest phenolics content in EHM followed by EHA and EHE. However, the flavonoids content was highest in EHA followed by EHM. The flavonoids content in EHE was very less. From this we can conclude that the methanolic and aqueous extracts possess a good amount of phenolics and flavonoids which confers antioxidant potential to these extracts.

DPPH assay is one of the most widely used methods for screening antioxidant activity. DPPH shows a strong absorption band at 517 nm in the visible spectrum (deep violet color). The color changes to yellow due to the scavenging of DPPH radicals by antioxidant compounds via donation of hydrogen atom (Ambardekar et al., 2009). The absorption decreases and coincides with the number of electrons taken up. The maximum antioxidant potential in this study was observed for EHM which was followed by the other two extracts i.e., EHA and EHE respectively. The results were compared with the reference sample BHA and V<sub>c</sub>.

The scavenging of DPPH radicals can be correlated with the number of available hydroxyl groups. Thus, from the results we can infer that the antioxidant
activity of EHM may be due to the presence of molecules with an available hydroxyl group.

$H_2O_2$ is a molecule that causes oxidative stress to cells. It is produced by dismutation of superoxide anion by univalent reduction. It is highly important because of its ability to penetrate biological membranes. It causes breaking up of DNA, resulting in single strand breaks and formation of DNA-protein cross-links (Singh and Singh, 2008). Hence, the elimination of $H_2O_2$ is very essential for protecting the in vivo systems. In Hydrogen peroxide scavenging assay the activity is assessed on the basis of decrease in absorbance caused by consumption of $H_2O_2$ by antioxidant moieties. Thus, we can say that EHM has highest $H_2O_2$ scavenging potential followed by EHA and EHE respectively.

The total reduction capability assay tests the capability of extracts to convert potassium ferricyanide ($Fe^{3+}$) complex to potassium ferrocyanide ($Fe^{2+}$) complex, which then reacts with ferric chloride to form ferric-ferrous complex which absorbs maximally at 700nm. Thus, the measurement of increase in color leads to the estimation of the total reduction capability or antioxidant potential of the antioxidant compound (Chung et al., 2002; Gulcin et al., 2010). The results for this assay revealed that the extracts of *Eremurus himalaicus* lead to the formation of potassium ferrocyanide ($Fe^{2+}$) complex. The activity was found to be concentration dependent and the results ascertained that the extracts of *Eremurus himalaicus* possess moderate reducing capacity with EHM having the highest potential.

### 5.3 ANTIMICROBIAL ACTIVITY

Infectious diseases are one of the top reasons of mortalities in the World (McNeil et al., 2001; WHO, 2002). In spite of the recent advances in the field of microbiology, humans fall prey to a number of microbial infections. A large spectrum of drugs is available in the market for their treatment; however, the development of drug resistance has become a huge hurdle in the treatment of these diseases and the need for new drugs has increased enormously (Giamarellou, 2010; Marasini et al., 2015).

A number of reports are available on the antibacterial and antifungal properties of plants (Samy and Ignacimuthu, 2000; Palombo and Semple, 2001;
It has been reported that the antimicrobial activity reflects the constituents of the plant extracts (Hegazi, 1998; Hegazi, 2000). Flavonoids and phenolics are mostly found to be responsible for the antimicrobial activity of plants (Hemandez et al., 1990; Sforcin et al., 2000). However, many plants have shown similar antimicrobial activity with different phytocompound combinations (Kujumgiev et al., 1999). Different plant secondary metabolites exercise various mechanisms in order to exert antimicrobial activity e.g., some secondary metabolites increase the permeability of the inner membrane, destroy the membrane structure and inhibit membrane embedded enzymes, some may decrease the ATP production through loss of membrane potential and cause energy depletion, some may cause inhibition of the enzyme DNA gyrase which is the bacterial enzyme for nucleotide synthesis, others may boost immune-modulator organs and cause killing of the microorganisms, while others may cause inhibition of protein synthesis by formation of irreversible complexes with proline-rich proteins (Cox et al., 2000; Tsuchiya and Inuma, 2000; Cox et al., 2001). Thus, the plant secondary metabolites can be very useful for the development of new drugs in the treatment of microbial infections.

In the present study, antimicrobial activity of various extracts of *Eremurus himalaicus* was evaluated against six bacterial and four fungal strains. The antimicrobial efficacy was determined using agar diffusion method. In the present study, the inhibition of growth of bacteria by EHE and EHM was seen at high concentrations only. The antifungal activity was also shown by EHE at high concentrations only. The possibility for the limited antibacterial potency may be due to soxhlet extraction method and use of crude extracts (Prashant et al., 2011). Instead of it, percolation extraction, subfraction, semipure compound, or pure compounds isolated from these plants might exhibit better antibacterial activity.

5.4 ACUTE TOXICITY STUDY

The plant bioactive constituents may have positive as well as negative physiological effects on living organisms. Thus, in order to ascertain the nontoxic effect
Discussion of the plants, toxicity testing is done prior to the experimental elucidation of biological activities in test organisms (Aneela et al., 2011). Toxicity testing provides knowledge about the extent to which the bioactive compounds present in the plant can harm the organism (Rajalakshmi et al., 2014). The assessment of toxicity can be done by two different methods; acute toxicity testing and chronic toxicity testing. In acute toxicity testing the harmful effects of the test substance are assessed by exposing the organism to a single dose or multiple doses of the test substance in a small period of time (Rajalakshmi et al., 2014). In chronic toxicity testing the exposure to the test substance is given repetitively for a longer duration of time (Sireeratawong et al., 2016). In the present study, acute toxicity testing was performed and the results revealed that this plant was non-toxic to the experimental animals at the highest dose tested (2000 mg kg\(^{-1}\)bw), proving that the plant could be used for further in-vivo studies.

5.5 ANTI-INFLAMMATORY ACTIVITY

The assessment of anti-inflammatory activity of various extracts from *Eremurus himalaicus* was done by two methods, initially by carrageenan induced rat paw edema test and secondarily by croton oil induced mouse ear edema. Carageenan induced rat paw edema is one of the widely used methods in screening of anti-inflammatory activity of plants (Mascolo et al., 1987; Segura et al., 1998).

The croton oil induced mouse ear edema test comes with some advantages; firstly, in this model only a small amount of test substance is needed and secondly, only the skin of the ear is involved, which prevents its metabolism and excretion in the body (Jacobs et al., 1985; Segura et al., 1998). Carrageenan induced inflammation is generally measured by measuring the increase in paw size of the rats at different time intervals after the sub-plantar injection of carrageenan. Carrageenan induced rat paw edema for acute inflammation is considered to be biphasic. The initial phase which includes first 1-2 h is mediated through the release of histamine, serotonin and increase in the synthesis of prostaglandins and in the second phase the prostaglandins are released and inflammation is mediated by bradykinin, leukotrienes, polymorphonuclear leukocytes and prostaglandins (Kavimani et al., 2000; Gupta et al., 2006; Nivsarkar et al., 2009; Marlous et al., 2014; Chatterjee et al., 2015; Kapewangolo et al., 2015).
Nonsteroidal anti-inflammatory drugs (NSAID) are known to reduce swelling caused by an inflammatory stimulus (Otterness and Bliven, 1985; Mohsin and Kurup, 2011). In the present study, the standard drug indomethacin and EHM significantly inhibited the edema formation, induced by carrageenan, in rats. However, indomethacin showed the anti-edematous effect in both first and second phases, whereas EHM showed anti-edematous effect in the second phase only. The anti-edematous activity shown by indomethacin in the first phase is likely due to the inhibition of histamine receptor and histamine signaling and also the gene transcriptions of histidine decarboxylase gene (Tripathi et al., 1979; Baruah et al., 2000; Shashidhara et al., 2008; Nurul et al., 2011). The anti-edematous effect in the second phase shown by indomethacin and EHM and also by EHA to some extent can be attributed to inhibition of the release of prostaglandin and kinin (Pramanik et al., 2005).

Croton oil single application test is widely used in the study of anti-inflammatory activity of plants in case of acute inflammation (Stanley et al., 1991). The inflammatory response generated by croton oil is due to the presence of 12-o-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters. The mechanism by which TPA leads to acute inflammatory response is that TPA causes the activation of Protein Kinase–C (PK–C). The activated PK–C causes activation of various enzymatic cascades which in turn lead to the increase in vascular permeability and vasodilation, cause migration of polymorphonuclear leukocytes and release of histamine and serotonin. It also leads to the activation of cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes, which are responsible for the synthesis of prostaglandins (Ferrandiz et al., 1996; Wang et al., 2001; Murakawa et al., 2006). In croton oil-induced mouse ear edema prostaglandins, histamine and serotonin are known to be the most important mediators of inflammation (Chen et al., 1994).

The inhibition of COX pathway by NSAID is responsible for a decrease in the production of prostaglandins which are the major components involved in the process of inflammation (Szolcsanyi, 1988; Natalia et al., 2014). In the present study, the standard drug indomethacin and EHM efficiently reversed the edema induced by the topical application of croton oil, which could be suggested to be due to nonselective
inhibition of the COX isoforms (COX-1 and COX-2), as described by Gabor (2000) and Carlson et al. (1985).

Different researchers have demonstrated that the secondary metabolites found in the plants like phenolics, flavonoids, etc. possesses anti-inflammatory activity (Arslan et al., 2010; Sannigrahi et al., 2011). It has been reported that flavonoids inhibit the enzyme prostaglandin synthetase and thus produce anti-inflammatory effect (Chatterjee et al., 2015). Hence, it is assumed that the anti-inflammatory potential of Eremurus himalaicus could be due to the presence of flavonoids, phenolics and other secondary metabolites.

5.5 ANTI-HYPERLIPIDEMIC ACTIVITY

An increased level of lipids in the blood is termed as hyperlipidemia. It is characterized by elevated level of Triglycerides (TG), Total cholesterol (TC), Low density lipoprotein cholesterol (LDL-C), and very low density lipoprotein cholesterol (VLDL-C) and decreased levels of high density lipoprotein cholesterol (HDL-C) in the blood (Kaliora et al., 2006). Hyperlipidemia characterized by hypercholesterolemia is known to be the most predominant indicators of vulnerability to cardiovascular diseases which is the principal cause of death in both technologically advanced and developing nations (Dhuley et al., 1999). The prime risk factors for initiation and advancement of these diseases are disorders of lipid metabolism following oxidative stress. Hyperlipidemia can be considered responsible for the development of atherosclerosis and various other conditions which arise due to atherosclerosis (Hardman et al., 2001). The main purpose of treatment in patients with hyperlipidemia is to condense the risk of developing ischemic heart disease or the occurrence of further cardiovascular or cerebrovascular disease (Smith et al., 1992).

The development of cardiovascular diseases is known to be hastened up by an increase in the lipid levels in the body (Lusis et al., 2000). Verlecar et al. reported that coronary artery disease (CAD) is one of the most important reasons of premature death and it is likely to be the supreme cause of mortality in India (Verlecar et al., 2007). Many medicinal plants have been and are being used for the treatment of hyperlipidemia in Unani and Ayurvedic systems of medicine. Also, the traditional system of medicine relies on plants and plant based products (Harshitha et al., 2013;
Alam et al., 2015). The current study has been carried out to determine the effect of various extracts of *Eremurus himalaicus* on physical (body weight and liver histology) and biochemical parameters (serum TC, TG, LDL-C, VLDL-C and HDL-C) of hyperlipidemia induced rats. The inducer of hyperlipidemia used in the study was high cholesterol diet and coconut oil. Coconut oil is a well known saturated fatty acid containing oil which, along with dietary cholesterol, results in buildup of intracellular cholesterol and cholesteryl esters in the body tissues. The results revealed that the extracts of *Eremurus himalaicus* prevent this accumulation of cholesterol and its esters. Hence, these possess significant antihyperlipidemic potential in cholesterol induced hyperlipidemia. The results were quite comparable to that of the standard drug atorvastatin. The antihyperlipidemic potential of *Eremurus himalaicus* extracts may be due to various reasons which include decrease in intestinal cholesterol absorption, effect on production of lipoproteins, amplification in hepatic LDL-C catabolism through hepatic receptors etc. (Brown et al., 1981; Brown and Goldstein, 1981; Brown and Goldstein, 1986; Augusti, 2001; Khanna et al., 2002; Pooja and Priscilla, 2009; Khyati et al., 2010). All these events may be considered responsible for the decrease in blood cholesterol and LDL-C levels.

The extracts of *Eremurus himalaicus* also lead to an increase in the serum levels of HDL-C. HDL-C, also called “good cholesterol” helps in the transfer of triglycerides and cholesterol from the peripheral cells to the liver cells where it is catabolised and eliminated from the body (Austin, 1994). HDL-C levels and the incidence of cardiovascular diseases share an inverse relationship (Anila and Vijayalaksmi, 2002).

It has been reported that LDL-C and VLDL-C is highly atherogenic. The increased TG levels are known to increase their level (Guerin et al., 2001). Thus, the decrease in the levels of TG by the standard and various extracts of *Eremurus himalaicus* may be attributed to increased lipolytic activity of Plasma Lipoprotein Lipase (Perez et al., 1999; Alam et al., 2015).

It is well known that the atherogenic index (AI) and LDL-C: HDL-C ratio are important indicators of risk of coronary heart disease, irrespective of the serum cholesterol levels (Treasure et al., 1995; Dhuley et al., 1999; Lakshmi et al., 2011). The effect of *Eremurus himalaicus* extracts on AI and LDL-C:HDL-C ratio was also in
favour of their antihyperlipidemic activity. The histopathological studies also favored the same, proving it to be a potential antihyperlipidemic plant.

5.6 ANTIHEPATOTOXIC ACTIVITY

The liver is an essential organ in our body which plays various roles in our body. One of its major roles is detoxification which makes it fall prey to a huge number of toxicants. To overcome the effect of these toxicants the liver has its inherent detoxification systems e.g., glutathione enzyme which scavenges the toxicants. However, at certain times these are overrun and the result is hepatotoxicity (Qureshi et al., 2007; Howida et al., 2016). Any damage to the liver, which is related to altered or impaired functioning of the liver and is caused due to drug exposure or exposure to other noninfectious agents is defined as hepatotoxicity (Navarro and Senior, 2006; Grattagliano et al., 2009).

In order to study the mechanism of hepatotoxicity and/or drug discovery for prevention of hepatotoxicity, various chemical toxins are used as the mock-up substances for causing hepatocyte damage. These substances include carbon tetrachloride, acetaminophen, thioacetamide and galactosamine (Ledda-Columbano et al., 1991; Kucera et al., 2006; Domenicali et al., 2009; Rousar et al., 2009). The mechanisms by which these substances produce liver damage are different; however, the damage caused by these hepatotoxins is evaluated through the same parameters.

The carbon tetrachloride induced hepatotoxicity has probably been most extensively studied from both biochemical and pathological perspectives which provides an idea about its mechanism of toxicity (Rubinstein, 1962; Chaterrjee, 2000; Akram et al., 2012). CCl₄ exerts its effect through trichloromethyl radical which gets bound to the cellular macromolecules and causes damage to the endoplasmic reticulum membrane lipids and causes leakage of enzymes (Kaplowitz et al., 1986; Johnson and Kroening., 1998). Elevated levels of serum enzymes indicate that there is loss of functional integrity of the cell membrane and hence cellular leakage due to toxicity produced by CCl₄ (Drotman and Lawhan, 1978).

The amelioration of CCl₄ induced hepatotoxicity by *Eremurus himalaicus* plant has not been evaluated yet and this study is the first step in this direction. In the present
work, CCl₄ induced hepatocyte damage which was evident from the increase in the serum marker enzymes SGOT, SGPT, ALP and bilirubin and decrease in total protein and uric acid levels. The administration of methanolic extract of *Eremurus himalaicus* and the standard drug Liv 52 caused a significant (P<0.0001) restoration of serum levels of these enzymes. The ethyl acetate extract also showed some restoration of the enzyme levels. This reversal of increased levels of serum enzymes by *Eremurus himalaicus* extracts may be due to various reasons which lead to membrane stabilization and hence prevent the leakage of intracellular contents. Another reason may be that it prevents hepatocyte damage and induces hepatocyte regeneration. This can be assumed on the basis of histopathological reports in which it was clear that the CCl₄ treated group showed degenerative changes, whereas, the standard and extract treated groups showed not as much of degenerative changes. This provided further insight into the hepatoprotective effect of *Eremurus himalaicus*. Moreover, this plant has been reported to contain various phytochemicals which are known to possess hepatoprotective activity (*Seevola et al., 1984; Wegner et al., 1999*). Also, various studies have revealed that the plant extracts having antioxidant activity may protect against CCl₄ induced hepatotoxicity; this occurs via inhibition of lipid peroxidation and enhancement of activity of antioxidant enzymes (*Sheweita et al., 2001; Shahjahan et al., 2004*). Therefore, this may be considered as another reason for hepatoprotective activity of this plant. Further studies are in progress to characterize the bioactive principles from this plant.