CHAPTER IV

Pharmacological Screening
4. PHARMACOLOGICAL SCREENING

4.1 Introduction

Screening of the plant extracts and their bioactive compounds are done to authenticate the pharmaceutical properties of the medicinal plants.

The study which deals with the effects of chemical agents of therapeutic value or with the potential toxicity on biological systems is known as pharmacology. It is a multi-disciplinary branch of science consisting of several sub branches such as behavioural pharmacology, neuropsychopharmacology, pharmacoconomics, clinical pharmacology, pharmacogenetics and cardiovascular pharmacology. The interactions of chemical substances within living systems are being examined in order to comprehend the properties of drugs and their actions. It also includes the interactions among the drug receptors and drug molecules and mechanism how these interactions bring out the effect. For a multiple unique applications like the use of drugs in medicine as therapeutic agents, the development and regulation of pharmaceuticals, the study of drug actions in the health sciences or as tools in scientific research; all these represents scientific basis and principles of pharmacology.

The other related subject of pharmacology is toxicology. Toxicology is the study of the adverse effects of chemicals & drugs and the means to avoid or eradicate such effects in living systems is called as toxicology. Toxicologists examine various chemical compounds, environmental agents as well as therapeutic agents that originate in nature or are synthesized by humans. The range of toxic effects of these agents includes discomfort, disease or death of individual organisms or whole ecosystems and disturbances in growth patterns etc. There are several subclasses of toxicology which
includes forensic toxicology, occupational toxicology, risk assessment, regulatory toxicology and clinical toxicology (where both of these are found in the toxicology and pharmaceutical industry).

Pharmacology and toxicology are cognate disciplines which require the knowledge of basic properties and actions of chemical compounds. Moreover, toxicology deals with the adverse effects and risk assessment of chemicals; while pharmacology emphasizes on the therapeutic effects of chemicals more particularly the drugs.

Pharmacology consists of two closely associated areas namely Pharmacodynamics and Pharmacokinetics. The absorption, distribution, metabolism and excretion of drugs is the Pharmacokinetics and the study of the physiological, biochemical and molecular effects of the drugs on cellular systems and their mechanisms of action is the Pharmacodynamics.

4.1.1 Antimicrobial activity

Identification of active principles, dosage formulations, comprehensive biological assays and clinical studies which establishes the efficacy, safety and pharmacokinetic summary of the new drug are essential in the development of pharmaceuticals (Iwu et al., 1999; Ncube et al., 2008). To ensure the efficacy and safety of plant extracts, systematic biological evaluation is crucial. All these factors are necessary to accept the plant extracts as justifiable medicinal drugs. In the field of science, an elemental and indispensable technique most commonly applied is the antimicrobial susceptibility test (AST) which is used in pathology to determine the antimicrobial resistance of microbial strains and to determine the efficacy of novel antimicrobial agents against microorganisms of medical importance in ethnopharmacology research. AST is the first step towards the drug
development in testing a new anti-infective agent. The researchers are practicing various AST methods and thus leads to variations in the results generated (Lampinen, 2005). Natural-product chemists and microbiologists expressed an opinion that there is a risk of large number of potentially useful phytochemicals which are synthesized chemically are being lost irreversibly due to the extinction of potential plant species (Cowan, 1999).

A number of defense mechanisms are developed by bacteria have been evolved against presently occurring antimicrobial agents and thus drug-resistant pathogens are ever-increasing which is presented by membrane translocases and multidrug resistance pumps (MDRs) that extrude toxins having structural dissimilarity from that of the microbial cell which protect them from both natural and synthetic antimicrobial drugs (Stermitz et al., 2000). Ligands, signal transduction molecules, hormones, neurotransmitters and endogenous metabolites resembles secondary metabolites due to their recognition in potential target sites which are having beneficial medicinal effects on humans (Parekh et al., 2006). Evaluation of plants for antimicrobial activity by standard criteria are still lacking and the results are significantly fluctuating between researchers because a number of parameters such as choice of plant extracts, choice of extraction method, the environmental and climatic conditions under which the plant grows, test microorganisms and antimicrobial effect expressed due to extract treatment (Nostro et al., 2000; Hammer et al., 1999).

The plant materials provide a beneficial medicinal effect which is characteristically due to secondary products present in the plant. This effect usually attributed to combination of metabolites and not by a single compound. The plants
contain rich antimicrobial agents particularly after the flowering stage of growth and the plants which have grown in stressful environment. (Mitscher et al., 1972).

Usually two standard tests such as the diffusion and dilution methods are followed to test the AST. Other conventional methods include agar bioautography, disk diffusion and agar well diffusion while dilution methods are agar dilution and broth micro & macrodilution. Most commonly conducted methods for AST are the agar and broth based methods (Tenover et al., 1995). A potentially useful technique for determining the MIC of large number of test samples is the micro-titre plate or method. Broth dilution method has various advantages than diffusion techniques such as ability to distinguish between bacteriostatic and bactericidal effects; quantitative determination of the MIC and increased sensitivity even for minute amount of the extract which is vital if the antimicrobial is rare as is the case of natural products (Langfield et al., 2004). The minimum bactericidal concentration (MBC) is governed by sub culturing the preparations that have shown no visible growth in the MIC determination assay that are made either in agar plates or in broth. The MBC is regarded in the broth as the lowest concentration of the extract which does not produce any absorbance reading at 620 nm compared to the negative control and on agar MBC is the lowest concentration showing lack of growth (Salie et al., 1996).

4.1.2 Antioxidant activity

Free radical is a molecule that contains an unpaired electron in an atomic orbital and is capable of independent existence. Most radicals share certain common properties due to the presence of an unpaired electron (Lobo et al., 2010). In many disease state the free radicals containing oxygen are are hydrogen peroxide, hydroxyl radical, oxygen
singlet, superoxide anion radical, peroxynitrite radical, nitric oxide radical and hypochlorite. They are highly reactive species present in the nucleus and in the membranes of cells which are capable of damaging biologically important molecules such as lipids, carbohydrates, DNA and proteins (Young and Woodside, 2001). ROS and free radicals are the derivatives of normal metabolic processes taking place in the human body or from external sources such as exposure to air pollutants, ozone, X-rays, industrial chemicals and cigarette smoking (Bagchi and Puri, 1998). The existence of both enzymatic and nonenzymatic antioxidants is to detoxify ROS in the intracellular and extracellular environment (Frie et al., 1988). There are two standard mechanisms of action for antioxidants: the first is a chain-breaking mechanism where the primary antioxidant donates the electron to the free radicals present in the systems and the second mechanism involves secondary antioxidants in which quenching of chain-initiating catalyst occurs to remove the ROS/reactive nitrogen species initiators (Rice and Diplock, 1993). Different mechanisms exist such as co-antioxidants, metal ion chelation, electron donation and regulation of gene expression by which antioxidants exert their effect on biological systems (Krinsky, 1992).

There are different levels in the defense systems by which antioxidants act at including de novo, repair, preventive and radical scavenging. The first level of defense which suppresses the formation of free radicals is the preventive antioxidants. Even though the in vivo site of radical formation and precise mechanism are not well elucidated but one of the important sources of hydrogen peroxide is the metal-induced decomposition. Peroxidase, glutathione-s-transferase, phospholipid hydroperoxide-glutathione peroxidase (PHGPX) and glutathione peroxidase are recognized to
Pharmacological screening

decompose lipid hydroperoxides to subsequent alcohols. PHGPX has exceptional nature which can reduce the hydroperoxides present in phospholipids integrated into biomembranes. Further, glutathione peroxidase and catalase reduce hydrogen peroxide to water.

The antioxidant will scavenge the active free radicals which suppress the chain initiation and break the chain propagation reactions, it forms the second level of defense. Hydrophilic and lipophilic type of endogenous radical-scavenging antioxidants are known commonly which involves bilirubin, Vitamin C, albumin, uric acid and thiols as hydrophilic radical-scavenging antioxidants, while ubiquinol and vitamin E are lipophilic radical-scavenging antioxidants. Vitamin E is accepted as the most potent radical-scavenging antioxidant which belongs to lipophilic group.

The third line of defense is the de novo and repair antioxidants. The cytosolic and mitochondrial enzymes such as proteolytic enzymes, proteases, peptidases and proteinases present in the mammalian cells that recognize, degrade and remove oxidatively modified proteins, thus preventing the accumulation of proteins. An important role in the total defense system against oxidative damage is played by the DNA repair systems which involve various enzymes such as nucleases and glycosylases.

Enhancement of protection against free radicals is made by sufficient intake of dietary antioxidants. Foods containing antioxidants particularly the antioxidant nutrients are of chief importance in the prevention of diseases. However, a growing consensus among researchers report is that combination of antioxidants instead of individual entities is more effective over the long term. To improve the quality of life, antioxidant has a remarkable importance for prevention or postponement of the onset of degenerative
diseases. Furthermore, they possess the potential in the expense of health care delivery for considerable savings. To investigate the antioxidant property of samples such as plant extracts, diets and commercial antioxidants, a variety of methods are being used. Both *in vitro* and *in vivo* antioxidant models provide productive benefits to the investigators for effective results by reducing the time to review literature and development of methods (Alam et al., 2013).

Butylated hydroxytoluene (BHT), *ter*-butyl hydro quinone (TBHQ) and butylated hydroxyanisole (BHA) are synthetic compounds used as potential inhibitors of lipid peroxidation to stabilize fat-containing foodstuffs essential in the protection of unsaturated fats and oils. The use of these synthetic antioxidants in foods is not encouraged as they may cause liver swelling and influence liver enzyme activities and also due to the toxicity and carcinogenicity (Shahidi, 1997; Jeong et al., 2004; Siddhuraju and Becker, 2003). As natural antioxidants are the constituents of many fruits and vegetables having great attention of public and scientific research community due to their anti-carcinogenic property and other health promoting activity. Hence they are preferred. Diet rich in fruits and vegetables specifically with natural antioxidants such as plant flavonoids, vitamin C and polyphenols are directly related with reduction of cardiovascular, certain cancers and other chronic diseases is indicated through recent epidemiological studies (Zuo et al., 2002; Laandrault et al., 2001; Gheldof et al., 2003; Liu et al., 2000).

Based on a single test model, the antioxidant activity should not be inferred. Various *in vitro* test systems are conducted to evaluate the antioxidant activities with the samples of interest which vary in results in several aspects. Consequently, it is hard to
compare one method with other methods due to complexity. Badarinath et al. (2010) have made a comparison among different in vitro methods at some level. ABTS decolorization method is relevant for both lipophilic and hydrophilic antioxidant tests. However, various free radical scavenging methods are applied for testing antioxidants among which DPPH method is more rapid, simple with few steps and cost effective when compared to other test models. Bhandarkar and Khan (2004); Raja et al., (2007) and Soetan and Aiyelaagbe (2009) have used crude extracts of plants have been used to treat injuries caused to the liver which is diagnosed with marked alteration in liver chemistry. Herbal medicine perform a significant function as reliable liver protective drugs in the management of various liver disorders that is absent in allopathic medicinal system (Sadeghi et al., 2008). Several plants have been tested and reported having hepatoprotective property (Ulican et al., 2003; Aniya et al., 2005; Sethuraman et al., 2003; Scott- Luper, 1998; Hewawasam et al., 2004).

The pharmacological effect of plants is basically due to the presence of secondary metabolites which are distinctive to particular plant species (Kaufman et al., 1999). The potential medicinal properties of herbal drugs for humans includes defensive mechanism through cytotoxicity against microbial pathogens which prove to be useful as antimicrobial and antioxidant agents (Briskin, 2000). Beneficial effects of phytomedicine is exerted by additive or synergistic action of numerous phytomedicinal compounds acting at single or multiple target sites is associated with particular physiological process.

4.1.3 Anti-inflammatory activity

Inflammatory disease is commonly known as the ‘King of Human. It is produced by the host defense mechanism as a reaction against foreign substance, infection or
irritation which is involved in the production of prostaglandins, bradykinin, fluid extravasations, histamine, cell migration, infection and also symptoms of neurodegenerative conditions, cancer, repair and tissue breakdown (Dandiya and Kulkarni, 1995; Kumar et al., 2011; Azeem et al., 2010). These are aimed at host defense system usually activated in disease condition. Inflammatory disease includes diverse category of rheumatic diseases which are the main cause of morbidity throughout the world. The main manifestations of inflammation are leukocyte infiltration, edema and granuloma formation (Mitchell and Cotran, 2000). A synergism between various inflammatory mediators that increase the vascular permeability and blood flow results in edema formation in the paw (Tian et al., 2011). Ramachandran et al., (2011) and Sakat et al., (2010) have studied on the evaluation of anti-inflammatory and analgesic potential of methanolic extracts of *Tectona grandis* flowers and have concluded that carrageenan-induced paw edema is the most commonly used method to determine the biphasic stage of inflammation. In the early phase of carrageenan-induced inflammation, histamine, 5-hydroxytryptamine and bradykinin are the first detectable mediators while in the late phase of inflammation prostaglandins are detectable.

The functional and safer natural antioxidants having broad spectrum in action is needed, hence, it is essential to determine the antioxidant activity of plant extracts by using several analytical methods. In this context, a preliminary pharmacological study for assessing the potentiality of *Feronia limonia* Linn plant is done by selecting the methanolic extract at the doses of 200 and 400 mg/kg body weight based on the results exhibited by phytochemical studies.
4.2 Materials and Methods

4.2.1 Antimicrobial screening

Test microorganisms

The test organisms used in the study includes four bacterial strains namely *Salmonella typhimurium* (MTCC 98), *Klebsiella pneumonia* (MTCC 432), *Escherichia coli* (MTCC 45), *Pseudomonas aeruginosa* (MTCC 647), and two fungal strains namely *Aspergillus niger* (MTCC 282), *Aspergillus flavus* (MTCC 277) were procured from IMTECH, Chandigarh, India. They were subcultured in recommended media purchased from Hi-Media, India private Ltd, Mumbai and stored in 4°C for further use.

Culture media and antibiotics

Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), Potato dextrose agar (PDA) and Potato dextrose broth (PDB) media manufactured by HiMedia Laboratories Ltd., India were used. Streptomycin was used as the standard antibiotic for bacteria and Nyastatin was used for fungi.

Screening for antibacterial activity

Antibacterial activity of all the extracts was tested by agar well diffusion method. The bacterial isolates were first grown in a Mueller-Hinton broth for 18 h before use and standardized to 0.5 McFarland standards (10^6 CFU/ml). The culture plates were prepared by pouring 20 ml of Mueller-Hinton agar medium into sterile petri-plates. The test bacteria were then swabbed over the agar media using sterile cotton swabs to get uniform distribution of the bacterial cultures. After the agar in each plate solidified, 6mm diameter wells were made using sterile cork borer. The wells were filled with 50 μl of plant extracts at concentration (50μgml⁻¹) of the sample extracts as well as the standard
antibiotic solution. A sterile antibiotic disc was placed on another end. For each bacterial strain, Streptomycin (50μgml⁻¹) served as positive control and DMSO as negative control. The assay plates were then incubated in 37°C for 24 hours. The diameter of the zone of inhibition around each well by using a transparent ruler was taken as a measure of antibacterial activity. The readings were taken for six experiments and the average values were recorded.

\[
\text{Control - Treated} \\
\text{% inhibition} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100
\]

\[
\text{Activity index (AI)} = \frac{\text{Zone of inhibition (ZI)} \text{ of test sample}}{\text{Zone of inhibition (ZI)} \text{ of standard}}
\]

**Screening for antifungal activity**

The procured fungal isolates were allowed to grow on Potato dextrose agar (PDA) at 25°C until they are sporulated. Activated fungal cultures were adjusted to \(1 \times 10^8\) cfu/m as per McFarland standard of 0.1 at OD at 600nm. After the agar in each plate solidified, wells of 6mm each were bored using a cork borer. 50 μl of plant extracts at concentration (50μgml⁻¹), as well as the standard antibiotic solution was loaded into the wells. Control experiments were set up using Nystatin (50μgml⁻¹). The plates were incubated at 25°C for 48h. The transparent ruler was used to calculate the zone of inhibition. The experiment was repeated for six times and mean readings were noted.

**Minimum inhibitory concentration**

The minimum inhibitory concentration (MIC) of plant extracts was determined using the broth dilution method. In this method, 1ml of the extract solution at the
concentration of 50mgml⁻¹ was added to 1ml of nutrient broth and subsequently transferred to make solutions of varying concentrations (25, 12.5, 6.25, 3.12, 1.56 mgml⁻¹) in different test tubes. Then 1ml of bacterial and fungal suspensions and 0.1ml of plant extracts were added to each test tube and incubated at 37°C, 24h for bacteria and 25°C, 48h for fungi. The test tube with particular concentration of plant extract at which no detectable growth was observed was considered as the MIC. All experiments were performed in six replicates.

**Determination of MBC**

The bactericidal concentration (MBC) was determined by serial sub-culture of 2 μl of broth culture from MIC tube into the first test tube containing 100 μl of broth; serially diluted into corresponding successive tubes upto the 10⁶ dilution and incubated for 72 hours. The lowest concentration with no visible growth was defined as the MBC, indicating 99% killing of the original inoculum. The optical density of each tube was measured at a wavelength of 655 nm by spectrophotometer and compared with the standards Streptomycin for bacteria as the positive control.

**Determination of MFC**

The fungicidal concentration (MFC) was determined by serial sub-culture of 2 μl into test tube containing 100 μl of broth and was incubated for 72 hours at 28°C. The lowest concentration with no visible growth was defined as MFC indicating 99% killing of the original inoculum. Commercial standard Nyastatin was used as positive control for fungi. The experiment was repeated for six times and mean readings were noted.
4.2.2 Antioxidant activity

4.2.2.1 Invitro antioxidant activity

DPPH scavenging method

1, 1-diphenyl-2-picrylhydrazyl (DPPH) is depicted as a stable free radical by the delocalization of the extra electron over the molecule as a whole, as a result the molecule do not dimerize like that of other free radicals. The delocalization of electron results in the formation of deep violet color described by an absorption band centered at 517 nm in ethanol solution. When a solution of DPPH is mixed with the substrate that can donate a hydrogen atom, then this gives rise to the reduced form with the decolorization of the violet color.

In order to evaluate the antioxidant potential through free radical scavenging of methanol extract of *Feronia limonia*, the change in optical density of DPPH radicals was carried out according to the method of Manzocco et al., (1998). The sample extract (0.2 ml) was first diluted with methanol and then 2 ml of DPPH solution (0.5 mM) is added. After 30 min of incubation, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging was calculated using the following equation:

\[
\% \text{ inhibition of DPPH radical} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where \(A_0\) is the absorbance before the reaction; \(A_1\) is the absorbance after the reaction has taken place.

Trolox equivalent antioxidant capacity (TEAC) method/ ABTS radical cation decolorization assay

The ABTS method uses a diode-array spectrophotometer to measure the decolorization of color when an antioxidant is added to the blue–green chromophore called 2,2-azino-bis-3-ethylbenzthiazoline- 6-sulfonic acid (ABTS+). The antioxidant
reduces ABTS+ to ABTS and decolorizes it. ABTS+ is a stable radical that is absent in the human body. Antioxidant activity by ABTS was measured using the procedure as described by Seeram et al. (2006).

The ABTS reagent was prepared freshly by mixing 7mM ABTS and 2.45 mM potassium persulfate and incubated for 16 h at 37°C. The ABTS cations diluted with ethanol to set O.D at 0.7 (±0.02). 3.9 ml of reagent was added to 0.1 ml of extract. The mixture was incubated at 37°C and absorbance was measured at 734 nm after 2 min. Trolox is used as a standard reference. Results were expressed in μM Trolox Equivalents (TE)/g of plant extract.

\[
\% \text{ Inhibition} = \left(\frac{A_0 - A_t}{A_0}\right) \times 100
\]

where \( A_0 \) is the absorption of blank sample; \( A_t \) is the absorption of test sample.

**Phosphomolybdenum method**

Total antioxidant capacity assay is a spectroscopic method for the quantitative determination of antioxidant capacity by the formation of phosphomolybdenum complex. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. The total antioxidant capacity of MEFL was determined using ascorbic acid as standard following the phosphomolybdate method of (Prieto et al., 1999).

0.1 ml of extract solution was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a water bath at 95°C for 90 min. A typical blank contained 1 ml of reagent solution and appropriate volume of solvent incubated under the same conditions.
The tubes were cooled to room temperature and absorbance was measured at 765 nm against the blank. The antioxidant capacity was estimated using following formula:

\[
\text{Antioxidant effect (\%)} = \left(\frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\right) \times 100
\]

**Determination of total phenolic content (TPC)**

The amount of total phenolics in MEFL was determined according to the Folin-Ciocalteu method. 200 µL of samples were introduced into test tubes; 1.0 ml of Folin-Ciocalteu’s reagent and 0.8 ml of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorbance was measured at 765 nm (Perkin-Elmer UV-vis spectrophotometer, Norwalk, CT). The total phenolic content was expressed as Gallic acid equivalents (GAE) in milligrams per gram dry material.

**Statistical analysis of data**

Data were presented as mean ± SD of three experiments. Analysis of variance was performed on the data obtained. Significance of differences between means was determined by least significant differences (LSD) at \( P \leq 0.05 \).

**Animals**

Study was performed as per Organization for Economic Cooperation and Development (OECD) guidelines 423. Experiments were carried out using healthy young adult wistar strain Albino rats of inbred colony weighing about 150 – 175 g. The protocol was approved by the Institute’s Animal Ethical Committee (IAEC Reg No. 34800/ CPCSEA Dated: 19.08.2001). Animals were kept in animal house at an ambient temperature of 25\(^{\circ}\)C with 45 – 55% relative humidity, at 12 h each of dark and light
cycles. They were fed with a balanced diet as described by Central Food and Technological Research Institute (CFTRI, Mysore) and water ad libitum.

**Acute toxicity experiment**

Albino rats were divided into control and test groups (6 animals each). Control group received the vehicle (3% Tween 80) while the test groups received graded doses (200–4000 mg/kg) of MEFL orally and were observed for mortality till 48 h and the LD$_{50}$ was calculated. They were identified by the markings in different body part. Animals were marked on head, body, tail, head and body, body and tail and one mouse with no marking to ease the observation.

**Mode of administration and dosing**

The methanol extract of *Feronia limonia* was administered in a single dose by oral catheter. Animals were fasted 3 h prior to dosing (only food was suspended for 3 h but not water). Following the period of fasting, animals were weighed and the extract was administered orally at a dose of 200, 500, 1000, 2000 and 4000 mg/kg body weight. After the administration of extract, food was withheld for 2 h. Though the concentration of the drug varies, but the volume of the drug administered was 1ml/kg body weight (bw) of the animal. Based on the body weight of the animal on the day of treatment, the quantity of the test drug was calculated.

**Physical observation**

All the animals were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioral changes body weight, food and water consumption. The body weights of each animal were noted once in 5 days and the differences in the body weights were recorded. The amount of extract to be given was
calculated based on the body weights of the experimental rats to ensure a constant dose
volume/kg body weight of the extract given to the rats. The amount of food and water
intake was recorded.

4.2.2.2 Invivo antioxidant activity in rat liver

Chemicals

CCl₄ were purchased from Sigma-Aldrich (Mumbai). Liv 52 from Himalaya
drugs Ltd. All other chemicals and reagents were purchased of analytical grade, and they
are used as received.

Induction of experimental hepatotoxicity

30% CCl₄ was prepared in olive oil. Animals of group 2, 3, 4 and 5 were given a
single dose of CCl₄ at 1ml/kg body weight (bw) intraperitoneally (i.p). Methanolic extract
of Feronia limonia at the dose level of 200 mg/kg bw and 400 mg/kg bw as low dose and
high dose were administered to animals of group 4 and 5 orally with the aid of an
intragastric catheter for 7 days. Liv 52 (50 mg/kg bw) was given orally as a standard drug
to group 3 animals at a single dose and treated as standard control.

Rats were divided into five groups according to the following protocol.

GROUP 1: Normal control (n=6, these animals received saline at 1ml/kg body weight).

GROUP 2: Hepatotoxic control (n=6, these animals received CCl₄ at 1ml/kg body
weight).

GROUP 3: Positive control (n=6, these animals received CCl₄ + Liv 52 for 7 days).

GROUP 4: Treatment group (n=6, these animals received CCl₄ + 200 mg/kg MEFL for 7
days).
GROUP 5: Treatment group (n=6, these animals received CCl₄ + 400 mg/kg MEFL for 7 days).

At the end of the experimental period, blood sample from each rat (2 ml) was withdrawn by cardiac puncture and collected in previously labeled non-heparinized centrifuge tubes and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 10,000 rpm for 5 min.

**Assessment of hepatotoxicity**

Liver functions were evaluated by measuring the serum activity of ALT and AST by following the method of Reitman and Frankel (1957) while the activities of ALP and LDH were estimated by using the procedure of Babson et al., (1996). The serum concentration of TB was estimated according to the method described by Walter and Gerarade (1970) and TP as described by Henary et al., (1974).

**Assessment of oxidative stress**

Liver tissue was homogenized in 10 volume of 100 mM KH₂PO₄ buffer containing 1 mM EDTA (pH 7.4) and centrifuged at 12,000 rpm for 30 min at 40°C. The activities of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were assayed in the hepatic tissue homogenate of the control and experimental rats according to the methods of Sun and Zigman (1978) and Chance and Maehley (1995). GSH tissue content was also measured using Moron et al., (1979) method.

**Histopathological study**

Liver was dissected out and divided into two parts. One part was kept in liquid nitrogen for determination of antioxidant status and the other part was immediately fixed.
in 10% buffered formalin and was used for histopathological examination using the standard micro technique.

**Statistical analysis of data**

Data is presented as means ± SD of six experiments. Analysis of variance was performed on the data obtained. Significance of differences between means was determined by least significant differences (LSD) at $P \leq 0.05$.

4.2.2.3 *Invivo* antioxidant activity in rat testis

**Chemicals**

6-hydroxy-2, 5, 7, 8- tetramethylchromane- 2-carboxylic acid (trolox) was purchased from Sigma Aldrich, Bangalore. All other chemicals and reagents used were of analytical grade, and they are received as and when required.

**Experimental design**

30% CCl$_4$ was prepared in olive oil. Animals of group 2, 3, 4 and 5 were a given single dose of CCl$_4$ at 1ml/kg body weight (bw) intraperitoneally (i.p). Methanolic extract of *Feronia limonia* at the dose level of 200 mg/kg bw as low dose and 400 mg/kg bw as high dose was administered to the animals of group 4 and 5 orally with the aid of an intragastric catheter for 7 days. Silymarin (50 mg/kg bw) was used orally as a standard drug to group 3 at a single dose. Rats were divided into five groups, each group consisting of six animals.

GROUP 1: Normal control group received saline 1ml/kg body weight.

GROUP 2: Toxic control treated with CCl$_4$ at 1ml/kg body weight.

GROUP 3: Positive control received CCl$_4$ + Silymarin for 7 days.
GROUP 4: The CCl$_4$ treated animals were given 200 mg/kg of MEFL for 7 days.

GROUP 5: The CCl$_4$ treated animals were given 400 mg/kg of MEFL for 7 days.

At the end of the experimental period, blood samples from each rat (2 ml) was withdrawn by cardiac puncture, collected in previously labeled non-heparinized centrifuge tubes, and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 10,000 rpm for 5 min and used for enzyme analysis. The testis of each animal was dissected out, freed from adipose tissue, weighed up to nearest milligram on electronic balance. The testis from one side of each animal is processed for estimation of LPO and antioxidant activities. The testis of other side from each animal was fixed in buffered formalin and processed for histopathological study.

**Assessment of testicular lipid peroxidation and antioxidant activities**

50 mg of testis was homogenized in 10 volume of KH$_2$PO$_4$ (100 mmol) buffer containing EDTA (1 mmol, pH 7.4) and centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected and used for enzymatic studies. Protein concentration of tissue supernatant was determined by the method of Lowry et al using BSA as standard. Various antioxidant enzymes including CAT and SOD, TBARS, GST, GSR and GSH were carried out (Mishra and Fridovich, 1972; Yagi, 1984; Mohandas et al., 1984; Wu et al., 2005; Turner and Lysiak, 2008).

**Histopathological examination**

Testis tissues were fixed in buffered formalin for 48 h, dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned at 5-μm thicknesses and stained with Mayer’s haematoxylin & eosin.

**Statistical analysis of data**
To determine the effects of treatment, the data was calculated by applying one way analysis of variance using software SPSS 13.0. Level of significance among the various treatments was determined by LSD at 0.01% level of probability.

4.2.3 Anti-inflammatory activity

Chemicals and reagents

The chemicals used in the present study are carrageenan (S. D. Fine Chemicals Limited, Bombay) and Indomethacin (IPCA, Bombay).

Investigation of acute anti-inflammatory effects by carrageenan induced paw oedema

The rats were divided into 5 groups of six animals each (n = 6) and all the animals were deprived of food and water for 24 hours before the experiment. They were marked and numbered for identification. 0.1 ml sterile saline was given to group 1 animals and maintained as control group. Paw oedema was induced by injecting 2mg/kg of 1% carrageenan into the rat hind paw to the animals of group 2, 3, 4 and 5. Group 2 animals were given single dose of carrageenan only and maintained as toxic control group. Rats were treated with test compound methanolic extract at the dose level of 200 mg/kg bw and maintained as group 3 and 400 mg/kg bw as group 4. Standard drug Indomethacin was administered orally to group 5 animals and maintained as standard group.

GROUP 1: Normal control group received saline 1ml/kg body weight.

GROUP 2: Toxic control treated with Carrageenan at 2 mg/kg body weight.

GROUP 3: Positive control received Carrageenan + Indomethacin.

GROUP 4: The Carrageenan treated animals were given 200 mg/kg of MEFL.

GROUP 5: The Carrageenan treated animals were given 400 mg/kg of MEFL.
The volume of the paw was measured by a vernier caliper immediately after the injection. Successive readings were carried out at one-hour intervals up to 4h and compared to the initial readings. The increase in paw volume was taken as oedema volume. The percentage of inhibition of inflammation was calculated and compared in all the treated and control group animals. The anti-inflammatory effect of MEFL was calculated by the following equation:

\[
\text{Anti-inflammatory activity (\%)} = (1 - \frac{D}{C}) \times 100.
\]

where D is the percentage difference in paw volume after MEFL administration; C is the percentage difference in volume of the control group.

**Biochemical assays and hematological studies**

Blood from all the group animals 24 h after the last dose was obtained from the tail vein in a non-heparinized tube and centrifuged at 3000 rpm at 4°C for 10 minutes to separate the serum. The activities of serum glutamate oxaloacetate transaminase level (SGOT) and serum glutamate pyruvate transaminase (SGPT) were assayed by the method of Bergmeyer and Bernt (1974). The alkaline phosphatase activity in the serum was measured according to the procedure of King (1965a). The blood was also used to estimate other hematological parameters in all control and treated groups. On 15th day, the liver was excised, rinsed in ice-cold normal saline followed by cold 0.15 M Tris-HCl (pH 7.4), blotted and weighed. The homogenate was processed for estimation of lipid peroxidation and GSH (Turner and Lysiak, 2008). Proteins were estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin as the standard.
4.3 RESULTS

4.3.1 Antimicrobial assay (Table 4.1; Graph 4.1 & 4.2)

The antimicrobial activities of *F. limonia* fruit pulp extracts tested against the microorganisms such as *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Aspergillus flavus* were qualitatively and quantitatively assessed by the expression of inhibition zones and MIC values. The results are represented in (Table 4.1). The extracts have shown a potent antimicrobial activity against Gram-positive, Gram-negative bacteria and fungal strains.

Chloroform extract has shown the highest activity against *E. coli* exhibiting 12 mm of inhibition zone with MIC value of 12.5 mg/ml; moderate activity against *P. aeruginosa* and *S. typhimurium*. The zone of inhibition for *P. aeruginosa* was 11 mm with MIC value of 25 mg/ml and for *S. typhimurium* zone of inhibition was 8 mm with MIC of 12.5 mg/ml. There was no activity against *K. pneumonia*, *A. niger* and *A. flavus*.

Whereas methanol extract has shown the highest activity against *A. niger* and *P. aeruginosa*. It exhibited 22 mm of zone of inhibition with MIC of 6.25 mg/ml for *A. niger* & 21 mm zone of inhibition with MIC of 6.25 mg/ml for *P. aeruginosa*. Methanol extract displayed moderate activity against *S. typhimurium* (16 mm, 12.5 mg/ml), *E. coli* (19 mm, 3.125 mg/ml), *K. pneumonia* (15 mm, 6.25 mg/ml) and *A. flavus* (19 mm, 6.25 mg/ml) with zone of inhibition and MIC values respectively (Table 4.1).

However, aqueous extract has shown the highest activity against *A. niger* exhibiting 18 mm of inhibition zone with 6.25 mg/ml of MIC value; moderate activity against *E. coli* (16 mm, 6.25 mg/ml), *P. aeruginosa* (17 mm, 12.5 mg/ml) and *A. flavus* (16 mm, 12.5 mg/ml); least activity against *S. typhimurium* (12 mm, 12.5 mg/ml) and no
activity against *K. pneumonia*. The results were compared with standards Streptomycin for bacteria and Nyastatin for fungi which were used as the positive controls and have shown a complete inhibition (no turbidity) against all the organisms for MIC. 1% Tween 80 was used as a negative control which has shown no inhibitory effect against the tested organisms. Among all the three extracts, methanol extract has shown good activity against tested organisms in comparison to the standard drug.

Methanol extract has shown lowest MBC value against *E. coli* at 6.25 mg/ml concentration; the highest value of MBC against *S. typhimurium* at 25 mg/ml and moderate value against *K. pneumonia, P. aeruginosa, A. flavus* and *A. niger* was observed at 12.5 mg/ml concentration. Chloroform extract has shown MBC value of 25 mg/ml against *S. typhimurium, P. aeruginosa* and *E. coli*. Furthermore it is clear from the results that chloroform extract possessed no activity against *K. pneumonia, A. flavus* and *A. niger*. However aqueous extract has shown MFC value of 6.25 mg/ml against *A. flavus* and 12.5 mg/ml against *A. niger*. Further aqueous extract has shown moderate value of MBC against *E. coli* at 12.5 mg/ml; *S. typhimurium* and *P. aeruginosa* have shown MBC value of 25 mg/ml; there was no activity observed against *K. pneumonia* (Table 4.1). The results were compared with the standards Streptomycin and Nyastatin to evaluate the % of inhibition (Graph 4.1). The maximum % inhibition value was observed in chloroform extract which has shown 58.97%; moderate inhibition against *P. aeruginosa* with 45.45% & *E. coli* with 47.61%; no inhibition against *K. pneumonia, A. flavus* and *A. niger*. Methanol extract has shown 17.94% against *S. typhimurium*, 13.63% against *E. coli*, 18.91% against *K. pneumonia*, 4.34% against *A. niger*, 5% against *A. flavus* and no inhibition against *P. aeruginosa*. Aqueous extract has shown 38.46% against *S.
typhimurium, 27.27% against E. coli, 19.04% against P. aeruginosa, 21.73% against A. niger, 20% against A. flavus and no inhibition against K. pneumonia.

The highest activity index (AI) value was 1.04 mm for methanolic extract against A. niger, 0.02 mm against S. typhimurium, 0.86 mm against E. coli, 0.81 mm against K. pneumonia, 1 mm against P. aeruginosa and 0.95 mm against A. flavus. Chloroform extract has exhibited 0.41, 0.54 and 0.52 mm of AI against S. typhimurium, E. coli and P. aeruginosa respectively. No activity was shown by chloroform extract against Klebsiella pneumoniae, Aspergillus niger & Aspergillus flavus. Further aqueous extract exhibited 0.61, 0.72, 0.8, 0.78 and 0.8 mm of AI against S. typhimurium, E. coli, P. aeruginosa, Aspergillus niger & Aspergillus flavus respectively and no activity was observed against Klebsiella pneumoniae (Graph 4.2) which were obtained by comparing with the standards Streptomycin for bacteria and Nyastatin for fungi correspondingly. The results of the study indicates that among all the tested extracts, methanol extract has exhibited the highest antimicrobial activity and least activity is observed by chloroform extract and the aqueous extract has shown moderate activity.
4.3.2 Antioxidant activity

4.3.2.1 *Invitro* antioxidant activity (Graph 4.3, 4.4, 4.5; Table 4.2)

DPPH radical scavenging activity of MEFL (Graph 4.3)

Concentration dependent DPPH radical scavenging activity was observed in both MEFL and standard test experiment. The decrease in concentration of DPPH radical is shown due to the significant free radical scavenging activity of MEFL with 52.74%, 58.43%, 67.15% & 72.23% inhibition at the concentrations of 1.25, 2.5, 5 & 10 mg/ml correspondingly and BHT which has shown the radical scavenging inhibition activity of 67.15%, 77.88%, 83.56% & 90.45% respectively at 1.25, 2.5, 5 & 10 mg/ml.

ABTS radical scavenging activity of MEFL (Graph 4.4)

ABTS radical scavenging activity of MEFL ranged from 43.13%, 54.08%, 61.47% & 76.21% at the concentrations of 1.25, 2.5, 5 & 10 mg/ml and for standard inhibition was 67.15%, 71.38%, 82.11% & 89.75% in the concentrations of 1.25, 2.5, 5 & 10 mg/ml respectively (Graph 4.4).

Phosphomolybdate assay of MEFL (Graph 4.5)

The quantitative assay of phosphomolybdate was carried out where the total antioxidant capacity (TAC) is expressed as ascorbic acid equivalents. The results are indicated that the antioxidant activity was in dose dependent manner at concentrations of 1.25, 2.5, 5 & 10 mg/ml with values of 15%, 42%, 66% & 78% for MEFL and 27%, 58%, 81% & 97% for standard (Graph 4.5).

Total phenolic content (Graph 4.6)

Total phenolic content was determined by Folin Ciocalteu method is expressed as Gallic acid equivalents (GAE)/g dry material. It was noted that MEFL had a significant
total phenolic content of about 76.77, 118.5, 222 and 351.45 mg GAE/g dry material at concentrations ranging from 1.25, 2.5, 5 and 10 mg/ml respectively (Graph 4.6).

**Relationship between phenolic content, radical scavenging and total antioxidant capacity (Table 4.2)**

Correlation analysis (Table 4.2) has exhibited positive and strong correlation of TPC with DPPH \( (r = 0.98) \), ABTS \( (r = 0.99) \) and TAC \( (r = 0.95) \). This analysis also shows a positive and strong correlation of DPPH with ABTS \( (r = 0.99) \) and TAC \( (r = 0.98) \); ABTS with TAC \( (r = 0.97) \). The findings suggest the strong involvement of phenolics in the antioxidant activity of MEFL. The high degree of correlation in the simple spectrophotometric assay showed that it would be a useful technique for rapid evaluation of antioxidant activity in this plant.

**4.3.3 Acute toxicity study (Table 4.3, 4.4)**

The dose selection for MEFL study was based on the observation of acute toxicity. The study did not show any adverse effect of doses up to 4000 mg/kg. Accordingly, experimental oral doses of 200 and 400 mg/ kg equal to one-twentieth and one-tenth of the feasible dose of the extract that did not cause mortality in rats are selected for all pharmacological studies such as antioxidant activity in rat liver & testis and also anti-inflammatory activity. No significant change has been observed in the body weight of animals and their mortality.

**Physical observation**

No signs of toxicity was observed in the rats at the tested dose levels. Physical observation of the animals treated with the extract throughout the study indicated that none of the them have showed the signs of toxic effect such as changes on skin and fur,
eyes and mucus membrane, behaviour pattern, tremors, salivation, diarrhoea, sleep and coma. No mortality was observed in any of the experimental rats.

**Body weight, food and water intake (Table 4.3, 4.4)**

The body weight of the treated and control animals are as shown in Table 4.3. There was a gradual increase in body weight of treated and control group rats is observed. The body weight of the treated rats were not significantly different as compared to the control. The food and water consumption of the treated animals was also not significantly different as compared to the control group was observed throughout the study (Table 4.4).
4.3.4 Invivo antioxidant activity in rat liver (Table 4.5, 4.6; Figure 4.1)

Assessment of hepatotoxicity (Table 4.5)

The effects of methanol extract of *Feronia limonia* at a dose levels of (200 and 400 mg/kg bw) on serum marker enzymes are shown in Table 4.5. The marker enzyme levels in CCl$_4$ induced rats such as ALT, AST, ALP, TP and TB with the values of 64.18 ± 0.23, 81.56 ± 5.54, 107.69 ± 1.33, 8.14 ± 0.37 and 0.64 ± 0.04 IU/L respectively. Hepatic injury induced by CCl$_4$ has caused significant ($P < 0.01$) rise in the marker enzymes such as ALT, AST and ALP with values of 170.22 ± 0.14, 134.91 ± 7.33, 152.56 ± 1.07 and decrease in serum TP and TB with values of 4.11 ± 0.3, and 1.81 ± 0.09 IU/L in CCl$_4$ group compared to the control group. Administration of methanol extract of *Feronia limonia* at two different dose levels (200 and 400 mg/kg bw) shown the increased levels of the serum enzymes, produced by CCl$_4$, and caused a subsequent recovery towards normalization almost like that of Liv 52 treated rats. The level of ALT is 142.10 ± 0.25, AST is 126.72 ± 7.16, ALP is 144.30 ± 1.66, TP is 5.01 ± 0.35 and TB is 1.43 ± 0.06 for 200 mg/kg bw of extract. Whereas for 400 mg/kg bw of extract the level of ALT is 117.35 ± 0.31, AST is 115.80 ± 7.43, ALP is 132.78 ± 2.01, TP is 6.25 ± 0.65 and TB is 1.09 ± 0.05 which are in coordination with the standard Liv 52 treated group animals having values of 91.31 ± 0.42 for ALT, 96.07 ± 4.38 for AST, 120.01 ± 1.25 for ALP, 7.00 ± 0.24 for TP and 0.72 ± 0.05 for TB.

Assessment of oxidative stress (Table 4.6)

CCl$_4$ treatment also resulted in the depletion (P<0.01) of the hepatic antioxidant enzymes such as GSH, CAT and SOD the activities are depleted to 1.01 ± 0.12, 23.16 ± 1.27 and 17 ± 0.17 respectively in the hepatotoxic control group when compared to the
normal control group having the values of 1.47 ± 0.43, 45.02 ± 1.33 and 26.54 ± 0.33 respectively (Table 4.6). The decline in the activities is noticeable (P<0.01) after the MEFL administration at the dose level of 200 mg/kg bw and 400 mg/kg bw. The levels of GSH, CAT and SOD are 1.16 ± 0.22, 27.20 ± 1.74 and 18.21 ± 0.66 in 200 mg/kg bw MEFL treated rats; 1.24 ± 0.26, 34.10 ± 1.45 and 20.95 ± 0.21 in 400 mg/kg bw treated rats is observed. Treatment with MEFL has enhanced the liver antioxidant enzymes yet after CCl₄ treatment proving the antioxidant potentiality of the extract.

**Histopathology (Figure 4.1)**

The histopathological examination is represented in figure 4.1 which display the significant recovery of hepatocytes in the standard drug and MEFL treated animals, which is again correlated with the biochemical parameters. The results of the liver histopathological studies showed swelling of the hepatocytes with necrosis in CCl₄-treated rats when compared with normal control rat’s liver. Treatment with MEFL exhibited a significant protection against hepatocytes injury and showed a complete normalization of the tissues where no fatty accumulation or necrosis was seen in the liver. The central vein of the liver has appeared clearly indicating a potent anti-hepatotoxic activity. MEFL was found to exhibit a potent anti-hepatotoxicity compared with standard drug Liv 52., where the transverse section of the liver has shown the structure of the portal triad and a normal liver parenchyma, and the central vein appeared clearly. There was no lymphocytic infiltration and fatty deposition are representing a potent anti-hepatotoxicity effect of the MEFL treatment.
The mean values of body weight of rats treated with MEFL shown in graph 4.7 which indicates that there is no significant difference of the body weights after MEFL treatment in rats and also in the control group rats. Similarly, no significant difference was found in the weight of the testis after MEFL treatment either with 200 or 400 mg/kg bw for 7 days period when compared with the control group animals (Graph 4.8).

**Effect of MEFL on GST, GSR, GSH, TBARS (Table 4.7)**

The effects of methanol extract of *Feronia limonia* at the dose levels of (200 and 400 mg/kg bw) on tissue phase II metabolizing enzymes such as GST, GSR, GSH and TBARS are shown in Table 4.7. Testicular injury induced by 30% CCl₄ has caused a significant (*P* < 0.01) decrease in GST (14.25 ± 1.67), GSR (45.2 ± 1.17), GSH (1.09 ± 0.27) and increase in TBARS (25.17 ± 1.93) values in the hepatotoxic control group when compared to the normal control group having the values of 23.12 ± 1.83, 62.44 ± 2.33, 1.36 ± 0.33 and 16.87 ± 1.14 respectively. Administration of methanol extract of *Feronia limonia* has shown the increased levels of above all the enzymes, but decreased in TBARS produced by CCl₄ and resulted in subsequent recovery towards normalization almost like that of standard drug silymarin treatment. The levels of GST (19.02 ± 1.74), GSR (56.41 ± 2.66), GSH (1.11 ± 0.22) and TBARS (12.30 ± 1.19) was observed in 200 mg/kg bw of MEFL treated rats and the GST (21.14 ± 1.55), GSR (59.95 ± 2.21), GSH (1.20 ± 0.35) and TBARS (16.03 ± 1.20) was observed in 400 mg/kg bw of MEFL treated rats.

**MEFL and antioxidant profile (Table 4.8)**

CCl₄ treatment resulted in the depletion (P<0.01) of the testicular antioxidant enzymes activities such as SOD, CAT and amount of tissue protein which are depleted to
14.00 ± 1.57, 3.16 ± 1.27 and 1.95 ± 0.82 respectively in the toxic control group when compared to the normal control rats which has shown the values of 21.23 ± 2.33, 5.09 ± 1.43 and 2.07 ± 0.53 respectively (Table 4.8). The decline in the activities is noticeable (P<0.01) after administration of MEFL at the dose level of 200 mg/kg bw having the values of 18.61 ± 1.66, 4.20 ± 1.04 & 1.94 ± 0.22 and at 400 mg/kg bw of MEFL treatment the values are 20.95 ± 2.21, 4.41 ± 1.05 & 2.1 ± 0.26 correspondingly. Treatment with the MEFL has enhanced the testicular antioxidant enzymes even after CCl₄ induction; thus proving the antioxidant potential of the MEFL.

**Histopathology (Figure 4.2)**

The histopathological changes such as necrosis, degeneration, disorganization and reduction in germinal cells, thickening in basal membrane, interstitial oedema and congestion, multinuclear cell formation and spermatogenic arrest were observed only in CCl₄ treated group. Testis of the animals from CCl₄ induced group when compared to the control group contains a good amount of spermatogonia, but comparatively with few spermatocytes and spermatids. However, an increase in the number of spermatocytes and spermatids along with spermatogonia was observed in MEFL treated rat testis in comparison with the CCl₄ treated group. However, the administration of methanol extract to the CCl₄-treated animals has significantly (P < 0.001) improved the CCl₄-induced damages indicating the anti-oxidant potentiality of the MEFL (Figure 4.2).
4.3.6 Anti-inflammatory activity (Table 4.9, 4.10 and 4.11)

The present study establishes the potential effect of the MEFL for its anti-inflammatory activity. The methanol extract has exhibited significant anti-inflammatory activity at the tested doses of 200 and 400 mg/kg bw in a dose dependant manner. The methanol extract has shown maximum inhibition of edema with value of 54.60% at the dose level of 400 mg/kg, while at the dose level of 200 mg/kg bw the inhibition was 44.90% when compared with the standard drug which has shown 61.30% of inhibition of the edema at 3 hours. The results are summarized in table 4.9. The rats are also observed for the behavioral changes such as loss of appetite, inactiveness, dizziness, erection of hairs and hypothermia. After oral administration of the MEFL extract at the doses levels of 200 and 400 mg/kg bw has not indicated any abnormal behavioral responses in any of the rats. The body weight and also weight of the liver remained same in the treated groups when compared to the control group of animals.

The hematological profile and biochemical parameters are shown in table 4.10. Carrageenan induced group rats have exhibited slight elevations in all the blood parameters such as Hemoglobin (12.4 ± 1.10), Haematocrit (37.7 ±1.94), MCHC (33.4 ± 2.83), MPV (134.8 ± 7.36) and platelets (1358 ± 41.2) when compared to the normal control group whose values are 12.2 ± 1.00, 35.3 ±1.21, 31.0 ± 1.23, 131.9 ± 5.14 & 1344 ± 62.0 respectively. Oral administration of MEFL at the dose level of 200 mg/kg body weight for 7 days resulted in ameliorated levels of these blood parameters with the values of Hemoglobin (12.3 ± 0.83), Haematocrit (35.7 ± 1.43), MCHC (32.2 ± 2.17), MPV (132.4 ± 3.31) & platelets (1346.0 ±74.1). Similarly at 400 mg/kg body weight treatment
Pharmacological screening

has shown the values Hemoglobin (12.5 ± 1.04), Haematocrit (37.7 ± 1.23), MCHC (33.4 ± 0.77), MPV (138.6 ± 4.65) & platelets (1355 ± 57.13).

Liver functioning parameters such as SGOT, SGPT, ALP, protein, LPO and GSH were also assessed and shown in table 4.11. The results indicate that there was slight increase in enzymes of carrageenan induced rats with the values of about SGOT (114.9 ± 8.57), SGPT (20.7± 1.72), ALP (60.2 ± 3.04), protein (7.2 ± 0.04), LPO (0.97 ± 0.03) and GSH (2.38 ± 0.03) in comparison with normal control rats having the values of SGOT (112.0 ± 2.04), SGPT (18.8± 0.11), ALP (58.9 ± 4.07), protein (7.0 ± 0.24), LPO (0.96 ± 0.01) and GSH (2.36 ± 0.03) correspondingly. The levels of the enzymes after are 200 mg/kg bw of MEFL treatment in rats are SGOT (110.8 ± 6.44), SGPT (19.5 ± 2.21), ALP (59.9 ± 4.8), protein (6.92 ± 0.53), LPO (0.97± 0.04) & GSH (2.39 ± 0.12) and after 400 mg/kg bw of MEFL treatment the values are SGOT (114.5 ± 1.62), SGPT (20.1 ± 1.74), ALP (67.4 ± 4.65), protein (7.23 ± 0.65), LPO (0.98 ± 0.02) & GSH (2.46 ± 0.17) which clearly indicates the antioxidant property of the MEFL in acute induced oedema model and also signifies the safe use of the extract without any adverse effects.
4.4 DISCUSSION

4.4.1 Antimicrobial

Effort is being made to identify the antimicrobial compounds from natural sources that possess the potential antimicrobial drugs used to replace the use of synthetic drugs which have received much attention in recent times. In order to develop more effective and less toxic drugs that are helpful in controlling the growth of microorganism, the role of primary and secondary metabolites obtained from plant sources serve as a significant model prototype (Kelmanson et al., 2000; Ahmad and Beg, 2001). Significant therapeutic and medicinal applications are shown by phytocompounds against various human pathogens such as fungi, virus and bacteria. A number of studies have been reported previously on using of different plant extracts to screen for antimicrobial activity as well as for the invention of novel antimicrobial agents (Zakaria et al., 2007; Guleria and Kumar, 2006; Majid et al., 2013). Looking into the above problem many of the industries like neutraceuticals, pharmaceuticals, food and beverage are preferring to manufacture herbal products.

In the present investigation, different extracts such as chloroform, methanol and aqueous extracts of Feronia limonia are evaluated for the exploration of their antimicrobial property by agar well diffusion method against certain gram negative, gram positive bacterial and fungal strains which are regarded as human pathogenic microorganisms. Susceptibility of each extract was further tested by broth serial micro-dilution method (MIC). A large number of phytochemicals have been shown to possess inhibitory effects on wide range of microorganisms. The activity is probably due to the
ability of extracellular and soluble proteins to form the complex with bacterial cell walls (Cowan, 1999).

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit growth of organisms. Determination of the MIC is an important parameter in diagnostic laboratories because it helps in confirming resistance of micro-organism to an antimicrobial agent and it also monitors the activity of new antimicrobial agents. Plant extracts are claimed to be a broad-spectrum antimicrobial agents which are considered as a main source for the search of lead compounds. In the present investigation the fruit pulp extracts of *F. limonia* has shown a potential antimicrobial activity against the microorganisms tested. The zone of inhibition and MIC values were in the range of 15–21 mm and 3.125-12.5 mg/ml for the methanol extract, 12–18 mm and 3.125-12.5 mg/ml for the aqueous extract, 8-12 mm and 12.5-25 mg/ml for the chloroform extract respectively, which indicates that the methanol extract possess broad spectrum activity than other extracts such as chloroform and aqueous. This effect of the extract serves as a clue for development of multi-resistant drug. On the basis of zone of inhibition and MIC values, it was concluded that the *Pseudomonas aeruginosa* was more susceptible to the methanolic extract than all other bacteria with an inhibition zone of 21 mm and MIC value of 6.25 mg/ml respectively. However, among fungi, *A. niger* was susceptible to methanol extract with inhibition zone of 22 mm and MIC value of 6.25 mg/ml than *A. flavus*. The antimicrobial analysis of Indian ethnomedicinal plants against clinically isolated human pathogens using the agar well diffusion and MIC method has been performed and reported by many researchers (Arora and Kaur, 2007; Gurudeeban et al., 2010; Pavithra et al., 2010).
The MBC and MFC was determined by subculturing the test dilution used in MIC on to a fresh solid medium and incubated further for 24 h. The concentration of plant extract that completely killed the bacteria was taken as MBC and that of fungi as MFC respectively. The MIC index (MBC/MIC) for bacteria was calculated for each extract to confirm the extract is bactericidal if (MBC/MIC < 4) or bacteriostatic if (MBC/MIC > 4) and similarly for fungi using (MFC/MIC < 4) as fungicidal and (MFC/MIC > 4) as fungistatic was determined. In the present study the MIC index value (< 4) of the active plant extracts indicates that all the extracts are bactericidal (Kone et al., 2004; Chattopadhyay et al., 2007; Cutler et al., 1994). The study also revealed that aqueous extract has shown moderate activity and chloroform extract has shown minimum antimicrobial activity. However, methanol extract of plant has shown significant antimicrobial activity. Sen et al., (2012) have reported the antimicrobial activity by using MIC, MBC and MFC of the plant Melia azedarach L. (Pavithra et al., 2010; Srinivasan et al., 2005; Parekh and Chanda, 2007).

The antimicrobial activity of F. limonia is due to the presence of phytochemicals such as alkaloids, tannins, phenolics and flavonoids which are soluble in organic polar solvents. Thus, the variation in the antimicrobial activity of extracts used in the present study might be attributed to the different compounds present in various solvents. Similar results were observed by Preethi et al., (2010) where the alcoholic extract from the plants Leucas aspera and Holarrhena antidysenterica has shown the potent antimicrobial activity. Seyydnejad et al., (2010) have also studied the effect of ethanol and methanol extracts for antimicrobial activity exhibiting the difference in the activity which is due to the presence of various phytocompounds in the extracts (Yusha’u et al., 2008). However,
the high genetic variability of bacteria enables them to neutralize the action of antibiotics by developing antibiotic resistance (Guleria and Kumar, 2006; Zakaria et al., 2007). Thus there has been a rapid search for new and potent antibiotics is increasing. In our investigation, *F. limonia* is set to be a good candidate against multidrug resistant bacterial and fungal strains.

4.4.2 *In vitro* antioxidant activity

Several techniques have been adopted to determine the antioxidant activity in order to allow rapid screening of compounds, because compounds that have low *in vitro* antioxidant activity will also show less *in vivo* activity.

Due to the complexity of the antioxidant compounds and their mechanism, there is no single testing method which can able to provide a comprehensive antioxidant profile of sample was not available and thus a combination of methods is to be followed. Thus in the present study DPPH and ABTS scavenging activity are tested. DPPH assay is a sensitive method for primary antioxidant screening and in finding the novel antioxidant molecules (Koleva et al., 2002; Blois, 1958).

The method of scavenging of DPPH is based on the addition of the radical species or an antioxidant compound that has the ability to decolorize the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants used. A large decrease in the absorbance of the reaction mixture indicates the significant free radical scavenging activity of the compound. In the present study, methanol extract at the dose of 10 mg/ml concentration has shown significantly higher inhibition percentage of about 72.23% and exhibited dose dependent scavenging activity. DPPH free radical scavenging activity of the phenolic compound was reported by many
ABTS radical scavenging assay involves a method that generates a bluish green ABTS+ chromophore through the reaction of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate; its reduction in the presence of hydrogen donating antioxidants is measured spectrophotometrically at 745 nm. All the concentrations of the methanol extract has possessed a strong dose dependent ABTS scavenging activity but at the dose level of 10 mg/ml it has exhibited significant scavenging activity of 76.21% when compared with the standard BHT. Antioxidant activities was evaluated using ABTS radical scavenging method of *Rumex hastatus*, *Torilis leptophylla* and *Rubus idaeus* (Saeed et al., 2012; Sahreen et al., 2011; Re et al., 1999; Gulcin et al., 2011).

The phosphomolybdenum method was used to measure the antioxidant capacity of the MEFL extract by using spectrophotometer. The principle is based on the reduction of the Mo (VI) complex by the test sample MEFL to Mo (V) and resulting in formation of final compound green phosphate/Mo (V) which has a maximum absorption at 765 nm. The present study demonstrated that methanol extract exhibited a highest antioxidant capacity for phosphomolybdate reduction with 78% in comparison with 97% of the standard drug BHT. Also a significant positive correlation was found between the total phenolic content, DPPH, ABTS and TAC in methanol extract is observed in the present study. There is a great contribution of many flavonoids and related polyphenols in scavenging activity of medicinal plants significantly by using phosphomolybdate method.
which has been reported by recent studies (Sharififar et al., 2009; Khan et al., 2012; Saeed et al., 2012; Babu et al., 2001).

**4.4.3 Toxicity study of MEFL extract**

The acute toxicity study of *Feronia limonia* methanol extract was carried out by administering the extract orally to rats. The extract did not cause any adverse effect and mortality to the animals after treatment. The toxicological evaluation results revealed that the oral administration of the methanolic extract at the doses of 200, 500, 1000, 2000 and 4000 mg/kg bw did not produce any demonstrable acute toxic effect and did not cause death of the animals, suggesting that the oral LD<sub>50</sub> of the extract was higher than 4000 mg/kg bw. According to guidelines of the Organization for Economic Cooperation and Development; the substances possessing LD<sub>50</sub> dose of 2000 mg/kg bw or higher are categorized as unclassified and non-toxic. Therefore, the tested methanol extract of *Feronia limonia* is categorized as highly safe and non-toxic. In addition to the toxicity there was no significant difference was observed in the amount of feed consumed by the animals and the body weight gain in treated rats when compared to control group throughout the experimental period. A study on acute toxicity of methanolic extract of *Mesua ferrea* L. in swiss albino mice was performed by Thangavelu et al., (2014) and concluded that the acute toxicity study of *M. ferrea* leaf extract administered orally to mice did not cause any death or acute adverse effect on the clinical observation and mortality to the treatment animals. Similar studies were also reported by other researchers who tested the acute toxicity of medicinal plants to evaluate the safety of non-allopathic medicines (Halim et al., 2011; Gogtay et al., 2002).
4.4.4 *In vivo* antioxidant activity in rat liver

First report on CCl₄ induced liver injury in rats was made by Cameron et al., (1936) which is now extensively and effectively followed by many researchers. Carbon tetrachloride is metabolized in endoplasmic reticulum and mitochondria by the cytochrome P-450 with the formation of CCl₃O⁻, a reactive oxidative free radical which initiates the lipid peroxidation. In the presence of oxidative stress more of lipid peroxidation products are formed due to cell damage (Aitken and Roman, 2008; Turner and Lysiak, 2008).

In the present investigation the carbon tetrachloride damage to erythrocytes was confirmed by the increase in SOD, GSH and CAT activities, and decrease in membrane fluidity. In the antioxidant defense system, an enzymic antioxidant i.e. SOD helps in the reduction of reactive oxygen species (ROS) and peroxides which are produced in the living organism and also by detoxifying various compounds that are of exogenous origin. Thus in the maintenance of a balanced redox status, SOD has an important role. The increase of SOD activity even after CCl₄ induction in rats suggests that the MEFL has an efficient protective mechanism in response to ROS. Catalase is a very important component of the antioxidant defense system. The increased SOD activity resulted in the accumulation of hydrogen peroxide, which stimulates to increase in CAT activity (Farombi *et al.*, 2013). The MEFL treatment increases the activities of Catalase in CCl₄ induced liver damage in rats to prevent the accumulation of excessive free radicals and thus protects the liver from intoxication (Sumaira *et al.*, 2013). GSH is a naturally occurring substance abundant in many living creatures; reduction in the levels of GSH leads to the tissue disorder and injury by increasing the sensitivity of the cells to several
aggressions (Jollow, 1980). In the present study our results demonstrated the efficiency of the extract in CCl₄ induced rats and found that exogenous MEFL supplementation elevated GSH levels in rats with CCl₄ treatment and thus it might provide a mean of recovering reduced GSH levels to prevent tissue injury which is in accordance with the study conducted by Khan et al., (2012). Treatment of experimental animals with the MEFL exhibited an improved free radical scavenging activity ensuring decrease in the activities of enzymes towards normal which is in agreement with the studies of Khan & Ahmed (2009) and Singh et al., (2008).

The field of dietary modification and chemoprevention is the considerable effective approach against oxidative stress and are the focus of research in these days. In the assessment of liver damage by CCl₄, the determination of enzyme levels such as AST, ALP and ALT is largely considered. Bilirubin concentration has been used to evaluate chemically induced hepatic injury. The data of the present experiment showed that the control group has demonstrated a normal range of AST, ALP, ALT, protein and bilirubin levels, while the CCl₄-treated group showed elevated levels in all the enzymes confirming that CCl₄ caused liver injury, altered membrane integrity and as a result enzymes in hepatocytes leak out. However, after treatment with MEFL, the increase in ALT, AST and ALP were significantly restored. Increase in the level of TP by MEFL indicates hepatoprotective activity, as stimulation of protein synthesis which accelerates the regeneration process and production of liver cells. These results indicate that the extract has the ability to protect against CCl₄-induced hepatocyte injury. Xiao et al., (2007) reported the protective effect of polyphenolic compounds against CCl₄-induced liver cirrhosis. Sayyah et al., (2004) have reported on the effects of intraperitoneal
Pharmacological screening

administration of CCl₄ to rats induced the development of liver cirrhosis; whereas Hefnawy et al., (2013) have demonstrated the protective effects of Lactuca sativa ethanolic extract on carbon tetrachloride induced oxidative damage in rats.

4.4.5 Invivo antioxidant activity in rat testis

The results of the present study revealed that the CCl₄ treatment caused a slight decrease in the body and decrease in the testis weight of toxic control group. Administration of methanol extract of Feronia limonia at two different dose levels attenuated the increased body weight and decrease in testis weight produced by CCl₄, and also resulted in subsequent recovery towards normalization compared to the normal control group. The increase in the testis weight may be due to the increase in lipid peroxidation resulting from the oxidative damage induced in rat testis. Oxidative stress may result in overproduction of oxygen free-radical precursors and decreased efficiency of the antioxidant system. CCl₄ and oxygen free-radical generation is associated with impaired glutathione metabolism, alterations in the antioxidant status. The results of our investigation reveals that the CCl₄ administration in rats has caused significant reduction in the activity of antioxidant enzymes like GSH, GSR, GST and increased TBARS. Reduction of antioxidant enzymes activity in testicular tissue might be due to the accumulation of free radicals leading to enhanced lipid peroxidation or inactivation of the antioxidant enzymes (Adewole et al., 2007). Recover of increased testicular enzyme levels in CCl₄ treated group towards the normal levels by the administration of Feronia limonia methanolic extracts at the dose levels of 200 mg/kg bw and 400 mg/kg bw demonstrated the antioxidant potential of the plant. Improvement in the levels of antioxidant enzymes may be due to the presence of phenolic and polyphenolic
Pharmacological screening

constituents which may have different functional properties such as scavenging of active oxygen species, inhibition of the generation of free radicals and chain breaking activity (Bast et al., 1998; Auddy et al., 2003; Palace et al., 1999). Several conditions are associated with male infertility for inducing oxidative stress which results in germ cell apoptosis and subsequent hypospermatogenesis (Santos et al., 2004; Conn, 1986). The hazardous effects caused by the \( \text{CCl}_4 \) is minimized by the administration of \textit{Feronia limonia} methanolic extract, which exerts many health-promoting effects, including the ability to increase intercellular antioxidant levels, scavenge oxidants and free radicals.

In the present study, a marked histopathological changes are observed such as disorganization of cell, reduction in germinal cells, necrosis, degeneration, spermatogenic arrest and multinuclear cell formation in \( \text{CCl}_4 \) group when compared to the control group rats. Similar findings have been reported following long-term \( \text{CCl}_4 \) administration in rats (Kalla & Bansal, 1975; Horn et al., 2006).

4.4.6 Anti-inflammatory activity

Carrageenan is a sulphated polysaccharide acquired from sea weed (Rhodophyceae) and is generally used to induce acute inflammation and is assumed to be bi-phasic. The first phase is caused by the release of histamine and serotonin. The second phase is due to the release of bradykinin, protease, prostaglandin and lysosome (Crunkhorn and Meacock, 1971; Elias and Rao, 1988). The second phase of oedema is sensitive to most clinically effective anti-inflammatory drugs, which has been frequently used to access the anti-oedematous effect of natural products. Prostaglandins play a major role in the development of second phase of reaction that is measured at 3 hours time interval. These mediators take part in the inflammatory response and are able to stimulate
nociceptor and thus induce pain (D'Armour et al., 1965). Based on these reports, it can be inferred that the inhibitory effect of the extract of *Feronia limonia* on carrageenan-induced inflammation in rats may be due to inhibition of the mediators responsible for inflammation. The anti-inflammatory property of the ethanolic extract of *A. aureum* is due to its ability to inhibit the cyclooxygenase pathway by inhibition of prostaglandins. Inflammation in rats has been reported to be linked with elevation in serum enzymes (Dacie, 1958). Swingle & Shideman (1972) have reported that an increased level of serum alkaline phosphatase occurs along with the low hemoglobin concentration in rheumatoid arthritis and inflammation which leads to anorexia and weight loss.

MEFL possesses several phytocompounds such as alkaloids, cardiac glycosides, tannins, flavonoids, sterols and triterpenes which are responsible for anti-inflammatory effect in animals. Our findings after MEFL treatment prove that the anti-inflammatory effect of MEFL in carrageenan induced paw oedema is due to its ability to decrease capillary permeability which further results in decrease of fluid exudation. Previous studies have reported that mediators like bradykinin, histamine, prostaglandins and leukotrienes will play a significant role in increasing vascular permeability. It is possible that MEFL inhibits the release of these mediators and thus act as a potent anti-inflammatory drug. Similar studies for the inhibition of mediators was demonstrated by various *in-vitro* and *in-vivo* anti-inflammatory models (Hemayet et al., 2011; Vijender et al., 2012).

During present era there is incredible development in the field of synthetic drugs which is usually accompanied by numerous undesirable side effects. Whereas plants still hold their own unique place, with lesser side effects. Therefore, a systematic approach
should be made to find out the efficacy of plants against inflammation as herbal anti-inflammatory agents. The formation of mediators of inflammation including prostaglandins and leukotrienes is due to the action of phospholipase A2 enzyme. The enzyme act at the site of inflammation by the release of free radicals and attracting polymorphonuclear leucocytes, thus leading to the damage of tissue.

In the cell membrane, the enzyme phospholipase A2 converts phospholipids into the product arachidonic acid. This product is a highly reactive substance and further rapidly metabolized by the enzyme cyclooxygenase to yield prostaglandins. Prostaglandins are the major components that induce pain and inflammation (Manivannan et al., 2012; Higgs et al., 1984, Vane, 1971). Our results revealed that administration of methanolic extract has inhibited the oedema starting from the first hour and during all phases of inflammation, which is probably due to inhibition of different chemical mediators of inflammation. The anti-inflammatory drugs have not been used successfully in all cases due to adverse side effects such as gastric lesions caused by non-steroidal anti-inflammatory drugs (NSAIDs). Therefore, new drugs lacking these side effects are searched for all over the world. During this process, the investigation of the efficacy of plant-based drugs used in the traditional medicines for the treatment of pain, fever and inflammatory ailments have received attention as they are cheap, safe and have less side effects. Thus the present study provides a scientific evidence of the claimed ethnopharmacological properties of the plant by investigating the anti-inflammatory activity of the methanolic extract of *Feronia limonia* fruit.