4. Discussion

4.1 Study of the phytochemical profiles of the leaves extract of FR and FB Linn.

The medicinal plants selected for the present study, FR and FB Linn., are two of the most important ingredients of Indian folk medicine due to their pharmacological attributes. FR Linn., has been known to have numerous therapeutic uses in traditional medicine. It has been used for different disease conditions related to central nervous system, gastrointestinal tract, endocrine system, respiratory system, reproductive system and infectious disorders. According to ayurveda medicine system, FB Linn. is lessens inflammations, aphrodisiac, tonic and is useful in piles. Furthermore, the leaves of these plants are considered to their anti-oxidative properties [94]. Effects of dietary polyphenols on human health has strongly supports a role of polyphenols in the prevention of degenerative, cardiovascular diseases and cancers [95]. The antioxidant properties of plant polyphenols have been widely studied, but it has become clear that the mechanisms of action of polyphenols go beyond the modulation of oxidative stress [95].

Consumption of plant antioxidants as the herbal extracts or food is beneficial to health because of down-regulating of many degenerative processes [96]. Recovery of antioxidant compounds from different plants is typically accomplished through different extraction techniques. However, Solvent extraction is most frequently used
technique for isolation of plant antioxidant compounds [97]. The extract yields and resulting protective potential of the extracts are strongly dependent on the nature of extracting solvent, due to varied chemical characteristics and polarities of plant ingredients that may or may not be soluble in a particular solvent.

The most suitable polar solvents for extract making are aqueous, ethanol or methanol extracting methods [98]. Methanolic and ethanolic extracts have been extensively used to extract antioxidant compounds from various Ficus species plants such as FR and FB Linn. [99]. Maniana et al, obtained the maximum content of phenolic compounds from methanolic extract of FB Linn. [99,100]. Therefore, the first part of the study was conducted to compare the antioxidant potential of extracts, obtained from the three solvent techniques (aqueous, ethanolic or methanolic), from leaves of FR and FB Linn.

The primary phytochemical screening of different extracts of FR and FB Linn, leaves showed the presence of tannins, flavonoids, glycosides, carbohydrates and triterpenoids. These active constitutes are augment immune function, induce detoxification enzymes, and protecting the body against chronic diseases. The presence of these constitutes has reported previously in the aqueous leaf extract of these plants [100,101].

In the last decade, epidemiological studies had suggested the importance of plant polyphenols against degenerative diseases [102]. From the results of current study methanol extract of FR showed the highest levels of active constitutes, like phenolic component, compared to another plant extracts. The over abundance of phenolic compounds from several plant extracts have been reported to possess strong antioxidant activities [103]. The phenolic groups can accept an electron to form relatively stable phenoxy radicals, thereby disrupting chain oxidation reactions in cellular components [100]. There are increasing evidences that as antioxidants,
polyphenols may protect cell constituents against oxidative damage and, therefore, limit the risk of various diseases associated with oxidative stress [100,101, 102].

Flavonoids are the most common groups of polyphenols in the human diet which are found in plants and reported to be the efficient as antioxidants [104]. Major dietary sources of flavonoids include wine, plants extracts, vegetables, cereals and fruit juices. In this study the higher content of flavonoids was followed by higher radical scavenging activity and reducing power. The sequential reduction of oxygen through the addition of electrons leads to the formation of a number of ROS including superoxide and hydroxyl radicals. The potential of current extracts studied against the activity of ROS. The phytochemical constituents of current plant extracts, acts in synergism to increase the extract bioactivity such as anti-oxidative potential. In this study, the higher content of bioactive contents was followed by higher anti-oxidative power. Phytochemical investigation on Ficus species revealed the phenolic compounds as the major components of this species and reported the antioxidant activity of this species which attributed to the phenolic contents [105].

4.2 The acute toxicity studies on the different leaf extracts of FR and FB in mice

Hepato-toxicity and nephro-toxicity are the most common side effects of plant based-medicines. In the present study, activity of serum transaminases (ALT and AST) as the main markers of neuro and hepato-toxicity [106] and study of BUN and serum Cr as the markers of nephro-toxicity [107], were studied to investigate to test the current extracts for their potential tototoxicity (If any). For this purpose, normal mice treated with different extracts of FR and FB (aqueous, ethanol and methanol) in increasing dose levels (500 to 2000 mg/kg BW) for fifteen days, continuously. This dosage has calculated as the two times of the maximum dosage of current extracts in Siddha (The oldest Indian traditional medicine system), form the basis for further studies [99,100,103].
An acute toxicity study of the FR and FB Linn. leaves extract were publicized the non-toxic nature of the extracts. The different doses of the FR and FB Linn. leaves extract did not show any significance changes in toxicity markers of brain, liver and kidney or lethality at any of the doses selected until the end of the study period. There was no lethality found in the groups which received the different doses of the extract until the end of the experimental period. With reference to this part of study we decided to use the normal dosages of current extracts in Indian folk medicine (250, 500 and 1000mg/kg BW) which were four to two fold of the maximum dosages in primary toxicity assay.

4.3 The possible protective effects of the leaves extract of FR and FB Linn. on Cisplatin-induced mice

Cisplatin is a prominent member of anti-tumor drugs [108]. However, its clinical usage has restricted due to side effects, such as nephro-toxicity and neuro-toxicity [109, 110]. However, recent studies has suggested the hepato-toxicity as the another dose-limiting side effect of Cisplatin-based chemotherapy [108].

Previous studies have reported the importance of apoptosis and necrosis followed by DNA damages as the main Cisplatin mechanism of action. Furthermore, Yao at 2007 reported the importance of oxidative stress as the one of the main mechanisms involved in Cisplatin toxicity [110]. The role of oxidative stress in Cisplatin toxicity is additionally supported by the protective effect of several free radical scavengers and antioxidants. The in vitro studies had showed the cyto-protective effect of antioxidants on cells exposed to Cisplatin [109]. The free radical scavenging potential of natural antioxidants present in some herbal products has shown to reduce oxidative stress induced by chemotherapy-based drugs [111].

Serum transaminases are the most universally important markers for hepatic and brain tissue injury, indicators for studies ranging from early preclinical animal testing to post marketing patient monitoring [112]. Serum transaminases are the cytoplasmic
enzymes involved in amino acid metabolisms [113]. Liver marker enzymes are localized in the cytosol of hepatic cells and thus are extruded into the serum when cells are damaged or necrotic. In healthy subjects, serum transaminases level are low. However, when cells are damaged, transaminases may leak into the blood stream and reduced the organs levels of transaminases [114]. Therefore, determination of serum transaminases has great clinical and diagnostic significance [115].

In the present study, Cisplatin induced cyto-toxicity as manifested by decrease content of serum transaminases (ALT, AST) and ALP in different organs. This indicated the presence of necrotic cells that resulted leakage of these enzymes to serum. Our results are parallel to that of the previously reported by Palipoch et al., 2013 who reported that, Cisplatin administration induced significant decrease in tissue transaminases and ALP levels [116].

The extracts as obtained from FR Linn, elevated the reduced level of liver and brain transaminases and ALP. These results suggested the protective potential of FR and FB against leakage of transaminases from the cells. Furthermore, the increased level of liver transaminases suggested the hepatoprotective potential of FR and FB against Cisplatin toxicity. However, The FB extracts was not able to affect the Cisplatin toxicity in kidneys.

The level of total bilirubin in the serum of NCG mice was also significantly increased when compared to the normal control group. Administration of the different extracts of FB and FR once daily for 15 days, exhibited a significant hepatoprotective activity, resulting in reduction in the elevated serum activities of transaminases and level of total bilirubin when compared to NCG mice.

In this study, mice intoxicated with Cisplatin developed significant hepatic damage as manifested by a significant increase in the serum activities of ALT, AST and ALP that are indicators of hepatocyte damage and loss of functional integrity. Pretreatment of mice with the different extracts of FB and FR in doses of 500 and
1000 mg/kg effectively protected mice against Cisplatin-induced hepatic damage, resulting in reduction in serum activities of liver marker enzymes when compared to the intoxicated control mice. Decrease in the level of these enzymes with FB and FR is an indication of the stabilization of plasma membrane caused by Cisplatin. Furthermore, the rise in the level of total bilirubin in serum following Cisplatin intoxication is also a measure of hepatotoxicity and could be attributed to impaired hepatic clearance due to hepatic parenchymal damage and biliary obstruction [117].

The ability of the extracts of FB and FR to reduce the level of total bilirubin in the serum of intoxicated mice suggests its potential protective effect of current plants. The lowered serum levels of total bilirubin and albumin due to Cisplatin are attributed to the damage of the endoplasmic reticulum which results in the loss of P-450 leading to fatty liver [103,117]. Administration of the extract of FB and FR remarkably prevented Cisplatin-induced reduction of total protein and albumin in serum. This assures the hepatoprotective activity of this extract against damage by Cisplatin.

As the most important intracellular antioxidant, GSH serves as the major scavenger of ROS. [104,112]. The thiol group of GSH is a potent reducing agent, in body. GSH plays a role in the detoxification of a variety of peroxides via catalysis by glutathione S-transferases (GST) and glutathione peroxidases. In addition to detoxification, GSH plays a role in other cellular reactions, including, the gene expression via thiol disulfide exchange reactions [106]. The changes in GSH, GST and GPx levels, has been suggested to the developing of oxidative damages in different organs [108,111]. In the current study the changes in GSH related enzymes such as GST and GPx, in different organs, has suggested the oxidative damage induced by Cisplatin in brain, liver and kidneys.

Many cellular components, in the cytoplasm that have soft nucleophilic sites such as thiol-containing peptides and RNA, may react with Cisplatin. The GSH-Cisplatin bounds are supported the decreased activity of endogenous antioxidants in
Cisplatin oxidative stress cases [112,115]. The pre and post administration of the different extracts of FR and FB Linn, caused an elevated levels of GSH, GST and GPx content of all tissues significantly. This may indicate the possible protective effect of these medicinal plants as a safe guarding agent against Cisplatin-induced oxidative stress. Glutathione peroxidase (GPx), provides a mechanism for detoxification of free radicals in living cells. It plays a crucial role in protecting cells from damage by free radicals, which are formed by peroxide decomposition [110,112]. GPx enzymes also catalyze the reduction of a wide variety of organic peroxides to the stable alcohols and water using cellular glutathione as the reducing reagent [118].

The GR enzyme is a ubiquitous enzyme involved in the protection of cells from cell stress conditions. Glutathione reductase catalyzes the reduction of oxidized glutathione (GSSG) to GSH [111,115,116]. It is essential for the glutathione redox cycle that maintains adequate levels of reduced cellular GSH, which serves as an antioxidant reacting with free radicals and organic peroxides. Glutathione is also an electron donor for GPx and a substrate for GST contributing to the detoxification and elimination of toxic electrophilic metabolites and xenobiotics [117]. Results summarized in this study showed that Cisplatin treatment to control mice resulted in significant decrease in GR content in different organs. However, methanolic extract of FR and FB protected the GR activity of organs against oxidative estress induced by Cisplatin.

In the normal conditions organs possess a powerful antioxidant defense system, including enzymatic and non-enzymatic antioxidants such as SOD and CAT [107]. In the primary study on phyto-chemistry profiles of FR and FB, the highest concentration of extracts, showed the highest reducing power. However, in in vivo condition also the highest level of the current extracts reported the higher anti-oxidative potential against Cisplatin toxicity referenced to elevated levels of CAT, SOD and GSH in liver, kidneys and brain organs.
Chapter 4: Discussion

Natural antioxidants are appearing as a good candidate in the prevention of antioxidant status impairment in different organs [108]. In the present study, FR and FB Linn. diminished the Cisplatin toxicity. These effects can be because of the antioxidant potential of flavonoids presence in the current extract. Furthermore, the lower content of flavonoids was reported in the aqueous extracts which was the reason in lower protective effect of them against Cisplatin toxicity compared to another extracts.

Superoxide is believed to be the cause of other ROS formations such as hydrogen peroxide and hydroxyl radicals. Therefore, Superoxide scavenging capacity in the body is the first line of defense against oxidative stress. It has been reported that over-expression of superoxide dismutase in transgenic flies extended life-span by as much as one-third, perhaps, due to decreased oxidative stress reflected by lower protein carbonyl contents [112]. Toxicity by superoxide anions has been suggested as a major cause of Cisplatin toxicity [115]. In this study, Cisplatin decreases the SOD activity in liver, kidney and brain. Larry at 2001 has reported the effects of Cisplatin on lowering the enzymatic activities of both manganese-containing and copper-containing SOD [114,117]. The decreased activity of SOD in the present study might be the reason of oxidative damage in Cisplatin treated animals. Phytochemistry study of different extracts of current plants showed the presence of high quantity of tannins. However, higher content of tannins was available in methanolic extract of FR. Velayutham investigated the effect of tannin supplementation on attenuating oxidative stress in diabetic rats [118]. With reference to this study, the higher level of SOD activity was in the organs of mice which were treated with methanolic extract of FR. However, in the current study, both of the plants, extracts prevented the loss of SOD activity in the dose dependent manner, in Cisplatin treated mice.

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the hydrogen peroxide (H$_2$O$_2$) to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by ROS [119]. In the
present investigation it has been found that Cisplatin induced toxicity by damaging the antioxidant defense system of organs such as GSH, SOD and CAT in different organs of Cisplatin treated mice (NCG). Sadaa at 2009, demonstrated that Cisplatin induced ROS in renal epithelial by depletion the intracellular concentration of GSH and CAT [120]. However, treatment of mice, which induced toxicity by Cisplatin, with FR and FB showed a strong protective potential against oxidative stress caused by Cisplatin.

Lipid components of the cell are especially susceptible to reactions with free radicals, resulting in lipid per-oxidation. Lipid per-oxidation refers to the oxidative degradation of lipids. It is the process in which free radicals steal electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which methylene bridges (-CH2-) that possess especially reactive hydrogen [121]. However, in the current study Cisplatin- induced lipid per-oxidation along with damaging the antioxidant defense system of mice in NCG. From the other hand, the high dosage of FR and FB has protected the tissues from lipid per-oxidation of by improving the antioxidant potential of organs. Furthermore, decreased level of MDA in different organs could be because of antioxidant protective effect of leaf extract of the studied plants.

Nephro-toxicity is an undesired side effect of chemotherapy. A minimum dose of Cisplatin (5 mg/kg body weight) is sufficient to induce nephro-toxicity in mice [122, 123]. Although higher doses of Cisplatin are more efficacious for cancer chemotherapy, the high-dose therapy manifests sever toxicities such as nephro-toxicity [122]. A number of antioxidants have been reported to render protection against Cisplatin-induced nephro-toxicity [123]. Several lines of evidence indicate that free radicals are involved in the nephro-toxicity. The nephro-toxicity is caused by Cisplatin, and the damage is suggested to be the consequence of decreased renal
antioxidant enzyme activity with enhanced lipid per-oxidation. Administration of antioxidants has been shown to ameliorate nephro-toxicity of Cisplatin [121,123].

The liver produces urea in the urea cycle as a waste product of the digestion of protein. BUN is an indication of renal health. If glomerular filtration rate and blood volume decrease then BUN will increase. The Cr is a breakdown product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body. Serum Cr is also an important indicator of renal health because it is an easily-measured by-product of muscle metabolism and excreted unchanged by the kidneys [124]. Cr and BUN are removed from the blood by the kidneys, primarily by glomerular filtration and via proximal tubular secretion. There is little or no tubular re-absorption of Cr. If the filtration in the kidney is deficient, the level of Cr will rise [124]. Therefore, Cr levels in blood and urine may be used to calculate the Cr clearance, which correlates with the glomerular filtration rate. In the acute renal failure induced by Cisplatin, renal tubular cells suffer a cytotoxic injuries, ranging from mild sub-lethal changes to a necrotic death [125]. Increasing the serum levels of BUN and Cr in current study can be because of nephrons damaging potential of Cisplatin which has affected the glomerular filtration rate [126]. Furthermore, decreasing the nephro-toxicity markers and also increased level of antioxidant defense system of kidneys with different extracts of FR and FB Linn. are the good references to nephro-protective potential of these plants against oxidative damage of kidneys.

The FR and FB have a notable place in traditions of Indian folk medicine [127]. However, fewer attempts have been made to investigate the therapeutic potential of these plants. Our investigations have shown that FR and FB Linn, possessed the significant antioxidant properties in different organs. In this study, we report the preventive effect of the different extract of these plants occurring oxidative damage caused by the administration of a cancer chemo-preventive agent, Cisplatin.