CHAPTER IV
DISCUSSION

No treatment related clinical signs were observed in control and ethylene glycol - treated animals. However, there was 40% mortality in group 4 rats, administered with 1.0% of ethylene glycol in drinking water. Khan (1997) also reported mortality in Sprague Dawley rats treated with 1.0% ethylene glycol in drinking water.

The administration of ethylene glycol in drinking water caused a dose – dependent significant reduction in the body weight of rat (Table 3.1; Fig. 3.1). A significant loss in body weight correlates with the decrease in feed consumption. Similar decrease in body weight due to the decrease in the food consumption has also been reported in an earlier study (Ringold et al., 2005). A considerable reduction in body weight and increase in kidney weight in rats was also observed when treated with ethylene glycol in drinking water (Parmar et al., 2012).

Treatment with ethylene glycol caused a significant increase in absolute and relative kidney weight of rat (Table 3.2; Fig. 3.2), which could be due to increased crystal depositions in the kidney. Histopathological investigations in the present study revealed significant calcifications in the ethylene glycol - treated groups (Plate B; Figs. 1-2, Plate C; Figs. 1-2 and Plate D; Figs. 1-2). Whereas the kidney untreated control rats showed normal histopathology with no calcifications (Plate A; Figs. 1-2). High mineral contents in the kidney are responsible for the kidney weight gain in the ethylene glycol – treated rats. Wientarsih et al. (2012) have attributed the inflammatory reactions due to calcifications. The relative organ weight was
considered to be a marker of cell constriction and inflammation (Moore and Dalley, 1999).

Histopathological studies revealed CaOx crystal deposition, tubular damage and dilatations of lumen in kidneys of ethylene glycol - treated rats in a dose – dependent manner (Plate B; Figs. 1-2, Plate C; Figs. 1-2 and Plate D; Figs. 1-2). Betanabhatla et al. (2009) reported that ethylene glycol - induced hyperoxaluria results in crystalline depositions with tubular dilatations and inflammatory infiltrations. Many earlier reports have confirmed the massive tubular cell death exposes the basement membrane to the urine stream, thus increasing the opportunity for the degraded structure to act as a seed for CaOx crystal formation or deposition, which in turn causes the gradual disruption of the tubular structure and lumen dilatation (Khan, 1995; Huang et al., 2009). Several studies have shown that, crystal formation results in cell damage and cell detachment from the basement membrane and the released degradation products further promote nucleation of crystals (Hackett et al., 1990; Verkoelen et al., 1998).

Urinary chemistry is one of the important factors in determining the type of crystals formed and the nature of macromolecules included on the surface of the crystals. Hence, the study of the urinary chemistry related to the calculi forming minerals will provide a good indication of the extent of stone formation. The urinary supersaturation with respect to stone-forming constituents is generally considered to be one of the causative factors in calculogenesis. The biochemical mechanism for this process is related to an increase in the urinary concentration of calcium and oxalate. In the present study, the administration of ethylene glycol in rats caused a significant increase in calcium and oxalate excretion levels as compared to untreated control in a time - and dose - dependent manner (Table 3.3, Figs. 3.3-3.4). This was also
correlated well with the microscopic observations of the urine of the ethylene glycol treated rats which showed significant increase in the number of calcium oxalate monohydrate and dihydrate crystals (Plate R; Fig. 2). However, the microscopic observations of the urine of the untreated rats showed no calcium oxalate crystals (Plate R; Fig. 1). The high urinary calcium concentrations leads to increased urinary saturation of calcium salts and reduced urinary inhibitory activity by way of complexation with negatively charged inhibitors such as citrate (Zerwekh et al., 1988). The hyperoxaluria is also a major significant risk factor in the pathogenesis of renal stone. It has been reported that oxalate play an important role in stone formation and has about 15-fold greater effect than urinary calcium (Karadi et al., 2006; Soundararajan et al., 2006). In the present study, urinary oxalate was increased in ethylene glycol - induced urolithic rats (Table 3.3, Fig. 3.4). Similar prompt increase in calcium and oxalate excretion due to ethylene glycol treatment were also reported by Anand et al. (1994), Malini et al. (1995) and Bashir and Gilani (2009).

The administration of ethylene glycol caused a significant elevation in phosphate excretion, as compared to untreated control, in a dose- and time – dependent manner (Table 3.4, Fig. 3.5). The increased excretion of phosphate has been reported in stone formers (Soundararajan et al., 2006). The increased urinary phosphate excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces CaOx deposition (Selvam et al., 2001; Karadi et al., 2006; Soundararajan et al., 2006). In addition, the increased levels of phosphates in urolithic rats might be due to increased basal metabolic rate (Ajayi et al., 2007).

The treatment of ethylene glycol caused a significant reduction in magnesium excretion as compared to untreated control in a dose – and time – dependent manner.
Magnesium is a well-known inhibitor of calcium stone formation preventing crystal growth and aggregation. In a supersaturated CaOx solution, magnesium reduced CaOx particle number by 50% (Desmars and Tawashi, 1973). Magnesium can form complexes with oxalate and thus decreases urinary supersaturation. The oral intake of magnesium has been reported to decrease the oxalate absorption and urinary excretion, in a manner similar to calcium by binding to oxalate in the gut (Liebman and Costa, 2000).

An increased urinary total protein excretion was observed in ethylene glycol-induced urolithiatic rats in a dose-dependent manner (Table 3.5, Fig. 3.7). The proteinuria reflects proximal tubular dysfunction. The supersaturation of urinary colloids results in precipitation as crystal initiation particle which when trapped acts as a nidus leading to subsequent crystal growth. This is associated with proteinuria (Selvam et al., 2001). Many earlier reports have indicated that administration of ethylene glycol results in a prompt increase in total protein excretion (Betanabhatla et al., 2009; Divakar et al., 2010).

Ethylene glycol-induced changes in the creatinine (Fig. 3.23), uric acid (Fig. 3.24) and urea nitrogen (Fig. 3.25) in urine are presented in Table 3.19. The treatment of ethylene glycol results in a significant increase in the urinary excretion of creatinine, uric acid and urea nitrogen. The hyperuricosuria cause excessive supersaturation of the urine. Hyperuricosuria appears to cause calcium oxalate nephrolithiasis by promoting the formation of monosodium urate or uric acid crystals, which either act as seed crystals for calcium oxalate or adsorb normally occurring macromolecular inhibitors of CaOx crystallization (Coe et al., 1980).

In calculi-induced rats, there is significant reduction in serum calcium level due to ethylene glycol treatment in a dose- and time-dependent manner (Table 3.7,
It has been reported that the oxalic acid, a metabolite of ethylene glycol, chelates serum calcium and precipitates as crystals in renal tubules, thereby causing depletion of serum calcium level (Scalley et al., 2002). However, there is a significant increase in serum phosphate level in urolithiatic rats (Table 3.7, Fig. 3.9). These results are in accordance with the previous studies with ethylene glycol in various animal species (Betanabhatla et al., 2009; Divakar et al., 2010).

Results also indicated a dose-dependent decrease in magnesium content in serum after ethylene glycol treatment (Table 3.8, Fig. 3.10). Magnesium supplementation in subjects with magnesium deficiency has been reported to increase the excretion of citrate in urine, which in turn, binds with calcium thereby reducing the concentration of CaOx aggregation (Reungjui et al., 2002). The low levels of serum magnesium are also encountered in stone-forming rats as well as in stone formers (Gyawali et al., 2011).

Table 3.9 indicates the results of serum levels of total protein after ethylene glycol treatment (Fig. 3.11). There was considerable increase in serum total protein due to the ethylene glycol treatment in a dose-dependent manner. The main protein that is most likely to appear in urine is albumin (Yim et al., 2001). However, it has been reported that in CaOx urolithiasis, there has been a rise in oxalate binding protein in serum as well as in kidney thereby promoting the crystallization (Selvam and Kalaiselvi, 2003). Similar rise in serum protein due to ethylene glycol – induced urolithiasis has been observed in an earlier report (Bouanani et al., 2010).

Table 3.24 shows the results of ethylene glycol - induced changes in the serum electrolytes (sodium and potassium) in a time-dependent manner (Fig. 3.32). The present study showed hyponatremia due to ethylene glycol. Hyponatremia results when water intake exceeds the water excretion. The administration of ethylene glycol
in drinking water has been reported to increase the water intake and subsequent hyponatremia (Bashir and Gilani, 2009).

In the present study, ethylene glycol administration also results in a significant increase in the serum potassium concentration in a time-dependent manner (Table 3.24; Fig. 3.33). This increase could be due to the fact that ethylene glycol leads to the acidosis which results in hyperkalemia because of shifts of potassium from the intracellular to the extracellular compartment. Similar hyperkalemia due to ethylene glycol administration has also been reported in an earlier report (Perez et al., 1981).

Table 3.30 presents the increase in the activities of ALT and AST by the administration of ethylene glycol (Figs. 3.41-3.42). Hepatic function has been monitored by the evaluation of the serum levels of ALT and AST. Body cells contain more AST than ALT. Usually, about 80% of AST is found in the mitochondria whereas ALT is a purely cytosolic enzyme. The AST is also found in a large number of tissues, such as heart, lung, skeletal muscle and kidney, whereas ALT is primarily limited to liver. Thus the latter is considered as highly sensitive indicator of hepatotoxicity (Al-Mamary et al., 2002). The ALT in blood increases when the hepatocellular permeability is changed or when necrosis and cellular injury occur (Latha et al., 1998). Urolithiasis and hepatotoxicity induction by ethylene glycol was established by many researches (Christina et al., 2002; Huang et al., 2006; Celik and Suzek, 2007).

Ethylene glycol treatment results in a significant increase in the activity of alcohol dehydrogenase (ADH) (Table 3.31, Fig. 3.43). The major cause of toxicity is not the ethylene glycol itself but its metabolites. Initially it is metabolized by ADH in liver to glycolaldehyde, which is then oxidized to glycolic acid and finally to oxalic
acid, leading to hyperoxaluria as well as increased retention of oxalate in kidney (Brent, 2001). Therefore, in studies in which inhibitors of ADH have been administered in both animals and humans, toxicity has been minimized.

The deposition of the crystalline components in the renal tissue, namely oxalate, phosphate and calcium were significantly increased in ethylene glycol - treated groups in a dose – dependent manner (Table 3.11, Figs. 3.12-3.14). The increase in calcium level in renal tissue might be due to the increased bioavailability of nitric oxide (NO) which in turns activates cGMP (3´, 5´ cyclic guanosine monophosphate) that controls the increase in intracellular calcium levels. Previous studies reported that NO donors have the capacity to control the intracellular rise in calcium levels (Divakar et al., 2010). The ethylene glycol treatment results in increased oxalate production by way of increase substrate availability that induces the activity of oxalate synthesizing enzyme. Glycolic acid oxidase and lactate dehydrogenase catalyses the oxidation and reduction of glyoxalate results in formation of glycolate and oxalate (Soundararajan et al., 2006). These changes facilitate the hyperoxaluria and subsequent CaOx crystal adherence and retention in renal tubules (Khan, 2005).

The present study also indicated that the administration of ethylene glycol for 28 days caused a significant increase in total protein content in kidney tissue (Table 3.11, Figs. 3.15). The high molecular weight proteins derived from hyperoxaluric rat kidney showed greater promoter activity than the low molecular weight proteins. Molecular changes in proteins derived from stone-formers, have been reported to exert a greater tendency to self-aggregation and thereby loss of inhibitory potential takes place (Kalaiselvi and Selvam, 2001).
The Pearson correlation analysis in the present study showed a significant positive correlation among calcium, oxalate, phosphate and total protein whereas magnesium is found to be negatively correlated with them (Table 3.12). Furthermore, there are strong correlations between the values of urine, serum and kidney parameters (Table 3.13). Similar kind of correlations has been reported by Erdamar et al. (2007).

Oxidative stress plays a significant role in the pathogenesis of many diseases and antioxidants have emerged as key therapeutic agents in the management of various associated disorders. Oxalate increases the production of free radicals, which can induce cell death process, crystal deposition in the renal tubules, which further leads to growth of calcium oxalate stones. The human, animal, explants and in vitro studies indicate that raised oxidative stress is often present in urolithiasis and oxalate promotes oxidative stress, which is substantially retarded by antioxidants (Kubo et al., 1997).

In this regard, the significant reduction was seen in the activity of antioxidative enzyme that is SOD and also in total antioxidant capacity (TAC) with a concomitant increase in lipid peroxidation after 28 days treatment with ethylene glycol suggesting oxidative stress (Table 3.28, Figs. 3.40-3.42). The determination of the total antioxidant capacity of serum or plasma is an efficient biomarker because of the difficulty in measuring each antioxidant component separately and the interactions among them (Miller et al., 1993). The effects of ethylene glycol on serum antioxidant defense systems and lipid peroxidation were investigated earlier and it has been proven that ethylene glycol results in significant increase in lipid peroxidation with a concomitant decrease in activities of antioxidant enzymes like SOD and CAT (Celik and Suzek, 2007; Thamilselvan et al., 1997).
Moreover, ethylene glycol treatment also caused extensive CaOx crystal deposition in kidney of rats accompanied by oxidative damage as reflected from increased levels of markers of oxidative injury such as MDA and protein content (Table 3.34, Figs. 3.47-3.48). Recent studies have provided evidence that CaOx kidney stone patients excrete significantly higher amounts of MDA and protein in their urine, indicating ROS in kidneys of CaOx stone patients (Huang et al., 2003; Puntel et al., 2007). Malonyldialdehyde (MDA) is a major end product of lipid peroxidation. Oxidative tissue damage caused by ROS resulting in structural alteration of membrane with release of cell and organelle contents, loss of essential fatty acids with formation of cytosolic aldehyde and peroxide products (Kato et al., 2007). These generates toxic responses in renal epithelial cells, including altered membrane surface properties, changes in gene expression (NF-κB), disruption of mitochondrial function and formation of ROS (Jonassen et al., 2005). Mitochondria are a major site of ROS formation and oxalate-induced activation of NADPH oxidase, is another source of ROS in renal cells. Animal model studies have provided evidence for the hyperoxaluria-induced activation of the renin-angiotensin system (RAS) and angiotensin II; implicated in causing oxidative stress by activating membrane associated NADPH oxidase, which leads to the production of ROS (Antus et al., 2001; Khan, 2004, 2005).

The ethylene glycol administration in drinking water also results in significant decrease in activities of enzymatic antioxidants like SOD, CAT, GPx and GR (Table 3.36, Figs. 3.51-3.54) as well as non-enzymatic antioxidants like GSH and TAA level (Table 3.35, Figs. 3.49-3.50). Free radical scavenging enzymes such as CAT, SOD and GPx are the cellular defense enzymes against oxidative injury, decomposing superoxide and peroxide before their interaction to form the more reactive hydroxyl
radical. Under oxidative stress conditions, ROS are reduced by conjugation with GSH directly or by means of GSH-related enzymes, which decrease GSH levels (Pastore et al., 2003). Glutathione reductase also plays a key role in cellular detoxification by catalyzing the reaction of reducing glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant (Meister, 1988). All the above parameters were reported to decrease in hyperoxaluria induced urolithiasis (Rodrigo and Bosco, 2006; Puntel et al., 2007).

The pathophysiology of calcium oxalate stone formation is due to hyperoxaluria as evidence suggests that hyperoxaluria increases the production of free radicals with subsequent lipid peroxidation in the kidneys, leading to renal tubular epithelial cell injury and calcium oxalate stone formation (Moriyama et al., 2009). In this regard, the sodium oxalate induced oxidative stress were also ascertained by determining lipid peroxidation level and activities of superoxide dismutase and catalase in sodium oxalate treated kidney homogenate from rat in vitro condition (Table 3.40; Figs. 3.82-3.87). The sodium oxalate elicits a significant increase in lipid peroxidation and concurrent decrease in SOD and CAT activities (Figs. 3.82-3.87). Oxalate toxicity is mediated in part by activation of lipid signaling pathways, which in turn can disrupt mitochondrial function by increasing the production of ROS such as superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radicals. Reactive oxygen species mediate cell injury by modifying lipids, proteins, and DNA, and this can result in the promotion of calcium oxalate crystal formation (Hackett et al., 1990; Moriyama et al., 2005). Oxidative damage due to oxalate is therefore considered to play an important part in stone formation. Sodium oxalate has been reported to significantly pronounce the release of malonyldialdehyde in liver and
kidney homogenates and reduced the activity of SOD and catalase (Selvam and Kurien, 1987).

Herbal medicine is gaining popularity. In the last few years there has been an exponential growth in the field of herbal medicine and these drugs are gaining popularity both in urban and rural India because of their natural origin and less side-effects. Therefore, it has been of great interest to determine better alternative treatment options through herbal medicine. This has given rise to stimulation in the search for investigating natural resources showing antiurolithiatic activity. During the present study, two plants – rhizomes of *Bergenia ciliata* (Family - Saxifragaceae) and seeds of *Dolichos biflorus* (Family - Fabaceae) were selected for the study. The mid dose (MD) of ethylene glycol was found to be the most effective dose in inducing urolithiasis with no mortality in rat. Hence, it was chosen for further study along with the two plant extracts and marketed polyherbal drug i.e., cystone.

Phytochemicals occur in various parts of plants. Qualitative and quantitative analysis which are considered as parameters for development of preliminary quality control standards in plant extracts were performed. The quantitative analysis revealed higher percentage of phenolic compounds in *B. ciliata* while flavonoids were found to be more in *D. biflorus*. Tannins content was high in *B. ciliata* while it was found to contain a very less amount of ascorbic acid.

Antioxidants have been proposed as therapeutic agents for the prevention and treatment of diseases. Recent interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products, because they possess multifacetedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance (Djeridane et al., 2006; Wannes et al., 2010). Free radical scavenging activities of the both the plant extracts were
determined by using 1, 1-diphenyl - 2 - picrylhydrazyl free radical (DPPH), superoxide and hydrogen peroxide - induced free radicals (Figs. 3.64-3.66). Moreover, nitric oxide radical scavenging activity and reducing power assays were performed and compared with known positive controls like ascorbic acid (Figs. 3.63, 3.67). The plant extracts significantly scavenge the effect of these free radicals in a dose - dependent manner. However, the effect of the plant extracts was found to be more potent than cystone and ascorbic acid. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals implicated in several diseases (Bravo, 1998).

The demand for complementary and alternative medicines is increasing among patients with kidney stones. Currently strict rules are imposed on herbal preparations by many regulatory agencies worldwide and presence of sufficient quantity of any known marker compound in them is considered mandatory. Chromatographic fingerprinting of plant extracts is as a holistic method for detection and quality control of active ingredients. The phenomenon of ‘Standardization’ has now been accepted as an essential part of medicinal plant research.

The HPLC analysis showed that the major active component of the hydro-alcoholic extract of rhizomes of *B. ciliata* is gallic acid which is a phenolic compound (Figs. 3.74-3.76). Similarly, hydro-alcoholic extract of seeds of *D. biflorus* showed the presence of a biflavonoid, quercetin as their major constituent (Figs. 3.77-3.79).

Notable work in the process of extraction, separation and characterization of different constituents of *Bergenia* (Tucci *et al*., 1969; Haslam, 1969; Bahl *et al*., 1974; Dixit *et al*., 1989; Reddy *et al*., 1999; Vaishali *et al*., 2008) and *Dolichos* species (Kawsar *et al*., 2009; Kawsar *et al*., 2010; Morris *et al*., 2013) has been well documented in previous reports. Subsequently, Chauhan *et al*. (2000) have unambiguously isolated
and elucidated the structure of gallic acid from *Bergenia* species. Morris et al. (2013) have analyzed *D. biflorus* seeds and have determined quercetin and myricetin by gas chromatography and then reverse-phase high performance liquid chromatography.

Table 3.14 presents the results of plant extract treatment on ethylene glycol-induced changes in the body weight of rat in a time-dependent manner. Plant extracts alone treated rats did not show any significant difference in body weight as compared to untreated control. However, ethylene glycol administration caused a significant reduction in the body weight of rat. Results indicated a significant increase in body weight of both the plant extracts plus ethylene glycol-treated rat (Table 3.14, Fig. 3.16). The effects were dose-dependent and maximum for BCE, followed by DBE and CST. The rhizomes of *B. ciliata* have been reported to contain a large number of polyphenolic constituents, including gallic acid, methyl gallate, bergenin, afzelechin, leucocyanidine, (+)-catechin, 11-*O*-galloyl bergenin, paashanolactone and sterols like β-sitosterol and β-sitosterol-D-glucoside (Reddy et al., 1999). These phenolic constituents are responsible for the protective effect of *B. ciliata*. Patel and Goyal (2011) have evaluated the cardioprotective effect of gallic acid and reported that, administration of this compound caused a gradual increase in the body weight of rats. Nanta and Kale (2010) observed a significant increase in body weight in 7,12-dimethylbenzanthracene-induced carcinogenic mice after treatment with the seeds of *D. biflorus*.

Table 3.15 shows the mitigatory effect of plant extract treatment on ethylene glycol-induced changes in the absolute and relative kidney weights of rats (Fig. 3.17). This effect could be due to the reduced calcifications in kidney of rats with plant extract treatment. These results were also confirmed by the histopathological analysis of the kidney. The plant treatment results in milder calcifications in the
kidneys, indicating a reduction of the extent of damage done at the tissue level (Wiessner et al., 2001). Patel et al. (2012) have evaluated antiurolithiatic activity of Solanum xanthocarpum in ethylene glycol - induced lithiatic rats and confirmed the similar results with reduced kidney weights due to reduced calcifications in plant extract treated rats. It suggests that the effect of the extracts could be advantageous in preventing renal stone retention by reducing renal necrosis and thus inhibit crystal retention.

Renal histopathology and Pizzolato staining also supports the above results as evident from CaOx crystal deposition, glomeruli and tubular damage in kidneys of ethylene glycol - induced rats (Plate I; Figs. 1-2). The calcifications in the kidney of ethylene glycol - induced rats were also confirmed by the von Kossa staining and further showed as calcium oxalate crystallization by Pizzolato staining (Plate P; Fig. 2 and Plate Q; Fig. 2). However, co-treatment of ethylene glycol along with plant extracts inhibited crystal deposition and ameliorates renal injury with maximum potency was achieved with B. ciliata followed by D. biflorus and cystone (Plate L; Figs. 1-2, Plate M; Figs. 1-2, Plate N; Figs. 1-2 and Plate O; Figs. 1-2). Similar trend was observed in von Kossa and Pizzolato staining which showed reduced calcifications by the treatment of both the plant extracts (Plate P; Figs. 3-5 and Plate Q; Figs. 3-5). No significant changes were observed in the kidney of untreated control rat (Plate A; Figs. 1-2, Plate P Fig. 1 and Plate Q; Fig. 1). The tissue injury, loss of membrane integrity and inflammation in kidney of these animals are due to hyperoxaluria - induced lipid peroxidation and depletion of antioxidant enzymes (Mani and Selvam, 2003; Thamilselvan and Menon, 2005; Itoh et al., 2005). The renal epithelial injury promotes crystal retention, as epithelial injury exposes a variety of crystal adhesion molecules on epithelial surfaces (Bijarnia et al., 2008). These
changes facilitate CaOx crystal adherence and retention in renal tubules (Khan, 2005). Previous studies indicate that both individual cell injury (loss of lipid asymmetry) and generalized cell monolayer injury (loss of cell polarity) result in the presentation of different cell surface, and that both form of injury result in an increased affinity of crystal attachment (Wiessner et al., 2001).

Table 3.16 and 3.17 represents the results of plant extract treatment on ethylene glycol – induced changes in calcium, oxalate, phosphate and magnesium excretion levels (Figs. 19-22). A significant and dose-dependent decrease was observed in urinary excretion of calcium, oxalate and phosphate when the plant products were administered on a co-treatment regimen along with ethylene glycol. This was also correlated with the microscopic observation of the urine which showed reduced number of CaOx crystals due to the plant extracts treatment along with the ethylene glycol (Plate R; Figs. 1-8). The high urinary calcium concentrations lead to increased urinary saturation of calcium salts and reduced urinary inhibitory activity by way of complexation with negatively charged inhibitors such as citrate (Zerwekh et al., 1988). Thus, treatment strategies aimed at reducing urinary calcium levels are beneficial in reduction of stone recurrence rates.

The decrease in oxalate excretion due to plant extracts (Table 3.16; Fig. 3.19) might be due to the inhibition of formation of oxalate by the plant extract. In this regard, the treatment of plant extracts also resulted in the significantly reduced activity of ADH in liver of ethylene glycol - induced urolithiatic rats (Table 3.31; Fig. 3.43). Thus, both the plant extracts were found to be potent inhibitors of ADH which in turn might results in the inhibition of the metabolism of ethylene glycol to oxalate. Similar inhibition of ADH activity was also reported by Dawidek et al. (1998) who have reported four inhibitors (pyrazole, 4-methylpyrazole, cimetidine and
theophylline) which inhibit ADH activity *in vitro* at physiological pH 7.4 using human enzyme hepatic fraction. The extract of *Aerva lanata* decreases the oxalate excretion, in ethylene glycol fed rats, by decreasing the formation of oxalate synthesizing enzymes like glycolic acid oxidase or alcohol dehydrogenase in liver and lactate dehydrogenase in liver and kidney (Soundararajan *et al.*, 2006). Gallic acid, a polyphenyl present in the *B. ciliata* has been reported to inhibit lactate dehydrogenase in liver of rats with acute alcohol intoxication (Kartkaya *et al.*, 2013). *Dolichos biflorus* contains a number of flavonoids like quercetin, streptogenin, beta-sitosterol, a phyto-haemagglutinin, beta-N-acetylglucosaminidase, alpha and beta galactosidases, alpha mannosides and beta glucosides (Khare, 2003). Waheed and Mohammed (2012) have reported that quercetin inhibit lactate dehydrogenase in liver of rats with fenvalerate - induced hepatotoxicity. Shirfule *et al.* (2013) also reported that Gokshuradi polyherbal ayurvedic formulation inhibited lactate dehydrogenase, the oxalate synthesizing enzyme the activity of which was increased on ethylene glycol administration. Similar results were also reported in the extract of *Tribulus terrestris* (Sangeeta *et al.*, 1994).

The increased urinary phosphate excretion due to ethylene glycol was significantly protected by the plant extracts at both the dose levels tested (Table 3.16, Fig. 3.20). Ethylene glycol - induced calcium oxalate crystals has been shown previously to nucleate epitaxially calcium phosphate crystals from a supersaturated solution of CaOx due to the similarity of lattice structure of both the type of crystals (Meyer *et al.*, 1977). This was prevented by both the plant extracts treatment with *B. ciliata* being most potent followed by *D. biflorus* and cystone at the same dose levels. Similar results were also obtained with crude aqueous - methanolic extract of
Holarrhena antidysenterica against ethylene glycol - induced urolithiasis (Khan et al., 2012).

Consistent with some previous reports (Divakar et al., 2010; Khan et al., 2012), stone induction by ethylene glycol caused a decrease in urinary magnesium excretion which was prevented by the plant extracts in a time-dependent manner at both the dose levels (Table 3.17, Fig. 3.21). Magnesium oxide has been shown to decrease the saturation of CaOx and inhibit crystal nucleation, growth and aggregation, while reduced crystallization in urine of stone forming patients (Kato et al., 2004). Similarly, the ethanolic extract of leaves of Achyranthus aspera Linn has been screened for antilithiatic activity and decreases the urinary concentration of ions that is calcium, oxalate and phosphate and subsequently increases the magnesium excretion (Awari et al., 2009).

The plant extracts treatment significantly reduces the elevated levels of the total protein (Fig. 3.22), creatinine (Fig. 3.23), uric acid (Fig. 3.24) and urea nitrogen (Fig. 3.25) in urine of ethylene glycol – treated rats (Table 3.18 and 3.19). This action of the plant extracts could be due to the increase in the proximal tubule reabsorption. Moreover, the reduction of uric acid level by plant extracts also suggests that both the extracts reduce the excessive supersaturation of the urine by uric acid crystals. Divakar et al. (2010) also reported that hydro-alcoholic extract of roots of Rubia cordifolia caused a dose-dependently decrease in the total protein, uric acid, creatinine and urea nitrogen excretions in ethylene glycol – induced urolithiasis.

The results of the present study revealed significant increase in the total bilirubin and unconjugated bilirubin content in urine in ethylene glycol - treated rats which was protected by the plant extracts (Table 3.20, Figs. 3.27 and 3.29). Urobilinogen is a byproduct of hemoglobin breakdown. An increase in unconjugated
bilirubin occurs due to the increased breakdown of RBCs (Thapa and Walia, 2007). An elevation in unconjugated bilirubin level that is urobilinogen might be due to the inadequate vitamin D which is needed in active form for the development of bone. Elkousy et al. (2012) have investigated the metabolic abnormalities of patients with calcium oxalate urolithiasis and have concluded that the patients presenting with urolithiasis were found to have a high prevalence of inadequate vitamin D. Aggarwal et al. (2013) have reported that phytocompounds like lupeol results in similar decrease in bilirubin levels in ethylene glycol - treated rats.

Table 3.22 presents the results of plant extract treatment on ethylene glycol - induced changes in the calcium (Fig. 3.29) and phosphate levels (Fig. 3.30) in the serum. The results of the present study indicated that both the extracts reversed the changes in serum levels of calcium and phosphate. It has already been discussed earlier that the oxalate chelates serum calcium and thereby results in depletion of serum calcium level (Scalley et al., 2002). Therefore, this reversion by the plant extracts could be due to reduction in the ADH activity (Table 3.31) thereby reducing the generation of oxalate which in turn results in reduced chelation of serum calcium. Bouanani et al. (2010) have reported similar kind of results when aqueous and butanolic extracts of aerial parts of Paronychia argentea were tested for antiurolithiatic effects.

A significant decrease were observed in magnesium levels in serum due to ethylene glycol treatment which was most significantly prevented by the extract of B. ciliata followed by the D. biflorus and then cystone in a time – and dose – dependent manner (Table 3.23, Fig. 3.31). Magnesium has been reported to inhibit CaOx crystal nucleation (Li et al., 1985), growth (Doremus et al., 1978; Li et al., 1985) and aggregation (Ryall et al., 1981). In 1932, Cramer showed that renal calcification and
tubular degeneration occurred in rats fed on a diet deficient in magnesium, an observation that has since been confirmed by other investigators (Rushton and Spector, 1982). Thus, magnesium has a long historical association with calcium stone disease, principally as a result of its ability to form ion complexes with oxalate and magnesium oxalate is considerably more soluble than CaOx. Interference with crystal growth and aggregation, therefore, seems a possible therapeutic strategy for the prevention of recurrent stone disease. The effect of the plant extracts on magnesium levels in serum might be due to the presence of polyphenolic compounds present in them as they have been reported to increase magnesium levels (Ghodasara et al., 2010). Freitas et al. (2002) investigated the effect of an aqueous extract of *Phyllanthus niruri*, a plant used in folk medicine to treat lithiasis, on the urinary excretion of endogenous inhibitors of lithogenesis, magnesium and glycoaminoglycans. Their results showed that *Phyllanthus niruri* has an inhibitory effect on crystal growth, which might be related to the higher incorporation of magnesium into the calculi.

Table 3.24 shows the results of plant extracts treatment on ethylene glycol-induced changes in the serum electrolytes (sodium and potassium) in a time-dependent manner. The administration of plant extracts significantly reduces potassium level and increases sodium level in serum of ethylene glycol rat model of urolithiasis (Fig. 3.32-3.33). Similarly, wood bark extracts of *Cassia fistula* efficiently reversed the decreased sodium and increased potassium levels in serum (Ramesh et al., 2010). Similar kind of results were found with aqueous and butanolic extracts of aerial parts of *Paronychia argentea* (Bouanani et al., 2010).

Ethylene glycol treatment caused impairment of renal functions of the rats as evident from total protein loss (Fig. 3.37) and raised blood urea nitrogen (Fig. 3.36),
serum creatinine (Fig. 3.34) and uric acid (Fig. 3.35), which was prevented in the animals treated with the plant extracts (Table 3.25 and 3.26). The restoration of the marked increase in serum protein level by plant extracts in our study shows that these extracts help in minimizing the extent of tubular dysfunction. In renal stones, the serum urea accumulates (resulting in uremia) because the rate of serum urea production exceeds the rate of clearance (Mayne, 1994). In urolithiasis, the glomerular filtration rate decreases due to the obstruction of the outflow of urine by stones in urinary system. Due to this, the waste products particularly nitrogenous substances such as urea, creatinine and uric acid accumulate in blood (Grover and Resnick, 1995), which was reversed by the administration of the plant extracts. Gallic acid, a well known polyphenolic compound present in *B. ciliata*, significantly attenuated the oxytetracycline-induced nephrotoxicity by decreasing levels of serum urea and creatinine with the significant normalization of creatinine clearance (Balagangadharan, 2012). Similarly, quercetin, a bioflavonoid present in *D. biflorus*, is a well known renoprotective agent as it significantly reduces serum levels of these nitrogenous substances in ischemic renal injury (Shoskes, 1998), myoglobinuric acute renal failure (Chander *et al.*, 2005) and organochlorine compounds-induced renotoxicity (Padma *et al.*, 2012). Similar kinds of results were also obtained with other plant extracts by many researchers (Bouanani *et al.*, 2010; Khan *et al.*, 2012; Patel *et al.*, 2012).

The Pearson correlation analysis was performed and it was observed that there were positive correlations between calcium, oxalate, phosphate and renal function parameters that are urea, uric acid and creatinine (Table 3.21). Fellstrom *et al.* (1982) with calcium lithiasis and Dumoulin *et al.* (1984) with pure and mixed CaOx lithiasis found a positive correlation between urine values of calcium, phosphorus, oxalate and
uric acid. Both Tefekli et al. (2003) and Ogava et al. (2003) found that levels of calcium are strongly correlated with citrate, magnesium and creatinine. Gyawali et al. (2011) have observed a positive correlation between calcium, phosphorus, uric acid and magnesium levels in serum and 24 hours urine.

Table 3.31 presents the decrease in the activities of ALT and AST by the co – treatment of plant extracts which was increased significantly by the administration of ethylene glycol (Figs. 3.39-3.40). In this view, the reduction of ALT and AST levels with the B. ciliata followed by D. biflorus and cystone, is a stabilization indication of plasma membrane as well as repair of hepatic tissue damage caused by oxalate crystal deposition. The seeds of D. biflorus have already been reported to reduce AST and ALT in a dose - dependent manner in D-galctosamine and paracetamol - induced hepatotoxicity in rats. Similarly, bergenin, as earlier described, one of the major constituent of B. ciliata, substantially reduced the AST and ALT levels in CCl₄ – intoxicated rats (Lim et al., 2000).

Increase in calcium, oxalate and phosphate levels in the renal tissue of ethylene glycol treated rats was observed (Table 3.32, Figs. 3.45-3.47). The CaOx crystal agglomerate tends to retain in kidney by trapping in renal tubules and develop into renal stones, which damage the renal tissue and deteriorate the renal function. The plant extracts treatment suppresses the increase in intracellular calcium. Thus, plant extract could effectively control the levels of both the salts by either inhibiting the synthesis of oxalate or by increasing the bioavailability of NO to sequester calcium through the cGMP pathway (Pragasam et al., 2005).

The protective capacity by the plant extracts were also determined in terms of modulation of oxidative stress induced by ethylene glycol administration. The significant reduction was seen in the activity of SOD and TAC with a concomitant
increase in lipid peroxidation by ethylene glycol which was significantly mitigated by both the plant extracts (Table 3.28, Figs. 3.38-3.40). In an earlier report, there was a significant increase in MDA and significant decrease in vitamin E and β-carotene (p<0.001) levels in patients with urolithiasis (Bharathi et al., 2013). The elevation of serum MDA level indicates increased peroxidative stress in renal stone formers and recurrent stone formers (Baxi et al., 1994). Thus antioxidants like gallic acid could protect against renal damage by recovery of serum antioxidative enzymes and decrease of serum LPO (Padma et al., 2011). Similarly, quercetin has also been reported to decrease serum LPO against gamma radiation-induced oxidative stress (Das et al., 2013).

Furthermore, the plant extracts significantly increase the activity of enzymatic antioxidants like SOD, CAT, GPx and GR (Table 3.36, Figs. 3.51-3.54) as well as non-enzymatic antioxidants like GSH and TAA level (Table 3.35, Figs. 3.49-3.50) in kidney of ethylene glycol - treated rats.

Similarly, antioxidant constituents (polyphenols) of B. ciliata and flavonoids of D. biflorus effectively scavenge the 1, 1-diphenyl - 2 - picrylhydrazyl (DPPH), superoxide, nitric oxide and peroxide radicals and protect the renal cell from oxidative stress - induced injuries as discussed earlier (Figs. 3.60-3.64), which is also evident from restoration of SOD and CAT level (Figs. 3.65-3.71) as well as concomitant decrease of LPO in sodium oxalate - treated kidney homogenate of rat (Figs. 3.65-3.71), thus confirming the antioxidative potential of the both the plants. The effect was more significant with B. ciliata than D. biflorus and polyherbal drug, cystone showed least potency. Several in vitro studies have demonstrated that exposure to high level of oxalate results in greater production of superoxide and peroxide free radicals, leading to redox imbalance and have been manifested as antioxidant
depletion, peroxidation of lipid and oxidation of protein (Thamilselvan and Selvam, 1997). Recent studies evidenced that antioxidant therapy like vitamin E prevents CaOx deposition in the rat kidney and reduced renal cell injury by restoring these enzymes (Thamilselvan and Menon, 2005). Similarly, herbal decoction from *Rubus idaeus* had significantly more superoxide dismutase, catalase and glutathione reductase activities with reduced malondialdehyde than the hyperoxaluria group (Ghalayini et al., 2011).

The results of the present study confirmed the protective role of *B. ciliata* and *D. biflorus* against ethylene glycol - induced urolithiatic changes. *Bergenia ciliata* was found to be the most potent plant in combating the pathology of ethylene glycol – treated rats as shown by biochemical and antioxidative parameters, histopathological analysis, von Kossa staining and Pizzolato staining.

Moreover, plant extracts also efficiently reduce the levels of MDA and protein content in kidney of ethylene glycol - treated rats (Table 3.34, Figs. 3.47-3.48). This action of the plant extracts might be due to the hyperoxaluria-induced activation of the renin-angiotensin system. Reduction of angiotensin II production by inhibiting angiotensin converting enzymes (ACE) or blocking angiotensin receptors has been shown to significantly reduce renal CaOx crystal deposition as well as the development of interstitial inflammation (Toblli et al., 2002). Quercetin isolated from *D. biflorus* has already been shown to inhibit ACE *in vitro* (Loizzo et al., 2007). Häckl et al. (2002) have also demonstrated that both oral and intravenous administration of quercetin in Wistar rats have 31% inhibition of ACE activity with baseline, suggesting that quercetin acted as an ACE inhibitor. Goretta et al. (2006) have attributed the inhibition in ACE activity by isolated polyphenols including gallic
acid, chlorogenic acid, quercetin, (+) - catechin and (-) -epicatechin in rat kidney membranes.

Reactive oxygen species also culminate in phospholipase A$_2$ activation through transcription factor NF-κB (Lappas et al., 2004), as NF-κB can be activated by the stress of oxidants (Siebenlist et al., 1994) and oxalate exposure promotes rapid degradation of IκBα, an endogenous inhibitor of the NF-κB transcription factor (Jonassen et al., 2005). The inhibition of the lipid peroxidation (decreased MDA level) after post-treatment of plant extracts can be attributed to scavenging the ROS and indirect inhibition of phospholipase A$_2$ through inactivation of NF-κB. Gallic acid, an important constituent of the B. ciliata, is accredited with NF-κB inactivation activity (Hsun et al., 2010).

Modern medicines are proved to target only one aspect of urolithiatic pathophysiology whereas herbal remedies have been shown to exert effectiveness at different stages of stone pathophysiology. Herbal remedies produce multiple mechanism of action and therefore might have crystallization inhibition activity in addition to inhibition of stone formation. In the present study, the plant extracts significantly inhibit nucleation as well as aggregation of calcium oxalate crystallization in vitro (Figs. 3.56-3.58). The microscopic photographs also showed reduced number and size of calcium oxalate crystals by both the plant extracts in a dose – dependent manner (Fig. 3.59). The crystal aggregation is the most critical step as it occurs very fast and has a considerable effect on particle size and aggregated crystals are commonly found in the urine and renal stones (Masao, 2008). As earlier described, B. ciliata and D. biflorus have a number of polyphenolic constituents like alkaloids, flavonoids, saponins, terpenoids and tannins. Saponins are known to possess anticrystallization property by disaggregating the suspension of mucoproteins,
promoters of crystallization (Gurocak and Kupeli, 2006). Saponin rich fraction of *Herniaria hirsuta* has also been found to be potent inhibitor of CaC$_2$O$_4$ stone formation in animal model as well as in CaOx crystal formation *in vitro* (Fouada *et al*., 2006). Saponin derivatives appear as component of a great number of medicinal herbs with claimed antiurolithiatic properties (Lakshminarasimhan *et al*., 2002). A 98 kDa protein isolated from the seeds of *D. biflorus* has been reported to inhibit calcium oxalate crystal growth (Bijarnia *et al*., 2009). Similar inhibition of CaOx monohydrate stones was also reported by *Tamarindus indica* pulp (Choudhary *et al*., 2008). Sharifa *et al*., (2012) have isolated a terpenoid from the methanol extract of *Plantago major* and proven its inhibition on calcium oxalate crystals *in vitro*.

Moreover, Bhandari *et al*., (2008) reported that the extract of *B. ciliata* have no toxic effect till the dose of 3000 mg/kg body weight in acute oral toxicity studies. Similarly, extract of *D. biflorus* has also been reported to have Ld$_{50}$ value greater than 5000 mg/kg body weight (Sengupta *et al*., 2012). Therefore, the administration of these plant extracts doesn’t cause any toxicity or lethal effect.

The present study clearly indicates that both the plants namely, *B. ciliata* and *D. biflorus* have antiurolithiatic property which is higher than the marketed polyherbal drug, cystone. It could be due to the antioxidants present in them like gallic acid in *B. ciliata* and quercetin in *D. biflorus*, respectively and many others. Thus the antiurolithiatic activity of these plants are mediated by

a) Inhibition of metabolism of ethylene glycol

b) Antioxidant property of plant extracts and/or

c) Anticrystallization property of plant extracts