Chapter 5

DISCUSSION
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Urolithiasis is a common medical condition affecting people all across the globe, though the rate of incidence and prevalence may vary. The last five decades have witnessed a gradual increase in renal stone incidence rate in industrialized countries. The greater incidence rates in those parts of the world might be attributed to changing eating habits (Saha and Verma, 2015). An established therapy is lacking for the reduction of urinary oxalate excretion in calcium oxalate stone patients and also its relapses. Herbal therapies of preventing kidney stones have gained importance in the last decade (Saha et al., 2014). Recent studies have highlighted the potential of several medicinal herbs and natural compounds for the treatment of nephrolithiasis. Numerous scientific literatures are available suggesting that herbal therapies are effective and at the same time potentially safer alternative to other forms of management. There are varieties of herbal formulations available commercially for the treatment of urolithiasis, although the scientific data showing their advantage over others is missing in most of the cases. Moreover, various phytotherapeutic agents have also been proposed as useful alternative or complementary therapies for the management of urolithiasis, in part due to their anti-oxidative effects (Aggarwal et al., 2013a).

Formation of oxalate-containing kidney stones in animal model involves the administration of low doses of ethylene glycol (EG) which is known to induce chronic hyperoxaluric state. Hyperoxaluria-induced generation of oxidative stress is considered as the initial trigger for a vicious cycle of nephrolithiasis. Gradually this chronic state leads to a large accumulation of CaOx crystal deposits in the kidney and leads to renal damage (Green et al., 2005). Interestingly, different animal models respond differently to EG toxicity and Wistar strains have been reported to have the highest susceptibility among different rat strains (Cruzan et al., 2004). After absorption through the intestinal epithelial lining, liver is the primary site of EG metabolism (Figure 5.1). The metabolic intermediates produced by EG metabolism in liver are schematically represented in figure 5.1. As shown in the figure, EG is metabolized via alcohol dehydrogenase/aldehyde
dehydrogenase enzymes to produce glycolic acid. The glycolic acid produced is further oxidized to glyoxylic acid. The oxalic acid is the oxidised product of glyoxalic acid by either glycolate oxidase or lactate dehydrogenase (Jonassen et al., 2003).

**Figure 5.1** Metabolism of ethylene glycol in liver and excretion of CaOx crystals in kidney

The excess EG intake can result in renal failure due to CaOx crystal formation and accumulation in kidneys. Microscopically, the presence of crystals has been linked with necrotic damage in kidney tissues. Also, in primary hyperoxaluria, renal accumulation of CaOx has been shown to be involved in renal parenchymal damage. The toxic effects of oxalate may also involve attachment of calculi to the plasma membrane (Lieske et al., 1997), activation of enzyme activity (Miller et al., 2000) and production of free radicals.
and lipid peroxidation (Thamilselvan et al., 2003). These detrimental cellular events lead to the activation of cellular triggers for cell necrosis and apoptosis (Miller et al., 2000).

*Bergenia ligulata*, commonly known as ‘Pashaanbhedha’ has a great medicinal values. It has been used since ancient times in ayurveda. This plant’s rhizome is an active constituent of various marketed antiurolithiatic herbal formulations like Cystone, Calcuri, Neeri and uriflow. The antiurolithiatic potential of this valuable medicinal plant has not been reported systematically based on scientific experiments or clinical trials. Bashir and Gilani (2009) have beautifully reported the *in vitro* and *in vivo* potential of B. ligulata rhizome in treating hyperoxaluria. However, to our knowledge the study of active constituent(s) of B. ligulata in relation to its anti-urolithiatic activity has not been reported. The rationale of the present study was to inspect the role of *B. ligulata* in preventing urolithiasis, to look into the active component(s) of B. ligulata rhizome extract and to recognize the cellular mechanism of action of active metabolite for preventing urolithiasis.

**In vitro efficacy**

The inhibitory potency of this plant was evaluated *in vitro* on calcium oxalate and calcium phosphate crystallisation. The gradual accumulation of CaOx leads to supersaturation of urine and is an important risk factor of kidney stones. This gives a clue, suggesting a therapeutic window of averting kidney stones by preventing supersaturation (Coe et al., 2010). In the present study, the ethanolic extract of *B. ligulata*’s rhizome (BLE) showed 85% inhibition of CaOx crystal growth with 30μl of 0.5% solution of sample. Although the literature about the role of *B. ligulata* in inhibiting CaOx crystal growth is inadequate, the results of the present study are in accordance with previous published studies (Bashir and Gilani, 2009; Joshi et al., 2005a; Joshi et al., 2005b). It has been postulated that the mechanism of stone formation is a complex physicochemical process occurring as a result of an imbalance between promoting and inhibiting factors of crystallization and aggregation in urine. Also, in idiopathic stone formers, calcium phosphate deposits originate in the basement membrane of the loops of Henle and from there continuously grow outward reaching the papillary surface. The calcium phosphate
deposits on papillary surface then become focal points for the development of CaOx kidney stones (Evan, 2010).

As a number of compounds are likely to be present in the crude extract of B. ligulata rhizome, it was further subjected to sequential extraction using soxhlet apparatus in order to isolate pure metabolite(s). Chromatographic procedures were employed for the isolation of the pure metabolite. The most active sub fraction 1 was isolated in the form of white crystalline material and it was characterized by using NMR, FTIR and LC-MS. The results revealed the presence of purified bergenin (BRG) molecule (De Abreu et al., 2008). The UV spectrum of SFR1 illustrated two characteristic absorption bands at 220 and 275 nm. Qin et al., (2010) has reported similar absorption peaks for BRG.

The antilithiatic potential of BRG and crude ethanolic extract (BLE) were compared with Cystone®. BRG showed the highest activity in comparison with BLE and Cystone® towards in vitro CaOx crystal growth inhibitory assay. This activity of BRG could be attributed to its structural property, being a gallic acid derivative. Lee et al., (2011) had shown that 1,2,3,4,6-penta-O-galloyl-β D-glucose (PGG), a water soluble Gallotannin can alter the CaOx crystal adhesion to cells and oxalate induced renal cell injury. Role of Gallotannin from green tea has also been assessed and found to be a protectant of renal cells against CaOx induced injury. Bergenin is a C-glycoside of 4-O-methyl gallic acid and its hydrolytic product contains 4-O-methyl gallic acid (4-OMG) as the main moiety (Figure 5.2). 4-OMG has acidic properties and hence may undergo ionization in neutral and alkaline physiological environments (Kobayashi and de Mejia, 2005). The ionising ability of BRG in buffer under neutral pH could explain its mechanism of interference with the precipitation and growth of CaOx crystals.

Taller et al., (2007) had reported that a linear aspartic acid rich peptide preferentially binds to faces of CaOx crystals. The acidic amino acids having negatively charged side chains are attracted to positively charged calcium ions of CaOx crystals (Bijarnia et al., 2009). It is argued that proteins rich in γ-carboxy glutamic acid possessing two negative carboxylate groups have better binding ability with calcium sites.
Discussion of CaOx crystal (Sheng et al., 2005). These reports supported that the hydrolysis of BRG to 4-OMG having free carboxylate group makes it capable of actively regulating and inhibiting CaOx crystal growth and also explains its mechanism of interference with the growth of CaOx in the *in vitro* assay system. Crystal growth inhibitors are believed to act by adsorbing onto sites at which lattice-ion addition is energetically favoured (Bijarnia et al., 2009). Hydrogen bonding with freely available growing sites of CaOx crystals *i.e.* oxalate ion (carbon and oxygen) as shown in figure 5.2 implicates the modulation of calcium oxalate crystal growth.

![Diagram](image)

**Figure 5.2** Possible way of hydrolysis of bergenin into C-glycoside of 4-O-methyl Gallic acid and interaction of bergenin with calcium oxalate crystal (Aggarwal et al., 2014b)

Chen et al., (2010) have shown that phenolic group rich tea extract can form hydrogen bonds with electronegative oxalate in CaOx crystal. The phenolic groups of BRG might also be able to effectively bind via hydrogen bonding with oxalate in a similar manner.
Oxidative stress develops due to overproduction of ROS and/or a reduction in cellular antioxidant capacity with down regulation of the expression of antioxidant enzymes (Hovda et al., 2010). Previous studies have demonstrated that both oxalate and CaOx crystals directly induce renal epithelial cell injury through lipid peroxidation and involve free radicals (Tsujihata et al., 2006). Tissue culture and animal model studies have provided enough evidence that generation of ROS leads to renal epithelial cells injury in the presence of high levels of oxalate and CaOx crystals (Khan, 2004). It has also been shown that both oxalate and calcium oxalate crystals independently increase free radical generation in a time and concentration dependent manner. Oxalate alone increases oxidative cellular injury while CaOx crystals potentiate injury. Oxalate induced oxidative stress disrupts structural integrity of the renal epithelial membrane (Thamilselvan et al., 2009). Development of oxidative stress following oxalate load in renal tissue is a pivotal step in pathophysiology of kidney stone and various antioxidants have been employed to reduce its consequences. Bashir and Gilani, (2009), in their study shown that the potential of the ethanolic extract of B.ligulata to ameliorate renal dysfunction in hyperoxaluric rats. This anti-hyperoxaluric activity was due to its antioxidant capability.

Previous reports suggesting the antioxidant potential of bergenin accentuated to explore its ability to reduce ROS (Nazir et al., 2011; Roselli et al., 2012). The free radical scavenging capacity of BRG could be primarily attributed to the high reactivity of hydroxyl substituents on benzene ring as they form aromatic conjugated dienes. This conjugated electron rich structure can stabilize free radicals by resonance. Bergenin can also form complexes with Fe (II) and hence prevent the production of hydroxyl radicals in Fenton reaction (De Abreu et al., 2008). In the present study total reducing potential of BRG was found to be comparable with α-tocopherol. Also, hydrogen peroxide (H₂O₂) scavenging capacity of BRG was similar to α-tocopherol. The galloyl moieties of the hydrolysed BRG could be responsible for chelating and radical scavenging properties of this compound. This implies that the antioxidant and antilithiatic capability of B. ligulata might be due to bergenin.
In Vivo efficacy

The *in vivo* efficacy of BRG was examined on rat hyperoxaluric (HYO) model and compared with BLE. Prasad *et al.*, (2007) had reported that urinary system of male rats resembles that of human and the stone deposition is a male dominant disease. The sex related demarcation is because of the role of androgens in increasing urinary oxalate excretion, plasma oxalate concentration and kidney calcium oxalate crystal deposition (Fan *et al.*, 1999). For adequate absorption of metabolites intra peritoneal administration is the preferred mode. In the present study intra peritoneal administration of the extract was used.

Body weight monitoring in the present study revealed that EG administration to rats caused significant reduction in body weight whereas the rats treated with BRG and BLE showed no significant difference between initial and final body weights. Our results are in concurrence with a previous study by Bayir *et al.*, (2011) in which similar effects of a potent herbal antiurolithiatic compound on body weight of animal model has been reported. As the body weight has been considered an important marker of well being, the *B. ligulata* has proven its beneficial ability in ameliorating harmful effect of hyperoxaluria in rat model. Similar effect of its alcoholic extract had been reported previously by Bashir and Gilani, *et al* (2009).

The kidney functions are affected in urolithiasis, since lowering of the glomerular filtration rate is observed due to obstruction to the outflow of the urine by calculi deposited along the urinary system. Thereby, the waste products particularly nitrogenous substances such as urea, creatinine and uric acid accumulate in blood. Hyperoxaluria leads to impairment of kidney function and levels of serum creatinine and urea have been reported to be increased under these conditions (Sayana *et al.*, 2014). In this scenario, marked renal damage is observed in calculi induced rats ascribed by virtue of the elevated serum levels of creatinine and urea in hyperoxaluric rats. However, normalization as caused in the levels of both serum urea and creatinine by BRG leads to dissolving preformed crystals. It also interrupts the process of crystal aggregation and deposition along the urinary system, as the plant extract has proved to be better in reverting the
damage caused by calculi induction. The significant lowering of serum levels of the accumulated waste products may be attributed to the enhanced glomerular filtration rate (GFR). BLE was also able to lower down the values of serum creatinine and urea but its effect was lower than BRG administration.

The urinary pH was found to be slightly acidic in HYO rats as compare to NRM rats. The similar urinary changes have been reported in hyperoxaluria in a recent report (Bodakhe et al., 2013). The pH of urine in BRG and BLE treated rats was found towards normal value. Urinary excretion of oxalate was found to be increased via administrating EG and NH$_4$Cl (Sharma et al., 2015). Calcium levels also increase in urine during hyperoxaluria (Patel et al., 2012; Sharma et al., 2015). In the present study, similar to that of previous reports, levels of urinary oxalate and calcium were found to be enhanced in HYO rats as compared to NRM. The BRG treatment was able to significantly lower down excretion of oxalate and calcium in urine. BLE also decreased the excretion but to a lesser extent. The levels of creatinine and urea in urine were found to be decreased in HYO rats as compared to NRM. In treatment groups the values were found to be significantly normalized.

Clinically, creatinine clearance (CrCl) is a useful measure for determining renal function. Renal dysfunction diminishes the ability to filter creatinine and increase serum creatinine levels. This lowers the CrCl. The impairment of renal function after exposure to EG and ammonium chloride is an outcome of CaOx crystals deposition in renal tissue. Administration of BRG was able to restore renal function by preventing the elevation of serum levels creatinine and also treat the damage caused on lithogenic treatment to the kidney. Similar reports are published pertaining to normalization of CrCl by plant metabolites, like a plant alkaloid-berberine treatment to hyperoxaluric rats, improved kidney functions reflected by the levels of CrCl (Bashir and Gilani, 2011).

Specific proteins excreted in the urine after injury to particular segments of the nephron can serve as biomarkers for assessing the site and severity of the renal damage. For several years, studies have demonstrated that excreted urinary enzymes may be useful biomarkers for evaluation and diagnosis of tubular dysfunction and injury. These markers
suggested that tubular damage most likely preceded glomerular damage reinforcing the observations that urinary enzyme excretion can be used as early markers (Gatua et al., 2011). Alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH) are two cytosolic enzymes. Their higher activity in the extracellular fluid indicates cell lysis. Rats treated with EG and ammonium chloride showed significant elevation of renal injury marker enzymes, ALP and LDH. This is because EG results in many other toxic metabolites in addition to oxalic acid and exposure to these toxins for long duration would result in higher order of renal injury (Khan, 2006). The enhanced urinary excretion of injury marker enzymes in urolithiatic rats suggests damage to the brush border membrane of renal tubules, which appears to be associated with the retention and deposition of crystals in the kidney. LDH is also partly involved in the synthesis of oxalate. It catalyzes the coupling of oxidation and reduction of glyoxylate, resulting in the formation of glycolic acid and oxalate. Increased activity of LDH activity has been reported in hyperoxaluria (Pragasam et al., 2005; Thamilselvan et al., 2009). Administration of BRG had profound effect on reducing the urinary enzyme excretion, in the form of decreasing ALP and LDH activity, thus preventing the nidus formation for nucleation and thereby minimising the extent of tubular dysfunction. Natural moieties having antioxidant potential like lupeol (Sudhahar et al., 2008) and phycocyanin (Farooq et al., 2004) showed similar trend of LDH and ALP reduction after hyperoxaluric stress. In addition, to ascertain the extent of injury in renal tissue, LDH release in urine was observed. As explained earlier that BRG interferes with crystallization, no CaOx crystals form and that may be linked with reduced renal injury and lesser expression of LDH in urine in BRG treated rats.

Crystalluria (presence of crystals in urine) is an important index to measure the effectiveness of antilithiatic agents. Crystalluria can predict the severity and risk of stone recurrence in calcium stone formers, if it is repeatedly found in early morning urine samples (Daudon et al., 2005). The present study witnessed the raised excretion of CaOx in the urine samples of hyperoxaluric rats that enhance the crystalluria. In BLE treated rats only crystal debris was found. However, in BRG treated rats the urine samples were devoid of crystals. This effect of BRG is consistent with its in vitro antilithiatic potential and proves that BRG interferes with CaOx crystal formation and decrease crystalluria.
This shows that BRG was able to protect and heal the renal damage in vivo. Thus, the probable mechanism by which BRG has reduced renal injury might be due to its ability to inhibit CaOx crystal formation during this damage.

Microscopic examination of kidney sections derived from calculi induced HYO rats showed polymorphic irregular crystal deposits in the tubules accompanied by cast formation which causes dilation of proximal tubules which might be attributed to oxalate formation. Also, administration of EG and ammonium chloride caused severe damage to the medulla, glomeruli, tubules and interstitial spaces. Similar changes were observed in a study by Ahmed et al., (2013) in an identical hyperoxaluria model. There was mild degree of damage observed in glomeruli, tubules, and interstitial spaces in BLE treated groups. The damage was found to be almost reversed in BRG treated rats except mild blood vessel proliferation, edema and glomerular damage. The damage to the capsule, tubules, and interstitial spaces was completely corrected and the tissues looked almost similar to NRM rats.

In vivo antioxidant potential of BRG was also investigated in the hyperoxaluric rat tissues. Similar to the previous reports (Naghii et al., 2015), present study also demonstrate that EG causes an increase in lipid peroxidation and decrease in activities of antioxidant enzymes in kidney. The levels of Malondialdehyde (MDA, a marker of lipid peroxidation) have been found to be enhanced in HYO rats. Similar to the present study, Saha et al., (2014) have also seen the enhanced MDA levels in hyperoxaluric conditions. Treatment groups were able to significantly reduce the MDA levels towards normal value. Reduced glutathione (GSH), a tripeptide, is an excellent natural antioxidant and a biomarker of oxidative stress. Levels of glutathione have been reported to be decreased under hyperoxaluric conditions (Sharma et al., 2015). Superoxide dismutase (SOD), Catalase and gluthathione peroxidase (GPx) are also the key antioxidant enzymes whose activities are crucial in maintaining the cellular redox balance. The activities of these enzymes also has been reported to fall under hyperoxaluric conitions (Lee et al., 2011). The results revealed the potential of BRG as a free radical scavenger. The antioxidant
potential of BRG along with its direct binding to CaOx crystals might be the mechanism of its antilithiatic activity figure (5.3).

![Diagram of potential target sites of BRG during EG induced hyperoxaluria]

**Figure 5.3** Potential target sites of BRG during EG induced hyperoxaluria

It has been shown in recent studies that the cytotoxic effects are due to CaOx crystals and not the oxalate ions. The necrotic cell death due to CaOx crystals may involve the oxidative stress generation and depleted ATP levels due to mitochondrial dysfunction. The uptake of CaOx crystals into the cytoplasm may result in their direct interactions with mitochondria (or mitochondrial membranes). Also, CaOx crystals may induce the mitochondrial membrane transition and loss of the proton-motive force and leads to cell death (Hovda *et al.*, 2010). Though, mitochondria are the prime site of generation of superoxide and \( \text{H}_2\text{O}_2 \) they also have an impressive array of antioxidant enzymes like SOD and GPx (Khan, 2013). The elevated oxidative stress, either due to enhanced lipid peroxidation or diminished antioxidants levels has been reported to be
associated with loss of complex IV activity that consequently leads to mitochondria dependent apoptosis. The decline in ATP production might have severe impact on kidney functions. Therefore, complexes involved in electron respiratory chain might be the main mitochondrial targets of chronic hyperoxaluria (Cao et al., 2004). It has been reported that urolithiasis increases dysfunction of antioxidant systems and increases oxidative stress. It has been proposed that reactive intermediates can damage the critical active sites of the respiratory complex enzymes which indicate that mitochondria might serve as a source as well as target for reactive species. Moreover, deposition of crystals during hyperoxaluric condition, results in angiotensin II (Ang II) activation (Yoshioka et al., 2011). NADPH Oxidase is stimulated by activated Ang II, and through phosphorylation of the former’s cytosolic subunit p47phox and translocation to the membrane assembling the catalytic complex of active oxidase leading to ROS production, which can damage renal cells. Significant correlation has been seen between CaOx crystal-induced up regulation of p22phox and p47phox and NADPH Oxidase activation and associated cell injury (Khan et al., 2014).

Under pathological conditions the activity of mitochondrial superoxide dismutase and glutathione peroxidase enzymes is highly reduced (Pigeolet et al., 1990). Antioxidant defense systems against mitochondrial ROS (in particular, H$_2$O$_2$) include thiol-reducing systems \textit{i.e.} thioredoxin (Trx), glutaredoxin, and glutathione system. Limited quantities of glutathione in the mitochondria suggest that maintenance of adequate thiol disulfide redox status is essential to protect against the injurious effects of ROS. Hence, GSH depletion could lead to an oxidative stress condition which is supported by the presence of increased levels of TBARS concentration in GSH depleted mitochondrial fractions. Inhibition of antioxidant enzymes, SOD and GPx by oxygen radicals further can exaggerate the situation, resulting in the loss of protein thiols. In the present study in HYO animals, the increased LPO levels indicate a state of oxidative stress. Also, both the non-enzymatic (GSH) and enzymatic antioxidant (SOD) levels were diminished in the diseased model. The enhanced oxidative stress shown in the present study is in accordance with previous studies from our lab (Sharma et al., 2015) and other groups (Veena et al., 2008). However, the enzymatic activities of SOD and GPx have been
restored successfully in both the treatment groups, the BRG treatment showed better outcomes than BLE.

Mn-SOD is an inducible enzyme that protects mitochondria from oxidative stress. The diminished Mn-SOD activity in hyperoxaluric mitochondria suggested impaired dismutation of superoxide radicals resulting in increased $\text{H}_2\text{O}_2$ levels (Macmillan-Crow and Cruthirds, 2001). The restoration of Mn-SOD activity by BRG reported in the present study might involve induction of enzyme as reducing agents have been shown to induce Mn-SOD expression through activation of NF-κB (Tucci et al., 2008). BRG treatment was able to protect mitochondria from hyperoxaluria induced oxidative stress. The onset of LPO may result in loss of critical phospholipids like cardiolipin that are critical to mitochondrial enzymes/function. The reversal of mitochondrial enzymes by BRG and hence the increased antioxidant ability might offer protection by scavenging ROS in mitochondrial proteins. The present study confirmed that mitochondrial oxidative stress and dysfunctions contribute in part in to the pathogenesis of hyperoxaluria-induced nephrolithiasis. In addition, the findings of the present study provide evidence that BRG exert protective effects in hyperoxaluria through mitochondrial protection that involve attenuation of oxidative stress.

As discussed earlier, EG metabolism initiates in liver and results in formation of oxalic acid and other metabolites. Hence, oxidative stress may also generate in hepatic cells. There was increase in the levels of MDA observed in liver tissue in case of HYO rats in the present study. Liver injury in case of EG treatment has also been reported in a study conducted by Bouanani et al. (2010). The levels were found to be normalized in BRG treated rats. B. ligulata has also been reported to exhibit hepatoprotective activity (Singh et al., 2009). The status of antioxidant enzymes were also studied in liver of HYO rats and were found to be under stress. Similar reduction in activity of antioxidant enzymes under hyperoxaluric conditions have been reported previously by Bijarnia et al., (2007). Both the treatment groups showed significant elevation in levels of antioxidant enzymes and restoration of antioxidant defence.
Present study firmly supported the role of bergenin for combating calcium oxalate crystallization and reducing free radical damage both *in vitro* and *in vivo*. The animal study put forth the idea that bergenin is the active constituent of *B. ligulata* possessing activity to maintain renal functionality following hyperoxaluric exposure.