Chapter 4
Discussion
Due to significant side effects and failure to prevent recurrence of kidney stones by the present day treatment procedures for urolithiasis, alternative treatment modalities by herbal products have assumed importance. Recent years have seen dramatic advances in phytotherapy for urolithiasis and many investigators have proposed to implicate scientific study on its efficacy [149]

There are various herbal drugs commercially available which are prescribed for treatment of kidney stones. The various drugs like Cystone, Uriflow, Renacare and Neeri are some examples of such drugs. These drugs are herbal composites and are prepared by mixing of various plant extracts in different proportions. Although, all these drugs have mixtures of more or less same plant extract but still they are not equally effective in all patients. In addition, some times the two different plants show antilithiatic effect individually but when they are mixed together, their efficacy is reduced. Like it is shown that Bergenia ligulata and Dolichos biflorus effectively inhibit calcium phosphate mineralization individually but together their efficacy is reduced [186] Since, alternative medication is not fully studied, so it is possible that the herbal plants known to have kidney stone inhibitory potential may also have certain toxins. Salvia miltiorrhiza, is a Chinese herb which is used to decrease the incidence of kidney stones. A small dose of this herb is usually recommended. In a case report by Wang and Yang [217], prescription of this herb in large amount by an unauthorized practitioner to a 15 years old boy for removal of kidney stone lead to severe neurological problems. He had severe dystonia in the trunk and limbs together with evident posture tremors. Chemical analysis showed that Salvia miltiorrhiza has a toxin named, Coumarin, which is toxic, mainly to the basal ganglia, and is possibly responsible for neurological symptoms in this patient. Clear analysis of herbs for their chemical component and testing each of them to determine what causes these clinical symptoms is of the utmost importance for preventing such tragedies. Identification of CaOx inhibitory biomolecules from herbs together with the chemical analysis for toxin compounds is very important to formulate an effective treatment for kidney stones.
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Till date various plants extract have been studied to reduce the incidence of calcium stone deposition both in vivo and in vitro [218,219,220] but the identification of naturally occurring CaOx inhibitory biomolecules from plants was hampered in past by limitations in identification method. In the present research, an effective protein biomolecule from the seeds of *Dolichos biflorus* was isolated which has antilithiatic properties under both in vitro and in vivo conditions.

4.1. In vitro antilithiatic properties

On comparing the antilithiatic ability of four plants viz. *Achyranthes aspera, Dolichos biflorus, Terminalia chebula, Cocos nucifera* using in vitro calcification assay, the seeds of *Dolichos biflorus* showed highest percentage of inhibition towards growth & demineralization of calcium phosphate and a significantly good inhibition towards initiation of CaP mineral phase formation (Figure 3.1, 3.2, 3.3). *Dolichos biflorus* seeds are common dietary food of north India and are known to have antilithiatic proficiency. There have been very few systematic studies on the antilithiatic properties of this plant [221,186,187]. Still, the constituents of *Dolichos biflorus* possessing antilithiatic property have not yet been identified. Based on in vitro antilithiatic potential of *Dolichos biflorus*, this study was aimed at purifying and characterizing the most potent biomolecule from it.

4.2. Protein from the seeds of *Dolichos biflorus*

Various CaOx crystal growth inhibitors mostly proteins and glycosaminoglycans have been reported in humans to play an important role in renal stone diseases for several decades [222,223]. Most of these proteins have been isolated from CaOx kidney stones matrix itself in their active form [224,225]. Likewise, many plants are also known to produce CaOx as crystalline deposits [74,75] having an organic matrix constituting different proteins [116]. These proteins are believed to play an important role in the control of crystal growth and modification of crystal form [215]. More recently [117] four proteins from the organic matrix of CaOx crystals present in the seeds of *Phaseolus vulgaris*, have been isolated which inhibited the nucleation of CaOx crystallization in solutions. It was also shown that the isolated proteins modified the morphology of CaOx
crystals mainly at \{120\} face (fastest growing face). A well known CaOx inhibitor, citrate, has also shown to slow the growth of \{120\} face [226].

In the present study, an antilithiatic protein was isolated from the seeds of *Dolichos biflorus* inhibiting both calcium oxalate and calcium phosphate crystallization. The *Dolichos biflorus* antilithiatic protein (DAP) (98 kDa, pI 4.79) showed two bands of molecular weight 58 kDa & 34 kDa which clearly indicate its dimeric nature (Figure 3.9). Previous studies claim non-protein part to be responsible for antilithiatic nature [186, 187]. But our study showed the most active antilithiatic component to be a protein. There were certain other fractions which showed some extent of CaP and CaOx inhibition. Our focus was to find the most potent biomolecule, so we proceeded with the characterization protein fraction (DAP) having highest inhibitory potency. The MALDI–TOF MS analysis of DAP showed maximum similarity (35% sequence coverage) with calnexin (CNX) protein of *Pisum sativum* (CAA76741). Although molecular weight of CNX (62kDa) is not similar with DAP protein, but the pI of DAP is comparable with CNX (Figure 3.17). Since, many plant databases are still largely incomplete, many proteins present in *Dolichos biflorus* were found to be absent in those databases. So, DAP may not be homologous to CNX, but probably is a CNX like protein.

Calnexin, a type 1 membrane protein is as an interacting protein in the biogenesis of class I histocompatibility molecules in ER [227]. Calnexin is a predominant integral membrane protein of the ER and was first identified by its ability to bind Ca\(^{2+}\) [228]. It has a long amino-terminal domain (460 amino acids) localized in the lumen of the ER, a single hydrophobic transmembrane domain, and a short, acidic cytosolic domain (91 amino acids). Calnexin may promote the proper assembly of protein complexes during transit through the ER. The retention and folding of such complexes is Ca\(^{2+}\) dependent [229]. Calnexin has also been identified in nuclear membrane preparations [230], suggesting that it may also play a role in nuclear membrane trafficking or the regulation of nuclear Ca\(^{2+}\) transients. Calnexin shares significant sequence similarity with calreticulin [231,232], the major Ca\(^{2+}\) binding protein found in the lumen of the ER. Both calreticulin and calnexin lack "EF hand", a Ca\(^{2+}\) binding motif found in the calmodulin
family of Ca\textsuperscript{2+}-regulated proteins [233]. However, these molecules contain unique subdomains that can be distinguished by charge, repeating motifs, and predicted secondary structure. A recently reported X-ray crystal structure shows that CNX consists of two domains, a globular domain and a long extended arm domain. Also, there are two disulfide bonds in the CNX structure, one in the globular domain, shown to be labile, and the second near the tip of the arm domain [234]. A lectin binding site was found within the concave surface of the globular domain and this it makes its structural similarity to legume lectin family. Additionally, a calcium binding site was also identified within the globular domain [235].

On exploring the inhibitory potency of DAP on calcium phosphate mineralization, again an effective inhibition was observed, thus clearly indicating that DAP is probably imparting its effect by binding to calcium ions. Probably DAP, which is CNX like protein has a calcium binding site, which might also be responsible for its ability to inhibit CaOx as well as CaP crystallization (Figure 3.11 & 3.12).

Lectins are particularly abundant in the seeds of legumes and they constitute upto 10% of soluble proteins in their seeds extract. Dolichos biflorus, a leguminous seed, has abundant lectins [236]. A report showed that adhesion of calcium phosphate and calcium oxalate crystals was inhibited by polyanions found in tubular fluid and by polycations and specific lectins that act on the apical cell surface of renal epithelial cells. They showed that lectins exerted their effect on the cell [237]. Treatment of cells with neuraminidase inhibited binding of crystals, suggesting that anionic cell surface sialic acid residues function as crystal receptor sites that can be blocked by specific cations or lectins [238]. This further strengthens the perspectives that DAP which is CNX like protein, possibly has a lectin domain that might be accountable for its calcium crystallization inhibitory properties as is observed during in vivo experiments.

It has been suggested that acidic amino acid residues such as Asp and Glu, that are expected to be deprotonated and negatively charged at urinary pH, are attracted to positively charged calcium ions of calcium stones [239]. Our data of amino acid analysis suggests that both CNX and DAP have higher acidic amino acids (Asp and Glu) content.
Thus, it could be argued that acidic amino acids present in the DAP, may possess the capability to inhibit calcium crystallization. A recent study by Wang et al. [216] presented that addition of serine spacer in poly-aspartate peptide increased its ability to inhibit COM crystallization. They suggested that the hydroxyl groups (-OH) of serine may have contributed in the interaction by directly binding to calcium ions and formation of hydrogen bonds. Another study says that phosphorylation of osteopontin is required for inhibition of calcium oxalate crystallization [252]. A significant amount of serine amino acids in DAP further ascertain its ability to inhibit CaOx crystallization.

4.3. Modeling of known kidney stone inhibitory proteins with COM crystal

The use of molecular modeling and docking of compounds into the target sites of molecular models derived directly from resolved crystal structures has already proven valuable for discovering new ligands [240]. In the present study we have incorporated docking simulations to analyze the interactions between calcium oxalate monohydrate and proteins which are known to inhibit its growth. Docking energies are calculated as the sum of the intermolecular interactions (electrostatic + Van der Waals) plus the conformational energy of the ligand-domain complex determined by molecular mechanics [241]. More negative the docking score, stronger is the binding between ligand (COM in our study) and protein’s active binding site. These strong interactions between protein active binding site and COM crystal, specifically at the growing sites would predict inhibition of COM crystal.

Each protein possessing the property of inhibiting COM crystallization taken in the present study showed diverse kind of interactions, supporting the fact that most of the kidney stone inhibitory proteins are multifunctional proteins. The property common to them is the ability to strongly interact with the free available growing sites (Ca, C and O atom) (Figure 2.5) of COM. It is known that COM crystal growth is slow in some directions since certain macromolecules adsorb on it and prevent formation of crystal lattice. Face (-101) of COM crystals is more active as it presents more closely packed calcium atoms and has significantly more adsorptive characteristics for many macromolecules (proteins) [242]. In the present investigation, it was observed that
calcium ions of COM form hydrogen bonding with varied amino acids of active binding sites and carbon of oxalate group gets involved in hydrophobic interactions. It is a fact that hydrogen-bonding are of primary significance in establishing strong complex between ligand and protein active binding site, but nevertheless hydrophobic interactions also act as a stabilizing factor and addition of a hydrophobic group not only allows hydrophobic bonding but also strengthens existing hydrogen bonds and the increased hydrogen bond strength can be an important factor in determining the overall binding energy [243].

The interaction of amino acids is also dependent on the conformation of the active sites, same amino acid in one instance is effectively involved in hydrogen bonding and in other instance same amino acid is involved in weak hydrophobic interactions as is shown in the case of active binding site bikunin-1 (Figure 3.18). This dependence is purely steric hindrance, thus suggesting that not all amino acids which could strongly bind with calcium ions, although repeatedly present in the active binding site, interact with COM. Thus there is no advantage of repeated Glu residues until they are structurally available to interact. Strong hydrogen bonding of Glu and Asp with calcium ion of COM crystal supports the hypothesis that negatively charged acidic amino acids which are attracted to positively charged calcium ions [244]. The results presented by CD59-1 binding site supports the findings of Wesson et al. [245] that the charge of the side group was not the sole determinant to cause this effect, as Glu and Asp present in this binding site do not interact with COM. It is further perceived that proteins rich in gamma carboxy glutamic acid (CGU) possess two negative carboxylate groups have better binding to calcium ions of COM [246]. Here again it was observed from the results that although active binding site uripro-1 of protein urinary Prothrombin have repeated CGU monomers, still all the CGU are not involved in COM interactions (Figure 3.21). This evidence further suggests that conformational and interface chemistries interact in a complex manner to inhibit aggregation of COM and an understanding of such interactions may help to determine and control the factors affecting kidney stone formation.
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In addition to inhibition of COM by acidic amino acids, certain other amino acids also showed optimal binding with COM unit cell. Osteonectin-2 and uripro-1 active binding site, showed formation of hydrogen bonding by Tyr and Ser, both form hydrogen bond through the hydroxyl group of their side chain with calcium ion. A recent study presented that addition of serine spacer in poly aspartate peptide increased their ability to inhibit COM crystallization [216]. They suggested that the hydroxyl groups (-OH) of serine may have contributed in the interaction by directly binding to calcium ions and form hydrogen bonds. This hypothesis is supported by our finding that the formation of hydrogen bonds is involved between the -OH group of both serine and tyrosine with calcium ion.

Hence, it can be concluded that amino acids interact with calcium ion by forming hydrogen bonds as well as hydrophobic interactions. Although acidic amino acids are primarily involved in establishing strong binding with COM but involvement of other amino acids is also important as it strengthen this interaction.

4.4. In vivo antilithiatic properties of DAP

The antilithiatic activity of DAP was further confirmed in rat model to evaluate its efficacy in vivo. The protein was injected intraperitonealy (1 mg/kg and 2 mg/kg body weight) in stone forming animals and both the doses showed positive results towards decreasing COM crystals in a dose-dependent manner.

Determination of creatinine clearance more consistently predicts renal insufficiency than serum creatinine determination alone [247]. Creatinine clearance measures the volume of blood plasma that is cleared of creatinine per unit time. Clinically, creatinine clearance is useful measure for estimating the glomerular filtration rate of the kidneys, which is clinically important factor in determining renal functioning. The proper functioning of kidney after EG and NH₄Cl dose is found to get hampered. As observed by Yamaguchi et al [248], the combination of high doses of EG and NH₄Cl in the drinking water induced crystalluria and hyperoxaluria, along with calcium oxalate deposits in the kidney. In addition, deterioration of renal function was observed,
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especially more after 11 days. Thus, the renal functioning was more vulnerable after 15 days dose than 9 days dose. To see the effect of DAP after long duration of its dosage and to see its ability to restore renal functioning after a marked deterioration, DAP was given for both 9 and 15 days at the dose of 1 mg and 2 mg per kg body weight. From these experiments, DAP was found to restore renal functioning in stone forming rats in a dose-depandant manner.

Renal dysfunction further diminishes the ability to filter urea and increases serum urea level [249]. The EG treated rats showed more urinary volume. This is an adjustment to filter toxic metabolites from the blood. But still the level of urea in serum was found high in EG and NH$_4$Cl exposed animals for both 9 and 15 days treatment. Depending on the magnitude of renal dysfunction, the level of serum urea was more after 15 days treatment than after 9 days treatment. After EG and NH$_4$Cl exposure the serum urea level increased, indicating renal dysfunctioning due to crystal deposition. Here, again rebalancing of serum urea further unveils the potential effect of DAP on maintaining renal functioning. It has been found that external prophylactic agents restore renal functioning by maintaining creatinine clearance and serum urea level in hyperoxaluric rats [250].

Alkaline phosphatase (AP) and lactate dehydrogenase (LDH) are two cytosolic enzymes and their higher activity in the extracellular fluid indicates cell lysis. Similarly, higher amount of AP and LDH in the urine is an indicator of renal cell injury and these enzymes are the injury marker enzymes. The enhanced urinary excretion of renal injury marker enzymes like AP and LDH in urolithiatic animals suggests damage to the brush border membrane of the renal tubules. This damage also appears to associate with the retention and deposition of crystals in the kidneys [251]. Recent in vitro studies have suggested that proximal tubule cells, when compared to distal tubule or collecting duct cells, are more sensitive to the toxic effects of both oxalate and calcium oxalate at pathological level [253].

The tissue injury which occurred upon administration of EG and NH$_4$Cl resulted in increase of COM deposition in kidney tissue (Figure 3.33). The animals given EG and
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NH₄Cl dose for 15 days showed much higher excretion of renal injury markers enzymes than 9 days treatment (Figure 3.26 & 3.27). This is because EG results in many other toxic metabolites in addition to oxalic acid and exposure to these toxins for longer duration would results in higher order of renal injury [251]. DAP restored renal injury to the EG and NH₄Cl exposed animals after treatment of both 9 and 15 days. This shows that in addition to reducing COM crystal and oxalate in rat kidneys, DAP has some additional properties of reducing toxic effects of other metabolites of ethylene glycol.

Khan [242] has hypothesized that inflammation in kidney tissue recruit macrophages to the sites of crystal deposition to reduce renal crystal burden and acts as an early defense against stone formation. Increased inflammation in stone forming animals as observed in renal histology is a protective mechanism against crystal deposition. Reduced inflammation in DAP treated stone forming animals depict fewer sites for crystal deposition. Crystal deposition which is mostly present in renal tubules [251], is also shown in our results. Crystal retention within the renal tubules is promoted by renal epithelial injury, which exposes a variety of crystal adhesion molecules like CD44 on its surface [254]. Decreased renal injury upon DAP administration further decreases sites for calcium oxalate deposition which is evident from the polarization microscopy of renal tissue (Figure 3.33).

It has been documented that COM crystals either in free or aggregated form are found in the urine of urolithic patients [255,256]. COM crystals have greater affinity for renal tubular cells than COD and are responsible for the formation of stone in kidneys [257]. In the present study, administration of DAP reduced supersaturation of COM crystals in the urine as compared to the stone forming rats (Figure 3.28 & 3.29). In addition, a significant reduction in the size of COD crystals was also observed in animals given higher dose of DAP (2 mg/kg body weight). The formation of COD crystals in preference to COM crystals is propitious because it protects against stone disease by reducing the attachment of crystals to renal tubular cells.

Our results as shown in urolithic group A2 and B2 are in conformity with the dose selection of EG and NH₄Cl based on the model developed by Yamaguchi et al [248]
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where the crystal deposition in the kidney and stones attached to the papillary tip were found to be COM in nature, whereas COD were observed in the urine after giving the prescribed dose. This is due to the fact that COM crystals are prevalent in urolithiasis. After nine days treatment with DAP, the crystals excreted with the urine were less in number and that too only COD crystals were present but after 15 days treatment of urolithiatic rats with DAP, more COM crystals were excreted out instead of COD (Figure 3.28 & 3.29). This shows that after giving DAP for longer duration, it flush out COM crystal retained in the kidney tissue.

From above results, it can be strongly emphasized that DAP has an ability to reduce the incidence of crystal deposition in vivo. In addition to decreasing COM and COD crystal retention in kidney and their excretion in urine, DAP maintains proper renal functioning. DAP was also found to reduce renal injury which is evident from the restoration of renal marker enzymes (Figure 3.26 & 3.27).

4.5. Antioxidant properties of DAP

Free radicals such as reactive oxygen species are formed during a variety of biochemical reactions and cellular functions such as mitochondria metabolism. Oxidative stress results from an imbalance between formation and neutralization of pro-oxidants. Various pathological processes disrupt this balance by increasing the formation of free radicals in proportion to the available antioxidants. Urinary excretion of renal tubular enzymes which are considered to be markers for renal injury [258] are the consequence of oxidative stress. AP and LDH (renal injury markers) excretion was normalized following DAP dosing in stone forming rats. This shows that DAP reduced renal injury, which further provides sites for CaOx deposition.

Oxalate is readily filterable at glomerulus and secreted by proximal tubules [259,260]. The damage of glomerulus and its capsule following oxalate exposure as shown in figure 3.31, might have been caused by oxalate itself or its derivative(s) acting as free radicals. In DAP treated stone forming animals the normal morphology of
glomeruli was restored. This effect could be attributed to antioxidant properties of DAP which might have reduced oxalate induced free radical damage.

The antioxidant properties of DAP were studied after inducing hyperoxaluria by sodium oxalate dose for 24 hrs. The dose (70 mg/kg body weight) of sodium oxalate was chosen because it is known to cause oxidative stress in kidney tissue [261], before the formation of calcium oxalate crystals. Thus, we could see the effect of DAP on oxidative stress, prior to calcium oxalate formation. In addition, the activity of DAP was also compared to a known antioxidant N-acetylcysteine (NAC) which was taken as a positive control. After 24 hrs treatment, the kidney tissue of rats were homogenised and various oxidative stress marker enzymes and lipid peroxidation were studied.

By definition lipid peroxidation is a process whereby free radicals “steal” electrons from the lipids in our cell membranes, resulting in cell damage and increased production of free radicals. Lipid peroxidation (LP) is the mechanism by which lipids are attacked by ROS that have sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the side chain. The greater the number of double bonds in the molecule, the easier is the removal of the hydrogen atom. Thus, polyunsaturated fatty acids are in particular susceptible to lipid peroxidation. All the biological membranes are characterized by the large amount of polyunsaturated fatty acids associated with amphipathic lipids and a variety of membrane proteins. Lipid peroxidation of biological membranes results in loss of membrane fluidity, changes in membrane potential and increased membrane permeability.

In the present investigation, the level of malondialdehyde (MDA), which is an indirect index of lipid peroxidation, was found to be significantly elevated following oxalate exposure (Figure 3.34). So, it can be suggested that oxalate is injurious to renal tissue and the injury is caused by production of reactive free radicals. Oxalate is also known to induce free radical production like hydroxyl radical and peroxyl radical in renal tissue [262]. The reactive oxygen species culminate in phospholipase A2 activation through NF-κB (nuclear factor κB) DNA-binding activity [263], as NF-κB can be activated by the stress of oxidants [264]. NAC having a thiol group reduces the level of
free radicals responsible for the lipid peroxidation and thus decreases the level of malondialdehyde, the end product of lipid peroxidation. Since, DAP also showed decrease in lipid peroxidation, this implies that antioxidant ability of DAP is potent enough to quench oxalate-induced free-radical reaction.

The free radicals like hydroxyl radical, superoxide, hydrogen peroxide and peroxynitrite are generated during normal metabolism and cells contain multiple protective systems, which limit their damaging effects. These include network of protective enzymes and antioxidants, which prevent and intervene in the injurious oxidative reaction initiated by these species. The first line of defense against superoxide is superoxide dismutase (SOD). This enzyme dismutase the superoxide ion to H$_2$O$_2$ and O$_2$ [265]. Because, SOD enzyme generates H$_2$O$_2$, it works in collaboration with H$_2$O$_2$ removing enzymes. Catalase converts H$_2$O$_2$ to water and oxygen. Catalase is present in the peroxisomes of mammalian cells, and probably serves to destroy H$_2$O$_2$ generated by oxidase enzymes located within these subcellular organelles. Catalase is a hemoprotein and is responsible for the decomposition of hydrogen peroxide.

The increase in SOD activity after oxalate exposure could be an adaptive response of this enzyme to increased production of superoxide ions produced by activation of NAD(P)H oxidase via cytokine TGF-β1 (transforming growth factor β 1) induction [266]. NAC treatment is known to cause a decrease of TGF-β1 in mouse mesangial cells [267]. Because TGF-β1 activates NAD(P)H oxidase, which is a potent source of superoxide ion, the decrease in TGF-β1 level would result in decreased superoxide radicals and subsequently normalized enzyme activity. DAP also reduced the activity of SOD enzymes which further suggests that DAP could have reduced production of superoxide anion and hence lower the damage likely to result from its activity.

Further evidence of the efficacy of DAP in relieving oxalate-induced oxidative stress includes rebalancing of catalase activity. Because SOD enzyme generates H$_2$O$_2$, it works in collaboration with H$_2$O$_2$-removing enzyme catalase. The increased SOD activity in hyperoxaluric rats leads to production of hydrogen peroxide, which is further decomposed by catalase. Thus, catalase activity was also found to increase after oxalate
exposure to abate increased hydrogen peroxide. It has been reported that NAC supplementation reduces H₂O₂-induced damage of epithelial cells in vitro [268], suggesting that the decrease in catalase activity after NAC dose is an outcome of reduced H₂O₂ level. Subsequently, the decrease in catalase activity after DAP administration shows that DAP might have acted akin to NAC. The rebalancing of elevated antioxidant enzyme’s activity by DAP treatment further substantiated the protective nature of DAP against free radical induced oxidative stress.

Glutathione (γ-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. Glutathione is ubiquitous in animals, plants, and microorganisms, and being water soluble is found mainly in the cell cytosol and other aqueous phases of the living system [269]. Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants. Glutathione exists in two forms the antioxidant reduced glutathione tripeptide, conventionally abbreviated as GSH and the oxidized form sulfur-sulfur (S-S) linked compound, known as glutathione disulfide or GSSG.

The GSSG/GSH ratio may be a sensitive indicator of oxidative stress. The reduced glutathione molecule consists of three amino acids - glutamic acid, cysteine, and glycine covalently joined end-to-end. The sulfhydryl (-SH) group, which gives the molecule its electron-donating character, comes from the cysteine residue. Glutathione is present inside cells mainly in its reduced (electron-rich, antioxidant) GSH form. In the healthy cell GSSG, the oxidized (electron-poor) form, rarely exceeds 10 percent of total cell glutathione. Intracellular GSH status appears to be a sensitive indicator of the cell's overall health, and of its ability to resist toxic challenge. Experimental GSH depletion can trigger suicide of the cell by a process known as apoptosis [270,271].

Hyperoxaluria induces oxidative stress and it is found that treatment with methionine [142] or glutathione monoester [143] reduced renal CaOx crystal deposits in the kidneys of hyperoxaluric rats. Both methionine and glutathione have sulfhydryl (-SH) group. N-acetylcysteine too has an -SH group. It is also found to scavenge oxidants directly and increase intracellular GSH levels [272]. Thus, it was found that the dose of
NAC to hyperoxaluric rats resulted in an increase in the levels of GSH (Figure 3.38). Since, DAP dose also displayed an increase in GSH levels thus it can be stated that DAP might also possess antioxidant properties similar to NAC.

As stated above that the GSSG/GSH ratio (redox-ratio) is treated as a sensitive indicator of oxidative stress, this ratio was found to be increased in hyperoxaluric rats. Animals given NAC and DAP dose exhibited a decrease in this ratio (Figure 3.40) clearly indicating that DAP has similar antioxidant potential in reducing hyperoxaluria induced manifestations in rat kidney as that of NAC.

In a recent report, α-tocopherol was found to increase protein level of calnexin in the renal tissues and transfection of calnexin was protective against oxidative stress in vitro in rat renal tubular cells [273]. In another report, a protein restricted diet based on soy protein isolate has been found to increase hepatic expression of calnexin in pigs with superior oxidative stress response [274]. These studies suggest role of calnexin conferring oxidative stress resistance by its induction. The mechanism by which DAP might have reduced renal injury is not yet elucidated but may be related to the fact that DAP probably acted against oxidative stress perhaps in the same way as calnexin.