Kidney diseases rank third among the most prevalent life threatening disorders after cancer and cardiac ailments. Kidney stones, which refer to mineral depositions in the kidney, are one of the most prevalent and painful of urological disorders affecting human beings since ancient times. Nephrolithiasis is the medical term used to describe kidney stone disease. Archaeological evidences from ancient Egypt revealed that kidney and bladder stone diseases were common and regimens for their treatment were found in the papyrus Ebers (1500 BC), which provides the link to traditional Egyptian medicine. Even in the 4th century BC, Hippocrates referring to kidney stones wrote in his Hippocratic oath”… I will not cut, even for stone, but leave such procedures to the practitioners of the craft.” Detailed medical literature from India by Susruta and Charaka mentions stones in the urinary tract, treatment regimens and their removal. “Stein Schneiders” or professional “stone cutters” as they were called were employed for the surgical removal of stones in ancient times (Shah & Whitfield, 2002; Eknoyan, 2004)

The regions of the world with high incidence of kidney stones are the Scandinavian countries, Mediterranean countries, British Isles, northern Australia, central Europe, North Africa, southern states of USA, portions of the Malayan Peninsula, China, Pakistan and north western India. In Asia, the stone-forming belt is reported to stretch across Sudan, Saudi Arabia, the United Arab Emirates, the Islamic Republic of Iran, Pakistan, India, Myanmar, Thailand, Indonesia and Philippines (Hussain et al, 1995). The
prevalence of kidney stones ranges from 4% to 20% in these areas. In India, approximately 5-7 million people suffer from stone disease and at least 7-10 in 1000 of Indian population needs hospitalization due to kidney stone. Kidney stones are more prevalent in the northern regions of India particularly Maharashtra, Gujarat, Punjab, Haryana, Delhi and Rajasthan (Abbagani et al, 2010). There is a lack of data from the southern states of India with regard to the prevalence of kidney stones. However a report based on hospital admissions by Bharathi & Amirthaveni (2007) concluded that there was a considerable increase in incidence of kidney stone cases year by year in Tamil Nadu.

There are four major types of kidney stones, calcium stones, uric acid stones, struvite stones and cystine stones. Of these, the most predominant type is calcium oxalate kidney stones accounting for 60-80% of the stones in the total stone forming population (Asplin, 2002). A study conducted at Amrita Institute Kochi, Kerala showed that out of the 176 stone patients studied, 74% had calcium oxalate kidney stones (Surinder et al, 2015). The treatments for kidney stones depend on their size. Dietary intervention, life style changes and increasing the fluid intake is generally recommended for small stones less than 2 mm. Oral medicines are used for stones up to 4 mm size which can pass through the urinary tract. These include drugs like thiazides, alkaline citrate and sodium cellulose phosphate. Thiazide diuretics like hydrochlorothiazide, chlorothalidone and indapamide act by reducing calciuria. Hence these drugs induce positive calcium balance and thereby increase the bone mineral density (Mikawlrwng & Kumar, 2014). Eructation, bloating, gaseousness or frank diarrhoea are associated with alkaline citrate administration which has limited its usage. For larger stones up to 20 mm in diameter, a non invasive procedure called extra corporeal shock wave lithotripsy (ESWL) is used which employs
ultrasound waves to disrupt stones. It is however associated with adverse effects such as vascular and renal tissue damage, haemorrhages, hypertension and kidney fibrosis (McAteer & Evan, 2008). Ureteroscopy is the preferred treatment in pregnant and morbidly obese people, as well as those with bleeding disorders. Percutaneous nephrolithotomy (PCNL) is an invasive procedure used for the treatment of stones greater than 20 mm. In this procedure, a small flank incision is made, through which a telescopic instrument called nephroscope is inserted. This is used to pull the stone out and is broken into small pieces using laser or pneumatic force. PCNL has risk of severe bleeding, possibility of septicaemia, the necessity of open surgery if kidney cannot be accessed and damage to other organs. Open surgery is preferred for very large stones. This involves the inconvenience of long term hospitalisation and the risks associated with the surgical protocol. The annual medical expenditure involved in the management of nephrolithiasis is estimated to be about 5 billion US dollars in developed countries. Thus it is apparent that cost effective, non invasive treatment procedures with minimum side effects are the need of the hour in the treatment of kidney stones.

Kidney stone formation is a collective process of nucleation, growth, aggregation of crystals and attachment of these crystals to renal cells. A fundamental prerequisite for the calcium oxalate stone crystallisation is the supersaturation of these crystal forming constituents either in final urine or in the nephrons. Low fluid intake, hyperoxaluria, hypercalciuria, hypocitraturia and environmental factors like increased temperature and fluid loss may cause calcium oxalate kidney stones. Hypercalciuria may be caused by rare genetic abnormalities like Dent disease which is an X-linked hypercalciuric stone-forming disorder or Bartter syndromes which arise from transport defects that affect calcium reabsorption (Taugner et al, 1988; Thakkar,
The association of crystals with the renal tubular cells is considered a potentially important factor in the process of renal stone formation (Menon & Koul, 1992). Renal tubular epithelial cell injury as a result of the crystal attachment helps in the crystal-cell interaction process. Renal cell injury also increases the affinity for crystal adhesion (Wiessner et al., 2001).

Reactive oxygen species production leading to lipid peroxidation of the renal epithelial cells and oxidative stress in the renal tissue are implicated in the pathogenesis of calcium oxalate stone formation. Oxidative stress is caused by an imbalance between generation of reactive oxygen species and antioxidant defence system (Khan, 2005).

Antioxidant deficit with increased lipid peroxidation is a common feature observed in calcium oxalate stone formers (Huang et al., 2000; Holoch & Tracy, 2011). Most of the present therapies for kidney stones are targeted to the dissolution and removal of the existing stones. They have not been instrumental in protecting the renal cell membranes against the damage caused by the crystal deposition. Antioxidants are known to confer membrane protective effects. Antioxidant treatment has also been extensively investigated in alleviating the detrimental effects of oxidative stress (Finkel, 2005). Carotenoids especially of natural origin are found to be better antioxidants than synthetic ones due to their increased absorption. This is attributed to the presence of oxygenated functional groups in their structure (Yeum & Russell, 2002).

Astaxanthin (3, 3′-dihydroxy-β-carotene-4, 4′-dione) is a xanthophyll carotenoid, which is attracting more attention due to its multiple effects and high antioxidant potential. It is a red pigment found in plants, algae and many marine animals like shrimps, lobsters, and crayfish. The US Food and drug administration (FDA) awarded “generally recognized as safe” status to
astaxanthin extracted from *Haematococcus pluvialis* in 2010. Astaxanthin is a more potent quencher of singlet oxygen (Shimidzu et al, 1996) than other antioxidants and its polar properties allows strategic placement in cell membranes which enhances its antioxidant potential (McNulty et al, 2008). Orally administered astaxanthin has satisfactory bioavailability and efficacy at reducing oxidative stress and inflammation. It is dubbed as “super vitamin” referring to its superior antioxidant status almost 500-times that of α-tocopherol (Kurashige et al, 1990). Astaxanthin was found to confer a protective effect on the renal tissue on mercuric chloride induced renal toxicity (Augusti et al, 2008). Further it offers the advantage of being a nutritional supplement of natural origin accompanied with no side effects reported so far. As per our knowledge, astaxanthin has not yet been evaluated for its antlithiatic potential. Therefore, the topic undertaken in this study is to assess the protective potential of the natural compound astaxanthin on calcium oxalate nephrolithiasis.

The chlorophyte alga *H. pluvialis* is the richest source of natural astaxanthin with a capacity to accumulate astaxanthin up to 4–5% of dry weight as per reported sources (Boussiba, 2000). Therefore, we chose biomass from *H. pluvialis* as the source of astaxanthin used in the current study. The doses of astaxanthin used in this study were calculated based on the guidelines of U.S. Department of Health and Human Services, Food and Administration Centre for Drug Evaluation and Research (2005) which recommended 3.8-7.6 mg/day astaxanthin. The FDA has increased this to 12 mg per day in 2011. The doses used in the study were extrapolated to human doses (Preuss et al, 2009). The administration of astaxanthin was found to be effective in within three to eight weeks in human trials improving blood rheology (Miyawaki et al, 2008) controlling the blood
pressure, reducing glycosylated haemoglobin (HbA1c) in healthy volunteers in risk of metabolic syndrome (Uchiyama et al, 2002) and significantly reducing the oxidative stress biomarkers (Choi et al, 2011) in overweight and obese subjects. The durations used in our study were based on these observations.

Oral administration of 0.75% v/v ethylene glycol daily and vitamin D_3 on alternate days has hence been used as the experimental regimen in many studies to generate a rat model of calcium oxalate nephrolithiasis (De Water et al, 1995; Mo et al, 2007). Kidney is the predominant and most sensitive target of ethylene glycol toxicity. Following absorption through the gastrointestinal tract, ethylene glycol is initially metabolized to glycolaldehyde by alcohol dehydrogenase which is rapidly converted to glycolate and glyoxal by aldehyde oxidase and aldehyde dehydrogenase. The metabolism of glycolate by glycolate oxidase or lactate dehydrogenase results in the formation of glyoxylate, which may be further metabolized to formate, oxalate, glycine, and carbon dioxide (Jacobsen & McMartin, 1986). Hyperoxaluria, defined as an increased oxalate excretion exceeding the normal range, is an important risk factor for the pathogenesis of calcium oxalate stone disease. The solubility of oxalate is found to be reduced in the physiological system in the presence of calcium (Schepers et al, 2005). Administration of vitamin D_3 is found to promote hypercalciuria. Hyperoxaluria with elevated calcium levels are shown to promote the supersaturation of calcium oxalate and favour the precipitation of calcium oxalate crystals. Hence a major feature of ethylene glycol toxicity is the marked accumulation of calcium oxy monohydrate crystals in kidney tissue and oxalate excretion (both ionic and crystalline) in the urine (Cruzan et al, 2004). The urinary system of male rats resembles that of
humans and the extent of calcium oxalate deposition is more in male rats when compared to females (Karadi et al, 2006). The sensitivity to ethylene glycol was reported to be more for male rats than for females. Also, Wistar rats were found to be more sensitive than other strains. (Cruzan et al, 2004). Hence male Wistar rats were preferably selected as the experimental models.

The hallmark of calcium oxalate stone disease is an altered renal function. Hence the renal function markers have to be assessed in the study to evaluate the effect of astaxanthin treatment on the nephrolithiatic animals. The urinary albumin excretion is a general clinical prognostic marker of kidney disorders. Moderately increased albumin levels found in both initial and repeat urine tests indicate an early phase of developing kidney disease and very high levels are an indication that kidney disease is present in a more severe form (Alter et al, 2012). The onset of calcium oxalate stone disease brings about changes in serum and urine calcium, phosphorous and magnesium and urine oxalate and thereby disturbing the ionic pattern. Hence the serum and urine electrolyte balance will be a useful prognostic tool to evaluate the effect of astaxanthin administration.

N-acetyl-β-D-glucosaminidase (NAG) is the most active glycosidase found in proximal tubular epithelial cell lysosomes. An increase in the urinary activity of this enzyme is a sensitive and reasonably specific measure of renal tubular damage, since its relatively large molecular weight (>130 kD) precludes filtration by the glomerulus (Dittrich et al, 2000). Alanine aminopeptidase (AAP) is another integral brush border enzyme indicative of renal cell integrity. The evaluation of these enzymes will provide an index of renal tubular damage and the effect of astaxanthin on the tubular cells.

Liver plays a central role in biotransformation and disposition of both exogenous and endogenous xenobiotics (Miyayi, 1991). The smooth
endoplasmic reticulum of the liver is the principal ‘metabolic clearing house’ for both endogenous and exogenous substances.

Liver cell destruction shows its effects mostly as increase in the liver cell membrane permeability, which results in the leaking out of tissue content into the blood stream. In several organs, cell membrane damage is followed by release of a number of cytoplasmic enzymes into the blood, a phenomenon that provides the basis for clinical diagnosis. Alanine transaminase (ALT) activity is the most frequently relied biomarker of hepatotoxicity. It is a liver enzyme that plays an important role in amino acid metabolism and gluconeogenesis. It catalyzes the reductive transfer of an amino group from alanine to \( \alpha \)-ketoglutarate to yield glutamate and pyruvate. This enzyme detects hepatocellular necrosis. Aspartate aminotransferase (AST) is another liver enzyme that aids in producing proteins. It catalyzes the reductive transfer of an amino group from aspartate to \( \alpha \)-ketoglutarate to yield oxaloacetate and glutamate (Dufour et al, 2000). Alkaline phosphatise (ALP) is a hydrolase enzyme that is eliminated in the bile. It hydrolyzes monophosphates at an alkaline pH. It is particularly present in the cells which line the biliary ducts of the liver. It is also found in other organs including bone, placenta, kidney and intestine. Hepatotoxicity leads to elevation of the normal values of this enzyme due to the body’s inability to excrete it through bile due to the congestion or obstruction of the biliary tract (Ramaiah, 2007). The assessment of the levels of the marker enzymes ALT, AST and ALP will point out the possible hepatotoxicity of astaxanthin administration in the nephrolithiatic animals if any.

Oxidative stress can result in damage to important biological molecules, such as DNA, proteins, and lipids, leading to mitochondrial dysfunction, embryo-cell block, ATP depletion, and apoptosis (Guerin et al,
Lipid peroxidation refers to the functional impairment of cellular components by reactive oxygen species such as superoxide radicals, hydroxyl free radicals and hydrogen peroxide. The process is initiated by the hydroxyl radical formed through the extraction of a hydrogen atom from unsaturated fatty acids of membrane phospholipids, the resulting chain reaction yielding several types of secondary free radicals and a large number of reactive compounds, culminating in the destruction of cellular membranes (Farber et al., 1990). The extent of lipid peroxidation can be studied by measuring the thiobarbituric acid reactive substances (TBARS) in liver and kidney of the astaxanthin treated animals.

The antioxidant system is composed of a group of enzymes and non-enzymatic antioxidants responsible for the control of free radicals and minimizing the adverse cellular effects resulting from excessive exposure to reactive oxygen. Super oxide dismutase (SOD) is an important initial component in the cellular defence against oxygen toxicity. SOD is widely distributed in cells exhibiting high level of lipid peroxidation and scavenges the deleterious superoxide anion. It converts superoxide anions into hydrogen peroxide and oxygen. Catalase and glutathione peroxidase (GPx) are enzymes responsible for hydrogen peroxide degradation into water and oxygen, reducing toxicity of this compound in the tissues. In this reaction, the GPx oxidizes reduced glutathione (GSH) to oxidised glutathione (GSSG), donating an electron to hydrogen peroxide. The GSSG is reconverted to GSH by the action of the enzyme glutathione reductase (GR), a flavoprotein containing FAD (Mates, 2000). Glutathione-S-transferase (GST) helps on the transport and elimination of reactive compounds. Glutathione (GSH) is a major intracellular antioxidant with multiple biological functions, including the maintenance of the thiol moieties of
proteins and the reduced from of many other biologically active molecules (Ushio-Fukai et al, 1999). Vitamin E plays a vital role in protecting cell membranes against oxidative damage through trapping and scavenging free radicals. Vitamin C is a water-soluble antioxidant that reduces radicals from a variety of sources and also serves to recycle oxidized vitamin E (Chen et al, 2012).

A fostaxanthin has been shown to be one of the most effective antioxidants against lipid peroxidation and oxidative stress in in vitro and in vivo systems (Kurashige et al, 1990; Barros et al, 2001). It acts as a strong antioxidant by donating the electrons and reacting with free radicals to convert them to more stable products and terminates free radical chain reaction in a wide variety of living organisms (Yuan et al, 2011). The level of SOD, catalase, GPx, GST, GR, GSH and vitamins C and E in the liver and kidney can be measured to study the effect of astaxanthin on renal and hepatic antioxidant status in calcium oxalate nephrolithiatic condition.

Histopathological analysis of the liver and the kidney tissue will help to observe the severity of crystal deposition and the effect of astaxanthin on renal and hepatic cells in nephrolithiasis. The extent of calcium deposition in the kidney can be quantified by flame photometry and scanning electron microscopy energy dispersive X-ray (SEM-EDX) analysis. The SEM analysis also will help to have a closer examination of calcium oxalate crystal deposition in the renal tubular lumens and the possible ameliorative effect of astaxanthin treatments on crystal dissolution. Pizzolato staining is specifically done to differentiate calcium oxalate deposits. The crystal deposition can therefore be observed and assessed by this specific staining procedure.
Proteins make up 1–5% of the weight of kidney stones. Extraction of the proteinaceous fraction from calcium oxalate stones using ethylene diamine tetra acetic acid has shown that osteopontin is the major constituent (Kohri et al, 1992). To find out if astaxanthin exerts any regulatory effect on stone matrix proteins an examination of the proteins and their regulatory mechanisms involved in the calcium oxalate stone formation will be helpful. Increased osteopontin (OPN) expression in renal proximal tubular cells is modulated through reactive oxygen species generation, renin angiotensin system activation and transforming growth factor-β1 (TGF-β1) expression. (Hsieh et al, 2006). Increased nucleation of calcium oxy monohydrate crystals in the nephron lumen and the crystal cell interactions up regulates osteopontin expression and increases its secretion by the renal tubular cells by stimulating the renal renin angiotensin system (Yasui et al, 2001). Angiotensin-I converting enzyme, the enzyme which catalyses the conversion of angiotensin I to angiotensin II, is abundant in the rat kidney and has been located in the proximal and distal tubules, the collecting ducts, and renal endothelial cells (Casarini et al, 1997). Angiotensin-I converting enzyme participates in various renal functions by regulating the circulating level of angiotensin II, which in turn affects the expression of genes including OPN and TGF-β1 locally. Oxidative stress also plays an important role in the proinflammatory effect of angiotensin II. Thus any molecule which can disrupt the renin angiotensin system can be assumed to disrupt renal stone formation and exert an antilithiatic effect. With this hypothesis in mind and supported by a study in Zucker fatty rats in which astaxanthin influences the RAS system (Preuss et al, 2009) it is assumed that estimation of the levels of renal and urinary ACE and the correlation of the renal enzyme with renal OPN and TGF-β1 expressions will help to unravel a
possible mechanism of action of astaxanthin in calcium oxalate nephrolithiasis.

The objectives of the study were to conduct the following investigations in calcium oxalate nephrolithiasis.

1) To assess the effect of astaxanthin on the liver and kidney tissue

2) To study the efficacy of astaxanthin in dissolving the calcium oxalate crystals deposited in the kidney of the nephrolithiatic rats.

3) To evaluate the effect of astaxanthin on stone matrix protein OPN and the cytokine TGF-\(\beta\)1 expressions in the renal tissue.

4) To elucidate the mechanism of action of astaxanthin by studying the correlation of renal and urinary ACE levels with renal OPN and TGF-\(\beta\)1 expressions and calcium oxalate deposition.

5) To compare the efficacy of astaxanthin treatment over potassium citrate administration on renal stone formation and regulation of OPN and TGF-\(\beta\)1 expressions.