In vitro and *In vivo* Studies on Nephroprotective and Antiurolithic Effect of *Biophytum sensitivum* in Different Toxicity Models

Thesis submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai
Chennai – 600032

In partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY
in
Faculty of Pharmacy

Submitted By
B.R. ABHIRAMA, M.Pharm.,
(Reg. No. 141440001)

Under the guidance of
Dr. R. SHANMUGA SUNDARAM, M.Pharm., Ph.D.,

J.K.K. Nattraja College of Pharmacy
Komarapalayam, Namakkal - 638183, Tamil Nadu. India.

DECEMBER - 2017

TABLE OF CONTENTS

\

1. INTRODUCTION

1.1. Back ground of the proposed work

Kidney receives rich blood flow of 525 mL/min and plays a prominent role in the filtration, concentration, excretion and detoxification of drug, chemicals or their metabolites. This makes kidney, a major organ vulnerable to drug-related toxic responses and also become a routinely assessed organ during the preclinical safety evaluations or in toxicological studies. Drug-induced kidney injury is a common condition present even among the infants, young children, often require multiple interventions, hospitalization and exert financial burden.[1] Previous literature has prescribed that the concurrent administration of nephroptotective herbal drugs along with different nephrotoxic agents may reduce its toxicity.[2] Several research studies have proved that various chemical and natural substances with antioxidant activity reduce the toxic response of drug having nephrotoxic potential and enhance its efficacy.[3]

1.2. The Kidney

1.2.1. Structure of the kidney

Adult human kidney weighs about 160 g, 11.25 cm long, 2.5 cm thick and 5.5-7.7 cm wide. This retroperitoneal organ are paired, bean shaped, reddish brown, located at the level of the twelfth thoracic and third lumbar vertebrae on either sides of the vertebral column. It is protected by three layers of tissue. These layers consists of (1) renal capsule, an innermost strong fibrous layer which protects kidney from shock and infections (2) the adipose capsule, middle layer of fatty tissue which keeps kidney in right position (3) the renal fascia, outermost fibrous connective tissue which bind kidney with the abdominal wall. Cut surface of a bisected kidney shows two anatomically

distinct regions: the pale outer region called renal cortex and the inner dark region known as renal medulla. [4, 5]

Figure 1: Structure of kidney & nephron

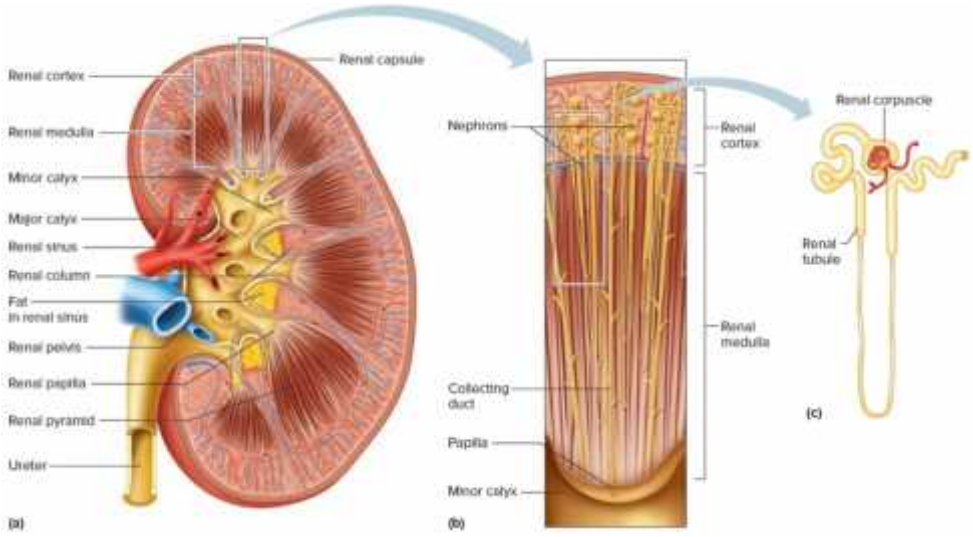


Table 1: Kidney structure and functions based on different parts

Parts of the kidney	Functions
Hilus	Renal vein and ureter exit and the renal artery enters the kidney
Renal capsule	Maintain the kidney's shape and protect the kidney from damage
Renal cortex	Bowman's capsules, glomeruli, proximal and distal convoluted tubules and blood vessels are found.
Renal medulla	Loop of Henle and collecting ducts are found
Renal pyramids	Transport urine from the cortical or outer part of the kidney where urine is produced to the calyces in which urine collects.
Renal pelvis	Receives urine drained from the nephrons through the collecting ducts and papillary ducts
Renal artery	Blood vessel that carry oxygen-rich blood to the kidney
Renal vein	Blood vessel that collects deoxygenated blood from the kidney
Interlobular artery	Blood vessel that delivers oxygen-rich blood to the glomerular capillaries
Interlobular vein	Blood vessel that receives deoxygenated blood from the glomeruli and the loops of Henle
Nephrons	Functional units where the kidney's main functions are performed
Collecting duct	Collects urine and drains finally into the ureter and urinary bladder
Ureter	Structure which conveys urine from the kidney to the urinary bladder.

1.2.2. Functions of the kidney

Table 2: Major functions of the kidney[6]

Basic functions of kidney	
Excretory functions	Excretion of nitrogenous waste products of protein metabolism Excretion of most drugs or toxins Regulation of extracellular fluid volume and blood pressure by altering sodium excretion. Regulation of osmolality by altering water excretion Regulation of plasma electrolyte concentration within normal range Regulation of plasma pH by eliminating excess hydrogen and bicarbonate ions
Non-excretory functions	**Synthesis and activation of hormones** Erythropoietin which stimulate red blood cell synthesis by bone marrow Renin which control blood pressure, salt and water balance. Prostaglandins which act as vasodilators and prevent renal ischemia. *1,25*-dihydroxyvitamin D3, potent form of vitamin D which maintain bone health
	Degradation of polypeptide hormones Antidiuretic hormone, gastrin, glucagon, growth hormone, insulin, parathormone, prolactin, vasoactive intestinal poly peptide

1.3. Nephrotoxicity

Kidney plays a prominent role in the metabolism and excretion of many exogenously administered drugs, diagnostic agents and their metabolites. Nephrotoxins are drugs or chemicals that produce toxic effect on kidney.[7, 8] Through renal arteries nephrotoxins reach the functional units of kidney known as nephrons. Hence cells of nephrons are more susceptible to drug related toxic responses as it is exposed to high concentration of drug and metabolites. Previous research studies have reported that the acute kidney injury have been a serious adverse effect of various drugs, compounds used in industries and diagnostic agents like radio contrast media.[9]

Kidney injury associated with certain drug administration may cause either cumulative dose-dependent toxicity or idiosyncratic dose-independent toxicity. The

morphological examination of the kidney biopsy due to nephrotoxic insult exhibited alterations in the normal structures of the nephron, indicating damage to the renal infrastructure. Drug induced renal failure can be further classified based upon the renal compartments principally affected with toxicity as acute tubular necrosis, interstitial nephritis, glomerulonephritis, renal vascular damage and intrarenal obstruction. Acute tubular necrosis is the most common form of intrarenal failure associated with prolonged exposure of kidney to nephrotoxin or due to ischaemia, sepsis, diabetes and artherosclerosis.[10] Interstitial nephritis is related with nephrotoxin-induced hypersensitivity reaction or inflammation affecting interstitium. Common cause of glomerular nephritis is inflammatory response due to the deposition of immune complexes (exogenous or endogenous antigens) in glomerulus.[11] Damage to the visceral layer of the Bowman's capsule-podocytes may cause a disorder known as nephritic syndrome, which further leads to glomerulonephritis. Occlusion of renal artery or vein also causes alterations in intrarenal vasculature. Deposition of debris provoke intrinsic renal damage.

Lithium chloride, usually indicated for psychotic disturbances like bipolar disorder causes nephrogenic diabetes insipidus, due to the decreased expression of aquaporin-2 channels on the apical surfaces of the proximal convoluted tubule. The prolonged administration of lithium containing drugs also promote development of chronic tubular interstitial nephritis and tubular microcysts. Most of the analgesics are available in combination dosage forms. The large cumulative doses of analgesics may lead to a more serious form of nephrotoxicity known as analgesic nephropathy. Pre-renal failure also occurs due to diminished cardiac output related to heart diseases and

alterations in fluid volume distribution associated with sepsis or burns.[12] Urolithiais, prostatic hypertrophy, blood clots and neoplasm are the various mechanism associated with obstruction of urinary tract resulting in post renal failure. Certain nephrotoxic drugs induce tubular injury, interstitial inflammation and obstruction due to the formation of crystal like substances in the kidney. Most common type is calcium-containing stones (75-90%), followed by magnesium ammonium phosphate or struvite stones (10-15%), uric acid crystals (3-10%) and cystine stones (0.5-1%). Other types of stones include silicate stones, protease related stones and dihydroxy adenine crystals.[13]

1.3.1. Epidemiology of drug induced nephrotoxicity

Drug-induced acute kidney injury accounted for 20% of all acute renal failure cases in an Indian study.[14] Prospective cohort studies have reported that the incidence of drug related kidney toxicity is about 14-16% of adult population.[15,16] Nephrotoxicity linked with the administration of aminoglycoside antibiotics elevated from 3% in 1969 to 10-20% in 2010.[9,17] Another study reported that 27 million persons are suffering from chronic kidney disease. Drastic increase in drug related nephrotoxicity (about 30%) has been observed over the last 10 years.[18] Drug related renal injury is a common condition present even in children and estimated about 16% of hospitalized acute renal failure events.[19] Incidence and prevalence of chronic kidney disease and end-stage renal disease is dramatically increasing globally.[20] Kidney stones is a painful disorder and still it remain as common worldwide problem. Recurrent rate is high, more than 50% in 5-10 years. It is accounted that 12% of the world population is affected by kidney stones and male/female ratio is 3:1. Relapse rate is more in male (70 to 80%) than in females (47 to 60%).[21]

1.3.2. Etiology

Various factors are involved which enhance the drug induced nephrotoxicty. An easiest approach to know about the vulnerability of kidney nephrotoxins entails the classification of risk factors into three major categories as patient-specific, kidney-related, and drug-related factors. Usually, more than one risk factors are involved in drug related renal injury affecting all renal compartments and cause one or more renal syndromes.

Table 3: Risk factors of drug induced nephrotoxicity

Major risk factors of drug induced nephrotoxicity[22]	
Patient-related factors	Sex- female Age - children and age over 65 years Co-morbidities and polypharmacy Volume depletion Reduced glomerular filtration rate Increased proximal tubular toxin reabsorption Decreased distal tubular urine flow rates Metabolic perturbations Alkaline or acid urine pH Immune response genes Gene mutations in hepatic and renal P450 system Gene mutations in renal transporters and transport proteins Acute/Chronic kidney disease Nephrotic syndrome Cirrhosis/obstructive jaundice
Kidney-related factors	High perfusion rate Enhanced toxin level Biotransformation of substances to reactive oxygen species Elevated metabolic rate of tubular cells Proximal tubular absorption of toxins
Drug-related factors	Prolonged dosing periods Potent direct nephrotoxic effects of the drug or compound Combinations of toxins/drugs promoting enhanced nephrotoxicity Competition between endogenous and exogenous toxins for transporters Increasing toxin accumulation within the tubular cell Intratubular crystal precipitation of insoluble parent compound or metabolite.

Table 4: Risk factors of drug induced urolithiasis

Major risk factors of drug-induced urolithiasis [23]	
Patient-related factors	Sex- Male Age-age over 40 years Co-morbidities and polypharmacy Family history Volume depletion High concentration of minerals and chemicals within the urine Living in warm climate Drinking insufficient amount of water or excess sweating High dietary intake of animal protein, sodium, refined sugars, fructose High body mass index (BMI), large waist size and weight gain Alkaline or acid urine pH Increased calculi-induced toxin reabsorption Hyperparathyroidism, hypercalcemia and hyperuricosuria Insulin resistant states, surgical menopause, history of gout, urinary tract infections, cystic fibrosis, hypertension, gastric bypass surgery, inflammatory bowel disease or chronic diarrhoea, Crohn's disease
Kidney-related factors	High perfusion rate Enhanced drug level which induce calculi Biotransformation of substances to reactive oxygen species Decreased urine volume Increased excretion of stone-forming components Changes in the acid-base balance (pH) Decrease in urinary citrate levels Excess vitamin D, deficiency of vitamins A or C Intratubular crystal precipitation of insoluble dug or metabolite
Drugs-related factors	Prolonged dosing periods Combinations of toxins/drugs promoting kidney stone formation Increase toxin accumulation within the tubular cell Urinary supersaturation of drug or its metabolite Drug-Induced metabolic calculi

1.3.3. Mechanism of drug induced nephrotoxicity

Most of the drug exert nephrotoxicity through more than one pathogenic mechanism but some drugs exert toxicity through direct effect or their accumulation in renal tubule.[24] Renal blood flow is disproportionately higher in renal cortex (90%)

compared to renal medulla (6-10%) or papillae (1-2%), hence concentration of blood borne toxins are more in this region. Mechanism involved in urine formation and reabsorption process of nephron also promote accumulation of nephrotoxins and often causes precipitation of poorly soluble substances. High permeability and active transport increase the possibility of drug accumulation in proximal tubule of kidney than in the distal tubular region.[5, 25] Therefore, most research works related to drug induced kidney injury is focusing mainly on proximal tubular part of kidney.[26] Sometimes, kidney metabolically convert protoxicants to nephrotoxicants.[4]

Changes in glomerular hemodynamics, tubular cell toxicity, inflammation, crystal nephropathy, rhabdomyolysis, and thrombotic microangiopathy are the general mechanisms leading to renal dysfunction following the exposure to nephrotoxins.[27, 28] Kidney maintain constant glomerular filteration rate (120 mL per min) and urine output by adjusting intraglomerular pressure and regulating blood flow in afferent and efferent arteries Prolonged administration of nonsteroid anti-inflammatory drugs (anti-prostaglandin drugs) angiotensin converting enzyme inhibitors, angiotensin receptor blockers may induce changes in glomerular hemodynamics and thereby induce toxicity in glomerulus.[29].

Proximal tubular cell toxicity is associated with mitochondrial damage, disturbed tubular transport system and oxidative stress by free radical generation.[30] Gentamicin (aminoglycoside antibiotics), cisplatin and foscarnet (anticancer) drugs, amphotericin B (antifungal agents), adefovir (anti-retroviral) are the common drugs which induce tubular cell toxicity.[26,30]

Certain nephrotoxic drug often produce inflammation in the kidney tissue inducing acute interstitial nephritis, chronic interstitial nephritis or/and glomerulonephritis. Gold, penicillin, phenytoin are certain drugs which induce inflammation in glomerulus (glomerulonephritis). Prolonged use of non-steroid anti-inflammatory drugs and rifampicin may induce acute interstitial nephritis, where as administration of calcineurin inhibitors, lithium, anticancer drugs or analgesics causes chronic interstitial nephritis.[31, 32]

Administration of certain drugs ampicillin (antibiotics), acyclovir (antiviral agents) or chemicals (ethylene glycol) cause crystal nephropathy (crystallisation of stone forming constituents in human urine).[26,30] Alcoholism or prolonged intake of heroin, methadone, methamphetamine and statins induce rhabdomyolysis or disintergration of renal muscle cell which causes release of myoglobin and serum creatine kinase into the blood.[33]

Some drugs including cyclosporin, mitomycin-C and quinine have potential to induce drug-induced thrombotic microangiopathy through inflammation or direct toxic effect in renal epithelial cells.[34]

1.3.3.1. Gentamicin-induced nephrotoxicity

Degree of renal damage varies from one drug to another. Gentamicin (GM) is a typical aminoglycoside antibiotic which provides an effective treatment against life-threatening infections caused by gram-negative organisms.[35] However, it has been reported that nephrotoxicity was observed in 30% of patients treated with GM, for more than 7 days.[36] Possibility of developing nephrotoxicity increases with duration of therapy,

as 50% of patient treated with genatmicin for more than 14 days exhibited acute kidney injury symptoms.[37] GM-induced nephrotoxicity related to the clinical features of acute tubular necrosis such as proximal tubular dysfunction, enzymuria, proteinuria, glycosuria, hypokalemia, hypocalcemia and hypomagnesemia. Still this drug is widely used because of its low cost and efficacy, even though less nephrotoxic antibiotics are available.[38]

The usual dose of aminoglycoside antibiotic like gentamicin is less than 80 to 100 mg/kg for about 5 days. At physiological pH, this aminoglycoside antibiotic become highly charged, water soluble and impermeable through biological membranes. These factors causes the accumulation of high amount of drug in the renal cortex,[39] then filtered through glomeruli, reabsorbed actively via lysosomes in the brush border membrane of proximal tubular cells resulting in the formation of myeloid bodies/secondary lysosomes. The gentamicin drug molecules enter the tubular cells by absorptive/ receptor mediated endocytosis after binding to acidic phospholipids and megalin receptors. Rats administered even with less and therapeutic doses of gentamicin exhibited phospholipidosis and apoptosis in proximal tubular cells.[40] Prolonged administration of gentamicin causes alterations in structural architecture of lysosomes, followed by the release of lysosomal enzymes from the proximal convoluted tubule. This also causes lowering absorption of protein, electrolytes (potassium, magnesium, calcium, sodium), phospholipid urea, fall in creatinine clearance, glucose wasting, and appearance of casts (solidification of protein) in the urine.[41]

The histopathological examination of the kidney tissue exposed to gentamicin in animals exhibited signs of focal necrosis and apoptosis in the tubular epithelium. This supports that the aminoglycoside exerts its toxicity due to its high concentration in the

tissues. Metabolic alterations are observed within the tubular cells due to the accumulation and release of excess aminoglycosides from the lysosomes. This causes lipid peroxidation, decreased rate of mitochondrial respiration or cell death.[42]

The aminoglycoside released from lysosomes of the tubular cells act indirectly as nephrotoxic. GM-induced hydrogen peroxide production causes the release of iron from mitochondrial membranes. The gentamicin chelates with iron forming oxidant molecule, the ferric-gentamicin complex which causes necrosis of tubular hair cells in the proximal convoluted tubule. The changes in the sodium-glucose symporters and sodium-hydrogen antiporters in tubular cells of the nephrons is mainly due to fluidity of the membrane caused by gentamicin. Gentamicin also causes the direct inhibition on sodium-potassium-ATPase of proximal convoluted tubule.[41]

1.3.3.2. Cisplatin-induced nephrotoxicity

Cisplatin is chemically known as cis-diamine dichloro platinum (II), CDDP. It is the most potent, highly effective and currently available alkylating agent used in the treatment of solid tumours like breast cancer, head cancer, neck cancer, testes and ovarian cancers etc.[43] However, clinical utilization of this heavy metal complex is restricted due to the adverse reactions including ototoxicity, gastrotoxicity, myelosuppression, allergic reaction and nephrotoxicity.[44] Cisplatin induced nephrotoxicity occurs in about 20-30% of patients.[45] Factors which promote cisplatin induced nephrotoxicity include high perfusion rate, drug uptake of free-form cisplatin in the proximal kidney tubule, metabolism and disproportionate accumulation of platinum concentrations in the kidneys greater than other organs.[46] Major clinical manifestations of cisplatin induced

nephrotoxicity include erythropoietin deficiency, proteinuria, renal salt wasting, hyperuricemia, renal concentrating defect, hypocalcemia, hypomagnesemia and hypokalemia.

Through glomeruli cisplatin enter the tubular lumen of the nephron either by passive process or facilitated transport mechanism. Cisplatin administration may precipitate acute renal failure as it causes vasoconstriction of afferent arterioles, leading to the decreased blood flow to the Bowman's capsule. Cisplatin stimulates various signaling pathways (mitogen-activated protein kinase, tumor protein p53, reactive oxygen species) of the tubular cells and induces tumor necrosis factor-alpha formation which leads to inflammatory response, tubular cell injury or necrosis. The intrinsic mitochondrial and extrinsic death receptor may also gets activated which further leads to caspase-dependent or independent apoptosis. Inequilibrium between cyclin-dependent kinase (CDK2) or cyclin-dependent kinase inhibitor proteins (p21) is the major causative factor which induce cell apoptosis and acute renal failure. Cisplatin administration causes induction of inhibitory protein p21 and the activation of CDK2 protein which results in renal tubular cell toxicity. DNA injury stimulate ATR (ataxia telangiectasia and Rad3-related protein) which leads to stimulation of inhibitory protein p53 and phosphorylation. This inhibitory protein further causes transcription of p53 upregulated modulator of *apoptosis (*PUMA) and p53-inducible death-domain-containing protein, (PIDD, apoptosis genes) in the neuron cells. PIDD genes promote caspase-2 activation and secretion of apoptosis-inducing factor (AIF) from the mitochondria of the tubular cells and causes caspase-independent apoptosis. The PUMA-alpha translocates to the cellular power house, interacts with cellular components, neutralizes anti-apoptotic

protein called B-cell lymphoma-extra large (Bcl-XL) and form oligomer pores on the outer surface of the mitochondria. This process is followed by the release of cytochrome c into the cytoplasm and stimulates the caspases leading to caspase-dependent apoptosis.[47]

1.3.3.3. Ethylene glycol-induced urolithiasis

Drugs can cause renal stone formation either by raising excretion rates of naturally occurring stone forming components or by directly precipitating within the urinary tract. Urinary supersaturation of the agent may also lead to metabolic abnormalities that facilitate the formation of stones and eventually grow large enough to cause problems. Ureteral obstruction causes post renal azotemia, hydronephrosis and spasm of the ureter. This leads to renal colic pain (most commonly felt in the flank, lower abdomen, and groin), nausea, vomiting, fever, hematuria, pyuria, painful urination and secondary infections.

Urolithiasis can be induced by using variety of agents like ethylene glycol, sodium oxalate, ammonium oxalate, glyolic acid etc. Accumulation of glycolic acid, a metabolite of ethylene glycol in the body is responsible for the toxicity.

Alcohol dehydrogenase converts ethylene glycol initially into glycoaldehyde which is then metabolized by acetaldehyde dehydrogenase, producing glycolic acid. Cytochrome P450 2E1 (CYP2E1) metabolize glycoaldehyde to glyoxal. Glyoxal is then further metabolized into glycolic acid or glyoxylic acid. Glycolate oxidase or lactate dehydrogenase convert glycolic acid into glyoxylic acid. Glyoxylic acid is further converted into oxalic acid either by the action of lactate dehydrogenase or glycolate

oxidase. Oxalic acid can form crystals with calcium, producing calcium oxalate, which is the most common type of kidney stone causing renal injury.

Urolithiasis or crystallisation of the calcium oxalate is a multifaceted process involving several physicochemical events which begins with increased urinary super saturation with one or more calculogenic (crystal-forming) substances. Supersaturation of the urine with respect to a calculogenic compound depends on urinary pH, ionic strength, solute concentration and complexations. Driving force for crystallization is a reduction in the potential energy of the atoms or molecules that bonds to each other. This is followed by formation of seed crystal by which stone-forming salts in supersaturated urinary solution coalesce into clusters (nucleation) and attaches to cell surface of renal papilla. Growth of insoluble particles and agglomeration is the next major step of stone formation. A seed crystal can grow by combining with other crystals in solution, ultimately retained and accumulated in the kidney.[48] Crystal retention promotes renal injury and the development of a stone nidus on the renal papillary surface and further supports crystal nucleation even at lower supersaturation levels.

In most individuals, the crystallization inhibition capacity of urine prevent stone formation whereas, this natural inhibition is lacking in stone formers.[49] When inhibitors of stone formation fall below their normal proportions, urolithiasis can occur. Normal urine contains chelating agents such as citrate, magnesium and orthophosphate which inhibit the nucleation, growth and aggregation of calcium-containing crystals. Other endogenous inhibitors include calgranulin , Tamm–Horsfall protein, glycosaminoglycans, uropontin (a form of osteopontin), nephrocalcin (an acidic glycoprotein), prothrombin F1 peptide, and bikunin (uronic acid-rich protein).[50] Sufficient dietary intake of magnesium

and citrate also inhibit the formation of calcium oxalate and calcium phosphate stones. Nephrolithiasis causes a variety of changes in the renal epithelial cells, such as an increase in free radical production and a decrease in antioxidant status, followed by inflammation, cell injury and cell death.

1.3.4. Assessment of kidney function

Periodic assessment of renal function is a part of health care especially for chronically sick patients.[51] Conventional parameters widely employed for assessing kidney function and monitoring progression and treatment of renal disease are serum blood urea nitrogen and creatinine level.[52] Urea is the waste product of protein catabolism produced in the liver from ammonia, dissolved in the blood and finally excreted by renal tubules. Creatinine is waste product of metabolism formed from a high energy molecule known as creatinine kinase used by skeletal muscles.[53] However these parameters are nonspecific to kidney injury as various non-renal related factors exhibit alteration from normal serum level of these nitrogenous waste products without producing any negative effect on kidney. Other parameters that indicate renal dysfunction include lowered glomerular filtration rate (<60%), proteinuria, hematuria, increased renal excretion and serum level of uric acid, altered enzymes, urinary and serum electrolytes from normal level.[54] Reabsorption power for endogenous components like small proteins, sugars or metabolites is highly diminished and the presence of low molecular weight proteins (β2-microglobulin, α1-macroglobulin and retinol binding protein) in urine indicate tubular injury.[55] Presence of high molecular weight proteins like albumin,[56] transferrin and immunoglobulin G in urine represent renal dysfunction.[57] Imaging tests like X-ray,

computed tomography, ultrasound etc are used to confirm the size and location of the kidney stone.

1.3.5. Prevention and treatment strategies

Close monitoring of renal function and vital signs is important especially when administering nephrotoxic drug to patients at risk. Age, co-morbidities and polypharmacy are the major risk factors. Gentamicin, cisplatin, polyethylene glycol etc cause acute tubular necrosis, predominantly in early or late segments of the proximal tubule. Initial therapy for drug-induced nephrotoxicity is basically supportive which include examining of serum drug levels and renal function twice or thrice per week, extended-interval dosing or prefer once-daily doses, using lowest effective dose of causative drug, limit duration of therapy or stopping the causative drug, avoid drug combination having nephrotoxic potential, maintaining fluid and electrolyte balance, and controlling sepsis . Hemodialysis may be required in certain occasions.[58] It is reported that the hydration (intravenous saline-about 150 to 250 mL/hr before, during and after chemotherapy) and intravenous mannitol decreases the appearance of cisplatin induced nephrotoxicity. It acts by decreasing the uptake of cisplatin into the renal tubular cells. However, there is still significant incidence of renal insufficiency despite routine hydration and frequent use of mannitol before cisplatin administration. Urine alkalinization, adequate hydration, establishing high urine flow and reducing dose of nephrotoxic drug may prevent crystalluria. Diuretics such as hydrochlorothiazide, potassium citrate are used to decrease calcium excretion. Allopurinol decreases high blood uric acid levels. In most cases, renal function slowly recovered within 7 days after stopping the offending drug. Steroid therapy (oral prednisone, 1 to 2 mg/kg/day for 4 to 6 weeks) is suggested if renal failure

persists.[59] If renal dysfunction persist even after steroid therapy, immune suppressive agents such as cyclophosphamide can be indicated. However, no randomized trial data is available supporting the efficiency of use of these drugs in this situation.

1.4. Importance of herbs in nephroprotection

Plant drugs have been used for treating numerous health problems all over the world as it is generally considered to be free from side effects due to their natural origin. The trend of using herbal products has increased and the active plant extracts are frequently screened for new drug discoveries. It has been observed that modern medicine used for the treatment of many diseases like kidney diseases, liver disorders, cardiovascular diseases, arthritis, asthma and skin diseases associated with serious adverse effects. Hence in recent years numerous research works have been carried out on medicinal plants for their claimed activities.

Several research studies confirmed that the generation of free radicals and diminished antioxidant activity are implicated in development of several life limiting chronic diseases and xenobiotics-induced nephrotoxicity.[60,61] Inhibition of protein synthesis, DNA damage, mitochondrial injury, apoptotic cell death are the various mechanism associated with oxidative stress in drug induced nephrotoxicty. Endogenous antioxidant defense system of body generally provide insufficient protection against the generation of reactive oxygen species, hence antioxidant are needed to supplement from external sources.

Most synthetic drugs used for the treatment of nephrotoxicity associate with higher incidence of adverse drug reactions. Currently, there is no satisfactory drug for the

treatment, prevention or recurrence of kidney stone formation.[62] Invasive procedures including extracorporeal shock wave lithotripsy (ESWL), ureteroscopy, nephro-lithotomy etc, are effective but they are costly and may reduce renal functions. It also causes acute renal injury, infections and recurrence of kidney stone formation.[63]

Prolonged exposure of kidney to drugs (gentamicin, cisplatin, NSAIDs and cyclosporine), chemical reagents (ethylene glycol, carbon tetra chloride, sodium oxalate) and heavy metals (lead, mercury, arsenic and cadmium) lead to renal injury. Nephrotoxicity is a multifactorial process linked with various etiological factors, hence the treatment is aimed at multiple targets. Medicinal plants have curative properties on nephrotoxicity due to the presence of various chemical constituents aiming at multiple targets and may offer effective, inexpensive and safe remedy. Early literatures have prescribed numerous herbal drugs for the treatment of renal disorders and also reported that concurrent administration along with different nephrotoxic agents reduced the toxic effects. Many herbs have been proven to be effective as nephroprotective agents while many more are claimed to be nephroprotective but there is lack of scientific evidence to support such claims.

1.4.1. Various mechanism of action of nephrocurative plant drugs

Research conducted on multiple species of *Lespedeza capitata* rich with phenolic and polyphenolic compounds like flavonoids, proanthocyanidins exhibited improvement in renal function.[64] *Lespedeza virgata* attenuated kidney damage due to doxorubicin injection.[65] Glabridin, a flavonoid from *Glycyrrhiza glabra* showed protective effect against proteinuria in a mice model of glomerulonephritis.[66]

Moringa oleifera and *Rubia cordifolia* significantly lowered serum levels of

accumulated waste products blood urea nitrogen and creatinine and enhanced glomerular filtration rate.[67] *Berginia ciliate* possessed diuretic, antioxidant, hypermagnesuric effect and also showed highest dissolution of both calcium oxalate and calcium phosphate stones.[68] *Phyllanthus niruri*, *Solanum xanthocarpum* (Solanaceae), decreased the stone forming constituents such as calcium in urine along with an increase in glycosaminoglycans (crystallization inhibitors) concentration.[69]

Presence of flavonoids, quercitrin and afzelin, the major components in *Copaifera langsdorffii* leaves showed protective effect against nephrotoxicity due to their antioxidant properties.[70] Pomegranate (*Punica granatum*) juice was found to be nephroprotective which increased inducible nitric oxide synthase (iNOS) and nuclear factor , NF-kappa-B (transcription factor 65) expression emphasizing its antioxidant potential. These plant drugs also showed inhibition on crystal growth.[71] *Orthosiphon stamineus* showed good diuretic, antiseptic and litholytic properties and its flavonoids were found to possess adenosine A1 receptor binding activity, which promote diuresis and sodium excretion.[72]

Tribulus terrestris rich with saponin exhibited a significant decrease in peristaltic movements of rabbit jejunum preparation in a dose-dependent manner. This effect may be useful for colic pain or smooth muscle spasms.[73] Patients treated with Cystone showed a symptomatic relief from abdominal pain, dysuria, urinary tract infection and infective stones.[74]

Rubia cordifolia, Aerva lanata, Moringa oleifera and Cystone (polyherbal formulation) maintain crystalloid-colloid balance by decreasing excretion of urinary calcium, oxalate, uric acid, phosphorus and protein in urolithiasis.

Phytoconstituents khellin and visnagin isolated from *Ammi visnaga* fruits are found to be effective against hyperoxaluria. The mechanism of action of *Crataeva nurvala* produce a reduction in glycolate oxidase, with hypermagnesuric effect.[75]

'Pashanabheda' is the Sanskrit term used for a group of plants with diuretic and antiurolithic activities (Pashana = stone; Bheda = break). The term has been indicated in various literature in indigenous system of medicine. During the last two decades extensive research has been carried out to elucidate the chemistry, biological activities, and medicinal application of *Biophytum sensitivum* (*B. sensitivum*). The flower of this plant is considered as one of the ten sacred plants which is called as Dasapushpam in tradition and culture of Kerala state in India.[76] This drug is commonly known as 'Nagbeli,' a folk medicine against 'Madhumeha' (diabetes mellitus), particularly in Eastern Nepal. Tracing the history of traditional practice of *B. sensitivum* reveals its key action; the plant is bitter tonic, expectorant and stimulant in Ayurveda. It has been used as traditional medicine for various ailments. Specifically, the leaves of *B. sensitivum* showed diuretic effect and the powdered form is indicated for urolithiasis. Extract of these bioactive compound have been known to possess various pharmacological activities mainly antioxidant and anti- inflammatory and the phytochemical analysis have shown the presence of beneficial compounds which include bioflavonoids like amentoflavone, cupressoflavone and isorientin. Aforementioned reasons suggests that this plant may have some therapeutic effect as nephroprotective and antiurolithic. Currently, the search for nephroprotective and antiurolithic drug possessing significant antioxidant activities from natural sources has gained immense potential. Hence, in this study an attempt has been made to investigate these activities and this could serve as a source of information on

the present trends in research on plants accredited with nephroprotective and antiurolithic activity.

2. AIM AND OBJECTIVES

In the present scenario, most of the people above sixty years of age, have higher incidence of co-morbidities, and are exposed more to diagnostic or therapeutic procedures having nephrotoxic potential.[77-79] Prolonged exposure of kidney to nephrotoxic drugs, chemical reagents and heavy metals lead to renal injury. Gentamicin and cisplatin, still widely prefered in clinical practice because of its high efficacy, but limits their clinical relevance due to their nephrotoxicity.

Various etiological factors are involved in nephrotoxicty and hence effective treatment mainly focus on drugs acting on multiple targets. However, satisfactory synthetic drugs for the treatment of kindey injury are not available in the market. Currently available allopathic medicines for the treatment of nephrotoxicity and urolithiasis associates with higher incidence of adverse drug reactions. Accumulation of nephrotoxic drug or chemicals at higher concentration in renal tubule may induce apoptosis, necrosis and oxidative stress.[80] Lipid peroxidation mediated by reactive oxygen species has been suggested as a major causative agent of cell death.[81]

Globally, the use of medicinal plants for the prevention and treatment of various diseases is constantly developing owing to their natural origin, low cost, easy accessibility and minimal side effects. Large volumes of preclinical research studies have been carried out on herbal drugs for their claimed activities. World health organisation reported that 80% of the world's population still depends exclusively on traditional medicine for their primary health care, despite the advancement in allopathic medicines.

Early literatures have prescribed many herbal drugs for the treatment of nephrotoxicity and urolithiasis. The curative properties of these medicinal plants are mainly due to the presence of various complex chemical substances aiming at multiple targets such as antispasmodic, anti-inflammatory, diuretics, antibiotics, muscle relaxants, analgesics and anti-oxidant activities. Research studies also confirmed that co-therapy with nephroprotective herbal drugs along with nephrotoxic agent may reduce its toxicity. Traditionally many more herbal plants are claimed to be nephroprotective but there is lack of scientific evidence to support such claims. Previous research works also confirmed that the presence of high content of phenolic and polyphenolic compounds (flavonoids, proanthocyanidins) in medicinal plants preserved renal function.[64, 82] Among polyphenols, phenolic and flavonoids are naturally occuring aromatic compounds which have excellent antioxidant and free radical scavenging activity, thereby reducing the nephrotoxic effect. Plants rich with saponins possess anti-crystallisation properties by disaggregating the suspension of mucoproteins, the promoters of crystallization.[83]

Biophytum senitivum is a well known medicinal plant widely used in traditional Ayurvedic and Siddha systems. This auspicious herbs is also included in the group of "Dasapushpam", an Ayurvedic formulation. Phytochemical investigations of this plant had revealed the presence of large amount of phenolic and poly phenolic compounds, saponins, polysaccharides, pectin and essential oils. The principle bioactive constituents are bioflavonoids like amentoflavone with trace amounts of cupressoflavone, luteolin, isoorientin and isovitexin.[84, 85] Grounded leaves exhibited diuretic effect and powdered form is indicated for urolithiasis.[86] This plant was investigated for various

pharmacological properties which include antidiabetic, anti-inflammatory, hypocholesterolemic, antibacterial, antiulcer, antioxidant, anthelmintic and many more.

However none of the studies have compared the combined nephroprotective, antiurolithic and antioxidant activities with ethanol extract of *B. sensitivum.* Presently, the search for nephroprotective and antiurolithic drug possessing significant antioxidant activities from natural sources has gained immense potential. Hence, this study was designed to investigate nephroprotective activity of *B. sensitivum* on gentamicin and cisplatin-induced nephrotoxicity and antiurolithic activity on ethylene glycol-induced urolithiasis in Wistar albino rats.

The specific objectives of the study were to determine the following:

1. Various phytoconstituents present in the ethanol extract of *B. sensitivum* (EEBS)
2. *In vitro* antioxidant and free radical scavenging activity of EEBS.
3. *In vitro* antiurolithic activity of EEBS
4. *In vitro* cytoprotective activity of EEBS on kidney cell lines
5. Nephroprotective activity of EEBS on gentamicin-induced nephrotoxicity in rats.
6. Nephroprotective activity of EEBS on cisplatin-induced nephrotoxicity in rats
7. Antiurolithic activity of EEBS on ethylene glycol-induced urolithiasis in rats
8. *In vivo* enzymatic and non-enzymatic antioxidant studies in kidney homogenate

3. REVIEW OF LITERATURE

3.1. Plant Profile

3.1.1. Botanical description

Biophytum sensitivum (Linnaeus) DC (*B. sensitivum*), within the Biophytum genus belongs to the Oxalidaceae family and have been used as traditional medicine in Ayurvedic and Siddha system to alleviate various ailments. This plant is considered as one among ten sacred plants, known as 'Dasapushpam' in tradition and culture of Kerala.[76, 87] Life plant, little tree plant are the common name used for this plant. This auspicious herb is also known as sensitive plant and in Sanskrit as 'Lajjaluka,' due to an interesting nastic movement in leaves in response to touch, similar to touch-me- not plant. In Eastern Nepal, this plant is commonly known as "Nagbeli," which is used as a folk medicine for the treatment of "Madhumeha" (Diabetes mellitus). In Ayurveda, powdered form of this drug is indicated for urolithiasis, gonorrheal infection and grounded leaves with water exhibited diuretic activity. Ayurveda also recommends decoction of this plant for amenorrhea and dysmenorrhea. In Siddha system, this plant is advised for diarrhea, cough, epilepsy and wound healing.[88]

3.1.2. Geographical distribution

This genus is commonly found in wetlands, under shades of trees, at low and medium altitude. Most of the species occur in plains of tropical Africa and Asia, mainly Philippines. In Asia, this plant is widely distributed throughout the hotter parts of Nepal, Thailand, Malaysia, Indonesia, Sri Lanka and India.[89] *B. sensitivum* is abundantly seen in South India , especially in Eastern and Western Ghats.[90]

3.1.3. Common names of *Biophytum sensitivum* (Linn.) DC [91, 92]

Language		Name
French	:	*Alleluya*
Bengal	:	Jhalai
Hindi	:	Lajalu, Lajjaalu, Lakshmana, Zarer
Indo-China	:	Chua me
Kannada	:	Haramuni, Jalapushpa
Marathi	:	Jharera, Lajwanti, Lahanmulaka
Sanskrit	:	Jhullipuspa, Lajjaluka, Panktipatra, Pitapushpa
Telugu	:	Attapatti, Chumi, Jala pupa
Malayalam	:	Mukkutti
Tamil	:	Nilaccurunki, Tintaanaalee

3.1.4. Scientific classification of *Biophytum sensitivum*[92]

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Class	:	Magnoliposida
Order	:	Oxalidales
Family	:	Oxalidaceae
Genus	:	*Biophytum*
Species	:	*sensitivum*
Botanical name	:	*Biophytum sensitivum* (L.) DC

Figure 2: Pictorial presentation of *Biophytum sensitiv*um (Linn.) DC

3.1.5. Morphology[93]

This is a slender, erect, annual herb, looks like a miniature palm. Plants can grow upto to 25 cm.

***B. sensitivum* leaves**: The leaves are sensitive, compound, abruptly pinnate, crowded into rosette on top of stem, total length less than 5- 12 cm with short petiole, rachis slender, glabrous or hairy with 6-12 pairs of leaflets. The leaflets are opposite, oblong, obliquely rounded, apiculate at apex, subsessile, glabrous with short petiole, measure upto 4-5 mm and 1.5 cm long, the terminal pair the biggest and the pairs becoming smallest downwards.

***B. sensitivum* flowers:** The flowers are dimorphic, many and crowded, 8 mm across yellow peduncle, length varies up to 10 cm, slender, pubescent or glabrous, pedicles many, bracts lanceolate, crowded beneath the pedicles. Sepals are 5, subulate-lanceolate, striate, about 7 mm long, imbricate, and acute with parallel nerves. Petals are 5, yellow with red marking in the centre of petals, corolla connate into salver shaped exceeding the sepals, lobes rounded, spreading. Stamens are 10, distinct filament free, the five inner ones are longer and styles 5. Fruits are ellipsoidal capsule, apiculate, exceed the sepals. Seeds are ovoid and transversely striate.

***B. sensitivum* stem:** The stems are short**,** unbranched, erect, glabrous or hairy stems, cylindrical, woody, apical part greenish and basal part usually brownish, ligneous when old.

***B. sensitivum* root:** Typical tap root with few root nodules in the lateral branches, hard and woody, cylindrical, external surface brown.

3.1.6. Ethnomedicinal /traditional uses

B. sensitivum is a well known auspicious medicinal plant and the ethno-medicinal values of this plant has been recommended in various indigenous system of medicine. This plant has been used for the treatment of various diseases in Ayurveda and Siddha system.

The whole plant of this plant has been used traditionally in the treatment of urinary calculi, gonorrhea, asthma, pthisis, stomachalgia, chest complaints, hyperdipsia in bilious fever, wound, inflammatory-tumours, burns, cramps, snake bite, stangury and in abcesses.[94] It is also used as bitter tonic, stimulant, thermogenic, expectorant, suppurative, diuretic and lithonotriptic.[92] Leaf extract of this plant has been used as

traditional folk medicine for various diseases, such as diabetes, ashma, pthisis and decoction of leaf for tuberculosis.[95] Leaf extract also applied on wound to arrest bleeding, also act as bitter tonic, antiseptic, astringent, diuretic, styptic, expectorant and stangury.[93] Powdered form of seed is used as antiseptic and also to arrest bleeding, hence used to apply on wounds. Decoction of root of this plant is used in gonorrhea, fever, styptic in pthisis and lithiasis.[94, 95]

3.1.7. Phytochemistry

Major group of phytoconstituents present in *B. sensitivum* include phenolic and polyphenolic compounds, saponin, essential oil, polysaccharides and pectin.[96]

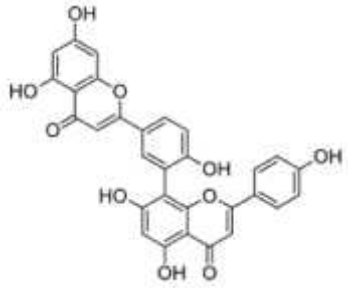
Methanolic extract of the leaves showed the presence of phenolic compounds including isoorientin, orientin, isovitexin, isoorientin-7-O-glucoside, isoorientin-2”-O-rhamnoside.[85]Ariel part of this plant has been reported to contain three flavonoids, luteolin-7-methyl ether, isoorientin and 3'-methoxyluteolin-7-o-glucoside, and two acids, 4-caffeoylquinic acid and 5-caffeoylquinic acid.[84]

Methanolic extract of roots, stems and leaves revealed the presence of two bioflavonoid, amentoflavone which is the main bioactive constituent with small amount of cupressoflavone.[97]

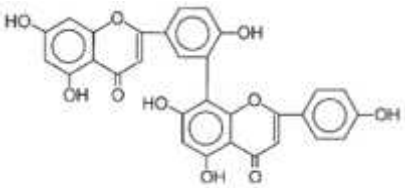
Highest amount of epicatechin and epicatechin-(4β-8)-epicatechin (proanthocynidin B2, condensed form of tannins) were found in roots and leaves possess C-glycosyl flavones.[85] Around 69 compounds were identified in the essential oil of air dried plant. Major constituents are benzene derivatives including 1,4-dimethoxy benzene, 1,2-dimethoxy benzene, 2-methoxy-4-methyl phenol, the monoterpenes (Z)-linalool oxide, (E)-linalool oxide, linalyl acetate , 1-octen-3-ol and isophorone.[86]

Aqueous extract of aerial parts confirmed the presence of a bioactive polysaccharide, BP100 III which is composed of galacturonic acid and rhamnose.[98]

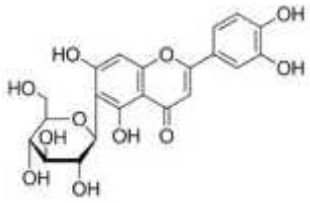
Figure 3: Chemical structures of some of the phytochemicals present in *B. sensitivum*



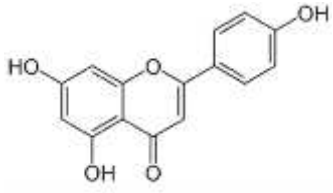
Amentoflavone



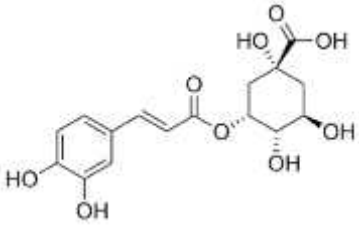
Cupressoflavane



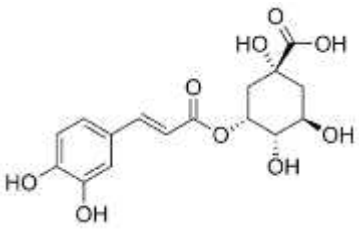
Isoorientin



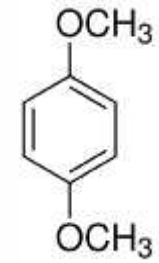
Luteolin 7-methyl ether



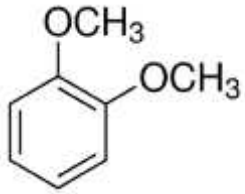
4-Caffeoylquinic acid



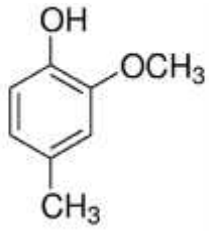
5-Caffeoylquinic acid



1,4-dimethoxy benzene



1,2-dimethoxy benzene



2-methoxy-4-methyl phenol

3.2. Review of published literature on *B. sensitivum*

B. sensitivum is a very popular and a sacred plant in Indian medical system and has been used for the prevention or treatment of wide spectrum of diseases. Scientifically

unproven pharmacological activities of this plant helped researchers to investigate the validity of these traditional claims. This species were investigated for multifarious pharmacological activities including antidiabetic, anti-inflammatory, hypocholesterolemic, antibacterial, antiulcer, antioxidant, anthelmintic, antiangiogenic, chemoprotective, apoptotic, larvicidal, immuno- modulatory and antitumour activities. Some compounds have been isolated and characterized from this plant species.

Inngjerdingen KT *et al*., (2006)[98] isolated polysaccharide fractions, BP100 III, from the aqueous extract of aerial parts of *B. petersianum* [syn. *Biophytum sensitivum* (L.) DC]. This fraction was then degraded by endo-alpha-d-(1-->4)-polygalacturonase and collected three fractions by gel filtration. BP100 III.1, highest molecular weight fraction which consists of galacturonic acid and rhamnose showed dose-dependent complement fixating activity, compared to other two lower molecular weight fractions.

Bucar F *et al*., (1998)[97] isolated amentoflavone from the roots of *B. sensitivum* DC and showed a selective inhibition on cycloxygenase (COX)-1 catalyzed prostaglandin biosynthesis, indicating anti-inflammatory activity of *B. sensitivum*.

Guruvayoorappan C *et al*., (2007)[99] demonstrated antitumour activity of amentoflavone, a biflavonoid from *B. sensitivum.* Intraperitoneal injection of *Ehrlich ascites* carcinoma cells to BALB/c mice resulted in tumour formation and by administration of amentoflavone significantly elevated the natural killer cell activity and antibody-dependent cellular cytotoxicity in tumour bearing animals, compared to untreated tumour-bearing control animals.

Guruvayoorappan C *et al*., (2008)[100] displayed that amentoflavone showed significant reduction in the production of various endogenous factors such as interleukin-1 beta,

interleukin-6, tumour necrosis factor-alpha, granulocyte-macrophage colony-stimulating factor, and vascular endothelial growth factor that control the process of angiogenesis.

3.2.1. Antidiabetic activity of *B. sensitivum*

Puri D *et al.*, (1998)[101] investigated antidiabetic activity of leaf extract of *B. sensitivum* in alloxan induced hyperglycemia in male rabbits which were categorized into three groups based on the severity of disease as sub-diabetic, mild-diabetic and severe-diabetic. Hypoglycaemic activity was assessed based on the improvement in the oral glucose tolerance test and fasting plasma glucose (FPG) lowering effect. FPG lowering was observed in 1 hour and 2.5 hour showing glucose values by 25.9% and 27.4% respectively in the subdiabetic rabbits, and by 36.9% and 37.7% in the mild diabetic rabbits. Improvement in glucose tolerance test (GTT) response was also observed in the subdiabetic rabbits and mild diabetic rabbits. These results support the use of this plant as an antidiabetic agent.

Prabu *et al.*, (2012) [102] investigated the effect of aqueous solution of *B. sensitivum* leaf extract on normal and streptozotocin-nicotinamide-induced diabetic models. Diabetic rats were treated with extract (200 mg/kg) for 28 days. Antidiabetic effect of extract was assessed by evaluating the blood glucose, plasma insulin, total haemoglobin, glycosylated haemoglobin, liver glycogen and carbohydrate metabolism regulating enzymes of liver. The study concluded that this plant possessed significant antidiabetic activity as it showed reduction in blood glucose, glycosylated haemoglobin levels, glucose-6-phosphatase, fructose-1,6-bisphosphatase activities and significant elevation in total haemoglobin, plasma insulin and liver glycogen levels and the hexokinase activity in diabetic rats.

3.2.2. Anti-inflammatory activity of *B. sensitivum*

Chandrasekharan *et al.*, (2008)[103] examined the methanolic extract of *B. sensitivum* for its anti- inflammatory activity and the findings revealed that the plant extract could suppress the inducible nitric oxide synthase, cyclo-oxygenase-2 mRNA expression and production of proinflammatory cytokines in lipopolysaccharide (LPS) or Concanavalin (Con) A-stimulated primary macrophages. Hence this plant may provide beneficial effect for the treatment of endotoxic shock or sepsis.

Jachak SM *et al.*, (1999)[104] assessed the anti-inflammatory activity of aqueous and methanol extracts of aerial parts and aqueous and ultrafiltration fractions of methanol extract of roots of *B. sensitivum* in carrageenan-induced rat paw edema. The test extracts significantly suppressed edema except the methanol extract of aerial parts. This study confirmed that the aqueous extract of *B. sensitivum* exhibited better anti-inflammatory effect compared to methanol extract.

3.2.3. Hypocholesterolemic activity of *B. sensitivum*

Puri D, (2003)[105] demonstrated hypocholesterolemic effect of aqueous extract of leaves of *B. sensitivum* in male albino rabbits. Animals were divided into three groups, containing six animals each, designated as Group I, II, III. Group I served as healthy control, group II of untreated hypercholesterolemic rabbits and group III of hypercholesterolemic rabbits co-treated with the aqueous extract. The result suggested that animals co-administered with the extract lowered the elevated serum triglycerides, very low density lipoprotein plus low density lipoprotein cholesterol and the ratios of total cholesterol to high density lipoprotein cholesterol level.

3.2.4. Antibacterial activity of *B. sensitivum*

Natarajan D *et al*., (2010)[106] demonstrated that the leaves extract of *B. sensitivum* can be used as an anti-infective agent. Antibacterial activity of petroleum ether, chloroform, acetone and methanol extract of *B. sensitivum* were evaluated against human pathogenic bacterial strains (*Bacillus subtillis, Staphylococcus aureus, Streptococcus pneumonia, Klebsiella pneumonia, Salmonella typhi, Proteus vulgaris* and *Escherichia coli*). Antibacterial activity was remarkably higher with acetone extract compared to other extracts. Methanol and chloroform extracts were found to be better than petroleum ether extract.

3.2.5. Anthelmintic activity of *B. sensitivum*

Rajanikant TK *et al*., (2013)[107] evaluated the anthelmintic activity of *B. sensitivum* leaves and stems by extracting with petroleum ether, ethyl acetate and methanol. All the extracts were evaluated for anthelmintic activity with Indian earthworm (*Pheretima posthuma*). This study concluded that the ethyl acetate extract showed excellent anthelmintic activity (at concentration of 25 mg/mL, 50 mg/mL, 100 mg/mL with paralysis time 85.0±1.9, 66.0±1.3, 40.8±1.0 and death time 91.3±1.1, 81.2±1.7, 50.5±0.8 respectively), compared to other extracts and this may be due to presence of bioflavonoids and tannins.

3.2.6. Antioxidant activity of *B. sensitivum*

Guruvayoorappan C *et al*., (2006)[108] investigated *vitro* and *in vivo* antioxidant potential of *B. sensitivum.* The study result suggested that the plant extract scavenged superoxide radicals, hydroxyl radicals, nitric oxide radicals and inhibit lipid peroxidation with an IC_{50} value of 50, 95, 100, and 120 µg/mL respectively. Administration of *B. sensitivum* to

mice significantly inhibited superoxide generation in macrophages and increased the catalase activity, glutathione-S transferase and glutathione reductase levels, thereby clearly indicated its antioxidant potential.

3.2.7. Immunomodulatory and antitumour activity of *B. sensitivum*

Guruvayoorappan C *et al.*, (2007)[109] assessed immunomodulatory and antitumour activity of alcoholic extract of *B. sensitivum*. This study fortifies that extract was found to be cytotoxic at a concentration of 0.1 mg/mL to L929 cells lines and 100% toxic at a concentration of 0.5 mg/mL to Dalton's lymphoma ascites (DLA) and *Ehrlich ascites* carcinoma (EAC) cells. Co-administration of extract (500 µg/dose/animal) to mice induced with DLA cells, inhibited tumour development. Life span of mice bearing *Ehrlich ascites* carcinoma tumours was increased. Moreover, extract significantly ($p<0.001$) lowered the tumour cell glutathione levels, serum gamma glutamyl transpeptidase (GGT) and nitric oxide (NO) levels in ascites tumour bearing animals. The total WBC count, bone marrow cellularity and β esterase positive cells were increased by the administration of *B. sensitivum* extract. This study provided evidences suggesting its immunomodulatory and antitumour potential.

Bhaskar *et al.*, (2010)[110] investigated the possible antitumour effects of aqueous extract of *B. sensitivum* leaves against Dalton's Ascitic lymphoma bearing Swiss albino mice. Study concluded that *B. sensitivum* exhibited significant antitumour activity.

3.2.8. Antiulcer activity of *B. sensitivum*

Sakthivel KM *et al.*, (2014)[111] demonstrated antiulcer activity of methanol extract of aerial parts of *B. sensitivum* in Wistar rats. Ulcerative colitis was induced by intracolonic injection of 3% acetic acid. The study result confirmed the antiulcer activity

of extract (50 mg/kg) as it inhibited colitis by lowering macroscopic score (up to 3.66±0.77) and reducing ($p<0.01$) colonic tumor necrosis factor-α (TNF-α), interleukin-1-β (IL-1β) and IL-6 levels. The expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were lowered in extract treated animals, compared with rats in disease control group. Significant reduction ($p<0.01$) in lactate dehydrogenase, myeloperoxidase activities and lipid peroxidation (LPO) were also observed.

Anindya B *et al.*, (2014)[112] communicated the anti-ulcer profile of ethanolic extract of leaves of *B. sensitivum* in aspirin induced peptic ulcer model. This study offered anti-ulcer activity in comparison with the rats treated with standard drug Omeprazole.

3.2.9. Anti-angiogenic activity of *B. sensitivum*

Guruvayoorappan C *et al.*, (2007)[113] investigated antiangiogenic effect of *B. sensitivum* using B16-F10 melanoma cell-induced capillary formation in C57BL/6 mice. Intraperitoneal administration of the extract at a concentration of 50 mg/kg controlled the production of proinflammatory cytokines such as interleukin-1 beta, interleukin-6, tumor necrosis factor-α, granulocyte macrophage-colony stimulating factor and vascular endothelial growth factor involved in angiogenesis, thereby indicated its anti-angiogenic activity.

3.2.10. Chemoprotective activity of *B. sensitivum*

Guruvayoorappan C *et al.*, (2006)[114] demonstrated that alcoholic extract of *B. sensitivum* could diminish cyclophosphamide induced intestinal damage in Swiss albino mice. This study result showed significant improvement in relative organ weight of the spleen and thymus and elevation of total WBC count, bone marrow cellularity and α-esterase positive cells in animals treated with extract and cyclophosphamide, in

comparison with control mice treated with cyclophosphamide alone. Administration of *B. sensitivum* extract also reduced pro-inflammatory cytokine, tumor necrosis factor-alpha and elevated cytokines interferone-gamma, interleukin-2 and granulocyte macrophage-colony stimulating factor.

3.2.11. Cytotoxicity and larvicidal potential of *B. sensitivum*

Johnson M *et al*., (2015) [115] assessed cytotoxicity, larvicidal, and antioxidant activity of various extract of *B. sensitivum*. Total phenolics were maximum in methanolic extracts of *B. sensitivum* (1399.84 ± 215.79 mg of gallic acid equivalent/g) and showed strongest phosphomolybdenum reduction (202.24 ± 11.44 g of ascorbic acid/100 g). Excellent DPPH radical scavenging activity was observed in acetone extracts (IC_{50} value 30.12 µg/mL). The methanolic extracts of *B. sensitivum* exhibited highest larval mortality against *Culex quinquefasciatus* with LC_{50}= 215.34 mg/mL and brine shrimp nauplii with LC_{90}= 66.34 mg/mL. This study report confirmed that extracts showed good antioxidant, larvicidal and cytotoxic activity.

3.2.12. Wound healing activity of *B. sensitivum*

Saritha1 B *et al*., (2015) [116] investigated the wound healing potentials of methanolic extracts of *B. sensitivum* at dose level of 1g and 2g in excision wound models. The wound treated with plant extract demonstrated better wound contraction, elevated level of hydroxy proline, hexosamine content, superoxide dismutase and diminished lipid peroxidation. On 15th day, complete wound healing activity was observed in animals treated with 2g of plant extract.

3.2.13. Apoptotic effect of *B. sensitivum*

Guruvayoorappan C *et al.*, (2007)[117] have evaluated apoptotic effect and regulatory effect of *B. sensitivum* on nitric oxide and cytokine production in B16F-10 cells, tumour-associated macrophages, and peritoneal macrophages. This result suggested that extract induced apoptosis in B16F-10 melanoma cells by reducing the production of proinflammatory cytokines.

3.2.14. Radioprotective activity of *B. sensitivum*

Guruvayoorappan C *et al.*, (2008)[118] demonstrated radioprotective effect of methanolic extract of *B. sensitivum in* mice models. Animals were pretreated with *B. sensitivum* (50 mg/kg body wt.), before exposing whole body to gamma irradiation and observed protection against radiation-induced hemopoietic damage and free radical scavenging activity.

3.2.15. Antiurolithic activity of *B. sensitivum*

Anil TP *et al.*, (2015)[119] reported the antiurolithic activity of standardized methanolic extract of whole plant of *B. sensitivum* in rats. Kidney stone formation was induced by surgical implantations of zinc disc in the urinary bladders of rats, followed by the administration of various doses (100, 200, and 400 mg/kg; *p.o.)* of plant extract for a period of 7 days. Stone size and various biomarker levels were estimated in urine and serum. Extract administration caused significant improvement in glomerular filtration rate and also altered the elevated levels of nitrogenous waste product, indicating its antiurolithic activity.

Anil TP *et al.*, (2016) [120] also evaluated antiurolithic activity of some fractions of methanolic extract of whole plant of *B. sensitivum*, by extracting with dichloromethane,

ethyl acetate, ethanol and water. Rats were treated with 20 & 40 mg/kg. *p.o*. of the fractions and after 1 hr urolithiasis was induced by injecting sodium oxalate (70 mg/kg, *i.p.*) for 7 days. Histological changes and various biochemical changes in urine, serum and kidney homogenate were observed. Ethyl acetate fraction exhibited significant antiurolithic activity compared to other two fractions.

Anil TP *et al.*, (2015)[121] also investigated methanolic extract of whole plant of *B. sensitivum* at three dose level (100, 200, 400 mg/kg) against calcium oxalate induced urolithiasis in rats. The study assessed antiurolithic c activity by evaluating histological and biochemical changes in urine, serum and kidney and the result confirmed that the extract attenuated drug induced urolithiasis.

3.2.16. Nephroprotective activity of *B. sensitivum*

Sachin C *et al.*, (2017)[122] evaluated nephroprotective activity of whole plant *B. sensitivum* by extracting with dichloromethane, ethyl acetate, ethanol and water. Rats were treated with gentamicin (40mg/kg. *i.p*) for seven days. Serum level of urea and creatinine were determined. Study concluded that this medicinal plant showed excellent nephroprotective activity.

4. MATERIALS AND METHODS

4.1. Phytochemical studies

4.1.1. Collection of plant materials and authentication

Well grown whole plant, *Biophytum sensitivum* were collected between the months of December 2015 and February 2016 from Shevaroy Hills, Salem District, Tamil Nadu. The plant was taxonomically identified and authenticated by Dr. A. Balasubramanian, Executive Director, ABS Botanical conservation, Research and Training Centre, Kaaripatti, Salem. The authentication number is T.N. (Ref. No.-AUT/JKK/095).

4.1.2. Preparation of crude ethanol extract of *Biophytum sensitivum* (EEBS)

After authentication, the collected whole plants of *B. sensitivum* were washed and dried in shadow for about three weeks. Dried plants were crushed in to coarse powder, passed through pharmaceutical sieve No. 40 and stored in air tight container at room temperature. For defatting the drug, 500 gms of powdered plant material was sequentially extracted with 2L of petroleum ether using Soxhlet apparatus for 36 hrs at a temperature of 60-80°C with occasional shaking and filtered. The marc was again dried in hot air oven below 50°C and extracted using 70% v/v ethanol for 72 hrs at a temperature of 50°C using Soxhlation method. The extracted solvents were concentrated by vacuum distillation and evaporated to dryness in a water bath. After weighing, the final extract obtained from this procedure were stored in air tight container and kept in refrigerator for further phytochemical and pharmacological studies.[123]

4.1.3. Qualitative analysis of phytochemicals present in whole plant *B. sensitivum*

Crude EEBS was subjected to various qualitative tests for screening the presence of phytochemical constituents. The extract was analyzed for carbohydrate, alkaloids, steroids, saponins, proteins, aminoacids, flavonoids, glycosides, terpenes, tannins and phenolic compounds according to the standard testing procedures.[93, 124]

4.1.3.1. Tests for carbohydrates and reducing sugars

Small quantity of extract (about 50 mg) was dissolved in 5 mL of distilled water. After filtration the solution was subjected to the following procedures.

a. Molisch's test (General test): Two mL of the filtrate was subjected to Molisch's test by adding three drops of alcoholic solution of alpha napthol, shaken well and added 1 mL of concentrated sulphuric acid through the sides of test tube. Formation of violet colour ring at the junction of two liquids indicates the presence of carbohydrates.

b. Fehling's test: Boiled 1 mL of Fehling's solution A and Fehling's solution B taken in test tube for 1 min. Added I mL of the filtrate and heated the solution further for 5-10 mins by keeping it in boiling water. Formation of yellow colour, followed by brick red precipitate indicates the presence of reducing sugar.

c. Benedict's test: Mixed thoroughly 5 mL of Benedict's reagent and 1 mL of the filtrate in a test tube and heated in a boiling water bath for 2 mins. Appearance of green or yellow colour, followed by the formation of red precipitate upon cooling indicates the presence of reducing sugar.

d. Barfoed's test: Mixed thoroughly equal volume (2 mL) of Barfoed's reagent and the filtrate in a boiling water bath and heated for 2 mins. Formation of red precipitate after cooling indicates the presence of monosaccharides.

e. Tollen's test: Added one mL of the filtrate to 2 mL of Tollen's reagent and boiled. Formation of silver colour on the inner walls of the test tube indicates the presence of aldose sugar.

f. Seliwanoff's test: About one mL of the filtrate was heated with 1 mL of hydrochloric acid and resorcinol. Formation of red colour indicates the presence of glucose.

g. Bromine water test: Small quantity of the filtrate was mixed with bromine water. Decolorization indicates the presence of aldose sugar.

4.1.3.2. Tests for alkaloids

A small portion of the solvent free extract (50 mg) was mixed with few drops of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents such as:

a. Mayer's reagent: One mL of the filtrate was treated with 1 mL of Mayer's reagent (potassium mercuric iodide solution). Appearance of cream colour precipitate indicates the presence of alkaloids.

b. Dragendorff's reagent: One mL of the filtrate was treated with 1 mL of Dragendroff's reagent (potassium bismuth iodide solution). Appearance of reddish brown or orange precipitate indicates the presence of alkaloids.

c. Hager's reagents: One mL of the filtrate was treated with 3 mL of Hager's reagent (aqueous solution of picric acid). Appearance of yellow colour precipitate indicates the presence of alkaloids.

d. Wagner's reagent: One mL of the filtrate was mixed with 1 mL of Wagner's reagent (iodine and potassium iodide solution). Appearance of reddish brown colour precipitate indicates the presence of alkaloids.

e. Tannic acid test: One mL of filtrate was mixed with 1 mL of 10% tannic acid solution. Appearance of buff colour indicates the presence of alkaloids.

4.1.3.3. Tests for saponins

a. Foam test: Small amount of the extract was shaken vigorously with 20 mL distilled water in a graduated cylinder for 15 mins. Formation of foam layer (about 1 cm) indicates the presence of saponins.

b. **Hemolytic test:** Few drops of the extract solution was mixed thoroughly with a drop of blood placed on glass slide. Formation of hemolytic zone indicates the presence of saponins.

c. Lead test: One mL of the filtrate was mixed with 1% lead acetate solution. Appearance of white precipitate indicates the formation of saponins.

4.1.3.4. Tests for flavonoids

Small quantity of the extract was shaken with few mL of water and the resulting mixture was subjected to the following tests:

a. Shinoda's test: A small quantity of test sample was dissolved in 5 mL of alcohol (95%) and treated with 2-3 pieces (0.5 gm) of magnesium metal. Development of pink colour within two mins after the addition of few drops of concentrated hydrochloric acid indicates the presence of flavonoids.

b. Alkaline reagent test: Small quantity of the filtrate was mixed with sodium hydroxide. Appearance of yellow colour indicates the presence of flavonoids.

c. **Lead acetate test:** Small quantity of the filtrate was treated with lead acetate solution. Appearance of yellow colour which further decolorized on addition of acid indicates the presence of flavonoids.

d. Sulphuric acid test: Appearance of yellow colour on treating an aliquot quantity of crude extract with concentrated sulphuric acid indicates the presence of flavonoids.

4.1.3.5. Tests for phenolic compounds

a. Ferric chloride test: Dissolved about 50 mg of extract in distilled water and added 3-4 drops of neutral 5% ferric chloride solution. Appearance of green colour indicates the presence of phenols.

b. Lead acetate test: Small quantity of extract dissolved in distilled water was mixed with 3 mL of 10% lead acetate solution. Appearance of white precipitate indicates the presence of phenols.

4.1.3.6. Tests for tannins

a. Ferric chloride test: Dissolved about 50 mg of extract in distilled water and to this added 3-4 drops of neutral 5% ferric chloride solution. Appearance of bluish black colour which disappears on addition of few drops of concentrated sulphuric acid without the formation of yellow or brown precipitate indicates the presence of tannins.

b. Gelatin test: One mL of the filtrate was treated with 1% gelatin solution containing 10% sodium chloride. Appearance of cream precipitate indicates the presence of tannins.

c. Lead acetate test: Small amount of the filtrate was treated with 10% lead acetate solution. Appearance of white precipitate indicates the presence of tannins.

4.1.3.7. Tests for glycosides

a. Keller-killiani test: An aliquot quantity of extract was dissolved in 2 mL of ethanol and 0.5 mL of strong lead acetate solution. Filtered and then the filtrate was further mixed thoroughly with chloroform. Chloroform layer was separated and solvent was evaporated. Residue was dissolved in 2 mL of glacial acetic acid and few drops of 5%

ferric chloride solution were added. This solution was then transferred to a test tube containing concentrated sulphuric acid. Formation of red ring at the junction of two liquids indicates the presence of glycosides.

b. Baljet's test: One mL of the filtrate was mixed with 1 mL of sodium picrate solution. Yellow colour changing to orange colour indicates the presence of glycosides.

c. Legal's test: A small portion of the extract was hydrolyzed with dilute hydrochloric acid for few minutes on a heating water bath. To the hydrolysate, 1 mL of pyridine and few drops of sodium nitroprusside solution were added and then made alkaline with sodium hydroxide solution. Appearance of pink to red colour indicates the presence of cardenolide glycosides.

d. Borntrager's test: Boiled 3 mL of extract with 2 mL dilute sulphuric acid and filtered. To the filtrate added equal volume of chloroform. Shaken well and the chloroform layer was separated. To this added equal quantity of dilute ammonia solution. Appearance of rose pink colour in the ammoniacal layer indicates the presence of anthraquinone glycoside.

4.1.3.8. Tests for steroids

a. Libermann Burchard test: Two mL of the extract was treated with chloroform solution and 2 mL of acetic anhydride. Mixed well and then added two drops of concentrated sulphuric acid through the sides of the test tube. Appearance of bluish green color indicates the presence of steroids.

b. Salkowski test: Two mL of the extract was dissolved in 2 mL of chloroform solution and the resulting solution was then shaken with 2 mL of concentrated sulphuric acid.

Appearance of red colour in chloroform layer and greenish yellow flouresence in acid layer indicates the presence of phytosterol.

4.1.3.9. Tests for proteins and amino acids

Small quantity of the extract was shaken with few mL of water and the resulting mixture was subjected to the following tests:

a. Biuret test: Two mL of test sample was treated with 3 drops of 4% of sodium hydroxide solution and few drops of 1% copper sulphate solution. Appearance of pink to purple colour indicates the presence of proteins.

b. Ninhydrin test: Two mL of test sample was heated with 3 drops of 5% of ninhydrin solution in boiling water bath for 10 mins. Appearance of violet, blue or purple colour indicates the presence of proteins or amino acid.

c. Xanthoprotein test: One mL of concentrated nitric acid was added to 2 mL of test sample. After heating and cooling, sodium hydroxide (40% w/v) was added to make the solution alkaline. Formation of white precipitate initially, then on boiling changed to yellow and then to orange colour indicates the presence of amino acids.

d. Millon's test: One mL of the test sample was heated with 3 drops of Millon's reagent (mercuric nitrate solution), appearance of pink or dark red colour indicates the presence of protein.

e. Lead acetate test: Three mL of the filtrate was mixed with 10% lead acetate and 40% sodium hydroxide solution. Formation of black precipitate indicates the presence of amino acids.

4.1.3.10. Tests for terpenoids

a. Hirschonn reaction: Two mL of the filtrate was added with 2 mL of tetra nitro methane. Appearance of a yellow color indicates the presence of triterpenoids.

b. Knoller's test: To a small quantity of the extract taken in a dry test tube, added few tin granules and 1 mL of thionyl chloride and shaken well. Appearance of a pink color indicates the presence of triterpenoids.

4.1.3.11. Tests for fixed oil and fat

a. Spot test: Small quantity of the extract was pressed between two filter papers. Appearance of oil stains on the filter paper indicates the presence of fixed oil.

b. Saponification test: Few drops of 0.5 N alcoholic potassium hydroxide were mixed with 2 mL of the extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hrs. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

c. Solubility test: Oils are soluble in benzene, ether and chloroform but insoluble in water and 90% ethanol.

4.2. Quantitative estimation of total phenolic and flavonoid content of EEBS

After phytochemical screening, EEBS was subjected to quantitative analyses of total phenolic content and flavonoids.

4.2.1. Estimation of total phenolic content[125]

Total phenolic content was estimated by calibration curve method using gallic acid (standard), Folin- Ciocalteu reagent and 20% sodium carbonate solution. 1mL of crude extract was mixed with 1mL of 10% Folin-Ciocalteu reagent. After keeping it for 3 mins in darkness, 1mL of sodium carbonate (7.5% w/v) was added and adjusted to 10

mL with distilled water. Incubated for 90 mins in darkness . Absorbance was measured at 725 nm by using Shimadzu UV-1700 spectrophotometer. Standard calibration curve of absorbance against gallic acid concentration was prepared and used for estimation of total phenolic content in test sample. Assays were carried out in triplicate and the results were mean values ± standard deviation, expressed as gallic acid equivalents.

4.2.2. Estimation of total flavonoid content[126]

Flavonoid content present in EEBS were evaluated according to spectrophotometric method. Test sample and rutin (standard) were prepared with the methanol to make a final concentration of 100 mg/mL. One mL of test solution was treated with 1 mL of 2% aluminium chloride in ethanol, taken in a 10 mL volumetric flask and volumes were made up to 10 mL with methanol. After keeping this solution for an hr at room temperature, absorbance was measured at 415 nm using spectrophotometer. Assays were conducted in triplicate and the mean value of absorbance were obtained. Calibration curve was constructed using standard solution of rutin by following the same assay procedure. Based on the measured absorbance the concentration of flavonoid was estimated from the calibration line. The content of flavonoids in extract were expressed in terms of rutin equivalents.

4.3. Assessment of *in vitro* antioxidant activity of EEBS

4.3.1. DPPH (1,1-Diphenyl-2-picryl hydrazine) radical scavenging activity

DPPH free radical scavenging activity was evaluated using the method described by Blois.[127] Various concentrations of EEBS and ascorbic acid (standard) ranging (10-100 µg/mL) were mixed with 1 mL of freshly prepared 0.3 mM DPPH ethanol solution and 2 mL of 0.1M acetate buffer. Incubated at room temperature for 30 mins and the

Absorbance of resulting solutions were then measured colorimetrically at 517 nm. DPPH solution (1.0 mL, 0.3 mM) treated with 1mL ethanol, served as negative control. Ascorbic acid was used as positive control under the same assay condition. Samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The antioxidant activity of the extract was expressed as IC_{50}. Higher absorbance indicates lower free radical scavenging activity. The percentage of DPPH radical scavenging activity of extract was calculated from decrease in absorbance in comparison with control by using formula:

$$\text{Percentage inhibition (\%)} = [(Abs_{control} - Abs_{test}) / Abs_{control}] \text{ X } 100$$

4.3.2. Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity of EEBS was determined using method described by Benzie and Strain.[128] 0.5 mL of EEBS at different concentration (10-100 µg/mL) was mixed with 900 µL of FRAP reagent, incubated for 6 mins at room temperature and the absorbance was measured at 593 nm. Methanol solution of ferrous sulphate ranging from 100 to 2000 μM were prepared and used for the preparation of the calibration curve of known Fe^{2+} concentration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol ferrous sulphate equivalents per gram of sample. Ascorbic acid at various concentrations were used as standard. Increased absorbance of reaction mixture indicates increase in reducing power.

4.3.3. ABTS [2, 2'-azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) diammonium salt] radical scavenging assay

Total antioxidant potential of extract was determined based on the procedure described by Re *et al.*[129] 5 mL of 4.9 mM ammonium per sulphate solution was mixed with 5 mL of 14 mM ABTS stock solution and kept for 16 hrs in dark at room temperature that resulted in the formation of fresh ABTS radical. The resultant $ABTS^{*+}$ solution was diluted with ethanol (99.5%) until an absorbance of 0.70 ± 0.02 at 734 nm was obtained. One mL of different concentration of EEBS or standard ascorbic acid (10-100 μg/mL) were allowed to react with 900 μL of ABTS radical solution. Reaction mixture was vortexed for 10 secs and after 6 mins, the absorbance was recorded at 734 nm. Negative control was prepared without adding extract or standard. Mean values were obtained from triplicate analysis. The extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

4.3.4. Hydroxyl radical scavenging activity

Scavenging activity of the extract on hydroxyl radical was determined according to the 2-deoxyribose assay reported by Halliwell and Gutteridge. [130] Hydroxyl radical was generated from Fe^{3+} -ascorbate, EDTA-hydrogen peroxide system. 0.4 mL of EEBS at concentration ranging (10-100 μg/mL) were treated with 2-deoxy-D- ribose (1.4 mM), ferric chloride (100 μM), EDTA (1.04 mM) and hydrogen peroxide (1 mM). Volume was made upto 1.6 mL using potassium phosphate buffer (10 mM, pH 7.4). Incubated the solution for 10 mins at 37ºC. 0.4 mL of 0.2 mM phenyl hydrazine hydrochloride was added, incubated for 1hr and then 1 mL each of 2.8% tricarboxylic acid (TCA) and

thiobarbituric acid (0.5% TBA in 0.025 M sodium hydroxide containing 0.02% butylated hydroxyanisole) were added to 0.5 mL of the reaction mixture. Reaction mixture was then heated in a water bath at 95ºC for 15 mins. The reaction mixture was centrifuged at 5000 rpm for 15 mins, after cooling by keeping the mixture on ice. Absorbance of the supernatant was measured at 532 nm. The hydroxyl radical scavenging capacity of the extract was compared with that of ascorbic acid which was used as positive control under the same assay condition. Negative control was prepared without adding extract or standard and was considered as 100% deoxy oxidation. The percentage hydroxyl radical scavenging activity of extract was determined in terms of IC_{50} value, in comparison with negative control.

4.3.5. Superoxide radical scavenging activity

Super oxide anion radical scavenging assay was determined according to the method described by Nishimiki *et al*.,[131] with slight modification. 0.1 mL of EEBS/standard at different concentrations (10-100 µg/mL) were mixed with 1 mL of 156 µM nitroblue tetrazolium (NBT) solution in phosphate buffer (100 mM, pH 7.4) and 1 mL 468 µM nicotinamide adenine dineucleotide hydrogen (NADH) in phosphate buffer (100 mM, pH 7.4). The reaction was started by adding 100 mL of 60 mM phenazine methosulphate (PMS) in phosphate buffer (100 mM, pH 7.4), then incubated the reaction mixture at 25ºC for 5 mins and the absorbance was measured at 560 nm against the standard solution (ascorbic acid). Radical scavenging activity of the extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting inhibition percentage versus extract concentration.

4.3.6. Nitric oxide radical scavenging activity

This assay was done according to the method of Garatt *et al* .[132] Griess Ilosovy reagent was modified by using naphthyl ethylenediamine dihydrochloride (0.1% w/v) instead of the use of 1-naphthyl amine (5%). 2mL of 10 mM sodium nitroprusside prepared in 0.5 mM phosphate buffer saline (pH 7.4) was added to 0.5 mL of sample at various concentration (10-100 µg/mL). The solution was incubated at 25ºC for 2.5 hrs. Then 1.5 mL of mixture was mixed with 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid, 0.1% of naphthyl ethylenediamine di-hydrochloride) and incubated at room temperature for 5 mins. The absorbance was read at 546 nm. Ascorbic acid was taken as standard. Percentage inhibition was calculated.

4.3.7. Reducing power activity

The reducing power activity was estimated according to the method of Oyaizu.[133] 2.5 mL of various concentrations of EEBS (10-100 µg/mL) were mixed with sodium phosphate buffer (2.5 mL, pH 6.6) and 1% potassium ferricyanide (2.5 mL). Incubated at 50ºC for 20 mins and 2.5 mL of 10% trichloroacetic acid (w/v) was added and then centrifuged for 10 mins. The upper layer of solution 5 mL was treated with 5 mL deionized water and 1mL of 0.1% ferric chloride.. Reducing ability of extract was compared with that of ascorbic acid which was used as positive control. A blank was prepared without adding extract or standard. Absorbance was measured at 700 nm. Assays were carried out in triplicate and the results were expressed as mean values ± SD. Increased absorbance of reaction mixture indicates increase in reducing power.

4.4. Assessment of *in vitro* antiurolithic activity of EEBS

Chemicals: Calcium chloride dihydrate, Tris-buffer, Sodium oxalate (Sisco Research Laboratories Pvt. Ltd., Mumbai, India).

Stock calcium chloride and sodium oxalate solutions (respectively $20x10^{-3}$M and $1.0x10^{-3}$M) were buffered at pH 5.5 with 9 mM sodium di-methyl arsinate and brought to an ionic strength of 0.15 M.

4.4.1. Nucleation assay

Inhibition capacity of plant extract on calcium oxalate (CaOx) crystallization was determined according to the classical method described by Hennequin *et al.*[134] 1 mL of 0.025 M calcium chloride solution, 2 mL of 0.05 mol/L Tris-buffer, 1 mL of EEBS/ standard compound cystone at various concentrations (10-100 mg/mL) were added to test tube and then 1 mL of 0.025 M sodium oxalate was added. Solution was transferred to a beaker and then constantly stirred at room temperature. Procedure was repeated for six duplicates for each sample. The rate of nucleation was determined by comparing appearance of crystals that reached critical or optically detectable size in the presence of extract and that of control with no extract. [135] The absorbance was recorded at 620 nm and the percentage inhibition was calculated by using the formula:

$$\text{Percentage inhibition (\%)} = \text{Abs}_{\text{test}} / \text{Abs}_{\text{control}} \times 100$$

4.4.2. Aggregation assay

Aggregation of CaOx crystals was determined by following the method of Atmani *et al.*[136] The CaOx crystals were prepared by mixing 1 mL of 0.025 M calcium chloride and 1mL of 0.025 M sodium oxalate. Both solutions were then equilibrated at 60ºC in a water bath for 1 hr and then cooled to 37ºC overnight. The formed crystals were

harvested by centrifugation for 5 mins and then evaporated at 37ºC. The crystals were used at final concentration of 0.8 mg/mL, buffered with Tris-hydrochloride 0.05 mol/L and sodium chloride 0.15 mol/L at pH 6.5. Experiments were conducted at 37ºC by adding 1mL of EEBS or cystone (standard) at various concentrations (10-100 mg/mL). The rate of aggregation was estimated by comparing turbidity in presence of EEBS/standard with that of control. The absorbance at 620 nm was recorded spectrophotometrically. The percentage inhibition rate (Ir) was calculated by the following formula:[69]

$$\text{Percentage inhibition (Ir)} = 1 - (\text{Turbidity}_{\text{test}} / \text{Turbidity}_{\text{control}}) \times 100$$

4.4.3. Image analysis of calcium oxalate (CaOx) crystal morphology (Microscopic assay)

Incubation of metastable solutions of calcium chlorie and sodium oxalate resulted in the formation of CaOx crystals. The harvested crystals were centrifuged and placed on a petriplate glass slide. Various concentration of EEBS (20, 40, 80, 160 µg/mL) and control were then applied directly to the crystals. Change in structure of CaOx crystals were compared with the control by observing under microscope after 30 mins to determine how crystals were dissolved by extract. Crystal size was observed under Leica stereo zoom dissecting microscope with digital imaging system at 4X and the photographs were taken.[135]

4.5. *In vitro* pharmacological studies

4.5.1. Assessment of cytotoxic activity of EEBS on kidney cell lines

Therapeutic usefulness of plant drugs for various diseases were normally based on accidental discovery. Hence cytotoxicity testing with herbal extracts are vital for the

safety evaluation. Prior to *in vivo* pharmacologic studies, cell viability or cell toxicity of EEBS were determined by Trypan blue dye exclusion aasay and MTT assay method. Lactate dehydrogenase (cytosolic enzyme) levels were also determined which is an important indicator of cellular cytotoxicity. Additionally, DNA fragmentation assay was also done for further evaluating the nature of cell death.

The percentage cell viability of EEBS was determined on human embryonic kidney 293 (HEK 293) cell line by using Trypan blue dye exclusion and MTT assay method.

Chemicals: Trypan blue, 3-(4, 5 dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate buffered saline (PBS), bovine serum albumin (BSA), Dulbecco's modified eagle's medium (DMEM), Trypsin (Sigma Aldrich Co., St Louis, USA) ethylenediaminetetraacetic acid (EDTA), antibiotics, isopropanol and dimethyl sulphoxide (DMSO)

Cell line

The human embryonic kidney cell line (HEK 293) was obtained from National Centre for Cell Science (NCCS), Pune and were grown in DMEM containing 10% inactivated FBS, penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (5 µg/mL). The cells were maintained at 37ºC, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly and the culture medium was changed twice a week. Stocks were maintained in 25-cm^2 tissue culture flasks at 37ºC in 5% CO_2 incubator. Each experimental culture was performed with a culture density of 1×10^6 cells in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of test solutions

For cytotoxicity studies, different concentrations of EEBS (12.5, 25, 50,100, 200 μg/mL) were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/mL concentration and sterilized by filtration. A serial of two fold dilution of different concentration of plant extracts were prepared and added to the culture medium. All preparations were sterilized by passing through the membrane filter 0.45 μm (Schleicher & Schuell, Germany).

4.5.1.1. Determination of cell viability by trypan blue exclusion assay [137, 138]

Trypan blue is an essential blue acid dye, consist of two azo chromophore groups used in determining viability of cells. For the determination of cell viability, HEK 293 cells were plated at a density of (1×10^6 cells/well) in 96 well tissue culture plates and cultured for 48 hrs at 37ºC. Cells were then treated with various concentrations of EEBS (12.5, 25, 50, 100 and 200 μg/mL) for 48 hrs. The cultures were harvested, washed twice with PBS and then cell pellets were suspended in 0.5 mL PBS. 50 μL of cell suspension was mixed thoroughly with an equal volume of 0.4% trypan blue in sterile microfuge tube. Washed hemacytometer and coverslip with 70% isopropanol, dried and transferred 10 μL mixture of the cell culture/trypan blue mixture from microfuge tube using micropipette into a notch of the haemacytometer and covered it with a cover slip. Placed the haemocytometer on the stage of an inverted microscope. Focused light on the haemocytometer grid using 100X magnification and observed live cells which appeared as clear. The medium without samples were served as control and triplicate was

maintained for all concentrations. Calculated the percentage of cell viability by using following formula:

Percentage cell viability (%) = Viable cell count / Total cell count X 100

4.5.1.2. Determination of cell viability by MTT assay[139-141]

Procedure

The monolayer cells were detached with Trypsin phosphate Versene Glucose (TPVG) solution (0.1% trypsin, 0.02% EDTA, 0.05% glucose in PBS) to make single cell suspensions (HEK 293 cells) and one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well. Incubated for cell attachment at 37º C, 5% CO_2, 95% air and 100% relative humidity. Aliquots of 100 µL of serial concentrations of the test sample (12.5, 25, 50, 100, 200 µg/mL), which was initially dissolved in dimethyl sulfoxide were added to the appropriate wells already containing 100 µL of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 24 hrs at 37ºC, 5% CO_2, 95% air and 100% relative humidity. The viable cells were counted using hemocytometer. The medium containing no samples were served as control and triplicate were maintained for all concentrations. After 48 hrs of incubation, 15 µL of MTT (5 mg/mL) in phosphate buffered saline (PBS) were added to each well and incubated at 37ºC for 4 hrs. The plates were wrapped in aluminum foil**.** The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µL of DMSO and absorbance was measured at 570 nm using micro plate reader. The percentage cell growth was then calculated with respect to control.

Percentage cell growth (%) = $Abs_{test} / Abs_{control}$ X 100

4.5.1.3. Determination of cytotoxicity of EEBS by lactate dehydrogenase (LDH) assay

[LDH, EC 1.1.1.27]

Chemicals: Sodium pyruvate, NADH, foetal bovine serum, RPMI (Roswell Park Memorial Institute medium), penicillin (100 IU/mL), streptomycin (100 μg/mL).

Cell lines and culture medium

The human kidney adenocarcinoma cell line (A498) was obtained from National Centre for Cell Science (NCCS), Pune. Cell culture A498 cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum and penicillin (100 IU/mL), streptomycin (100 μg/mL). Cultures were maintained in a humidified incubator containing 21% O_2 and 5% CO_2 in air.

Procedure

Lactate dehydrogenase assay was carried out according to the method of Wroblewski and Ladue.[142] The LDH leakage assay is a simple reliable and fast cytotoxicity assay based on the measurement of lactate dehydrogenase activity in the extracellular medium. A498 cells (1×10^6 cells/well) were plated in 100 μL of medium/well in 96-well plates and were allowed to attach to the plate for 24 hrs. After cell attachment, cells were treated with various concentrations of EEBS (5, 10, 20, 40, 80,160, 320 μg/mL). Following treatment, the culture medium was aspirated and centrifuged at 3000 rpm for 5 mins in order to obtain a cell free supernatant. The activity of LDH in the medium was determined by adding 100 μL of cell free supernatant of the control and EEBS treated A 498 cells to 1-mL cuvette containing 0.9 mL of the reaction mixture of pyruvate, 0.15 mmol/L NADH and 104 mmol/L phosphate buffer (pH 7.4).

After thorough mixing, the absorbance of the solution was measured at 340 nm. LDH activity was expressed as μ moles of NADH used per minute per well. All experiments were repeated for three duplicates for each sample.

LDH activity (U/L) = (Abs.@ 340 nm/min) X 16030.

4.5.1.4. Determination of nature of cell death by DNA fragmentation assay[143]

Chemicals: Lysis buffer, Tris-EDTA (TE) buffer, Trypsin, ethanol, Sodium acetate, Ribo- nuclease A (RNase A), Isopropanol

Procedure

A498 cells were seeded at a concentration of 10^6 per 35 mm dish incubated at 37^oC/ 5 % CO_2 for 24 hrs. The confluent cells grown after 24 hrs of incubation were treated with EEBS extract at 160 and 320 μg/mL. After treatment, cells were trypsinized and both adherent and floating cells were collected by centrifugation at 2000 rpm for 5 mins. The cell pellet was suspended in 0.5 mL lysis buffer [Tris-HCl 10 mM, pH 8; EDTA 20 mM, pH 8.0; Triton X-100 (0.2%), 4M NaCl], vortexed vigorously and incubated at 50°C for 5 mins. To the lysate, 0.5 mL of phenol-chloroform-isoamylalcohol were added and mixed for 2-3 mins. It was centrifuged at 10000 rpm for 15 mins at 4^oC. The upper aqueous layer was taken in a tube, to which double the volume of cold 100% ethanol and 3M sodium acetate were added and incubated for 5-10 mins at room temperature. The supernatant was removed, the DNA pellet was air dried and was finally dissolved in TE buffer (Tris-HCl 10 mM, pH 7.4, EDTA 1 mM, pH 8.0), and separated by 2% agarose gel electrophoresis at 100 V for 50 mins.

4.6. Pharmacological studies

4.6.1. Experimental animals

Adult male albino rats of Wistar strain, weighing between 150-200 gms were used for the nephroprotective and antiurolithic studies. They were obtained from Sree Venkateswara Enterprises Pvt. Ltd., Bangalore and housed in clean polypropylene cages covered with stainless steel filter tops under standard laboratory condition of 12 hr light/12 hr dark cycle, 50% humidity and temperature 25±2ºC. They were fed with standard commercial rat feed pellets (SAI animal feed Ltd., Bangalore, India) and water *ad libitum* throughout the study. The animals were acclimatized to laboratory condition before the commencement of experiments.

4.6.2. Ethical committee approval

All animal procedures in this study were performed after obtaining ethical clearance from Institutional Animals Ethics Committee (IAEC) of KMCH College of Pharmacy, Coimbatore, (KMCRET/Ph.D/12/2015-16). The rats received human care according to the guideline of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

4.6.3. Acute toxicity studies

Acute oral toxicity studies were not performed as studies of ethanolic extract of whole plant *B. sensitivum* have already been reported. Previous study by Anidya *et al.*,[112] used 5000 mg/kg dose of EEBS as higher dose in albino rats. Hence, one tenth of this dose, 500 mg/kg (higher dose) and lower dose as 250 mg/kg have been selected for the present study.

4.7. Evaluation of nephroprotective activity of EEBS

4.7.1. Animal grouping

Thirty albino rats were used in each drug induced toxicity models. Animals were divided into five groups with 6 animals in each group, designated as Group I, II, III, IV and V. Animals in group I served as control and group II served as toxic control. Rats in Group II to V were treated with drugs which induce toxicity. Animals in group III, IV and V also received standard drug, low dose of EEBS (250 mg/kg) and high dose EEBS of (500 mg/kg) respectively.

4.7.1.1. Gentamicin-induced nephrotoxicity [144, 145]

Table 5: Grouping of animals in gentamicin (GM)-induced nephrotoxicity model

Gentamicin (GM)-induced nephrotoxicity	
Group	Treatment
Group I	Normal saline1mL/day; for 8 days; *i.p.*
Group II	GM 100 mg/kg; for 8 days; *i.p*
Group III	GM+Quercetin 50 mg/kg; for 8 days*; p.o.*
Group IV	GM+Extract 250 mg/kg; for 8 days*; p.o.*
Group V	GM+Extract 500 mg/kg*;* for 8 days*; p.o.*

4.7.1.2. Cisplatin-induced nephrotoxicity [146, 147]

Table 6: Grouping of animals in cisplatin (CDDP)-induced nephrotoxicity model

Cisplatin (CDDP)-induced nephrotoxicity	
Group	Treatment
Group I	Normal saline 1mL/day; for 9 days; *i.p*
Group II	CDDP single dose of 8 mg/kg on fifth day; *i.p.*
Group III	CDDP+Quercetin 50 mg/kg; for 9 days; *p.o.*
Group IV	CDDP+Extract 250 mg/kg; for 9 days; *p.o.*
Group V	CDDP+Extract 500 mg/kg; for 9 days; *p.o.*

4.7.1.3. Ethylene glycol-induced urolithiasis [148-150]

Table 7: Grouping of animals in ethylene glycol (EG)-induced urolithic model

.

Ethylene glycol (EG)-induced urolithiaisis	
Group	Treatment
Group I	Normal control
Group II	EG (0.75% v/v) for 28 days; *p.o.*
Group III	EG+Cystone 750 mg/kg; from 15th day till 28th day; *p.o.*
Group IV	EG+Extract 250 mg/kg; from 15th day till 28th day; *p.o.* (Curative regimen)
Group V	EG+Extract 500 mg/kg; from 1st day till 28th day; *p.o.* (Preventive regimen)

4.8. Parameters studied in drug induced nephrotoxicity and urolithic models

After treatment period, 24 hr urine samples were collected. Urine was then analyzed for total protein, albumin, sodium, potassium, calcium and magnesium in animals induced with nephrotoxicity, whereas in calculi-induced rats total protein, albumin, calcium, phosphate and magnesium levels were determined. After collecting the urine, the rats in all groups were anaesthetized with 80 mg/kg ketamine hydrochloride. Blood was collected from tail vein under mild anesthesia and determined various haematological parameters. For the estimation of different biochemical parameters, serum was separated and analyzed for total protein, albumin, sodium, potassium, calcium, magnesium, blood urea nitrogen (BUN), uric acid, and creatinine levels in CDDP and GM-induced nephrotoxicity models. Total protein, albumin, calcium, phosphate, magnesium, BUN, uric acid, and creatinine levels were determined in EG-induced urolithic rats.The rats were sacrificed after the administration of last dose of toxicant and separated kidneys were weighed, homogenized and used for the estimation of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and malondialdehyde (MDA) levels.

4.8.1. Assessment of general parameters

Body weight of all experimental animals were recorded at the beginning and end of the treatment schedule. After the last dose of drug administration, change in the body weight, kidney weight and water intake (mL/24 hr) were measured. Total urine volume (mL/24 hr) and the acidity of the urine were also recorded.

4.8.2. Assessment of hematological parameters

After the treatment period, experimental animals in drug induced toxicity models were fasted over night and blood was collected for the estimation of different hematological parameters including, red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular hemoglobin (MCH), white blood corpuscles (WBC), lymphocyte, monocyte, polymorphs and eosinophils.

For determining red blood cells, EDTA anti-coagulated blood was drawn using a red blood cell pipette of haemocytometer and thoroughly mixed with RBC diluting fluid (Turks solution). The mixture was transferred onto the counting chamber (neubar chamber) and placed under the microscope. The total number of RBC (millions per cubic millimetre) were counted uniformly in the smaller four corner squares.[151] Haemoglobin was converted into acid haematin by the action of dilute hydrochloric acid. Blood drawn upto 20 µL in the Sahli's pipette was mixed with 0.1 N hydrochloric acid taken up to the lowest marking in the haemoglobinometer. Allowed the mixture to stand at room temperature for 10 mins. The acid haematin solution was further diluted with distilled water until its colour matched exactly with that of permanent standard in the haemoglobinometer. The lower meniscus of the fluid was noted in g/100 mL. (Sahli's acid hematin method). The total white blood cells were enumerated according to the method described by John,[152] using Turk's fluid (WBC diluting fluid). Blood was collected using a white blood cell pipette of haemocytometer and mixed with WBC diluting fluid. The mixture was transferred onto the counting chamber (neubar chamber) placed under the microscope and viewed under 10X objective. The total number of WBC in thousands per cubic millimeter were counted uniformly in the four larger corner

squares. The haematological parameters like PCV (%), MCH (Pg) and differential leukocytes (%) were measured using instrument celldyn 1700 haematology analyser (automated method).

4.8.3. Determination of total protein and albumin level

Total protein content in urine/serum were determined by the method of Lowry *et al*.[153] Briefly, 4.5 mL of alkaline copper sulphate reagent and 0.5 mL of Folin's phenol reagent were added to all the test tubes containing 0.2 mL of test solution and 0.2-1 mL of Bovine serum albumin (working standard, 100 mg/mL). Volume of the sample and standard were made upto 1mL with distilled water, before adding the reagent. Distilled water of 1mL served as blank. Thoroughly mixed the reagent with sample or test solution and the blue colour developed was read at 640 nm. From the standard graph the amount of protein in the given unknown solution was calculated. Total protein present in the sample is expressed in g/dL.

Albumin level was determined by Reinhold's method using biuret reagent.[154] Pipetted out 0.2 mL of serum/urine in a centrifuge tube and added 5.8 mL sodium sulphite solution and 3 mL ether. Mixed well, kept for 5 mins and then centrifuged. Albumin appears in the bottom layer of the tube. 3 mL of biuret reagent was added to test tubes containing 3 mL globulin free test solution. 3 mL of biuret reagent was added to 0.1 mL standard solution (albumin solution) mixed with 2.9 mL of distilled water. For blank, 3 mL of distilled water was mixed with 3mL of biuret reagent. Mixed well and kept all test tubes in a water bath for 10 mins at 37ºC. Allowed to cool and observed readings of test and standard against the blank at 540 nm in a spectrophotometer. Total albumin content present in the sample is expressed in g/dL.

4.8.4. Determination of urinary and serum electrolyte level

4.8.4.1. Determination of calcium level

The serum/urine calcium levels were estimated based on method of Lorentz using a diagnostic kit supplied by Clini chem calcium kit manufacturer, Robonik Pvt. Ltd., Mumbai, India.[155] 24-hr collected urine sample was diluted with distilled water (1:3). The pH-value of the urine was adjusted to pH 3-4 with dilute hydrochloric acid. 0.5 mL of buffer solution (2-amino-2-methyl-1-propanol, 400 mmol/L) mixed with 0.5 mL of chromogen reagent (o-cresolphthalein 0.62 mmol/L, 8-Hydroxy quinoline 69 mmol/L), served as working reagent. 1 mL of working reagent was added to each test tube containing 20 µL sample and 20 µL standard solution. 1 mL working reagent added to 20 µL distilled water was used as blank. After mixing thoroughly incubated the reaction mixtures at room temperature for 5 mins. The absorbance of sample and standard were measured at 570 nm against the blank reagent.

$$\text{Conc. of calcium (mg/dL)} = \text{Abs}_{\text{sample}} / \text{Abs}_{\text{blank}} \text{ X Conc. of sample}$$

4.8.4.2. Determination of phosphate level

Phosphate levels were determined by the method of Fiske and Subbarow.[156] 5mL serum/urine or 5 mL standard containing 0.04 mg phosphate, were mixed with 1mL molybdic acid reagent, and added 0.4 mL amino-naphthoL-sulphonic acid (0.25%). For blank , 1mL molybdic acid reagent was mixed with 5mL distilled water and added 0.4 mL amino- naphthoL-sulphonic acid. Finally volume was made upto 10 mL mark with distilled water. The absorbance of sample and standard were measured at 680 nm against the blank reagent after keeping the reaction mixtures at room temperature for 5 mins.

$$\text{Conc. of Phosphate (mg/dL)} = \text{Abs}_{\text{test}} / \text{Abs}_{\text{standard}} \text{ X } 0.04 \text{ X } 100$$

4.8.4.3. Determination of magnesium level

Urinary and serum level of magnesium were determined according to the method of Heaton.[157] 1 mL serum/urine was mixed with 6 mL trichloroacetic acid (10%). Centrifuged after keeping the solution for 10 mins at room temperature. 0.5 mL polyvinyl alcohol (0.2%), 2.5 mL titan yellow (15mg%) and 1 mL sodium hydroxide (20%) were added to the 2mL of supernatant, mixed the solution carefully and allowed to stand for 20 mins at room temperature. For standard, 1 mL magnesium sulphate solution was used and followed same assay procedure of test solution. 1mL distilled water was used as blank. The absorbance of sample and standard were measured at 680 nm against the blank.

$$\text{Conc. of magnesium (mg/dL)} = \text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}} / \text{Abs}_{\text{std}} - \text{Abs}_{\text{blank}} \text{ X Conc. of std}$$

4.8.4.4. Determination of sodium level

Urinary and serum level of sodium was estimated by the modified method of Trinder and Maruna[158] using a diagnostic reagent kit supplied by Lab care diagnostics pvt Ltd., Sarigam, Valsad, India. 1 mL filtrate reagent was added to each of sample (50 µL serum/urine) and 50µL sodium standard. Mixed well and allowed to stand for 5 mins at room temperature. 50 µL supernatant of standard sodium solution or 50 µL supernatant of test solution, after centrifugation at high speed for 10 mins was mixed with 1 mL acid reagent and 2 drops of color reagent. 50 µL filtrate reagent mixed with 1 mL acid reagent and 2 drops color reagent were used as blank. Absorbance of standard and test against reagent blank were measured at 546 nm.

$$\text{Conc. of sodium (mmol/L)} = \text{Abs}_{\text{blank}} - \text{Abs}_{\text{test}} / \text{Abs}_{\text{blank}} - \text{Abs}_{\text{std}} \text{ X } 150$$

4.8.4.5. Determination of potassium level

Potassium was estimated by the modified method of Trinder and Maruna[159] using a diagnostic reagent kit supplied by Lab care diagnostics pvt Ltd, Sarigam, Valsad, India. 1 mL potassium reagent was added to each of the sample (20 µL serum/urine), potassium standards (20 µL) and blank (20 µL distilled water). After incubating the mixture at room temperature for 15 mins absorbance of the standard and test were measured against reagent blank at 630 nm.

$$\text{Conc. of potassium (mmol/L)} = \frac{\text{Abs}_{test} - \text{Abs}_{blank}}{\text{Abs}_{std} - \text{Abs}_{blank}} \times 5$$

4.8.5. Determination of nitrogenous waste product levels in serum

4.8.5.1. Determination of serum blood urea nitrogen (BUN) level

The blood urea was estimated by Berthelot method (Fawcett and Scott)[160] using the commercially available kit (Kamineni Life Sciences Pvt. Ltd., Hyderabad, India). 1000 µL of working reagent-I containing urease reagent and a mixture of salicylate, hypochlorite and nitroprusside were added to each of the sample (10 µL of serum), standard (10 µL of urea) and blank (10 µL of purified water). Mixed well and incubated at 37°C for 5 mins. Then 1000 µL of reagent-II containing alkaline buffer, was added to all the test tubes, which were incubated at 37°C for 5 mins. The intensity of blue-green colour produced is directly proportional to the concentration of urea in the sample and absorbance was measured spectrophotometrically at 578 nm. The blood urea was calculated using the following formula:

$$\text{Conc. of blood urea (mmol/L)} = \text{Abs}_{test} - \text{Abs}_{std} \times 40$$

$$\text{Blood urea nitrogen (mg/dL)} = \text{Serum urea} \times 0.467$$

4.8.5.2. Determination of serum uric acid level

Uric acid content was estimated by following the method of Caraway.[161] After centrifugation of serum, 0.6 mL of phosphotungstic acid and 0.6 mL of sodium carbonate were added to each of 2 mL supernatant of sample and standard. Uric acid standards with graded volumes were also prepared. A blank was setup with 3.0 mL of water. After 10 mins the colour developed was measured at 640 nm spectrophotometrically.

$$\text{Conc. of uric acid (mg/dL)} = \text{Abs}_{\text{sample}} / \text{Abs}_{\text{std}} \times 0.02$$

4.8.5.3. Determination of serum creatinine level

The serum creatinine concentration was estimated using the commercially available kit (Cresent biosystems, Goa, India) by alkaline picrate method (Bonsnes and Taussky).[162] 2.0 mL of picric acid reagent was added to 0.2 mL of serum, mixed well and centrifuged at 3000 rpm to obtain a clear supernatant. 100 µL of buffer reagent was added to 1.1 mL of supernatant, 0.1 mL of standard creatinine and 0.1 mL of distilled water to prepare test, standard and blank, respectively. 1.0 mL of picric acid reagent was added to blank and standard. Mixed well and kept all solutions at room temperature for 20 mins. The intensity of orange colour formed was read at 520 nm spectrophotometrically. The serum creatinine concentration was calculated using the following formula:

$$\text{Conc. of creatinine (mg/dL)} = \text{Abs}_{\text{test}} / \text{Abs}_{\text{std}} \times 2$$

4.8.6. Determination of antioxidant enzymes and lipid peroxidation

Preperation of tissue homogenate

The rats were sacrificed after the administration of last dose of toxicant by euthanasia method. Both the kidneys were removed immediately, washed with normal

saline and stored for 12 hrs to carry out *in vivo* antioxidant studies. The separated left kidney was homogenized with a motor driven Teflon coated homogenizer with 0.1M Tris-hydrochloride buffer (pH 7.4) to get 10% w/v homogenate. The homogenate was centrifuged at 10,000 rpm for 10 mins at 5°C, then the collected clear supernatant was used for the estimation of superoxide dismutase, catalase activity, glutathione peroxidase, reduced glutathione and lipid peroxidation.

4.8.6.1. Estimation of superoxide dismutase (SOD)

SOD was estimated according to the modified procedure described by Kakkar *et al*. [163] 0.5 mL of kidney homogenate was diluted with distilled water (0.5 mL) and treated with 0.25 mL of ethanol and 0.15 mL of chloroform. Supernatant of kidney homogenate was removed after centrifuging the mixture for 1 min. 1.5 mL of buffer was added to 0.5 mL of kidney homogenate and the reaction was started by adding 0.4 mL of epinephrine. Change in optical density per minute was determined at 480 nm in double beam UV-VIS spectrophotometer. Activity of SOD was expressed as unit/min/mg of protein.

4.8.6.2. Estimation of catalase (CAT)

The catalase activity was assayed by colourimetric method of Sinha.[164] Kidney homogenate (0.1 mL) was mixed with 1.0 mL of phosphate buffer and 0.50 mL of hydrogen peroxide. 1 mL of the reaction mixture was withdrawn and 0.2 mL dichromate/acetic acid reagent was blown at the interval of 1 min to arrest the reaction. The standard hydrogen peroxide in the range of 4 to 20 µL were taken and treated similarly. The tubes were heated in a boiling water bath for 10 mins. The green color developed was read at 570 nm by using a double beam UV-VIS spectrophotometer. The

activity of catalase was expressed as μmol of hydrogen peroxide consumed/min/mg protein.

4.8.6.3. Estimation of glutathione peroxidase (GPx)

The glutathione peroxidase activity was measured according to the method of Rotruck *et al.*[165] 0.2 mL each of EDTA, sodium azide, reduced glutathione, hydrogen peroxide, 0.4 mL of buffer and 0.1 mL of enzyme (kidney homogenate) were mixed and incubated at 37°C for 10 mins. The reaction was arrested by the addition of 0.5 mL of tricarboxylic acid and the tubes were centrifuged. To 0.5 mL of supernatant, 3mL of sodium hydrogen phosphate and 1 mL of 5,5′-dithiobis(2-nitrobenzoic acid) were added and the color developed was read at 412 nm immediately by using UV-VIS spectrophotometer. Glutathione peroxidase activity is expressed as μ moles of glutathione oxidized/min/mg protein.

4.8.6.4. Estimation of reduced glutathione (GSH)

GSH was estimated by Ellman's procedure.[166] Tissue homogenate (250 μL) was treated with 1 mL of 5% tricarboxylic acid in a 2 mL Eppendorf tube and precipitate was removed by centrifugation at 3000 rpm for 10 mins at room temperature. To 250 μL of the above supernatant, 1.5 mL of 0.2 M phosphate buffer was added and mixed well. 250 μL of 0.6 mM of Ellman's reagent [5,5′-Dithiobis(2-nitrobenzoic acid) solution] was added to the above mixture and the absorbance was measured at 412 nm within 10 mins. A standard graph was plotted using glutathione reduced (1.0 mg/mL) and GSH content present in sample was calculated by interpolation. Amount of glutathione expressed as μg/mg of protein.

4.8.6.5. Estimation of lipid peroxidation (LPO)

Lipid peroxidation (LPO) was estimated by thiobarbituric reaction method of Okhawa *et al*. [167] 1 mL of kidney homogenate was mixed with 0.2 mL 4 % w/v sodium dodecyl sulfate, 1.5 mL 20% acetic acid in 0.27 M hydrochloric acid (pH 3.5) and 15 mL of 0.8% thiobarbituric acid (pH 7.4). The mixture was heated in a hot water bath at 85˚C for 1 hr. The intensity of the pink colour developed was read against a reagent blank at 532 nm, followed by centrifugation at 1200 rpm for 10 mins. The concentration was expressed as *n* moles of malondialdehyde per mg of protein using 1,1,3,3-tetra-ethoxypropane as the standard.

4.9. Histopathological studies[168]

The right kidney of experimental animals from all groups were removed, washed with the normal saline and fixed in 10% neutral buffered formalin for 48 hrs. After washing for 1 hr in running tap water, dehydrated the tissue and cleaned the tissues with xylene. Embedded cleaned tissues in melted paraffin wax at 56ºC. Longitudinal and transverse sections were taken and incubated the tissue sections for 2 hrs at 40ºC. After rehydration, the sections were stained with haematoxylin and eosin (H and E) and observed under light microscope. Photographs were obtained to study the histopathological changes.

4.10. Statistical analysis

***In vitro studies* of EEBS:** All results were expressed as mean value ± standard deviation (SD). Student's t-test was used for comparison between values of samples and standards. Difference was considered statistically significant when $P<0.05$.

***In vivo* studies of EEBS:** The results were expressed as mean ± standard error of mean (SEM). Statistical significance between means was analyzed by one-way analysis of variance (ANOVA) followed by "Dunnett's test." Pvalue<0.05 were considered statistically significant. The statistical program used was Graph Pad Prism 7.02 version for Windows (GraphPad Soft ware Inc., San Diego, California, USA).

5. RESULTS

5.1. Phytochemical studies

5.1.1. Preparation of crude EEBS Linn

The authenticated whole plant of *B. sensitivum* were washed, air dried, subjected to size reduction and passed through sieve No. 40. Uniformly powdered plant material was initially extracted with petroleum ether (60-80ºC) for defatting the drug and then with 70% ethanol by using Soxhlation method. The obtained solvent extract was filtered, evaporated to dryness and weighed. The percentage yield was calculated in terms of air dried weight of the plant material subjected to the extraction process. The percentage yield of petroleum ether and ethanolic extract of *B. sensitivum* were 4.92% w/w and 12.54% w/w respectively and are presented in table 8.

Table 8: Percentage yield of solvent extraction of *B. sensitivum* (Linn.) DC

Sl.No.	Solvents	Colour & Consistency	Plant material taken (gms)	Weight of extract (gms)	% yield (w/w)
1	Petroleum ether	Greenish yellow Sticky solid	500	24.6	4.92
2	Ethanol	Brownish black Semisolid	500	62.7	12.54

5.1.2. Qualitative analysis of phytochemicals

The preliminary phytochemical investigations of crude EEBS were carried out and the results confirmed the presence of carbohydrates, alkaloids, steroids, saponins, proteins, aminoacids, falvonoids, tannins, phenolic compounds and fixed oils. The details of phytochemical investigations are demonstrated in table 9.

Table 9: Preliminary phytochemical investigation of EEBS

Sl.No.	Phytochemical constituents	Phytochemical tests	EEBS
1	Carbohydrate	Molisch's	+ve
		Fehling's	+ve
		Benedict's	+ve
		Barfoed's	-ve
		Tollen's	-ve
		Seliwanoff'S	+ve
		Bromine water	-ve
2	Alkaloids	Mayer's	+ve
		Dragendorff's	+ve
		Wagner's	+ve
		Hagner's	+ve
		Tannic acid	+ve
3	Saponin	Foam	+ve
		Hemolytic	+ve
		Lead acetate	+ve
4	Flavonoids	Shinoda's	+ve
		Alkaline reagent	+ve
		Lead acetate	+ve
		Sulphuric acid	+ve
5	Phenolic compounds	Ferric chloride	+ve
		Lead acetate	+ve
6	Tannins	Ferric chloride	+ve
		Gelatin	+ve
		Lead acetate	+ve
7	Glycoside	Keller-killiani	-ve
		Baljet	-ve
		Legal's	-ve
		Borntrager's	-ve
8	Steroids	Libermann-Burchard's	+ve
		Salkowsky's	+ve
9	Protein	Biuret	+ve
		Ninhydrin	+ve
		Xanthoprotein	-ve
		Million's	+ve
		Lead acetate	-ve
10	Terpenoid	Hirschonn reaction	-ve
		Knoller's	-ve
11	Fixed oil	Spot	+ve
		Saponificaton	+ve
		Solubility	+ve

+ve - present, -ve - absent

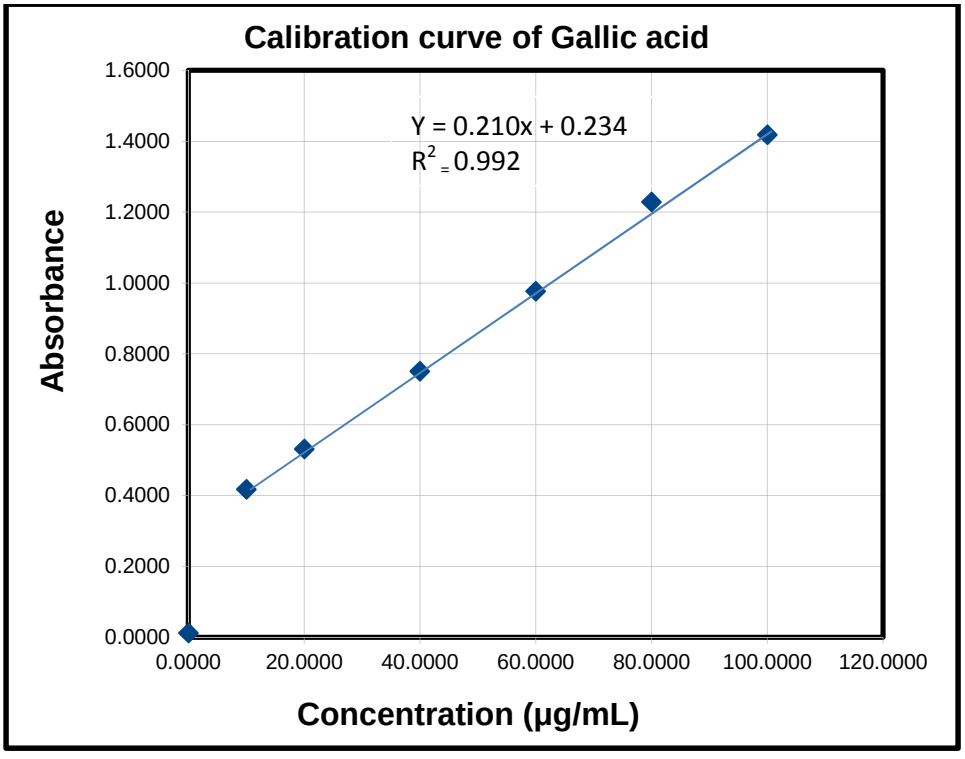
5.2. Quantitative estimation of phytoconstituents present in EEBS

Major phytoconstituents contributing to antioxidant activity includes total phenolic compounds and total flavonoid compounds. Calibration curve for the determination of total phenolic content and flavonoid content of EEBS are illustrated in figure 4 and 5.

5.2.1. Total phenolic content of EEBS

Total phenolic content of EEBS were expressed in terms of gallic acid equivalents. The concentration of total phenolic content is expressed as mg/g of gallic acid equivalent. Absorbance increases as concentration of phenolic compounds increases. The total phenolic content in the extract of *B. sensitivum* were 53.55 mg/g of gallic acid equivalents (figure 4).

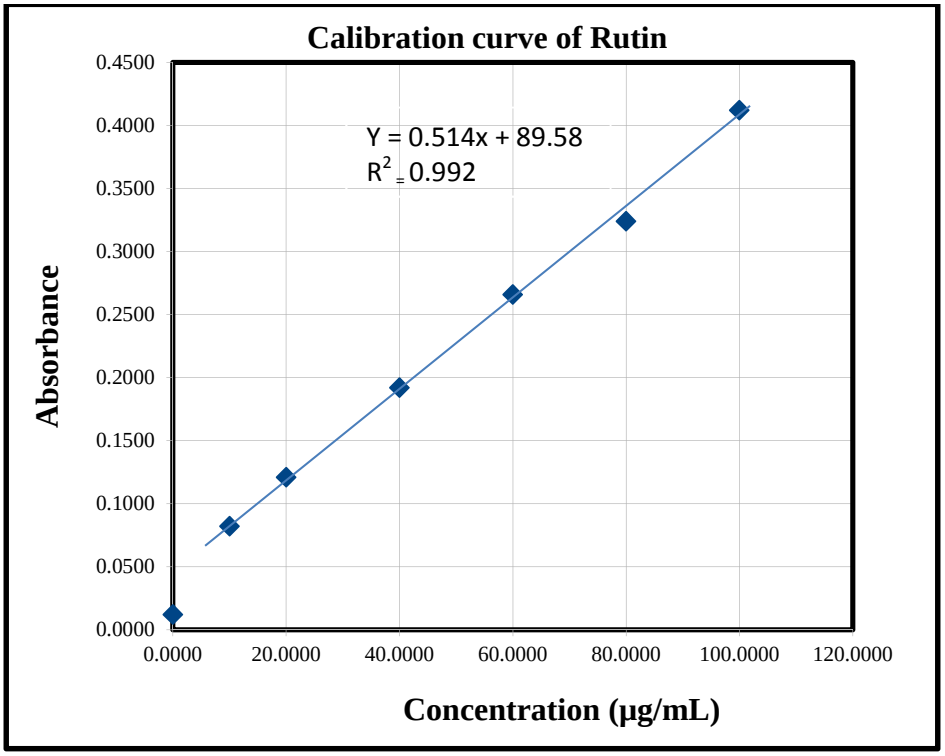
Figure 4: Calibration curve of gallic acid showing linearity over concentration range of 10-100 µg/mL



5.2.2. Total flavonoid content of EEBS

Total flavonoid content of EEBS were expressed in terms of rutin equivalents. The concentrations of total flavonoids were expressed as mg/g of rutin equivalents. Maximum absorption depends on the concentration of flavonoids. The total flavonoid content in the extract of *B. sensitivum* were 153.08 mg/g of rutin equivalents (figure 5).

Figure 5: Calibration curve of rutin showing linearity over concentration range of 10-100 µg/mL



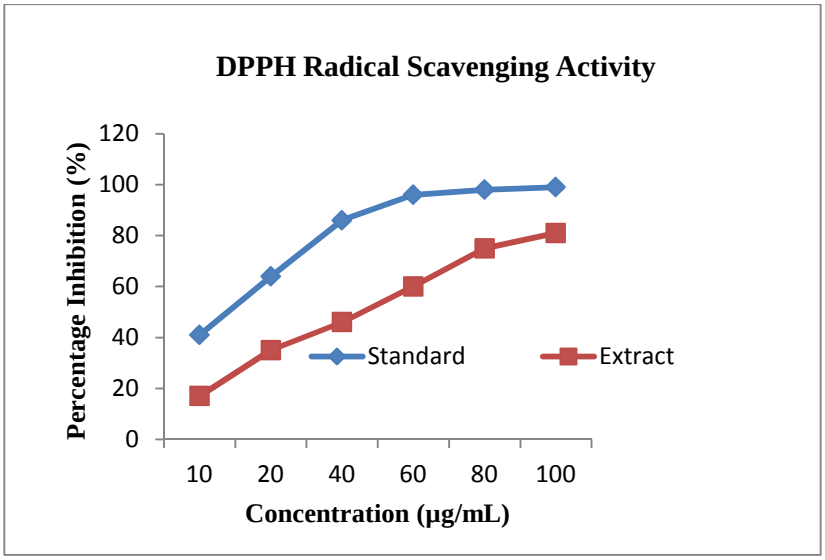
5.3. *In vitro* antioxidant and free radical scavenging activity of EEBS

5.3.1. DPPH radical scavenging activity

Radical scavenging activity of extract was observed from decrease in absorbance of DPPH with increase in concentration. Absorbance value of EEBS had shown as 1.52, 1.19, 0.98, 0.72, 0.45 and 0.34 and standard ascorbic acid exhibited as 1.28, 0.78, 0.30, 0.07, 0.04 and 0.03 at 10, 20, 40, 60, 80 and 100 µg/mL respectively. EEBS at a

concentration of 10-100 µg/mL inhibited production of DPPH radical by 17-81% and showed significant ($P<0.05$) scavenging effects on DPPH radical compared to standard ascorbic acid which exhibited 41-99% of inhibition. IC_{50} value of EEBS was found to be 46.34 µg/mL and that of ascorbic acid (standard) was 14.12 µg/mL. This result indicated that EEBS contained sufficient phytochemical constituents capable to donate 'H' for the conversion of free radical DPPH to non-free radical DPPH-H (figure 6).

Figure 6: **Percentage inhibition of DPPH radical scavenging activity of EEBS, in comparison to standard ascorbic acid**

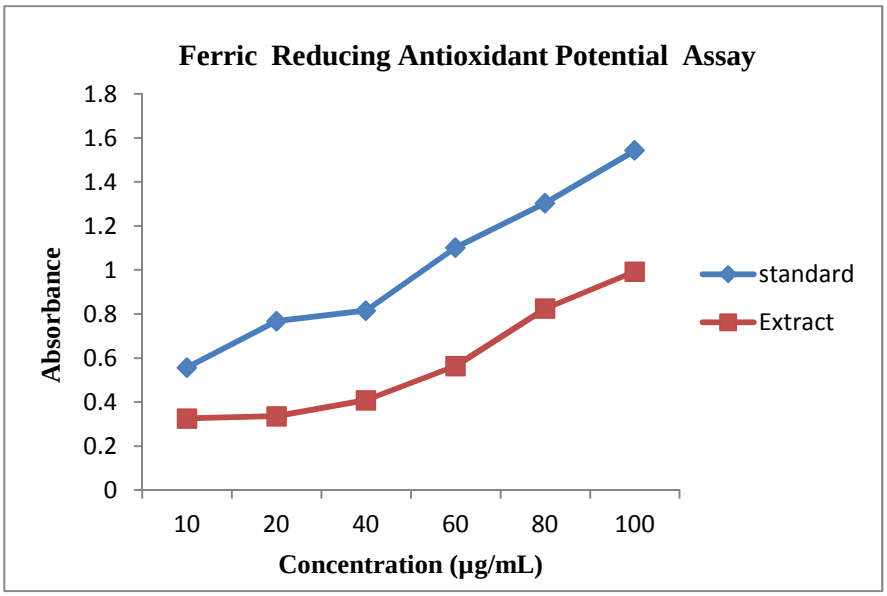


5.3.2. Ferric reducing antioxidant power (FRAP Assay)

The trend for ferric ion reducing activities of EEBS and standard had shown in figure 7. Ferric reducing antioxidant power was determined according to the ability of sample to reduce Fe^{3+} to Fe^{2+} ions. Increased absorbance of reaction mixture indicates increase in reducing power. Absorbance value of EEBS at 10, 20, 40, 60, 80 and 100 µg/mL had shown as 0.32, 0.33, 0.40, 0.56, 0.82 and 0.99 respectively. Ascorbic acid at the same concentration range (10-100 µg/mL) exhibited absorbance value as 0.55, 0.76,

0.81, 1.10, 1.30 and 1.54 respectively. This result indicated that EEBS exhibited ferric reducing ability in a concentration dependent manner and was significant with that of standard ($p<0.01$). The ferric reducing antioxidant powers of EEBS are depicted in figure 7.

Figure 7: Absorbance of ferric reducing ability of EEBS, in comparison to standard ascorbic acid.

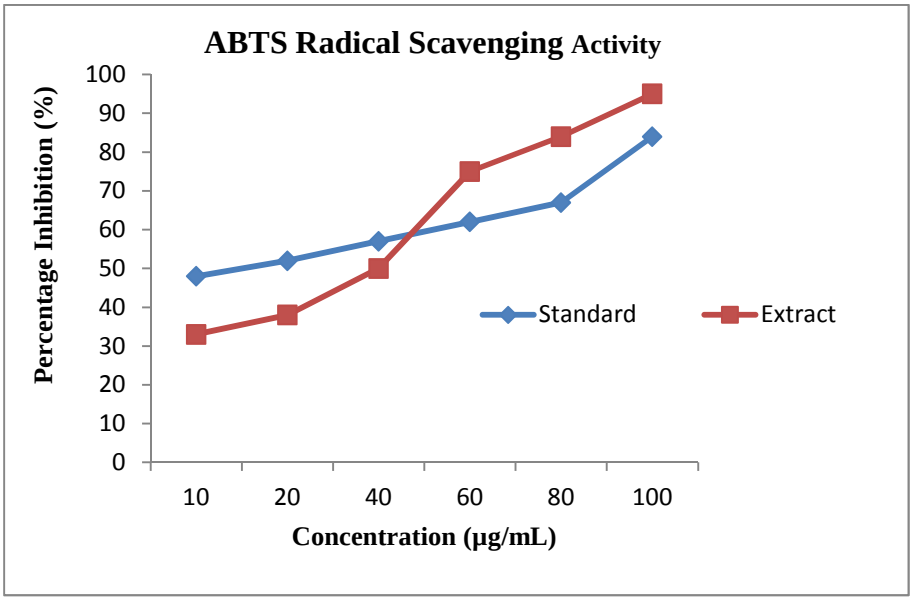


5.3.3. ABTS radical scavenging activity

Radical scavenging activity of EEBS was determined by comparing with ascorbic acid standard reference. Absorbance value of EEBS had shown as 0.26, 0.24, 0.19, 0.09, 0.06 and 0.02 and standard ascorbic acid exhibited as 0.32, 0.30, 0.26, 0.23, 0.20 and 0.09 at 10, 20, 40, 60, 80 and 100 µg/mL respectively. EEBS inhibited production of ABTS radical by 33-95%, where as ascorbic acid showed 48-84% of inhibition. EEBS scavenged ABTS radical in a concentration dependent manner and was comparable with that of ascorbic acid. The concentration of extract needed to produce 50% inhibiton on ABTS production was found to be 42.01 µg/mL, where as 18.43 µg/mL was needed for

ascorbic acid. Scavenging effect of extract was found to be less compared to reference compound. This antioxidant activity reflects free radical scavenging capacity of EEBS, by donating electron or hydrogen atom to inactivate this radical cation (fig 8).

Figure 8: Percentage inhibition of ABTS radical scavenging activity of EEBS, in comparison to standard ascorbic acid.

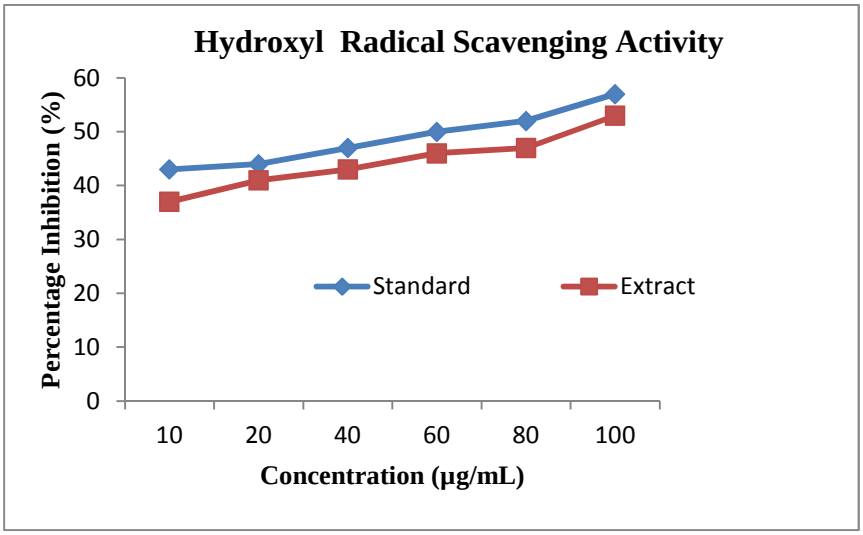


5.3.4. Hydroxyl radical scavenging activity

The extract inhibited hydroxyl radical induced deoxyribose degradation in a concentration dependent manner and maximum inhibition at concentration of 100 µg/mL was found to be 53%, whereas ascorbic acid (standard) had shown as 57%. Absorbance value of EEBS had shown as 0.215, 0.201, 0.194, 0.185, 0.181 and 0.172 and standard ascorbic acid exhibited as 0.161, 0.155, 0.148, 0.141, 0.136 and 0.120 at 10, 20, 40, 60, 80 and 100 µg/mL respectively. The extract showed hydroxyl radical scavenging activity with an IC_{50} value of 94.42 µg/mL in comparison to that of ascorbic acid (60.31 µg/mL). These results indicated that EEBS can act as a hydroxyl radical scavenger and also inhibit

lipid peroxidation. The hydroxyl radical scavenging activity of EEBS are presented in figure 9.

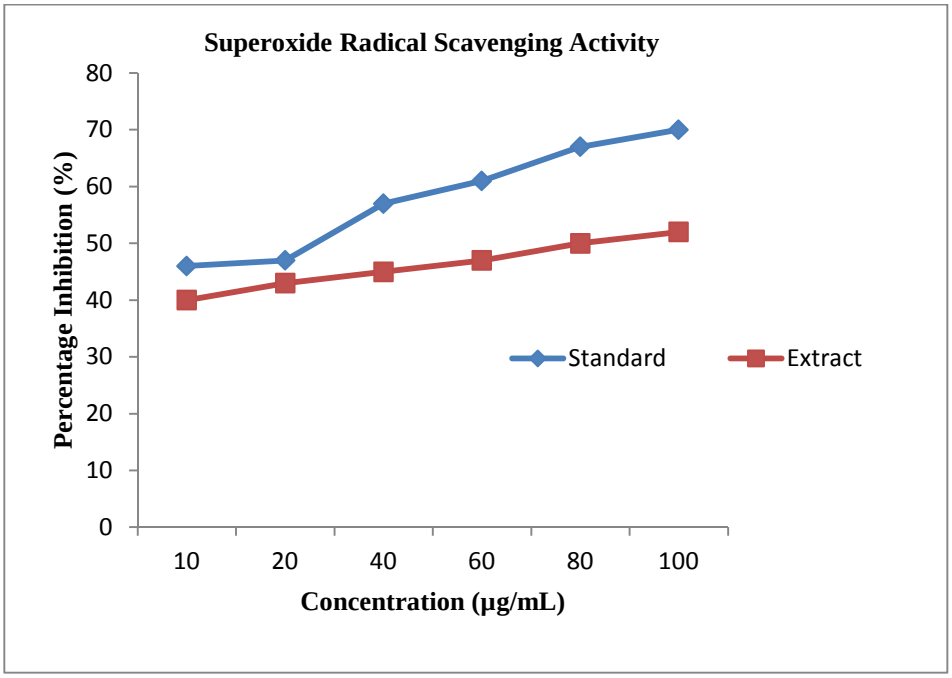
Figure 9: Percentage inhibition of hydroxyl radical scavenging activity of EEBS, in comparison to standard ascorbic acid



5.3.5. Superoxide radical scavenging activity

Superoxide radical scavenging activity of EEBS was compared with the same dose of ascorbic acid ranging from 10-100 µg/mL. Absorbance value of EEBS had shown as 0.178, 0.169, 0.167, 0.159, 0.158 and 0.146 and standard ascorbic acid exhibited as 0.434, 0.420, 0.343, 0.311, 0.312 and 0.242 at 10, 20,40,60,80 and 100 µg/mL respectively. Ic_{50} value of ascorbic acid and EEBS were found to be 23.64 µg/mL and 72.12 µg/mL respectively. Radical scavenging effect of extract was found to be significantly ($p<0.01$) less compared to reference compound. EEBS inhibited production of superoxide anion radical by 40-52% whereas reference compound had shown 46-70% of inhibition. The super oxide radical scavenging activities of EEBS are presented in figure 10.

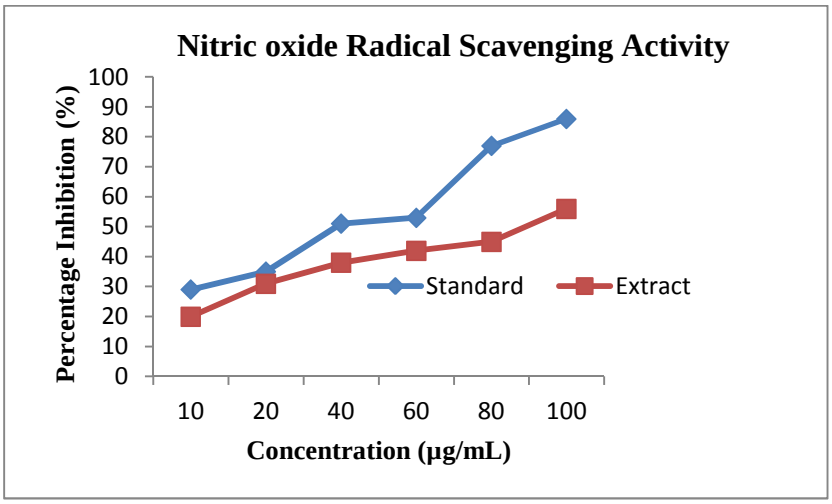
Figure 10: Percentage inhibition of superoxide radical scavenging activity of EEBS, in comparison to standard ascorbic acid



5.3.6. Nitric oxide radical scavenging activity

Absorbance value of EEBS had shown as 0.56, 0.48, 0.43, 0.40, 0.38 and 0.31 and standard ascorbic acid exhibited as 0.65, 0.60, 0.45, 0.43, 0.21 and 0.13 at 10, 20, 40, 60, 80 and 100 µg/mL respectively. IC_{50} value of EEBS was found to be 90.12 µg/mL and that of ascorbic acid (standard) was 37.23 µg/mL. Result indicated that EEBS had significant scavenging effect on nitric oxide radicals in a concentration dependent manner. Standard ascorbic acid at a concentration of 10-100 µg/mL inhibited production of nitric oxide radical by 29-86% whereas EEBS inhibited nitric oxide radical generation by 20-56%, thereby exhibited radical scavenging activity. Nitric oxide radical scavenging assay are depicted in figure 11.

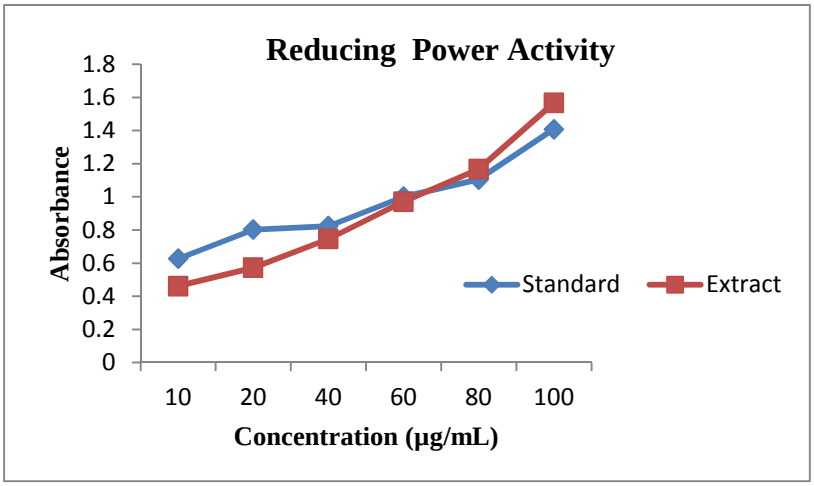
Figure 11: Percentage inhibition of nitric oxide radical scavenging activity of EEBS, in comparison to standard ascorbic acid



5.3.7. Reducing power

Reducing power of extract increased with increase in the concentration and it was found to be 0.46, 0.57, 0.74, 0.97, 1.16 and 1.56 at 10, 20, 40, 60, 80 and 100 µg/mL respectively. Ascorbic acid at same concentration exhibited absorbance value as 0.62, 0.80, 0.82, 1.00, 1.10 and 1.40 respectively. EEBS at 80 µg/mL and 100 µg/mL showed higher reducing activities than standard and differences were comparable (figure 12).

Figure 12: Absorbance of reducing ability of EEBS, in comparison to ascorbic acid



5.4. *In vitro* antiurolithic activity of EEBS.

Small particle size of stone forming constituents in urine reduces the possibility of nucleation, aggregation and retention in the urinary tract. In this study inhibition capacity of plant extract on calcium oxalate (CaOx) crystallization were determined using nucleation, aggregation and microscopic assay.

5.4.1. Nucleation assay

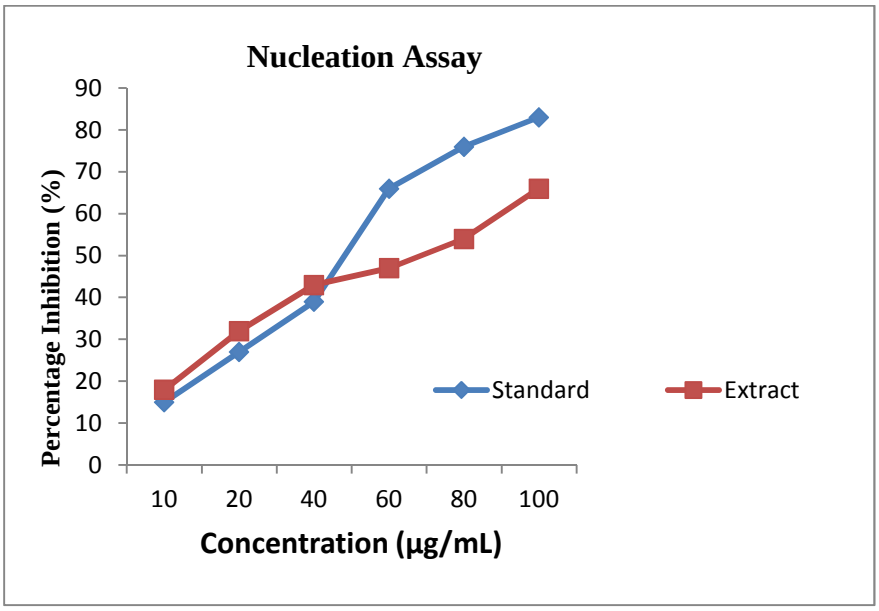
The percentage inhibition of extract on nucleation of CaOx crystals were found to be 18-66%, whereas with Cystone (standard) it was 15-83%. IC_{50} value of the EEBS was 68.82 mg/mL, compared with 52.41 mg/mL for Cystone. Absorbance value of EEBS at 10, 20, 40, 60, 80 and 100 mg/mL had shown as 0.76, 0.63, 0.52, 0.49, 0.42 and 0.31 respectively. Cystone (standard) at same concentration (10-100 mg/mL) exhibited absorbance value as 0.81, 0.69, 0.58, 0.32, 0.22 and 0.16 respectively. Inhibition on nucleation of CaOx crystals increased with increasing concentration of the extract and was comparable with that of Cystone (table 10, figure 13).

Table 10: Percentage inhibition capacity of EEBS and Cystone on nucleation of CaOx crystals

Sl.No.	Concentration (mg/mL)	Percentage inhibition Cystone*	Percentage inhibition EEBS*
1	10	15±0.020	18±0.001
2	20	27±0.001	32±0.008
3	40	39±0.000	43±0. 006
4	60	66±0.006	47±0.000
5	80	76±0.001	54±0.005
6	100	83±0.105	66±0.006
Ic_{50} values		Ic_{50}= 52.41 mg/mL	Ic_{50}= 68.82 mg/mL

*Values are obtained from average of 6 determinants. EEBS-ethanol extract of *B.sensitivum*

Figure 13: Percentage inhibition of EEBS on nucleation of CaOx crystals, in comparison to Cystone (standard)



5.4.2. Aggregation assay

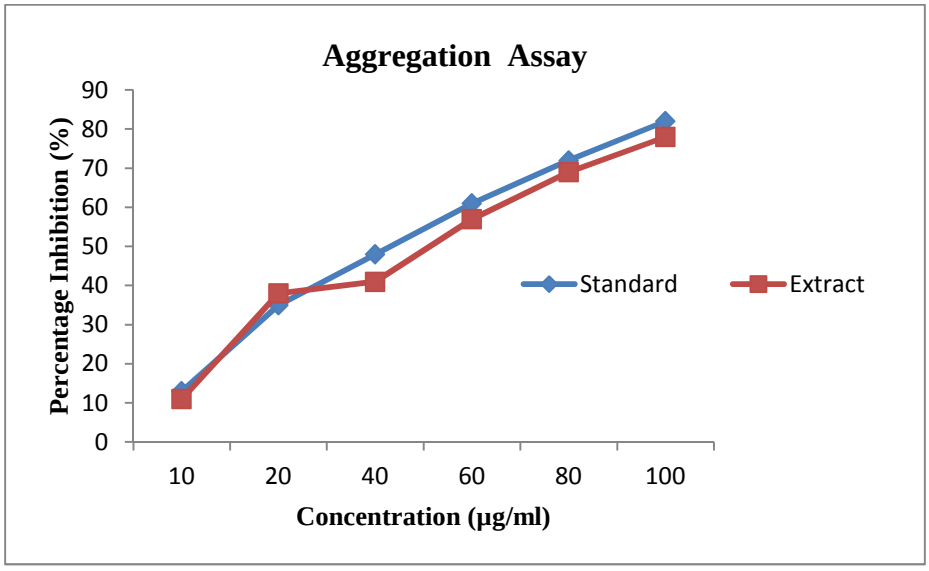
Percentage inhibition of EEBS was found to be 11-78% whereas with Cystone it was 13-82%. IC_{50} of the plant extract was 52.39 mg/mL and for Cystone it was found to be 41.62 mg/mL. Absorbance value of EEBS at 10, 20, 40, 60, 80 and 100 mg/mL had shown as 0.65, 0.45, 0.36, 0.27, 0.19 and 0.12 respectively. Cystone at same concentration (10-100 mg/mL) exhibited absorbance value as 0.74, 0.52, 0.49, 0.35, 0.26 and 0.18 respectively. EEBS showed a significant dose-dependent inhibition on the aggregation of CaOx crystals. Higher concentrations of EEBS indicated lower aggregation. The rate of inhibition capacity of various concentration of EEBS and Cystone (standard) on CaOx aggregation are depicted in table 11 and figure 14.

Table 11: Percentage inhibition capacity of EEBS and Cystone on aggregation of CaOx crystals

Sl.No.	Concentration (mg/mL)	Percentage inhibition Cystone*	Percentage inhibition EEBS*
1	10	13±0.005	11±0.003
2	20	35 ±0.002	38± 0.003
3	40	48 ±0.000	41 ±0.001
4	60	61±0.005	57 ±0.002
5	80	72 ±0.001	69 ±0.001
6	100	82±0.005	78±0.003
Ic_{50} values		Ic_{50}= 41.62 mg/mL	Ic_{50}= 52.39 mg/mL

*Values are obtained from average of 6 determinants. EEBS-ethanol extract of *B.sensitivum*

Figure 14: Percentage inhibition of EEBS on aggregarion of CaOx crystals comparison to Cystone (standard)



5.4.3. Image analysis of calcium oxalate (CaOx) crystal morphology (Microscopic assay)

In microphotographic study, incubation of metastable solutions of calcium chloride and sodium oxalate resulted in the formation of CaOx crystals. The corresponding size of CaOx crystals treated with control and various concentration of EEBS are illustrated in figure 15-19. Addition of EEBS at various concentration of 20, 40, 80, 160 µg/mL had reduced crystal size as 1275.49 µm, 1080.73 µm, 905.23 µm and

812.68 µm respectively. CaOx crystal size treated with control was 1398.05 µm (figure 15-19).

Figure 15-19: The size of CaOx crystals, observed under microscope (4X), in the absence of plant extract (control) and in presence of EEBS at concentration of 20, 40, 80 and 160 µg/mL

Figure 15: CaOx crystal treated with control

Figure 16:CaOx crystal treated with EEBS (20 µg/mL)

Figure 17: CaOx crystal treated with EEBS (40 µg/mL)

Figure 18: CaOx crystal treated with EEBS (80 µg/mL)

Figure 19: CaOx crystal treated with EEBS (160 µg/mL)

5.5. Pharmacological studies

5.5.1. Cytotoxic activity of EEBS on kidney cell lines

5.5.1.1. Trypan blue exclusion assay

In trypan blue exclusion method, treatment with EBBS reduced cell viability of human embryonic kidney 293 (HEK 293) cells in a dose-dependent manner as compared to cells treated with control which exhibited 100% viability. EBBS at lower dose of 12.5 µg/mL showed 98.34% of cell viability, whereas dose at which 25 µg/mL showed 97.02% viability. Exposure of cells to EEBS at dose of 50 µg/mL exhibited 95.40% of cell viability. EEBS at 100 µg/mL and 200 µg/mL showed significant ($p<0.01$) reduction in the number of viable cells compared to control and the percentage of inhibition was higher 92.20% and 88.07% respectively. The percentage cell viability of various concentration of EEBS are illustrated in table 12 and figure 20.

Figure 20: Cytotoxic effect of EEBS against HEK-293 cell line by trypan blue exclusion assay

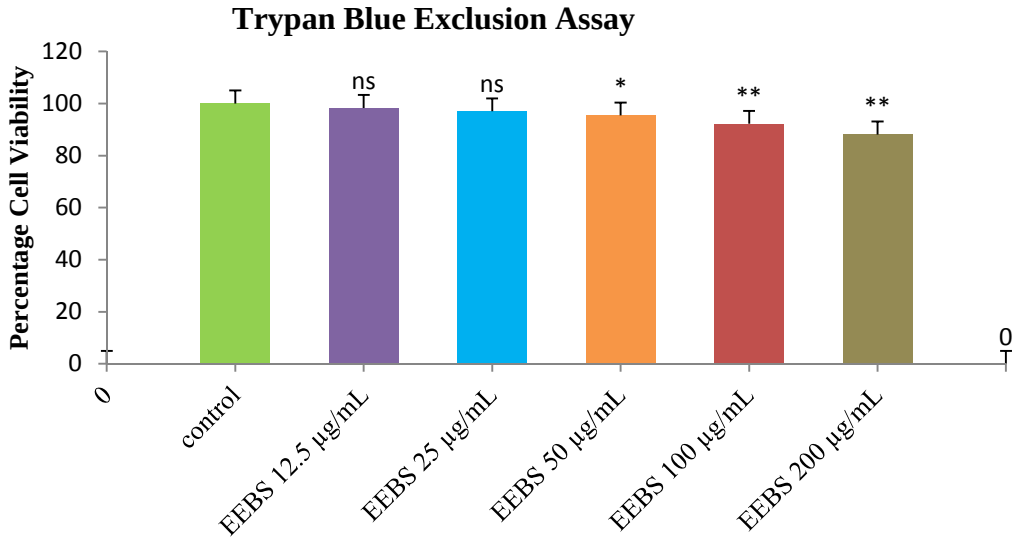


Table 12: Percentage of viable cell determination by trypan blue exclusion method

Name of test sample	Test Conc. (µg/mL)	No. of viable cells	No of dead cells	Cell viability* (%)
EEBS	12.5	99.66±0.332	1.66±0.310	98.34±0.326
	25	97.66±0.320	3.00±0.577	97.02±0.548
	50	96.66±0.811	4.66±0.333	95.40±0.321
	100	90.66±0.310	7.66±0.333	92.20±0.260
	200	88.00±0.577	12.00±1.15	88.07±0.981

*****Values are obtained from average of 3 determinants. EEBS-ethanol extract of *B. sensitivum*

5.5.1.2. MTT cell viability assay

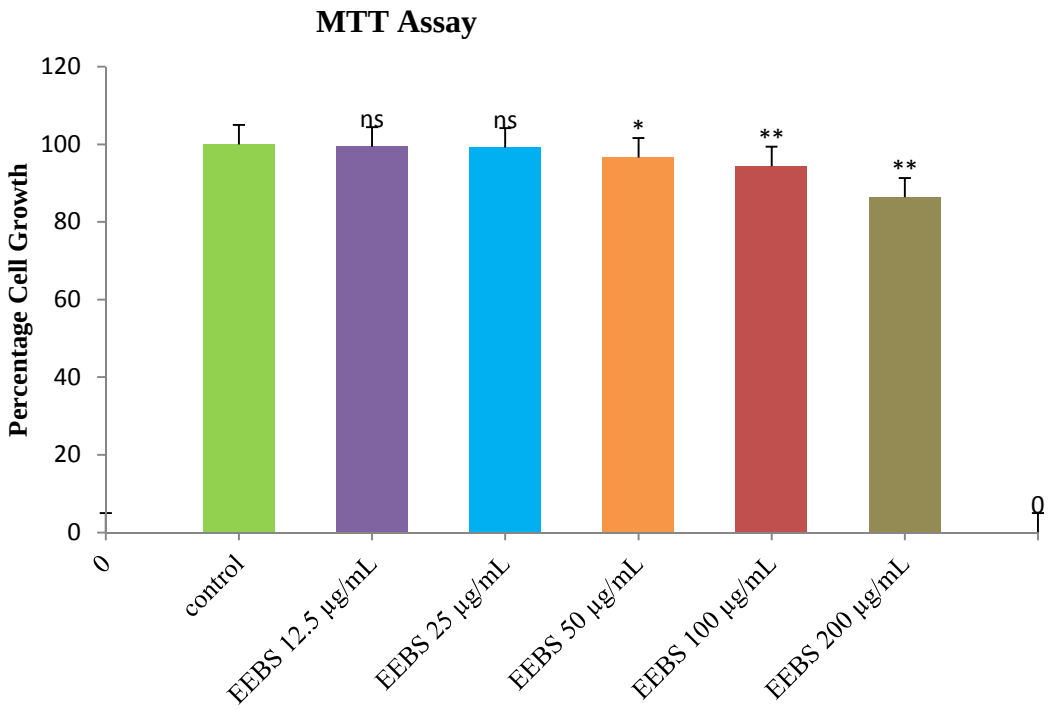
Cytotoxicity activity of various concentration of EEBS against human embryonic kidney 293 (HEK 293) cell lines were determined. Cells treated with control showed 100% cell viability. EBBS at dose of 12.5, 25, 50, 100, 200 µg/mL showed 99.39%, 99.16%, 96.59%, 94.40%, 86.34% of cell growth respectively. Significant ($P < 0.01$) cell growth inhibition was observed only on cells which were treated with higher concentration of EBBS, compared to control. Graph plotted with percentage of viable cells against the concentration showed a significant dose-dependent inhibition on cell growth (table 13, figure 21).

Table 13: Percentage of viable cell determination by MTT assay method

Name of test sample	Test Conc. (µg/mL)	Absorbance value	Cell growth* (%)
EEBS	12.5	0.319±0.005	99.395±1.76
	25	0.319 ±0.004	99.162±0.36
	50	0.310±0.008	96.593±2.93
	100	0.303±0.004	94.402±1.63
	200	0.274±0.003	86.34±1.45

*****Values are obtained from average of 3 determinants. EEBS-ethanol extract of *B. sensitivum*

Figure 21: **Cytotoxic effect of EEBS against HEK-293 cell line by MTT assay**



5.5.1.3. Lactate dehydrogenase (LDH) assay

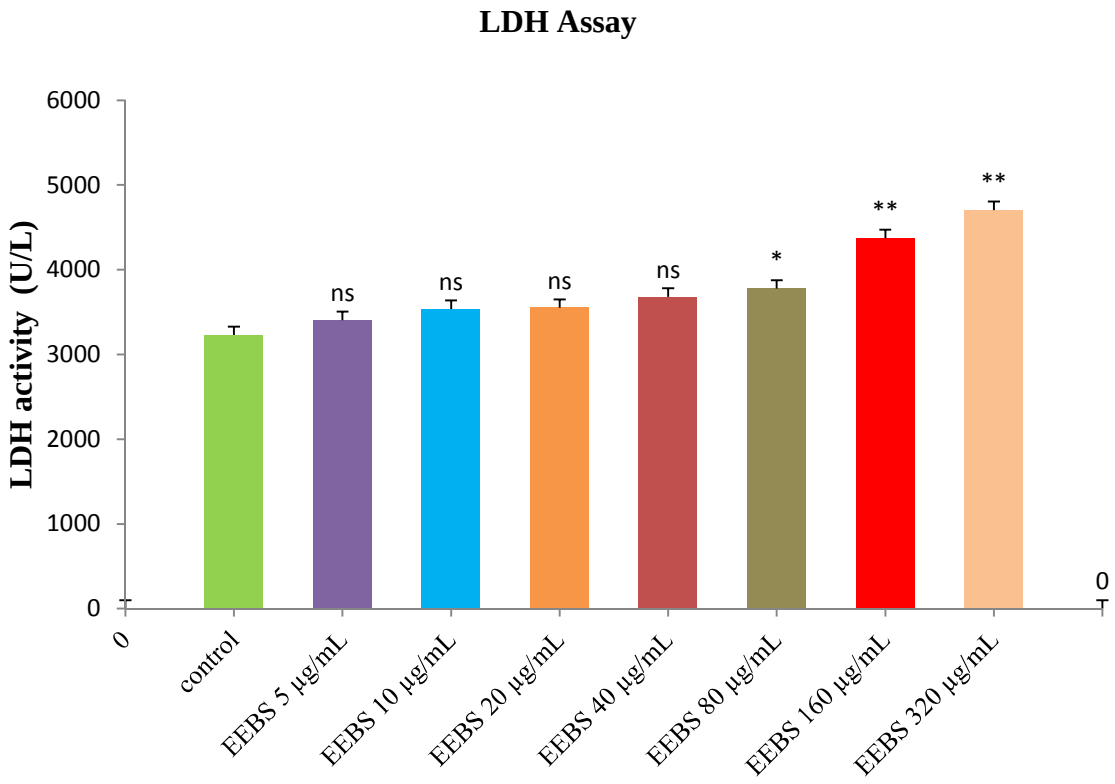
EEBS at different concentrations (5, 10, 20, 40, 80, 160, 320 µg/mL) were screened for its cytotoxic activity against A498 cell lines by lactate dehydrogenase assay. Cell lines treated with control showed no significant difference with that of mild concentration of EEBS (5, 10, 20, 40 and 80 µg/mL). EEBS treated cells at higher concentration (160 & 320 µg/mL) showed significant ($P<0.01$) increase in release of LDH. Release of lactate dehydrogenase due to the effect of different concentration of EEBS and control were shown in table 14 and figure 22.

Table 14: **Cytotoxic effect of EEBS against A498 cell line by lactate dehydrogenase assay**

Name of test sample	Test Conc. (µg/mL)	Absorbance value	LDH activity (U/L)*
EEBS	Control	0.2015±0.001	3230.05
	5	0.2125±0.024	3406.38
	10	0.2207±1.433	3537.82
	20	0.2215±3.333	3550.65
	40	0.2296±1.006	3680.49
	80	0.2357±2.432	3778.27
	160	0.2728±4.231	4372.98
	320	0.2935±0.008	4704.81

*****Values are obtained from average of 3 determinants. EEBS-ethanol extract of *B.sensitivum*; LDH- lactate dehydrogenase

Figure 22: Cytotoxic effect of EEBS against A498 cell line by lactate dehydrogenase assay



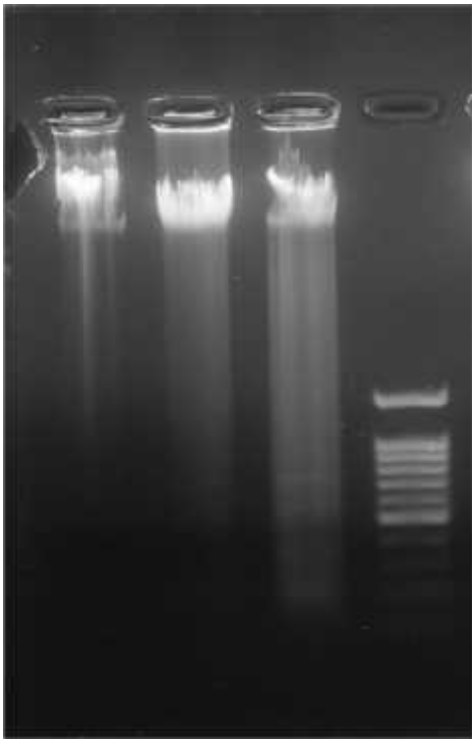
5.5.1.4. DNA fragmentation assay

DNA fragmentation was analyzed by gel electrophoresis. DNA laddering was observed when A498 cells were incubated with 160 and 320 µg/mL of EEBS for 24 hrs. (Lane 1, 2 and 3 stands for control, 160 µg/mL, and 320 µg/mL respectively. Lanes 4 stands for 100 bp DNA Marker). The figure depicted, EEBS treated A498 cells showing significant DNA damage that was observed as a ladder pattern by agarose gel electrophoresis. More evident DNA fragmentation was seen on A498 cells treated with EEBS at 320 µg/mL concentration when compared to control (culture without EEBS

treatment). DNA fragmentation on A498 cells treated with 160 µg/mL of EEBS was found to be less. Thus the result suggested that higher concentration of EEBS induces DNA fragmentation and persuades significant apoptotic activity (figure 23).

Figure 23: **Agarose gel electrophoresis of the chromosomal DNA of A498 cells.**

1 2 3 4



5.6. In vivo pharmacological studies

Nephroprotective activity of EEBS at concentration of 250 mg/kg (low dose) and 500 mg/kg (high dose) were investigated using various general, urinary, blood, serum and kidney homogenate parameters against gentamicin-induced nephrotoxicity in Wistar albino rats.

5.6.1. Gentamicin (GM)-induced nephrotoxicity

5.6.1.1. Effect of EEBS on general parameters in control and experimental animals

Table 15 illustrated the effect of EEBS on general parameters of GM-induced nephrotoxicity in control and experimental animals that were obtained at the end of the experiment in each group. Body weight was recorded before commencing the experiment. This results revealed that body weight of animals in group II were significantly ($p<0.01$) reduced after treatment, compared to control group, where as body weight of animals in group III ($p<0.001$), group IV ($p<0.01$) and group V ($p<0.01$) were significantly increased compared to animas in toxic control group. In GM-treated group, the kidney weight was found to be significantly increased ($p<0.001$) compared to normal control group. Significant reduction in kidney weight were observed in animals co-treated with low dose of EEBS ($p<0.01$), high dose of EEBS ($p<0.001$) and quercetin ($p<0.001$). 24-hr urine volume in the GM-treated group were found to be significantly ($p<0.01$) reduced in comparison to control group. Supplementation of quercetin, low dose of EEBS ($p<0.01$) and high dose of EEBS ($p<0.001$) to GM-treated rats increased urine output. However, water intake was significantly ($p<0.05$) increased only in animals co-administered with 500 mg/kg of EEBS. Urinary pH in GM-treated group was found to be 6.6 ± 0.04 which revealed no significant ($p>0.05$) changes in urinary pH compared to animals co-treated with extract/standard.

Table 15: Effect of EEBS on general parameters in GM-induced nephrotoxicity in rats

Parameters Studied (Unit)	Group I Normal control	Group II GM 100 mg/kg	Group III GM+QTN 50 mg/kg	Group IV GM+EEBS 250 mg/kg	Group V GM+EEBS 500 mg/kg
Change in body wt. (g)	3.06±0.23	1.50±0.11^{**a}	3.38± 0.14^{***b}	2.58± 0.17^{**b}	2.52± 0.22^{**b}
Kidney wt. (g)	0.64±0.00	0.97±0.05^{***a}	0.70±0.08^{***b}	0.73±0.00^{**b}	0.71±0.00^{***b}
Water intake (mL/24 hr)	12.35±0.57	14.53±0.76^{ns}	17.35±0.82^{ns}	17.35± 0.82^{ns}	17.87±0.86^{*b}
Urine volume (mL/24 hr)	7.73±0.14	5.74± 0.20^{**a}	7.62±0.29^{**b}	7.24±0.15^{**b}	7.88± 0.08^{***b}
Urine pH	6.8±0.05	6.6±0.04^{ns}	6.8±0.01^{ns}	6.7±0.06 ^{ns}	6.8±0.06 ^{ns}

Ethanol extract of *B. sensitivum*, GM: gentamicin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared o control group (group I), ^{b}significant compared with GM-induced group (group II), ^{ns} not significant.

5.6.1.2. Effect of EEBS on urinary total protein and albumin levels in experimental rats

GM-treated rats showed a marked increase in urinary protein and albumin excretion (p<0.01). Animals co-treated with EEBS/quercetin significantly (P<0.01) reduced the incidence of proteinuria, however, significant (P<0.01) reduction in albuminuria were observed only in rats co-treated with quercetin or high dose of EEBS, compared to group II rats (table 16).

Table 16: Effect of EEBS on urinary total protein and albumin levels in GM-induced nephrotoxicity in rats

Urinary parameters (Unit)	Group I Normal control	Group II GM 100 mg/kg	Group III GM+QTN 50 mg/kg	Group IV GM+EEBS 250 mg/kg	Group V GM+EEBS 500 mg/kg
Total protein (g/dL)	3.65 ±0.01	4.52±0.02^{**a}	3.55±0.01^{**b}	4.05±0.04^{**b}	3.53±0.05^{**b}
Albumin (g/dL)	0.77±0.01	0.84±0.06^{**a}	0.63±0.01^{**b}	0.81±0.08^{ns}	0.71±0.00^{**b}

EEBS: Ethanol extract of *B. sensitivum,* GM: gentamicin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with GM-induced group (group II), ^{ns} not significant

5.6.1.3. Effect of EEBS on urinary electrolyte levels in experimental rats

Urinary excretion of calcium and magnesium were found to be much higher (p<0.01) in group II animals, compared to control group. Co-treatment with EEBS at 250 mg/kg (p<0.01), quercetin (p<0.001) and EEBS at 500 mg/kg (p<0.001) significantly decreased urinary calcium excretion compared to GM-treated animals. Urinary magnesium excretion were significantly (p<0.01) lowered in animals co-administered with standard/ extract. However, significant differences were not observed in sodium and potassium levels among animals in different groups (table 17).

Table 17: Effect of EEBS on urinary electrolyte levels in GM-induced nephrotoxicity

Urinary parameters (Unit)	Group I Normal control	Group II GM 100 mg/kg	Group III GM+QTN 50 mg/kg	Group IV GM+EEBS 250 mg/kg	Group V GM+EEBS 500 mg/kg
Sodium (mmol/L)	74.16±1.86	75.16±1.87^{ns}	71.66±0.33^{ns}	74.33±1.92^{ns}	74.30±1.49 ^{ns}
Potassium (mmol/L)	3.20±0.02	3.21±0.02^{ns}	3.20±0.02^{ns}	3.14±0.01^{ns}	3.21±0.02^{ns}
Calcium (mg/dL)	8.93±0.01	10.90± 0.01^{**a}	6.47±0.02^{***b}	8.05±0.05^{**b}	7.81±0.06^{***b}
Magnesium (mg/dL)	0.78±0.01	0.83±0.07^{**a}	0.76±0.04^{**b}	0.79±0.05^{**b}	0.77±0.06^{**b}

EEBS: Ethanol extract of *B. sensitivum,* GM: gentamicin, QTN: quercetin.Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a}significant compared with control group, ^{b}significant compared with GM-induced group, ^{ns}not significant.

5.6.1.4. Effect of EEBS on hematological parameters in experimental rats

Table 18 demonstrated that hemoglobin (Hb), packed cell volume ($p<0.001$), red blood cells (RBCs) and mean corpuscular hemoglobin (MCH) levels were significantly ($p<0.01$) lowered in rats treated with GM than those in the control group. White blood corpuscles ($p<0.01$) and polymorphs ($p<0.001$) were found to be significantly elevated in rats in group II, compared to animals in group I. Results revealed that the animals co-treated with standard/extract caused a significant ($p<0.01$) increase in RBCs, haemoglobin and MCH level. Packed cell volume (PCV) were found to be increased only in animals treated with quercetin/high dose of EEBS ($p<0.01$). Total WBC and polymorphs were found to be significantly reduced in animals co-treated with standard/ extract ($p<0.01$).

Table 18: Effect of EEBS on hematological parameters in GM-induced nephrotoxicity

Parameters studied (Unit)	Group I Normal control	Group II GM 100 mg/kg	Group III GM+QTN 50 mg/kg	Group IV GM+EEBS 250 mg/kg	Group V GM+EEBS 500 mg/kg
RBC (million/mm^3)	7.35±0.14	6.81±0.31**a	7.22±0.21**b	6.85±0.31**b	7.10±0.43**b
HB (g/dL)	14.11± 0.28	11.76± 0.58***a	14.02±0.13**b	13.96±0.21**b	13.83±0.31**b
PCV (%)	43.66±0.52	38.27±0.81***a	39.58±0.61**b	38.29±0.42ns	38.53±0.20**b
MCH (pg)	20.67±0.12	20.19±0.69**a	20.84±0.27**b	20.45±0.49**b	20.67±0.51**b
WBC ($1X10^3/mm^3$)	8.22± 0.41	8.43±0.33**a	8.21±0.41**b	8.24±0.21**b	8.15±0.10**b
Lymphocytes (%)	60.05± 0.01	59.96 ±0.08 ns	59.97±0.01 ns	59.96±0.02ns	59. 96±0.05 ns
Monocytes (%)	4.97±0.00	5.00±0. 01 ns	4.96±0.01 ns	4.97± 0.06 ns	4.96±0.06 ns
Polymorphs (%)	12.22±0.21	15.84 ±0.11***a	14.65±0.32**b	14.19±0.20**b	14.05±0.11**b
Eosinophils (%)	1.93±0.01	2.02±0.01 ns	1.97±0.08 ns	1.97±0.02 ns	1.96±0.01 ns

EEBS: Ethanol extract of *B. sensitivum*, GM: gentamicin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001, [a]significant compared with control group, [b]significant compared with GM-induced group, [ns]not significant.

5.6.1.5. Effect of EEBS on serum total protein and albumin levels in experimental rats

Serum total protein and albumin level ($p<0.05$), were found to be significantly decreased in GM-treated animals, compared to rats in group I. However, significant differences ($p>0.05$), were not observed on *p.o.* administration of 250 and 500 mg/kg of EEBS in group IV & V, whereas rats in group III showed significant change in total protein ($p<0.05$) and albumin ($p<0.01$) level, compared to the animals in group II (table 19).

Table 19: Effect of EEBS on serum total protein and albumin levels in GM-induced nephrotoxicity in rats

Serum parameters (Unit)	Group I Normal control	Group II GM 100 mg/kg	Group III GM+QTN 50 mg/kg	Group IV GM+EEBS 250 mg/kg	Group V GM+EEBS 500 mg/kg
Total protein (g/dL)	7.18±0.01	7.14±0.05^{*a}	7.18±0. 09^{*b}	7.16±0.09^{ns}	7.17±0.07^{ns}
Albumin (g/dL)	4.36±0.00	4.33±0.04^{*a}	4.37±0.00^{**b}	4.34±0.00^{ns}	4.35±0.03^{ns}

EEBS: Ethanol extract of *B. sensitivum,* GM: gentamicin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with GM-induced group (group II), ^{ns} not significant

5.6.1.6. Effect of EEBS on serum electrolyte levels in experimental rats

Sodium, magnesium ($p<0.01$) and calcium ($p<0.001$) levels were found to be significantly decreased in GM-treated animals, compared to group 1 animals. Co-administration of standard/ extract significantly attenuated ($p<0.01$) changes in calcium and magnesium levels, however, no significant differences ($p>0.05$) were observed in sodium and potassium levels, compared to group II rats (table 20).

Table 20: Effect of EEBS on serum electrolyte levels in GM-induced nephrotoxicity in rats

Serum parameters (Unit)	Group I Normal control	Group II GM 100 mg/kg	Group III GM+QTN 50 mg/kg	Group IV GM+EEBS 250 mg/kg	Group V GM+EEBS 500 mg/kg
Sodium (mmol/L)	138.7±0.07	138.1±0.05^{**a}	138.5±0.15^{ns}	138.3±0.11^{ns}	138.5±0.14^{ns}
Potassium(mmol/L)	5.75±0.00	5.75±0.07	5.73±0.07^{ns}	5.76±0.01^{ns}	5.78±0.01^{ns}
Calcium (mg/dL)	10.60±0.13	8.17±0.00^{***a}	9.04±0.00^{**b}	9.25±0.08^{**b}	9.43±0.06^{**b}
Magnesium(mg/dL)	2.44±0.01	2.23±0.02^{**a}	2.54±0.01^{**b}	2.34±0.09^{**b}	2.44±0.01^{**b}

EEBS: Ethanol extract of *B. sensitivum,* GM: gentamicin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with GM-induced group (group II), ^{ns} not significant.

5.6.1.7. Effect of EEBS on serum BUN, creatinine and uric acid levels in rats

Group II animals treated with GM demonstrated a significant (p<0.001) elevation in the serum levels of creatinine, uric acid and blood urea nitrogen (BUN). Co-treatment with quercetin significantly (p<0.001) decreased creatinine, uric acid and BUN, compared to group II rats. Animals co-administered with extract significantly (p<0.01) decreased creatinine, and uric acid. BUN levels were significantly lowered in animals co-treated with extract at low concentration (p<0.01) and high concentration (P<0.001), compared to toxic control group (table 21).

Table 21: Effect of EEBS on serum BUN, creatinine and uric acid levels in GM-induced nephrotoxicity in rats

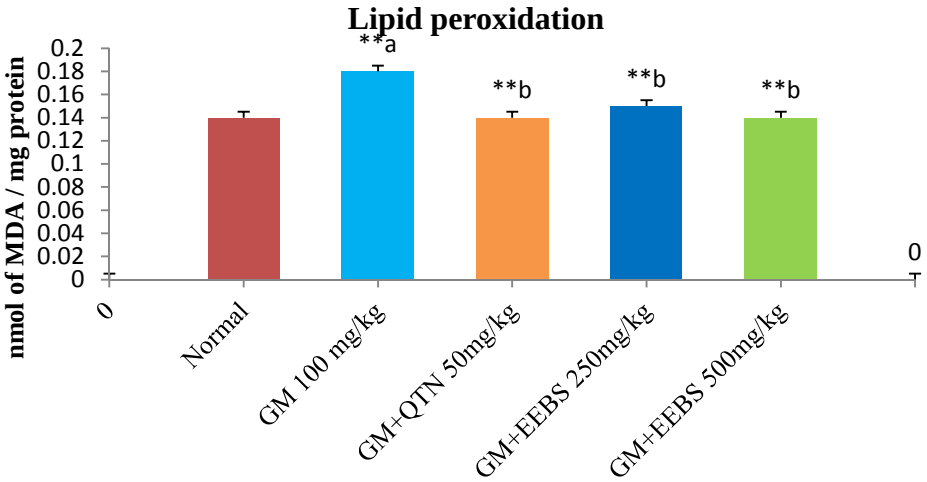
Serum Parameters (Unit)	Group I Normal control	Group II GM 100 mg/kg	Group III GM+QTN 50 mg/kg	Group IV GM+EEBS 250 mg/kg	Group V GM+EEBS 500 mg/kg
BUN (mg/dL)	15.83±0.19	28.89±0.02^{***a}	12.13±0.02^{***b}	18.92±0.01^{**b}	14.74±0.04^{***b}
Creatinine (mg/dL)	0.68±0.00	1.98±0.02^{***a}	0.81±0.00^{***b}	1.51±0.01^{**b}	0.94±0.01^{**b}
Uric acid (mg/dL)	2.13±0.08	3.22±0.01^{***a}	2.03±0.01^{***b}	3.04±0.08^{**b}	2.95±0.05^{**b}

EEBS: Ethanol extract of *B. sensitivum,* GM: gentamicin, QTN: quercetin, BUN: blood urea nitrogen. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with GM-induced group (group II), ^{ns} not significant.

5.6.1.8. Effect of EEBS on markers of oxidation in control and experimental animals

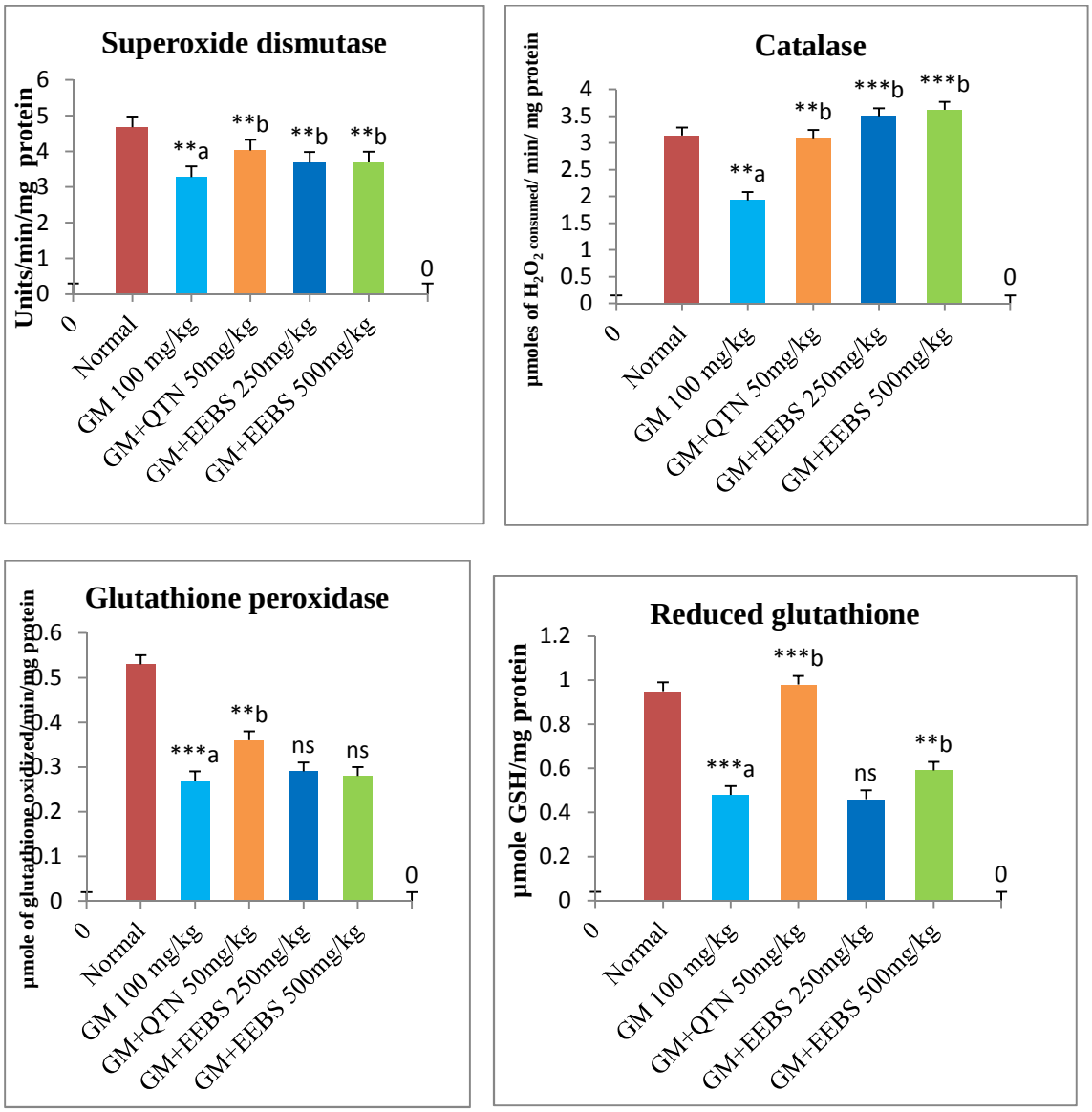
This study also revealed the association of GM-induced oxidative stress and nephrotoxicity**.** Antioxidant status in rats exposed to GM showed a significant diminution of activity of superoxide dismutase (SOD), catalase ($p<0.01$), reduced glutathione (GSH) and glutathione peroxidise ($p<0.001$), compared to group I animals. Co-administration of quercetin to group III rats showed a significant elevation of SOD, CAT, GPx ($p<0.01$) and GSH ($p<0.001$) activity. EEBS at dose level of 250 mg/kg to group IV and dose level of 500 mg/kg to group V significantly increased the SOD ($p<0.01$) and catalase ($p<0.001$). GSH level were significantly increased only in rats co-administerd with high dose of extract, however, no significant differences were observed in GPx activity in animals co-treated with extract, compared to animals in group II. A significant ($p<0.01$) increase in production of malondialdehyde (MDA) levels were observed in group II toxic control animals compared to group I rats. Co-administration of quercetin/EEBS significantly ($p<0.01$) decreased MDA levels (figure 24 & 25).

Figure 24: Effect of EBS on lipid peroxidation in GM-induced nephrotoxicity



EEBS: Ethanol extract of *B. sensitivum*, GM: gentamicin, QTN: quercetin. Units: LPO (nmol of MDA formed/ mg protein). Values are expressed in mean ± standard error of mean (n=6), p<0.05, **p<0.01, ***p<0.001 [a] significant compared with control group (group I), [b]significant compared with GM-induced group (group II), [ns] not significant.

Figure 25: Effect of EEBS on oxidative stress parameters in GM-induced nephrotoxicity in rats



EEBS: Ethanol extract of *B. sensitivum*, GM: gentamicin, QTN: quercetin. a) superoxide dismutase b) catalase c) glutathione peroxidase d) reduced glutathione.; Values are expressed in mean ± standard error of mean (n=6), p<0.05, **p<0.01, ***p<0.001 [a] significant compared with control group (group I), [b]significant compared with GM-induced group (group II), [ns] not significant

5.6.2. Cisplatin (CDDP)-induced nephrotoxicity

Renoprotective activity of EEBS at concentration of 250 and 500 mg/kg were determined using different general, urinary, blood, serum and kidney homogenate parameters against cisplatin- induced nephrotoxicity in Wistar albino rats.

5.6.2.1. Effect of EEBS on general parameters in control and experimental animals

Table 22 illustrated the effect of EEBS on general parameters of CDPP-induced nephrotoxicity in control and experimental animals that were obtained at the end of the experiment. Body weight was recorded before starting the experiment. After treatment period, body weight was significantly reduced in rats in CDDP-treated group ($p<0.001$), compared to animals in control group. Significant weight gain was observed in animals co-treated with quercetin ($p<0.001$), high dose of EEBS ($p<0.001$) and low dose of EEBS ($p<0.01$) compared to animals in the CDDP-treated group. Kidney weight was significantly increased in CDDP-treated group ($p<0.05$) compared to normal control group. Significant reduction in kidney weight were observed in animals in group III ($p<0.01$), group IV ($p<0.05$) and group V ($p<0.01$), compared to rats in group II. 24 hr urine volume were measured in the CDDP-treated group and found to be significantly ($p<0.01$) increased in comparison to control group. However, there were no significant ($p>0.05$) change in water intake and urine volume in groups supplemented with quercetin /EEBS, compared to group II animals. This result also revealed a significant change in urine pH in rats co-treated with extract ($p<0.01$) or standard ($p<0.001$), compared to rats in CDDP-treated group.

Table 22: Effect of EEBS on general parameters in CDDP-induced nephrotoxicity in rats

Parameters Studied (Unit)	Group I Normal control	Group II CDDP 8 mg/kg	Group III CDDP+QTN 50 mg/kg	Group IV CDDP+EEBS 250 mg/kg	Group V CDDP+EEBS 500 mg/kg
Change in body wt.(g)	3.56±0.98	1.92±0.87^{***a}	3.74±3.44^{***b}	3.34±2.50^{**b}	3.72±2.16^{***b}
Kidney wt.(g)	0.85±0.01	0.89±0.01^{*a}	0.76±0.01^{**b}	0. 83±0.00^{*b}	0.81±0.01^{**b}
Water intake (mL/24 hr)	10.54±0.45	11.00±0.21^{ns}	10.56±0.29^{ns}	10.78±0.22^{ns}	10.76±0.07^{ns}
Urine volume (mL/24 hr)	7.82±0.25	8.84± 0.09 ^{**a}	8.40±0.30^{ns}	8.35±0.14^{ns}	8.23± 0.13^{ns}
Urine pH	6.80± 0.10	5.93±0.12^{**a}	6.95±0.05^{***b}	6.80±0.081^{**b}	6.86±0.088^{**b}

EEBS: Ethanol extract of *B. sensitivum,* CDDP: cisplatin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 [a] significant compared with control group (group I), [b] significant compared with CDDP-induced group (group II), [ns] not significant.

5.6.2.2. Effect of EEBS on urinary total protein and albumin levels in experimental rats

CDDP-treated rats showed a marked increase in urinary total protein excretion (p<0.05). Concurrent administration of EEBS (500 mg/kg)/ standard significantly (p<0.01) reduced the incidence of proteinuria. Lowering of albumiuria were observed in rats co-treated with high dose of EEBS/quercetin (p<0.05), compared to CDDP-treated rats (table 23).

Table 23: Effect of EEBS on urinary total protein and albumin levels in CDDP-induced nephrotoxicity in rats

Urinary parameters (Unit)	Group I Normal control	Group II CDDP 8 mg/kg	Group III CDDP+QTN 50 mg/kg	Group IV CDDP+EEBS 250 mg/kg	Group V CDDP+EEBS 500 mg/kg
Total protein (g/dL)	1.41±0.02	1.79±0.12^{*a}	1.13±0.05^{**b}	1.50±0.10^{ns}	1.16±0.10^{**b}
Albumin (g/dL)	0.39±0.01	0.40±0.07^{ns}	0.37±0.02^{*b}	0.39±0.05^{ns}	0.38±0.03^{*b}

EEBS: Ethanol extract of *B. sensitivum,* CDDP: cisplatin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 [a] significant compared with control group (group I), [b]significant compared with CDDP-induced group (group II), [ns] not significant.

5.6.2.3. Effect of EEBS on urinary electrolyte levels in experimental rats

Urinary excretion of calcium ($p<0.001$), magnesium ($p<0.05$) and potassium ($p<0.01$) levels were found to be significantly higher in group II animals compared to control group. Significant ($p<0.01$) reduction in urinary excretion of electrolyte levels were observed in group treated with quercetin. Concurrent administration with extract significantly ($P<0.01$) lowered urinary calcium excretion, however, there were no significant ($P>0.05$) differences in urinary excretion of sodium, potassium and magnesium levels, compared to nephrotoxic control group (table 24).

Table 24: Effect of EEBS on urinary electrolyte levels in CDDP-induced nephrotoxicity

Urinary parameters (Unit)	Group I Normal control	Group II CDDP 8 mg/kg	Group III CDDP+QTN 50 mg/kg	Group IV CDDP+EEBS 250 mg/kg	Group V CDDP+EEBS 500 mg/kg
Sodium (mmol/L)	51.28±0.02	51.34±0.01^{ns}	50.75±0.02^{**b}	51.29±0.02^{ns}	51.31±0.02^{ns}
Potassium (mmol/L)	3.20±0.00	3.26±0.01^{**a}	3.13±0.00^{**b}	3.23±0.07^{ns}	3.22±0.01^{ns}
Calcium (mg/dL)	15.53±0.03	25.16± 0.01^{***a}	18.18±0.02^{**b}	18.94±0.01^{**b}	18.78±0.24^{**b}
Magnesium (mg/dL)	1.34±0.01	1.36±0.00^{*a}	1.33±0.09^{**b}	1.34±0.08^{ns}	1.34±0.08^{ns}

EEBS: Ethanol extract of *B. sensitivum*, CDDP: cisplatin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with CDDP-induced group (group II), ^{ns} not significant.

5.6.2.4. Effect of EEBS on hematological parameters in experimental rats

Table 25 illustrated the effect of EEBS on haematological parameters in control and experimental animals. Total white blood cells (WBC), monocytes and polymorphs were significantly ($p<0.01$) elevated in CDDP-treated animals compared to control group. Co-treatment with quercetin/EEBS significantly ($p<0.01$) decreased total WBC. Polymorphs level were significantly reduced only in animals treated with standard

($p<0.01$) or high dose of extract ($p<0.05$). Significant reduction in total red blood cells (RBCs), hemoglobin (Hb), mean corpuscular hemoglobin ($p<0.01$), packed cell volume (PCV), lymphocytes ($p<0.001$) levels were observed in animals treated with CDDP, in comparison with control group. Animals co-administered with quercetin/ EEBS significantly increased total RBC, PCV and Hb levels ($p<0.01$), however, MCH levels were elevated only in animals in group III and V ($p<0.01$), compared to toxic control animals (table 25).

Table 25: Effect of EEBS on hematological parameters in CDDP-induced nephrotoxicity

Parameters Studied (Unit)	Group I Normal control	Group II CDDP 8 mg/kg	Group III CDDP+QTN 50 mg/kg	Group IV CDDP+EEBS 250 mg/kg	Group V CDDP+EEBS 500 mg/kg
RBC (million/mm^3)	6.81±0.27	5.89±0.32**a	6.56±0.22**^{b}	6.85±0.41**^{b}	6.86±0.12**^{b}
HB (g/dL)	12.22± 0.49	11.61± 0.12**^{a}	13.25±1.08**^{b}	13.77±0.45**^{b}	13.62±0.31**^{b}
PCV (%)	44.38±0.62	32.38± 0.48***^{a}	39.51±0.37**^{b}	38.29±0.31**^{b}	38.22±0.52**^{b}
MCH (pg)	23.62±0.12	22.85±0.13**^{a}	23.79±0.09**^{b}	22.64±0.08^{ns}	23.82±0.09**^{b}
WBC ($1X10^3/mm^3$)	10.46± 0.33	10.69±0.31**^{a}	10.46±0.32**^{b}	10.25±0.52**^{b}	10.08±0.51**^{b}
Lymphocytes (%)	81.02± 0.42	75.32±0.31***^{a}	77.32±0.32**^{b}	75.37±0.24^{ns}	75.33±0.42^{ns}
Monocytes (%)	5.89±0.03	5.98±0.03**^{a}	5.91±0.01**^{b}	5.95±0.01^{ns}	5.94±0.02^{ns}
Polymorphs (%)	12.82±0.22	12.92 ±0.11**^{a}	12.22±0.30**^{b}	12.86±0.11^{ns}	12.85±0.21*^{b}
Eosinophils (%)	2.33±0.01	2.38±0.00^{ns}	2.31±0.01*^{b}	2.32±0.02^{ns}	2.33±0.01^{ns}

EEBS: Ethanol extract of *B. sensitivum,* CDDP: cisplatin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *$p<0.05$, **$p<0.01$, ***$p<0.001$ ^{a}significant compared with control group (group I), ^{b}significant compared with CDDP-induced group (group II), ^{ns} not significant.

5.6.2.5. Effect of EEBS on serum total protein and albumin levels

Significant difference in protein and albumin levels were not observed in animals in CDDP-treated group in comparison with the control group. However, concurrent administration of quercetin /high dose of EEBS ($p<0.05$) showed signifnicant elevation in serum total protein level. Marked elevation in albumin levels were observed in rats treated with standard ($p<0.01$), but not in animals treated with EEBS, compared to toxicity induced rats in group II (table 26).

Table 26: Effect of EEBS on serum total protein and albumin levels in CDDP-induced nephrotoxicity in rats

Serum parameters (Unit)	Group I Normal control	Group II CDDP 8 mg/kg	Group III CDDP+QTN 50 mg/kg	Group IV CDDP+EEBS 250 mg/kg	Group V CDDP+EEBS 500 mg/kg
Total protein (g/dL)	3.39±0.01	3.38±0.01^{ns}	3.41±0.08^{*b}	3.40±0.07^{ns}	3.42±0.07^{*b}
Albumin (g/dL)	1.38±0.00	1.37±0.00^{ns}	1.41±0.01^{**b}	1.38±0.04^{ns}	1.39±0.04^{ns}

EEBS: Ethanol extract of *B. sensitivum,* CDDP: cisplatin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with CDDP-induced group (group II), ^{ns} not significant.

5.6.2.6. Effect of EEBS on serum electrolyte levels in experimental rats

Sodium, potassium, and calcium levels (p<0.01) were found to be significantly decreased in CDDP-treated animals. Co-administration of quercetin, significantly increased serum electrolyte levels except magnesium level. Significant (p<0.01) change in calcium levels were observed in group IV and V animals, whereas serum sodium levels were found to be significantly (p<0.01) increased in rats co-treated with 500 mg/kg of EEBS. No significant differences (p>0.05) were observed in potassium and magnesium levels in group IV and V rats, compared to animals in group II (table 27).

Table 27: Effect of EEBS on serum electrolyte levels in CDDP-induced nephrotoxicity

Serum parameters (Unit)	Group I Normal control	Group II CDDP 8 mg/kg	Group III CDDP+QTN 50 mg/kg	Group IV CDDP+EEBS 250 mg/kg	Group V CDDP+EEBS 500 mg/kg
Sodium (mmol/L)	125.58±1.67	106.05±2.06^{**a}	125.84±1.5^{***b}	109.06±1.37^{ns}	120.80±1.48^{**b}
Potassium (mmol/L)	7.18±0.01	6.12±0.05^{**a}	6.48±0.02^{**b}	6.09±0.08^{ns}	6.13±0.07^{ns}
Calcium (mg/dL)	17.20±0.00	12.61± 0.01^{**a}	15.70±0.01^{**b}	15.03±0.01^{**b}	15.84±0.04^{**b}
Magnesium (mg/dL)	4.35±0.05	4.35±0.01	4.38±0.09	4.36±0.09^{ns}	4.38±0.04^{ns}

EEBS: Ethanol extract of *B. sensitivum,* CDDP: cisplatin, QTN: quercetin. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with CDDP-induced group (group II), ^{ns} not significant.

5.6.2.7. Effect of EEBS on serum BUN, creatinine and uric acid levels in experimental rats

Group II animals treated with CDDP, demonstrated a significant elevation in the serum levels of creatinine, uric acid ($p<0.01$) and BUN ($p<0.001$). Co-treatment with quercetin/extract (group III, IV, &V) significantly ($p<0.01$) decreased BUN and creatinine levels. However, significant decrease in uric acid levels were observed only in group co-treated with quercetin ($p<0.01$), compared to animals in group II (table 28).

Table 28: Effect of EEBS on serum levels of BUN, creatinine and uric acid in CDDP-induced nephrotoxicity in rats

Serum Parameters (Unit)	Group I Normal control	Group II CDDP 8 mg/kg	Group III CDDP+QTN 50 mg/kg	Group IV CDDP+EEBS 250 mg/kg	Group V CDDP+EEBS 500 mg/kg
BUN (mg/dL)	30.54±0.01	39.97±0.00^{***a}	35.53±0.02^{**b}	38.69±0.00^{**b}	37.92±0.07^{**b}
Creatinine (mg/dL)	1.04±0.01	2.55±0.01^{**a}	1.09±0.007^{*b}	0.96±0.01^{**b}	0.93±0.08^{**b}
Uric acid (mg/dL)	2.31±0.02	3.15±0.01^{**a}	2.36±0.01^{**b}	3.13±0.01^{ns}	3.15±0.02^{ns}

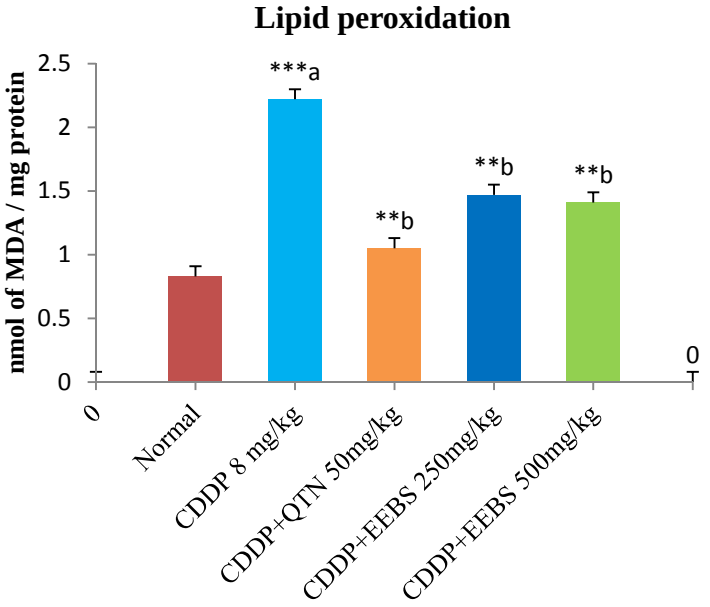
EEBS: Ethanol extract of *B. sensitivum*, CDDP: cisplatin, QTN: quercetin, BUN: blood urea nitrogen. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with CDDP-induced group (group II), ^{ns} not significant.

5.6.2.8. Effect of EEBS on markers of oxidation in control and experimental animals

This result also confirmed the link between CDDP- induced oxidative stress and nephrotoxicity**.** Antioxidant status in rats exposed to CDDP showed a significant diminution of activity of SOD, CAT ($p<0.01$), GSH and GPx ($p<0.001$), compared to group I animals. Administration of EEBS to animals induced with nephrotoxicity significantly increased SOD activity ($p<0.01$). Evaluation of catalase activity revealed that rats co-treated with low dose of extract ($p<0.01$) and high dose of extract ($p<0.001$) presented significant elevation, however, significant ($p<0.01$) improvement in GPx levels were observed only in rats co-treated with high dose of extract. GSH levels were also

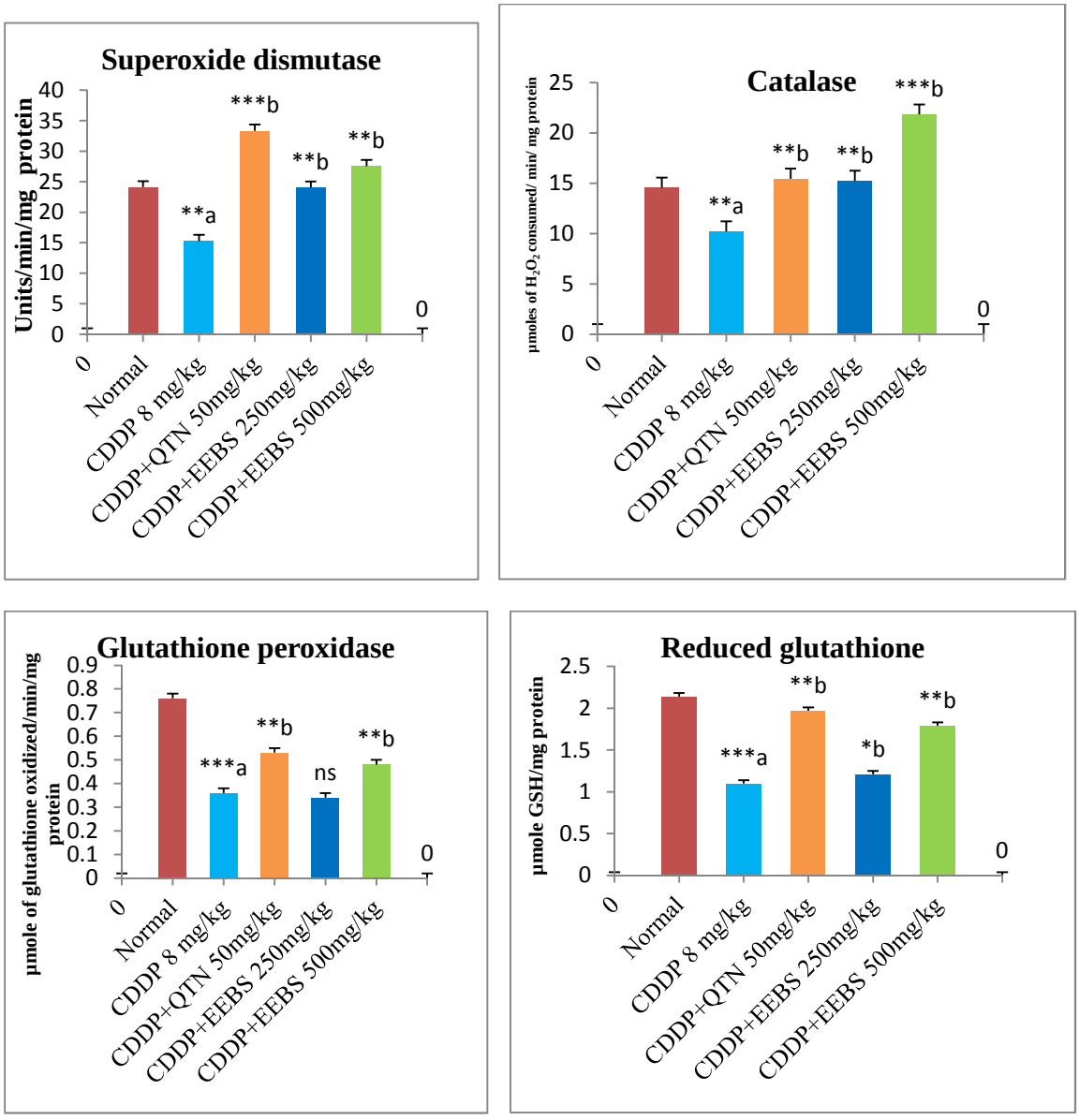
significantly improved in rats co-administered with 250 mg/kg of extract ($p<0.05$) and 500 mg/kg of extract ($p<0.01$). Rats co-administered with standard significantly restored depleted levels of SOD ($p<0.001$), CAT, GSH and GPx ($p<0.01$). A significant ($p<0.001$) increase in production of MDA were observed in group II toxic control animals compared to group I normal animals. Co-administration with standard to group III, EEBS to group IV and group V animals significantly ($p<0.01$) decreased MDA levels (figure 26 &27).

Figure 26: Effect of EEBS on lipid peroxidation in CDDP-induced nephrotoxicity in rats



EEBS: Ethanol extract of *B. sensitivum*, CDDP: cisplatin, QTN: quercetin. Units: LPO (nmol of MDA formed/ mg protein).Values are expressed in mean ± standard error of mean (n=6), $p<0.05$, **$p<0.01$, ***$p<0.001$ [a] significant compared with control group (group I), [b]significant compared with CDDP-induced group (group II), [ns] not significant

Figure 27: Effect of EEBS on oxidative stress parameters in CDDP-induced nephrotoxicity in rats



EEBS: Ethanol extract of *B. sensitivum*, CDDP: cisplatin, QTN: quercetin. a) superoxide dismutase b) catalase c) glutathione peroxidase d) reduced glutathione. Values are expressed in mean ± standard error of mean (n=6), $p<0.05$, **$p<0.01$, ***$p<0.001$ [a] significant compared with control group (group I), [b]significant compared with CDDP-induced group (group II), [ns] not significant

5.6.3. Ethylene glycol (EG)-induced urolithiasis

Antiurolithic activity of EEBS at concentration of 250 and 500 mg/kg, were determined using different general, urinary, blood, serum and kidney homogenate parameters against ethylene glycol-induced urolithiais in Wistar albino rats.

5.6.3.1. Effect of EEBS on general parameters in control and experimental animals

Table 29 depicted the effect of EEBS on general parameters of EG-induced urolithiasis in control and experimental animals that were obtained at the end of the experiment in each group. Body weight was measured before starting the experiment and other general parameters were recorded on 28^{th} day during the experimental period. Significant (P<0.001) weight reduction were observed in group II animals (toxic control) compared to control group. This results confirmed that there was significant (P< 0.001) gain in body weight in animals treated with EG+Cystone (group III), EG+ EEBS (group IV and V), compared to calculi-induced group. Kidney weight was significantly increased in group II rats (p<0.001) compared to normal control group. Significant kidney weight reduction were observed in animals in group III, IV (p<0.01) and V (p<0.001), compared to group II rats. Water intake and urine output in EG-treated group were significantly (p<0.01) reduced. Urine volume was significantly higher in group co-treated with standard, low dose of EEBS, (P<0.01) or high dose of EEBS (P< 0.001). No significant (p>0.05) change in water intake were observed in rats treated with extract, however significant (P<0.01) improvement was observed in rats co-treated with standard, compared to animals in group II. Urine pH was significantly (P<0.01) elevated in calculi-induced rats, compared to animals in group I. The urine pH were reduced significantly (p<0.01) in extract/Cystone-treated rats in comparison with group II rats.

Table 29: Effect of EEBS on general parameters in EG-induced urolithiasis in rats

Parameters studied (Unit)	Group I Normal control	Group II EG 0.75% v/v	Group III EG+Cystone 750 mg/kg	Group IV EG+EEBS 250 mg/kg	Group V EG+EEBS 500 mg/kg
Change in body wt. (g)	5.50±1.02	2.60±0.87^{***a}	5.25±2.94^{***b}	4.81±1.26^{***b}	4.90±1.92^{***b}
Kidney wt. (g)	0.63±0.01	1.06±0.01^{***a}	0.71±0.00^{**b}	0.82±0.00^{**b}	0.69±0.02^{***b}
Water intake (mL/24 hr)	17.67±0.02	17.22±0.01^{**a}	18.62±0.16^{**b}	17.45± 0.04^{ns}	17.50±0.01^{ns}
Urine volume (mL/24 hr)	17.83±0.00	10.83± 0.08^{**a}	16.40±0.00^{**b}	17.98±0.00^{**b}	21.85±0.08^{***b}
Urine pH	6.8±0.05	8.2±0.11^{**a}	6.86±0.11^{**b}	7.10±0.05^{**b}	6.90±0.10^{**b}

EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a}significant compared with control group (group I), ^{b}significant compared with EG-induced group (group II), ^{ns}not significant.

5.6.3.2. Effect of EEBS on urinary total protein and albumin levels in experimental rats

Total protein content in urine increased significantly (p<0.001) in EG-induced group, compared to animals in control group. Oral administration of 250 and 500 mg/kg of EEBS (p<0.01) and cystone (p<0.001) attenuated changes in proteinuria. However, there were no significant (p>0.05) change in urinary albumin levels among animals treated with EG, EG+Cystone or EG+EEBS (table 30).

Table 30: Effect of EEBS on urinary total protein and albumin levels in EG-induced urolithiasis in rats

Urinary parameter (Unit)	Group I Normal control	Group II EG 0.75% v/v	GroupIII EG+Cystone 750 mg/kg	Group IV EG+EEBS 250 mg/kg	Group V EG+EEBS 500 mg/kg
Total protein (g/dL)	2.02±0.07	5.13±0.08^{***a}	2.45±0.01^{***b}	3.38±0.00^{**b}	3.03±0.00^{**b}
Albumin (g/dL)	1.02±0.00	1.01±0.07^{ns}	1.04±0.00^{ns}	1.01±0.01^{ns}	1.02±0.02^{ns}

EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with EG-induced group (group II), ^{ns} not significant.

5.6.3.3. Effect of EEBS on urinary electrolyte levels in experimental rats

Urinary calcium and phosphate excretion were significantly ($p<0.001$) elevated in EG-induced group compared to control. Phosphate levels ($p<0.01$) were found to be increased only in group III rats, whereas, concurrent administration with Cystone /extract ($p<0.01$) attenuated changes in calcium levels. Magnesium excretion were significantly ($p<0.05$) reduced in EG-treated group compared to control group. Co-treatment with Cystone at the dose of 750 mg/kg ($p<0.01$)/ EEBS at 500 mg/kg ($p<0.001$) significantly increased magnesium excretion, compared to lithiatic control group (table 31).

Table 31: Effect of EEBS on urinary electrolyte levels in EG-induced urolithiasis in rats

Urinary parameters (Unit)	Group I Normal control	Group II EG 0.75% v/v	Group III EG+Cystone 750 mg/kg	Group IV EG+EEBS 250 mg/kg	Group V EG+EEBS 500 mg/kg
Calcium (mg/dL)	8.02±0.00	14.68±0.03^{***a}	12.02±0.07^{**b}	11.93±0.06^{**b}	10.23±0.03^{**b}
Phosphate (mg/dL)	3.23±0.01	5.46±0.01^{***a}	4.12±0.07^{**b}	5.40±0.03^{ns}	5.39±0.01^{ns}
Magnesium (mg/dL)	2.17±0.01	2.04± 0.01^{*a}	2.43±0.04^{**b}	2.06±0.05^{ns}	2.66±0.06^{***b}

EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. Values are expressed in mean ± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 ^{a} significant compared with control group (group I), ^{b}significant compared with EG-induced group (group II), ^{ns} not significant.

5.6.3.4. Effect of EEBS on hematological parameters in experimental rats

Significant reduction in total red blood cells (RBCs), lymphocytes ($p<0.001$), hemoglobin (Hb), packed cell volume (PCV) and mean corpuscular hemoglobin (MCH) levels ($p<0.01$) were observed in calculi-induced animals compared to control group. Animals co-administered with Cystone/ EEBS significantly increased levels of total RBC ($p<0.001$), MCH, lymphocytes, and Hb ($p<0.01$), compared to toxic control animals. PCV levels were significantly ($p<0.01$) improved in animals co-treated with Cystone but not in animals co-administered with extract ($p>0.05$). Total WBCs, polymorphs

($p<0.001$) and eosinophils ($p<0.01$) were significantly elevated in EG-treated animals compared to control group. Co-treatment with quercetin/EEBS significantly ($p<0.001$) decreased total WBC compared to lithiatic control rats. No significant differences were observed in eosinophil level, however, polymorphs levels were significantly elevated only in rats co-treated with standard Cystone ($p<0.01$), compared to calculi-induced rats (table 32).

Table 32: Effect of EEBS on hematological parameters in EG-induced urolithiasis in rats

Parameters Studied (Unit)	Group I Normal control	Group II EG 0.75% v/v	GroupIII EG+Cystone 750 mg/kg	Group IV EG+EEBS 250 mg/kg	Group V EG+EEBS 500 mg/kg
RBC(million/mm^3)	6.12±0.10	3.92±0.42***[a]	6.95±0.23***[b]	6. 70±0.42***[b]	6.87±0.52***[b]
HB (g/dl)	10.47±0.46	8.23±0.36**[a]	10.82±0.24**[b]	10.08±0.23**[b]	10.94±0.42**[b]
PCV (%)	37.26±0.24	35.15±0.34**[a]	37.20±0.33**[b]	35.01±0.36[ns]	35.19±0.42[ns]
MCH (pg)	21.61±0.13	20.94±0.12**[a]	22.72±0.09**[b]	21.64±0.08**[b]	22.79±0.13**[b]
WBC ($1X10^3/mm^3$)	8.87±0.12	9.91±0.22***[a]	8.27±0.34***[b]	9.12±0.33***[b]	9.19±0.12***[b]
Lymphocytes (%)	86.21±0.12	78.94±0.34***[a]	83.22±0.28**[b]	81.19±0.44**[b]	82.41±0.46**[b]
Monocytes (%)	3.55± 0.02	3.54±0.13[ns]	3.52±0.02[ns]	3.49±0.02[ns]	3.48±0.11[ns]
Polymorphs (%)	12.28±0.53	18.27±0.24***[a]	13.66±0.14***[b]	18.25±0.13[ns]	18.26±0.09[ns]
Eosinophils (%)	3.03±0.02	3.30±0. 01**[a]	3.28±0.02[ns]	3.24±0.03[ns]	3.23±0.00[ns]

EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. Values are expressed in mean ± standard error of mean (n=6), *$p<0.05$, **$p<0.01$, ***$p<0.001$ [a] significant compared with control group (group I), [b]significant compared with EG-induced group (group II), [ns]not significant.

5.6.3.5. Effect of EEBS on serum total protein and albumin levels in experimental rats

Table 33 illustrated the effect of EEBS on serum total protein and albumin levels in control and experimental animals in each group. Serum total protein levels were significantly lowered ($p<0.001$) in calculi-induced rats in comparison with group I rats. However, significant elevation in serum levels of total protein were observed in animals in group III ($p<0.01$), group IV and V ($p<0.001$), compared to group II rats. Significant

change in serum albumin levels were not observed among animals co-treated with standard/extract, compared to group II rats ($p>0.05$).

Table 33: Effect of EEBS on serum total protein and albumin levels in EG-induced urolithiasis in rats

Serum parameters (Unit)	Group I Normal control	Group II EG 0.75% v/v	Group III EG+Cystone 750 mg/kg	Group IV EG+EEBS 250 mg/kg	Group V EG+EEBS 500 mg/kg
Total protein (g/dL)	7.46±0.02	$5.76±0.03^{***a}$	$7.20±0.01^{**b}$	$7.43±0.01^{***b}$	$7.86±0.07^{***b}$
Albumin (g/dL)	4.32±0.00	$4.31±0.00^{ns}$	$4.32±0.00^{ns}$	$4.29±0.03^{ns}$	$4.30±0.00^{ns}$

EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. Values are expressed in mean± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 [a] significant compared with control group (group I), [b]significant compared with EG-induced group (group II), [ns]not significant.

5.6.3.6. Effect of EEBS on serum electrolyte levels in experimental rats

The serum levels of calcium, phosphate ($p<0.01$) and magnesium ($p<0.05$) were significantly elevated in calculi-induced animals compared to control group, indicating impaired renal function. Co-therapy with Cystone/extract significantly ($p<0.01$) decreased calcium level. However, significant reduction in serum phosphate levels were observed only in animals co-treated with Cystone ($p<0.01$)/high dose of EEBS ($p<0.05$). Animals administered with EG+standard (group III), attenuated changes in magnesium level ($p<0.01$), but not in animals co-treated with EEBS ($p>0.05$), compared to group II rats (table 34).

Table 34: Effect of EEBS on serum electrolyte levels in EG-induced urolithiasis in rats

Serum parameters (Unit)	Group I Normal control	Group II EG 0.75% v/v	GroupIII EG+Cystone 750 mg/kg	Group IV EG+EEBS 250 mg/kg	Group V EG+EEBS 500 mg/kg
Calcium (mg/dL)	9.29±0.07	$11.16±0.01^{**a}$	$9.39±0.02^{**b}$	$10.42±0.00^{**b}$	$9.15±0.01^{**b}$
Phosphate (mg/dL)	6.58±0.06	$8.06±0.01^{**a}$	$7.18±0.01^{**b}$	$8.02±0.01^{ns}$	$8.00±0.01^{*b}$
Magnesium (mg/dL)	2.30±0.00	$2.33±0.00^{*a}$	$2.51±0.00^{**b}$	$2.31±0.00^{ns}$	$2.32±0.00^{ns}$

EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. Values are expressed in mean± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 [a] significant

compared with control group (group I), [b]significant compared with EG-induced group (group II), [ns] not significant.

5.6.3.7. Effect of EEBS on serum BUN, creatinine and uric acid levels in experimental rats

The serum levels of creatinine, uric acid ($p<0.01$) and BUN ($p<0.001$) were significantly elevated in calculi-induced animals compared to control group, indicating impaired renal function. . Animals co-treated with EEBS 250 mg/kg (curative regimen) or EEBS 500 mg/kg (preventive regimen) exhibited significant reduction on BUN, creatinine ($p<0.001$) and uric acid ($p<0.01$) levels. Animals treated with standard also showed significant reduction on BUN ($p<0.001$), creatinine and uric acid level ($p<0.01$), compared to calculi-induced group (table 35).

Table 35: Effect of EEBS on serum BUN, creatinine and uric acid levels in EG-induced urolithiasis in rats

Parameters Studied (Unit)	Group I Normal control	Group II EG 0.75% v/v	Group III EG+Cystone 750 mg/kg	Group IV EG+EEBS 250 mg/kg	Group V EG+EEBS 500 mg/kg
BUN (mg/dL)	36.09 ±0.01	58.53±0.01^{***a}	34.51±0.00^{***b}	41.75±0.01^{***b}	39.87±0.03^{***b}
Creatinine (mg/dL)	0.57±0.00	0.88±0.00^{**a}	0.60±0.01^{**b}	0.49±0.00^{***b}	0.46±0.00^{***b}
Uric acid (mg/dL)	2.75±0.01	3.91±0.00^{**a}	3.34±0.01^{**b}	3.66±0.27^{**b}	3.68±0.01^{**b}

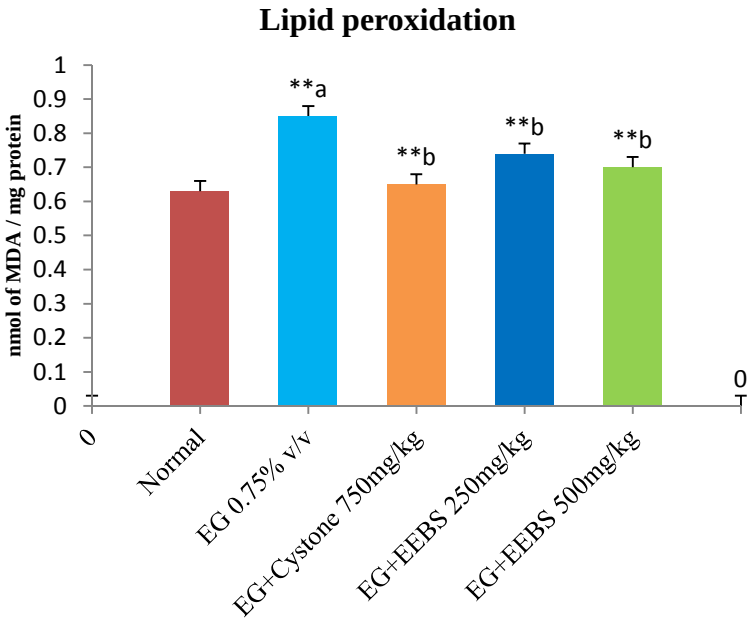
EEBS: Ethanol extract of *B. sensitivum,* EG: ethylene glycol, BUN: blood urea nitrogen. Values are expressed in mean± standard error of mean (n=6), *p<0.05, **p<0.01, ***p<0.001 [a] significant compared with control group (group I), [b]significant compared with EG-induced group (group II), [ns] not significant.

5.6.3.8. Effect of EEBS on markers of oxidation in control and experimental animals

The effect of EEBS on lipid peroxidation and antioxidant status in rats exposed to EG-induced urolithiasis showed a significant decrease of SOD, CAT, GSH and GPx ($p<0.01$) activity, compared to group I normal animals. Co-administration of standard (cystone) to group III rats, EEBS (250 mg/kg) to group IV rats and EEBS (500 mg/kg) to group V rats, significantly increased SOD levels ($p<0.01$). GPx activity was found to

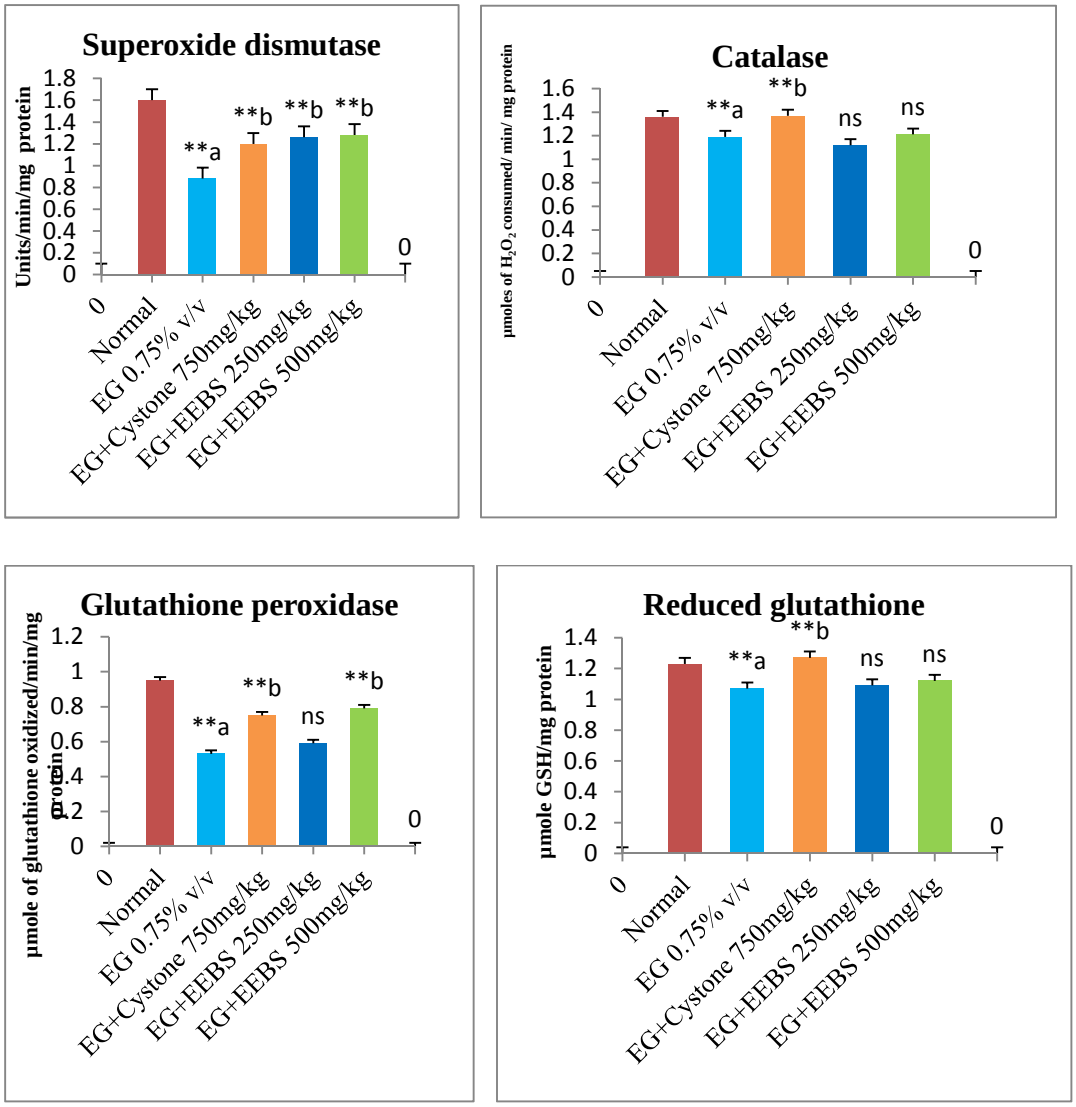
be increased in rats co-treated with standard/ high dose of extract ($p<0.01$), however, no significant differences were observed in catalase and GSH activity in animals co-treated with low/ high dose of extract, compared to calculi-induced rats. A significant ($p<0.01$) increase in production of MDA were observed in group II toxic control animals compared to group I normal animals. Co-administration of cystone to group III, EEBS to group IV rats and group V rats significantly ($p<0.01$) decreased MDA levels, compared to calculi-induced rats (figure 28, 29).

Figure 28: Effect of EEBS on lipid peroxidation in EG-induced urolithiasis in rats



EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. Units: LPO (nmol of MDA formed/ mg protein). Values are expressed in mean ± standard error of mean (n=6), $p<0.05$, **$p<0.01$, ***$p<0.001$ [a] significant compared with control group (group I), [b]significant compared with EG-induced group (group II), [ns] not significant.

Figure 29: Effect of EEBS on oxidative stress parameters in EG-induced urolithiasis in rats



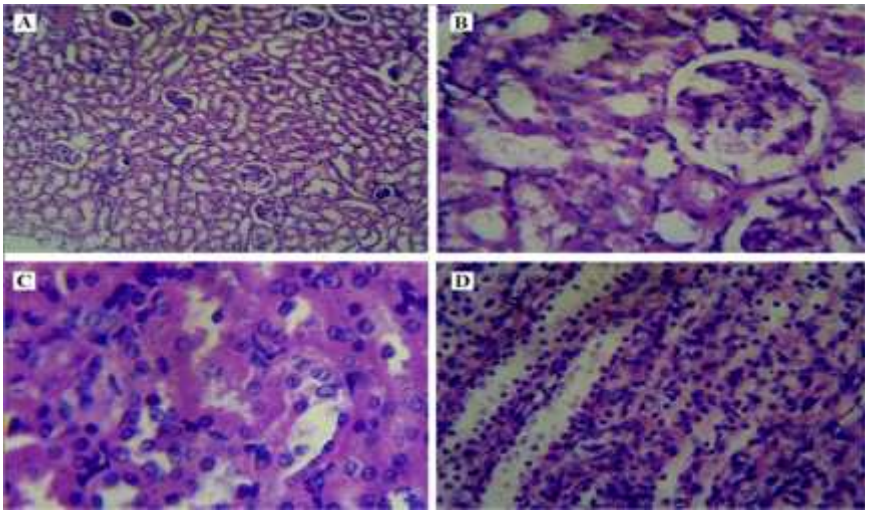
EEBS: Ethanol extract of *B. sensitivum*, EG: ethylene glycol. a) superoxide dismutase b) catalase c) glutathione peroxidase d) reduced glutathione. Values are expressed in mean ± standard error of mean (n=6), $p<0.05$, **$p<0.01$, ***$p<0.001$ [a] significant compared with control group (group I), [b]significant compared with EG-induced group (group II), [ns] not significant

5.7. Histopathological studies

5.7.1.Histopathological studies of EEBS on gentamicin (GM)-induced nephrotoxicity

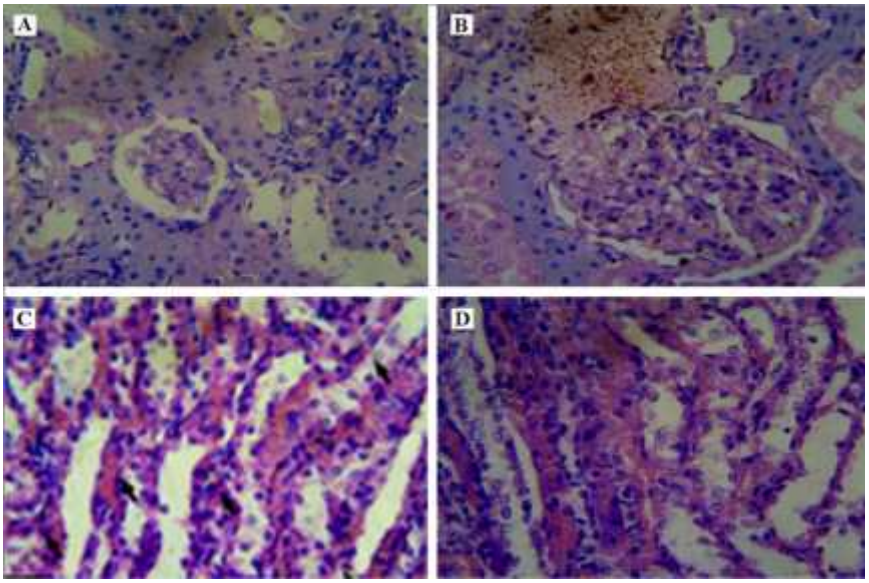
Histopathological analysis of kidney in group I rats revealed normal morphology of glomeruli, tubules, blood vessels and interstitium. Rats administered with GM (100 mg/kg; for 8 days; *i.p.*) caused acute renal damages which were evident by marked mesangial hypercellularity in glomerulus, tubular necrosis, tubular degeneration and blood vessel congestion. Rats administered with GM+low dose of EEBS (250 mg/kg) also showed mesangial hypercellularity and cytoplasmic vacuolation in the proximal tubules. These changes were reduced in rats co-treated with high dose of EEBS (500 mg/kg) which were evident only by mild epithelial vacuolation in the proximal tubules, thus showing protective effect of extract on drug-induced nephrotoxicity. Histopathological presentation of kidney in normal, toxic, standard and extract treated animals are presented in figure 30-34.

Figure 30: Histopathology results of kidney of normal rats (control) in GM-induced nephrotoxicity model



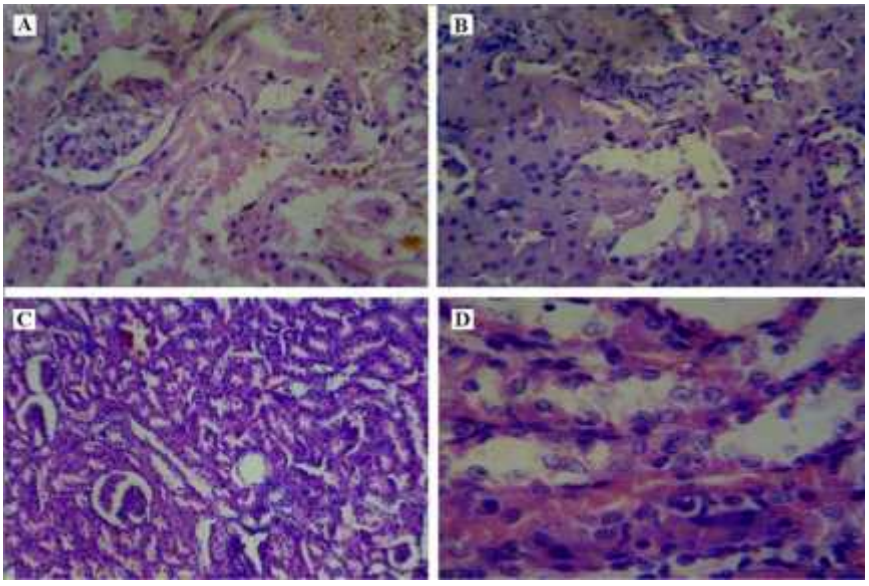
(A) 10X shows normal cortex and medulla; (B) 40X shows normal glomeruli; (C) 40X shows normal tubule; (D) 40X shows normal interstitium.

Figure 31: Histopathology results of kidney in GM-treated rats (toxic control)



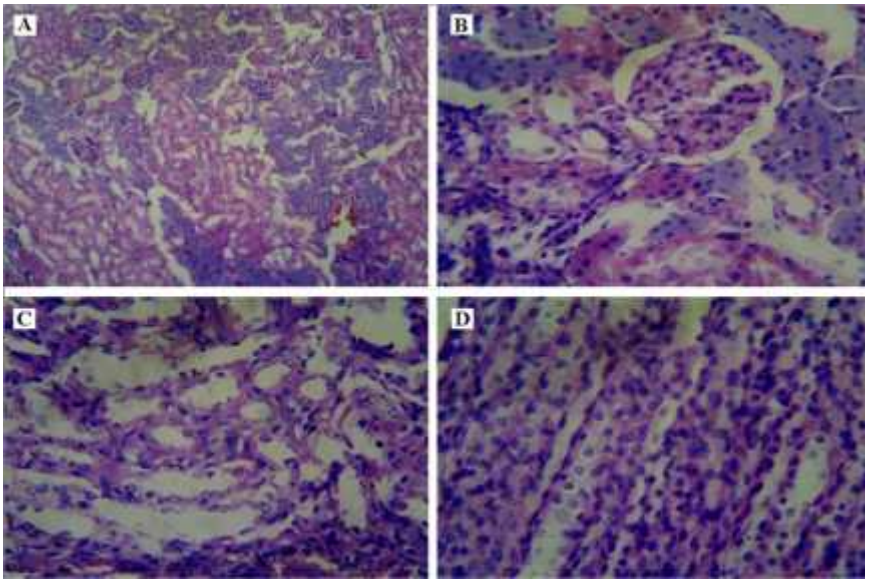
A) 40X shows cortex and medulla; (B) 40X shows marked mesangial hypercellularity in glomeruli; (C) 40X shows focal tubular epithelial loss; (D) 40X shows blood vessel congestion.

Figure 32: Histopathology results of kidney in GM+quercetin-treated rats



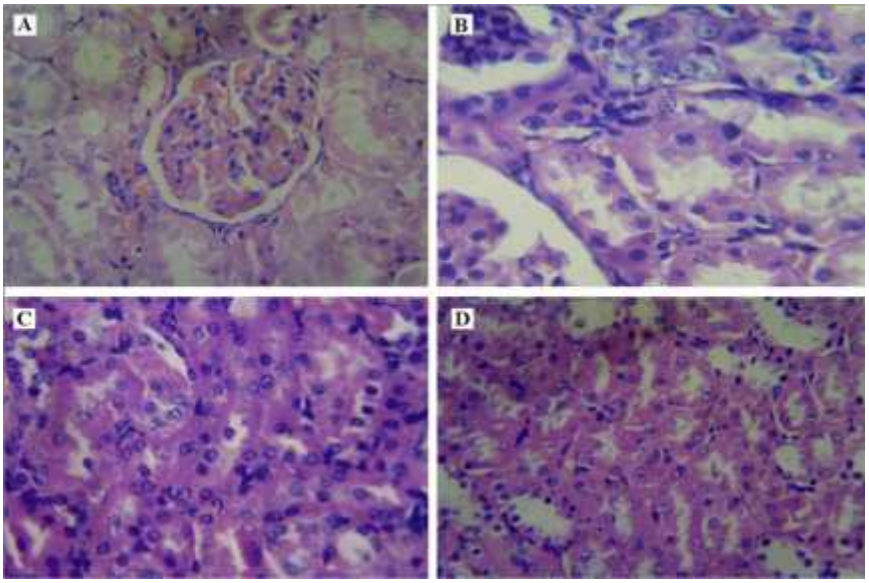
(A) 40X shows normal cortex and medulla; (B) 40 X shows mild mesangial hypercellularity in glomeruli; (C) 40X shows normal tubules; (D) 40X shows mild blood vessel congestion.

Figure 33: Histopathology results of kidney in GM+EEBS (250 mg/kg)-treated rats



(A) 10X shows cortex and medulla with mild mesangial hypercellularity; (B) 40X shows cortex and medulla with mild mesangial hypercellularity; (C) 40X shows tubular degeneration, cytoplasmic vacuolation in the proximal tubules; (D) 40X shows normal interstitium.

Figure 34: Histopathology results of kidney in GM+EEBS (500 mg/kg)-treated rats

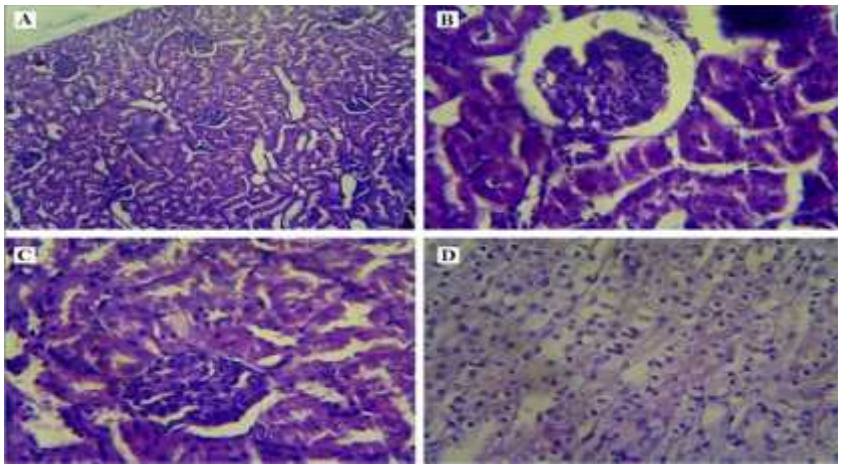


(A) 40X shows normal glomeruli with tubules; (B) 40X shows normal glomeruli; (C) 40X shows mild cytoplasmic vacuolation in tubules; (D) 40X shows normal interstitium.

5.7.2. Histopathological studies of EEBS on cisplatin (CDDP)-induced nephrotoxicity

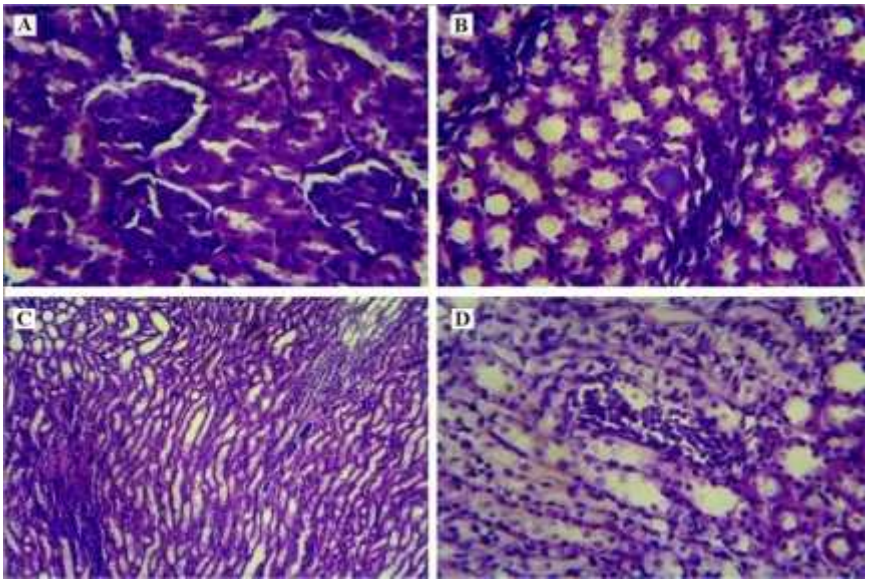
Histopathological analysis of kidney section of animals in control group revealed normal morphology of glomeruli, tubules, blood vessels and interstitium . Treatment with CDDP caused acute renal damages which were evident by mild mesangial hypercellularity in glomerulus, tubular casts, mild interstitial inflammation, cytoplasmic vacuolations and swelling in glomerulus, as compared to control. Animals co-administered with low dose of EEBS (250 mg/kg) revealed mild mesangial hypercellularity and matrix expansion in glomerulus, focal nuclear loss and epithelial thinning in tubular region. These changes were reduced in group V animals co-administered with high dose of EEBS (500 mg/kg) thus showing nephroprotective effect of drug. Histopathological presentation of kidney in normal, toxic, standard and extract treated animals are presented in figure (35-39).

Figure 35: Histopathology results of kidney of normal rats (control) in CDDP-Induced nephrotoxicity model



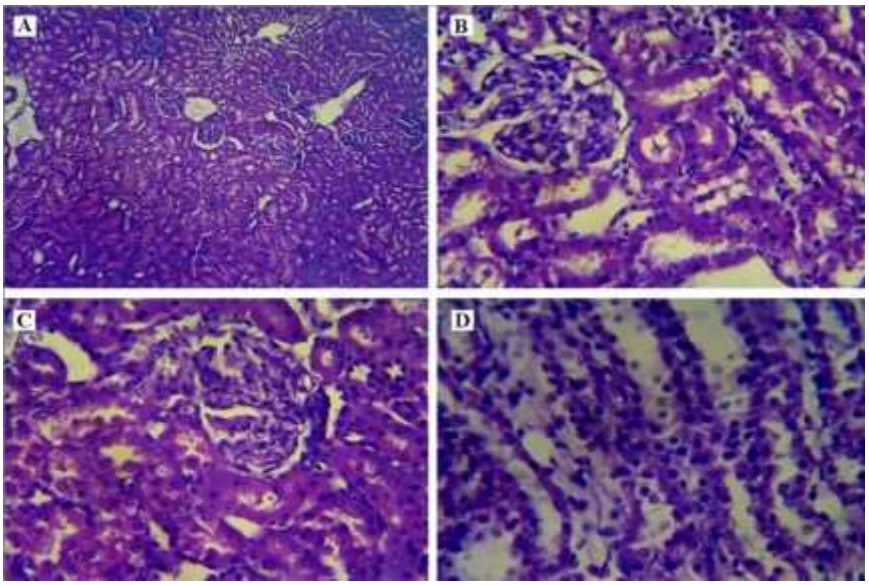
(A) 10X shows normal cortex and medulla; (B) 40X shows normal glomeruli; (C) 40X shows normal tubule; (D) 40X shows normal interstitium.

Figure 36: Histopathology results of kidney in CDDP-treated rats (toxic control)



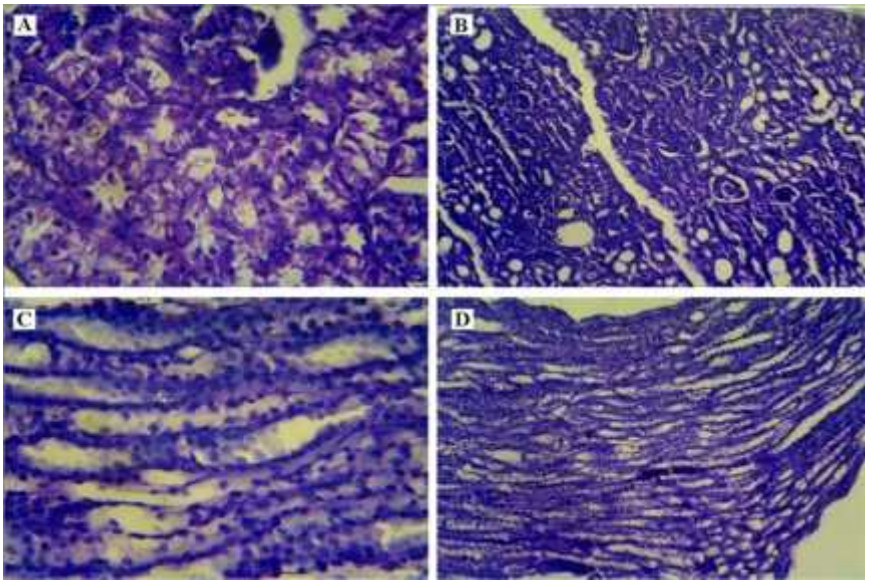
(A) 40X shows marked mesangial hypercellularity in glomeruli; (B) 40X shows casts in tubules; (C) 10X shows mild inflammatory infilterate in interstitium; (D) 40X shows mild inflammatory infilterate and cytoplasmic vacuolation in interstitium.

Figure 37: Histopathology results of kidney in CDDP+quercetin-treated rats



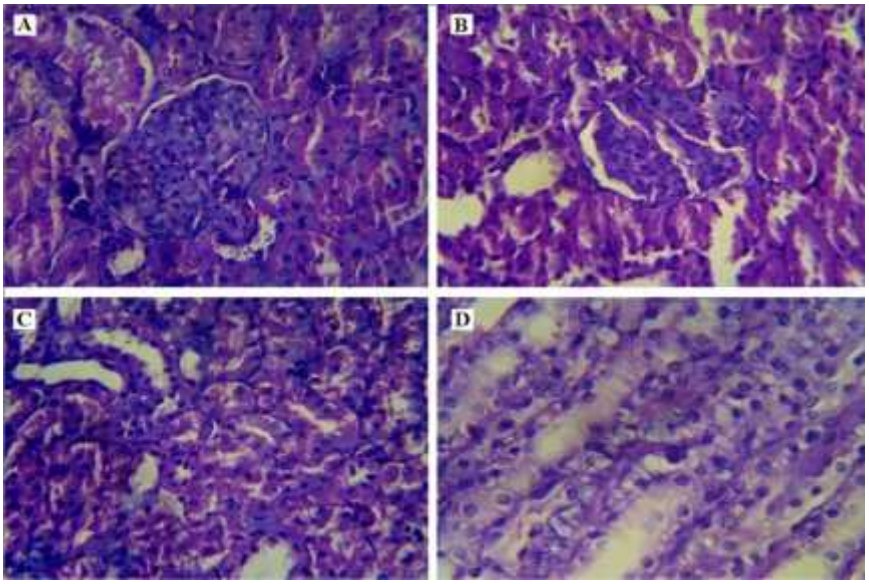
(A) 10X shows normal cortex and medulla; (B) 40X shows normal glomeruli; (C) 40X shows normal glomeruli and tubule; (D) 40X shows normal interstitium.

Figure 38: Histopathology results of kidney in CDDP+EEBS (250 mg/kg)-treated rats



(A) 40X shows mild mesangial hypercellularity and matrix expansion in glomerulus; (B) 10X shows mild mesangial hypercellularity and matrix expansion in glomerulus; (C) 40X shows focal nuclear loss and epithelial thinning; D) 40X shows normal interstitium.

Figure 39: Histopathology results of kidney in CDDP+ EEBS (500 mg/kg)-treated rats

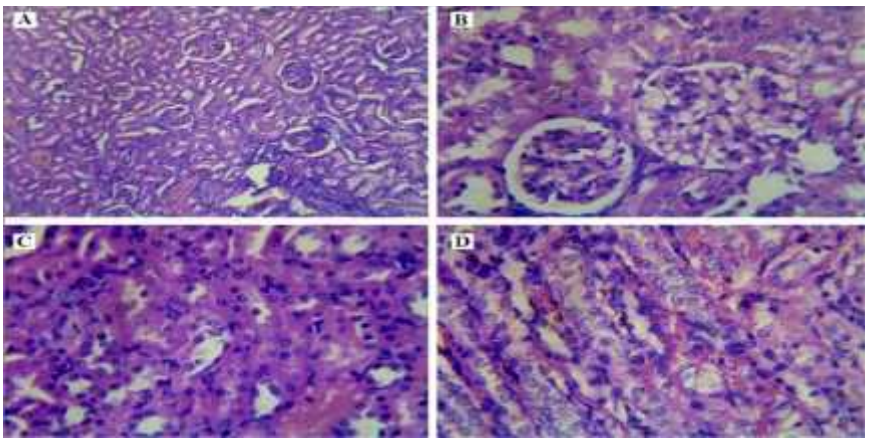


(A) 40X shows normal glomeruli; (B) 40X shows normal glomeruli and tubules; (C) 40X shows normal tubules; (D) 40X shows normal interstitium.

5.7.3. Histopathological studies of EEBS on ethylene glycol (EG)-induced urolithiasis

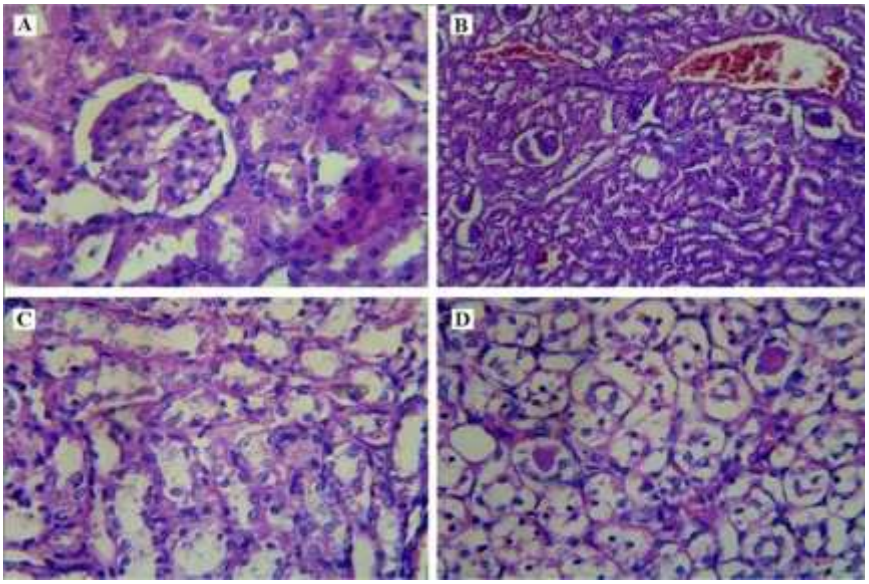
Histopathological analysis of kidney section of glomeruli, tubules, blood vessels and interstitium in control group rats revealed normal morphology. There were no abnormalities or calcium oxalate deposits inside tubles. Rats in toxic control (group II) exhibited mesangial hypercellularity in glomerulus, interstitial inflammation, mild congestion in blood vessels, tubular epithelial loss, vacuolation and dilation (features of acute tubular necrosis), along with crystal deposits. Animals co-administered with low dose of EEBS (250 mg/kg) revealed tubular epithelial loss, vacuolization and dilation, along with crystal deposits. These changes were reduced in group V animals co-administered with high dose of EEBS (500 mg/kg) thus showing antiurolithic effect of drug. Histopathological presentation of kidney in normal, toxic, standard and extract treated animals are presented in figure 40-44.

Figure 40: Histopathology results of kidney of normal rats (control) in EG-induced urolithiasis model



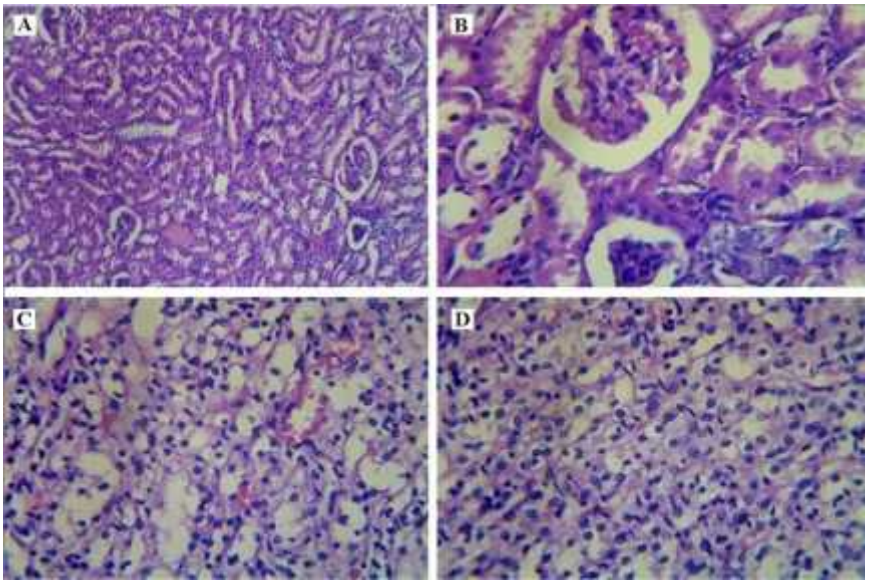
(A) 10X shows normal cortex and medulla; (B) 40X shows normal glomeruli; (C) 40X shows normal tubule; (D) 40X shows normal interstitium.

Figure 41: Histopathology results of kidney in EG-induced urolithic rats (toxic control)



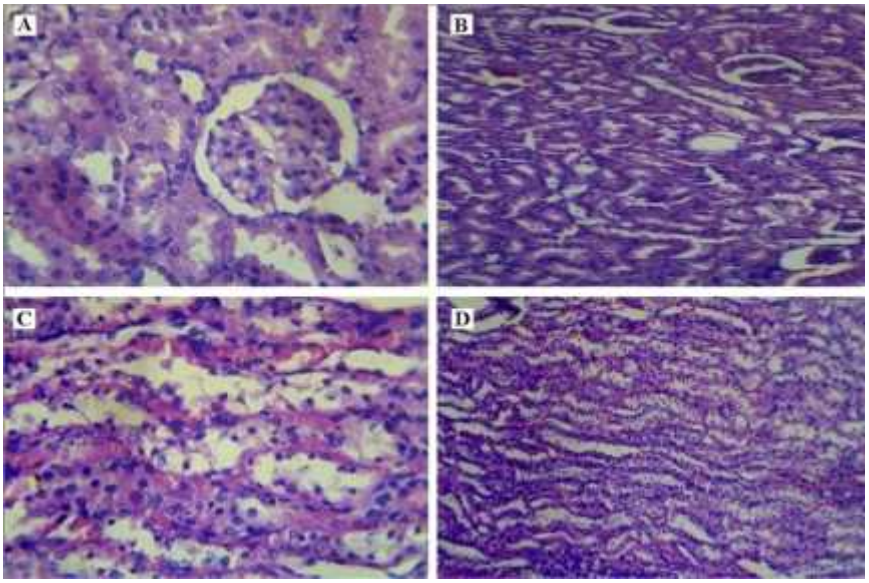
(A) 40X shows marked mesangial hypercellularity in glomeruli; (B) 40X shows proximal tubular dilation along with crystal; (C) 40X shows tubular loss, vacuolation and necrosis; D) 40X shows interstitial inflammation.

Figure 42: **Histopathology results of kidney in EG+ cystone-treated rats**



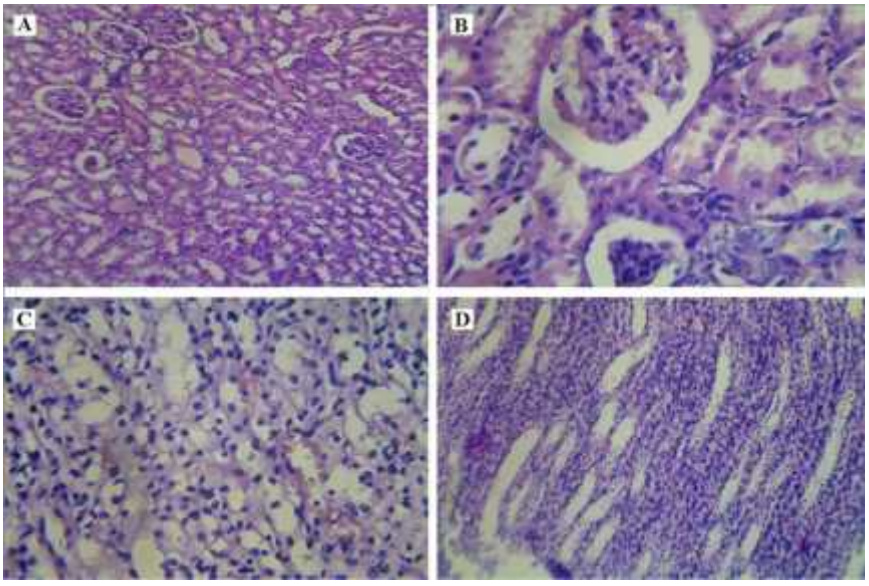
(A) 10X shows normal cortex and medulla; (B) 10X shows normal glomeruli; (C) 40X shows focal dilatation of the tubules; (D) 40x shows normal interstitium.

Figure 43: Histopathology results of kidney in EG+EEBS (250 mg/kg)-treated rats



(A) 40X shows normal glomerulus; (B) 10X shows tubular dilation with crystal deposit; (C) 40X shows tubular epithelial loss and vacuolation; (D) 10X shows normal interstitium.

Figure 44: Histopathology results of kidney in EG+EEBS (500 mg/kg) -treated rats



(A) 10X shows normal cortex and medulla; (B) 40X shows normal glomeruli; (C) 40X shows mild tubular dilation; (D) 10X shows normal interstitium2

6. DISCUSSION (Inferences)

The term herbalism, herbology or phytotherapy refers to the use and importance of herbal plants based on their therapeutic values. Medicinal plants are the oldest form of health care, ever since the birth of mankind. In prehistoric period, the primitive men depended only on natural resources (plants and animals) for curing their ailments. They started observing the relationship between life, disease and plants and found that large number of plants possess therapeutic activities.[169] The collected knowledge acquired through their experiences about medicinal value of herbal plants were transferred from generation to generation. The world health organisation (WHO) has recorded about 21,000 medicinal plants all over the world, out of which 2500 species are from India, therefore India is known as the botanical garden of the world.[170] Herbal drugs constitutes the major biosource for use as food supplements, folk medicines, modern medicine, pharmaceutical aids, nutraceuticals and chemical entities for synthetic drugs. It is also recorded that about one fourth of prescribed drugs contain active ingredients derived from medicinal plants. Previous research studies reported that the curative property of medicinal plants for various ailments are perhaps due to the presence of secondary metabolites including alkaloids, phenols, flavonoids, glycosides, tannins, saponins and terpenoids.[171]

Globally, renotoxicity remains a common challenging side effect of various classes of drug and and exerts substantial burden to the society.[1] Hence, there is an urgent need for providing safe and successful treatment to prevent drug-induced renal complications. Large volume of research works with different medicinal plants were performed to prove their claimed effects on drug induced nephrotxicity or urolithiasis. Several studies have proved

that co-therapy with nephroprotective medicinal plants along with different nephrotoxic agents minimized its toxicity.[2] Several chemical and natural substances with antioxidant activity also enhanced the therapeutic efficacy of drug having nephrotoxic potential.[3] Therefore, investigating new pharmacological agents from plant source with different phytoconstituents may provide safe and effective treatment for drug induced nephrotoxicity.

The whole plant *B. sensitivum* is a well-known auspicious medicinal plant used in the Indian medicine for treating several diseases. This plant was evaluated for various pharmacological activities which included antioxidant, antibacterial, antidiabetic, antitumour, immunomodulation, radioprotective, anti-inflammatory, cardioprotective and many more. In this current study, the investigation covered phytochemical, cytoprotective, antioxidant, nephroprotective and antiurolithic activities of whole plant *B. sensitivum.*

6.1. Phytochemistry

6.1.1. Collection, identification and preliminary phytochemical studies

In the present study, the shade dried whole plant *B. sensitivum* were subjected to size reduction by passing through sieve No. 40. The uniformly powdered plant material were then subjected to solvent extraction process using petroleum ether for defatting the drug and then with 70% ethanol. Shade drying of plant material protects phytoconstituents so that therapeutic properties would not be destroyed. Extraction of plant material is the initial process used for screening, isolation and standardization of phytoconstituents present in the plant material. Solvent extractions are widely accepted procedure due to their efficiency, availability, applicability and ease of use. The yield of extractable compound depends on various factors including temperature, solvent polarity, duration, sample

characteristics, ratio of sample and solvent. 70% ethanol is generally considered as safe and better solvent for extraction. In this study, percentage yield of extraction were found to be 12.54% w/w with ethanol extract and 4.92% w/w with petroleum ether extract of *B. sensitivum* (Table 8). Highest amount of total extractable compounds were obtained with 70% ethanol in comparison with petroleum ether.

Preliminary phytochemical analysis would be useful in the identification of chemical constituents with therapeutic potential and their subsequent use in drug development and discovery. Phytochemical screening results of our study have confirmed that whole plant of *B. sensitivum* contains carbohydrates, alkaloids, steroids, saponins, proteins, aminoacids, falvonoids, tannins, phenolic compounds and fixed oils (Table 9). Previous research studies have reported that bioflavonoid, the primary antioxidant and free radical scavenger are responsible for wide range of therapeutic activities mainly antioxidant, anti-inflammatory and anticancer properties.[100,104,108,110] Our phytochemical screening result s were in agreement with the earlier findings by Uma *et al.,* [172] but lacked the presence of steroids. Findings of Shibila *et al.*,[173] showed the presence of steroids. In contrast to our findings, Uma *et al*.,[172] also reported the presence of cardiac glycoside in EEBS. These secondary metabolites perhaps are responsible for the pharmacological activities of plant extract.

6.2. Quantitative estimation of total phenolic content and flavonoids

Polyphenols are the large and diverse class of compounds which occur naturally in wide range of plants. Among polyphenols, phenolic compounds and flavonoids are the best studied aromatic compounds which protect organism from damage caused by free

radical induced oxidative stress. Polyphenols possess diverse therapeutic activities including antioxidant, anti-mutagenic, anti-carcinogenic and anti-inflammatory effects.

6.2.1. Total phenolic content

Several methods have been used for the estimation of total phenolic contents. Among all, Folin-Ciocalteu assay are most widely used method, which was preferred in the present study for the estimation of total phenolics due to its sensitivity and speed. The Folin-Ciocalteu assay relies on the ability of phenolic compounds to transfer reducing equivalents (electrons), to phosphomolybdic/phosphotungstic acid complexes in the alkaline medium, resulted in the formation of blue colour complexes and absorbance was measured at 725 nm.[125] In this study, total phenolic content in EEBS were 53.55 mg/g of gallic acid equivalents.

6.2.2. Total flavonoid content

Flavonoids are large sub groups of plant phenolics which were estimated using colorimetric method based on the ability of aluminium chloride to form acid stable complexes with C-4 ketogroups, either with the C-3 or C-5 hydroxyl group of flavones and flavonols, or acid labile complexes with orthodihydroxyl groups in the A-or B-ring of flavonoids.[126] Absorbance was measured at 415 nm. Result of this study demonstrated that EEBS posessed highest flavonoid content *i.e.* 153.08 mg/g of rutin eqiuvalents. Our findings were consistent with the result of study performed by Sulaiman CT & Balachandran I.[174]

6.3. *In vitro* antioxidant and free radical scavenging activities of EEBS

Free radicals are highly reactive and chemically unstable atoms or molecules capable of initiating oxidative reactions due to the presence of unpaired electrons in

outermost orbital. Accumulation of free radicals are responsible for about 95% of pathologies in people above 35 years of age.[175] Overwhelming research reports suggest that ample generation of reactive oxygen species in any biogenic redox process may leads to pairing of free radicals by interacting with specific biomolecules in the body such as cell protein, lipid, carbohydrate, lipoprotein and eventually cause induction of lipid peroxidation, cell damage or cell death. In biochemical reaction, oxygen and hydrogen peroxide react together to form hydroxyl radical which is the most reactive free radical known to initiate lipid peroxidation and DNA fragmentation. Nitric oxide react with superoxide radical and form highly reactive peroxy nitrite. Excessive generation of free radicals has also been implicated in development of several chronic diseases.[176-181]

Antioxidants, containing one or more free electrons are capable of neutralizing reactive oxygen species (ROS) and convert them into less harmful or harmless species. Endogenous antioxidant defense system generally provide insufficient protection against ROS, hence antioxidants are needed to supplement from external source. At present, there has been a great interest on naturally occurring antioxidant owing to harmful side effects of most extensively used commercially available synthetic antioxidants. Several experimental data indicates there is a close association between consumption of antioxidant rich food and prevention of diseases.

Various *in vitro* methods have been developed to measure the antioxidant potential of pure compounds or plant extracts. Ferric reducing antioxidant power (FRAP), α, α-diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), superoxide anion radical scavenging assay, hydroxyl radical scavenging assay, nitric oxide radical scavenging assay and total phenol assay are most widely used *in vitro* methods[182] due to

their sensitivity and speed. The concentration of antioxidants needed to decrease free radical concentration by 50% (IC_{50}) is a parameter widely used to measure the antioxidant activity. Lower IC_{50} value indicates a higher antioxidant power or excellent free radical scavenging activity.

In this current study, we have determined the antioxidant or free radical scavenging activity of EEBS using different *in vitro* models which included ferric reducing antioxidant power assay, reducing power assay, 1,1-Diphenyl-2-picryl hydrazine (DPPH) radical scavenging assay, ABTS radical scavenging assay, hydroxyl radical scavenging assay, superoxide radical scavenging assay and nitric oxide radical scavenging assay.

6.3.1. DPPH (1, 1-Diphenyl-2-picryl hydrazine) radical scavenging activity

DPPH radical scavenging assay is widely used method[183] based on the reduction of DPPH, a stable organic nitrogen radical to non-radical form DPPH-H. It is rapid, simple, reliable, inexpensive and reproducible type of assay. The dose dependent inhibition of DPPH radical indicated that odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from phenolic OH group of antioxidant to corresponding hydrazine. In this study EEBS exhibited significant scavenging effect on the DPPH radical in comparison to standard ascorbic acid and suggested that the phytochemical constituents present in this extract are capable to prevent oxidative injury to tissues. Our findings were similar to the report of Sreena R *et al*.[184]

6.3.2. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was employed to estimate the reducing ability of antioxidants *in vitro* against oxidative effects of reactive oxygen species. This assay method is based on the reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to form ferrous at low pH which produce intense blue colour. This reaction can be monitored by measuring the change in absorption which is directly related to the reducing power of antioxidants present in the reaction mixture. They exert their action by donating a hydrogen atom after breaking the free radical chain. Ferrous sulphate is used for calibration. Butylated hydroxyanisole, butylated hydroxytoluene**,** ascorbic acid, quercetin, catechin and trolox can be used as positive control.[128] Reducing ability of extract might be due to the presence of flavonoids or polyphenolic compounds. Our findings were correlated with the result of Pal TP *et al*.[185]

6.3.3. ABTS [2, 2'-azino-bis (ethylbenzthiazoline-6-sulphonic acid)] radical scavenging activity

ABTS radical decolorization study is an excellent method to measure the antioxidant activity of phenolic compounds. This assay is based on the ability of sample to scavenge 2, 2'-azino-bis (ethylbenzthiazoline-6-sulphonic acid) or ABTS radical.[129] Blue coloured ABTS radical generated in presence of free radical inducer was quenched by EEBS and demonstrated oxygen radical absorbance capacity in a concentration dependent manner. EEBS possessed good hydrogen-donating capacity, as evidenced by its IC_{50} value (42.01 µg/mL).

6.3.4. Hydroxyl radical scavenging activity

Hydroxyl radical is the most potent reactive oxygen species which react with lipid, polypeptides, proteins, DNA and are capable of initiation of lipid peroxidation by extracting hydrogen atoms from unsaturated fatty acid.[130] Hydroxyl radical scavenging activity was quantified based upon the competition between deoxy ribose and antioxidant component in the plant extract for hydroxyl radicals generated by Fe^{3+}-ascorbate, EDTA-hydrogen peroxide system (Fenton) system. Hydroxyl radicals formed in free solution or in presence of transition metal ions such as iron causes degradation of sugar into malondialdehyde which form a pink chromogen on heating with thiobarbituric acid.[186] Effect of EEBS on hydroxyl radicals scavenging activity was measured by determining the degree of deoxyribose degradation, an indicator of thiobarbituric acid-malonaldehyde (TBA-MDA) adduct formation. Absorbance value decreases with increase in hydroxyl scavenging activity. In this study, polyphenolic compounds present in EEBS may be performed as metal chelators or hydrogen donors which inhibit complexing of hydroxyl radical with deoxy ribose, thereby demonstrated hydroxyl radical scavenging activity. Our results were consistent with the findings of Guruvayoorappan C *et al*.[108]

6.3.5. Superoxide radical scavenging activity

Superoxide anion (O_2^-) is one electron reduced form of molecular oxygen. This free radical generates under condition of oxidative stress have potential of reacting with biological macromolecules. Superoxide anion also promote generation of powerful and dangerous hydroxyl radicals and singlet oxygen which play an important role in contributing oxidative stress.[187] Recent studies have shown that phenolic compounds particularily flavonoids are important superoxide anion scavengers. Superoxide anion

derived from dissolved oxygen by phenazine methosulfate/reduced nicotinamide adenine dinucleotide (PMS/NADH) coupling reaction reduces nitroblue tetrazoliumchloride (NBT) resulting in the formation of blue formazan. The intensity of colour decreases proportional to the superoxide radical scavenging activity of extract.[131] Decrease of absorbance at 560 nm indicated the consumption of superoxide anion in the reaction mixture.[188] Hence, from the result it can be concluded that the extract possessed significant superoxide scavenging activity and this ability depends on the concentration of phenolic compounds, number of hyrdroxyl group in aromatic ring. Our findings were consistent with the report of Guruvayoorappan C *et al*.[108]

6.3.6. Nitric oxide radical scavenging activity

Nitric oxide is an important reactive oxygen species generated by endothelial cells, macrophages, stress, toxins etc and have been implicated in various diseases. Excess nitric oxide reacts with oxygen and generates stable nitrate and peroxy nitrite ions under aerobic condition. The method used for the determination of nitric oxide is based upon the inhibition of nitric oxide radical generated from sodium nitroprusside in aqueous solution at physiological pH. The inhibitory potentials of EEBS against this highly reactive compound may be attributed to their ability to compete with oxygen for nitric oxide leading to reduced production of nitrite ions, thereby demonstrated its protective role against oxidative stress. Nitric oxide scavenging activity exhibited by EEBS was found to be less compared to standard ascorbic acid. Our findings were in accordance with the report of Guruvayoorappan C *et al*.[108]

6.3.7. Reducing power activity

Previous research reports have revealed that there is a direct correlation exist between antioxidant activities and reducing power of certain plant extracts.[189] Antioxidant having reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide(Fe^{2+}), further react with ferric chloride to form ferric ferrous complex and can be monitered by measuring the absorbance at 700 nm. The change in absorbance is directly related to total reducing power of the electron donating antioxidants present in the reaction mixture. Phytoconstituents with reducing power, serving as primary and secondary antioxidants can oxidize intermediates of lipid peroxidation processes.[190] In this study, EEBS at concentration of 80 and 100 µg/mL showed reducing capacity higher than that of standard ascorbic acid, indicating its potency to donate electron to reactive free radicals and converting them into more stable non-reactive species. This result also implies that poly phenolic compounds present in EEBS may be the contributing factor towards antioxidant activity or quenching ability of free radicals in a dose dependent manner.

Previous studies have reported the antioxidant properties of medicinal plants or foods which are rich in phenolic compounds.[191] However, it is required to depend more than one antioxidant assay when plant extracts are being evaluated for their antioxidant potential because of the complex nature of phytochemicals.[192] In this study ability of EEBS to scavenge free radicals were found to be different in different testing system. Overall, the present *in vitro* antioxidant and free radical scavenging assay indicated that the whole plant *B. sensitivum* are potential source of bioactive compounds which exhibits excellent antioxidant and free radical scavenging activity.

6.4. *In vitro* antiurolithic activity of EEBS.

Related to urinary tract diseases, renal calculi is the third most common affliction, which is exceeded by the urinary tract infections and prostate diseases.[193] Urolithiasis begins with urinary super saturation of solid crystalline particles followed by nucleation, growth, aggregation and retention within the kidneys. Calcium oxalate monohydrate , calcium oxalate dihydrate and basic calcium phosphate are the most commonly occurring stones. Accumulation of thermodynamically stable calcium monohydrate promotes stone formation in kidney than calcium dihydrate form. [194] Crystal size is a limiting factor in stone formers as large particle occludes, less likely to pass through urinary tract and subsequently induce injury on urinary tract. Therefore, antiurolithic activity is mainly associated with the dissolution of kidney stones or calcium oxalate crystals which further prevent kidney stone formation and its recurrence. Previous clinical and preclinical studies reported that the formation of urinary stones leads to oxidative stress in patients.[195,196] Ureteric calculus disappeared within 55 days of treatment with 'Cystone' a herbo-mineral composition mainly by inducing diuresis.[197] In this current study, we have determined the antiurolithic activity of EEBS using *in vitro* models such as nucleation, aggregation and microscopic assay.

6.4.1. Nucleation assay

In this study, the *in vitro* inhibitory effect of EEBS and Cystone (standard) on calcium oxalate (CaOx) crystallisation were determined at various concentrations (10-100 mg/mL) and compared it with control. The findings of the present study exhibited dose dependent inhibitory effect of EEBS on nucleation of crystals.

6.4.2. Aggregation assay

In the aggregation assay, EEBS at various concentration (10-100 mg/mL) showed a significant dose-dependent inhibition on aggregation of crystals (11-78%). Cystone showed inhibitory activity on crystal aggregation more potent than the plant extract at the same concentration range (13-82%).

6.4.3. Microscopic assay

EEBS at different concentrations were applied directly on the crystals. This process resulted in number and size reduction of crystals in concentration dependent manner, which showed the ability of EEBS to prevent super saturation and precipitation of the CaOx crystal.

These results further confirming that EEBS might contain some phytochemicals that inhibit nucleation, aggregation or growth of CaOx crystal. EEBS promoted dispersion of particles in solution which favour excretion from the kidney and thereby reducing the chance of retention in the urinary tract. Previous research studies mentioned the significance of polyphenols and flavonoids in the antioxidant and antiurolithic activity of different plant extracts.[198,199] Several clinical and preclinical studies were also confirmed that the flavonoids present in medicinal plants inhibit calcium oxalate and crystal deposition in human urine as well as in animal models.[200, 201] *Herniaria hirsuta,* a plant rich with saponin, disaggregate the suspension of mucoproteins, which promote crystallization.[136] The present findings revealed similar inhibition on formation of kidney stones as reported for *Adiantum capillus,*[202] *Terminalia arjuna,*[203] *Phyllanthus niruri* [204]. Overall, *in vitro* antiurolithic activity of EEBS indicated that the phytoconstituents especially phenolic compounds, flavonoids and saponin present in the

whole plant *B. sensitivum* might inhibit nucleation, aggregation and growth of CaOx crystals, thereby reducing the possibility of kidney stone formation and injury.

6.5. Cytoprotective activity of EEBS

Ethnomedical usefulness of herbal drugs for various ailments were normally based on accidental discovery. Hence cell viability or cytotoxic testing with medicinal plant extracts is vital for safety evaluation. Several mechanisms are responsible for cytotoxicity including oxidative stress, inflammation, fibrogenesis and apoptosis. Lipid peroxidation mediated by reactive oxygen species inhibit DNA inter-strand and intra-strand cross-linking process which subsequently leads to cell death.[205] Cytotoxic compounds often compromise cell membrane integrity. Hence assessing cell membrane integrity is one of the most common ways to measure cytotoxic effects.

Trypan blue exclusion method, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) assays are well-established methods to determine the percentage cell viability, mitochondrial competence, cell membrane integrity respectively which provide an indirect measurement of cell death.[206] Apoptosis or necrosis compromise cell membrane integrity, hence additionally DNA fragmentation assay (biochemical hallmark of apoptosis) was performed.

In this study, cell protective activity of EEBS were evaluated using Trypan blue exclusion and MTT assay method on human embryonic kidney 293 (HEK 293) cell lines. These methods are highly popular, simple and convenient way of determining cell viability. HEK 293 cells are preferred in cell biology research for many years, because of

their reliable growth and propensity for transfection.[207] Lactate dehydrogenase assay was also carried out to determine cytoprotective activity of extract using A498 cell lines. A498 cells are widely used in cytotoxic research. DNA laddering assay was performed in A498 cell lines for further evaluating the nature of cell death.

6.5.1. Trypan blue exclusion assay

Dye exclusion assays are based on the exclusion of certain dyes by intact cell membrane. Cell suspension is mixed with the dye and visually examined to determine whether cells take up or exclude. Dye penetrates when membrane integrity of the cells is compromised. Affinity of trypan blue is increased for basic proteins as it is a weak acid, hence its nuclei uptake is generally high due to the presence of histones. Therefore viable cells remain unstained with clear cytoplasm and a refractile ring around them where as nonviable cells appear as dark blue color with no refractile ring around them.[208] In this study, cell lines treated with different concentration of EEBS (12.5-200 µg/mL), demonstrated that the percentage cell viability decreases as concentration of EEBS increases. Our findings were in accordance with the study done by Jetva K *et al*.[209]

6.5.2. MTT assay

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5 dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT), a water soluble tetrazolium salt. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble dark purple colored formazan product by mitochondrial enzyme succinate-dehydrogenase.The amount of formazan produced is directly proportional to the number of viable cells, as cleavage of the tetrazolium ring of MTT can only occur in metabolically active cells. It was clear from our study that MTT assay method produced

almost similar results as that of trypan blue dye exclusion method. EEBS exhibited cytotoxic effects only at high concentration (100 & 200 µg/mL) and percentage cell viability was decreasing in dose dependant manner in both assay method. Our findings were correlated with the report of Sreena R *et al.,*[184] Guruvayorappan C and Girija K.[109]

6.5.3. Lactate dehydrogenase assay

Lactate dehydrogenase is an oxidative enzyme widely concentrated in cell membranes and cytoplasm and plays an important step in energy production in cells. When cell membrane ruptures, lactate dehydrogenase release and catalyse the conversion of pyruvate to lactate. This is a simple, reliable and fast cytotoxicity assay based on the level of lactate dehydrogenase (LDH) in the extracellular medium. This cytoplasmic enzyme level indicating cytotoxicity was measured by assessing nicotinamide adenine dinucleotide (NADH) that remained in mixture.[210] Result clearly indicated that the volume of enzyme activity measured in this assay had progressed in a dose-dependent manner. However, significant LDH release was observed only in cell lines treated with EEBS at dose level of 160 µg/mL and 320 µg/mL, compared to untreated cells indicating irreversible cell death. Apoptosis or necrosis process may compromise cell membrane integrity that resulted in the release of LDH into the medium. Our findings were in accordance with the study done by Gayathri G *et al.,*[211] Kangas L *et al.* [212]

6.5.4. DNA fragmentation assay

Cell membrane injury is milder incase of apoptosis, compared to necrosis. Hence DNA laddering was done for determining the nature of cell death. The conventional agarose gel electrophoresis was performed on A498 cell lines by treating with the 160

and 320 µg/mL of EEBS for 48 hrs. Apoptotic DNA fragmentation is a natural fragmentation of cell or programmed cell death, characterized by cytoplasmic condensation, plasma membrane blebbing, nuclear pyknosis, leading to nuclear DNA breakdown into multiple fragments. In dying cells, DNA is cleaved by endonuclease that fragments the chromatin into nucleosomal units, which are multiples of about 180 bp (base pair) oligomers and appear as a DNA ladder when run on an agarose gel. This study result revealed the degradation of chromosomal DNA into smaller internucleosomal fragments and produced a ladder pattern. [213] Evident DNA fragmentation was seen on A498 cells treated with EEBS at 320 µg/mL concentration indicating apoptosis, however, at 160 µg/mL lesser fragmentation was observed. Our findings were correlated with the report of Guruvayoorappan C *et al*.[117]

Overall, this result suggested that the EEBS at lower concentration did not show any cytotoxic effect. Phytoconstituents present in this plant especially phenolic compounds and flavonoids may be attributed to its cell protective activity. However, EEBS at higher concentration demonstrated cytotoxic and apoptotic effect.

6.6. *In vivo* pharmacological studies

Nephrotoxicity is the most common kidney problem generally observed among people who widely use potent therapeutic drugs cisplatin (chemotherapeutic agent) and aminoglycoside antibiotics. However, these drugs still widely used in clinical practice because of its low cost and efficacy. In preclinical studies, gentamicin or cisplatin-induced nephrotoxicity are widely used experimental models. Hence the present study has been undertaken to determine nephroprotective activity of EEBS by selecting these experimental models. Nephroprotective effect of EEBS were determined by assessing

various general, hematological, urinary and serum parameters. *In vivo* antioxidant enzyme levels in kidney homogenate were also determined. In present study, EEBS at concentration of 250 mg/kg (low dose) and 500 mg/kg (high dose) were selected to investigate the nephroprotective activity based on the study report of Anidya *et al.*[112] Natural flavonoid, quercetin (50 mg/kg) was used as standard. Large amount of this flavonoid present in various fruits and vegetables exhibited excellent antioxidant, cytoprotective and nephroprotective effects.[214, 215]

6.6.1. Gentamicin-induced nephrotoxicity

Gentamicin (GM), is an aminoglycoside antibiotic widely used in the treatment of gram-negative infections. However, its clinical use is highly constrained due to its adverse effects, mainly nephrotoxicity and ototoxicity.[216] Poon *et al*., have reported that GM at dose level of 100 mg/kg/bw is nephrotoxic to experimental animals.[217]

In this sudy, progressive weight loss of animals were observed in the negative control group treated with GM (100 mg/kg;*i.p.*) and this might be associated with increased catabolism, physiological imbalance, mental stress, poor food or water intake. Co-administration with EEBS/quercetin (standard) promoted food intake and cause subsequent increase in body weight. Nairuti *et al.*,[218] demonstrated significant reduction in body weight, urine volume and increase in kidney weight in animals treated with GM. In current study, significant weight reduction of kidney was observed in animals co-treated with EEBS/quercetin compared to rats in toxic control group and this might be due to its potential to reduce inflammation. Urine output was markedly reduced in animals in toxic control compared to animals in normal group. This might be due to the

drug-induced change in release of antidiuretic hormone or thirst center activity in the cerebral cortex.[219] Supplementation of EEBS to GM-treated animals significantly improved water intake and urine output. Our findings were consisitent with the study of Nairuti *et al*.[218] Usually urinary pH value decreases consistently with degree of kidney injury.[220] However, in our findings there were no significant difference in urinary pH among animals in toxic control group, standard/extract-treated rats. In contrast to our findings, Aparna RM *et al*.,[221] reported that elevated urinary pH value observed in GM-treated group were significantly lowered in animals co-administered with ethanolic extract of *Annona reticulata*.

Incidence of elevated levels of urinary excretion of total protein and albumin have been suggested as an index of renal damage and proximal tubular dysfunction.[222] In this study, proteinuria and albuminuria observed in animals in toxic control group were significantly reduced in EEBS/quercetin-treated rats. These observations indicated protective role of EEBS against GM-induced nephrotoxicity. The results obtained were consistent with the findings of Anitha T and Mohan DS,[223] based on the investigation of nephroprotective activity *of Morinda citrifolia* extract against GM-induced renal damage in rats.

Drug induced nephrotoxicity has been associated with fall in glomerular filtration rate. Drug induced rise in urinary excretion of sodium, potassium, calcium and magnesium have been reported previously.[224, 225] In this study urinary excretion of calcium and magnesium levels were significantly increased. Hypermagnesiuria and hypercalciuria reveal specific membrane or transport system abnormality.[226, 227] In current study, no significant changes were observed in urinary excretion of sodium and

potassium levels among animals treated with GM, standard or extract. However, co-administration of EEBS/quercetin showed a significant change in elevated urinary level of calcium and magnesium observed in GM-treated animals. Thus, prevention of hypercalciuric and hypermagnesuric effect may probably be attributed to the protective effect of EEBS in GM-induced nephrotoxicity. Previous findings of Anitha T and Mohan DS,[223] confirmed that administration of *Morinda citrifolia* reverted the urinary excretion of these biochemical constituents to near normal suggesting nephroprotective activity of this plant extract.

Determination of haematological parameters are considered as earliest indicator of deleterious effect produced by the nephrotoxic drug and its metabolite. [228] Exposure of human body to toxic substances may affect multiple organ systems and cause morphological, biochemical and physiological changes, including impaired kidney function and hematological disorders.[229-231] Hematopoietic system is the main target of some drug which adversely affects heme synthesis enzymes, normal production rate of erythropoietin, normal range, morphology and distribution of various blood cells.[232, 233] Toxic drugs damage erythrocyte and affect its oxygen carrying capacity thereby increase risk of developing hypertension and cardiac arrest.[234] Factors contributing to pathogenesis of anaemia include, shortened RBC survival, marrow suppression by uraemic toxins, iron or folate deficiency. Damage of peritubular cells especially due to the exposure to toxins resulted in inadequate secretion of erythropoietin. In this study, it was observed that haematological parameters were significantly affected due to the administrtion of GM. Fall in the hemoglobin content, red blood cells counts count, packed cell volume, mean corpuscular hemoglobin level and elevation of white blood

cells count and polymorphs were observed in animals in toxic control group. These effects may cause defective hematopoiesis, anemia and weakness in experimental rats. Overall, results of this study suggested that the animals co-administered with standard/extract showed significant reversal in the hematological parameters that observed in animals in toxic control group and showed an improvement in hematological status**.** Ebtihal *et al*.,[235] narrated that that concurrent administration of *Acacia senegal* with GM reversed reduction in haemoglobin, red blood cells count, packed cell volume that observed in toxic control animals.

This study further demonstrated significant elevation of serum levels of creatinine, uric acid, blood urea nitrogen (BUN) and lowering of serum level of total protein, albumin, sodium, calcium and magnesium in GM-treated rats compared to animals in control group. This result revealed failure of kidney's ability to filter waste products or to conserve cations adequately. Preclinical studies in agreement with the present findings, Chatterjee *et al.*,[236] Qadir *et al.*,[237] Vidya *et al.*,[7] reported significant elevation of serum creatinine, BUN, uric acid levels in animals in toxic control group. Drug induced nephrotoxicity associates with decreased activity of sodium-potassium-ATPase and disturbances in the electrolytes which are manifested by hyponatremia, hypomagnesaemia, hypocalcaemia and hypokalemia. Drug induced nephrotoxicity induces destruction of proximal and distal tubules leading to decreased reabsorption, increased vascular resistance and this caused the elevation in BUN, uric acid and creatinine levels.[238] In current study significant change in serum total protein and albumin levels were not observed in GM+EEBS-treated group, however co-therapy with quercetin exhibited significant elevation, compared to the rats in toxic control group. In

contrast to our findings, elevated level of serum total protein and albumin in GM-treated group were reduced significantly in animals treated with aqueous extract of *Khaya senegalensis*.[239] In this study significant changes in serum level of sodium and potassium were not observed in EEBS/quercetin-treated group, compared to animals in toxic control. These findings were similar to the study conducted by Cekmen M *et al.*[240] Co-therapy with EEBS/quercetin in GM-treated animals restored the elevated serum level of BUN, creatinine, uric acid and diminished calcium and magnesium levels. These results confirmed that EEBS possessed significant protective effect against drug induced nephrotoxicity in a dose dependent manner. Co-administration of *M. piperita* with gentamicin successfully elevated serum calcium level compared to GM-treated animals.[241] Vamsi *et al*.,[242] reported that GM intoxication resulted in elevated serum urea, BUN, uric acid, and creatinine levels which was found to be significantly lowered in dose-dependent manner in groups received *Daucus carota* L extract.

Gentamicin is highly charged and water soluble at physiological pH and hence, exhibits less penetration through biological membranes.[39] This leads to the accumulation of drug in proximal tubules and enhance hydrogen peroxide production. This causes release of iron from mitochondria membranes and promote complex formation with gentamicin which further hasten the production of reactive oxygen products.[42] This further resulted in lipid peroxidation and decrease in antioxidant enzyme levels.[243] Endogenous antioxidative enzymes like superoxide dismutase, catalase, glutathione peroxidase and reduced glutathione possess excellent antioxidant activity by neutralizing or scavenging various free radicals. Decrease in these enzyme activities cause accumulation of lipid peroxides and induce oxidative stress.[244-246]

Superoxide dismutase associate with the convertion of superoxide radicals to oxygen and hydrogen peroxide and considered as a major defense against reactive oxygen species.[247] Catalase involved in the conversion of hydrogen peroxide to water and oxygen.[248] Soluble tripeptide reduced glutathione (GSH) acts as an antioxidant and a redox buffer.[249] Drug induced nephrotoxicity induce oxidative degradation of lipids which leads to a process called lipid peroxidation.[250] The extend of lipid peroxidation can be measured based upon the amount of malondialdehyde (MDA) formed. MDA level increases with degradation of lipid. In this study, it was observed that co-therapy with EEBS in GM-treated animals significantly restored the level of SOD, CAT, GSH and MDA, thereby inhibited the changes associated with oxidative stress. The results of our study demonstrated that EEBS exhibited significant antioxidant activity in both *in vitro* and *in vivo* studies. Our results were in accordance with the previous studies of Guruvayoorappan *et al*. [108]

6.6.2. **Cisplatin-induced nephrotoxicity**

Renotoxicity is a common adverse effect of chemotherapy and degree of toxicity depends on accumulation and binding of the substance in kidney. Nephrotoxicty is a common complication of cisplatin (CDDP), a platinum compound used to treat cancer. Inhibition of protein synthesis, DNA damage, mitochondrial injury, inflammation, oxidative stress and apoptosis are the major mechanism associated with CDDP-induced nephrotoxicity.

In this sudy, statistically significant decrease in body weight and an increase in kidney weight were observed in the negative control group treated with single dose of CDDP (8 mg/kg;*i.p.).* Weight loss of animals might be closely related with poor food

intake, stress, increased catabolism or physiological imbalance. Rats co-treated with EEBS/quercetin demonstrated subsequent increase in body weight. The increase in kidney weight of animals in negative control group treated with CDDP may be associated with inflammation or edema due to drug induced nephrotoxicity. Significant weight reduction of kidney was observed in animals co-treated with EEBS/ standard which might be due to its anti-inflammatory action. Our findings were consistent with the report of Fatemeh M *et al*.,[251] during the investigation of nephroprotective activity of Pomegranate flower extract against CDDP-induced nephrotoxicity.

In our study, animals treated with CDDP showed a significant increase in urine output. This study result revealed CDDP-induced non-oliguric acute renal failure and this effect perhaps due to reduction in the gene expression of aquaporines and their density in the proximal tubule.[252] Administration of the EEBS and quercetin to CDDP-treated rats for nine days caused reduction in urine volume, but statistically not significant. Our findings were similar to the report of Md-Azmat *et al*.,[253] during the investigation of nephroprotective activity of etahnolic extract of *Bauhinia purpurea* extract against CDDP-induced nephrotoxicity.

In this study urinary pH value was markedly lowered (5.93±0.12) in animals treated with CDDP. Urinary pH value decreases with increase in degree of kidney injury. In our study significant change in urine pH were observed among animals co-supplemented with extract/ standard, compared to group II animals. Ashish M and Ashutosh P,[254] reported that urine pH was higher in CDDP-treated group and rats treated with extract of *Ocimum sanctum* showed significant reduction in urinary pH.

Proteinuria observed in animals treated with CDDP indicating renal damage and proximal tubular dysfunction. [222] In current study, proteinuria and albuminuria were significantly reduced in animals co-administered with high dose of EEBS indicating the nephroprotective role of extract. Our findings were similar to the study report of Kaveripakam S and Adikay S.[255]

Previous study reports demonstrated significant elevation in urinary excretion of sodium, potassium, calcium and magnesium level in animals induced with nephrotoxicity, [224, 225] however, disturbance of this electrolyte homeostasis is less well documented . In current study urinary electrolyte level (potassium, calcium and magnesium) were significantly elevated in animals treated with CDDP, revealing specific membrane or transport system abnormality.[226, 227] Concurrent administration of EEBS with CDDP showed a significant reduction in calcium excretion. Thus, prevention of hypercalciuria may probably be attributed to the protective effect of EEBS in CDDP-induced nephrotoxicity. Hassan *et al.*, reported that pretreatment with grape seed extract and fish oil significantly decreased CDDP-induced elevation of urinary excretion of sodium, potassium and calcium levels in urine.[256]

In our study, total WBCs and polymorphs were significantly ($p<0.01$) elevated in CDDP-treated animals indicating infections in the body due to kidney injury. The elevation of WBC and polymorphs level may be due to its adverse effects on haemopoietic system.[257] Co-treatment with quercetin/EEBS significantly ($p<0.01$) decreased total WBC and polymorphs level. Lowering level of red blood cells (RBC), hemoglobin (Hb), mean corpuscular haemoglobin (MCH), packed cell volume (PCV), lymphocytes in rats treated with nephrotoxic drug may be due to the imbalance between

the rate of normal production of erythropoietin and their catabolism/ destruction. Lowering of RBC count indicated that these nephrotoxic drug might induce direct toxic effect on red blood cells thereby, reducing its life span. These deleterious effects perhaps as consequence of generation of reactive oxygen species. However animals co-administerd with EEBS/standard mitigated toxic effect of drugs by protecting the hemopoietic stem cells from oxidative damage, thereby restored fuctional capacity of blood. EEBS treatment produced a significant reversal in the haematological parameters exhibited by toxic control animals in group II, which signify the protective activity of extract. These effects on haematology may be attributed to the antioxidant activities of the phytochemicals, mainly phenolic compound and flavonoids present in EEBS. Present study results were consistent with the results of Shelkea T *et al.*,[258] in which oral treatment with *Crataeva nurvula* reversed the lowering level of Hb, RBC and PCV recorded in CDDP-induced hematotoxicity.

In this study, we found that a single dose of CDDP administration caused significant elevation in serum levels of creatinine, uric acid and BUN and lowering of serum concentration of sodium, potassium and calcium compared to control group, indicating renal dysfunction. This toxic effects of CDDP in our study were consisitent with the study of Badary *et al*.,[259] showing significant elevation in urea and creatinine levels in CDDP-treated rats, compared to control group. We confirmed that co-administration of EEBS had beneficial effect on kidney as it restored the elevated serum level of BUN, creatinine and diminished total protein, calcium and sodium levels. These findings were in accordance with the results described previously by Kusumoto.[260]

Oxidative stress is the major mechanism involved in CDDP-induced nephrotoxicity through elevation of reactive oxygen species and reduction of the antioxidant defense system.[261, 262] Previous research studies proved that CDDP administrations resulted in increased formation of free radicals, and heavy oxidative stress.[263-265] As a marker of oxidative stress and lipid peroxidation we evaluated superoxidedismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and malondialdehyde (MDA) levels in kidney homogenate. In this study, it was observed that treatment with EEBS in CDDP-induced animals significantly restored the levels of SOD, CAT, GPx, GST and MDA, thereby inhibited the changes associated with oxidative stress. Our findings were in accordance with the results demonstrated previously by Yogesh CY *et al.*[266]

6.6.3. Ethylene-glycol induced nephrotoxicity

Urolithiasis is a painful disease that afflicts human population since ancient times and still remain a common problem globally. Relapse rate is high and more prevalent in age between 20-40 in both sexes. Kidney stone is a hard mass composed of inorganic and organic crystals amalgamated with proteins. Stone forming minerals and chemicals that are excreted in the urine sometimes combine and grow to form very small invisible sized to large sized crystals. If stone size is small, it will flush out and leave the body through the urine stream without causing symptoms. If stones grow to sufficient size (at least 3 millimeters) causes obstruction to the ureter. Ureteral obstruction causes post renal azotemia, hydronephrosis, spasm of the ureter and renal colic. Renal colic is associated with nausea, vomiting, fever, hematuria, pyuria, and painful urination. Urolithiasis also induce free radical production and decrease in antioxidant status followed by cell injury

and cell death. Ethylene glycol, sodium oxalate, ammonium oxalate, glyolic acid are common agents used for inducing urolithiasis. [267] In this study, male albino rats were used for inducing urolithiasis, as the urinary system of this experimental animals resembles that of human. [268] In our study, ethylene glycol (EG) was used to induce renal stone formation. Accumulation of glycolic acid, a metabolite of EG in kidney is responsible for toxicity.[269] Cystone, a herbomineral formulation, favourably reported as an effective drug for the treatment of urolithiasis, crystalluria, urinary tract infections and edema. It acts on the mucin that binds the stone forming components together. It relaxes the smooth muscles of the ureter and the urinary tract and enables easy passage of small calculi. It has no toxic effect and also relieves the pain. Previous studies have proven that this drug is significantly effective in 80% patients with urolithiasis.[270] Hence cystone was selected as standard drug in this study.

In this study, decreased quantity and frequency of food or water intake were found to be the major factors involved in weight loss of animals in calculi-induced group. Physiological imbalance and/or mental stress may be the other factors influencing weight reduction.[271] Co-administration of EEBS/Cystone (standard) improved diet (frequency and quantity), water intake and this cause subsequent increase in body weight. Kidney weight was significantly increased in calculi-induced group compared to normal control group. Significant weight reduction of kidney was observed in animals co-treated with EEBS/Cystone, which might be due to the increased excretion of stone forming chemicals or due to the anti-inflammatory action. Decreased urine output plays a prominent role in inducing urolithiasis. [272] In current study, rats co-administered with curative dose of EEBS (250 mg/kg;from 15th day till 28th day; *p.o.*), preventive dose of

EEBS (500 mg/kg; from 1st day till 28th day; *p.o.*) and Cystone (standard) increased urine output in a dose dependent manner along with normalization of pH. Effect of EEBS in experimental animals confirmed that the diuretic activity promotes flushing out of deposits thereby inhibiting saturation and precipitation of stone forming constituents. The results obtained were consistent with the findings of Aseesh KG *et al*.,[273] during the evaluation of antiurolithic potential of *Kigelia africana* fruits. Nucleation and aggregation of crystals in urine is pH dependent. Acidic urine favours uric acid stones formation, whereas calcium oxalate and calcium phosphate stones occur in highly alkaline urine.[274, 275] In this study, pH of urine was found to be 8.2±0.11 in EG-treated animals, which favoured formation of kidney stones. Treatment with plant extracts (EEBS 500 mg/kg) significantly reduced urine pH to 6.9±0.10. Our results obtained were similar to the study done by Lulat SI *et al*.,[276] during the evaluation of lithocare against ethylene glycol induced urolithiasis.

Proteinuria observed in our study indicates renal tubular dysfunction. Total protein levels were significantly reduced in animals treated with EEBS/Cystone, however no statistical differences were observed in albumin level among rats treated with extract/ standard, compared to rats in calculi-induced group. Sharma I *et al*.,[277] also demonstrated that albuminuria observed in calculi-induced rats were not significantly lowered in experimental rats treated with aqueous extract of *Bergenia ligulata*, as compared to toxic control rats.

28 days of ethylene glycol (0.75% v/v) treatment, increased the risk of kidney stone formation by raising the level of stone forming chemicals in urine. High level of stone forming constituentss in the urine induce toxic effects on the renal epithelial cells

leading to alteration in membrane integrity, generation of reactive oxygen species, development of oxidative stress, lipid peroxidation and depleted source of antioxidant enzymes.[278] Renal epithelial injury exposed to a variety of crystal adhesion molecules further promotes retention of stone forming constituents. High concentration of urinary calcium promotes nucleation, aggregation or retention of calcium oxalate or calcium phosphate.[279] Recurrent kidney stone formation have been prevented in patients treated with potassium magnesium citrate. Magnesium deficiency promote the nucleation and retention of renal tubular calcium. High magnesium level favours binding with oxalate to form soluble complex, and reduce the availability of oxalate to form precipitate with calcium oxalate.[280] Excess urinary excretion of calcium and phosphate observed in calculi induced group in our study indicating an environment appropriate for calcium phosphate crystals formation. Supplementation with Cystone, EEBS (500 mg/kg) served as preventive regimen and EEBS (250 mg/kg) served as the curative regimen, significantly restored the alteration in the level of urinary calcium. Magnesium levels were increased in animal group treated with high dose of extract, thereby clearly indicated antiurolithic activity of EEBS. Lulat SI *et al*.,[276] demonstrated that the treatment with lithocare showed a significant decrease in elevated urinary calcium and phosphate level observed in EG-treated rats and similar to our study significant improvement in magnesium levels were also observed.

Haematological parameters were significantly affected by the administration of EG (0.75% v/v) for 28 days. In present study, lowering of red blood cells count (RBC), haemoglobin (Hb), packed cell volume (PCV), lymohocytes and mean corpuscular hemoglobim (MCH) levels were more pronounced in ethylene glycol induced urolithiasis

rats, indicating anaemia. WBC and polymorphs were increased showing that the immune system of animal is affected which might be due to the presence of infections associated with kidney injury. Animals co-administerd with EEBS showed significant reverseal in the haematological parameters (RBC, Hb, MCH, lymphocytes and WBC) that observed in animals in urolithiasis control group, signifying the protective activity of extract. Our results were consisitent with the study report of Badrinathan S *et al*.[281]

Hypoalbuminemia and hypoproteinemia represent nephrotic syndrome, in which protein is lost through urine due to kidney injury.[282] In our findings, decreased serum total protein level observed in urolithiais control rats were significantly elevated in rats treated with standard/ extract, where as significant change in albumin level were not observed among animals in toxic control, standard or extract-treated rats . Sharma I *et al*.,[277] demonstrated that significant changes were not observed in total protein level in calculi-induced rats, compared to rats in normal control group. However, elevated serum albumin level observed in urolithiasis rats were reduced in experimental animals treated with aqueous extract of *Bergenia ligulata*.

Glomerular filtration rate decreases due to the obstruction of urine outflow by stones in the urinary system or due to the injury of renal parenchyma. This promotes accumulation of the waste products, particularly nitrogenous substances such as urea, creatinine, and uric acid in the blood.[283] This result also confirmed a significant increase in serum levels of uric acid, creatinine, blood urea nitrogen, calcium, magnesium and phosphate in animals treated with EG, compared to animals in normal control. These nitrogenous substances and serum biochemicals (calcium, phosphate) levels were found

to be lowered following treatment with EEBS in dose dependent manner. Our findings were consistent with the study of Shah JG *et al*.,[284] Hussain G *et al.*[285]

Previous research study have confirmed enhanced lipid peroxidation and lowering levels of antioxidant potential in rats supplemented with a calculi-producing diet.[286] In this study EEBS treated animals served both as preventive and curative regimens, significantly decreased malondialdehyde levels and increased activity of antioxidant enzyme level of superoxide dismutase and glutathione peroxidase in comparison with animals in calculi-induced group, thereby inhibiting the changes associated with oxidative stress. The results obtained were consisitent with the findings of Dixit P *et al.*[287]

6.7. Histopathological studies

Histopathological examination of kidneys substantiated the results obtained from *in vivo* evaluation of antiurolithic and nephroprotective activity in animals. Renotoxicity in preclinical study can be confirmed by determining the pathological symptoms such as tubular degeneration, desquamation, necrosis, intertubular hemorrhage, presence of hyaline casts in tubules, congestion and swelling in glomerulus.[288] In this study, administration of GM (100 mg/kg) and single dose of cisplatin (8 mg/kg) caused acute kidney injury which were evident by marked mesangial hypercellularity in glomerulus, tubular necrosis, tubular degeneration, cytoplasmic vacuolation in the proximal tubules, intertubular hemorrhage and swelling in glomerulus, as compared to animals in control group.` These pathological symptoms were lowered in EEBS-treated groups which were evident from mild epithelial vacuolation in the proximal tubules and mild blood vessels congestion, thus showing protective effect of drug. It was observed that, amelioration of

GM/CDDP-induced renal injury was more insightful in rats treated with 500 mg/kg of EEBS. Our histopathological findings were correlated with those of Sahar M *et al*.[289]

Microphotographic analysis of animals treated with EG for 28 days resulted in dilation of renal tubule, glomerular injury which might be due to the presence of crystals which causes obstruction in tubular blood flow. Preclinical studies have reported that 28 days of EG (0.75%, v/v) administration in rats resulted in the formation of renal calculi.[290] Group V animals co- administered with EEBS at a dose of 500 mg/kg, markedly decreased crystal deposition in renal tubules with mild tubular dilation and blood vessel congestion. Our histopathological results were correlated with those of Gilhotra U *et al.*[291]

7. SUMMARY AND CONCLUSION

Kidney performs various essential functions in the body including metabolism and excretion of exogenously administered drugs, chemicals and their metabolites. Hence kidney is considered as a major organ vulnerable to develop various forms of toxic effects due to drug administration. Today, people have more comorbidties and are exposed more to therapeutic and diagnostic procedures having nephrotoxic potential. Therefore, incidence of drug induced kidney injury is dramatically increasing worldwide and present even among young children and exerts substantial burden to the people. Urolithiasis is a painful disorder and also leads to morbidities and end stage renal failure. Several mechanisms are involved in developing drug induced kidney injury including oxidative stress, inflammation, fibrogenesis and apoptosis. Reactive oxygen species are considered as the important mediators in drug induced nephrotoxicity. Previous research studies also reported that formation of urinary stones leads to oxidative stress in patients. Endogenous antioxidant defense system often produce insufficient protection against free radicals, hence it is required to supplement antioxidants from external source.

Drug induced kidney injury is a multifactorial process, hence indigenous system of medicine and traditional herbal remedies focusing on multiple targets are considered as effective and safe with minimal side effects. Various herbal medicines are esteemed all over the world as a source of therapeutic agent for prevention and treatment of nephrotoxicity and urolithiasis. Clinical and preclinical reports indicated that flavonoids inhibit calcium oxalate and crystal deposition in urine. Polyphenols especially phenolic and flavonoids are aromatic compounds which possess excellent free radical scavenging

activity. *Plants* rich with saponin, disaggregate the suspension of mucoproteins, which promote crystallization.

In this present study, we have evaluated the nephroprotective and antiurolithic activity of ethanol extract of whole plant *B. sensitivum* by selecting experimental models which included gentamicin-induced nephrotoxicty, cisplatin-induced nephrotoxicity and ethylene glycol-induced urolithiasis in rats.

Phytochemical screening indicated the presences of carbohydrates, alkaloids, steroids, saponins, proteins, aminoacids, falvonoids, tannins, phenolic compounds and fixed oils in ethanol extract of *B. sensitivum.*

Our study also proved that this drug possess considerable amount of flavonoid and phenolic compounds. *In vitro* antioxidant and antiurolithic study illustrated that EEBS possessed significant free radical scavenging activity and excellent inhibiton on nucleation and aggregation of stone forming constituents in urine, thereby confirming its lithotriptic effect. Image analysis of CaOx crystals using microscopic assay, further confirmed its lithotriptic activity. Our study also demonstrated cytoprotective activity of EEBS. Cytotoxicity and DNA fragmentation were observed only at high concentration. Supplementation of EEBS to drug induced nephrotoxic and urolithiasis rats significantly restored changes in body weight, kidney weight, urine pH, indicating its nephroprotective and antiurolithic activity. Diuretic effects were observed in gentamicin-induced nephrotoxicity and ethylene glycol-induced urolithiasis model. Increased urine output promotes flushing out of stone forming constituents through urine. Prevention of proteinuria, albuminuria and hypercalciuria in gentamicin and cisplatin-treated rats,

hypermagnesuria in gentamicin-treated rats, indicating its nephroprotective activity. Hypocalciuric and hypermagnesuric effect observed in the ethylene+EEBS-treated animals, clearly indicating its antiurolithic activity.

Co-therapy with EEBS showed significant reversal in the hematological parameters that observed in animals in toxic control group and exhibited improvement in hematological status. Treatment with EEBS in GM-induced rats restored the elevated serum level of nitrogenous waste products and diminished calcium and magnesium levels. CDDP-treated animals restored the elevated serum level of BUN, creatinine and diminished total protein, calcium and sodium level. In urolithiasis induced animals, serum level of calcium, phosphate and nitrogenous substances were lowered, where as total protein levels were increased in animals co-administered with EEBS. Effect of *B. sensitivum* on oxidative stress were evaluated using estimation of enzymatic (superoxide dismutase, catalase, and glutathione peroxidase) and non enzymatic (lipid peroxidation and reduced glutathione) constituents in kidney homogenate. Co-treatment with EEBS significantly restored the level of enzymatic and nonenzymatic antioxidants, thereby inhibited the changes associated with oxidative stress. Histopathological examination of kidneys from toxicity induced animals substantiated the results obtained from *in vivo* evaluation of nephroprotective, antiurolithic and antioxidant activity.

Thus the results obtained from nephroprotective and antiurolithic investigation provided sufficient scientific evidences about the use of *B. sensitivum* for the treatment of drug induced kidney injury. These activities attributed to the presence of phytoconstituents present in *B. sensitivum*.

Hence, we may conclude that the whole plant *B. sensitivum* used alone or in combination with other herbal or synthetic drugs may have promising nephroprotective and antiurolithic effect.

8. IMPACT OF THE STUDY

Current study highlighted the nephroprotective activity of ethanol extract of *B. sensitivum* in gentamicin and cisplatin-induced nephrotoxicity and antiurolithic effect in ethylene glycol-induced urolithiasis in rats. These activities of EEBS can be confirmed further by doing research works using different toxic agents or solvent extracts.

Our findings warrant advanced analytical research on isolation, identification and purification of phytoconstituents present in *B. sensitivum* responsible for the nephroprotective and antiurolithic activity.

We also recommend to evaluate pharmacological studies, molecular mechanism of action and potential usefulness of *B. sensitivum* as an alternative nephroprotective and antiurolithic agent.

Further pharmacokinetic, toxicological studies and clinical evaluation are needed before usage in human.

9. BIBILOGRAPHY

1. Choudhury D, Ahmed Z. Drug-associated renal dysfunction and injury. *Nat Clin Pract Nephrol*. 2006; 2(2): 80-91.

2. Rodrigues FA, Prata MM, Oliveira IC, Alves NT *et al.* Gingerol fraction from *Zingiber Officinale* protects against gentamicin-induced nephrotoxicity. *Antimicrob Agents Chemother*. 2014; 58(4): 1872-8.

3. Miller RP, Tadagvadi RK, Ramesh G, Reeves WB. Mechanisms of Cisplatin nephrotoxicity. *Toxins (Basel)*. 2010; 2(11): 2490-518.

4. Van Der GK. *Human Anatomy*. 6th ed. New York, USA: McGraw-Hill Companies; 2001. pp. 678- 83.

5. Scanlon VC, Sanders T. *Essentials of Anatomy and Physiology.* 5th ed. Philadelphia, USA: FA Davis Company; 2007. pp. 421-32.

6. Russell JG, Norman DH. Pathology and Therapeutics for pharmacists. A basic for clinical pharmacy practice. 3rd ed. London: Pharmaceutical press; 2008: 132-5.

7. Vidya S, Ramesh A, Rajashekar G, Meghana D. The nephroprotective activity of methanolic extracts of *Phyllanthus acidus* Leaves against gentamycin-induced nephrotoxicity in experimental rodents. *Int J Pharm Pharm Sci*. 2013; 5(4): 209-13.

8. Galley HF. Can acute renal failure be prevented. *J R Coll Surg Edinb*. 2000; 45(1): 44-50.

9. Rajitha P, Joyamma V, Dinoop DR. Are statins nephroprotective? A Dose in Albino rats. *Int J Pharm Pharm Sci*. 2013; 5(3): 182-90.

10. Liberthal W, Nigam SK. Acute renal failure. Experimental models of acute renal failure: imperfect but indispensible. *Am J Renal Physiol*. 2000; 278(1): 1-12.

11. Kashtan CE. Glomerular disease. *Semin Nephrol.* 1999; 19(4): 353-63.

12. Wardle EN. Acute renal failure and multiorgan failure. *Nephrol Dial Transplant.* 1994; 9(4): 104-7.

13. Jehti RK, Duggal B, Sahota RS, Gupta M, Sofat JB. Important plants used in stone. *India J Med Res*. 1983; 78(3): 422-5.

14. Jha V, Chugh KS. Drug induced renal disease. *J Assoc Physicians Drug India*. 1995; 43(6): 407-21.

15. Uchino S, Kellum JA, Bellomo R, Doig GS *et al*. Acute renal failure in critically ill patients: a multinational, multicenter study. *J Am Med Assoc*. 2005; 294(7): 813-8.

16. Hoste EA, Bagshaw SM, Bellomo R, Cely CM *et al*. Epidemiology of acute kidney injury in critically ill patients: the multinational AKI-EPI study. *Intensive Care Med*. 2015; 41(8): 1411- 23.

17. Eslami SH, Ebrahimzadeh MA, Hajizadeh AD, Nabavi SF *et al*. Renoprotective Effect of *Eryngium caucasicum* in gentamicin-induced nephrotoxic mice. *Arch Biol Sci.* 2011; 63(1): 157-60.

18. Coresh J, Selvin E, Stevens L A, Manzi J *et al*. Prevalence of chronic kidney disease in the United States. *J Am Med Assoc*. 2007; 98(17): 2038-47.

19. Moffett BS, Goldstein SL. Acute kidney injury and increasing nephrotoxic medication exposure in non critically-ill children. *Clin J American Soc Nephrol*. 2011; 6(4): 856-63.

20. Bommer J. Prevalence and socio-economic aspects of chronic kidney disease. *Nephrol Dial Transplant.* 2002; 17(11**)**: 8-12.

21. Curhan GC, Willett WC, Rimm B, Stampfer M. A prospective study of the intake of vitamins C and B6, the risk of kidney stone in men. *J Urol*. 1996; 155(6): 1847-51.

22. Mark AP. Renal vulnerability to drug toxicity. *Clin J Am Soc Nephrol.* 2009; 4: 1275-

    83.

23. Margaret SP, Elizabeth C, Gary CC. Urologic disease in America project: Urolithiasis. *J Urol.* 2005; 173(3): 848-57.

24. Portilla D, Safar AM, Shanon ML, Penson RT. Cisplatin Nephrotoxicity. *Wolters Kluwer Health*. 2013; 2: 39-57.

25. Davies DM, Ferner RE, De-Glanville H. *Adverse Drug reactions*. 5th ed. New Zealand: Wellington Publishers; 2008. pp. 300-12.

26. Perazella MA. Crystal-induced acute renal failure. *Am J Med.* 1999; 106(4): 459-65.

27. Schetz M, Dasta J, Goldstein S, Golper T. Drug-induced acute kidney injury. *Curr Opin Crit. Care*. 2005; 11(6): 555-65.

28. Ferguson MA, Vaidya VS, Bonventre JV. Biomarkers of nephrotoxic acute kidney injury. *Toxicology*. 2008; 245(3): 182-93.

29. Palmer BF. Renal dysfunction complicating the treatment of hypertension. *N Engl J Med.* 2002; 347(16): 1256-61.

30. Markowitz GS, Perazella MA. Drug-induced renal failure: a focus on tubulointerstitial disease. *Clin Chim Acta*. 2005; 351(1-2): 31-47.

31. Isnard BC, Deray G, Baumelou A, Le Quintrec M *et al*. Herbs and the kidney. *Am J Kidney Dis.* 2004; 44(1): 1–11.

32. Rodriguez IB, Garcia GG. The role of tubulointerstitial inflammation in the progression of chronic renal failure. *Nephron Clin Pract.* 2010; 116 (2): 81-8.

33. Coco TJ, Klasner AE. Drug-induced rhabdomyolysis. *Curr Opin Pediatr*. 2004; 16(2): 206–10.

34. Pisoni R. Ruggenenti P. Remuzzi G. Drug-induced thrombotic microangiopathy: incidence, prevention and management. *Drug Saf.* 2001; 24(7): 491-501.

35. Noorani AA, Gupta K, Bhadada K, Kale M K. Protective Effect of methanolic leaf extract of *Caesalpinia Bonduc* (L.) on Gentamicin-induced hepatotoxicity and nephrotoxicity in rats. *Iranian J Pharmacol & Ther.* 2010; 10(1): 21-5.

36. Lerner SA, Schmitt BA, Seligsohn R, Matz GJ. Comparative study of ototoxicity and nephrotoxicity in patients randomly assigned to treatment with amikacin or gentamicin. *Am J Med*. 1986; 80(6): 98-104.

37. Luft FC. Clinical significance of renal changes engendered by aminoglycosides in man. *J Antimicrob Chemother*. 1984; 13(suppl A): 23-30.

38. Edson RS, Terrell CL. The aminoglycosides. *Mayo Clin Proc*. 1999; 74(5): 519-28.

39. Alfthan O, Renkonen OV, Sivonen A. Concentration of gentamicin in serum, urine and urogenital tissue in man. *Acta Pathol Microbiol Scand B Microbiol Immunol*. 1973: 241(Suppl): 92-4.

40. Kakasaheeb JK, Rajkumar VS, Apsari JP. Effect of *Abutilon indicum* extract in gentamicin induced nephrotoxicity. *IJPRD.* 2011; 3(1): 73-9.

41. Marie-Paule, Mingeot-Leclercq, Paul MT. Aminoglycoside: Nephrotoxicity. *Antimicrob Agents Chemothe*r. 1999; 43(5): 1003-12.

42. Afeefa T, Uzma S, Saeed M, Furqan KH *et al.* Evaluation of protective and curative role of α-lipoic acid and selenium in gentamicin induced nephrotoxicity in rabbits. *Pak J Pharm Sci*. 2012; 25: 103-10.

43. Mohammed SA, El-Gerbed. Ameliorative effect of fish oil on the cisplatin-induced hepatotoxicity and nephrotoxicity in rats. *Res J Pharm Biol Chem Sci.* 2013; 4(4): 479-91.
44. Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. *Kidney Int*. 2008; 73(9*)*: 994-1007.

45. Ronald PM, Reghu KT, Ganesan R, William BR. Mechanism of nephro toxicity. *Toxins (Basel)*. 2010; 2(11): 2490-518.

46. Kim ES, Lee JS, Akram M, Kim KA *et al*. Protective activity of *Dendropanax morbifer*a against cisplatin- induced acute kidney injury. *Kidney and Blood Press Res*. 2015; 40(1): 1-12.

47. Amala R, Laurent P, Patrick M, Ganeshan R. Netrin-1 overexpression in kidney proximal tubular epithelium ameliorates cisplatin nephrotoxicity. *Lab invest*. 2011; 91(12); 1717-26.

48. Yadav RD, Jain SK, Alok S, Mahor A *et al.* A review herbal plants used in the treatment of urolithiais. *Int J Pharm Res Re*. 2011; 2(6): 1412-20.

49. Tiselius HG, Hallin A, Lindback B. Cystallization properties in stone forming and normal subject's urine dilution using a standardized produce to match the composition of urine in the distal part of the distal tubule and the middle part of the collecting duct. *Urol Res.* 2001; 29: 75-82.

50. Gupta M, Bhayana S, Sikka SK. Role of urinary inhibitors and promoters in calcium oxalate crystallization. *IJRPC*. 2011; 1(4): 793-8.

51. Stevens LA, Coresh J, Greene T, Levey AS. Assessing kidney function measured and estimated glomerular filtration rate. *N Engl J Med*. 2006; 354(3): 2473- 83.

52. Summer D. Blood urea nitrogen-diacetyl monoxime method, Procedure No. 1750. *Chemistry, M LAB*. 2012; 2360: 220-81.

53. Nelson DL, Cox MM, Lehninger. *Principles of Biochemistry.* 4th ed. New York, USA: WH Freeman and Company; 2005. pp. 667-9.

54. Delanghe JR, Speeckaert MM. Creatinine determination according to Jaffe-what does it stand for. *NDT Plus*. 2011; 4(2): 83-6.

55. Herget RS, Poppen D, Husing J, Margraf G *et al*. Prognostic value of tubular proteinuria and enzymuria in nonoliguric acute tubular necrosis. *Clin Chem*. 2004; 50(3): 552-8.

56. Morgensen CE. Urinary albumin excretion in early and long term juvenile diabetes. *Scandinavian J Clin Lab Invest*. 1971; 28: 101-9.

57. Schurek HJ, Neumann KH, FlorH, ZehM *et al.* Diagnostic and prognostic significance of proteinuria selectivity indexing glomerular diseases. *Clinica Chimica Acta*. 2000; 297: 73-8.

58. Cynthia N. Drug induced nephrotoxicity. *Am Fam Physician*. 2008; 78(6): 743-50.

59. Yoshimura E, Fujii M, Koide S. A case of Chinese herbs nephropathy in which progression of renal dysfunction was slowed by steroid therapy. *Jap J Nephrol*. 2000; 42: 66-72.

60. Aslam M, Dayal R, Javed R, Parray SA *et al*. Nephroprotective effects of *Peucedanum grande* against accute renal failure induced by potassium dichromate in rats. *Int J Pharma Sci Drug Res*. 2013; 5(2): 45-9.

61. Pedraza-Chaverri J, Paola YC, Yolanda IC, Dolores JSG *et al.* Protective effects of garlic powder against potassium dichromate- induced oxidative stress and nephrotoxicity. *Food Chemical Toxicol.* 2008; 46(2): 619-27.

62. Moe OW, Pearle MS, Sakhaee K. Pharmacotherapy of urolithiasis: evidence from clinical trials. *Kidney Int*. 2011; 79(4): 385-92.

63. Pak CY. Prevention and treatment of kidney stones. Role of medical prevention. *J Urol*. 1989; 141(3): 798-801.

64. Kwon DJ, Bae YS. Flavonoids from aerial parts of *Lespedeza cuneata*. *Biochem Syst Ecol*. 2009; 37(1): 46-8.

65. Wang CY, Deng HZ, Li H. Experimental study on treatment of minimal change nephropathy with *Lespedeza michx. Zhongguo Zhong Yao Za Zhi*. 2005; 30: 614-7.

66. Fukai T, Satoh K, Nomura T. Preliminary evaluation of antinephritis and radical scavenging activities of glabridin from *Glycyrrhiza glabra*. *Fitoter.* 2003; 74(7-8): 624-9.

67. Divakar K, Pawar AT, Chandrasekhar SB, Dighe SB *et al*. Protective effect of the hydro-alcoholic extract of *Rubia cordifolia* roots against ethylene glycol induced urolithiasis in rats. *Food Chem Toxicol.* 2010. 48(4): 1013-8.

68. Bhayatti VV, Pai KV, D'Souza MG. Effect of phenolic compounds from *Berginia* ciliata (Haw.). Leaves on experimental kidney stones. *Ancient Science of Life*. 2010; 30(1): 14-7.

69. Patel PK, Patel MA, Vyas BA, Shah DR. Antiurolithiatic activity of saponin rich fraction from the fruits of *Solanum xanthocarpum* Schrad. & Wendl. (Solanaceae)

against ethylene glycol induced urolithiasis in rats. *J Ethnopharmacol*. 2012; 144(1): 160-70.

70. Akanae W, Tsujihata M, Yoshioka I, Nonomura N *et al*. *Orthosiphon grandiflorum* has a protective effect in a calcium oxalate stone forming rat model. *Urol Res*. 2010; 38(2): 89-96.

71. Tugcu V, Kemahli E, Ozbek E, Arinci YV *et al*. Protective effect of a potent antioxidant, pomegranate juice, in the kidney of rats with nephrolithiasis induced by ethylene glycol. *J Endourol*. 2008; 22(12): 2723-31.
72. Yuliana ND, Khatib A, Link-Struensee AM, Ijzerman AP *et al.* Adenosine A1 receptor binding activity of methoxy flavonoids from *Orthosiphon stamineus. Planta Med.* 2009; 75(2): 132-6.

73. Arcasoy HB, Erenmemisoglu A, Tekol Y, Kurucu S *et al*. Effect of *Tribulus terrestris* L. saponin mixture on some smooth muscle preparations: A preliminary study. *Boll Chim Farm*. 1998; 137(11): 473–5.

74. Kumaran MGS, Prasad SR, Mitra SK. Evaluation of efficacy and safety of cystone syrup in lower ureteric calculus. *Indian J Clin Prac*. 2007; 18(4): 31-6.

75. Varalakshmi P, Shamila Y, Latha. Effect of *Crataeva nurvala* in experimental urolithiasis. *J Ethnopharmacol*. 1990; 28(3): 313-21.

76. Varghese KJ, Anila J, Nagalekshmi R, Resiya S, Dasapushpam JS. The traditional uses and the therapeutic potential of ten sacred plants of Kerala state in India. *Int J Pharm Sci Res*. 2010; 1(10): 50-9.

77. Hoste EA, Kellum JA. Acute kidney injury: epidemiology and diagnostic criteria. *Curr Opin Crit Care*. 2006; 12(6): 531-7.

78. Weinberg JM. The cellular basis of nephrotoxicity & diseases of the Kidney. In: Schrier RW, Gottschalk CW, editors. *Boston Little Brown*. 1993; pp. 1031-7.

79. Zager RA. Pathogenetic mechanisms in nephrotoxic acute renal failure. *Semin Nephrol*. 1997; 17(1): 3-14.

80. Abdel-Raheem IT, Abdel-Ghany AA, Mohamed GA. Protective effect of quercetin against gentamicin-induced nephrotoxicity in rats. *Biol Pharm Bull*. 2009; 32(1): 61-7.

81. Bhattacharjee S. Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plants. *Curr Sci*. 2005; 89(7): 1113-21.

82. Wagner H, Elbl G. ACE-inhibitory procyanidins from *Lespedeza capitata. Planta Med*. 1992; 58: 27-9.

83. Gurocak S, Kupeli B. Consumption of historical and current phytotherapeutic agents for urolithiasis: a critical review. *J Urol*. 2006; 176(2): 450-5.

84. Yun-Lian L, Wan-Yi W. Chemical constituents of *Biophytum sensitivum*. *Chin Pharm J*. 2003; 55(1): 71-5.

85. Bucar F, Jachak SM, Schubert ZM, Kartnig T. Phenolic compounds from *Biophytum sensitivum*. *Pharmazie*. 1998; 53: 651-3.

86. Leopold J, Gerhard B, Mohamed SP, Beena J, Andrea W. Medicinally used plants from India: Analysis of the essential oil of air-dried *Biophytum sensitivum* (L.) DC. *Sci Pharm*. 2004; 72(1): 87-96.

87. Joy PP, Thomas J, Mathew S, Skaria BP. *In: Medicinal Plants: Tropical Horticulture*. Bose TK, Kabir J, Das P, Joy PP, editors. Vol. 2. Calcutta: Naya Prakash; 2001. pp. 449-632.

88. *Biophytum sensitivum* or Mukkutty. Available from: http://siddham.in/mukkutty-*Biophytum-sensitivum*. [Last updated on 2010 June 26; cited on 2010 Aug 9]

89. Jirovetz L, Buchbauer G, Jose B. Medicinal used plants from India: Analysis of essential oil of air- dried *Biophytum sensitivum* (L.) DC. *Sci Pharm*. 2004; 72: 87-96.

90. Nadkarni AK. *The Indian materia medica*. 3rd ed. Mumbai: Popular Prakasan Pvt. Ltd; 2002. 1; pp. 99-107.

91. Editorial Committee of the Flora of Taiwan. 2nd ed. *Flora of Taiwan*; 1993.

92. Arya Vaidya Sala. *Indian medicinal plants*. Chennai: Orient longman Ltd; 2002. 1: 271

93. Kirtikar KR, Basu BD. *Indian medicinal plants*. 2nd ed. Vol. I, Dehradun: International Book Distributors; 1999. pp. 1045-8.

94. Agarwal VS. *Drug plants of India*. Ist ed. New Delhi: Kalyani publishers; 1997. 1; pp. 222-3.

95. Chatterjee A, Pakrashi SC. *The treatise on Indian medicinal plants*. *NISCAIR CSIR*. 2003. 3: 118.

96. Bharati AC, Sahu AN. Ethnobotany, phytochemistry and pharmacology of *Biophytum sensitivum* DC. *Pharm Rev*. 2012; 6(11): 68-73.

97. Bucar F, Jachak SM, Noreem Y, Karting T *et al*. Catalysed prostaglandin M. Amentoflavone from *Biophytum sensitivum* and its effect on COX-1/COX-2 biosynthesis. *Planta Med*. 1998; 64(4): 373-4.

98. Inngjerdingen KT, Coulibaly A, Diallo D, Michaelsen TE, Paulsen BS. A complement fixing polysaccharide from *Biophytum petersianum Klotzsch*, a medicinal plant from Mali, West Africa. *Biomacromolecule*s. 2006; 7(1): 48-53.

99. Guruvayoorappan C, Kuttan G. Amentoflavone a bioflavonoid from *Biophytumm sensitivum*, augments lymphocyte proliferation, natural killer cell, and antibody dependent cellular cytotoxicity through enhanced production of IL-2 and IFN-gamma restrains and restrains sialic acid and gamma glutamyl transpeptidase production in tumour bearing animals. *J Exp Ther Oncol.* 2007; 6(4): 285-95.

100. Guruvayoorappan C, Kuttan G. Inhibition of tumour specific angiogenesis by amentoflavone. *Biochemistry (Mosc)*. 2008; 73: 209-18.

101. Puri D, Baral N. Hypoglycemic effect of *B. sensitivum* in the alloxan diabetic rabbits. *Indian J Physiol Pharmacol.* 1998; 42(3): 401-6.

102. Prabu KA, Kumarappan CT, Christudas S, Kalaichelvan VK. Effect of *B. sensitivum* on streptozotocin and nicotinamide induced diabetic rats. *Asian Pac J Trop Med*. 2012; 2(1): 31-5.

103. Chandrasekharan C, Kuttan G. Methanol extract of *Biophytum sensitivum* alters the cytokine profile and inhibits iNOS and COX-2 expression in LPS/Con A stimulated macrophages. *Drug Chem Toxicol*. 2008; 31(1): 175-88.

104. Jachak SM, Bucar F, Kartnig T. Anti-inflammatory activity of extracts *Biophytum sensitivum* in carrageen in-induced rat paw oedema. *Phytother Res*. 1999; 13(1): 73-4.

105. Puri D. Hypocholestrolemic effect of *Biophytum sensitivum* leaf water extract. *Pharm Biol*. 2003; 41(4): 253-8.

106. Natarajan D, Shivakumar MS, Srinivasan R. Antibacterial activity of leaf extract of Biophytum sensitivum (L.) DC. *J Pharm Sci Res*. 2010; 2: 717-20.

107. Rajanikant TK, Nagesh S, Senthilkumar KL. *In vitro* anthelmintic activity of leaves and stem extracts of *Biophytum sensitivum* (Linn.). *Asian J Plant Sci Res.* 2013; 3(6): 64-8.

108. Guruvayoorappan C, Afira AH, Kuttan G. Antioxidant potential of *Biophytum sensitivum* extract *in vitro* and *in vivo*. *J Basic Clin Physiol Pharmacol*. 2006; 17(4): 255-67.

109. Guruvayoorappan C, Kuttan G. Immunomodulatory and antitumor activity of *Biophytum sensitivum* extract. *Asian Pac J Cancer Prev*. 2007; 8(1): 27-32.

110. Bhaskar VH, Rajalakshmi V. Anti tumour activity of aqueous extract of *Biophytum sensitivum* Linn. *Ann Biol Res*. 2010; 3: 76-80.

111. Sakthivel KM, Guruvayoorappan C. Modulating effect of *Biophytum Sensitivum* extact on rats with acetic acid induced ulcerative colitis. *Pharm Biol.* 2014; 1: 1-11.

112. Anindya B, Abdur R, Mohammed ALR, Tapas P. Screening of ethanolic extract of *Biophytum sensitivum* DC leaves on peptic ulcer induced by aspirin in Wistar albino. *Int J Pharm Phytopharmacol Res*. 2014; 3(6): 418-22.

113. Guruvayoorappan C, Kuttan G. Anti-angiogenic effect of *Biophytum sensitivum* is exerted through its cytokine modulation activity and inhibitory activity against VEGF mRNA expression and endothelial cell migration and capillary tube formation. *J Exp Ther Oncol.* 2007; 6(3): 241-50.

114. Guruvayoorappan C, Kuttan G. Evaluation of the chemoprotective effect of *Biophytum sensitivum* (L.) DC extract against cyclophosphamide induced toxicity in Swiss albino mice. *Drug Metabol Drug Interact.* 2006; 22(2-3): 131-50.

115. Johnson M, Shibila T, Revathy I, Uchimahali M *et al*. Biopotency of *Biophytum sensitivum*. *Res Pharm.* 2015; 5: 42-8.

116. Saritha B, Brindha P. Wound healing potential of *Biophytum sensitivum* (L.) DC. An ayurvedic drug. *J Chem Pharml Res.* 2015; 7(3): 87-94.

117. Guruvayoorappan C, Kuttan G. Apoptotic effect of *Biophytum sensitivum* on B16F-10 cells and its regulatory effects on nitric oxide and cytokine production on tumor-associated macrophages. *Integr Cancer Ther*. 2007; 6(4): 373-80.

118. Guruvayoorappan C, Kuttan G. Protective effect of *B. sensitivum* (L.) DC on radiation- induced damage. *Immunopharmacol Immunotoxicol*. 2008; 30(4): 815-35.

119. Anil TP, Niraj SV. Anti-urolithiatic activity of standardized extract of *Biophytum sensitivum* against zinc disc implantation induced urolithiasis in rats. *J Adv Pharm Technol Re.* 2015; 6(4); 176- 82.

120. Anil TP, Niraj SV. Protective effect of ethyl acetate fraction of *Biophytum sensitivum* extract against sodium oxalate- induced urolithiasis in rats. *J Tradit Complement Me*. 2017; 7(4): 476-86.

121. Anil TP, Niraj SV. Protective effect of standardized extract of *Biophytum sensitivum* extract against calcium oxalate urolithiasis in rats. *Bull fac pharm, (Cairo univ).* 2015; 53(2): 161-72.

122. Sachin C, Girendra G, Mamle SN. Nephroprotective activity of different extracts of *Biophytum sensitivum* (Linn.) DC. *Int J Herb Med*. 2017; 5(1): 31-4.

123. Cooper JW, Gunn C. In; Infusion and maceration processes. *Tutorial Pharmacy*. 5th ed. London: Pitman Medical; 1957. pp. 308-17.

124. Harborne AJ. Phytochemical methods. A guide to modern techniques of plant analysis. 3rd ed. *Springer Sciences and Business Media*. 1998. pp. 334.

125. Singleton VL, Rossy JA. Colourimetric of total phenolics with phophomolybdic acid- phosphotungistic acid reagents. *Am J Enol and Vitice*. 1965; 16 (3): 144-58.

126. Quitter DC, Gresseir B, Vasseur J, Dine T *et al*. Phenolic compounds and antioxidant activities of buck wheat (*Fagopyrum esculentum* Moench) hull and flour. *J Ethnopharmacol*. 2000; 72(1-2): 35-42.

127. Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature*. 1958; 181(4617): 1199-1200.

128. Benzie IFF, Strain JJ. Ferric reducing ability of plasma (FRAP) as a measure of antioxidant power. The FRAP assay. *Anal Biochem*. 1996; 239(1): 70-6.

129. Re R, Pellegrini N, Proteggente A, Pannala A *et al*. Antioxidant activity applying an improved ABTS radical cation decolourizationn assay. *Free Radic Biol Med.* 1999; 26 (9-10): 1231-7.

130. Halliwell B, Gutteridge JMC. *Free Radicals in biology and medicine*. 3rd ed. Oxford: Clarendon Press; 1989. pp. 617-783.

131. Nishmiki NA, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem Biophys Res Commun*. 1972; 46(2): 849-54.

132. Garrat D. The quantitative analysis of drugs. 3rd ed. USA: *Springe*r; 1964. pp. 926.

133. Oyiazu M. Studies on product of browning reaction prepared from glucose amine. *Jpn J Nature*. 1986; 44: 307-15.'

134. Hennquin C, Lalanne V, Lacour B, Drueke T. A new approach to studying inhibitors of calcium oxalate crystal growth. *Urol Res*. 1993; 21(2): 101-8.

135. Prachi K, Jaya S, Arun K. *In-vitro* evalution of *Coleus aromaticus* leaves for antilithatic activity. *Int J pharmacol*. 2014; 1(1): 45-50.

136. Atmania F, Slimania Y, Mimounib M, Aziz M, Hachtb B. Effects of aqueous extract from *Herniaria hirsute* L. on experimentally nephrolithiatic rats. *J Ethnopharmacol.* 2004; 95: 87-93.

137. Phillips HJ, Terryberry JE. Counting actively metabolizing tissue cultured cells. *Exp Cell Res.* 1957; 13: 341-7.

138. Patel S, Gheewala N, Suthar A, Shah A. *In-vitro* cytotoxicity activity of *Solanum nigrum* extract against HELA cell line and VERO cell Line. *Int J Pharm Pharm Sci*. 2009; 1(1): 38-46.

139. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65(1-2): 55-63.

140. Van MJ, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay Methods. *Mol Biol*. 2011; 731: 237-45.

141. Denizol F, Lang R. Rapid colourimetric assay for cell growth and survival: Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods*. 1986; 89(2): 271-7.

142. Wroblewski F, La Due JS. Lactic dehydrogenase activity in blood. *Proc Soc Exp Bio Med*. 1955; 90(1): 210-3.

143. Takada M, Noguchi A, Sayama Y, Kurohane KY. Inositol 1,4,5-trisphosphate receptor-mediated initial $Ca(2^{+})$ mobilization constitutes a triggering signal for hydrogen peroxide-induced apoptosis in INS-1 β-cells. *Biol Pharm Bull*. 2011; 34(7): 954-8.

144. Azhar-Alam MM, Javed K, Jafri MA. Effect of *Rheum emodi* (Revand Hindi) on renal functions in rats. *J Ethno Pharmacol*. 2005; 96(1-2): 121-5.

145. Rajesh KS, Rajani GP, Vivek S, Komala N. Effect of ethanolic and aqueous extracts of *Bauhinia Variegata* Linn. on Gentamicin-induced nephrotoxicity in rats. *Ind J Pharm Edu Res*. 2011; l45(2): 192-8.

146. Sonja I, Nenad S, Milica V, Slavimir V, Gordana S. Protective effect of quercetin on cisplatin-induced nephrotoxicity in rats. *Med Biol*. 2014; 16(2): 71-5.

147. Oluwatosin AA" Adesola FA, Olufunke EO. Ameliorative effects *of* chloroform fraction *of Cocosnucifera* L. husk fiber against cisplatin induced toxicity *in* rats. *Pharmacog Res.* 2016; 8(2): 89-96.

148. Soundararajan P, Mahesh R, Ramesh T. Biopotency of *Aervalanata* on membrane bound ATPase and marker enzymes in urolithiatic rats. *IRJP*. 2007; 1(4): 221-8.

149. Surendra K, Pareta, Kartik CP. *Boerhaavia diffusa* Linn aqueous extract as curative agent in ethylene glycol induced urolithiasis. *Pharmacolonline.* 2010; 3(2): 112-20.

150. Atmani F, Slimani Y, Mimouni M, Hacht B. Prophylaxis of calcium oxalate stones by *Herniaria hirsuta* on experimentally induced nephrolithiasis in rats. *BJU Int.* 2003; 92(1): 137-40.

151. Ramnic S. *Text book of medical lab technology*. 1st ed. New Delhi: Jypee; 2007. pp. 204-21.

152. John MB. *Laboratory medicine hematology.* 4th ed. ST Louis: CV Mosby Co; 1972. pp. 1198- 204.

153. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin- phenol reagent. *J Biol Chem*. 1951; 193(1): 265-75.

154. Reinhold JG. Total protein, albumin and globulin. Stand. Methods. *Clin Chem*.1953; 1: 88-97.

155. Lorenz K. Improved determination of serum calcium with ortho-cresolphthalein complexone. *Clinica Chimica Acta*. 1982; 126(3): 327-34.

156. Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem*. 1925; 66(1): 375-81.

157. Heaton FW. Determination of magnesium by the Titan yellow and ammonium phosphate methods. *J ClinlPathol.* 1960; 13(4): 358-60.

158. Maruna RF, Trinder SR. Determination of serum sodium by colorimetric method. *Clinica Chimica Acta*. 1957; 2(6): 581-5.

159. Maruna RFL. Determination of serum potassium by colorimetric method. *Clinica Chemica acta*. 1957; 2(2): 131-3.

160. Fawcett JK, Soctt JE. A rapid and precise method for the determination of urea. *J Clin Pathol*. 1960; 13(2): 156-9.

161. Caraway WT. Uric acid. In: *Standard methods of clinical chemistry*, 4th ed, Edited by Seligson D. London: Academic Press; 1963. pp. 239- 47.

162. Bonsnes RW, Taussky HH. On the colorimetric determination of creatinine by the Jaffee reaction. *J Biol Chem*. 1945; 158(3): 581-91.

163. Kakkar P, Das B,Viswanathan PN. A modified spectrophotometric assay of SOD. *Ind J Biochem Biophy*. 1984; 21(2): 130-2.

164. Sinha AK. Colorimetric assay of catalase. *Anal Biochem*. 1972; 47(2): 389-94.

165. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hateman DG. Selenium, biochemical roles as a component of glutathione peroxidase. *J Sci*. 1973; 179(4073): 588-90.

166. Ellman GL. Tissue sulphydryl groups. *Arch Biochem Biophys*. 1959; 82(1): 70-7.

167. Okhawa H, Ohishi N, Yagi. Assay for lipid peroxidase in animal tissue by thiobarbituric acid reaction. *Anal Biochem*. 1979; 95(2): 351-8.

168. Drury RE, Walligton E. *Histological techniques*. 5th ed. New York Pronto: Carltons, Oxford University Press; 1980. pp. 206.

169. Lyons AS, Pertrucelli RJ. *Medicine: an illustrated history*. New York: Harry N Abram Publishers; 1987.

170. Seth SD, Sharma B. Medicinal plants of India. *Indian J Med Res*. 2004; 120: 9-11.

171. Rohit KB. Preliminary test of phytochemical screening of crude ethanolic and aqueous extract of *Moringa pterygosperma* Gaertn. *J Pharmacogn Phytochem*. 2015; 4(1): 7-9.

172. Uma C, Sekar KG. Elemental analysis of *Biophytum sensitivum* DC. *Int J advances in Pharm Biol Chem*. 2014; 3(3): 583-8.

173. Shibila T, Johnson M, Revathy I, Narayani M *et al*. Phytochemical profile of *Biophytum sensitivum* DC (Oxalidaceae). *World J Pharm Pharm Sci*. 2014; 3(8): 1885-94.

174. Sulaiman CT, Balachandran I. Total phenolics and total flavonoids in selected Indian medicinal plants. *Indian J Pharm Sci*. 2012; 74(3): 258-60.

175. Gunalan G, Myla N, Balabhaskar R. In vitro antioxidant analysis of selected coffee bean varieties. *J Chem Pharm Res*. 2012; 4(4): 2126-8.

176. Kinnula VJ, Crapo JD. Superoxide dismutases in malignant cells and human tumors. *Free Radic Biol Med.* 2004; 36(6): 718-44.

177. Sas K, Robotka R, Toldi J, Vécsei L. Mitochondria, metabolic disturbances, oxidative stress and the kynurenine system, with focus on neurodegenerative disorders. *J Neurol Sci.* 2007; 257(1-2-): 221-39.

178. Ramakrishna BS, Varghese R, Jayakumar S, Mathan M, Balasubramanian KA. Circulating antioxidants in ulcerative colitis and their relationship to disease severity and activity . *J Gastroenterol Hepatol.* 1997; 12 (7): 490-4.

179. Hyun DH, Hernandez JO, Mattson MP, De- Cabo R. The plasma membrane redox system in aging. *Ageing Res ReV.* 2006; 5(2): 209-220.

180. Singh U, Jialal I. Anti-inflammatory effects of alpha-tocopherol. *Ann Newyork Acad Sci.* 2004; 1031(1): 195-203.

181. Mondal SK, Chakurborty G, Mazumbder UK, Gupta M. In vitro antioxidant activity of *Diospyros malabarica* Kostel bark. *Indian J Exp Biol.* 2006; 44(1): 39-44.

182. Haung D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. *J Agric Chem*. 2005; 53(6): 1841-56.

183. Yokozawa T, Chen CP, Dong E, Tanaka T *et al.* Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2 picrylhydrazyl radical. *Biochem Pharmacol.* 1998; 56(2): 213-22.

184. Sreena R, Manon B, Merlene AB, Karthikeyan S *et al*. Antioxidant, antibacterial and anti-proliferative activity and phytochemical analysis of selected medicinal plants. *Int J Pharm Sci Rev Res*. 2013; 23(1): 172-9.

185. Pal TP, Kalita P, Barman TK , Chatterjee TK. Quantification of total flavonoid content and antioxidant activity in comparison to a reference flavonoid as *in vitro* quality evaluation parameter for assessing bioactivity of biomarkers in herbal extracts or formulations. *JPR Bio Med Rx.* 2013; 1(8): 757-66.

186. Paya M, Halliwell B, Hoult JRS. Interactions of a series of coumarins with reactive oxygen species. *Biochem Pharmacol*. 1992; 44(2): 205-14.

187. Dahal MK, Richardson T. Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and aminoacids. *J Dairy Sci*. 1978; 61: 400-7.

188. Robak J, Gryglewski RJ. Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol*. 1988; 37(5): 837-41.

189. Yildirim A, Mavi A, Kara AA. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *J Agric Food Chem*. 2001: 49: 4083-9.

190. Gow-Chin Yen, Hui-Yin Chen. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem.* 1995; 43(1): 27-32.

191. Wang H, Gao XD, Zhou GC, Cai L, Yao WB. *In vitro* and *in vivo* antioxidant activity of aqueous extract from *Choerospondiasaxillaris* fruit. *Food Chem*. 2008; 106(3): 888-95.

192. Chanda S, Nagani K. Antioxidant capacity of *Manilkarazapota* L. leaves extracts evaluated by four *in vitro* methods. *Nat Sci*. 2010; 8(10): 260-6.

193. Hamid M, Mohammad MN, Ghanea L. Evaluation of the *Raphanus sativus* effect on urinary pH. *J Res Med Sci.* 2007; 12(2): 58-61.

194. Tricheri A. Epidemiological trends in urolithiasis: impact on our health care systems. *Urol Res*. 2006; 34(2): 151-6.

195. Thamilselvam S, Hackett RL, Khan SR. Lipid peroxidation in ethylene glycol induced hyperoxaluria and calcium oxalate. *Nephrolithiasis J urol*. 1997; 157(3): 1059-63.

196. Huang HS, Ma MC, Chen CF, Chen J. Lipid peroxidation and its correlations with urinary levels of oxalate, citric acid, and osteopontin in patients with renal calcium oxalate stones. *Urology.* 2003; 62(6): 1123-8.

197. Muthusamy VV, Muthu P. Usefulness of history-taking, physical examination and diagonistic scoring in acute renal colic. *Probe*. 1980; 19: 130-1.

198. Sarmistha S, Ramtej JV. Inhibition of calcium oxalate crystallization *in vitro* by an extract of *Bergenian ciliate. Arab J U rol*. 2013; 11(12): 187-92.

199. Aslam K, Saeed RK. Anwar HG. Studies on the *in vitro* and *in vivo* antiurolithic activity of *Holarrhena antidysenterica. Urol Res*. 2012; 40(6): 671–81.

200. Zhong YS, Yu CH, Ying HZ, Wang ZY, Cai HF. Prophylactic effects of *Orthosiphon stamineus* Benth. extracts on experimental induction of calcium oxalate nephrolithiasis in rats. *J Ethnopharmacol*. 2012; 144(3): 761-7.

201. Noorafshan A, Doust SK , Karimi F. Diosmin reduces calcium oxalate deposition and tissue degeneration in nephrolithiasis in rats: a stereological study. *Korean J Urol*. 2013; 54(4): 252-7.

202. Ahmed A, Jahan N, Wadud A, Bilal A, Hajera S. *In vitro* effect of hydroalcoholic extract of *Adiantum capillus* veneris Linn. on calcium oxalate crystallization. *Int J Green Pharm*. 2013; 7(2): 106-10.

203. Mitta A, Tandon S, Singla SK, Tandon C. *In vitro* studies reveal antiurolythic effect of *Terminalia arjuna* using quantitative morphological information from computerized microscopy. *Int Braz J Urol*. 2015; 41(5): 935-44.

204. Barros ME, Schor N, Boim MA. Effects of an aqueous extract from *Phyllantus niruri* on calcium oxalate crystallization *in vitro*. *Urological research*. 2003; 30(6): 374-9.

205. Chvalova K, Brabec V, Kasparkova J. Mechanism of the formation of DNA- protein cross -links by antitumor cisplatin. *Nucleic Acids Research*. 2007; 35(6): 1812-21.

206. Freshney RI. Culture of animal cells. *A manual of basic technique*. 4th ed. New York: Wiley-Liss; 2000.

207. Graham FL, Smiley J, Russell WC, Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *The Journal of general virology*. 1977: 36(1): 59-74.

208. Strober W. Trypan blue exclusion test of cell viability. *Curr Protoc Immunol*. 2015; 2 (1-3): 111.

209. Jethva K, Bhatt D, Zaveri M. *In-vitro* cytotoxicity activity of some selected ethano-medicinal plants against Vero cell line. *Int J Pharm Sci Rev Res*. 2016; 37(2): 130-3.

210. Xia M, Huang R, Witt KL, Southall N *et al*. Compound cytotoxicity profiling using quantitative high-throughput screening. *Environ Health Perspect*. 2008; 116(3): 284-91.

211. Gayathri G, Bindu RN, Babu V. Anti-Proliferative and cytotoxic effects of *Azima tetracantha* Lam. on cervical cancer cell line (HeLa) and human peripheral lymphocyte (HPL). *J Pharmacog Phytochem*. 2015; 4(4): 161-4.

212. Kangas L, Grönroos M, Nieminen AL. Bioluminescence of cellular ATP: A new method for evaluating cytotoxic agents *in vitro*. *Med Biol.* 1984; 62(6): 338-43.

213. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol*. 2007; 35(4): 495-516.

214. Renugadevi J, Prabu SM. Quercetin protects against oxidative stress-related renal dysfunction by cadmium in rats. *Toxicol Pathol.* 2010; 62(5): 471-81.

215. Choi YJ, Kang JS, Park JH, Lee YJ *et al*. Polyphenolic flavonoids differ in their antiapoptotic efficacy in hydrogen peroxide-treated human vascular endothelial cells. *J Nutr*. 2003; 133(4): 985-91.

216. Luft FC, Kleit SA. Renal parenchymal accumulation of aminoglycoside antibiotics in rats. *J Infect Dis*. 1974; 130(1): 656-9.

217. Poon PY, Michel KT, Chiu H, Leung Y *et al.* A Yang-invigorating' Chinese herbal formula protects against gentamicin-induced nephrotoxicity in rats. *Phytother Res*. 2008; 22: 131-3.

218. Nairuti MP, Vrusabendra SBM, Viswanath SKM. Protective Activity of *Psidium Guajava* Linn. leaves extract against gentamicin induced nephrotoxicity in rats. *RGUHS J Pharm Sci*. 2012; 2(4): 66-72.

219. Desouza VB, de-Oliveira RFL, de-Lucena HF *et al*. Gentamicin induces renal morpho pathology in wistar rats. *Int J Morphol*. 2009; 27(1): 59-63.

220. Li CC, Finley DS, Uribe C, Eichel L *et al*. Effect of urine specific gravity on effectiveness of shockwave lithotripsy. *J Endourol*. 2005; 19(2): 167-9.

221. Aparna RLD, Yaso DM , Nagaraju B, Prasad K. Evaluation of nephroprotective activity of ethanolic extract of *Annona reticulata* in Gentamicin and Cisplatin induced nephrotoxicity in rats. *J Pharm Sci & Res*. 2016; 8(9): 995-1007.

222. Wolf G, Ziyadeh FN. Cellular and molecular mechanisms of proteinuria in diabetic nephropathy. *Nephron Physiol*. 2007; 106(2): 26-31.

223. Anitha T, .Mohan D. *Morinda citrifolia* extract attenuates Gentamicin induced renal damage in rats. *Bull Env Pharmacol Life Sci*. 2014; 3(2): 32-8.

224. Derakhshanfar A, Bidadkosh A, Kazeminia S. Vitamin E protection against gentamicin induced nephrotoxicity in rats: a biochemical and histopathologic study. *Iran J Vet Res*. 2007; 8(3): 231-8.

225. Foster JE, Harpur S, Garland HO. An investigation of the acute effect of gentamicin on the renal handling of electrolytes in the rat. *J Pharmacol Exp Ther*. 1992; 261(1): 38-43.

226. Khoshnoud MJ, Moghbel BNA, Geramizadeh B, Niknahad H. Effect of Simvastatin on Cisplatin-induced Nephrotoxicity in Male Rats. *Indian J of Pharm Sci.* 2011; 7(3): 165-173.

227. Akram A, Amin AF, Mohammad KG, Taha J, Iran R. Hydrogen sulfide ameliorates the kidney dysfunction and damage in cisplatin-induced nephrotoxicity in rat. *Veterinary Research Forum Spring*. 2014; 5(2): 121-7.

228. Paprika MV, Sharma BB. Protective effect of Sesame oil against lead acetate induced hematao-biochemical toxicity in albino rats. *J cell tissue Res*. 2003; 3(1): 12-7.

229. Patocka J, Cerný K. Inorganic lead toxicology. *Acta Medica (Hradec Kralove)*. 2003; 46(2): 65-72.

230. Lavicoli I, Carelli G, Stanek EJ *et al*. Effects of low doses of dietary lead on red blood cell production in male and female mice. *Toxicol Lett.* 2003; 137: 193-9.

231. Ashour AA, Yassin MM, Abu Aasi NM, Ali RM. Blood, serum glucose and renal parameters in lead-loaded albino rats and treatment with some chelating agents and natural oils. *Turk J Biol*. 2007; 31: 25-34.

232. Sharma R, Panwar K, Mogra S. Alterations in developing RBCs after prenatal and postnatal exposure to lead acetate and Vitamins. *Int J Pharm Sci Res*. 2013; 4(8): 3214-24.

233. Adikwu E, Deo O, Geoffrey, OBP, Enimeya DA. Lead organ and tissue toxicity: Roles of mitigating agents (Part 1). *British J Pharm Toxicol*. 2013; 4(6): 232-40.

234. Vaziri ND, Liang K, Ding Y. Increased nitric oxide inactivation by reactive oxygen species in lead- induced hypertension. *Kidney Int*. 1999; 56: 1492-8.

235. Ebtihal ET, Sania AS, Muddathir AK, Muha MEA. Nephro-protective effect of *Acacia senegal* against gentamycin-induced renal damage in rats. *World J Pharm Res*. 2016 5(5): 294-303.

236. Chatterjee P, Mukherjee A, Nandy S. Protective effects of the aqueous leaf extract of *Aloe barbadensison* gentamicin and cisplatin-induced nephrotoxic rats. *Asian Pac J Trop Biomed.* 2012; S1754-63.

237. Qadir MI, Tahir M, Lone KP, Munir B, Sami W. Protective role of ginseng against gentamicin induced changes in kidney of albino mice. *J Ayub Med Coll Abbottabad*. 2011; 23(4): 53-5.

238. Daugaard G, Abildgaard U. Cisplatin nephrotoxicity: a review. *Cancer Chemother Pharmacol*. 1989; 25(1): 1-9.

239. Abdel G, Bakhiet EH, Amel O, El B, Samia. Haemato-biochemical effects of aqueous extract of *Khaya senegalensis* stem bark on gentamicin-Induced nephrotoxicity in Wistar Rats. *J Bio Sci*. 2012; 12(6); 361-6.

240. Cekmen M, Otunctemur A, Ozbek E, Cakir SS *et al*. *P*omegranate extract attenuates gentamicin-induced nephrotoxicity in rats by reducing oxidative Stress. *Ren Fail*. 2013; 35 (2): 268-7.

241. Waqar A, Afzal HA, Mir AK, Taous K, Naveed U. *Mentha piperita* in nephrotoxicity - a possible intervention to ameliorate renal derangements associated with gentamicin**.** *Indian J Pharmacol.* 2014; 46(2); 166-70.

242. Vamsi S, Latha P, Raviteja M, Saisudheer M. Carrot (*Daucus carota* L.): Nephroprotective against gentamicin-induced nephrotoxicity in rats. *Indian J Pharmacol*. 2016; 48(2): 122-7.

243. Gowrisri M, Vikram KS, Sarita K, Vrushabendra SB. Anti-oxidant and nephroprotective activities of *Cassia occidentalis* leaf extract against gentamicin induced nephrotoxicity in rats. *Res J Pharm Biol Chem Sci*. 2012; 3(3): 684-94.

244. Pinto AF, Rodrigues JV, Teixeira M. Reductive elimination of superoxide: structure and mechanism of superoxide reductases. *Biochim Biophys Acta*. 2010; 1804(2): 285-97.

245. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot*. 2003; 91(2): 179-94.

246. Punitha1SR, Rajendran K, Arun S, Annie S. Alcoholic stem extract of *Coscinium fenestratum* regulates carbohydrate metabolism and improves antioxidant status in streptozotocin–nicotinamide induced diabetic rats. *Evid Based Complement Alternat Med.* 2005; 2(3): 375-81.

247. Abreu I, Caballi DE. Superoxide dismutases- a review of the metal-associated mechanistic variations. *Biochimica Biophysica Acta.* 2010; 1804 (2): 263-74.

248. Sivanandham, V. Free Radicals in health and diseases-A Mini Review. *Pharmacology online*, 1. 2011; 1062-77.

249. Main PAE, Angley MT, O-Doherty CE, Thomas P, Fenech M. The potential role of the antioxidant and detoxification properties of glutathione in autism spectrum disorders: a systematic review and meta-analysis. *J Nutr Metab.* 2012; 9 (35): 1-37.

250. Girotti AW. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J Lipid Res.* 1998; 39(8): 1529-42.

251. Fatemeh M, Mehdi N, Ramesh M, Zahra P *et al*., Effect of Pomegranate flower extract on cisplatin induced nephrotoxicity in rats. *J Nephropath*ol. 2014; 3(4):133-8.

252. Kim SW, Lee JU, Nah MY, Kang DG *et al*., Cisplatin decreases the abundance of aquaporin water channels in rat kidney. *J Am Soc Nephrol*. 2001; 12(5): 875-82.

253. Md-Azmat R, Rahat AK, Mohammad N, Aijaz AK. Amelioration of cisplatin-induced nephrotoxicity by ethanolic extract of *Bauhinia purpurea*: An *in vivo* study in rats. *Saudi J Kidney Dis Transpl*. 2016; 27(1): 41-8.

254. Ashish M, Ashutosh P. Protective properties of dietary inclusion of *Ocimum sanctum* on cisplatin-induced nephrotoxicity in rats. *Int J Green Pharm*. 2017; 11 (3): S579-83.

255. Kaveripakam S, Adikay S**.** Amelioration of Cisplatin-induced nephrotoxicity by roots of *Anthocephalus cadamba* **.** *Biomed Pharmacol J*. 2017; 10(3): 1433-9.

256. Hassan HA, Edrees GM, El-Gamel EM, El-Sayed EA. Amelioration of cisplatin-induced nephrotoxicity by grape seed extract and fish oil is mediated by lowering oxidative stress and DNA damage. *Cyto technology*. 2014; 66(3): 419-29.

257. Fenninger LD, Mider GB. Energy and nitrogen metabolism in cancer. *Adv Cancer Res*. 1954; 2: 229-53.

258. Shelkea TT, Bhaskarb VH, Adkara PP, Jhaa U, Oswala RJ. Nephroprotective activity of ethanolic extract of stem barks of *Crataeva nurvula* Buch Hum. *Int J Pharm Sci Res.* 2011; 2(10): 2712-17.

259. Badary OA, Abdel-Maksoud S, Ahmed WA, Owieda GH. Naringenin attenuates cisplatin nephrotoxicity in rats. *Life Sci.* 2005; 76(18): 2125-35.

260. Kusumoto M, Kamobayashi H, Sato D, Yoshimurs M *et al.* Alleviation of cisplatin-induced acute kidney injury using phytochemical polyphenols is accompanied by reduced accumulation of indoxyl sulfate in rats. *Clin Exp Nephrol*. 2011; 15(6): 820-30

261. Shimeda Y, Hirotani Y, Akimoto Y, Shindou K *et al*. Protective effects of capsaicin against cisplatin-induced nephrotoxicity in rats. *Biol Pharm Bull*. 2005; 28(9):1635-8.

262. Koyner JL, Sher AR, Murray PT. Antioxidants. Do they have a place in the prevention or therapy of acute kidney injury? *Nephron Exp Nephrol*. 2008; 109(4): 109-17.

263. Weijl NI, Cleton FJ, Osanto S. Free radicals and antioxidants in chemotherapy-induced toxicity. *Cancer Treat Rev*. 1997; 23(4): 209-40.

264. Antunes LM, Darin JD, Bianchi MD. Protective effects of vitamin c against cisplatin-induced nephrotoxicity and lipid peroxidationin adult rats: a dose-dependent study. *Pharmacol Res*. 2000; 41(4): 405-11.

265. Antunes LM, Darin JD, Bianchi NL. Effects of the antioxidants curcumin or selenium on cisplatin-induced nephrotoxicity and lipid peroxidation in rats. *Pharmacol Res*. 2001; 43(2): 145-50.

266. Yogesh CY, Srivastav DN, Seth VS, Balaraman R, Ghelan TK. *In vivo* antioxidant potential of *Lepidium sativum* l. seeds in albino rats using cisplatin-induced nephrotoxicity. *Int J Phytomedicine*. 2010; 2: 292-8.

267. Khan SR. Animal models of kidney stone formation: An analysis. *World J Urol*. 1997; 15(4): 236-43.

268. Selvam R, Kalaiselvi P, Govindaraj A, Murugan V, Sathishkumar AS. Effect of *A. lanata* leaf extract and Vediuppu chunnam on the urinary risk factors of calcium oxalate urolithiasis during experimental hyperoxaluria. *Pharmacol Res*. 2001; 43(1): 89-93.

269. Brent J. Current management of ethylene glycol poisoning. *Drugs*. 2001; 61(7): 979-88.

270. Karamakar P, Patki. Evaluation of efficacy and safety of a herbal formulation Cystone in the management of urolithiasis: Meta-analysis of 50 clinical studies. *Internet J Altern Med*. 2008; 8(1); 1-10.

271. Morley JE, Levine AS, Rowland NE. Stress induced eating. *Life Sci*. 1983; 32(19): 2169- 82.

272. Ravindra DH, Sunil SJ. Effect of hydro-alcoholic extract of *Vernonia cinerea* less. against ethylene glycol-induced urolithiasis in rats. *Indian J pharmacol*. 2016; 48(4): 434- 40.

273. Asheesh KG, Preeti K, Suneel P. Evaluation of antiurolithiatic potential of *Kigelia africana* fruits in albino rats. *FABAD J Pharm Sci*. 2011; 36: 197-205.

274. Moe OW, Abate N, Sakhaee K. Pathophysiology of uric acid nephrolithiasis. *Endocrinol Metab Clin North Am*. 2002; 31(4): 895-914.

275. Ebrahimpour A, Perez L, Nancollas GH. Induced crystal growth of calcium oxalate monohydrate at hydroxyapatite surfaces. The influence of human serum albumin, citrate, and magnesium. *Langmuir*. 1991; 7(3): 577-83.

276. Lulat SI, Yadav YC, Balaraman R, Maheshwari R. Antiurolithiatic effect of lithocare against ethylene glycol-induced urolithiasis in Wistar rats. *Indian J Pharmacol*. 2016; 48(1): 78-82.

277. Sharma I, Khan W, Parveen R, Alam MJ *et al*. Antiurolithiasis activity of bioactivity guided fraction of *Bergenia ligulata* against ethylene glycol induced renal calculi in rat. *Biomed Res Int*. 2017; 2017: ID1969525; 1-11.

278. Shukkur MF, Abdul SE, Karthik HS, Ramasamy S, Ganesh R *et al*. Oxalatemediated nephronal impairment and its inhibition by c-phycocyanin: A study on urolithic rats. *Mol Cell Biochem*. 2006; 284(1): 95-101.

279. Roger K, Low MD, Stoller ML. Uric acid nephrolithiasis. *Urol Clin North Am*. 1997; 24(1): 135-48.

280. Grases F, Genestar C, Conte A, March P, Costa B A. Inhibitory effect of pyrophosphate, citrate, magnesium and chondroitin sulfate in calcium oxalate urolithiasis. *Br J Urol*. 1989; 64(3): 235-7.

281. Badrinathan S, Shiju TM, Ramachandran A, Selvaraj MR. Beneficial effect of *Citrus limon* peel aqueous methanol extract on experimentally induced urolithic rats. *Pharmaceutical Biology*. 2016; 54(5): 759-69.

282. Donald A, Adams MD. The Pathophysiology of the Nephrotic Syndrome. *Arch Intern Med.* 1960; 106(1): 117-42.

283. Ghodkar PB. Chemical tests in kidney disease. *Text Book of Medical Laboratory Technology*. Mumbai: Bhalani Publishing House; 1994. pp.18-32.

284. Shah JG, Patel BG, Patel SB, Patel RK. Antiurolithiatic and antioxidant activity of *Hordeum vulgare* seeds on ethylene glycol-induced urolithiasis in rats. *Indian J Pharmacol*. 2012; 44(6): 672-7.

285. Hussein G, Fayed AY, Hassan I , Salah A, Afrah A. Evaluation of ethanolic seed extract of Parsley on ethylene glycol induced calcium oxalate experimental model. *Inter J Sci Res.* 2017; 6(3): 1683-9.

286. Bashir S, Gilani AH. Antiurolithic effect of *Bergenia ligulata* rhizome: An explanation of the underlying mechanisms. *J Ethno pharmacol*. 2009; 122(1): 106-16.

287. Dixit P, Koti BC, Vishwanathswamy AHM. Antiurolithiatic activity of Crashcal on ethylene glycol induced urolithiasis in rats. *RGUHS J Pharm Sci*. 2014; 4(1): 30-5.

288. Polat A, Parlakpinar H, Tasdemir S, Colak C *et al*. Protective role of aminoguanidine on gentamicin-induced acute renal failure in rats. *Acta Histo Chem*. 2005; 108: 365-71.

289. Sahar M. El-Sheshtawy, Naglaa F, Al-Shap. Effect of Vitamin C on gentamicin induced nephrotoxicity in broiler chicks. *Egypt J Chem Environ Health*. 2015; 1(1): 715-28.

290. Kurien TB, Selvam R. Induction of lipid peroxidation in calcium oxalate stone formation. *Indian J Exp Biol*. 1989; 27(5): 450-3.

291. Gilhotra UK, Christina AJM. Effect of *Rotula aquatica* Lour. on ethylene-glycol induced urolithiasis in rats. *Inter J of Drug Develop Res*. 2011; 3(1): 1273-80.