Summary and conclusion

The present research work is a sincere effort to find an answer for age old disorder called kidney stone. Literature review concluded that pathophysiology of renal calculi disease is not well established. Mechanism of calculi formation is still evolving. Lack of information is available on mechanism of stone formation and genetic involvement (if any) in the disease. Genetic involvement in the disease is not conclusive, though certain people tend to form kidney stone much likely. Disease has characteristic steps such as, saturation of urine with crystal salts followed by nucleation and aggregation in the urine.

In the present study, an attempt has been made to validate scientific claim of Ayurvedic class of herbs called ‘Pashanbhed’ for kidney disorder. One of the identity of pashanbhed plant is, they grows in the crevices of rocks. Plants of the genus lepidagathis, grows in the interstice of rocks, were selected for the antiurolithiatic study. Four plants were screened to get, most active plant. Plants screened were *Lepidagathis cristata* Willd, *Lepidagathis prostrata* Dalz, *Lepidagathis incurva* Buch.-Ham. ex D. Don., and *Lepidagathis pungens* Nees.

All the plants were authenticated from botanist before initiating study. A voucher specimen *L. prostrata* (PP609) has been deposited in the herbarium of our institute, Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal for future reference. *L. cristata* (225/2013), *L. incurva* (256/2014), and *L. pungens* (259/2014) were received as a gift samples from Natural remedies Pvt. Ltd., Bengaluru, Karnataka, India.

Whole plants were used for the study. Methanol extract was taken to screen antiurolithiatic activity. An established and validated *in vitro* nucleation and aggregation assay was performed at 620 nm to assess, calcium oxalate inhibition in the presence of extract. Among the screened plants *L. prostrata* was found to be the most active plant in inhibiting calcium oxalate stone formation *in vitro*. Plant showed IC$_{50}$ of 807.27 ± 69.63 µg/mL and 627.97 ± 39.14 µg/mL in nucleation and aggregation assay respectively.

*L. prostrata* was considered for, pharmacognostic studies viz. macroscopy, microscopy, physicochemical constants, powder analysis and fluorescence analysis, as a tool for plant identification.
The plant *L. prostrata* can be identified morphologically as a rigid prostrate undershrub with woody rootstock. Sessile leaves (2.5×0.8 cm), plicate, rigid, oblong-lanceolate with pointed apex. Flowers are simple, erect, pubescent spikes, usually terminal on short lateral ascending branches. Corolla pinkish about 2 cm long. Capsules are ovoid-lanceolate, glabrous and 2-seeded.

Microscopically leaf of *L. prostrata* shows prominent upper epidermis consisting of elongated columnar parenchymatus cells. Mesophyll consists of compact two layered palisade cells continuous over midrib. Loosely arranged polygonal spongy parenchyma followed by lower epidermis. Midrib shows the flat ventral surface and convex dorsal surface with group of collenchyma. Collateral vascular bundles had xylem on the ventral side and phloem on the dorsal side. Beneath this prominent collenchyma with polygonal lower epidermis detected.

Transverse section of *L. prostrata* (stem) shows outer epidermis, collenchyma, cortex, endodermis, internal phloem, phloem, pith, rosette calcium oxalate crystals, and xylem vessels. Powder analysis of the plant shows stone cells, xylem vessels, unicellular covering trichomes.

Preliminary phytochemical screening of the plant extract showed presence of sterols, triterpenoids, phenols, flavonoids, alkaloids, saponins, and tannins.

HPTLC standardization of the plant extract and fraction was performed. Bioactive marker compounds rutin, lupeol, β-sitosterol, quercetin, and gallic acid were estimated. These marker compounds may serve an important role in establishing quality of the extract.

Methanol extract of *L. prostrata* (LPM) was subjected to bioactivity guided fractionation (BAGF) in different solvents of variable polarity. Methanol extract was partitioned to get petroleum ether (LPPE), ethyl acetate (LPEA), butanol (LPBU) and aqueous (LPAQ) fractions. All the fractions along with marketed antiurolithiatic formulation ‘Cystone’ (Himalaya drug company, India) were subjected to antiurolithiatic screening. LPEA was found to be most active fraction in the study. LPEA showed IC$_{50}$ of 336.23 ± 30.79 µg/mL and 149.63 ± 10.31 µg/mL in nucleation and aggregation assay respectively. Hence bioactivity was expected from some relatively non polar to mid polar compounds.
Free radical scavenging activity plays an important role in urolithiasis. Antioxidants found to protect epithelial cells of kidney from stress associated with oxalic acid in the urine. Different in vitro antioxidant assays were performed on plant extract/fractions. Order of activity was found to be LPBU>LPEA>LPM>LPPE>LPAQ in ABTS and DPPH assay. The nitric oxide scavenging activity was assessed by the Griess reagent. Incsavenging nitric oxide,polar fractions were found more active in the sequence of (LPAQ>LPBU>LPM>LPPE>LPEA). Iron chelating activity was estimated using the o-phenanthroline method. The activity was attributed to mid polar compounds (LPBU>LPEA>LPPE>LPAQ>LPM). Total antioxidant capacity was reported as ascorbic acid equivalent/mg of extract (fraction).The progression of activity was found to be LPBU>LPEA>LPAQ>LPPE>LPM. Butanol fraction was found to be most active, followed by ethyl acetate fraction in antioxidant assays.

Total phenolic and flavonoid content was evaluated for plant extract and fraction. An attempt has been made to study Pearson’s correlation between antioxidant and total phenol and flavonoid content. Correlation analysis showed a slightly higher correlation between ABTS scavenging potential and total flavonoid content of the extract/fractions (R = −0.864) indicating that flavonoids present in the plant extract/fractions constitute the major free radical scavenging compounds of L. prostrata. Pearson’s correlation analysis revealed a moderate correlation between DPPH scavenging and total flavonoid content (R = −0.817). Lower correlation was observed with total phenolic content (R = −0.595) suggesting that flavonoids were mainly responsible for the DPPH scavenging activity. Correlation analysis did not reveal a strong correlation between total phenolic, flavonoid content, and nitric oxide scavenging indicating that components other than phenols are responsible for the nitric oxide scavenging activity. A moderate correlation was also observed between iron chelating activity and total phenolic (R = −0.603, p > 0.05) and flavonoid content (R = −0.608, p > 0.05) of plant extract/fractions suggesting the role of phenolics in iron chelating ability. A moderate correlation was observed between total antioxidant capacity and phenolic content (R = 0.765, p > 0.05) and flavonoid content (R = 0.596, p > 0.05).

Ethyl acetate fraction was further subjected to isolation using vacuum liquid chromatography and column chromatography to isolate bioactive compounds. Silica was used as an adsorbent with different grades. The column was eluted with linear increment
of ethyl acetate in petroleum ether and methanol in ethyl acetate. Column elution was monitored using TLC.

GCMS was used to identify and quantify compounds from successive fractions. Total of 48 compounds were identified and quantified by GCMS. Four compounds were isolated and characterized using melting point, IR, Mass, NMR spectroscopy. These compounds were designated/identified as, R1: Lupeol, R2: Stigmasterol, R3: Quercetin, and R4: Chlorogenic acid. Compound R1 (Lupeol) 25 mg, R2 (Stigmasterol) 27 mg, R3 (Quercetin) 21.3 mg, were subjected to nucleation and aggregation assay. Compound R4 (Chlorogenic acid) 17.6 mg, was not enough to perform any assay. Stigmasterol showed an IC$_{50}$ < 100 µg/mL and quercetin IC$_{50}$ ≤ 400 µg/mL. Lupeol was found to be most active compound among all. The IC$_{50}$ value of lupeol in nucleation was found to be 46.12 ± 1.87 µg/mL and in aggregation 37.30 ± 2.20 µg/mL.

Ethyl acetate fraction (LPEA) at the dose of (100, 200 mg/kg) was subjected to in vivo antiurolithiatic activity in prophylactic and curative regimen. Ethylene glycol (0.75%v/v) and ammonium chloride (0.5%w/v) induced urolithiasis in rat model was used for the efficacy study. Isolated compounds were not sufficient in quantity to perform in vivo studies, hence other compounds which were present in LPEA, subjected to in vivo antiurolithiatic study. These compounds were gallic acid and quercetin. In animal study urine parameters such as volume, pH, calcium, magnesium, oxalate, creatinine and uric acid were studied. Serum parameters such as calcium, creatinine, uric acid, AST, ALT, ALP, and BUN were explored. Kidney homogenate was used for antioxidant assays and left kidney was subjected for histopathological evaluation.

In the study ethylene glycol and ammonium chloride induced urolithiasis was evidenced by a significant (p < 0.05) variation in urine, serum and tissue homogenate parameters. Administration of ethylene glycol (0.75 %v/v) and ammonium chloride (0.5 %w/v) in drinking water for 14 days, produced urolithiasis as characterized by an increase in urine/serum, calcium, creatinine, uric acid. Other elevated serum biomarkers were AST, ALT, and BUN. Urine parameters revealed that increase in oxalate content and decrease in pH of diseased animals. Antioxidant defense systems were compromised as indicated by decreased glutathione, catalase and increased MDA, total protein in the kidney tissue. Section of the kidney showed marked tubular necrosis, calcium oxalate calculi and peritubular inflammation.
In the prophylactic regimen the ethyl acetate fraction (LPEA) was administered from first day to 28 days of study. LPEA at dose level of 100 and 200 mg/kg (Figure 7.1) were found to normalize the urine calcium, uric acid, and oxalate. LPEA at 200 mg/kg (Figure 7.1, 7.2) was found to be effective in normalizing urine creatinine and pH, serum calcium and AST. Serum BUN was normalized at both the dose levels in the treatment group. A significant recovery in the animal tissue was characterized by elevated glutathione and catalase (Figure 7.3). LPEA at 200 mg/kg (Figure 7.3) was effective in depleting elevated kidney calcium and total protein. Kidney tissue sections showed no evidence of calculi or peritubular inflammation at 100 mg/kg dose.

In the curative regimen, treatment with LPEA was initiated after induction of urolithiasis i.e. from day 15 of the study till 28th day. LPEA at dose level of 100 and 200 mg/kg (Figure 7.1, 7.2) were found to reduce elevated urine calcium and serum biomarker AST. LPEA at the dose level 100 mg/kg was efficient in normalizing urine pH, creatinine, and oxalate. Tissue antioxidant (Figure 7.3) catalase was found to be elevated by treatment group at both the dose levels. Section of tissue showed no renal calculi, necrotic debris, peritubular fibrosis and interstitial edema. However glomerular congestion was detected in the treatment group, thereby suggesting that L. prostrata has greater antiurolithiatic potential as prophylactic rather than as a curative.

Antioxidant quercetin was tested for its efficacy at 50 mg/kg from day 1 till 28th day of the study. Quercetin (Figure 7.1, 7.2) showed significant reduction in urine calcium, creatinine, uric acid and serum AST, calcium levels. Kidney parameters were found unaffected by quercetin (Figure 7.3). Reduction in renal calculi, tubular swelling and edema was observed in tissue sections.

Gallic acid was tested for its efficacy at 40 mg/kg from day 1 till 28th day of the study. Gallic acid reduced urine calcium, creatinine, uric acid, and oxalate with normalization of urine pH (Figure 7.1). Serum calcium (Figure 7.2) and all other biomarkers were normalized with the treatment. Antioxidant (Figure 7.3) glutathione, MDA were found to be elevated and kidney calcium, total protein depleted, in the treatment. Kidney section was deprived of evidence of calculi, tubular swelling, peritubular inflammation, and necrotic debris, thereby suggesting that gallic acid is better candidate as an antiurolithiatic than quercetin.
Oxidative stress caused tissue injuries were assessed by quantifying DNA damage. LPEA at the dose of 200 mg/kg (prophylactic) and the gallic acid significantly reduced the DNA damage (Figure 7.4), establishes their protective effect in ethylene glycol, ammonium chloride induced urolithiasis in rats.

To conclude, studies have shown that, *L. prostrata* possess marked antiurolithiatic activity with minimal signs of tissue toxicity and thus have a promising role in the treatment of urolithiasis caused by calcium oxalate. Gallic acid is promising antiurolithiatic candidate. Further isolation of active compounds and evaluation of its antiurolithiatic activity have to be carried out.