DISCUSSION

As Urolithiasis is serious disorder and there is lack of non invasive treatment comprising of specific drugs for calculi formed in urinary tract, *Aerva lanata* (L) is selected for the study. This plant is used as *Pashanabheda* in Ayurveda and widely available. Hence this plant cultivated in Western Ghats of Khanapur region from Belagavi district of Karnataka was selected for the present study.

A detailed literature review was carried out regarding the pharmacological uses of *Aerva lanata* and it was found that the plant extract was screened for antiurolithiatic potentials at different parts of India and the plant available from Western Ghats was not exclusively studied for Urolithiasis. As there is no specific drug available for Urolithiasis, there was need to isolate active constituents and develop new lead molecules for Urolithiasis. Based on the literature review the present study was designed to isolate, characterize and develop new lead molecules from *Aerva lanata* for Urolithiasis.

The whole plant *Aerva lanata* from Western Ghats was identified, collected and authenticated by RMRC, Belagavi by Botanist Dr. Harsha Hegde (RMRC species authentication no-507). The morphological study, ash value determination was carried out. Literature survey revealed that Soxhlet extraction with hydro alcohol (80-20) would give maximum possible yield of different constituents. Therefore successive Soxhlet method of extraction was followed first by using non polar solvent- petroleum ether (40-60ºC) and then polar solvent hydro alcohol. Petroleum ether (40-60ºC) was used to extract hydrocarbons present in plant and hydro alcohol was used to extract polar constituents present in plant.
The hydro alcoholic extract was subjected to fractionation with different solvents based on their polarity (higher to lower polarity) such as dichloromethane, ethyl acetate and n-butanol with an idea that targeted polar constituents would get dissolved more in polar solvents. Phytochemical investigation and spectral studies like UV/VIS and FT-IR study of ethyl acetate and n-butanol fractions showed presence of primary and secondary metabolites which mainly includes saponin glycosides, flavanoids, triterpenoids, steroids and phenolic compounds.

Based on the results of Phytochemical tests and spectral study of fractions, it was found that the targeted compounds which were reportedly responsible for antiurolithiatic activity present in fractions II and III. These two fractions ethyl acetate (II) and fraction n-butanol (III) were subjected to antimicrobial study and antiurolithatic study.

As Urolithiasis is also reportedly associated with urinary tract infections, the two fractions (II and III) were screened for their antimicrobial potency against the bacteria that are found to be responsible for urinary tract infection such as – *E. coli* (ATCC no. 25922), *S. aureus* (ATCC no. 12598), *Proteus vulgaris* (ATCC no. 49565) and *Bacillus subtilis* (ATCC no. 6051). Serial dilution method was followed for antimicrobial study with Ciprofloxacin as standard drug. The MICs were recorded and it was noticed that the treated fractions showed significant antimicrobial potency at higher dilutions (100 µg/ml) against all the tested microorganisms, *S. aureus* and *Bacillus sp* showed sensitivity against ethyl acetate fraction at lower dilutions (50 and 25 µg/ml). This study results shows the significant antimicrobial potency of the tested fractions and in turn that of the whole plant in UTIs due to the tested microbes. As it was reported that UTIs were associated with Urolithiasis, these findings of
antimicrobial potency of fractions II and III signifies the potency of the fractions and in turn that of plant in these infectious conditions. (68-75).

Later, the two fractions (II and III) were screened for antiurolithiatic potency based on the methodology as per the protocol (79-81). This study was designed for 28 days on ethylene glycol induced male Wistar albino rats. The animals (male Wistar albino rats) were divided into 7 groups of 6 each. Gr 1 was as normal, 2nd group was administered with 0.75 % v/v ethylene glycol for 28 days to induce calculi and identified as disease induced group. 3rd to 7th group of animals were administered with 0.75% v/v ethylene glycol for 14 days to induce calculi. Later, 15th day to 28th day, these groups were administered with drug to know the curative effect and treated groups were compared with disease induced groups to know the curative effect of the fraction II and III.

As per the protocol, fraction II and fraction III were administered to group 4 to 7 in two doses (20 mg/kg and 40 mg/kg).

Urine volume was measured on 14th and 28th day. At the end of 28th day, urine was collected and analyzed for the parameters like calcium, phosphate and oxalate and magnesium which are reportedly responsible for calculi as promoter or inhibitor (Fig 7) (85 & 88). The urine collected was also analyzed for the calculi under electronic microscope. Urine study of calculi under electronic microscope has revealed the reduction in the size of the calculi in treated group (III to VII) compared with disease induced group. This signifies the effect of the fractions on the calculi in the experimental animals.

Kidneys were isolated and Histopathological study was carried out for the calculi. The serum analysis showed significant changes (p<.001) in reduction of the
levels of elements like Ca, phosphate, which may be responsible for calculi formation and significant reduction in the level of BUN in treated animals (Fig 7) which indicate curative effect of fractions and also significant elevation (p<.001) of Mg level which is considered as calculi inhibitor.

Histopathology of the kidneys has revealed the absence of calculi in the kidney sections of the treated animals which indicates the possible effect of the fractions (Fig 9). These results have shown significant changes made by fractions II and III in animals when compared with disease induced animals. Scanning Electron Microscopic (SEM) examination of kidney showed significant and ultra structurally detectable changes in the proximal convoluted tubular epithelial cells (PCT) in the test groups, compared to the control group. The glomeruli in all three groups (treated with standard drug and n- butanol fraction of both high and low doses) did not show any ultra structural changes. The PCT epithelial cells were considered normal when the brush border was found to be intact and clear. The cell organelles were also intact in the epithelial cells\textsuperscript{77-78}.

From the Histopathological data it is evident that there is slight inflammation in the renal tubules (Fig 9). This gives an indication that there is a slight possibility that the fractions may not be successful to reduce the inflammation at the dose administered and further study is needed to evaluate the anti-inflammatory effects of the fractions. Also the Histopathological data revealed the effect of the fractions on the calculi which are compared with the standard drug (table no 2). The data revealed that there is slight hemorrhage seen which may be due to the calculus. PCT is responsible for reabsorption of 60-65% of filtered water from the tubular lumen. Disrupted brush border may affect the reabsorption of solutes from the tubular lumen.
Discussion

leading to a reduction in the passive reabsorption of water at the level of PCT. The structural changes that we have observed in the pictures are unlikely due to ischemia.

Based on the In vivo study results of fractions, the potent fractions (II and III) were subjected to bioactivity guided isolation of active constituents using column chromatography followed by preparative TLC.

Two compounds were isolated from two fractions, AEF1 and AEF 2.3 from ethyl acetate fraction and n-butanol fraction respectively. These two compounds AEF 1 and AEF 2.3 were characterized as Quercetin and Betulin respectively from the data obtained from IR, $^1$H NMR, $^{13}$C NMR and LC MS.

These two isolated compounds AEF 1 (Quercetin) and AEF 2.3 (Betulin) were subjected to molecular docking studies using ETE 2 from Oxalate oxidase downloaded from pdb (www.rcsb.org/pdb) $^{111-115}$. This study was aimed to understand the possibility of binding and inhibition of amino acids of Oxalate oxidase which has reportedly participated in calculi formation and to know the probable mechanism of action of AEF 1 (Quercetin) and AEF 2.3 (betulin) with the help of BIOPREDICTA from V LIFE, Pune. The results obtained from this study showed significant binding ability of the isolated compounds which is very essential for antiurolithiatic activity (Fig 30 and 31). These two compounds binded to important amino acids of 2 ETE which shall help in preventing the enzyme Oxalate oxidase from participating in Urolithiasis cycle. The study has shown 6 hydrogen bonds which signify the hydrophilic interactions in the binding process and less lipophilic interaction involved. These research findings are preliminary, however, significant in understanding the mechanism of action of Antiurolithiatic agents in blocking the biochemical pathway involving the enzyme inhibition.
Later, these two compounds Quercetin and Betulin were subjected to screening of antiurolithiatic potentials using animal’s model as per protocol. Male Wistar albino rats (30) were divided into 5 groups of 6 each as per protocol. Group 1 was identified as normal, group II was disease induced group and ethylene glycol (0.75% v/v) was administered for 1 to 28 days and other groups (III to V) were administered with ethylene glycol (0.75% v/v) for 1 to 14 days. Equivalent dose for Quercetin and Betulin (2 mg/k.g) was administered to the groups (IV to V) respectively from 15th day to 28th day to study the curative effect of the compounds in the calculi induced animals. Urine volume of the animals was recorded every fortnight and a significant increase was noticed in the urine level which indicates the diuretic effect of the compounds.

After 28th day, urine sample of the treated animals was analyzed by qualitative tests for the elements like calcium, oxalate, phosphate which help for calculi formation and magnesium which is calculi inhibitor. The results revealed that there was significant reduction in the level of Ca, PO$_4$ and oxalate where as the level of magnesium was significantly enhanced (p<.001) which is considered as added effect. The level of BUN in serum analysis was reduced in treated groups (gr 4 to gr 5) compared with disease induced group (p<.001) which is considered as effect of the drug on kidneys. The P$^H$ of the urine was maintained at 6. The urine sample was analyzed for the calculi under electronic microscope and it was noted that size of the calculi was significantly reduced in urine sample of treated animals which signifies the effect of Quercetin and Betulin in animals (Fig 29).

Urine biochemistry has revealed that calcium, oxalate, phosphate levels were reduced (p<0.001) significantly (Fig 25) and serum creatinine level was also reduced
significantly (p<0.001) compared with disease induced animals (Fig 26). The reduction in creatinine gives indication about healing effect of Quercetin and Betulin in treated animals. The significant increased level of Magnesium is an indication about the efficacy of the isolated compounds which has elevated the level and shall be useful for reducing the calculi by interfering with calcium oxalate complex. The serum analysis has significantly increased level of BUN (p<0.001) which is an indication of the curative effect of Quercetin and Betulin on the kidneys compared to disease induced group (Fig 26).

Scanning Electron Microscopic (SEM) examination of kidney showed significant changes in the tubular epithelial cells in the test groups, compared to the control group. The glomeruli in kidneys of the groups treated with Quercetin did not show major structural changes whereas there were minor structural changes found in the animals treated with Betulin. The cell organelles were also intact in the epithelial cells. From the Histopathological data it is evident that there is slight congestion in the renal tubules of the animals treated with betulin as well as hemorrhage. This gives an indication that there is a slight possibility of congestion and damage to the internal organs of kidneys. PCT is responsible for reabsorption of 60-65% of filtered water from the tubular lumen. Microscopic study of internal organs of kidney sections have helped us to understand the deposition of micro crystals in tubules which may be responsible for the inflammation in disease induced rats and this has been significantly restored to normal by isolated compounds. Deposition of disrupted brush border may affect the reabsorption of solutes from the tubular lumen leading to a reduction in the passive reabsorption of water at the level of PCT. These two isolated compounds have increases the excretion of calcium oxalate by dissolution in urine which is helpful for the restoration of internal structures of kidneys. However
Quercetin and Betulin are equally effective in reducing the amount of calculi and their removal from kidneys by diuretic activity. Both the isolated compounds have produced alkalinizing effect in urine\textsuperscript{88-91}.

Based on the Histopathological results, Quecetin was subjected to analytical method development and validation of the method as per ICH guidelines. This study was conducted by using UFLC (Shimadzu). The compound was compared with commercial formulation of Quercetin (QUERCETIN COMPLEX from Solgar, USA) which had a label claim of 250 mg\textsuperscript{118-122}.

The column used was C18 (150 mm x 4.6mm x 5u), mobile phase developed – (0.3%) Formic acid : Acetonitrile : methanol (HPLC grade) 40:20:40, temp: ambient (22\textdegree C), Injection Volume : 10 uL, R\textsubscript{T} -4.9, Wavelength at 370 nm by using PDA Detector.

The linearity range was found to be 2-20 µg/ml, the theoretical plates were 4929 and tailing factor was 1.217 which is well within the range and signifies the system suitability. The intraday and interday RSD values were within the range and hence the method is precise. The percentage recovery with 50%, 100 %and 150% of the drug was carried out in three levels and recovery was accurate. Limit of detection and limit of quantification was carried out. The flow rate was changed and checked under different conditions for robustness and it was found that the method was robust (Table 10).

The developed method was validated in accordance with International Conference on Harmonization (ICH) guidelines for different parameters such as system suitability, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) and robustness. The chromatographic separation was performed
on Shiseido Capcell Pak C<sub>18</sub> column (250 X 4.6 mm, 5 µm) and the detection was performed at 370 nm using PDA detector. The mobile phase consists of a mixture of acetonitrile, methanol and 0.3 % formic acid (20: 40:40 v/v) at a flow rate of 1.0 ml/min. The injection volume was 10 µl. The retention time was found to be 4.9 min and the total run time was 8 min. The system suitability parameters such as theoretical plate count, tailing factor and % relative standard deviation was within the acceptance criteria. The calibration curve was linear from 2-20 µg/ml with regression coefficient value > 0.999. Accuracy (99.91-102.65%) and precision was found to be satisfactory.

The limit of detection (LOD) and limit of quantification (LOQ) was found to be 0.816 µg/ml and 2.473 µg/ml. The robustness of the developed method was evaluated by deliberately altering the chromatographic conditions. The developed method can be used for the quality control and standardization of different plant extracts and herbal formulations containing Quercetin. From the assay the purity of the natural Quercetin was found to be 105%<sup>124</sup>.

The analytical method developed is simple, accurate, robust and the method can be used to determine the purity of any formulation containing Quercetin.