6. DISCUSSION

In the present study, AqE and EtE of *H. indicus* (roots), *V. vinifera* (fruits) and *B. ceiba* (fruits) were prepared and evaluated for pharmacological activities in two phases. In the first phase evaluation for diuretic activity and in the later phase evaluation of antiurolithiatic activity was carried out.

Male rats were selected in the present study because the urinary system of male rats resembles that of humans\textsuperscript{169} and also earlier studies have shown that the amount of stone deposition in female rats was significantly less\textsuperscript{13}.

Diuresis occurs by mainly two phenomena including, net increase in urine volume (water excretion) and elevated excretion of electrolytes (soluters) in the urine\textsuperscript{170}. These processes result from suppression of renal tubular reabsorption of water and electrolytes into the blood stream. The thiazide diuretics inhibit Na\textsuperscript{+}/Cl\textsuperscript{−} symporter (co-transporter system) in the distal convoluted tubule, by competing for the Cl\textsuperscript{−} binding site and thereby increasing the excretion of Na\textsuperscript{+} and Cl\textsuperscript{−}, while the loop diuretic reference drug, frusemide, increases the urine output and urinary excretion of Na\textsuperscript{+} by inhibiting Na\textsuperscript{+}/K\textsuperscript{+}/Cl\textsuperscript{−} symporter in the thick ascending limb of Loop of Henle\textsuperscript{170}.

In the first phase of the present study, 25 mg/kg dose of frusemide and HTZ, each, showed significant diuresis in rats over a period of 24 h. In comparison, similar increase in urine excretion was found with the AqE and EtE of *H. indicus*, *V. vinifera* and *B. ceiba*, when administered orally in dehydrated rats. While, different results obtained on electrolytic excretion by both extracts suggesting a difference in their comparative diuretic profile. AqE and EtE of *H. indicus* and *B. ceiba* showed a gradual rise in excretion of electrolytes (Na\textsuperscript{+}, K\textsuperscript{+} and Cl\textsuperscript{−}) in a dose-dependent manner. Particularly the EtE of *H. indicus* and *B. ceiba* show unsubstantial rise in urinary K\textsuperscript{+} levels. Furthermore, no alkalization of urine was seen with these extracts. Collectively, these observations suggest that the EtE of *H. indicus* and *B. ceiba* act as potassium-sparing diuretics in agreement with their natriuretic indices\textsuperscript{171-173}.

Although the thiazide diuretics, like HTZ, increase only the urinary K\textsuperscript{+} level and alter the urinary Na\textsuperscript{+}/K\textsuperscript{+} ratio\textsuperscript{171,172}, the administration of AqE of *H. indicus* and *B. ceiba* in experimental subjects of the present study showed increase in urinary K\textsuperscript{+} levels along with urinary Na\textsuperscript{+} and Cl\textsuperscript{−} levels without significant alteration in the Na\textsuperscript{+}/K\textsuperscript{+} ratio. Such, activity of AqE reflect that is unlikely to predict the modus operandi of AqE of *H. indicus* and *B. ceiba* as thiazide or loop diuretics. Moreover, the evidences suggest that especially the
higher dose (400 mg/kg) of AqE of *H. indicus* and EtE of *B. ceiba* act by kaliuretic action in conjunction with their saluretic activity. Further, the intensity of aquaresis and accompanied marked increase in urinary Na\(^+\) and K\(^+\) levels by the AqE of *H. indicus* roots and EtE of *B. ceiba* fruits were similar to that of frusemide and HTZ. Alterations in urinary pH were also non-significant. These features strongly suggest that the AqE of *H. indicus* and EtE of *B. ceiba* act more as a loop diuretic than as thiazide diuretic, mainly due to their higher natriuretic, kaliuretic and saluretic actions. Loop diuretics mainly inhibit the Na\(^+\)/K\(^+\)/Cl\(^{-}\) symporter in the thick ascending Loop of Henle; thereby cause to increase natriuresis and kaliuresis\(^{171-173}\). Nevertheless, moderate acidification of urine is also seen with these diuretics\(^{171,172,174}\). Besides, another important index (the ion quotient) as exhibited by experimental groups in the range between 0.8 and 1.0, suggests their unequivocal association with carbonic anhydrase inhibition\(^{154}\). Notably, the AqE and EtE of *V. vinifera* (roots) could hardly exhibit any significant alteration in the urinary excretion of electrolytes. Hence, the strong diuretic effects shown by these extracts can be attributed solely to their high aquaretic properties.

After the oral administration of the single dose, the onset of the diuretic activity exhibited by the AqE and EtE of *H. indicus*, *V. vinifera* and *B. ceiba* was gradual till first 5 h which is in conformity with clinically used synthetic loop diuretics\(^{171,172}\). Interestingly, in spite of the heavy loss of urinary Na\(^+\) and K\(^+\), there was a significant reduction in the osmolarity of urine in AqE and EtE treated rats. Considering the fact that inhibition of ADH causes polyurea with low osmolarity\(^{174,175}\), it is possible that the AqE and EtE induced diuresis in the present study, may also be due to the impaired basal secretion of ADH and/or diminished sensitivity of uriniferous tubules to the action of ADH.

High ceiling loop diuretics are clinically used in patients with salt and water overload due to host of conditions. The observed mode of action of AqE and EtE in the present study, indicate that traditional practitioners may find *H. indicus* (roots), *V. vinifera* (fruits) and *B. ceiba* (fruits) being useful as a non-toxic natural therapeutic agent in the treatment of conditions such as pulmonary oedema, cardiac oedema, hypertension etc.\(^{171,172}\). Besides the only limitation of increased risk of hypokalaemia, as with other therapeutically used loop diuretics, the diuretic actions of crude extracts *H. indicus* roots and *B. ceiba* fruits conclude the plants as an appealing alternatives to presently available diuretic drugs. Though the onset of the diuretic action of the AqE and EtE was fairly gradual, it had a substantially prolonged duration of action. Thus, it is probable that it
would curtail the frequency of administration of a diuretic drug.

In urolithiasis, urinary chemistry is one of the important factors in determining the type of crystals formed and the nature of molecules occluded on to the surface of the crystals and stones. Substantially low urine volume, pH, mild hyperoxaluria, hypercalciuria, hypocitraturia, hyperuricosuria and hypomagnesuria are major risk factors in stone formers. Urine pH (<5.5 for uric acid stones and >6 for calcium stones) is a surrogate marker for determining the type of calculi. Particularly, in CaOx urolithiasis, the remarked basicity of the urine beyond pH 7.2 initiates the nucleation of phosphate and oxalate with calcium.

In the second phase of the present study, young male albino rats show alkalization of urinary pH (7.82) and form renal calculi composed mainly of CaOx in response to 14 days oral supplement of ethylene glycol. Urinary supersaturating with respect to stone-forming constituents is generally considered to be one of the causative factors in calculogenesis. Evidence in previous studies indicate that an increase in the urinary concentration of oxalate account for the biochemical mechanisms in this process. The biochemical mechanisms for this process are related to an increase in the urinary concentration of oxalate. The ready conversion of glycolate to oxalate renders the ethylene glycol fed animals to a state of hyperoxaluria, which causes increased renal retention and excretion of oxalate. Similar results have been obtained when rats were treated with ethylene glycol and ammonium oxalate.

In calculi-induced rats, the urinary excretion of calcium is also progressively increased, which may be attributed to defective tubular reabsorption in kidneys. However, the changes in urinary oxalate levels are relatively much more important than those of calcium, since it is accepted that hyperoxaluria is far more significant risk factor in the pathogenesis of renal stones than hypercalciuria. Increased urinary calcium favors nucleation and precipitation of CaOx or apatite (CaPh) from urine and subsequent crystal growth. However, in present study the curative treatment with AqE and EtE of H. indicus (root), V. vinifera (fruit) and B. ceiba (fruit) lowers the levels of oxalate as well as calcium excretion.

A progressive increase in urinary phosphate as observed in calculi-induced rats, along with oxalate load seems to provide further an environment appropriate for calculi formation by forming a nidus of CaPh and triple phosphate crystals with epitaxial deposition of CaOx. However, supplementation of H. indicus (root), V. vinifera (fruit) and B. ceiba (fruit) extracts restore phosphate level, thereby reducing the further risk of
Chapter 6  

Discussion

Stone formation.

The kidney function is affected in urolithiasis, since lowering of the glomerular filtration rate (GFR) is observed due to the obstruction to the outflow of urine by calculi deposited along the urinary system. Thereby, the waste products particularly nitrogenous substances such as urea, creatinine and uric acid, accumulate in blood\(^{185}\). It is well established that the glycolate feeding causes increased lipid peroxidation and decreases levels of antioxidant potential in kidneys\(^{186,187}\). Oxalate, being the precursor molecule to induce lipid peroxidation, further causes renal tissue damage by reacting with polyunsaturated fatty acids in cell membrane\(^{188}\). In this scenario, marked renal damage is observed in calculi-induced rats ascribed by virtue of the elevated serum levels of creatinine and uric acid, and BUN. Significant proteinuria and hematuria also substantiate the extent of nephritic damage. However, the diuresis induced by the AqE and EtE of \textit{H. indicus} (root), \textit{V. vinifera} (fruit) and \textit{B. ceiba} (fruit) causes to accelerate the process of dissolving preformed calculi and interrupts the process of crystal aggregation and deposition along the urinary system. The significant lowering of serum levels of accumulated waste products may be attributed to the enhanced GFR and the antioxidant properties exhibited by extracts of \textit{H. indicus}\(^{46}\), \textit{V. vinifera}\(^{90,99,100,109,113}\) and \textit{B. ceiba}\(^{134,189}\).

The histopathological studies also support the biochemical findings. Markedly elevated serum levels of BUN, creatinine and uric acid in stone-forming animals indicated the prominent necrosis of renal epithelia. In the calculi induced animals, there was damage to the last part of nephron and collecting system. Elevated levels of oxalate in urine and even its retention in kidney may be one of the causative factors for the peroxidative degeneration of renal epithelia. However, the curative treatment with AqE and EtE of \textit{H. indicus} (root), \textit{V. vinifera} (fruit) and \textit{B. ceiba} (fruit) prevents oxalate induced lipid peroxidation and causes regeneration of renal epithelium.

The preliminary phytochemical study of \textit{H. indicus} (roots), \textit{V. vinifera} (fruits) and \textit{B. ceiba} (fruits) extracts showed presence of glycosides, saponins and triterpenoids. Results of phytochemical investigations from other researchers indicate the possible role of secondary plant metabolites (particularly saponins and triterpenoids) for possessing antiurolithiatic activity\(^{190,191}\). Lupeol, a pentacyclic triterpenes, which exists widely in \textit{Crataeva nurvala} (Buch Ham.) mainly as aglycone of triterpenoid saponins, have been found to inhibit glycolic acid induced the urolithiasis\(^{192,193}\). Thus, similarity in biological activities of the bioactive extracts [i.e. AqE of \textit{H. indicus} (root), EtE of \textit{V. vinifera} (fruit) and \textit{B. ceiba} (fruit)] may be reciprocated to similar classes of phytoconstituents present in
these plants.

The fractionation of these bioactive extracts shows separation of different classes of phytoconstituents on TLC/HPTLC plates. The compounds giving characteristic colour due to specific chemical reaction with Vanillin-Sulphuric acid reagent (VR-7) were selected for isolation. The visualizing reagent VR-7 gives characteristic colour reactions with terpenoids (mono-, di- and triterpenoids) and saponins\textsuperscript{164-167}.

Further, the separation, isolation and purification using preparative HPTLC technique yielded five compounds of >80 % purity. The DSC chromatographic data reveals the isolated compound ICH-1 and ICV-1 to be the pure compounds having stable modifications. The FTIR spectra of ICH-1, ICV-1 and ICV-2 confirms the presence of more polar hydroxyl (–OH, alcoholic) groups/substitutions due to transmission minimum at 3400 cm\textsuperscript{-1}. While, the FTIR spectra ICV-1, ICV-2 and ICB-2 the characteristic transmission minimum at 1749 cm\textsuperscript{-1} confirms the presence of carbonyl (=C=O) groups\textsuperscript{168}.

The mass spectra of isolated compounds confirm their molecular weight as ICH-1 (281), ICV-1 (126), ICV-2 (166), ICB-1 (428) and ICB-2 (337). This data suggests that ICV-1 and ICV-2 may be monoterpene derivatives (molecular weight ~140), ICH-1 and ICB-1 may be a diterpene derivative (molecular weight ~280), and ICB-2 may be a triterpene derivative (molecular weight ~420).

The \textsuperscript{1}H-NMR data shows the proton environment for each molecule. However, further experiments with \textsuperscript{13}C-NMR and 2-D NMR spectroscopy shall provide more insight to the carbon atoms and their association with protons in the molecule. Thus, there is ample scope to extend the present study, using activity-guided preparative isolation followed by purification and characterization to elucidate the chemical structure of bioactive phytoconstituent(s).