Chapter 4

Establishment for anti-urolithiatic property through in vivo tests
INTRODUCTION

Urolithiasis is an extremely painful disease that afflicts the human population since ancient times (Grases et al., 1998) and can persist, with serious medical consequences, throughout a patient's lifetime. Urolithiasis is a urinary tract disorder characterized by the presence of solid deposits such as urinary calculi (also known as stones) or excessive amounts of crystals in the urinary tract. These solid deposits/calculi can form anywhere in the urinary collecting system, but most often calculi arise in the kidney (nephrolithiasis) (Kumar et al., 1992). Calculi refer to the solid nonmetallic minerals in the urinary tract. Stone formation is one of the most widespread diseases known to man. Urinary calculi have been found in the tombs of Egyptian mummies dating back to 4000 B.C. (Riches, 1968) and in the graves of North American Indians from 1500-1000 B.C. (Beck & Mulvane, 1996). Reference to stone formation is made in the early Sanskrit documents in India between 3000 and 2000 B.C. (Butt, 1957). There are only a few geographical areas in which stone disease is rare, e.g., in Greenland and in the coastal areas of Japan (Tiselius, 2003). The incidence of urinary stones has been increasing over the last years while the age of onset is decreasing (Devuyst & Pirson, 2007). The etiology of this disorder is multifactorial and is strongly related to dietary lifestyle habits or practices (Taylor et al., 2005). Increased rates of hypertension and obesity, which are linked to urolithiasis, also contribute to an increase in stone formation (Obligado & Goldfarb, 2008).

Urolithiasis in its different forms is a frequently encountered urological condition that has been at the forefront of urology. It occurs more frequently in men than women but rare in children (Smith, 1978), affecting approximately 12% of men and 5% of women by the age of 70. The formation of these stones involves several physicochemical events, beginning with crystal nucleation, growth and aggregation, and ending with retention within the urinary
tract. The etiology of stone formation is a multifactorial process which may relate to diet, urinary tract infection, altered urinary solutes and colloids, decreased urinary drainage and urinary stasis, prolonged immobilization, Randall's plaque, microliths, etc. (Fowler, 1995). The principle causative factor of the formation of stones is attributed to the supersaturation of precipitating salts. One of the most important phenomena that characterize urolithiasis is its high recurrence. Urolithiasis is worldwide in distribution with a recurrence rate of 70-80% on males and 47-60% females (Smith & Guay, 1992). The recurrence of urolithiasis represents a serious problem as patients who have formed one stone are more likely to form another. Once recurrent, the subsequent relapse risk is raised and the interval between recurrences is shortened. Features associated with recurrence include a young age of onset, positive family history, infection stones and underlying medical conditions (Moe, 2006).

The majority of stones, up to 80%, are composed mainly of calcium oxalate (Daudon et al., 1993). Many remedies have been employed during ages to treat renal stones. Most of remedies were taken from plants and proved to be useful, though the rational behind their use is not well established except for a few plants and some proprietary composite herbal drugs and they are reported to be effective with no side effects (Nadkarni, 1976).

Kidney stone formation or urolithiasis is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidneys (Daudon & Jungers, 2001). Not all standard pharmaceutical drugs used to prevent urolithiasis are effective in all patients, and many have adverse effects that compromise their long-term use (Atmani et al., 2003).

Renal calculi can be broadly classified in two large groups: tissue attached and unattached (Grases et al., 2002). Attached calculi are mainly integrated by calcium oxalate monohydrate
(COM) renal calculi, with a detectable attachment site to the renal papilla and basically consisting of a core located near to the attachment site (concave zone) and radially striated concentrically laminated peripheral layers. Unattached calculi, with no detectable site of attachment to papilla, are developed in renal cavities of low or reduced urodynamic efficacy and can exhibit diverse composition and structures. Several reports have been published since Randall's first description of papillary calcifications and their possible active role in the genesis of COM papillary calculi (Low et al., 2000; Kuo et al., 2003; Kim et al., 2005). At present, it seems clear that renal epithelial cell injuries play a decisive role in such a type of renal calculi development (de Water et al., 2000; Muthukumar & Selvam, 1997), and in fact the lithogenic effect caused by ethylene glycol (EG) must be mainly attributed to the oxidative damage caused by the high level of oxalate generated by EG. The first studies on experimental EG renal lithiasis appeared in the 60th decade (Vaille et al., 1963; Debray et al., 1964) but the importance of the oxidative damage caused by hyperoxaluria was not clearly proposed until the end of the century (Thamilselvan et al., 1997). From this last period it appeared several prophylaxis proposals on EG induced urolithiasis using herbal extracts and antioxidants (Atmani et al., 2003; Itoh et al., 2005; Thamilselvan & Menon, 2005; Farooq et al., 2005; Huang et al., 2006; Veena et al., 2006; Laroubi et al., 2007; Hadjzadeh et al., 2007; Karadi et al., 2006).

Ethylene glycol is an intermediate in the synthesis of a number of commercial chemical products, including Polyethylene Terephthalate (PET) resins, unsaturated polyester resins and polyester fibers and films. It is also a constituent in antifreeze, deicing fluids, surface coatings, heat transfer fluids and industrial coolants, hydraulic fluids, surfactants and emulsifiers (Lockely et al., 2002). Exposure occurs primarily from the use of ethylene glycol in automotive antifreeze. There have been a number of acute human poisonings from
accidental or intentional ingestion of antifreeze, with the kidney being the most sensitive target organ. Regimens for the treatment of acute ethylene glycol poisoning are designed to prevent metabolism to the toxic acidic metabolites, to treat acidosis and to prevent kidney damage (Brent et al., 1999). Ethylene glycol has in itself a low toxicity, but is in vivo broken down to four organic acids: Glycoaldehyde, glycolic acid, glyoxylic acid and oxalic acid. The metabolites are cell toxins that cause central nervous system depression and cardiopulmonary and renal failure. Glycolic acid causes severe acidosis and oxalate is precipitated as calcium oxalate in the kidneys and other tissues (Leth & Gregersen, 2005).

The present day medical management of urolithiasis is either costly or not without side effects. Invasive procedures for the treatment of urolithiasis may cause serious complications and they also impose a great load to the healthcare system. Hence the search for antilithiatic drugs from natural sources has assumed greater importance (Verma et al., 2009). Thus, appropriate management is required to treat urolithiasis and therefore, it is worth looking for an alternative to those conventional methods, such as the use of medicinal plants.

Medicinal plants are of great economic importance in the Indian subcontinent. Plants remain the basis for a large proportion of the medications used today for the treatment of variety of diseases. A number of researchers have documented the use of traditional medicinal plants in India (Dey, 1998). A variety of plants including those used by traditional medical practitioners grow luxuriantly in Manipur (24° 49’ N and 93° 52’ E), a region in the northeastern part of India which happens to be within the Indo-Burmese mega-biodiversity hotspot (Myers et al., 2000). The Indian indigenous system of medicine provides abundant data on plants available for the treatment of urolithiasis. Among the vast number of medicinal plants that are claimed to be anti-urolithiatic, Hibiscus sabdariffa L. is one for which
systematic pharmacological studies have not been carried out on its calyces to support the claim made. Hence, the present study aims at systematically evaluating the calyces of this plant to establish its scientific validity for anti-urolithiatic property using ethylene-glycol induced hyperoxaluria model in rats and to assess the genotoxicity of this plant. Thus, scientific investigations on the indigenous medicine prepared from plant products used by the Tribals and Meiteis of Manipur may prove to be of great pharmacological importance leading to the advent of novel drugs, which could be at par with the modern allopathic medicines in terms of efficacy, minimal side-effects and cost affordability. Despite considerable progress in medical therapy, there is no satisfactory drug to treat kidney stones. So, the present study was designed to investigate the antilithiatic activity of *Hibiscus sabdariffa* supplementation on ethylene glycol induced urolithiasis in male albino rats.

*Hibiscus sabdariffa* commonly known as Roselle or Jamaican Sorrel is an annual erect shrub that grows to 6 feet or more and is mostly branched. Stems are robust and glabrous. Leaves are alternate, long-petiolate, palmately divided into 3-7 lobes with serrate margins and bear short-peduncled light-yellow flowers with a reddish centre at the base of the staminal column. The flowers are in axillary or in terminal racemes. The calyx becomes fleshy when enlarged at maturity creating a bright fleshy red, acid fruit of about 1 1/4 inches. *Hibiscus sabdariffa* is cultivated in the hotter regions of India as a monsoon (April-November) crop. Besides the pleasant aroma and beauty of the plant itself, it possesses qualities of traditional medicinal plants. As a medicine, it is used as a therapeutic, laxative, chemopreventive (Lin *et al.*, 2007), anti-hypertensive, cholesterol lowering medicine (Farombi & Ige, 2007) and an antioxidant agent (Tseng *et al.*, 1997; Farombi & Ige, 2007). It also lowers hepatotoxicity and reduces fever. Almost all the parts are considered diuretic and antiscorbutic
(Moussisamy et al., 2002; Wright et al., 2007) in their action. The fibrous part of Hibiscus is used in the production of twins and cord known as “Roselle hemp”.

Safe use of herbal medicine is a burning question at present. WHO collaborating centre for International Drug monitoring in Sweden is classifying common toxic ingredients of herbal medicine. In UK and USA, government funded agencies have been appointed to investigate of complaints from the consumers and to recommend removal of harmful products from the market. With the increase in the popularity of herbal medicine, it is necessary to ensure safety of the patients.

Plants contain different entities which may be mutagenic, carcinogenic, clastogenic, genotoxic, immune suppressive drugs, antifertility alkaloids, etc. As for example, alcoholic extract of 100 g leaves of Eupatorium ayapana showed genotoxic effect on the bone marrow chromosomes of Rattus norvegicus. Rats treated with different doses of extract produced various kinds of chromosomal aberrations. The types and quality of aberrations were increased with increase in the doses of the extract (Chakrabarti, 2001). Intraperitoneal injection of crude aqueous extracts of stembark, leaf and seed kernel of yellow oleander, Thevetia peruvian to albino rats weighing about 150-200 g or exposed to bait prepared with the dry extracts of the plant parts were toxic, and produced marked poisoning symptoms that culminated in death. Poisoning symptoms indicated serious cardiac, neuromotor and mental malfunctioning and manifested as tachycardia, arrhythmia, paralysis, ataxia and disorientation (Oji & Okafor, 2000).

Cycasin, a water soluble compound present in the nuts, leaves and roots of Cycas, such as the palm Cycas circinalis is carcinogenic in hamsters, guinea pigs, rats, mice and rabbits (Hirono
et al., 1971; International Agency for Cancer Research, 1976). It induces chromosomal aberrations in onion root tip cells (Teas et al., 1965). Extracts of Krameria izina have been reported to induce sarcoma in rats (Ogara, 1974). Some plant extracts have also been observed to induce chromosomal damage in CHO cells and human lymphocytes (Kanaya et al., 1992).

Studies on medicinal plants in various perspectives are currently an active field of research throughout the world. In India also, studies on medicinal plants in various perspectives are going on. Elaborate treatise on medicinal plants has recently been produced, namely, Medicinal plants and folklore (Singh et al., 1990). Medicinal plants – New vistas of research (Govil et al., 1993) and Herbal drugs of Himalayas: Medicinal plants of Garhwall (Singh & Ali, 1998). Considering the prevalence of traditional system of using plants in medicine in large section of our societies, it is important to analyze the cytogenetic effects of the plants used in traditional system of medicines.

There are various test systems for screening genetic toxicity of chemicals. But, cytogenetic test system is one of the most useful short-term systems. Cytogeneticists have employed a variety of test materials and test methods to measure the effect of mutagens on chromosomes. Chromosome damage constitutes a set of efficient, reliable and economical criteria to measure genetic toxicity.

Nowadays, it has become mandatory for a new drug to undergo genetic toxicity testing in order to determine whether the drug will lead to any genetic disorder or not. Traditional medicinal plants also need to be subjected to systematic screening for genotoxicity testing. Since traditional medicinal plants are not used with strict regulation of dosage and timing,
the risk posed by these medicines is more than that of approved drugs. Therefore, the cytogenetic assays for genotoxicity testing of plant and plant products are undertaken.

Urolithiasis is generally encountered through medical practices such as surgery, lithotripsy and the use of allopathic medicine system, which are rather risky or with numerous side effects. Therefore, the normal target is towards the advent of phytotherapeutic agents that could prevent and control the pertinent problem of stone case and urine crystallization. Another goal to be achieved is the cost effectiveness of the naturopathic drug, which aims at the affordability of the commons. Thus seeing the high incidence of urinary stones in our country, this study would show the potential and healing powers of medicinal plants and will surely be a great boon to the human society.
MATERIALS AND METHODS

Chemicals and reagents

Giemsa stain was purchased from Sigma-Aldrich, St. Louis, USA. RPMI-1640 (without L-glutamine and sodium bicarbonate) and foetal calf serum were purchased from Hi-Media, India. Ethylene glycol and ammonium chloride were obtained from Merck, India. All other chemicals and reagents were of analytical grade obtained from Merck, India.

Plant samples

The fresh calyces of *Hibiscus sabdariffa* were collected from various areas of Imphal-West (24° 37’ N and 93° 30’ E) district, India. A voucher number (Deb 1377) was assigned to it after depositing in the Herbarium of Manipur University, Imphal.

Preparation of plant extract

The plant samples were washed in fresh running tap-water and air-dried for about 10 minutes. For preparation of aqueous extract, about 10 g of the plant sample was extracted in 100 mL of deionized water with the help of a mortar and pestle and homogenized for uniformity. Then, the homogenate was centrifuged at 3000g for 10 minutes to get a clear supernatant. Finally the clear supernatant was decanted and filtered through Whatman No. 1 filter paper and stored at 4 °C for future use.

Pharmacological screening for antiurolithiatic activity

Animal selection

Male albino rats (*Rattus norvegicus*) weighing about 150-200 g were selected for the study [Fig. 4.1]. The animals were maintained under 12 hrs. dark/light cycle in well ventilated polypropylene metabolic cages at (25 ± 2 °C) and fed with standard pellet diet and had free
access to drinking water. The animal care and the experimental protocols were performed in accordance with the guidelines of the Institutional Animal Ethical Committee (IAEC). Experiments on albino rats were conducted with the approval (No. MU/8-199/06/UGC, Dated 03-09-2008) of IAEC.

**Acute toxicity studies**

Maximum tolerated dose (MTD) was determined by treatment of the test system (albino rat, *Rattus norvegicus*) with various concentrations of plant extracts as described by Carrol (1952). For this purpose, male albino rats weighing about 150-200 g of 5 animals each were taken for each dose. Different doses of aqueous extract of *Hibiscus sabdariffa* were administered orally with a plastic disposable syringe, fitted with a feeding needle [Fig. 4.2]. All the groups of rats were kept under observation for 24 hours.

**Ethylene-glycol induced urolithiasis model**

The effect of oral administration of aqueous extract of *Hibiscus sabdariffa* on calcium oxalate urolithiasis induced by ethylene glycol was studied using male albino rats. Animals were divided into nine groups of 5 each. Group I – Normal/control rats given normal regular diet and water *ad libitum*; Group II – Urolithic rats given drinking water containing a mixture of 0.75% ethylene glycol [v/v] (EG) and 2% ammonium chloride [w/v] (AC); Group III - Urolithic rats given standard antiurolithic drug, cystone (750 mg/kg body weight); Groups (IV – VI) - Urolithic rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight; Groups (VII – IX) – Normal rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight. All the groups of rats were given drinking water *ad libitum* and the treatment duration was for 28 days.
Fig. 4.1: Normal male albino rat (*Rattus norvegicus*)

Fig. 4.2: Administration of the plant extract with a plastic disposable syringe to the experimental animal
Assessment of antiurolithiatic activity

Serum analysis

After the experimental period, the blood was collected from the retro-orbital region of the rat under anesthetic conditions. Serum was separated by centrifugation at 10,000 x g for 10 minutes (Karadi et al., 2006) after which the levels of calcium (Medeiros & Mustafa, 1985), phosphorus (Fiske & Subbarow, 1925), urea (Raghuramulu et al., 1983) and creatinine (Raghuramulu et al., 1983) were assessed.

Kidney analysis

The anesthetized rats (with chloroform) were sacrificed by cervical dislocation. The abdomen was dissected and both the kidneys were removed. The isolated kidneys were cleaned off extraneous tissues. The left kidney was oven-dried at 80 °C for 24 hours, after which the kidneys were weighed. About 1 g of the dried kidney sample was boiled in 100 mL of 1 N hydrochloric acid for 30 minutes and homogenized. The homogenate obtained was again centrifuged at 2000 x g for 10 minutes and the supernatant was separated (Chow et al., 1975) after which the levels of calcium (Medeiros & Mustafa, 1985) and phosphorus (Fiske & Subbarow, 1925) in the kidney were assessed.

Histological analysis

The tissue pieces taken from the kidney of the rats were fixed by neutral buffered formalin (10%) and subsequently embedded in paraffin. The sections (about 5 μm thick) were stained using hematoxylin and eosin stains (Cuzzolin et al., 1995) to study the histopathological changes and to check calcium oxalate crystal deposition. General procedure for obtaining tissue sections is given as illustration in [Fig. 4.3]. Tissues slices were photographed using a
Fig. 4.3: Generalised procedure for obtaining tissue sections
CCD camera fitted to trinocular phase contrast microscope - UNILAB (GE-52TRH) with 400 x magnification.

Genotoxicity testing

Chromosomal aberrations

The anesthetized rats were sacrificed by cervical dislocation and the bone marrow was taken out from either of the femur to perform cytogenetic analysis. The metaphase chromosomal plates were prepared according to the conventional air-drying method of Uma Devi et al., (1998) with minor modifications. In brief, the bone marrow taken out from one of the femurs was aspirated, flushed with normal saline (0.9% NaCl), treated hypotonic solution (0.54% KCl) for about 20-30 minutes, centrifuged at 3000 rpm for 5 minutes and the pellet was fixed in freshly prepared chilled Carnoy’s fluid (3 methanol : 1 glacial acetic acid) after 2-3 changes with this fixative along with centrifugation. The slides were prepared by standard air drop method. They were then dried and stained with 10% Giemsa (Sigma) prepared in Sorrenson’s buffer, pH 5.6. The stained slides were rinsed with distilled water. Chromosomal aberrations per 200 cells were scored under 400 x magnifications. Chromosomal aberrations such as chromatid breaks, centromeric gaps, fragments and deletions were scored. Aberrations were identified according to criteria given by Savage (1976).

Micronucleus assay

The micronucleus assay affords a procedure for the detection of aberrations involving metaphase chromosome behaviour utilizing the bone marrow erythroblast (Schmid, 1973). This assay is based on the formation of micronuclei from particles of chromatin material that, due to chromosome breakage or spindle disfunction, do not migrate to the poles during
anaphase and are not incorporated into the telophase nuclei of the dividing cell. Such chromatin fragment or even whole chromosomes in the case of chromosome lag results in the formation of one or more satellite nuclei in the cytoplasm of the daughter cells.

Micronucleus assay was performed according to the method of Schmid (1979) with minor modifications. Briefly, bone marrow taken out from the femur was aspirated out into 1 mL of RPMI-1640 (without L-glutamine and sodium bicarbonate), centrifuged at 700 rpm for 5 minutes and the pellet was resuspended in 0.5 mL of foetal calf serum (heat inactivated at 56 °C for 30 minutes). The smears of cells were prepared on precleaned, precoded dried slides and fixed in absolute methanol. The slides containing the cells were stained with 10% Giemsa (Sigma) prepared in Sorrenson's buffer, pH 5.6 according to the method of Vives Corrons et al., (2004) with minor modifications and examined under a microscope. 1000 cells were counted blind for each animal. In this analysis, both the Polychromatric micronucleated erythrocytes (PCEs) and Normochromatric micronucleated erythrocytes (NCEs) were scored and expressed as polychromatric micronucleated erythrocytes or normochromatric micronucleated erythrocytes/1000 cells. Finally, the results were expressed in terms of the percentage of micronucleus. Photographs were taken using a CCD camera fitted to trinocular phase contrast microscope - UNILAB (GE-52TRH) using oil immersion at 1000-fold magnification.

**Statistical analysis**

Data were subjected to one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Values are presented as means ± standard error (S.E.). P values < 0.05 were considered significant.
RESULTS

Based on the results obtained for acute toxicity testing, the maximum tolerated dose (MTD) of the plant extract, *Hibiscus sabdariffa* was found to be 2010 mg/kg body weight. So, the therapeutic doses of the plant extract were taken as 250, 500 and 750 mg/kg body weight (Table 4.1).

**Table 4.1**: Exploratory trials for determination of MTD (Maximum Tolerated Dose) of aqueous extract of *Hibiscus sabdariffa* L.

<table>
<thead>
<tr>
<th>Dose (mg/kg body weight)</th>
<th>Mortality (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>250</td>
<td>0</td>
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<tr>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>750</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>1250</td>
<td>0</td>
</tr>
<tr>
<td>1500</td>
<td>0</td>
</tr>
<tr>
<td>1750</td>
<td>0</td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
</tr>
<tr>
<td>2010</td>
<td>0</td>
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<tr>
<td>2020</td>
<td>20</td>
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<td>2030</td>
<td>40</td>
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<tr>
<td>2040</td>
<td>80</td>
</tr>
<tr>
<td>2050</td>
<td>100</td>
</tr>
</tbody>
</table>

In the present study, chronic administration of aqueous solution of a mixture of 0.75% ethylene glycol and 2% ammonium chloride in drinking water to male albino rats significantly increased the deposition of calcium oxalate stones. Serum and kidney analyses revealed that the levels of renal stone components were increased grossly in urolithiatic rats (Table 4.2, Group II) when compared with the control group (Table 4.2, Group I). This shows strong indication of renal and hepatic impairment. The decrease in the serum calcium
concentration indicates utilization of calcium for calcium oxalate formation ultimately depositing in the kidney site, thereby subsequently elevating its level in kidneys of urolithic rats. However, supplementation of aqueous extract of *Hibiscus sabdariffa* at different doses (250, 500 and 750 mg/kg body weight) significantly lowered the deposition of stone forming constituents in the kidneys and serum in both the treated sets when compared to the cystone-treated rats (Table 4.2, Group III). The treatment of aqueous extract of *Hibiscus sabdariffa* at different doses significantly reduced the deposition of the crystalline components in both the sets (Table 4.2, Groups IV-VI and Groups VII-IX). The serum urea and phosphorus levels were remarkably increased in urolithic rats (Table 4.2, Group II), while the level of creatinine in the serum was only slightly elevated in Group II indicating marked renal damage. However, treatment of *Hibiscus sabdariffa* extract in both the sets (Table 4.2, Groups IV-VI and Groups VII-IX) significantly lowered the elevated serum levels of urea, phosphorus and creatinine.

**Table 4.2:** Effect of aqueous extract of the calyces of *Hibiscus sabdariffa* L. on kidney and serum parameters in control and experimental animals*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Kidney (mg/g)</th>
<th>Serum (mg/dl)</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calcium</td>
<td>Phosphorus</td>
<td>Calcium</td>
</tr>
<tr>
<td>Group I</td>
<td>3.41 ± 0.03a</td>
<td>2.59 ± 0.09a</td>
<td>4.51 ± 0.17b</td>
</tr>
<tr>
<td>Group II</td>
<td>9.09 ± 0.04d</td>
<td>4.06 ± 0.35b</td>
<td>3.00± 0.16a</td>
</tr>
<tr>
<td>Group III</td>
<td>3.61 ± 0.12ab</td>
<td>2.59 ± 0.07a</td>
<td>4.50 ± 0.17b</td>
</tr>
<tr>
<td>Group IV</td>
<td>4.14 ± 0.24c</td>
<td>2.71 ± 0.70a</td>
<td>4.40 ± 0.08b</td>
</tr>
<tr>
<td>Group V</td>
<td>4.13 ± 0.05c</td>
<td>2.71 ± 0.64a</td>
<td>4.42 ± 0.09b</td>
</tr>
<tr>
<td>Group VI</td>
<td>4.13 ± 0.05c</td>
<td>2.65 ± 0.30a</td>
<td>4.48 ± 0.06b</td>
</tr>
<tr>
<td>Group VII</td>
<td>4.11 ± 0.03c</td>
<td>2.66 ± 0.30a</td>
<td>4.47 ± 0.07b</td>
</tr>
<tr>
<td>Group VIII</td>
<td>3.97 ± 0.07bc</td>
<td>2.66 ± 0.30a</td>
<td>4.48 ± 0.09b</td>
</tr>
<tr>
<td>Group IX</td>
<td>3.95 ± 0.08bc</td>
<td>2.61 ± 0.33a</td>
<td>4.49 ± 0.06b</td>
</tr>
</tbody>
</table>

*Values are expressed as means ± standard error (n = 5). For each column, values followed by the same letters (a-d) are not statistically different at p < 0.05 as measured by Tukey HSD test.

where Group I - Normal/control rats given normal regular diet and water *ad libitum*; Group II - Urolithic rats given drinking water containing a mixture of 0.75% ethylene glycol [v/v] (EG) and 2% ammonium chloride [w/v] (AC); Group III - Urolithic rats given standard antiurolithic drug, cystone (750 mg/kg body weight); Groups (IV -- VI) - Urolithic rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight; Groups (VII -- IX) - Normal rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight.
Physical verification of the kidneys also throws light towards deposition of crystals in urolithic conditions [Fig. 4.4]. Histological investigations revealed that rats treated with ethylene glycol and ammonium chloride had large deposits of calcium oxalate crystals in all parts of the kidney. Tubular dilation and degeneration of epithelial lining also occurred [Fig. 4.5 (b), Group II]. Such deposits were not present in normal rats [Fig. 4.5 (a), Group I], cystone-treated rats [Fig. 4.5 (c), Group III], normal rats treated with the extract at all doses (Groups VII-IX) and urolithic rats treated with extract at the dose of 750 mg/kg body weight [Fig. 4.5 (f), Group VI]. Slight traces of crystals were observed in urolithic rats treated with extract at the doses 250 and 500 mg/kg body weight [Figs. 4.5 (d) & 4.5 (e), Groups IV & V]. There is less degeneration of epithelial lining and tubules thereby indicating marked improvement in these groups too.
Fig. 4.4: Physical verification of the kidneys (arrows indicate stone deposition)
**HISTOLOGICAL SECTIONS**

**Fig. 4.6 (a):** Kidney section of control rat with normal epithelial lining and tubules.

**Fig. 4.6 (b):** Kidney section of urolithic rat showing dilated tubules, degenerated epithelial lining and crystal deposits.

**Fig. 4.6 (c):** Kidney section of urolithic rat treated with cystone (100 mg/kg body weight) showing epithelial lining and tubules comparatively similar to that of normal rats.
Fig. 4.5 (d): Kidney section of urolithic rat treated with aqueous extract of *H. sabdariffa* (250 mg/l kg body weight) showing less degeneration of epithelial lining and tubules with lower rate of crystal deposition.

Fig. 4.5 (e): Kidney section of urolithic rat treated with aqueous extract of *H. sabdariffa* (500 mg/l kg body weight) showing less degeneration of epithelial lining and tubules with very low rate of crystal deposition.

Fig. 4.5 (f): Kidney section of urolithic rat treated with aqueous extract of *H. sabdariffa* (750 mg/l kg body weight) showing epithelial lining and tubules comparatively similar to that of normal rats.
The observed structural abnormalities in bone marrow cells in various groups of treated rats were illustrated in tables 4.3 and 4.4.

Table 4.3: Effect of aqueous extract of *Hibiscus sabdariffa* L. on rat bone marrow

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal metaphases/200 cells</th>
<th>Centromeric gaps</th>
<th>Chromatid Breaks</th>
<th>Deletions</th>
<th>Fragments</th>
<th>Total aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>196.00 ± 0.70</td>
<td>1.20 ± 0.49</td>
<td>0.80 ± 0.49</td>
<td>1.40 ± 0.40</td>
<td>0.60 ± 0.24</td>
<td>4.00 ± 0.71</td>
</tr>
<tr>
<td>Group II</td>
<td>192.60 ± 0.67</td>
<td>2.00 ± 0.32</td>
<td>1.60 ± 0.25</td>
<td>2.20 ± 0.37</td>
<td>1.60 ± 0.24</td>
<td>7.40 ± 0.67</td>
</tr>
<tr>
<td>Group III</td>
<td>194.60 ± 0.51</td>
<td>1.40 ± 0.40</td>
<td>1.20 ± 0.49</td>
<td>1.80 ± 0.58</td>
<td>1.00 ± 0.45</td>
<td>5.40 ± 0.51</td>
</tr>
<tr>
<td>Group IV</td>
<td>193.40 ± 0.68</td>
<td>1.80 ± 0.49</td>
<td>1.40 ± 0.40</td>
<td>2.00 ± 0.32</td>
<td>1.40 ± 0.50</td>
<td>6.60 ± 0.68</td>
</tr>
<tr>
<td>Group V</td>
<td>193.80 ± 0.80</td>
<td>1.60 ± 0.25</td>
<td>1.40 ± 0.24</td>
<td>1.80 ± 0.49</td>
<td>1.40 ± 0.40</td>
<td>6.20 ± 0.80</td>
</tr>
<tr>
<td>Group VI</td>
<td>194.40 ± 0.40</td>
<td>1.60 ± 0.24</td>
<td>1.20 ± 0.20</td>
<td>1.60 ± 0.24</td>
<td>1.20 ± 0.37</td>
<td>5.60 ± 0.40</td>
</tr>
<tr>
<td>Group VII</td>
<td>194.60 ± 0.40</td>
<td>1.40 ± 0.51</td>
<td>1.20 ± 0.58</td>
<td>1.60 ± 0.24</td>
<td>1.20 ± 0.37</td>
<td>5.40 ± 0.40</td>
</tr>
<tr>
<td>Group VIII</td>
<td>194.20 ± 0.49</td>
<td>1.20 ± 0.49</td>
<td>1.40 ± 0.50</td>
<td>1.80 ± 0.58</td>
<td>1.40 ± 0.51</td>
<td>5.80 ± 0.49</td>
</tr>
<tr>
<td>Group IX</td>
<td>194.00 ± 0.32</td>
<td>1.60 ± 0.24</td>
<td>1.20 ± 0.58</td>
<td>1.80 ± 0.32</td>
<td>1.40 ± 0.25</td>
<td>6.00 ± 0.32</td>
</tr>
</tbody>
</table>

*No significant changes were observed as determined by Tukey’s post hoc test. Values are expressed as means ± standard error (n = 5)*

where Group I – Normal/control rats given normal regular diet and water *ad libitum*; Group II – Urolithic rats given drinking water containing a mixture of 0.75% ethylene glycol [v/v] (EG) and 2% ammonium chloride [w/v] (AC); Group III - Urolithic rats given standard antiurolithic drug, cystone (750 mg/kg body weight); Groups (IV – VI) - Urolithic rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight; Groups (VII – IX) – Normal rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight

Table 4.4: Effect of aqueous extract of *Hibiscus sabdariffa* L. on rat bone micronuclei

<table>
<thead>
<tr>
<th>Groups</th>
<th>Polychromatic micronucleated erythrocytes</th>
<th>Normochromatic micronucleated erythrocytes</th>
<th>Micronucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>2.20 ± 0.20</td>
<td>0.80 ± 0.37</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>Group II</td>
<td>3.80 ± 0.58</td>
<td>2.60 ± 0.50</td>
<td>0.64 ± 0.07</td>
</tr>
<tr>
<td>Group III</td>
<td>2.60 ± 0.24</td>
<td>1.20 ± 0.37</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>Group IV</td>
<td>3.60 ± 0.75</td>
<td>2.20 ± 0.20</td>
<td>0.58 ± 0.09</td>
</tr>
<tr>
<td>Group V</td>
<td>3.60 ± 0.60</td>
<td>2.00 ± 0.32</td>
<td>0.56 ± 0.06</td>
</tr>
<tr>
<td>Group VI</td>
<td>3.40 ± 0.24</td>
<td>1.80 ± 0.37</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>Group VII</td>
<td>3.00 ± 0.71</td>
<td>1.40 ± 0.60</td>
<td>0.44 ± 0.12</td>
</tr>
<tr>
<td>Group VIII</td>
<td>3.20 ± 0.58</td>
<td>1.60 ± 0.51</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td>Group IX</td>
<td>3.20 ± 0.49</td>
<td>2.20 ± 0.37</td>
<td>0.54 ± 0.07</td>
</tr>
</tbody>
</table>

*No significant changes were observed as determined by Tukey’s post hoc test. Values are expressed as means ± standard error (n = 5)*

where Group I – Normal/control rats given normal regular diet and water *ad libitum*; Group II – Urolithic rats given drinking water containing a mixture of 0.75% ethylene glycol [v/v] (EG) and 2% ammonium chloride [w/v] (AC); Group III - Urolithic rats given standard antiurolithic drug, cystone (750 mg/kg body weight); Groups (IV – VI) - Urolithic rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight; Groups (VII – IX) – Normal rats given aqueous extract of *Hibiscus sabdariffa* at the doses of 250, 500 and 750 mg/kg body weight
Results of *in vivo* genotoxicity testing showed no significant chromosomal aberrations (centromeric gaps, deletions, chromatid breaks and fragments) in bone marrow cells of ethylene glycol-induced rats [Figs. 4.6(d)-4.6(f)] although, there is a slight increase in the micronuclei formation. The plant extracts at the doses administered showed mild chromosomal aberrations which were not significant and thus, it could be inferred that the plant extracts induced neither toxic nor lethal effects [Fig. 4.6(c)] when compared to that of the control groups [Fig. 4.6(a)] and with that of cystone-treated male albino rats [Fig. 4.6(b)]. There is no significant micronuclei formation [Fig. 4.7(a) & 4.7(b)] in these groups and hence, it can be concluded that oral administration of *Hibiscus sabdariffa* did not have any genotoxic effect. The present results indicated that aqueous extract of *Hibiscus sabdariffa* is safe whereas, they did not induce mutagenic activity in bone marrow cells.
Fig. 4.6 (a): Normal metaphase plate of male albino rat

Fig. 4.6 (b): Chromosomes of urolithiatic male albino rat treated with cystone (750 mg/kg body weight)

Fig. 4.6 (c): Chromosomes of urolithiatic male albino rat treated with *Hibiscus sabdariffa* L. (750 mg/kg body weight)
Fig. 4.6 (d): Chromosomes of male albino rat showing centromeric gap

Fig. 4.6 (e): Chromosomes of urolithiatic male albino rat showing deletion

Fig. 4.6 (f): Chromosomes of urolithiatic male albino rat showing chromatid break and fragments
Fig. 4.7(a): Normochromatic micronucleated erythrocyte in bone marrow cells of urolithiatic albino rat

Fig. 4.7 (b): Polychromatic micronucleated erythrocyte in bone marrow cells of urolithiatic albino
DISCUSSION

Urolithiasis is a stone-disorder due to an imbalance between inhibitors and promoters in the kidneys and human kidney stones are mainly composed of calcium oxalate type of stones (Daudon & Jungers, 2001). Many in vivo models have been developed for investigating the mechanisms underlying in stone formation and ascertaining the effect of various therapeutic agents on the development and progression of urolithiasis (Khan, 1991; Lee et al., 1992; Boeve et al., 1993; Khan & Glenton, 1995; Fan et al., 1999; Atmani et al., 2004).

Rats are the most frequently used animal models of calcium oxalate deposition in the kidneys. a process that mimics the etiology of kidney stone formation in humans (Atmani et al., 2004). Rat models of calcium oxalate urolithiasis induced by either ethylene glycol alone or in combination with other drugs such as ammonium chloride, were often used to study the pathogenesis of kidney crystal deposition (Fan et al., 1999). In the present study, male albino rats were also treated with solutions containing a mixture of 0.75% ethylene glycol and 2% ammonium chloride for 28 days. All positive control rats (Group II) developed calcium oxalate depositions during that time. The present results also showed that the administration of ethylene glycol caused statistical increase in the levels of phosphorus, urea and creatinine and a decrease in the level of calcium in the calculi-induced or urolithiatic rats.

Selvam (2001) reported that stone formation was also caused by hyperoxaluria which resulted in increased renal retention and excretion of calcium and phosphate. The
decrease in serum calcium concentration indicates an increase of urinary calcium and calcium oxalate stone formation. This suggestion is in agreement with several studies like Rajagopal et al., (1977) who reported that the level of serum calcium was decreased and urinary calcium increased in rats treated with ethylene glycol. Soundararajan et al., (2006) showed that calcium oxalate excretion was significantly increased in urine of ethylene glycol induced urolithiatic rats. They also stated that ethylene glycol disturbed oxalate metabolism by increasing the substrate availability resulting in an increase in the activity of oxalate synthesizing enzymes in these rats. Moreover, several investigations demonstrated that ethylene glycol treatment increased urinary calcium excretion significantly in urolithiatic rats (Christina et al., 2002; Karadi et al., 2006; Verma et al., 2009).

It has been reported that administration of ethylene glycol to rats increased excretion of phosphorus in stone formers (Ettinger, 1986) and hyperoxaluric rats (Rengaraju & Selvam, 1987; Subha & Varalakshmi, 1993). It seems that increased urinary phosphorus excretion along with oxalate stress provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition (Roger et al., 1997). However, the phosphorus level was brought down to normal after treatment with aqueous extract of Hibiscus sabdariffa thereby, reducing the risk of stone formation.

It has also been observed that there is a marked decrease in the glomerular filtration rate (GFR) due to the obstruction of urine outflow by stones in urinary system during
uroolithiasis, resulting in the accumulation of waste products, particularly the nitrogenous substances such as urea and creatinine in the blood (Ghodkar, 1994). It has also been reported that there is an increased lipid peroxidation and decreased antioxidant potential in the kidneys of rats supplemented with a calculi-producing diet (Sumathi et al., 1993; Saravanan et al., 1995). Elevated serum levels of urea and creatinine indicates that there is a marked renal damage. However, the curative and prophylactic treatment with aqueous extract of *Hibiscus sabdariffa* causes diuresis and hastens the process of dissolving the preformed stones and prevention of new stone formation in the urinary system. The significant lowering of serum levels of accumulated waste products is attributed to the enhanced GFR and the anti-lipid peroxidative property of *Hibiscus sabdariffa*.

Histopathological investigations revealed the presence of polymorphic irregular calcium oxalate crystals in Lumina of tubules accompanied by edema and cast formation which caused dilation of proximal tubules along with interstitial inflammation in both the ethylene glycol induced urolithiatic groups. This might attribute to oxalate formation and also caused extensive intertubular hemorrhages and congestion of blood vessels. Atmani et al., (2004) have reported that crystal deposits were intensely birefringent, polycrystalline, and arranged in rosette characteristic of calcium oxalate crystals which proved adhesion and retention of pentides within renal tubules. These histological observations support the presence and growth of renal calculi in renal medulla region as observed in human urolithiasis. Supplementation of aqueous extract of *Hibiscus sabdariffa* at the doses of 250 and 500 mg/kg body weight revealed the presence of
moderate to few crystals along with mild appearance of edema and dilation in tubules, and crystals were present focally indicating the ability of aqueous extract of *Hibiscus sabdariffa* to dissolve the pre-formed stones to some extent. Similarly, when anti-ureolithiatic drug, cystone or *Hibiscus sabdariffa* extract was administered at the dose of 750 mg/kg body weight, no crystals were observed in both the cases indicating the ability of extract of *Hibiscus sabdariffa* to dissolve pre-formed stones to a greater extent as that of cystone. These histological studies support the calcium oxalate deposition data in kidneys by ethylene glycol induction and its treatment by supplementation with aqueous extract of *Hibiscus sabdariffa*.

*In vivo* mammalian test is widely used for monitoring genotoxicity of chemicals and plant extracts because of many advantages such as absorption, pharmacokinetics, tissue distribution, metabolism, age or sex effects and species specificity. In the present study, genotoxicity testing of the medicinal plant extract of *Hibiscus sabdariffa* was carried out using bone marrow cells of albino rat.

A rapid method for qualitative assessment of chromosomal breakage has been proposed as a method of screening chemicals for mutagenicity (Heddle, 1973; Matter & Schmid, 1971). This method depends upon the scoring of the micronuclei which arise from those chromosomal fragments that are not incorporated daughter nuclei, about 20% of the total (Carrano & Heddle, 1973) i.e., micronucleus originates from chromatin which for different reasons has been lagging in anaphase. Such micronuclei are quite distinct, easily scored structures that persist in the cytoplasm of cells and thus can be scored at any stage.
of the cell cycle. Under appropriate test conditions measurement of the frequency of newly formed micronucleated erythrocytes in the bone marrow provides a convenient index of chromosomal damage in nucleated erythrocyte precursor cells.

Bone marrow micronucleus (BMM) test is highly specific for clastogenic effects but its sensitivity is determined to a great extent by the substance tested, particularly by their metabolism. Some compounds such as unstable mutagens or those which generate short-lived metabolites are not detected in this test because the metabolites produced in liver do not reach the bone marrow. Bone marrow, the commonly studied target organ has a low drug metabolizing capacity. As a result, some drug such as those with electrophilic metabolites which combine locally with DNA or which generate short-lived metabolites, gave negative results in the BMM test. The bone marrow micronucleus test is one of the least expensive in vivo assays for genotoxic effects. The results are suitable for an initial screening. However, its application is not wide since only chromosome breaks or laggards can be scored and not even the nature or types of break. Therefore, analysis of traditional chromosomal aberrations was employed in conjunction with BMM test for comprehensive results of mutagenicity testing.

The use of herbal medicine is an old age practice in north-east India. Use of extracts from various plant sources such as trees, herbs and shrubs were mentioned in Ayurveda. Many plants contain different entities which may be mutagenic or carcinogenic and also which may be anti-mutagenic or anti-carcinogenic (Nandi et al., 1998).
Results of cytogenetic investigations demonstrated that aqueous extract of *Hibiscus sabdariffa* did not produce any type of toxic effects in bone marrow cells at the doses administered as the chromosomal aberrations occurred were minimal and thus, it can be said that no significant aberrations were observed in the chromosomes of experimental rats. *Hibiscus sabdariffa* contains several beneficial compounds (Zarrabal *et al.*, 2005) which contribute to the overall biological activity of the extract. The best response was observed in experimental rats administered with *Hibiscus sabdariffa* extract at a dose of 750 mg/kg body weight. Possible reasons for this may be related to the phenolic contents in *Hibiscus sabdariffa* which have the ability to complex with proteins. The present results thus indicate that the plant, *Hibiscus sabdariffa* is not genotoxic and is safe for consumption for treating urolithiasis.
REFERENCES


Carrol SW (1952). Tables for Convenient Calculation of median effect dose (LD₅₀ or ED₅₀) and instruction in their use. Biometrics. pp. 249-263.


