Effect of a Combination of *Piper cubeba* L.f and *Cyperus rotundus* L. in Experimentally Induced Urolithiasis in Rats

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Abstract

The present study was undertaken to investigate the effect of a combination of Kabab Chini *(Piper cubeba*) and Sad Kufi *(Cyperus rotundus*) in urolithiatic rats. Thirty male Sprague Dawley rats divided into 5 groups of 6 animals each were used. Group I served as control and received regular rat food and drinking water *ad libitum*. The animals of group II to V were treated with Ethylene glycol (EG) 0.75% (V/V) and ammonium chloride (AC) 1% (W/V) by adding in their drinking water for 7 days to induce urolithiasis. The animals of group II were sacrificed just after 7 days of administration of EG and AC and served as Positive control A, while group III was left untreated till 14 days, and served as Positive control B. The animals of group IV were treated with Cystone (750 mg/kg) and served as Standard control, while the animals of group V were treated with hydro-alcoholic extract of the combination of *Piper cubeba* and *Cyperus rotundus* (135mg/kg) and served as Test group. The treatment continued further for next14 days. At the end of treatment all the animals were sacrificed after 24 hrs of fasting. Highly significant calcium oxalate stones were found in renal tissue as well as in the urine along with increase in urine and serum Calcium, Chloride, Sodium, Phosphorus and creatinine. Test drug significantly decreased the quantity of calcium oxalate deposited in the kidneys and also reverted all the biochemical changes induced by calcium oxalate. The effect of the test drug was found to be comparable to the standard drug Cystone. The study demonstrated a significant lithotriptic effect produced by the combination of *Piper cubeba* and *Cyperus rotunda*.

Keywords: *Kabab Chini, Sad Kufi,* Calcium oxalate, Ethylene glycol, Ammonium chloride, Lithotriptic.

Introduction

Urolithiasis is a common disease worldwide leaving no age, cultural, geographical or racial group spared (Moe, 2006). Calcium oxalate stones are most common occurring in more than 80% of urolithiatic patients. They are found either alone or mixed with calcium phosphate (Tiselius, 2003). A number of medicinal plants are used as medicine for the treatment of urolithiasis in Unani system of medicine. Badiyan *(Foeniculum vulgare),* Barge Turb (leaves of *Raphanus sativus),* Duqoo *(Pencedanum grande),* Habbul qilt *(Dolichos biflorus),* Khar khask *(Tribulus terrestris),* Darchini *(Cinnamomum zeylanicum*), Muqil *(Commiphora mukul),* Parsiaoshan *(Adiantum capillus veneris)* etc. are some of the drugs used commonly as single drugs (Razi 2001; Ali, 2004; Qamri, 2008), while some of the

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formulations possessing antilithiatic activity include *Sharbate Aloo Baloo, Sharbate Buzoori, Kushta Hajrul Yahood, Majoon Aqrab* etc (Anonymous, 2007; Hussain, 1844). Among them only few have been evaluated on scientific parameters and shown to possess antilithiatic activity (Hadjzadeh *et al*., 2007; Ahmed *et al.,* 2013; Shah *et al.,* 2011; Hussain, 2010; Shah *et al.,* 2012).

Kabab Chini (KC) and *Sad Kufi* (SK) are two important drugs used commonly in Unani System of Medicine for antilithiatic activity. Both the drugs possess *mufattite hisat* (lithotriptic), *muhallil* (resolvent), *mufattehe sudad* (deobstruent) *and mudirre baul* (diuretic) properties (Ghani, YNM; Chughtai and Chughtai, YNM; Baitar, 2002), and have been investigated for various pharmacological activities. In a recently concluded study KC and SK have been shown to possess dose dependent antilithiatic activity (Humairah *et al*., 2014). In view of the attributed lithotriptic effect of these two drugs in Unani literature, and the findings of the recent study, the present study was undertaken to find out the synergistic effect, if any, between the two drugs in combination form in experimentally induced urolithiasis in male Sprague Dawley rats.

Materials and Methods

Animals

Male Sprague Dawley (SD) rats weighing 200-250 gm were procured from registered breeders. Prior to experiment the animals were allowed to get acclimatized for one week. They were maintained under standard laboratory conditions and provided standard diet and water *ad libitum* (unless stated otherwise). They were housed in clean polypropylene cages in a room, maintained at a temperature of 25±2^{0}C and humidity of 45-55% with 12 hour light and 12 hour dark cycle. The study was undertaken in the department of Ilmul Advia, National Institute of Unani Medicine (NIUM), Bangalore after getting the approval of Institutional Animal Ethics Committee (IAEC) of the institute vide registration no IAEC/IX/05/IA.

Chemicals and Reagents

Cystone ® manufactured by the Himalaya Drug Company was procured from the market. Ethylene glycol and gum acacia of analytical grade were purchased from NICE chemicals Pvt. Ltd., Kerala and Ammonium chloride from CDH Pvt. Ltd., New Delhi. Reagents used for Biochemical analysis, kidney homogenate preparation and for urinary parameters were Calcium (Arsenazo III method), Sodium (mono test) and Chloride Reagent set (mercuric Theocynaate method). These items were procured from Lab-care Diagnostics (India) Pvt. Ltd. Gujarat. Phosphorus (UV Molybdate method) was obtained from Pathozyme Diagnostic,

EURO diagnostic systems Pvt. Ltd. Chennai, India. Magnesium kit (Calmagite method) from Coral Clinical Systems, Uttarakhand, India and Urea kit (Urea kin-GLDH) was procured from Prism Diagnostics Pvt. Ltd. Thane, India. Creatinine (Modified Jaffe's method) was procured from AD, India, EURO diagnostic systems Pvt. Ltd.

Plant Material and Preparation of Extract

The fruit of KC was procured from the pharmacy of NIUM, Bangalore. The fresh rhizomes of SK were collected from Herbal garden of NIUM, Bangalore. The drugs were identified by a renowned Botanist. The voucher specimen was deposited in the herbarium of NIUM, Bangalore with reference number 15/IA/Res/2014.

The fruits of KC were kept in drying chamber at 40^0C for about 30 min to dry the moisture if any, and powdered coarsely in an electrical grinder. The rhizomes of SK were carefully washed and rinsed with tap water for at least 30 min. Dead parts were removed. Roots were separated from the rhizomes, and shade dried at room temperature of 28°C ± 1°C for 15 days. Dried rhizomes were pulverized in electric grinder in the form of coarse powder. The powder of both the drugs was extracted in Soxhlet's extractor separately with hydroalcoholic solvent (distilled water and ethanol 1: 1) for about 6 hours at a fixed temperature of 80^0C. The liquid extract was cooled and filtered by Whatman filter paper 40, the filtrate was then concentrated over steam bath (80^0C) till it dried. The resulting dark colour residue was collected and stored in refrigerator for further use.

Dosage of the Drug

The human therapeutic dose of KC and SK is 3 gm and 7 gm, respectively (Kabeeruddin 2007). The dose of the crude drug for Sprague Dawley rat was calculated by factor seven (Freirich et al., 1966) and found to be 350 mg/kg for KC and 816 mg/kg for SK. Since the hydroalcoholic extract of the test drugs was used so the dose of extract was calculated with respect to its yield percentage (10.06% for KC and 12.79% for SK). Thus the dose of extract was found to be 35 mg/kg for KC and 104.8 mg/kg for SK which was rounded off to 100 mg/kg. In order to know synergistic effect the calculated dose of the combination both the doses was taken in the study i.e. 135 mg/kg. The extract of test drug was reconstituted a fresh in water with 5% gum acacia, daily before the administration.

Ethylene Glycol and Ammonium Chloride Induced Urolithiasis

The experiment was carried out by the method of Fan *et al.*, 1999 and Aziz *et al.*, 2012 with some modification in the treatment schedule. Thirty male Sprague

Dawley rats divided into 5 groups of 6 animals each were used. Group I served as Plain control and received regular rat food and drinking water *ad libitum*. While the animals of group II to V were treated with Ethylene glycol 0.75% (V/V) and ammonium chloride 1% (W/V) added to their drinking water for 7 days to induce urolithiasis. All the animals received regular rat food. The animals of group II were sacrificed after 7 days of administration of ethylene glycol and ammonium chloride and served as Positive control A. While the animals in group III were left untreated after seven days administration of EG and AC till 14 days and served as Positive control B, thereafter they were sacrificed. From 8^{th} day the animals of group I received 1 ml of 5% gum acacia, while the animals of group IV were treated with Cystone in the dose of 750 mg/kg, and served as Standard control. The animals of group V were treated with hydroalcoholic extract of the test drug and served as Test group. The test and standard drugs were suspended in 5% gum acacia. The treatment continued further for next 14 days. At the end of treatment all the animals were kept on fasting for 24 hrs and sacrificed thereafter.

Urine Analysis

Urine analysis was done on 8^{th} day after administration of EG and AC and on 22^{nd} day after treatment with test drug. The animals of each group were placed singly in individual metabolic cage with water *ad libitum* for the collection of urine passed during 24 hours. The calcium oxalate crystals present in urine were identified and counted using the light microscope (40X) (Kaur *et al*., 2009). One ml of urine sample was centrifuged at 2500 rpm for 5 min (Dhaval and Arvind, 2013) and analyzed for presence of calcium, phosphorus, creatinine, sodium, chloride and magnesium by autoanalyser.

Serum Analysis

On 22^{nd} day rats were anaesthetized with Theopentone sodium (50 mg/kg IP). The abdomen was cut open and the blood samples were collected by cardiac puncture. Serum was separated by centrifugation at 10,000 rpm for 10 min (Dhaval and Arvind, 2013) which was analyzed for serum calcium, creatinine, urea and phosphorus.

Kidney Homogenate Analysis

After collection of blood both the kidneys were dissected out. Right kidneys from two animals of each group were sent for histopathological examination. While the left kidneys of all the rats were used for kidney homogenate analysis. Isolated left kidney was trimmed off from extraneous tissue then dried at 80^0C in a hot air oven for 30 min. A sample of 100 mg of the dried kidney was boiled in 10 ml of 1 N hydrochloric acid for 30 min and homogenized using a tissue homogeniser.

The homogenate was centrifuged at 2,000 x g for 10 min (Dhaval and Arvind, 2013) and 6 ml of supernatant was discarded. Remaining supernatant was used for the analysis of calcium and phosphorus content.

Statistical Analysis

The data was analyzed using Graph pad software. ANOVA repeated measure with Tukey-Kramer multiple pair comparison test was applied for the parameters which passed normality test.

Results

Urine Analysis

In all groups after 7 days administration of EG and AC a significant increase ($p<0.001$) in number of calcium oxalate crystals was found when compared with plain control. On intra group comparison of the findings of 22nd day from 8th day, significant reduction ($p<0.001$) was noted in all the groups. The same level of reduction ($p<0.001$) was observed in the test groups on 22nd day when compared with positive control B. The number of crystals in test group after 14 days treatment with test drug was found almost equal to plain control on 8th day and 22nd day (Table 1, Fig. 1-6). In EG and AC treated groups urine calcium and phosphorus increased while creatinine and magnesium level decreased significantly. On 22nd day after treatment with test drug Ca decreased significantly ($p<0.05$). Urine Phosphorus also decreased significantly but magnesium level increased significantly ($p<0.001$) (Table 2).

Table 1: Effect of the test drug on calcium oxalate crystals in urine in "Ethylene glycol-Ammonium chloride induced Urolithiasis" in Rats

Groups	Drug & dose	No. of CaOx crystals	
		8th day	22nd day
Plain control	1 ml of 5% GA	7.5 ± 0.88	6.66 ± 0.66
Positive control A	EG 0.75% and AC1%	131.7 ± 10.93^{a***}	–
Positive control B	EG 0.75% and AC1%	130.8 ± 10.83 ^{a***}	92.50 ± 8.24 ^{a***}
Standard control	Cystone 750 mg/kg	132.7 ± 16.98 ^{a***}	8.33 ± 1.87 ^{b***c***}
Test group	combination of KC & SK 135 mg/kg	124.2 ± 18.09 ^{a***}	6.00 ± 0.68 ^{b***} ^{c***}

***p<0.001 **a**- compared with plain control, **b**-compared with Positive control A, **c**- compared with Positive control B. n=6

CaOx crystals seen in urine under light microscopy (40X)

Fig. 1: Plain control on 8th day

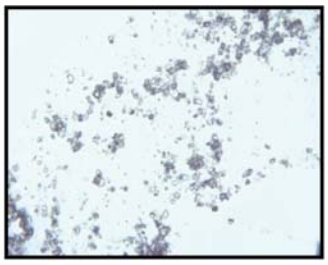


Fig. 2: Positive control A on 8th day

Fig. 3: Positive control B on 22nd day

Fig. 4: Standard control on 22nd day

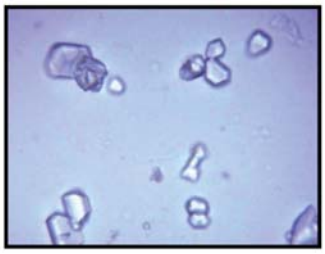


Fig. 5: Test group on 8th day

Fig. 6: Test group on 22nd day

Serum Analysis

It was found that concentration of serum creatinine and urea decreased significantly ($p<0.01$) and a significant reduction was observed in serum calcium ($p<0.001$). No significant change was observed in the serum phosphorus level (Table 3).

Kidney Homogenate Analysis

Calcium and phosphorus were found significantly increased in urolithiatic group. Phosphorus decreased significantly ($p<0.01$) in test group but no significant change was found in calcium (Table 4).

Histopathological Findings of Kidney

In urolithiatic group kidney architecture was found intact with mild increased hypercellularity in Bowman's space. Some tubules have shown irregular homogenous amorphous eosinophilic material. Few uncongested blood vessels were found with increase in mesengial cells and congested capillaries. Dilated and congested blood vessels were observed. Mononuclear inflammatory infiltration was found in interstitium. Changes reverted to the normal level in test group which were almost similar to the findings plain control (Fig. 8-12). On examination with polarized light, intratubular and interstitial crystal deposition was observed in positive controls. But in test and standard groups such type of deposition was not seen (Fig. 7).

Table 2: Effect of the test drug on urinary parameters in "Ethylene glycol-Ammonium chloride induced Urolithiasis" in Rats

Groups	Drug & Dose	Ca (mg/dl)	Creatinine (mg/dl)	P (mg/dl)	Na (mEq/dl)	Cl (mg/dl)	Mg (mg/dl)
Plain control	1 ml of 5% GA	1.568 ± 0.171	0.8798 ± 0.221	63.28 ± 1.53	141.9 ± 10.19	131.6 ± 14.84	1.502 ± 0.28
Positive control A	EG 0.75% and AC1%	5.806 ± 0.973^{a***}	0.1912 ± 0.138^{a*}	73.47 ± 0.86^{a***}	210.2 ± 21.05^{a**}	146.1 ± 6.66	0.371 ± 0.12^{a**}
Positive control B	EG 0.75% and AC1%	8.067 ± 0.206^{a***}	0.1657 ± 0.148^{a*}	70.88 ± 0.32^{a***}	182.8 ± 10.89	147.7 ± 17.17	0.446 ± 0.10 ^{a**}
Standard control	Cystone 750 mg/kg	1.824 ± 0.251$^{e*b*** c***}$	0.8193 ± 0.033^{b*c*}	69.71 ± 0.43 ^{b*}	142.4 ± 9.36 ^{b**}	133.8 ± 6.73	1.423 ± 0.12 ^{b**c*}
Test group	Combination of KC & SK 135 mg/kg	1.834 ± 0.189$^{b***e* c***}$	0.8185 ± 0.149	71.04 ± 0.346 ^{b*}	199.6 ± 27.14 ^{b**}	134.0 ± 11.39	1.563 ± 0.19 ^{c**b***}

*p<0.05, ** p<0.01, ***p<0.001 **a**- compared with plain control, **b**-compared with Positive control A, **c**- compared with Positive control B, **e**-compared with standard control. n=6

Table 3: Effect of the test drug on serum parameters in "Ethylene glycol and Ammonium chloride induced Urolithiasis" in Rats

Groups	Drug & Dose	Ca (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	P (mg/dl)
Plain control	1 ml of 5% GA	8.05 ± 0.415	1.31 ± 0.129	40.26 ± 3.510	2.14 ± 0.320
Positive control A	EG 0.75% and AC1%	10.40 ± 0.654^{a**}	3.05 ± 0.484 ^{a**}	53.65 ± 5.354	3.42 ± 0.453
Positive control B	EG 0.75% and AC1%	9.06 ± 0.888^{b*}	3.02 ± 0.603^{a**}	72.15 ± 9.827^{a**}	3.18 ± 0.355
Standard control	Cystone 750 mg/kg	8.35 ± 0.485^{b*}	1.39 ± 0.047^{b**c**}	42.80 ± 4.354^{c**}	2.41 ± 0.307
Test group	combination of KC & SK 135 mg/kg	7.949 ± 0.376^{b**}	1.404 ± 0.1093^{b**c**}	42.59 ± 2.55^{c**}	2.247 ± 0.214

*p<0.05, ** p<0.01 **a**- compared with Plain control, **b**-compared with Positive control A, **c**- compared with Positive control B. n=6

Table 4: Effect of the test drug on kidney homogenate in "Ethylene glycol Ammonium chloride induced Urolithiasis" in Rats

Groups	Drug and dose	Kidney homogenate analysis	
		Ca (mg/100 gm)	P (mg/100 gm)
Plain control	1 ml of 5% GA	0.834 ± 0.391	2.224 ± 0.348
Positive control A	EG 0.75% and AC1%	3.333 ± 1.037^{a*}	4.290 ± 0.294^{a*}
Positive control B	EG 0.75% and AC1%	2.115 ± 0.583	3.693 ± 0.609
Standard control	Cystone 750 mg/kg	1.054 ± 0.189	2.328 ± 0.318^{b*}
Test group	combination of KC and SK 135 mg/kg	0.9902 ± 0.155	2.058 ± 0.169^{b**}

*p<0.05, **p<0.01

a- compared with Plain_control, **b**-compared with Positive control A, n=6

Discussion

All the rats treated with EG and AC for 7 days developed calcium oxalate crystals in urine which is evident by the presence of crystals in urine microscopy on 8th day (Table 1, Fig. 1-6).Similar findings have been reported by other workers (Rathod et *al.*, 2012; Fan *et al.*, 1999; Divakar *et al.*, 2010). Further it has been

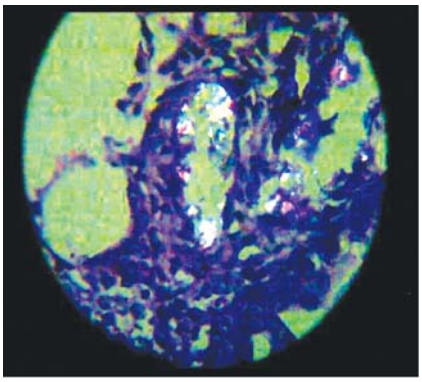


Figure 7: Calcium oxalate crystal deposition in rat kidney tissue seen under polarized microscope (40X) on 8th day

Histopathological reports of rat kidney under light microscopy (400X)

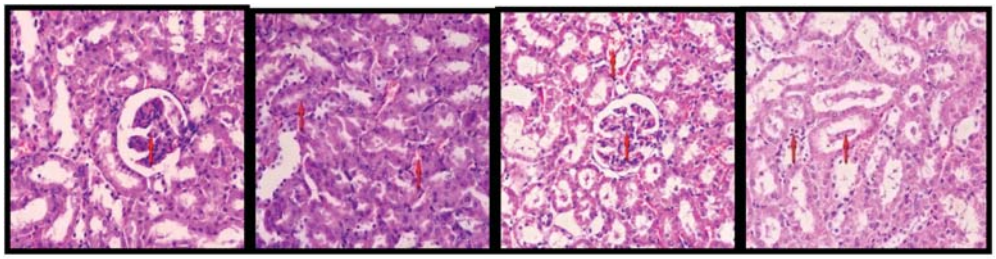


Fig. 8: (a) Plain control **8: (b)** Plain control **Fig. 9: (a)** Positive control A **Fig. 9: (b)** Positive control A

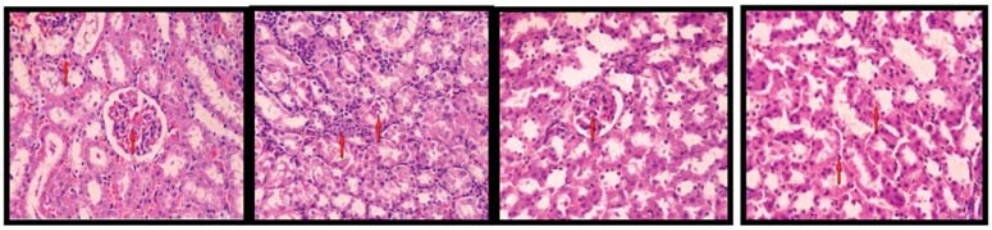


Fig. 10: (a) Positive control B: **(b)** Positive control B **Fig. 11: (a)** Standard control **11: (b)** Standard control

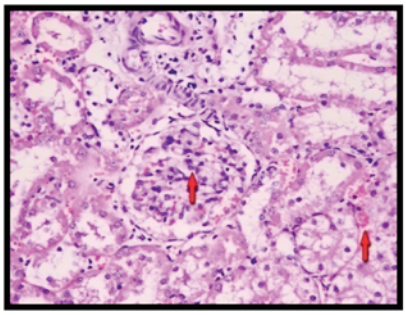
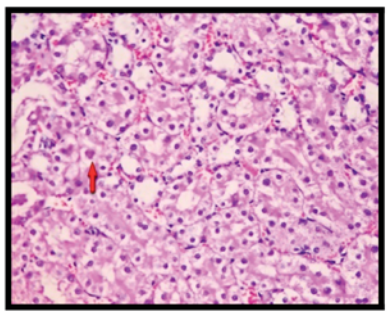


Fig. 12: (a) Test group



12: (b) Test group

reported that EG being oxidized into oxalic acid by non specific dehydrogenase leads to hyperoxalaurea which is considered to be a factor in the causation of lithiasis. EG metabolizes into CaOx monohydrate and produces renal mitochondrial

toxicity similar to clinical CaOx renal calculi (Mc Martin and Wallace, 1984). Renal function was assessed at the end of the study by estimating urinary and serum parameters.

In positive control A (treated with EG and AC for 7 days) and B (left untreated for 14 days after 7 days of treatment with EG and AC) urinary calcium was found increased significantly ($p<0.001$) when compared with plain control. It was also reported in previous studies that EG causes hypercalciuria, hyperphosphaturia and hyperoxalaurea. The increased urinary calcium is a factor favoring the nucleation and precipitation of calcium oxalate from urine and subsequently crystal growth (Lemann *et al*., 1991). Reduction in urinary calcium also reduces super saturation which is main risk factor for stone formation (Dadametirkee *et al*., 2007). Increased excretion of phosphorus has also been reported in stone formers and hyperoxaluric rats ((Rathod *et al*., 2012). After 14 days treatment with test drugs the phosphorus excretion was found to be decreased (although it was not found significant). Urinary sodium was found to be significantly ($p<0.01$) increased in positive control A when compared with plain control, while in positive control B, no significant elevation was observed. A significant reduction ($p<0.01$) was also noted in test and standard groups. The result showed that KC and SK in combined form were comparable with standard drug in reducing super saturation by decreasing the sodium concentration in urine (Table 2).

No significant difference in urinary chloride was observed when test group was compared with positive control groups. Urinary magnesium decreased significantly ($p<0.01$) in positive control A and B. Magnesium is considered an important inhibitor in stone formation ((Divakar *et al.,* 2010) and low level of magnesium has been noted in stone formers as well as stone forming rats (Soundarajan *et al.,* 2006; Rushton *et al.,*1980). In test group a significant elevation ($p<0.001$) in magnesium concentration was observed when compared with positive control A. The results were found even better than the standard drug. The positive control B however showed some elevation in magnesium level but it was less than the test group (Table 2).

Treatment with test drug significantly reduced calcium oxalate in urinary excretion, thus reducing the super saturation of urine. This might be responsible for preventing the formation and dissolving the preformed calcium oxalate type of stones by decreasing calcium and increasing the magnesium level in urine (Akanae *et al.,* 2010). An increase in the concentration of creatinine and urea are the markers of kidney and tubular damage. Glomerular filtration rate is decreased due to injury in kidney tissue. This decrease may also be due to the obstruction in urinary systems because of the stones. Due to this, the waste products, particularly nitrogenous substances may increase (Patel *et al*., 2012). Test drug showed significant decrease in serum creatinine and urea. Serum calcium was observed

to be decreased in test group; although it was non-significant, still it suggested that Test drug improved kidney tissue Ca disruption more than serum Ca.

In histopathological study of the kidneys, cellular derangement, dilated and congested blood vessels, hypercellularity in Bowman's capsule, mononuclear inflammatory infiltration and injured glomerulus were found in positive control groups. While in test drug treated group minimal changes were observed.

The combination of test drugs showed more pronounced effect in comparison to single drug (Humairah *et al*., 2014), indicating synergistic effect of the two test drugs of the combination. The effect of test drug was found to be comparable to the standard drug Cystone. On inter group comparison between test and standard groups no significant difference was observed, thus it can be concluded that the test and standard drugs are equally effective.

Exposure to high level of oxalate and calcium oxalate crystals has been reported to produce cellular injury mediated by membrane lipid peroxidation through intra cellular reactive oxygen species generation. Therefore, a reduction in renal oxidative stress could be one of the effective measures for the treatment of urolithiasis. It has been reported that flavonoids found in some of the plant drugs decreased oxidative injury in renal tubular cells and calcium oxalate deposition in rat kidney (Jeong *et al*., 2006; Akanae *et al*., 2010). SK has been reported to contain ample amount of flavonoid (Shamkuwar *et al*., 2012). This constituent along with certain other constituents such as saponins and alkaloid in SK and few alkaloids in KC (Parmar, 1997) may have exerted anti oxidant and thereby protective effect (Rathod *et al*., 2012). Stones are formed due to deficit in the crystallization inhibitory effect of urine and the presence of promoters. When these conditions favor stone formation the anti adherent layer of GAGs acts as a protective barrier against urinary stone disease. If this layer is damaged due to consequence of bacterial attack, a stone nucleus develops leading to full stone formation in urinary tract. At this point the drugs having anti microbial property may be effective (Akanae, 2010). The antimicrobial property of test drugs has already been reported (Aneja *et al*., 2010; Ahmad et al, 2012) therefore it may be considered as one of the possible mechanisms that the test drug may have evolved as a part of its action. According to Unani concept kidney disease occurs due to change in *mizaj* of kidney or a disturbance in the equilibrium of any of the five *Quwa* (faculties) i.e. *Quwwate Hazima* (digestive power), *Quwwate Jaziba* (absorptive power), *Quwwate Masika* (retentive power) and *Quwwate Dafiah* (evacuating power) and *Quwwate Mumayyizah* (separating power) the kidney is conferred with. In such a condition along with other forms of failure of function removal of viscous and morbid matters become difficult and these matters get entangled in some parts of kidney (Majoosi, 2010). The *hararat* of the kidney dries out these entangled morbid matters, which gradually convert into stone (Razi,

2001; Qamri, 2008; Ibn Sina, 2007). For proper excretion of waste products the *quwa* and the *mizaj* should be in *aitedal* (equilibrium) and *akhlaat* should have the normal viscosity. Therefore the drugs which are relatively less *har* are used to help dissolve the *ghaleez lesdar khilt* (viscid and sticky matter). The two ingredients of test drug are *har* and *yabis* in second degree (Ghani, ynm; Kabiruddin, 2007) and have *mulattif, mufattehe sudad, muhallil* and *mufattite hissat* properties (Ghani, ynm). By virtue of these actions the drugs may have transformed the *ghaleez* matter into *lateef* and made it easy to excrete.

However, since the two components of the test drug have been reported to possess different pharmacological effect, therefore it is likely that the lithotriptic effect was mediated through different mechanisms. Further, the two components appear to produce synergistic effect because the degree of response exerted by the combination was more than the reported effect of the two ingredients individually.

Conclusion

On the basis of results, it can be concluded that test the drug possesses significant lithotriptic effect probably mediated through diverse mechanisms. The ingredients of the test drug have synergistic effect because the combination produced more pronounced effect as compared to the effect reported in respect of its individual ingredients.

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