DISCUSSION
Urolithiasis (kidney stone) is a problem that has confronted clinicians since the time of Hippocrates (Andrew and Chandru., 2001). There is also recorded evidence a number of years ago of calcium, magnesium, oxalates, phosphates and uric acid stones in humans (Kassimi et al., 1986).

Stones in kidney are formed because of a complicated interaction of biological events that are most likely triggered by genetic susceptibility coupled with dietary factors. The process is not completely understood. Some of the factors include super-saturation of urine, deficiencies in protective factors against stone formation, changes in the acidity of the urine and factors that bind crystals to the kidney tubule and bacterial infection (http://www.afud.org; http://www.kidney.org/: Parks and Coe, 1996).

In most cases, presence of kidney stones is asymptomatic, generally detected during Masters Health Check-up or during ultra sonogram of stomach or pelvis on patients during investigation of a different problem. When stones are lodged in the ureter (the thin tubes between the bladder and the kidney) the symptoms can be very severe. Pain often usually begins abruptly on one side and then gets intense depending on the stone's location. There is considerable discomfort usually while standing, sitting, pacing or reclining or in the search in a vain for a position that will bring relief. Pain in the flank area, nausea and vomiting may occur coupled with bleeding, frequent urination with burning sensation with gastrointestinal symptoms, chills, fever, and blood in urine. Anatomic
abnormalities in the urinary tract, inherited factors, lifestyle factors (specific foods, weight considerations, stress, and sleep position), medical conditions (being bed ridden, high blood pressure, gout inflammatory bowel disease, urinary tract infection and hyperparathyroidism) kidney disease, chronic diarrhea, certain cancers eg., leukemia and lymphomas, and sarcoidosis are some of the clinical features of kidney stone (Pak 1987, 1999; http://www.asn-online.com). There is a specific relationship between stone locations to symptoms (Andrew et al., 1998).

The present biomedical treatment however involves mainly surgical removal of stones. The different treatment modalities for renal and ureteral calculi have its own complications viz., extracorporeal shock wave lithotripsy (ureteral obstruction by stone fragments prenephric hematoma), uretreoscopy (ureteral stricture of injury), uretreorenoscopy (ureteral stricture of injury), and percutaneous nephrolithotomy (bleeding injury to adjacent stricture (Andrew et al., 1998). The long term shock wave lithotripsy significantly carry higher risk of diabetes and high blood pressure years later (Krambeck, 2006).

There are several medicines in the form of chemicals and formulations cited which purportedly dissolve existing stones or prevent new stone formation (Atmani et al., 2003; Grover and Yadav, 2004; Koide et al., 1995; Ackermann 1997; Varalakshmi et al., 1990; Compos and Schor 1999; Kirdpon et al., 1994; Borisov et al., 2004; Premgamone et al., 2001). However evidence based
clinical trials to prove their claims is unfortunately absent. Ortho and alkaline phosphates, methylene blue, and magnesium oxide have been quoted to decrease the calcium crystal aggregation in kidneys (Victor, 1999). Allopurinol may prevent the concentration of uric acid in the urine. These claims have also not been put through extensive scientific rigor. Side effects including the perinephric bleeding of kidneys (Schafer, 1999; Knorr and Woodside, 1990) and peptic ulcers have however been recorded.

Several medicines from plant, mineral, or mixtures have been cited in a number of publications of Ayurveda, Siddha, Unani and Homeopathy systems (Lev and Dolev, 2002; Subramanian and Madhavan, 1984: Nambiraj et al., 2002; Gogtay et al., 2002). However these medicines have also not been validated scientifically through clinical trials, making it difficult for biomedical physicians to prescribe the formulations. Also many such formulations contain minerals and metals. Many single-plant drugs and their extracts that were published showed activities against animal models only. It is obvious that the drugs proved efficacious in animals need not show efficacious in humans. The concentrations employed by these researchers are very small and the number of animals used was also few in many of the studies. The physicians therefore desist recommending to the patients such medicines and the patients themselves also wish to refrain from taking them (Mathur and Ramamurthi, 2003).
Dalmia Centre for Research and Development (DCRD) had developed a formulation, DCBT5678 for the treatment of kidney stones with five plant extracts, inspired from literature and usage. The five medicinal plants are available abundantly and can be cultivated easily. The plants used by DCBT5678 formulation has also been found to be considerably mentioned in ancient literature in the treatment of several diseases including kidney stones (Chopra, 1982; Nadkarni, 1976; Satyavati et al., 1976; Asima and Satyesh, 1994; Rastogi and Mehrotra, 1993).

Currently, there is awareness on the quality of the raw material used in the preparation of various herbal and Ayurvedic formulations particularly for export out of India. The quality of the final product is determined through the input of the raw materials and the process of manufacturing. There have been numerous instances when the wrong plant has been used in formulations. It is therefore very important that plants are correctly identified before their parts are used. Plants used in DCBT5678 formulation have always been sent for authentication by Botanical Survey of India (Coimbatore) which has the second largest herbarium collection in India.

The respective plant part of each fresh plant were collected from natural resources and shade dried at room temperature (30 ± 2 °C), with <50% humidity. They were cut in to uniform size of 2mm before extraction with the specific
solvent (70 percent ethanol) for the drug preparation and for generating fingerprint profiles.

Also as per present day requirement, the plant material collected was analyzed for heavy metals like mercury, lead, chromium, and arsenic. The presence of heavy metals in Ayurvedic formulation, which are sold as food supplement in the United States of America, has led to a furor in scientific communities and the public have in recent times become a bit weary on the safety of such drugs, which have not undergone a basic heavy metal testing for inadvertent presence.

Recently, the Journal of American Medical Association has published a research article on the presence of heavy metals in alarming amounts in popular Ayurvedic medicines, manufactured by leading pharmaceutical companies in India and warned against using them (Saper et al., 2004).

To avoid inadvertent contamination of heavy metals, several countries have made mandatory testing of all herbal products that are sold in their respective markets. But, Ayurveda attributed important therapeutic roles to metals such as mercury and lead (Gogtay et al., 2003; Acharya and Joshi, 1998) where there is a deliberate introduction of these metals in their preparations through scientific processing. It is estimated that 35-40% of the approximately 6000 medicines in the Ayurvedic formulary intentionally contain at least one metal (Gogtay et al., 2003). Ayurvedic drug manufactures and physicians are assuring that metal containing HMPs are purportedly detoxified through multiple heating-cooling
cycles and the addition of specific herbs (Acharya and Joshi, 1998; Patwardhan et al., 2004).

The heavy metals like Hg, Cr, Pb, Cd and As in the plant parts used in DCBT5678 formulation were not detectable and well below the threshold limits as specified by World Health Organization (WHO, 1998) and the levels as described for the infant foods specified by Food Adulteration Act of Government of India (Prevention of Food Adulteration Act, 1954). No detectable quantities of major pesticides of organo chlorine and organo phosphorous were found, that indicated that these plant parts are safe to use as drug for human consumption.

Dalmia Center for Research and Development had earlier conducted a limited toxicology of the drug DCBT5678 on Wister rats and the LD$_{50}$ was found that there was no toxicity to animals even at dosages over 5000 mg/kg. No adverse symptoms were noticed. It was therefore possible to take the formulation to humans.

For the development of quality systems for an herbal drug, a standard operating procedure with reliable methods of quality control and standardization is of great importance. Screening natural product extracts for new biologically active compounds is increasing due to the high throughput screening methods, which will not only help to screen the compounds but also proper validation of the formulation for effective drug discovery. Internationally reliable methods of
quality control and development of standardization parameters is of great importance.

Polyherbal formulation is a biosynthetic laboratory for chemical compounds like polyphenols, glycosides, and alkaloids. These compounds exert physiological and therapeutic effect on human beings. With acceptance of alternative and complementary medicines, polyherbal medicine is making dramatic comeback (Singh, 2005; Etkin, 1990). But a quality standardization of poly herbal formulation is difficult. Individual plants and their extracts used in the formulation need to be consistent while making every batches of drug preparation. At least 1 or 2 biological markers for each of the ingredient used in the formulation to be followed beginning from the purchase of raw material to the storage stability of the final product. Quantification of these products through different chromatographical equipments like TLC, HPLC, HPTLC, GC, and GC-MS would be immensely helpful.

In this study, a basic HPTLC profile for each of the plant part used in the DCBT5678 formulation was standardized and fingerprint profiles of the compounds were generated for each of the extract (Chromatogram 1, 2 and 3). Marker compounds are characteristic phytochemicals found in a plant. They are often chosen to represent the standard for standardized extract, serve a useful function in terms of quality, such as the purpose of identification and ensuring appropriate drying, handling, and extraction of the herbal starting material.
However, these marker compounds are not necessarily been active compounds, as in *Ginkgo biloba* and St. John's wort (Butterweck *et al.*, 2003, 2000, 1998 and 1997) where the marker compounds are different from active constituents. If a marker compound is chosen which has no known useful pharmacological activity, it is advisable not to optimize this at the expense of other phytochemicals in the extract as suggested by Bone (2004).

Elaborate search in internet data bases like Medline, Pubmed, Cochrane Library and Embase, from inception to 2007 with search terms "kidney stone", "urolithiasis" and the name of the plant used in the study with or without key word "Clinical trial" were searched, yielding no major results of the plants' phytochemical profile or efficacy in kidney stones. The hydro alcoholic extract of DCBT5678 plant materials had proved efficacious in the earlier studies, and each plant material was extracted with 70: 30 ethyl alcohol : water extraction and the extracts were condensed yielding approximately 50 gm dry weight per 50 ml concentrate. Finger print profiles of these plants were prepared individually, starting with a less polar mobile phase, then with a medium and further with a highly polar mobile phase on Silica gel 60 F<sub>254</sub> pre-coated plates using HPTLC.

From the results it was observed that most of the compounds from the extracts of TT and BMS could be separated in the mobile phase of ethyl acetate : methanol : water (10 : 1.35 : 1) but major portions of the extracts of BM, BP and EM were left behind in the loading point itself (Chromatogram 1). Two higher
polar mobile phases, chloroform : glacial acetic acid : water (10 : 2.2 : 1.1 : 2.6) were employed in the separation of many compounds of BM, BP and EM (Chromatogram 3), successfully suggesting these compounds were polar. This characteristic finger print profiles of each plant was recorded and used as a standard for quality check in the different batches. The profiles of each extract were visualized in UV 254 nm and UV 366 nm. The \( R_f \) values are depicted in Table – 4. It has been known through observation for centuries that herbs harvested from different habitats, at different times of the growing cycle and even at different time of the day show a different therapeutic effect. Modern research has shown that these old findings about the right location and the right time to harvest an herb are indeed correct (http://www.herbalmedicine.co.nz). The traditional Materia Medica gives specific advice when an herb needs to be harvested for optimum therapeutic effectiveness and this has been practiced by the physicians. In St. John’s wort, \textit{Hypericum perforatum}, mid-summer collection was the best as its growing cycle is at peak and the phytochemicals profile showed high level of both hypericin and hyperforin, ensuring maximum therapeutic efficacy. The flower heads of St. John’s wort are gathered in the late morning until early afternoon as the time when the majority of plant juice is in the aerial parts. It is also important to avoid the morning and evening dew as plants with too much moisture are in danger of becoming mouldy and may be spoilt (http://www.herbalmedicine.co.nz).
Herbs contain a wide variety of phytochemicals which act together to achieve a desired therapeutic result. In the chemical approach of standardization, more and more active constituents in the plant species are discovered all the time, making standardization against all of them a chemist’s nightmare. A different approach was attempted in this study to understand the nature of the compounds in the extracts of the plants of DCBT5678 at different photoperiods in a day. Each plant material collected in different time was extracted with hydro-alcohol (30:70) several times and the pooled extracts were used for the separation of compounds in HPTLC silica gel 60 F254 plates. Interesting results, showing different concentration of several compounds in each of the plant used in DCBT5678 in different photoperiods (morning, noon, and night) were observed. Some compounds found in the morning where even absent in noon or in the night and some other compounds where found higher in night than in other two periods suggesting the necessity of quality control of these plants when used as drug.

Based on the presence of the quantity of compounds in different photoperiods as shown in Chromatograms 4, 5, 6, 7 and 8, B. monosperma flowers need to be collected in the noon, B. pinnatum leaves and flowers of B. malabaricum to be collected in the late evening, and E. microphylla plants need to be collected in morning. T. terrestris fruits can be collected at any time as most of the compounds were found to be same in all the period studied. This became the
standard for subsequent preparations and was used while the drugs were manufactured at every time during this study.

An important part of quality control of herbal preparations is the evaluation of the chemical stability of a finished product during the storage period. Measuring chemical stability is very challenging due to the complexity of a plant extract, which may contain thousands of different compounds and the presence of enzymes (glycosidases, esterases, or oxidases) also play an important role in the breakdown of secondary plant metabolites. The concomitant compounds like organic acids which enhances stability of phenolic compounds may influence the overall stability of the herbal formulation (Gafner and Bergeron, 2005; Jayesh, 2003 and Hamischfeger, 2005).

In this study also, the stability of the drug was analyzed for 3 years with sample drawn on 0, 6, 12, 24, and 36 months. From the results, it was known that there was no deterioration of any of the compounds suggesting that the drug was stable for at least 3 years. Such an exercise was not observed or practiced by any of the herbal or Ayurvedic or Siddha drug manufacturers.

Stability of a drug provides the evidence on how the quality of an active substance or medicinal produce varies with time under the influence of a variety of environmental factors such as temperature, humidity, light and enables recommended storage conditions and shelf life (Carstensen et al., 2000). The purpose of stability testing is to provide evidence on how the quality of an active
substance or finished product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light and to establish a re-test period for the active substance or a shelf life for the finished product. The stability tests envisage the manufacturer to store the product under strict storage conditions. Information on the stability on the active substance is an integral part of the systematic approach to stability evaluation in many of the synthetic pharmaceutical biomedical drugs.

Studies on the stability of the drug formulation is also mandatory for all drug manufactured under GMP and so the drug, DCBT5678 prepare for the clinical trial was also undergone the stability study for a period of three years. The samples were kept in room temperature (30 ± 1°C), drawn at regular intervals, and analyzed in HPTLC.

Chromatograms 9 and 10 described the analysis of DCBT5678 for a period of 3 years storage. The formulated drug was stored in room temperature and the samples were drawn after 0, 6, 12, 24, and 36 months. The capsules were broken and the drug was dissolved in 70:30 ethyl alcohol and water. Individual plant extracts were run with the drawn drug samples. One compound (band) specific to one plant was followed in the plant and drug extract. Though the concentration of each plant extract loaded and the respective plant extract concentration present in the drug were same, there was significant difference in the peak area of the extracts of the drug stored in different time intervals and the
individual plant extract. All individual plant extract had higher compounds than in the drug formulation. Even the mixing of liquid extract led to the same result only suggesting some compounds may got transformed while mixing the extracts and so their mobility (polarity) in the particular mobile phase/stationary phase was changed. However, it is to be noted that in dry form, these extracts were stable for the three-year period tested.

The efficacy of a biomedical drug is generally ascertained through several phase studies as described by USFDA 2004. The toxicity of the drug is ascertained in the preclinical studies through animals (oral or intra venous or intra peritoneal) along with absorption, distribution, metabolism and excretion (ADME) studies. Then phase I study is practiced, followed by Phase 2 and 3 and the details are mentioned in USFDA 2004. However, such an elaborative practice is not necessary in the case of many of the herbal formulations, as they were used as medicines for centuries.

Before this study pilot study on ten patients for a period of 30 days was carried out earlier by the Dalmia Centre for Research and Development to ascertain efficacy of the formulation DCBT5678. Reductions in size of stones were observed in five patients. In three patients, the stones were eliminated from their kidneys and in two patients, no change in size or increase was noted (personal communication, not a part of this study). This study with a larger population of 40 patients was carried out with patients that were asymptomatic, having stones
in one or both kidneys. The demographic data suggested no major difference in dietary pattern or of patients consumed alcohol. Female patients were also recruited in this study but the ratio was 1:5 (Female : Male). This is possibly due to distribution as conformed by earlier studies on the population of kidney stone distribution (Menon et al., 1998 and Uribarri et al., 1989). Seven percent of the patients were smokers and 93% of the patients did not take any narcotic substances. Only 10% of the patients were obese having higher BMI. 36 patients completed the study. 4 patients showed complete 'dissolution' of stones and 12 patients showed reduction in the number or size of the stones (Table-5). However, a significant number (20) of patients did not respond to the drug (no increase or decrease in the stone numbers or size)

The concentration of serum urea, serum uric acid, and uric acid in the urine were found higher in the baseline than the normal values (Table-6). The mean blood and urine chemistry values of the patients presented interesting information that these patients exhibited a significant reduction in the serum urea and uric acid concentrations at the end of the study. 24h. urine collected also revealed that there was excess excretion of uric acid. However, the mean serum calcium, serum phosphate, urine calcium and urine phosphate values obtained at the end of the trial did not differ from the respective baseline values. The importance in analyzing the biochemistry of serum and urine in the patients has been researched by several authors (Curhan et al., 1993; Andrew and Chandru, 2001). The increased concentration of serum calcium, serum phosphate, serum uric acid and blood
urea observed in kidney stone patients have been described earlier (Kirdpon et al., 1994; Christina et al., 2002 and 2005; Premgamone et al., 2001; Campos and Schor, 1999;).

Based on the results obtained in this study, it was noted that the plant ingredients of DCBT5678 decreased the concentrations of urea in the blood and serum uric acid levels in the patients. This reduction in the serum led to the increased excretion of uric acid in the urine (Table 6). The physiology or the mechanism of action of these ingredients on the kidneys of the patients to alleviate the high concentrations of urea and uric acid from the blood and serum respectively is not known. The reduced concentrations of uric acid in the serum and increased excretion in the urine prompted to study the role of the ingredients of DCBT5678 in removing the uric acid stones in the patients.

So, yet another study on a small population of patients that were diagnosed only with uric acid stones in either or both the kidneys was initiated. Patients having the uric acid stone(s) could be visualized in ultrasound as opaque crystals that differentiated with other stone types. These patients also exerted pH less than 5.5. These two criteria were utilized in the study in selecting the uric acid stone bearers alone. But it was extremely difficult in recruiting the uric acid stone bearers only as a population was less as observed by Shekariz and Stoller (2002), were 2 to 7% of the population kidney stone bearers is found to have uric stones. This study also screened more than 100 patients to recruit
10 patients. Among the 10 patients selected, 3 patients had mixed stones with uric acid. The recruitment period took 6 months and the patients who had uric acid stones in one or both the kidneys were selected for the study. This trial had one female patient and nine male patients. The mean age of these patients were relatively low (36 years). All the patients were laborers only and one was working in air-conditioned environment. Ninety percent of the patients were non-vegetarians, 10% of them were smokers, and 90% of them did not use any narcotic substance. No patients were observed obese.

Two patients were lost to follow up after one month of trial period and repeated reminders did not bring them back to the trial. When contacted these patients opined that they did not have any pain or discomfort after taking one month dose. They also said if they find any difficulty in urination they would come back to the same hospital and take the DCBT5678 formulation. Five out of eight patients (62.5%) had complete dissolution of stones in one or both kidneys suggested that the plant formulation DCBT5678 exhibited strong activity against uric acid stones. The comparative analysis on the mean number of stones in the patients between the baseline and end of the trial values suggested that there was a significance reduction in the number stones from $4.4 \pm 2.1$ to $0.7 \pm 0.5$ and in the size of the stones from $7.7 \pm 2.7$ mm to $4.8 \pm 1.9$ mm. These reductions in size and number of stones were gradual in every month (Table 8). The mean biochemical value also confirmed the earlier trial results and the above said conclusion. Patients who had high urea content in the blood had shown
reduction in the values at the end of the trial (Table 9). These patients were also diagnosed reduction in the serum uric acid levels at the end of the trial period. Most of the patients who had uric acid stones in their kidneys exhibited very low excretion of uric acid in the urine that was evidence from the 24h. urine analysis (Table 9) at the time of induction in to the trial. This is because they probably have metabolic disorders that could have induced the formation of stones in the kidneys. The mean values of the uric acid levels of the patients at the end of the trial period showed significant increase. At the same time, the patient did not exhibit any change in the levels of serum calcium, serum phosphate, urine calcium, urine phosphate that were maintained with in the normal limits throughout the study (Table 9).

Both the studies suggested that the drug formulation, DCBT5678 could be used as a potent formulation in the treatments of uric acid stones in kidneys. The clinical trials done earlier with cystone or other plant materials did not conclusively reveal on the action of the respective formulation in alleviating the specific stones types of kidney in the patients (Premgamone et al., 2001: Campos and Schor, 1999; McHarg et al., 2003; Kirdpon et al., 1994). The study by Borisov et al., (2004) suggested a food additive, Prolit was more effective (27.5% in the treatment of urolithiasis than Cystone (16%), but this study was also lacked in - depth analysis of the stones were the Prolit would function.
When individual plant extracts of DCBT5678 were tested for their antimicrobial activity against two urinary tract infective organisms, *Proteus* spp and *E. coli* collected from the laboratory of the hospital where the trials were conducted, *B. pinnatum* was found to be the best, followed by *E. microphylla* and *B. malabaricum*. This was in tune with the observations made by Mehta and Bhat (1952); Obaseiki-Ebor (1985); Satyavati *et al.*, (1987) and Rastogi and Mehrotra (1990) in these three plants. But the absence of activity of the extract of *T. terrestris* against these microbes can be explained as they were of clinical isolates and each isolate can show varied activities for different plant extracts collected from different locations. The antimicrobial activity of the three of the five plants of DCBT5678 suggested that this formulation can also work against urinary tract infection and stones formed due to infections.

Kidney stone disease is a painful disorder and surgery becomes an absolute necessity if the stones lodge in the ureter. Therefore, a double blind study was not possible. Consequently, all the trials had to be open and no placebo comparison could be carried out.

The mechanism of action of DCBT5678 formulation on the uric acid stone and development of new formulations for other types of stones, the elucidation, purification, and characterization of bioactive compounds of these medicinal plants studied and their drug formulation in alleviating the kidney stone disorder are the future missions.