Chapter 7

Miscellaneous

* Recommendation from Institutional Ethics Committee
* List of publications
OFFICE OF THE CHAIRMAN : ETHICS COMMITTEE
MANIPUR UNIVERSITY

N O T I C E .
September 03, 2008

No.MU/8-199/06/UGC : As recommended by the Head of the Department of Life Sciences, the following scholars are allowed to use albino rats for their research work as per the Ethics' guide-lines and to follow the maintenance procedure.

<table>
<thead>
<tr>
<th>S.I.No.</th>
<th>Name of the scholar</th>
<th>Name of the guide</th>
<th>Department</th>
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<tr>
<td>1</td>
<td>Moirangthem Deinesh Singh</td>
<td>Prof. Th. Bhagirath</td>
<td>Life Sciences</td>
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<tr>
<td>2</td>
<td>Dhanaraj Singh Thokchom</td>
<td>Prof. G. J. Sharma</td>
<td>Life Sciences</td>
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<tr>
<td>3</td>
<td>Ms. Reena Laikangbam</td>
<td>Dr. M. Damayanti Devi</td>
<td>Life Sciences</td>
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<td>4</td>
<td>Mr. Katingpou Panmei</td>
<td>Dr. M. Damayanti Devi</td>
<td>Life Sciences</td>
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( P. S. Yadav) 0/0/0/0
Dean, School of Sciences

Copy to :

1. A.R. to Vice-Chancellor
2. The Registrar, M.U.
3. Prof. Th. Bhagirath, Life Sciences Department
4. Prof. G. J. Sharma, Life Sciences Department
5. Dr. M. Damayanti, Life Sciences Department
6. Deputy Registrar-I, Manipur University
7. Notice Board / Book
8. Relevant file
LIST OF PUBLICATIONS


Anti-bacterial efficacy of elite medicinal plants on urolithiasis inducing flora

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Abstract

Medicinal plants are valuable sources of novel antibacterials which are associated with the prevention and control of urolithiasis. Seventeen plant species, namely, Kilaimulax semecarpifolia, Asparagus acutifolius (Willd.) Spreng., Averrhoa carambola (Linnaceae), Bambusa bambos (Burm.f.), Cynara scolymus (Burm.f.), Cissus discolor (Burm.f.), Curcuma longa (Linnaceae), Cynara scolymus (Burm.f.), Curcuma longa (Linnaceae), Cynara scolymus (Burm.f.), Cissus discolor (Burm.f.), and Cynara scolymus (Burm.f.), were screened for potential antibacterial activity against four selected urolithiasis inducing bacteria, namely Proteus mirabilis, E. coli, Staphylococcus aureus and Klebsiella pneumoniae subsp. pneumoniae. This study was based on antibacterial susceptibility test in which the antibacterial activities of aqueous and ethanol extracts of the medicinal plants were determined by standardized disc-diffusion technique. Observations were noted at the end of 24, 48 and 72 h of incubation. From the screening experiments, H. sabdariffa exhibited the highest antibacterial activity in almost all the tested organisms. Others, namely C. adhatoda, C. cymbarum, C. coriandum, C. longa and T. indica also exhibited significant antibacterial effect to a certain degree. Ethanol extracts showed more efficacy for almost all the plants studied. Thus, C. adhatoda, C. cymbarum, H. sabdariffa, O. vulgare and T. indica show promising roles in the prevention and cure of urolithiasis. This study shows the potential and healing powers of medicinal plants and will be a great boon to the human society. In fact, folk or traditional medicinal uses represent ‘leads’ that could Shortcut the discovery of modern medicines.

Keywords: Medicinal plants, urolithiasis, antibacterial activity, aqueous extracts, ethanol extracts, inhibition zone.

Introduction

One of the most common problems faced by the human society is the stone, out of which urolithiasis is one of them. Urolithiasis is synonymous to calculus formation at any level in the urinary collecting system but most often, calculi arises in the kidney. ¹ It is one of the commonest renal disorders in Manipur. It occurs more frequently in man than woman but rare in children. ² Recurrent stone formation is probably the most important problem in the prognosis of patients who have undergone operations for renal and ureteric calculi. The etiology is complex and is considered multifactorial, such as food habit, altered urinary solutes and collod, decreased urinary drainage and urinary stasis, prolonged immobilization, Randall’s plaque, reinfections, urinary tract infection etc. ³

When the urinary tract is infected by uro-epithelial organisms, the area which is being eroded in the urine splits resulting to the formation of amorphous as by-product which renders the urine alkaline. In this alkaline urine, there tends to be precipitated crystals of calcium and magnesium phosphate and calcium carbonate which are present in large amount in that medium. There is a strong tendency to the formation of calcium phosphate and calcium carbonate crystals when abundance crystalloids are found continually in the medium. ⁴ Another mechanism by which bacterial infection may induce stone formation is by crystal adherence. Thomson and Stamey ⁵ have confirmed the fact that struvite and other urinary calculi are caused by the action of bacteria on urine. They also contain numerous infectious bacteria within the structure. The majority of uro-epithelial organisms are of the species Proteus. On the other hand, organisms such as Pseudomonas, Klebsiella, Staphylococcus, occasionally E. coli and even Mycoplasma are capable of producing bacterial urease ⁶ ⁷. Robertson ⁸ observed that infected stones were associated with the organisms like E. coli, Proteus sp., Klebsiella sp., Streptococcus, Staphylococcus, Pseudomonas and Uropasyma aureofaciens. Griffith and Osborne ⁹ have shown that the end products of urea-splitting damage the glycansylglycoprotein layer of the renal tubular cells leading to the bacterial adherence, followed by biofilm formation and mineral entouragement. So, a careful microbiological investigation to find and treat the infection responsible for the stone formation is mandatory.

Medicinal plants are the local heritage with global importance. Out of the total 42,200 flowering plants reported from the world ¹⁰, more than 50,000 are used for medicinal purposes ¹¹. Medicinal plants have been the subjects of man’s curiosity since times immemorial ¹². Almost every civilization has a history of medicinal plant use ¹³. Utilization of plants for medicinal purposes in India has been documented long back in ancient Hindu scriptures like Rigveda (4500-1600 B.C.), Charak Samhita (1000-
800 B.C.), Sushruta Samhita (800-700 B.C.) and others. However, organized studies in this direction were initiated in 1956 and of late, such studies are gaining recognition and popularity due to loss of traditional knowledge and declining plant population. Therefore, the use of traditional medicine and medicinal plants in most developing countries, as a basis for the maintenance of good health, has been widely observed.

Manipur (24°49'N and 93°52'E) is a state embedded with very rich biotic resources representing a mixed flora and fauna of the Himalayan region and Malaian Archipelago. Manipur, which is a part of North East India, happens to be within the Indo-Burmese mega-biodiversity hot spot. This region is one of the eight hottest hotspots in terms of richness of edenic species diversity of both plants and vertebrates and high degree of threat. Many varieties of plants including those used by traditional medicinal practitioners grow luxuriantly in this region. Ayurveda is an ancient Indian form of medicine which deals with plants and plant extracts. This indigenous form of medicine uses the active ingredients present in plants for treating diseases. Medicinal plants have curative properties due to the presence of various complex chemical substances of different composition, which are generally secondary plant metabolites found in one or more parts of these plants. Many traditional medicinal plants have been identified as cures, retardants or sustenance of various health ailments. Interest in phytotherapy has exploded during the last few years. About 500 different plant species are used as key ingredients and many are still being collected from the wild. Phytochemicals are frequently considered to be less toxic and more free from side effects than synthetic ones.

Scientists throughout the world are trying to explore the precious assets of medicinal plants to help the suffering human population. Furthermore, in the world, more than 30% of the pharmaceutical preparations are herbal based. Ethnobotanical studies have brought light to numerous plants having significant medicinal properties which were earlier unknown to the scientific world. The World Health Organization (WHO) estimates that about 80% of the world’s population relies mainly on herbal medicine for primary healthcare and is reported to have minimal side effects and about 85% of traditional medicine involves the use of plant extracts. However, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants.

In India, as in many countries, recent interest has focused on the therapeutic potential of traditional plants in the context of various diseases by using scientific methods. The role of coliform bacilli in urinary tract infection has long been known in developed countries. The introduction of antibiotics for the chemotherapy of bacterial infections has been one of the most important medical achievements of the past 50 years. However, the emergence of bacterial resistance to antibiotics undermines the therapeutic utility of existing agents, creating a requirement for the discovery of new antibacterial drugs. The rising incidence in multi-drug resistance (MDR) amongst pathogenic microbes has further necessitated the need to search for newer antibiotic source. Plants remain the most common source of antimicrobial agents. Many of the existing synthetic drugs cause various side effects. Hence, drug development from plant-based compounds could be useful in meeting this demand for newer drugs with minimal side effects.

Scientific investigations on the indigenous medicines prepared from plant products used by the Tribals and Meities of Manipur may prove to be of great pharmacological importance leading to the advent of newer drugs, which could be at par with the modern allopathic medicines in terms of efficacy. The aim of the study was to screen medicinal plant species grown in Manipur for potential antibacterial activity against urolithiasis inducing flora.

**Materials and Methods**

**Collection of plant samples:** Fresh plants/plant parts were collected from various places in Imphal west district (24°37'N and 93°30'E), Manipur, India. The plant samples (Table I) were collected and deposited in the Herbarium of Manipur University, Imphal, and respective voucher numbers were assigned. Fresh plant samples were washed under running tap water and air-dried for about 10 minutes.

**Crude extraction of plant samples:** Aqueous extracts of the plant samples were prepared by crushing the fresh plants with the help of a mortar and pestle in the ratio of 0.1 g ml⁻¹ of distilled water. The plant extract thus obtained was filtered through Whatmann No.1 filter paper, sterilized and stored at refrigerated conditions for future use.

Thirty grams of the plant material was extracted in 300 ml of 80% ethanol using a mortar and pestle. The plant extract thus obtained was filtered through Whatmann No.1 filter paper, sterilized and stored in airtight bottles at 4°C for future use.

**Collection of urine samples:** The present study was performed on pre-operative urine. First voided morning mid-stream urine samples were collected aseptically from 25 urolithiasis patients admitted in the Urology Department, Regional Institute of Medical Sciences, Imphal, in sterile wide-mouth containers by giving proper guidelines for urine collection for both males and females. The subjects were chosen randomly consisting of mostly adults (aged 20-80), out of which 14 were males and 11 were females. Relevant information about the disease was collected from each patient after taking prior consent from the patient.

**Microorganisms:** In human beings, urinary tract infections are mostly caused by Gram-negative bacteria and rarely by Gram-positive bacteria. Microbial strains *Proteus mirabilis*, *Escherichia coli*, *Pseudomonas stutzeri* and *Klebsiella pneumoniae* subsp. *pneumoniae* were selected for the present study. All the test organisms were clinical isolates obtained from the urine samples of infected patients. These bacterial strains are potential human pathogens. Isolation and identification of the test organisms were performed as described by Cruckshank et al. 

The bacterial isolates were sub-cultured periodically on blood-agar plates and prepared for the assessment of plant extract activity.

**Antibacterial assay:** The antibacterial assay was carried out by using standard disc-diffusion technique. The *in vitro* antimicrobial activity was performed against overnight grown cultures of four selected bacteria, namely *P. mirabilis*, *E. coli*, *P. stutzeri* and *K. pneumoniae* subsp. *pneumoniae* on blood-agar media. This can be achieved by first thoroughly spreading the overnight grown bacteria (1 O.D.) on blood-agar plates. Then, the sterile filter discs were placed onto the inoculated plates. Twenty µg of
Table 1. Tested plant species, their voucher numbers, families and local names.

<table>
<thead>
<tr>
<th>Plant species (Voucher No.)</th>
<th>Family</th>
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</thead>
<tbody>
<tr>
<td>Allium odorum</td>
<td>Liliaceae</td>
<td>Marru-rukkipi</td>
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<td>Asparagus racemosus (Deb 253)</td>
<td>Liliaceae</td>
<td>Nanggarci-angubha</td>
</tr>
<tr>
<td>Avocado carica (Deb 2210)</td>
<td>Averrhoaceae</td>
<td>Heinsjoe</td>
</tr>
<tr>
<td>Banyan brachiate (Deb 573)</td>
<td>Scrophulariaceae</td>
<td>Keeloom</td>
</tr>
<tr>
<td>Cineraria adianta (Deb 450, 482 &amp; 543)</td>
<td>Vitaceae</td>
<td>Konguyen</td>
</tr>
<tr>
<td>Cissus discolor (Mukherjee 3522)</td>
<td>Vitaceae</td>
<td>Konguyen-laba</td>
</tr>
<tr>
<td>Cissus discolor</td>
<td>Passifloraceae</td>
<td>Channing</td>
</tr>
<tr>
<td>Barbosa cinerea (Deb 2341)</td>
<td>Apocynaceae</td>
<td>Jerm</td>
</tr>
<tr>
<td>Eupatorium bharanum (Deb 782 &amp; 832)</td>
<td>Asstercacea</td>
<td>Langshai</td>
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<td>Hedyotis marginatum (Deb 1377)</td>
<td>Zingiberaceae</td>
<td>Takhelle-angangha</td>
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<tr>
<td>Malvaceae</td>
<td>Sik-sengree</td>
<td></td>
</tr>
<tr>
<td>Mimosa pudica (Deb 2467)</td>
<td>Leguminosae</td>
<td>Kangphial-kakihabi</td>
</tr>
<tr>
<td>Orchis flavum (Kanjali 502)</td>
<td>Lamiaceae</td>
<td>Leikham</td>
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<tr>
<td>Oulaa corniculata (Mukherjee 2808)</td>
<td>Oxalidaceae</td>
<td>Meibi Yemii</td>
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<td>Piper longum (Deb 1265)</td>
<td>Piperaceae</td>
<td>Taboppi</td>
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<td>Pratia keganisifolia</td>
<td>Campanulaceae</td>
<td>Nongsai-penk</td>
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<tr>
<td>Tamarindus indica (Deb 2477)</td>
<td>Leguminosae</td>
<td>Mangue</td>
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</table>

Each plant extract (both aqueous and alcoholic) was added onto the discs. After addition of each of the plant extracts, the inoculated plates were kept at room temperature for about 1 hour to enable the diffusion of the plant extracts and incubated at 37°C. Microbial growth inhibition was determined by measuring the diameter of the zone of inhibition which was assessed at 24, 48 and 72 h incubation.

Results and Discussion

Successful prediction of bioactive compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Traditional healers use primarily water as the solvent but in our studies, we found that plant extracts in organic solvent (ethanol) provided more consistent anti-microbial activity compared to those extracted in water. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the medium used in the assay. The results of screening are presented in Figs 1-4. The aqueous and ethanol extracts of seventeen plant species belonging to fourteen different families were tested against four Gram-negative bacteria species using standard disc-diffusion technique.

Out of the 17 medicinal plant species analyzed, Fig. 1 projects that both the aqueous and ethanol extracts of C. adianta showed the highest efficacy of anti-bacterial activity against P. mirabilis. The aqueous and ethanol extracts of C. cineriana, H. sabdariffa, T. indica and P. longum also showed comparative efficacy against the microbe. P. mirabilis was resistant to the aqueous extracts of A. racemosa, E. bharanum and O. spiralis while their ethanol extracts were found to inhibit the organism. This shows that ethanol extracts exhibited higher antibacterial activity. This finding is in consistency with the report of Evans & that alcohol is a general solvent which tends to provide a more complete extraction of compounds with a variety of polarities and the aqueous extracts may not contain some of the less polar compounds. It was also reported that the ethanol extract of A. racemosa significantly reduced the elevated level of calculegenic ions present in urine and it elevated the urinary concentration of magnesium, which is considered as one of the inhibitors of crystallization.

Both the aqueous and ethanol extracts of H. sabdariffa exhibited the highest antibacterial activity against E. coli. The aqueous and ethanol extracts of C. adianta, O. coriaceae, T. indica and P. longum had also inhibitory effect on E. coli unlike C. discolor which showed least inhibitory effect (Fig. 2). Ruthone et al. reported that tamarind intake at a dose of 10 g showed significant beneficial effect in the inhibition of spontaneous crystallization in both normal subjects and in stone formers.

The extracts (aqueous and ethanol) of H. sabdariffa showed the highest potential in inhibiting the growth of K. pneumoniae subsp. pneumoniae, followed by O. coriaceae. The extracts of A. racemosa, C. adianta, C. cineriana, H. marginatum, P. longum and T. indica showed higher efficacy in alcoholic state in controlling the growth of the microbe. The ethanol extracts of C. discolor and E. bharanum couldn't inhibit the growth of the microorganism (Fig. 3).

Aqueous and ethanol extracts of H. sabdariffa significantly inhibited the growth of P. stutzeri. The plants that exhibited antibacterial activity to a certain degree were A. carica, C. adianta, H. marginatum, M. pudica, O. coriaceae, P. longum and T. indica (Fig. 4). Both the aqueous and ethanol extracts of A. odorum couldn't inhibit the growth of the microbe. The aqueous
extracts of *B. brasiliensis* and *C. glochidiata* showed least efficacy against the microbe.

Thus, the observations conferred that both the aqueous and ethanol extracts of *H. sabdariffa* showed the highest antibacterial activity in almost all the test organisms. Others, namely *C. adhatoda*, *C. communis*, *C. xylocarpa*, *P. longifolia* and *T. indica* exhibited significant antibacterial effect to a certain degree. From our investigation of screening different plant species, the results obtained confirmed the therapeutic potency of some plants used in traditional medicine.

The demonstration of antimicrobial activity by aqueous extracts provides the scientific basis for the use of these plants in the traditional treatment of diseases, since most traditional medicine systems employ water as their solvent in which the decoctions are prepared. Both the aqueous and ethanol extracts were effective against the four bacteria, namely *P. mirabilis*, *E. coli*, *K. pneumoniae*, *S. aureus*, *P. aeruginosa* and *P. stutzeri* in which ethanol extracts exhibited a higher degree of antibacterial activity.

The results of the present study form a good basis for selection of candidate plant species for further physicochemical and pharmacological investigation. The findings also support the folkloric usage of the studied plants and suggest that some of the plant extracts possess compounds with antibacterial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. It has been reported that physicochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds that are secondary metabolites of plants served as defense mechanisms against predation by many microorganisms, insects and herbivores. Thus, the most active extracts can be subjected to isolation of the active antimicrobial and undergo further pharmacological evaluation. Such investigations may lead to the discovery of novel bioactive molecules. Extensive work is in progress to identify compounds responsible for this biological activity.

**Conclusions**

Conclusion can be drawn that the medicinal plants viz., *C. adhatoda*, *C. communis*, *H. sabdariffa*, *O. coriandrum*, *P. longifolia* and *T. indica* could be employed for the eradication of bacterial flora associated with urolithiasis with reference to promising results obtained on antibacterial activity against the four bacterial isolates. This eventually would lead to a break-through in the prevention of urolithiasis. The exploitation of these potential herbs for the cause of urolithiasis prevention and cure would help in subsiding the existing problem of kidney stone formation up to an appreciable degree. Thus, this investigation would further open up new avenues to the use of these medicinal plants in drug development for the treatment of urolithiasis.

**Acknowledgement**

Authors are thankful to the staff of Regional Institute of Medical Sciences, Imphal, for their kind help and timely co-operation in undergoing the work and also to all the patients for their willingness and sensitivity shown to us.

**References**


Phytomedicine for Controlling Urolithiasis Agents

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ABSTRACT: Crystal aggregation and retention are critical events in the formation of kidney stones. Traditional plants are a valuable source of novel antibacterials and urine demulcent agents that are associated with prevention and control of urolithiasis. Urine demulcent and in vitro antibacterial activity of seventeen plant extracts were assessed. In vitro preparation of synthetic struvite and citrate crystals in the final weight of the stone were also determined after treatment with different ethanol extracts of the plants. Aquaphase extracts of dry plant samples were used to determine their capacity to control crystallization of urine. Antibacterial susceptibilities were also determined by standard filter-disc diffusion technique. Two known antibiotics were used as positive control. Out of the seventeen plants analyzed, Cissus quadrangularis, Cynodon dactylon L., Eupatorium birmannicum DC., Hibiscus sabdariffa L., Odax corniculata L., Piper longum L. and Tamarindus indica L. significantly prevented crystal formation in urine and exhibited strong antibacterial activity against four urolithiasis inducing flora. Ethanol extracts of H. sabdariffa L., T. indica L. and P. longum L. showed comparatively the highest efficiencies in dissolving the stone, thus, C. adhatoda, C. cyanus, E. birmannicum, H. sabdariffa, O. corniculata, P. longum and T. indica showed promising role in prevention and control of urolithiasis.

Keywords: Microbes, Inhibition zone, Urolithiasis, Decrystallization, Medicinal plants

Introduction
Urolithiasis is one of the most common diseases of the urinary tract which has been affecting human kind since antiquity. Urolithiasis is associated with calculus formation at any level in the urinary collecting system, but calculi often arise in the kidney (Kumar et al. 1991). It occurs more frequently in men than women but rare in children (Smith, 1970), affecting approximately 12% of men and 5% of women by the age of 70. Recurrent stone formation is probably the most important problem in the after care of patients who have undergone operations for renal and ureteric calculi. Urolith formation is a multifactorial process which may relate to diet, urinary tract infection, altered urinary solutes and colicants, decreased urinary drainage and urinary stasis, prolonged immobilization, Randall's plaque and microthelial, etc. (Fowler, 1995).

When the urea-splitting organisms infect the urinary tract, bacteria disintegrate the urea excreted in the urine in the presence of urease enzyme, which subsequently triggers the formation of ammonia rendering the urine alkaline. In alkaline states, urine tends to contain precipitated crystals of calcium and magnesium phosphate and calcium carbonate in large amount thereby leading to a strong tendency to form calcium phosphate and calcium carbonate calculi (Chute and Suby, 1943). Bacterial infection may induce stone formation by crystal adherence. Struvite and other urinary calculi are caused by the action of bacteria on urine (Thompson and Stanley, 1973). Most of the urea-splitting organisms belong to species Proteus but, organisms such as Pseudomonas, Klebsiella, Staphylococcus, Escherichia coli and even Mycoplasma were reported to be capable of producing urease (Friedlander and Braude, 1974; Griffith et al. 1975). Robertson in 1952, has reported that infected stones were associated with the organisms like E. coli, Proteus sp., Streptococcus, Staphylococcus, Pseudomonas and Ureaplasma urealyticum. There are increasing evidences that have been reported that the end products of urolithiasis damage the glycosaminoglycan layer of the renal urothelial cells thus leading to the bacterial adherence, biofilm formation and mineral encrustation (Griffin and Osborne, 1987). Exhaustive microbiological investigations are therefore necessary to diagnose and treat the infection responsible for the stone formation.

Medicinal plants are of great economic importance in the Indian subcontinent. The documentation of traditional knowledge especially on the medicinal uses of plants in the history has provided many important drugs of the modern day (Fabricant and Farnsworth, 2001). Even today, this area holds much more hidden treasure as at least 80% of the human population in developing countries is dependent on plant resources for health-care (Farnsworth et al. 1985). Herbal medicines offer conventional treatments, providing safe and well-tolerated remedies for chronic illnesses which typically result from the combinations of secondary plant metabolites that are synthesized and deposited in specific parts or in all parts of the plant. Since, many of the existing synthetic drugs cause various side effects, drugs synthesized from the higher plants continue to occupy an important niche in modern medicine and play an important role in the introduction of new therapeutic agents.

A variety of plants including those used by traditional medicinal practitioners grow luxuriantly in Manipur (24° 49' N and 93° 52' E), a region in the north-eastern part of India, which happens to be within the Indo-Tibetan mega-biodiversity hot spot (Myers et al. 2000). Since its civilization, the living population of this region has been using various medicinal plants for the treatment of stone cases. These plants are conventionally used to prevent formation of stone as well as to dissolve and remove them from the human body.

The screening of plant extracts for antimicrobial activity is necessary to throw insight knowledge to medicinal flora and get the molecules responsible for the activity which adds value to natural resources from tropical areas (Koss and Reito, 2005). Thus, scientific investigations on the plant based indigenous medicines prepared and used by the tribal and Melite population 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. This led us to investigate the screening of various medicinal plants for their potential activities in prevention and control of urolithiasis.

Materials and Methods
Collection of urine samples
First voided morning mid-stream (pre-operative) urine samples were collected aseptically from 25 Urolithiasis patients admitted at the Urology Department, Regional Institute of Medical Sciences, Imphal, for testing crystal formation using different plant extracts. The subjects were mostly adults (aged 20-80) chosen randomly, comprising of 14 males and 11 females. Detailed case histories, relevant information about occupation, family history of stone, onset of urolithiasis, previous urinary tract disease, age, sex, racial origin, environment, metabolic activities, dietary habit, obstructive uropathy, infection of urinary tract symptomatology (like dysuria, nocturia, hematuria, pyuria etc.), duration of attack, site of stone etc. were collected. Since the experiment does not involve human subjects directly, the approval from the ethics committee does not apply. Moreover, samples such as urine are considered wastes and excreta and urine samples were collected after taking consent from each patient by giving proper explanation.

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Collection and extraction of plant samples

The plant samples (Table 1) were collected randomly from various places of Imphal-West (24° 37' N and 93° 30' E) district, and deposited in the Herbarium of Manipur University. Imphal and voucher numbers were assigned. The plant samples were oven-dried at 50°C for 2-3 days and ground to fine powder. About 30 gm each of these powdered samples were crushed using mortar and pestle and homogenized in suitable solvent systems (distilled water/80% ethanol). The crude extracts obtained were centrifuged and then filtered through Whatmann No.1 filter paper, sterilized and stored at refrigerated conditions (4°C) for future use.

<table>
<thead>
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<th>Sl. No.</th>
<th>Plant species (Voucher No.)</th>
<th>Parts used</th>
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<tbody>
<tr>
<td>1</td>
<td>Azadirachta indica (L.) (002510)</td>
<td>Leaves</td>
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<tr>
<td>2</td>
<td>Asperula cearensis Wild. (Deb 253)</td>
<td>Roots</td>
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<tr>
<td>3</td>
<td>Aristolochia clematitis L. (Deb 2210)</td>
<td>Leaves</td>
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<td>4</td>
<td>Bauhinia purpurea L. (003420)</td>
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<td>5</td>
<td>Cistus monadelphus L. (Deb 573)</td>
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<td>14</td>
<td>Cyperus rotundifolius L. (Kangpui 2808)</td>
<td>Whole plant</td>
</tr>
<tr>
<td>15</td>
<td>Datura stramonium L. (Deb 1655)</td>
<td>Leaves</td>
</tr>
<tr>
<td>16</td>
<td>Ferula digyna Bl. (Deb 2477)</td>
<td>Whole plant</td>
</tr>
<tr>
<td>17</td>
<td>Ficus benghalensis L. (005060)</td>
<td>Leaves</td>
</tr>
</tbody>
</table>

Table 1: Names of plants along with their voucher numbers and parts used

Determination of the effects of different plant extracts on urine crystallization

For qualitative assessment of effect on urine crystallization, 15 mL each of pre-operative urine samples were treated with 5 mL each of the plant extracts. And 20 mL of pre-operative urine sample was taken in a beaker without any treatment to serve as control. In another beaker, 15 mL of pre-operative urine was taken and treated with 5 mL of distilled water. All the samples in the experiment were taken as triplicates. The samples were then incubated for 72 hrs. at room temperature for testing crystal formation.

Anti-microbial evaluation of different medicinal plant-extracts

In the urinary system of man, infections are mostly caused by gram-negative bacteria and rarely by gram-positive bacteria. Bacterial strains namely, Proteus mirabilis (MTCC 779), Escherichia coli (MTCC 729), Pseudomonas aeruginosa (MTCC 2499) and Klebsiella pneumoniae sub sp. pneumoniae (MTCC 432) which had been reported to be responsible for urinary tract infections were selected for the present study. The antibacterial activities of the plant extracts were determined by following standard filter disc diffusion technique. In this, the strains of bacteria were cultured on blood-agar plates. Overnight grown bacteria (1 O.D.) were thoroughly spread on blood-agar plates. The filter-discs (about 6 mm in diameter) were placed on the inoculated plates into which 50 μL each of the plant extract was added. The plates were then maintained at room temperature for about 1 hr. to allow diffusion of the plant extracts into the discs and the medium and subsequently incubated at 37°C. Inhibition was followed by measuring the diameter of the inhibition zone at the end of 24 hrs., 48 hrs., and 72 hrs. The experiments were done in replicates of three. Sensitivity of each organism to different plant extracts was assessed by observing zones of inhibition.

Preparation of synthetic struvite and its treatment using different plant extracts

When magnesium mixture [MgCl₂, NH₄Cl] and a little aqueous ammonia were added to a solution of sodium phosphate [Na₂HPO₄], a white crystalline precipitate of magnesium ammonium phosphate [Mg(NH₄)₂(PO₄)₂] was produced (Guity and Kapoor, 1963). The precipitate was repeatedly washed with distilled water to free the ammonium and chloride ions and its absence was confirmed through flame-test and silver-nitrate test respectively. The precipitate obtained was soluble in acetic acid and mineral acids and insoluble in dilute ammonia solution. The chemical reaction is depicted below:

\[ \text{Na}_2\text{HPO}_4 + \text{MgCl}_2 + \text{NH}_4\text{Cl} \rightarrow \text{Mg(NH}_4\text{)PO}_4 \cdot \text{6H}_2\text{O} \]

\[ \text{Na}_2\text{HPO}_4 \text{ and MgCl}_2 \text{ mixture} \]

\[ \text{Little aqueous ammonia} \]

\[ \text{Struvite} \]
This synthetically prepared struvite was treated with different quantities of ethanol extract of plants and incubated at 25 °C for 24 hours to see the effects of the plant extracts on the stone.

**Results**

Out of the 25 patients taken under study, 9 patients showed urine decrystallization in the control state (i.e. without any treatment of plant extract in their urine samples). So, their data were not taken into account for statistical analysis. Fig 1, shows the effects of different plant extracts on urine decrystallization and revealed that the aqueous extracts of C. ashwagandha, N. sabdariffa, O. comiculata and T. indica have significant positive effect on urine decrystallization in all the patients. Extracts of P. longum was also observed to have significant positive effect on urine decrystallization in almost all the patients, but extracts of A. carolanga showed the least efficacy on urine-decrystallization.

Fig 1: Prevention of urine crystallization using plant extracts

Plant extracts
Different plant extracts were found to possess the capability to alter the pH of the urine, by elevating or decreasing the pH of the urine, rendering it more alkaline or acidic conditions. The plants that could significantly lower the pH of the urine were found to have greater efficacy towards urine decrystallization (Fig 2). Out of the 17 plants analyzed, both the aqueous and ethanol extracts of H. sabdariffa, T. indica, A. carambola, A. racemosus and P. longum showed significant effect in controlling the growth of E. coli when compared with the antibiotic, Amikacin (0.25 μg) by observing the zones of inhibition most prominently seen in 72 hrs. incubated plates (Fig 3).
Aqueous and ethanol extracts of H. saladeniffa, T. indica, C. cymiferum, E. bicornicium and C. zatrata were found to show comparatively high anti-bacterial activity against Proteus mirabilis as compared to the effect of Amikacin (0.25 ml) (Fig 4).

Fig 4: Effect of plant extracts on Proteus mirabilis

Fig 5 shows that extracts of H. saladeniffa, M. pudica, A. cecumbola, H. marginatum, P. beganiifolia, C. cymiferum and T. indica could comparatively inhibit the growth of Pseudomonas stutzeri as compared to that of Gentamycin (0.25 ml).

Fig 5: Effect of plant extracts on Pseudomonas stutzeri

Extracts of H. saladeniffa, O. corniculata, C. cymiferum, O. spiralis, H. marginatum exhibited high efficacies in controlling the growth of K. pneumoniae sub sp. pneumoniae which were at par with 0.25 ml of Amikacin (Fig 6).
In almost all the cases, ethanol extracts have higher efficacies than aqueous extracts in controlling the growth of the microbes. Thus, the extracts prepared from dried parts of C. ashadha, C. cinnamus, E. bimanicum, H. sabdariffa, O. corniculata, P. longum and Y. indica exhibited a comparative anti-microbial action against the four selected bacteria, viz. E. coli, P. mirabilis, P. stutzeri and K. pneumoniae sub sp. pneumoniae. This finding is consistent with the results obtained from the extracts prepared from fresh parts of these plants in our earlier experiments (Lakangbam et al., 2009). Out of the seventeen plants investigated, ethanol extracts of H. sabdariffa L., Y. indica L. and P. longum L. showed comparatively higher efficacies than the others at the dose of 1 g/15 ml. In dissolving the stone whereas extract of A. odoratum showed the least efficacy in dissolving the stone (Table 2).

Table 2: Determination of the final weight of the struvite after treatment with different doses of ethanol plant extracts

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Plant samples (50% ethanol extracts)</th>
<th>Final wt. of struvite (1 g/5 ml)*</th>
<th>Final wt. of struvite (1 g/10 ml)*</th>
<th>Final wt. of struvite (1 g/15 ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A. odoratum L.</td>
<td>0.9997 ± 0.06</td>
<td>0.9994 ± 0.05</td>
<td>0.9981 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>A. odoratum W.</td>
<td>0.9997 ± 0.06</td>
<td>0.9987 ± 0.06</td>
<td>0.9983 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>A. odoratum L.</td>
<td>0.9999 ± 0.09</td>
<td>0.9997 ± 0.06</td>
<td>0.9995 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>A. odoratum L.</td>
<td>0.9618 ± 0.55</td>
<td>0.9571 ± 0.32</td>
<td>0.9371 ± 0.20</td>
</tr>
<tr>
<td>5</td>
<td>A. odoratum L.</td>
<td>0.9393 ± 0.23</td>
<td>0.9383 ± 0.23</td>
<td>0.9180 ± 0.13</td>
</tr>
<tr>
<td>6</td>
<td>A. odoratum L.</td>
<td>0.9704 ± 0.03</td>
<td>0.9738 ± 0.03</td>
<td>0.9690 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>A. odoratum L.</td>
<td>0.9899 ± 0.32</td>
<td>0.9891 ± 0.03</td>
<td>0.9890 ± 0.03</td>
</tr>
<tr>
<td>8</td>
<td>A. odoratum L.</td>
<td>0.9914 ± 0.40</td>
<td>0.9910 ± 0.05</td>
<td>0.9904 ± 0.03</td>
</tr>
<tr>
<td>9</td>
<td>A. odoratum L.</td>
<td>0.9065 ± 0.04</td>
<td>0.9080 ± 0.29</td>
<td>0.9060 ± 0.27</td>
</tr>
<tr>
<td>10</td>
<td>H. sabdariffa L.</td>
<td>0.9060 ± 0.07</td>
<td>0.9070 ± 0.06</td>
<td>0.9060 ± 0.07</td>
</tr>
<tr>
<td>11</td>
<td>O. corniculata L.</td>
<td>0.9616 ± 0.29</td>
<td>0.9585 ± 0.25</td>
<td>0.9485 ± 0.23</td>
</tr>
<tr>
<td>12</td>
<td>P. longum L.</td>
<td>0.9893 ± 0.12</td>
<td>0.9820 ± 0.16</td>
<td>0.9920 ± 0.12</td>
</tr>
<tr>
<td>13</td>
<td>Y. indica L.</td>
<td>0.9893 ± 0.12</td>
<td>0.9890 ± 0.17</td>
<td>0.9741 ± 0.09</td>
</tr>
<tr>
<td>14</td>
<td>P. longum L.</td>
<td>0.9904 ± 0.16</td>
<td>0.9969 ± 0.12</td>
<td>0.9731 ± 0.10</td>
</tr>
<tr>
<td>15</td>
<td>P. longum L.</td>
<td>0.9920 ± 0.17</td>
<td>0.9610 ± 0.09</td>
<td>0.9623 ± 0.07</td>
</tr>
</tbody>
</table>

* Values are expressed as means of three observations. ± standard error

Discussion
In India, as in many countries, recent interest has been focused on the therapeutic potential of traditional plants in the context of controlling various diseases by using scientific methods. The role of coliform bacilli in urinary tract infection has long been known in developed countries (Willet and Radajou, 1976; Morton and Lawande, 1982). Plants remain the most common source of antimicrobial agents.

It has been reported that acidic plant extracts helped in preventing crystal formation of urine, eventually leading to the prevention of kidney stones. In this way, diet and the food habit of the individual...
plays an important role in the formation of kidney stones or their prevention. Not only this, pH also has a key role in the different stone formation as the antibacterial activities of the extracts were found to be increased at an acidic pH. Moreover, phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds that are secondary metabolites of plants serve as defense mechanisms against predation by many microorganisms, insects and herbivores (Lutterotti et al. 1999; Marjorie, 1999). Increase in the activities of the phyto-constituents in the presence of acidic medium had also been reported (Molan, 1992). Contrary to this, the activity of plant extracts deteriorated considerably at alkaline pH.

Out of the 17 medicinal plants analyzed, C. adhatoda, H. sabdariffa, O. combretum, P. longum and T. indica significantly prevented crystal formation as compared to the aqueous extract. It has been reported that tamarin intake at a dose of 10 gm showed significant beneficial effect in the inhibition of spontaneous crystallization in both normal subjects and in stone formers (Rathore et al., 1993). The present study revealed that M. pudica had moderate effect on urine crystallization, our finding is in agreement with other (Joyamma, 1990), who reported that M. pudica was not effective in either preventing stone deposition or dissolving preformed stones. This report suggests the possibility of the involvement of exogenous factors such as environment, soil nutrients, stress etc. The aqueous extract of A. racemosa showed moderate efficacy in preventing crystal formation in urine while, the ethanol extract of A. racemosa was reported to significantly reduce the elevated level of Circulating ions in urine and elevated the urinary concentration of magnesium which is considered as one of the inhibitors of crystallization (Christina et al. 2005).

The demonstration of antimicrobial activities by aqueous extracts provide the scientific basis for the use of these plants in the traditional treatment of diseases, since most traditional medicine systems use water as their solvent in which the decoctions are prepared. In the present investigations, both the aqueous and ethanol extracts are found to be effective against the four bacteria i.e. P. mirabilis, E. coli, K. pneumoniae and P. stutzeri. But the ethanol extracts exhibited higher antibacterial activity as compared to the aqueous extract. It has been reported that alcohol is a general solvent and tends to provide a more complete extraction of compounds with a variety of polarities (Evans, 1996). Thus, aqueous extracts may not contain some of the less polar compounds.

Even though stability in pH contributes to the control of urolithiasis, it was observed that not only acidic pH, but alkaline pH also helped in controlling urolithiasis. Medicinal plants extracts such as C. cinerariifolium and E. bennamum controls urolithiasis even though they render the urine alkaline. In our present study, since the pre-operative urine samples were collected randomly from different patients, the nature of the stones may be of different types. And this might be one of the contributing factors in influencing varied effects of each plant extract on the urine decrystallization pattern and pH in the patients studied as found in the present investigations. As a result of this, the need of administering different types of medicinal plants to different types of stone i.e. calcium-oxalate stone, struvite, uric acid and urate stones, cystine stone etc. arises to get a promising result. It has been observed that plants which have an acidic pH help in preventing the stone, but struvite is a type of stones formed due to urinary tract infection in alkaline pH. It is still a general assumption that the plant extracts prepared through local traditional medicine systems have some positive effect on urolithiasis. Thus, control of urolithiasis largely depends upon the bioactive molecules present in the medicinal plants. At this juncture, attention is needed for carrying out chemical and ethno-pharmacological studies as a control measure for preventive measure for urolithiasis. Such investigations may lead to the discovery of novel bioactive molecules and several works are undertaking to identify the compounds responsible for this biological activity.

Conclusion

Biological evaluation of potential medicinal plants such as C. adhatoda, C. cinerariifolium, H. sabdariffa, O. combretum, P. longum and T. indica demonstrates promising results on urine decrystallization, antibacterial activity and dissolution of stone thereby serving as controlling agents of urolithiasis and which eventually would lead to a break-through in the prevention of urolithiasis. Thus, this investigation will further open up new avenues to the use of these medicinal plants in drug development for the treatment of urolithiasis.

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References


