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In-Vitro Anti-Microbial and Anti-Urolithiatic Models for Extract of *Parmelia Perlata* Lichen: An Evaluation of Prophylactic Management against Kidney Stone

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Abstract- Background: Phyto-medicine is regaining interest owing to its advantages over conventional drugs and increasing cases of drug resistance. Moreover, recurrence and persistent side effects of present-day treatment for urolithiasis restrict their use, so an alternate solution using phytotherapy is being sought. The lichen species *Parmelia Perlata (*family *Parmeliaceae)* mentioned in India Materia Medica are useful in treating several ailments, and they are being used in large quantities as a food supplements in India.

Objective: Up to date, the scientific documentation regarding *in-vivo* antiurolithiatic and antimicrobial activity of *Parmelia perlata* has been reported, although it has not reported for *in-vitro* antiurolithiatic as well as an antimicrobial activity up till now. The present study attempted to evaluate phytochemical screening, antimicrobial activity, and anti urolithiatic activity of methanolic lichen extracts of *Parmelia Perlata,* which called "Chandila" by *in vitro* model.

Material and Methods: In this study, the crude extracts were obtained from the *Parmelia perlata* by cold extraction method using methanol as a solvent. The phytochemical tests were being carried out on the extract of lichen. The antimicrobial efficacy was being investigated against various pathogenic bacterial and fungal strains. *In vitro* antiurolithiatic models were investigated through nucleation assay and aggregation assays by spectrophotometric technique. Cystone was being used as a standard drug in the *in vitro* model for anti urolithiatic activity.
Result: The result of the phytochemical tests showed the presence of several biologically active phytochemicals with the highest quantity of alkaloids, flavonoids, and phenols in methanolic extract. The methanolic extract had the highest activity against *P.vulgaris* and *C. tropicalis* at 160 mcg/mL concentration. The maximum percentage of dissolution of existing calcium oxalate crystal was found to be 35% at 40 mg/ml concentration.

Conclusion: Methanolic extract of *Parmelia Perlata* exhibited significant *in vitro* antimicrobial and anti-urolithiatic activity.

Keywords: parmelia perlata, lichen, phytochemicals, antimicrobial, anti-urolithiatic.

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I. Introduction

Urolithiasis, the formation of kidney stone presence of one or more calculi in any location within the urinary tract, is one of the oldest and wide spread diseases known to man.[1] It is a serious, debilitating problem in all societies throughout the world, affecting approximately 12% of the population and, men are three times more prone than women.[2] It is more prevalent between the ages of 20 and 40 in both sexes.[3] Etiology is multifactorial and is strongly related to dietary lifestyle habits or practices.[4] Increased rates of hypertension and obesity also contribute to an increase in stone formation.[5] Kidney stones are hard, solid particles that form in the urinary tract. In many cases, the stones are very small and can pass out of the body without any problems. However, if a stone (even a small one) blocks the flow of urine, excruciating pain may result, and prompt medical treatment may be needed. Recurrent stone formation is a common part of the medical care of patients with stone disease. Calcium-containing stones, especially calcium oxalate monohydrate, calcium oxalate dihydrate and, basic calcium phosphate are the most commonly occurring ones to the extent of 75-90% followed by magnesium ammonium phosphate (Struvite) to the extent of 10-15%, uric acid 3-10% and cystine 0.5-1% [6]. The present-day medical management of nephrolithiasis is either costly or not without side-effects. Invasive procedures for the treatment of nephrolithiasis may cause serious complications and also impose a burden of costs on the healthcare system [7].

Lichens are being used since ancient times as one of the natural drugs [8]. Lichens represent a symbiotic association of a fungus with an algal partner and are important constituents of the ecosystem. Lichens produce characteristic secondary metabolites such as aliphatic, aromatic, and terpenic components which have considerable biological activities such as antiviral, antibacterial, antifungal, antitumor, antioxidant, etc. [9, 10]. *Parmelia perlata* is a well-known lichen of family *Parmeliaceae*. A lichen is an association of an alga and fungus living together in a symbiotic relationship.

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Parmelia perlata is commonly called a Stone flower or Chadila. In India it is mainly found in Himachal Pradesh and West Bengal. It is used as food, fodder and medicine. It is a good pain reliever and is being used as a remedy for the early healing of wounds. It cures many skin diseases and is considered to be an expectorant, astringent, resolvent, laxative, carminative and, aphrodisiac. It is also used in the treatment of fever, cough, dysentery and, renal calculi. This lichen exhibits antimicrobial [11-12], antiviral [13], antitumor [14], antispasmodic [15], antioxidant [16] and antipyretic [17] activities. Its hepatoprotective action[18] has also been reported. Phytochemical studies of *Parmelia perlata* have led to the isolation of various chemical constituents such as atranorin, chloroatranarin, salazinic acid [19], lecanoric acid [20], imbricaric acid [21], lecanora. The study has been undertaken to evaluate *Parmelia perlata* different lichen extracts and cystone as a standard for their possible potential to dissolve experimental kidney stone using a modified in vitro model [22-25] to isolate the chemical constituent responsible for the activity.

II. Material and Methods

a) Source of Plant Materials

The plant material Parmelia perlata (lichen) was collected from the hills of Himachal Pradesh (India). The collected lichen species were identified as *Parmelia perlata* (Rajnegi and Gadgil, 1996) (Figure 1).

b) Source of microorganisms

The organisms studied, *Escherichia Coli* (MTCC No.40), *Pseudomonas aeroginosa* (MTCC No.424), *Staphylococcus aureus* (MTCC No.87), *Proteus vulgaris* (MTCC No.742), *Streptococcus mutans* (MTCC No.497), *Bacillus subtilus* (MTCC No.441), *Staphylococcus epidermidis* (MTCC No.9041), *Micrococcus luteus* (MTCC No.106), Saccharomyces cerevisiae (MTCC No.170), *Candida albicans* (MTCC No.183) and *Candida tropicalis* (MTCC No.1000). The organisms were obtained from MTCC Chandigarh and maintain according to specifications. Sub culturing was done at an interval of 15 days.

c) Extraction of lichen

The collected lichen materials were brought to the laboratory, air-dried for three days, cleaned free of any other plant materials or mosses, and then washed under running tap water. They were oven-dried at 40°C for 42 h and grounded into powder by using a mixer. The powdered samples were stored in sterilized specimen bottles until when needed. Lichen constituents were extracted by cold extraction.

d) Cold extraction

10 g of lichen powder was added to 200 ml of acetone. The mixture was timed thoroughly by using a shaker water bath for five hours, then left at room temperature overnight and filtered using Whatman No. 1 filter paper. The filtrate was collected, and the solvent was removed using rotary evaporator and about 200 mg residues were recovered. The lichen powder that remained on the filter paper was dried and again extracted using 200 ml methanol. From this solvent, about 162 mg of residue was recovered [26].

e) Phytochemical Analysis [27]

Phytochemical analysis for the qualitative detection of alkaloids, glycosides, reducing sugar flavonoids, tannins, and saponins were performed with the extracts.

f) Determination of Antimicrobial Activity

The antimicrobial activity of the lichen extracts was determined using the agar well diffusion method[28] by following the known procedure. Briefly, Nutrient agar was inoculated with the given microorganisms by spreading the bacterial, and fungal inoculums on the media. Wells were made in the agar using the stainless steel borer of 8 mm and filled with 80, 120 and 160 μl of plant extracts. Control wells containing neat solvents (negative control) were also run parallel on the same plate. The plates were incubated at 37°C for 72 hours, and the antimicrobial activity was assessed by measuring the diameter of the zone of inhibition.

g) Evaluation for Anti-Urolithiatic Activity

Behind this activity, the idea was to know the role of plant extract in dissolving the already formed stones nucleus in the renal system. For this artificial calcium oxalate crystals were prepared in the laboratory [29] and semi-permeable membrane was prepared from the egg using standard methods [30-32].

Step-1: Preparation of experimental kidney stones (Calcium oxalate stones) by homogenous precipitation

1.47gm of calcium chloride dihydrate was dissolved in 100 ml distilled water, and 1.34 gm of sodium oxalate was dissolved in 100 ml of 2N H_2SO_4. Both solutions were mixed equally in a beaker to precipitate out calcium oxalate with stirring. An equimolar solution of calcium chloride dehydrate (AR) in distilled water and Disodium hydrogen phosphate (AR) in 10 ml of (2N H_2SO_4) was allowed to react in a sufficient quantity of distilled water in a beaker. The resulting precipitate was calcium phosphate. Both precipitates freed from traces of H_2SO_4 by ammonia solution. Washed the precipitates with distilled water and dried at 60 ^{0}C for 4 hours.

Step- 2: Preparation of semi-permeable membrane from farm eggs

The semi-permeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin & yolk. The apex of eggs was punctured by a glass rod to squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker consisting of 2 M HCl for an

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overnight, which caused complete decalcification. Further, washed with distilled water, placed in ammonia solution for neutralization of acid traces in the moistened condition for a while & finally rinsed with distilled water (Figure 2) and stored in the refrigerator at a pH of 7-7.4.

Step-3: Estimation of Calcium oxalate by Spectrophotometrically:

Group I: 1ml of calcium oxalate (1mg/ml) + 1ml of distilled water

Group II: 1ml of calcium oxalate (1mg/ml) + 1ml of Cystone solution

Group III: 1ml of calcium oxalate (1mg/ml) + 1ml of the cold extract of *Parmelia perlata*

All groups were packed together in egg semi-permeable membrane tied with thread at one end and were suspended in a conical flask containing 150 ml, 0.1 M Tris buffer each. At another end of thread tied by a stick placed on the mouth of the conical flask and covered with aluminum foil. All groups were kept in an incubator, pre heated to 37°C for 4 hours, kept for three days. The entire content of each group was removed from the sutured semi permeable membrane and was transferred into the test tube individually. 4ml of 1N H_2SO_4 and 60-80µl of 0.02M $KMnO_4$ were added and kept aside for 2 hours. Color change from dark pink to colorless was observed after 2 hours. The change of color intensity was measured against 620 nm spectrophotometrically. The concentration of undissolved calcium was determined from a standard calibration curve of calcium oxalate by using the measured absorbance readings.

h) Nucleation assay (Turbidity method):

The inhibitory activity of the extracts on the nucleation of calcium oxalate crystals was determined by a spectrophotometric assay. Crystallization was initiated by adding 100 µl of 4 mM calcium chloride and 100 µl of 50 mM sodium oxalate solutions to 0.5 ml of normal human urine, both prepared in a buffer containing 0.5 ml of 0.05 mM Tris buffer and 0.5 ml of 0.15mM NaCl solution at pH 6.5 and 37°C and adjusted to volume by adding 1.5 ml of distilled water. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the extract and that of the control with no extract. The optical density (OD) was recorded at 620nm, and the percentage inhibition calculated as (1-OD (experimental)/OD (control))/100.

i) Aggregation assay

The rate of aggregation of the calcium oxalate crystals was determined by a spectrophotometric assay with slight modifications. The calcium oxalate monohydrate (COM) crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate of 50 mM each. Both solutions were then equilibrated. The solutions were then cooled to 37°C and then evaporated. The COM crystals were then dissolved with 0.5ml of 0.05mM Tris buffer and 0.5 ml of 0.15mM NaCl solution at pH 6.5 to a final concentration of 1 mg/ml. Absorbance at 620 nm was recorded. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract against control.

III. Results

a) Qualitative Phytochemical Analysis

The present study reveals that lichen extract shows the presence of phytochemical constituents like alkaloids, flavonoids, carbohydrates, glycosides, proteins, saponins, tannins, terpenoids, reducing sugar and volatile oil in solvent extracts as shown in Table 1.

b) Antimicrobial Activity

The potential sensitivity of the extract was obtained against all the microorganisms tested, and the zone of inhibition was recorded. The results obtained were compared against standard antibiotic kanamycin and amphotericin B and presented below in the tabulation drawn (Table 2).

c) Estimation of Calcium oxalate

In kidney stones formation, calcium oxalate and calcium phosphate or other chemicals in the urine form crystals on the inner surfaces of kidneys. This stage is called an initial mineral phase formation. Over the period, crystals may combine to form a small, hard mass called as stones, and the stage is referred to crystal growth. Calcium oxalate stones have been classified into two types, i.e., calcium oxalate monohydrate stones (COM) and calcium oxalate dihydrate stones (COD).

d) Spectrophotometric estimation of calcium oxalate

The extract of *Parmelia perlata* has greater capability to dissolve calcium oxalate as the foremost element for stone forming in urinary tract. Lower percentage indicates more potency in the dissolution of calcium oxalate crystals as shown in Table 3.

e) Nucleation assay

Urine supersaturation attributes to calcium oxalate particles crystallization within the urinary tract. This is a nucleation process where stone-forming salts begins to unite into clusters with the addition of new constituents. Cystone standard solution exhibited stronger inhibition activity than the extract of *Parmelia perlata* in the nucleation of calcium oxalate salts. As *in vitro* crystallization study was performed, since nucleation is an important first step for the initiation of crystals, which then grow and form aggregates, extract of *Parmelia perlata* inhibited crystallization by inhibiting the nucleation of calcium oxalate through disintegrating

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into smaller particles with increasing concentrations of the fraction. The results of nucleation assay confirmed that the extract contained nucleation-preventing agents (Figure 3).

f) Aggregation assay

Calcium oxalate crystals begin to grow, aggregate with other crystals and, retained in the kidney. This is an aggregation process that causes renal injury. The extract of *Parmelia perlata* inhibited formation of COD crystals slightly better compared to Cystone standard solution. COM has a stronger affinity with cell membranes; it may lead to become a higher potential risk for renal calculi formation. This is may be due to the high content of saponins present in *Parmelia perlata*. It has several polyphenolics, e.g., alkaloids, saponins, phenolics, flavonoids, and other phytoconstituents. Saponins are well known to have anti-crystallization properties by disaggregating the suspension of mucoproteins as crystallization promoters. The present investigation will be supportive as additional information to the scientific evidences regarding *in-vitro* studies (Figure 4). Since the mechanism of anti-urolitholytic activity in the extract is exactly unknown to date, the correlation between *in vitro* and *in vivo* studies should be further investigated to reveal the phytochemicals of the extract which are responsible for dissolving or disintegrating renal calculi and for knowing better understanding in the molecular mechanism of lithiolysis.

IV. Conclusion

The present study conclusively demonstrates that *Parmelia perlata* is a good source of various phytochemicals like alkaloids, flavonoids, carbohydrates, glycosides, saponins, tannins, Terpenoids. This study evaluates the antiurolithiatic and antimicrobial activity of methanolic extracts of *Parmelia perlata* Lichen. The work was performed by using an *in vitro* antiurolithiatic model for calculating the percentage dissolution of kidney stones. This study has given primary evidence for *Parmelia perlata* as the lichen which possesses lithotriptic property. From the result Tables, it is also clear that a positive correlation exists between individual extracts and concentration used, in the study. Out of four concentration used we can observe that activity increase as we increase the concentration and, at one point, further no increase in the activity observed. The plant used in the above study also showed good activity when it was compared with the standard drug cystone. The methanolic extract found to be more potent in terms of activity and the authors of the above work recommends the lichen extract for further studies by conducting the *in vivo* model.

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Figure 1: *Parmelia perlata* Lichen



Figure 2: Experimental Egg membrane

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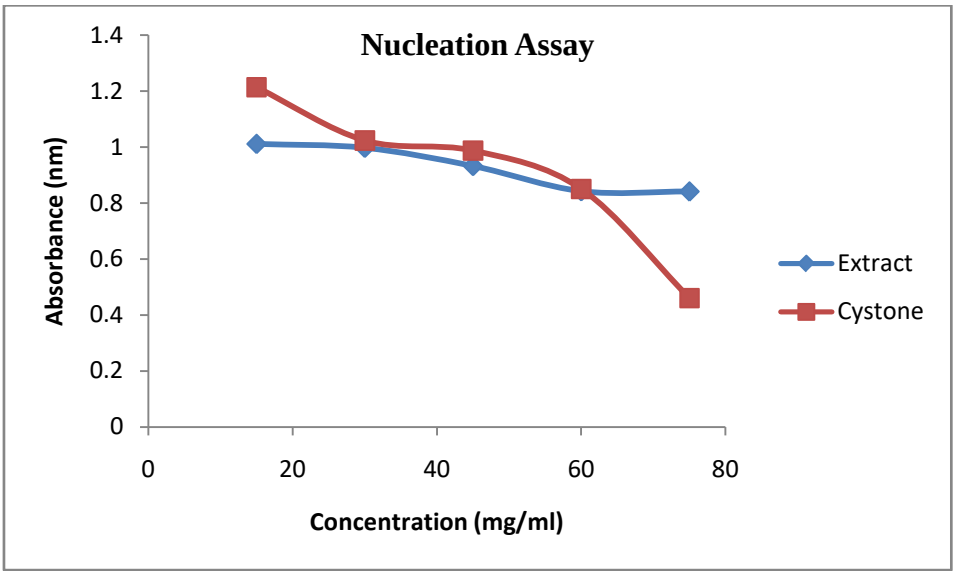


Figure 3: Nucleation assay showed that the various concentration of *Parmelia perlata* lichen extract and Cystone against absorbance at 620nm

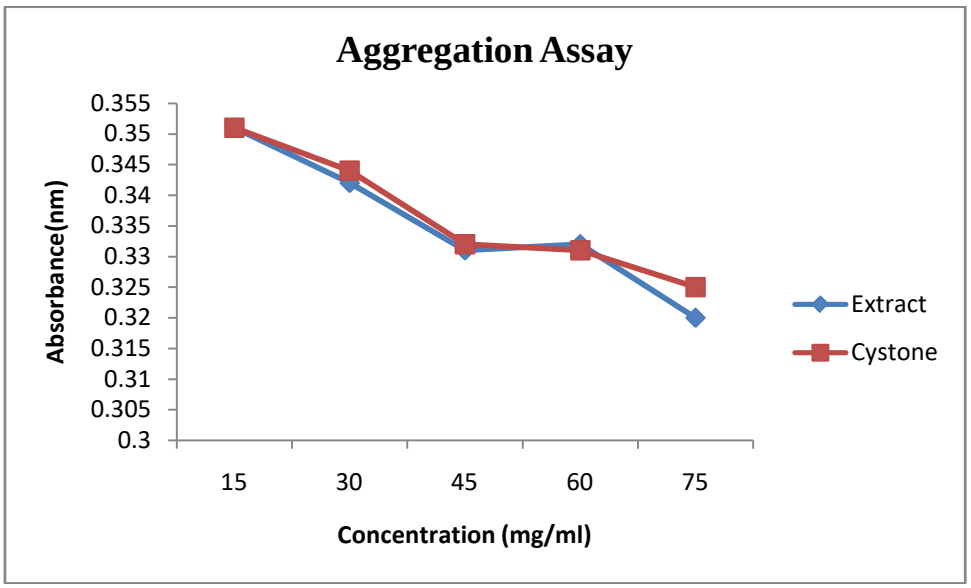


Figure 4: Aggregation assay showed that the various concentration of *Parmelia perlata* extract and Cystone against absorbance at 620nm

Table 1: Phytochemical constituent Present in Extracts

Extract	Alkaloid	Flavanoids	Saponin	Terprnoid	Tannin	Glycoside	Reducing sugar	Volatile oil
Methanolic	+	+	+	+	+	+	+	+

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Table 2: Zone of inhibition of extract and standard

Tested micro organism	Zone of Inhibition ± SD			
	Kanamycin for anti bacterial And Amphotericin B for anti fungal (20 mcg/ml)	80 mcg/ml	120 mcg/ml	160 mcg/ml
E.coli	18 mm ±0.23	10 mm±0.15	13 mm±0.11	15 mm±0.15
S.aureus	21 mm±0.11	13 mm±0.02	14 mm±0.24	18 mm±0.33
P.vulgaris	26 mm±0.16	-	13 mm±0.27	19 mm± 0.31
P.aerouginosa	14 mm±0.32	08 mm± 0.12	11 mm±0.41	15 mm±0.38
B.subtilis	19 mm±0.41	-	-	10 mm±0.21
S.epidermidis	20 mm±0.11	-	10 mm±0.72	16 mm±0.11
M.luteus	15 mm±0.56	-	-	10 mm±0.11
S. cerevisiae	20 mm±0.48	-	15 mm±0.71	17 mm±0.34
C. albicans	17 mm±0.52	-	10 mm±0.19	14 mm±0.36
C. tropicalis	24 mm±0.67	-	15 mm ±0.61	19 mm±0.38

Table 3: Dissolution of calcium oxalate

Groups	Mean ± SD	Weight of calcium oxalate reduced	Dissolution Percentage
Group I	0.130 ± 0.22		
Group II	0.076± 0.32	0.054	42
Group III	0.085± 0.11	0.045	35

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