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Research Article

ISOLATION OF PHYTOCONSTITUENTS AND *IN VITRO* ANTILITHIATIC BY TITRIMETIC METHOD, ANTIOXIDANT ACTIVITY BY 1,1- DIPHENYL -2- PICRYL HYDRAZYL SCAVENGING ASSAY METHOD OF ALCOHOLIC ROOTS & RHIZOMES EXTRACT OF *HEDYCHIUM CORONARIUM J.* KOENIG PLANT SPECIES

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ABSTRACT

Objective: The herbal medicines do not have any side effect and it is cost effective and safest medicine from an ancient periods. Urolithiasis or Lithiasis is a consequence of complex physical processes. The major factors are supersaturation of urine with the offending salt and crystallization. Crystals retained in kidney can become nucleus for stone formation. This process is synonymously known as Urolithiasis or Lithiasis or Nephrolithiasis or Kidney stones or renal calculi. Calcium-containing stones are the most common comprising about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%). In this study, ethanolic & aqueous extracts of roots and rhizomes of *Hedychium coronarium* J. Koenig plant were evaluated for their potential to dissolve experimentally prepared kidney stones like calcium oxalate by titrimetic method with an invitro model and Antioxidant activity performed by DPPH scavenging assay method. Phytoconstituents were also isolated by chromatographic techniques of this plant species.

Method: For performing invitro antilithiatic activity titrimetic method was adopted and for antioxidant activity 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging assay method was adopted. Phytoconstituents were isolated by column and thin layer chromatographic techniques.

Results & Conclusion: Ethanolic roots & rhizomes extract of this plant produced highest dissolution of stones when compare to standard drug cystone and at 10 mg. concentration. Also this study showed alcoholic extract of roots & rhizomes of *Hedychium coronarium* J. Koenig plant in higher concentration possess best antioxidant potential as compare to standard ascorbic acid with IC50 value 9.0 and 18.9 µg/ml. for ascorbic acid and alcoholic extract respectively. Isolated phytoconstituent from alcoholic extract of this plant was 8a, hydroxy hedychilactone and its structure was confirmed by IR, NMR and Mass spectroscopic datas.

Keywords: *Hedychium coronarium J*. Koenig, Urolithiasis, Kidney stones, Calcium oxalate, Antioxidants, 1,1- diphenyl-2-picryl hydrazyl etc.

INTRODUCTION

Lithiasis or Urolithiasis - is the medical term used to describe stones occurring in the urinary tract. Other frequently used terms are urinary tract stone disease and nephrolithiasis. Kidney stones may contain various combinations of chemicals. The most common type of stone contains calcium in combination with either oxalate or phosphate. These chemicals are part of a person's normal diet and make up important parts of the body, such as bones and muscles. A less common type of stone is caused by infection in the urinary tract [1]. This type of stone is called a struvite or infection stone. Another type of stone, uric acid stones, are a bit less common, and cystine stones are rare. For unknown reasons, the number of people in the United States with kidney stones has been increasing over the past 30 years. In the late 1970s, <4% of the population had the stone-forming disease [2]. By the early 1990s, the portion of the population with the disease had increased to more than 5%. The prevalence of kidney stones rises dramatically as men enter their 40s and continues to rise into their 70s. For women, the prevalence of kidney stones peaks in their 50s. Once a person gets more than one stone, other stones are likely to develop [3,4].

Kidney stones often do not cause any symptoms. Usually, the first symptom of a kidney stone is extreme pain, which begins suddenly when a stone moves in the urinary tract and blocks the flow of urine. Generally, a person feels a sharp, cramping pain in the back and side in the area of the kidney or in the lower abdomen. Sometimes nausea and vomiting occur [5,6].

Kidney stone formation is a complex process that results from a succession of several physicochemical events including supersaturation, nucleation, growth, aggregation, and retention within the kidneys. The incidence of urolithiasis is more in men (recurrence rate is 70-80%) then women (47-60%). Many remedial measures have been employed during ages to treat this condition. Most of them were from plants and proved to be useful. However, the rational behind their use is not well established except for a few plants and are reported to be effective with no side effects [7,8].

Antioxidants play an important role as health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease. Primary sources of naturally occurring antioxidants are whole grains, fruits, and vegetables. Plant sourced antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, etc. have been recognized as having the potential to reduce disease risk. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties [9]. A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity [10,11].

Hedychium coronarium J. koenig plant (Zingiberaceae)- is a small herbaceous plant. It is hard, perennial, erect, branched, annual weed up to 3-6 feet height. The leaves are simple arranged in an alternate manner with undulate margin [12]. The flowers are white in color and have pleasant fragrance; summer flowering; fall flowering. The trunk is green in color, very thick. It is widely distributed over the tropical and subtropical region of the Asia and Africa. It is an annual branching

herb which grows well on wastelands and in the tropical region after the rainy season. It contains many bioactive compounds including saponins, glycosides, flavonoids, fats and volatile oil [13-16].

This plant has been reported to possess antilithiatic and antioxidant properties. Hence, this study has been undertaken to evaluate *H. coronarium* J. koenig plant for their possible potential to dissolve experimental urinary stones/calcium oxalate using a modified *in vitro* model and antioxidant action by DPPH scavenging method [17-19].

METHODS

Plant material

H. coronarium J. koenig plant species along with roots and rhizomes (underground part) was collected in the month of May-June 2013 from the botanical garden of Shantikunj-Haridwar (Uttarakhand) India. Then, this plant was identified and authenticated from Botanical survey of India, Dehradun (Uttarakhand) under accession number - 114682.

Extraction

Shade dried underground parts of this plant were pulverized and about 100 g of powdered roots and rhizomes were extracted with increasing order of polarity solvents series starting from pet ether, chloroform, ethanol via soxhlet apparatus by successive hot continuous percolation method. Simultaneously, the same amount of the root part of this plant species was also macerated separately in chloroform water for preparing aqueous extracts for 72 hrs. At last, all extracts were concentrated in a rotary flash evaporator and the residue were dried in a desiccator over sodium sulfite.

Evaluation for antilithiatic activity (*in vitro*)

All four extracts *viz.* aqueous, pet. ether, chloroform and ethanol of root and rhizome parts of this plant were evaluated for antilithiatic potential by an *in vitro* model using calcium oxalate stones. Formulation cystone was used as a reference standard.

Preparation of experimental kidney stones (calcium oxalate stones by homogenous precipitation method)

Equimolar solution of calcium chloride dihydrate in distilled water and sodium oxalate in 10 ml of (2N sulfuric acid) were allowed to react in sufficient quantity of distilled water in a beaker. The resulting precipitate was calcium oxalate. The precipitate was freed from traces of sulfuric acid by Ammonia solution, washed with distilled water and dried at 60°C for 4 hrs.

Preparation of semi-permeable membrane from farm eggs

The semi-permeable membrane of eggs lies between the outer calcified shell and the inner contents such as albumin and yolk. Shell was removed chemically by placing the eggs in 2M HCl for an overnight, which caused complete decalcification. Further, washed with distilled water, and carefully with a sharp pointer a hole is made on the top and the contents squeezed out completely from decalcified egg. Washed thoroughly with water and stored in refrigerator at a pH of 7-7.4

Estimation of calcium oxalate by titrimetry

Exactly 1 mg of calcium oxalate and 10 mg of the extract/standard were weighed and packed it together in semi-permeable membrane by suturing. This was allowed to suspend in a conical flask containing a 100 ml 0.1 M TRIS buffer. One group served as negative control (contained only 1 mg of calcium oxalate). Now, the conical flask of all groups in an incubator were placed, preheated to 37°C for 2 hrs, for about 7-8 hrs and then, removed the contents of the semi - permeable membrane from each group into a test tube. It was added 2 ml of 1 N sulfuric acid and titrated with 0.9494 N $KMnO_4$ till a light pink color end point obtained (1 ml of 0.9494 N $KMnO_4$ equivalent to 0.1898 mg of calcium) [7,20-22].

The amount of undissolved calcium oxalate is then subtracted from the total quantity used in the experiment in the beginning, to know how much quantity of calcium oxalate actually the test substance could dissolve.

Evaluation of antioxidant activity by DPPH radical scavenging method

The free radical scavenging activity of different extracts of roots and rhizomes of *H. coronarium* plant were measured by DPPH. In brief, 0.1 mM solution of DPPH in ethanol was prepared. This solution (1 ml) was added to 3 ml. of different extracts in ethanol at different concentration (5, 10, 15, 20, 25, 30 µg/ml). The mixture was shaken vigorously and allowed to stand at room temp for 30 minutes. Then, absorbance was measured at 517 nm. by using spectrophotometer (UV-VIS Shimadzu). Reference standard compound being used was ascorbic acid and experiment was done in triplicate. The IC50 value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using Log dose inhibition curve. The Lower absorbance of the reaction mixture indicated higher free radical activity. The percent DPPH scavenging effect was calculated by using following equation:

DPPH scavenging effect (%) or percent inhibition = $A_0 - A_1/A_0 \times 100$

Where A_0 was the absorbance of control reaction and A_1 was the absorbance in presence of test or standard sample [23,24].

Isolation of phytoconstituent

To perform this procedure first of extraction of given grounded plant material was done by hot continuous successive percolation method using soxhlet apparatus and finally obtained alcoholic extract was concentrated and prepared as a slurry for column chromatographic technique. This extract was used for isolation of phytoconstituents because it has a lot of potentials [25-27]. Then, column and thin layer chromatographic techniques were followed, and the column was run by various ratios of n-hexane and ethyl acetate. Finally, on isolated samples in sufficient quantity various spectroscopic techniques *viz.*: Infrared (IR), C13 nuclear magnetic resonance (NMR), 1H NMR and Mass were applied [28-30]. This spectroscopic study reveals the presence of hydroxyl derivative of hedychilactone in one of the above ratio.

The compound was isolated as a yellow semi-solid, compound revealed a molecular ion peak corresponding to (M+H) at *m/z* 331. 2 indicating the molecular formula $C_{20}H_{26}O_4$. The 1H NMR spectrum of the compound showed all the features of labdane diterpene. The IR spectrum displayed absorption bands at 3400 cm^{-1} (OH), 1681 cm^{-1} (α, β-unsaturated C=O) and 1709 cm^{-1} (α, β- unsaturated γ-lactone). The 1H NMR spectrum displayed four quaternary methyl signals each integrating for three protons as singlets at δ 0.78, δ 0.99, δ 1.03, and δ 1.74. It has displayed a singlet at δ 2.86 for one proton (H-9) indicating the presence of one methine adjacent to the carbonyl (C-7) carbon atom. A sharp singlet integrated for 1 H at δ 5.88 is due to methine proton (H-14) in the lactone ring. A characteristic doublet for one proton at δ 2.97 (d, *J*=7.0 Hz) indicating the presence of methine (H-9) group adjacent to olefinic double bond. The presence of one trans double bond at δ 5.83 (1H, d, *J*=10.84) and δ 5.79 (1H, d, *J*=10.92Hz) was suggested by the 1H NMR. Another sharp singlet integrated for two protons at δ 4.69 (2H, s) is assigned to CH_2 group in the lactone ring, the 1H NMR spectrum also revealing that the trans double bond (C-11/C-12) is conjugated with lactone ring.

The 13C NMR spectrum of compound C showed the presence of 20 C-atoms. The 13C NMR spectrum indicated the presence of α, β-unsaturated ketone (δ 207.49), trisubstituted olefin (δ 143.86 and 156.10) and four methyl signals (δ, 33.57, 31.91, 19.33 and 21.87). Further, it also displayed signal at δ 167.84 is due to C=O of lactone ring, δ 128.83, 128.27 are corresponding to disubstituted olefin, and δ 69.71 is assignable to a methylene carbon in the lactone ring.

Characterization of isolated phytoconstituent

- Physical state of constituents: Semi-solid
- R_f value: 0.82 (n-hexane: Ethyl acetate in 85:15 ratio)
- Color: Yellowish red.

Spectral characteristics of isolated compound

1HNMR interpretation

Position	δH multiplicity
C1	1.51, 1, 55 (2H, m)
C2	1.57, 1.28 (2H, m)
C3	0.78-0.99 (6H, m)
C4	2.18 (1H, s)
C6	1.59-1.47 (2H, m)
C8	1.74 (3H, m)
C9	2.86 (1H, s)
C11	5.76 (1H, d)
C12	5.79 (1H, d)
C13	6.46 (1H, d)
C16	4.11-4.95 (1H, s)

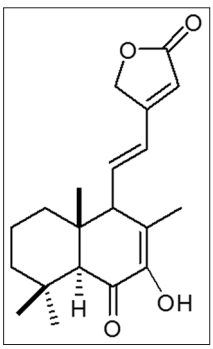
^{13}C NMR ($CDCL_3$) can be interpreted as

Position	Δc (ppm)
C1	19.33
C2	39.77
C3	33.5
C4	55.85
C5	22.91
C6	39.7
C7	207.49
C8	132.49
C9	128.1
C10	38.7
C11	130.93
C12	128.80
C13	167.84
C14	83.74
C15	167.84
C16	72.80
C17	33.5
C18	33.5
C19	14.05
C20	19.33

The IR spectra (Nujol oil) showed α and β unsaturated lactone ring and α - β unsaturated C=O bonds

Serial number	Peak (cm^{-1})	Interpretation
1	721.84	Monosubstituted (C-H)
2	1072	C-O str.
3	1376.91	C-H bending
4	1460	N-H bending
5	1681.96	α -β unsaturated C=O
6	1709.19	α -β unsaturated lactone
7	3400	O-H

Proposed structure of isolated constituents depending upon interpretation of IR, NMR both C13 and 1H spectral analysis:



It seems 8a, hydroxy hedychilactone. Attempts were also made to isolate some more compounds like 8a, ethoxy derivative of this compound, etc.

RESULTS

The alcoholic extract obtained by roots and rhizomes of this plant at 10 mg concⁿ produced the highest dissolution of calcium oxalate stones in comparison to other extracts. Cystone was to be equally effective found when compared to the alcoholic extract. Likewise, alcoholic extract of this part showed better antioxidant action on higher concentration as compare to standard ascorbic acid with absorbance 0.0390 and 0.0715 for ascorbic acid and alcoholic extract and IC 50 value were 9.0 and 18.9 µg/ml. for ascorbic acid and ethanolic extract respectively and isolated well-interpreted phytoconstituent was 8a, hydroxy hedychilactone from 85:15 ratio of n-hexane and ethyl acetate. Its structure was confirmed by spectral analysis (Tables 1-3, Graphs 1 and 2).

DISCUSSION

This study evaluates antilithiatic activity *in vitro* of different extracts of *H. coronarium* J. Koenig roots and rhizomes and a standard drug cystone. From the study results, it is observed that ethanolic roots and rhizomes extract produced the highest dissolution of calcium oxalate stones in comparison to other extract. This study has given primary evidence that this plant possess lithontriptic property. This *in vitro* study has given lead data, and shown that ethanolic root extract is quite promising for further work in this regard. Likewise, the ethanolic extract also showed best antioxidant activity by DPPH radical scavenging method as compared to standard ascorbic acid. Meanwhile a phytoconstituent was also isolated by column chromatography of ethanolic extract of this plant species known as 8a, hydroxy hedychilactone.

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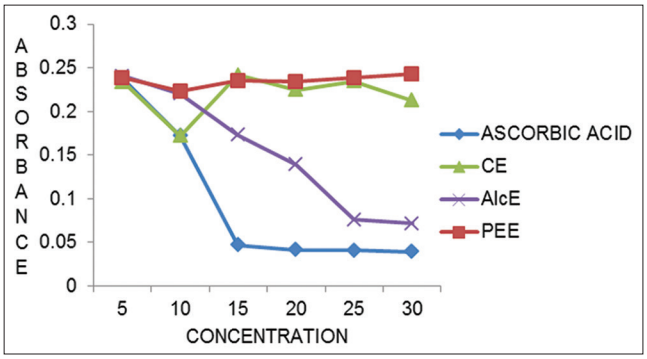
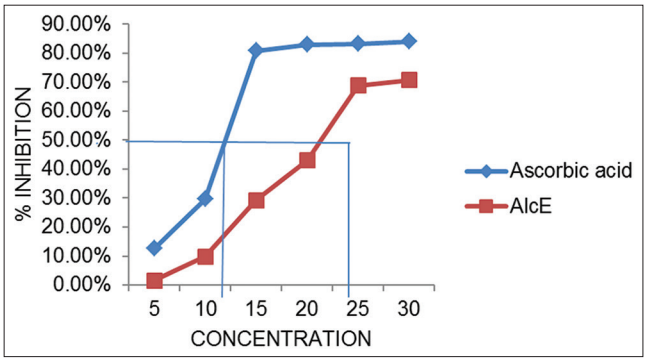


Graph 1: absorbance in different concentration



Graph 2: % inhibition in different concentration

Table 1: Calcium oxalate dissolution by extracts and cystone

Group	Volume of standard $KMnO_4$ (ml)	Weight of calcium estimated (mg)	Weight of calcium reduced (mg)	Percentage dissolution (%)
Control	4.6	0.8730	-	-
Standard (cystone)	2.4	0.4555	0.4175	47.82
Alcoholic extract of roots and rhizomes	2.6	0.4934	0.3796	43.48
Aqueous extract of roots and rhizomes	2.9	0.5504	0.3225	36.94

Correspond to 10 mg

Table 2: Absorbance of different extract of *H. coronarium* J. Koenig with ascorbic acid

Concentration (µg/ml)	Ascorbic acid (Abs)	PEE (Abs)	$CHCl_3$E (Abs)	AlcE (Abs)
5	0.2380	0.2386	0.2343	0.2405
10	0.1719	0.2232	0.1720	0.2204
15	0.0469	0.2350	0.2420	0.1729
20	0.0415	0.2341	0.2245	0.1393
25	0.0410	0.2386	0.2348	0.0761
30	0.0390	0.2428	0.2128	0.0715

Average control reading: 0.2444, % inhibition=$(Abs_{control}-Abs_{test})/Abs_{control}\times100$

Table 3: % inhibition of different extract of *H. coronarium* J. Koenig

Concentration (µg/ml)	Ascorbic acid (% inhibition)	PEE (% inhibition)	$CHCl_3$ E (% inhibition)	AlcE (% inhibition)
5	2.61	2.37	4.13	1.59
10	29.66	8.67	29.62	9.81
15	80.8	3.84	0.98	29.25
20	83.01	4.21	8.14	43
25	83.22	2.37	3.92	68.86
30	84.02	0.65	12.92	70.7

H. coronarium: Hedychium coronarium

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