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Isolation, Characterization and In vitro Antiurolithiatic activity of Cerpegin Alkaloid from *Ceropegia bulbosa* var. Lushii root

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Abstract

Ceropegia bulbosa var.*lushii* also known as Kadula, Haudlo, Khappar-kaddu, Bhuu-tumbi, Gilodya and Patalatumbi in local language, family Asclepiadaceae. The tuberous roots are used to treat several diseases like diarrhoea, dysentery, analgesic, antipyretic, kidney stone and other activities. The tuberous root contains steriods, polyphenols, fats, albuminoids, sugars, potassium and active constituent an alkaloid cerpegin.The present investigation was carried out to isolate, purify and characterize cerpegin from root of *Ceropegia bulbosa* var.*lushii*. The isolated cerepegin was further characterized with the help of Ultraviolet Spectroscopy, Thin Layer Chromatography, Attenuated Total Reflectance Spectroscopy, Mass Spectroscopy, Proton- Nuclear Magnetic Resonance Spectroscopy confirmed the identification. The antiurolithiatic activity of cerpegin was evaluated by using modified invitro model. The isolated compound cerpegin (A1) was showed maximum dissolution of both types of stones (calcium oxalate and calcium phosphate) in comparison to the all extract tested. Cystone was found to be more effective.

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Key words:
Antiurolithiatic activity, *Ceropegia bulbosa,* Cerpegin alkaloid, Spectroscopy, Calcium oxalate, Calcium phosphate.

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Introduction:

In developing countries-all over the world-80% of population continues to use traditional medicine in

primary medical problems. In the past decade, therefore, research has been focused on scientific evaluation of traditional drugs of plant origin. *Ceropegia bulbosa* is one such plant that has been used as medicine by tribal communities. Khadulo consists of dried tuberous root of *Ceropegia bulbosa Var. (Lushii)*. Family: Asclepiadaceae. It is twining herb and collected in July-October [1]. Active principle of tuberous roots contains an alkaloid cerpegin (Fig.1) which is active against diarrhoea, dysentery, kidney stone and other activities [3]. It is potentiated pentobarbitone hypnosis and exhibited analgesic and diuretic activities. The raw tubers are eaten by tribal ladies to promote fertility and vitality [4, 5, 6]. The paste of seeds is dropped in the ear to cure deafness. Decoction of tuber is taken orally to get rid of urinary bladder stone [7].

There is an urgent need to systematically evaluate the plants used in traditional medicine. Such research could lead to new drug discovery or advance the use of indigenous herbal medicines for orthodox treatment. Now a day a renewed interest in traditional medicine is observed and there has been an increasing demand for more and more drugs from plant sources. This revival of interest in plant derived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than the costly synthetic drugs many of which have adverse side effects. Therefore an attempt has been made to be isolated, purified, characterizes & to find out antiurolithiatic activity of cerpegin from *Ceropegia bulbosa* Var. Lushii root.

Material and Methods

Plant materials

Fresh plants of *Ceropegia bulbosa var.* Lushii were collected in the month of September, from local forests of the from Pali district (Jodhpur), Rajasthan and were authenticated by P. M. Padhye, Scientist 'E' and Head of office, Botanical survey of India, Jodhpur, Rajasthan, India. A herbarium specimen bearing voucher No. JNU/PH/2010/$Cb_{LE}C_2$ has been deposited in the Department of Pharmacognosy, Jodhpur Pharmacy College, Jodhpur, Rajasthan, India.

Isolation of cerpegin from *Ceropegia bulbosa* Var. Lushii root[8]

Shade-dried and coarsely powdered of tuberous root (850g) was exhaustively extracted with n-hexane, ethanol (90%) and water by a cold maceration method (72 hr). Ethanol (90%) extract was concentrated in vaccum to a syrupy mass and treated with H_2SO_4 (2%). The aqueous acidic extract was cooled and basified with aqueous NH_3 to pH 10 and extracted with $CHCl_3$ (3 x 200 ml). The $CHCl_3$ phases were bulked, dried over Na_2SO_4, concentrated and kept in refrigerator for overnight.White crystals formed, the crystals were recrystallized by dissolving in 90% ethanol.The substance thus obtained was referred to here as cerpegin (300mg).

Characterization of Cerpegin

Melting point

Melting point of isolated compound was determined by capillary method.

Chemical Test

Dragendroff's test:

To extract added few drops of Dragendroff's reagent, reddish brown precipitate is produced.

TLC identification test

Stationary Phase : Silica gel G
Mobile Phase : Chloroform: methanol:
Ammonia (aqueous) : (80: 40:1.5)
Chamber Saturation : 30 minutes
Test sample : n-hexane, ethanolic, water and isolated compound extract
Spraying reagent : Dragendroff' reagent and Iodine Chamber.

U.V. spectrum of Cerpegin

A UV spectrum was recorded on Model CECIL-7400, UV-VIS Spectrophotometer between wavelengths

400 to 200 nm. By taking ethanol as blank and preparing 100 µg ml^{-1} solution of Cerpegin in 90% ethanol.

ATR Spectrum of Cerpegin

ATR Spectra of isolated compound was recorded in BRUKER-ATR α-T, (Opus software), Jodhpur Pharmacy College, Jodhpur. 10 mg of cerpegin was accurately weighed and dried under IR lamp for 40-45 min. The sample was placed on zinc selanide crystal of ATR with help of spatula and scanned at 4000- 600 cm^{-1}.

1H-NMR and ^{13}C NMR of Cerpegin

^{1}H- NMR and ^{13}C NMR Spectra of isolated compound in (D2O) was recorded by employing BRUKER-AVANCE II 400 NMR SPECTROMETER SAIF, Punjab University, Chandigarh.

Mass Spectrum of Cerpegin

MS analysis of isolated compound was recorded by MALDI-TOF MS, M/Z (REL. INT. BASED) Punjab University, Chandigarh. The compounds were identified by comparison of their mass spectra with the published mass spectra.

In vitro Antiurolithiatic activity of cerpegin and root extracts

Estimation of Calcium oxalate by titrimetry[9,10]

Preparation of Calcium oxalate by homogenous precipitation

By taking equimolar solution of Calcium chloride dihydrate (A.R) was dissolved in distilled water and Sodium oxalate (A.R) was dissolved in 10 ml of 2N H_2SO_4 and distilled water, sufficient quantity is allowed to react in a beaker. The resulting precipitate was calcium oxalate which was freed from traces of sulfuric acid by ammonia solution .Washed with distilled water and dried at a temperature 60 °C for 4 hours.

Preparation of the Semi permeable membrane from farm eggs

The outer calcified shell was removed chemically by placing the eggs in 2 ml HCL for 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 the decalcified egg. Washed thoroughly with distilled water and placed it in ammonia solution, in the moistened condition for a while and then rinsed it with distilled water. Stored in refrigerator at a pH of 7- 7.4.

Method

Weighed exactly 1 mg of the calcium oxalate and 10 mg of the n-hexane extract, ethanolic extract, water extract, isolated compound and standard cystone were packed it together in semi permeable membrane by suturing as shown in Model design (Fig 2).

They were allowed to suspend in a conical flask containing 100 ml 0.1 M TRIS buffer. One group served as negative control (contained only 1 mg of calcium oxalate). Placed the conical flask of all groups in a incubator, pre heated to 37° C for 2 hours, for about 7-8 hours. Removed the contents of semi permeable membrane from each group into a test tube. Added 2 ml of 1 N sulfuric acid and titrated with 0.9494 N $KMnO_4$ till a light pink color end point obtained. 1ml of 0.9494 N $KMnO_4$ equivalent to 0.1898 mg of Calcium. Percentage dissolution of calcium oxalate by various groups is shown in (Table 1).

Estimation of Calcium phosphate by colorimetry[11]

Preparation of Calcium phosphate by homogenous precipitation

By taking equimolar solution of Calcium chloride dihydrate (A.R) dissolved in distilled water and Disodium hydrogen phosphate (A.R) dissolved in 10 ml of (2N H_2SO_4) and distilled water.

The resulting precipitate was calcium phosphate which was freed from traces of sulfuric acid by

ammonia solution .Washed with distilled water and dried at a temperature 60° C for 4 hours.

Preparation of the Semi permeable membrane from farm eggs

This is prepared in the same way as described earlier.

Preparation of Molybdate-sulphuric acid reagent

This was prepared by mixing 2 parts of 5% w/w solution of Sodium molybdate (A.R), 1 part of 10 N sulfuric acid and 1 part of distill water.

Preparation of Reducing solution

1 g. of *p*-Phenylene diamine was dissolved in 100 ml of 3 % w/w solution of Sodium bisulfite to get the required solution.

Method

Weighed exactly 1 mg of the calcium phosphate and 10 mg of the n-hexane extract, ethanolic extract, water extract, isolated compound and standard cystone were packed it together in semi permeable membrane by suturing.

This was allowed to suspend in a conical flask containing 100 ml 0.1 M TRIS buffer. One group served as negative control (contained only 1 mg of calcium phosphate). Placed the conical flask of all groups in incubator, pre heated to 37° C for 2 hours, for about 7-8 hours. Removed the contents of semi permeable membrane from each group into a test tube. Added 2 ml of 1 N sulfuric acid, 2.5 ml of Molybdic-sulphuric acid reagent, 1 ml of reducing solution and made up the volume to 10 ml using distill water. Standard dilutions of calcium phosphate were prepared, (200, 400, 600, 800 and 1000 µg/ml) containing 2.5 ml of Molybdic-sulphuric acid reagent, 1 ml of reducing solution and made up the volume to 10 ml using distilled water respectively. Measured the optical density of standard dilutions and for the groups under study in colorimeter using the Filter no.67 (Table 2). The undissolved calcium phosphate was determined from the standard calibration curve by extrapolation (Fig3). The results of the various groups interpretated as percentage dissolution are shown in (Table 3).

Result and discussion

Plant drugs are now receiving great attention for their therapeutics and because of this extensive research are now being carried out in this area. However, herbal drugs being a complex mixture of several phytoconstituents, it becomes difficult to decide that which component is responsible for activity. The isolation of the various constituents also is a tedious process. Characterization of cerpegin was done by Melting Point, UV, TLC, ATR, MS, 1H-NMR. Percent yield was 0.3 of the dried roots, white-creamish in colour and soluble in water. Giving positive Dragendroff's test. TLC of cerpegin with solvent system Chloroform: methanol: Ammonia (aqueous) (80: 40:1.5) showed Rf value 0.66 (Fig 4).

M.p.: 234-236°C (product decomposed), ATR Cm^{-1}: 1742.46 Cm^{-1} (carbonyl of lactone part), 1647.14 Cm^{-1} (carbonyl of lactam ring), 3050-3150 Cm^{-1} (aromatic CH stretching), 1456 Cm^{-1} (C=C stretching-aromatic), 2900 Cm^{-1} (CH stretching of three CH_3 groups).$UV_{\lambda\ max}$nm: 209.1. ^{1}H NMR: Taken AT 400 MHz δ 1.65-1.59 (s, 6 H, 2 CH_3 Groups of lactone side ring), δ 3.7 (d, 3H, CH_3 of cyclic amide), δ 7.2 (s, 1H, J=9.2Hz, H_a of aromatic ring), δ 8.1 (s, 1H, J=9.3 Hz, H_b of aromatic ring).

^{13}C NMR Interpretation: TAKEN AT 400 MHz δ 32-33 (s, 2C, two Methyl of lactone ring), δ 40 (CH_3 of amide attached part), δ 95.0 (Carbon of lactone ring from where two CH_3 attached), δ 170 (carbonyl group of lactone part), δ 121 (aromatic C of adjutant ring), δ 162 (aromatic C of adjutant ring) δ 155 (carbonyl of lactam ring/ cyclic amide part), δ 134 & 116 (2 C of aromatic region of lactam ring). $C_{10}H_{11}NO_3$ ($[M]^+$193) C=62.17%; H,=5.74%; N= 7.25%; O= 24.84% , MALDI-TOF MS, M/Z (REL. INT. BASED): 194.1 $[M+1]^+$, 164.9 $[M-(Me)_2+1]^+$, 179.05 $[(M-Me)+1]^+$.

Ethanolic extract, n-hexane extract, water extract and compound A1 were evaluated for antilithiatic activity

by modified invitro model. The compound A1 (at 10 mg concentration) showed maximum dissolution of both types of stones (calcium oxalate and calcium phosphate) in comparison to the all extract tested. However, it was more effective in dissolving calcium phosphate than calcium oxalate. Cystone was found to be more effective (Fig 5 and Fig 6).

Conclusion:

The present investigations provide useful information on antiurolithiatic activity of Cerpegin isolated from *Ceropegia bulbosa*. The isolated compound cerpegin (A1) was showed maximum dissolution of both types of stones (calcium oxalate and calcium phosphate) in comparison to the other all extracts. Further pharmacological and clinical studies are required to understand the mechanism and the actual efficacy of the cerpegin in treating various infections and diseases.

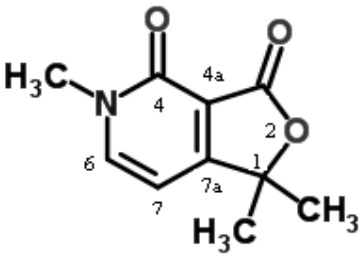

Fig 1: Structure of Cerpegin Alkaloid

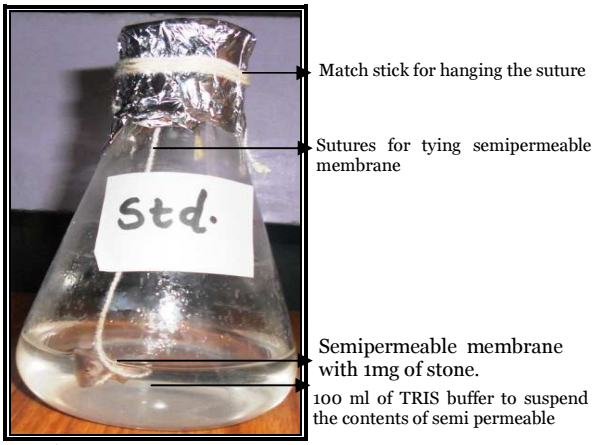

Fig 2: Invitro model to evaluate antiurolithiatic activity

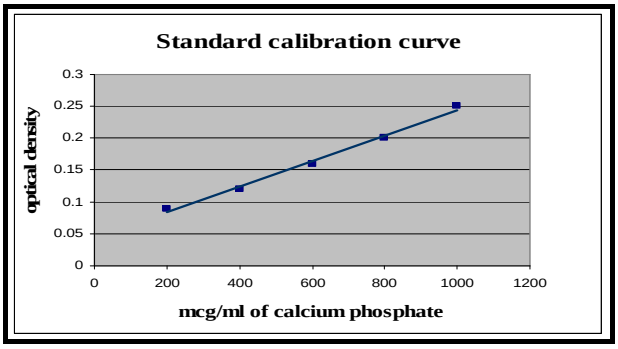

Fig 3: Histogram showing the standard calibration curve of calcium phosphate by colorimetry

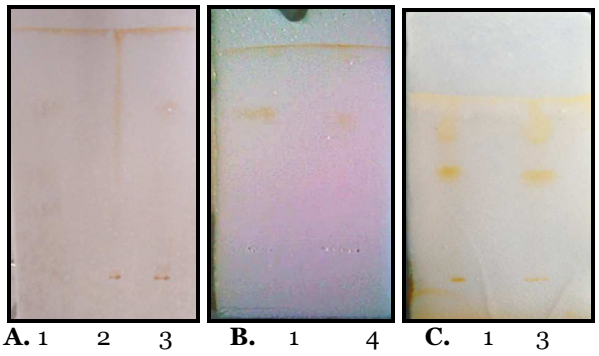

Fig 4: TLC of ethanolic, n-hexane, water extract and 4-isolated compound (A1)
A & B: After spraying with dragendroff's reagent. C: Iodine chamber.

Sample ID:

1- Ethanolic extract
2- n-hexane extract
3- Water extract

Isolated compound Cerpegin (A1)

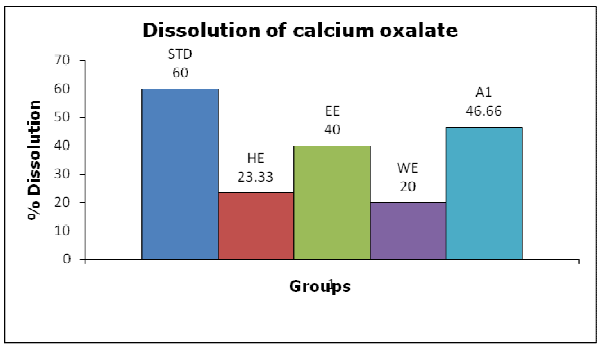

Fig 5: Percentage dissolution of calcium oxalate by various groups

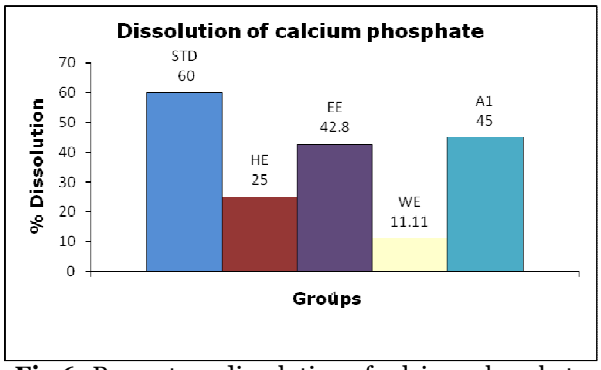

Fig 6: Percentage dissolution of calcium phosphate by various groups

Table: 1 Dissolution studies of Calcium oxalate by all extracts, A1 compound and Standard cystone

Group	Vol. of Standard $KMnO_4$(ml)	Wt. of Calcium Estimated	Wt. of Calcium Reduced	Percentage Dissolution
Negative*	3.0±0.1	0.5694	---	---
STD* (Cystone)	1.2 ±0.03	0.22776	0.34164	60.00
n-hexane*	2.3 ± 0.14	0.43654	0.13286	23.33
Ethanolic *	1.8 ± 0.6	0.34164	0.22776	40.00
Water *	2.4 ±0.01	0.45552	0.11388	20.00
A1* Compound	1.6 ± 0.41	0.30368	0.26572	46.66

(*Correspond to 10 mg)

Table 2: Dissolution studies of Calcium phosphate by by all extracts, A1 compound and Standard cystone

Standard	Molybdic-H_2SO_4 Reagent	Reducing solution	Distill water (q.s.)	Optical density
200 µg/ml	2.5 ml in each	1 ml in each	10 ml in each	0.09
400 µg/ml				0.12
600 µg/ml				0.16
800 µg/ml				0.2
1000 µg/ml				0.25
Groups	Molybdic-H_2SO_4 Reagent	Reducing solution	Distill water (q.s.)	Optical density
Negative*	2.5 ml in each	1 ml in each	10 ml in each	0.20
Standard* (Cystone)				0.08
Ethanolic extract *				0.14
Water extract*				0.17
n-hexane*				0.16
A1 compound*				0.09

Table 3: Dissolution studies of Calcium phosphate by all extracts, A1 compound and Standard cystone

Group	Wt. of Calcium Estimated	Wt. of Calcium Reduced	Percentage Dissolution
Negative*	0.20	---	---
STD* (Cystone)	0.08±0.04	0.12	60
n-hexane*	0.15±0.32	0.05	25
Ethanolic *	0.14±0.12	0.06	42.8
Water *	0.18±0.10	0.02	11.11
A1* Compound	0.11±0.06	0.09	45

(*Correspond to 10 mg)

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