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Determination of Total phenolic content and Antiurolithiatic activity of Sahasravatamulika (*Hibiscus cannabinus* Linn.) Extract and Fractions

Research Article

Sneha Wali[1], Sneha B Patil[2*], Mrityunjaya B Patil[3]

1. Research Scholar, 2.Assistant Professor, 3. Vice-Principal, KLE College of Pharmacy Belagavi, KLE Academy of Higher Education and Research (KAHER), Belagavi. India.

Abstract

The purpose of this study was to assess the total phenol content and *invitro* antiurolithiatic activity of *Hibiscus cannabinus* Linn. extract and various fractions. Kidney stones are one of the most painful and prevalent urinary tract disorders. Drug treatment and Extracorporeal Shock Wave Lithotripsy (ESWL) are used to treat kidney stones. Instead of dissolving the stone, these treatments may cause acute renal damage and an increase in stone production. Phenolic compounds show the best stone dissolution rate, i.e., they could dissolve calcium oxalate stones. In the present research *Hibiscus cannabinus* Linn. phenolic fraction was tested for its capacity to dissolve calcium phosphate stones, which is one of the many forms of kidney stones that can form. The highest phenolic content was found in the Ethyl acetate fraction of the plant, which was assessed using Gallic acid as a standard. This fraction was then tested for Antiurolithiatic activity in an *in-vitro* manner employing the egg's semipermeable membrane. The fraction can be evaluated for additional *in-vivo* and *in-vitro* investigations to determine its ability to dissolve Kidney stones developed in humans.

Keywords: *Hibiscus cannabinus Linn.,* Kidney stones, Calcium phosphate stones, Total Phenolic content, Semipermeable membrane, Antiurolithiatic activity.

Introduction

Ayurveda, or "Science of Life," is an ancient Indian herbal medicine system that plays an essential role in treating and preventing diseases. Plants with medicinal properties could be used to generate novel herbal medicines. Herbal medicine treatments are the most common type of Traditional Medicinal system, and around 7500 plants are utilised in India's rural and tribal areas for treating and preventing ailments, and they continue to play an important part in the health-care system. Secondary metabolites in plants are active compounds that have therapeutic effects and are used as medicine or drugs. (1)

Urolithiasis is a one of the common health problems with increasing prevalence of up to 20% all over the globe. The increased prevalence of the disease is due to the lifestyle changes such as lower dietary intake of vegetables or fruit, higher consumption of animal proteins, salt, sweetened beverages, and inadequate fluid intake. Calcium oxalate stones are the most common type of nephrolithiasis which is most painful urinary tract illnesses in which calcium is combined with either oxalate or phosphate. (2,3)

*** Corresponding Author:**

Sneha B Patil

Assistant Professor,
Department of Pharmacognosy,
KLE College of Pharmacy Belagavi,
KLE Academy of Higher Education and Research (KAHER), Belagavi. Karnataka. India.
Email Id: snehabpatil11@gmail.com

Medicinal plants serve as a raw material for drugs which are effective, reasonable and safer health care for people. Many plant-based remedies have proven to be effective in treating the kidney stones. Some of the medicinal plant extracts and phenolic fractions examined had the highest stone dissolving rate. (4)

Hibiscus cannabinus Linn., sometimes known as Kenaf, belongs to Malvaceae family and Hibiscus Genus, grows in Tropical and temperate climates, with high water demanding crop. (5)

Plant contains many bioactive molecules such as phenolic compounds and phytosterols that have anti-oxidant, anti-inflammatory, anti-hypertensive, cardiac protecting, and anti-proliferative properties. Peelings from the stems are also known to be used as a hematinic medication to treat exhaustion and anaemia. It is utilised as a vegetable, blood tonic, and in the treatment of liver disease. (6)

There have been no cited scientific evidences supporting the *in-vitro* pharmacological activity of *Hibiscus cannabinus* Linn. against renal calculi till date. The purpose of this study was to examine effect of *Hibiscus cannabinus* Linn. extract and its various fractions for *in-vitro* urolithiasis using a semi-permeable egg membrane as a dissolving model. (7)

Materials and Methods

Materials

Methanol, Ethyl acetate, n-hexane, Gallic acid, Ethanol, Calcium chloride dihydrate, Disodium hydrogen phosphate, Sulphuric acid, Ammonia solution, Hydrochloric acid, Potassium permanganate, tris buffer

were purchased from HIMEDIA. Cystone was purchased from nearby Pharmacy.

Methods

Collection and processing of plant material

Aerial part of *Hibiscus cannabinus* Linn. was collected from local areas of Belagavi, Karnataka, in the month of September-October and authenticated by ICMR, Belagavi with plant authentication Id: RMRC-1587. The plant material was cleaned, shade dried and later pulverised into coarse powder and stored in air tight container until further use.

Plant material was studied for various Pharmacognostical parameters like Morphological, Microscopical, Physicochemical and Phytochemical parameters as per standard procedure. (8)

Morphological evaluation

Various morphological characters of the plant material were studied such as colour, odour, size, shape, and taste by organoleptic evaluation (6,9) The results are shown in Figure 1.

Powder microscopy

Different microscopical characters were studied by powder microscopy method using Trinocular microscope. (10,11) The images are shown in Figure 2.

Physicochemical evaluation

The physicochemical parameters like Determination of Total Ash content, Water soluble ash, Acid insoluble ash, Loss on drying, Extractive value were determined as per standard procedures. (8,12,13) The results are mentioned in Table no 1.

Determination of Ash content

Ash value is used to determine quality, purity and to establish identity of a crude drug. The sample was analysed for Ash content as per Standard procedures.

Ash content was calculated from the formula given below;

$$\% \text{ Ash content} = \frac{\textit{Weight of ash}}{\textit{Original weight of sample}} \text{ X } 100$$

Loss on Drying

The amount of moisture present in the crude powdered drug was determined by loss on drying method. 1.5gm of powdered plant material was weighed in a flat thin porcelain dish, dried in oven at 100^{o}C then cooled in desiccators and weighed. The loss in weight is recorded as moisture.

Extractive value

Extractive value was determined by standard procedure using 1gm of coarse powder soaking with three different solvents viz. Methanol, Pet. Ether and Water for 24hrs with constant shaking for first 2hrs and allowed to stand for rest of the time. After 24hrs extract was filtered and filtrate was evaporated and the yield was calculated.

Preparation of Extract and fractions

The dried plant powder was subjected for cold maceration as per given standard procedure using methanol as a solvent. Methanolic Extract was concentrated in rotary evaporator to get a crude extract and it is stored in an airtight container at room temperature. The crude extract was subjected for fractionation as per the procedure given . The n-hexane, ethyl aetate, and water fractions were all evaporated to dryness and stored in an airtight container until further analysis. (8,14) The yield of extract and fractions obtained are mentioned in Table no 2 and Figure 3.

Phytochemical analysis

The phytochemical analysis was performed for screening various Phytocomponents from the crude extract. Then fractions were screened for Phenolics and Flavonoids.(15) The results are shown in Table no 3 and 4.

Estimation of Total Phenolic content

Total phenolic content of the Crude extract and three fractions were determined using Folin-Ciocalteau reagent method. An aliquot of the extract and fractions was mixed with F.C. reagent and saturated sodium carbonate solution to make the reaction mixture. The reaction mixture was incubated at room temperature for 90 minutes and the absorbance was measured at 765nm. For each assay, the samples were produced in triplicates, and the mean value of absorbance was calculated. A standard solution of Gallic acid (20 µg/ml to 100 µg/ml) was used for standard calibration curve. (16,17) The results are given in Table no 5,6 and Figure 4. By determining Total Phenolic content it was found that Ethyl acetate fraction has highest Phenolic content thus it was used for further study.

Evaluation for *Invitro* Antiurolithiatic activity

Preparation of experimental stones by homogenous precipitation (Calcium phosphate stones)

In a beaker with enough distilled water, an equimolar solution of calcium chloride dihydrate and disodium hydrogen phosphate in 2N H_2SO_4 was allowed to react which results in to formation of Calcium phosphate precipitate, which was cleared using ammonia solution to remove residues of sulphuric acid further it is washed with distilled water and dried in a hot air oven at 60°C for 4 hours. (18,19)

Preparation of semi-permeable membrane from eggs

The egg shell was chemically removed by soaking it in 2M HCl overnight, resulting in complete decalcification. The egg was washed with distilled water and a hole was carefully made in the top to squeeze out the contents completely from the decalcified egg to get a semipermeable membrane then the egg membrane was washed thoroughly with distilled water, placed in an ammonia solution to neutralise acid traces, and rinsed with distilled water. The membrane was stored in the refrigerator at a pH of 7-7.4 until further usage. (18,19). The Semi-permeable membrane is mentioned in Figure 5.

Preparation of Standard

Cystone tablets was placed in ethanol for removing colour coating of the tablet and it was then crushed in a motor and pestle to get powder form then dispersed into distilled water, filtered and then used for further study.

Estimation of Calcium phosphate

For the estimation of dissolution of Calcium phosphate stone, the semi-permeable membrane prepared was utilised and the following procedure was carried out.

10mg of calcium phosphate was suspended in 10ml of distilled water.

- Group1: 1ml of calcium phosphate (1mg/ml) + 1ml of distilled water = Negative control
- Group2: 1ml of calcium phosphate (1mg/ml) + 1ml cystone solution = Positive control
- Group3: 1ml of calcium phosphate (1mg/ml) + 1ml of extract (20mg/ml)

The contents mentioned above are packed together in the prepared egg semipermeable membrane by suturing as shown in the Figure 7 and were suspended in a conical flask containing 100ml, 0.1M Tris buffer. Conical flasks of all the three groups were placed in an incubator preheated to 37°C for 4 hours, for about 4 days. The contents were then removed and 4mL of 1N H_2SO_4 and 60µL of 0.02M $KMnO_4$ were added and kept for 2 hours, the solution becomes colourless from dark pink. Change in the intensity of colour was measured against 620nm spectrophotometrically. Concentration of undissolved calcium was determined from standard calibration curve of calcium phosphate by using measured absorbance readings. (20)

The Concentration of Undissolved Calcium was calculated by formula obtained by standard calibration curve of Calcium phosphate i.e.

$$y = 0.6658x + 0.0003 \quad \text{thus } x = \frac{y-0.0003}{0.6658}$$

The reduction in weight of calcium was calculated by subtracting the concentration obtained in previous column. % Dissolution was obtained by multiplying the weight of calcium reduced with 100. The results are mentioned Figure 6,7 and Table no 7,8.

Results

Morphological Evaluation

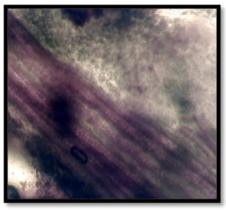
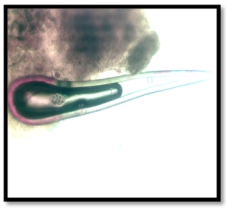
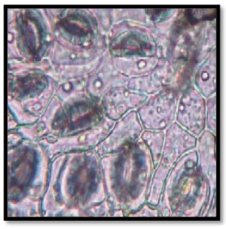
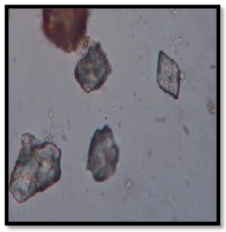
Morphological characters of the Plant are described by visually assessing the plant as given in the Figure**.**

Figure 1: Aerial Part of *Hibiscus cannabinus* Linn. Plant|

Powder microscopy

Various microscopical characters were studied and shown in Figure.

Figure 2: Microscopical characters

A: Pitted Xylem vessels	B: Lignified fibres	C: Trichomes	D: Pollen grain	E: Stomata	F: Crystals
					

Physicochemical evaluation

Powder was analysed for various physicochemical characters like total ash, acid insoluble, water-soluble ash, extractive value, and moisture content.

Table 1: Physiochemical parameters of *Hibiscus cannabinus* Linn

Parameters	Value % W/W
Total ash	5.10
Acid insoluble ash	1.2
Water soluble ash	2
Loss on drying	1
Methanol soluble extractive value	26
Petroleum ether soluble extractive	12
Water soluble extractive value	20

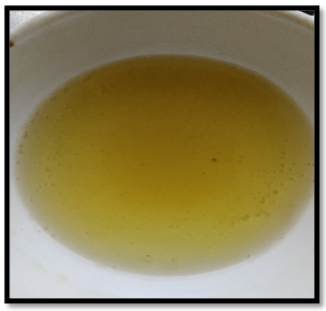
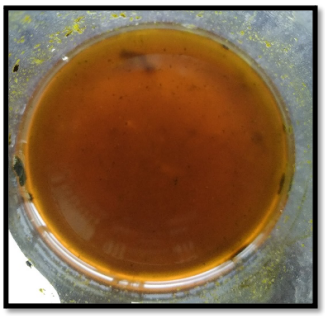
Preparation of Extract and fractions

The Methanolic extract, n-hexane and Ethyl acetate fractions obtained were studied for colour and consistency and yield was calculated.

Table 2: Physical appearance and Yield of extract and fractions

Extract	Consistency and colour	Yield % W/W
Methanolic extract	Sticky, Greenish black colour.	14%
Ethyl acetate fraction	Sticky, brownish colour.	40%
n-hexane fraction	Sticky, yellowish colour.	60%
Water fraction	Dry, brown colour.	45%

Figure 3

A: Crude Extract	B: n-Hexane Fraction	C: Ethyl acetate Fraction	D: Water Fraction
			

Phytochemical analysis

Table 3: Phytochemical evaluation of Raw plant material

Phytochemical compounds	Methanolic extract
Carbohydrates	+
Proteins	+
Saponin glycosides	+
Tannins	+
Flavonoids	+
Alkaloids	+

Table 4: Phytochemical evaluation for Phenolic Content in Fractions

Phytochemical compound	Ethyl acetate fraction	n-hexane fraction	Water fraction
Phenol	+	+	+

Estimation of Total Phenolic Content

Total phenolic content in methanolic Extract and its various Fractions was estimated. The TPC contents from the FC measurements are expressed as **µg/ml** of GAE as standard calibration. The Results obtained are as bellow.

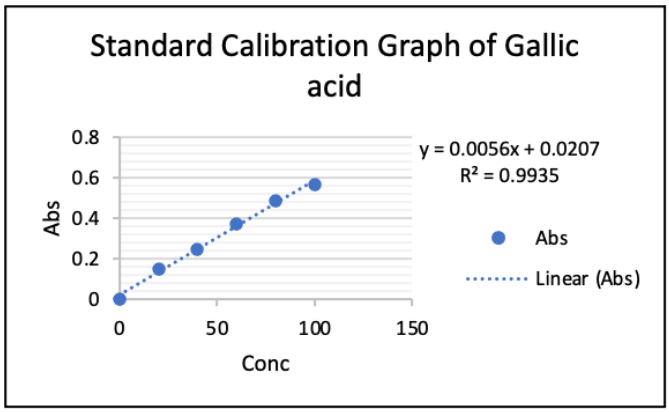
Table 5: Concentration and absorbance of Standard Gallic Acid

Concentration (Gallic acid) µg/ml	Absorbance
0	0
20	0.149
40	0.247
60	0.374
80	0.484
100	0.564

Table 6: Total Phenolic Content in Extract and Fractions

Concentration 100 µgm/ml	Absorbance	Total phenolics (µgm GAE/g)
n-hexane fraction	0.908	158.44 ± 1.24
Ethyl acetate fraction	1.614	284.51 ± 1.36
Water fraction	1.101	192.91 ± 0.98
Extract(methanol)	1.009	176.48± 1.56

Figure 4: Standard Calibration Curve Graph of Gallic acid



Evaluation for *Invitro* Antiurolithiatic activity for Ethyl acetate fraction

Preparation of Semi-permeable membrane from Eggs.

Figure 5: Semi-permeable egg membrane

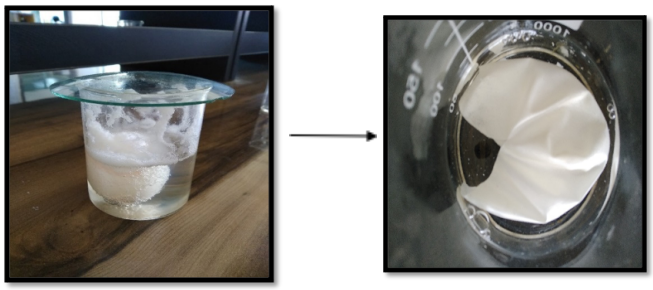
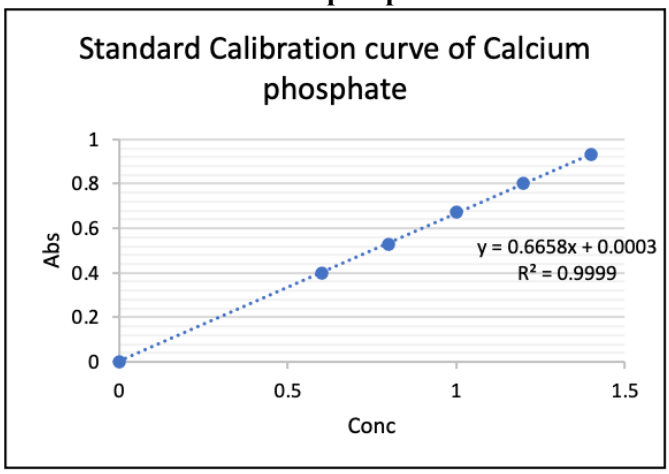


Table 7: Concentration and absorbance of Standard

Concentration (Calcium Phosphate)	Absorbance
0	0
0.6	0.40
0.8	0.53
1	0.67
1.2	0.80
1.4	0.93

Figure 6: Standard Calibration Curve of Calcium phosphate



Estimation of Stone Dissolution

Figure 7: Contents packed in semi permeable membrane

Table 8: Estimation of Calcium Phosphate stone Dissolution

Group	Absorbance	Concentration of stone undissolved (mg)	Weight of Calcium reduced (mg)	Dissolution
1	0.671	-	-	-
2	0.049	0.073	0.927	92.7%
3	0.182	0.272	0.728	72.8%

Discussion

The plant material was evaluated for its microscopic characteristics which showed the presence of Pitted Xylem vessels, Lignified fibres, Trichomes, Pollen grain.

The Physicochemical parameters were evaluated and the results were obtained as, the total ash 5.10%, water soluble ash 2 % and acid insoluble ash 1.2 %. Less amount of these three parameters indicate that the inorganic matter and silica was less in *Hibiscus cannabinus* Linn. plant material. The moisture content was found to be 1%. The extractive value of crude powder was maximum in methanol (26%) followed by water (20%) and minimum was in petroleum ether (12%).

The powdered plant material was subjected for maceration and the extract obtained with yield of 14%.The extract was subjected for fractionation using Ethyl acetate, n-hexane and water. Fractions obtained with the yield of 40%, 60% and 45% respectively

Phytochemical analysis revealed for presence of various phytoconstituents present like Carbohydrates, proteins, saponin glycosides, tannins, flavonoids and alkaloids. Various fractions were also evaluated for presence of Phenolic content and all three fractions showed presence of Phenolics.

The extract and fractions were determined for Total Phenolic content, in which Ethyl acetate fraction showed highest Phenolic content and the concentration was 284.51 (µgm GAE/g) Thus this fraction was used for further study.

Concentration of stone dissolved by Ethyl acetate fraction was compared with the standard cystone tablet by Spectrophotometric estimation. % of dissolution was calculated and the results revealed that ethyl acetate fraction showed the % dissolution of 72.8% and standard 92.7%.

Conclusion

As there is no Antiurolithiatic activity record of *Hibiscus cannabinus* Linn. which is a traditionally valued medicinal plant ,the present study focused on evaluating the same. Ethyl acetate fraction of the plant contains highest Phenolic content, which showed the ability to dissolve Calcium phosphate stone, thus it can be considered for further *in-vivo* and *in-vitro* studies to determine its ability to dissolve the Kidney stones formed in Human being.

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