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In Vitro Evaluation of Anti-Urolithiatic Activity of Aqueous Extract of *Bougainvillea glabra* (Leaves)

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ABSTRACT

The aim of the present study is to carry out phytochemical extraction, preliminary phytochemical screening, and in vitro anti urolithiatic studies on the aqueous extract (leaf) of *Bougainvillea glabra* (AEBG). The results of preliminary phytochemical screening indicated the presence of saponin glycosides, tropane alkaloids and acidic compounds .the anti urolithiatic activity was studied as percentage inhibition of stones by nucleation, growth aggregation assays for aqueous extract at 100-500µg/ml cystone is taken as standard. The results indicated that AEBG showed a dose-dependent inhibition of crystal growth.

1. INTRODUCTION:

The World Health Organization (WHO) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices[1] that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today. Alternatively, say, traditional medicine is the synthesis of therapeutic experience of generations of practicing physicians of indigenous systems of medicine. The traditional preparations comprise medicinal plants, minerals, organic matter, etc. Herbal drugs constitute only those traditional medicines, which primarily use medicinal plant preparations for therapy[2]. The earliest recorded evidence of their use in Indian, Chinese, Egyptian, Greek, Roman and Syrian texts dates back to about 5000 years. The classical Indian texts include Rigveda, Atharvanaveda, Charak Samhita and Sushruta Samhita. The herbal medicines/traditional medicaments have, therefore, been derived from rich traditions of ancient civilizations and scientific heritage.

Urolithiasis or Uroliths is the formation of stones in the kidney, bladder, ureter, urethra or any part of urinary tract[3]. It occurs due to inadequate urinary drainage, the presence of foreign bodies in the urinary tract, microbial infections, the diet rich with oxalates and calcium, vitamin deficiencies like vitamin A and metabolic disorders like hyperthyroidism, cystinuria, gout, etc. It affects about 10-12% of the world population mostly in the industrialized countries. It is the third most prevalent disorder in the urinary system with the recurrence rate of 50 %. Urolithiasis occurs because of successive physiological events like supersaturation of urine, nucleation, growth, aggregation, and retention of calculi within the renal tubules[4]. Renal calculi are composed of calcium oxalate, struvite, uric acid, and cysteine. The present study was carried in an objective to find anti-urolithiatic activity of AEBG by in-vitro evaluation.

2. MATERIALS AND METHODS:

2.1 Evaluation of standardization parameters of individual plant leaf powder

1. Organoleptic evaluation

In this study, the following organoleptic properties of the plant materials were surveyed including physical appearance, odour, and taste for the samples[7].

a. Colour:

Powdered plant material was poured in the watch glass and placed on a white background under a bright white light. Colour of the material was examined by naked eyes and reported.

b. Odor:

Powdered plant material was smelled with 2 min interval for 3 times to nullify the previous feel to smell.

c. Taste:

Powdered plant material was placed on the tongue, its taste was reported. Results were shown in table 1

2. Determination of foreign matter

Any dirt, stones, sand, dust, and other outside natural matter was removed before therapeutic plant materials were cut or ground for testing. Microscopic examination was utilized for the determination of a foreign matter in plant material.

3. Determination of bulk density and tapped density

a. Bulk density:

The bulk density of a powder, which is the ratio of the mass of an untapped powder sample to its volume including the interparticulate void volume, depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in gm/ml or kg/m^3.the bulk density of a powder is determined by measuring the volume of a known mass of powder sample that had been passed through a sieve, into a graduated cylinder.

Method:

Sufficient quantity of powder was passed through a sieve with apertures greater than or equal to 0.1mm, cautiously to break up agglomerates that may have formed during storage. Into a dry graduated cylinder of 250ml, 100gm of test sample weighed with 0.1% accuracy was gently introduced. Carefully, the powder was leveled without compacting and unsettled apparent

volume was noted to the nearest graduated unit. A method is carried in triplicate and the bulk density in (gm/ml) was calculated using the formula[8].

Bulk density=M/V_0

Where M=mass of the sample

V_0=unsettled apparent volume

B. Tapped density

The tapped density is an increased bulk density attained after mechanically tapping a container containing the powder sample. The tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing the powder sample[9]. After observing the initial powder volume or mass, the measuring cylinder or vessel is mechanically tapped, and volume or mass readings are taken until little further volume or mass change was observed.

Method

Unsettled apparent volume was determined(v_0). Secured the cylinder in the holder and carried out 10, 500 and 1250taps on the same powder sample and corresponding volumes were noted as *V10, V500* and *V1250* to the nearest graduated unit. Repeat in increments such as 1250 taps, until the difference between succeeding measurements is less than or equal to 2ml. The procedure was carried out in triplicate and calculated the tapped density (gm/ml) using formula.

Tapped density = *M/Vf*

Where M = mass of the sample

V_f = final volume

4. Ash content of the powder

Ash values are helpful in determining the quality and purity of crude drugs, especially in powder form. Incineration of crude drugs leaves an ash consisting of carbohydrates, phosphates, and silicates of sodium, potassium, calcium, and magnesium.

Determination of Ash values

a. Determination of Total Ash:

2gms of accurately weighed, a ground drug was taken in a tarred platinum or silica dish which was previously ignited and weighed. The ground drug was scattered increasing the heat not exceeding dull red heat until free from carbon, cooled and weighed[10]. Percentage of ash with reference to the air-dried drug was calculated.

$$\textbf{Total ash value of the sample (\%)} = \frac{z-x}{y} \times 100$$

Where z = weight of dish +ash

x = weight of empty dish

y = weight of drug is taken

b. Determination of water-soluble ash

The ash obtained by total ash was boiled for 10min for 25ml of water. The insoluble matter was collected in a Gooch crucible or on ashless filter paper. It was washed with hot water, ignited and weighed. The percentage of water-insoluble ash was collected with reference to the air-dried drug.

$$\textbf{Water soluble ash} = \frac{a}{b} \times 100$$

Where a = water soluble ash

b = air dried extract

c. Acid – Insoluble ash

Total ash was boiled for 5min with 25ml of diluted HCl; the insoluble matter was collected in a Gooch crucible, ignited and weighed. Percentage of acid-insoluble ash with reference to the air-dried drug was collected[11].

$$\textbf{Acid-insoluble ash} = \frac{a}{y} \times 100$$

Where a = weight of the residue

Y = air dried extract

5. Determination of loss on drying

A weight of 2gms of the selected plant material leaf powder and polyherbal formulation powder were taken into a clean, dry glass stoppered bottle. The loaded bottles were placed in a drying chamber and dried[12]. Upon opening the chamber, the bottle was closed promptly and allowed to come to the room temperature in a desiccator before weighing.

$$\textbf{Loss on drying} = \frac{\textbf{loss in}}{\textbf{weight of extract in gms}} \times 100$$

Results were shown in table 2.

2.2Phytochemical screening of individual plant leaf powder.

Identification of Phytoconstituents in the selected plant leaf extract was carried out using various qualitative chemical tests.

Test for carbohydrates

- **Molisch's test :**

The test solution is combined with a small amount of Molisch's reagent (α-naphthol dissolved in ethanol) in a test tube. A small amount of concentrated sulphuric acid is slowly added down to the slides of the scoping test tube, without mixing, to form a layer. A positive reaction indicates the appearance of the purple ring at the interface between the acid and test layers.

- **Bial's test:**

To the test sample (2ml), 2ml of Bial's reagent (a solution of orcinol, HCl, and ferric chloride) was added. The solution is then heated gently on a hot water bath. Formation of a bluish product indicates the presence of pentoses.

Test for Amino acids

- **Ninhydrin test :**

To the sample (1ml) 5 drops of ninhydrin reagent were added and heated on a boiling water bath for 2 min. The appearance of purple colour indicates in the presence of amino acids.

- **Xanthoproteic test :**

To the herbal extract (2-3ml), conc. HNO_3 (1ml) was added and heated for 2-3 min. After cooling, 0.5ml 0f NaOH was added. The appearance of red colour indicates the presence of aromatic amino acids.

- **Millon's test :**

The test sample and millions reagent were taken in equal proportions and heated for 3 min. $NaNO_3$ (1%) was added to the above solution. The appearance of cherry red colour indicates the presence of tyrosine.

Test for Alkaloids

- **Dragendroff's test:**

The test sample of 200mg was warmed with 2% sulphuric acid for 2 min. The solution was filtered and added few drops of Dragendroff's reagent. Orange-red precipitate indicates the presence of alkaloids.

- **Mayer's test :**

To the sample, the sides of the test tube added few drops of Mayer's reagent. Creamy white precipitate confirms the presence of alkaloids.

Test for Flavonoids

- **Alkaline reagent test :**

To the sample, 10% NaOH was added. Formation of yellow colour indicates the presence of flavonoids.

- **Ammonium hydroxide test :**

To the sample, 10% NH_4OH was added. Development of yellow colour fluorescence indicates the presence of flavonoids.

Test for phenolic compounds

- **Test with ferric chloride**

Herbal extract (500mg) was added to 5ml of distilled water. Few drops of neutral 5% $FeCl_3$ were added to it. Development of a dark green colour indicates the presence of phenolic compounds.

Test for fixed oils and fats

A drop of the concentrated herbal extract was pressed between two filter papers and kept aside. Presence of oil impression or stain indicates the presence of oil and fats.

1. **Test for glycosides**[6]

- **Keller – killiani test**

To the test sample, glacial acetic acid, 4to 5 drops of $FeCl_3$ and concentrated H_2SO_4 were added. Reddish brown colour at the junction of two layers and a bluish-green colour at upper layer indicates the presence of glycosides.

Test for steroids

- **Liebermann Burchard test**

For the herbal extract (20 gms) 2.5 ml of acetic anhydride and 2.5ml of chloroform were added. Further, conc.H_2SO_4 was added drop by drop. Development of violet colour or bluish green colour indicates the presence of steroids. Results were shown in table 3.

Thin layer chromatography evaluation

For alkaloids:

TLC was done with aqueous extract of APHP. 5gm of the sample in alcohol (25ml×3) under refluxed on a water bath for 30 min. Filtered, concentrated to 10ml and carried out the thin layer chromatography. 10µl of the extract was applied on TLC plate, develop the plate to a distance of 8cm using benzene: ethanol: methanol (3:4:3) as the mobile phase. After the development of the plate, allow it to dry in air and examine under UV light (254nm)[6].

For glycosides:

TLC was done with aqueous extract of APHP. 5gm of the sample in alcohol (25ml×3) under refluxed on a water bath for 30 min. Filtered, concentrated to 10ml and carried out the thin layer chromatography. 10µl of the extract was applied on TLC plate, develop the plate to a distance of 8cm using ethyl acetate: pyridine: water (3:2:4) as the mobile phase. After the development of the plate, allow it to dry in air and examine under UV light (254nm).

For steroids

TLC was done with aqueous extract of APHP. 5gm of the sample in alcohol (25ml×3) under refluxed on a water bath for 30 min. Filtered, concentrated to 10ml and carried out the thin layer chromatography. 10µl of the extract was applied on TLC plate; develop the plate to a distance of 8cm using benzene: ethyl acetate (7:3) as the mobile phase. After the development of the plate, allow it to dry in air and examine under UV light (254nm). Results were shown in table 4.

2.3 In vitro studies for the anti urolithiatic activity of *Bougainvillea glabra*

The effects of aqueous extract of *Bougainvillea glabra* on calcium oxalate crystallization were determined by measuring the turbidity which was due to the addition of 0.01M sodium oxalate solution to the artificial urine. The turbidity was measured by using UV/visible spectrophotometer (Shimadzu) at 37°C with pH 6.8 at 620 nm.

Preparation of artificial urine (AU)

The artificial urine was prepared according to Burns and Finalayson method[15]. It was prepared freshly each time and the pH has to be adjusted to 6.0.

Na oxalate assay:

This method of analysis was just modified as specified in Goyal Paveen Kumar *et al.* [16], and it proceeded in the following way.

To the 1ml of AU, add 0.5 ml of distilled water and blank reading was taken at 620 nm. Then to the above solution, 0.5ml of 0.01M sodium oxalate was added and incubated for 10 minutes. The absorbance was measured immediately for a period of ten minutes. For each experiment, three replicates were taken.

The aqueous extract of *Bougainvillea glabra* was made to dissolve in water, filtered through the Whatman's filter paper and then different concentrations of 50µgm/ml, 100µgm/ml, 150µgm/ml, 200µgm/ml, and 250µgm/ml were prepared for each extract. A blank reading was taken with 1ml of AU and 0.5ml of plant extract solution and then to the above, 0.5ml of 0.01M sodium oxalate solution was added and incubated for 10 minutes. The absorbance was observed to the above resulting solution at 620nm[14]. For each experiment, three replicates were taken. The percentage of inhibition was calculated by using the following formula. Results were shown in table 5.

Ca oxalate assay:

This method of analysis was just modified as specified in Goyal Paveen Kumar *et al.* [16], and it proceeded in the following way.

To the 1ml of AU, add 0.5 ml of distilled water and blank reading was taken at 620 nm. Then to the above solution, 0.5ml of 0.01M calcium oxalate was added and incubated for 10 minutes. The absorbance was measured immediately for a period of ten minutes. For each experiment, three replicates were taken.

The aqueous extract of *Bougainvillea glabra* was made to dissolve in water, filtered through the Whatman's filter paper and then different concentrations of 50µgm/ml, 100µgm/ml, 150µgm/ml, 200µgm/ml, and 250µgm/ml were prepared for each extract. A blank reading was taken with 1ml of AU and 0.5ml of plant extract solution and then to the above, 0.5ml of 0.01M calcium oxalate solution was added and incubated for 10 minutes. The absorbance was observed to the above resulting solution at 620nm[14]. For each experiment, three replicates were taken. The percentage of inhibition was calculated by using the following formula. Results were shown in table 5.

$$\textbf{Percentage of inhibition} = \left[\, 1 - \frac{absorbance\ of\ blank}{absorbance\ of\ sample}\right] 100$$

3 RESULTS

Table 3.1: Organoleptic properties of plant powder (leaf) of *Bougainvillea glabra*

Colour	Green
Odour	Aromatic
Taste	Bitter

Table 3.2: Physicochemical properties of plant powder (leaves) of *Bougainvillea glabra*

Bulk density	0.353gm/ml
Tapped density	0.43gm/ml
% carr's index	17.9%
Hausner's ratio	1.21
Total ash	0.75%
Water soluble ash	0.65%
Acid-insoluble ash	0.635%
Loss on drying	5.5

Table 3.3: Phytochemical screening of AEBG

Compounds	**Tests**	**AEBG**
Carbohydrates	Molisch test	+
	Bial 's test	+
Amino acids	Ninhydrin test	-
	Xanthoproteic test	-
	Millon's test	-
Alkaloids	Dragendroff's test	+
	Mayer's test	+
Flavonoids	NH_4OH test	+
	Alkaline reagent test	+
Phenolic compounds	Test with ferric chloride	+
Fixed oils and fats		+
Glycosides	Keller – killiani test	+
Steroids	Liebermann Burchard test	+
Tannins	Test with ferric chloride	+

Table 3.4: Phytochemical Analysis of AEBG by TLC

Compounds	**R_f value**
Alkaloids	0.206
Steroids	0.281
Glycosides	0.578

Table3. 5: Effect of AEBG on crystal growth (sodium oxalate assay)

Concentration(µg)	Mean ± standard deviation	% of inhibition
Control	0.0274±0.001140	0
Standard	0.0448±0.001357*	38.8
100	0.058± 0.001581*	52.75
250	0.064±0.001581*	57.1
500	0.0776±0.001140*	64.6

Each value is represented as mean ± S.D, analysed by t-test, followed by one way ANOVA,*p<0.05 was considered as significant

Table 3.6: Effect of AEBG on crystal growth (calcium oxalate assay)

Concentration(µg)	Mean ± standard deviation	% of inhibition
Control	0.0274±0.0008944	0
Standard	0.0498±0.0009203*	44.9
100	0.0718±0.0007071*	61.8
250	0.072±0.0008367*	61.9
500	0.0772±0.0008367*	64.5

Each value is represented as mean ±S.D, analysed by t-tests followed by one way ANOVA, *p<0.05 was considered as significant

4. DISCUSSION AND CONCLUSION

The present study had revealed the anti-urolithiatic activity of aqueous extract of *Bougainvillea glabra* by in vitro method .plant powder of *Bougainvillea glabra* was standardized as per WHO guidelines. The phytochemical investigation revealed the presence of alkaloids, glycosides, steroids etc. In-vitro studies had shown anti-urolithiatic activity in a dose-dependent manner (100-500µg). Cystone (100µg) was taken as standard. Aqueous extract of *Bougainvillea glabra* had shown better anti-urolithiatic activity compared to standard (p<0.05).

5. SCOPE OF STUDY

Based on the observation of the present study further studies on toxicological & in vivo studies can be proceeded. The present study forms the basis for an evidence-based report on the herbal

preparation of an aqueous extract of *Bougainvillea glabra* which further can be prepared as a formulation for treating urolithiasis.

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