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R E S E A R C H A R T I C L E

Evaluation of *In-Vitro* Anti Urolithiatic Activity of *Justicia Tranquebariensis*

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Abstract
The present investigation is to study about the preliminary phytochemical analysis of *Justicia tranquebariensis* indicated the presence of Flavonoids, quinones, glycosides, coumarins and steroids. *In-vitro* Calcium oxalate crystallization inhibition study was evaluated. From this study conclude that the ethanol, ethyl acetate, and hexane extracts of *Justicia tranquebariensis*. inhibits the calcium oxalate crystallization in the order of 58%, 46% and 39% respectively. *In-vitro* turbidity method was evaluated that these extracts inhibits the calcium oxalate nucleation in the order of 73%, 62% and 61% respectively. Among the various extracts, the ethanolic extract produces more potent anti urolithiatic activity. The study provides basic evidence that the *justicia tranquebariensis* has the beneficial effect for the treatment of urolithiasis. Further studies are required to elucidate the molecular mechanism of action and its therapeutic potential in the treatment of urolithiasis.

Keywords: Justicia tranquebariensis, calcium oxalate crystallization, turbidity method, urolithiasis

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Plants provide food, raw materials for medicine and various other requirements for the very existence of life from the origin of human beings. Even the current conventional medicine is using a lot of plant derived chemicals as therapuetic agents. The overuse of synthetic drugs results in higher incidence of adverse drug reactions has motivated humans to return to nature for safe remedies. Herbs and herbal drugs have created interest among the people by its clinically proven effects. Therefore, there is a compelling need for detailed scientific validation of all traditional medicinal plant drugs to establish their efficacy and safety in light of modern science.

Kidney stone disease is a multi-factorial disorder resulting from the combined influence of epidemilogical, biochemical and genetic risk factors. Urolithiasis is considered as the third most common affliction of the urinary tract. It refers to the solid non-metallic minerals in the urinary tract. It is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidney. The formation of kidney stones involves several phytochemical events beginning with crystal nucleation, aggregation and end with retention within the urinary tract. Among the several types of kidney stones, the most common are calcium oxalate stones representing upto 80% of the analyzed stones. Calcium containing stones may be in the form of pure calcium oxalate(50%) or calcium phosphate(5%) and a mixture of both(45%) followed by magnesium phosphate(15-20%),uric acid(10%) and cystine(1%). It is estimated that at least 10% of the population in the industrialized part of the world is afflicted by urinary tract diseases and among these kidney stones are common with an annual incidence of 0.5 -1.9%. About 12% of the population of India is expected to have urinary stones and out of that 50% of cases encounter loss of one or both kidneys with or without renal damage upto some extent2. Stone disease is 2-3 times more common in males, than in females. It has a reoccurence rate of 70-81% in males and 47-60% in females5.In spite of substantial progress in pathophysiolgy and treatment of urolithiasis, there is no satisfactory drug being used in clinical therapy. Kidney dialysis, endoscopic stone removal and extra corporeal shock wave lithotripsy are prohibitively costly and reoccurrence is quite common with theseprocedures1. Data from in vitro and in vivo clinical trials revealed that phytotherapeutic agents could be useful as alternative therapy in the management of urolithiasis. Medicinal plants and their products are more useful, because they promote the repair mechanism in natural way1.Pharmacological and phytochemical prospecting of medicinal plants based on traditional knowledge can lead to the discovery of new drug and development of pharmacologically important products for human health care6.Green medicines were safe and more dependable than the costly synthetic drugs, many of which have side effects7. Juices of leaves act as a cooling agent and aperients and also given to children in Small pox. Crushed leaves applied to contusions8. Paste made of the leaves applied externally on the swelling to reduce the pain. Root paste applied for tooth ache9.Leaf juice, about15-20 ml, is administered orally for every one hour up tohalf of the day and keeping of leaf paste externally on the sight of snake bite work as an antidote for Cobrabite10. Leaf juice is given orally to treat jaundice and leaf paste is applied over affected area to treat skin diseases11-13.

2. Methodology

Plant collection and identification:

The whole plant was collected near Tirumala Hills, Chittoor District, A.P (INDIA). It was authenticated by Dr. K Madhava Chetty, Assistant Professor, Department of Botany, S V University, Tirupati.

Drugs and chemicals

Hexane, Ethyl acetate, Ethanol, Cystone, Ethylene glycol.

Preparation of plant extracts

The powdered plant material (50g) was extracted by hot continuous soxhlet extraction method. The plant material was extracted with Hexane (500ml), Ethyl acetate (500ml) and Ethanol (99.9% v/v) (500ml), for four days in a soxhlet apparatus. It is a process of continuous extraction method in which the solvent can be circulated through the extractor for several times. The vapours from the solvent are taken to the condenser and the condensed liquid is returned to the extract for continuous extraction. The apparatus consist of body of extractor (thimble) attached with side siphon tube, lower end attached with distillation flask and the mouth of the extractor is fixed to the condenser by the standard joints.

Procedure

Weighed about 50g dried powdered plant and transferred into a thimble for packing. While packing, the content was wetted with Hexane, Ethyl acetate and Ethanol respectively and poured until the siphon tube was filled. A piece of porcelain was added into the round bottom flask to avoid bumping effect. After assembling the extractor, the plant material was extracted at about (40-45^{o}C), (35-40^{o}C), (40-45°C), (20-30^{o}C), temperature respectively until the colour of the solution in the siphon tube became pale. The extracts obtained were dried at room temperature and the yield was stored in air tight container. Further Phytochemical screening and invitro anti urolithiatic activity of the extracts has been performed[14-16].

In-Vitro Anti-Urolithiatic Activity By Turbidity Method AndTitrimetry Method

Turbidity Method[17-22]

Principle: *In vitro* anti-urolithiatic activity of *Justicia tranquebariensisa* whole plant extract were tested in terms of inhibition of calcium oxalate formation by the extracts in the presence (standard drugs and extract) and absence of inhibitors. The precipitation of calcium oxalate at 37^{o}C and pH 6.8 has been studied by the measurement of turbidity by UV/Vis spectrophotometer at 620nm. It was employed to measure the turbidity caused due to formation of calcium oxalate. To evaluate calcium oxalate inhibition of plant extracts by absorptions were noted and in microscopical study the comparison of un-controlled growth of the stone nucleus for the comparison of growth in the presence of the standard drugs and plant extracts were also observed.

Materials requirements

Calcium chloride dehydrate, Sodium oxalate, Tris buffer, Cystone 750mg, Hexane, ethyl acetate and ethanolic Plant extracts.

Procedure

Study without inhibitor: Volume of 1.0 ml of 0.025M calcium chloride dihydrate and 2ml of Tris-buffer (pH7.4) were added in a test tube. Then 1.0ml of 0.025M sodium oxalate was added. After mixing of above solution immediately due to the formation of turbidity and then up to the period of 10 minutes to measure the turbidity of solution by UV/Vis spectrophotometer at 620nm. This control experiment was done in three replications.

Study with inhibitor

In this experiment, 1ml of 0.025M calcium chloride dihydrate, 2ml Tris-buffer and 1ml (10 mg/ml solution) of Hexane, ethyl acetate and ethanolic plant extracts were added in a four sets of test tubes. Two more test tubes were

added 1ml of 0.025M calcium chloride dihydrate, 2ml Tris-buffer and poly herbal formulation, Cystone. Then 1ml of 0.025M sodium oxalate was added to each test tube and then up to the period of 10 minutes to measure the change in turbidity of the solution by UV/Vis spectrophotometer at 620nm. Each procedure was done in three times. Inhibition in stone nucleus formation was calculated by graphical method using the following formula:

Percentage inhibition = {1-[Si /Sc]} x 100

Where;

Si - slope of graph in the presence of inhibitors (drugs/plant extracts)

Sc - slope of without inhibitors (control).

Microscopic study

Light microscopy of the crystals formed in the solution with and without was also done. Photographs of calcium oxalate were taken using the objective of 40X.

Titrimetry Method[23-27]

Principle

The Titrimetric method is one of dissolution method, the artificial preparation of calcium oxalate crystal was taken in the egg semipermeable membranes act as control (without inhibitor) and added different plant extracts and standard Cystone (with inhibitor). Then it was immersed in the 0.1M Tris buffer solution and incubated at 37^{o}C for 2hours. After 2hours removal of content of semi permeable membranes and add 2ml of 1N sulphuric acid titrated against 0.9494N potassium permanganate till a light pink colour end point was obtained. The amount of remaining undissloved calcium oxalate is subtracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by various extracts.

Material required

Calcium chloride dehydrate, Sodium oxalate, Tris buffer, Egg semipermeable membrane, Cystone 750mg, Hexane, ethyl acetate and ethanolic Plant extracts, Sulphuric acid

Procedure

The dissolution method involved the three steps.

- Preparation of experimental kidney stones (calcium oxalate stones) by homogenous precipitation.
- Preparation of semi-permeable membrane from eggs.
- Estimation of calcium oxalate by Titrimetry.

Preparation of Calcium oxalate crystals (Kidney stones):

The experimental kidney stones of calcium oxalate (CaOx) were prepared in the laboratory by taking equimolar solution of calcium chloride dehydrate in distilled water and sodium oxalate in 10 ml of 2N H_2SO_4.Both 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 sulphuric acid by ammonia solution, washed with distilled water and dried at 60ºC.The dissolution percentage of calcium oxalate was evaluated by taking exactly 1mg of calcium oxalate and 10mg of the extract, packed it together in semipermeable membrane of egg as shown in the model designed given below. This was allowed to suspend in a conical flask containing 100 ml of 0.1M Tris buffer. First group served as blank containing only 1mg of calcium oxalate. The second group served as positive control containing 1mg of calcium oxalate, along with 10 mg standard drug, *i.e*.cystone. The 3 and 4th groups along with 1mg of calcium oxalate contain 10mg of aqueous and methanolic extracts. The conical flasks of all groups were kept in an incubator preheated to 37ºC for 2hrs. Remove the contents of semipermeable membranes from each group into separate test tubes, add 2 ml of 1N sulphuric acid to each test tube and titrated with 0.9494 N $KMnO_4$ till a light pink colour end point is obtained. The amount of remaining undissolved calcium oxalate is substracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by various solvent extracts.

Preparation of semi-permeable membrane from eggs

The semi - permeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin & yolk. Apex of eggs was punctured by a glass rod in order to squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker consisting 2M Hcl for an overnight, which caused complete decalcification. Further, washed with distilled water, placed it in ammonia solution for neutralization of acid traces in the moistened condition for a while & rinsed it with distilled Water. Stored in refrigerator at a pH of 7- 7.4.

Estimation of calcium oxalate by Titrimetry

The dissolution percentage of calcium oxalate was calculated by taking exactly 1 mg of calcium oxalate and 10mg of different plant extracts, packed it together egg semipermeable membrane. This was allowed to suspend in a conical flask containing 100ml of 0.1M Tris buffer. The1 mg of calcium oxalate in egg semipermeable membrane act as the control. The1 mg of calcium oxalate and 10 mg of Cystone (standard) and 10mg of Hexane, ethyl acetate and ethanolic Plant extracts in egg semipermeable membrane. All the conical flask containing semipermeable membrane were kept in an incubator to 37^{o}Cfor 2hours.Remove the contents of semipermeable membrane in to separate test tube, add 2ml of 1Nsulphuric acid to each test tube and titrated with 0.9494N $KMnO_4$ till a light pink colour end point obtained. The amount of remaining undissloved calcium oxalate is subtracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by various extracts. Each ml of 0.9494 N $KMnO_4$ equivalents to 0.1898mg of Calcium oxalate.

The percentage dissolution calculated follows

Dissolved calcium oxalate = (Total quantity used in the Experiment in the beginning) - (Undissloved calcium oxalate)

Percentage dissolution = Dissolved calcium oxalate X 100

3. Results and Discussion

Table 2: Phytochemical analysis of Hexane, Ethyl acetate and Ethanolicextracts of whole plant of *Justicia tranquebariensis*

S.no	Phytochemical Test	Hexane extract	Ethyl acetate extract	Ethanolicextract
1.	Carbohydrates	-	-	-
2.	Tannins	-	-	-
3.	Saponins	-	-	+
4.	Flavonoids	-	+	+
5.	Alkaloids	+	+	+
6.	Quinones	-	-	+
7.	Glycosides	-	-	+
8.	Cardiac glycosides	-	-	+
9.	Terpenoids	-	-	-
10.	Phenols	-	-	-
11.	Coumarins	-	+	+
12.	Phlobatannins	-	-	-
13.	Steroids &phytosteroids	+	-	+
14.	Anthraquinone	-	-	-

Table 3: *In-vitro* anti-urolithiatic activity

s.no	Concentration (µg/ml)	% inhibition			
		Hexane extract	Ethyl acetate extract	Ethanolic extract	Cystone Standard
1.	200	21.34±0.23	24.12±0.32	32.22±0.14	34.65±0.16
2.	400	24.54±0.76	31.18±0.23	43.34±0.76	49.56±0.23
3.	600	36.43±0.24	38.67±0.83	50.38±0.50	53.45±0.34
4.	800	43.46±0.82	46.30±0.45	62.44±0.23	67.55±0.22
5.	1000	61.42±0.65	62.42±0.56	73.82±0.22	76.65±0.43
	IC50(µg/ml)	**842.3**	**796**	**600.75**	**546.8**

Table 4: The Percentage dissolution of calcium oxalate

S.no	Extracts	Dissolved calcium oxalate(mg)	% Dissolution
1	Hexane extract	0.392	39.2
2	Ethyl acetate extract	0.468	46.8
3	Ethanolic extract	0.582	58.2
4	Cystone	0.658	65.8

Discussion

Preliminary phyto-chemical analysis of hexane, ethyl acetate and ethanolic extract of whole plant of *Justicia tranquebariensis* revealed the presence of various components flavanoids, glycosides, quinines, caumarins, sopponins and steroids. Active phyto chemical such as flavanoids and sapponins are known to responsible for the diuretic activity. The percentage inhibition of calcium oxalate crystallization of various extracts was calculated by using formula. when compared these three extracts, present study clearly indicates the ethanolic extract of *Justicia tranquebariensis* whole plant showed higher calcium oxalate crystallization inhibition (**73.82 %**) than the hexane (**61.42%**) and ethyl acetate (**62.42%**) extract of *Justicia tranquebariensis* whole plant for the turbidity method. While Cystone a prescribed medicine for renal calculi showed highest inhibition (**76.65%**) in terms of formation of calcium oxalate precipitation **(Table.3** and**Fig.2)**. The values are depicted in the table and the IC50 value of ethanolic extract of *Justicia tranquebariensis* whole plant showed significant IC50 value (**600.75µg/ml**) than the hexane (**842.3µg/ml**) and ethyl acetate **(796µg/ml)** extract of *Justicia tranquebariensis* whole plant. The IC50 value of the Cystone standard (**546.8 µg/ml**) **(Fig.3).** Kidney oxalate stone is the result of supersaturation of urine with certain urinary salts such as calcium oxalate. Since crystallisable oxalate species are pH independent, the crystallization of oxalate in the absence of an inhibitor, led to the formation of calcium oxalate monitored by light microscope the process of calcium oxalate crystallization in control without the addition of inhibitors is shown in (**Fig.3.2**). The % inhibition of turbidity (aggregation) in the presence of herb extracts was lower than in the control, showing that crystals were less aggregated. The inhibited aggregation associated with the extract increased with concentration. This inhibition was greatest with ethanolic extract of whole plant of *Justicia tranquebariensis*. (**Fig.3.3**). Ethanolic extract has a greater capacity to reduce all these crystallisation process as compared to hexane and ethyl

acetate extract. The ethanolic extract of *Justicia tranquebariensis* whole plant showed higher (**58.2%)** percentage dissolution of calcium oxalate than the hexane (**39.2%**) and ethyl acetate (**46.8%**) extract of *Justicia tranquebariensis* whole plant. While Cystone a prescribed medicine for renal calculi showed highest percentage dissolution (**65%**) of calcium oxalate **(Fig.4**and values depicted in the **table4)** by the Titrimetry method.

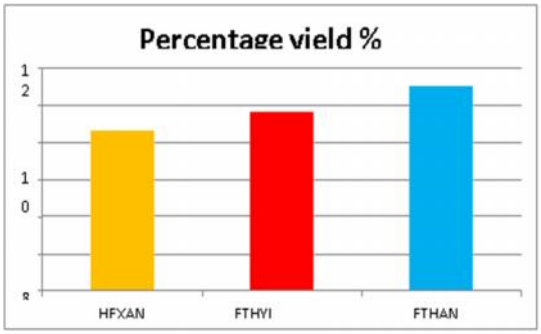

Fig.1: percentage yield of *Justicia tranquebariensis*

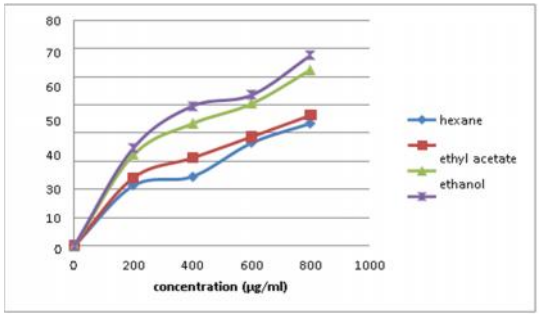

Fig.2: IC50 Value of Hexane, Ethyl acetate and Ethanolic extracts of *Justiciatranquebariensis*

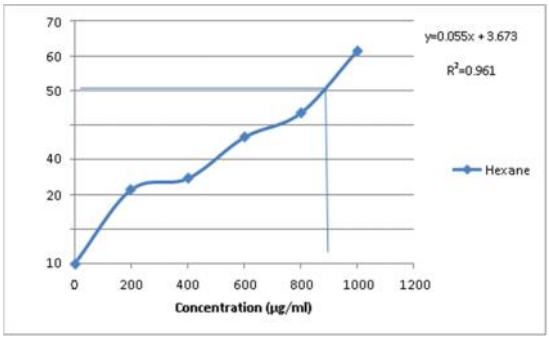

Fig.3: Hexane extract

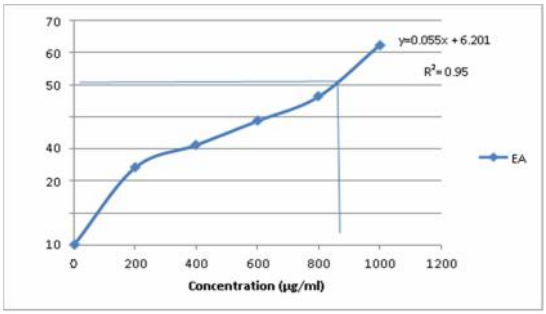

Fig.4: Ethyl acetate extract

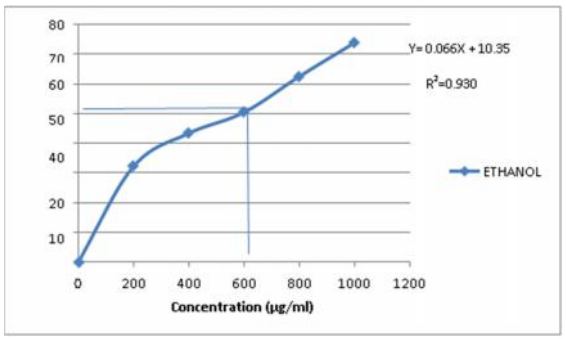

Fig.5: Ethanolic extract

4. Conclusion

In-vitro Calcium oxalate crystallization inhibition study was evaluated. From this study conclude that the ethanol, ethyl acetate, and hexane extracts of *Justicia tranquebariensis*. inhibits the calcium oxalate crystallization in the order of 58%, 46% and 39% respectively. *In-vitro* turbidity method was evaluated. From this study conclude that the ethanol, ethyl acetate, and hexane extracts of *Justicia tranquebariensis*. Inhibits the calcium oxalate nucleation in the order of 73%, 62% and 61% respectively. Among the various extracts, the ethanolic extract produce more potent anti urolithiatic activity. In the present study provided basic evidence that the *justicia tranquebariensis* has the beneficial effect for the treatment of urolithiasis. Further studies are required to elucidate the molecular mechanism of action and its therapeutic potential in the treatment of urolithiasis.

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