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ORIGINAL ARTICLE **OPEN ACCESS**

Evaluation of Flavonoid Rich Extract of *Tridax procumbens* Linn for Acute Toxicity Profile and Antiurolithiatic Activity

Birendra Shrivastava*[1], Rupali Patil[2], Rupali Tasgaonkar[3], Babita More [3]
[*1,2] Jaipur National University, Jaipur.
[3]YadavraoTasgaonkar Institute of Pharmacy, Bhivpuri, Karjat.
*Email ID : rupalikes7@gmail.com

ABSTRACT
Now-a-days interest of human in the use of traditional medicines has growing. To improve the acceptance, the variety of dosage forms were formulated and developed. In the present work Tridaxprocumben has been developed in the form of liquid dosage. The developed formulation evaluated for different parameters and antilithiatic activity. Flavonoid rich extract was obtained from Tridaxprocumben stem. The extract was further used to develop formulation of the syrup. The physicochemical properties of the syrup were studied. The syrup was evaluated for antiurolithiatic action. The accelerated stability of syrup was evaluated during the period 6 months. The product was light brown semi-transparent syrup with sweet taste and characteristic odor. The pH and density were found to be 5.39± 0.01, 1.061± 0.13 g/ml respectively for selected formulation (F2). There was no significant change observed in the evaluation parameters during the accelerated stability studies. The overall results concluded that the Tridax syrup formulated showed to have good antiurolithic property. This herbal syrup successfully reduced kidney stones by a non-toxic and convenient way.
Keywords: *Tridaxprocumbens, Acute toxicity, Antiurolithiatic activity, Flavonoid rich extract*

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INTRODUCTION

'Urolithiasis' is a problem world over, affecting people from thousands of years. It is also called as 'Nephrolithiasis', kidney stones or renal calculi. It is a condition wherein crystal formation occurs in the urinary tract eventually leading to stony structures. There are multiple factors contributing to the formation of these calculi/stones related to lifestyle or dietary habits of an individual. Even congenital tendencies or geographical impact cannot be denied in certain cases. Calculi are made up of deposits of polycrystalline aggregates. These aggregates are made up of varying amounts of crystalloid and organic matrix. These stones can be found in different sizes, shapes and colours. Stone formation and prevalence may be found in any part of the entire urinary tract, (renal area to the bladder) [1].Along with surgical and other conventional treatment management, Ayurveda treatment option has been explored over the past few years. Numerous Ayurvedic medicinal herbs as single drugs or combined formulations have become exposed to potential research and studies. These medicines are being used for management of urinary disorders since thousands of years as the history of Ayurveda dates back to. These drugs are known to have litholytic (disintegration of stones) and litho-preventive (non-formation of stones) properties. *T. procumbens* is a medicinal herb used since a very long time in Ayurveda and later in Unani, folklore or tribal traditional medicinal practices. The usage of plants in earlier system of medicine was based on using the whole plant whereas in the modern era, the technological advancements have made it possible to identify, isolate and validate active chemical principles from the medicinal plants. These discrete lead molecules may prove to more efficient that the whole plant extracts in the applicability in treatment of various disease conditions. The plant has been therapeutically found to be useful in the management of non-healing wounds [2], dysentery [3], epileptic seizures, malarial infection [4], stomach upset, diarrhoea, hypertension, diabetes mellitus [5] and metabolic syndrome [6]. It also known to possess antimicrobial, antiseptic and hepatoprotective properties. It also shows a strong depressant action on the respiratory system [7-9]. This paper aims to explore the actions of formulation containing *Tridax Procumbens* extract with respect to the management of renal calculi. The study also reveals the evaluation and standardization of the developed formulation.

MATERIAL AND METHOD

Material

The raw drug (stem and leaves) *Tridax procumbens* was collected from the college campus. The authentication was done at Blatter Herbarium St. Xavier's College in Mumbai (Herbarium No: NI- 5524). Quercetin was purchased from sigma Aldrich, Mumbai. Other excipients such as propylene glycol, glycerin, sucrose, sorbitol, sodium saccharin, sodium benzoate purchased from Rankem chemicals. All the chemicals used for the present work are of analytical grade. Cystone syrup manufactured by the Himalaya Drug Company, Bangalore, was purchased from the market of Bangalore. Kits used in this study for the determination of calcium, blood urea nitrogen(BUN), creatinine, and phosphorus were purchased from Lab Care Diagnostics (India) Pvt. Ltd.,Bangalore.

Experimental animals

The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of PES's RajaramAndTarabaiBandekar College Of Pharmacy, Goa India, approval no PESRTBCOP/IAEC/2020-21 R-98. Six to seven-week-old male Wistar albino rats weighing 150–200 g were purchased from Global bioresearch, Shirwal Maharashtra. The animals were housed individually in stainless steel wire meshed plastic cages in a temperature (23 ± 2°C) and humidity (55–60%) controlled room with a 12 h light-dark cycle. The animals were supplied with standard rat pellet diet and drinking water and ethylene glycol during the entire period of the study [10, 11]. Animals were maintained and the experiment was carried out according to the rules and regulations of ICH.

Methods of preparation

Tridax procumbens stems and leaves were obtained from college campus. In the shade, the stem and leaves were cleaned and dried. Both samples (leaves and stem) were powdered before being sieved coarsely (0.2mm).

Preparation of Flavonoid rich extract

Dried ground (50 g) *Tridax procumbens* leaves and shoots were defatted for 8 hours using the reflux method with 400 ml of n-hexane. Under reduced pressure, the n-hexane extract was filtered out and concentrated. The defatted plant was again refluxed for 8 hours with 400 ml methanol. Under vacuum, the whole methanol extract was concentrated until it was dry.Using the Soxhlet equipment, a portion of the methanol extract residue was fractionated against three distinct solvents: ethyl acetate, acetone, and isopropanol. Filters were used to separate the distinct fractions from the residual methanol extract, which was then concentrated and dried under decreased pressure.

Preparation of Tridax Syrup

Five distinct syrups were prepared using active extract at a concentration of 25% and a variety of excipients. Glycerin, Hydroxy propyl methyl cellulose (HPMC), and carbomer 940 were among the excipients used to achieve the necessary viscosity. To prevent microbial growth, sodium benzoate and potassium sorbate were used. The detailed composition of the formulations has been mentioned in the table 1 below.

Table 1: Tridax Syrup formulation

Materials	Category	Formulation code (%)				
		F1	F2	F3	F4	F5
Tridax stem flavonoid rich extract	API	25	25	25	25	25
Propylene glycol	Solubilizer	45	30	30	30	15
Sodium benzoate	Preservative	3	3	3	3	3
Potassium sorbate	Preservative	2	2	2	2	2
Sorbitol	Stabilizer and sweetener	10	10	10	10	10
Glycerin	Thickener/ diluent and sweetener	--	15	--	--	30
HPMC	Thickener/ diluent	--	--	15	--	--
Carbomer 940	Thickener/ diluent	--	--	--	15	15
Distilled water	Diluent	q.s to100	q.s to 100	q.s to 100	q.s to100	q.s to 100

Evaluation of tridax Syrup

Determination of pH

The syrup's pH was measured with a digital pH metre that had been calibrated and stabilised with buffer tablets. Steady readings in pH were documented. The stability and properties of the formulation were also determined by the pH of the syrup [12].

Determination of weight/ml

The syrup was added up to the mark in a 10 mL volumetric flask that had already been weighed. It was noted what the net volume was. The weight of the volumetric flask, as well as the syrup, was then recorded. The density of syrup formulations was evaluated based on their weight per millilitre [13].

Estimation of percent content of quercetin in Tridax syrup by High performance thin layer chromatography

HPTLC Instrumentation and Method development

The precoated silica gel aluminium plate $60F_{254}$ (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) was used as stationary phase. The test solutions were spotted in the form of bands of width 6 mm with a CAMAG microlitre syringe. The plates were pre-washed by methanol and activated at 60°C for 5 min prior to chromatography. The slit dimension was kept at 5 mm × 0.45 mm, bandwidth was set at 7mm, each track and 10 mm/s scanning speed was employed. The composition of the mobile phase was Toluene: ethyl acetate: methanol: glacial acetic acid (3: 5: 2: 0.5) v/v/v for quercetin was employed. The linear ascending development was carried out in a twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 10 minutes at room temperature (25 ± 2°C). The length of the chromatogram run was 80 mm. Subsequently, the plate was allowed to dry at room temperature. The separated bands on the HPTLC plates were scanned over the wavelength of 200 – 400 nm. The source of radiation utilized was the tungsten lamp (or deuterium Illumination). The plate was fixed on scannerstage (Camag TLC Scanner 3). The maximum absorbance was scan at 254 nm for quercetin.

Preparation of standard solution

The stock solution of quercetin was 1 mg/ml and working solution was 100 $\boldsymbol{\mu g}$/ml.

Sample preparation

Sample of prepared syrup formulation was prepared by adding 100gm of sample to 50 ml of methanol in volumetric flask. Further dilutions were made to produce 50 $\boldsymbol{\mu g}$/ml concentration.0.22 µ membrane filter was used to filter all samples.

Method validation [14]

The validation of the developed HPTLC method was carried out in accordance with ICH guidelines. The linearity was analysed for concentration ranging from 1- 1000 ng/spot by using Least-square regression analysis where, peak areas were plotted against the corresponding concentrations.The intra- day and inter- day precision was evaluated by triplicates of three different concentrations of each quercetin was spotted and analyzed on same day for intra-day study and two different days for inter-day study with respective chromatographic conditions.

Recovery study method was employed to evaluate accuracy. The samples were spiked with 80, 100 and 120 % of median concentrations of standards.

$$\text{Accuracy} = \frac{\text{spiked concentration} \ - \ \text{mean concentration}}{\text{spiked concentration}} \times 100$$

Robustness was carried out by making deliberate changes in the chamber saturation time, and wavelength their effect on the retention factor and quantitative analysis was determined. The estimation of LOD and LOQ were done by standard deviation method. Detection limit =3.3σ /S and quantitation limit=10 σ /S (σ isresidual standard deviation of a regression line and S is the slope of the calibration curve).

Antiurolithiatic Activity on Rats[15,16]

In vivo method

The ethylene glycol model was used to induce urolithiasis for 28 days. Twenty-four animals were randomly divided into four groups as Groups I, II, III, and IV containing six in each. Group I served as a vehicle-treated control and maintained on regular rat food and drinking water. All the remaining groups received calculi inducing treatment for 28 days which comprised 0.75%v/v ethylene glycol in drinking water ethylene glycol. Group II, which received ethylene glycol only, served as model control for 28 days. Group III was administered cystone syrup at a dose of 750 mg/kg body weight/day (The Himalaya Drug Company, India) served as standard. Group IV served as a treatment groups which received tridax syrup (F2) at a dose of 750 mg/kg body weight/day. Final formulation and standard cystone syrup formulation were suspended in distilled water and given once daily by oral route using the gastric tube.

Collection and analysis of urine

On the 28th day of calculi induction treatment, 24 hour urine samples were obtained from all animals in individual metallic cages. Volume, pH, and crystalluria were all calculated. Urine was acidified with a drop of strong hydrochloric acid and stored at 20°C for commercially available kits to determine calcium,

magnesium, phosphate, uric acid, and glycosaminoglycans (Span Diagnostics Ltd., India; Biocolor Ltd., UK). The oxalate and citrate concentrations were calculated using the method provided by Hodgkinson (1970) and Rajagopal (1984).

Collection and analysis of serum

Blood was obtained from the retro-orbital under light ether anesthesia after the experiment, and the animals were euthanized with a high dose of anesthetics. Serum was separated by centrifugation at 10,000 g for 10 minutes and evaluated for calcium, creatinine, uric acid, urea, and BUN with commercially available diagnostics, while oxalate was determined using Hodgkinson's 1970 technique.

Histopathological study

The purpose of histopathology on the harvested kidney part was to see how the medicine in the dosage form affected the calculi in the kidney as well as the interior structures of the kidney. It provides data about the drug's efficacy in dose form as well as its toxicity. This was done in accordance with established procedures. The staining of the kidney segment was done according to normal histological techniques.

Accelerated stability study [17, 18]

The final tridax syrup formulation was subjected to stability testing by storing at 40 ± 2°C and 75 ± 5% RH for three months. The samples were evaluated at 0, 30, 60, and 90 days for parameters such as appearance, pH, color, odor, and taste.

Freeze and thaw studies

This study was done by exposing the final formulation to 4°C and 40°C alternatively [19]. No precipitation and turbidity were observed in the final formulation.

Statistical analysis

The biochemical results were presented as mean ± standard error of the mean. The statistical significance of the findings from urine analysis (calcium and oxalate), serum creatinine, calcium, urea, and uric acid from blood serum analysis was determined using a one-way analysis of variance test followed by a Tukey's post-test, with a value less than $P < 0.05$ considered statistically significant. Semi-qualitative analysis was used to examine the histopathological findings.

Result and Discussion

Tridax syrup was prepared by the combinations shown in table 1. HPTLC method was developed and validated for testing the percentage of quercetin present in in the Tridax syrup formulations. The physicochemical parameters were evaluated.

Table 2: Evaluation of formulation

Sr. No.	Formulations	Parameters				
		F1	F2	F3	F4	F5
1.	Appearance	Light brown & semi-transparent	Light brown & semi-transparent	Light brown & semi-transparent	Light brown & semi-transparent	Light brown & semi-transparent
2.	Taste	Bitter sweet	Bitter sweet	Bitter sweet	Bitter sweet	Sweet
3.	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
4.	pH	5.22± 0.04	5.39± 0.01	5.19± 0.1	5.51± 0.07	5.49± 0.07
5.	Density	1.081± 0.01	1.061± 0.13	1.077± 0.04	1.073± 0.005	1.065± 0.1

All the formulations prepared (F1-F5) were in the acceptable range. Of all the prepared formulations, F2 showed promising results and was used further for conducting animal activity.

Marker based standardization of sample

Method optimization

The optimized HPTLC separation was achieved on TLC plateby employing CAMAG HPTLC system equipped with TLC scanner 3, and WinCATS 1.2.2 software (CAMAG, Muttens, Switzerland). The separation was carried on silica gel 60 F254 TLC plate using Toluene: ethyl acetate: methanol: glacial acetic acid (3:5:2:0.5 v/v/v) as a solvent system. The detection of wavelength was done at 254 nm. The optimized chromatographic method give good separation and resolution of the standard quercetin (R_f value = 0.61) given in figure 1A- B.

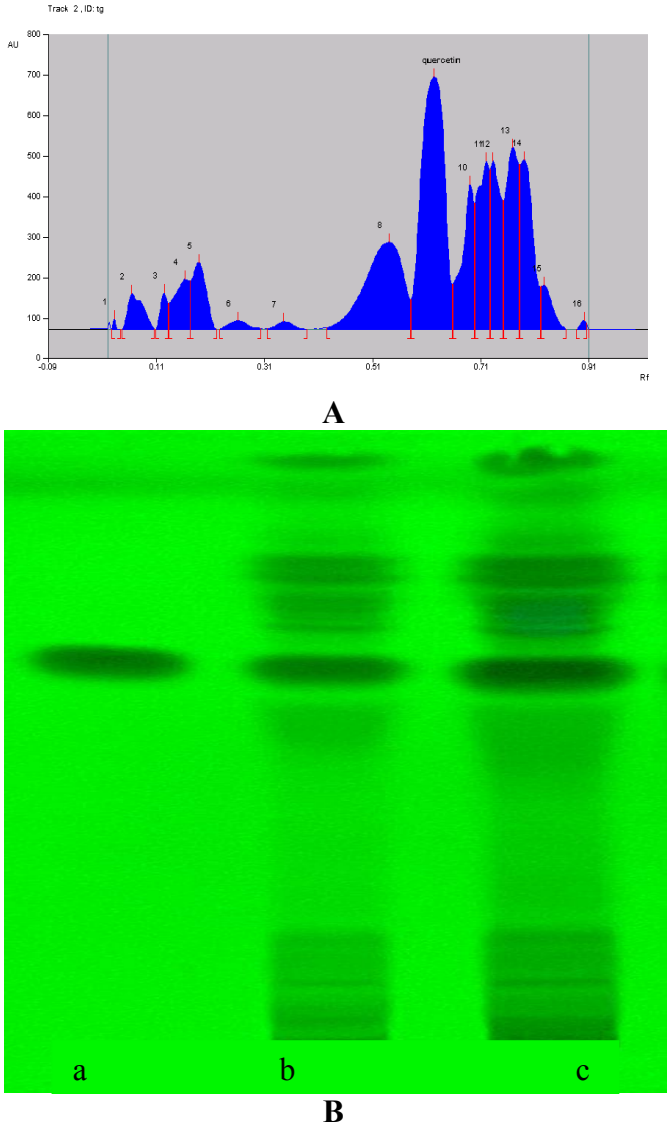

Figure 1: A- HPTLC profile of formulation F2, B- TLC of quercetin (a: Standard; b&c: Sample in duplicate)

Method Validation:

Linearity

The linear correlation between the peak area and the concentration (figure 2) was found in the range of 40-140 ng/spot for quercetin under the chromatographic conditions mentioned above.

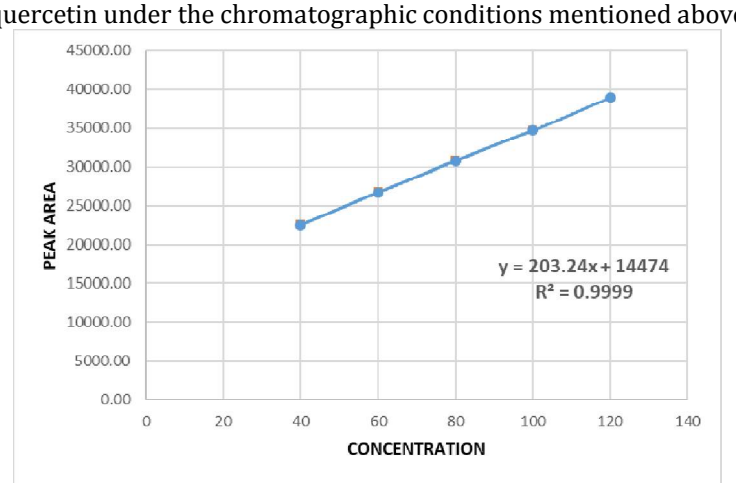

Figure 2: Linearity graph of Standard Quercetin.

Precision

Intraday and interday precision was done in triplicate at 3 distinct concentration levels. Data on repeatability and instrumental variation were obtained. RSD < 1% , proved that the method was highly precise.

Accuracy

This was done by recovery method at 3 concentrations of 80%, 100% and 120%. The recoveries were found to be in the range of 99.48- 109.40%. The results concluded that the HPTLC method was found to be accurate for quantification of quercetin.

Robustness

The saturation time was changed from 10min to 12min and wavelength was switched from 252nm to 256nm. The area under curve calculated in the both conditions was within the limit. There was no change in the peak shape and retention time for both. Resolution and separation was found to be have no change making the method robust.

LOD and LOQ

The LOD was found to be 0.79ng/spot and the LOQ was found to be 2.40ng/spot for quercetin.

Percentage of Quercetin in formulation:

The above method was validated and used for quantification of quercetin in the formulations prepared. The developed method provided a well resolved chromatogram, with no alterations in peaks of quercetin.

Table 3: Percentage of quercetin

Sr. No.	Formulation	Percent of quercetin
1.	F2	0.75%±0.23

Table 4: Antiurolithic activity on rats

Sr. No.	Group	Total urine volume (ml)	Urine pH	Body weight (g)	Kidney weight (g)
1	Normal	18.34±0.51	6.50±0.11	18.33±1.03	2.24±0.03
2	Control	15.61±0.43	8.18±0.05	5±1.09	3.20±0.04
3	Sample	18.87±0.49	7.19±0.07	10.17±2.13	2.72±0.12
4	Standard	18.70±0.61	6.35±0.08	9.33±2.25	2.36±0.06

Table 5: Evaluation of Serum parameters in rats

Serum parameters (mg/dL)	Groups			
	Normal	Control	Sample	Standard
Creatinine	0.76±0.04	3.64±0.32	1.08±0.18	1.10±0.15
Urea	26.81±1.06	73.75±1.19	52.82±7.12	37.90±1.67
Uric acid	3.85±0.23	8.93±0.19	5.10±0.51	4.76±0.45
Calcium	6.86±0.17	11.00±0.55	8.72±0.42	8.54±0.43
BUN	14.20±0.76	34.06±1.51	22.11±1.43	15.26±0.50
MDA	0.60±0.05	4.52±0.26	1.50±0.20	1.30±0.14
GSH	7.47±0.33	4.16±0.06	5.89±0.40	5.18±0.24
Catalase	37.68±1.20	17.39±0.65	30.94±0.55	32.68±1.18

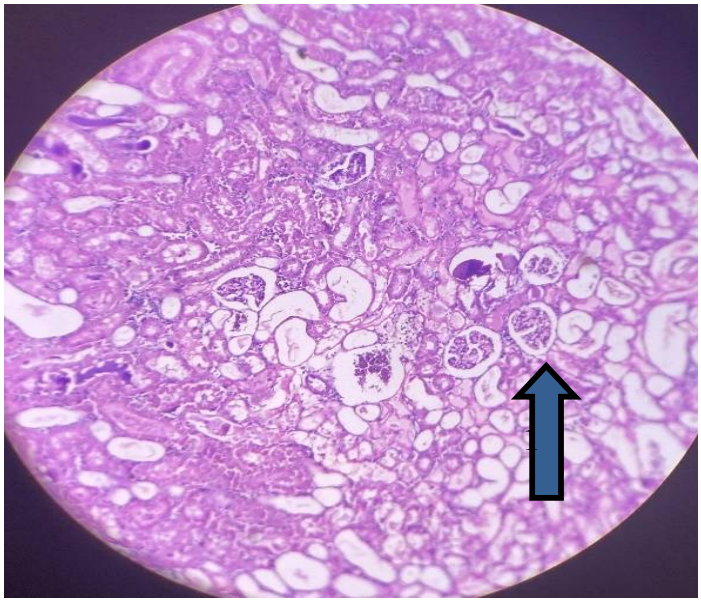

Figure 3: Microscopic examination of normal group (1- Glomeruli)

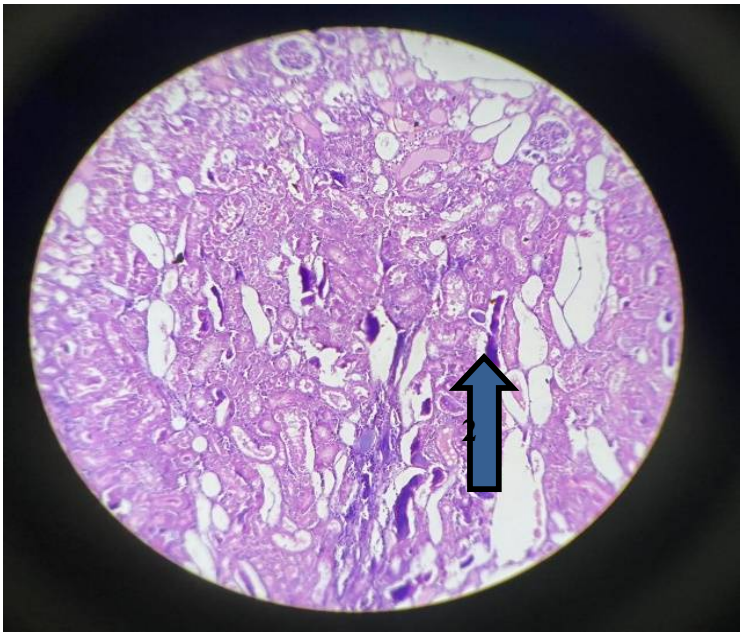

Figure 4: Microscopic view of Control group (2-Thyroidisation)

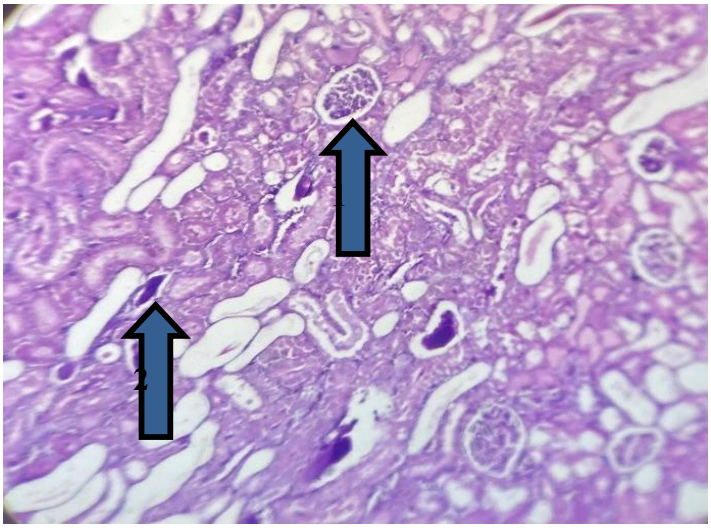

Figure 9: Microscopic view of Flavonoid rich extract

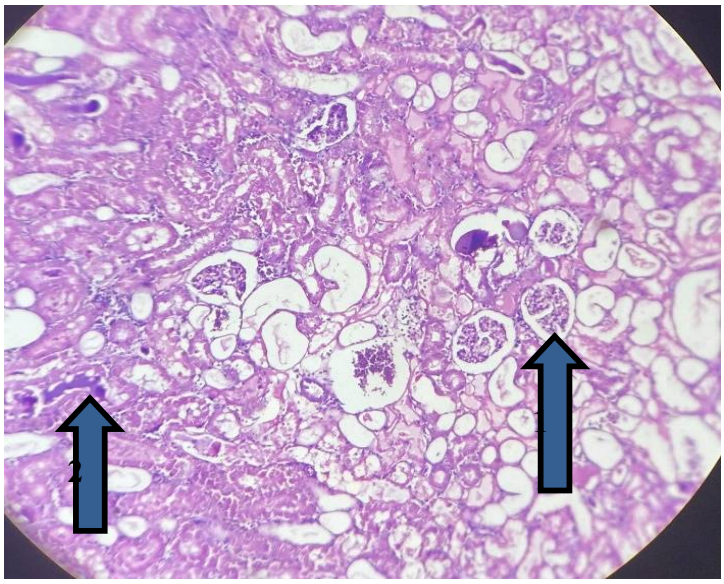

Figure 10: Microscopic view of Standard- Cystone group

Administration of 0.75 % (v/v) ethylene glycol aqueous solution to male albino wistar rats produced formation of renal calculi. This showed an increase in the level of oxalate, calcium and citrate excretion. Supplementation of Tridax syrup significantly lowered the levels of increased calcium, oxalate and citrate in urine when compared to the control group (II). Level of creatinine, urea and uric acid in serum also were increased drastically, this too was seen to be lowered by the use of Cystone (standard) and Tridax formulation (F2). Urine volumes were seen to be increased by the standard and the Tridax syrup as compared to the control group. Urinary pH showed an increase in pH value, when the animals were introduced to 0.75% ethylene glycol. There was a subsequent decrease in pH on administration of Tridax syrup and Cystone. Examination of kidney sections in control group showed no calculi formation or deposits, whereas there were huge amounts of deposits of calcium and oxalate in the groups treated with ethylene glycol.

In the histopathological studies, the occurrence of thyroidisation indicate a sign of deterioration of kidney which was found to be lowered in the group treated with the flavonoid rich extract (figure 9) compared with the control group and all the prepared extracts. There was also increase in the number of glomeruli was observed indicating the sample was found to be effective against the urolithiasis.

The Tridax syrup formulated showed to have good antiurolithic property. This herbal syrup successfully reduced kidney stones by a non-toxic and convenient way. The plant is cheap and easily available. The

presence of organic constituents like quercetin found in *Tridax procumbens* help in the reduction of renal calculi. This is a good natural remedy for kidney stones.

Stability study Data

Table 6: Accelerated stability study

Sr. No.	Parameters	Initial Data	3 months stability	6 months stability
1.	Appearance	Light brown, semi-transparent	Light brown, semi-transparent	Light brown, semi- transparent
2.	Taste	Sweet	Sweet	Sweet
3.	Odour	Characteristic	Characteristic	Characteristic
4.	pH	5.39± 0.01	5.36± 0.1	5.34± 0.05
5.	Density (g/ml)	1.061± 0.13	1.061± 0.07	1.061± 0.002

The outcomes of the accelerated stability test revealed no statistically significant changes ($p>0.05$) in the tested variables over the course of six months, confirming the product's stability. The developed syrup formulation exhibited sufficient stability in terms of physical properties and quality control assessments after 6 months at 40 °C, and was suitable for an oral use.

CONCLUSION

In Ayurveda, *Tridax procumben* is the well-known plant possesses antiseptic, hepatoprotective, antimicrobial, antihypertensive, antidiabetic action. The drug also used to cure disease conditions like dysentery, non-healing wounds, stomach upset, diarrhea, metabolic syndrome.In the present studies, different syrup formulations of *Tridax procumben* were prepared (F1 to F5). The prepared formulations were evaluated for different parameters. The F2 formulation was found to be better from all the prepared formulation.The marker i.e. quercetin was standardize the formulations and was then confirmed through HPTLC analysis where Rf value was found to be 0.61. The formulation F2 was analyzed for the identification of quercetin. The percent of quercetin was found to be 0.75%.The formulation further studied for Antiurolithiatic action. There was decreased in the level of calcium, oxalate and citrate in urine when compared to the control. There was also decreased in the level of creatinine, urea and uric acid was observed in serum when compared with standard (cystone). The overall results concluded that the administration of *Tridax procumbens* reduced and prevented growth of renal calculi.

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