Investigation Of Potential Anti-Urolithiatic Activity Of Some Herbs By Using Ethylene Glycol Induced Urolithiasis In Animals

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Abstract:
Common terms for urolithiasis include kidney stone. Urinary calculus is very old disease and its history dates back ancient to the earliest period of society. In order to treat kidney stone illness, the formed stone must be removed, either by dissolving it or breaking it up into little pieces so that urine may flush it out of the urinary tract. Therefore, it is necessary to create a potent polyherbal formulation for measuring urolithiatic activity. The potent antiurolithiatic herbs were chosen for this study's polyherbal tablet in order to evaluate its antiurolithiatic effectiveness. Using wistar rats, the antiurolithiatic activity of tablets was evaluated against renal calculi caused by ethylene glycol.
Keywords: Antiurolithiatic, Kidney stones, Calcium, Nephrolithiasis.

Introduction

The urolithiasis has common names such as renal calculus/ calculi or kidney stone. Stone may form at any level in the urinary tract, but most arise in the kidney[1]. Urinary calculus is very old disease and its history dates back ancient to the earliest period of society[2]. When urinary constituents normally in solution are getting precipitates, calculi or stones form in the kidney and bladder. The solutes involved in the formation of kidney stones are generally oxalates and phosphates. The age of 30 years in case of males are more prone to form kidney stone and the condition is often recurrent. Most of the times, it originates in the collecting tubules or renal papillae and then passes into the renal pelvis where they may increase in size. Kidney damage happens when some stones become too large to pass through the ureter and may obstruct the outflow of urine. Others pass to the bladder and are either excreted or increase in size and obstruct the urethra [3].

There are various abnormal constituents present in urine have chemical composition of kidney stones when the level of the constituents increases above the normal value, they become responsible for the kidney stone disease. Stones differ in dimension, character, and chemical compositions (mineralogy)[4]. Based on variations in mineral composition and pathogenesis, kidney stones are commonly classified into five types as follows:

Calcium stones (Calcium oxalate Calcium phosphates), Struvite stones, Cystic stones, Uric acid stones and Drug induced stones [4,5]. In the treatment of kidney stone disease there is need to remove the formed stone either by dissolving or by breaking it into small pieces and pass from urinary tract with urine. None of the surgical treatment produces satisfactory result. So, there is need to develop an effective polyherbal formulation for assessment of the urolithiatic activity.According to ayurveda system of medicine, *Tribulus terrestris, Musa balbisianacolla, Bryophyllum Pinnata* and Commiphora wightii were reported to be useful in the treatment of urinary stones [6,7].

In this present study the polyherbal tablet was prepared for the assessment of antiurolithiatic activity. The wet granulation method was used for the formulation of tablet. The prepared tablets were pass all the necessary test like weight variation test, hardness test, friability test disintegration test and dissolution test. The assessment of antiurolithiatic activity of tablets was carried out against ethylene glycol induced renal calculi by using wistar rats.

Materials and Methods

Extraction of Process of selected plants:

The pseudo stem of ***Musa balbisian***, fruits of ***Tribulus terrestris*****,** fresh leaves of *Bryophyllum pinnata* and powder of ***Commiphora wightii*** were procured from local market of Pune city. They were authenticated from Vidya Pratishthan's Arts Science and Commerce College, Botany Department, Baramati.

Extraction Process:

Extraction of Process of selected plants

a) Extraction procedure of *Musa balbisianacolla*: The juice was extracted from a pseudo stem by pressing using a sugarcane press machine and this was done within 24 h after harvesting. The juice extracted was filtered to remove solid materials. The filtered juice was then freeze dried using a freeze dryer. The freeze dried extracts were then collected[10].

b) Extraction procedure of *Tribulus terestris*: The dried and matured fruits of *Tribulu terrestris* were obtained from local areas of Pune. Aqueous extract was prepared by using the dried and matured fruit of *Tribulu terrestris* was ground into fine powder and the extraction was carried out at temperature of 23.5 °C for a period of 19.50 hours under constant stirring. Following this, the extract was filtered, and stored in air tight container[11].

c) Extraction procedure *of Bryophyllum Pinnata*: Fresh leaves of ***Bryophyllum Pinnata*** were collected from the botanical garden of the Seth Govind Raghunath Sable College of Pharmacy, Saswad. The leaves were air dried, pulverized and extracted exhaustively by cold maceration. The filtrate was subsequently evaporated to obtain the dry extract using a rotary evaporator [12,13].

d) Extraction procedure of *Commiphora wightii*: The stem barks of *Commiphora wightii* were collected from local market of Pune. The plant was washed, chopped in to small pieces and dried under shade then powdered coarsely with a mechanical grinder. The powder was passed through sieve No. 40 and stored in an airtight container for further use. Extraction of Plant Material of coarsely powdered plant material was extracted by Soxhlet extraction method using petroleum ether. All the extracts thus obtained were stored in air-tight bottles at 4ºC for further experiments[14].

Preparation of Tablet by Wet granulation method:

Weigh all the required ingredients and mix them by doubling up method. Then add little amount of water to the mixture to form a dough. Pass this dough through 44# mesh sieve to form granules. Dry the formed granules and again pass them through 80# mesh sieve. These granules are punched in tablet punching machine to form the tablets [15, 16, 17].

In vivo animal model: Anti-urolithiatic activity of polyherbal formulation by ethylene glycol induced urolithiasis in rats [18-22].

Selection of Animals:

Wistar rats, weighing 200- 250 gm of both sex and Swiss albino mice, weighing 25- 30 gm of either sex were obtained from animal house of PDEA's Seth Govind Raghunath Sable College of Pharmacy, Saswad. All the animals have free access of food pellets and water was provided *ad libitum*. The animals were housed in air-conditioned area where the temperature of in the room was maintained at 22 ± 2° C. and the relative humidity was 45 to 55 %. Also, the animals were kept under 12 hr light and 12 hr dark cycle in strict hygienic conditions.

Approval for research protocol by IAEC with Protocol Approval No. SGRS/IAEC/10/2021-22

Anti-urolithiatic activity of polyherbal formulation by ethylene glycol induced urolithiasis in rats

Urolithiasis is induced in either sex of wistar rats by administering 0.75 % ethylene glycol & 1 % ammonium chloride solution for 28 days for preventive control & 14 days for curative control study.

Preparation of ethylene glycol & ammonium chloride solution:

Ethylene glycol is a toxic drug specially used for the urolithiasis model in rats for antiurolithiatic activity. 0.75% solution of ethylene glycol and 1 % solution of ammonium chloride administered to Wistar rats through drinking water. Ammonium chloride solution was administered to rats simultaneous to ethylene glycol for uniformity in stone production.

Experimental design:

Table No. 1: Experimental design of in vivo ethylene glycol induced urolithiasis model

Group No.	**Title**	**Treatment**	**No. of animals**
I	**Normal control**	Normal saline	6
II	**Lithiatic control**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for next 24 days.	6
III	**Standard control**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for next 24 days + standard drug (Cystone) from 14^{th} to 28^{th}day.	6
IV	**Curative control (100 mg/kg)**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for next 11 days + 100 mg/kg dose of drug from 14^{th} to 28^{th} day.	6
V	**Curative control (200 mg/kg)**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for next 11 days + 200 mg/kg dose of drug from 14^{th} to 28^{th}day.	6
VI	**Curative control (400 mg/kg)**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for next 11 days + 400 mg/kg dose of drug from 14^{th} to 28^{th} day.	6
VII	**Preventive control (100 mg/kg)**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for 24 days + 100 mg/kg dose of drug from 1^{st} to 28^{th} day.	6
VIII	**Preventive control (200 mg/kg)**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for 24 days + 200 mg/kg dose of drug from 1^{st} to 28^{th} day.	6

IX	**Preventive control (400 mg/kg)**	Ethylene Glycol (0.75% v/v) + Ammonium Chloride (1% w/v) for 3 days, and only Ethylene Glycol (0.75%) in drinking water for 24 days + 400 mg/kg dose of drug from 1st to 28th day.	6

Parameters observed:

Collection and analysis of urine:

All animals were kept in individual metabolic cages and 24 h urine samples were collected on 0, 7, 14 and 28th day of calculi inducing treatment. After measurement of urine volume, all urine samples were analyzed for pH, calcium, oxalate,and phosphate content. The urine oxalate level was measured using the method of Hodgkinson.

Serum analysis:

After urine collection of 28th day, blood was obtained from the retro-orbital sinus under anesthetic condition and animals were sacrificed by cervical decapitation. Serum was separated by centrifugation at 10,000 rpm for 10 min and analyzed for creatinine, uric acid and blood urea nitrogen (BUN).

Kidney and Liver histopathology:

The abdomen was cut open to remove both kidneys & a liver from each animal. Isolated kidneys & liver were cleaned off extraneous tissue and rinsed in ice-cold physiological saline. One of the kidneys & a liver was fixed in 10% neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 μm and stained with H and E (Haematoxylin and Eosin) for examination under polarized light. The slides were also observed to estimate tubulointerstitial damage index.

Statistical analysis

All the results were expressed as mean ± SEM. The results were analyzed statistically using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test. P-values were calculated against vehicle and lithiatic control groups and $P < 0.05$ was considered significant. [9-13]

Results

Extraction of *Musa balbisianacolla*: The percentage yield obtained was1.64 % w/w. Phytochemical analysis of aqueous extract of *Musa balbisianacolla* was found Saponins, Xylose, galactose, mannose, arabinose, triterpenoids.

Extraction of *Tribulus terestris*: The percentage yield obtained was14.5 % w/w. The extract was reveals to presence of tannic acid, rutin, quercetin, catechin, gallic acid.

Extraction of *Bryophyllum Pinnata* : The percentage yield obtained was **7** % w/w**.** Phytochemical analysis of aqueous *Bryophyllum Pinnata* leaf extract showed the presence of alkaloids, carbohydrates, flavonoids, saponins, triterpenoid, tannins, phenols, anthraquinone and steroids.

Commiphora wightii:

The percentage yield obtained was 25.75% w/w**.** Phytochemical analysis of ***Commiphora wightii*** extract showed the presence of alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes and terpenoids.

Determination of LD_{50}:

Toxicity study was carried out using a starting dose of 2000 mg/kg body weight. Animals were observed individually after dosing at least once during the first 30 min. periodically during the first 24 h, with special attention given during first 4 h. OECD Guidelines, No. 425(para-36). A single dose of 5000 mg/kg produces some lethargic effects on the mice. So

the LD_{50} is lesser than the 5000 mg/kg that is 2000 mg/kg. All the five animals were survived. Thus, the one tenth dose of 2000 mg/kg i. e. 200 mg/kg was selected as a therapeutic dose and the sub-therapeutic and super-therapeutic dose were selected as 100 mg/kg and 400 mg/kg respectively.

In-vivo Model
Ethylene Glycol Induced Urolithiasis Model:
1. Preventive Study:

Table 2. Effect of on serum parameters of urolithiatic rats

Parameters	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
Creatinine	0.83±0.03	2.87±0.16###	1.74±0.02***	2.03±0.06***	1.32±0.03***	1.42±0.013***
BUN	13.15±0.10	24.59±0.33###	15.63±0.17***	21.73±0.25***	17.92±0.12***	14.23±0.17***
Uric Acid	1.63±0.07	7.01±0.15###	3.19±0.09***	5.11±0.19***	3.24±0.12***	2.61±0.16***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n= 6

Fig.1 Effect of tablet on Serum Creatinine in urolithiaticrats

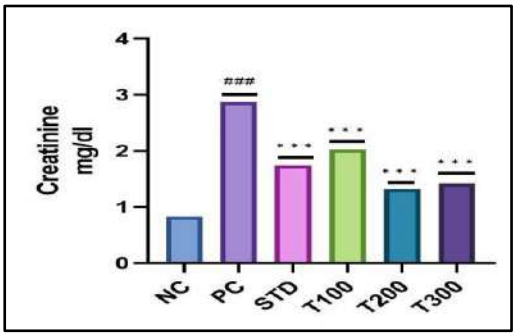


Fig.2 Effect of tablet on Serum BUN in urolithiatic rats

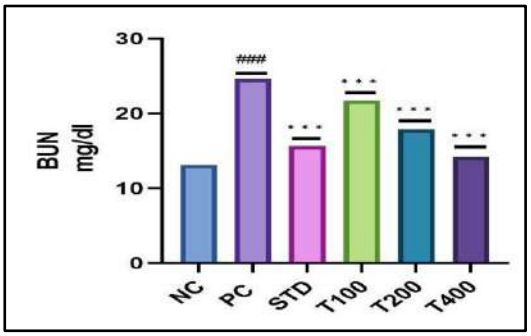


Fig. 3 Effect of on Serum Uric Acid in urolithiatic rats

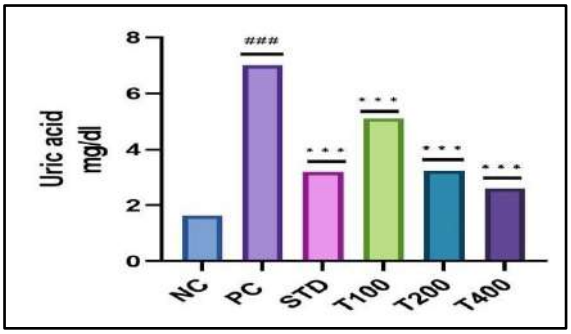


Table. 3 Effect of on urine volume of urolithiatic rats

Parameter Urine Volume (ml/24 hr)	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	2.23±0.07	2.59±0.02	2.47±0.03	3.05±0.03	2.11±0.02	2.17±0.02
7 Day	2.42±0.06	2.11±0.04###	1.86±0.03	1.92±0.02	2.17±0.02	1.89±0.03*
14 Day	2.51±0.07	1.67±0.03###	2.28±0.03***	2.01±0.03***	1.57±0.02***	2.07±0.02***
28 Day	2.37±0.03	1.29±0.03###	2.11±0.02***	2.05±0.04***	2.13±0.04***	2.29±0.02***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.4 Urine volume on 0 day of study

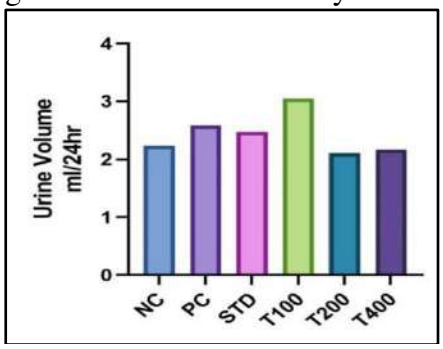


Fig.5 Urine volume on 7th day of study

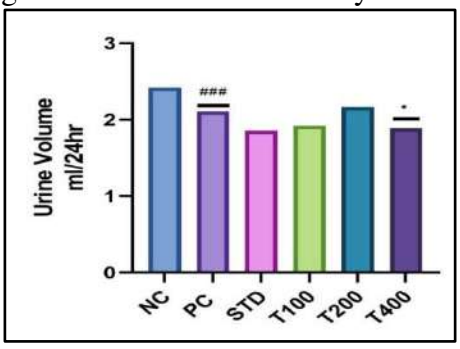


Fig.6 Urine volume on 14thday of study

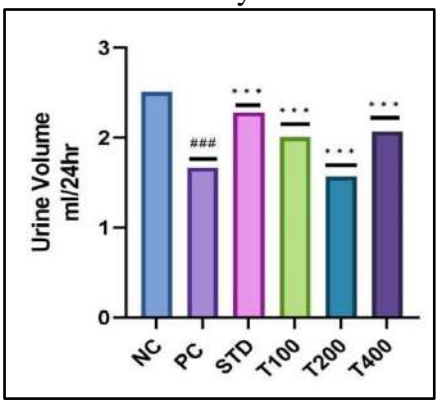


Fig.7 Urine volume on 28th day of study

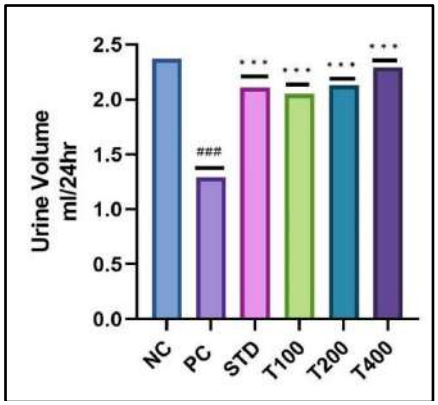


Table. 4 Effect of tablet on urine pH of urolithiatic rats

Parameter Urine pH	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	7.65±0.05	7.65±0.05	7.62±0.06	7.77±0.05	7.73±0.05	7.89±0.05

7 Day	7.59±0.06	6.79±0.07###	6.63±0.06	6.61±0.02	6.83±0.05	6.94±0.05*
14 Day	7.71±0.07	6.47±0.04###	7.09±0.04***	6.81±0.03***	7.17±0.03***	7.37±0.01***
28 Day	7.75±0.04	5.31±0.04###	7.52±0.02***	6.99±0.04***	7.38±0.02***	7.51±0.02***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.8 Urine pH on 0 day of study

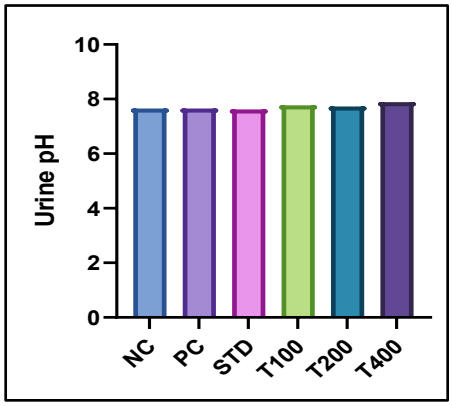


Fig.9 Urine pH on 7th day of study

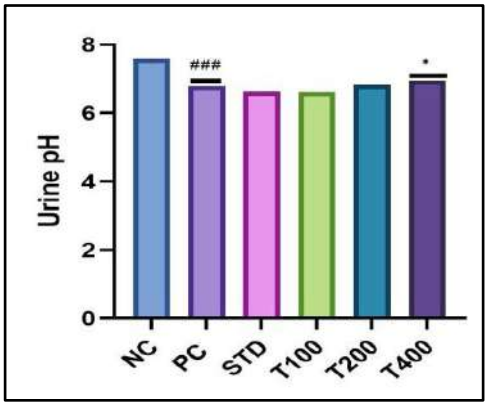


Fig.10 Urine pH on 14th day of study

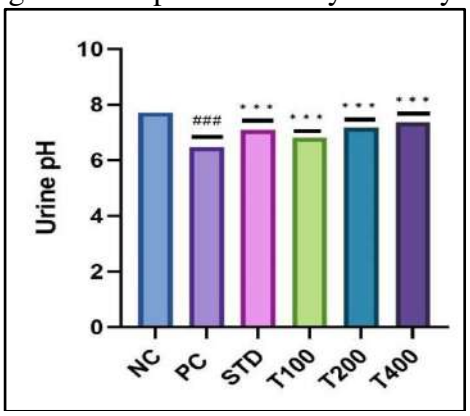


Fig.11 Urine pH on 28th day of study

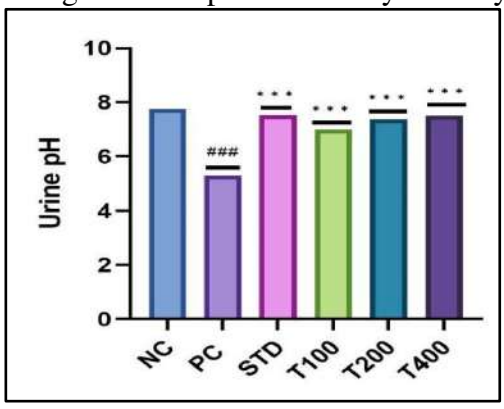


Table. 5 Effect of on urine calcium of urolithiatic rats

Parameter Urine Calcium	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	3.98±0.07	4.27±0.14	3.93±0.09	4.32±0.07	4.16±0.07	4.04±0.05
7 Day	4.10±0.07	6.09±0.16###	5.57±0.17**	6.05±0.01	5.53±0.02**	5.49±0.01***
14 Day	4.11±0.09	7.35±0.02###	4.98±0.07***	5.37±0.08***	5.27±0.09***	5.17±0.09***
28 Day	4.17±0.07	7.47±0.02###	5.54±0.10***	6.70±0.07***	5.49±0.12***	4.77±0.05***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.12 Concentration of calcium on 0 day of study

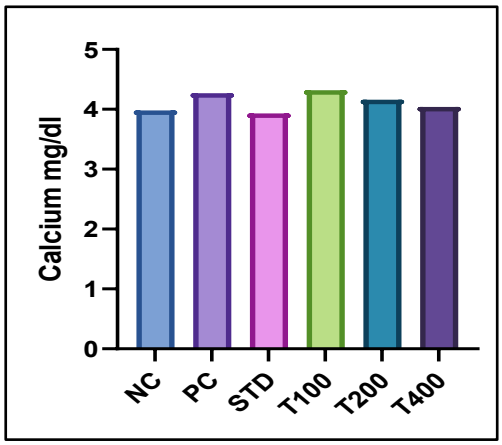


Fig.13 Concentration of calcium on 7th day of study

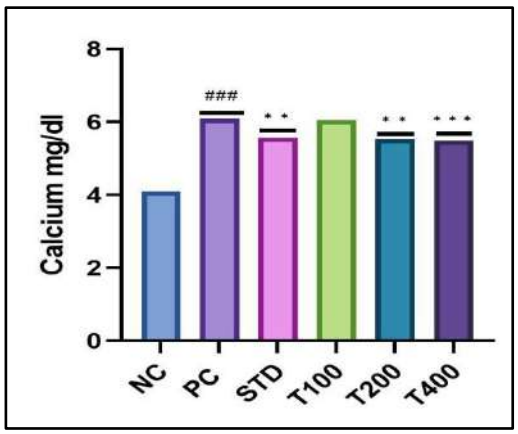


Fig.14 Concentration of calcium on 14th day of study

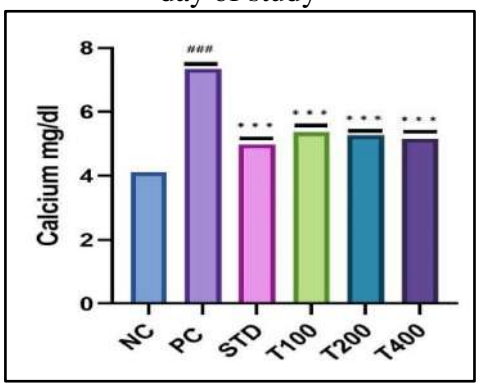


Fig.15 Concentration of calcium on 28th day of study

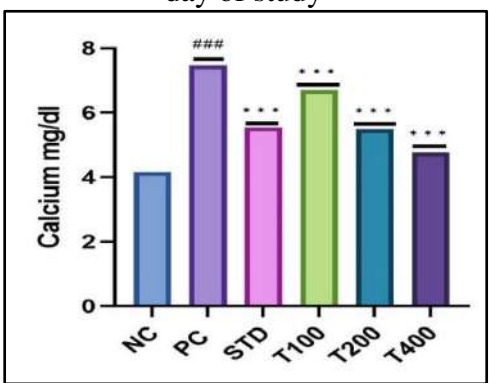


Table. 6 Effect of on urine oxalate of urolithiatic rats

Parameter Urine Oxalate	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	0.80±0.02	0.81±0.02	0.81±0.02	0.80±0.02	0.78±0.03	0.79±0.02
7 Day	0.81±0.02	1.39±0.03###	1.33±0.02	1.14±0.04***	0.97±0.01***	0.87±0.01***
14 Day	0.83±0.02	2.53±0.18###	1.27±0.02***	1.52±0.02***	1.24±0.02***	0.86±0.01***
28 Day	0.83±0.02	3.69±0.15###	1.07±0.05***	0.93±0.02***	0.83±0.02***	0.78±0.01***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.16 Concentration of oxalate on 0-day of study

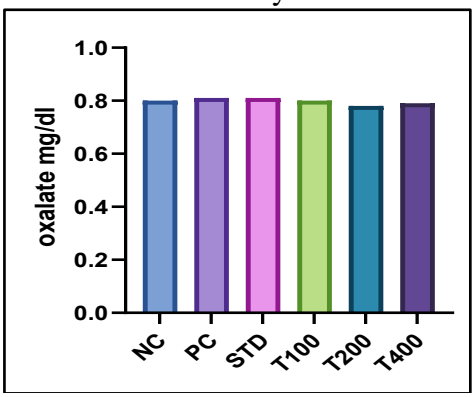


Fig.17 Concentration of oxalate on 7th day of study

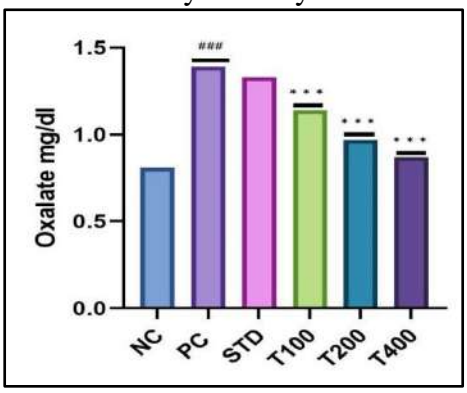


Fig.18 Concentration of oxalate on 14thday of study

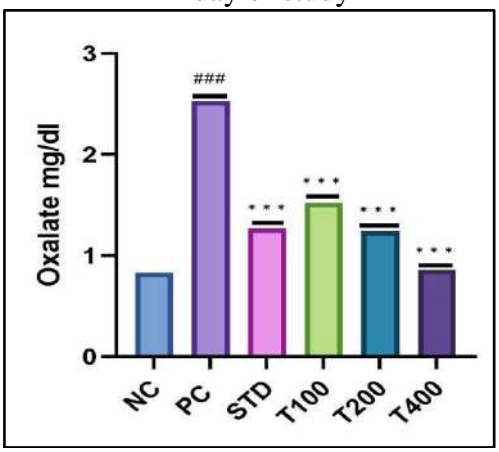


Fig.19 Concentration of oxalate on 28th day of study

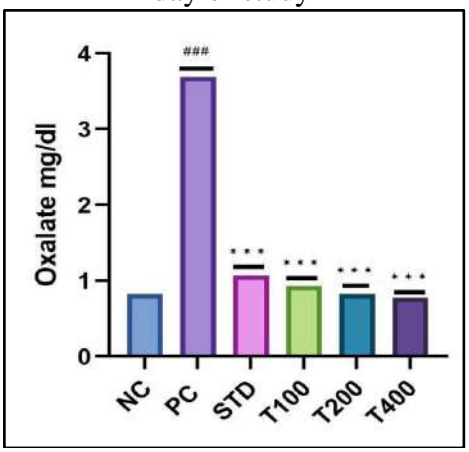


Table. 7 Effect of on urine phosphate of urolithiatic rats

Parameter Urine Phosphate	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	5.30±0.02	5.30±0.02	5.28±0.01	5.26±0.01	5.26±0.02	5.26±0.01
7 Day	5.28±0.02	6.36±0.05###	6.38±0.02	6.13±0.03***	5.81±0.02***	5.57±0.03***
14 Day	5.31±0.02	7.45±0.03###	6.17±0.03***	5.93±0.02***	5.62±0.01***	5.38±0.07***
28 Day	5.29±0.02	8.11±0.07###	6.19±0.03***	6.31±0.02***	5.82±0.02***	5.4±0.02***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.20 Concentration of phosphate on 0 day of study

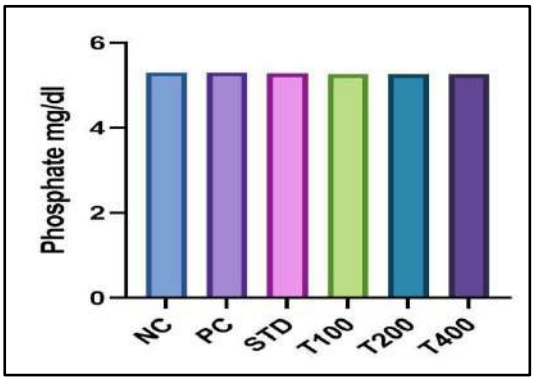


Fig.21 Concentration of phosphate on 7th day of study

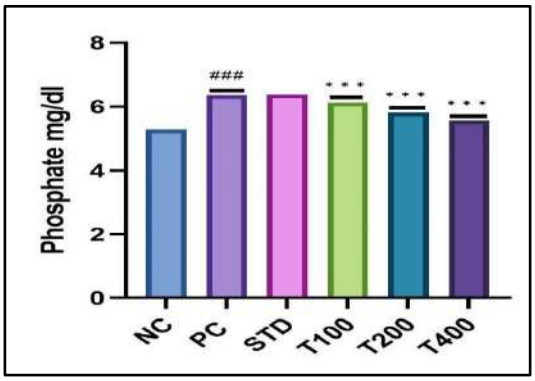


Fig.22 Concentration of phosphate on 14th day of study

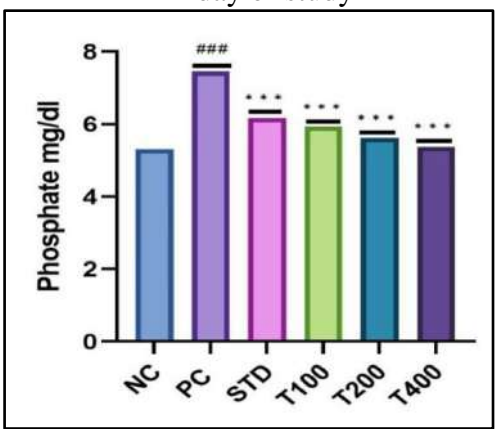
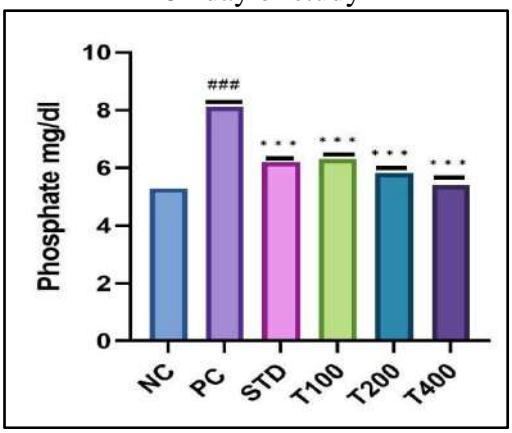
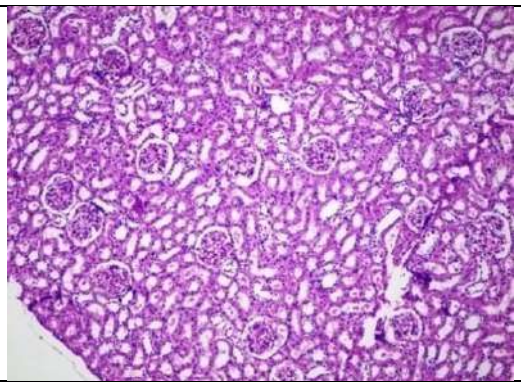


Fig.23 Concentration of phosphate on 28th day of study

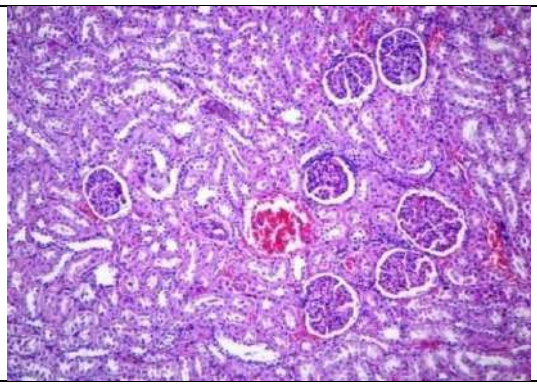


Histopathology Study:

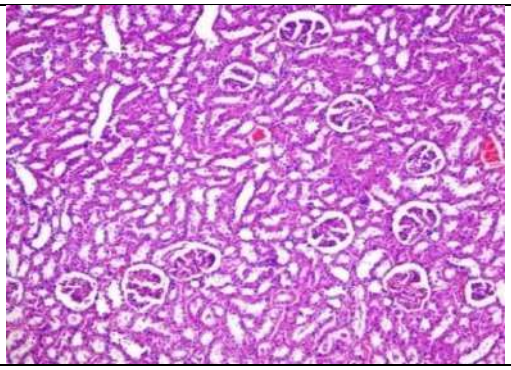
Fig.24 Effect of on histopathology of isolated kidneys of urolithiatic rats in ethylene glycol induced urolithiasis model.



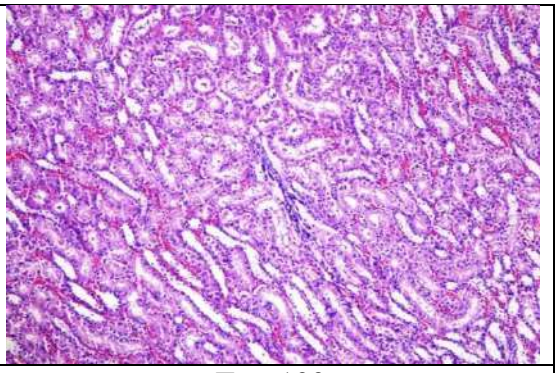
Normal Control



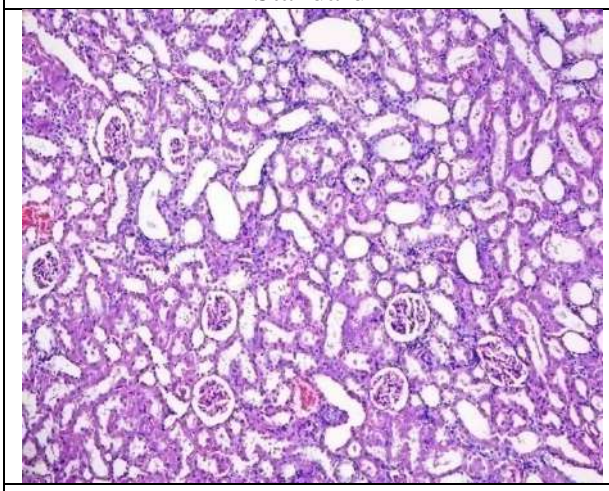
Positive Control



Standard



Test 100



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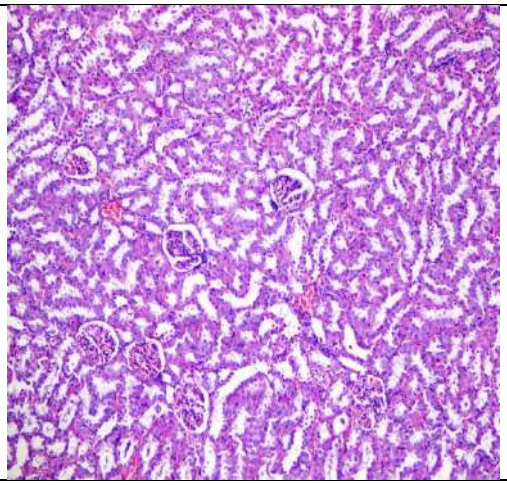


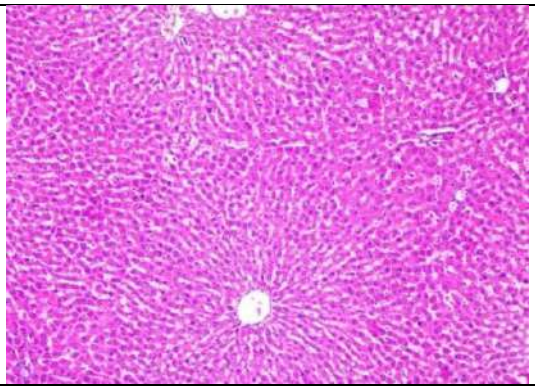
Test 400

Table. 8 Histopathology report of kidney (Preventive study)

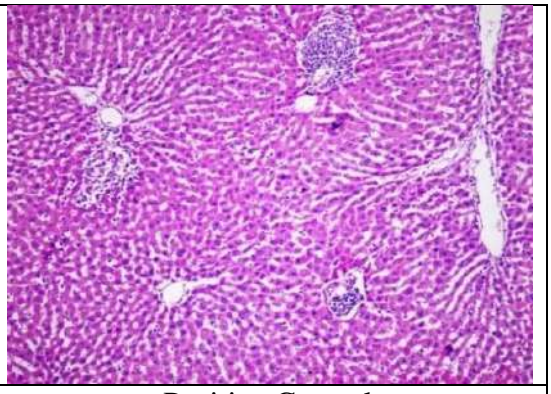
Histopathology Report of Kidney (Preventive study)			
Sr. No.	**Group**	**Histopathological observations**	**Overall pathological remark**
1	**Normal Control**	The microscopic observations of kidney sections showed normal renal tubules and glomeruli. The renal tubules showed normal epithelium. There was absence of any inflammatory or pathological changes in kidney.	**NAD**
2	**Positive Control**	The renal tubules showed diffuse degenerative changes with congestion and interstitial focal haemorrhages in the renal parenchyma. Renal tubules showed cellular swelling and focal accumulation of eosinophilic and cellular debris in lumen of tubules in the medullary region. Marked degenerative changes in renal tubules. Multiple foci of dilation of renal tubules with interstitial haemorrhages were observed.	**Moderate (+3)**
3	**Standard**	The microscopic observations of kidney sections showed normal renal tubules and glomeruli. The renal tubules showed focal degenerative changes in renal parenchyma.	**Minimal (+1) to NAD**
4	**Sub-therapeutic**	The renal tubules showed mild degenerative changes with congestion and focal haemorrhages in the renal parenchyma.	**Mild (+2)**

	Dose 100 mg/kg	Renal tubules showed cellular swelling and focal accumulation of eosinophilic and cellular debris in lumen of tubules in the medullary region. Multiple foci of dilation of renal tubules with interstitial haemorrhages were observed.	
5	**Therapeutic Dose 200 mg/kg**	The microscopic observations of kidney sections showed normal renal tubules and glomeruli in the cortex and medulla. Focal cellular swelling and focal degenerative changes in renal parenchyma with focal accumulation of eosinophilic debris in lumen of tubules.	**Minimal (+1)**
6	**Supper-therapeutic Dose 400 mg/kg**	The microscopic observations of kidney sections showed normal renal tubules and glomeruli in the cortex and medulla. Focal cellular swelling and focal degenerative changes in renal parenchyma with focal accumulation of eosinophilic debris in lumen of tubules.	**Minimal (+1)**
Note: Overall Grade score as- NAD =No Abnormality Detected, Minimal changes (+1), Mild changes (+2), Moderate changes (+3), Severe changes (+4).			

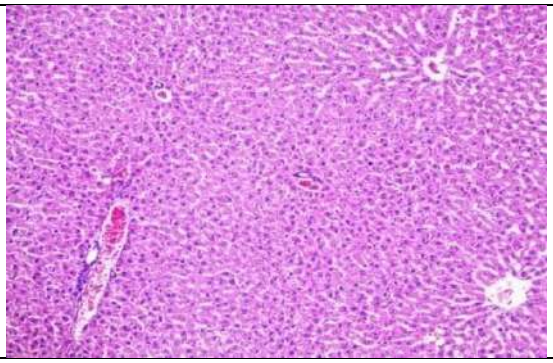
Fig. 25 Effect of on histopathology of isolated liver of urolithiatic rats in ethylene glycol induced urolithiasis model.



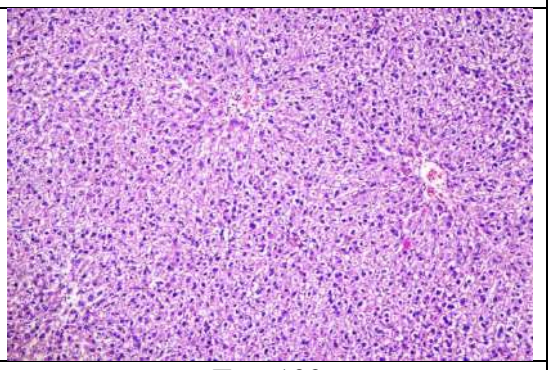
Normal Control



Positive Control



Standard



Test 100

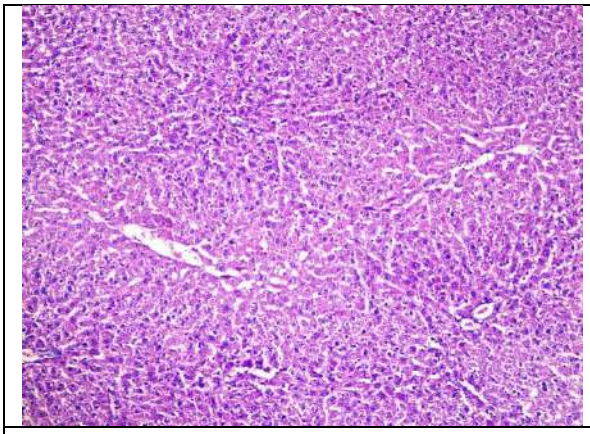
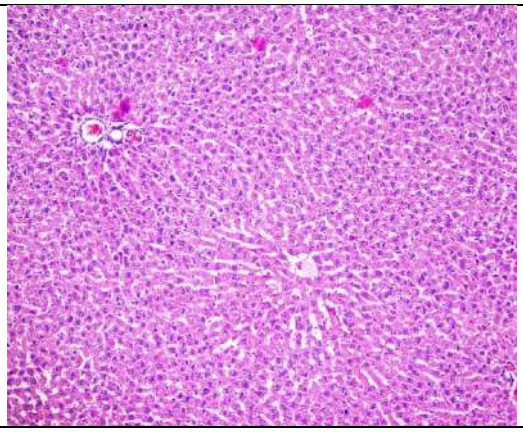
		
Test 200		Test 400

Table. 9 Histopathology report of liver (Preventive study)

Histopathology Report of Liver (Preventive study)			
Sr. No.	**Group**	**Histopathological observations**	**Overall pathological remark**
1	**Normal Control**	The microscopic examination showed normal cellular features of hepatocytes with intact nucleus and cell borders. The hepatic parenchyma showed hepatocytes arranged in strands around central vein. There was absence of any inflammatory or metabolic pathological changes in the hepatocytes.	**NAD**
2	**Positive Control**	The microscopic examination of tissue sections from positive control group showed congestion of hepatic vasculature with mild degenerative changes in hepatic parenchyma was observed. Hepatocytes showed cellular swelling with enlarged nucleus as well as diffuse degenerative changes with granular cytoplasm. Derangement of hepatic cords with loss of continuity was observed with focal necrotic changes of hepatocytes with loss of nucleus and cell borders was also noted.	**Moderate (+3)**
3	**Standard**	The microscopic examination of tissue sections from MID dose group showed focal congestion of central veins. Focal presence of degenerative changes of hepatocytes was noted.	**Minimal (+1)**
4	**Sub-therapeutic Dose 100 mg/kg**	The microscopic examination of tissue sections from Low dose group showed congestion of hepatic vasculature. Hepatocytes showed cellular swelling with	**Mild (+2) to Moderate (+3)**

		enlarged nucleus as well as diffuse degenerative changes with granular cytoplasmic changes in the parenchyma. Focal fatty infiltration was noted in hepatocytes.	
5	**Therapeutic Dose 200 mg/kg**	The microscopic examination of tissue sections from Mid dose group showed congestion of hepatic vasculature with mild degenerative changes in hepatic parenchyma. Hepatocytes showed cellular swelling with enlarged nucleus as well as diffuse degenerative changes with granular cytoplasm.	**Mild (+2)**
6	**Supper-therapeutic Dose 400 mg/kg**	The microscopic examination of tissue sections from HIGH dose group showed focal congestion of central veins. Focal degenerative changes were noted with focal cellular swelling in few of the hepatocytes only.	**Minimal (+1)**
Note: Overall Grade score as- NAD =No Abnormality Detected, Minimal changes (+1), Mild changes (+2), Moderate changes (+3), Severe changes (+4).			

Curative Study

Table. 10 Effect of on serum parameters of urolithiatic rats

Parameters	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
Creatinine	0.94±0.03	3.22±0.16###	1.40±0.02***	2.74±0.14**	1.99±0.02***	1.57±0.08***
BUN	11.83±0.10	24.57±0.33###	15.51±0.17***	22.83±0.43***	19.17±0.17***	15.11±0.20***
Uric Acid	1.64±0.07	6.61±0.15###	3.27±0.09***	4.93±0.16***	3.17±0.10***	2.81±0.12***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.26 Effect of on Serum Creatinine in urolithiatic rats.

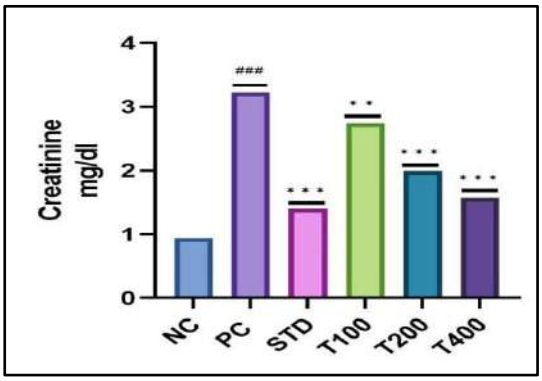


Fig.27 Effect of on Serum BUN in urolithiatic rats

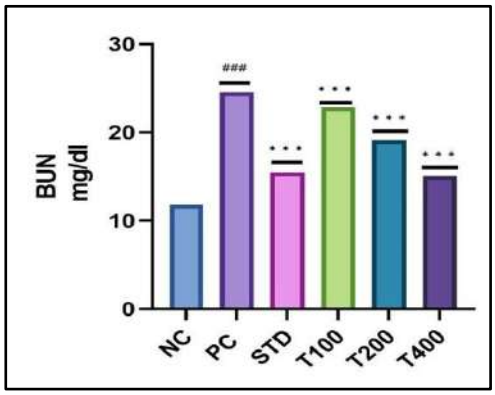


Fig. 28 Effect of on Serum Uric Acid in urolithiatic rats

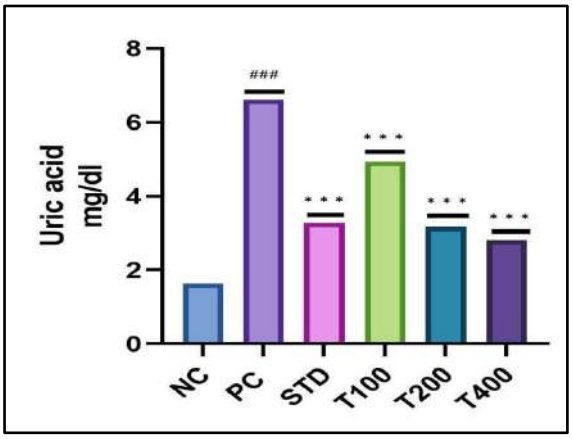


Table. 11 Effect of on urine volume of urolithiatic rats

Parameter Urine Volume (ml/24 hr)	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	2.19±0.07	2.27±0.02	2.33±0.03	2.29±0.02	2.25±0.03	2.30±0.03
7 Day	2.31±0.06	1.87±0.04###	1.81±0.02	1.72±0.03	1.83±0.03	1.78±0.03
14 Day	2.34±0.07	1.52±0.03###	2.10±0.03***	1.67±0.01**	1.84±0.01***	2.03±0.04***
28 Day	2.36±0.03	1.17±0.03###	2.04±0.02***	1.92±0.01***	2.03±0.04***	2.21±0.02***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.29 Urine volume on 0 day of study

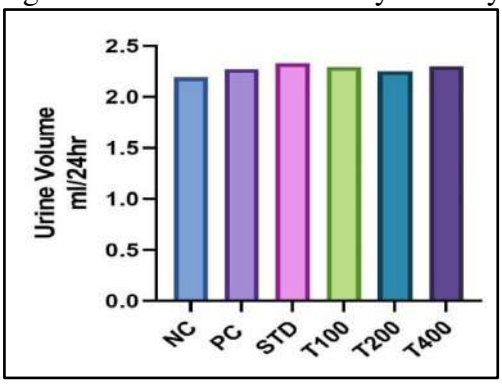


Fig. 30 Urine volume on 7th day of study

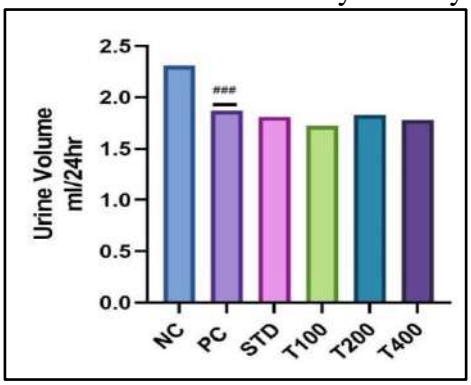


Fig.31 Urine volume on 14th day of study

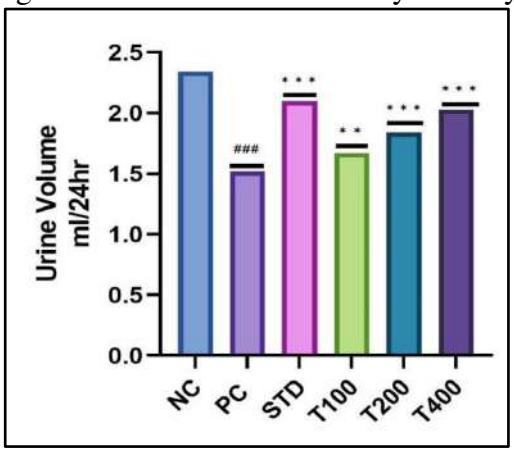


Fig.32 Urine volume on 28th day of study

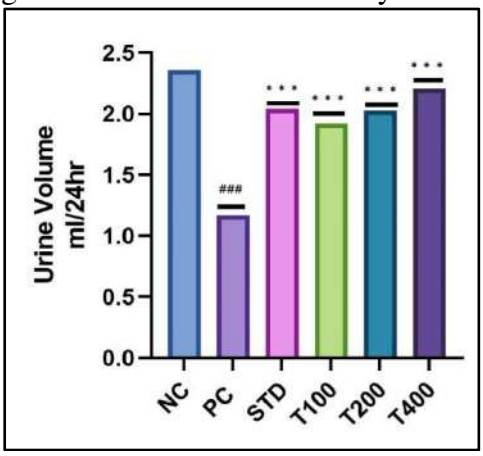


Table. 12 Effect of on urine pH of urolithiatic rats

Parameter Urine pH	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	7.65±0.05	7.64±0.05	7.71±0.06	7.69±0.03	7.60±0.03	7.62±0.04
7 Day	7.61±0.06	6.72±0.06###	6.63±0.06	6.73±0.07	6.67±0.02	6.67±0.03
14 Day	7.64±0.07	6.55±0.04###	7.15±0.04***	6.99±0.02***	7.19±0.03***	7.19±0.03***
28 Day	7.69±0.04	5.39±0.04###	7.49±0.02***	7.22±0.03***	7.42±0.03***	7.51±0.02***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.33 Urine pH on 0 day of study

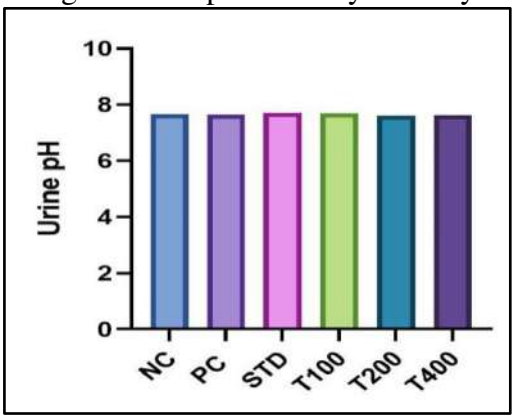


Fig.34 Urine pH on 7th day of study

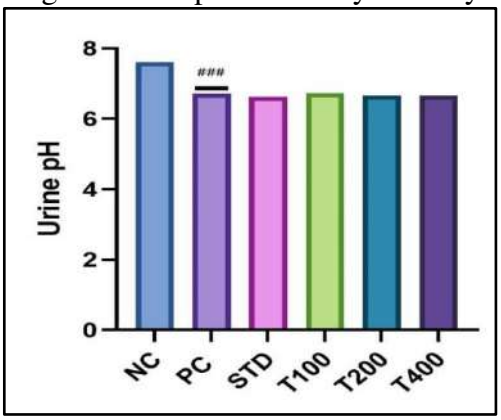


Fig.35 Urine pH on 14th day of study

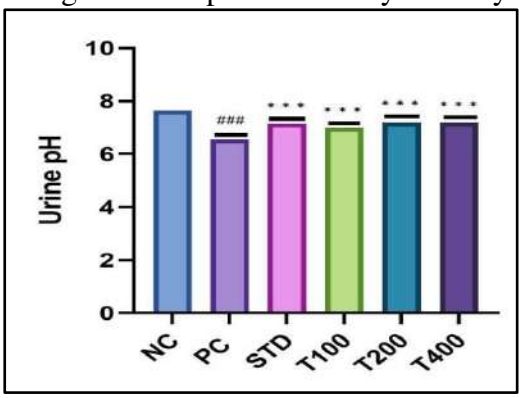


Fig.36 Urine pH on 28th day of study

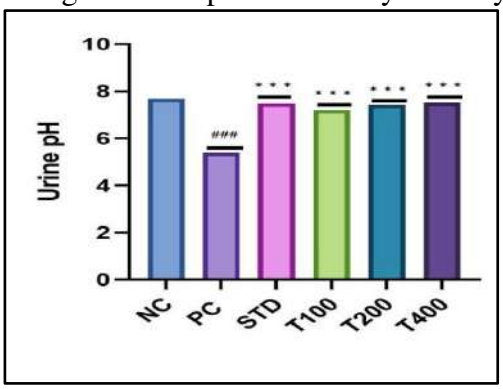


Table. 13 Effect of on urine calcium of urolithiatic rats

Parameter Urine Calcium	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	3.98±0.07	4.20±0.14	3.98±0.09	4.07±0.03	4.08±0.04	3.96±0.04
7 Day	4.15±0.07	6.10±0.16###	6.10±0.07	6.28±0.03	6.05±0.05	5.97±0.05
14 Day	4.16±0.09	7.35±0.02###	4.99±0.07***	6.14±0.08***	6.07±0.09***	5.96±0.09***
28 Day	4.18±0.07	7.45±0.02###	5.59±0.10***	6.18±0.06***	5.09±0.06***	4.81±0.05***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.37 Concentration of calcium on 0 day of study

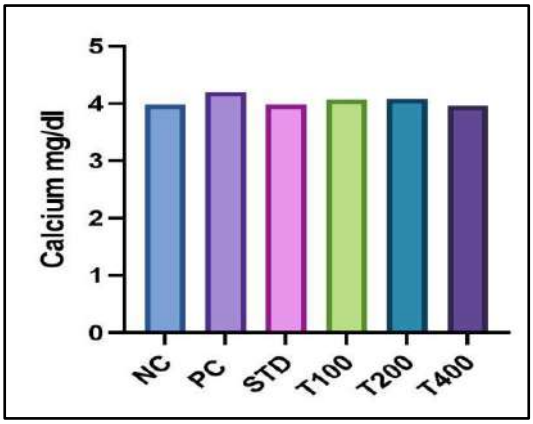


Fig.38 Concentration of calcium on 7th day of study

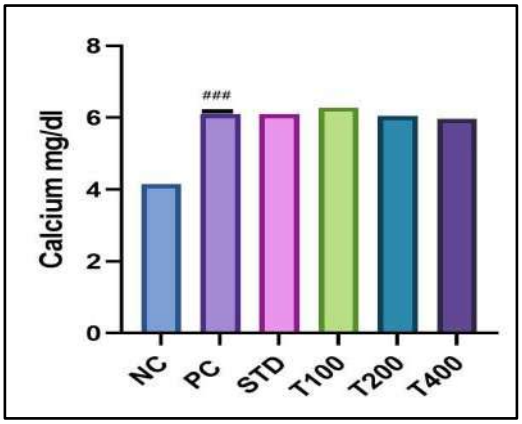


Fig.39 Concentration of calcium on 14th day of study

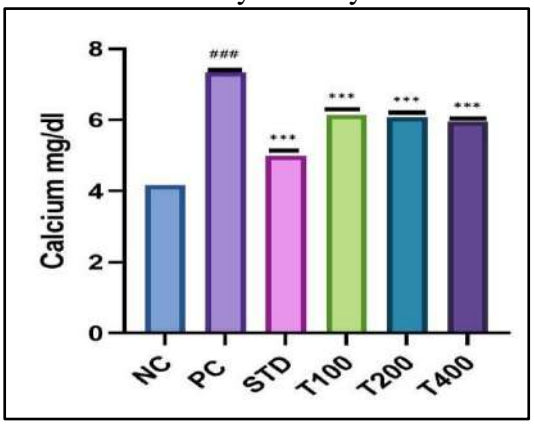


Fig.40 Concentration of calcium on 28th day of study

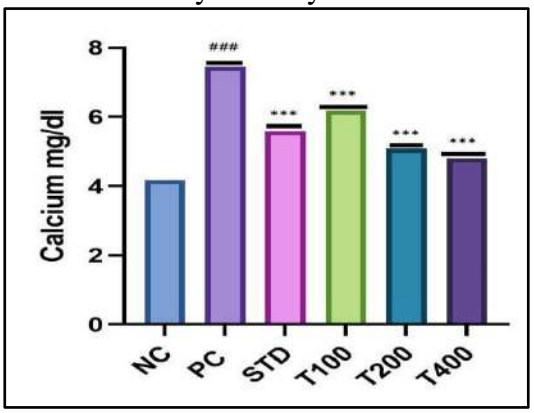


Table. 14 Effect of on urine oxalate of urolithiatic rats

Parameter Urine Oxalate	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	0.83±0.02	0.85±0.02	0.85±0.02	0.83±0.02	0.85±0.02	0.86±0.02
7 Day	0.85±0.02	1.37±0.03###	1.34±0.02	1.39±0.03	1.35±0.03	1.32±0.02
14 Day	0.86±0.02	2.55±0.18###	1.58±0.02***	1.54±0.02***	1.13±0.03***	0.93±0.02***
28 Day	0.86±0.02	3.70±0.15###	1.91±0.02***	1.28±0.01***	0.98±0.01***	0.88±0.02***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.41 Concentration of oxalate on 0 day of study

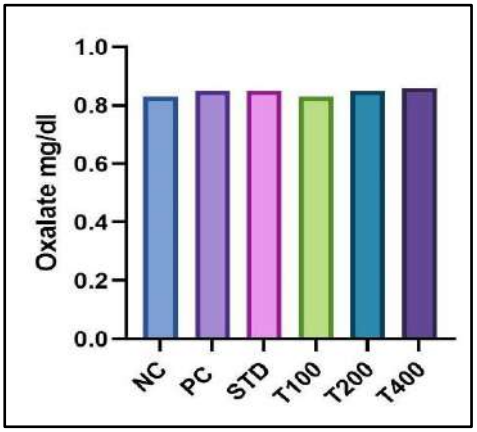


Fig.42 Concentration of oxalate on 7th day of study

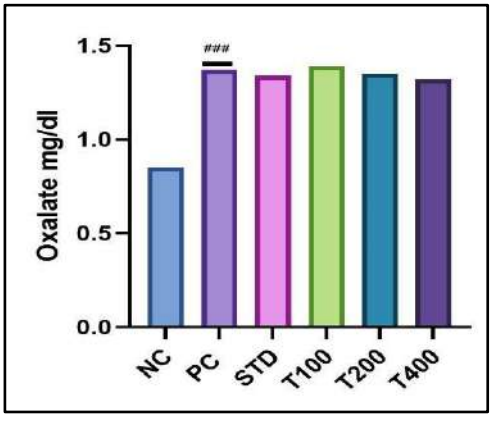


Fig.43 Concentration of oxalate on 14th day of study

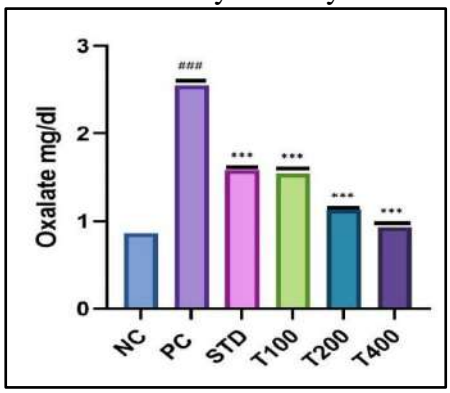


Fig.44 Concentration of oxalate 28th day of study

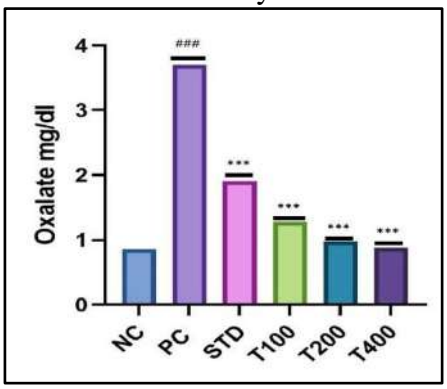


Table. 15 Effect of on urine phosphate of urolithiatic rats

Parameter Urine Phosphate	**Group I Normal**	**Group II Positive Control**	**Group III Standard (Cystone)**	**Group IV 100 mg/kg**	**Group V 200 mg/kg**	**Group VI 400 mg/kg**
0 Day	5.31±0.02	5.32±0.02	5.30±0.01	5.29±0.02	5.27±0.01	5.28±0.01
7 Day	5.29±0.02	6.37±0.05###	6.33±0.03	6.32±0.02	6.26±0.02	6.28±0.02
14 Day	5.31±0.01	7.46±0.03###	6.81±0.03***	6.53±0.01***	6.02±0.07***	5.85±0.04***
28 Day	5.30±0.02	8.12±0.07###	5.52±0.01***	5.82±0.02***	5.56±0.02***	5.38±0.03***

Statistical analysis done by using ANOVA followed by Dunnett's test, Group III, Group IV, Group V & Group VI compared with Group II. *** P<0.001, ** P<0.01, * P<0.05 Group II compared with Group I. ### P<0.001 n = 6

Fig.45 Concentration of phosphate on 0 day of study

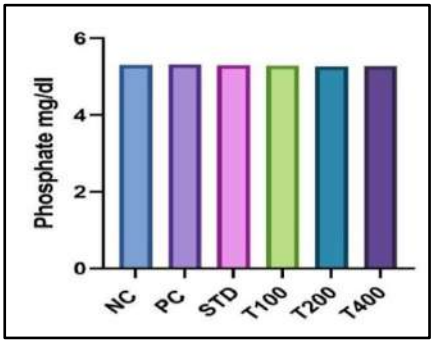


Fig.46 Concentration of phosphate on 7th day of study

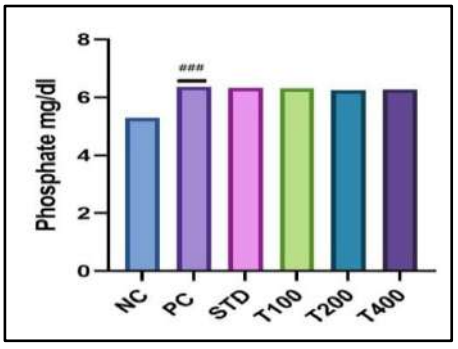


Fig. 47 Concentration of phosphate on 14th day of study

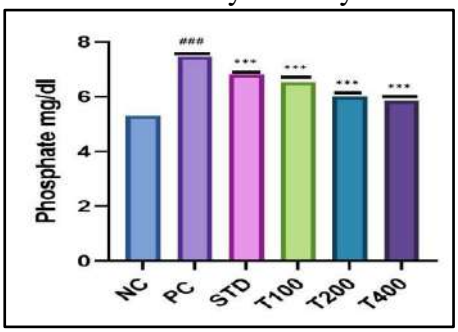
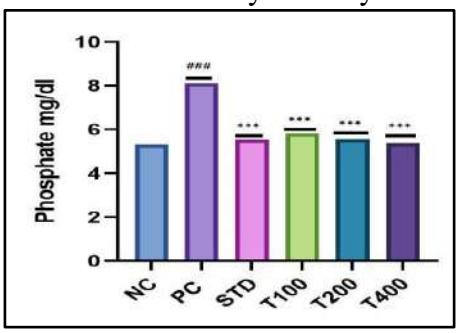
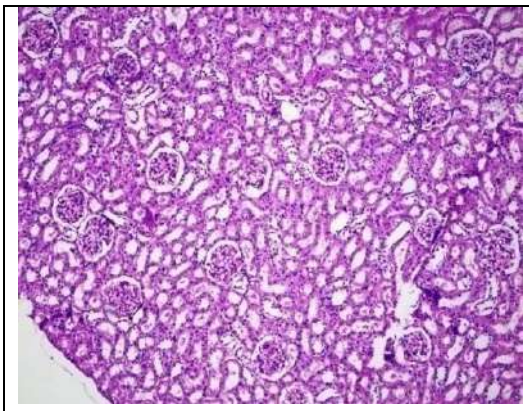
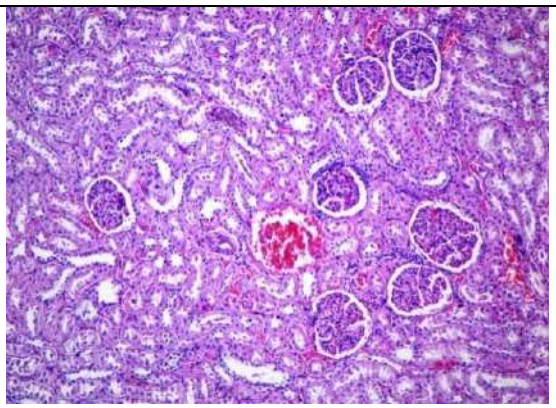
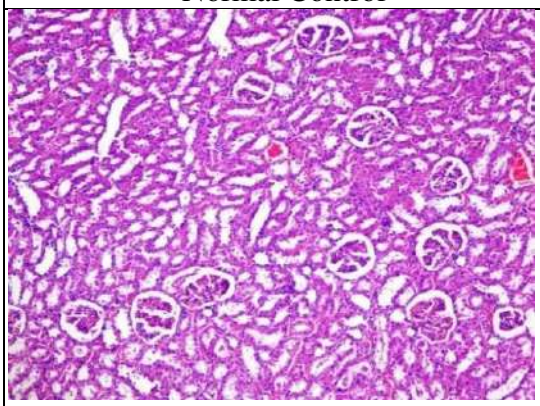
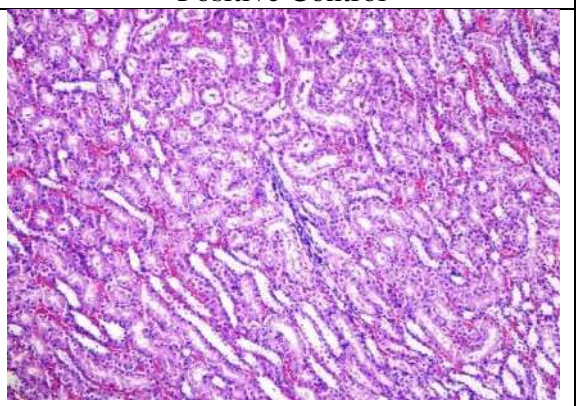


Fig.48 Concentration of phosphate on 28th day of study



Histopathology Study:

Fig.49 Effect of on histopathology of isolated kidneys of urolithiatic rats in ethylene glycol induced urolithiasis model

		
Normal Control		Positive Control
		
Standard		Test 100

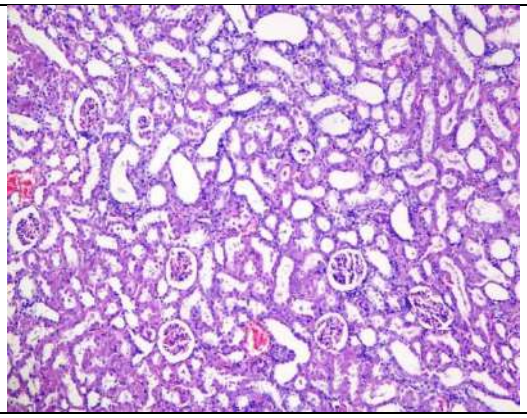
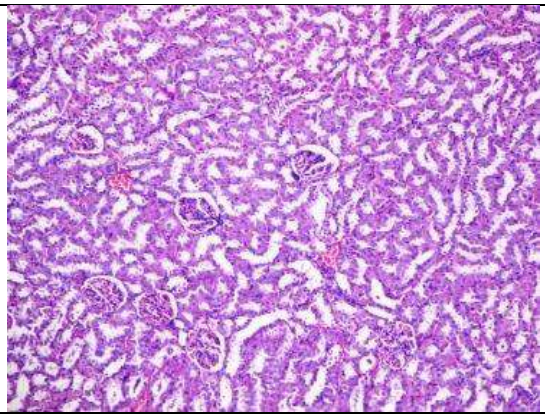
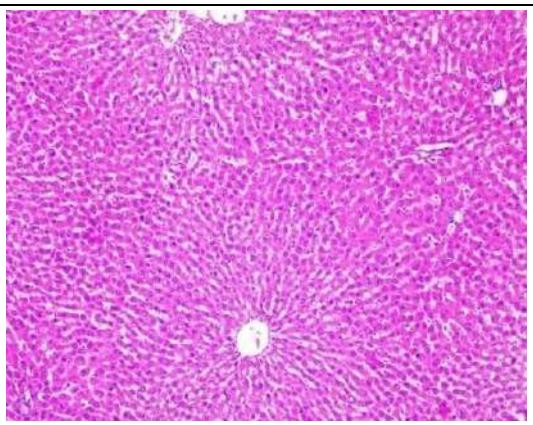
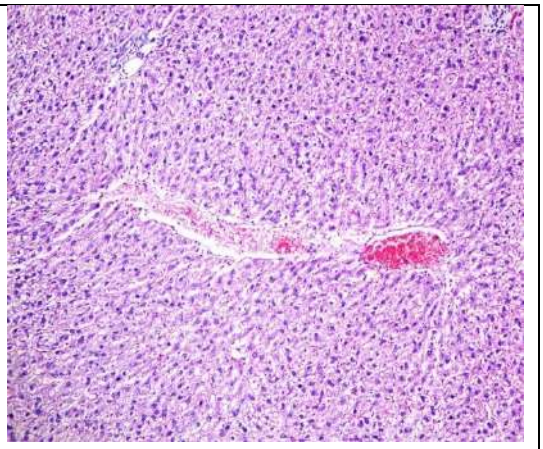
		
Test 200		Test 400

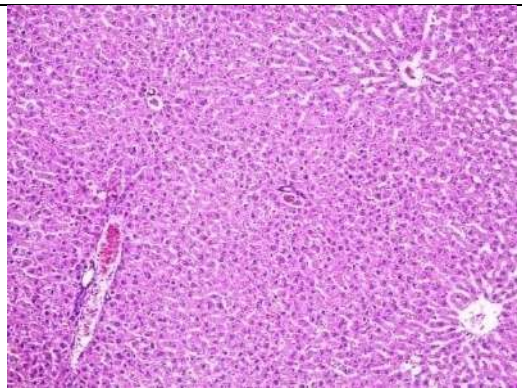
Table. 16 Histopathology report of kidney (Curative study)

Histopathology Report of Kidney (Curative study)			
Sr. No.	**Group**	**Histopathological observations**	**Overall pathological remark**
1	**Normal Control**	The microscopic observations of kidney sections showed normal renal tubules and glomeruli. The renal tubules showed normal epithelium. There was absence of any inflammatory or pathological changes in kidney.	**NAD**
2	**Positive Control**	The renal parenchyma showed prominent nephropathic changes of renal tubules with diffuse degenerative changes with congestion and interstitial focal haemorrhages in the renal parenchyma. Renal tubules showed cellular swelling and focal accumulation of eosinophilic and cellular debris in lumen of tubules in the medullary region suggestive of accumulation of toxic metabolites in the lumen of tubules leading to calculi formation. Sections showed degenerative changes in renal tubules with loss of nucleus and vacuolar changes of damaged tubules. Focal interstitial haemorrhages were also observed.	**Moderate (+3)**
3	**Standard**	The microscopic observations of kidney sections showed normal renal tubules and glomeruli. The renal tubules showed focal degenerative changes in renal parenchyma.	**Minimal (+1) to NAD**
4	**Sub-therapeutic Dose 100 mg/kg**	The renal tubules showed mild degenerative changes with congestion and focal haemorrhages in the renal parenchyma. Renal tubules showed cellular swelling and focal accumulation of eosinophilic and cellular debris in lumen of tubules in the medullary region suggestive of accumulation of toxic metabolites in	**Mild (+2)**

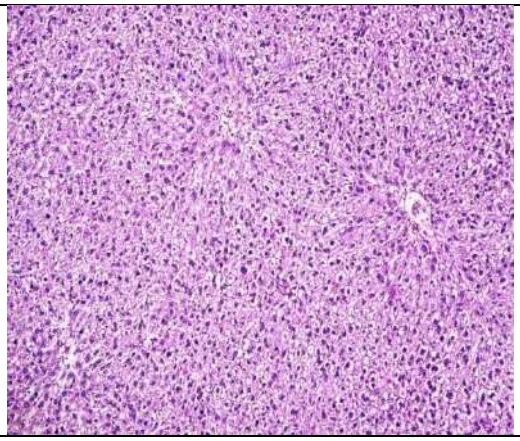
		the lumen of tubules leading to calculi formation. Multiple foci of dilation of renal tubules with interstitial haemorrhages were observed.	
5	**Therapeutic Dose 200 mg/kg**	The renal tubules showed mild degenerative changes with congestion and focal haemorrhages in the renal parenchyma. Renal tubules showed cellular swelling and focal accumulation of eosinophilic and cellular debris in lumen of tubules in the medullary region. Multiple foci of dilation of renal tubules with interstitial haemorrhages were observed. Degenerative and Nephropathic changes were lesser than Low dose group.	**Mild (+2)**
6	**Super-therapeutic Dose 400 mg/kg**	The microscopic observations of kidney sections showed normal renal tubules and glomeruli in the cortex and medulla. Very few areas in the kidney sections showed focal cellular swelling and focal degenerative changes of tubules with occasional accumulation of eosinophilic debris in lumen of tubules. Degenerative and Nephropathic changes were very minimal and were of significantly lesser than Low dose and Mid dose group.	**Minimal (+1)**
Note: Overall Grade score as- NAD=No Abnormality Detected, Minimal changes (+1), Mild changes (+2), Moderate changes (+3), Severe changes (+4).			

Fig.50 Effect of on histopathology of isolated liver of urolithiatic rats in ethylene glycol induced urolithiasis model.

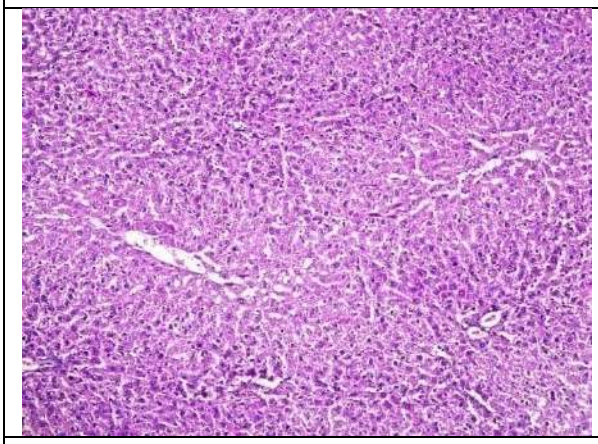
		
Normal Control		Positive Control



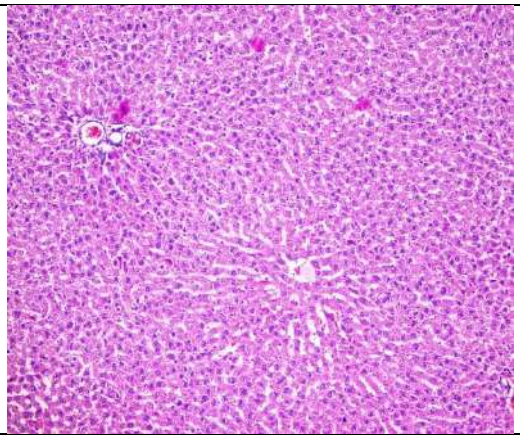
Standard



Test 100



Test 200



Test 400

Table. 17 Histopathology report of liver (Curative study)

Histopathology Report of Liver (CURATIVE study)			
Sr. No.	**Group Code**	**Histopathological observations**	**Overall pathological remark**
1	**Normal Control**	The microscopic examination showed normal cellular features of hepatocytes with intact nucleus and cell borders. The hepatic parenchyma showed hepatocytes arranged in strands around central vein. There was absence of any inflammatory or metabolic pathological changes in the hepatocytes.	**NAD**
2	**Positive Control**	The microscopic examination of liver tissue sections from positive control group showed mild degenerative changes in hepatic parenchyma and congested vessels. Hepatocytes showed cellular swelling with enlarged nucleus as well as diffuse degenerative changes with granular cytoplasm. Multi-focal areas of derangement of hepatic cords were observed. Liver tissue sections also showed focal necrotic	**Mild (+2) to Moderate (+3)**

		changes of hepatocytes with loss of nucleus and cell borders was also noted.	
3	**Standard**	The microscopic examination of tissue sections from MID dose group showed focal congestion of central veins. Focal presence of degenerative changes of hepatocytes was noted.	**Minimal (+1)**
4	**Sub-therapeutic Dose 100 mg/kg**	The microscopic examination of tissue sections from Low dose group showed congestion of hepatic vasculature. Hepatocytes showed cellular swelling with enlarged nucleus as well as diffuse degenerative changes with granular cytoplasmic changes in the parenchyma.	**Mild (+2) to Moderate (+3)**
5	**Therapeutic Dose 200 mg/kg**	The microscopic examination of tissue sections from Mid dose group showed focal and mild congestion of hepatic vasculature and minimal and focal degenerative changes in hepatic parenchyma. Hepatocytes showed cellular swelling with enlarged nucleus as well as diffuse degenerative changes with granular cytoplasm.	**Mild (+2)**
6	**Supper-therapeutic Dose 400 mg/kg**	The microscopic examination of tissue sections from HIGH dose group showed focal congestion of central veins. Focal degenerative changes were noted with focal cellular swelling in few of the hepatocytes only.	**Minimal (+1)**
Note: Overall Grade score as- NAD =No Abnormality Detected, Minimal changes (+1), Mild changes (+2), Moderate changes (+3), Severe changes (+4).			

Discussion and Conclusion

In present study for formulation of polyherbal formulation extracts of all selected herbs has done by using specific method of extraction. In case of *Musa balbisianacolla* juice was extracted from a pseudo stem by pressing using a sugarcane press machine. The aqueous extract of *Tribulus terrestris* was prepared by using the dried and matured fruit. The fresh leaves of *Bryophyllum Pinnata* which were air dried, pulverized and extracted exhaustively in distilled water for 72 h by cold maceration. Extraction of *Commiphora wightii* of coarsely powdered plant material was successively extracted by Soxhlet extraction method.

The tablet was prepared by using aqueous wet granulation method and all the in-process quality control tests were performed. According to pre-compression results, prepared granules have good flow property. Tablets pass the weight variation test. There hardness was found to be 6.14 kg/cm^2. Friability of tablets was found to be 0.020%i. e. within the official limit. Also tablet have disintegration time 30 sec to 1 min, dissolution time 25 min. and percentage release was 91.55% in 30 min. Hence, we conclude that tablets pass the all IPQC tests.

A tablet was evaluated for acute oral toxicity study. In this study, death was observed at 5000 mg/kg. There was no death observed at dose of 2000 mg/kg. One tenth dose of 2000 mg/kg i. e. LD_{50} 200 mg/kg was chosen as therapeutic dose. Similarly, 100 mg/kg and 400 mg/kg dose

was chose as sub-therapeutic and super-therapeutic dose for more precise effect of tablet. (Table 7.9) The preventative and curative both type of studies were performed for evaluation of polyherbal formulation for antiurolithiatic activity by using ethylene glycol animal model.

In the present study in-vivo antiurolithiatic study of tablet was carried out using ethylene glycol induced urolithiasis model. In the present study, 0.75% v/v ethylene glycol and 1% ammonium chloride up to 28 days in case of preventive study. The test drug (tablet) was administered orally at the dose of 100 mg/kg, 200 mg/kg & 400 mg/kg simultaneously with inducer drug for 28 days. In case of curative study 0.75% v/v ethylene glycol and 1% ammonium chloride was replaced with drinking water for 3 days and after that only 0.75% v/v ethylene glycol was given to rats up to 14 days. After that 15^{th} to 28^{th} day tablet was administered orally to animals. In the period of study, 24 hr urine was collected on 0, 7^{th}, 14^{th} and 28^{th} day and analysed for urine volume, pH, calcium, oxalate and phosphate content in urine. On 28^{th} day blood was collected by retro orbital puncture and the serum was examined for the presence of creatinine, blood urea nitrogen and uric acid. Serum creatinine level of all the drug treated groups i. e. 100 mg/kg, 200 mg/kg & 400 mg/kg tablet and also standard drug (Cystone) group was decreased significantly (*** $P<0.001$) as compared with positive control group. Similarly serum BUN was decreased significantly (*** $P<0.001$) in 100 mg/kg, 200 mg/kg & 400 mg/kg tablet and standard drug (Cystone) groups as compared with positive control group. Also serum uric acid level lowers significantly (*** $P<0.001$) in 100 mg/kg, 200 mg/kg & 400 mg/kg tablet and standard drug (Cystone) groups as compared with positive control group in both preventive and curative study. (Table.2 & 10, Fig. 1, 2, 3, & 26, 27, 28) respectively.

Urine volume of positive control group decreases due to deposition of calcium oxalate crystals in the kidney. When animals in groups III, IV, V, VI treated with standard drug (Cystone), 100 mg/kg, 200 mg/kg & 400 mg/kg tablet respectively the urine volume was increased significantly (*** $P<0.001$) from 14^{th}&28^{th} day in both preventive and curative study. (Table 3 & 11, Fig.4, 5, 6, 7, &29, 30, 31, 32) respectively.

Similarly, urine pH also decreased in positive control group and it was significantly (*** $P<0.001$) increased in groups III, IV, V, VI treated with standard drug (Cystone), 100 mg/kg, 200 mg/kg & 400 mg/kg tablet respectively on 14^{th}&28^{th} day in both preventive and curative study. (Table 4 & 12, Fig. 8, 9, 10, 11 & 33, 34, 35, 36) respectively.

As the ingestion of ethylene glycol in animals increased day by day the subsequent increase in urinary calcium takes place. When animals in groups III, IV, V, VI treated with standard drug (Cystone), 100 mg/kg, 200 mg/kg & 400 mg/kg tablet respectively urinary calcium was increased significantly (*** $P<0.001$) on 14^{th} and 28^{th} day in both preventive and curative study (Table 5 & 13, Fig. 12,13,14,15 & 37, 38, 39, 40) respectively.

Urinary oxalate was decreased significantly (### $P<0.001$) in positive control group as compared to normal control. Significant (*** $P<0.001$) rise in urinary BUN was takes place on 14^{th} and 28^{th} day in both preventive and curative study in groups III, IV, V, VI treated with standard drug (Cystone), 100 mg/kg, 200 mg/kg & 400 mg/kg tablet (Table 6 & 14, Fig. 16,17,18,19 & 41,42,43,44) respectively.

There was significant (### $P<0.001$) reduction of urinary phosphate content in positive control group as compared to normal control group. When animals in groups III, IV, V, VI treated with standard drug (Cystone), 100 mg/kg, 200 mg/kg & 400 mg/kg tablet respectively urinary phosphate content was increased significantly (*** $P<0.001$) on 14^{th} and 28^{th} day in both preventive and curative study (Table 7& 15, Fig. 20,21,22,23 & 45,46,47,48) respectively.

On 28^{th} day all animals were sacrificed and liver and kidneys of animals were isolated for histopathological findings. In this present study, animals in positive control group treated with ethylene glycol shows moderate (+++) pathological changes in kidney and liver in both

preventive and curative study. Animals in group III treated with standard drug (Cystone) shows minimal (+) to NAD pathological changes in kidney and liver in both preventive and curative study. In case of preventive and curative study, animals in group IV treated with 100 mg/kg dose of tablet shows mild (++) pathological changes in kidney and mild (++) to moderate (+++) pathological changes in liver.Animals in group V treated with 200 mg/kg dose of tablet shows minimal (+) pathological changes in kidney and mild (++) pathological changes in liver in case of preventive study. Similarly, in case of curative study, there are mild (++) pathological changes in both kidney and liver. Animals in group VI treated with 400 mg/kg dose of tablet shows minimal (+) pathological changes in kidney and liver in both preventive and curative study. (Table 8, 9, 16, 17 & 7.25, Fig. 24, 25, 49, 50) respectively.
In the present study, anti-urolithiatic activity of polyherbal tablet was evaluated by in-vivo model.Anti-urolithiatic activity of tablet was evaluated by in-vivo model using ethylene glycol induced urolithiasis. In the present study, it can be concluded that animals treated with standard (Cystone) drug and the tablet in dose of 200 mg/kg of body weight exerted significant anti-urolithiatic activity and protection against tubular interstitial damage in kidneys and liver in ethylene glycol induced urolithiatic rats.

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