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POLYHERBAL FORMULATION-MEDIATED INHIBITION OF CRYSTAL NUCLEATION AND AGGREGATION: AN EXPERIMENTAL STUDY ON ETHYLENE GLYCOL-INDUCED UROLITHIASIS IN WISTAR RATS

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ABSTRACT
Urolithiasis is the process of crystal nucleation, aggregation and retention in the urinary tract, and finally production of stones in the urinary tract. Urolithiasis has been effectively treated with conventionally used medicinal herbs. An herbal formulation containing a variety of plant extracts was assessed for its Antiurolithiatic activity. The present study showed the effect of polyherbal formulation on kidney stones by using the wistar rat model. The polyherbal formulation contained Aqueous extracts of *Tribulus Terrestris* fruit, *Boerhavia diffusa* root, *Piper longum* fruit, and *Crataeva nurvala* bark. The phytochemical analysis of plant extracts identified as alkaloids, carbohydrates, glycosides, saponins, phenols, and flavonoids. In the acute oral toxicity study at a dose of 2000 mg/kg no any changes were seen in behaviour, biochemical, haematological and body weight of rats.The efficacy of TBCP at dose of 200 and 400 mg/kg was studied in ethylene glycol-induced urolithiasis model and marketed cystone tablet at a dose of 750 mg/kg was used as the standard drug. The polyherbal formulation at a dose of 200 and 400 mg/kg significantly decreased $p < 0.01$ the amounts of calcium, oxalate, and phosphate in comparison to the untreated group.

KEYWORDS: *Piper longum, Boerhavia diffusa, Tribulus Terrestris, Crataeva nurvala* Antiurolithiatic action, herbal remedy.

INTRODUCTION
The kidney is the human body's most vital excretory organ. It has not only excreted metabolic waste products, but it has also maintained the acid-base balance and endocrine roles such as erythropoietin production.[1] People nowadays face numerous kidney problems, the most common of which are urolithiasis and renal toxicity.[2] The global incidence and prevalence are increasing, particularly as industrialization progresses.[3] Urolithiasis is the third most widespread urinary tract infection, affecting at least 12% of the world's population and having a higher recurrence rate in men than in women.[4] Etiology is multifactorial, with biochemical, epidemiological and genetic risk factors all playing a role.[5] Due to a lack of written records and generally low or no compensation for traditional herbal practitioners or "Vaidyas," this traditional knowledge of healthcare systems has begun to erode over time.

As a result, an herbal formulation Antiurolithiatic activity was developed, assessed, and researched. It was made from *Tribulus Terrestris* fruit, *Boerhavia diffusa* root, *Piper longum* fruit, and *Crataeva nurvala* bark. *T. terrestris* has antibacterial and antifungal properties throughout the entire plant. The best action, however, was that of fruits.[6] Due to the high quantity of potassium salts, it has diuretic properties. It is Antiurolithiatic and reportedly prevents the development of stones.[7,8] It has anti-inflammatory, analgesic[9], and immunomodulatory properties. Additionally, it eases smooth muscle spasms.[10]

The *B. diffusa* leaf has analgesic, anti-inflammatory, and antibacterial properties.[11] *C. nurvala* bark has Antiurolithiatic activity, antibacterial activity. *P. longum* has antimicrobial activity, immunomodulatory and antioxidant properties An increasing belief in herbal medicines, which have fewer or no adverse effects compared to the widely accessible allopathic system of medicine and promise patients speedy cure, has helped medicinal plants reclaim some of their former notoriety in recent years. Given the rapid changes taking place, the loss of traditional knowledge within civilizations is as irrevocable as the extinction of species.[12]

MATERIAL AND METHOD

Plants material and preparation of polyherbal formulation Aqueous extract

The dried and matured fruits of *T. Terrestris* (TT) and *P. longum* (PL), dried roots of *B. diffusa* (BD), and dried bark of *C. nurvala* (CN) have been provided by Vikalp herbal.

Preparation of *Polyherbal formulation* extract

The root, fruit, and bark were washed with water, and shade dried at room temperature for a period of 1 month for proper removal of moisture, after the dried root, fruit, and bark was grinded with a mixer grinder in 1:1:1:1 of four plants that are 25mg of each plant. The plant material was powdered to a coarse powder and properly stored in an air-dried container before extraction. Solid-liquid extraction technique was used to extract a known amount of sample using a solid- liquid ratio of 1:8 ml. The Polyherbal formulation's aqueous extract was produced after the solvent had been completely removed, and it was kept at 4°C in a moisture free firmly closed container until usage.

Preliminary Phytochemical Screening

To assess the quality of the phytoconstituents in TBCP, a recently made aqueous extract was made for oral administration. The collected roots, fruit, and bark were washed with water, and shade dried at room temperature for a period of 1month for proper removal of moisture, after dried these roots, fruit, and bark was grinded and converted to a coarse powder and extraction was carried out by hot percolation method at a temperature 100℃ and then the Polyherbal formulation's aqueous extract was produced after the solvent had been completely removed, and it was kept at 4°C in moisture free firmly closed container until usage.

Experimental protocol

All animals were divided into five groups each group contained six animals. Aqueous extract infusion of the polyherbal formulation was administered orally to all test groups daily for a period of 28 days. The detail of the doses is mentioned in the table. Every day, the animals were checked for toxicity signs and mortality. Furthermore, rats were subjected to a thorough clinical examination in accordance with OECD guidelines, during which any changes in eyes, fur, mucous membranes, the emergence of peculiar excretions and secretions, as well as modifications in an autonomic activity like lacrimation, pupil size, piloerection, respiration were noted. All animal's body weights were measured before and after the treatment began (day 0), once a week (on 7, 14, 21, and 28 days), and also weekly food consumption was recorded. All animals' blood and plasma samples were examined for clinical chemistry and haematology at the conclusion of the treatment. As an antimicrobial and preservative agent, 20% sodium azide was mixed into the urine. Then the urine calcium, urine oxalate, and urine phosphate analysis were done by using an autoanalyzer (Erba chem). The urine pH was discovered to be where uric acid crystals formed most frequently. Thus, a pH meter was used to measure the urine'sacidity.[13]

All were euthanized at the end of treatment for necropsy. All animals' organ weights (brain, testis, uterus, adrenal glands, liver, heart, kidney, and spleen) were also recorded, and histopathological evaluations were performed for each group.

Histopathology Assessment

Histopathology modifications were measured by eosin staining and hematoxylin. Each animal's abdomen was sliced open in order to remove both kidneys and preserved in 10% formalin then transferred to a container that would allow reagents to easily interact with the tissue inside. Sections were examined with a 20X and 40X Ti microscope, a Nikon eclipse, and multiple fields were analyzed for all sections. prepared extract of a polyherbal formulation was subjected to preliminary phytochemical screening. According to accepted procedures, phytochemical analysis of the carbohydrates, flavonoids, alkaloids, proteins, saponins, glycosides, phenols, and tannins was conducted.[14]

Acute oral toxicity

Acute oral toxicity testing was done in accordance with OECD guideline (423). The extract was applied according to standard guideline and the dose was given up to 2000mg/kg. The rat showed no signs of toxicity. Two doses 200mg/kg and 400mg/kg were further used for the study.

Animals

The in-vivo study was carried out by using 180-250 gm Wistar rats of either sex. The departmental animal house of Kumaun University provided the rats. The creatures were split up into six groups each group carrying six animals (n=6). They were kept in poly-acrylic cages under standard experimental settings at a temperature of 25 ± 2°C, a relative humidity of 60–70%, and exposureto a 12-hour cycle of light and darkness. One week before the start of the experiment, the animals were maintained to acclimate to the lab environment. The experiment was approved by the ethics committee and carried out in compliance with established norms for the care and use of animals in scientific research by the (IAEC) with protocol Number KUDOPS/159.

Induction of kidney stone and experimental pattern

A solution of ethylene glycol 0.75% with water was given for 28 days to Wistar rats for the formation of kidney stones to perform in-vivo experiments. Five groups of animals each containing 6 animals, group 1 functioned as the control and was provided with food and water ad- libitum. To cause renal calculi, groups II to V received ethylene glycol EG (0.75%) for 28 days in drinking water. From the 15th to the 28th day, Group 3 got the standard anti-urolithiatic medication Cystone (750mg/kg body weight). From day 15 to day 28, Groups

4th and 5th received a polyherbal formulation 200 mg and 400 mg/kg of body weight, respectively. The experimental pattern of the study is shown in the figure.

Dosage

Water and one oral dose were administered every day of prepared experimental groups. Cystone is manufactured by Himalaya drug company, Bangalore (India). Plant extracts in Cystone, such as *Didymocarpus pedicellate*, *Saxifraga ligulata*, and *Gokshura*.

It has been demonstrated that *Didymocarpus pedicellate*, *Saxifraga ligulata*, and *Gokshura* possess antilithic and diuretic properties. It was previously observed that 750 mg/kg b.wt. per oris (p.o.) cystone protected against the buildup of calcium oxalate crystals and oxidative stress caused by hyperoxaluria. As a result, this concentration was used for the cystone-treated positive control group.

Statistical Analysis

The results were presented as the mean standard error of the mean (SEM) of six animals (n=6), and statistical analysis was carried out using Dunnett's test, a one-way analysis of variance (ANOVA), and Tukey's multiple comparison test using GraphPad Prism 9.0.0, which was used to compare the results with the control group.

Phytochemical investigation of a polyherbal formulation

Preliminary phytochemical analysis of the polyherbal formulation revealed the presence of alkaloids, carbohydrates, glycosides, saponins, phenols, and flavonoids.

Polyherbal formulation acute oral toxicity test

According to the OECD guidelines, all mice were free of any toxicity up to a dosage of 2000 mg/kg. Using these data and the results of the pilot research, two distinct dosages of 200, and 400 mg/kg of the polyherbal formulation were selected for this study.

Urolithiasis caused by calcium oxalate

When compared to the normal control group, oxalate-induced urolithiasis caused significant changes in urinary parameters. There was an increase in urinary oxalate, urinary volume and calcium levels and an increase in urinary phosphate levels the results are shown in Table.1. When compared to the control group, these changes were significantly reduced in the polyherbal formulation 200, and 400 mg/kg treated groups. The 400 mg/kg dose was more significant (P<0.01) than the 200 mg/kg doses in terms of reducing urine magnesium, while the opposite was observed in reducing urinary calcium.

RESULTS

S. No	Groups	Treatment	Urinary parameters		
			Calcium	Urine oxalate (mg/dl)	urine phosphate
1.	Group I	Normal control	3.45±0.14	2.35±0.11	2.88±0.07
2.	Group II	Negative control	5.66±0.10****	4.86±0.04****	6.45±0.14****
3.	Group III	Cystone 750mg/kg	3.45±0.10****	2.50±0.11****	3.08±0.26****
4.	Group IV	TBCP 200mg/kg	4.10±0.26****	3.48±0.03****	4.33±0.09****
5.	Group V	TBCP 400mg/kg	3.73±0.07****	2.98±0.17****	3.51±0.10****

All values are n = 6, mean ± SEM, p <0.05, p <0.01.

The data was analyzed by comparing Group I to Group II using the Student T-test and Group II tothe other groups using the GraphPad programme after a two-way analysis of variance (ANOVA): ##P<0.01, *P<0.05, ****P<0.001.

Urine pH

The concentration of acidic urine pH was discovered to be where uric acid crystals formed mostfrequently. Thus, a pH meter was used to measure the urine's acidity.

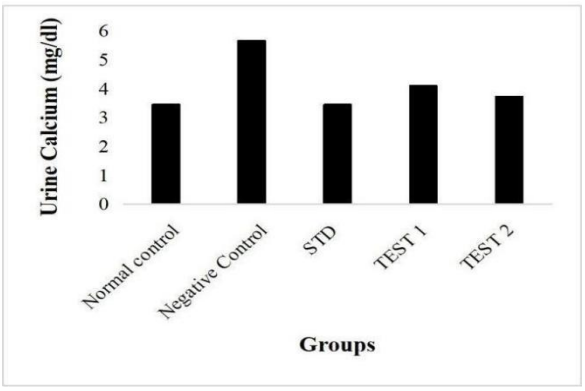


Figure. 1: Effect on urine calcium of polyherbal Formulation.

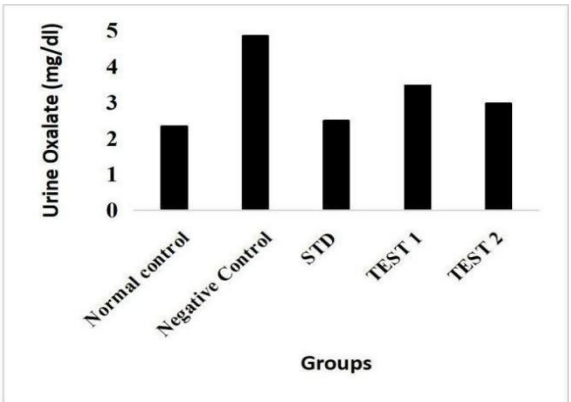


Figure. 2: Effect on urine oxalate of polyherbal Formulation.

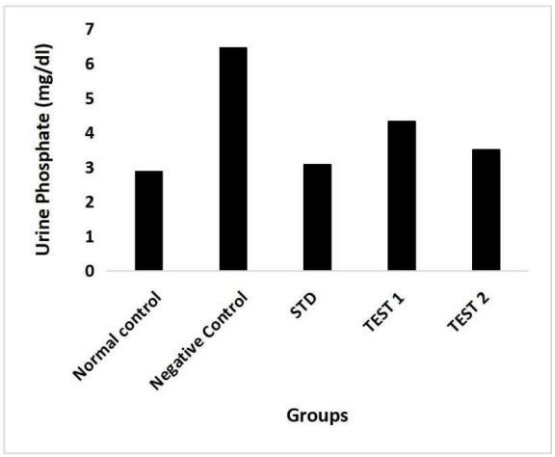


Figure. 3: Effect on urine phosphate of polyherbal formulation.

Collection of blood and serum analysis

The blood samples from rats were collected from retro-orbital puncture route by penetrating the retro-orbital sinus in rats with a sterile hematocrit capillary tube. The samples were kept aside for 30 minutes at room temperature. They were centrifuged at 3000 rpm, 20º C for 15 minutes, and analyzed for urea, uric acid, and creatinine. The rats were sacrificed, and kidneys were removed and used for histopathological examination results are shown in the figure and Table.2.

S.No.	Groups	Treatment	blood and serum parameters		
			Serum Creatinine	Serum uric acid	Blood urea
1.	Group I	Normal control	0.55±0.07	0.76±0.05	15.17±0.30
2.	Group II	Negative control	1.65±0.04****	2.33±0.04****	27.33±1.05****
3.	Group III	Cystone 750mg/kg	0.61±0.06****	1.33±0.06****	16.8±0.30****
4.	Group IV	TBCP 200mg/kg	1.16±0.09***	1.76±0.05****	18.17±0.30****
5.	Group V	TBCP 400mg/kg	0.75±0.06****	1.53±0.06****	17.33±0.33****

All values are n = 6, mean ± SEM, p <0.05, p <0.01

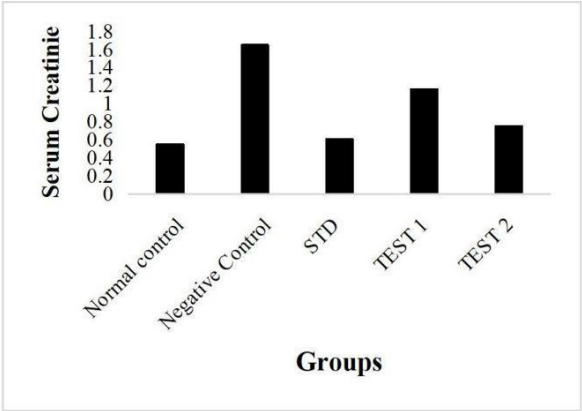


Figure. 4: Effect on Serum Creatinine of polyherbal formulation.

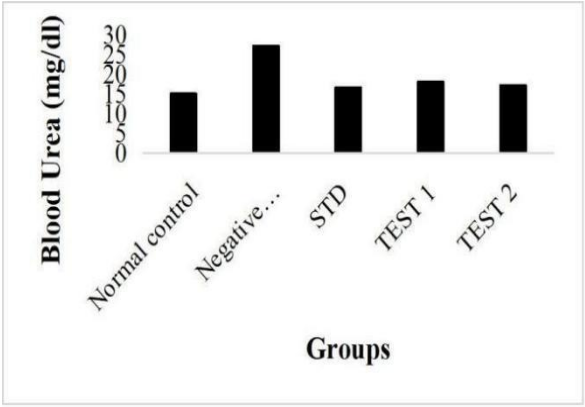


Figure. 4: Effect on Blood Urea of polyherbal formulation.

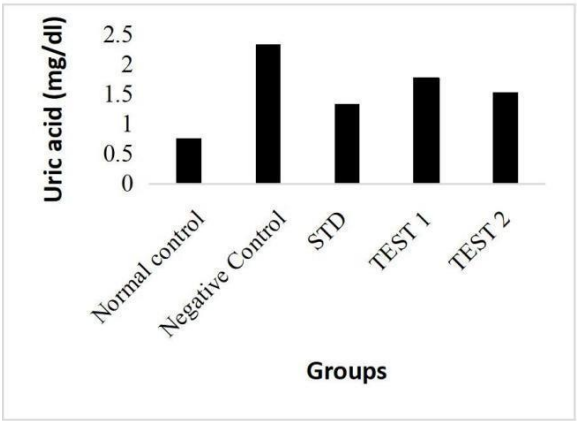
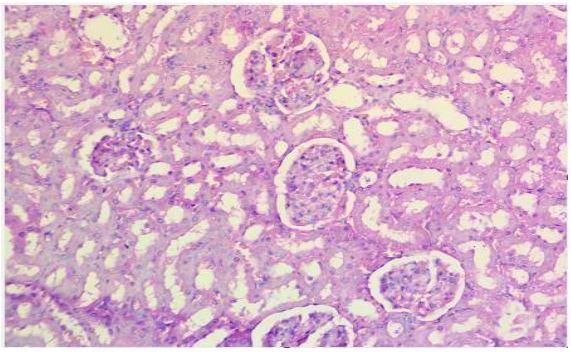


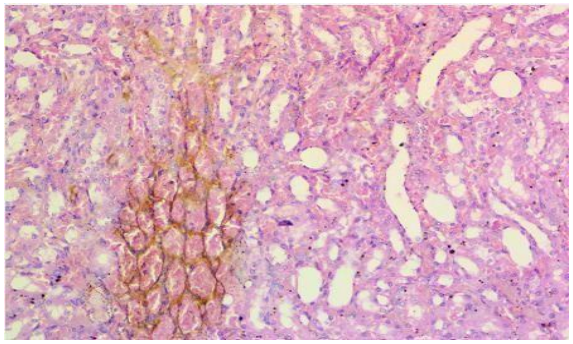
Figure. 4: Effect on uric acid of polyherbal formulation.

Histology of sections of the kidney for Antiurolithiatic activity

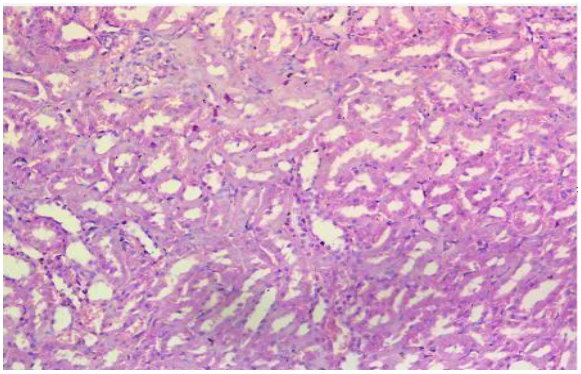
Histopathology studies were undertaken to clarify the pathology of the disease and to compare the treatment at two different dosages 200 mg/kg and 400 mg/kg body weight. Urinary stones were induced by using ethylene glycol. There was a considerable reduction in stone formation by the kidney with the treatment group.



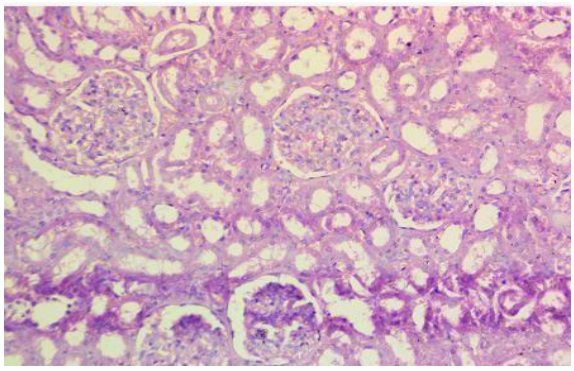
(a) Normal control



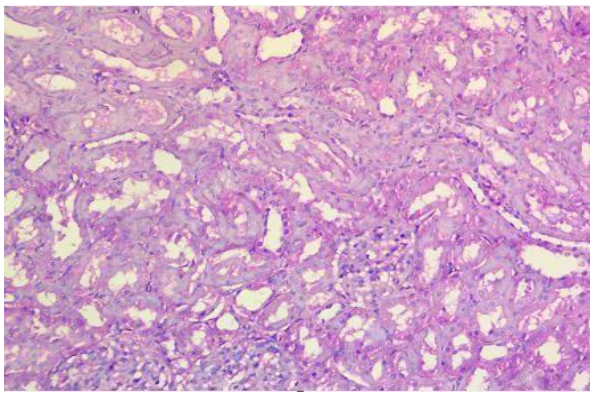
(b) Disease control



(c) Standard



(d) Low dose 200mg/kg



(e)High Dose 400mg/kg

(a) Normal Control Kidney showing unremarkable tubular histology, architecture of tubules is somewhat maintained. Glomeruli appear normal.

(b) Disease Control showing focal tubular necrosis, mainly epithelial lining necrosis, the architecture of tubules is somewhat maintained. moderate congestion noted. moderate lymphoplasmacytic Infiltration noted. Glomeruli also show inflammatory changes and congestion.

(c) Standard Control shows unremarkable tubular histology, and the architecture of tubules is somewhat maintained. Congestion noted. Mild lymphoplasmacytic Infiltration was noted. Glomeruli appear normal.

(d) Low Dose showing focal tubular necrosis, mainly epithelial lining necrosis, the architecture of tubules is somewhat maintained. moderate congestion noted. moderate lymphoplasmacytic Infiltration noted. Glomeruli also show inflammatory changes and congestion.

(e) High Dose showing unremarkable tubular histology, the architecture of tubules is somewhat maintained. moderate congestion noted. mild lymphoplasmacytic Infiltration was noted. Glomeruli also showinflammatory changes and congestion.

DISCUSSION

Kidney stone disease is characterized by the formation of crystal concretions within the kidneys. It is a growing urological disorder that affects approximately 12% of the world's population.[15] Various factors which are responsible for kidney formation such as Diet and lifestyle alone cannot fully account for the variations, and a number of other variables, including genetics, and the impact of sex hormones, problems with urine pH and acid-base handling, variations in calcium tubular reabsorption, and differences in stone composition

between men and women, may also play a role.[16] Kidney stone formation is complicated, and it is triggered by high levels of calcium-oxalate or calcium-phosphate in the urine.[17] The steps that must be taken to maximize the ability of medicinal plants to dissolve stones are covered in the current article. A great way to get rid of all kidney stone-related problems is to combine herbal treatments with allopathic care.[18] The present study of that polyherbal formulation contained the dried and matured fruits of T. Terrestris (TT) and P. longum (PL), dried roots of B. diffusa (BD), and dried bark of C. nurvula (CN) contained various phytochemicals which are responsible for the treatment of kidney stone problem. Following a polyherbal formulation investigation that revealed the presence of phenols, carbohydrates, glycosides, saponins, and alkaloids, and flavonoids. The overall presence of phytochemicals is consistent with previously published reports in which antiurolithiatic activity was demonstrated[19] This contributes to the selection of appropriate plants. All rats were free of any oral toxicity of polyherbal formulation that is investigated during the experiment in accordance with the OECD guidelines' permissible range, up to a dosage of 2000 mg/kg.

Stones formation as a result of changes in uric acid, urine flow rate excretion rate, and urine pH. Calcium oxalate kidney stones are caused by increased bone resorption, hypercalciuria, and hyperphosphaturia.[20] Serum creatinine and BUN levels are frequently measured to assess renal function. While serum creatinine increases only with nephron damage, BUN is influenced by hydration, hepatic protein metabolism, and decreased glomerular filtration rate. Significant increases in blood and urine protein, urea, creatinine, and uric acid levels are also indicators of nephrotoxicity.[21] The administration of polyherbal formulation had no effect on alertness, touch response, or any locomotor activity. Based on this result two different doses were administered 200mg/kg & 400mg/kg for pharmacological evaluation. Urolithiasis was induced in Wistar albino rats for 28 days through introducing 0.75% v/v ethylene glycol (EG) into their water supply.[22] After induction urolithiasis shows various changes in the urinary and blood parameters increases in the Disease control group animals such as in urine pH, calcium, urine oxalate & urine phosphate as well as blood parameters such as serum creatinine, serum uric acid & blood urea. These changes were considerably less pronounced in the groups treated with the polyherbal formulation (200 and 400 mg/kg) than in the disease control group.

Test-2 group (400 mg/kg) administered the dose more effectively than the 200mg/kg administered dose and 400mg/kg worked the same as the standard marketed drug. The histopathology results show that a section of the normal control rat kidney showed normal organization of tubular epithelial cells and glomeruli, whereas urolithic control rat kidney tissue showed crystal deposition. Furthermore, tissue sections from the control group demonstrated degenerative changes in the tubules and glomeruli. Similar histological observations previously reported support crystal deposition and consistency of epithelial cells in tubules and glomeruli when treated with polyherbal formulation.[23]

CONCLUSION

In the overall study, the polyherbal formulation 400mg/kg was more effective and worked the same as the standard formulation cystine when administered for 28 days. Further analysis of these extracts is required to predict the phytochemicals of Polyherbal formulation responsible for antiurolithiatic activity.

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