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PHYTOCHEMICAL EVALUATION & PHARMACOLOGICAL SCREENING OF DIDYMOCARPUS PEDICELLATA AND ASHWAGANDHA FOR ANTIUROLITHIATIC ACTIVITY

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Abstract:

The kidney stones are one of the most widely spreading disorders in the world. The present study was undertaken to evaluate the efficacy of ethanolic extract of *Didymocarpuspedicellata* and *Ashwagandha* for its antiurolithiatic activity in rats. Urolithiasis was induced in adult male albino wistar rats by 0.75% of ethylene glycol for 28 days. The effect of the oral administration of the ethanolicextracts has been studied and is compared with the effect of oral administration of Cystone(Himalaya) as a standard on Wistar rat. Ethylene glycol feeding resulted in hyperoxaluria as well as increased renal excretion of calciumand phosphate. Supplementation with ethanolic extract of the plants significantly reduced the elevated urinary oxalate, showing a regulatory action on endogenous oxalate synthesis. Both the plant extract showed significant antiurolithiatic activity.

Key words: Urolithiasis, *Didymocarpuspedicellata, Ashwagandha*, Ethylene glycol.

Introduction:

Urolithiasis is also called as urinary calculi, urinary stones, kidney stones, renal stones and renal calculi refer to growth of hard, nonmetallic mineral calcifications that form in the urinary system, primarily in the kidney or ureter and may also migrate into the lower urinary system or located anywhere in the urinary system.[1] The formation of stones in the urinary tract is mainly due to crystal nucleation, crystal aggregation, crystal retention which further result in precipitation of certain substances within urine. Thus super saturation acts as a driving force for stone formation.[2] Urinary stones are typically classified by their location or by their chemical composition (calcium-containing, struvite, uric acid, or other compounds).Renal calculi are characterized clinically by colicky pain (renal colic) as they pass down along the ureter and manifest by hematuria.[3]

Materials:

Plant: *Didymocarpuspedicellata (A)* and *Ashwagandha (B)* plants were collected from Botanist from Heritage bionaturals. The plants were chopped and dried in shade. It was then subjected to size reduction by mechanical grinder. The powdered material was subjected to soxhlet extraction with ethanol; the extract obtained was dried and used for preclinical studies.

Chemicals: All the chemicals: Ethanol (China-Changshu Yangyuan Chemical), Ethylene glycol (Qualigen Fine Chemicals, Mumbai, India), Cystone (Himalaya Herbal Health Care, India) and all other chemical used for the study were of analytical grade.

Methodology:

Acute toxicity study:

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Cooperation and Development (OECD) received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).The acute toxicity will be determined on spraguedawley rats by **fixed dose method** of OECD **Guide line no. 420.**[4]

Method:

Male spraguedawley rats were accommodated in polypropylene cages and temperature was maintained between 22°C with 12 h each of dark and light cycle. The rats were fed with standard laboratory pelleted feed. The rats were fasted overnight before and 3 h after the administration. A single dose of plant extract 2000 mg/kg orally was administered to one of the animals included in the study. Based on whether the animal survived or died, suitable tests were performed. (If died main test was performed and if survived, same test was continued with the remaining animals.).If three animals died, the limit test was terminated and main test was performed. The drug treated animals were carefully observed for mortality and clinical signs for first 10 min, 30 min, 1h, 2h, 4h, and 6 h after dosing and thereafter twice daily for mortality and once a day for clinical signs, for 14 days. Animals were sacrificed at the end of the study period of 14 days.

Direct observation parameters include tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. Skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and central nervous systems, motor activity and behavior pattern are the other parameters observed. The time of death, if any, was recorded. After administration of the test substance, food was withheld for further 1-2 h. The number of survivors was noted

after 24 h and then these were maintained for a further 14 days with a daily observation. The animals were observed continuously for 2h for general behavior studies, neurological profile and autonomic profile.[4]

Preclinical Study Model:

Ethylene glycol (0.75%V/V) induced urolithiasis model

Experimental animals:

Fifty four (54) albino Male Sprague Dawley rats weighing between 120-200 gms were used for the study. They were kept in polypropylene cages and allowed to acclimatize to the environment for 1 week before the commencement of the experiment. The animals were fed with standard animal pellet grower mash and allowed access to water ad libitum

Experimental induction of urolithiasis:

Stone were induced in experimental animals by 0.75% (0.75ml of ethylene glycol in 100 ml of drinking water) in rats for a period of 28 days for the production of calcium oxalate stones.

Preparation & administration of doses:

All the doses were prepared in normal saline using 10% tween 20 as suspending agent. The test substances (2000mg/kg b.w) and standard drug Cystone (750mg/ kg b.w) will be administered orally in a single dose by using oral gavage.

Ethylene glycol (0.75%V/V) induced urolithiasis model

Rats will be divided into nine groups (6 in each group) and all rat groups will be maintained using commercial pelleted feed and to induce Calcium Oxalate crystals, animals will be exposed to 0.75% Ethylene glycol in their drinking water for 28 days. The experimental groups are summarized below.[5-30]

Group in the Study:

Group 1 **(Healthy animals)**	Control rats will be given regular food and drinking water *adlibitum*for 28^{th} day.
Group 2 **(Untreated group)**	Ethylene glycol (**0.75%V/V**) in drinking water is fed to induce renal calculi till 28^{th} day.
Group 3 **(Standard group)**	Ethylene glycol (0.75%V/V) in drinking water is fed and treated with standard drug, Cystone (**750mg/kg body wt**) from 15^{th} day to 28th day.

Group 4 **(Test group –I) (Preventive regimen)**	Ethylene glycol (0.75%V/V) in drinking water is fed and treated with extract of *Didymocarpuspedicellata* (2000mg/kg b.w) from 1st day to 28th day.
Group 5 **(Test group-II) (Preventive regimen)**	Ethylene glycol (0.75%V/V) in drinking water is fed and treated with extract of *Ashwagandha*from 1st day to 28th day.
Group 6 **(Test group-III) (Preventive regimen)**	Ethylene glycol (0.75%V/V) in drinking water is fed and treated with extract of *Didymocarpuspedicellata* and *Ashwagandha*from 1st day to 28th day.
Group 7 **(Test group-IV) (Curative regimen**)	Ethylene glycol (0.75%V/V) in drinking water is fed and treated with extract of *Didymocarpuspedicellata*(2000mg/kg b.w)from 15th day to 28th day.
Group 8 **(Test group-V) (Curative regimen)**	Ethylene glycol (0.75%V/V) in drinking water is fed and treated with extract *Ashwagandha*from 15th day to 28th day.
Group 9 **(Test group-VI) (Curative regimen)**	Ethylene glycol (0.75%V/V) in drinking water is fed and treated with extract *Didymocarpuspedicellata* and *Ashwagandha*from 15th day to 28th day.

The Group 1 animals will be given tap water as drinking water for 28 days. Ethylene glycol (EG) (0.75%) in drinking water will be given to Group 2 to Group 9 for induction of renal calculi till 28th day. Group 3 (Standard group) will be treated with standard antiurolithiatic drug, Cystone (750 mg/kg body wt) from 15th day till 28th day. Test group I, II, and Test group III will serve as Preventive regimen. Test group I, II and Test group III will receive test dose of ethanolic extract from 1st day till 28th day .Test group IV, V and Test group VI will serve as Curative regimen. Test group IV, V and Test group VI will receive test dose of ethanolic extract from 15th day till 28th day. All extracts will be given once daily by oral route.

Assessment of anti urolithitic activity

Collection &analysis of urine:

All the animals will be kept in individual metabolic cages and urine samples of 24 hrs will be collected on 28th day. Animals will have free access to drinking water during the urine collection period. A drop of Con HCL will be added to the urine before being stored at 4^0C.

Urine will be analyzed for its volume then pH, glucose, protein will be measured by dip strips method then calcium, phosphate, content will be measured on the same day by spectrophotometric method.

Serum analysis:

After the experimental period, blood will be collected from the retro-orbital under anaesthetic conditions and animals would be sacrificed by cervical decapitation. Serum samples would be separated by centrifugation at 3,000rpm for 10 min and analyzed for Creatinine, Uric acid and Urea.

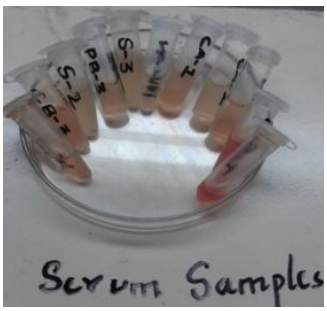


Figure 1: Serum samples for biochemical estimation of various groups.

The change in body weight of each animal is measured from the starting day till the end of the experiment i.e., till the 28th day. On the 28th day of the experiment period, all the rats were sacrificed by cervical dislocation. They were dissected by opening the abdomen and both the kidneys of each rat were removed and weighed.

At the end of the experiment, on day 28th the rats will be sacrificed by cervical decapitation and kidneys excised, isolated kidneys will be cleaned off extraneous tissue and rinsed in ice cold physiological saline. The left kidney will be washed with normal saline and weighed, the kidneys will be dried at 80^{0}C in a hot air oven and dried weight will be noted. The right half of the kidney will be fixed in 10% neutral buffered formalin, processed in a series of alcohol and xylene, embedded in paraffin wax, sectioned at 5µm and stained with haematoxylin and eosin for histopathological examination. The slides will be examined under light microscope and also in polarized light to study architecture of the kidney and calcium oxalate deposits and their photomicrographs are taken.[5-30]

Results

Yield calculation

The percentage yield of Plant A and Plant B was found to be 12% and 10% respectively.

Phytochemical evaluation

The ethanolicextract of *Didymocarpuspedicellata, Ashwagandha,* was subjected to preliminary phytochemical screening for the presence of different phytoconstituents with their respective reagents and the results are summarized below.[31]The presence of Carbohydrate was confirmed by Fehling's test, Benedict test,

Seliwanoff's test, Tollen's phloroglucinol test. The presence of protein & aminoacids were confirmed by Millon's test, Ninhydrin test, Tyrosin test & Cysteinetest.While alkaloids by Dragendroff's test, Mayer's test, Hager's test & Wagner's test. The presence of Steroids by Salkowski reaction, Libermannburchard reaction& Tannins by 5% $FeCl_3$ solution while of Glycoside by Legal's test, Keller killani test and Flavanoids by lead acetate test. As, the ethanolic extract shows the presence of most of these compounds, these extract were selected for the study.

Table I: Phytochemical investigation of extracts.

S.No	Name of the compound	Ethanolic extract of *Didymocarpus Pedicellata*	Ethanolic extract of *Ashwagandha*
1.	**Carbohydrates**		
	I. Test for reducing sugars		
	Fehling's test	+++	+++
	Benedict test		
	II. Test for monosaccharide		
	Barfoed's test		
	III. Test for pentose sugars	++	++
	Bial'sorcinol test		
	IV. Test for hexose sugar		
	Seliwanoff's test Tollen'sphloroglucinol test	-	-
	V. Test for non-reducing sugars	-	+
	Iodine test		
	Tannic acid test		
		++	++
		++	++

2.	**Protein**		
	Biuret test	-	-
	Millontest	+	++
3.	**Aminoacids**		
	Ninhydrin	+	+
	Test for tyrosin	+	+
	Test for cysteine	+	+
4.	**Alkaloids**		
	Dragendroff's test	++	++
	Mayer 's test	++	++
	Hager's test	++	++
	Wagner's test	++	++
5.	**Steroids**		
	Salkowski reaction	+	+
	Libermannburchard reaction	+	+
6.	**Tannins**		
	5% $FeCl_3$ solution	++	-
7.	**Glycosides**		
	Legal's test	++	+
	Keller killani test	++	+
8.	**Flavanoids**	++	++

+ = slightly present; ++ = moderately present; +++ = highly present; - = absent

Acute toxicity studies

Acute toxicity was studied byfixed dose method, showed that animals were found to be normal at a maximum dose of 2000 mg/kg b.w. with no noticeable behavioural, neurological and autonomic changes in all groups. Therefore, the maximum tolerated dose 2000 mg/kg b.w. was selected for antiurolithiatic activity.

Antiurolithiatic activity

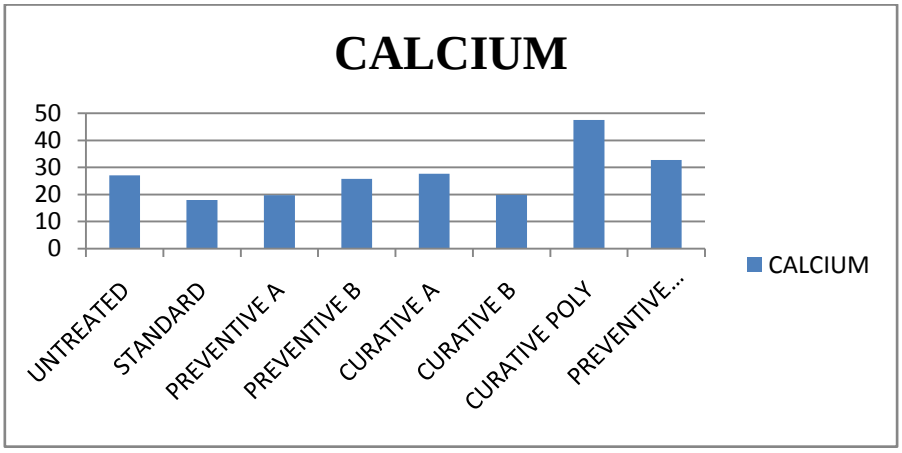
Table II: Urine analysis on 14th and 28th day

Urine analysis on 14th day									**Urine analysis on 28th day**						
S. No	**Groups**	**Water Intake (ml)**	**Vol (ml)**	**Ph**	**Color**	**Appearance**	**Protein**	**Glucose**	**Water Intake (Ml)**	**Vol (ml)**	**Ph**	**Color**	**Appearance**	**Protein**	**Glucose**
1.	NORMAL	2	2	7	Yellow	Clear	-	-	2	2	7	Yellow	Clear	-	-
2.	UNTREATED	1.3	1.0	7.3	Dark yellow	Clear	93.3+	-	1.3	0.4	6	Dark yellow	Clear	30+	-
3.	STANDARD	1.6	1.0	6.6	Light yellow	Clear	Trace	-	1.5	1.0	6	Light yellow	Clear	Trace	-
4.	PREVENTIVE A	1.3	1.1	6.3	Dark yellow	Clear	Trace	-	1.1	1.1	6	Dark yellow	Clear	Trace	-
5.	PREVENTIVE B	4	3.5	6	Dark yellow	Clear	30+	-	1.6	0.5	6	Dark yellow	Clear	Trace	-

6.	PREVENTIVE POLY	1.6	1.0	6	Dark yellow	Clear	Trace	-	1.2	1.0	6	Dark yellow	Clear	Trace	-
6.	CURATIVE A	0.8	0.4	6	Dark yellow	Turbidity	Trace	-	0.8	0.4	6	Dark yellow	Clear	Trace	-
7.	CURATIVE B.	1.6	1.0	6	Dark yellow	Clear	30+	-	1.6	1.4	6	Dark yellow	Clear	30+	-
8.	CURATIVE POLY	1.2	1.1	5	Dark yellow	Clear	30+	-	1.2	1.1	6	Dark yellow	Clear	30+	-

Table III: Determination of calcium level in urine on 28th day.

S.NO	GROUPS	CALCIUM (MEAN±SD)
1	UNTREATED	27.1 ± 1.25
2	STANDARD	18± 0.95
3	PREVENTIVE A	19.7± 0.52
4	PREVENTIVE B	25.8± 0.71
5	PREVENTIVE POLY	32.75± 0.61
6	CURATIVE A	27.7± 0.54
7	CURATIVE B	19.8± 0.45
8	CURATIVE POLY	47.5± 0.75

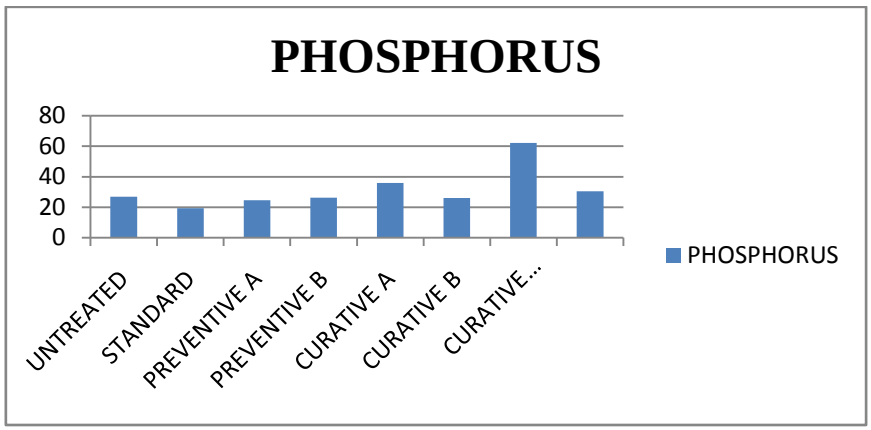


Graph 1: Determination of Calcium level in urine on 28th day.

Table IV: Determination of phosphorus level in urine on 28th day.

S.NO	GROUPS	PHOSPHORUS (MEAN±SD)
1	UNTREATED	26.9± 1.75
2	STANDARD	19.3± 0.55
3	PREVENTIVE A	24.5± 0.65
4	PREVENTIVE B	26.3± 0.9
5	PREVENTIVE POLY	30.5± 0.41

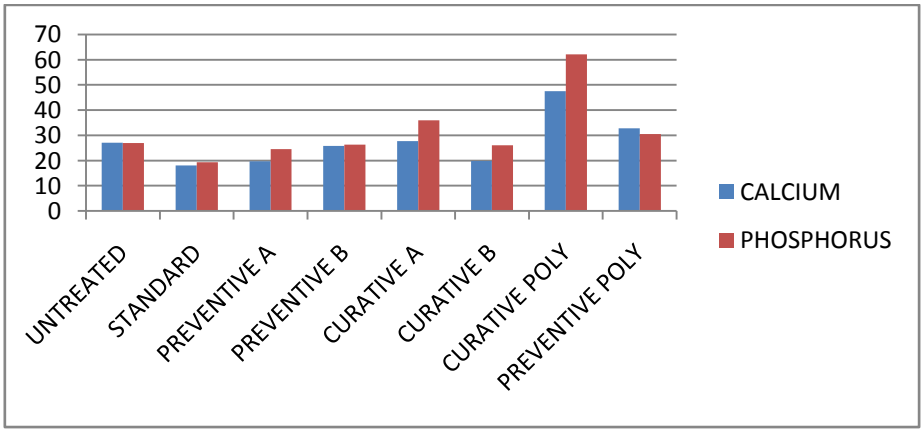
6	CURATIVE A	36± 0.13
7	CURATIVE B	26.1± 0.25
8	CURATIVE POLY	62.1±0.31



Graph 2: Determination of Phosphorus level in urine on 28th day.

Table V: Comparison between calcium and phosphorus.

S.NO	GROUPS	CALCIUM	PHOSPHORUS
1	UNTREATED	27.1	26.9
2	STANDARD	18	19.3
3	PREVENTIVE A	19.7	24.5
4	PREVENTIVE B	25.8	26.3
5	PREVENTIVE POLY	32.75	30.5
6	CURATIVE A	27.7	36
7	CURATIVE B	19.8	26.1
8	CURATIVE POLY	47.5	62.1

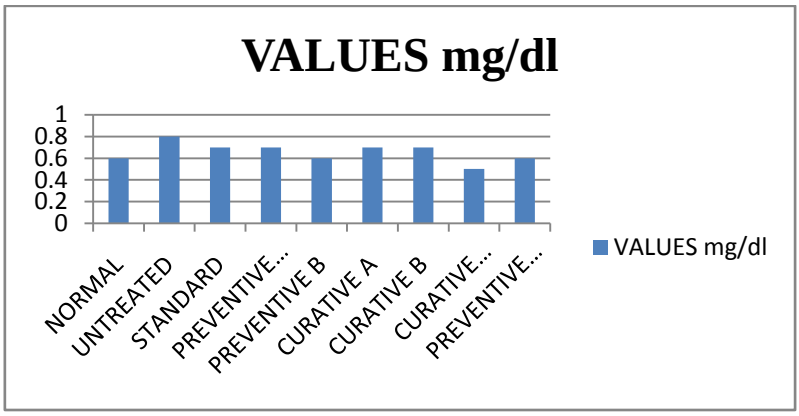


Graph 3: Comparison between calcium and phosphorus level in urine.

Serum analysis on 28th day

Table VI: Estimation of creatinine.

S.NO	GROUPS	SERUM CREATININE VALUES (mg/dl)(MEAN±SD)
1	NORMAL	0.6± .91
2	UNTREATED	0.8± 0.54
3	STANDARD	0.7± 0.45
4	PREVENTIVE A	0.7± 0.12
5	PREVENTIVE B	0.6± 0.65
6	CURATIVE A	0.7± 0.31
7	CURATIVE B	0.7± 0.45
8	CURATIVE POLY	0.5± 0.35
9	PREVENTIVE POLY	0.6±0.9



Graph 4: Estimation of Creatinine.

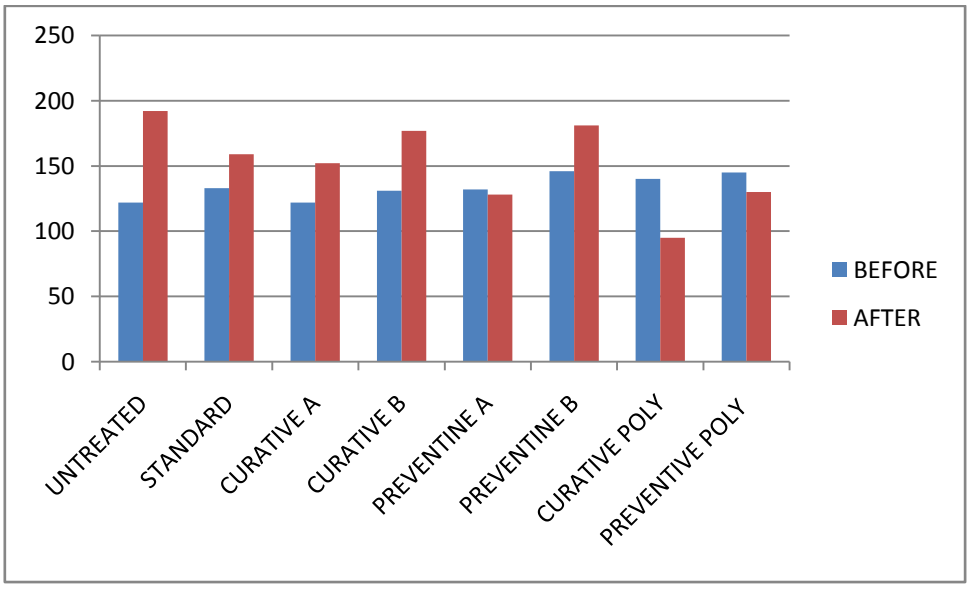
Table VII: Estimation of uric acid.

S.NO	GROUPS	SERUM URIC ACID VALUES (mg/dl)(MEAN±SD)
1	NORMAL	1.8± 0.25
2	UNTREATED	2.4± 0.56
3	STANDARD	2.1± 0.75

4	PREVENTIVE A	2.2± 0.45
5	PREVENTIVE B	1.8± 0.23
6	CURATIVE A	1.7± 0.33
7	CURATIVE B	1.9± 0.21
8	CURATIVE POLY	3.5± 0.44
9	PREVENTIVE POLY	2.0± 0.65

Table VIII: Body weights.

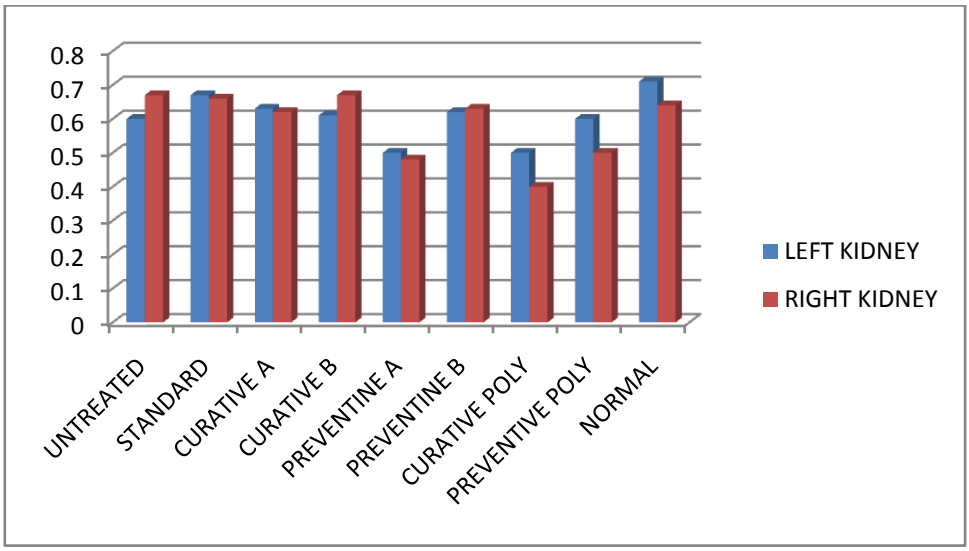
S.NO	GROUPS	BEFORE	AFTER
1	UNTREATED	122	192
2	STANDARD	133	159
3	CURATIVE A	122	152
4	CURATIVE B	131	177
5	CURATIVE POLY	140	95
6	PREVENTINE A	132	128
7	PREVENTINE B	146	181
8	PREVENTIVE POLY	145	130



Graph 5: Body weights.

Table IX: Kidney weights.

S.NO	GROUPS	LEFT KIDNEY	RIGHT KIDNEY
1	NORMAL	0.71	0.64
2	UNTREATED	0.6	0.67
3	STANDARD	0.67	0.66
4	CURATIVE A	0.63	0.62
5	CURATIVE B	0.61	0.67
6	PREVENTINE A	0.5	0.48
7	PREVENTINE B	0.62	0.63
8	CURATIVE POLY	0.5	0.4
9	PREVENTIVE POLY	0.6	0.5



Graph 6: Kidney weights.

Histopathological Evaluation

The histopathological changes in the kidney of urolithiatic group which were treated with ethylene glycol (0.75% v/v) show high renal damage with great nephrotic damage. The histopathological changes in the kidneys treated with the extract of Plant A were found to have intertubular mild congestion with no damage to the histology showing a protective effect. The histopathological changes in the kidneys treated with the extract of Plant B showed mild congestion and inter tubular haemorrhage with degenerative changes in 20x.The

histopathological changes in the kidneys treated with the extract of poly formulation both preventive and curative group shows better protective effect.

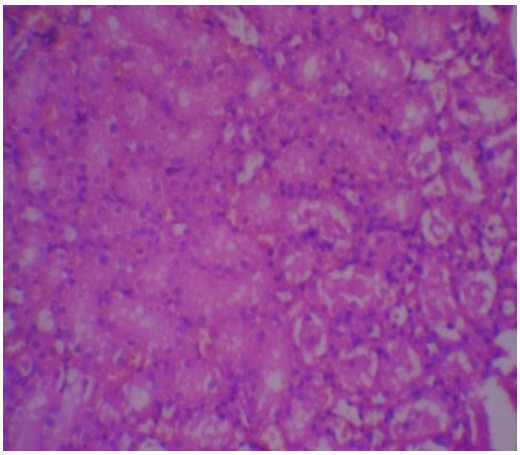


Figure 2:Urolithiatic group.

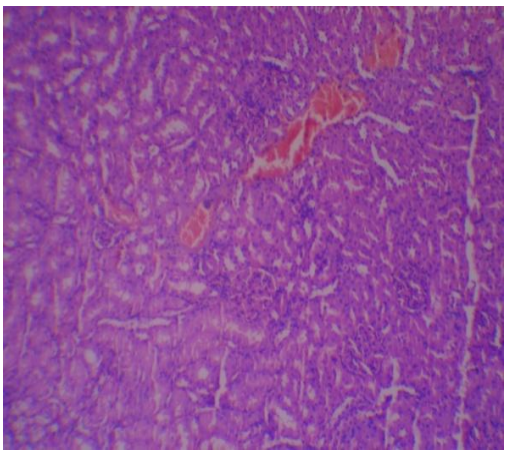


Figure 3: Plant A treated Group.

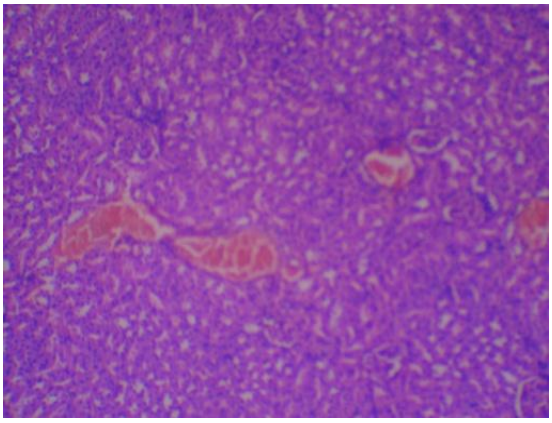


Figure 4: Plant B treated Group.

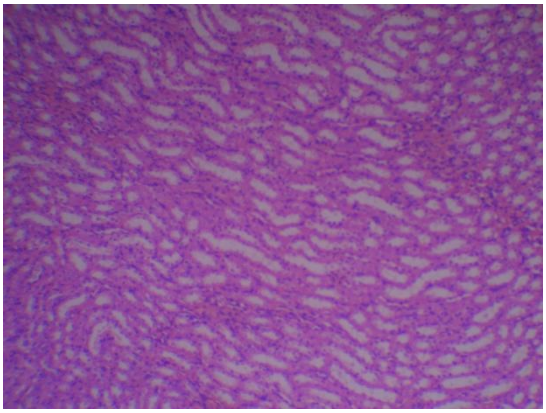


Figure 5: Poly formulation treated Group.

Discussion

In the present study the leaves of *Didymocarpuspedicellata* and roots of *Ashwagandha* were shade dried, powdered and then extracted with ethanol by soxhlet extraction method. The extracts were subjected to preliminary phytochemical screening. The phytochemical results showed the presence of carbohydrate, proteins, alkaloid, amino acids, steroids, tannins, glycoside and flavanoid in *Didymocarpuspedicellata* extract. Carbohydrate, proteins, alkaloid, aminoacids, steroids, glycoside and flavanoid in *Ashwagandha* extract.

The ethanolic extracts of selected plants were analyzed for their acute toxicity profile with reference to behavioural aspects. In this study, the acute toxicity was carried out using Male Sprague dawley rats. The acute toxicity by "Fixed dose method" showed LD_{50}cut off doses at 2000mg/kg body weight indicating that the extracts of *Didymocarpuspedicellata* and *Ashwagandha*are much safe. Hence this dose is selected for antiurolithiaticactivity. The neuropharmacological profile of all animals was observed. It was found that general

behavior studies, neurological and autonomic profile of ethanolic extract of *Didymocarpuspedicellata* and *Ashwagandh*a do not possess any neurotoxicity and found to much safer.

Conflict of Interest

The authors do not have any conflict of interest.

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