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CONTRIBUTION TO THE PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF TRADITIONAL PLANT *TABERNAEMONTANA DIVARICATA* IN THE TREATMENT OF INFLAMMATION AND UROLITHIASIS

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ABSTRACT

This study determined the phytochemical screening, acute toxicity studies, anti-inflammatory and anti urolithiatic of the aqueous and methanolic extracts of *Tabernaemontana divaricata* (carnation of India*)* belonging to the family *Apocynaceae*. Natural products which are of plant origin are found to be very useful and contain active constituents that cure or prevent many diseases. These natural products are believed to be safer when compared with synthetic drugs as they are less toxic with minimal side effects. *Tabernaemontana divaricata* (TD) is a shrub or small tree, usually glabrous, found in Konkan/Kanara regions of India and Bangladesh. The crude aqueous (AETD) and methanolic (METD) extracts of *Tabernaemontana divaricata* were tested for acute toxicity, anti-

inflammatory activity by carrageenan induced edema and anti urolithiatic potential against ethylene glycol induced urolithiasis model. From growing evidences it was suggested the presence of phytoconstituents like carbohydrates, flavonoids, alkaloids, glycosides, steriods, saponins and tannins. Both the medicinal plant extracts were found effective against carrageenan induced edema and ethylene glycol induced urolithiasis. The results found in our current research might help the folklore botanical significance of the screened medicinal plant *Tabernaemontana divaricata* for the treatment of inflammation and urolithiasis.

Key words: *Tabernaemontana divaricate*, *Apocynaceae*, Konkan, Kanara, acute toxicity, carrageenan, anti-inflammatory, ethylene glycol, urolithiasis

INTRODUCTION

Inflammation is a protective mechanism by the body to remove the harmful stimuli such as pathogens, damaged cells or irritants and initiate the healing process in the body. Acute inflammation is the initial response of the body which begins within seconds to minutes after a tissue injury caused by the harmful stimuli. Acute inflammation will turn chronic if injurious foreign substances persist for longer time and cannot be removed by the body **[1]**.

Urolithiasis is the third prevalent disorder of the urinary system that is approximately 2–3% in the general population. Urinary calculi, if untreated, may cause serious medical consequences such as extreme obstruction, hydronephrosis, infection, and hemorrhage in the urinary tract system **[2]**.Urinary supersaturation, which is due to stone forming minerals, is the first step in urinary calculi formation which leads to crystal nucleation, crystal growth, crystal aggregation and finally, crystal retention. Calcium stones crystallization occurs more commonly in the urinary tract **[3]**. Surgical operation, lithotripsy, and local calculus disruption using high-power laser are commonly used techniques to remove the calculi. However, these procedures are associated with the risk of acute renal injury leading to decrease in renal function **[4]**.

In recent years, more people are considering alternative therapies for complete cure of chronic ailments. Medicinal plants have been a host of a variety of biological compounds with therapeutic properties **[5]**. Scientists are regarding it as a safe source of active compounds for treatments of various diseases compared to synthetic drugs **[6]**. Hence, plants are considered to be a prolific resource for modern day drugs **[5]**. Some medicinal plants and proprietary composite herbal preparations are reported to be effective in the treatment as well as

prevention of recurrence of renal calculi with minimal side effects **[7]**.

Tabernaemontana divaricata commonly called as crepe Jasmine, a glabrous, evergreen, dichotomously branched shrub mostly grown in tropical countries. The plant grows up to a height of about 6-feet, bears attractive, white colored flowers having five-petal pinwheels gathered in small clusters on the stem tips. The leaves are large, shiny and deep green in color and the size is about 6-inches in length and 2-inches wide **[8]**. The flower juice is used in the treatment of eye infection. The root is acrid and bitter in taste; the milky juice mixed with oil is rubbed on to the head to cure pain in the eye. Chewing of root relieves the tooth ache and applied to wounds with water to prevent inflammation **[9]**. It has been used in chinese ayurvedic and Thai traditional medicine for the treatment of fever, pain and dysentery **[10, 11]**. It is reported that the plant have various medicinal and therapeutic properties viz, anti-tussive, anti-asthmatic **[12]**, anti-inflammatory **[13]**, anti-bacterial **[14]**, hypolipidemic **[15]**, gastro protective effect **[16]**, anti-nociceptive **[17]**, anti-convulsant **[18]** and anti-diabetic activity **[19]**. In view of the medicinal applications of this plant, the present study was designed for activities of aqueous extracts to evaluate the preliminary phytochemical studies, acute toxicity studies, anti-inflammatory and anti-urolithiatic and methanolic extracts of *T. divaricata* leaves.

MATERIALS AND METHODS

Plant material:

The fresh leaves of *Tabernaemontana divaricata* were collected from local areas of Guntur, Andhra Pradesh, India and authenticated by Prof. M. Raghu Ram, Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur. The plant specimen was certified as *Tabernaemontana divaricata* of family *Apocynaceae*.

Methanolic extract:

Tabernaemontana divaricata leaves were dried in shade at room temperature and powdered. 50g of the powder was extracted with 500 ml of methanol on Soxhlet apparatus. Then, the filtrate was concentrated separately to a thick paste and desiccated under vacuum by using rotary evaporator.

Aqueous extract:

Tabernaemontana divaricata leaves powder was macerated using distilled water for 24 hrs with occasional shaking. The extract was filtered through muslin cloth then the filtrate was evaporated under reduced pressure and vacuum dried. The extract was concentrated in rotary flash evaporator and dried in desiccator.

Phytochemical Screening:

The obtained aqueous and methanolic extracts were subjected for phytochemical evaluation to detect the presence of different phytoconstituents (carbohydrates, proteins, steroids, flavonoids, alkaloids, glycosides, saponins and tannins) **[20-25]**.

Animals:

The experiment was carried out on healthy male wistar rats (200–250 g). The animals were acclimatized to the standard laboratory conditions, fed with standard pellet diet and water *ad libitum* during the study. The study protocol was approved by the Institutional Animal Ethics Committee (Reg. No. 1048/PO/Re/S/07/CPCSEA) as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, India.

Acute oral toxicity study:

The acute oral toxicity study was evaluated as per the Organization for Economic Cooperation and Development (OECD) guidelines no. 425, on male wistar rats (200-250 g). Before the experiment, animals were fasted overnight with water *ad libitum*. Six animals per group were selected which received a geometric dose sequence of 50, 300, 500, 2000 and 5000 mg/kg, body weight. Animals were observed individually for any sign of toxicity, behavioral changes, and mortality after dosing, with special supervision given during the first 4 hours, and periodically thereafter for 24 h, for a total period of 7 days [26].

Anti-Inflammatory activity:

Carrageenan-induced paw edema model **[27]** with modifications was used for evaluating anti-inflammatory property of aqueous and methanolic extracts of T.D. The rats were divided into six groups. Each group of rats was given orally with vehicle (control), Indomethacin (5mg/kg), aqueous extract of *TD* (AETD, 250mg/kg body weight), AETD (500mg/kg body weight), methanolic extract of *TD* (METD, 250mg/kg body weight) and METD (500mg/kg body weight).

Edema was produced by sub-plantar injection of carrageenan (0.1 ml of 1% w/v) in the hind paw of each rat one hour after the administration of corresponding drugs. The paw volume was measured by water displacement method using the Digital plethysmometer at the time intervals of 30, 60 and 180 min.

Anti-Urolithiatic activity:

Ethylene glycol (EG) induced hyperoxaluria model **[28]** was used to assess the anti-urolithiatic activity in wistar rats. Animals were divided into 7 groups of each containing six animals. All the extracts were given once daily by oral route.

Group-I (Control)	**:**	**All the rats received regular food and drinking water ad libitum for 28 days.**
Group-II (Positive Control)	**:**	**EG (0.75%v/v) + ammonium chloride (1%) in drinking water was fed for 28 days to induce renal calculi.**
Group-III (Standard)	**:**	**EG (0.75%v/v) + ammonium chloride (1%) in drinking Water was fed for 28 days and treated with Cystone (750mg/kg body wt) from 15th day to 28thday.**
Group-IV (AETD-250 mg/kg):		**EG (0.75%v/v) + ammonium chloride (1%) in drinking water was fed for 28 days and treated with TD 15thday to 28thday.**
Group-V (METD-250 mg/kg)	**:**	**EG (0.75%v/v) + ammonium chloride (1%) in drinking water was fed for 28 days and treated with TD 15thday to 28thday.**
Group-VI (AETD-500 mg/kg):		**EG (0.75%v/v) + ammonium chloride (1%) in drinking water was fed for 28 days and treated with TD 15thday to 28thday.**
Group-VII (METD-500 mg/kg):		**EG (0.75%v/v) + ammonium chloride (1%) in drinking water was fed for 28 days and treated with TD 15thday to 28thday.**

Collection and analysis of urine:

Individual metabolic cages were allotted for all animals and 24 h urine samples were collected on 28thday of calculi induction treatment. Animals had free access to drinking water during urine collection period. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4^{0}C.Urine was analyzed for calcium, phosphate and oxalate content **[29-31]**.

Serum analysis:

The blood was collected from the retro-orbital sinus under anesthetic condition and serum was separated by centrifugation at 10,000 g for 10 min and analyzed for creatinine, uric acid and blood urea nitrogen **[32, 33]**.

Kidney homogenate analysis:

The abdomen was cut opened to remove both kidneys from each animal. Isolated kidneys were cleaned off extraneous tissue and preserved in 10% neutral formalin. The kidneys were dried at 80^{0}C in a hot air oven. A sample of 100mg of the dried kidney was boiled in 10ml of 1N hydrochloric acid for 30min and homogenized. The homogenate was centrifuged at 2000×g for 10min and the supernatant was separated **[34]**. The calcium, phosphate and oxalate content in kidney homogenate were determined.

Statistical Analysis:

All the values are expressed as mean ± SEM. The data were statistically analyzed by Two-way ANOVA followed by Tukey's multiple comparisons test. P values < 0.0001 (n=6) were considered significant.

RESULTS AND DISCUSSION

Phytochemical examination revealed that methanolic extract answer positively with carbohydrates, saponins, sterols, tannins, alkaloids, glycosides and flavonoids where in aqueous extract noted with all constituents present in methanolic extract except sterols.

Acute toxicity studies:

From the acute toxicity study, the LD_{50} cut-off dose was found to be 2000mg/kg body weight for both extracts. Hence, the therapeutic doses were taken as 250mg/kg

& 500mg/kg body weight for both METD and AETD.

Anti-inflammatory activity:

Carrageenan-induced paw edema is a commonly used primary test for the screening of new anti-inflammatory agents and is believed to be biphasic. The first phase (1-2 hr) is due to the release of histamine or serotonin and the second phase of edema is due to the release of prostaglandins. Supplementation with AETD and METD significantly reduced the paw volume compared to the control **(Table 1)**.

Antiurolithiatic activity:

In the present study, ethylene glycol-induced hyperoxaluria model was used to assess the antiurolithiatic activity in wistar rats. Chronic administration of 0.75% (v/v) ethylene glycol aqueous solution to male albino rats resulted in hyperoxaluria. Oxalate, calcium and phosphate excretion were increased in the calculi-induced (Group II) animals. The biochemical mechanism involved in this process is associated with a raise in the urinary concentration of oxalate. Stone formation in ethylene glycol-fed animals is caused by hyperoxaluria, which causes increased renal retention and excretion of oxalate.

Hyperoxaluria and hypercalciuria are major risk factors for the pathogenesis of renal stones. Since hyperoxaluria is a far more significant risk factor, the changes in urinary oxalate levels are comparatively much more imperative than those of calcium. Increased urinary and kidney calcium is a factor stimulating the nucleation and precipitation of calcium oxalate or apatite (calcium phosphate) from urine and following crystal growth. However, in the present study, supplementation with AETD and METD and Cystone restored oxalate and calcium in urine and kidney in curative regimens and preventive regimens as compared to calculi-treated animals **(Table 2)**.

The glomerular filtration rate (GFR) is an important parameter for ensuring renal function and it gets decreased in urolithiasis due to the obstruction to the outflow of the urine by stones in urinary system, which leads to a rise in nitrogenous waste products like urea, creatinine, and uric acid in blood. In calculi-induced rats (Group II), marked renal damage was seen by the elevated serum levels of creatinine, uric acid and Blood Urea Nitrogen (BUN). However, the treatment with AETD and METD restored the elevated serum levels of creatinine, uric acid and BUN **(Table 3)**.

The deposition of the crystalline components in the renal tissue, namely oxalate, phosphate and calcium, was increased in the urolithiasis induced rats

(positive control). Treatment with AETD and METD significantly (P <0.0001) reduced the deposition of crystals (oxalate, phosphate and calcium) **(Table 4)**.

The general architectures of the kidney were normal in control group **(Fig.1- 1)**. Extensive in tratubular crystal depositions and degenerative tubular structures were found in urolithiasis induced rats **(Positive control, Fig.1– 2)**. There were no intratubular crystal depositions and histopathological changes found in Cystone and METD (500 mg/Kg) treated rats **(Fig.1- 3 and 7)**.

Table 1: Effect of METD and AETD on Carrageenan induced paw edema in rats

Group	Treatment	Paw volume (ml as measured by water displacement method using digital plethysmometer)			
		0 min	30 min	60 min	120 min
I	Control	0.12±0.011	0.265±0.18	0.438±0.023	0.668±0.046
II	Indomethacin (5 mg/kg)	0.130±0.009	0.135±0.006****	0.138±0.009****	0.127±0.011****
III	AETD (250mg/kg)	0.170±0.009	0.172±0.009****	0.177±0.007****	0.178±0.010****
IV	METD (250mg/kg)	0.180±0.004	0.185±0.013****	0.192±0.005****	0.190±0.004****
V	AETD (500mg/kg)	0.157±0.005	0.168±0.005****	0.175±0.003****	0.169±0.005****
VI	METD (500mg/kg)	0.170±0.004	0.178±0.006****	0.185±0.006****	0.183±0.009****

AETD= Aqueous extract of TD; METD = Methanolic extract of TD; Values are expressed as mean ± SEM (n=6); Data are analyzed by Two-way ANOVA followed by Tukey's multiple comparisons test, ****P<0.0001 *vs* positive control

Table 2: Effect ofAETD and METD on urine parameters in urolithiasis-induced rats

Group	Treatment	Levels in urine		
		Calcium(mg/dl)	Phosphate(mg/dl)	Oxalate(mg/dl)
I	Control	1.33±0.015	3.69±0.02	0.41±0.001
II	Positive control	4.78±0.003	7.47±0.017	3.73±0.03
III	Cystone (750 mg/kg)	1.59±0.007****	3.82±0.03****	0.58±0.002****
IV	AETD (250mg/kg)	2.27±0.02****	4.75±0.003****	1.91±0.009****
V	METD (250mg/kg)	1.88±.0.006****	4.04±0.009****	1.72±0.009****
VI	AETD (500mg/kg)	1.93±0.005****	4.16±0.005****	1.30±0.01****
VII	METD (500mg/kg)	1.72±0.007****	4.02±0.004****	0.95±0.004****

AETD= Aqueous extract of TD; METD = Methanolic extract of TD; Values are expressed as mean ± SEM (n=6); Data are analyzed by Two-way ANOVA followed by Tukey's multiple comparisons test, ****P<0.0001 *vs* positive control

Table 3: Effect ofAETD and METD on serum parameters in urolithiasis-induced rats

Group	Treatment	Serum parameters (mg/dl)		
		Uric acid	BUN	Creatinine
I	Control	37.75±0.02	0.72±0.03	1.51±0.05
II	Positive control	50.37±0.02	0.96±0.01	3.64±0.02
III	Cystone (750 mg/kg)	39.42±0.19****	0.80±0.01****	1.69±0.03****
IV	AETD (250mg/kg)	43.69±0.04****	0.90±0.002****	2.23±0.05****
V	METD (250mg/kg)	42.23±0.12****	0.86±0.005****	2.01±0.01****
VI	AETD (500mg/kg)	41.23±0.11****	0.85±0.01****	1.93±0.03****
VII	METD (500mg/kg)	40.47±0.21****	0.83±0.02****	1.88±0.003****

AETD= Aqueous extract of TD; METD = Methanolic extract of TD; Values are expressed as mean ± SEM (n=6); Data are analyzed by Two-way ANOVA followed by Tukey's multiple comparisons test, ****P<0.0001 *vs* positive control

Table 4: Effect of AETD and METD on constituents in kidney of urolithiasis induced rats

Group	Treatment	Levels in kidney homogenate mixture		
		Calcium (mg/g)	Phosphate (mg/g)	Oxalate (mg/g)
I	Control	2.92±0.02	2.13±0.03	1.47±0.009
II	Positive control	4.67±0.01	5.14±0.03	3.71±0.013
III	Cystone (750 mg/kg)	3.65±0.02****	2.47±0.017****	1.56±0.02****
IV	AETD (250mg/kg)	4.29±0.001****	3.13±0.07****	2.91±0.011****
V	METD (250mg/kg)	3.83±0.003****	2.86±0.08****	2.65±0.02****
VI	AETD (500mg/kg)	3.76±0.01****	2.62±0.025****	1.98±0.02****
VII	METD (500mg/kg)	3.68±0.004****	2.44±0.015****	1.87±0.02****

AETD= Aqueous extract of TD; METD = Methanolic extract of TD; Values are expressed as mean ± SEM (n=6); Data are analyzed by Two-way ANOVA followed by Tukey's multiple comparisons test, ****P<0.0001 *vs* positive control

Histopathological studies:

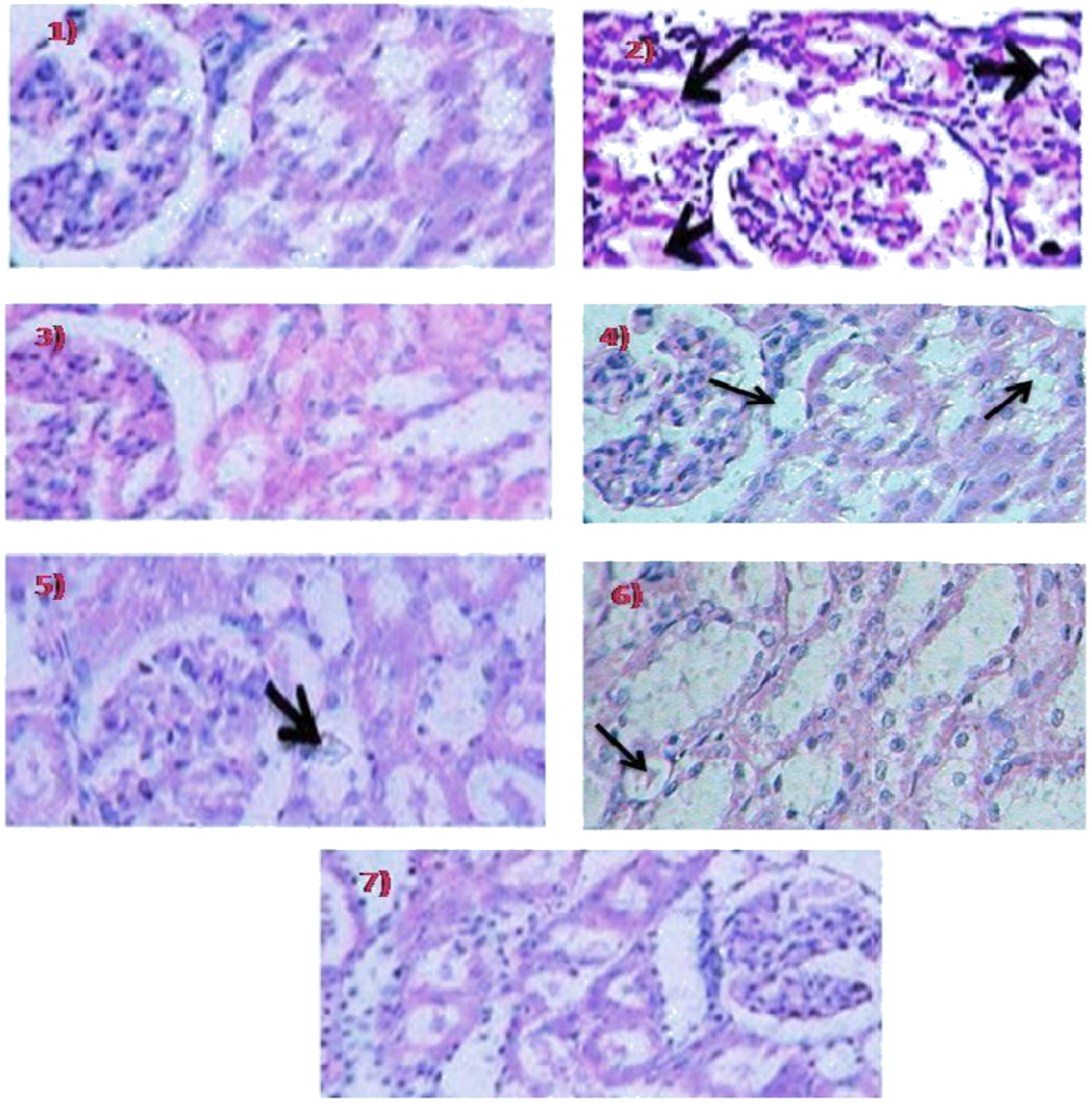

Figure 1: Histology of rat kidneys [1-Control, 2-Positive control, 3-Standard, 4-AETD (250 mg/Kg); 5-METD (250 mg/Kg); 6-AETD (500 mg/Kg); 7-METD (500 mg/Kg)]

CONCLUSION

In the present study, the extraction for the plant *Tabernaemontana divaricata* was carried out and tested for anti-inflammatory and antiurolithiatic activity. Preliminary phytochemical analysis proved the presence of alkaloids, saponins, sterols and flavonoids. From this study, it is concluded that *T. divaricata* plant shows efficient anti-inflammatory and anti-urolithiatic activity. Thus, the plant *T. divaricata* could be used as a prolific resource for drugs.

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