INVESTIGATION OF THE ANTIUROLITHIATIC ACTIVITY OF *Ixora pavetta* ON SODIUM OXALATE INDUCED UROLITHIASIS IN ALBINO RATS

Kalasani Krishna Veni, K. Sravanth & D. Rama brahma Reddy
Nalanda institute of Pharmaceutical Sciences, Siddharth Nagar, Kantepudi(Village), Sattenapalli(Mandal), Guntur(Dist)-522438
E.Mail: prasannab970@gmail.com

**ABSTRACT**

*Ixora pavetta* (torch tree) is a plant in the Rubiaceae family most widely used by the tribals of andhrapradesh and Karnataka for the various purposes and having traditional values. preliminary phytochemical screening of methanolic root extract of *Ixora pavetta* revealed the presence of Carbohydrates, alkaloids, flavonoids, triterpenes, tannins ,phenols, saponins, phytosterols, proteins, aminoacids. In group of Male wister Albino rats weighing 200-250g treated with sodium oxalate (0.164 ml, IP, single dose) a significant increase (P<0.05) in serum biochemical parameters sodium, potassium, chloride, uric acid ,creatine, urea levels (136.5±5.071,72±2.67,91.3±3.86,1.945±0.17,31.1±2.79,50.3±2.9) was observed when compared to normal group (80.3±3.62,72.09±2.67,78.6±3.15,1.505±0.107,11.1±1.125,12.5±0.87) and urine parameters like oxalate , uric acid, creatinine (4.101±0.433,34.92±2.841,4.793±0.222) was observed when compared to normal group(1.90±0.309,15.78±1.512,1.535±0.167) This indicates NaOx induces urolithiasis in rats. In group that received standard drug (Cystone, 500 mg/kg, p.o, once daily) there was significant decrease (P<0.05) in the serum sodium, potassium, chloride, uric acid, creatinine, urea and urine oxalate, uric acid, creatinine levels when compared to NaOx treated group. On administration of MEIP (200mg/kg, 400 mg/kg, p.o, once daily) there was a significant decrease (P<0.05) in the serum sodium, potassium, chloride, uric acid ,creatine, urea and urine oxalate, uric acid, creatinine levels (338.71±8.79) when compared to negative control group. A dose related effect was not observed in groups treated with the test drug. These results suggest that MEIP has better Anti urolithiatic activity.

INTRODUCTION

Many medicinal plants provide relief of symptoms comparable to that obtained from allopathic medicines. *Ixora pavetta* (torch tree) is a plant in the Rubiaceae family commonly seen in Ballari district of Karnataka and Tirumala hills, Andhrapradesh, India. It is called as Goravi in Telugu. People use the branches of this tree for making walls and paste with mud for their thatched huts in villages, but now this practice has become obsolete owing to modern housing materials. Summer is the fruiting season and the fruits are globose, 2-seeded, become black when ripened. Indian Sloth Bears eat the fruits and the seeds are dispersed through its scat. Urolithiasis, which is referred to as the process of formation of calculi (singular calculus) in the urinary system. By extensive literature review, we have concluded that, the above said plant was traditionally used for the treatment of gall stones and the aim of our study is to investigate the antiurolithiatic activity of *Ixora pavetta* on sodium oxalate induced urolithiasis in albino rats. [1, 2]
Materials and Method

Collection and Authentication of plant material:

*Ixora pavetta* roots were collected from thirupati hills. The plant was identified and authenticated by Dr.K.Madhava chetty, Department of Pharmacognosy, S.V.university,thirupati.

Preparation of extract:

Roots are collected, washed properly and dried under shade for few days. They are powdered coarsely and subjected for cold maceration with pet ether for 48hrs and filtered. The dried mass obtained were subjected for soxhlet extraction with methanol at 65ºc for 6hrs.the extract was Made to dried under reduced vacuum. The yield of root in 42gm/kg.

Preliminary Phytochemical screening:

The methanolic root extract of *Ixora pavetta* were subjected to phytochemical screening for identification of various phyto constituents present in it. [3]

ANTUROLITHIATIC ACTIVITY

Experimental Animals

Male Wistar albino rats of weighing between (200-250 g) used for the experimentation.animals were obtained from the animal house attached to our institute. Six animals were housed in each Cage made up of poly-propylene with stainless steel top grill. After randomization into various groups, animals were acclimatized for period of 7 days under standard husbandry conditions. Room temperature 27 ± 30C, Relative humidity 65 ± 10%, 12 hrs light/dark cycle. All the animals were fed with rodent pellet diet (Malla Reddy institute of pharmaceutical sciences, hydearbad) and water was allowed ad-libitum under strict hygienic condition. Ethical clearance for performing experiments on animals was obtained from Institutional Animal Ethical Committee (Regd No:1662/PO/a/CPCSEA,2013) and were in accordance with the guidelines of CPCSEA.[4]

Acute Toxicity Study

The acute oral toxicity procedure was followed by using OECD 423 guidelines. The acute toxic class method is a step wise procedure with 6 animals of a single sex per step (OECD,2001). Depending on the mortality and /or moribund status of the animals, on the average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance. This procedure results in the use of minimal number of animals while allowing for acceptable data based scientific conclusion. The method used defined doses (5, 50, 300, 2000 mg/kg b.wt.) and the results allow a substance to be ranked and classified according to the Globally Harmonized System for the classification of chemicals which cause acute toxicity.

After oral administration of the test, animals were observed individually at least once during the first 30 minutes and periodically during the first 24 hrs, with special attention given during the first 4 hrs and daily thereafter for total of 14 days. Body weight of the rats before and after treatment were noted and any changes in the skin, fur, eyes, mucous membranes, salivation, nasal discharge, urination, behavioural,
neuromuscular, cardiovascular, lethargy, sleep and coma were noted for 14 days. Test compounds were not mortal for rats even at 200 mg/kg dose hence, 1/20th of this dose was selected for further study.

EXPERIMENTAL DESIGN

Evaluation of Antiurolithiatic activity in Sodium oxalate induced urolithiasis

Male wister Albino rats weighing 200-250g were selected and divided into five groups consisting of six animals each and they are grouped as follow.

Group I - Animal received 2% v/v Tween 80, p.o. vehicle for 10 days and served as Normal group.

Group II - Animal received Sodium oxalate 70mg/kg,i.p. for 10 days and served as disease control group.

Group III - Cystone 500mg/kg, p.o.suspended in 2%v/v Tween 80 and Sodium Oxalate 70mg/kg,i.p. for 10 days and served as standard group.

Group IV - Methanolic extract of Ixora pavetta (MEIP) 200mg/kg,p.o. suspended in 2%v/v Tween 80 and sodium oxalate(NaOx) 70mg/kg,i.p. for 10 days and served as Test group-I.

Group V - Methanolic extract of Ixora pavetta (MEIP)400mg/kg,p.o.suspended in 2% Tween 80 and Sodium oxalate(NaOx) 70mg/kg,i.p. for 10 days and served as Test Group-II.

Collection of blood and urine samples:

At the end of 10th day blood was collected from Retro orbital plexus and the serum was separated by centrifuge at 800rpm for 20 min and serum was subjected to biochemical parameters. after collected blood sample from each group placed in individual cages and the Urine was collected for 24hrs.the volume is measured in ml. the urine was subjected for estimation of urinary parameters.

ESTIMATION OF PARAMETERS [5-10]

Estimation of sodium by colorimetric method

The sodium and the proteins are precipitated simultaneously by means of reagent containing magnesium uranyl acetate containing alcohol. The precipitate is separated by centrifugation. The content of sodium is calculated from the loss in concentration of magnesium uranyl acetate in the reagent solution in comparison to standard sodium solution treated similarly. The residual amount of magnesium uranyl acetate is estimated by forming brown (dark) ferrous uranyl acetate, which is read in a colorimeter.

Reagents:

1. Sodium Precipitating Reagent - 33ml
2. Standard Sodium/Potassium - 3ml
3. Sodium colour Reagent - 10ml

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>End point with std.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Slope</td>
<td>Increasing</td>
</tr>
<tr>
<td>Wave length</td>
<td>540nm</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Incubation time</td>
<td>10min</td>
</tr>
<tr>
<td>Standard conc</td>
<td>150m.Mol/L</td>
</tr>
<tr>
<td>Linearity</td>
<td>200m.Mol/L</td>
</tr>
<tr>
<td>Unit</td>
<td>m.Mol/L</td>
</tr>
</tbody>
</table>
Procedure:
Pipette into clean dry test tubes, labeled standard(S) and test (T).

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard PPT Reagent</td>
<td>1.0ml</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Standard sodium/potassium</td>
<td>0.02ml</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>0.02ml</td>
</tr>
</tbody>
</table>

Mix well on vortex for one minute and wait for 5 minutes at room temperature. Centrifuge for one minute at 3000 rpm.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3ml</td>
<td>3ml</td>
<td>3ml</td>
</tr>
<tr>
<td>Supernatant from step-1</td>
<td>-</td>
<td>0.05ml</td>
<td>0.05ml</td>
</tr>
<tr>
<td>Sodium PPT Reagent</td>
<td>0.05ml</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium Colour Reagent</td>
<td>0.2ml</td>
<td>0.2ml</td>
<td>0.2ml</td>
</tr>
</tbody>
</table>

Pipette into 3 clean dry test tubes labelled Blank (B) Standard (S) and Test (T).
Mix well and allow it to stand at room temperature for 5 minutes then measure absorbance of Blank, Standard and Test against distilled water on photocolorimeter at 540 nm within 10 minutes

Calculations:
Sodium in m.Mol/L = \frac{\text{Absorbance of B-T}}{\text{Absorbance of B-S}} \times 150 \times (\text{Standard concentration})

Estimation of potassium by colorimetric method

Principle:
Potassium can be determined by a number of different methods. It can be directly estimated by flame photometry, colorimetry. It can also be measurement of turbidity of the reaction mixture containing Sodium Tetra phenyl Boron, alkaline EDTA, formaldehyde and sample containing potassium (or) standard potassium salt. The method accurate within concentration of 2.0 to 7.0 m.M/L, there is a good agreement with flame photometry.

Reagents:
1. Potassium Reagent - 45ml
2. Standard Sodium/Potassium – 3ml

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction type</td>
<td>End point</td>
</tr>
<tr>
<td>Reaction slope</td>
<td>Increasing</td>
</tr>
<tr>
<td>Wave length</td>
<td>620nm</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Incubation time</td>
<td>5min</td>
</tr>
<tr>
<td>Standard concentration</td>
<td>5m.Mol/L</td>
</tr>
<tr>
<td>Linearity</td>
<td>7m.Mol/L</td>
</tr>
</tbody>
</table>
Procedure:

Pipette into two clean dry test tubes labelled Standard (S) and test (T).

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Reagent</td>
<td>1.0ml</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Standard Sodium/Potassium</td>
<td>0.05ml</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>0.05ml</td>
</tr>
</tbody>
</table>

Mix gently wait for 5 minutes at room temperature and read the absorbance of standard and test against distilled water on a photo colorimeter at 620nm within 10 minutes.

Calculation:

\[
\text{Potassium in mMol/L} = \frac{\text{Absorbance of T}}{\text{Absorbance of S}} \times 5 \text{ (standard concentration)}
\]

Estimation of chloride by Colorimetric method

Principle:

Chloride ions form a coloured complex when reacting with mercury (II) thiocyanate solution. The intensity of the colour is proportional to chloride concentration.

Reagents:

1. Chloride Reagent
2. Standard chloride

<table>
<thead>
<tr>
<th>Reaction type</th>
<th>End point with std.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Slope</td>
<td>Increasing</td>
</tr>
<tr>
<td>Wavelength</td>
<td>505nm</td>
</tr>
<tr>
<td>Incubation temperature</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Incubation time</td>
<td>2 min</td>
</tr>
<tr>
<td>Standard concentration</td>
<td>100m.Mol/L</td>
</tr>
<tr>
<td>Linearity</td>
<td>130m.Mol/L</td>
</tr>
</tbody>
</table>

Chloride Assay:

Pipette into three clean dry test tubes labelled Blank (B), Standard (S) and Test (T).

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride Reagent</td>
<td>1.0ml</td>
<td>1.0ml</td>
<td>1.0ml</td>
</tr>
<tr>
<td>Standard chloride</td>
<td>-</td>
<td>0.005ml</td>
<td>-</td>
</tr>
<tr>
<td>Serum</td>
<td>-</td>
<td>-</td>
<td>0.005ml</td>
</tr>
</tbody>
</table>

Mix well wait for 2 minutes at room temperature and read the absorbance of Blank, Standard and Test against distilled water on a photo colorimeter at 505nm.
Calculation:

Chloride in m.Mol/L = \frac{\text{Absorbance of } T-B \times 100}{\text{standard concentration}}

\text{Absorbance of } S-B

Estimation of Creatinine by Jaffes’s method:

**Principle:** Creatinine reacts with alkaline picrate to produce a red coloured complex; the rate of red coloured complex formation is directly proportional to the creatinine concentration^52.

**Reagents Composition:**

1) Picric acid Reagent
2) Alkaline Buffer Reagent
3) Standard Creatinine (2mg %)
4) Acid Reagent

**Preparation of working reagent:** Mix equal volumes of picric acid reagent & alkaline buffer reagent.

**Sample:** Serum, plasma, urine

Dilute urine specimen 1:100 using distilled water before use.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Type</td>
<td>Kinetic with std</td>
</tr>
<tr>
<td>Wavelength</td>
<td>520 nm (green filter)</td>
</tr>
<tr>
<td>Temperature</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>30 sec</td>
</tr>
<tr>
<td>Read Time</td>
<td>60 sec</td>
</tr>
<tr>
<td>No of Readings</td>
<td>2</td>
</tr>
<tr>
<td>Sample Volume</td>
<td>100µl</td>
</tr>
<tr>
<td>Reagent Volume</td>
<td>1000µl</td>
</tr>
<tr>
<td>Standard Concentration</td>
<td>2 mg%</td>
</tr>
<tr>
<td>Linearity</td>
<td>20 mg%</td>
</tr>
<tr>
<td>Unit</td>
<td>mg%</td>
</tr>
</tbody>
</table>

**Procedure: KINETIC METHOD**

1. Pipette into a clean dry cuvette

   Working reagent 1ml
   Serum/dilute urine/standard 0.1ml

2. Mix well and start stopwatch. Read initial absorbance $A_0$ exactly after 30 seconds for test and standard.

3. Read another absorbance $A_1$ for test and standard exactly 60 secs later.

4. Calculate change in absorbance for test and standard.

   For test \[ \Delta A_T = A_1 - A_0 \]
   For standard \[ \Delta A_S = A_1 - A_0 \]

**Calculation:**

a) Serum creatinine in mg% = \frac{\Delta AT}{\Delta AS} \times 2 \text{ std conc}

b) Urine creatinine in gm/lit = \frac{\Delta AT}{\Delta AS} \times 2 \text{ std conc}

c) Urine creatinine in gm/24 hr = (b) \times 24 \text{ hrs urine volume in liters}

SI conversion factor

μ/L = \text{mg%} \times 88.4

<table>
<thead>
<tr>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Reagent</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Standard</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Serum/Diluted urine</td>
<td>---</td>
</tr>
<tr>
<td>Acid Reagent</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

1. Mix well and allow it to stand at RT for 5 mins.

2. Read absorbance for standard As₀ and Test At₀ against distilled water at 520 nm (or) green filter in spectrophotometer.

3. Read absorbance for standard As₁ and Test At₁ against distilled water at 520nm (or) green filter in spectrophotometer.

**Calculation**

\[ \Delta AS = AS0 - AS1 \]
\[ \Delta AT = AT0 - AT1 \]

a) Serum Creatinine = \frac{\Delta AT}{\Delta AS} \times 2 \text{ (Std. Conc)}

**Estimation of uric acid by end point assay**

**Principle:**

Uric acid is oxidized to allantoin & hydrogen peroxide by the enzyme uricase. In presence of peroxidase, released hydrogen peroxide is coupled with aniline derivative and 4-amino antipyrine (4-AAP) to form colored chromogen complex. Absorbance of colored dye is measured at 550nm and is proportional to uric acid concentration in the sample.

\[ \text{Uricase} \]

\[ \text{Uric acid} + 2 \text{H}_2\text{O} \rightarrow \text{Allatonin} + \text{CO}_2 + \text{H}_2\text{O}_2 \]

\[ \text{POD} \]

\[ \text{H}_2\text{O}_2 + \text{Alanine derivative} + 4\text{-AAP} \rightarrow \text{chromogen complex} + \text{H}_2\text{O}_2 \]
Mix well and incubate at 37°C for 5 minutes

1. Blank the analyzer with reagent blank
2. Measure the absorbance of standard followed by test
3. Calculate results

**Mode**

<table>
<thead>
<tr>
<th>End point</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum/plasma/urine</td>
<td>-</td>
<td>-</td>
<td>20µL</td>
</tr>
<tr>
<td>Reagent 2</td>
<td>-</td>
<td>20µL</td>
<td>-</td>
</tr>
<tr>
<td>Reagent 1</td>
<td>1000 µL</td>
<td>1000 µL</td>
<td>1000 µL</td>
</tr>
</tbody>
</table>

**Calculation**

```
Serum/plasma uric acid mg/dL = Absorbance of test
                           --------------------------- x 6
                           Absorbance of standard

Urine uric acid (mg/day) = Absorbance of test
                           --------------------------- x 6 x dilution
                           Absorbance of standard x 24 hrs urine volume in dL
```

Conversion factor in mmol/L = uric acid concentration in mg/dL x 0.059

**Estimation of Urea by Berthelot method**

**Principle:**

Urease catalyses the conversion of Urea to Ammonia and Carbon dioxide. The Ammonia released reacts with a mixture of salicylate, hypochlorite and Nitroprusside to yield a blue-green colored compound.
(Indophenol). The intensity of color produced is proportional to the concentration of urea in the Sample and is measured photometrically at 570nm or with yellow filter.

**Reaction:**

\[
\text{Urea} + \text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{CO}_2
\]

\[
\text{Urease} \quad \text{Nitroprusside}
\]

\[
\text{NH}_3 + \text{salicylate} \rightarrow 2\text{-2-Dicarboxy Indophenol}
\]

\[
\text{Hypochloride}
\]

**Reagents composition:**

<table>
<thead>
<tr>
<th></th>
<th>2×50ml</th>
<th>2×100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Urease Reagent</td>
<td>1×50ml</td>
<td>1×100ml</td>
</tr>
<tr>
<td>2) Enzyme Concentrate</td>
<td>1Vial</td>
<td>1Vial</td>
</tr>
<tr>
<td>3) Color Reagent</td>
<td>1×50ml</td>
<td>1×100ml</td>
</tr>
<tr>
<td>4) Urea Standard (40 mg%)</td>
<td>1×2ml</td>
<td>1×2ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Type</td>
<td>End point with std</td>
</tr>
<tr>
<td>Wavelength</td>
<td>578 nm (yellow filter)</td>
</tr>
<tr>
<td>Incubation Temp</td>
<td>37°C</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>10 mins</td>
</tr>
<tr>
<td>Standard</td>
<td>40 mg%</td>
</tr>
<tr>
<td>Linearity</td>
<td>300 mg%</td>
</tr>
<tr>
<td>Unit</td>
<td>mg%</td>
</tr>
</tbody>
</table>

**Procedure:**

**Working Reagent Preparation:** Transfer the entire enzyme Concentrate (2) into Urease Reagent (1) with dropper or micro tip, rinse the Enzyme Concentrate Vial with little Urease Reagent and transfer the residual enzyme to ensure better reconstitution.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease Reagent (working)</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Urea Standard</td>
<td>---</td>
<td>0.01 ml</td>
<td>---</td>
</tr>
<tr>
<td>Specimen</td>
<td>---</td>
<td>---</td>
<td>0.01 ml</td>
</tr>
<tr>
<td><strong>Incubate for 5min at 37°C/10 min at RT</strong></td>
<td>Colour reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>
1. Pipette in a clean, dry test tubes labelled Blank (B), Standard(S), and Test(T)
2. Mix and incubate for 5min at 37°C/10min. at RT
3. Read absorption of standard(S) and Test (T) against Blank (B) at 570nm or with yellow filter.
4. The final color is stable for 30min at RT.

**Sample:** Serum, Heparin, Urine (dilute 1:100 with distilled water)

**Calculation:**

\[
\text{Urea in mg\%} = \frac{A_{of\ (T)}}{A_{of\ (S)}} \times 40 \text{(Std. Conc)}
\]

b) Urine Urea in gm/24hrs = a × 24 hrs urine vol. in lits.

**Estimation of oxalate by colorimetric method:**

The enzymatic reactions involved in the assay procedure are as follows:

\[
\text{Oxalate Oxidase} \\
\text{Oxalate + O}_2 \xrightarrow{\text{peroxidase}} 2\text{CO}_2 + \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + \text{MBTH} + \text{DMAB} \xrightarrow{\text{indamine dye +H}_2\text{O}}
\]

Oxalate is oxidized to carbon dioxide and hydrogen peroxide by oxalate oxidase. The hydrogen peroxide reacts with 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-dimethylamino benzoic acid (DMAB) in the presence of peroxidase to yield an indamine dye which has an absorbance maximum at 590 nm. The intensity of the color produced is directly proportional to the concentration of oxalate in the sample.

**Reagents**

| OXALATE REAGENT A | 3.2mmol/L |
| DMAB             | 0.22mmol/L |
| MBTH             | 3.1±0.1    |
| Buffer pH        |            |
| Nonreactive ingredients and stabilizers |          |
| OXALATE REAGENT B | 3000 u/L   |
1. Warm oxalate reagents to assay temperature (any temperature between ambient and 37°C).
2. Label tubes for Reagent Blank, Standard, urine Control and urine Sample. Pipette 1 ml Oxalate Reagent A into each tube.
3. Pipette 50 μl of Supernatants or Filtrates ("Sample Preparation" section, Step 9), to respective tubes. Add 50 μl deionized water to Reagent Blank tube and 50 μl standard to tube labeled standard.
4. Pipette 0.1 ml of Oxalate Reagent B into each tube and immediately mix by gentle.
5. Incubate the tubes at desired temperature (18-37°C) for 5 minutes.
6. Read absorbances (A) of Blank, Standard, Control and urine Sample at 590 nm.
7. Determine the corrected absorbances (ΔA) of Standard, Control and Sample by subtracting Reagent Blank absorbance from the absorbance readings of Standard, Control and urine Sample.
8. To determine oxalate concentration in urine Sample, refer to "Calculations" section.

**Calculations**

Determine oxalate concentration in sample as follows:

\[
\text{Oxalate (mmol/L)} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \times 0.5 \times 2
\]

Where: 0.5 = Concentration (mmol/L) of oxalate in standard

2 = Dilution factor

Quantity of Oxalate Excreted During 24-Hour Period = Oxalate (mmol/L) \times \text{Volume of Urine Voided during 24 hours (L)}

**ESTIMATION OF INVIVO ANTI OXIDANT ACTIVITY**

**Preparation of mitochondrial supernatant (PMS):**
The kidney were perfused with ice cold saline (0.9% sodium chloride) and homogenized in cold potassium chloride (1.17%) using a homogenizer. The homogenates were centrifuged at 800rpm for 5 minutes at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500rpm for 20min at 4°C to get the PMS.

**Invivo antioxidant activity**
The anti-oxidant activity of *ixora pavetta* root extract has been determined by subjecting the PMS to the following invivo methods.

1. Lipid peroxidation method (LPM)
2. Catalase method (CAT)

**Lipid peroxidation method** [11]

**Principle**

Oxidative stress is associated with peroxidation of cellular lipids, which is determined by measurement of thiobarbituric acid reacting substance (TBARS). The concentration of LPO products may reflect the degree of oxidative stress. The increased level of TBARS results in increase of OFR’s, which attacks the polyunsaturated fatty acids in cell membranes and cause LPO. The malandialdehyde (MDA) content, a measure of lipid peroxidation was assayed in the form of TBARS.

**Reagents used**

1. 1.0 M Tris HCl buffer
2. 2.10% w/v Tris chloro acetic acid
3. 3.067% w/v Thio barbituric acid

**Procedure**

0.5 ml of PMS was taken and to it 0.5 ml of Tris hydrogen chloride buffer was added and incubated at 37ºc for 2hrs and then 1 ml of ice cold Trichloro acetic acid was added and centrifuged of 1000rpm for 10min. From the above, 1 ml of supernatant was take and added to 1 ml of thiobarbituric acid and the tubes were kept in boiling water bath for 10 min. the tubes were removed and brought to room temperature and 1 ml of distilled water was added. absorbance was measured at 532nm by using spectrophotometer.

**Calculation**

\[
\text{3} \times \text{Absorbance of sample} \quad \frac{\text{-------------}}{\text{50.156 X mg of tissue taken}} = \text{µM/ mg tissue}
\]

2. Catalase method (CAT) [12]

Catalase is a hemoprotein localized in the microperoxisomes. It reduces hydrogen peroxide produced by dismutation reaction and prevents generation of hydroxyl radicals, thereby protecting the cellular constituents from oxidative damage in peroxisome. The enzyme catalyses the decomposition of \( \text{H}_2\text{O}_2 \) to water and oxygen and thus protects the cell from oxidative damage by \( \text{H}_2\text{O}_2 \).

**Reagents used**

1. 1.50 mM phosphate buffer (pH 7.0)
2. 2.12.5 mM \( \text{H}_2\text{O}_2 \) in phosphate buffer

**Procedure**

Catalase activity was assayed by the method of claiborne et al. The assay mixture consisted of 1.95 ml phosphate buffer (0.05M, pH 7.0), 1.0 ml hydrogen peroxide (\( \text{H}_2\text{O}_2 \))(0.019), and 0.05 mL of PMS(10%)
in a final volume of 3mL, changes in absorbance were recorded at 240nm. Catalase activity was calculated in terms of k.

HISTOPATHOLOGICAL STUDIES:

Processing of isolated kidneys:

At the end of the study, the animals were sacrificed and the kidneys of each animal was isolated and was cut into small pieces, preserved and fixed in 10% formalin for two days. Then the kidneys piece was washed in running water for about 12 hours to remove the formalin and was followed by dehydration with isopropyl alcohol of increasing strength (70%, 80% and 90%) for 12 hours each. Then finally dehydration is done using absolute alcohol with about three changes for 12 hours each.

Dehydration was performed to remove all traces of water. Further alcohol was removed by using chloroform and chloroform is removed by paraffin infiltration. The clearing was done by using chloroform with two changes for 15 to 20 minutes each. After paraffin infiltration the pancreas pieces were subjected to automatic tissue processing unit.

Embedding in paraffin vacuum:

Hard paraffin was melted and the hot paraffin was poured into L-shaped blocks. The kidney pieces were then dropped into the molten paraffin quickly and allow it to cool.

Sectioning:

The blocks were cut using microtome to get sections of thickness of 5µ. The sections were taken on a micro slide on which egg albumin i.e., sticking substance was applied. The sections were allowed to remain in an oven at 60°C for 1 hour. Paraffin melts and egg albumin denatures, thereby fixing tissue to slide.

Staining:

Eosin is an acid stain, hence it stains all the cell constituents pink which are basic in nature i.e., cytoplasm. Haematoxylin, a basic stain which stains all the acidic cell components blue i.e. DNA in the nucleus.

Statistical analysis:

Results were expressed as mean ± SEM, (n=6). Statistical analyses were performed with one way analysis of variance (ANOVA) followed by Dunnet t-test using Graph pad Instat software. P < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Phytochemical screening

It was observed from the preliminary phytochemical screening of methanolic root extract of Ixora pavetta revealed the presence of Carbohydrates, alkaloids, flavonoids, triterpenes, tannins, phenols, saponins, phytosterols, proteins, aminoacids.
Serum parameters

In group treated with sodium oxalate (0.164 ml, IP, single dose) a significant increase (P<0.05) in serum biochemical parameters sodium, potassium, chloride, uric acid, creatinine, urea levels 

(136.5±5.071,72±2.67,91.3±3.86,1.945±0.17,31.1±2.79,50.3±2.9) was observed when compared to normal group 

(80.3±3.62,72.09±2.67,78.6±3.15,1.505±0.107,11.1±1.125,12.5±0.87) and urine parameters like oxalate, uric acid, creatinine 

(4.101±0.433,34.92±2.841,4.793±0.222) was observed when compared to normal group

(1.90±0.309,15.78±1.512,1.535±0.167) This indicates NaOx induces urolithiasis in rats. In group that received standard drug (Cystone, 500 mg/kg, p.o, once daily) there was significant decrease (P<0.05) in the serum sodium, potassium, chloride, uric acid, creatinine, urea and urine oxalate, uric acid, creatinine levels when compared to NaOx treated group.

On administration of MEIP (200mg/kg, 400 mg/kg, p.o, once daily) there was a significant decrease (P<0.05) in the serum sodium, potassium, chloride, uric acid, creatinine, urea and urine oxalate, uric acid, creatinine levels (338.71± 8.79) when compared to negative control group. A dose related effect was not observed in groups treated with the test drug. These results suggest that MEIP has better Anti urolithiatic activity. The effect of MEIP on serum and urine parameters levels is given in table-17, 18 and graph-

Table 17: Effect of MEIP on Serum parameters in Sodium oxalate induced urolithiasis

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Sodium (meq/l)</th>
<th>Potassium (meq/l)</th>
<th>Chloride (meq/l)</th>
<th>Creatinine (mg/dl)</th>
<th>Uricacid (mg/dl)</th>
<th>Urea (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Group</td>
<td>80.3±3.621</td>
<td>29.5±3.43</td>
<td>78.6±3.15</td>
<td>1.505±0.107</td>
<td>11.1±1.125</td>
<td>12.5±0.87</td>
</tr>
<tr>
<td>Disease Control Group</td>
<td>136.5±5.071</td>
<td>72.0±2.67</td>
<td>91.3±3.86</td>
<td>3.968±0.18</td>
<td>31.1±2.79b</td>
<td>31.1±2.7a</td>
</tr>
<tr>
<td>Standard Group</td>
<td>92.5±4.080b</td>
<td>31.8±2.26b</td>
<td>76.1±3.89b</td>
<td>1.901±0.20b</td>
<td>13.6±1.52b</td>
<td>13.1±0.9b</td>
</tr>
<tr>
<td>Drug treated group 1</td>
<td>106.6±3.765b</td>
<td>49.9±2.98b</td>
<td>83.1±2.48b</td>
<td>2.926±0.22b</td>
<td>17.3±1.85b</td>
<td>17.3±1.8b</td>
</tr>
<tr>
<td>Drug treated group 2</td>
<td>101.6±4.006b</td>
<td>41.4±2.43b</td>
<td>80.6±3.40b</td>
<td>2.105±0.23b</td>
<td>14.3±1.33b</td>
<td>15.8±1.1b</td>
</tr>
</tbody>
</table>

Note: Statistical significance test wa done by ANOVA followed by Dunnest’s’ t’-test; 
a = P<0.05 compared to normal group. 
b= P<0.05 compared to disease control group. 
Values are expressed as Means±SEM of 6 animals.
MEIP-Methanolic extracts of Ixora pavetta roots.
Graph 1: Effect of MEIP on serum Sodium parameters

Graph 2: Effect of MEIP on serum potassium parameters

Graph No-3: Effect of MEIP on serum chloride parameters

Graph No-4: Effect of MEIP on serum creatinine parameters

Graph No-5: Effect of MEIP on serum uric acid parameters

Graph No-6: Effect of MEIP on serum Urea parameters

**Note:**
a = P<0.05 compared to normal group
b = P<0.05 compared to disease control group

**Table No-18:** Effect of MEIP on urine parameters of Sodium oxalate induced urolithiasis
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Oxalate (mg/dl)</th>
<th>Uric acid (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Urine volume (ml)</th>
<th>Urine pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Group</td>
<td>1.90±0.309</td>
<td>15.782±1.512</td>
<td>1.535±0.167</td>
<td>4.10±0.355</td>
<td>7.53±0.297</td>
</tr>
<tr>
<td>Disease Control Group</td>
<td>4.101±0.433</td>
<td>34.929±2.841</td>
<td>4.793±0.222</td>
<td>1.10±0.243</td>
<td>5.17±0.406</td>
</tr>
<tr>
<td>Standard Group</td>
<td>1.702±0.314</td>
<td>16.152±1.679</td>
<td>1.920±0.180</td>
<td>3.54±0.308</td>
<td>7.98±0.213</td>
</tr>
<tr>
<td>Drug treated group 1</td>
<td>2.365±0.383</td>
<td>23.713±1.363</td>
<td>2.413±0.377</td>
<td>3.14±0.313</td>
<td>7.74±0.346</td>
</tr>
<tr>
<td>Drug treated group 2</td>
<td>1.737±0.338</td>
<td>19.450±1.363</td>
<td>2.041±0.267</td>
<td>3.41±0.267</td>
<td>7.85±0.356</td>
</tr>
</tbody>
</table>

**Note:** Statistical significance test was done by ANOVA followed by Dunn’s ‘t’-test; a = P<0.05 compared to normal group. b= P<0.05 compared to disease control group. Values are expressed as Mean±SEM of 6 animals.

MEIP-Methanolic extracts of Ixora pavetta roots.

**Graph No-7:** Effect of extract of MEIP on urine oxalate parameters

**Graph No-8:** Effect of MEIP on urine uric acid parameters

**Graph No-9:** Effect of MEIP on urine creatinine parameters

**Graph No-10:** Effect of MEIP on urine volume parameters

**Graph No-11:** Effect of extract of MEIP on urine pH

**INVIVO ANTI-OXIDENT PARAMETERS**
In the present study various anti-oxidant parameters were assessed in the kidneys of NaOx induced urolithiatic rats at the end of the study on 10th day.

**Estimation of Malondialdehyde by lipid peroxidase:**

Rats treated with only NaOx (disease control group) had MDA levels of (1.7±0.068µmoles/mg tissue) when measured on day 10. This was significantly higher (p<0.05) when compared to MDA levels in normal group (0.51±0.05µmoles /mg tissue).

Rats treated with standard drug (cystone, 500mg/kg, orally, once daily) had MDA levels of (0.39±0.03µmoles/gm tissue) when measured on day 10. This was significantly lower (p<0.05) when compared to the disease control group. The groups treated with different doses of MEIP (200mg/kg and 400mg/kg, orally, once daily) also exhibited a significant decrease (p<0.05) in the MDA levels (0.47±0.034 and 0.43±0.04µmoles/gm tissue) when compared to the disease control group respectively.

**Estimation of Catalase:** A significant decrease in the levels of catalase was observed in the disease control group (0.37±0.020µmoles/gm tissue) when compared to the normal group (0.52±0.03µmoles/gm tissue). The group III receiving standard drug (cystone, 500mg/kg) had significant (p<0.05) increase in the catalase levels (0.89±0.023µmoles/gm tissue) when compared to the disease control group. The groups treated with different doses (200mg/kg and 400 mg/kg , orally, once daily) of MEIP also exhibited a significant increase (p<0.05) in the catalase levels (0.52±0.03 and 0.62±0.03 µmoles/gm tissue) when compared to the disease control group respectively. The results are given in Table no 5 and Graph no.12 & 13.

**Table No-5: Catalase and lipid peroxidation method**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment groups</th>
<th>In vivo anti-oxidant parameters (mean± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Catalase method</td>
</tr>
<tr>
<td>1</td>
<td>Normal group</td>
<td>0.52 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>Disease control group</td>
<td>0.37 ±0.02</td>
</tr>
<tr>
<td>3</td>
<td>Standard group</td>
<td>0.89 ± 0.023</td>
</tr>
<tr>
<td>4</td>
<td>Test group 1</td>
<td>0.52 ±0.03</td>
</tr>
<tr>
<td>5</td>
<td>Test group 2</td>
<td>0.62 ±0.03</td>
</tr>
</tbody>
</table>

**Graph No- 12: Catalase method**

**Graph No-13: Lipid peroxidation method**

**Note:**
a = \text{P}<0.05 \text{ compared to normal group.}

b = \text{P}<0.05 \text{ compared to disease control group.}

**Histopathological studies**

Histopathological studies of kidneys of normal group (Group-I) animals showed normal glomerulus, collecting tubules, and particular interstitium, while disease control group (Group-II) showed tubules applied focally ecstatic and are surrounded by inflammatory infiltration, focal hyperplasia, flattened epithelium with focal vascular degeneration and single cell necrosis and bordered the tubules which focally contained hyelin casts. Irregular crystals were present inside the tubules and in the peritubular interstitium along with nephron at papillary level. Group treated with standard drug (G-3) showed normal glomeruli mild inflammation of nephrons, more prominent epithelial recovery and group treated with MEIP at 200mg/kg showed normal glomeruli, edema of tubular cells slight epithelial recovery compare to the disease control group while MEIP treated at the dose of 400mg/kg showed normal glomeruli, prominent epithelial recovery slight oedema of the tubular cells. Histopathological studies revealed treatment by MEIP reduced kidney damage caused by sodium oxalate. Results were shown in fig 3,4,5,6,7.

![Fig. 3: Normal group focally ecstatic and surrounded by inflammatory infiltration](image1)

![Fig. 4: Disease control Group Normal glomeruli, normal tubule](image2)
DISCUSSION

Previous reports showed that sodium oxalate administration results in hyperoxoluria in untreated group [13,14] since it is accepted that hyperoxoluria is a major risk factor in the pathogenesis of renal stones [15]. The observation that oxalate levels were significantly decreased by MEIP at 400mg/kg dose.

In urolithiasis, the glomerular filtration rate decreases due to the obstruction of out flow of the urine by stones in kidney. The waste products, particularly creatinine and uric acid, accumulate in the blood [16]. In NaOx alone group urine and serum creatinine were increased, which indicated that there was a marked renal damage; however, in MREP-treated group urine creatinine was significantly decreased (P<0.05) and also serum creatinine was lowered, which indicated that there was arrest of breakdown of protein due to renal damage in MREP group. Hyperoxaluria is a more significant risk factor in the pathogenesis of renal stone.
has been reported that oxalate plays an important role in stone formation and has about 15-fold greater effect than urinary calcium [17]. In earlier studies cystone was used as standard drug for composition of antiurolithiatic activity of plant Crataeva magna Lour. Bark, Mimosops elengi and Bergenia ciliate leaves [18-19]. Clinically study on cystone described the efficacy and safety in the management of urolithiasis in human[20]. In this study, cystone was used as a standard drug. There was increase in urinary oxalate after NaOx administration decreased level of oxalate

In cystone and MREP group was seen. This effect may be due to the inhibition of formation of oxalate by the MREP treatment increased excretion of uric acid has been reported in stone formers and hyperoxaluric rats. Uric acid interferes with CaOx solubility and it binds and reduces

The inhibitory activity of glycosaminoglycans [21], the urine and serum uric acid levels of NaOx group increased, which indicated that there was a renal damage; however, in MREP-treated group urine and serum uric acid were significantly lower, which confirm arrest of renal damage.

Phytochemical constituents, as triterpenes [22-24] and e-glycosyl flavonoids containing plants showed anti urolithiatic effect. The mechanism underlying this effect is still unknown, but it is apparently related to its diuretic properties and lowering of urinary concentrations of stone forming constituents, which may be attributed to the presence of triterpenoids and flavonoid.

It is thus apparent that the flavonoids, triterpenes present in MREP might have been responsible for reduction of CaOx crystal aggregation and stone formation in kidney as observed in present study. The results support the use of MREP as an effective alternative in treating NaOx-induced urolithiasis.

CONCLUSION

In conclusion, the results indicate that administration of MREP reduced and prevented the growth of urinary stones. The underlying mechanism could be due to its diuretic activity, nephron protective effect and lowering the concentration of urinary stone forming constituents. Further experimental and clinical studies are required to elucidate the chemical constituents of extract with potent antiurolithiatic activity.

REFERENCES

2. http://indiabiodiversity.org/species/show/31460/?max=8&offset=0&classification=265799&taxon=29693&view=grid