ANNEXURE
Inhibitory effect of *Hemidesmus indicus* R. Br. on calcium oxalate crystal growth *in vitro*

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**ABSTRACT**

*Hemidesmus indicus* R.Br. (Fam. Asclepiadaceae) commonly known as "Nannari" in Tamil and "Ananta" in Sanskrit is used as a substitute for "Sarsaparilla". Roots are diaphoretic, demulcent, diuretic and blood purifier. They are also used for skin diseases, syphilis, rheumatism, scorpion - sting and snake - bite. The present work deals with the inhibitory studies of the ethanolic and aqueous extracts of the root of *H. indicus* on calcium oxalate crystal growth *in vitro*. Calcium oxalate crystals were grown in silica gel media in Hane's tubes by single diffusion method. The crystal growth after the addition of the ethanolic and aqueous extracts; in 20 mg/5 ml and 10 mg/5 ml doses was studied. In both the extracts there were reductions in sizes of calcium oxalate crystals when compared with that of the control. Individual calcium oxalate crystals when viewed under microscope showed considerable reduction in size when compared with that of the control. Also the higher dose was found to be more effective when compared to the lower dose.

**Keywords**: Inhibitory effect, *Hemidesmus indicus*, calcium oxalate, *in-vitro*.

**INTRODUCTION**

Urinary stone disease (Urolithiasis) is common world wide. Most urinary stones probably begin as minute aggregates in the small collecting ducts of the kidney. They then migrate to the pelvicalyceal system, where they increase in size and may then move into the ureters and bladder. The general factors responsible for urinary stone formation are; environmental, genetic, the concentration of the relevant ionic species in the urine, the availability and chemical nature of the macromolecular components of the stone matrix and the concentration of inhibitory physiological inhibitors of crystallization (Werness et al., 1981; Schrier *et al.*, 1978; Drach *et al.*, 1990). The types of urinary stones are calcium oxalate, calcium phosphate, magnesium – ammonium – phosphate, uric acid, sodium and ammonium urates, cystine, xanthine etc. (Prien, 1949). The higher incidence of urolithiasis in men than in women is due to their higher excretion rates of oxalate, calcium, uric acid and generally lower citrate excretion as well as the anatomical differences (Rajendran *et al.*, 1989). Occupations likely to cause either under hydration or increased vitamin D biosynthesis due to prolonged exposure to sunlight will predispose to urinary tract stones (Parry and Lister, 1975). Higher dietary intakes of dairy products, oxalate, vitamin C, refined carbohydrates,
fats, purines, animal proteins and sodium chloride, together with low intakes of fibre and pyridoxine, have been suggested as factors that increase the incidence of stone formation (Clifford and Story, 1976; Robertson et al., 1981).

The root in combination with other drugs is prescribed in snake bite and scorpion sting but the root is not an antidote to either snake – venom or scorpion – venom (Kirtikar et al., 1993). An aqueous extract of H. indicus roots exhibited bacteriostatic activity in mice infected with *mycobacterium leprae* (Gupta, 1981). Hydrodistilled essential oil isolated from *H. indicus* was found to exhibit anti – bacterial activity against both gram – negative and gram – positive organisms even at the concentration of 0.2% (Rajendra Prasad et al., 1983). Two pregnane glycosides had been isolated from the dried stem (Prakash et al., 1991) and three coumarinolignoids from the root of this plant (Mandal et al., 1995). A new triterpene lactone was isolated from the hexane soluble portion of the ethanol extract of the stem of *H. indicus* (Gupta et al., 1992). Chloroform and ethanol extracts of *H. indicus* root showed antifungal activity against *Aspergillus niger* (Hiremath et al., 1997). Cell culture extract of *Hemidesmus indicus* prevented hypercholesterolemia in rats (Bopanna et al., 1997). Oral treatment with the ethanol extract of the root significantly prevented rifampicin and isoniazid – induced hepatotoxicity in rats. The activity has been attributed to a free radical scavenging activity of the coumaranolignoids present in the extract (Prabakan et al., 2000). Hexatriacontane, lupeol octacosanoate, β- amyrin acetate, lupeol acetate, α- amyrin, lupeol, β- amyrin and sitosterol (Padhy et al., 1973) were obtained from the petroleum ether extract of *H. indicus* root. 2-hydroxy-4-methoxy benzoic acid isolated from the methanolic extract of *H. indicus* root significantly neutralized the viper venom-induced lethality and hemorrhagic activity in albino rats and mouse (Alam and Gomes, 1998).

**MATERIALS AND METHODS**

The roots of *H. indicus* were collected from Thenmalai, Tirunelveli District, Tamil Nadu, India during the month of September. The botanical identity was confirmed by comparing the sample with the Herbarium specimens preserved in the Dept. of Botany, by Dr. V. Chelladurai, Govt. Siddha Medical College, Palayamkottai, Tirunelveli District, Tamil Nadu, India. A voucher specimen of the plant had been deposited at the Herbarium, Department of Chemistry, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, India [Voucher specimen number MSU 052]. The plant material was thoroughly washed with water, cut into small pieces, dried under shade and powdered.

The powdered root was successively extracted with petroleum ether (40° – 60°C), benzene, chloroform, ethanol and water and they were condensed and evaporated to dryness under vacuum. Ethanolic and aqueous extracts alone had been subjected to the inhibitory studies.
Calcium oxalate crystals were grown in-vitro in silica gel media in Hane's tubes by single diffusion method. In this method, gel was prepared by treating sodium metasilicate solution of density 1.03 g/ml with 3M acetic acid and the pH was adjusted to 6.5. One of the reactants, calcium chloride (1 M) was incorporated inside the gel. After gelation, 1 M oxalic acid was slowly added over the gel along with alcohol/water as the supernatant solution for control group and alcoholic/aqueous extracts of the test plant for other groups. The crystals appeared as cloudy precipitate and in due course, the depth of the column was found to increase. The thickness (length) of crystal column was measured in centimetre on days 1, 3, 7, 10, 15, 20, 25 and 30. The sizes of the crystals formed were noted in microns (mm) on days 1, 3, 7, 14, 21 and 28 using an optical microscope and the values thus obtained were compared with each other. The whole experiment was carried out at room temperature (27° ± 3°C). The inhibitory effect of alcoholic and aqueous extracts were studied at 20 mg/5 ml and 10 mg/5 ml doses. The experiments were repeated six times and the average value was taken. All the results were expressed as means ± SE. The test of significance was statistically analyzed using Student’s t-test (Fisher, 1950). After the crystal growth, the crystals were cleared off the gel by repeated washing with distilled water, filtered and air-dried. The purity of the crystals thus obtained was assessed by infra-red spectroscopy using FT - IR - 410 JASCO - Japan instrument (KBr pellet method). Scanning electron microscopy was used for studying the morphology of the crystals. For SEM studies a JEOL JSM 35 -C scanning electron microscope was used.

RESULTS AND DISCUSSION

The inhibitory effect of the alcoholic and aqueous extracts of the roots of *H. indicus* on in-vitro oxalate crystal growth have been studied at 20 mg/5 ml and 10 mg/5 ml doses and the results are presented in Tables 1 and 2. Concentration dependent inhibitory effect was observed in both alcoholic and aqueous extracts. In the case of alcoholic extracts both these concentrations did not produce any significant change on crystal growth upto the 3rd day. On the third day the growth was almost equal in control and drug added tubes. From the 5th day onwards significant decrease in growth rate was observed in tubes containing alcoholic extracts. Aqueous extracts did not produce significant reductions in crystal columns. The mean lengths of crystal columns in the control set up were 3.48 cm and 3.97 cm respectively on the 30th day when alcohol and distilled water were used. The mean lengths of crystal columns in the extract set up were 3.00 cm (20 mg/ 5 ml) and 3.13 cm (10 mg / 5 ml) for alcoholic extracts and 3.87 cm (20mg / 5ml) and 3.90 cm (10 mg / 5 ml) for aqueous extracts. The degree of inhibition was more with 20 mg / 5 ml concentration in both the extracts.

Drug treated calcium oxalate crystals when viewed under microscope showed considerable reduction in size when compared to that of the control. The mean size of the grown crystals on 28th day in the control set up were 180 mm and 294 mm respectively when
Table 1. Effect of alcoholic extract of *Hemidesmus indicus* root on calcium oxalate crystal growth in vitro.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>H. indicus</em> 20 mg/5 ml</th>
<th><em>H. indicus</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.783 ± 0.08</td>
<td>1.783 ± 0.01</td>
<td>1.800 ± 0.00</td>
</tr>
<tr>
<td>3</td>
<td>2.200 ± 0.05</td>
<td>2.166 ± 0.03</td>
<td>2.583 ± 0.08</td>
</tr>
<tr>
<td>7</td>
<td>2.716 ± 0.01</td>
<td>2.450 ± 0.02**</td>
<td>2.766 ± 0.08</td>
</tr>
<tr>
<td>10</td>
<td>3.016 ± 0.03</td>
<td>2.616 ± 0.01**</td>
<td>3.016 ± 0.08</td>
</tr>
<tr>
<td>15</td>
<td>3.266 ± 0.03</td>
<td>2.866 ± 0.01**</td>
<td>3.116 ± 0.08*</td>
</tr>
<tr>
<td>20</td>
<td>3.483 ± 0.04</td>
<td>3.000 ± 0.00**</td>
<td>3.133 ± 0.08*</td>
</tr>
<tr>
<td>25</td>
<td>3.533 ± 0.04</td>
<td>3.000 ± 0.00**</td>
<td>3.133 ± 0.08*</td>
</tr>
<tr>
<td>30</td>
<td>3.483 ± 0.04</td>
<td>3.000 ± 0.00**</td>
<td>3.133 ± 0.08*</td>
</tr>
</tbody>
</table>

Lengths of the crystal columns are given in centimetre; All values are mean ± SE of six experiments in each set* P < 0.05; ** P < 0.005 versus the control group.

Table 2. Effect of aqueous extract of *Hemidesmus indicus* root on calcium oxalate crystal growth.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th><em>H. indicus</em> 20 mg/5 ml</th>
<th><em>H. indicus</em> 10 mg/5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.733 ± 0.02</td>
<td>1.666 ± 0.02</td>
<td>1.700 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>2.566 ± 0.02</td>
<td>2.550 ± 0.03</td>
<td>2.566 ± 0.04</td>
</tr>
<tr>
<td>7</td>
<td>3.133 ± 0.02</td>
<td>3.150 ± 0.03</td>
<td>3.116 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>3.616 ± 0.02</td>
<td>3.583 ± 0.04</td>
<td>3.616 ± 0.04</td>
</tr>
<tr>
<td>15</td>
<td>4.016 ± 0.02</td>
<td>4.000 ± 0.03</td>
<td>4.016 ± 0.04</td>
</tr>
<tr>
<td>20</td>
<td>4.683 ± 0.04</td>
<td>4.516 ± 0.02</td>
<td>4.600 ± 0.06</td>
</tr>
<tr>
<td>25</td>
<td>3.966 ± 0.07</td>
<td>3.866 ± 0.03</td>
<td>3.916 ± 0.04</td>
</tr>
<tr>
<td>30</td>
<td>3.966 ± 0.07</td>
<td>3.866 ± 0.03</td>
<td>3.900 ± 0.05</td>
</tr>
</tbody>
</table>

Lengths of the crystal columns are given in centimetre; All values are mean ± SE of six experiments in each set* P < 0.05; ** P < 0.005 versus the control group.

alcohol and distilled water were used. The mean size of the crystals in the extracts set up were 150 mm (20mg/5ml) and 162 mm (10mg/5ml) for alcoholic extracts and 240 mm (20mg/5ml) and 240 mm (10mg/5ml) for aqueous extracts on the same day. The results are shown in Figs. 1 and 2.
The drug treated crystals are found to exhibit IR spectra (Figs. 3 - 6) similar to that of the control, indicating no possibility of any type of chemical bonding between the drug and the oxalate crystals. Hence the crystals were subjected to SEM analysis for understanding the morphological changes if any by the plant extract. Figs. 7-10 show the SEM of in vitro grown calcium oxalate crystal in silica gel media.

The crystals from control experiments contained individual prismatic crystals, twinned crystals and some aggregates. Crystals grown with alcoholic extract of *H. indicus* showed less defined edges, cracks, crevices and dots of corrosion which could mean a tendency to reduce the size by crushing the stones into small bits.

Addition of aqueous extract of *H. indicus* resulted in an increase in the number of single crystals and decreased the tendency of agglomerate formation.
Fig. 7a. Calcium oxalate crystals on adding alcohol

Fig. 7b. Calcium oxalate crystals on adding alcohol showing sharp edges

Fig. 8a. Calcium oxalate crystals on adding alcoholic extract

Fig. 8b. Calcium oxalate crystals on adding alcoholic extract showing less defined edges

Fig. 9a. Calcium oxalate crystals on adding water showing twinning

Fig. 9b. Calcium oxalate crystals on adding water showing aggregation
The agglomeration of calcium oxalate crystals plays a major role in urinary stone formation and has the potential to produce very large crystals in a short period of time with no reduction of supersaturation (Hounslow et. al., 1988).

The SEM study reveals that the drug treated oxalate crystals are smaller in size and show a tendency for chipping away of the parts as compared to that of the controls. Thus the results of in–vitro experimental models, IR and SEM together attribute an inhibitory capacity with respect to calcium oxalate crystals for \textit{H. indicus} extracts.

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