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# INVESTIGATION OF PLAUSIBLE ANTI-UROLITHIATIC POTENTIAL OF METHANOLIC EXTRACT OF ACACIA NILOTICA BARK

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**Abstract**
In the present work, the effect of methanolic extract of bark of *Acacia nilotica* on nucleation and aggregation of calcium oxalate crystals and on ethylene glycol induced lithiasis was evaluated. The yield of the methanolic extract was found to be 6.21 % (7.079 g). The findings of phytochemical analysis suggest the presence of of glycosides, phenolics and tannins, flavonoids and carbohydrates in the bark of the plant. The phenolic content in the methanolic extract of *Acacia nilotica* was found to be 26.9 ± 1.33 % w/w. The administration of methanolic extract of *Acacia nilotica* bark caused an inhibition of the slope of rate of nucleation as well as the rate of aggregation of calcium oxalate crystallization. The methanolic extract was significant in preventing the formation of crystal and was further tested in animal using ethylene glycol induced lithiasis. The methanolic extract was able to exert diuretic action in rats and also decreased the activity lactate dehydrogenase and alkaline phosphatase, the biomarkers of the biomarkers of lithiasis.

**Keywords**
Urolithiatic, *Acacia nilotica*, ethylene glycol, alkaline phosphatase, bark extract

**Introducion**

Kidney stone disease is common, worldwide, often debilitating disorder that has differing etiologies and pathophysiology [1,2]. Its treatment and morbidity are sources of considerable health care expenses. This condition has plagued humans for centuries, affecting populations of almost every region, culture, and race [3,4]. Medicinal plants are major parts of traditional systems in developing countries. Herbal medicine is defined as the branch of science in which plant used formulations are used to alleviate the diseases. It is known as botanical medicine or phytomedicine. Many infectious diseases are known to be treated with herbal remedies throughout the history of mankind. Even today plant materials continue to play a major role in primary health care as therapeutic remedies in many developing countries [5]. The plant based therapies have been shown to be effective at different stages of stone pathophysiology using various mechanism [6].

*Acaica nilotica* (Babul) is an evergreen plant of family Leguminoseae with thick trunk has been widely used as demulcent, astringent, antimicrobial, antiulcer, anticancer and wound healing

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plant. Several pharmacological actions of the plant have been experimentally explored [7-11]. The present study was therefore undertaken to investigate the role of methanolic extract of bark of *Acacia nilotica* in prevention of urolithiasis.

**Material and Methods**

The bark of *Acacia nilotica* has been collected from the surroundings of Gwalior. Calcium chloride dihydrate, sodium oxalate, sodium chloride and all other reagents and chemicals were purchased from Oxford Fine Chemicals, Mumbai. All chemicals were of analytical grade and used as obtained without any purification.

**Extraction of bark and phytochemical analysis**

The stem bark of *Acacia nilotica* was dried in shade and then powdered with a mechanical grinder. 114 g of the bark powder was equitably filled in the extractor of the soxhlet apparatus and extracted with methanol by hot consistent extraction process for around 7 h. The extracted material was filtered while hot through Whatman filter paper to eliminate any contamination. The extract was evaporated on a water bath under careful observation to obtain a thick syrupy concentrate which was then allowed to dry in air [12].

The testing for presence of alkaloids, glycosides, tannins and phenolics, flavonoids, proteins, carbohydrates and sterols was done using various previously reported procedures.

**Total Phenolic content [13]**

One gram of the extract was added to 15 ml of methanol (50% v/v) and extracted for three times by maceration for 2h, then filtered and made up the volume with methanol (50% v/v) in volumetric flask upto 100 ml. One ml aliquot of the sample was taken in a test tube and diluted with 10 ml of distilled water. Then 1.5 ml Folin Ciocalteu's reagent was added and allowed to incubate at room temperature for 5 min. Four ml of 20% (w/v) $Na_2CO_3$ was added, adjusted with distilled water up to the mark of 25 ml, agitated and left to stand for 30 min at room temperature. Absorbance of the sample was measured at 765 nm against blank, i.e., distilled water.

Standard solutions of gallic acid (10-100 ppm) were similarly treated to plot the analytical curve. The control solution contained 200 µL of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples.

The total phenolic content (%w/w) was calculated using the formula:

Total phenolic content (%w/w) = $GAE*V*D*10^{-6}*100/W$

Where GAE – Gallic acid equivalent (µg/mL), calculated from calibration curve equation and absorbance of sample; V –Total volume of sample (mL); D – Dilution Factor; W- Sample weight

***In vitro* inhibition of calcium oxalate crystallization [14, 15]**

The precipitation of calcium oxalate at 37°C and pH 5.7 has been studied by measuring the turbidity of the solution at 620 nm. A UV-Visible spectrophotometer (Labtronics, LT2201) was used to measure the turbidity that developed due to the formation of calcium oxalate.

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***Stock solution***

Solution A: 10.0 mM calcium chloride ($CaCl_2$) in 200 mM sodium chloride (NaCl) and 10 mM sodium acetate, pH 5.7

Solution B: 1.0 mM sodium oxalate ($Na_2C_2O_4$) in 200 mM sodium chloride (NaCl) and 10 mM sodium acetate, pH 5.7.

Solution A and B were filtered through 0.22 µm cellulose acetate filter and warmed to 37°C before use.

***Sample preparation***

Methanolic extract of *Acacia nilotica* bark was dissolved in 200 mM sodium chloride and concentration of 10% was prepared by suitable dilution with sodium chloride solution.

***Experimental protocol***

The study was done in the presence and absence of the extract of *Acacia nilotica* to aid the formation of calcium oxalate crystals and study the effect of extract of *Acacia nilotica* on crystallization.

***Without extract (Control)***

1.0 mL of calcium chloride dihydrate was transferred to the quartz cuvette and 1.0 mL of sodium oxalate solution was added to it to obtain concentration of 5.0 mM for calcium and 0.5 mM for oxalate, respectively. The measurement of turbidity was done every 30 seconds for a period of 10 min by measuring the absorbance at 620 nm using UV-Visible spectrophotometer. Each observation was taken in triplicate.

***With extract***

1.0 mL of calcium chloride dihydrate was transferred to the quartz cuvette and 1.0 mL of sodium oxalate solution was added to it to obtain concentration of 5.0 mM for calcium and 0.5 mM for oxalate, respectively. 1.0 mL of extract sample solution in 200 mM NaCl was added to the cuvette. The measurement of turbidity was done every 30 seconds for a period of 10 min by measuring the absorbance at 620 nm using UV-Visible spectrophotometer. Each observation was made in triplicate.

Percentage inhibition produced by the extract was calculated by the formula

$$[1 - (T_Ni/T_Nc)] \times 100 \text{ for the rate of nucleation;}$$

$$\& \; [1 - (T_Ai/T_Ac)] \times 100 \text{ for the rate of aggregation}$$

Where, i stand for slope of inhibitor (extract) and c for slope of control.

***In vivo* antiurolithiatic action**

***Experimental Animals***

Wistar rats of either sex, weighing 150-200g were maintained in the animal house. The selected animals were grouped and housed in polypropylene cages in standard environmental conditions at 23 ± 2°C with 12 h dark and light cycle. The animal had free access to food and water *ad libitum*. All animals were housed standard hygienic laboratory condition one week prior to testing.

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***Experimental Design***

Rats were divided into nine groups of three animals each (n = 5). CaOx stones were induced in rats by administering 0.75% v/v of EG and 1 % w/v of AC in drinking water for 15 days. Cystone (750 mg/kg) was used as a standard drug, and extract was administered at dose of 200 mg/kg. All groups were maintained on commercial pellet diet for 28 days. The dosing schedule and grouping of animal is presented in table 1.

**Table 1 Experiment design**

| Group | Treatment | No. of animals |
|---|---|---|
| I | Normal Control | 5 |
| II | Ethylene Glycol (0.75% v/v) for 28 days | 5 |
| III | Ethylene Glycol (0.75% v/v) for 28 days + standard drug Cystone 750mg/kg, p.o (15-28$^{th}$ day) | 5 |
| IV | Ethylene Glycol (0.75% v/v) for 28 days + Methanolic extract 200mg/kg, p.o (15-28$^{th}$ day) | 5 |
| V | Ethylene Glycol (0.75% v/v) for 28 days + Methanolic extract 400mg/kg, p.o (15-28$^{th}$ day) | 5 |

***Induction of urolithiasis***

Urolithiasis was induced by oral administration of ethylene glycol (0.75% v/v) in drinking water.

***Determination of antiurolithiaitic activity***

On 28$^{th}$ day all animals which were kept in metabolic cages are taken and urine samples were collected. Animals had free access to drinking water during the urine collection period. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4°C.

The collected urine samples were measured for urine volume, urine pH, lactate dehydrogenase (LDH) and alkaline phosphatase (ALP).

**Urine volume:** measured using the measuring cylinder.

**Urine pH:** the pH of urine was measured using pH meter.

**Lactate dehydrogenase:** The activity of Lactate dehydrogenase (LDH) was estimated by the method of Vassault [16].

***Reagents:***

1. Substrate - 3.5 g $K_2HPO_4$, 0.45 g $KH_2PO_4$, 5.35 g NaCl (pH 7.2) and 31 mg of sodium pyruvate were dissolved in 450 mL distilled water
2. NADH - 42 mg NADH was dissolved in 4.5 mL 1% $NaHCO_3$

***Procedure:***

To a cuvette, 3 mL substrate, 50 µL NADH and 200 µL sample was added. The solution was mixed rapidly and a decrease in absorbance was measured at 340 nm.

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***Calculations:***

The activity of Lactate Dehydrogenase was calculated using the following formula:

$$\text{LDH Activity (Units/min/mg protein)} = \frac{\Delta A_{340} / \text{min}}{6.22 \text{ X mg protein/ml sample}}$$

**Alkaline Phosphatase:** The activity of enzyme alkaline phosphatase (ALP) was measured by the method of Bessey et al. [17].

***Reagents:***

1. ALP Reagent (*Reagent* 1) – Contains p - nitrophenyl phosphate, $Mg^{2+}$ in Tris/Carbonate buffer (pH 10.2)

***Procedure:***

To 20 µL sample, add 1.0 mL of Reagent 1. Mix well and measure the increase in absorbance was at 405 nm with time.

***Calculations:***

$$\text{ALP Activity (IU/L)} = \frac{(\Delta A_{405} / \text{min}) \text{ X T.V. X } 10^3}{\text{S.V. X Absorbtivity X P}}$$

Where, T.V. – total reaction volume in µL, S.V. – sample volume in µL, Absorptivity – 18.8, P – Cuvette path length (1 cm)

**Results and Discussion**

**Extraction Yield and phytochemical analysis**

The extraction yield of the crude extract obtained using methanol was calculated as the percentage of dry weight of the powdered bark. The yield of the methanolic extract was found to be 6.21 % (7.079 g). The findings of phytochemical analysis suggest the presence of glycosides, phenolics and tannins, flavonoids and carbohydrates in the bark of the plant (Table 2).

**Table 2 Phytochemical screening of *Acacia nilotica* bark methanolic extract**

| Chemical Tests | Observation | Methanolic extract |
|---|---|---|
| **Alkaloids** | | |
| *Mayer's catalyst* | cream colour precipitate | - |
| *Hager's catalyst* | yellow colour precipitate | - |

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| | | |
|---|---|---|
| *Wagner's catalyst* | reddish brown precipitate | - |
| *Dragendorff's catalyst* | reddish brown precipitate | - |
| **Glycosides** | | |
| *Froth test* | Frothing is seen | - |
| *Kedde's Test* | No color | - |
| *Bontrager's Assay* | Rose pink or red color in the ammonical layer not found | + |
| *Keller-Kiliani* | No color in acetic acid layer | - |
| **Phenols/Tannins** | | |
| *Ferric chloride* | Blue green color | + |
| *Gelatin Solution* | White precipitate | + |
| *Alkaline reagent test* | Yellow to red precipitate | + |

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| | | |
|---|---|---|
| *Vanillin HCl Assay* | Purplish red colour | + |
| **Flavonoids** | | |
| *Shinoda Assay* | red color | + |
| *Alkaline reagent Assay* | Yellow colour that turns red on acidification | + |
| *Zinc HCl reductino test* | red color | + |
| **Proteins** | | |
| *Millon's Test* | white precipitate, turns red on heating | - |
| *Ninhydrin Test* | Voilet color | - |
| **Sterols/triterpenoids** | | |
| *Liberman-Burchard Test* | Brown ring at junction Upper layer turns green | - |
| *Salkowski Test* | Yellow color in lower layer | - |
| **Carbohydrates** | | |
| *Molischs Test* | Purple color | - |
| *Barfoed Test* | Red precipitate | - |

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**Total Phenolic Content**

The results of the total phenolic content of the extracts examined, using Folin-Ciocalteu method. The total phenolic content in extract, expressed as percent w/w. The phenolic content in the methanolic extract of *Acacia nilotica* was found to be 26.9 ± 1.33 % w/w.

***In vitro* inhibition of calcium oxalate crystallization**

The effect of the extracts on various phases of calcium oxalate crystallization was determined by time course measurement of turbidity in the sodium chloride solution. The absorbance according to the time in the absence or presence of extracts was represented by plotting the time against absorbance. The rate of crystallization of oxalate is presented in figure 1.

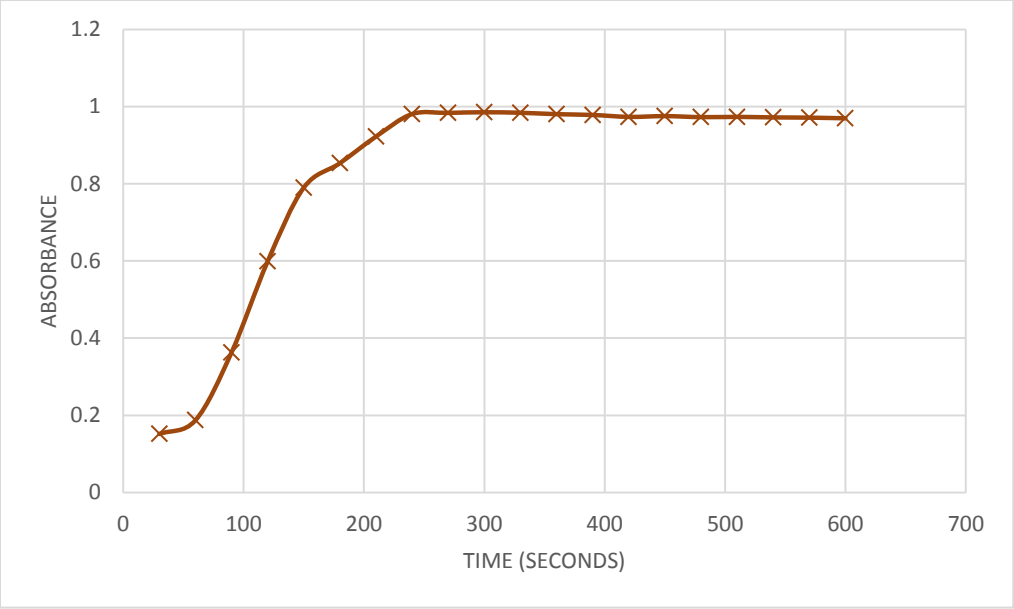


**Figure 1 Rate of formation of calcium oxalate crystals (control)**

The maximum slope of increase of absorbance with time, determined by linear regression analysis, reflects an increase in particle number as a function of time. Since absorbance is a measure of particle concentration, it may also reflect the growth in particle size. Therefore, the maximum slope of increase of absorbance with time represents crystal nucleation. Once saturation has been reached, crystals can neither nucleate nor grow; hence a rather decrease of absorbance with time is observed the slope of which reflects the rate of decrease in particle number, due to crystal aggregation [18,19].

The administration of methanolic extract of *Acacia nilotica* bark caused an inhibition of the slope of rate of nucleation as well as the rate of aggregation of calcium oxalate crystallization (Figure 2).

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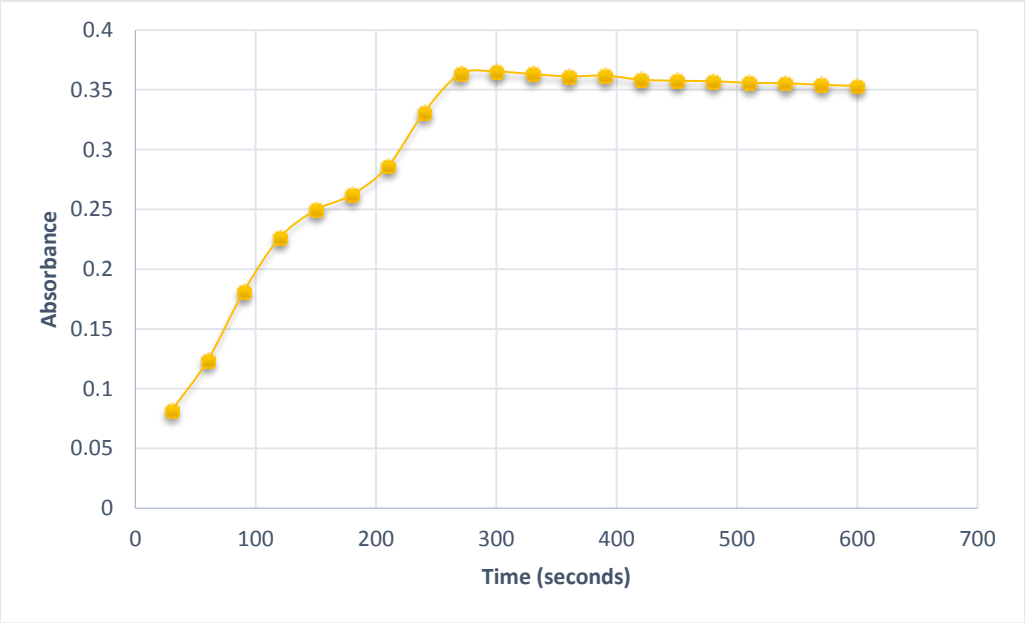


**Figure 2 Rate of formation of calcium oxalate crystals (Methanolic extract)**

The results reveal that the rate of nucleation of calcium oxalate crystals decreased on the administration of the extract as compared to that of control, suggesting an anti-urolithiatic effect.

***In vivo* antiurolithiatic action**

The methanolic extract was significant in preventing the formation of crystal. Hence it was further tested using *in vivo* studies.

Ethylene glycol is used as urolithiasis induction agent because it induces calcium oxalate crystalluria without severe renal damage in rats and it mimics the etiology of stone formation in human. Induction of Calcium oxalate type of urolithiasis by Ethylene glycol is a well-validated and clinically relevant model. Ethylene glycol is metabolized to form acidic metabolites such as oxalic acid, benzoic acid, formic acid, and hippuric acid, which causes metabolic acidosis. Subsequently, this acidic condition favours Calcium oxalate nucleation followed by growth, aggregation and crystallization, then finally retention at the renal tissue to cause renal mitochondrial toxicity similar to clinical Calcium oxalate renal calculi.

Urine volume also played a major role in the Calcium oxalate stone formation. In this study, a decrease in urine output was observed in the ethylene glycol treated group (Lithiatic) indicating an obstruction in the urinary flow due to the presence of the Calcium oxalate stones. An increase in the urinary output was observed on treatment with cystone and extract, indicating its diuretic action. Furthermore, it dilutes the urinary electrolytes concentration and therefore decreases the chance of stone development (Table 3).

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**Table 3 Effect of treatment of Urine volume and pH**

| Group | Urine volume | pH |
|---|---|---|
| I | 2.23 ± 0.043 | 7.09 ± 0.055 |
| II | 1.16 ± 0.071 | 6.57 ± 0.130 |
| III | 2.59 ± 0.055 | 7.34 ± 0.109 |
| IV | 2.33 ± 0.1 | 7.07 ± 0.045 |
| V | 2.21 ± 0.122 | 7.05 ± 0.054 |

Values are expressed as mean ± SD; n = 5

***Lactate Dehydrogenase***

Lactate dehydrogenase (LDH), a cytosolic enzyme, released on cell injury, is a biomarker for kidney injury. The activity of urinary LDH were highly elevated in lithiatic group II up to 415.2437%. Cystone (positive control), showed a significant restoration in the activity of LDH (93.72% increase was observed compared to group I animals). Moreover, with the treatment of methanolic extract resulted in a reduction of LDH activity with an increase upto 194.31% at higher extract dose (400 mg/kg) compared to the control group.

***Alkaline Phosphatase***

Renal calculi is known to cause damage to renal epithelium causing the release of enzymes like alkaline phosphatase (ALP), which is a potent biomarker. The treatment of ethylene glycol has increased the activity of ALP by about 508.98% as compared to control group. With the administration of cystone, the activity of ALP was decreased significantly exhibiting an increase of 153.33% in comparison to group I animals. The percentage increase observed in the activity of ALP in methanolic extract treated animals was 349.47% at the higher dose compared to control.

**Table 4 Effect of treatment on ALP and LDH activity**

| Group | LDH | % increase in LDH* | ALP | % increase in ALP* |
|---|---|---|---|---|
| I | 0.0215 ± 0.0003 | - | 154.627 ± 5.0751 | - |
| II | 0.1108 ± 0.0074 | 415.24 | 941.655 ± 13.5562 | 508.98 |
| III | 0.04162 ± 0.0185 | 93.72 | 391.723 ± 7.8154 | 153.33 |
| IV | 0.0760 ± 0.0098 | 253.31 | 836.074 ± 12.3423 | 440.7 |
| V | 0.0633 ± 0.0003 | 194.31 | 695.010 ± 3.5370 | 349.47 |

Values are expressed as mean ± SD; n = 5. *compared to control (group I)

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