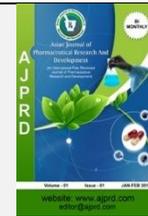


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Research Article

Assessment of antiurolithiatic potential of gallic acid on calcium oxalate crystallization and ethylene glycol induced lithiasis

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ABSTRACT

In the present work, the effect of gallic acid on nucleation and aggregation of calcium oxalate crystals and on ethylene glycol induced lithiasis was evaluated. Gallic acid is a natural phenolic compound found in several fruits and medicinal plants. It is reported to have several health-promoting effects. The presence of peaks at 3700, 1715, 1550-1650 are indicative of the presence of hydroxyl, carbonyl, and aromatic ring respectively. The results of solubility analysis indicate that gallic acid is soluble in organic solvents easily and is partially or sparingly soluble in aqueous solution. Gallic acid was able to inhibit the rate of nucleation as well as aggregation of calcium oxalate crystals in the in vitro experiment. It is exerted diuretic action in rats and was able to decrease the activity of the biomarkers of lithiasis viz. lactate dehydrogenase and alkaline phosphatase in ethylene glycol induced lithiatic rats. It could be concluded from the study that gallic acid is a potent anti urolithiatic compound and can be optimized for treatment of kidney stones.

Keywords: Gallic acid, lithiasis, calcium oxalate, ethylene glycol, lactate dehydrogenase, alkaline phosphatase

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INTRODUCTION

Urolithiasis is a multi factorial disorder that results due to the formation of urinary calculi in renal tubules. It is caused by several anatomical abnormalities such as increased electrolyte concentration (calcium and phosphorous ions) and presence or absence of endogenous inhibitors and complex formers.^[1]

Kidney stones are very common (the commonest urologic disorder) with 7-17% prevalence in the general population. Lifetime risk of stone formation is exceeding 12% in men and 6% in women.^[2] In India, 12% of the population is expected to suffer from urinary stones, of which 50% may end up with severe renal damage or kidney loss. Also, nearly 15% of the population of northern India suffers from kidney stones. Fewer occurrences of urinary calculi are found in southern India, which may be due to regular dietary intake of tamarind.^[3]

Reactive oxygen species (ROS) often cause oxidative stress which may lead to oxidative tubular damage. This damage

accelerates the nucleation and aggregation of crystals in urine, increase crystal retention and cause formation of stones.^[4] Gallic acid is a natural phenolic compound found in several fruits and medicinal plants. It is reported to have several health-promoting effects. It is potent antioxidant known to reduce the ROS and thereby oxidative stress. The objective of the present work was to investigate and establish the antiurolithiatic effect of gallic acid on experimentally induced urolithi as is in rats.

Material and Methods

Material

Gallic acid was purchased from Central Drug House, Mumbai. 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.

Methods

Physicochemical characterization of gallic acid

The color of the gallic acid sample was visually evaluated and the texture was evaluated by its touch. The infrared spectrum of gallic acid was obtained and compared to the reference spectrum for confirming the identity of the sample. The solubility of the sample was evaluated in various solvents ranging from polar to non polar, qualitatively. Melting point of the procured gallic acid was determined using open capillary method.

In vitro inhibition of calcium oxalate crystallization ^[5,6]

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.

Stock solution

Solution A: 10.0 mM calcium chloride (CaCl₂) in 200 mM sodium chloride (NaCl) and 10 mM sodium acetate, pH 5.7

Solution B: 1.0 mM sodium oxalate (Na₂C₂O₄) 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

Gallic acid was dissolved in 200 mM sodium chloride and concentrations of 10% and 50% were prepared by suitable dilution with sodium chloride solution.

Experimental protocol

The study was done in the presence and absence of gallic acid to aid the formation of calcium oxalate crystals and study the effect of gallic acid on crystallization.

Without gallic acid (Control)

1.0 mL of calcium chloride dehydrate (10.0 mM) in 200 mM NaCl and 10 mM sodium acetate, pH 5.7 was transferred to the quartz cuvette and 1.0 mL of sodium oxalate solution (1.0 mM) in 200 mM NaCl and 10 mM sodium acetate, pH 5.7 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 gallic acid

1.0 mL of calcium chloride dehydrate (10.0 mM) in 200 mM NaCl and 10 mM sodium acetate, pH 5.7 was transferred to the quartz cuvette and 1.0 mL of sodium oxalate solution (1.0 mM) in 200 mM NaCl and 10 mM sodium acetate, pH 5.7 was added to it to obtain concentration of 5.0 mM for calcium and 0.5 mM for oxalate, respectively. 1.0 mL of gallic acid 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 (gallic acid) 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.

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 gallic acid 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 + Gallic acid 200mg/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 antiurolithiatic activity

On 28th 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).

Determination of LDH activity

The activity of Lactate dehydrogenase (LDH) was estimated by the method of Vassault.^[7]

Reagents:

- Substrate - 3.5 g K₂HPO₄, 0.45 g KH₂PO₄, 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% NaHCO₃

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.

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

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

Determination of ALP activity

The activity of enzyme alkaline phosphatase (ALP) was measured by the method of Bessey et al. [8].

Reagents:

ALP Reagent (Reagent 1) – Contains p - nitrophenyl phosphate, Mg²⁺ 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. The ALP activity was determined by the following formula

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

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

RESULTS AND DISCUSSION

The purchased gallic acid was of yellow color and the texture appeared to be fine and crystalline. It exhibited partial solubility in water and was soluble in ethanol, acetone and ether. The melting point was found to be 258-262°C. The FTIR spectra revealed stretching vibrations for hydroxyl, carbonyl and aromatic carbon-carbon groups.

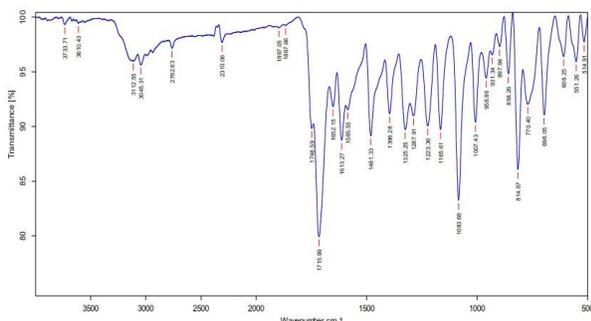


Figure 1: FTIR Spectra of gallic acid

In vitro inhibition of calcium oxalate crystallization

The effect of gallic acid 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 gallic acid was represented by plotting the time against absorbance.

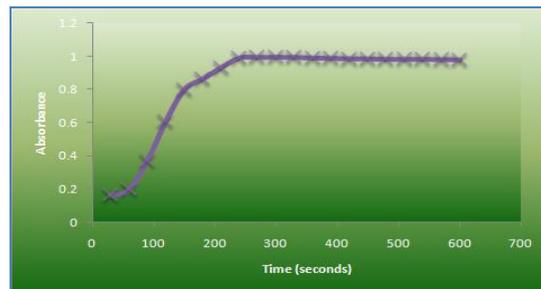


Figure 2: Rate of formation of calcium oxalate crystals (control)

The maximum slope of increase of absorbance with time was determined by linear regression analysis. It presents 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 decrease in absorbance with time is observed, the slope of which exhibits the rate of decrease in particle number, due to crystal aggregation. [9,10]

The administration of gallic acid caused an inhibition of the slope of rate of nucleation as well as the rate of aggregation of calcium oxalate crystallization at both the dose levels of 10% and 50% (figure 3 and 4).

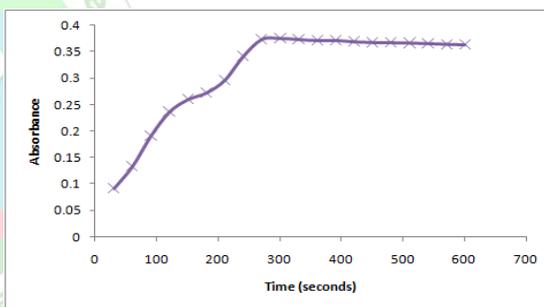


Figure 3: Rate of formation of calcium oxalate crystals (Gallic acid 50%)

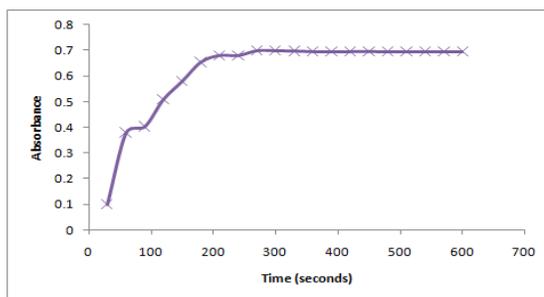


Figure 4: Rate of formation of calcium oxalate crystals (Gallic acid 10%)

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

In vivo antiurolithiatic action

The LD₅₀ of gallic acid has been reported to be more than 28g/kg [11] when administered orally, hence a therapeutic dose of 200 mg/kg was taken for the present investigation.

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. Urine volume plays 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 crystals. An increase in the urinary output was observed on treatment with cystone and gallic acid, suggesting their diuretic action. Furthermore, increased urine volume might dilute the urinary electrolytes and hence decrease the chance of stone formation (table 2).

Table 2: Effect of treatment of Urine volume and pH

Group	Urine volume	pH
I	2.07 ± 0.043	7.14 ± 0.055
II	1.2 ± 0.071	6.48 ± 0.130
III	2.66 ± 0.055	7.42 ± 0.109
	2.3 ± 0.1	7.08 ± 0.045

Values are expressed as mean ± SD; n = 5

Table 3: Effect of treatment on ALP and LDH activity

Group	LDH	% increase in LDH*	ALP	% increase in ALP*
I	0.023 ± 0.0003	-	180.12 ± 5.625	-
II	0.112 ± 0.0074	386	990.70 ± 4.457	450
III	0.042 ± 0.0199	84	445.97 ± 7.815	147
IV	0.066 ± 0.0009	190	722.13 ± 3.537	300

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

CONCLUSION

Gallic acid has demonstrated promising anti-urolithiatic potential by *in vitro* method suggesting the possible mechanism of interfering with the nucleation of calcium oxalate crystallization. It also decreased the occurrence of calcium oxalate crystals in urine of experimental animals as witnessed from increased urine volume and normalized pH of the urine. It could be concluded from the study that gallic acid is a potent anti urolithiatic compound and can be optimized for treatment of kidney stones.

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LDH activity

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 386%. Cystone (positive control), showed a significant restoration in the activity of LDH (84% increase was observed compared to group I animals). Moreover, with the treatment of gallic acid reduction of LDH activity was found with an increase in 190% compared to the control group.

ALP activity

A renal calculus 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 450% as compared to control group. With the administration of cystone, the activity of ALP was decreased significantly exhibiting an increase of 147% in comparison to group I animals. The percentage increase observed in the activity of ALP in gallic acid treated animals was 300% compared to control.