

Effects of *Trachyspermum ammi* L. (Apiaceae) on serum, urine and hepatic uric acid levels in oxonate-induced rats and *in vitro* xanthine oxidase inhibition assay

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Invention of many of the new synthetic drugs is based on the knowledge of traditional system of medicine. *Trachyspermum ammi* L. (Apiaceae) is traditionally used to treat gout and rheumatism. The study was comprised of *in vitro* determination of total polyphenolic and flavonoid content, xanthine oxidase inhibition assay and *in vivo* hypouricemic activity of *T. ammi* in oxonate induced hyperuricemic rat model. *In vivo* activity consisted of 11 groups of rats each having 6 animals. All the groups except normal control were treated with potassium oxonate. Allopurinol, *T. ammi* 250 and 500 mg/kg were administered for one, three and seven days (single dose/day) in different study groups (3×3=9 groups). *T. ammi* has total phenolic content of 145.17 ± 1.70 gallic acid equivalents/g, and the total flavonoid content of 35 ± 2.20 rutin equivalents/g. *T. ammi* significantly inhibited xanthine oxidase *in vitro* ($IC_{50} = 68.6 \pm 0.2 \mu\text{g/mL}$). Oral administration of *T. ammi* significantly reduced serum and hepatic urate levels in hyperuricemic rats in a time-dependent manner. Furthermore, fractional excretion of uric acid was increased. The results suggested that hypouricemic effects of *T. ammi* could be explained, at least partly, by inhibiting xanthine oxidase *in vitro* and due to presence of phenolic and flavonoid contents.

Keywords: Hyperuricemic model of rats, Serum and hepatic uric acid level, *Trachyspermum ammi*, Uricosuric, Xanthine oxidase

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Hyperuricemia is a metabolic disorder that appears to be increasing worldwide. It is characterized by increased Serum uric acid (sUA) levels up to 6.8 mg/dL, about saturation level of uric acid solubility at 7.4 pH and 37°C temperature¹. It is a precursor of gout, a rheumatic inflammatory disease characterized by the deposition of uric acid in the form of monosodiumurate (MSU) crystals in joints². There are various marketed drugs used for the treatment of hyperuricemia³. Currently used hypouricemic medicines have some side effects such as gastrointestinal problems, which limit their use for patients. Therefore new hypouricemic agents are required^{4,5}.

Uric acid is a final product of purine metabolism. Uric acid is catalyzed by hydroxylation of hypoxanthine and xanthine in the presence of xanthine oxidase (XOD) and xanthine dehydrogenase

(XDH). Both enzymes are the target for the treatment of hyperuricemia and gout¹. There are two major categories of hypouricemic drugs, firstly the agents that decrease the synthesis of uric acid and secondly the agents that increase the excretion of uric acid (uricosuric)⁶. The basis of use of medicinal plants in the treatment of hyperuricemia or gout is based on the knowledge of traditional system of medicine^{7,8}. However, the use of these medicinal plants lacks scientific evidences. Moreover, there has been increasing trends on identifying plants having phytochemicals that could inhibit xanthine oxidase, decrease serum uric acid or increase excretion of uric acid for controlling hyperuricemia and its related disorders⁹. Various phenolic compounds and flavonoids have significant ability of xanthine oxidase inhibition and have hypouricemic effect¹⁰. Traditionally *Trachyspermum ammi* L. (Apiaceae) is used to treat gout, rheumatism, diuresis and anti-lithiasis effects^{11,12}.

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Urate oxidase (uricase), an enzyme present in many mammalian species, plays important role in the excretion of uric acid by breakdown of uric acid into allantoin, a more excretive form of uric acid but in human, the gene has lost its function due to mutation¹³. Potassium oxonate blocks the effect of hepatic uricase and causes hyperuricemia in rodents¹⁴. The oxonate-treated rats can serve as useful animal model to evaluate drugs that can affect serum and liver UA and to evaluate therapeutic agents in disorders associated with uric acid. The aim of the current study was to evaluate hypouricemic effects of *T. ammi* serum, urine and hepatic uric acid levels in the hyperuricemic rats induced by oxonate. The effects of *T. ammi* on XOD assay and quantitative total phenolic and flavonoid contents *in vitro* were also evaluated.

Methodology

Reagents

Potassium oxonate, xanthine oxidase and xanthine were purchased from Sigma-Aldrich. Allopurinol was procured from Mega Pharmaceutical, Lahore. All the other solvents and chemicals were obtained from Merck, Germany. Ketamine and Xylazine were obtained from Prix Pharmaceutica, Lahore.

Plant extract Preparation

Trachyspermum ammi seeds were collected from Bahawalpur, Pakistan and were authenticated by the botanist and the voucher specimen (2215/L.S) was deposited in Botany department, The Islamia University of Bahawalpur (IUB), Pakistan. The seeds were dried in shade at room temperature for 15 days. The seeds were powdered after proper cleaning. The seeds were macerated in hydro-ethanol 30/70 V/V for 15 days with occasionally stirring. The process was repeated 3 times for collection of maximum contents¹⁵. The solvent was evaporated through rotary evaporator. The residue was used for the quantification of phenolic, flavonoid contents and the determination of *in vitro* and *in vivo* biological activities.

Total Phenolic and flavonoid Contents

Total phenolic content of the hydro-ethanolic extract was estimated by Folin Ciocalteu reagent by using methodology of McDonald *et al*^{16,17}. 5 μ L volume of extract and 50 μ L of 1mM sodium carbonate was added and then Folin Ciocalteu 20% was added to make the volume up to 150 μ L in 96

wells plate and after incubating at 40°C, the absorbance was checked at 765nm. The total phenolic content was expressed as mg gallic acid equivalent per g dry weight.

Total flavonoid content of the hydro-ethanolic extract was estimated by aluminium chloride colorimetric method¹⁸. 200 μ L volume of extract, 75 μ L of 5% NaNO₂, 150 μ L of 10% AlCl₃ was added and the mixture was allowed to stand for 6 min. Then 500 μ L of 1 mol/L NaOH solution and the final volume was made to 2.5 mL by adding double distilled water. The mixture was allowed to stand for 15 minutes; the absorbance was checked at 510 nm. The total flavonoid content was expressed as mg rutin equivalent per g dry weight.

In vitro XOD inhibition activity

Xanthine oxidase (XOD) inhibition assay was performed according to the method reported by Khan¹⁹.

In vivo antihyperuricemic activity

Experimental Animals

Male Wistar albino rats (170-200g) were purchased from animal house of the Faculty of Pharmacy and Alternative Medicine, IUB, Pakistan. Rats were housed in polycarbonate cages of size 47 × 34 × 18 cm³ with a maximum of 6 animals per cage. The standard conditions of humidity (56 ± 5%) and temperature (25 ± 1°C) along with 12/12 hour light dark cycle were maintained throughout the study period. Rats were acclimatized to laboratory environment for one week prior to study and fed standard diet and water *ad libitum*. Only well trained and skilled individuals were involved with the care and use of animals. The whole manuscript complied according to the ARRIVE guidelines²⁰. The experimental protocol was approved by animal ethics committee (Pharmacy Research Ethics Committee), IUB Ref No. (37-2015/PREC) and were in accordance with the guidelines of committee for the purpose of experiments on animals.

Hyperuricemia model in rats

Hyperuricemia was induced in rats by administration of potassium oxonate by following the previously described methods¹. In short, potassium oxonate was injected intraperitoneally (250 mg/kg) before 1 h to the last drug administration to enhance hepatic and sUA levels.

Drug Administration

Experiment was divided into three portions 1 day, 3 day and 7 day experiment. In each experiment animals were divided into 6 groups which contain 6 animals in each group. Sample size was calculated through "resource equation" method and was sufficient for statistical analysis²¹. Animals were kept fasting (only food) for one hour prior to administration of drug. All drugs including allopurinol at different concentrations were dissolved in distilled water. The quantity of drug suspension were given to animals depends upon the body weight. 6 groups (normal control and hyperuricemic control) received distilled water orally for 1, 3, 7 day respectively. Standard control (3 groups) had received allopurinol 10 mg/kg orally and 2×3 groups had received *T. ammi* at concentration 250 mg/kg and 500 mg/kg for 1, 3 and 7 day respectively. The dose of *T. ammi* was selected by an effective dose fixation study method with slight modification²². All drugs were administered orally once daily via gavage at 9:00 AM -10:00 AM.

Collection of samples

Urine, blood and liver tissue were collected 2h after the administration of last dose. Urine was collected as mentioned by Murugaiyah and Chan²³. After final dose of drug administration, animals were held in metabolic cages for 2h while water ad libitum was given. Urine was collected and stored in tubes at -20°C till they were assayed. After urine collection, rats were anesthetized by intraperitoneal ketamine HCl & xylazine (4:1). Blood samples were collected via cardiac puncture and allowed to clot for 1 h. Serum was obtained by centrifugation (5000 rpm for 15 min) of clotted blood and stored at -20°C till assayed. Simultaneously liver tissues were also excised and separated on ice plate carefully. Liver tissues were stored at -70°C till further process.

Determination of uric acid and Creatinine

The liver tissues were homogenized with the help of homogenizer in 10 volume of ice cold potassium phosphate buffer (7.4 pH), then centrifuged at 12,000 rpm at 4°C for 15 min. Supernatant was separated carefully, and was intended for the determination of uric acid²⁴. Serum and urine samples were subjected to biochemical assays on the day of collection. Hepatic, serum and urine urate levels were determined by diagnostic Uric Acid FS TBHBA kit. Urine and serum urate levels were determined in mg/dL, whereas hepatic urate level was determined in mg/g of wet

tissue. Creatinine of urine and serum (mg/dL) were measured by using creatinine FS assay kit.

FEua Determination

Fractional excretion of uric acid (FEua) was calculated by the following formula.

$$\text{FEua} = \frac{\text{Uric acid (Urine)} \times \text{Creatinine (Serum)}}{\text{Uric acid (Serum)} \times \text{Creatinine (Urine)}}$$

Statistical analysis

Results were analyzed by SPSS (IBM SPSS. statistics.v20_32bit_oxava.com). Data was expressed as mean $\pm$ standard error of mean (SEM). For *in vivo* activity results, Student's *t*-test for independent means was applied for calculating statistical significance of the differences between the groups. $p \leq 0.05$ (Two tailed values) was considered significant.

Results

Total polyphenol and flavonoid contents

The total phenolic content of the hydro-ethanolic *T. ammi* seeds extract was calculated as 145.17 $\pm$ 1.70 gallic acid equivalents/g and the total flavonoid content was 35 $\pm$ 2.20 rut in equivalents/g (Table 1).

Effects of different doses of *Trachyspermum ammi* in normal rats

Dose fixation studies showed non-toxic nature of *T. ammi* seeds extract from the dose range of 1 and 5 g/kg body weight in normal rats. There was no mortality or side effect in the rats treated with the different dosages of extracts. 500 mg/kg body weight was selected as the highest dose (1/10th of highest dose of 5g/kg) for antihyperuricemic activity. The animals were also treated with 250 mg/kg body weight of *T. ammi* for estimating its effects on lower doses.

Effects of *Trachyspermum ammi* on xanthine oxidase (*in vitro*)

T. ammi seeds extract showed marked inhibition of *in vitro* xanthine oxidase with IC₅₀ of 68.6 $\pm$ 0.2 μ g/mL

Table 1 — Total Phenolic and Flavonoid Contents (*In vitro*)

Samples tested	TPC	TFC
<i>T. ammi</i>	145.17 $\pm$ 1.70 gallic acid equivalents/g	35 $\pm$ 2.20 rutin equivalents/g

Data represent mean $\pm$ S.E.M of triplicate testing.

Table 2 — Xanthine oxidase inhibition assay (*In vitro*)

Samples tested	% inhibition	IC ₅₀
<i>T. ammi</i>	72 $\pm$ 0.5 (0.5 mg/mL) ^a	68.6 $\pm$ 0.2 μ g/mL
Allopurinol	92 $\pm$ 0.5 (0.5mmol) ^b	24.3 $\pm$ 1.8 μ mol

Data represent mean $\pm$ S.E.M of triplicate testing. For statistical significance, One way ANOVA followed by LSD post hoc test was used. Different superscripts showed that the values are statistically significant ($p \leq 0.05$)

that was comparable to allopurinol, the standard comparison of study (Table 2).

Time and dose dependent effects of *Trachyspermum ammi* on serum and liver uric acid levels in the hyperuricemic rats

Administration of potassium oxonate, uricase inhibitor, caused hyperuricemia in rats, as shown by drastic increase in serum and liver uric acid levels. As shown in (Table. 3), the serum uric acid levels were time-dependent after *T. ammi* treatment. One-day administration of *T. ammi* (250 and 500 mg/kg) effectively reduced serum uric acid levels in rats compared to the hyperuricemic control group, though still higher than the normal control level. 3-or 7-day pretreatment with *T. ammi* at 250 and 500 mg/kg reversed the elevated serum uric acid levels to the normal value. *T. ammi* at 500 mg/kg showed a slight more pronounced effect as compared to 250 mg/kg in all time groups. Moreover, *T. ammi* at 250 and 500 mg/kg was as effective as allopurinol 10 mg/kg, standard comparison of study, after 3-and 7-day pre-treatment.

Similar to the above results, a time and dose dependent decrease in liver uric acid levels was also observed (Table 4). One-day administration of *T. ammi* at 250 and 500 mg/kg significantly reduced liver urate levels in rats, though still above the normal control level, while 3-or 7-day treatment with *T. ammi* at 250 and 500 mg/kg normalized hepatic urate levels. Similar to serum uric acid levels, *T. ammi* at 500 mg/kg showed a slight more pronounced effect as compared to 250 mg/kg in all time groups. Allopurinol at 10 mg/kg normalized hepatic urate levels in one day study group and further significant

Table 3 — Time and dose dependent effect of *T. ammi* on serum uric acid levels in hyperuricemic rats

Treatment	Dosage (mg/kg)	Serum uric acid Level (mg/dL)		
		1 day study	3 day Study	7 day Study
Normal animal	---	2.6±0.1	2.7±0.09	2.9±0.07
Negative control (Vehicle)	---	5.33±0.1 ^{###}	6.1±0.1 ^{###}	7.5±0.7 ^{###}
<i>T. ammi</i>	250	4.1±0.4 ^{*#}	2.7±0.2 ^{**}	2.5±0.1 ^{**}
	500	3.8±0.1 ^{**###}	2.5±0.1 ^{**}	2.1±0.1 ^{**}
Allopurinol	10	2.8±0.2 ^{**}	2.54±0.1 ^{**}	2.51±0.2 ^{**}

Data represent mean ± S.E.M. of 6 animals. For statistical significance, Student's t-test was used between control and study groups.

**p* < 0.01 (compared to hyperuricemia control group)

***p* < 0.001 (compared to hyperuricemia control group)

[#]*p* < 0.05 (compared to normal control group)

^{##}*p* < 0.01 (compared to normal control group).

^{###}*p* < 0.001 (compared to normal control group)

decrease in the level was achieved in 3-and 7 day treatment groups.

Effect of *T. ammi* on FEua

T. ammi at 250 and 500 mg/kg significantly increased urinary urate excretion in 24 h resulting in a remarkable elevation of FEua in time and dose dependent manner (Table 5). The effects of *T. ammi* on FEua were greater than allopurinol.

Discussion

In the present study, it was revealed that *T. ammi* contain phenolic and flavonoid contents. Moreover, xanthine oxidase was inhibited by *T. ammi* *in vitro*. XO inhibitors are considered the first-line therapy for long-term management of hyperuricemia and gout²⁵. Phenolics and flavonoids have significant ability of

Table 4 — Time and dose dependent effects of *T. ammi* on liver uric acid levels in hyperuricemic rat

Treatment	Dosage (mg/kg)	Liver uric acid Level (mg/g wet tissue)		
		1 day study	3 day Study	7 day Study
Normal animal	---	0.23±0.009	0.23±0.01	0.24±0.009
Negative control	---	0.5±0.005 ^{###}	0.6±0.006 ^{###}	0.7±0.005 ^{###}
<i>T. ammi</i>	250	0.34±0.02 ^{###}	0.23±0.01 ^{**}	0.21±0.007 ^{**#}
	500	0.31±0.05 ^{###}	0.22±0.01 ^{**}	0.21±0.01 ^{**}
Allopurinol	10	0.23±0.01 ^{**}	0.19±0.001 ^{**}	0.16±0.01 ^{**}

Data represent mean ± S.E.M. of 6 animals. For statistical significance, Student's t-test was used between control and study groups.

**p* < 0.01 (compared to hyperuricemia control group)

***p* < 0.001 (compared to hyperuricemia control group)

[#]*p* < 0.05 (compared to normal control group)

^{##}*p* < 0.01 (compared to normal control group)

^{###}*p* < 0.001 (compared to normal control group)

Table 5 — Effects of *T. ammi* on FEua in potassium oxonate induced hyperuricemia in rats

Treatment	Dosage (mg/kg)	FEua		
		1 day study	3 day Study	7 day Study
Normal animal	---	24.7±2.2	23.6±1.8	21.3±1.5
Negative control (Vehicle)	---	7.2±0.5 ^{###}	6.2±0.4 ^{###}	7±0.8 ^{###}
<i>T. ammi</i>	250	16.2±2.1 ^{###}	25.6±2.9 ^{**}	27±2.9 ^{**#}
	500	21.9±0.8 ^{**}	30.4±2.7 ^{**}	30.3±1.5 ^{**###}
Allopurinol	10	17.9±2.2 ^{**#}	18.2±1.6 ^{**#}	19±1.3 ^{**}

Data represent mean ± S.E.M. of 6 animals. For statistical significance, Student's t-test was used between control and study groups.

**p* < 0.01 (compared to hyperuricemia control group)

***p* < 0.001 (compared to hyperuricemia control group)

[#]*p* < 0.05 (compared to normal control group)

^{##}*p* < 0.01 (compared to normal control group)

^{###}*p* < 0.001 (compared to normal control group)

xanthine oxidase inhibition and have hypouricemic effect¹⁰. *In vivo* studies showed a significant increase in serum UA levels in all the groups compared to normal control group indicating that the hyperuricemia rat model was effectively established. This finding was matched to the previous studies reporting hyperuricemia induced by potassium oxonate intra peritoneal in rats and mice^{1,14}. Impaired renal excretion or the increased production of uric acid causes hyperuricemia and subsequently gout²⁶.

Antihyperuricemic drugs reduced hyperuricemia by inhibiting xanthine oxidase and action on renal or extra-renal urate transporters. In the present study, orally administered allopurinol (10 mg/kg) significantly decreased potassium oxonate induced hyperuricemia in rats. It has action on a urate transporter and Cl/urate transporter and inhibits xanthine oxidase^{27,28}. Results are similar to the several studies presented the reduction of hyperuricemia with allopurinol at same dosage^{1,29,30,31}.

This is the first study demonstrating that *T. ammi* has uricosuric and xanthine oxidase inhibitory potential. These biochemical data may provide evidence for the clinical application of *T. ammi* in the treatment of hyperuricemia and its related disorders in traditional herbal medicine. *T. ammi* has serum and hepatic uric acid reduction and FEua elevation in oxonate induced hyperuricemic rats. However, *T. ammi* slowly elicited its hypouricemic effects than that of allopurinol. Allopurinol normalized the increased serum and liver uric acid levels in only one day. After 3 day and 7 day administration of *T. ammi*, there was almost no significant difference between *T. ammi* and allopurinol in decreasing serum and liver uric acid levels in hyperuricemic rats. Moreover, *T. ammi* has increased urinary urate excretion, resulting in a significant FEua elevation in oxonate-induced hyperuricemic rats. In many species, major quantity of extracellular urate is transported into hepatocytes. Uricase in hepatic peroxisomes has degraded uric acid into allantoin i.e., excreted by kidneys. Liver uric acid is partially transformed into allantoin and rest is exported to the blood¹. *T. ammi* might show its hypouricemic action by enhancing the ability to transform hepatic uric acid into allantoin before entering the systemic circulation via the hepatic vein. In other words, *T. ammi* might have potency similar to uricase function in rat liver. The possible uricase function of *T. ammi* must be further investigated. However, humans lack functional uricase and urate is the end product of the purine metabolism in humans.

In rodents, serum uric acid level is also regulated by kidneys. Some pharmacological studies have suggested that uric acid produced in liver is transported to proximal tubules. Hyperuricemia is produced when basolateral uptake of uric acid from peritubular plasma is defected. An electrogenic urate transporter (UAT) is present in renal proximal tubule that regulates blood uric acid levels and has some similarities to the enzyme uricase³². Many studies confirmed that oxonate blocks channel activity of UAT in mammalian³²⁻³⁴. Moreover, urate transporter 1 in rodent kidney transported urate similar to human urate transporter 1, the gene responsible for hereditary renal hypouricemia. So, it can be speculated that potassium oxonate induced hyperuricemia in rats was due to either blocking of UAT activity or inhibition of uricase activity. *T. ammi* showed the reduction of serum and liver uric acid levels and elevated the fractional excretion of uric acid. It can also be assumed that urate excretion by *T. ammi* may lead to a decrease of serum urate levels in rats. Current study presented the dual action of *T. ammi* on regulation of uric acid i.e., it decreased serum and liver uric acid as well as increased the excretion of uric acid.

T. ammi may show its hypouricemic action due to presence of significant flavonoid and phenolic contents as various phenolic compounds and flavonoids have significant ability of xanthine oxidase inhibition and have hypouricemic effect¹⁰. However, specific chemical constituents in *T. ammi* responsible for hypouricemia are required to be explored.

The current study demonstrated the serum and liver urate-lowering and uricosuric effect of *T. ammi* in hyperuricemic rats caused by potassium oxonate in a time dependent manner. These hypouricemic effects of *T. ammi* could be explained, at least partly, by inhibition of xanthine oxidase *in vitro* and due to presence of phenolic and flavonoid contents. However, further studies are required to evaluate the secretion mechanisms in regulation of serum uric acid levels by *T. ammi* and specific constituents responsible for its hypouricemic effect. It is suggested that *T. ammi* may be a potent uric acid lowering agent in the diet as it is a commonly used spice and/or have an effective hypouricemic effect in medicine.

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