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Effect of the fractions of *Erythrina stricta* leaf extract on serum urate levels and Xo/Xdh activities in oxonate-induced hyperuricaemic mice

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ABSTRACT

The present study was aimed at investigating the *in vivo* hypouricaemic activity of the various fractions of the hydromethanolic extract of the leaves of *Erythrina stricta* roxb (papilionacea) using oxonate-induced hyperuricaemic mice. The leaves of this species was used in traditional medicinal system for the treatment of gout, rheumatism, jaundice, bronchitis, fever, skin eruptions, wounds, etc. The pet-ether, chloroform, ethyl acetate and residual fractions at a dose of 200 mg/kg *b.w.* orally, were tested for their ability to reduce the serum urate level and inhibitory actions on the XO/XDH enzyme activities in the mouse liver and intestine. Potassium oxonate (280 mg/kg, *i.p.*), an uricase inhibitor was used to induce hyperuricaemia. Allopurinol (10 mg/kg, *p.o.*) was used as the positive control. The pet-ether, chloroform and ethyl acetate fractions when administered to hyperuricaemic mice produced a significant reduction in serum urate levels. In addition, these fractions elicited significant inhibitory actions on the XO/XDH enzyme activities in the mouse liver. The hypouricaemic activity may be due to the inhibition of XO/XDH enzymes. The effect of the fractions was less potent than allopurinol. The activity produced by the residual fraction was insignificant ($P>0.05$). Phytochemical screening of the leaves of *Erythrina stricta* revealed the presence of tannins, flavonoids, alkaloids and terpenoids. The presence of phytochemical constituents may be partly responsible for the beneficial effect of the fractions on hyperuricaemia and gout. These results suggest that the leaves of *Erythrina stricta* could be used as a potential source to treat gout and other inflammatory disorders.

Keywords: *Erythrina stricta*, gout, hyperuricaemia, uric acid, XO/XDH.

INTRODUCTION

Xanthine oxidoreductase (XOR) is a highly versatile enzyme that is widely distributed among species (from bacteria to man) and among the various tissues of mammals. It is a member of the group of enzymes known as molybdenum-iron-sulfur-flavin hydroxylases. XOR has two interconvertible forms, xantine oxidase (XO) and xanthine dehydrogenase (XDH). Both the enzymes catalyze the oxidation of hypoxanthine to xanthine and then to uric acid, the final reactions in the metabolism of purine bases (Fukunari, *et al.*, 2004). There is substantial evidence that overactivity of this enzyme leads to a condition, generally called as gout (Liote *et al.*, 2003).

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Gout is one of the most common metabolic disorders with a worldwide distribution and continues to be a major health problem. It affects around 13% of the male population and 5% of the female population (Arromede, *et al.*, 2002). Gout is characterized by an excessive concentration of uric acid in the blood, causing the accumulation of monosodium urate crystals in the joints and kidneys leading to acute gouty arthritis, tophi of the joints and extremities, and uric acid nephrolithiasis (Kramer and Curhan 2002). Elevated levels of uric acid not only leads to gout, but also results in the development of hypertension, cardiovascular disorders, diabetes, obesity, hyperlipidemia and cancer (Lin, *et al.*, 2000). The therapeutic approach to treat gout is to use either uricosuric agents or xanthine oxidase inhibitors (XOI). XOIs block the synthesis of uric acid from purines and they are much useful when compared to other drugs, since they possess lesser side effects. Allopurinol remains to be the dominant clinically used xanthine oxidase inhibitor, however, adverse effects limits its therapy (Burke A, *et al.*, 2006). Thus, there is a need to develop compounds with XOI activity which is devoid of the undesirable side effects of allopurinol. A potential source of such compounds can be obtained from medicinal plants (Theoduloz, *et al.*, 1991; Gonzalez, *et al.*, 1995; Kong, *et al.*, 2000). Many Indian medicinal plants have been used for the prevention and treatment of gout and related inflammatory disorders, but they lack sufficient scientific evidence (Kirthikar and Basu, 1987; Wasantwisut *et al.*, 2003).

Flavonoids and polyphenolic crude extracts have been reported to possess xanthine oxidase inhibitory activity (Iio, *et al.*, 1985; Chang, *et al.*, 1993). Preliminary phytochemical screening of the hydromethanolic extract of the leaves of *Erythrina stricta* in our laboratory revealed the presence of many phytochemical constituents such as flavonoids, saponins, tannins, phenolics and triterpenoids. Hence, in the present study, The leaves of *Erythrina stricta* belonging to the family Papilionaceae has been used in traditional Indian medicine for the treatment of rheumatism, jaundice, bronchitis, fever, skin eruption, wound, the various fractions of the hydromethanolic extract of the leaves of *Erythrina stricta* was screened for its ability to reduce serum urate levels and to inhibit XO/XDH enzyme activities in mouse liver and intestine using oxonate-induced hyperperuricaemic mice.

MATERIALS AND METHODS

Experimental animals

Swiss albino mice of either sex weighing between 25-30 g were used for the study. The animals were housed in polypropylene cages inside a well-ventilated room. The room temperature was maintained at $23 \pm 2^\circ\text{C}$ with a 12 h light / dark cycle. The animals were fed with commercial feed pellets and provided with drinking water. All animal procedures have been approved by ethical committee in accordance with animal experimentation and care (817/04/AC/CPCSEA).

Drugs and chemicals

Xanthine oxidase (microbial origin) and oxonic acid potassium salt were obtained from Sigma – Aldrich, USA.

Xanthine, allopurinol, 2-deoxy-2-ribose, thiobarbituric acid, trichloro acetic acid, butylated hydroxyl toluene, oxidized glutathione, epinephrine and 5, 5- dithiobis nitrobenzoic acid (DTNB) were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Uric acid level was determined using commercial kits obtained from Agappe Diagnostics Pvt. Ltd., Kerala, India. 2 – 2' dipyrindyl and *O*-dianisidine were obtained from Himedia Laboratories Ltd., Mumbai. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

Plant material

The plant material consists of dried powdered leaves of *Erythrina stricta* Roxb belonging to the family Papilionaceae.

Plant collection and authentication

The leaves of *Erythrina stricta* were collected from Pathananthatta district in Kerala, India during the month of April 2007. The plant was identified and authenticated by Mr. P. Saathyanarayana, Scientist, C-I/C, Botanical survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BSI/SC/5/23/07-08/Tech-456.

Preparation of the extract and fractionation

Fresh leaves of the plant were collected, dried in shade under room temperature, powdered mechanically and sieved through No.20 mesh sieve. The finely powdered leaves were kept in an airtight container until the time of use.

About 500g of the leaf powder was soaked with 2.5 l of methanol : water (7 : 3) for 12 h and then macerated at room temperature using a mechanical shaker for 4 h. The extract was filtered off and the marc was again soaked with the same volume of methanol : water for 12 hour and then further extracted for 4 hour and filtered. The filtrates were then combined, concentrated under reduced pressure and evaporated at 40°C . The hydromethanolic extract of *Erythrina stricta* was partitioned separately against petroleum ether, chloroform and ethyl acetate separately in the order of increasing polarity. Around 100 ml of the hydromethanolic extract was mixed with 200 ml of organic solvent in a separating funnel and shaken for 30 minutes. The organic layer was separated and fresh solvent was added until recovery of clear organic layer. Each fraction was then concentrated under reduced pressure to obtain the pet-ether fraction (PEF), chloroform fraction (CF) and ethyl acetate fraction (EAF) and the material left over was labelled as the residual fraction (RF) and stored in a refrigerator until the time of use. The petroleum ether (PEF), chloroform (CF), ethyl acetate (EAF) and residual fractions (RF) of *Erythrina stricta* were used for the *in vivo* and *in vitro* experiments.

Acute toxicity studies

Swiss albino mice weighing between 20-30 g maintained under standard laboratory conditions was used. Animals were divided into four groups consisting of 5 each; the animals received

a single oral dose (2000 mg/kg, body weight) of each fraction. Animals were kept overnight fasting prior to drug administration. After the administration of the fraction, food was withheld for further 3-4 hours. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention during the first 4 hours) and daily thereafter for a period of 14 days. Once daily cage side observations included changes in skin and fur, eyes and mucous membrane (nasal), and also respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and CNS (ptosis, drowsiness, gait, tremors and convulsions) changes (OECD, 2000).

Selection of dose of the extract

LD₅₀ was done as per OECD guidelines for fixing the dose for biological evaluation. The LD₅₀ of the fractions as per OECD guidelines falls under category 4 values with no signs of acute toxicity at doses of 2000 mg/kg. The biological evaluations of the fractions were carried out at a dose of 200 mg/kg body weight.

In vivo hypouricemic activity

Mice were divided into seven groups consisting of six animals each. Group I received 0.5% carboxyl methylcellulose (10 ml/kg *b.w.*, orally) and served as the solvent control. Group II received potassium oxonate (280 mg/kg *b.w.*, *i.p.*) and served as hyperuricemic control. Groups III to VI received the petroleum ether, chloroform, ethyl acetate and residual fractions of *Erythrina stricta* respectively at a dose of 200 mg/kg orally. Group VII received allopurinol (10 mg/kg, orally) and served as the positive control. The fractions and the reference drug were suspended in 0.5% carboxyl methylcellulose and administered orally for 7 days. On the 7th day potassium oxonate (280 mg/kg *b.w.*) was injected intraperitoneally one hour before the final drug administration to induce hyperuricemia (Zhu *et al.*, 2004; Wang *et al.*, 2004).

After one hour of the final drug administration, blood was collected by retro-orbital puncture under mild ether anesthesia. The blood was allowed to clot for approximately 1h at room temperature and then centrifuged at 5000 g for 10 min to obtain the serum. The serum uric acid level was determined using standard kits obtained from Agappe Diagnostics Pvt. Ltd., Kerala, India.

Determination of serum uric acid level

Procedure

About 20 µl of serum was added to a test tube containing 1 ml of reagent mixture was kept aside for 5 min at 37°C and the absorbance was taken against the reagent blank at 546 nm. Absorbance of the standard was taken by using uric acid standard (6mg/dl) as mentioned above. Concentration of uric acid was determined as follows,

$$\text{Uric acid concentration (mg/dl)} = \frac{\text{Absorbance of standard}}{\text{Absorbance of sample}} \times 6$$

Preparation of liver homogenate

On the 7th day after blood collection, animals were killed by cervical dislocation and the liver was excised, washed in cold 0.15 M KCl separately and homogenized (10% w/v) using 0.05M potassium dihydrogen phosphate buffer pH (7.5) in 0.5 mM EDTA. The homogenate was then centrifuged at 5000 g for 10 min, the lipid layer was carefully removed and the resulting supernatant was further centrifuged at 5000 g for 10 min. The supernatant was used for the assay of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) enzymes activities (Wang *et al.*, 2004; Zhu *et al.*, 2004).

Assay of XDH/XO activities in mouse liver

The reaction mixture contained 3.5 ml of 0.05 M phosphate buffer (pH 7.5), 0.5 ml of tissue homogenate and 1 ml of 1 mM potassium oxonate to avoid the oxidation of uric acid to allantoin (for the assay of XDH, 0.5 ml of 200 µM NAD⁺ was added in addition to the above mixture). After preincubation for 15 min at 37°C, the reaction was initiated by the addition of 1 ml of 250 µM xanthine (dissolved in phosphate buffer, pH 7.5). After 10 min, the reaction was stopped by the addition 0.5 ml of 0.58 M Hcl and the solution was centrifuged at 5000 g for 5 min. The absorbance of the supernatant was measured at 290 nm against the blank (50 mM potassium dihydrogen phosphate buffer pH 7.5, containing 0.5 mM EDTA). XO/XDH activities were expressed as nM uric acid formed/ min/ mg protein (Wang *et al.*, 2004; Zhu *et al.*, 2004).

Estimation of total protein

The amount of total protein present in the tissue homogenate was estimated by the method of Lowry *et al.*, 1951. To 0.1 ml of tissue homogenate, 4.0 ml of alkaline copper solution was added and allowed to stand for 10 min. To the mixture 0.4 ml of phenol reagent was added rapidly, mixed quickly and incubated at room temperature for 30 min for colour development. Reading was taken against blank prepared with distilled water at 610 nm in an UV-Visible spectrophotometer. The protein content was calculated from the standard curve prepared with bovine serum albumin (BSA) and expressed as µg/mg liver tissue.

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's test. Results are expressed as mean ±SEM from six mice in each group. P values < 0.05 were considered significant.

RESULTS

Effect of the fractions of *Erythrina stricta* on serum urate level in mice

Administration of the uricase inhibitor, potassium oxonate resulted in significant (P<0.01) hyperuricaemic in mice, as indicated by an increase in the serum uric acid levels when compared to the control group. Pre-treatment with the pet-ether, chloroform and ethyl acetate fractions of *Erythrina stricta* for

seven days significantly ($P < 0.01$) reduced the serum urate levels, when compared with the hyperuricaemic control group. The reduction in urate levels produced by chloroform fraction was more potent than that of the other fractions. Administration of residual fraction of *Erythrina stricta* did not produce any significant ($P > 0.05$) reduction in serum urate levels when compared to the oxonate-treated group. The standard drug allopurinol at a dose of 10 mg/kg elicited significant ($P < 0.01$) reduction of serum urate level compared to hyperuricaemic mice. The activity produced by the pet-ether fraction was almost similar to that of the allopurinol treated group (Table 1).

Table 1: Effect of the fractions of *Erythrina stricta* on serum urate levels in control and experimental animals.

Treatment	Dose (mg/kg)	Serum urate levels (mg/dl)
0.5% CMC	10 ml/kg	3.72 ± 0.17
Potassium oxonate	280	7.39 ± 0.04 ^a
Pet-ether fraction	200	5.23 ± 0.01 ^b
Chloroform fraction	200	4.74 ± 0.18 ^b
Ethyl acetate fraction	200	6.02 ± 0.23 ^b
Residual fraction	200	6.62 ± 0.25 ^c
Allopurinol	10	3.05 ± 0.01 ^b

All drugs were given orally and potassium oxonate injected intraperitoneally. Values are mean ± SEM; n = 6 in each group. ^a $P < 0.01$, when compared to control; ^b $P > 0.05$ and ^c $P < 0.01$ when compared to hyperuricaemic control (one way ANOVA followed by Dunnett's test).

Effect of the fractions of *Erythrina stricta* on XO/XDH activities in mouse liver

Animals treated with potassium oxonate produced a significant ($P < 0.01$) increase in XO/XDH enzyme activities in mouse liver compared to control group. Pretreatment of mice with pet-ether, chloroform and ethyl acetate fractions at a dose of 200 mg/kg *b.w.* For seven days produced significant ($P < 0.01$) inhibition towards XO (29.88%, 35.75% and 15.97%) and XDH (31.56%, 37.18% and 27.5%) respectively, when compared with the hyperuricaemic control. The inhibition of the XO/XDH activities of the chloroform fraction was found to be the highest among the fractions tested, followed by petether and ethyl acetate fractions. On the other hand, the action of the residual fraction on the inhibition of XO (13.01%) and XDH (8.43%) was non significant ($P > 0.05$) at the same dose. Allopurinol inhibited both XO (63.9%) and XDH (65.93%) activities at a dose of 10 mg/kg, exhibiting more potent activity than the fractions (Table 2).

Effect of fractions of *Erythrina stricta* on tissue protein in mice

There was a significant ($P < 0.01$) decrease in the total protein content in the liver tissues of mice treated with the uricase inhibitor, potassium oxonate when compared to the normal control. Mice pre-treated with the pet-ether and chloroform fractions of *Erythrina stricta* orally for seven days produced a significant ($P < 0.01$) increase in total protein when compared to oxonate control. Treatment with the ethyl acetate fraction significantly ($P < 0.05$) increased the total protein when compared to oxonate control. The effect produced by the residual fraction on the above parameters was insignificant ($P > 0.05$) when compared to the oxonate control. The activity produced by the standard drug

allopurinol was found to be highest among the groups tested (Table 3).

Table 2: Effect of the fractions of *Erythrina stricta* on serum urate levels and liver XO/XDH activities in control and experimental animals.

Treatment	Dose (mg/kg)	XO		% Inhibition	
		(nM Uric acid formed / min/mg protein)	XDH	XO	XDH
0.5% CMC.	10 ml/kg	1.33 ± 0.07	1.511 ± 0.04	-	-
Potassium oxonate.	280	3.38 ± 0.11 ^a	3.20 ± 0.12 ^c	-	-
Pet-ether fraction.	200	2.37 ± 0.10 ^b	2.19 ± 0.22 ^a	29.88	31.56
Chloroform fraction.	200	2.17 ± 0.04 ^b	2.02 ± 0.07 ^a	35.79	37.18
Ethyl acetate fraction.	200	2.84 ± 0.14 ^b	2.32 ± 0.34 ^a	15.97	27.5
Residual fraction.	200	2.94 ± 0.30 ^c	2.93 ± 0.08 ^b	13.01	8.43
Allopurinol.	10	1.22 ± 0.08 ^b	1.08 ± 0.03 ^a	63.9	65.93

All drugs were given orally and potassium oxonate injected intraperitoneally. Values are mean ± SEM; n = 6 in each group. ^a $P < 0.01$, when compared to control, ^b $P < 0.01$ and ^c $P > 0.05$ when compared to hyperuricaemic control (one way ANOVA followed by Dunnett's test).

Table 3: effect of *erythrina stricta* on tissue protein in control and experimental animals.

Groups	PROTEIN
Normal control (0.05% CMC)	94.98 ± 3.6
Control Pot-oxonate (280 mg/kg, <i>b.w.</i> , <i>i.p.</i>)	44.48 ± 2.3 ^a
PEF (200 mg/kg <i>b.w.</i> , <i>p.o.</i>)	56.39 ± 2.1 ^b
CF (200 mg/kg <i>b.w.</i> , <i>p.o.</i>)	61.33 ± 2.4 ^b
EAF (200 mg/kg <i>b.w.</i> , <i>p.o.</i>)	55.18 ± 2.6 ^c
RF (200 mg/kg <i>b.w.</i> , <i>p.o.</i>)	47.5 ± 3.2 ^d
Allopurinol (10mg/kg <i>b.w.</i> , <i>p.o.</i>)	87.14 ± 2.8 ^b

All drugs were given orally and potassium oxonate injected intraperitoneally. Values are mean ± SEM; n = 6 in each group. Protein mmoles/min/mg wet tissue. ^a $P < 0.01$, when compared to solvent control, ^b $P < 0.01$, ^c $P < 0.05$ and ^d $P > 0.05$ when compared to hyperuricaemic control (one way ANOVA followed by Dunnett's test).

DISCUSSION

The enzyme XO catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid, which plays a crucial role in gout (Bowman and Rand, 1980). XO is an important source of oxygen derived free radicals. The enzyme catalyzes the reduction of oxygen (during reperfusion phase), leading to the formation of superoxide anion radicals and hydrogen peroxide, as well as hydroxyl radicals (Halliwell and Gutteridge, 2001). It has been proposed as a central mechanism of oxidative injury in some situations like gout, ischaemia, renal damage, hypertension, diabetes, etc. (Mazzali, *et al.*, 2001; Berry and Hare, 2004; Nakagawa, *et al.*, 2006).

Gout is characterized by high level of uric acid in the blood, which can result from a number of genetic disorders that cause over production or impaired excretion of uric acid. An acute attack of gout occurs as a result of inflammatory reactions to crystals of monosodium urate that is deposited in the joint tissue (Rang, *et al.*, 2001). The risk of gout increases with the degree and duration of hyperuricaemia. If the serum urate level exceeds 9.0 mg/dl, the annual incidence of gout rises to around 4.9-5.7% (Emmerson, 1996). Recent findings show that the occurrence of gout is increasing worldwide, possibly due to the changes in

dietary habits like intake of high-purine foods viz., organ meats, yeast, beer and other alcoholic beverages (Lewis and Doisy). The main therapeutic approach for gout is the use of XO inhibitors such as allopurinol, which block the final step in the synthesis of uric acid from purines. However, it has been observed that allopurinol induce side effects such as skin allergy, Steven-Johnson's syndrome and kidney damage (Kumar, *et al.*, 1996; Wallach 1998). An alternative to allopurinol is the use of medicinal plants which possess phytochemical constituents. We thus began our program to look for xanthine oxidase inhibitors of phytochemical origin from the leaves of *Erythrina stricta*. Phytochemical screening of the leaves revealed the presence of flavonoids, phenolics, saponins and triterpenoids accounting for its antioxidant property. Flavonoids are a group of polyphenolic compounds which exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic, anti-ulcer activities, etc. They have also been reported to inhibit the enzyme xanthine oxidase (Costantino, *et al.*, 1992). The leaves of *Erythrina stricta* (Papilionaceae) are being frequently used in Indian traditional medicinal system for the treatment of gout and rheumatism. We have previously reported the *in vitro* xanthine oxidase inhibitory activity of the *Erythrina stricta*. The hypouricaemic activity may be due to the inhibition of the XO/XDH enzyme activities *in vivo*, and hence the present study was carried out. Serum uric acid levels were elevated to approximately two-fold in oxonate-induced hyperuricaemic mice. The groups treated with the fractions of the leaf extract (except the residual fraction) and allopurinol for three days decreased the urate level to almost normal value. Among the fractions examined, the pet-ether fraction effectively reduced the urate levels than did the other fractions after oral administration. In addition, we observed that the pet-ether fraction exhibited more potent inhibitory action on the XO/XDH enzyme activities in the mouse liver and intestine, preferentially inhibiting XDH activity over XO activity. The potency of the fractions at 200 mg/kg on the inhibition of the XO/XDH activities was in the order of pet-ether > chloroform > ethyl acetate > residual fraction. The inhibition of XO/XDH activities by the residual fraction was non significant ($P > 0.05$). Allopurinol, at a dose of 10 mg/kg, exhibited highest inhibition on the enzyme activities. Although, the potency of the pet-ether fraction on enzyme inhibition was less than allopurinol, its effect in reduction of the serum urate level was similar to that of allopurinol. These results suggest that the fractions were capable of reducing the accumulation of purine metabolites in blood following oxonate induction. Our unpublished data suggests that the pet-ether fraction has highest phenolic and flavonoid content as determined by pyrocatechol and quercetin equivalents respectively. In conclusion, the data reported in the present study indicates that the fractions of *Erythrina stricta* have significantly reduced the serum urate levels in hyperuricaemic animals. This may be due to the inhibition of XO/XDH activities and the presence of phytochemical constituents. Further investigations on the isolation and identification of active compounds in the leaves are in progress to identify the potential chemical entity for clinical use in the

prevention and treatment of gout and related inflammatory disorders.

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