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Research Article

IN VITRO XANTHINE OXIDASE INHIBITORY ACTIVITY OF LEAVES, FRUITS AND PEEL EXTRACTS OF *CITRUS AURANTIUM, CITRUS LIMETTA* AND *CITRUS LIMON*

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Abstract
Aim of the study: To evaluate the *in vitro* xanthine oxidase inhibitory activity of the extract of leaves, fruits and peel of *Citrus aurantium, Citrus limetta* and *Citrus limon*.
Materials and Methods: Xanthine oxidase inhibitory activity was assayed spectrophotometrically under aerobic conditions and the degree of enzyme inhibition was determined by measuring the increase in absorbance at 295nm associated with uric acid formation.
Results: Among the extracts tested, the *C. limetta* peel extract exhibited highest potency of xanthine oxidase inhibition (IC_{50} 40.16±0.88μg/ml). This was followed by *C. aurantium* peel (IC_{50} 51.50±2.05μg/ml), *C. limon* peel (IC_{50} 64.90±1.24μg/ml), *C. aurantium* leaf (IC_{50}73.50±1.26μg/ml), *C. limetta* leaf (IC_{50} 74.83±2.42μg/ml), *C. limon* leaf (IC_{50} 76.83±2.02μg/ml), *C. limetta* fruit (IC_{50} 95.16±0.60μg/ml) extracts compared with the IC_{50} value of standard allopurinol was 6.6μg/ml.
Conclusion: Recent findings show that the occurrence of gout is increasing worldwide, possibly due to the changes in dietary habits like intake of food rich in nucleic acids, such as meat, sea foods, etc. Xanthine oxidase inhibitors such as allopurinol is the drug of choice, however it has been observed more side effects. An alternative to allopurinol is the use of medicinal plants; we thus began our program to look for xanthine oxidase inhibitors of phytochemical origin. In conclusion, the study suggests that the leaves and peel extracts of *Citrus aurantium, Citrus limetta* and *Citrus limon* possess xanthine oxidase inhibitory activity that might be helpful in preventing or slowing the progress of gout and related disorders.
Keywords: *Citrus aurantium; Citrus limetta; Citrus limon;* Gout; xanthine oxidase

1. Introduction

Gout and hyperuricemia are the common metabolic disorders in human, associated with an elevated uric acid level in the blood, leading to the deposition of urate crystals in the joints and kidneys, leading to gouty arthritis and uric acid nephrolithiasis. Xanthine oxidase and xanthine dehydrogenase are the two interconvertible forms of same gene product known as xanthine oxidoreductase. It catalyses the oxidation of hypoxanthine to xanthine and then to uric acid, the final reactions in the metabolism of purine bases with generation of reactive oxygen species, either superoxide anion radical or hydrogen[1]. The deposition of needle shaped monosodium urate crystals in the synovial fluid of the major joints produces an extremely painful acute arthritis with repeated attacks of gout. The increased risk of hyperuricemia has been also linked with the development of hypertension, hyperlipidaemia, cancer, diabetes and obesity [2,3]. Xanthine oxidase may also be involved in pathogenesis of neutronphil mediated lung injury [4], congestive heart failure [5] and may contribute to cerebral ischemia, inflammation and neuro degenerative disorders [6].
The treatment for gout is either increasing the excretion of uric acid or reducing the uric acid production. Xanthine oxidase inhibitors are much useful, since they possess lesser side effects compared to uricosuric and anti-inflammatory agents. Allopurinol is the only clinically used Xanthine oxidase inhibitor, which also suffers from many side effects such as hypersensitivity syndrome, Stevens Johnson syndrome and renal toxicity [7,8]. Thus, there is a need to develop compounds with Xanthine oxidase inhibitory activities which are devoid of the undesirable side effects of allopurinol. A potential source of such compounds can be obtained from medicinal plants [8]. Flavonoids and polyphenolic crude extracts have been reported to possess xanthine oxidase inhibitory activity [9,10].

Many Indian medicinal plants have been used for the prevention and treatment of gout and related inflammatory disorders, but they lack sufficient scientific evidence. *Citrus aurantium, Citrus limetta and Citrus limon* belonging to the family Rutaceae, which is traditionally used by the local people and tribals in India for the treatment of gout, liver disorders, stomachic, brain troubles, etc., [11]. Hence, the objective of the present study is to determine the *in vitro* xanthine oxidase inhibitory activity of the extract of leaves, fruits and peel of *Citrus aurantium, Citrus limetta* and *Citrus limon.*

2. Materials and Methods

2.1 Plant material: The plant material consists of dried powdered leaves, peel and fresh fruit juices of *Citrus aurantium, Citrus limetta* and *Citrus limon* belonging to the family Rutaceae. The leaves, fruits and peel of the plants were collected from Madurai district, Tamilnadu, India during the month of June 2009. The plant was identified and authenticated by Mr. G.V.S. Murthy, Joint Director, C-I/C, Botanical survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BSI/SC/5/23/09-10/Tech-451.

2.2. Preparation of the extract: Fresh leaves and peel of the above plants 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 120g of the dried powder was soaked with 1200 ml of ethanol: water (6: 4) 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 ethanol: 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 40ºC. About 50g of peel powder was taken in soxhlet apparatus, and extracted with 450 ml of ethanol: water (6:4). After extraction, filtered and the filtrate was concentrated and evaporated at 40ºC. The fresh juice was collected from the fruit and dried at 40^{o}C.

Table1. Percentage of yield of extracts

Extract	Leaf	Peel	Fruit
C.Aurantium	17.5 %	22.12%	10%
C.Limetta	19.38 %	24%	11.32%
C.Limon	22.67%	20.52%	10.37

2.3. Drugs and chemicals: Xanthine oxidase, xanthine and allopurinol were purchased Himedia Labs., Pvt. Ltd., Mumbai, India. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

2.4. In vitro xanthine oxidase inhibitory (XOI) activity: The xanthine oxidase inhibitory activity was assayed spectrophotometrically under aerobic conditions using xanthine as the substrate. The extract and the standard drug allopurinol (1 mg/ml) were prepared by dissolving in dimethyl sulphoxide (not exceeding more than 5% of total volume) initially and then made up to the required volume with potassium dihydrogen phosphate buffer (pH 7.5). The assay mixture consisted of 1 ml of extract at different concentrations (5-100 µg/ml), 2.9 ml of potassium dihydrogen phosphate buffer (pH 7.5) and 0.1 ml of xanthine oxidase enzyme solution (0.1U/ml in potassium dihydrogen phosphate buffer, pH 7.5, prepared immediately before use). After preincubation at 25°C for 15 min, the reaction was initiated by the addition of 2 ml of substrate solution (150 µM xanthine in phosphate buffer, pH 7.5). The assay mixture was incubated at 25°C for 30 minutes the reaction was then stopped by the addition of 1N HCl. The absorbance was measured at 290 nm against blank. Allopurinol (5–100µg/ml), a known inhibitor of XO,was used as the positive control. One unit of XO is defined as the amount of enzyme required to produce 1mmol of uric acid/min at 25◦C [12,13]. XOI activity was expressed as the percentage inhibition of XO in the above assay system calculated as

$$\textbf{\% Inhibition} = \{(A\text{-}B) - (C\text{-}D) / (A\text{-}B)\} \times 100$$

Where *A* is the activity of the enzyme without the fraction, *B* is the control of *A* without the fraction and enzyme; *C* and *D* are the activities of the fraction with and without XO, respectively. The assay was done in triplicate and IC_{50} values were calculated from the percentage inhibition.

3. Results

***In vitro* xanthine oxidase inhibitory activity:** All the extracts elicited a dose-dependent inhibition of xanthine oxidase enzyme activity. Inhibition of xanthine oxidase resulted in a decreased production of uric acid, which was measured spectrophotometrically. Of the 9 extracts assayed, 7 extracts (leaf and peel extracts of *C.aurantium, C.limetta* and *C.limon* and the fruit extract of *C.limetta*) demonstrated XOI activity at a concentration of 100µg/ml, showing an inhibition greater than 50%. *C.limetta* peel extract was found to have the highest activity (55.33±0.84%) at a concentration of 50µg/ml. All the 7 extracts showed IC_{50} values below 100µg/ml concentration. Fruit extracts of *C.aurantium* and *C.limon* did not show prominent xanthine oxidase inhibitory activity. In general, the peel extracts were found to be more active than the leaf and fruit extracts. The IC_{50} values of *C.limetta*, *C.aurantium,* and *C.limon* peel extracts were found to be 40.16±0.88, 51.50±2.05 and 64.90±1.24 µg/ml respectively. These results were compared with that of the standard drug allopurinol, which showed 91.02±1.28 % inhibition at 100 µg/ml concentration with an IC_{50} value of 6.6±0.20µg/ml (Table 2).

Table 2. *In vitro* xanthine oxidase inhibitory activity of the extract of leaves, fruits and peel of *Citrus aurantium, Citrus limetta* and *Citrus limon*

Extract		Percentage xanthine oxidase inhibition					IC_{50} (µg/ml)
		5µg/ml	10µg/ml	25µg/ml	50µg/ml	100µg/ml	
C. aurantium	Leaf	14.77±1.25	25.33±0.19	34.66±0.51	43.44±0.67	56.66±0.69	73.50±1.26
	Peel	15.21±0.44	26.10±0.90	36.66±0.38	48.77±0.90	72.22±0.78	51.50±2.05
	Fruit	08.22±0.22	20.33±0.38	28.44±0.11	37.21±0.44	45.55±0.48	-
C. limetta	Leaf	13.44±0.29	25.88±0.22	34.55±0.22	44.22±0.58	55.44±0.98	74.83±2.42
	Peel	17.44±0.58	27.44±0.48	42.66±0.50	55.33±0.84	75.55±0.67	40.16±0.88
	Fruit	13.10±0.22	18.55±0.29	28.21±0.86	41.54±0.91	51.44±0.22	95.16±0.60
C .limon	Leaf	14.55±0.77	23.44±0.11	31.66±0.83	43.22±1.16	54.22±0.40	76.83±2.02
	Peel	14.55±0.39	26.88±0.96	35.55±0.22	44.33±0.38	65.88±0.22	64.90±1.24
	Fruit	7.66±0.50	15.33±0.39	23.18±0.72	31.88±0.94	40.77±0.61	-
Allopurinol		44.87±0.32	65.38±0.55	75.63±0.84	84.61±0.55	91.02±1.28	6.66±0.20

Values are mean ± SEM of three parallel measurements.

4. Discussion and conclusion

Recent findings show that the occurrence of gout is increasing worldwide, possibly due to the changes in dietary habits like intake of food rich in nucleic acids, such as meat, sea foods, etc. [9]. Hypouricemic agents are commonly employed for the treatment of chronic gouty arthritis, which includes xanthine oxidase inhibitors and uricosuric agents [14]. Xanthine oxidase inhibitors such as allopurinol interfere with the conversion of hypoxanthine to xanthine and then to uric acid. In general, allopurinol is the drug of choice; however it has been observed that allopurinol induces side effects such as hepatitis, nephropathy and allergic reaction.

Thus, new alternatives with an increased therapeutic activity and less side effects are desired. An alternative to allopurinol is the use of medicinal plants, which possess phytochemical constituents like flavonoids are a group of polyphenolic compounds, which have been reported to possess xanthine oxidase inhibitory activity. We thus began our program to look for Xanthine oxidase inhibitors of phytochemical origin from the extracts of the leaves, fruits and peel of *Citrus aurantium, Citrus limetta* and *Citrus limon* which are traditionally used by the local people and tribals in India to treat scurvy, acute rheumatism, gout, diarrhea, liver disorders, stomachic, brain troubles, etc.[11].

Literatures suggest that the fruit of *C. aurantium* possess antianxiety activity [15] and antiobesity activity [16]. Seed of *C.limon* possess free radical scavenging activity [17], and its peel extract possess cytotoxic [18] and antioxidant activities [19]. In conclusion, the study suggests that the leaves and peel extracts of *Citrus aurantium, Citrus limetta* and *Citrus limon* possess xanthine oxidase inhibitory activity that might be helpful in preventing or slowing the progress of gout and related disorders. Further investigations on the isolation of active compounds present in the extracts are necessary to identify a potential chemical entity for clinical use in the prevention and treatment of gout and related inflammatory disorders.

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