

EVALUATION OF XANTHINE OXIDASE INHIBITORY ACTIVITY BY FLAVONOIDS FROM
*PONGAMIA PINNATA LINN*VANISHREE BAMBRANA¹, DAYANAND CD¹, SHEELA SR^{2*}¹Department of Biochemistry, Sri Devaraj Urs Medical College, SDUAHER, Kolar, Karnataka, India. ²Department of Obstetrics and Gynecology, Sri Devaraj Urs Medical College, SDUAHER, Kolar, Karnataka, India. Email: co.ahs@sduu.ac.in

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ABSTRACT

Objective: Flavonoids from the crude seeds extract of *Pongamia pinnata* L., dried fruit powder of *Morinda citrifolia* L., bark of *Mangifera indica* L., and rhizome of *Zingiber officinale* Rosc. were screened for xanthine oxidase (XO) inhibition at different concentration. The inhibitory potential of quercetin and allopurinol were used for the determination of 50% inhibitory concentration (IC₅₀) and K_i values.

Methods: Isolation of flavonoids from the plant extracts was processed by column chromatography and tested for XO inhibitory activity in the range of 6-800 µg/ml.

Results: The results demonstrated that optimized flavonoids extract of *P. pinnata* L. exhibited promising XO inhibition. *P. pinnata* L., *M. indica* L., and *Z. officinale* Rosc. had IC₅₀ in the concentration of 8.74 mM, 1.09 mM, 5.4 mM and Ki 0.35 mM, 1.73 mM, 2.7 mM, respectively.

Conclusion: The study showed that plant species under investigation exhibited XO inhibition by optimized flavonoid extract. *P. pinnata* L. indicated promising XO inhibition compared to other plant extracts. Flavonoids can be used as a potent inhibitor of XO an alternative to allopurinol.

Keywords: Xanthine oxidase, Quercetin, Allopurinol, *Pongamia pinnata*, Oxidative stress.

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INTRODUCTION

Traditional system of medicine comprises use of variety of bioactive compounds from the plants. The expeditions in search of medicinal values of plants are continuous process for therapeutic benefit. Plants sources are rich in pharmacologically active compounds such as phenolics, tannins, flavonoids, and lignin [1]. Such kind of active constituents distributed in different parts of the plants and possess properties of antimicrobial, antimutagenic, anticarcinogenic, anti-inflammatory, vasodilatation, and enzyme inhibitory activity [2].

Pongamia pinnata L. is an angiosperm also known as Indian beach tree belongs to family Fabaceae. It is known to have anti-inflammatory, antiulcer, antidiarrheal, antibacterial, anticonvulsion, wound healing, hypoglycemic, etc. [3].

Morinda citrifolia L. also known as Indian mulberry or noni belongs to family Rubiaceae. Different parts of this plant used as antioxidant, anti-inflammatory, antihypertensive, antidiarrheal, antithrombotic, anticancerous, xanthine oxidase (XO) inhibitory and hypoglycemic, etc. [4,5].

Mangifera indica L. is well known as mango that belongs to family Anacardiaceae. The different parts of the plants used as remedial measure for infection, hypertension, insomnia, asthma, rheumatism, hemorrhage, and anemia [6,7].

Zingiber officinale Rosc. is called as ginger belongs to family Zingiberaceae. The rhizome contains zingiberene and gingerols. Precisely 6-gingerol is a constituent responsible for pungent taste. It is used as antipyretic, analgesic, hypotensive, antiplatelet aggregation, and thromboxane synthesis [8].

The aim of the study was to screen, *in vitro* efficacy of flavonoid components isolated from the seeds of *P. pinnata* L., dried fruit powder

of *M. citrifolia* L., bark of *M. indica* L., and rhizome of *Z. officinale* Rosc. However, there is a need to determine XO enzyme inhibition to evaluate the protective effect on inflammation and oxidative stress.

Increased purine catabolism produces more reactive oxygen species as hydrogen peroxide and elevated uric acid level [9]. Furthermore, increased uric acid could be due to elevated XO activity. Hence, this has become subject of active research to screen for natural ingredients for therapeutics has become the need for the study. *In vitro* screening for XO inhibitory activity using vitamin C, vitamin E, flavonoid extracted from Indian conventional plants, along with allopurinol might be useful in extrapolating reduction of oxidative stress.

METHODS

Collection of plant material

Screening of XO inhibition was carried out using Indian conventional plants known to have medicinal property. They are *P. pinnata* L. (seeds), *M. citrifolia* L. (fruit), *M. indica* L. (bark), *Z. officinale* Rosc. (rhizome) were collected and authenticated from the Horticulture College, Tamaka, Kolar.

Preparation of plant extract

The methanolic extraction of *P. pinnata* L., *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. were carried out by following procedure. The plant materials were cleaned, air dried at room temperature in dark, ground to fine powder using pestle and mortar. Until further processing, the powder was stored in sterile amber colored bottle in dark at room temperature.

5 g of powder obtained was dissolved in 50 ml absolute methanol and subjected for filtration using whatman number 1 filter paper. The filtrate was concentrated in vacuum evaporator under reduced pressure and air dried. Thus obtained powder was stored in sterile bottles at 4°C until further use [10].

Isolation and purification of flavonoids from the plant extracts by column chromatography

A glass column measuring 50×2 cm dimension developed using methanol with silica gel adsorbent on glass wool and allowed to settle by gravity flow. Column was allowed to equilibrate with suitable methanol as elution solvent. The even surface of the silica gel was protected by placing whatman number 1 filter paper disc. In this process, 1 g/ml of processed crude extract was applied for separation. All the eluted fractions were tested for flavonoid content, the active fractions were pooled and air dried under sterile conditions. The concentrated dried powder subjected for qualitative confirmation of flavonoids using dimethyl sulfoxide as a dissolving solvent and quercetin as an internal standard [11].

Qualitative detection of flavonoids

2 ml of above extract was treated with few drops of 20% sodium hydroxide which produced intense yellow color and on further addition of dilute hydrochloric acid became colorless confirming the presence of flavonoids [12].

XO inhibition assay

XO inhibition assay was done as per the method described by Bergmeyer. Test solution contained 1.9 ml of phosphate buffer, 0.5 ml of xanthine substrate, and 0.5 ml of inhibitor of interest. Contents were mixed by inversion and equilibrated to 25°C. To this equilibrated solution, added 0.1 ml of XO enzyme obtained from Sigma-Aldrich, USA. Blank contained 0.1 ml of distilled water in place of enzyme solution. Immediately mixed the contents and recorded the change in absorbance per minute for approximately 5 minutes at 290 nm in PerkinElmer Lambda 35 spectrophotometer. The maximum linear rate of absorbance change obtained per minute of test considered for calculation [13]. Concentration of inhibitors such as quercetin, allopurinol, vitamin C, vitamin E, and flavonoid extract of plants used in the range of 6-800 µg/ml were tested for XO inhibition.

5% methanol was used to solubilize vitamin E, quercetin, and flavonoids extract of plants except vitamin C and allopurinol which was dissolved in water. The distinct percentages of inhibition, 50% inhibitory concentration (IC₅₀) and Ki were calculated.

RESULTS

Table 1 illustrating XO inhibition by pure chemical allopurinol, quercetin flavonoid, vitamin C and E and flavonoid components extracted from plants such as *P. pinnata* L., *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. The percentage of inhibition was calculated. In the similar way quercetin treated as internal standard flavonoid to compare the inhibitory activity of isolated flavonoids from the plants in the study. During the inhibition study, the results obtained using inhibitory components on XO activity was in the range of 6-800 µg/ml. It was evident that all the compounds exhibited ascending kind of inhibition from the concentration ranging from 50 to 800 µg/ml.

The percentage of inhibition of XO under assay condition by vitamin C and E clearly evinced that these vitamins had 50-60% of inhibition

compared to allopurinol. The result showed that these vitamins have property of bringing minimal inhibition on enzyme activity irrespective of their concentration.

The results on percentage of inhibition of XO by isolated flavonoids of *P. pinnata* L. along with quercetin had similar inhibition property. However, *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. have less inhibitory effect on XO compared to *P. pinnata*. This observation suggested that flavonoid content of *P. pinnata* L. is almost similar to quercetin in action compared to others. Percentage of inhibition by quercetin on XO activity, when compared to allopurinol, showed approximately 40% less inhibition.

The percentage of inhibition at 800 µg/ml concentration for allopurinol was found 93.25%, quercetin 71.1%, vitamin C 50.7%, vitamin E 54%, *P. pinnata* L 69.1%, *M. citrifolia* L. 46.3%, *M. indica* L. 53.6%, *Z. officinale* Rosc. 51.6%. Results indicated other than allopurinol and quercetin, *P. pinnata* L. had maximum inhibitory activity.

The investigation on the efficacy of quercetin, vitamin E and vitamin C on comparison with optimized flavonoid extract from the above plants on XO inhibitory activity were calculated and expressed concentration that reduces half of enzyme activity (IC₅₀) and inhibitory constant (Ki). Allopurinol a well-known competitive inhibitor of XO considered as positive control for comparison that showed IC₅₀ value 0.4 mM and Ki 0.13 mM. Inhibitory effect of quercetin on XO showed IC₅₀ value 2.38 mM and Ki 0.37 mM. The vitamin C and E inhibition on XO in the concentration of IC₅₀ 10.6 mM, 1.19 mM and Ki 3.37 mM, 0.76 mM, respectively. *P. pinnata* L., *M. indica* L., and *Z. officinale* Rosc. showed XO inhibition in the concentration of IC₅₀ 8.74 mM, 1.09 mM, 5.4 mM and Ki 0.35 mM, 1.73 mM, 2.7 mM, respectively. However, *M. citrifolia* L. did not show effective XO inhibition.

DISCUSSION

Flavonoids are closely related polyphenolic compounds with flavone ring structure, ubiquitously distributed in various parts of the plants in the wide range. Flavonoids are classified into flavones, flavonols, flavonoids, chalcones, anthocyanins, tannins, and aurones. They are reported to have antioxidant, enzymes inhibition related to inflammation, cardioprotective and bactericidal property, etc. [14]. Flavonoids known to have potential inhibitory action on XO responsible for oxidative injury.

P. pinnata L., *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. subjected in the study for isolation of flavonoids compounds to test the inhibitory property on XO enzyme. *P. pinnata* L. seeds material reported to have inhibition on α-amylase and α-glucosidase activity [15]. Furthermore, inhibition of XO measured using commercially obtained pure flavonoids compounds and suggested the planar flavones (chrysin, luteolin, and flavones), flavonol (quercetin, myricetin, kaempferol, rhamnetin, tangeretin and rutin) were having strong inhibitory effect on XO activity (Nagao et al., 1999).

Information is limited on XO enzyme inhibition by *P. pinnata* L. seeds hence this study reported 74% of enzyme inhibition with reference to

Table 1: Comparison of percentage of xanthine oxidase inhibition by allopurinol, pure and isolated flavonoids and vitamins at different concentrations

Compounds	Percentage of inhibition at different concentration of inhibitor							
	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml
Allopurinol	17.1±2.7	35.37±4.9	48.3±2.7	56.7±3.8	74.2±1.2	81.2±5.1	92.2±4.7	93.3±4.3
Quercetin	3.95±1.1	10.99±1.9	27.5±4.9	45.9±4.5	52.2±4.5	61.3±0.59	65.8±3.3	71.1±4.4
Vitamin C	8.94±1.3	18.3±2.5	25.1±2.7	26.8±4.6	31.8±1.8	46.4±0.68	48.8±3.9	50.7±2.8
Vitamin E	12.2±1.7	18.3±0.84	28.6±3.9	33.9±4.6	42.8±1.3	52.3±6.5	53.5±7.1	54±2.6
<i>P. pinnata</i>	3.3±1.8	6.7±0.5	27.5±4.4	45.9±3.4	52.2±1.0	62.9±2.1	65.9±2.6	69.1±1.1
<i>M. citrifolia</i>	8.9±0.9	15.6±72.8	23.9±1.7	33.9±4.1	39.5±1.8	42.9±2.8	45.1±0.9	46.3±1.1
<i>M. indica</i>	8.9±3.9	14.6±5.7	25.1±0.8	30.8±1.3	34.1±1.8	47.4±14.6	50.4±7.2	53.6±1.4
<i>Z. officinale</i>	7.3±0.59	12.2±0.8	21.02±1	27.7±2.6	37.6±3.7	44.5±2.8	49.7±4.2	51.6±0.6

P. pinnata: *Pongamia pinnata* L., *M. citrifolia*: *Morinda citrifolia* L., *M. indica*: *Mangifera indica* L., *Z. officinale*: *Zingiber officinale* Rosc.

allopurinol and identified *P. pinnata* L. seeds flavonoids as the member of XO inhibitors from plant origin.

Palu *et al.* reported 64% of XO inhibition using a fine powder of fruit of *M. citrifolia* L. after processing [4]. Our research findings are similar with the study and able to obtain 50% of XO enzyme inhibition when compared to allopurinol. Mangiferin is a component of leaf known as xanthone C-glycoside of *M. indica* L. stands as a first report to state about inhibitory activity on XO [16]. Our study investigated to explore similar property in the bark and reports nearly 58% of inhibition with allopurinol used as standard inhibitor.

Gouticin a coded herbal formulation contains one of the ingredient is *Z. officinale* Rosc. which is reported to have potential inhibition on XO activity. The effective percentage of inhibition found to be similar with allopurinol an allopathic drug for gouty arthritis. In addition to this, gouticin also contains *Apium graveolens*, *Colchicum autumnale*, *Tribulus terrestris*, and *Withania somnifera* along with *Z. officinale* Rosc. [17]. Our study showed that nearly 55% of inhibition from the flavonoid extract obtained from the rhizome of *Z. officinale* compared with allopurinol.

In systemic meta-analysis of randomized control trials of various research findings reported that vitamin C supplementation as lowering serum uric acid level [18]. In an *in vitro* study on XO inhibition by L-ascorbic acid in trace amounts resulted decreased XO activity [19]. In our study, L-ascorbic acid reported to have 51% of XO inhibition at the concentration of 1.0 mg/ml when compared with allopurinol. The findings of our study are apparently similar by means of exhibiting 54% of inhibition at the concentration of 0.8 mg/ml [20].

In a randomized controlled animal study reported that the vitamin E inhibitory effect on XO related to gastric lesion prevention [21-23]. In cholestasis induced hepatocellular injury, supplementation of vitamin C and E as antioxidants exerted protective benefit through the mechanism of inhibition of XO and xanthine dehydrogenase reported [16]. In the same line of investigation our research findings supported this view by showing 58% of inhibition by vitamin E on pure form of XO.

Nevertheless several reports emphasized vitamin C and E inhibits XO activity. None of the report presented remarkable percentage of inhibition other than 52-55% range. In support of this WHO 2012 report described supplementation of vitamin C and E has no significance in reducing the risk of oxidative stress.

CONCLUSION

Flavonoids can also be used as natural inhibitor of XO. *P. pinnata* L. had maximum inhibitory activity. However, *M. citrifolia* L. did not show effective XO inhibition.

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