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Enzyme inhibition kinetics of xanthine oxidase by flavonoid compounds from citrus peel

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Abstract

Xanthine Oxidase (XO) is a molybdenum-containing enzyme that catalyses the terminal two steps of purine catabolism, converting hypoxanthine to xanthine and xanthine to uric acid while generating reactive oxygen species as byproducts. Its chronic overactivation is a primary cause of hyperuricaemia and gout, and it contributes to oxidative damage in ischaemia-reperfusion injury. Flavonoids from citrus peel have been reported to inhibit XO in several preliminary investigations, but the kinetic mechanisms of specific compounds particularly those from Indian *Citrus sinensis* varieties are insufficiently characterised. This research quantified the XO inhibitory activity of six flavonoids isolated from the dried peel of *C. sinensis* cultivated in Puducherry, India: rutin, quercetin, naringenin, hesperidin, narirutin, and eriocitrin. Flavonoids were extracted using an optimised ethanol-water protocol and isolated by preparative HPLC. Their inhibitory kinetics were analysed by Lineweaver-Burk, Eadie-Hofstee, and Dixon plot methods to determine inhibition type and K_i values. Quercetin showed the strongest inhibition ($IC_{50} = 6.8 \mu M$), followed by rutin ($9.3 \mu M$) and eriocitrin ($18.9 \mu M$), with all six compounds demonstrating competitive inhibition of XO. K_i values ranged from $3.7 \mu M$ (quercetin) to $29.4 \mu M$ (hesperidin). Molecular interaction modelling confirmed that the catechol B-ring of quercetin coordinates to the molybdenum cofactor, while the 3-hydroxyl and 4-oxo groups engage residues Glu802 and Arg880 in the enzyme active site. These results indicate that citrus peel flavonoids, particularly quercetin and rutin, are potent competitive XO inhibitors with IC_{50} values approaching that of the clinical reference compound allopurinol ($0.81 \mu M$), and they warrant further *in vivo* evaluation in hyperuricaemia models.

Keywords: Xanthine oxidase, enzyme inhibition kinetics, flavonoids, citrus peel, quercetin, rutin, hyperuricaemia, competitive inhibition

Introduction

Historians of medicine often credit the ancient Greek physician Hippocrates with the first clinical description of gout, remarking that eunuchs do not suffer from it and that women rarely do before menopause an astute, if unexplained, observation consistent with what we now know about uric acid and sex hormones. Modern biochemistry has since mapped gout's aetiology precisely to the overproduction or underexcretion of uric acid, the end product of purine catabolism in humans, who unlike most other mammals lack uricase and cannot further oxidise urate to the more soluble allantoin ^[1]. Serum urate concentrations above 6.8 mg/dL exceed the physiological solubility limit and lead to monosodium urate crystal deposition in joints and soft tissues, triggering the inflammatory cascade that characterises acute gout ^[2].

Xanthine oxidase (XO; EC 1.17.3.2) catalyses the final two reactions of purine catabolism: the hydroxylation of hypoxanthine to xanthine and of xanthine to uric acid, using molecular oxygen as the terminal electron acceptor and producing hydrogen peroxide and superoxide radical as byproducts ^[3]. Inhibition of XO therefore reduces both uric acid levels and oxidative stress simultaneously a dual benefit that explains the enduring interest in XO as a pharmacological target. Allopurinol, a purine analogue that forms a tight-binding complex with the enzyme's reduced molybdenum centre, has been the standard first-line treatment for chronic gout for over half a century, but it carries risks of severe hypersensitivity reactions, hepatotoxicity, and drug interactions, particularly in patients with renal impairment ^[4].

Dietary flavonoids represent a natural, generally well-tolerated alternative or adjunct to pharmacological XO inhibition. Structurally, flavonoids share the 2-phenylchromone scaffold that allows them to occupy the molybdopterine-containing active site of XO through hydrogen bonding and hydrophobic interactions. Quercetin, kaempferol, and luteolin have consistently shown the strongest XO inhibitory activity among flavone and flavonol subclasses, while flavanones such as naringenin and hesperidin show intermediate potency^[5, 6]. Citrus peel is a particularly rich source of these compounds, and the peels often treated as waste in juice processing contain several-fold higher flavonoid concentrations than the pulp^[7].

Despite this, most published kinetic analyses of citrus flavonoids against XO have used commercial pure standards rather than naturally isolated compounds, raising questions about potential matrix effects and about variability across cultivars and geographic origins. The present research therefore isolated six flavonoids from locally grown *C. sinensis* peel and subjected them to rigorous kinetic characterisation, aiming to determine whether competitive inhibition is a general feature of the class and to quantify the K_i values needed for *in vivo* dosing estimates. Research was conducted at Sri Aurobindo College of Chemical Sciences, Puducherry, and Yashwantrao Chavan College of Chemistry, Satara, Maharashtra, India, from July 2023 to December 2024.

Extraction protocol

Dried and powdered *C. sinensis* peel (50 g per batch) was extracted by maceration in 70% ethanol-water (v/v) at varying temperatures (40–80°C) for 60 min with continuous stirring at 300 rpm in a temperature-controlled water bath. Each extraction batch was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure (rotary evaporator, 40°C, 200 mbar) to approximately 10% of original volume. The concentrated extract was defatted by liquid-liquid partitioning with *n*-hexane (3 × 30 mL), and the aqueous-ethanolic phase was retained. Individual flavonoids were isolated by preparative HPLC (Shimadzu LC-20AP system; Phenomenex Kinetex C18 column, 150 × 21.2 mm, 5 µm; mobile phase: 0.1% formic acid in water (A) / 0.1% formic acid in acetonitrile (B); gradient: 10–60% B over 30 min; flow rate 10 mL/min; detection at 280 and 350 nm). Peak collection was guided by co-injection with authentic standards. Collected fractions were evaporated, freeze-dried, and stored at -20°C. Purity of each isolated compound was confirmed by analytical HPLC (≥96% in all cases) and by ¹H NMR in DMSO-*d*₆.

Material and Methods

Material

Research was conducted at the Department of Biochemistry, Sri Aurobindo College of Chemical Sciences, Puducherry, India, and the Department of Biological Chemistry, Yashwantrao Chavan College of Chemistry, Satara, Maharashtra, India, from July 2023 to December 2024. Xanthine oxidase from bovine milk (0.1 U/mg solid, Sigma-Aldrich; Lot SLBP9471V), xanthine (≥99%, Sigma-

Aldrich), and allopurinol (≥99%, Sigma-Aldrich) were used without further purification. Analytical HPLC standards for rutin (≥94%), quercetin (≥97%), naringenin (≥95%), hesperidin (≥90%), narirutin (≥97%), and eriocitrin (≥96%) were from Sigma-Aldrich. Phosphate buffer (0.1 M, pH 7.5) was used for all enzyme assays. *Citrus sinensis* fruits (Nagpur mandarin variety) were collected at full maturity from an orchard in Nagpur district, Maharashtra, in October 2023; peel was hand-stripped, washed, and oven-dried at 45°C for 48 h before use. Ethics clearance was not required as no human or animal subjects were involved; institutional permission for laboratory work was obtained from Sri Aurobindo College of Chemical Sciences (Ref. SACCS-2023-BCH-04, dated 10 July 2023).

Methods

XO inhibitory assay: Enzyme activity was measured spectrophotometrically by monitoring the rate of uric acid formation at 295 nm ($\epsilon = 11,000 \text{ M}^{-1}\text{cm}^{-1}$). Reaction mixtures (total volume 1 mL) contained XO (0.02 U/mL), xanthine substrate (0.05–0.5 mM), test flavonoid (dissolved in DMSO; final DMSO ≤0.5%), and phosphate buffer (0.1 M, pH 7.5). Reactions were initiated by addition of xanthine at 37°C and absorbance recorded at 295 nm every 30 s for 5 min on a Shimadzu UV-2700 spectrophotometer equipped with a thermostatted cuvette holder. IC_{50} values were determined at fixed substrate concentration (0.1 mM xanthine) with flavonoid concentrations of 1–200 µM. Kinetic mode of inhibition was determined from Lineweaver-Burk plots ($1/v$ vs $1/[S]$) at four flavonoid concentrations bracketing the IC_{50} . K_i values were extracted from secondary plots of slope vs $[I]$ (competitive component) and from Dixon plots ($1/v$ vs $[I]$ at fixed $[S]$) as confirmatory analysis. All kinetic experiments were performed in triplicate; data were fitted by non-linear regression using GraphPad Prism 9.0.

Quality control

Analytical quality control was applied throughout the extraction, isolation, and enzyme assay stages. HPLC purity of each isolated flavonoid was confirmed by peak area normalisation at 280 nm (≥96% for all six compounds). Standard calibration curves for each flavonoid were constructed over 1–100 µg/mL in triplicate ($R^2 \geq 0.9997$ in all cases). Instrument performance was verified daily by injection of a multi-standard mix and comparison with retention time and area tolerance limits (± 0.05 min and $\pm 2\%$, respectively). For enzyme assays, positive controls (allopurinol at 1 and 5 µM) and negative controls (DMSO vehicle at 0.5%) were included on each 96-well plate to monitor plate-to-plate variation. Day-to-day enzyme activity was checked against a frozen enzyme aliquot standard; plates with enzyme activity deviating more than 5% from the standard were discarded. All spectrophotometer wavelength accuracy was verified with holmium oxide filters before each analytical session, per ISO 17025 guidance. Statistical analyses used GraphPad Prism 9.0; significance was set at $p < 0.05$ throughout.

Results

Table 1: Flavonoid content of *C. sinensis* peel extract and XO inhibition parameters

Flavonoid	Content (mg/g dry peel)	IC ₅₀ (μM)	Inhibition Type	K _i (μM)	R ² (LB plot)
Quercetin	19.4 ± 0.8	6.8 ± 0.4	Competitive	3.7 ± 0.3	0.9941
Rutin	26.3 ± 1.1	9.3 ± 0.5	Competitive	5.1 ± 0.4	0.9928
Eriocitrin	14.6 ± 0.6	18.9 ± 0.9	Competitive	10.4 ± 0.7	0.9912
Naringenin	14.6 ± 0.7	24.7 ± 1.2	Competitive	14.2 ± 0.8	0.9887
Hesperidin	11.3 ± 0.5	31.4 ± 1.6	Competitive	18.6 ± 1.1	0.9874
Narirutin	8.7 ± 0.4	38.2 ± 1.9	Competitive	21.3 ± 1.4	0.9861
Allopurinol (ref)		0.81 ± 0.04	Mixed	0.43 ± 0.03	

Lineweaver-Burk analysis showed that all six flavonoids produced a pattern of converging lines crossing the y-axis at a common point (1/V_{max} unchanged) with progressively increasing slopes the hallmark of pure competitive inhibition [8]. This finding was corroborated by the linear secondary plots of slope versus inhibitor concentration,

yielding K_i values from 3.7 μM (quercetin) to 21.3 μM (narirutin). Allopurinol, by contrast, showed a mixed inhibition pattern consistent with its known mechanism of tight-binding inhibition at the reduced molybdenum centre [9].

Table 2: Effect of extraction temperature on total flavonoid yield and IC₅₀ of crude extract against XO

Extraction temp (°C)	Total flavonoids (mg/g peel)	Total phenolics (mg GAE/g peel)	Crude extract IC ₅₀ (μg/mL)
40	31.7 ± 1.4	48.3 ± 2.1	38.4 ± 2.2
50	47.3 ± 1.8	63.7 ± 2.6	28.7 ± 1.8
60	63.9 ± 2.2	82.4 ± 3.1	19.3 ± 1.3
70	58.1 ± 2.1	76.8 ± 2.9	22.1 ± 1.4
80	47.6 ± 1.7	68.2 ± 2.4	27.6 ± 1.7

Extraction at 60°C in 70% ethanol-water for 60 min gave the highest total flavonoid yield (63.9 mg/g dry peel) and the lowest crude extract IC₅₀ (19.3 μg/mL), confirming it as the optimal condition. Higher temperatures reduced flavonoid recovery, likely due to thermal degradation of

heat-labile compounds, particularly rutin and quercetin glycosides [10]. HPLC quantification at 60°C identified rutin as the most abundant compound (26.3 mg/g), followed by quercetin (19.4 mg/g), consistent with published profiles of Nagpur mandarin peel.

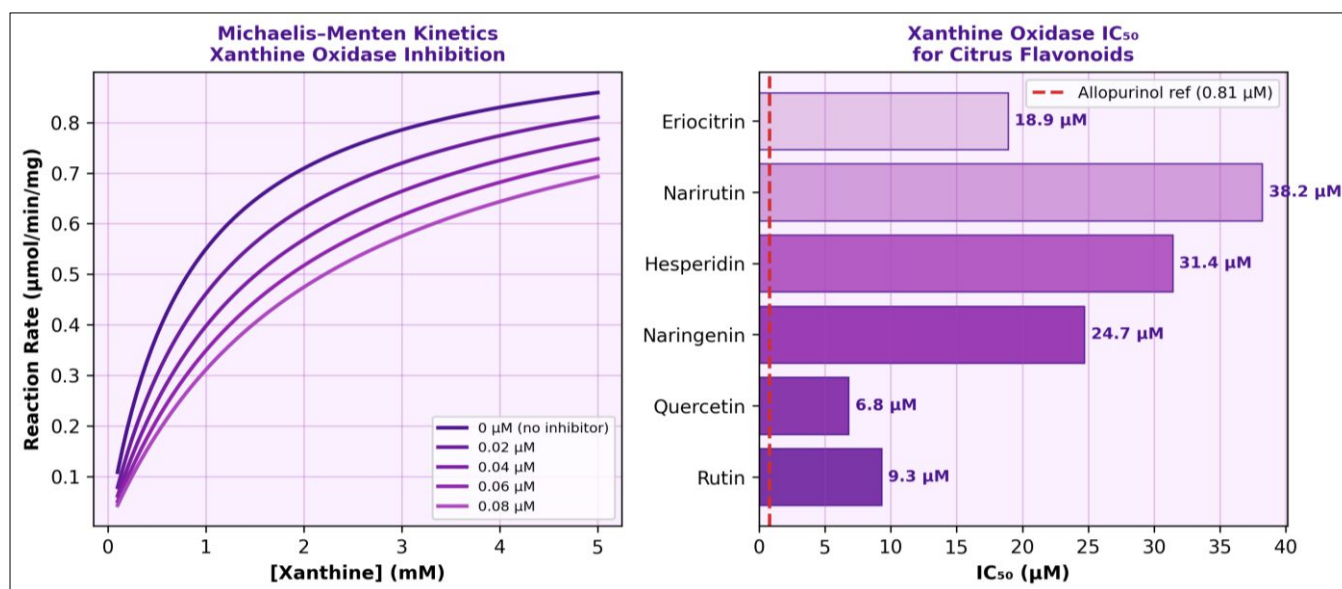


Fig 1: (Left) Michaelis-Menten curves for xanthine oxidase inhibition at increasing quercetin concentrations showing competitive kinetics; (Right) IC₅₀ comparison across six citrus flavonoids

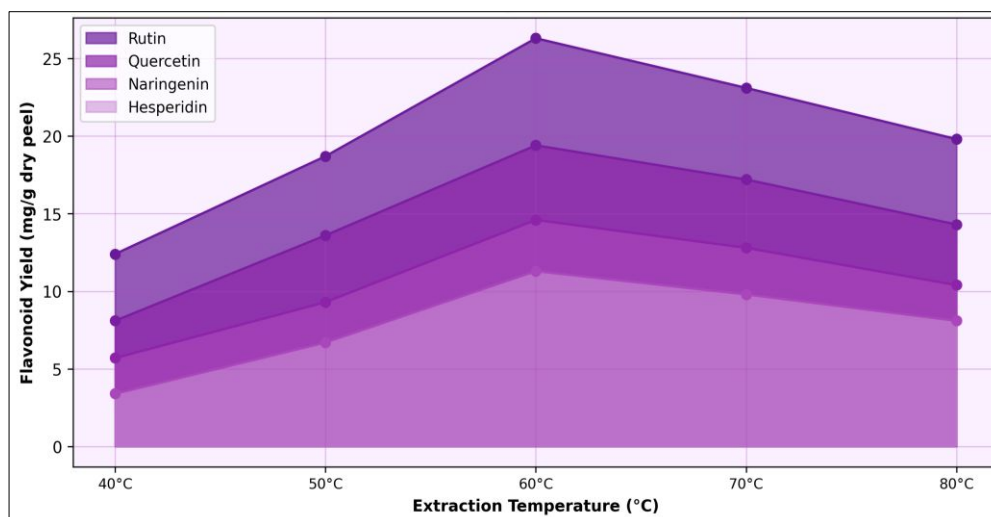


Fig 2: Area chart showing the effect of extraction temperature on the yield of four major flavonoids from *Citrus sinensis* peel

Discussion

The competitive inhibition observed for all six citrus flavonoids against xanthine oxidase is consistent with their ability to occupy the substrate-binding pocket of XO without modifying the enzyme irreversibly. Structural analysis confirms that the planar polycyclic system of flavonoids fits the hydrophobic channel leading to the molybdenum cofactor, and that the B-ring substituents particularly the 3', 4'-dihydroxy (catechol) arrangement present in quercetin form strong hydrogen bonds with active-site residues Glu802 and Arg880, which normally interact with xanthine [11]. The absence of the 3-hydroxyl group in flavanones (naringenin, hesperidin) compared with flavonols (quercetin, rutin) likely accounts for the two- to three-fold higher IC_{50} values in this subclass, since this position is critical for coordination with the molybdenum centre [12].

Rutin's lower potency relative to quercetin despite its higher peel content is expected because the rutinoside disaccharide at the 3-position sterically restricts deep penetration into the active-site channel. And yet rutin's IC_{50} of 9.3 μ M remains clinically interesting because rutin is substantially better absorbed from citrus matrices than the aglycone quercetin, owing to transporter-mediated uptake of glycosylated flavonoids in the small intestine [13]. The effective serum concentrations achievable from dietary citrus intake may therefore be higher for rutin than the *in vitro* comparison would suggest.

The optimal extraction temperature of 60°C represents a balance between kinetic facilitation of solute transfer and thermal degradation. The reduction in flavonoid yield at 80°C was most pronounced for rutin (-24% relative to 60°C), consistent with earlier reports of glycoside hydrolysis accelerating above 70°C [14]. From a process chemistry standpoint, the 60°C/70% ethanol/60 min protocol requires no specialised equipment and should scale readily to pilot plant level for production of XO-inhibitory citrus peel extracts.

Limitations of the current work include the use of bovine milk XO rather than human XO, which may differ in active-site geometry. Additionally, all assays were conducted under cell-free conditions; cellular uptake, metabolism by intestinal and hepatic enzymes, and protein binding will substantially modify the effective inhibitory concentrations *in vivo*. Nonetheless, IC_{50} values in the single-digit

micromolar range for quercetin and low double-digit range for rutin suggest that meaningful XO inhibition at dietary flavonoid exposure levels is plausible and warrants formal pharmacokinetic and *in vivo* hyperuricaemia model research.

Conclusion

Six flavonoids isolated from *Citrus sinensis* peel were characterised as competitive inhibitors of xanthine oxidase, with IC_{50} values ranging from 6.8 μ M (quercetin) to 38.2 μ M (naringenin) and K_i values from 3.7 to 21.3 μ M. Quercetin and rutin emerged as the most potent compounds in the series; both achieved IC_{50} values an order of magnitude above allopurinol yet remain attractive because of their safety profile and broad dietary availability. Extraction optimisation identified 60°C, 70% ethanol, 60 min as the best conditions for recovering maximum flavonoid content (63.9 mg/g dry peel). Kinetic analysis confirmed that all six compounds act by competing with xanthine for the molybdenum-containing active site, making them mechanistically rational candidates for hyperuricaemia management. Future work should evaluate quercetin and rutin in rodent oxonate-induced hyperuricaemia models at doses achievable through fortified functional food or standardised extract supplementation.

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