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In vitro investigation of xanthine oxidase inhibitory and antioxidant activities of 3,4,5-trihydroxycinnamic acid

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ABSTRACT

Introduction: Xanthine oxidase inhibitors with strong antioxidant activity are promising candidates for the treatment of gout and reactive oxygen species (ROS)-related disorders. 3,4,5-Trihydroxycinnamic acid (THCA), a natural hydroxycinnamic acid, exhibits strong antioxidant activities. This study investigated its xanthine oxidase inhibitory and antioxidant activities in comparison with sinapic acid, caffeic acid, and allopurinol.

Methods: *In vitro* xanthine oxidase inhibitory assay and a Lineweaver-Burk plot were used to measure enzyme inhibition activity and pattern. A docking study was used to explore hydroxycinnamic acid-xanthine oxidase interactions. Antioxidant activity was determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

Results: THCA (IC_{50} = 61.60 ± 8.00 µM) inhibited xanthine oxidase more potently than sinapic acid and caffeic acid (IC_{50}s = 117.00 ± 4.00 and 214.00 ± 5.00 µM), but less than allopurinol (IC_{50} = 2.84 ± 0.41 µM) (P < 0.05). THCA and allopurinol were competitive xanthine oxidase inhibitors with inhibition constants (K_i) of 170 and 2.12 µM, respectively. The docking investigation revealed that hydroxycinnamic acids occupied the enzyme active site near the molybdopterin core. THCA formed one more hydrogen bond with the enzyme active site than the other hydroxycinnamic acids, which could account for its higher inhibitory potency. THCA had significantly the strongest DPPH-radical scavenging activity (IC_{50} = 16.45 ± 3.35 µM) and higher than ascorbic acid (IC_{50} = 33.16 ± 7.38 µM) (P < 0.05).

Conclusion: THCA inhibits xanthine oxidase and has good antioxidant properties even more than sinapic acid and caffeic acid. Thus, it is a promising natural active compound that should be further investigated for the treatment of gout and other ROS-related disorders.

Implication for health policy/practice/research/medical education:
3,4,5-Trihydroxycinnamic acid displays substantial xanthine oxidase inhibition and antioxidant activity. Thus, it could be used as a biomarker for herbal plant screening and a lead compound for structural modification to discover a highly active molecule.

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Introduction

Xanthine oxidase is an important enzyme in purine catabolism that catalyzes the oxidation of hypoxanthine to xanthine and subsequently to uric acid. The enzymatic reaction also generates reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2). Overexpression of xanthine oxidase in pathological tissues can cause hyperuricemia, leading to gouty arthritis. In addition, the overproduction of ROS may lead to various pathological conditions, including cardiovascular diseases, diabetes mellitus, and cancer (1,2).

Xanthine oxidase inhibitors are widely used for the treatment of gouty arthritis and other ROS-related pathological conditions, such as hypertension, heart failure, and cardiovascular inflammation (1,3-6). Allopurinol, a competitive xanthine oxidase inhibitor, has been used clinically for the treatment of gout for many

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decades. However, allopurinol use has some limitations because it is a purine analog that can cause severe adverse drug reactions and serious hypersensitivity, especially Steven-Johnson syndrome. Non-purine analog xanthine oxidase inhibitors have been developed to overcome the limitations of purine analogs. Febuxostat and topiroxostat are non-purine analogs and potent xanthine oxidase inhibitors that have been clinically approved for the treatment of gouty arthritis. However, significant adverse drug reactions, specifically liver dysfunction, have been reported during their use. The search for novel xanthine oxidase inhibitors with low toxicity is in progress. These novel agents are aimed to be used in patients who are intolerant to currently available drugs or as a combination therapy to reduce their potential adverse reactions (1,2).

Hydroxycinnamic acids are a group of phenolic acids derived from many natural sources (7,8). Various pharmacological activities of hydroxycinnamic acids, including xanthine oxidase inhibition, have been investigated (9). The xanthine oxidase inhibitory effect of sinapic acid is higher than that of caffeic acid, ferulic acid, and *p*-coumaric acid (10,11). Most previous studies using the Lineweaver-Burk plot have indicated that hydroxycinnamic acids inhibit xanthine oxidase via competitive inhibition (12-14). However, Lin et al indicated that these compounds might act as non-competitive mixed-type inhibitors of xanthine oxidase (10).

3,4,5-Trihydroxycinnamic acid (THCA) is a hydroxycinnamic acid found in natural sources (15-17). It can also be synthesized through a biocatalytic process using substrates derived from palm oil mill effluents (18,19). Several pharmacological activities of THCA have been reported, including antioxidant, anti-inflammatory, neuroprotective, and anticancer (16,20-29). Previous studies have shown that THCA has the strongest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity among free hydroxycinnamic acids and their alkyl esters (20). However, the xanthine oxidase inhibitory activity of this compound has not been investigated.

The structure-xanthine oxidase inhibitory activity relationships of hydroxycinnamic acids have been examined previously. 3,4-Dihydroxy functional groups on the aromatic ring of hydroxycinnamic acid promote H-bond formation with amino acid residues at the active site of xanthine oxidase (12). A study conducted by Nishiwaki et al showed that a 3,4,5-trihydroxyphenolic derivative, 5-allylpyrogallol, inhibited xanthine oxidase with a higher potency than its 3,4-dihydroxy analog, hydroxychavichol, and allopurinol by approximately 13- and 24-folds, respectively (30). A docking study of 5-allylpyrogallol and hydroxychavicol with the xanthine oxidase-xanthine complex showed that the trihydroxyphenolic compound formed a higher number of H-bonds and had a more stable binding energy than its 3,4-dihydroxy phenolic analog (30). These studies and other reports (31-34) encouraged us to investigate the xanthine oxidase inhibitory and antioxidant activities of THCA compared to those of allopurinol and other hydroxycinnamic acids, including sinapic acid and caffeic acid. If THCA is a highly potent xanthine oxidase inhibitor with strong antioxidant activity, it might be a promising active compound in herbal plants for future development in the treatment of gouty arthritis, hyperuricemia, and other ROS-related diseases. Furthermore, it might be used as a lead compound for structural modification to obtain more active compounds.

Materials and Methods

Materials

The chemical compounds were purchased from the following chemical reagent companies: THCA from Carbosynth (Newbury, Berkshire, United Kingdom), sinapic acid from ACROS ORGANICS (Geel, ANTWERP, Belgium), caffeic acid and uric acid from Sigma-Aldrich, Merck (St. Louis, Missouri, United States), xanthine and allopurinol from Wako Pure Chemical Industries, Ltd. (Chuo-ku, Osaka, Japan), xanthine oxidase enzyme (Buttermilk) from Calbiochem® (Merck, San Diego, California, United States), and sodium dihydrogen phosphate dihydrate (AR grade) from EMSURE® Reag. Ph Eur; Merck, (Darmstadt, Germany).

Methods

Preparation of chemical and enzyme solutions

The same process was used to prepare stock solutions of 20 mg/mL of THCA, sinapic acid, caffeic acid, and allopurinol in DMSO. NaOH (0.1 M) was used to prepare the xanthine stock solution. Prior to the assay, all stock solutions were diluted in 100 mM sodium dihydrogen phosphate buffer (pH 7.5). The purchased enzyme solution was diluted to 1:20 in 100 mM sodium dihydrogen phosphate buffer (pH 7.5) and examined for enzyme activity. Xanthine was diluted in 100 mM sodium dihydrogen phosphate buffer (pH 7.5) to a final concentration of 100 µM for enzyme testing. We mixed 5 and 10 µL of xanthine oxidase solution with the xanthine solution at room temperature (25 °C). A UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan) was used to measure uric acid production rate (ΔAbsorbance at 295/min or ΔAbs_{295}/min). A standard curve of uric acid was used to calculate enzyme activity as mUnit/mL (1 Unit = 1 µmol of uric acid production/minute).

Solutions of 20, 30, 40, and 60 µM uric acid in sodium dihydrogen phosphate buffer solution (pH 7.5) were prepared. The absorbance of the solutions at 295 nm was measured in triplicates. A standard curve of uric acid was constructed. The linear equation was determined to be $y = 0.01176x - 0.01270$ ($r^2 = 0.9967$) (Figure S1).

Xanthine oxidase inhibitory assay

The assay was adapted from Gawlik-Dziki et al and

Shintani (35,36). The reaction proceeded at 25 °C. A 1 mL solution of 0.57 mUnit/mL xanthine oxidase and 100 µM sample in 100 mM sodium dihydrogen phosphate buffer (pH 7.5) was incubated at 25 °C for 10 minutes. Xanthine solution was added to start the reaction at a given concentration. A UV-visible spectrophotometer was used to monitor the process at 295 nm. Enzyme activity was monitored as the initial rate of uric acid production at 295 nm (ΔAbs_{295}/min). The negative control used sodium dihydrogen phosphate buffer (pH 7.5) instead of the sample solution. %Xanthine oxidase inhibition was determined using equation 1. All samples were tested in triplicate, and the %xanthine oxidase inhibition was presented as mean ± SD.

$$\%xanthine\ oxidase\ inhibition = \left(\frac{\left(\frac{\Delta Abs_{295}}{min}\right)_{control} - \left(\frac{\Delta Abs_{295}}{min}\right)_{sample}}{\left(\frac{\Delta Abs_{295}}{min}\right)_{control}}\right) \times 100 \quad (1)$$

$(\Delta Abs_{295}/min)_{control}$ and $(\Delta Abs_{295}/min)_{sample}$ are the initial velocities of the enzyme reactions of the control and sample, respectively.

An inhibitor concentration inhibitory curve was created using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA) to calculate the compound IC_{50}. Three concentration inhibition curves were measured for each inhibitor. IC_{50} values were presented as mean ± SD.

Investigation of xanthine oxidase inhibition pattern

The xanthine oxidase inhibition pattern was investigated using the Lineweaver-Burk plot, as shown in equation 2 (37).

$$\frac{1}{v_0} = \frac{k_m}{v_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{v_{max}} \quad (2)$$

v_0 and v_{max} are the initial velocity (ΔAbs_{295}/min) and maximum velocity of the enzyme reaction, respectively. k_m is the substrate concentration, which results in a half-maximal velocity for the enzymatic reaction. [S] is the substrate concentration.

A specific inhibitor concentration was used to inhibit xanthine oxidase at varying substrate concentrations. The xanthine oxidase solution (0.57 mUnit/mL) was mixed with an inhibitor at a specific concentration in a buffer of 100 mM sodium dihydrogen phosphate (pH 7.5) and incubated for 10 minutes at 25 °C . Xanthine was added and mixed to initiate the reaction. For a reaction with allopurinol, xanthine concentrations were 0.25 to 2.00 µM while allopurinol concentrations were 0 to 1.25 µM. For the reaction with THCA, xanthine concentrations ranged from 20 to 100 µM, while the acid concentration ranged from 0 to 51 µM. A UV-visible spectrophotometer was used to measure the enzyme reaction rate as ΔAbs_{295}/min. Triplicate assays were performed using the given xanthine and inhibitor concentrations. The Lineweaver-Burk plot for each inhibitor concentration was generated using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

The Lineweaver-Burk plot was constructed by plotting 1/(ΔAbs_{295}/min) and 1/[xanthine (nM)] on the Y- and X-axes, respectively. The v_{max} and k_m of each Lineweaver-Burk plot were calculated from the 1/Y-intercept and Y-intercept/slope. At different inhibitor concentrations, the Lineweaver-Burk plot, k_m, and v_{max} were used to interpret the enzyme inhibition pattern (37). In triplicate, the Lineweaver-Burk plot experiment was used to determine the k_m and v_{max} mean and SD values.

The results of the inhibition pattern study suggested that allopurinol and THCA were competitive inhibitors of xanthine oxidase. The calculated k_m and v_{max} of the Lineweaver-Burk plots are shown in Table S2. To determine the inhibition constant (K_i), the Lineweaver-Burk plot equation for competitive inhibition was represented by equation 3 (37).

$$\frac{1}{v_0} = \frac{k_m}{v_{max}}\left(1 + \frac{[I]}{K_i}\right)\left(\frac{1}{[S]}\right) + \frac{1}{v_{max}} \quad (3)$$

v_0 and v_{max} are the initial velocity (ΔAbs_{295}/min) and maximum velocity of the enzyme reaction, respectively. k_m is the substrate concentration, which results in a half-maximal velocity for the enzymatic reaction. [S] and [I] are the substrate concentration and inhibitor concentration. K_i is The inhibition constant or the equilibrium constant for the binding of the inhibitor to xanthine oxidase.

The inhibition constant (K_i) is the equilibrium constant for the binding of the inhibitor to xanthine oxidase. K_i was determined from the secondary plot by plotting the mean values of the slope obtained from the Lineweaver-Burk plot in the ordinate against the inhibitor concentrations in the abscissa, as shown in equation 4. K_i was calculated from the Y-intercept/slope (10,37).

$$Slope = \frac{k_m}{v_{max}} + \frac{k_m[I]}{v_{max}K_i} \quad (4)$$

v_{max} is the maximum velocity of the enzyme reaction. k_m is the substrate concentration, which results in a half-maximal velocity for the enzymatic reaction. [I] is the inhibitor concentration. K_i is The inhibition constant or the equilibrium constant for the binding of the inhibitor to xanthine oxidase.

DPPH radical scavenging assay

DPPH solution (180 µL of 0.1 mM) in absolute ethanol was mixed with 20 µL of sample at a given concentration (or control solution), in a 96-well plate. The mixture was shaken and left in a dark place at room temperature for 15 minutes. The absorbance of DPPH was monitored at 517 nm using a UV-visible spectroscopic microplate reader (Varioskan Lux; Thermo Fisher Scientific, Vantaa, Finland). %DPPH radical scavenging activity was calculated using equation 5.

$$\%DPPH\ radical\ scavenging = \frac{(Abs_{517control} - Abs_{517sample})}{Abs_{517control}} \times 100(\%) \quad (5)$$

$Abs_{517\ control}$ and $Abs_{517\ sample}$ are the absorbance at 517 nm of the control and sample.

To determine the IC_{50}, the %DPPH radical scavenging values were plotted against various sample concentrations (μg/mL) using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Ascorbic acid was used as a positive control. The measurements were performed in triplicate.

Molecular docking

The method consisted of three steps: ligand preparation, receptor preparation, and docking method validation.

Ligand preparation: Discovery Studio was used to build molecular models of xanthine oxidase inhibitors in this study. Gaussian 09 D.01 software (Gaussian Inc., Wallingford, UK) employed density functional theory with M062X/6-31G** to optimize the minimized structures (38,39). The following docking studies used fully geometrically optimized structures.

Receptor preparation: Enzyme selectivity and inhibitor-enzyme interactions were investigated using molecular docking. Templates for this investigation were the three-dimensional coordinates of the bovine xanthine oxidase enzyme (PDB ID code: 1FIQ, Resolution = 2.5 Å) from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). Salicylic acid and water molecules were removed from the protein crystal structure prior to docking. The Discovery Studio tool was used to identify and fill the side chains and missing loops to restore the protein. The molecular docking calculations were performed using AutoDock software. The protein's Kollman charges were computed and polar hydrogen was included using the AutoDock Tools (ADT) graphical user interface (40). Subsequently, the PDBQT file was saved for docking analysis. The non-covalent interaction between a small molecule (ligand) and a macromolecule (receptor) was effectively predicted by the computational molecular docking technique.

Docking method validation: An empirical free energy function and the Lamarckian genetic method were employed for docking in AutoDock 4.2.6 (Scripps Research Institute, USA). This study used a sample size of 150 randomly selected individuals. It conducted 2.5×10^6 energy evaluations, with a mutation rate of 0.02% and a crossover rate of 0.80%. The dimensions of the grid box were 60 Å in the x-, y-, and z-directions. The grid points were evenly distributed at 0.375 Å intervals. All ligands underwent 100 separate docking simulations. Redocking of the major ligand receptor on the active site validated docking. The results that had a positional root-mean-square deviation less than 2.0 Å were categorized and their binding free energy was used to represent them.

Statistical analysis

Mean ± SD is used to express the data on % inhibition against enzyme activity and IC_{50}. One-way ANOVA and the Scheffe post hoc test were used in the statistical analysis, which was carried out using the IBM SPSS Statistics software (IBM SPSS Statistics. Version 29. IBM Corp., Armonk, NY, USA). A P value of less than 0.05 indicated that there were significant differences between the data.

Results

Xanthine oxidase inhibition of THCA, sinapic acid, caffeic acid, and allopurinol

The structures of the tested compounds are shown in Figure 1. To evaluate the degree of xanthine oxidase inhibition, each compound was initially tested at a concentration of 100 μM. The results are presented in Table 1. THCA inhibited xanthine oxidase more effectively than sinapic and caffeic acids. However, its inhibitory efficacy was significantly lower than that of allopurinol (n=3; $P<0.05$).

THCA inhibited xanthine oxidase at 51-174 μM, compared to the negative control (Table S1). The concentration inhibitory curves and IC_{50} values for xanthine oxidase inhibition by allopurinol, THCA, sinapic acid, and caffeic acid are presented in Figure 2A-D and Table 1, respectively. Consistent with the previous screening at 100 μM, THCA showed a considerably stronger xanthine oxidase inhibitory action than sinapic acid and caffeic acid ($P<0.05$). However, its inhibitory potency was lower than that of allopurinol ($P<0.05$).

The xanthine oxidase inhibition pattern of THCA in comparison with allopurinol using the Lineweaver-Burk plot

The Lineweaver-Burk plots of allopurinol and THCA at defined concentrations are shown in Figures 3A and 3B. The k_m and v_{max} values of allopurinol and THCA derived from Lineweaver-Burk are presented in Table S2. The v_{max} values of both reactions with THCA and allopurinol were

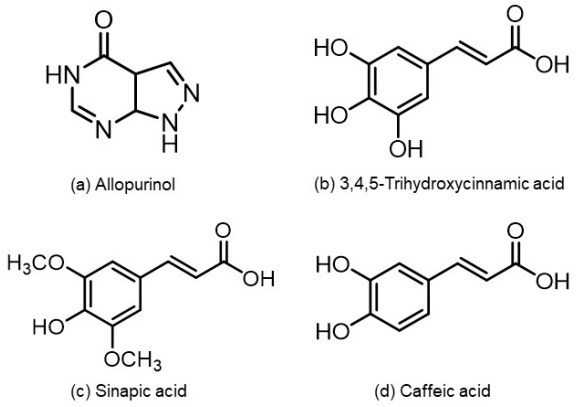

Figure 1. Chemical structures of (a) allopurinol, (b) 3,4,5-trihydroxycinnamic acid, (c) sinapic acid, and (d) caffeic acid.

Table 1. Xanthine oxidase inhibition and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of 3,4,5-trihydroxycinnamic acid and its comparators

Samples	Xanthine oxidase inhibition				DPPH radical scavenging activity
	%Xanthine oxidase inhibition (at 100 μM)[a]	IC_{50} (μM)[a]	Inhibition pattern	K_i (μM)	IC_{50} (μM)[a]
THCA	87.12 ± 0.50*	61.60 ± 8.00*	Competitive	170	16.45±3.35*
Sinapic acid	32.04 ± 0.89*	117.00 ± 4.00*	N/A	N/A	30.22±3.66
Caffeic acid	27.15 ± 0.76*	214.00 ± 5.00*	N/A	N/A	22.97±0.06
Allopurinol	100.00 ± 0.00*	2.84 0.41*	Competitive	2.12	- [b]
Ascorbic acid	N/A	N/A	N/A	N/A	33.16±7.38*

THCA: 3,4,5-trihydroxycinnamic acid; N/A: not analyzed.
[a] Mean ± SD (n = 3); *P < 0.05, when compared to every other agent tested; One-way ANOVA test followed by Scheffe post hoc test.
[b] No activity at 500 µM.

unchanged, but the k_m values increased with inhibitor concentration. Thus, THCA and allopurinol competitively inhibited xanthine oxidase (Table 1).

The xanthine oxidase inhibition constants (K_i) of THCA and allopurinol

Figures 4A and 4B show secondary plots of the Lineweaver-Burk slopes against the inhibitor concentrations (equation 4). Allopurinol and THCA inhibition constants (K_i) were calculated from the secondary plot of Y-intercepts and slopes (Figures 4A and 4B). The K_i values for allopurinol and THCA were 2.12 µM and 170 µM, respectively (Table 1).

Molecular docking of THCA, sinapic acid, caffeic acid, and allopurinol with xanthine oxidase active site

In silico molecular docking research utilizing the AutoDock program examined the binding interactions between xanthine oxidase and its inhibitors, THCA, sinapic acid, caffeic acid, and allopurinol. The optimal docking pose for the co-crystallized ligand salicylic acid was compared to its bound conformation to test the docking technique. The root means square deviation (rmsd) of 0.53 Å indicates that the docking technique could accurately anticipate. We considered the lowest energy docked conformation of the best cluster, which was the most populous. Molecular docking showed that THCA, sinapic acid, and caffeic acid were located near the molybdopterin core in the salicylic acid-xanthine oxidase enzyme (Figure 5). The minimum xanthine oxidase binding energies of THCA, sinapic acid, caffeic acid, and allopurinol were -6.08, -6.12, -5.94, and -5.80 kcal/mol, respectively (Table 2). The numbers of hydrogen bonds formed between these substances and the residues in the enzyme's active site are displayed in Table 2. 5, 4, 4, and 6 hydrogen bonds were generated, respectively by THCA, sinapic acid, caffeic acid, and allopurinol. The hydrogen bond interactions between each inhibitor and the xanthine oxidase active site are shown in Table S3.

DPPH radical scavenging assay of THCA, sinapic acid, caffeic acid, and allopurinol

Table 1 displays the IC_{50} values for the DPPH radical

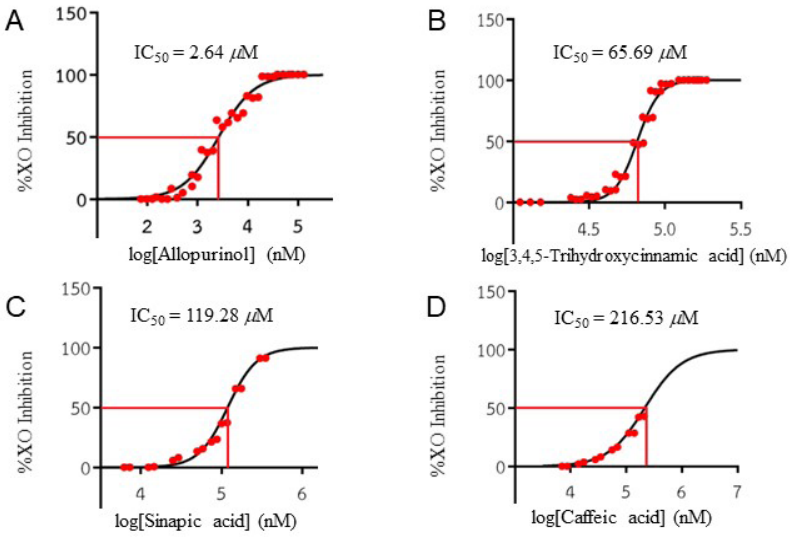

Figure 2. Representatives of %xanthine oxidase (XO) inhibition-concentration (nM) curves and IC_{50} values of (A) allopurinol, (B) 3,4,5-trihydroxycinnamic acid, (C) sinapic acid and (D) caffeic acid determined by the in vitro xanthine oxidase inhibitory assay.

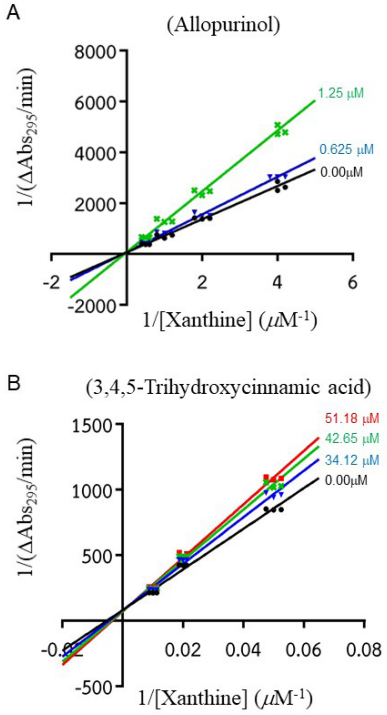

Figure 3. Representatives of Lineweaver-Burk plots of xanthine oxidase reactions in the presence of (A) allopurinol and (B) 3,4,5-trihydroxycinnamic acid.

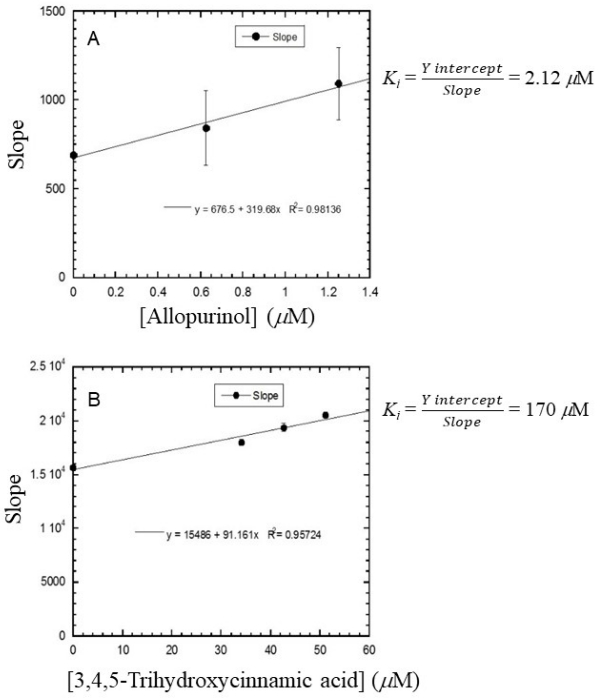

Figure 4. The secondary plot of the slopes (mean values) from the Lineweaver-Burk plots of (A) allopurinol and (B) 3,4,5-trihydroxycinnamic acid against their concentrations.

scavenging activity of THCA, caffeic acid, sinapic acid, caffeic acid, allopurinol, and ascorbic acid. The antioxidant activity of THCA (IC_{50} = 16.45±3.35 µM) was significantly greater than that of ascorbic acid (IC_{50} = 33.16±7.38 µM) (P<0.05), but it was not significantly different from caffeic acid (IC_{50} = 30.22 ±3.66 µM) or sinapic acid (IC_{50} = 22.97 ±0.06 µM). At a concentration of 500 µM, allopurinol did not show any DPPH radical scavenging action.

Discussion

This study explored THCA's xanthine oxidase inhibition for the first time. The IC_{50} values of the xanthine oxidase inhibitory assay (Table 1 and Figure 2) revealed that THCA inhibited xanthine oxidase 1.9 and 3.5-folds better than sinapic acid and caffeic acid, respectively. Previous investigations demonstrated that sinapic acid could inhibit xanthine oxidase with a lower IC_{50} than caffeic, ferulic, and coumaric acids (10,11). This study showed that THCA was the most powerful inhibitor of xanthine oxidase among free hydroxycinnamic acids. But compared to allopurinol, its xanthine oxidase inhibitory action is less potent.

The Lineweaver-Burk plot (Figure 3A-B, Tables 1 and 2SI) demonstrated that both allopurinol and THCA were competitive xanthine oxidase inhibitors. Allopurinol and free hydroxycinnamic acids competitively inhibit xanthine oxidase at the xanthine-binding site, as shown in most previous experiments (12-14). However, Lin et al found that free hydroxycinnamic acids bound to both free and xanthine oxidase-xanthine complexes as a non-competitive mixed-type inhibitors (10). The inhibition constants (K_i) of THCA (170 µM) and allopurinol (2.12 µM) were measured using the Lineweaver-Burk plot (Figure 4A-B and Table 1). This showed that trihydroxycinnamic acid has lower enzyme binding affinity and potency than allopurinol. Previous investigations revealed a K_i range of 0.34-9.22 µM for allopurinol (13,31). Sinapic and caffeic acids have K_i values of 24.59 and 47.83 mM, respectively (10). The K_i values from this study showed that trihydroxycinnamic acid binds to the enzyme more strongly than sinapic acid and caffeic acid.

The interconvertible forms of xanthine oxidoreductase include xanthine oxidase and xanthine dehydrogenase. The metalo-flavoprotein enzymes are homodimers with two identical 145-kDa subunits. Each subunit operates independently and has two iron-sulfur (Fe/S) clusters in the N-terminal domain, an FAD cofactor in the intermediate domain, and a molybdopterin cofactor in the C-terminal domain. Mammalian dehydrogenases can be transformed into oxidized forms by the oxidation of sulfhydryl residues and/or proteolysis in diseased tissues (1,2). Xanthine oxidase hydroxylates xanthine at the molybdopterin center of the enzyme active site. This region contains Phe649, Asn768, Glu802, Leu873, Arg880, Phe914, Phe1009, Thr1010, Leu1014, and Glu1261 residues. Glu802, Arg880, and Glu1261 are essential for xanthine hydroxylation (1,2).

The docking investigation of THCA, sinapic acid, and caffeic acid with oxidized (Mo^{6+}) xanthine oxidase

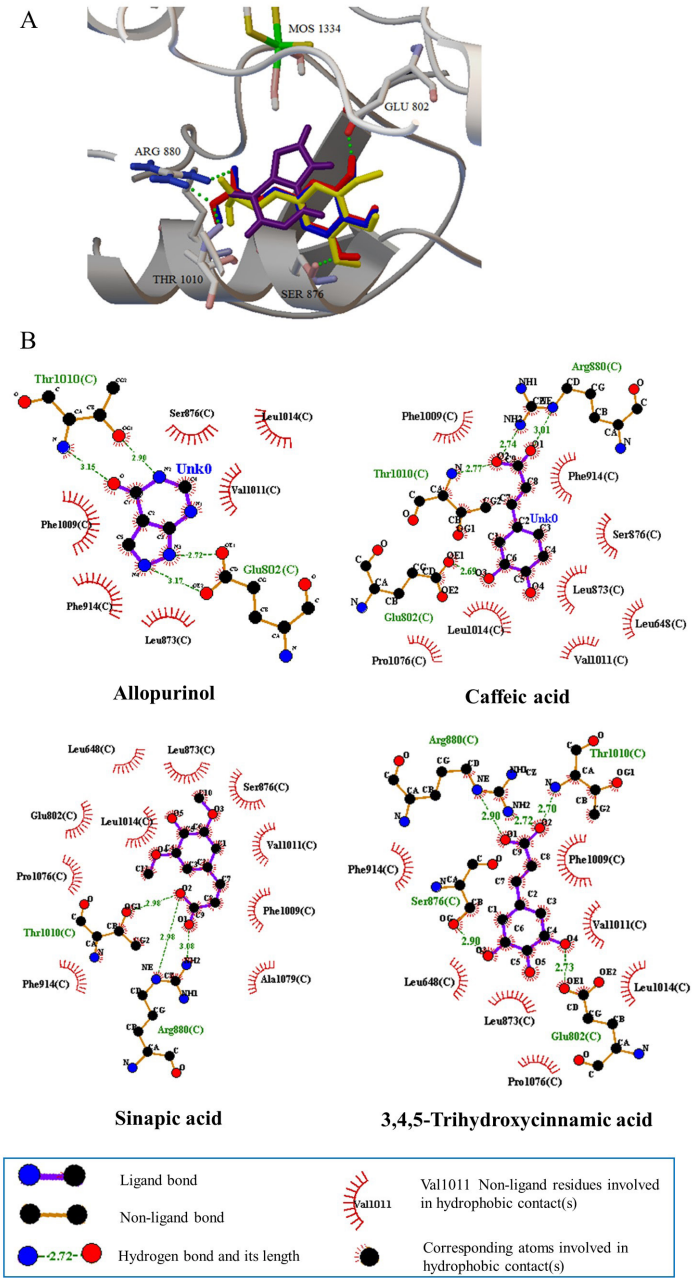

Figure 5. The interaction between the oxidized (Mo^{6+}) xanthine oxidase's active site (PDB ID code: 1FIQ, Resolution = 2.5 Å) with ligands from the docking study. (A) Superimposition of the best docking solutions for allopurinol (purple structure), caffeic acid (blue structure), sinapic acid (yellow structure), and 3,4,5-trihydroxycinnamic acid (red structure); (B) Protein-ligand interaction of allopurinol, caffeic acid, sinapic acid and 3,4,5-trihydroxycinnamic acid (Using LigPlot).

indicated that they were overlaid at the same binding location adjacent to the molybdopterin core (Figure 5). These chemicals bind similarly to allopurinol. These docking results agreed with the Lineweaver-Burk plot (Figure 3 and Table S2) showing that THCA competitively inhibits xanthine oxidase. The enzyme binding energies of THCA (-6.08 kcal/mol) were comparable to sinapic acid (-6.12 kcal/mol) but stronger than caffeic acid (-5.94 kcal/mol) (Table 2). THCA formed one more H-bond with the enzyme active site than sinapic and caffeic acids (Tables 2 and S3). This may explain why THCA had a lower IC_{50} than sinapic and caffeic acids (Table 1 and Figure 2). Nishiwaki et al reported similar findings. In their study, 5-allylpyrogallol, a 3,4,5-trihydroxyphenolic analog, formed one more H-bond with xanthine oxidase and had a lower IC_{50} and more stable binding energy than its 3,4-dihydroxyphenolic analog (30).

This study found that allopurinol bound the oxidized xanthine oxidase (Mo^{6+}) with the highest binding energy (-5.87 Kcal/mol). However, this interaction occurred with the greatest number of H-bond interactions (6 H-bonds). Because allopurinol is a mechanism-based xanthine

Table 2. Binding energy values and the number of hydrogen (H)-bond formation from the docking study

Compound	Binding energy (kcal/mole)	No. of H-bond formation
Allopurinol	-5.87	6
3,4,5-Trihydroxycinnamic acid	-6.08	5
Sinapic acid	-6.12	4
Caffeic acid	-5.94	4

oxidase inhibitor, the oxidized enzyme (Mo^{6+}) can oxidize its C-6 position to oxypurinol. This powerful xanthine oxidase inhibitor is predicted to covalently attach to the molybdopterin core of the reduced enzyme (Mo^{4+}) during oxidation (32,33).

To explain why THCA had a lower IC_{50} than sinapic acid and caffeic acid, hydrophobic contact and H-bond formation between the acids and xanthine oxidase active sites were investigated. The hydroxycinnamic acids are surrounded by hydrophobic residues (<5 Å) such as Phe914, Phe1009, Val1011, Leu1014, and Leu873 (Figure 5B). The amino acids Glu802, Ser876, Arg880, and Thr1010 surrounded all examined compounds (Figures 5A and 5B, Table S3). Glu802 and Arg880 are essential for catalytic oxidation of xanthine substrate (1,2). The carboxyl and 4-hydroxyl groups of these hydroxycinnamic acids interacted in a similar manner. The carboxylate group coupled with Arg880's guanidinium and Thr1010's hydroxyl groups, whereas the 4-hydroxyl groups might form van der Waals interactions with nearby amino acids. However, their 3,5-disubstituent groups have different H-bond interactions. At position-3, THCA and caffeic acid 3-hydroxyl H atoms establish hydrogen bonds with Glu802 carboxyl O atom (~1.7 Å), whereas sinapic acid 3-OCH_3 forms van der Waals interactions with Glu802 (~2.7 Å) and Leu1014 (~3.36 Å). An O atom at position-5 of THCA and a 5-methoxyl group of sinapic acid can establish a hydrogen bond with the hydroxyl H atom of Ser876, at 2.18 and 2.04 Å, respectively. THCA binds to the xanthine oxidase active site with one more H-bond than that of sinapic and caffeic acids. The lower IC_{50} of THCA compared to sinapic acid and caffeic acid was due to its enzyme binding interaction. The xanthine oxidase inhibition and docking investigation (Tables 1 and 2) also supports recent studies (10,11) revealed that sinapic acid inhibits the enzyme better than caffeic acid. These data showed that 3,4,5-trioxycinnamic acids bind and inhibit xanthine oxidase better than 3,4-dioxycinnamic acids.

When there is an imbalance between the production of ROS and the capacity of antioxidant defenses to combat them, oxidative stress results. ROS consist of several molecules that contain oxygen, including $O_2^{\bullet-}$, H_2O_2, and hydroxyl radical ($^{\bullet}OH$). These molecules are naturally produced as byproducts of the biological metabolism. Elevations in ROS have the potential to damage cells by oxidizing macromolecules like proteins, lipids, and DNA, which in turn can lead to the onset of many disorders. On the other hand, antioxidants act as scavengers of ROS, reducing oxidative damage and preserving cellular balance. The DPPH free radical scavenging assay was employed in this study to assess the capacity of hydroxycinnamic acids to counteract free radicals, like their role in quenching ROS in biological systems. This assay can offer useful information into the possible therapeutic uses of hydroxycinnamic acids in improving diseases related to oxidative stress (41).

A previous study demonstrated that THCA exhibits greater antioxidant activity compared to caffeic and sinapic acids (20). In this study, the DPPH radical scavenging activity of THCA was investigated and compared to that of ascorbic acid, sinapic acid, caffeic acid, and allopurinol (Table 1). The IC_{50} values of all hydroxycinnamic acids examined were lower than that of ascorbic acid. When compared to ascorbic acid, only THCA had noticeably more antioxidant activity. The results of the investigation demonstrated that at 500 μM, allopurinol exhibited negligible DPPH radical scavenging action.

It was found that THCA is the most potent xanthine oxidase inhibitor and antioxidant among the hydroxycinnamic acids. Therefore, it may potentially lower uric acid and reduce ROS from the xanthine oxidase-catalyzing process and by radical scavenging simultaneously. Overall, THCA could be a potential compound for treating gouty arthritis and other pathological disorders involving ROS overproduction (1,2).

THCA has been studied for several pharmacological properties such as antioxidative (20-22), anti-inflammatory (16,23-27), neuroprotective (28), and anticancer activities (21,29). Nonetheless, its toxicity has not been fully studied. It was found that the trihydroxycinnamic acid did not cause cytotoxicity in BJ normal cell lines at concentrations of 50 and 100 μM (21). Unlike allopurinol, it might not induce hypersensitivity because it is a non-purine molecule. In addition, our experiment showed that the viability of RAW 264.7 cells incubated with 500 μM THCA for 24 h, was approximately 100% (data not shown). More investigation is required to modify the chemical structure of THCA to create safer and more potent derivatives.

Caffeic acid's structural modification as ester derivatives, such as caffeic acid phenethyl ester, enhanced the inhibition of xanthine oxidase and preserved high levels of antioxidant activity. Caffeic acid phenethyl ester had a one-tenth lower IC_{50} value for xanthine oxidase inhibition (6.26±1.60 μM) compared to caffeic acid (65.58±2.71 μM), while both compounds had similar %DPPH radical scavenging activities (73.93±3.24% and

83.59±2.94%) (12,14,34). This study verified that THCA is a stronger xanthine oxidase inhibitor and antioxidant than caffeic acid, suggesting that it could be a promising lead molecule for the discovery of novel highly active drugs. Furthermore, THCA could be employed as an active marker to evaluate natural plants for their xanthine oxidase inhibition and antioxidant activity. For instance, the caffeic acid content has been determined and used to assess various plants traditionally used to treat gout in Malaysia (42).

Conclusion

THCA is a potent xanthine oxidase inhibitor and antioxidant. It inhibits the enzyme more effectively than sinapic acid and caffeic acid, but less effectively than allopurinol. This may be due to its ability to form one more hydrogen bond with the enzyme's active site than the other hydroxycinnamic acids. Furthermore, by competitive enzyme inhibition, THCA inhibits xanthine oxidase in a manner like allopurinol. Taken together, THCA is a promising naturally derived compound for the treatment of gouty arthritis and ROS-related disorders.

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Conflict of interests

The authors have no conflicts of interest to declare.

Data availability statement

The data included in this manuscript and Supplementary Information are openly available.

Ethical considerations

Animal and human models were not used in this study. The authors considered ethical issues, including plagiarism, misconduct, data fabrication, falsification, double publication, and redundancy.

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Supplementary files

Supplementary file 1 contains Figure S1 and Tables S1-S3.

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