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Identification and Mode of Action of 5-Hydroxymethyl-2-furfural (5-HMF) and 1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic Acid (MTCA) as Potent Xanthine Oxidase Inhibitors in Vinegars

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ABSTRACT: Vinegars have been used as an alternative remedy for treating gout, but the scientific basis remains to be elucidated. In this study, seven commercial vinegars and one laboratory-prepared red-koji vinegar were evaluated for the inhibitory activity of xanthine oxidase (XO), a critical enzyme catalyzing uric acid formation. Red-koji vinegar exhibited potent xanthine oxidase inhibitory (XOI) activity and was used for isolating active compounds. The substances under two peaks with XOI activity from HPLC were identified as 5-hydroxymethyl-2-furfural (5-HMF) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA), by LC-MS-MS and NMR. The XO half-maximal inhibitory concentrations (IC_{50}) of 5-HMF and MTCA were 168 and 860 μg/mL, respectively. In further mode-of-action analysis, the inhibitory mechanism of each compound was elucidated at the IC_{50} level in the presence of various concentrations of xanthine as the substrate. The following Michaelis–Menten kinetics analysis of XO inhibition revealed uncompetitive and competitive patterns for 5-HMF and MTCA, respectively.

KEYWORDS: *vinegar, xanthine oxidase inhibitor, 5-HMF, MTCA, red-koji vinegar*

INTRODUCTION

Gout is one of the most common forms of arthritis caused by hyperuricemia, a high level of uric acid in the blood. Xanthine oxidase (XO) is a critical enzyme for uric acid production, catalyzing the conversions of hypoxanthine to xanthine and xanthine to uric acid.[1] Uric acid is the final metabolite of purine degradation in the human body and is eventually excreted by the kidneys and, to a lesser extent, through the gastrointestinal tract. Hyperuricemia, due to the overproduction or/and underexcretion of uric acid, is an essential prerequisite for gout.[2] Uric acid has a very low solubility in biological milieu and is considered to be an endogenous danger signal that initiates inflammatory response through Nacht, leucine-rich repeat, and pyrin domain containing protein 3 (NLRP3) inflammasome.[3] Hyperuricemia leads to the deposition of monosodium urate (MSU) in articular and periarticular tissues, triggering characteristic intermittent acute inflammation and chronic cartilage injury of the gouty joint.[2,3]

In addition to the pivotal role in the etiology of gout, hyperuricemia is associated with various pathophysiological conditions such as heart failure, pulmonary disorder, and metabolic syndrome and has been proposed as an adverse prognostic marker for hypertension, and an indicator for predicting cardiac death in stroke patients.[1,4] Furthermore, the increase of XO activity promotes an inflammatory state in macrophage by suppressing the anti-inflammatory transcription factor peroxisome proliferator-activated receptor γ (PPARγ).[5] Treatment with allopurinol, an XO inhibitor, thus alleviates tissue injury in inflammatory diseases, such as Crohn's disease, acute pancreatitis, and atherosclerosis.[1]

For more than four decades, allopurinol has been the only clinically available agent with XO inhibitory (XOI) activity and remains as a mainstay drug for treating hyperuricemia and gout, regardless of its well-documented hepatic and renal toxicity.[1,6] Therefore, continuing the search for new XO inhibitors for nutraceutical and pharmaceutical applications is necessary.[7−9]

Vinegar is mostly prepared by indigenous fermentation using various regional materials and is predominately used as a seasoning ingredient to flavor foods. Along with extensive discoveries of the health benefits of consuming traditional fermented foods, the biological activities of vinegars in enhancing metabolic activities, releasing fatigue, modulating hypertension and blood sugar levels, and preventing cancer have been addressed in previous studies.[10−14] High antioxidant activity has also been observed in various vinegars.[13−16] Moreover, vinegar has been used as an alternative remedy for treating gout. As conceptually noted and communicated by gouty individuals, relieving gout-caused discomfort by ingesting indigenously fermented vinegars is highly effective, resulting in increased attention in research. From the scientific perspective, it is of worth to conduct intensive investigations to examine and identify active compounds with XOI activity in vinegars.

In this study, seven commercial vinegar samples and one laboratory-prepared red-koji vinegar were examined for XOI activity. As compared, the laboratory-prepared red-koji vinegar exhibited more potent XOI activity and was used for further isolation, structural identification, and mode of action characterization of the active compounds.

MATERIALS AND METHODS

Vinegar Sample Preparation. Seven commercial vinegars and one laboratory-prepared red-koji vinegar were used in this study. The commercial vinegars, including one seasoned vinegar, two fruit

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vinegars, and four rice vinegars, were purchased from local markets. The red-koji vinegar was produced in the laboratory by following the indigenous practices. Briefly, red-koji wine (6% ethanol) was inoculated with an *Acetobacter* starter, and the acetic fermentation proceeded for 30 days in a glass jar without agitation at ambient temperature. The vinegar was then collected by filtering the fermented broth through four layers of cheesecloth into glass bottles and pasteurized at 70 °C for 10 min in a water bath. All vinegar bottles were wrapped with heavy duty paper to eliminate light exposure and were stored at refrigeration temperature (4−5 °C).

For pH value measurement, 30 mL of vinegar was transferred to a 100 mL beaker, and the pH value was determined using a pH-meter (MP220, Mettler Toledo GmbH, Greifensee, Switzerland). To determine acidity, 1 mL of vinegar was diluted with 40 mL of deionized water and titrated with 0.05 N NaOH solution to the pH value of 8.6 by using an automatic titrator (Mettler DL12 Titrator, Mettler Toledo AG). The acidity was expressed as the percentage of acetic acid (w/v).

The vinegars were further centrifuged at 16000*g* for 20 min at 4 °C (Sigma Labrozentrifugen 2K15, Osterode, Germany), and the resulting supernatant was filtered through a 0.45 μm membrane into brown vials and stored at −20 °C for chromatographic analyses.

Determination of XOI Activity. XO activity was assayed spectrophotometrically.[17] Fifty microliters of a vinegar sample, or saline (as a blank), was mixed with 675 μL of $NaHCO_3$ buffer (50 mM, pH 9.4), 100 μL of 1 mM xanthine (Sigma Chemical Co., St. Louis, MO, USA), 100 μL of 1 mM EDTA, and 75 μL of XO (200 mU/mL) (Sigma Chemical Co.). XO activity was determined on the basis of the production of uric acid in a 2 min reaction, which was monitored by the increase of absorbance at 290 nm (Δ_{Abs}) in a spectrophotometer (U-2001 spectrometer, Hitachi Co., Tokyo, Japan). XOI activity was expressed as the percentage inhibition and was calculated using the following equation:

$$[(\Delta_{Abs}\text{ of saline} - \Delta_{Abs}\text{ of sample})/\Delta_{Abs}\text{ of saline}] \times 100\%$$

To determine the half-maximal inhibitory concentration (IC_{50}), a series of sample solutions with known concentrations (i.e., 0, 31.25, 62.5, 125, and 250 μg/mL of 5-HMF or 0, 500, 750, 1000, and 1500 μg/mL of MTCA) were subjected to XOI activity determination, and the data obtained were plotted against sample concentration levels to generate a concentration-dependent inhibition curve. The IC_{50} value of each compound was then calculated on the basis of the corresponding inhibition curve. Allopurinol (1,5-dihydro-4*H*-pyrazolo-[3,4-*d*]pyrimidin-4-one) (Sigma Chemical Co.) solutions at concentrations of 0, 10, 25, 50, and 100 μg/mL were used to construct a standard inhibition curve for equivalency estimation of sample XOI potency.

Solid-Phase Extraction (SPE) Fractionation of XOI Active Fractions. For screening XOI-bearing components, an aliquot of 15 mL of membrane-filtered (0.45 μm) red-koji vinegar was loaded into a C18 SPE cartridge (ODS-18, 50 μm, 5 g, 20 mL, Supelco, Bellefonte, PA, USA) and eluted stepwise with 15 mL of deionized water and 20, 40, 60, 80 and 100% of methanol (assigned as F1−F6). Each fraction was subjected to rotary evaporation (Eyela Rotary Evaporatory N-1000, Eyela Co., Tokyo, Japan) before dehydration by freeze-drying (Lyvotac GT2, Finn-Aqua, Heraus, Germany). The obtained residues were dissolved in deionized water to a concentration of 5000 μg/mL before XOI activity determination.

Semipreparative HPLC Collection of XOI Components in Vinegar. The fraction of red-koji vinegar eluted from SPE fractionation with 40% methanol, fraction 3 (F3), displayed the highest XOI activity and was further subjected to semipreparative HPLC separation (L-2130 pump, L-2420 UV detector, D-2500 Chromato-Integrator, Hitachi Co. Ltd., Tokyo, Japan). A semi-preparative column (Hyperprep HS C18 column, 250 × 10.0 mm, 8 μm, Thermo Electron Corp. Hypersil Ltd., Cheshire, UK) was used and run with a gradient dual-solvent system composed of methanol (solvent A) and deionized water (solvent B). Each run was initiated from 28 to 55% of A in 7 min, increased to 100% of A in 3 min, maintained for an additional 2 min and then decreased to 28% A in 2 min and maintained for 2 min prior to the next injection. The injection volume, flow rate, and monitoring wavelength were 0.1 mL, 3.0 mL/min, and 280 nm, respectively. The active peak fractions were each collected, pooled, and lyophilized (Lyvotac GT2, Finn-Aqua). The obtained oily substances (approximately 3 mg) were designated compounds **I** and **II** and stored at −20 °C for further structural identification.

Structural Identification by LC-MS-MS and NMR Analyses. The chemical structures of XOI compounds in the vinegar were identified by performing LC-MS-MS and NMR analyses and an HPLC retention time comparison with authentic compounds. Two purified compounds (peaks 1 and 2) collected from semipreparative HPLC were respectively subjected to LC-MS analysis (LXQ System, Thermal Fisher Scientific Inc., Pittsburgh, PA, USA) using a C18 HPLC column (Thermo Biobasic-C18 column, 2 mm × 15 cm, 5 μm, Thermo Electron Corp. Hypersil Ltd.). Each analysis was conducted by using a gradient dual-solvent system composed of methanol (solvent A) and 0.1% acetic acid (solvent B). Each run was initiated from 20% A for 10 min, increased to 50% A in 5 min and to 100% A in 2 min, maintained for an additional 4 min, then decreased to 20% A in 1 min, and maintained for 2 min before the next injection. The injection volume and flow rate were 10 μL and 0.7 mL/min, respectively. Mass spectroscopy was analyzed by electron spray ionization (ESI) and calibrated by using Xcalibur software with a positive mode (ESI+) for compound **I** and a negative mode (ESI−) for compound **II**. The related parameters were helium gas flow rate, 80 arb; auxiliary gas flow rate, 20 arb; ion spray voltage, 4.50 kV; capillary temperature, 300 °C; capillary voltage, 7.0 V; and tube lens, 55.0 V.

For nuclear magnetic resonance (NMR) analyses, the purified compounds were respectively dissolved in CD_3OD. Compound **I** was subjected to ^1H NMR (Bruker Avance III 400 MHz NMR spectrometer, Bruker BioSpin GmbH, Rheinstetten, Germany) and ^{13}C NMR (100 MHz NMR spectrometer) analyses by using Topspin 2.1 software. Compound **II** was subjected to ^1H NMR (Bruker Avance III 500 MHz NMR spectrometer) and ^{13}C NMR (125 MHz NMR spectrometer) analyses equipped with a 5-mm broad probe and Topspin 2.1 software.

On the bases of LC-MS-MS and NMR analyses, compound **I** was elucidated as 5-hydroxymethyl-2-furfural (5-HMF) with MW 126, and compound **II** was 1-methyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxylic acid (MTCA) with MW 230. Therefore, authentic 5-HMF (Acros Organics Co., Fair Lawn, NJ, USA) and MTCA (Sigma Chemical Co.) were subjected to HPLC analysis for retention time comparison with compounds **I** and **II** run concurrently.

Kinetic Mode of Action Analyses for 5-HMF and MTCA. In the attempt to construct a Michaelis−Menten equation, XO activity was determined under a series of different levels of xanthine (substrate) solutions (i.e., 0, 0.125, 0.25, 0.5, 0.75, 1.0, and 1.5 mM), with or without the addition of 5-HMF or MTCA at IC_{50} concentrations. Each reaction mixture contained 100 μL of xanthine solution, 50 μL of 5-HMF or MTCA solution, 775 μL of 50 mM $NaHCO_3$ buffer consisting of 1 mM EDTA, pH 9.4, and 75 μL of XO solution, and the absorbance at 290 nm was monitored for 2 min spectrophotometrically (U2001 Spectrometer, Hitachi Co.) to determine XOI activities as affected by xanthine concentration. Michaelis−Menten graphs and Lineweaver−Burk plots were then constructed. Modes of action of 5-HMF and MTCA in enzymatic inhibition of XO were characterized according to the graphs and equations.

Statistics Analysis. Data are presented as the mean with standard deviation and were statistically analyzed by conducting ANOVA and Tukey's HSD test using SPSS software (Statistical Package for Social Science, 12.0, SPSS Corp., Chicago, IL, USA).

RESULTS AND DISCUSSION

Acidities and XOI of Vinegars. The pH values of the eight vinegars tested ranged from 2.61 to 3.54 (Figure 1I). As reported, the pH values of 17 traditional balsamic vinegars collected in Italy ranged from 2.37 to 3.48.[18] The acidities of

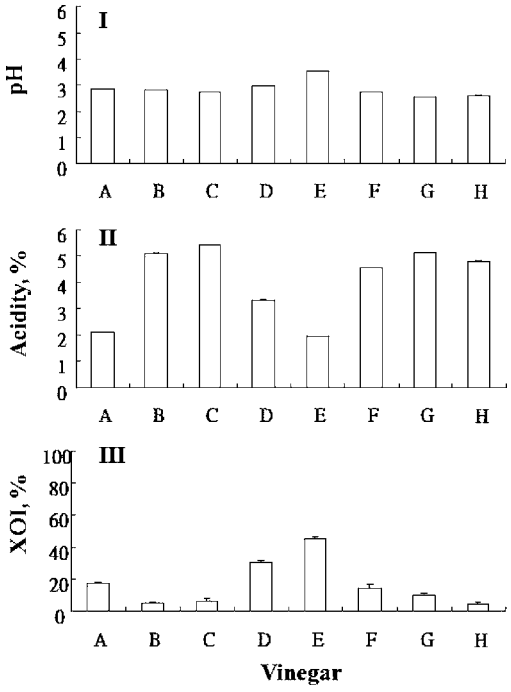


Figure 1. Determinations of pH value (I), acidity expressed as percent acetic acid (w/v) (II), and xanthine oxidase inhibitory (XOI) activity (III) of seven commercial vinegars (A–D, F–H) and one laboratory-prepared red-koji vinegar (E). Each value represents the mean ± SD (n = 3).

the vinegar samples ranged from 1.97 to 5.43% (% acetic acid, w/v) (Figure 1II), which also corresponded with those observed in Italian balsamic vinegars, 0.72–5.08%.[11] When a comparison was made on a single product basis, a closely correlated trend of the lower pH value of higher acidity was not observed.

The vinegars possessed different capacities of XOI depending on the product characteristic and source. The laboratory-prepared red-koji vinegar (sample E) exhibited the highest XO inhibition rate, 45.4%, as compared with other types of vinegar (Figure 1III). Interpolation calculation based on the allopurinol standard curve gave the XOI potency of 50 μL/mL of red-koji vinegar, equivalent to 10.6 μg allopurinol/mL.

XOI Fraction of Red-Koji Vinegar. The laboratory-prepared red-koji vinegar exhibited comparatively high XOI and, thus, was used as the source for the active compound isolation. Six fractions of the red-koji vinegar were collected from SPE fractionation with gradient methanol solutions. The residues of each fraction were dissolved in deionized water to an identical concentration (5000 μg/mL) and screened for XOI. On the basis of the fact that XOI was detected in every stepwise-fractionated solution collected, extensive distribution of the XOI active compounds in the vinegar was apparent. However, F3 (eluted with 40% methanol) exhibited the highest XOI activity (Figure 2) and was further separated by a semipreparative HPLC and monitored at 280 nm. Two distinct peaks at retention times of 5.87 and 11.36 min were detected (Figure 3I). Two oily substances under each peak (peaks 1 and 2) were obtained after the fractions were repeatedly collected, pooled, rotary evaporated, and lyophilized.

Identification of Compounds with XOI. The substance of peak 1 from semipreparative HPLC was analyzed using LC-MS-MS band with an ESI+ mode. The obtained spectrum

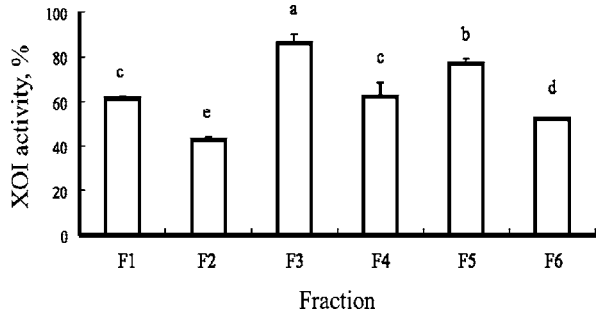


Figure 2. Xanthine oxidase inhibitory (XOI) activity of the fractions of red-koji vinegar eluted from SPE fractionation in order with water (F1), 20% methanol (F2), 40% methanol (F3), 60% methanol (F4), 80% methanol (F5), and 100% methanol (F6). Each value represents the mean ± SD (n = 3). Bars with different letters are significantly different ($p < 0.05$).

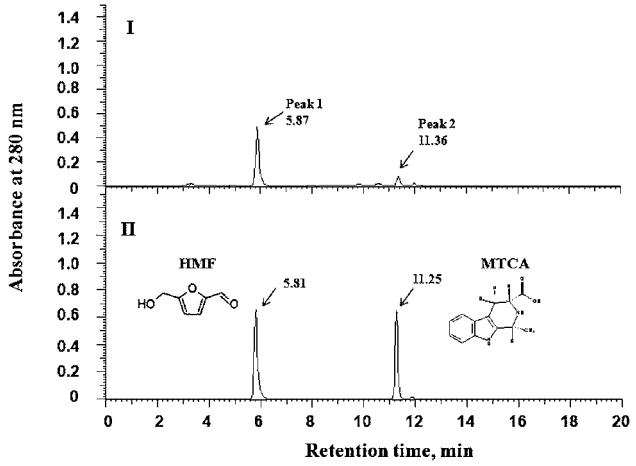


Figure 3. HPLC chromatograms of peaks 1 and 2 isolated from the red-koji vinegar (I) and HPLC chromatograms of authentic 5-HMF and MTCA (II).

showed the MS (**I**) major peak at m/z 127.13 and MS (**II**) fragment at m/z 109.06 (Figure 4), implying that peak 1 contained a water moiety [127.13 − 109.06 = 18.07 (H_2O)]. Recently, Kitts et al. identified 5-HMF in the anti-inflammatory fraction of Maillard reaction products (MRPs) generated by heating a glucose–lysine mixture based on the LC-ESI-MS/MS spectrum that also showed a positive molecular ion $[M + H^+]^+$ at m/z 127 and a fragment at m/z 109.[19] Teixidó et al. obtained similar results from LC-MS-MS analysis of 5-HMF by using an ESI+ mode, which revealed m/z 127, 109, and 81 fragments and a molecular weight of 126.[20] Accordingly, the assumptive structure of peak 1 corresponded to that of 5-HMF, and the MW was 126.

For further structural identification, the isolated peak 1 substance was subjected to one-dimensional NMR spectrometric analysis (Avance III 400 MHz) by using a 5 mm broad probe and tuned with CD_3OD as a solvent to detect ^1H resonance at 400 MHz; a clean proton spectrum was obtained by collecting data at 25 °C using the Topspin 2.1 software package. The ^1H spectrum indicated that the peak 1 substance possessed three methine protons [δ_H = 7.38 (H-3), 6.58 (H-4), and 9.53 (H-6)] and one methylene proton [δ_H = 4.61 (H-7)] (Table 1). The spectrum also showed a formyl group at δ_H 9.52, two mutually coupled aromatic protons at δ_H 7.37 (d, J = 3.5 Hz) and δ_H 6.57 (d, J = 3.5 Hz), and a CH_2OH group that

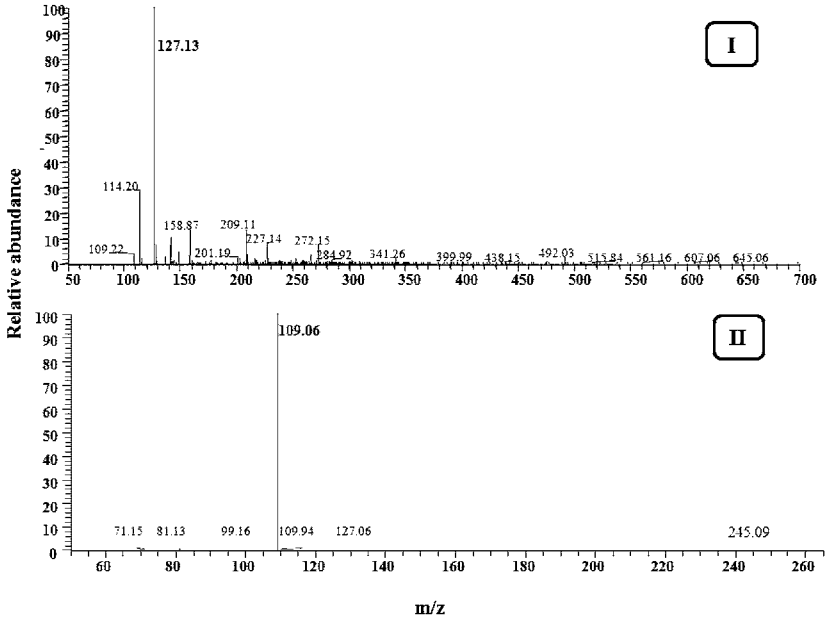
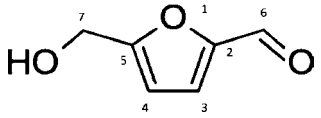


Figure 4. Mass spectra of peak1 subjected to LC-MS (I) and LC-MS-MS (II) analyses.

Table 1. ^{1}H NMR Chemical Shift Data of Peak 1 in Comparison with the Data of 5-Hydroxymethylfurfural (5-HMF) Reported by Li et al.[21]

C position	δ	δ (Li et al.[a])
3	7.38 (1H, d, J = 3.5 Hz)	7.22 (1H, d, J = 3.6 Hz)
4	6.58 (1H, d, J = 3.5 Hz)	6.52 (1H, d, J = 3.6 Hz)
6	9.53 (1H, s)	9.60 (1H, s)
7	4.61 (2H, s)	4.72 (2H, d, J = 2.1 Hz)

[a]Chemical structure of 5-hydroxymethylfurfural (5-HMF):



resonated at δ_H 4.61 (CH_2). In comparison with the ^{1}H NMR data of 5-HMF reported by Li et al. (Table 1), it is very supportive to identify peak I as 5-HMF.[21] As further analyzed by ^{13}C NMR, six carbons were detected, that is, three methine carbons [δ_C = 124.9 (C-3), 111.0 (C-4) and 179.5 (C-6)], one methylene carbon [δ_C = 57.7 (C-7)], and two quaternary carbons [δ_C = 154.0 (C-2) and 163.3 (C-5)]. It was further confirmed by the identical retention time and UV−visible spectra of purified peak 1 and authentic 5-HMF, which were concurrently subjected to HPLC analysis (Figure 3II). Therefore, there is no doubt that peak 1 was 5-HMF with $C_6H_6O_3$ and MW 126.

Subjecting peak 2 to LC-ESI-MS (I) analysis with a negative mode revealed the major peak at m/z 229 and MS (II) peak at m/z 185 (Figure 5). Accordingly, a molecule with a molecular weight of 230 containing a carboxylic group [229 − 185 = 44 (COO^-)] was proposed. Adachi et al., who analyzed MTCA by LC-ESI-MS analysis using a positive mode, showed the MS (I) major peak at m/z 231.[22] Gutsche and Herderich analyzed MTCA with LC-ESI-MS analysis using a positive mode and found the MS (I) major peak m/z 231 and m/z series of the fragments 214, 202, 187, 170, and 158.[23] Thus, it was likely that peak 2 contained a carboxylic group [231 − 187 = 44 (COO^-)] and had a MW of 230.

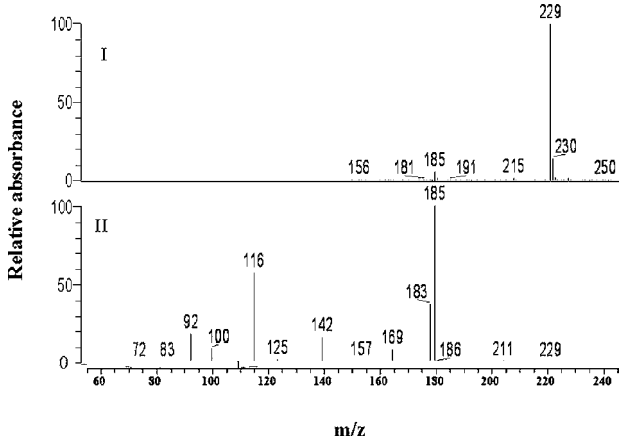


Figure 5. Mass spectra of peak 2 subjected to LC-MS (I) and LC-MS-MS (II) analyses.

Nine protons were detected by NMR analysis in the collected substance of peak 2, namely, six methine protons [δ_H = 4.70 (H-1), 3.96 (H-3), 7.47 (H-5), 7.04 (H-6), 7.13 (H-7), and 7.33 (H-8)], two methylene proton [δ_H = 3.02 (H-4a), 3.44 (H-4b)], and one methyl proton [δ_H = 1.75 (H-1′)] (Table 2). As shown by ^{13}C NMR analysis, 13 carbons were detected, that is, 6 methine carbons [δ_C = 51.2 (C-1), 59.7 (C-3), 119.1 (C-5), 120.5 (C-6), 123.3 (C-7), and 112.3 (C-8)], 1 methylene carbon [δ_C = 24.3 (C-4)], 1 methyl carbon [δ_C = 17.1 (C-1′)], and 5 quaternary carbons [δ_C = 173.6 (C-3′), 107.8 (C-4′), 127.4 (C-4″), 138.6 (C-8′), and 131.3 (C-9′)]. In comparison with the similar ^{1}H NMR data of MTCA reported by Piacente et al., peak II was 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) with $C_{13}H_{14}O_2N_2$ and a MW of 230.[24] The structure was further confirmed according to the match of the HPLC retention time with an authentic MTCA run concurrently (Figure 3II).

XOI Kinetics of 5-HMF and MTCA. Both 5-HMF and MTCA inhibited XO activity in a dose-dependent manner within the tested concentration ranges (Figure 6). The linear regression equations of the corresponding inhibition curve were

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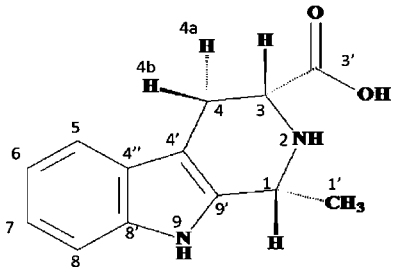
Table 2. ^{1}H NMR Chemical Shift Data of Peak 2 in Comparison with the Data of 1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) Reported by Piacente et al.[24]

C position	δ	δ (Piacente et al.[a])
1	4.70 (1H, dd, J = 6.8 Hz)	4.69 (1H, m)
1′	1.75 (3H, d, J = 6.8 Hz)	1.75 (3H, d, J = 6.0 Hz)
3	3.96 (1H, dd, J = 12.0, 5.2 Hz)	3.93 (1H, dd, J = 12.0, 5.2 Hz)
4a	3.02 (1H, ddd, J = 16.2, 12.0, 2.4 Hz)	3.03 (1H, ddd, J = 16.0, 12.0, 2.5 Hz)
4b	3.44 (1H, ddd, J = 16.2, 5.2, 1.2 Hz)	3.47 (1H, ddd, J = 16.0, 5.2, 1.3 Hz)
5	7.47 (1H, d, J = 8.0 Hz)	7.49 (1H, dd, J = 7.8, 1.5 Hz)
6	7.04 (1H, td, J = 8.4, 1.2 Hz)	7.10 (1H, td, J = 7.8, 1.5 Hz)
7	7.13 (1H, td, J = 8.0, 1.2 Hz)	7.16 (1H, td, J = 7.8, 1.5 Hz)
8	7.33 (1H, d, J = 8.4 Hz)	7.35 (1H, dd, J = 7.8, 1.5 Hz)

[a]Chemical structure of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA):



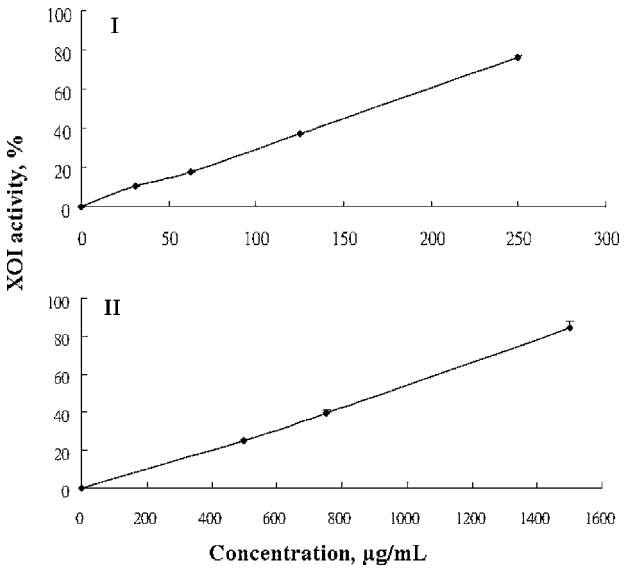


Figure 6. Xanthine oxidase inhibitory activities of 5-HMF (I) and MTCA (II) determined by reaction with various inhibitor concentrations. Each value represents the mean ± SD (n = 3).

$y = 0.0030x - 0.0029$ ($R^2 = 0.9990$) and $y = 0.0006x - 0.0162$ ($R^2 = 0.998$). The IC_{50} values were 168 and 860 μg/mL for 5-HMF and MTCA, respectively. The enzyme inhibitory mechanisms of 5-HMF and MTCA were elucidated at IC_{50} levels with different concentrations of xanthine solutions. The obtained Michaelis–Menten kinetics and Lineweaver–Burk plots, as shown in Figure 7, revealed that 5-HMF inhibited XO activity through an uncompetitive mechanism, whereas MTCA acted in a competitive way. The observed varied modes of action of 5-HMF and MTCA can be attributed to the nature of the structure–activity relationship. Allopurinol (1,5-dihydro-4*H*-pyraxolo[3,4-*d*]pyrimidin-4-one), the best-known XO inhibitor, is a structural analogue of hypoxanthine and inhibits XO activity by competitive mechanism.[25] As compared to chemical structures, there are varied extents of structural similarity among allopurinol, MTCA, and xanthine. Thus, MTCA plays a role as a xanthine analogue in the competition of substrate–enzyme complex formation.

Several research groups have widely explored XO inhibitors from natural sources, such as medicinal plants or herbs that are used in traditional medicine.[7,8] Pauff et al. found that luteolin and quercetin were competitive inhibitors, whereas silibinin was a mixed-type inhibitor of XO.[9] In this study, we revealed that 5-HMF and MTCA were XOI components in vinegar. Both 5-HMF and MTCA have been detected in different types of foodstuffs, medicinal plants, and herbs at various levels.[26,27] 5-HMF is formed by direct dehydration of hexose under an acidic condition or from the Maillard reaction of reducing sugars and amino acids during heat processing.[19,28,29] Few biofunctional studies for 5-HMF have been conducted. However, 5-HMF is a potent antioxidant and has been shown to exert beneficial effects in preventing hydrogen peroxide-induced hepatotoxicity and sickle cell anemia and to prolong the survival of forebrain ischemia animals.[30−32] The anti-inflammatory potential of 5-HMF has recently been demonstrated as well by the newly published work from Kitts et al., proving that 5-HMF-containing MRP fractions suppressed the expression of nitric oxide synthase 2 (NOS 2) and interleukine-8 (IL-8) in cultured human colorectal adenocarcinoma cells, likely through the down-regulation of nuclear factor-kappa B (NFκB).[19] NFκB is a reactive oxygen species (ROS) sensitive transcription factor, targeting various inflammation-related genes. XO is a main endogenous source of superoxide ($O_2^{\bullet-}$) that plays a critical role in NFκB activation during innate immune response.[33,34] Therefore, the XO inhibitor properties make 5-HMF a potential agent for reducing the risk of hyperuricemia and relieving gouty inflammation.

MTCA is formed by a condensation reaction of tryptophan and an aldehyde and is present in fermented foods such as beers, soy sauce, and miso and has been detected in body fluids including urine and human milk as well.[22,35,36] MTCA is a member of the β-carboline alkaloids, which are a group of compounds valued for their diverse biological activities.[37,38]

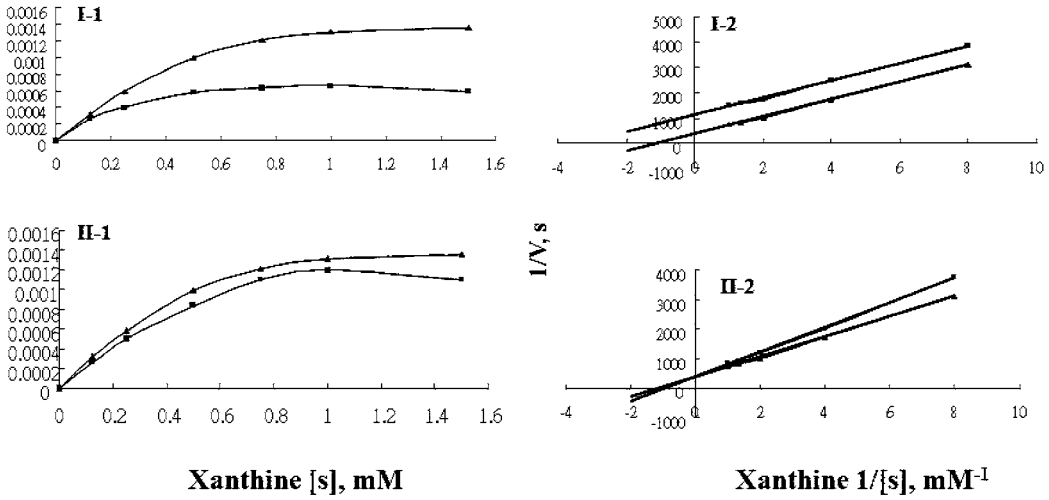


Figure 7. Lineweaver–Burk plots of xanthine oxidase inhibitory activities of 5-HMF (I) and MTCA (II) determined by reaction with various xanthine concentrations. Each value represents the mean ± SD (n = 3).

MTCA is a potent hydrogen peroxide scavenger and has been shown to protect against oxidative stress in human tissues.[39,40] MTCA has also exhibited inhibitory effects in lipopolysaccharide (LPS)-induced NO production by macrophages and aggregation response of human platelets.[39−41] Therefore, the results of this study augment the potential usefulness of 5-HMF and MTCA in health-promoting and therapeutic interventions.

In conclusion, XOI activity was detected in the collected seven commercial vinegars and the laboratory-prepared red-koji vinegar. From the laboratory-prepared red-koji vinegar, 5-HMF and MTCA were isolated and identified as the bioactive XOI compounds. Their IC_{50} levels for XO were 168 and 860 μg/mL. As supported by Michaelis–Menten kinetics analysis, 5-HMF was further characterized as an uncompetitive inhibitor and MTCA as a competitive inhibitor for XO. XOI activity was detected in the fermented fruit and rice vinegars and 5-HMF and MTCA were identified as two of the active compounds, comprising the first scientific evidence given to support the relief of gout-caused discomfort by the ingestion of vinegars. In the future, the related in-depth investigation and product development deserve research interest and industrial attention.

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