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Three new isopimaric acid diterpenoids from the bark of *Cryptomeria japonica* and their xanthine oxidase inhibitory activity

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ABSTRACT

Three new isopimaric acid diterpenoids, 6-oxoisopimaric acid (**1**), 6α-hydroxyisopimaric acid (**2**), and isopimara-7,9(11),15-trien-18-oic acid (**4**), together with two known isopimaric acid diterpenoids, isopimaric acid (**3**), and 8(14),15-isopimaradien-18-oic acid (**5**), were isolated from the bark of *Cryptomeria japonica* D. Don. Their structures were determined by analysis of spectroscopic data and comparison with the spectral data of known analogues. At the concentration of 50 μM, compounds **1**–**5** inhibited xanthine oxidase activity by 17.3, 16.5, 2.6, 30.5, and 24.5 %, respectively.

1. Introduction

Cryptomeria japonica D. Don belongs to the monospecific genus *Cryptomeria* in the cypress family Cupressaceae. It is endemic to Japan, known as sugi (Japanese cedar) in Japanese (Gan, 1958), and is a massive evergreen coniferous tree, growing up to in height. This plant exhibits aromatic reddish-pink wood with the good wood workability, such as soft, lightweight but strong, and waterproof properties and thus became one of the most representative plantation tree species in Asia. Its wood has been commonly used as a building material for Japanese-style houses and other wood products. Due to its excellent wood properties, it has been widely cultivated as an important plantation coniferous tree species in Taiwan since 1906. The leaves, heartwood, and barks of this plant is reported to contain various terpenoids, including monoterpenoids, sesquiterpenoids, and diterpenoids (Arihara et al., 2004; Chen et al., 2001; Kofujita et al., 2001, 2002; Morita et al., 1995; Nagahama et al., 1993, 1998; Narita et al., 2006; Shibuya, 1992; Shieh et al., 1981; Shimizu et al., 1988; Su et al., 1996; Morisawa et al., 2002; Yoshikawa et al., 2006), some of which showed antibacterial (Li et al., 2008), antifungal (Kofujita et al., 2001), cytotoxic (Kofujita et al., 2002), anti-inflammatory (Shyur et al., 2008), anti-androgenic (Tu et al., 2007), and insect antifeedant (Wu et al., 2008) and repellent (Morisawa et al., 2002) properties by researchers. A continuous search for bioactive compounds from the bark of *C. japonica*, we have already reported three sesquarterpenes (Chen et al., 2010; Chang et al., 2017c) and thirteen abietane-type diterpenoids (Chang et al., 2016, 2017a; Chang et al., 2017b, 2018). Herein, we describe the structure elucidation of three new compounds (**1**, **2**, and **4**), and the isolation and enzyme inhibitory

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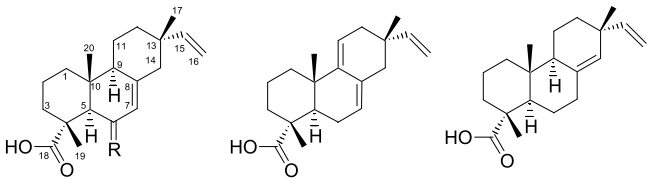
activity of compounds **1**–**5** (Fig. 1).

2. Results and discussion

The EtOAc soluble portion partitioned from methanol extract of the bark of *C. japonica* was subjected to repeated chromatography on silica gel followed by semipreparative NP-HPLC. Three new isopimaric acid diterpenoids, 6-oxoisopimaric acid (**1**), 6α-hydroxyisopimaric acid (**2**), and isopimara-7,9(11),15-trien-18-oic acid (**4**), together with two known isopimaric acid diterpenoids, isopimaric acid (**3**) (Li et al., 2008) and 8(14),15-isopimaradien-18-oic acid (sandaracopimaric acid, **5**) (Venditti et al., 2017) were obtained.

The HR-EI-MS of **1** gave a molecular ion at *m/z* 316.2029, consistent with the molecular formula of $C_{20}H_{28}O_3$, implying seven degrees of unsaturation. The UV maxima (241 nm) and IR absorptions (1666 cm^{-1}) of **1** indicated the presence of an α,β-unsaturated ketone functionality. The IR absorptions for a carboxylic acid group (3400−2500, 1690 cm^{-1}) was also observed. The ^1H and ^{13}C NMR spectra of **1** (Table 1) demonstrated three tertiary methyls [δ_H 0.90, 0.98, 1.44 (each 3H, s, Me-17, Me-20, and Me-19)], a α,β-unsaturated ketone group [δ_H 5.79 (1H, s, H-7); δ_C 126.5 (d, C-7), 160.3 (s, C-8), and 197.6 (s, C-6)], and a vinylic ABX system [δ_H 4.92 (1H, dd, J = 11.6, 0.8 Hz), 4.96 (1H, dd, J = 18.4, 0.8 Hz), and 5.79 (1H, dd, J = 18.4, 11.6 Hz); δ_C 110.3 (t, C-16), 148.4 (d, C-15)], and one isolated carboxyl group [δ_C183.3 (s, C-18)]. 20 carbon signals were found in the ^{13}C NMR spectrum of **1** and were assigned by a DEPT experiment as three aliphatic methyl, six aliphatic methylene, two aliphatic methine, three aliphatic quaternary, one olefinic methylene, two olefinic methine, one quaternary olefinic, and two quaternary carbonyl carbons. From the above structural characteristics, compound **1** was thus tentatively proposed to be a pimarane or isopimarane-type diterpene (Li et al., 2008). The ^{13}C NMR resonances of **1** were very similar to the corresponding signals of isopimaric acid in the rings A and C (Li et al., 2008), except for the signals of C-5–10 in the ring B. The HMBC correlations between H-5 (δ_H 2.98)/C-4, C-6, C-9, C-10, C18, C-19 and C-20 and H-7 (δ_H 2.81)/C-9 and C-14 indicated that a α,β-unsaturated ketone located at C-6 (δ_C 197.6), C-7 (δ_C 126.5), and C-8 (δ_C 160.3) in ring B (Fig. 1). Additionally, the HMBC correlations between H-15 (δ_H 5.79)/C-12 and C-13; Me-17 (δ_H 0.90)/C-12 and C-15; Me-19 (δ_H 1.44)/C-3, C-4 and C-18; and Me-20 (δ_H 0.98)/C-1, C-5, and C-10 helped to construct the planar structure of **1**. The relative configurations of sterogenic C-atoms in the tricyclic rings were determined by significant NOE correlations between H-5/H-9 (δ_H 2.21), H-9/H_α-12 (δ_H 1.52), H_β-12 (δ_H 1.63)/Me-17 (δ_H 0.90), H-14 (δ_H 2.17)/H-15 (δ_H 5.79), and Me-19 (δ_H 1.44)/Me-20 (δ_H 0.98) in the NOESY spectrum (Fig. 2). Besides, the ^{13}C NMR signal of Me-17 appeared at δc 22.3 in **1**, which was further confirmed to situate in axial orientation by the following reasons. Compound **1** showed high structure similarity and similar ^{13}C NMR chemical shift of Me-17 to that of isopimaric acid. By the comparison of ^{13}C NMR signal of Me-17 of **1** with several similar isopimane and pimrane compounds, such as isopimaric acid (Li et al., 2008) and 13-epimer of isopimaric acid, 7,15-pimaradien-18-oic acid (Zgoda-Pols et al., 2002). When the Me-17 was posited in axial orientation in isopimaric acid would receive the γ-gauche effect from both C-8 and C-11,



1 R = O
2 R = α-OH, β-H
3 R = H_2

4 **5**

Fig. 1. Structures of compounds **1-5**.

Table 1
^1H NMR data for compounds **1**, **2**, and **4**. ($CDCl_3$, δ in ppm, J in Hz, 400 MHz for ^1H NMR, 100 MHz for ^{13}C NMR).

	1		2		4	
No.	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	37.9	1.27 m, 1.79 br d (14.0)	38.7	1.15 m, 1.65 m	39.5	1.45 br d (12.4), 1.62 m,
2	17.7	1.50 m, 1.58 m	17.8	1.51 m	18.5	1.60 m, 1.63 m
3	37.5	1.71 m, 1.74 m	38.0	1.76 m	36.9	1.62 m, 1.84 m
4	42.8		43.8		47.5	
5	59.2	2.98 s	52.1	2.08 d (10.4)	40.5	3.56 dd (12.8, 2.8) 1.94 ddd (12.8, 3.6,
6	197.6		67.6	4.07 d (10.4)	28.6	2.8), 2.10 td (12.8, 3.6)
7	126.5	5.79 s	125.8	5.33 s	110.7	4.64 t (3.6)
8	160.3		137.0		152.6	
9	51.9	2.21m	51.7	1.80 m	169.2	
10	39.1		36.9		42.8	
11	19.7	1.60 m, 1.76 m	19.8	1.34 m	105.5	4.92 dd (8.0, 5.6)
12	35.2	1.52 m, 1.63 m	36.1	1.33 m, 1.48 m	35.6	1.58 dd(13.6, 8.0), 2.69 dd (13.6, 5.6)
13	37.0		36.2		36.3	
14	45.7	2.17 s	45.6	1.92 d (14.4), 1.99 d (14.4)	47.1	1.93 d (12.8), 2.40 d (12.8)
15	148.4	5.79 dd (18.4, 11.6)	149.7	5.78 dd (17.6, 10.8)	149.2	5.83 dd (17.2, 10.8)
16	110.3	4.92 dd (11.6, 0.8), 4.96 dd (18.4, 0.8)	109.4	4.86 dd (10.8, 1.2), 4.91 dd (17.6, 1.2)	109.8	4.92 dd (10.8, 1.2), 4.98 dd (17.2, 1.2)
17	22.3	0.90 s	21.7	0.84 s	21.8	0.88 s
18	183.3		185.5		184.5	
19	17.5	1.44 s	17.0	1.35 s	21.8	1.18 s
20	15.3	0.98 s	15.9	0.91 s	19.9	1.18 s

a) Coupling constants are presented in Hz.

and thus its ^{13}C NMR signal appeared in higher field region (δc 21.7) than that of 7,15-pimaradien-18-oic acid (δc 30.1). From the above evidences, the structure of **1** was determined as 6-oxoisopimaric acid.

The HR-EI-MS of compound **2** showed an $[M]^+$ ion at *m/z* 318.2180, which was consistent with the molecular formula $C_{20}H_{30}O_3$, indicating six degrees of unsaturation. The IR spectrum indicated the presence of carboxyl group (3400−2500 and 1692 cm^{-1}). In the ^1H and ^{13}C NMR spectra of **2** (Table 1), the signals for a vinylic ABX system [δ_H 4.86 (1H, d, J = 10.8, 1.2 Hz), 4.91 (1H, dd, J = 17.6, 1.2 Hz), and 5.78 (1H, dd, J = 17.6, 10.8 Hz); δ_C 109.4 (t), 149.7 (s)] and a trisubstituted double bond moiety [δ_H 5.33 (1H, s); δ_C 125.8 (d), 137.0 (s)], one carbinol methine [δ_H 4.07 (1H, d, J = 10.4 Hz); δ_C 67.6 (d)], and one isolated carboxyl group [δ_C185.5 (s, C-18)] were found. The ^{13}CNMR spectrum of **2** revealed twenty skeletal carbon resonances, including three aliphatic methyl, six aliphatic methylene, two aliphatic methine, three aliphatic quaternary, one olefinic methylene, two olefinic methine, one quaternary olefinic, one oxygenated methine, and one quaternary carbonyl carbons. From the above evidences, compound **2** was proposed to be also a isopimarane-type diterpene. The ^1H and ^{13}C NMR resonances of **2** were very similar to the corresponding signals of **1**, except for a carbinol group [δ_H 4.07 (1H, d, J = 10.4 Hz); δ_C 67.6 (s)] located at C-6 in **2**, instead of a ketone carbonyl group [δ_C197.6 (s)] in **1**. The carbinol methine proton, H-6, with a large coupling constant, 10.6 Hz, showed the NOESY correlations with Me-19 and Me-20, as well as H-5 showed both the HMBC correlations with C-4, C-6, C-7 C-9, C-10, and C-18, and the NOESY correlation with H-9, which confirmed that the hydroxy group attached on C-6 in α-equatorial orientation (Fig. 2). The Me-

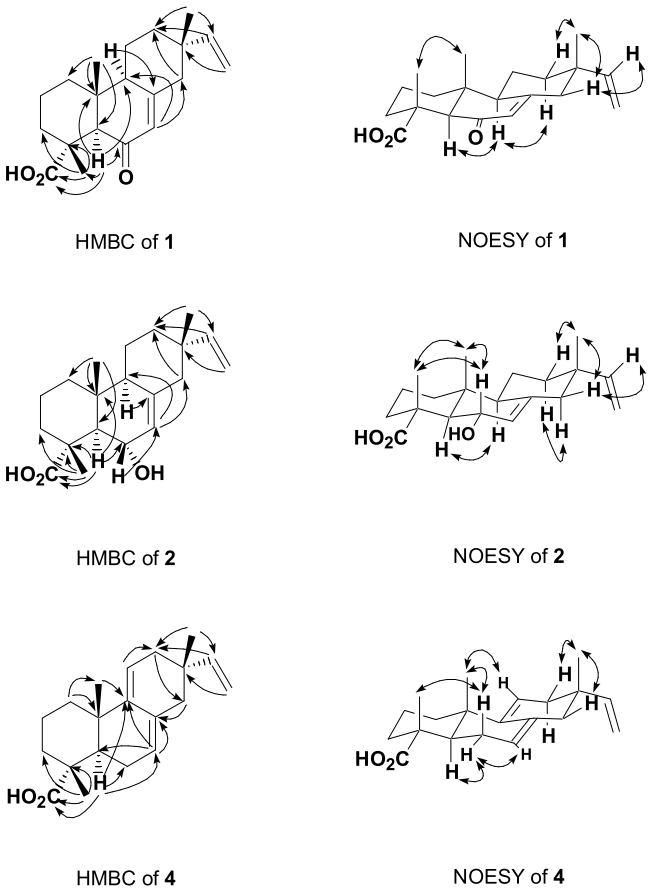


Fig. 2. Selected HMBC and NOE correlations of **1**, **2**, and **4**.

17 was assigned to be in axial orientation in **2**, which was supported by both its upfield ^{13}C NMR chemical shift (δ_C 21.7) and the NOESY correlations between Me-17/H_β-12 (δ_H 1.48). Thus, the structure of **2** was elucidated as 6α-hydroxyisopimaric acid.

The molecular formula of compound **4** was determined as $C_{20}H_{28}O_2$ from the HR-EI-MS $[M]^+$ *m/z* 300.2079, indicating seven degrees of unsaturation. The IR spectrum displayed absorption bands for a carboxylic acid group (3400−2500, 1692 cm^{-1}) and vinyl group (1645, 976, 910 cm^{-1}). The UV maxima at 220 nm indicated the presence of a conjugated diene functionality. The ^1H and NMR spectra of **4** (Table 1) exhibited signals for the presence of the signals for a vinylic ABX system [δ_H 4.92 (1H, d, $J = 10.8$, 1.2 Hz), 4.98 (1H, dd, $J = 17.2$, 1.2 Hz), and 5.83 (1H, dd, $J = 17.2$, 10.8 Hz); δ_C 109.8 (t), 149.2 (s)] and two trisubstituted double bond moieties [δ_H 4.64 (1H, t, $J = 3.6$ Hz); δ_C 110.7 (d), 152.6 (s) and δ_H 4.92 (1H, dd, $J = 8.0$, 5.6 Hz); δ_C 105.5 (d), 169.2 (s)], and one isolated carboxyl group [184.5 (s)]. The ^{13}C NMR spectrum of **4** revealed 20 resonances including three aliphatic methyl, six aliphatic methylene, one aliphatic methine, three aliphatic quaternary, one olefinic methylene, three olefinic methine, two quaternary olefinic, and one quaternary carbonyl carbons. By comparison of the ^1H and ^{13}C NMR data with those of the known compound, isopimaric acid (**3**) (Li et al., 2008), indicated that both compounds exhibited identical structure in ring A. Thus, the conjugated diene moiety would be located at C-7–9 and C-11, which was confirmed by the HMBC correlations between H-7/C-5, C-8, and C-9 and H-11/C-10 and C-12. The relative configurations of sterogenic C-atoms in the tricyclic rings were determined by significant NOE correlations between H-5/H_α-6 (δ_H 1.94), H_α-6/H-7, H_β-6 (δ_H 2.10)/Me-19, H_β-6/Me-20, and H_β-12 (δ_H 1.58)/Me-17 in the NOESY spectrum (Fig. 2). Thus, compound **4** was characterized as isopimara-7,9(11),15-trien-18-oic acid. Complete ^1H and ^{13}C NMR chemical shifts were established by ^1H- ^1H COSY, HMQC, HMBC, and NOESY spectra.

Xanthine oxidase is a key enzyme in the pathway of purine metabolism. It catalyzes the oxidation of hypoxanthine to xanthine, and of xanthine to uric acid and plays an important role in causing gout (Cos et al., 1998). Lin et al. have reported that abieta-8,11,13-triene diterpene derivatives exhibit inhibitory activity toward xanthine oxidase. (Lin et al., 2010). Therefore, compounds **1**-**5** were evaluated for their xanthine oxidase inhibitory potential in this study (Chen et al., 2009). At the concentration of 50 μM, compounds **1**–**5** inhibited xanthine oxidase activity by 17.3, 16.5, 2.6, 30.5, and 24.5 %, respectively, whereas the two positive controls, quercetin and allopurinol, inhibited xanthine oxidase activity by 35.1 and 100 %, respectively.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Jasco-DIP-180 polarimeter. UV and IR spectra were recorded on a Shimadzu UV-1601PC and a Perkin-Elmer spectrophotometer, respectively. ^1H and ^{13}C NMR and 2D NMR spectra were acquired on a Varian-Unity-Plus-400 spectrometer. Chemical shifts are referenced to residual solvent signals. EI-MS and HR-EI-MS were obtained on a Jeol-JMS-HX300 mass spectrometer. Merck Silica gel 60 (230–400 mesh; Merck KgaA) was used for column chromatography (CC). Thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (silica gel 60 F_{254}; Merck KgaA). Semi-preparative HPLC was performed using a normal phase column (Purospher STAR Si, 5 μm, 250 × 10 mm; Merck KgaA) on a LDC Analytical-III system.

3.2. Plant material

The bark of *C. japonica* D. Don was collected in Sitou, Taiwan in June 2000. A voucher specimen (TCF13443) has been deposited at the Herbarium of the Department of Forestry, NCHU, Taiwan. Species identification was confirmed by Dr. Yen-Hsueh Tseng, Department of Forestry, National Chung-Hsing University (NCHU).

3.3. Extraction and isolation

The air-dried bark of *C. japonica* (16.0 kg) was extracted by soaking in MeOH (100 L × 3) at room temperature for 7 days each time in a closed container. The combined MeOH extracts was concentrated under reduced pressure at 45 ℃ to produce a brown crude residue (480 g), which was suspended in H_2O (1 L), and then partitioned sequentially with EtOAc (1 L) and *n*-BuOH (1 L) to afford EtOAc, *n*-BuOH, and H_2O soluble fractions, respectively. The EtOAc fraction (430 g) was chromatographed over a silica gel (4.0 kg) column, eluted with a *n*-hexane–EtOAc gradient followed by a EtOAc–MeOH gradient of increasing polarity to obtain 11 fractions, fr. 1 (2.6 g), 2 (29.4 g), 3 (47.8 g), 4 (92.4 g), 5 (21.6 g), 6 (18.1 g), 7(22.5 g), 8 (35.8 g), 9 (19.2 g), 10 (44.2 g), and 11 (72.2 g). Fr. 4 from *n*-hexane–EtOAc (4:1) elution was further purified through a silica gel column (7 × 60 cm), eluted with a gradient mixture of CH_2Cl_2–EtOAc (100:1 to 0:1) to obtain sixteen fractions, 4A - 4 P. Further purification of subfraction 4 N by semi-preparative HPLC afforded **5** (20.2 mg, t_R = 31.2 min) using *n*-hexane–EtOAc (7:3) at the flow rate of 2.0 mL/min. Subfraction 4O was further purified by semi-preparative HPLC to afford **3** (32.3 mg, t_R = 33.9 min), eluting with *n*-hexane–EtOAc (7:3) at the flow rate of 2.0 mL/min. Fr. 5 from *n*-hexane–EtOAc (7:3) elution was further purified over a silica gel column (5 × 45 cm), eluted with *n*-hexane−CH_2Cl_2–EtOAc (8:8:1 to 0:1:1) to yield fifteen fractions, 5A - 5O. Further purification of subfraction 5 K by semi-preparative HPLC gave **1** (2.7 mg, t_R = 38.2 min) using *n*-hexane–EtOAc (7:3) at the flow rate of 2.0 mL/min. Further purification of subfraction 5 L by HPLC gave **4** (2.5 mg) using *n*-hexane–EtOAc (7:3). Fr. 6 from *n*-hexane–EtOAc (1:1)

elution was further purified over a silica gel column (5 × 45 cm), eluted with CH_2Cl_2–EtOAc (50:1 to 0:1) to yield ten fractions, 6A - 6 J. Further purification of subfraction 6 F by semi-preparative HPLC gave **2** (1.5 mg, t_R = 28.2 min) using *n*-hexane–EtOAc (3:2) at the flow rate of 2.0 mL/min.

3.3.1. 6-oxoisopimaric acid (1)

Gum; [α] +9.1 (*c* 0.5, $CHCl_3$); IR ν_{max} 3436, 3400−2500, 1690, 1666, 1560, 1460, 1381, 1255, 1129, 996, 903, 857 cm^{-1}; UV (MeOH) $_{max}$ (log) 241 (3.64) nm; ^1H and ^{13}C NMR data, see Table 1; EI-MS (%) *m/z* 316 (4) $[M]^+$, 298 (50) $[M\text{-}H_2O]^+$, 283 (24) $[M\text{-}H_2OCH_3]^+$, 270 (20), 255 (93), 239 (92), 227 (36), 213 (48), 199 (63), 187 (39), 105 (54), 91 (100). HR-EI-MS $[M]^+$ *m/z* 316.2029 (calcd for $C_{20}H_{28}O_3$ 316.2039).

3.3.2. 6α-hydroxyisopimaric acid (2)

Gum; [α] +29.7 (*c* 0.5, $CHCl_3$); IR ν_{max} 3396, 3400−2500, 1692, 1460, 1387, 1255, 1155, 1029, 990, 903, 738 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EI-MS (%) *m/z* 318 (3) $[M]^+$, 300 (100) $[M\text{-}H_2O]^+$, 285 (92) $[M\text{-}H_2OCH_3]^+$, 272 (22), 257 (26), 255 (40), 239 (83), 229 (38), 185 (26). HR-EI-MS $[M]^+$ *m/z* 318.2180 (calcd for $C_{20}H_{30}O_3$ 318.2196).

3.3.3. isopimara-7,9(11),15-trien-18-oic acid (4)

Gum; [α] +43.7 (*c* 0.5, $CHCl_3$); IR ν_{max} 3400−2500, 1692, 1660, 1645, 1454, 1381, 1275, 1162, 1082, 976, 910, 824, 731 cm^{-1}; UV (MeOH) $_{max}$ (log) 220 (4.67, sh) nm; ^1H and ^{13}C NMR data, see Table 1; EI-MS (%) *m/z* 300 (70) $[M]^+$, 285 (45), 229 (21), 217 (26), 203 (38), 189 (100), 91 (31), 55 (31). HR-EI-MS $[M]^+$ *m/z* 300.2079 (calcd for $C_{20}H_{28}O_2$ 300.2090).

3.4. Xanthine oxidase inhibition assay

The XO inhibition assay was performed according to the procedure as described by Chen et al. with slight modifications (Chen et al., 2009). Briefly, 20 μL of the sample solution (final concentration was 50 μM) was added to 35 μL of 0.1 mM phosphate buffer (pH = 7.5) and 30 μL of enzyme solution (0.01 units/mL in 0.1 mM phosphate buffer, pH = 7.5). The solution was mixed thoroughly by vortexing and preincubated for 15 min at, and then the reaction was initiated by the addition of 60 μL of substrate solution (150 mM xanthine in the same buffer). The reaction mixture was incubated for further 30 min at The reaction was stopped by adding 50 μL of 2 N HCl, and the absorbance was measured at 290 nm by using a UV spectrophotometer. The percentage activity of xanthine oxidase was calculated according to the following equation: XO Inhibition (%) = (1-B/A) × 100, where A and B are the activities of the enzyme without and with test sample. Quercetin and allopurinol were used as positive controls, whereas negative control was performed without any inhibitor.

Declaration of Competing Interest

The authors declared that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phytol.2021.09.005.

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