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Original Research Article

Limonoid Constituents of *Xylocarpus granatum* Fruits from Vietnam: Antioxidant, Anti-Inflammatory Activities, and Molecular Docking Studies

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ABSTRACT

Xylocarpus granatum J. Koenig, belonging to the Meliaceae family, is known for its various phytochemical constituents and significant biological activities. This study aimed to determine the limonoid components of *X. granatum* fruits and investigate their *in vitro* and *in silico* bioactivities. These limonoids were isolated by using chromatographic methods. Their structures were determined based on NMR and ESI-MS spectra and compared to the published data. Moreover, these compounds were assayed for *in vitro* antioxidant and anti-inflammatory activities. Molecular docking study was applied to understand better how these four compounds influence their anti-inflammatory capabilities. Phytochemical investigation of *X. granatum* fruits led to the isolation of four limonoids, including hainangranatumin A (**1**), xylogranatin C (**2**), xylocarpin F (**3**), and xyloccensin K (**4**). In addition, their antioxidant and anti-inflammatory activities were assayed and evaluated for the first time. All isolates expressed no remarkable antioxidant activity determined by the DPPH and ABTS tests. Meanwhile, the anti-inflammatory assay demonstrated that four limonoids showed significant inhibitory activity in LPS-induced RAW 264.7 macrophages with IC_{50} values ranging from 7.36 ± 0.23 to 12.71 ± 0.71 µg/mL (dexamethasone as the positive control, IC_{50} = 5.41 ± 0.46 µg/mL). *In silico* study shows that four limonoids (**1**-**4**) exhibited binding affinities ranging from -8.540 to -8.178 kcal/mol for TNF-α and from -9.924 to -7.014 kcal/mol for COX-2, compared to the reference compounds. The present work demonstrates that limonoids isolated from *X. granatum* are potential candidates for drug research aimed at treating inflammatory diseases.

Keywords: *Xylocarpus granatum*, Limonoid, Antioxidant activity, Anti-inflammatory activity, Docking.

Introduction

The genus *Xylocarpus* (Meliaceae family) contains at least three distinct species, including *X. moluccensis*, *X. granatum*, and *X. Rumphii*.[1] Among these, *X. Granatum* (Vietnamese name: su ổi, xương cá) is commonly found over the shores of Southeast Asia, Australia, East Africa, and Indian Ocean.[2] Traditionally, people have used *X. granatum* to treat diarrhea, cholera, and feverish diseases such as malaria, and also as an antifeedant.[3] Previous phytochemical studies have indicated that *X. granatum* contains various constituents, including limonoids,[4-8] lactones, steroids, and flavonoids.[9] Limonoids are the major natural compounds (with more than 130 compounds and derivatives being identified) from the different parts of the plant with compounds that have shown various biological activities.[10]
Nowadays, research on the antioxidant and anti-inflammatory activities of plant-based components has received increased attention.[11-16] In the present work, four limonoids from *X. granatum* fruits growing wild in Vung Tau province, Vietnam were isolated, purified and structure elucidated.

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The antioxidant and anti-inflammatory activities of these compounds were also investigated. The in vitro anti-inflammatory assay was supported by molecular docking study.

Materials and Methods

Plant materials
Fresh fruits of *X. granatum* were collected from Vung Tau city, Ba Ria - Vung Tau province, Vietnam, in August 2019. The scientific name of the plant was authenticated by Dr. Nguyen Quoc Binh (Vietnam National Museum of Nature, Vietnam Academy of Science and Technology, VAST). A voucher specimen (XG-082019) was deposited at the Natural Products Laboratory, Department of Chemistry, Vinh University, Vietnam.

General experimental procedures
The NMR spectra were acquired on a Bruker AV-III 500 NMR spectrometer using $CDCl_3$ as solvent. Chemical shifts are given as δ values with tetramethylsilane (TMS) as the internal reference, and coupling constants are given in Hertz (Hz). Electrospray Ionization Mass Spectrometry (ESI-MS) was performed using a Shimadzu LCMS-8045 mass spectrometer (Shimadzu Corp., Japan). Preparative high-performance liquid chromatography (Prep-HPLC) was performed on an Agilent 218 Purification System with ZORBAX SB-C_{18} columns (size: 100 mm × 21.2 mm, 5 µm, and size: 250 mm × 9.4 mm, 5 µm). Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70-230 mesh, Merck), C_{18} reverse-phased silica gel (RP-18, 15-25 µm, Merck) and Sephadex LH-20 (GE Healthcare). Thin-layer chromatography (TLC) was carried out on precoated Kieselgel 60 F_{254} plates (Merck) and detection was visualized by

spraying with aqueous H_2SO_4 (10%) followed by heating. All the chemicals and reagents used were of analytical grade.

Extraction and isolation

The dried and powdered fruits of *X. granatum* (10 kg) were extracted using ultrasound-assisted equipment in MeOH at 45 ℃ (15L solvent × 90 min × three times) and concentrated in a vacuum to yield MeOH extract (185 g). The MeOH extract was suspended in water and successively partitioned with *n*-hexane and ethyl acetate to afford *n*-hexane (20 g), ethyl acetate (95 g) residues, and the water layer (30g). The ethyl acetate residue (90 g) was subjected to CC, eluting with *n*-hexane/acetone (30/1, 25/1, 20/1, 10/1, 5/1, 1/1, v/v) to give six fractions (XGE1-XGE6). Fraction XGE2 (10.5 g) was subjected to CC, eluting with *n*-hexane/ethyl acetate (12/1, 10/1, 8/1, v/v) to give five subfractions (XGE2.1-XGE2.5). Fraction XGE2.4 (38 mg) was purified by Prep-HPLC (ZORBAX SB-C_{18} column, 250 mm × 9.4 mm, 5 μm) eluting with CH_3CN/H_2O (55/45, v/v, a flow rate of 12 mL/min) to afford compound **1** (13.5 mg). Fraction XGE4 (19 g) was separated by a Sephadex LH-20 CC, eluting with MeOH to yield four subfractions (XGE4.1-XGE4.4). Fraction XGE4.3 (45 mg) was purified by Prep-HPLC (ZORBAX SB-C_{18} column, 100 mm × 21.2 mm, 5 μm) eluting with CH_3CN/H_2O (12/13, v/v, a flow rate of 15 mL/min) to give compound **2** (16.0 mg). The XGE5 fraction (15 g) was repeatedly chromatographed on an RP-18 CC eluting with MeOH/H_2O (3/7, 4/6, v/v) to afford seven subfractions (XGE5.1-XGE5.7). Fraction XGE5.4 (56 mg) was purified by Prep-HPLC (ZORBAX SB-C_{18} column, 250 mm × 9.4 mm, 5 μm) eluting with CH_3CN/H_2O (23/27, v/v, a flow rate of 10 mL/min) to obtain compounds **3** (10.5 mg) and **4** (14.2 mg).

Assessment of antioxidant activity

The antioxidant activity of four isolates from *X. granatum* fruits was measured by using DPPH and ABTS methods. The experiments were carried out following the procedure outlined in the previous report with minor modifications.[17]

For the DPPH assay, the isolates were dissolved in DMSO at various concentrations and subsequently mixed with the 3 mM DPPH solution and incubated for 30 min, after which the mixture was spectrophotometrically measured at 517 nm. DMSO was used as the negative control (NC), while ascorbic acid was used as a positive control. The DPPH scavenging activity (%) was calculated using the following equation:

$$\text{DPPH Scavenging Activity (\%)} = [(A_{NC} - A_t)/A_{NC}] \times 100 \text{ (\%)}.$$

Where A_{NC} stood for the absorbance of the negative control and A_t stood for the absorbance of the test samples.

For the ABTS assay, 50 μL of the tested sample was mixed with the working ABTS solution (200 μL) and incubated for 30 min, after which the absorbance was determined at 734 nm. Using the same formula as the DPPH scavenging activity, the ABTS scavenging activity was determined and represented as a percentage of reduced ABTS radicals.

Assessment of anti-inflammatory activity

The anti-inflammatory activity of the isolates from *X. granatum* fruits was investigated based on the inhibitory effect of these compounds on NO production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Dexamethasone was used as a positive control. The details of the experiment were carried out according to the method described in the literature,[18] with some minor modifications. In brief, 10% FBS was added to DMEM during the culture of the murine macrophage RAW 264.7 cell line. The RAW 264.7 macrophages were seeded in 96-well culture plates (100 mL/well) at a density of 4×10^4 cells/well. They were subsequently treated with LPS (0.1 mg/mL) in the presence or absence of test samples. The culture supernatant was exposed to Griess reagent for 10 min at room temperature and in the dark after 24h. The absorbance (A) of the mixtures was then recorded at a wavelength of 540 nm.

$$\text{Inhibition (\%)} = 100 \times (A_{\text{LPS treated}} - A_{\text{LPS + sample treated}})/(A_{\text{LPS treated}} - A_{\text{untreated}}).$$

Statistical analysis

Each compound's antioxidant and anti-inflammatory activities were measured in triplicate (n = 3). The results are reported as the mean ± standard deviation (SD) using Microsoft Excel (Microsoft, 2018).

Molecular docking

The structures of the limonoids were drawn using the Marvin JS software and then converted to 3D structures using PyMOL software. The docking studies of these isolated compounds were conducted using the AutoDockVina v1.2.3 program on the Ubuntu operating system, which is considered one of the fast and accurate programs used for docking.[19] The protein structures prepared for docking were downloaded from the RCSB protein data bank with the crystal structure models of human cyclooxygenase (COX-2) (PDB ID: 5KIR) and tumour necrosis factor-alpha (TNF-α) (PDB ID: 2AZ5).[20,21] These structures were loaded into AutoDockTools and prepared by removing unnecessary molecules for docking, adding hydrogen atoms, and Kollman partial charges. The grid parameters for the simulation were set as reported previously.[22] After the docking simulation, the highest-ranking poses were generated for each ligand, and finally, the interpretation and analysis of the docking results were completed. Discovery Studio Visualizer software was used to display the various interactions between the ligands and the residues in the active sites of the target proteins.

Results and Discussion

Characterization of the isolated compounds

Hainangranatumin A (compound **1**): white amorphous powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 7.55 (1H, s, H-21), 7.45 (1H, brs, H-23), 6.97 (1H, s, H-3), 6.47 (1H, brs, H-22), 6.46 (1H, s, H-30), 6.17 (1H, s, H-15), 5.30 (1H, s, H-17), 3.88 (1H, s, 8-OH), 3.68 (3H, s, 7-OCH_3), 3.04 (1H, dd, *J* = 20.0, 6.5 Hz, H-11a), 2.60 (1H, m, H-12a), 2.55 (1H, m, H-11b), 2.50 (1H, m, H-6a), 2.40 (1H, m, H-2′), 2.28 (1H, m, H-10), 2.27 (1H, m, H-6b), 2.25 (1H, m, H-5), 1.68 (1H, m, H-3′a), 1.63 (1H, m, H-12b), 1.44 (1H, m, H-3′b), 1.18 (3H, s, H-28), 1.15 (3H, d, *J* = 7.5 Hz, H-5′), 1.12 (3H, s, H-29), 1.04 (3H, d, *J* = 5.5 Hz, H-19), 0.97 (3H, s, H-18), 0.87 (3H, t, *J* = 7.5 Hz, H-4′); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 208.7 (C-9), 198.8 (C-1), 175.3 (C-1′), 173.4 (C-7), 163.4 (C-16), 163.3 (C-14), 161.6 (C-3), 143.3 (C-23), 141.5 (C-21), 128.8 (C-2), 119.6 (C-20), 118.9 (C-15), 109.8 (C-22), 80.2 (C-17), 80.1 (C-8), 67.0 (C-30), 52.0 (7-OCH_3), 45.3 (C-5), 42.8 (C-10), 41.3 (C-2′), 38.4 (C-13), 36.9 (C-4), 34.6 (C-6), 33.0 (C-11), 27.9 (C-28), 26.1 (C-3′), 25.7 (C-12), 20.4 (C-29), 18.6 (C-18), 17.0 (C-4′), 11.9 (C-5′), 11.5 (C-19); ESI-MS: *m/z* 583.4 $[M-H]^-$.

Xylogranatin C (compound **2**): white amorphous powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 7.55 (1H, t, *J* = 0.5 Hz, H-21), 7.44 (1H, brs, H-23), 7.00 (1H, s, H-3), 6.53 (1H, s, H-30), 6.46 (1H, brs, H-22), 6.18 (1H, s, H-15), 5.33 (1H, s, H-17), 3.88 (1H, s, 8-OH), 3.69 (3H, s, 7-OCH_3), 3.03 (1H, m, H-11a), 2.62 (1H, m, H-12a), 2.51 (1H, m, H-11b), 2.48 (1H, m, H-6a), 2.29 (1H, m, H-5), 2.28 (1H, m, H-10), 2.27 (1H, m, H-6b), 2.10 (3H, s, H-2′), 1.64 (1H, m, H-12b), 1.21 (3H, s, H-28), 1.12 (3H, s, H-29), 1.04 (3H, d, *J* = 6.0 Hz, H-19), 0.98 (3H, s, H-18); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 208.6 (C-9), 198.8 (C-1), 173.4 (C-7), 170.0 (C-1′), 164.2 (C-14), 163.4 (C-16), 162.1 (C-3), 143.3 (C-23), 141.5 (C-21), 128.5 (C-2), 119.6 (C-20), 118.2 (C-15), 109.8 (C-22), 80.2 (C-17), 80.1 (C-8), 67.4 (C-30), 52.0 (7-OCH_3), 45.3 (C-5), 42.9 (C-10), 38.4 (C-13), 36.9 (C-4), 34.6 (C-6), 33.0 (C-11), 27.9 (C-28), 25.6 (C-12), 21.1 (C-2′), 20.6 (C-29), 18.5 (C-18), 11.7 (C-19); ESI-MS: *m/z* 541.4 $[M-H]^-$.

Xylocarpin F (compound **3**): white amorphous powder; ESI-MS: *m/z* 585.3 $[M-H]^-$; ^{1}H-NMR (500 MHz, $CDCl_3$) and ^{13}C-NMR (125 MHz, $CDCl_3$) were reported in our previous study.[23]

Xyloccensin K (compound **4**): white amorphous powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (ppm): 7.56 (1H, brs, H-23), 7.45 (1H, brs, H-21), 6.49 (1H, brs, H-22), 6.28 (1H, s, H-17), 4.23 (1H, d, *J* = 5.5 Hz,

H-3), 3.70 (3H, s, 7-OCH_3), 3.15 (1H, d, *J* = 18.0 Hz, H-15a), 3.08 (1H, dd, *J* = 10.5, 2.0 Hz, H-5), 2.98 (1H, t, *J* = 6.0 Hz, H-2), 2.53 (1H, d, *J* = 17.5 Hz, H-15b), 2.52 (1H, dd, *J* = 12.5, 6.5 Hz, H-30a), 2.25 (1H, dd, *J* = 17.0, 11.0 Hz, H-6a), 2.14 (1H, m, H-6b), 2.12 (1H, m, H-11a), 2.04 (1H, d, *J* = 12.5 Hz, H-30b), 1.95 (1H, dd, *J* = 12.5, 5.0 Hz, H-9), 1.68 (1H, td, *J* = 14.5, 4.0 Hz, H-12a), 1.50 (1H, dt, *J* = 14.5, 3.5 Hz, H-12b), 1.47 (1H, m, H-11b), 1.10 (3H, s, H-28), 0.99 (3H, s, H-18), 0.95 (3H, s, H-19), 0.67 (3H, s, H-29); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (ppm): 214.8 (C-1), 174.4 (C-7), 169.8 (C-16), 143.1 (C-23), 140.9 (C-21), 120.7 (C-20), 110.1 (C-22), 91.5 (C-3), 85.3 (C-17), 76.5 (C-8), 74.7 (C-14), 52.3 (C-9), 51.9 (7-OCH_3), 51.2 (C-10), 49.1 (C-2), 43.1 (C-5), 42.6 (C-30), 40.1 (C-13), 37.4 (C-4), 37.2 (C-15), 32.7 (C-6), 28.9 (C-12), 28.2 (C-28), 20.2 (C-29), 18.0 (C-11), 16.9 (C-19), 16.2 (C-18); ESI-MS: *m/z* 485.4 $[M-H]^-$.

Structure elucidation of the isolated compounds

Compound **1** was isolated as a white amorphous powder. Its molecular formula, $C_{32}H_{40}O_{10}$, was confirmed by the ESI-MS peak at *m/z* 583.4 $[M-H]^-$, indicating 13 indices of hydrogen deficiency. Analysis of the ^{1}H-NMR data revealed the presence of a characteristic β-substituted furan ring (δ_H 7.55, 7.45, and 6.47, each 1H), a methoxy group (δ_H 3.68, 3H, s), and six methyl groups (δ_H 1.18, 1.15, 1.12, 1.04, 0.97, and 0.87, each 3H). Additionally, the ^{13}C-NMR and DEPT spectra combined with HSQC showed thirty-two carbon resonances corresponding to seven methyls (a methoxy group at δ_C 52.0), four methylene groups, ten methine groups (including two oxygenated groups at δ_C 80.2 and 67.0), two ketone carbonyl groups (δ_C 208.7 and 198.8), three ester carbonyl groups (δ_C 175.3, 173.4, and 163.4), and six quaternary carbons (three sp^2 carbons, an oxygenated sp^3 quaternary carbon). The 2D spectrum (COSY, HSQC, and HMBC) of **1** showed the presence of 2-methylbutyroxy group (δ_H 0.87/H-4′, 1.15/H-5′, 1.41 and 1.68/H_2-3′, 2.40/H-2′; δ_C 175.3/C-1′, 41.3/C-2′, and 26.1/C-3′). The HMBC correlations between H-30 (δ_H 6.46) and C-1′ (δ_C 175.3) confirmed the position of 2-methylbutyroxy group was at C-30. A comparison of the spectral data of **1** and those of literature values suggested the chemical structure of **1** was hainangranatumin A.[24]

Compound **2** was isolated as a white amorphous powder. Its molecular formula, $C_{29}H_{34}O_{10}$, was confirmed by the ESI-MS peak at *m/z* 541.4 $[M-H]^-$, indicating 13 indices of hydrogen deficiency. The ^{1}H-NMR spectrum of **2** displayed signals for four methyl groups (δ_H 1.21, 1.12, 1.04, and 0.98, each 3H), an acetoxy group (δ_H 2.10, 3H, s), a methoxy group (δ_H 3.69, 3H, s), and a typical β-furan ring (δ_H 7.55, 7.44, and 6.46, each 1H). The ^{13}C-NMR and DEPT spectra showed that **2** has six methyls (a methoxy group at δ_C 52.0), three sp^3 methylenes, four sp^3 methines (two oxygenated methines at δ_C 80.2 and 67.4), five sp^2 methines (δ_C 162.1, 143.3, 141.5, 118.2, and 109.8), an sp^3 oxygenated quaternary carbon at δ_C 80.1, five quaternary carbons (three sp^2 carbons at δ_C 164.2, 128.5, and 119.6), two carbonyl carbons (δ_C 208.6 and 198.8), and three carboxyl carbons (δ_C 173.4, 170.0, and 163.4). Analyses of the 1D- and 2D-NMR data for **2** were similar to those of compound **1**. The only difference was the presence of an acetoxy group (δ_H 2.10/H-2′; δ_C 170.0/C-1′ and 21.1/C-2′) in **2** instead of the corresponding 2-methylbutyroxy group in **1**, which was confirmed by HMBC correlations between H-2′ (δ_H 2.10) and C-1′ (δ_C 170.0)/C-30 (δ_C 67.4), between H-30 (δ_H 6.53) and carbonyl carbon C-1′ (δ_C 170.0). Based on the above evidence and compared to published literature,[25] compound **2** was identified as xylogranatin C.

Compound **3** was obtained as a white amorphous powder. By comparing the ESI-MS and NMR data of this compound with those reported,[26] its structure was concluded as xylocarpin F, as reported in our previous study.[23]

Compound **4** was isolated as a white amorphous powder. The ESI-MS spectrum of **4** showed a quasimolecular ion peak at *m/z*485.4 $[M-H]^-$, consistent with a molecular formula of $C_{27}H_{34}O_8$ incorporating 11 degrees of unsaturation. All twenty-seven carbon resonances were well resolved in the ^{13}C-NMR spectrum of **4** and classified by chemical shifts and HSQC spectrum as four methyl groups, a methoxy group (δ_H 3.70/δ_C 51.9), five sp^3 methylenes, five sp^3 methines (two oxygenated sp^3 methines at δ_C 91.5 and 85.3), three sp^2 methines (δ_C 143.1, 140.9, and 110.1), and nine quaternary carbons (a ketone carbonyl at δ_C 214.8, two ester carbonyls at δ_C 174.4, 169.8, and two oxygenated quaternary carbons at δ_C 76.5 and 74.7). Further, the proton signals of four tertiary methyls (δ_H 1.10, 0.99, 0.95, 0.67, each 3H, s), a methoxy (δ_H 3.70, 3H, s), two oxygenated sp^3 methines (δ_H 6.28 and 4.23, each 1H), and one β-substituted furan ring at δ_H7.56 (1H, brs), 7.45 (1H, t, *J* = 1.5 Hz), and 6.49 (1H, brs) were also observed by extensive analysis of the ^{1}H-NMR spectrum. These data indicated that compound **4** was likely a mexicanolide-type limonoid. Several important HMBC correlations were confirmed such as the correlations between H-17 (δ_H 6.28) and C-20 (δ_C 120.7)/C-21 (δ_C 140.9)/C-22 (δ_C 110.1); the HMBC correlations between H_3-18 (δ_H 0.99) and C-12 (δ_C 28.9)/C-13 (δ_C 40.1)/C-14 (δ_C 74.7); the HMBC correlations between H_3-19 (δ_H 0.95) and C-1 (δ_C 214.8)/C-9 (δ_C 52.3)/C-10 (δ_C 51.2); the HMBC correlations between H_3-28 (δ_H 1.10) and C-3 (δ_C 91.5)/C-4 (δ_C 37.4)/C-5 (δ_C 43.1)/C-29 (δ_C 20.2); the HMBC correlations between H_3-29 (δ_H 0.67) and C-3/C-4/C-5/C-28 (δ_C 28.2); the HMBC correlations between proton of 7-OCH_3 group (δ_H 3.70)/H_2-6 (δ_H 2.25 and 2.14) and C-7 (δ_C 174.4). The correlations in the ^{1}H–^{1}H COSY spectrum of **4** displayed connectivities between H-23 (δ_H 7.56) and H-22 (δ_H 6.49), between H-3 (δ_H 4.23) and H-2 (δ_H 2.98), between H-5 (δ_H 3.08) and H_2-6 (δ_H 2.25 and 2.14), between H-2 and H-30a (δ_H 2.52). Based on spectroscopic analysis combined with literature data,[27]compound **4** was elucidated as xyloccensin K. The structures of these limonoids are shown in Figure 1.

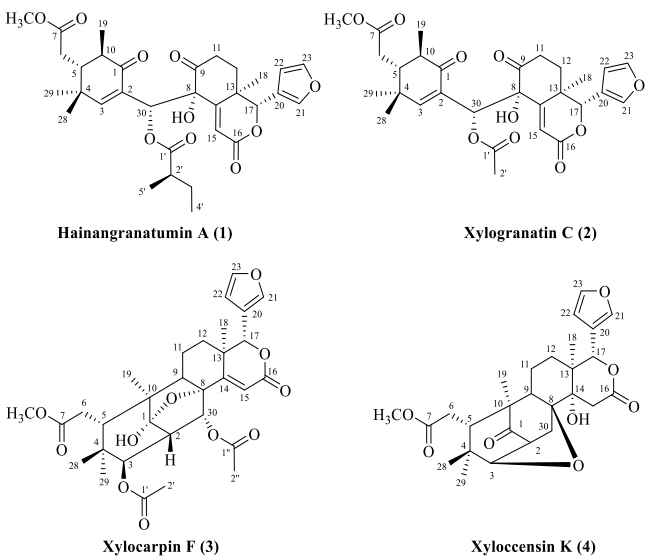


Figure 1: Structures of the isolated compounds (**1-4**) from *X. granatum* fruits

Antioxidant activity of the isolated compounds

The antioxidant activity of the purified limonoids from *X. granatum* fruits was measured using the DPPH and ABTS methods, the results of which are presented in Table 1. In particular, xylocarpin F (**3**) (DPPH assay: IC_{50} = 5.43 ± 0.72 and ABTS assay: IC_{50} = 122.64 ± 1.39 mg/mL), and xyloccensin K (**4**) (DPPH assay: IC_{50} = 6.59 ± 0.69 and ABTS assay: IC_{50} = 120.34 ± 1.34 mg/mL) had higher antioxidant activity than hainangranatumin A (**1**) (DPPH assay: IC_{50} = 12.58 ± 0.73 and ABTS assay: IC_{50} = 145.52 ± 0.54 mg/mL) and xylogranatin C (**2**) (DPPH assay: IC_{50} = 10.24 ± 0.86 and ABTS assay: IC_{50} = 135.78 ± 0.48 mg/mL). However, none of the isolated limonoids exhibited significant antioxidant properties (compared with ascorbic acid used as a positive control, DPPH assay: IC_{50} = 0.01 ± 0.00 and ABTS assay: IC_{50} = 0.05 ± 0.01 mg/mL).

Anti-inflammatory activity of the isolated compounds

In this work, all isolates were tested for their inhibitory activity against NO production in RAW 264.7 macrophages, and these limonoids exhibited notable NO production inhibitory activity. Xylogranatin C (**2**) and hainangranatumin A (**1**) were the most active compounds with IC_{50} values of 7.36 ± 0.23 and 8.76 ± 0.31

µg/mL. Their NO inhibitory effects were slightly higher than those of the positive control, dexamethasone, with an IC_{50} value of 5.41 ± 0.46 µg/mL. Besides, xylocarpin F (**3**) and xyloccensin K (**4**) had moderate effects (IC_{50} = 11.90 ± 1.28 and 12.71 ± 0.71 µg/mL, respectively). Our results suggest that *X. granatum* is a potential natural source of anti-inflammatory limonoids.

Molecular docking

Molecular docking is known as a powerful and important tool in supporting more efficient drug development processes. This method has been adopted by many researchers, helping to identify natural compounds in plants with medicinal potential, thereby exploiting natural resources for drug development.[22,28] Based on the promising *in vitro* activity results, the isolated limonoids have demonstrated a significant ability to inhibit NO production. To further explore the anti-inflammatory mechanisms of these isolates, we conducted molecular docking simulations for limonoids **1**-**4**.

Table 1: Antioxidant activity of the isolated compounds (**1**-**4**) from *X. granatum* fruits

Compounds	IC_{50} (mg/mL)	
	DPPH	ABTS
Hainangranatumin A (**1**)	12.58 ± 0.73	145.52 ± 0.54
Xylogranatin C (**2**)	10.24 ± 0.86	135.78 ± 0.48
Xylocarpin F (**3**)	5.43 ± 0.72	122.64 ± 1.39
Xyloccensin K (**4**)	6.59 ± 0.69	120.34 ± 1.34
Ascorbic acid	0.01 ± 0.00	0.05 ± 0.01

Results are presented as means ± SD (n = 3).

Table 2: Anti-inflammatory activity of the isolated compounds (**1**-**4**) from *X. granatum* fruits

Compounds	RAW264.7 (IC_{50}; µg/mL)
Hainangranatumin A (**1**)	8.76 ± 0.31
Xylogranatin C (**2**)	7.36 ± 0.23
Xylocarpin F (**3**)	11.90 ± 1.28
Xyloccensin K (**4**)	12.71 ± 0.71
Dexamethasone	5.41 ± 0.46

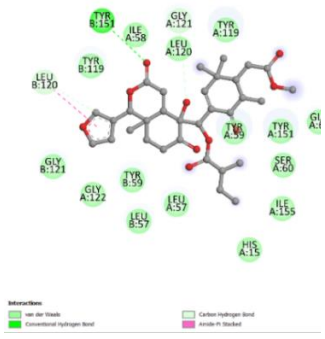
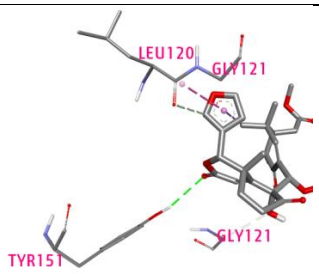
Results are presented as means ± SD (n = 3).

These simulations targeted two key inflammation-related enzymes, COX-2 and TNF-α, toassess their interaction capabilities and binding affinities. The findings are detailed in Table 3.

When docked with TNF-α, the limonoids exhibited binding affinities ranging from -8.540 kcal/mol to -8.178 kcal/mol, while the control inhibitor SPD304 had an ΔG of -8.710 kcal/mol. Among these, limonoid **1** showed the strongest affinity with an ΔG value of -8.540 kcal/mol, contributed by two hydrogen bonds with the amino acid residues TyrB151 and LeuD55. Similarly, limonoid **2** also formed hydrogen bonds with these residues. Moreover, limonoid **1** created an amide-pi stacked interaction with LeuB120, while limonoid **2** established an interaction with TyrB59. Limonoid **3** formed bonds with amino acid residues TyrB151 and Gly121. Additionally, it established pi-sigma and pi-pi T-shaped interactions with Tyr119. Unlike the previously mentioned compounds, limonoid **4** forgoes forming hydrogen bonds. Instead, it engages in alkyl and pi-alkyl interactions with LeuD55, TyrB119, and TyrA119, as well as pi-pi T-shaped and amide-pi stacking interactions with GlyA121 and TyrB59. Notably, the important amino acid residues TyrB119, TyrB59, TyrB151, and GlyA121 were also observed in the inhibitor complex SPD304, suggesting that the studied limonoids could become potential TNF-α inhibitors.

For the enzyme COX-2, the isolated limonoids exhibited binding affinities ranging from -9.924 kcal/mol to -7.014 kcal/mol. Among them, limonoid **3** showed the strongest affinity (ΔG = -9.924 kcal/mol), followed by limonoids **1** (ΔG = -9.618 kcal/mol), **2** (ΔG = -9.011 kcal/mol), and **4** (ΔG = -7.014 kcal/mol). Notably, limonoids **1** and **3** had stronger affinities than the control inhibitor rofecoxib (ΔG = -9.338 kcal/mol). Interaction analysis revealed the interactions of the limonoids with the COX-2 enzyme. Three amino acid residues, HisA351, SerA581, and Glu346, formed hydrogen bonds with **3**, significantly contributing to the binding affinity. Limonoid **1** formed hydrogen bonds with Asn350, while limonoid **2** established a bond with Gln565. Additionally, limonoid **1** formed pi-anion and pi-alkyl interactions with Lys358. Meanwhile, limonoid **4** established two hydrogen bonds with Phe580 and His356, along with a pi-alkyl interaction with His351. Previous reports have highlighted the notable COX-2 inhibitory activity of limonoids. For instance, secotrijugin B, a new limonoid from twigs and leaves of *Trichilia sinensis*, has demonstrated down-regulated COX-2 protein expression.[29] To our knowledge, the anti-inflammatory activities of limonoids **1**-**4**, specifically their effects on TNF-α and COX-2 inhibition, have not been previously reported. Therefore, the current study results will serve as a basis for further *in vitro* and *in vivo* biological testing.

Table 3: The docking results of isolated compounds (**1**-**4**) with the selected proteins

Protein	Compounds	2D interactions	3D interactions	ΔG
TNF-α	**1**			-8.540

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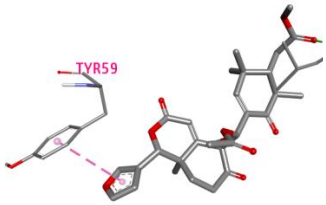
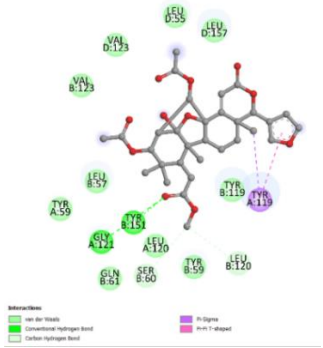
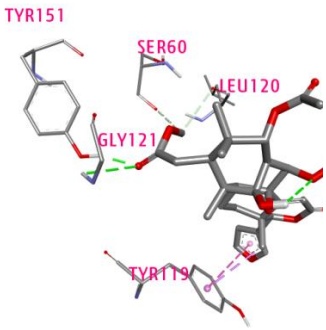
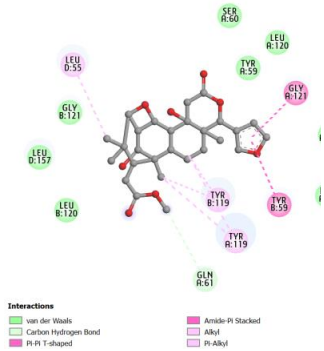
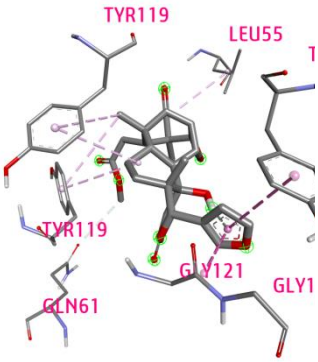
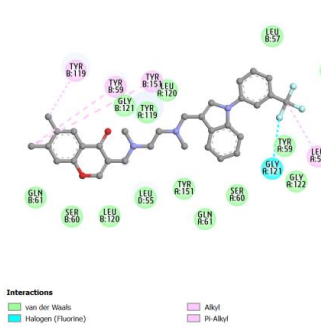
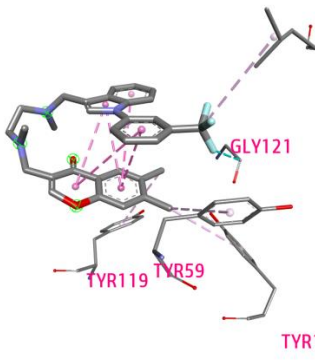
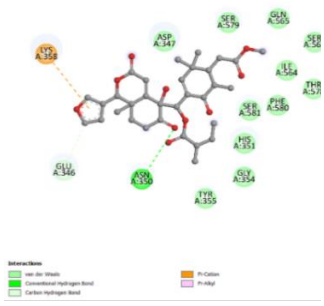
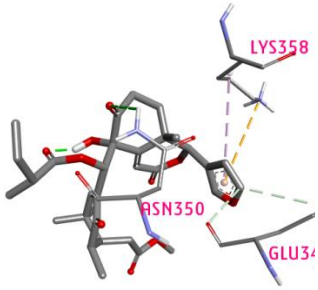
	2			-8.500
	3			-8.178
	4			-8.474
	SPD304			-8.710
COX-2	**1**			-9.618

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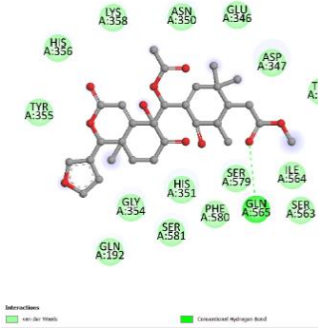
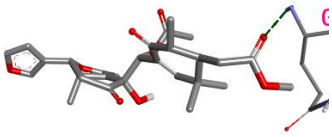
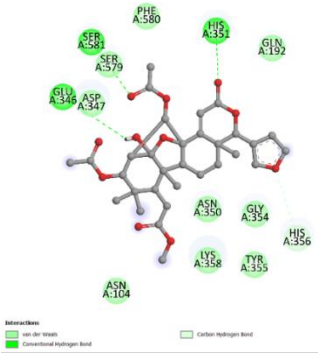
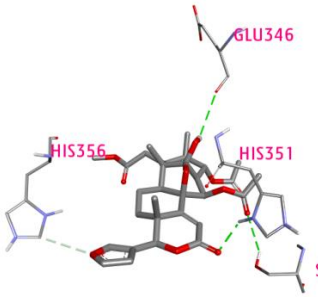
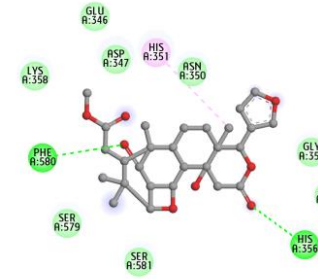
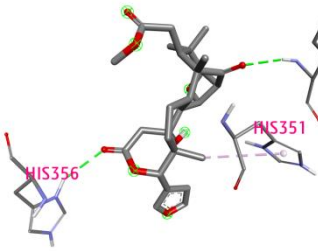
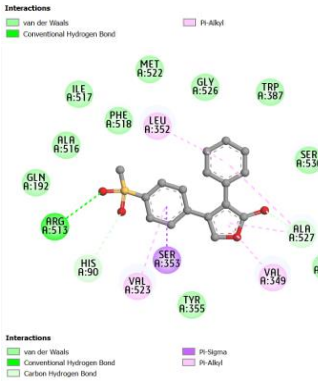
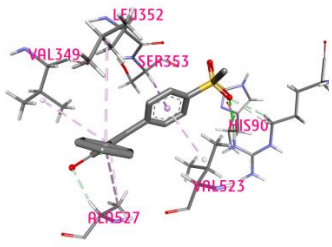
2			-9.011
3			-9.924
4			-7.014
Rofecoxib			-9.338

Conclusion

In conclusion, four limonoids, including hainangranatumin A (**1**), xylogranatin C (**2**), xylocarpin F (**3**), and xyloccensin K (**4**), were isolated from the ethyl acetate extract of *X. granatum* fruits. These compounds' antioxidant and anti-inflammatory activities were evaluated for the first time. Among them, xylogranatin C (IC_{50} = 7.36 ± 0.23 µg/mL) and hainangranatumin A (IC_{50} = 8.76 ± 0.31 µg/mL) showed significant inhibitory activity against LPS-induced NO production in RAW 264.7. The molecular docking showed that limonoid **1** had the highest affinity for TNF-α, while limonoid **3** exhibited the strongest affinity for COX-2. Specific interactions of the isolates were analyzed, highlighting the significant contributions of hydrogen bonds and hydrophobic interactions. This work offers valuable insights into the phytochemical constituents of *X. granatum* and their biological activities.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors here by declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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3*β*,8*β*-epoxymexicanolides from the fruit of a Chinese mangrove *Xylocarpus granatum*. Magn Reson Chem. 2006; 44(1):87-89. DOI: 10.1002/mrc.1713.
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