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Benzofurans nor-sesquiterpenoids from *Petasites hybridus* rhizomes and absolute configuration by circular dichroism

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ABSTRACT

Petasites hybridus (L.) G.Gaertn., B.Mey. & Scherb. has been used as a medicine for different illnesses. A phytochemical study on hexane extract has been carried out using normal phase silica gel column and RP-HPLC chromatography. Five sesquiterpenoids were isolated, purified, and identified from hexane extract. The structures were elucidated by applying extensive 1D and 2D NMR and HRESI-TOFMS spectroscopy. In addition, circular dichroism (CD) spectra were applied to determine absolute configurations of constituents. Two never described benzofuran nor-sesquiterpenes (2*S*,3*S*)-5′-acetyl-(*Z*)-3-(acetoxymethyl) but-2-enoate tremetone) (**4**) and (2*R*,3*R*)-5′-acetyl-(*Z*)-3-(acetoxymethyl) but-2-enoate-7-hydroxytremetone (**5**) as well as euparin (**1**), (2*S*,3*R*)-3,6-dihydroxytremetone (**2**), and (2*S*,3*S*)-3,6-dihydroxytremetone (**3**) were isolated from the rhizome of *Petasites hybridus*, a species in Flora of Iran. Also, the euparin content of the MeOH extracts of seven populations of Iran was measured by HPLC-PDA. Notably, isolated euparin was the main constituent of Iranian *P. hybridus* varying between 2.7 and 9.7 µg/mg among samples.

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1. Introduction

The genus *Petasites* Mill. (Asteraceae) species have a broad distribution in the Northern hemisphere, including North Africa, Asia, and Europe. *Petasites hybridus* (L.)"G. Gaertn., B. Mey. & Scherb."is the most common species in Poland (Ożarowski et al., 2013). *P. hybridus* and *P. albus* are two species of the *Petasites* genus that have been found in the highlands of northern forests of Iran (Khaleghi et al., 2011). The genus *Petasites* has a long history of use in alternative medicine in the treatment of certain illnesses, *e.g.*, respiratory, gastrointestinal, and urogenital diseases, and it is also traditionally used for the treatment of hay fever and migraine (Kim et al., 2012). In recent years the antinociceptive, anti-inflammatory, antioxidant, antiallergic activities and relaxant action of selected compounds such as some sesquiterpenes isolated from different *Petasites* species have been considered in numerous studies (Ko et al., 2001; Wang et al., 2001; Lee et al., 2019). *P. hybridus*, commonly known as butterbur, is the main medicinal plant in the *Petasites* genus used in European phytotherapy (Ożarowski et al., 2013). The traditional uses of *P. hybridus* for the treatment of headaches, gastrointestinal spasms, respiratory disorders such as bronchial asthma, and many other illnesses have been reported (Eaton, 1998; Lin et al., 1998; Mauskop, 2000; Lipton et al., 2004a; Agosti et al., 2006). Extracts of *P. hybridus* were reported to represent a new class of antiallergic drugs because of blocking LT synthesis and histamine binding to H1-receptors. Also, the anti-inflammatory effect of the extract was reported (Khaleghi et al., 2011). The presence of different classes of natural compounds, including pyrrolizidine alkaloids and sesquiterpenes was reported in *P. hybridus*. Four sesquiterpene derivatives, including petasine, isopetasine, and *S*-petasine, *iso*-*S*-

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petasine were extracted and identified from this plant by two groups of Switzerland scholars (Debrunner et al., 1995). Moreover, a total of 21 natural products such as seven sesquiterpenes, 9-hydroxyisobakkenolide, tsoongianolide B, ligularenolide, eremophilanolide, and *oxo*-furanopetasin were isolated from supercritical CO_2 extract of the root of *P. hybridus* (Bodensieck et al., 2007). Moreover, 16 sesquiterpenes, including petasol, isopetasol, and neopetasol have been isolated and identified in this plant (Neuenschwander et al., 1979). Also, some pyrrolizidine alkaloids (Bodensieck et al., 2007), steroids, and terpenes (Ito et al., 1982) were also isolated from *P. hybridus*. In contrast to other investigated *P. hybridus* species, the major constituent of the Persian species is not petasine. Euparin has been reported as the main substance of this plant, which showed moderate anticancer activity in the MCF-7 cell line. *P. hybridus* is one of these species that contain pyrrolizidine alkaloids. However, this work aimed to isolate and structurally elucidate phytochemical constituents of the rhizome of *P. hybridus* *n*-hexane extract by column chromatography and preparative high-performance liquid chromatography (prep-HPLC) led to the discovery of two *never described* euparin derivatives. Also, we determined the absolute configuration of two compounds and extracted a large amount of euparin as the main compound. In this study, the phytochemical composition of seven populations of *P. hybridus* was investigated.

2. Experimental

2.1. General experimental procedures

Technical grade solvents were used for extraction and column chromatography (CC) and redistilled before use; the purities of solvents were checked by gas chromatography. HPLC-grade solvents and deionized water were used for reverse-phase chromatography. Deuterated solvents (100 atoms %D) were from Armar Chemicals. Silica gel for CC (0.063-0.2 mm) and pre-coated silica gel 60 F_{254} (20 × 20 cm) plates were from Merck. TLC plates were visualized under UV light or by spraying with 5% phosphomolybdic acid in EtOH and subsequent heating. The semi-preparative HPLC was performed on a Knauer system with a PDA detector. Also, a SunFire C_{18} (5 µm, 10 × 150 mm i.d.) column fitted with a pre-column (10 × 10 mm i.d.) (Waters) was used (for quantification of euparin). The UV and ECD spectra were recorded in MeOH using a Chirascan spectrometer with 110 QS precision cells (1 mm path, Hellma Analytics), and data were analyzed with Pro-Data V2.4 software. NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. Spectra were measured at 18 °C in a 1 mm TXI probe (^{1}H and 2D NMR) and at 23 °C in a 5 mm BBO probe (^{13}C) with a Z-gradient. The obtained data were processed with Bruker TopSpin 3.5 software.

2.2. Plant material

Rhizome and leaf samples of *P. hybridus* were collected in the northern regions of Iran across seven natural locations in Golestan, Mazandaran, and Gilan provinces (Table 1). Dr. Ali Sonboli identified the plant materials at the herbarium of the Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. Materials were dried at ambient temperature and coarsely ground before extraction. A voucher specimen (MPH-1856-1 to 1856-7) is deposited in the herbarium of the Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. Sampling sites and geographic specifications have been shown in Table 1 and Fig. 1.

2.3. Extraction and isolation

The shade-dried rhizomes of *P. hybridus* (300 g) were cut into small pieces and successively extracted by maceration method at room temperature with three different solvents *n*-hexane (3 × 5 L, 48 h), EtOAc (3 × 5 L, 48 h), and MeOH (3 × 5 L, 48 h), respectively. After filtration, the solvents were evaporated under reduced pressure to afford the *n*-hexane (15.1 g), ethyl acetate (2.5 g), and methanol extracts (9.5 g). The *n*-hexane extract was subjected to silica gel open column chromatography (150 g, 3.5 × 50 cm) with a step gradient of *n*-hexane-EtOAc (100:0 to 0:100) as the mobile phase, followed by eluting with EtOAc:MeOH (100:0) up to (85:15) solvent mixture. Fractions with similar TLC patterns were combined to yield 29 main fractions. Compound **1** (1.2 g) was obtained as the yellow needle crude crystals from fraction F_3 [1.06 g, eluted with *n*-hexane-EtOAc (95:5)]. Fraction F_{15} [1.7 g, eluted with *n*-hexane-EtOAc (90:10)] was separated on an open silica gel column (50 g, 2.5 × 45 cm) using the *n*-hexane-EtOAc gradient. Compound **5** (0.3 mg) as a yellow viscose liquid and sub-fractions $F_{15\text{-}14}$ were obtained from this faction. Fraction F_{21} [410 mg, eluted with *n*-hexane-EtOAc (85: 15)] was separated by silica gel open column chromatography (30 g, 2.0 × 35.0 cm) using a gradient $CHCl_3$-MeOH with 0.5% formic acid. Fraction F_{21} led to compound 4 (8 mg) isolation as yellow viscose liquid and sub-fractions $F_{21\_1\text{-}8}$. Fraction F_{22} [600 mg eluted with hexane-EtOAc (70:30)] was submitted to semi-preparative HPLC [H_2O (A), MeCN (B); 50% B (0-3 min), 50% → 100% B (3-13 min), 100% B (13-26 min), 100% → 50% B (26-28 min), 50% B (28-36 min), flow rate 4 ml/min, detection at 280 nm] to afford sub-fractions $F_{22\_1\text{-}8}$. Sub-fraction F_{22_4} (18 mg) was further purified on preparative TLC [7.0× 5.0 cm, CH_2Cl_2-EtOAc as mobile phase (95: 5)] to isolate compounds **2** (1.0 mg) and **3** (0.4 mg).

2.4. Computational methods

Conformational analyses of compounds **2**-**5** were performed with MacroModel 9.1 software (Schrödinger LLC) employing the OPLS 2005 (Optimized Potential for Liquid Simulations) force field in H_2O. Conformers within a 2 Kcal/mol energy window from the global minimum were selected for geometrical optimization and energy

Table 1
Sample sites and geographic specification of *P. hybridus* evaluated populations.

Codes	Population	Province	Altitude (E)	Latitude(N)
1	Paghaleh	Golestan	55° 5′ 29.15″	36°54′ 43.17″
2	Ziarat waterfall	Golestan	54° 27′ 54.83″	36°40′ 35.49″
3	Kordkuy	Golestan	54° 8′ 6.99″	36°42′ 13.63″
4	Behshahr	Mazandaran	53° 48′ 56.04″	36°38′ 21.95″
5	Zirab	Mazandaran	53° 2′ 43.41″	36° 15′ 56.40″
6	Lonak waterfall	Gilan	49°51′ 49.21″	37° 0′ 17.74″
7	Masuleh	Gilan	48°58′ 53.07″	37° 9′ 19.27″

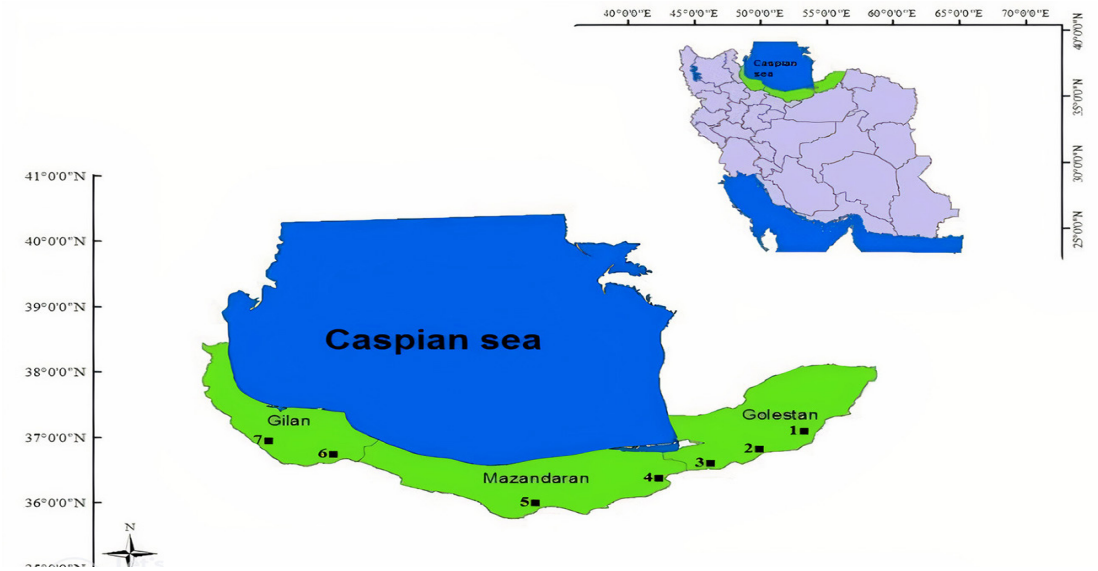

Fig. 1. The locations of *P. hybridus* populations are collected.

calculation using Density Function Theory (DFT) with Becke's nonlocal three-parameter exchange and correlation functional and the Lee-Yang-Parr correlation functional level (cam-B3LYP) using the B3LYP/6-31G** basis set in the gas phase with the Gaussian 09 program package. A vibrational evaluation was done at the same level to confirm minima. Excitation energy (denoted by wavelength in nm), rotatory strengths, dipole velocity (R_{vel}), and dipole length (R_{len}) were calculated in MeOH by TD-DFT/cam-B3LYP/6-31G**, using the SCRF method, with the CPCM model. ECD curves were obtained based on rotatory strengths with a half-band of 0.3-0.4 eV and UV shift using SpecDis v1.64 (Bruhn et al., 2013). ECD spectra were calculated using the spectra of individual conformers according to their contribution calculated by Boltzmann weighting.

2.5. Quantitative analysis of euparin

2.5.1. Sample preparation

For quantification of major compounds in the different parts of the plant, rhizomes and leaves were crushed and grounded. The 500 mg plant material was extracted by 10 mL of MeOH using an ultrasonic bath for 30 min. Then the extract was separated by centrifuging and filtered for HPLC analysis.

2.5.2. Liquid chromatography

A Waters H-Class with PDA apparatus (Waters, Milford, MA, USA) containing a vacuum solvent degassing part, a quaternary high-pressure gradient pump, an automatic specimen injector, and a column thermostat were applied in this investigation. Chromatographic dissociation was achieved on a Sunfire C18 column (3.5 µm, 150 × 5.0 mm) (Waters, Manchester, UK). The mobile phase includes water + 0.1% formic acid, acetonitrile (A) and MeOH (B). The primary gradient condition was 90% A and 10% B linearly converted to 34% B over 8 min, followed by a step to 90% B until 25.0 min, and held until 30.0 min. Then the initial gradient condition was repeated, and the sample was washed by this method until 35.00 min. The column temperature was set at

40°C. The flow was 0.5 mL/min and the injection volume was 10 µL. The sample was monitored at 350 nm.

2.6. Spectroscopic data of isolated compounds

(2*S*,3*R*)-3,6-Dihydroxytremetone (**2**) (1.0 mg): White powder; UV λ_{max} (MeOH) (log ε) 220 (3.5), 237 (3.0), 278 (3.0), 318 (2.5) nm; ECD (MeOH, *c* 4.3 x 10^{-4} M); λ_{max} $(\Delta\varepsilon)_{201}$ - 5101, $(\Delta\varepsilon)_{220}$ + 15811, $(\Delta\varepsilon)_{275}$ - 4809, $(\Delta\varepsilon)_{317}$ + 1816; ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.93 (1H, s, OH), 7.76 (1H, s, H-4), 6.42 (1H, s, H-7), 5.20 (1H, d, *J* = 6.3 Hz, H-2), 5.10 (1H, d, *J* = 6.3, H-3 overlapped H-11), 4.90 (1H, brs, H-11), 2.60 (3H, s, H-14), 1.76 (3H, s, H-12). ^{13}C NMR ($CDCl_3$, 125.77 MHz): δ 203.3 (C-13), 167.2 (C-6), 141.5 (C-10), 128.8 (C-4), 120.8 (C-9), 115.2 (C-5), 113.1 (C-11), 95.9 (C-2), 98.6 (C-7), 75.8 (C-3), 26.9 (C-14), 17.8 (C-12). ESI-TOFMS *m/z* $[M + H]^+$ = 235.0955 (calcd. 235.0965).

(2*S*,3*S*)-3,6-Dihydroxytremetone (**3**): White powder (0.4 mg), UV λ_{max} (MeOH) (log ε) 220 (3.5), 237 (3.0), 278 (3.0), 318 (2.5) nm; ECD (MeOH, *c* 4.3 x 10^{-4} M); λ_{max} $(\Delta\varepsilon)_{201}$ + 14555, $(\Delta\varepsilon)_{222}$ - 5895, $(\Delta\varepsilon)_{278}$ - 3674, $(\Delta\varepsilon)_{300}$ + 2686; ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.96 (1H, s, OH,), 7.79 (1H, s, H-4), 6.46 (1H, s, H-7), 5.10 (1H, d, *J* = 3.4, H-2), 5.09 (1H, brs, H-11), 4.97 (1H, d, *J* = 3.2, H-3), 4.94 (1H, brs, H-11), 2.60 (3H, s, H-14), 1.91 (3H, s, H-12). ^{13}C NMR ($CDCl_3$, 125.77 MHz): δ 203.3 (C-13), 167.2 (C-6), 141.5 (C-10), 129.4 (C-4), 120.8 (C-9), 115.2 (C-5), 112.1 (C-11), 99.1 (C-7), 95.9 (C-2), 75.8 (C-3), 26.9 (C-14), 18.0 (C-12). ESI-TOFMS *m/z* $[M + H]^+$ = 235.0955 (calcd. 235.0965).

(2*S*,3*S*)-5′-Acetyl-(*Z*)-3-(acetoxymethyl) but-2-enoate tremetone (**4**) yellow viscose liquid (8 mg), UV λ_{max} (MeOH) (log ε) 202 (4.08), 276 (3.53) nm; ECD (MeOH, *c* 0.8 × 10^{-3} M) λ_{max} $(\Delta\varepsilon)_{202}$ - 4797, $(\Delta\varepsilon)_{214}$ + 16593, $(\Delta\varepsilon)_{230}$ -12377, $(\Delta\varepsilon)_{292}$ - 2351; ^{1}H NMR (500 MHz, MeOD-d6)) 7.98 (1H, d, *J* = 1.7 Hz, H-4), 7.9 (1H, dd, *J* = 8.5, 1.9 Hz , H-6), 6.88 (1H,d, *J* = 8.5 Hz, H-7), 6.38 (1H, q, *J* = 7.2 Hz, H-3′), 6.19 (1H, d, *J* = 2.8 Hz, H-3), 5.05 (1H, d, *J* = 2.5 Hz, H-2), 5.01 (1H, brs, H-11), 4.90 (1H, brs, H-11), 4.62 (3H, s, H-2″), 2.45 (3H, H-9), 2.01 (3H, d, *J* = 7.3 Hz, H-4′), 1.88 (3H, s, H-2′′).^{13}C NMR (MeOD-d6(, 125.77 MHz): δ 196.9 (C-13), 170.8 (C-1′′), 165.3 (C-1′ overlapped C-8), 144.8 (C-3′), 133.1 (C-5), 131.6 (C-6), 128 (C-4), 127.7 (C-2′), 114.1 (C-11), 110.4 (C-7), 91.7 (C-2), 77.7 (C-3), 65.6 (C-5′), 26.9 (C-9), 20.4 (C-2′′), 17.5 (C-12), 15.7 (C-4′). ESI-TOFMS *m/z* $[M + H]^+$= 359.1479 (calcd. 359.1489).

(2*R*,3*R*)-5′-Acetyl-(*Z*)-3-(acetoxymethyl) but-2-enoate-7-hydroxytremetone, compound **5** yellow viscose (0.3 mg). UV λ_{max} (MeOH) (log ε) 222 (4.08), 237 (3.3), 276 (2.8), 319(2.5) nm; ECD (MeOH, *c* 0.8 × 10^{-3} M) λ_{max} $(\Delta\varepsilon)_{207}$-16003, $(\Delta\varepsilon)_{225}$ + 24866, $(\Delta\varepsilon)_{244}$ - 123788, $(\Delta\varepsilon)_{269}$ - 4939; ^{1}H NMR (500 MHz, MeOD-d6) 7.95 (1H, s, H-4), 6.49 (1H, q, *J* = 7.3 Hz, H-3′), 6.40 (1H, s, H-6), 6.12 (1H, d, *J* = 2.2 Hz, H-3), 5.16 (1H, s, H-2), 5.07 (1H, brs, H-11), 4.97 (1H, brs, H-11), 4.71 (3H, s, H-2″), 2.07 (3H, d, *J* = 7.3 Hz, H-4′), 1.95 (3H, s, H-2′′).^{13}C NMR (MeOD-d6, 125.77 MHz): δ 196.9 (C-13), 170.8 (C-1′′), 165.3 (C-1′ overlapped C-8), 144.8 (C-3′), 133.1 (C-5), 131.6 (C-6), 128 (C-4), 127.7 (C-2′), 114.1 (C-11), 110.4 (C-7), 91.7 (C-2), 77.7 (C-3), 65.6 (C-5′), 26.9 (C-9), 20.4 (C-2′′), 17.5 (C-12), 15.7 (C-4′). ESI-TOFMS *m/z* $[M + H]^+$= 375.1438 (calcd. 375.1426).

3. Results and Discussion

3.1. Characterization of lipophilic compounds from *n*-hexane extract of *P. hybridus* rhizomes

In this study, we focused on isolation of lipophilic compounds from *n*-hexane extract. Fractionation of *n*-hexane extract of *P. hybridus* rhizomes was carried out by combination of the open column chromatography on silica gel, preparative and semi-preparative RP-HPLC afforded to isolation and purification of three known (**1**-**3**) together with two never described (**4**-**5**) sesquiterpenoids (Fig. 2). Compound **1**, euparin, was first isolated from *Eupatorium purpureum* and its chemical structure was defined as a benzofuran in 1939 (Kamthong and Robertson, 1939). It has been reported to show antidepressant activities (Han et al., 2020). Euparin was also isolated from *Eupatorium buniifolium* as the major constituent and responsible for the anti-poliovirus activity of this plant (Visintini Jaime et al., 2013). Euparin also showed moderate antiprotozoal activity against epimastigotes (Elso et al., 2021). This compound exhibited a moderate antioxidant activity (Mohammadi et al., 2012). Moreover, it acts as an energy transfer by inhibiting phosphorylation in chloroplasts (Castañeda et al., 1998).

The structures of isolated compounds were elucidated by the application of extensive 1D, 2D NMR and HRMS spectroscopy. Also, the absolute configuration of **2**-**5** was achieved by comparing experimental ECD spectra with simulated ECD data for possible stereoisomers by using time-dependent density functional theory (TDDFT). The ^{3}J coupling values between the resonance of H-2 and H-3 were 3.2 Hz for compound **3** and 6.3 Hz for compound **2**. The obtained results indicated that H-2 is located on the opposite side of the dihydofuran ring, while H-3 and the dihydofuran ring are on the same side. The ^{1}H-NMR spectra of compounds **2** and **3** displayed hydroxyl groups by signals at 12.93 (1H, s) and 12.96 (1H,s), showing no connectivity with carbons at HMQC. While, in the HMBC spectrum, correlations from OH (δ_H 12.93) to the C-5 (δ_C 115.2), C-6 (δ_C 167.2) 115.2, and C-7 (δ_C 95.6) and from OH (δ_H 12.93) to C-5 (δ_C 115.2), C-6 (δ_C 167.2) 115.2, and C-7 (δ_C 99.1) were detectable. To determine the absolute configuration of compounds **2** and **3**, ECD spectra of the four possible stereoisomers for these compounds, including (2*S*,3*S*), (2*S*,3*R*), (2*R*,3*S*), and (2*R*,3*R*) (Fig. 3C), were calculated and compared with their experimental spectra. The observed ECD spectrum for compound **2** showed two sequential positive cotton effects (CEs) at 223 and 250 (shoulder) and a negative CE at 273, resulting from the $\pi \rightarrow \pi^*$ transitions of the aromatic ring and double bond moieties (Fig. 3A). The experimental ECD spectrum fits well with the calculated spectrum for the (2*S*,3*R*) stereoisomer (positive CEs at 223 and 251 nm, and a negative CE at 277). Thus, compound **2** was identified as (2*S*,3*R*)-3,6-dihydroxytremetone (Liu et al., 2010). The calculated ECD spectrum (Fig. 3B) of compound **3** for the stereoisomer (2*S*,3*S*) showed an excellent match with the experimental spectrum with

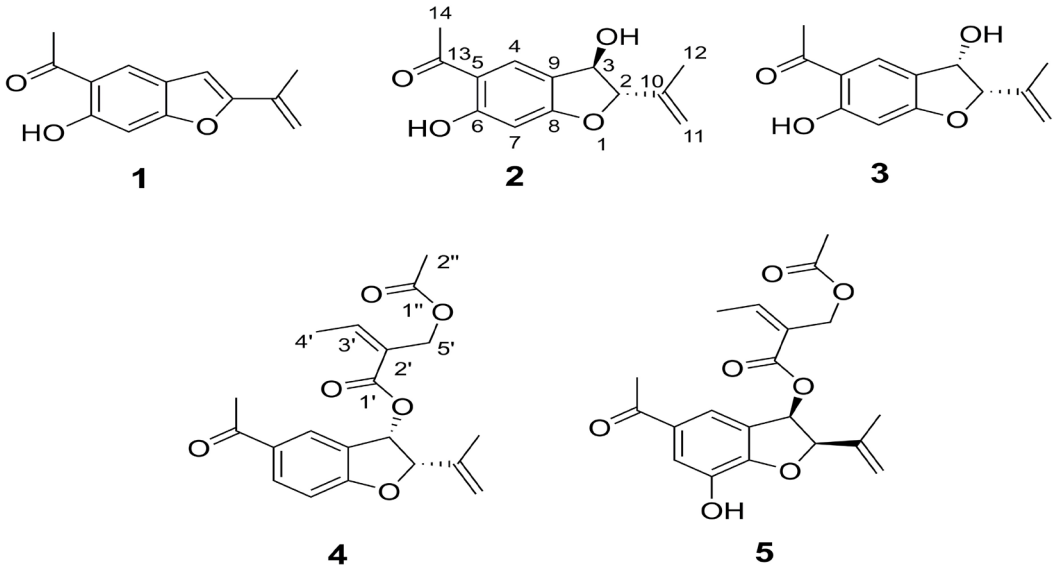

Fig. 2. Chemical structures of compounds **1-5** isolated from *Petasites hybridus*.

a positive CE at 249 nm and two negative CEs at 218 and 275 nm. Therefore, the structure of compound 3 was defined as (2S,3S)-3,6-dihydroxytremetone. Compounds **2** and **3** were isolated as white powders. The HRESI-TOFMS of compound **2** showed a molecular ion at *m/z* 235.0955 $[M + H]^+$ (calcd. 235.0965), indicating a molecular formula of $C_{13}H_{14}O_4$ and also the HRESI-TOFMS of compound **3** showed a molecular ion at *m/z* 235.0959 $[M + H]^+$ (calcd. 235.0965), indicating the same molecular formula. Analysis of the NMR data of compounds **2** and **3** confirmed that these compounds have a planar structure similar to the previously reported compound, named 3,6-dihydroxytremetone, from *Fitchia speciosa* (Bohlmann and Zdero, 1977). The NMR data of compounds **2** and **3** (Table 2) were almost superimposable, but significant differences were observed in their ^3J ($H\text{-}_2$, $H\text{-}_3$) couplings. excellent match with the experimental spectrum with a positive CE at 249 nm and two negative CEs at 218 and 275 nm. Therefore, the structure of compound **3** was defined as (2*S*,3*S*)-3,6-dihydroxytremetone. The HRESI-TOFMS of compound **4** showed a molecular ion at *m/z* 359.1479 $[M + H]^+$(calcd. 359.1490), indicating the molecular formula of $C_{20}H_{22}O_6$. The NMR data of **4** (Table 2) was similar to compounds **2** and **3**. However, the presence of an ester chain and the absence of hydroxyl group in position 6 of compound **4** were notable difference, suggesting that compound **4** was an ester analog of compounds **2** and **3**. A series of signals [δ_C 165.3 (C), 127.7 (C), 144.8 (C)] indicated the presence of one α,β-unsaturated carbonyl group in this side chain. The HMBC correlations from H-4′ (δ_H 2.01) to the carbonyl carbon C-1′ (δ_C 165.3) and C-2′ (δ_C 127.7), from H_2-5′ (δ_H 4.62) to C-5′ (δ_C 65.6), C-2′ (δ_C 127.7) and C-1′′ (δ_C 170.8), and from H-3 (δ_H 6.19) to C-1′ (δ_C 165.3) confirmed the presence of recommended ester group at C-3. The coupling constants $^3J_{(H\text{-}2,\ H\text{-}3)}$ = 3.6 Hz between H-2 and H-3 corresponded to a dihedral angle of ca. 60° and indicated the co-facial orientation of these protons. Also, this result was supported by the NOESY correlations between H-2 (δ_H 5.05) and H-3 (δ_H 6.19). In the experimental ECD spectrum of compound **4**, two positive CEs at 213 and 248 nm along with two negative CE at 227 and 277 nm due to π → π* transitions of α,β-unsaturated carbonyl moieties and aromatic ring were observed (Fig. 4). The obtained data agreed with the calculated ECD spectrum for the (2*S*,3*S*) stereoisomer (positive CEs at 213 and 265 and negative CE at 229 and 285 nm). The literature survey indicated that this benzofuran nor-sesquiterpene is *never described* before and identified as (2*S*,3*S*)-5′-acetyl-(*Z*)-3-(acetoxymethyl) but-2-enoate tremetone and has not been reported previously.

Compound **5** (Table 2) was obtained as a white powder, ^{1}HNMR spectrum of compound **5** showed noticeable similarities with that of compound **4**. HRESI-TOFMS data revealed the molecular formula of $C_{20}H_{22}O_7$. Also, the HRESI-TOFMS of compound **5** showed a molecular ion at *m/z* 375.1426 $[M + H]^+$(calcd. 373.1438) that differed by 15.9947 amu from that of compound **4** due to the replacement of a hydroxyl group by a hydrogen group. The OH was located at C-6 (δ_C 167.1), as deduced from HMBC correlations of H-6 (δ_H 6.40) and C-6. According to the NOESY spectrum of compound **5**, H-4 (δ_H 7.95) correlated with H-14 (δ_H 2.55), H-3 (δ_H 6.12), H-3 (δ_H 6.12) correlated with H-2 (δ_H 5.16), H-4 (δ_H 7.95), H-12 (δ_H 1.75) and H-3′ (δ_H 6.49) correlated with H-4′ (δ_H 2.07), H-2′ (δ_H 4.71). The absolute configurations at C-2 and C-3 were determined as (2*R*, 3*R*) based on the abovementioned method for compounds **1** to **4**. The experimental ECD showed two positive CEs at 225 and 285 and two negative CEs at 213 and 245 nm due to the π → π* and n → π* transitions of the aromatic ring, α,β-unsaturated carbonyl, double bond, and carbonyl group (Fig. 4) and showed an excellent match with the calculated spectrum for the (2*S*, 3*R*) stereoisomer

Table 2
^{1}H and $^{13}CNMR$ spectroscopic data (500 and 125 MHz) of compounds **2-5** (δ in ppm, J in Hz)[a]. For compound **2** and **3** $CDCl_3$, and **4** and **5** MeOD-d6 were used for dissolving samples.

Position	Compound 2		Compound 3		Compound 4		Compound 5	
	δH (*J* in Hz)	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)	δC	δH (*J* in Hz)	δC
2	5.20 (d, 0.7 Hz)	95.9, CH	5.10, (d, 3.2 Hz)	95.9, CH	5.05, (d, 2.5 Hz)	91.7, CH	5.16, s	92.1, CH
3	5.10, (d, 3.2 Hz)	75.8, CH	4.97 (d, 3.2 Hz)	75.8, CH	6.19, (d, 2.8 Hz)	77.7, CH	6.12, (d, 2.2 Hz)	77.3, CH
4	7.76, s	128.8, CH	7.79, s	129.4, CH	7.98, (d, 1.7 Hz)	128.0, CH	7.95, s	130.8, C
5	-	115.2, C	-	115.2, C	-	133.1, C	-	116.4, C
6	-	167.2, C	-	167.2, C	7.90,	131.6, CH	6.40, s	145.6 C
					(d,d,8.5, 1.9 Hz)			
7	6.42, s	98.6, CH	6.46, s	99.1, CH	6.88, (d, 8.5 Hz)	110.4, CH	-	98.2, CH
8	-	167.2, C	-	167.2, C	-	165.3, C	-	167.2, C
9	-	120.8, C	-	120.8, C				
10	-	141.5, C	-	141.5, C	-	140.3, C	-	140.3, C
11	5.10, (brs)	113.1, CH_2	5.09, (brs)	112.1, CH_2	5.01, (brs)	114.1, CH2	5.07, (brs)	113.6, CH_2
	4.90, (brs)		4.94, (brs)		4.90, (brs)		4.97, (brs)	
12	1.76, s	17.8, CH3	1.91, s	18.0, CH3	1.69, s	17.5, CH3	1.75, s	17.5, CH3
13	-	203.3, C	-	203.3, C	-	196.9, C	-	204.0, C
14	2.60, s	26.9, CH_3	2.60, s	26.9, CH_3	2.46, s	25.4, CH_3	2.55, s	25.64, CH_3
1′	-	-	-	-	-	165.3, C	-	165.8, C
2′	-	-	-	-	-	127.7, C	-	127.1, C
3′	-	-	-	-	6.38, (q, 7.2 Hz)	144.8, CH	6.49, (q, 7.3 Hz)	144.8, CH
4′	-	-	-	-	2.01, (d, 7.3 Hz)	15.7, CH_3	2.07, (d, 7.3 Hz)	15.7, CH_3
5′	-	-	-	-	4.62 (q, 12.4 Hz)	65.6, CH_2	4.71, (q, 12.3 Hz)	65.6, CH_2
1″	-	-	-	-	-	170.8, C	-	171.5, C
2″	-	-	-	-	1.88, s	20.4, CH_3	1.95, s	20.4, CH_3
OH	12.93, s	-	12.96, s	-	-	-		-

[a] $^{13}CNMR$ data of compounds were extracted from HSQC-DEPT and HMBC spectra due to a low of samples.

(positive CEs at 224 and 285 nm and negative CEs at 213 and 245 nm). Thus, the absolute configuration of compound **5** was established as (2*R*, 3R). The compound was benzofuran nor-sesquiterpene named (2*R*,3*R*)-5'-acetyl-(*Z*)-3-(acetoxymethyl) but-2-enoate-7-hydroxytremetone. The presence of one α,β-unsaturated carbonyl group in the side chain was confirmed as a series of signals [δ_C 165.8 (C), 127.1 (C), 144.8 (C)] were detected. Moreover, correlations from H-4′ (δ_H 2.07) to the carbonyl carbon C-1′ (δ_C 165.3), C-3′ (δ_C 144.8), and C-2′ (δ_C 127.7), from H-3 (δ_H 6.12) to C-1′ (δ_C 165.3), and from H_2-5′ (δ_H 4.71) to C-1′ (δ_C 165.3), C-2′ (δ_C 127.7), C-1′′ (δ_C 170.8), and C-3′ (δ_C 144.7) confirmed the presence of recommended ester group at C-3.

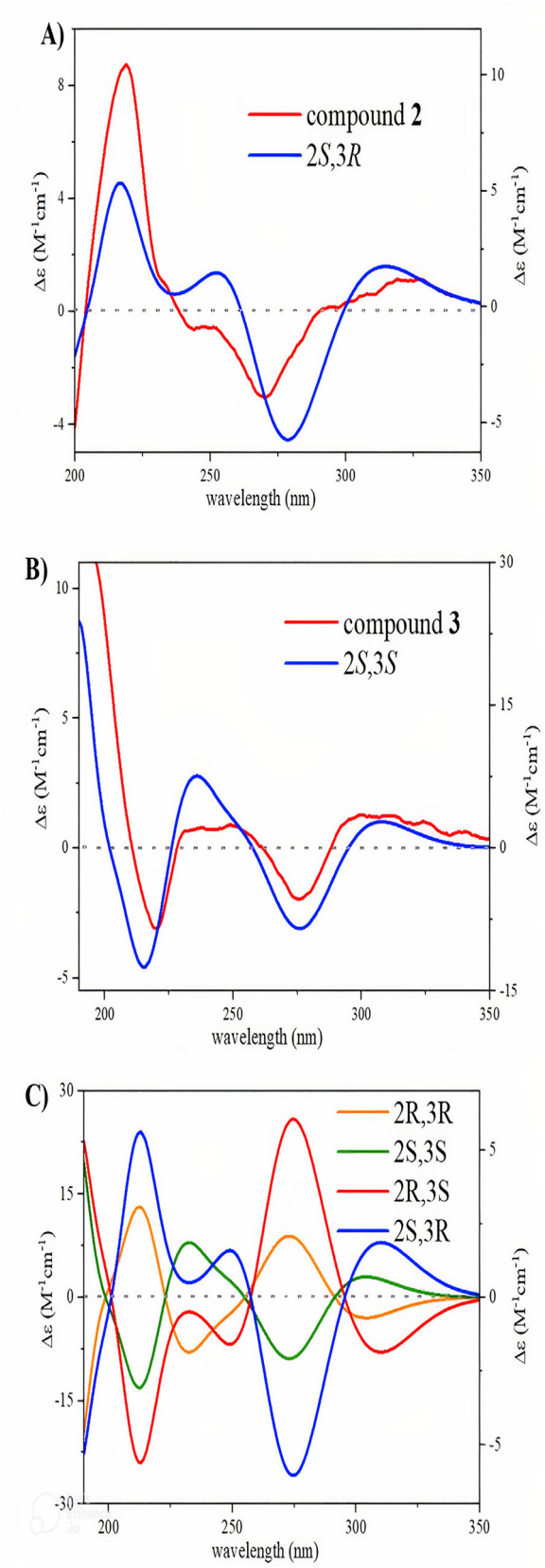

Fig. 3. Experimental and TDDFT calculated ECD spectra at in MeOH were used. A) Comparison of calculated ECD spectra of 2*S*,2*R* and compound **2**, B) Comparison of calculated ECD spectra of 2*S*,2*S* and compound **3**, C) Calculated ECD spectra for all possible stereoisomers.

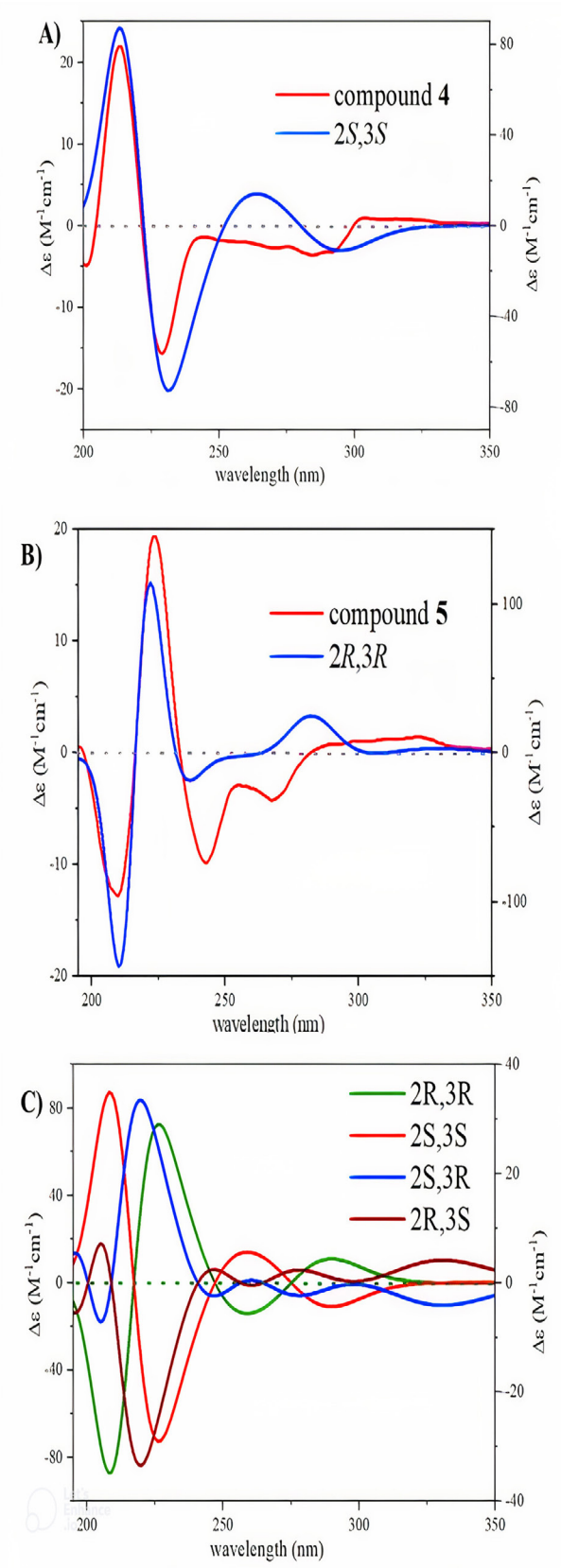

Fig. 4. Experimental and TDDFT calculated ECD spectra at in MeOH were used. A) Comparison of calculated ECD spectra of 2*S*,2*S* and compound **3**, B) Comparison of calculated ECD spectra of 2*R*,2*R* and compound **5**, C) Calculated ECD spectra for all possible stereoisomers.

3.2. Quantitative analysis of euparin

The euparin content of the MeOH extracts of the evaluated populations showed a high variability (Table 3, Fig. 5). The maximum and the minimum euparin contents in leaf samples were observed in the Ziarat waterfall population (2.7 µg/mg) and the Lonak waterfall population (8.4 µg/mg), respectively. The amount of this substance in rhizome samples of the Lonak waterfall population was the lowest (5.2 µg/mg) and was the maximum (9.7 µg/mg) in the Kordkuy population. Kamthong and Robertson (1939) determined and isolated euparin from the roots of *Eupatorium purpureum* for the first time. Also, Mohammadi et al. (2012) reported a high amount of euparin (73.0%) in *Petasites albus* essential oil.

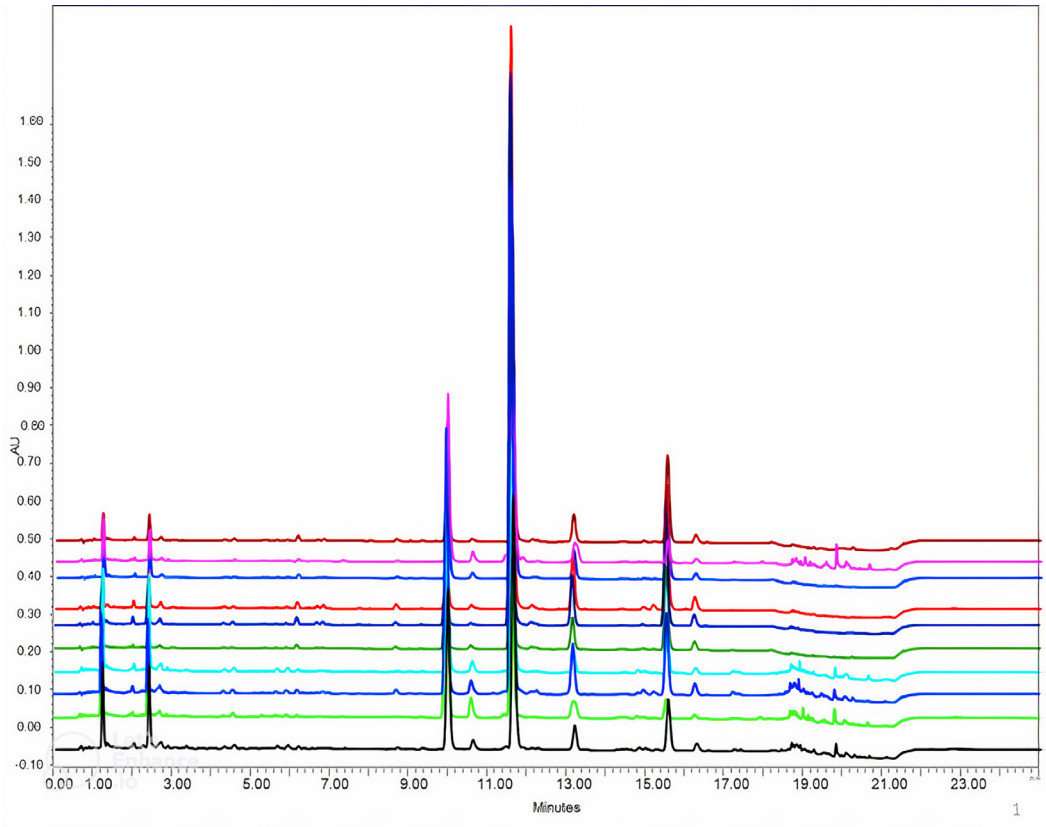

Fig. 5. HPLC chromatograms of the MeOH extracts of *Petasites hybridus* populations at 350 nm.

4. Concluding remarks

The obtained results from an investigation showed 75 mg of standardized *P. hybridus* extract could be used as a preventive therapy for migraine (Lipton et al., 2004b). Moreover, the effectiveness of *P. hybridus* leaf extract for the treatment of early allergic and late inflammatory symptoms of allergic rhinitis has been confirmed (Blosa et al., 2021). In this research, extensive 1D and 2D NMR, HRESI-TOFMS spectroscopy and circular dichroism (CD) were applied to investigate *P. hybridus* hexane extract. According to the obtained results, five sesquiterpenoids with benzofuran rings were isolated and identified. Compound **1**, known as euparin, was isolated and identified from the roots of *P. hybridus* (Khaleghi et al., 2011). Also, four derivatives of compound **1** were isolated from this plant, two of which (compound **4** and **5**) were *never described* and reported for the first time. Different sesquiterpenes and pyrrolizidine alkaloids were isolated and identified from the rhizome of *P. hybridus* collected from various locations in Europe (Bodensieck et al., 2007; Avula et al., 2012). While based on our results, benzofurans and euparin were identified as major compounds of *P. hybridus* rhizome collected from Iran. Numerous studies also have previously reported Iranian *P. hybridus* as a rich source of benzofurans and euparin (Khaleghi et al., 2011; Khaleghi et al., 2014). Also, in numerous phytochemical studies, the distribution of sesquiterpenes in different organs and different seasons, as well as different collection sites, was investigated. For instance, the profile of isopetasin, neopetasin, petasin, *iso*-S-petasin, *neo*-S-petasin and S-petasin in the other plant organs (rootstocks, leaves, stems) of *P. hybridus* was studied. The obtained results also showed that the content of petasins was significantly higher in rhizome compared with leaves and stems (Kulinowski et al., 2022). Moreover, the rhizome of *P. hybridus* collected in four different habitats in Bulgaria were reported to be

richer in sesquiterpenes, including petasin, isopetasin, neopetasin, S-petasin, than the leaves (Uzunova et al., 2020). Consequently, different samples grown in other locations have various chemotaxonomical characteristics.

In recent years, benzofuran compounds have received widespread attention from researchers. In this research, euparin was isolated from *P. hybridus*. As mentioned previously, this compound exhibited some pharmacological activities (Khaleghi et al., 2014). Accordingly, this plant can be domesticated and cultivated to produce valuable pharmaceutical compounds. Moreover, further studies are required to evaluate the biological activities of isolated compounds.

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Conflict of Interest

The author declares that there is no conflict of interest.

Supporting Information

1D and 2D NMR spectra of compounds can be found as supporting information.

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