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Research Article

Cytotoxic acylphloroglucinols from the aerial parts of *Hypericum hirsutum* L. (Hypericaceae)

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Abstract

A phytochemical investigation of the dichloromethane extract of the aerial parts of *Hypericum hirsutum* L. led to isolating the main polyprenylated acylphloroglucinols (**1** and **2**). The compounds were identified employing spectral methods (HRMS, 1D, and 2D NMR, IR, UV) as adsecohyperforin (**1**) and secohyperforin (**2**), respectively. The MS/MS fragmentation of both compounds and NMR spectral data of **1** are described here for the first time. These natural products were found for the second time in a plant source. The cytotoxicity of isolated compounds was established on a panel of tumor cell lines (MDA-MB, EJ, and HL-60) and was determined using MTT-based assays. Both components showed higher cytotoxicity (IC_{50} = 0.27–4.85 μM) than the positive control etoposide (IC_{50} = 1.27–8.42 μM), making them promising candidates for new chemotherapeutic drugs.

Keywords

Hypericum hirsutum, poly-prenylated acylphloroglucinols, adsecohyperforin, secohyperforin cytotoxicity

Introduction

The *Hypericum* L. (Hypericaceae) includes more than 480 species on every continent except Antarctica (Crockett and Robson 2011). *Hypericum hirsutum* L. is one of 28 species in section 18 of Taeniocarpium Jaub. & Spach is a resident of Northern Africa, Europe, and temperate Asia (Robson 2010). It is a perennial herb with hirsute green parts and can be found in the Bulgarian mountains. In previous phytochemical studies of this plant, the presence of phenolic compounds such as (+)-catechin, (-)-epicatechin, hyperoside (Kitanov et al. 1978), orientin, homoorientin, luteolin, myricetin (Kitanov et al. 1979b), 2”-acetyl orientin (Kitanov et al. 1979a), phenolic acids, isomangiferin, and miquelianin (Kitanov 1988) has been established. The essential oil of *H. hirsutum* was studied, revealing that its major constituents were n-undecane, patchoulene, and caryophyllene oxide (Gudžic et al. 2007). Additionally, 19 homoadamantane and adamantane acylphloroglucinols were isolated from the aerial parts of *Hypericum hirsutum* (Max and Heilmann 2021). As part of our ongoing research on the phytochemistry and pharmacology of *Hypericum* species, we isolated the two main polyprenylated acylphloroglucinols **1** and **2** (Fig. 1) from the dichloromethane extract of the aerial parts of *H. hirsutum*. In addition, we evaluated the cytotoxicity of the isolated compounds on a panel of tumor cell lines (MDA-MB, EJ, and HL-60) using MTT-based assays.

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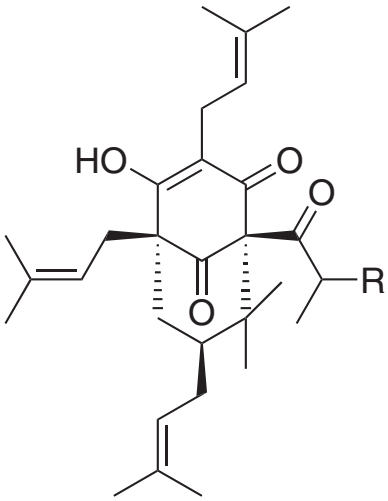

1: R = CH_2CH_3
2: R = CH_3

Figure 1. Structures of compounds **1** and **2**.

Materials and methods

General experimental procedures

Optical rotations (OR) were measured on a Rudolph Research Analytical Autopol VI (Hackettstown, NJ, USA). UV spectra were obtained on a UV-VIS Biochrom Libra S70 (Cambourne, United Kingdom). HRESI-MS analyses were conducted on a Thermo Scientific Q Exactive Plus (Bremen, Germany) mass spectrometer. NMR spectra were measured on a Bruker AVANCE II+ 600 spectrometer operating at 600 MHz for (^{1}H) and 150 MHz for (^{13}C) in CD_3OD. The 1D and 2D NMR experiments (HMBC and HSQC) were processed using Bruker Topspin 3.0 software. Column chromatography (CC) was carried out with Diaion HP-20 (Supelco, USA). Flash chromatography was performed using a Büchi Reveleris X2 flash system on a Büchi FlashPure EcoFlex C18 80 g column (Flawil, Switzerland) at 25 mL min^{-1}. Semi-preparative HPLC was performed on a Waters (Milford, MA, USA) Breeze2 high-pressure binary gradient system consisting of a pump model 1525EF, a manual injector 7725i, and a UV detector model 2489. Separations were achieved on a semi-preparative HPLC column Kromasil C18 (250×10 mm, 5 µm) purchased from Eka Chemicals AB (Bohus, Sweden) at 18 mL min^{-1}. All solvents were HPLC grade and purchased from Merck or Sigma-Aldrich (Taufkirchen, Germany).

Plant material

The aerial parts of *Hypericum hirsutum* L. were collected near Barzia village, Stara Planina, Bulgaria, in July 2023. Dr. P. Nedialkov identified the plant. A voucher specimen from the plant population (No. SOM-177782) was deposited at the National Herbarium, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Extraction and isolation

The aerial parts of *H. hirsutum* L. were dried in the shade, and powdered plant material (10 g) was extracted with CH_2Cl_2 (5×200 mL) at room temperature. The CH_2Cl_2 extract gives a dark green, waxy residue of 2.73 g. The CH_2Cl_2 extract was subjected to CC over Diaion HP-20 (5×6 cm) and was subsequently eluted with 90% aq. MeOH (1 L), MeOH (500 mL), and CH_2Cl_2 (1 L) to obtain three pooled fractions (A-C). For further purification, the fr. A (1.54 g, 90% MeOH) was subjected to flash chromatography using a Reveleris X2 flash system. The mobile phase was a binary gradient of solution A (0.05% TFA in 80% MeOH) and B (0.05% TFA in 90% MeOH), and the elution was performed according to the following gradient program: 0–2 min, 100% A; 2–2.1 min, 100→75% A and 0→25% B; 2.1–4.1 min, 75% A and 25% B; 4.1–4.2 min, 75→50% A and 25→50% B; 4.2–6.2 min, 50% A and 50% B; 6.2–6.3 min, 50→25% A and 50→75% B; 6.3–8.3 min, 25% A and 75% B; 8.3–8.4 min, 25→0% A and 75→100% B; 8.4–10.4 min, 100% B; 8.4–10.5 min, 100% B. Eighty-four fractions were collected and were combined into 12 pooled subfractions (I-XII) according to their similarities. An isocratic semi-prep. HPLC purification of subfraction VII (481 mg) with A-B (78:22, 18 mL min^{-1}, 280 nm), where 84% AcCN (A) and 0.1% TFA (B), gave pure **1** (132 mg) and **2** (180 mg).

Compound data

Adsecohyperforin (**1**): Colorless oil. $[\alpha]^{20}_D$ = −4.97 (*c* 0.17, MeOH). UV (MeOH) λ_{max} (log ε) 281 nm (3.98). ^{1}H NMR (600 MHz, CD_3OD) δ 0.78 (t, J 6.5 Hz, 3H, H-13), 0.97 (d, J 6.5 Hz, 3H, H-14), 1.15 (s, 3H, H-30), 1.20 (m, 1H, H-12b), 1.32 (s, 3H, H-31), 1.39 (m, 1H, H-7), 1.54 (s, 3H, H-29), 1.62 (s, 3H, H-23), 1.63 (s, 3H, H-18), 1.66 (s, 3H, H-19), 1.67 (s, 3H, H-28), 1.70 (s, 3H, H-24), 1.77 (m, 1H, H-11), 1.94 (m, 1H, H-12a), 1.99 (m, 1H, H-6b), 2.02 (m, 1H, H-25b), 2.10 (m, 1H, H-25a), 2.10 (m, 1H, H-6a), 2.42 (dd, J 14.5, 6.7 Hz, 1H, H-20b), 2.49 (dd, J 14.5, 7.0 Hz, 1H, H-20a), 3.03 (dd, J 14.5, 7.7 Hz, 1H, H-15b), 3.08 (dd, J 14., 6.7 Hz, 1H, H-15a), 4.90 (m, 1H, H-26), 4.97 (m, 1H, H-21), 5.18 (m, 1H, H-16). ^{13}C NMR (150 MHz, CD_3OD) δ 12.3 (C-13), 18.2 (C-19), 18.3 (C-29), 18.4 (C-24), 18.5 (C-14), 23.0 (C-15), 23.2 (C-31), 26.1 (C-18), 26.2 (C-28), 26.4 (C-23), 27.1 (C-30), 28.0 (C-12), 30.7 (C-25), 31.7 (C-20), 39.0 (C-6), 48.4 (C-8), 49.1 (C-7), 49.8 (C-11), 58.8 (C-5), 80.8 (C-1), 121.0 (C-21), 121.8 (C-3), 122.2 (C-16), 126.4 (C-26), 133.3 (C-27), 133.8 (C-17), 134.9 (C-22), 209.8 (C-9), 210.8 (C-10). HRMS–ESI (*m/z*): $[M+H]^+$ calcd for $C_{31}H_{47}O_4$, 483.3469; found, 483.3465. HRMS/MS *m/z* (composition, relative intensity): 427.2825 ($C_{27}H_{39}O_4$, 10), 415.2831 ($C_{26}H_{39}O_4$, 13), 359.2205 ($C_{22}H_{31}O_4$, 54), 343.2257 ($C_{22}H_{31}O_3$, 64), 291.1581 ($C_{17}H_{23}O_4$, 100), 287.1629 ($C_{18}H_{23}O_3$, 4), 275.1633 ($C_{17}H_{23}O_3$, 15), 235.0955 ($C_{13}H_{15}O_4$, 5), 219.1010 ($C_{13}H_{15}O_3$, 14).

Secohyperforin (**2**): Colorless oil. $[\alpha]^{20}_D$ = −14.07 (*c* 0.19, MeOH). UV (MeOH) λ_{max} (log ε) 282 nm (3.90). ^{1}H NMR (600 MHz, CD_3OD) δ 0.97 (d, J 6.5 Hz, 3H, H-13), 1.03

(d, J 6.5 Hz, 3H, H-12), 1.15 (s, 3H, H-29), 1.32 (s, 3H, H-30), 1.39 (m, 1H, H-7), 1.54 (s, 3H, H-28), 1.63 (s, 3H, H-17), 1.64 (s, 1H, H-22), 1.67 (s, 3H, H-27), 1.68 (s, 3H, H-18), 1.69 (s, 1H, H-23), 2.00 (m, 2H, H-6b), 2.01 (m, 1H, H-24b), 2.08 (m, 2H, H-6a), 2.09 (m, 1H, H-11), 2.10 (m, 1H, H-24a), 2.42 (dd, J 14.7, 7.0 Hz, 1H, H-19b), 2.48 (dd, J 14.5, 7.0 Hz, 1H, H-19a), 3.06 (d, J 7.8 Hz, 1H, H-14), 4.90 (m, 1H, H-25), 4.98 (m, 1H, H-20), 5.14 (m, 1H, H-15). ^{13}C NMR (150 MHz, CD_3OD) δ 18.3 (C-18), 18.4 (C-28), 18.5 (C-23), 21.4 (C-12), 22.4 (C-13), 23.2 (C-14), 23.2 (C-30), 26.2 (C-17), 26.3 (C-27), 26.5 (C-22), 27.3 (C-29), 30.9 (C-24), 31.8 (C-19), 39.1 (C-6), 42.7 (C-11), 48.5 (C-8), 49.1 (C-7), 80.6 (C-1), 121.1 (C-20), 121.8 (C-3), 122.2 (C-15), 126.5 (C-25), 133.4 (C-26), 134.0 (C-16), 135.0 (C-21), 210.0 (C-9), 211.3 (C-10). HRMS–ESI (*m/z*): $[M+H]^+$ calcd for $C_{30}H_{45}O_4$, 469.3312; found, 469.3311. HRMS/MS *m/z* (composition, relative intensity): 413.2683 ($C_{26}H_{37}O_4$, 25), 401.2675 ($C_{25}H_{37}O_4$, 45), 345.205 ($C_{21}H_{29}O_4$, 40), 343.2257 ($C_{22}H_{31}O_3$, 5), 287.1634 ($C_{18}H_{23}O_3$, 5), 277.1427 ($C_{16}H_{21}O_4$, 100), 275.1635 ($C_{17}H_{23}O_3$, 9), 221.0804 ($C_{12}H_{13}O_4$, 19), 219.1013 ($C_{13}H_{15}O_3$, 21).

Cell lines and culture conditions

The panel of cell lines used in this study consisted of tumor cell lines MDA-MB (breast carcinoma), EJ (urinary bladder carcinoma), and HL-60 (acute pre-myeloid leukemia) that were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were maintained in a controlled environment – cell culture flasks at 37 °C in a "Heraeus" incubator with a 5% CO_2 humidified atmosphere, and the growth medium was 90% RPMI-1640, supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine.

Cytotoxicity determination

Stock solutions of the tested compounds (**1** and **2**) were prepared in 95% EtOH and diluted with RPMI-1640 medium to yield the desired final concentrations. At the final dilutions obtained, cells were never exposed to EtOH concentrations exceeding 0.5%. For the cytotoxicity determination, cells were seeded into 96-well plates (100 µL/well at a density of 3×105 cells/mL) and exposed to various concentrations of the test compounds for 72 h. Cell survival was determined with the MTT dye-reduction assay, as previously described, with some modifications (Mosmann 1983; Konstantinov et al. 1999). After the incubation with the test compound, MTT solution (10 mg/mL in PBS) was added (10 µL/well). Plates were further incubated for 4 h at 37 °C, and the formazan crystals obtained were dissolved by adding 100 µL/well of 5% formic acid in 2-propanol. Absorption was measured on a microprocessor-controlled microplate reader (Labexim LMR1®) at 540 nm. For each concentration, at least 4 wells were used. As a blank solution, 100 µL RPMI 1640 medium with 10 µL MTT stock and 100 µL 5% formic acid in 2-propanol were used.

Statistics

The cytotoxicity assays were carried out in 4 separate experiments. The MTT data were fitted to sigmoidal concentration-response curves, and the corresponding IC_{50} values were calculated using non-linear regression analysis (GraphPad Prism software package). Statistical processing exploited the Student's t-test with $p \leq 0.05$ set as the significance level.

Results and discussion

A recent study indicated that two compounds were the primary constituents of the CH_2Cl_2 extract obtained from the aerial parts of *Hypericum hirsutum* L. (Ilieva et al. 2023). A thorough chromatographic procedure resulted in the isolation of these two compounds. Compound **1** was obtained as an optically active ($[\alpha]^{20}_D = -4.97$) colorless oil. The HRMS-ESI spectrum of **1** showed a protonated molecule $[M+H]^+$ at *m/z* 483.3460 (mass error Δppm = −1.9) corresponding to a molecular formula $C_{31}H_{46}O_4$ with nine degrees of unsaturation. The UV spectrum of **1** in MeOH showed a specific absorption maximum at 281 nm.

The MS/MS spectrum of the protonated molecule $[M+H]^+$ of compound **1** showed neutral losses characteristic of alkenes. Ion fragmentation in the collision cell leads to neutral losses of isobutene (-56), isoprene (-68), and methylethylketene (-84). The fragments and their corresponding product ions, as well as the proposed fragmentation pathways, are shown in Fig. 2. The presence of these neutral losses in the MS/MS spectra of the precursor ion is highly indicative of a compound structurally close to the hyperforin molecule (Liu et al. 2005).

The ^{1}H-NMR spectrum of compound **1** showed the presence of three proton singlets at δ_H 1.70, 1.67, 1.66, 1.63, 1.62, 1.54, 1.32, and 1.15, corresponding to 8 methyl groups. The last two signals (at δ_H 1.32 and 1.15) have the same correlations in the HMBC experiment (Fig. 3) with the signals of carbon atoms at δ_C 80.8 (C-1), 49.1 (C-7), and 48.4 (C-8), which define their C-8 position in the molecule of **1**. According to the MS/MS spectrum, prenyl groups (hydrocarbon chains) in the compound **1** molecule are expected, and the remaining six methyl groups are part of them. In the ^{1}H-NMR spectrum, the single proton multiplets of three methine groups at δ_H 4.90, 4.97, and 5.18 ppm exhibited COSY correlations (Fig. 3) with the signals of methylene protons at δ_H 2.10 and 2.02, 2.49 and 2.42, 3.08, and 3.03 ppm, respectively. The HSQC experiment showed that the signals of these methylene protons exhibit cross-peaks with the signals of the carbon atoms at δ_C 30.7 (C-25), 31.7 (C-20), and 23.0 (C-15), respectively. Based on available literature data (Nedialkov et al. 2015) and HMBC experiment correlations, the prenyl groups are attached to C-7, C-5, and C-3, respectively. The difference between the shifts of the methylene group at the sixth position is Δppm = 0.11, and the C-7 signal is at δ_C 49.1, indicating that the prenyl group at the seventh position is in the *endo* orientation (Grossman and Jacobs 2000).

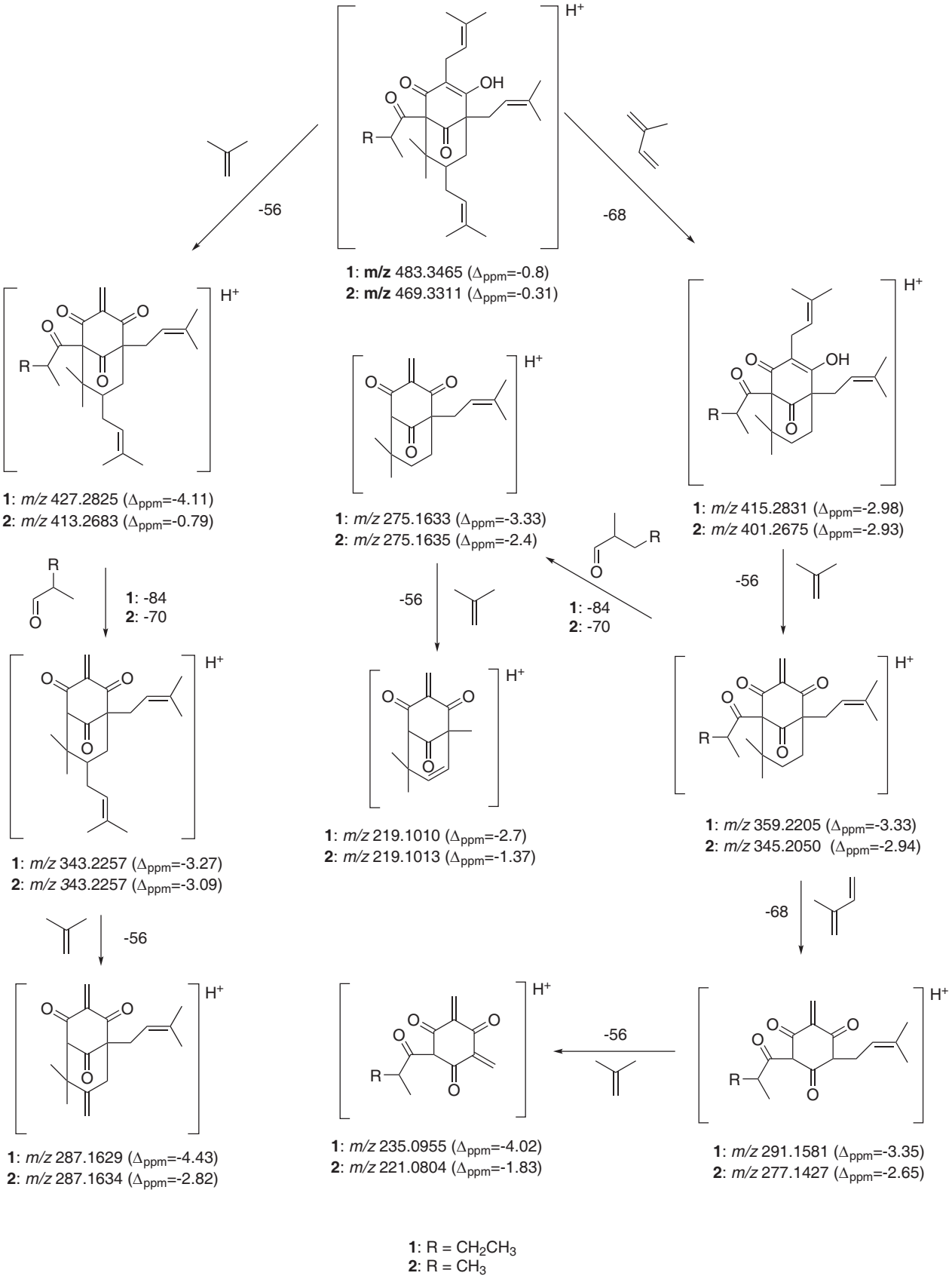

Figure 2. Plausible MS/MS fragmentation of the protonated molecules $[M+H]^+$ of **1** and **2**.

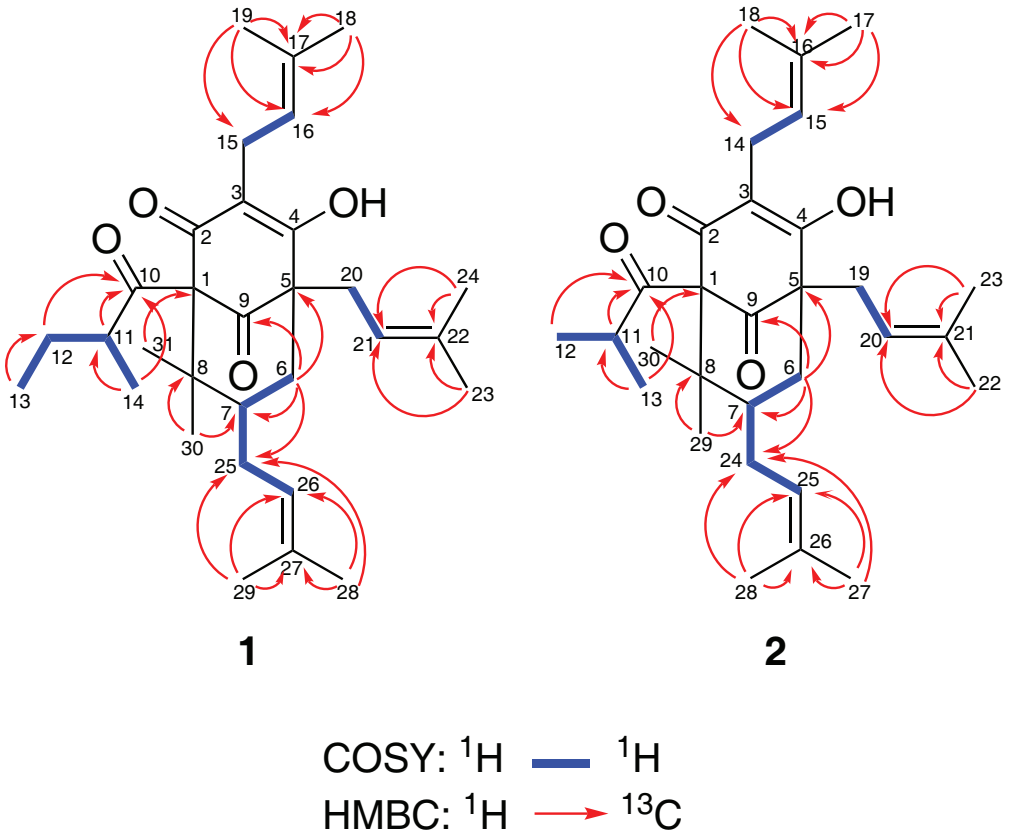

Figure 3. Selected COSY and HMBC correlations of compounds **1** and **2**.

The ^{1}H and ^{13}C-NMR spectra of **1** are typical of a type A polyprenylated acylphloroglucinol with a bicyclo[3.3.1] nonane-2,4,9-trione skeleton (Nedialkov et al. 2015). In addition, signals of two methyl groups are observed in the ^{1}H-NMR spectrum of compound **1**. The signal of the first methyl group appears as a three-proton triplet at δ_H 0.78 (J = 6.5 Hz), which shows a cross-peak with the signals of the methylene group at δ_H 1.20 and 1.94, while that of the second one is in the form of a three-proton doublet at δ_H 0.97 (J = 6.5 Hz), correlating with the methine proton signal at δ_H 1.77. In the HMBC experiment, some of these signals correlate with those of the carbonyl group at δ_C 210.8 (C-10). These signals are typical of a 2-methylbutyryl group, which is attached to the first position in the molecule of compound **1** (Nedialkov et al. 2018). Based on these spectral data, compound **1** was identified as 4-hydroxy-6,6-dimethyl-1,3,7-tris(3-methylbut-2-en-1-yl)-5-(2-methylbutanoyl)bicyclo[3.3.1]non-3-ene-2,9-dione, also known as adsecohyperforin. This substance was reported for the first time in cell cultures of *Hypericum perforatum* L. (Charchoglyan et al. 2007), but the proposed structure was not supported by NMR spectral data. This is the second report of the presence of adsecohyperforin in a plant source.

Compound **2** was isolated as an optically active colorless oil, and the reported specific optical rotation is $[\alpha]^{20}_D$ = −14.07. The HRESIMS spectrum of **2**, taken in a positive ion mode, showed a protonated molecule $[M+H]^+$ at *m/z* 469.3311 (mass error Δ_{ppm} = −0.31) corresponding to a molecular formula $C_{30}H_{44}O_4$ with nine degrees of unsaturation. Similarly to compound **1**, the MS/MS spectrum of the protonated molecule shows neutral losses characteristic of alkenes: isobutene (-56), isoprene (-68), and dimethylketene (-70). The plausible fragment structures, as well as the proposed fragmentation pathways, are shown in Fig. 2. The presence of these neutral losses in the MS/MS spectra of the selected $[M+H]^+$ ion suggests a compound structurally close to the hyperforin molecule (Liu et al. 2005). The ^{1}H and ^{13}C-NMR spectral data of compound **2** were typical of type A polyprenylated acylphloroglucinol with a bicyclo[3.3.1]nonane-2,4,9-trione skeleton (Nedialkov et al. 2015) and were very similar to those of compound **1**. In the ^{1}H NMR spectrum are observed two three-protone doublets with a spin-spin coupling constant *J* = 6.5 Hz at δ_H 1.03 and 0.97 ppm, which in the COSY experiment (Fig. 2) exhibit cross-peaks with the methine proton signal at δ_H 2.09 ppm. All these three signals in the HMBC experiment (Fig. 3) gave cross-peaks with the signal at δ_C 211.3 ppm. These signals are characteristic of an isobutyl group attached to C-1 in the molecule of this compound (Nedialkov et al. 2015). Based on this spectral evidence, compound **2** was identified as 4-hydroxy-1-isobutyryl-8,8-dimethyl-3,5,7-tris(3-methylbut-2-en-1-yl)bicyclo[3.3.1] non-3-ene-2,9-dione, also known as secohyperforin. This compound was found for the first time in cell cultures of *H. perforatum* (Charchoglyan et al. 2007).

The antineoplastic potential of compounds **1** and **2** was assessed in a panel of human tumor cell lines (MDA-MB,

EJ, and HL-60), using the standard MTT-dye reduction assay, following 72 h exposure and using the topoisomerase II inhibitor etoposide as a positive control. The tested compounds exerted concentration-dependent cytotoxicity against the tumor cell lines, causing a 50% reduction of cellular viability at low micromolar concentrations (the corresponding IC_{50} values are summarized in Table 1). All three cell lines showed that both substances exhibited greater cytotoxicity than the positive control, etoposide. The IC_{50} values of compounds **1** and **2** ranged from 0.27 to 4.85 μM, and those of etoposide from 1.27 to 8.4 μM. The results showed that compounds **1** and **2** are promising candidates for the development of new chemotherapeutic agents applicable in the treatment of tumor diseases.

Table 1. Cytotoxic activity of compounds **1** and **2** on some tumor cell lines after 72 h exposure (MTT test) expressed as IC_{50} (μM). Etoposide was used as a positive standard.

Compound	MDA-MB	EJ	HL-60
1	0.59 ± 0.16	0.72 ± 0.19	0.37 ± 0.05
2	2.13 ± 0.29	4.85 ± 0.67	0.27 ± 0.03
Etoposide	8.42 ± 1.3	5.4 ± 1.3	1.27 ± 0.4

Conclusion

A phytochemical investigation of the dichloromethane extract of the aerial parts of *Hypericum hirsutum* L. led to the isolation and identification of the main polyprenylated acylphloroglucinols adsecohyperforin (**1**) and secohyperforin (**2**). The MS/MS fragmentation of both compounds and NMR spectral data of adsecohyperforin (**1**) are described here for the first time. These natural products are established for the second time in a plant source. In a study involving a panel of tumor cell lines (MDA-MB, EJ, and HL-60), an MTT-based assay demonstrated that both substances showed greater cytotoxicity than the positive control, etoposide. The IC_{50} values for the substances ranged from 0.27 to 4.85 μM, while etoposide had IC_{50} values between 1.27 and 8.42 μM. These results suggest that the substances are promising candidates for developing new chemotherapeutic drugs.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

The authors declared that no experiments on animals were performed for the present study.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

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Author contributions

Z. Kokanova-Nedialkova – experiments: extraction and isolation; writing and editing.

G. Momekov – experiments: in vitro tests; writing and editing; funding.

P. Nedialkov – conceptualization, experiments: plant material collection and identification, structural elucidation, writing and editing, funding.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supporting information

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