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Antiseizure potential of the ancient Greek medicinal plant *Helleborus odorus* subsp. *cyclophyllus* and identification of its main active principles

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ABSTRACT

Ethnopharmacological relevance: Ethnopharmacological data and ancient texts support the use of black hellebore (*Helleborus odorus* subsp. *cyclophyllus*, Ranunculaceae) for the management and treatment of epilepsy in ancient Greece.

Aim of the study: A pharmacological investigation of the root methanolic extract (RME) was conducted using the zebrafish epilepsy model to isolate and identify the compounds responsible for a potential antiseizure activity and to provide evidence of its historical use. In addition, a comprehensive metabolite profiling of this studied species was proposed.

Materials and methods: The roots were extracted by solvents of increasing polarity and root decoction (RDE) was also prepared. The extracts were evaluated for antiseizure activity using a larval zebrafish epilepsy model with pentylenetetrazole (PTZ)-induced seizures. The RME exhibited the highest antiseizure activity and was therefore selected for bioactivity-guided fractionation. Isolated compounds were fully characterized by NMR and high-resolution tandem mass spectrometry (HRMS/MS). The UHPLC-HRMS/MS analyses of the RME and RDE were used for dereplication and metabolite profiling.

Results: The RME showed 80% inhibition of PTZ-induced locomotor activity (300 μg/ml). This extract was fractionated and resulted in the isolation of a new glucopyranosyl-deoxyribonolactone (**1**) and a new furostanol saponin derivative (**2**), as well as of 20-hydroxyecdysone (**3**), hellebrin (**4**), a spirostanol glycoside derivative (**5**) and deglucohellebrin (**6**). The antiseizure activity of RME was found to be mainly due to the new furostanol saponin (**2**) and hellebrin (**4**), which reduced 45% and 60% of PTZ-induced seizures (135 μM, respectively). Besides, the aglycone of hellebrin, hellebrigenin (**S34**), was also active (45% at 7 μM). To further characterize the chemical composition of both RME and RDE, 30 compounds (**A7**-**33**, **A35**–**37**) were annotated based on UHPLC-HRMS/MS metabolite profiling. This revealed the presence of additional bufadienolides, furostanols, and evidenced alkaloids.

Conclusions: This study is the first to identify the molecular basis of the ethnopharmacological use of black hellebore for the treatment of epilepsy. This was achieved using a microscale zebrafish epilepsy model to rapidly quantify *in vivo* antiseizure activity. The UHPLC-HRMS/MS profiling revealed the chemical diversity of the extracts and the presence of numerous bufadienolides, furostanols and ecdysteroids, also present in the decoction.

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1. Introduction

Epilepsy is one of the oldest and most critical central nervous system (CNS) disorders that affects more than 50 million people worldwide (Megiddo et al., 2016; WHO, 2019). Despite the availability of over 25 antiseizure drugs (ASDs), one third of epileptic patients suffer from pharmacoresistance and heavy side effects (Ngugi et al., 2010; Santulli et al., 2016).

The intense manifestations of epilepsy in the form of seizures have been described in numerous ancient texts dating to 2000 BC. Vivid descriptions of epileptic seizures have been found in inscriptions from Mesopotamia, ancient Egypt, the Babylonian and Roman empires, and ancient Greece (Magiorkinis et al., 2010). In antiquity, epilepsy was considered to have a divine emanation. Hippocrates of Kos (c. 460-370 BC) clearly identified the disease, contested its sacred nature and set the foundation for a scientific approach to find a cure. Several treatments for the prevention of seizures were proposed, such as shaving the head, walking, exercising, vomiting, use of diuretics, phlebotomy, and even sneezing (Magiorkinis et al., 2010).

The Hippocratic Corpus provided little insight for the treatment of epilepsy, although about 1500 recipes are proposed against various ailments (Totelin, 2009). Both Hippocrates and Theophrastus (372-287 BC) (Hort, 1916) acknowledged the purgative and potentially dangerous properties of the roots of black hellebore, but none of them associated its use with conditions consistent with what we call today "psychiatric disorders". Explicit mentions of the therapeutic use of black hellebore roots against epilepsy are found later in *De materia medica* of Dioscorides (c. 40-90 AD). His descriptions revealed that in fact, black hellebore was a popular treatment for epilepsy and "the insane" (Beck, 2005). Black hellebore is also acknowledged by the important Roman encyclopedist Celsus (25-50 AD), as effective against "melancholia" or the "black bile disease" which could result in madness (Spencer, 1935). The popular use of black hellebore as a treatment for epilepsy is also evident in Greek myths that share one common characteristic: the treatment of sudden outbursts of "madness". In the myth of Melampus, a famous clairvoyant and healer, black hellebore was used to cure the daughters of King Proteus who were stricken by divine "madness" and wandered in the wild believing they were cows (Olivieri et al., 2017). In the myth of Heracles, the famous hellebore from Antikyra healed Heracles of the "madness" caused by Hera who drove him to kill his wife and children. Hellebore growing in Antikyra, a fishing village located in the Gulf of Corinth, was so renowned for its strong pharmacological activity against mental disorders that it became part of the proverb "Go to Antikyra!", which means "You need a good dose of hellebore!" (Maieron, 2018).

In modern pharmacology, the use of *Helleborus* species has been cited for tooth pain relief (*H. foetidus*), abortion (*H. orientalis*), anticystitis (*H. thibetanus*), skin diseases (*H. odorus*), and sore joints (*H. niger*) (Maior and Dobrotă, 2013; Zhang et al., 2017). More recently, the pharmacological effects of *Helleborus* species included anti-rheumatic, anti-inflammatory, anti-cancer, anti-diabetic, anti-bacterial, and antioxidant properties (Apetrei et al., 2011; Erdemoglu et al., 2003; Lindholm et al., 2002; Puglisi et al., 2009; Seifarth et al., 2011). In addition, the CNS antidepressant effect has been linked to saponins (sarsasapogenin and its glycosylated form) contained in *H. niger* (Olivieri et al., 2017; Ren et al., 2007). Nevertheless, all the above-mentioned pharmacological properties of hellebore are overshadowed by the high cardiotoxic and cytotoxic properties of most species (Gomes et al., 2009; Moreno et al., 2013; Schmutz, 1949), whose dangerous results have been known since antiquity.

Black hellebore, which was widely used in Greece during antiquity, has now been clearly identified as *Helleborus odorus* subsp. *cyclophyllus* (A. Braun) Maire & Petitm., Ranunculaceae) (Dimopoulos et al., 2013; Strid, 2016). The plant is native to the Balkan peninsula, including Greece (Tutin et al., 1993), while it is the only subtaxon of *Helleborus* present in Greece (Dimopoulos et al., 2013, 2016). Previous phytochemical investigation of the species reported palmitic/linoleic acids, phospholipids, β-sitosterol, uridine, small phenolic analogs, bufadienolides (hellebrin, hellebrigenin, deglucohellebrin), furostanols (caucasicoside A, helleboroside B), ecdysteroids (20-hydroxyecdysone and polypodine B), and flavonoid derivatives (Philianos, 1967; Philianos et al., 1983; Tsiftsoglou et al., 2018).

The use of black hellebore in ancient Greece for the treatment and soothing of various forms of "madness" has not yet been correlated with modern pharmacological antiseizure assays. To address this, we used an epilepsy assay based on zebrafish larvae (*Danio rerio*), whose homology to humans (80% similarity of nucleotides with human), small size and rapid development, make this vertebrate model very convenient to use (Crawford et al., 2008). Zebrafish screening platforms are now well established to rapidly detect antiseizure compounds. In this study, the seizures were induced by the $GABA_A$ antagonist pentylenetetrazole (PTZ) and the locomotor activity was quantified by behavioral analysis (Afrikanova et al., 2013). This zebrafish epilepsy model was found well adapted for the bioactivity-guided isolation of antiseizure natural products (Brillatz et al., 2018, 2020; Challal et al., 2014; Orellana-Paucar et al., 2012).

Here, we describe the potential antiseizure activity of the extracts and decoction of *H. odorus* subsp. *cyclophyllus* for the investigation of its ancient use as a treatment for epilepsy. Besides, UHPLC-HRMS/MS metabolite profiling of root methanolic extract (RME) and root decoction extract (RDE) was performed to obtain a more thorough description of the specialized metabolites composition.

2. Materials and Methods

2.1. Reagents

Hellebrigenin (**S34**) was purchased from Biopurify (Chengdu Biopurify Phytochemicals Ltd., Chengdu, Sichuan, China), which estimated its purity at 99.4% by LC-UV (296 nm).

2.2. General experimental procedure

NMR spectroscopic data were recorded on a Varian (Palo Alto, CA, USA) Unity Inova 500 MHz spectrometer or on a Bruker Avance III HD 600 MHz NMR spectrometer equipped with a QCI 5 mm Cryoprobe and a SampleJet automated sample changer (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts (δ) are measured in parts per million (ppm) and coupling constants (*J*) are reported in hertz (Hz). Complete assignment was performed based on two-dimensional experiments (COSY, NOESY, HSQC and HMBC). High resolution tandem mass spectrometry (HRMS/MS) data were obtained on a Q Exactive Focus quadrupole-orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) using heated electrospray ionization (HESI-II) in the positive and negative modes. Semi-quantitative analysis was carried out using a Corona™ Veo™ RS Charged Aerosol Detector (CAD) (Thermo Scientific, Bremen, Germany). HRMS fingerprint data were obtained on a Micromass LCT Premier Time-of-Flight (TOF) mass spectrometer from Waters with an ESI interface (Waters, Milford, MA, USA). Ultra-high performance liquid chromatography (UHPLC-PDA-ELSD) measurements were performed using an Acquity UPLC system, coupled to a photodiode array (PDA) and a Sedex 85 detector (Sedere, Alfortville, France). Small scale pressurized liquid extraction was performed on an accelerated solvent extraction ASE 300 apparatus (Dionex, Sunnyvale, CA, USA) with 100 ml stainless steel vessels. Fractionation was performed on a medium pressure liquid chromatography (MPLC) preparative chromatographic system using Büchi sepacore system equipment (Büchi, Flawil, Switzerland). Further purification was performed on a semi-preparative HPLC equipment (Armen modular spot prep II, Saint-Avé, France).

2.3. Plant material extraction

Helleborus odorus subsp. *cyclophyllus* (A. Braun) Maire & Petitm. (Dimopoulos et al., 2013; Strid, 2016) was collected at mount Dirfys, Evia, Greece in May 2015. Botanical identification of the plant material was performed by Dr. E. Kalpoutzakis and a voucher specimen (Eb015) was deposited at the herbarium of the National and Kapodistrian University of Athens, Faculty of Pharmacy, Athens, Greece.

For the initial evaluation of the antiseizure activity of extracts, the dried roots and the aerial parts of *H. odorus* subsp. *cyclophyllus* were ground separately (20 g, < 1 mm particle size) and were subjected to a small-scale pressurized liquid extraction with ethyl acetate and methanol on an ASE system with 100 ml stainless steel vessels. The cell was pressurized, heated, and extracted statically under the following conditions: temperature 70 °C; pressure 120 bar; preheating time 1 min; two extraction cycles of 5 min each; flush volume 100%; purge 2 min. Methanol and ethyl acetate extracts of roots and aerial parts were dried under rotavapor and submitted to the zebrafish epilepsy assay (Fig. 1;

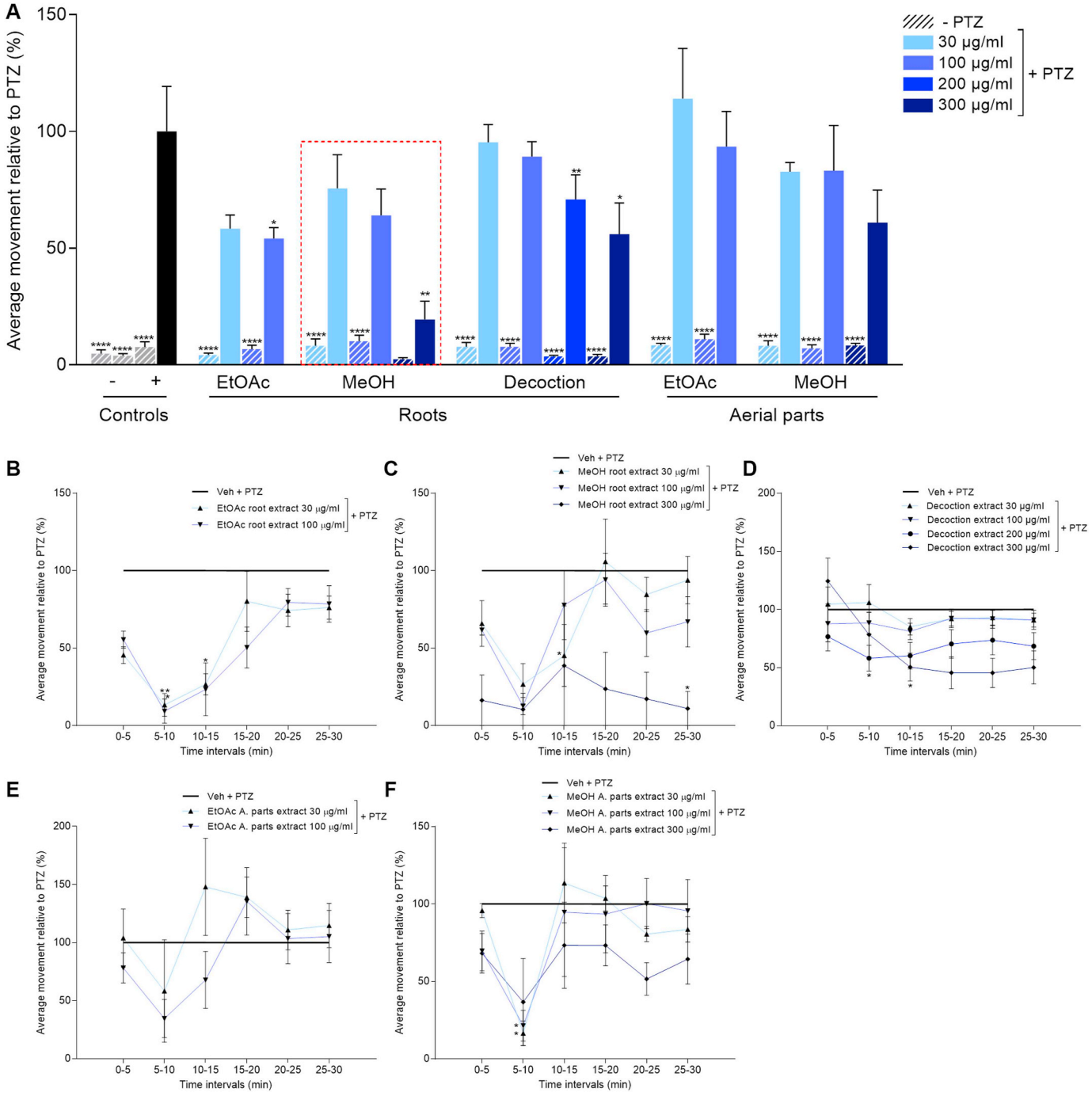

Fig. 1. Effects of *H. odorus* subsp. *cyclophyllus* extracts on larval locomotor activity. (A) Ethyl acetate (EtOAc), methanol (MeOH) and decoction extracts of the roots and EtOAc and MeOH extracts of the aerial parts. (B–F) PTZ-induced locomotor activity curves for a 30 min tracking period with 5 min average time periods after 18 h pretreatment with each extract. Striped bars represent the average movement before the induction of seizure (- PTZ). PTZ was used at 20 mM as proconvulsant agent (black bar/curve). Controls are described as "-" for the vehicle control and "+" for the positive control. Data are represented by the average % ± SEM (n ≥ 5) against Veh + PTZ. Statistical analysis was performed by one-way ANOVA (bar graph) and two-way ANOVA (movement/time graphs) with Dunnett's test to compare samples with positive control, with *P* value of < 0.05 (*), < 0.01 (**), < 0.0001 (****).

Fig. 1S, Supplementary information). Extracts were concentrated under vacuum until dryness to yield 2.5 g of root ethyl acetate extract (12.5% w/w), 7.2 g of root methanolic extract (35.8% w/w), 1.1 g of aerial parts ethyl acetate extract (5.5% w/w), and 5.5 g of aerial parts methanolic extract (27.5% w/w).

A large-scale extraction was performed on 300 g of root powder by solvents of increasing polarity (cyclohexane, dichloromethane, methanol, and water) for the preparative isolation of the potential bioactive compounds. All extracts were concentrated under vacuum until dryness to yield 15.9 g of cyclohexane extract (5.3% w/w), 8.3 g of dichloromethane extract (2.8% w/w), 42.0 g of methanol extract (14.0% w/w), and 29.8 g of aqueous extract (9.9% w/w) and evaluated in the zebrafish epilepsy assay (Fig. 2S, Supplementary information).

An aqueous decoction was prepared with 20 g of ground roots and 200 ml of distilled water, boiled during 2 min and left overnight at 40 °C, to yield 3 g of dry extract (15% w/w). This decoction is the closest formulation of what could have been used traditionally to treat epilepsy in ancient Greece (Beck, 2005).

2.4. Fractionation of the root methanolic extract and purification of compounds

The root methanolic extract (RME) obtained at large-scale was subjected to fractionation by preparative chromatography (Fig. 3S, Supplementary information). To ensure the same chromatographic selectivity, the extract was fractionated by MPLC-UV-ELSD after the use of a geometrical gradient transfer from analytical HPLC to preparative conditions (Challal et al., 2015). Fractionation of the RME (5 g) was performed using a C18 Zeoprep column (460 × 49 mm I.D., 15–25 µm spherical; Zeochem, Uetikon am See, Switzerland) with water (A) and methanol (B) containing both 0.1% formic acid, following a linear gradient of 5–100% of B in 263 min held during 65 min. The flow rate was fixed at 30 ml/min. The UV detection was recorded at 210, 254, 280 and 366 nm and the ELSD set at 40 °C, 2.3 bar N_2 and gain 1. Fractionation of the RME resulted in 40 fractions combined in 16 fractions (F1–F16) according to their UV and ELSD signals. All fractions were analyzed by UHPLC-PDA-ELSD and UHPLC-TOF HRMS and then tested in the zebrafish epilepsy assay to localize the bioactive fractions (Fig. 3S, Supplementary information). For the isolation, selected fractions were further purified on a semi-preparative HPLC equipment using a C18 X-bridge column (150 × 19 mm, 5 µm, Waters, Milford, MA, USA), with water (A) and methanol (B) containing both 0.1% formic acid. The purification was performed using: a step gradient for F3 (20–25% of B in 40 min, then 25–100% of B in 5 min held during 10 min), a linear gradient for F7 (50% of B in 20 min, then 50–100% of B in 5 min held during 10 min), a linear gradient for F8 (50% of B in 40 min, then 50–100% of B in 5 min held during 10 min) and a step gradient for F10 (50–80% of B in 40 min, then 80–100% of B in 5 min held during 10 min). The flow rate was fixed at 10 ml/min. The UV detection was set at 210–366 nm and ELSD detection at 40 °C, 3.1 bar N_2 and gain 8. Using this method, fraction F3 yielded **1** (2.0 mg) and **2** (4.2 mg), F7 yielded **3** (5.1 mg), F8 yielded **4** (6.8 mg), F10 yielded **5** (2.1 mg) and **6** (1.2 mg).

2.5. Description of the isolated compounds

(4*R*,5*S* or 4*S*,5*R*)-4-hydroxy-5-((β-D-glucopyranosyloxy)methyl)dihydrofuran-2(3H)-one (**1**). White amorphous solid. $[\alpha]_D^{25}$ -8.70 (c. 1.0, MeOH); UV (MeOH) λ_{max} 250 (2.87); ^{1}H NMR (CD_3OD, 600 MHz) δ 2.35 (1H, dd, *J* = 17.9, 2.2 Hz, H-3b), 3.03 (1H, dd, *J* = 17.9, 6.7 Hz, H-3a), 3.15 (1H, dd, *J* = 9.2, 7.8 Hz, H-2′), 3.26 (2H, m, H-4′, H-5′), 3.33 (1H, m, H-3′), 3.65 (1H, m, H-6′b), 3.81 (1H, dd, *J* = 11.6, 3.4 Hz, H-6b), 3.86 (1H, dd, *J* = 11.9, 1.8 Hz, H-6′a), 4.10 (1H, dd, *J* = 11.6, 3.4 Hz, H-6a), 4.28 (1H, d, *J* = 7.8 Hz, H-1′), 4.50 (1H, td, *J* = 3.4, 2.2 Hz, H-5), 4.52 (1H, dt, *J* = 6.7, 2.2 Hz, H-4); ^{13}C NMR (CD_3OD, 151 MHz) δ 39.0 (C-3), 62.7 (C-6′), 69.9 (C-4), 70.3 (C-6), 71.5 (C-4′), 75.1 (C-2′), 78.1 (C-3′, C-5′), 88.6 (C-5), 104.8 (C-1′), 178.9 (C-2); ESI (−)-HRMS *m/z* 293.0880 $[M-H]^-$ (calcd. for $C_{11}H_{17}O_9^-$, 293.0878, Δppm = 0.67). Data for MS/MS spectra are available in the GNPS library spectrum as CCMSLIB00005716784.

Helleodoraside (**2**). Amorphous solid. $[\alpha]_D^{25}$ -3.53 (c. 0.1, MeOH); UV (MeOH) λ_{max} 265 (2.56); ^{1}H NMR (CD_3OD, 500 MHz) δ 0.81 (3H, s, H-18), 0.89 (1H, m, H-9), 0.91 (1H, m, H-7α), 0.95 (3H, s, H-19), 1.00 (3H, d, *J* = 7.0 Hz, H-21), 1.03 (1H, m, H-5), 1.11 (1H, m, H-14), 1.12 (1H, m, H-12α), 1.25 (1H, m, H-15β), 1.31 (2H, m, H-4β, H-11β), 1.35 (1H, m, H-6α), 1.40 (1H, m, H-2β), 1.42 (1H, m, H-6β), 1.47 (1H, m, H-4α), 1.55 (1H, m, H-8), 1.66 (2H, m, H-7β, H-12β), 1.69 (1H, m, H-17), 1.81 (1H, m, H-23b), 1.90 (1H, m, H-23a), 1.95 (1H, m, H-15α), 2.12 (1H, m, H-24b), 2.19 (2H, m, H-2α, H-20), 2.21 (1H, m, H-24a), 2.64 (1H, dd, *J* = 14.2, 3.3 Hz, H-11α), 3.21 (1H, t, *J* = 8.7 Hz, H-2″), 3.25 (1H, m, H-5″), 3.28 (1H, m, H-4″), 3.35 (1H, t, *J* = 8.7 Hz, H-3″), 3.48 (2H, m, H-2′, H-4′), 3.49 (1H, m, H-5′b), 3.51 (1H, m, H-3), 3.56 (1H, dd, *J* = 11.8, 4.5 Hz, H-1), 3.66 (1H, dd, *J* = 12.0, 5.5 Hz, H-6″a), 3.78 (1H, d, *J* = 2.5 Hz, H-3′), 3.86 (1H, m, H-6″b), 3.87 (1H, m, H-5′a), 4.11 (1H, d, *J* = 12.3 Hz, H-26b), 4.26 (1H, d, *J* = 3.1 Hz, H-1′), 4.28 (1H, d, *J* = 8.7 Hz, H-1″), 4.33 (1H, d, *J* = 12.3 Hz, H-26a), 4.34 (1H, m, H-16), 4.93 (1H, d, *J* = 4.3 Hz, H-27b), 5.09 (1H, d, *J* = 4.3 Hz, H-27a); ^{13}C NMR (CD_3OD, 126 MHz) δ 8.4 (C-19), 16.2 (C-21), 17.2 (C-18), 24.4 (C-11), 28.8 (C-24), 29.7 (C-6), 32.3 (C-23), 33.0 (C-15), 33.4 (C-7), 37.0 (C-2), 37.5 (C-8), 39.3 (C-4), 41.2 (C-20), 41.6 (C-13), 41.6 (C-12), 42.2 (C-10), 43.9 (C-5), 56.2 (C-9), 57.7 (C-14), 62.9 (C-6″), 65.5 (C-17), 67.6 (C-5′), 68.8 (C-3), 70.2 (C-3′), 71.7 (C-4″), 72.7 (C-26), 72.8 (C-4′), 74.6 (C-2′), 75.1 (C-2″), 78.0 (C-5″), 78.1 (C-3″), 82.0 (C-1), 82.5 (C-16), 101.3 (C-1′), 103.3 (C-1″), 112.3 (C-27), 113.6 (C-22), 147.2 (C-25); ESI(−)-HRMS *m/z* 741.4054 $[M-H]^-$ (calcd. for $C_{38}H_{61}O_{14}^-$, 741.4067, Δppm = −1.72). Data for MS/MS spectra are available in the GNPS library spectrum as CCMSLIB00005716785.

20-hydroxyecdysone (**3**). $[\alpha]_D^{20}$ +58.9 (c. 0.3, MeOH); UV (MeOH) λ_{max} 246 (2.02) nm; For NMR spectra, see supplementary information.

Hellebrin (**4**). $[\alpha]_D^{20}$ -23.4 (c. 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 300 (2.57) nm; For NMR spectra, see supplementary information.

(*23S,24S*)-21-hydroxymethyl-24-{[*O*-β-D-glucopyranosyl-(1→4)-β-D-fucopyranosyl]oxy}-3β,23-dihydroxyspirosta-5,25(27)-diene-1β-yl *O*-(α-L-rhamnopy-ranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside (**5**). $[\alpha]_D^{25}$ -38.8 (c. 0.5, MeOH); UV (MeOH) λ_{max} 254 (3.61) nm; For NMR spectra, see supplementary information.

Deglucohellebrin (**6**). $[\alpha]_D^{17}$ -0.245 (c. 0.001, MeOH); UV (MeOH) λ_{max} 299 (2.90) nm; For NMR spectroscopic data, see supplementary information.

The purity of the isolated compounds was estimated by ^{1}H proton NMR, through visual estimation of the area integrals of the compound signals compared to all detected signals (see Supplementary information). With this method, the level of purity of **1** was at 70%, **2** at 70%, **3** at 60%, **4** at 80%, **5** at 85%, and **6** at 60%.

2.6. UHPLC metabolite profiling

2.6.1. UHPLC-PDA-ELSD, and UHPLC-*TOF-HRMS* analysis

Fractions fingerprinting data were acquired by UHPLC-PDA-ELSD and UHPLC-TOF HRMS according to standard protocols of the laboratory (Brillatz et al., 2018). Detailed protocols are provided in supplementary information (Annex A, Supplementary information).

2.6.2. UHPLC-PDA-CAD-HRMS/MS analysis

Metabolite profiling of the root methanolic and root decoction extracts was performed by UHPLC-PDA-CAD-HRMS/MS to obtain qualitative (PDA-HRMS/MS) and semi-quantitative (CAD) information in a single analysis (Fig. 5; Fig. 4S, Supplementary information). The optimized HESI-II parameters were as follows: source voltage, 3.5 kV (pos), 2.5 kV (neg); sheath gas flow rate (N_2), 55 units; auxiliary gas flow rate, 15 units; spare gas flow rate, 3.0; capillary temperature, 266 °C (pos), 320 °C (neg); S-Lens RF Level, 45. The data-dependent MS/MS events

were performed on the three most intense ions detected in full scan MS (Top 3 experiment). After being acquired in a MS/MS scan, parent ions were placed in a dynamic exclusion list for 1.0 s. The MS/MS isolation window width was 1.5 Da, and the normalized collision energy (NCE) was set to 15, 30 and 45 units. Full scans were acquired at a resolution of 35 000 FWHM (at *m/z* 200) and MS/MS scans at 17 500 FWHM with a maximum injection time of 119 ms and 55 ms, respectively. Separation was achieved on an Acquity BEH C18 column (2.1 × 100 mm I.D.; 1.7 μm).The temperature in the autosampler and column oven were fixed at 10 and 40 °C, respectively. The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% formic acid; separation was performed with a linear gradient from 2 to 100% of B in 30 min followed by a 10 min isocratic step at 100% of B and then 10 min isocratic step at 2% of B for column reconditioning. Injection volume was set at 4 μL, the flow rate was fixed at 0.46 ml/min. In positive ion mode, the di-isooctyl phthalate $C_{24}H_{38}O_4$ $[M+H]^+$ ion (*m/z* 391.28429) was used as an internal lock mass. An Acquity UPLC photodiode array detector was used to acquire UV spectra which were detected from 200 to 500 nm and extracted from 254 to 300 nm to obtain the broadest peak resolution (Fig. 5A). UHPLC-CAD parameters were set at 40 °C, split 9:1, 5 bar N_2 and power function 1.

2.6.3. MZmine parameters

After conversion to .mzXML format with Proteowizard (Chambers et al., 2012), MZmine (v2.51) was used to perform the UHPLC-HRMS/MS data processing (Pluskal et al., 2010). During the mass detection step, the ions kept were those above a noise level set at $4.0E^4$ for MS1 (and 0 for MS2). Chromatogram builder was employed with a minimum height of $4.0E^4$ and *m/z* tolerance of 0.004 (15 ppm). The chromatogram deconvolution was performed using the wavelets ADAP algorithm (Myers et al., 2017) with a single noise (S/N) threshold of 10, the wavelet scales from 0.00 to 0.08 min and a peak duration range from 0.10 to 1.50; the MS2 scan pairing was set at *m/z* 0.001 Da (0.5 min). The chromatograms were deisotoped using the isotope peak grouper with an *m/z* tolerance of 0.01 or 10 ppm, a retention time (RT) tolerance of 0.1 min and a maximum charge of 1, while the representative isotope used was the most intense. Peak alignment was applied using the join aligner method with an *m/z* tolerance of 8 ppm, an RT absolute tolerance at 0.03 min and a weight for *m/z* at 10. An adduct search in both positive and negative ionization modes was performed on the peak list with a RT tolerance set at 0.01 min (*m/z* 0.001 or 5 ppm) and the maximum relative peak height at 50%. A complex search was performed on the peak list as well, with an *m/z* tolerance of 0.001 (5 ppm), an RT tolerance of 0.05 min, and the maximum complex peak height at 50%. The peak list was gap-filled using the "same RT and *m/z* range gap filler" module with a *m/z* tolerance of 0.001 or 5.0 ppm. The peak list was reduced to a minimum peak area of $4.0E^4$ for data processing. A custom database of monocots and dicots (123 905 compounds) was extracted and created from the Dictionary of Natural Products for dereplication (DVD version 26:2) (Chapman, 2019).

2.6.4. Molecular network analysis

The MZmine files were exported in .mgf format for the processing of the molecular network in the Global Natural Products Social (GNPS) platform (Wang et al., 2016). To maintain the retention time and exact mass information and to allow the isomer separation, the feature-based molecular networks were created using the .mgf file resulting from the MZmine pretreatment step detailed above (Nothias et al., 2019). Spectral data were uploaded on the GNPS molecular networking platform. A network was then created where edges were filtered to have a cosine score above 0.65 and at least 6 matched peaks between the two nodes. Furthermore, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS spectral libraries. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. All spectra were searched against the ISDB-DNP database (Allard et al., 2016). A top 50 of the chemical structures was provided for each node according to the *in silico* MS/MS fragmentation.

Secondly a taxonomic reweighting step allowed the candidate structures to be re-ranked with a weight inversely proportional to the distance between the candidate biological sources and the one of the analyzed sample (family, Ranunculaceae; genus, *Helleborus*; species, *Helleborus odorus* subsp. *cyclophyllus*) and a top 6 of candidates was provided (Rutz et al., 2019). The output was visualized using Cytoscape v3.7.0 (Shannon et al., 2003). Peak areas of the different analyses were represented as pie chart-diagrams, where green was coded for root methanolic extract, blue for root decoction extract and white for methanol blank (Figs. 5–6S, Supplementary information). The size of the nodes was proportional to the surface area of the peaks in the methanolic extract. The full MS data set is uploaded and accessible on the GNPS servers as Massive Data sets N° MSV000084842 (https://doi.org/10.25345/C5K405).

2.7. Zebrafish seizure assay

The zebrafish seizure assay, based on the induction of epileptic seizures in zebrafish larvae by the PTZ, was performed as previously described (Afrikanova et al., 2013; Brillatz et al., 2018, 2020). Details and adaptations can be found in the supplementary information (Annex B, Supplementary information). The determination of the maximum-tolerated concentration (MTC) in the zebrafish assay was performed for each sample and is described in Annex B for extracts and isolated compounds. In the zebrafish experiments, the antiseizure drug topiramate was used as reference standard (data not shown) and demonstrated comparable reduction of PTZ-induced locomotor activity at similar concentration reported by Afrikanova et al. (2013). The Luxembourg Centre for Systems Biomedicine (LCSB) Zebrafish Core Facility and the experiments involving zebrafish embryos and larvae have been allowed, with regard to European Directive 2010/63/EU, by the relevant agencies of the Government of Luxembourg, both the "Ministère de la Santé et Ministère de l'Agriculture, de la Viticulture et du Développement Rural (Arrêté ministériel du 20 Décembre 2012 et 20 Janvier 2016)".

2.8. Statistical analysis

Data were expressed as mean ± SEM ($n \geq 3$) for the zebrafish epilepsy assay. Statistical differences among the data were assessed by one-way (bar graphs) or two-way (movement/time graphs) ANOVA with Dunnett's test to compare samples with positive control, with *P* values of < 0.05 (*), < 0.01 (**), < 0.001 (***), and < 0.0001 (****), respectively. Analyses were performed using the statistical software Prism v8.1.1 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

This work was designed to provide an insight into the potential antiseizure activity and a detailed survey of the chemical composition of *H. odorus* subsp. *cyclophyllus*. To this end, a series of polar and non-polar extracts of roots and aerial parts were prepared and evaluated for antiseizure activity, and the chemical composition of the decoction was investigated.

3.1. Preparation of extracts and decoction

Ethnopharmacological data indicated the use of black hellebore root as an antiseizure remedy (Beck, 2005). In order not to miss possible antiseizure compounds of this plant, which has never been studied for this kind of activity, complementary polar and non-polar extracts of the roots and aerial parts have been prepared in a first step to evaluate the

activity of the largest possible range of compounds.

3.2. Antiseizure activity of extracts and decoction

The roots and aerial parts of *H. odorus* subsp. *cyclophyllus* were extracted with ethyl acetate and methanol and tested in the zebrafish epilepsy assay (Fig. 1). The root methanolic extract (RME) demonstrated a significant antiseizure activity with a reduction of 80% of PTZ-induced locomotor activity ($p < 0.01$ at 300 µg/ml; Fig. 1A, C), while the ethyl acetate root extract was toxic at this concentration but exhibited a 45% reduction in seizures at 100 µg/ml ($p < 0.05$) (Fig. 1A and B). The methanolic extract of the aerial parts reduced PTZ-induced seizures up to 40% (300 µg/ml, $p < 0.01$) (Fig. 1A, F) but in this case, the ethyl acetate extract was inactive (Fig. 1A, E). The root decoction extract (RDE) was less potent than the RME and reduced seizures up to 45% (300 µg/ml, $p < 0.05$) (Fig. 1A, D), which is probably due to the presence of inactive components such as sugars. The information found on the use of hellebore in antiquity was not precise on how to administer the roots for the treatment of epilepsy (Beck, 2005). However, in antiquity, it was usual practice to prepare herbal wines and decoctions, which were an ideal method for physicians to control extraction through temperature (decoction) and polarity (ethanol vs. water). In this context, the mild antiseizure activity of the RDE and the significant activity of the RME may suggest that wine-based preparations would be more effective than decoctions. In this regard, the composition of the RME, which highlights compounds that are extractable in alcoholic/water solvents, is also documented in more detail in this study.

Based on these results, the activity of the RME was tested in an independent series of measurements and confirmed the trend observed for a potential antiseizure activity detected between 100 and 300 µg/ml (Fig. 1S, Supplementary information). Furthermore, the RME was prepared in large scale for further bioactivity-guided fractionation. In this case the very lipophilic part was removed by preliminary extraction with hexane and ethyl acetate. This enriched methanolic extract was re-evaluated in the zebrafish assay and also displayed a similar antiseizure activity trend (Fig. 2S, Supplementary information). Therefore, it was submitted to a large-scale fractionation by MPLC and was profiled by UHPLC-HRMS/MS.

3.3. Bioactivity-guided fractionation and isolation of compounds

Since the chemical composition of the plant is relatively well described (Philianos, 1967; Tsiftsoglou et al., 2018), a rapid bioactivity-guided fractionation was performed on the RME prepared at large scale

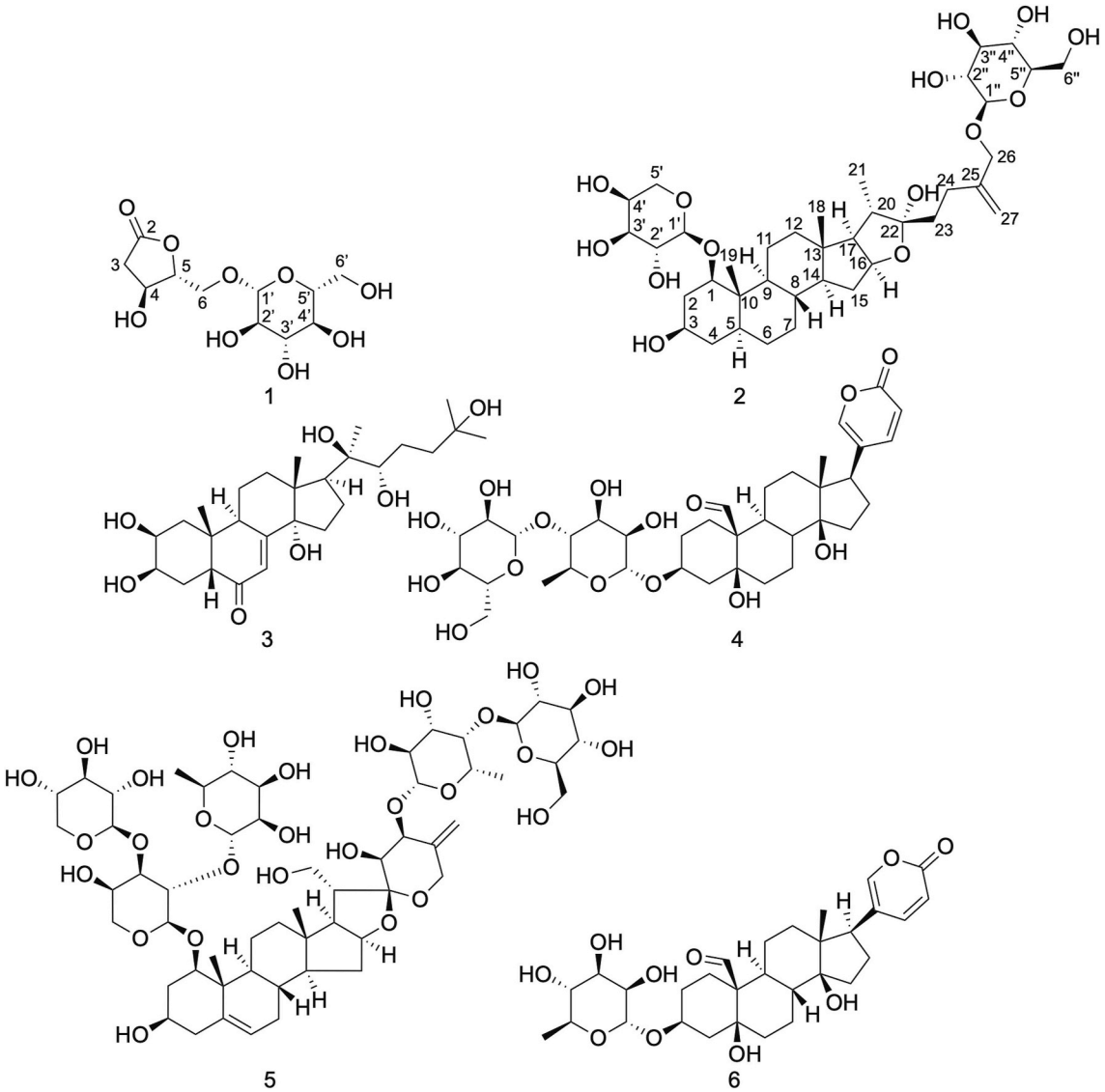

Fig. 2. Isolated compounds of the root methanol extract of *H. odorus* subsp. *cyclophyllus*. Compounds **1** and **2** are new chemical structures.

to localize and isolate the potential bioactive compounds. To ensure the same chromatographic selectivity, the extract was fractionated by MPLC-UV-ELSD after the use of a geometrical gradient transfer from analytical to preparative conditions (Fig. 3S, Supplementary information) (Challal et al., 2015). Forty fractions were collected, profiled by UHPLC-UV-ELSD and combined according to their common UV and ELSD signals to give 16 fractions. These fractions were evaluated in the zebrafish epilepsy assay and fraction F8 showed the most promising activity and reduced PTZ-induced seizures up to 60% at 300 µg/ml (Fig. 3S, Supplementary information). The UHPLC-ELSD analysis of F8 revealed the presence of a major compound, along with the trace of a minor one, and was therefore subjected to semi-preparative HPLC for further purification in order to yield **4**, which was found responsible for the activity (see *3.4*). Fractions F3, F7 and F10 were also purified to further support the comprehensive composition assessment of RME by metabolite profiling (see *3.4*).

This yielded four known compounds (**3–6**) previously reported in *Helleborus* species as 20-hydroxyecdysone (**3**) (Vokac et al., 1998), hellebrin (**4**) (Muhr et al., 1995; Tsiftsoglou et al., 2018), one spirostanol glycoside derivative (**5**) (Zhang et al., 2016) and deglucohellebrin (**6**) (Kissmer and Wichtl, 1986). It should be noted that for hellebrin (**4**), a consistent shift was observed with the original reference (Muhr et al., 1995) but in good agreement with Tsiftsoglou et al. (2018). Additionally, two new natural products were identified *de novo* by NMR and HRMS as a glucopyranosyl-deoxyribonolactone (**1**) and a furostanol saponin derivative (**2**) (Fig. 2).

Compound **1** was isolated as a white amorphous solid and had a molecular formula of $C_{11}H_{18}O_9$ (*m/z* 293.0880 $[M-H]^-$, calcd. for $C_{11}H_{17}O_9^-$, Δppm = 0.67). Analysis of the ^{1}H and HSQC NMR spectra included typical resonances of a glucopyranosyl moiety at δ_H/δ_C 4.28/104.8 (H/C-1′), 3.15/75.1 (H/C-2′), 3.33/78.1 (H/C-3′), 3.26/71.5 (H/C-4′), 3.26/78.1 (H/C-5′), 3.65 and 3.86/62.7 (H/C-6′) (Table 1S; Figs. 7–13S, Supplementary information). In addition to these signals, two oxymethines were observed at δ_H 4.50 (1H, td, *J* = 3.4, 2.2 Hz, H-5) and 4.52 (1H, dt, *J* = 6.7, 2.2 Hz, H-4), one oxymethylene was noted at δ_H 4.10 (1H, dd, *J* = 11.6, 3.4 Hz, H-6a) and 3.81 (1H, dd, *J* = 11.6, 3.4 Hz, H-6b), and lastly one methylene was identified at δ_H 3.03 (1H, dd, *J* = 17.9, 6.7 Hz, H-3a) and 2.35 (1H, dd, *J* = 17.9, 2.2 Hz, H-3b). COSY correlations allowed to link H-3 to H-4, H-4 to H-5 and H-5 to H-6. HMBC correlations of H-3, H-4 and H-5, with the carbonyl group at δ_C 178.9 (C-2), indicated a lactonization to a dihydrofuranone. HMBC correlation of the anomeric glucose proton (H-1) with C-6 linked the dihydrofuranone to the glucose. The relative stereochemistry of **1** was determined with the aid of NOESY experiment. NOE correlations between H-4 and H-6 indicated a *cis* configuration of these protons and thus a *trans* configuration between H-4 and H-5. Thus, the structure of **1** was identified as (4*R*,5*S* or 4*S*,5*R*)-4-hydroxy-5-((β-D-glucopyranosyloxy)methyl)dihydrofuran-2(3H)-one.

Compound **2** was isolated as a white amorphous solid and was assigned a molecular formula of $C_{38}H_{62}O_{14}$ (*m/z* 741.4054 $[M-H]^-$, calcd. for $C_{38}H_{61}O_{14}^-$, Δppm = −1.72). The ^{1}H NMR showed the characteristic signals of methyl protons at δ_H δ 0.81 (3H, s, H-18), 0.95 (3H, s, H-19), 1.00 (3H, d, *J* = 7.0 Hz, H-21), and two *exo*-methylene protons at δ_H/δ_C 4.26/101.3 (H/C-1′) and 4.28/103.3 (H/C-1″) from which a steroidal skeleton could be deduced (Table 2S; Figs. 14–20S Supplementary information). The edited-HSQC showed the following type of carbon substitutions: three methyl groups at δ_C 8.4 (C-19), 16.2 (C-21) and 17.2 (C-18); nine methylenes at δ_C 24.4 (C-11), 28.8 (C-24), 29.7 (C-6), 32.3 (C-23), 33.0 (C-15), 33.4 (C-7), 37.0 (C-2), 39.3 (C-4), 41.7 (C-12); six methines at δ_C, 37.5 (C-8), 41.2 (C-20), 43.9 (C-5), 56.2 (C-9), 57.7 (C-14) and 65.5 (C-17); three oxymethylenes at δ_C 62.9 (C-6″), 67.6 (C-5′) and 72.7 (C-26); ten oxymethines at δ_C 68.8 (C-3), 70.2 (C-3′), 71.7 (C-4″), 72.8 (C-4′), 74.6 (C-2′), 75.1 (C-2″), 78.0 (C-5″), 78.1 (C-3″), 82.0 (C-1) and 82.5 (C-16); two anomeric carbons at δ_C 101.3 (C-1′) and 103.3 (C-1″); one *exo*-methylene carbon at δ_C 112.3 (C-27). The HMBC correlations (Fig. 3A) from the methyl protons H_3-19 to the oxymethine C-1, the methines C-5 and C-9 and the quaternary carbon C-10 at δ_C 42.2 indicated that the sterol is oxygenated in C-1. The COSY correlation from H_2-2 to H-1 and H-3 showed the presence of a hydroxyl in C-3. The presence of the furostanol is supported by the following HMBC correlations: i) the methyl H_3-18 to C-12, C-14, C-17 and C-13 at δ_C 41.6; ii) the methyl H_3-21 to C-17, C-20 and C-22 at δ_C 113.6; iii) the methylene H_2-15 and methine H-17 to C-16. HMBC correlation from the *exo*-methylene H_2-27 to the methylene C-24, the oxymethylene C-26 and the quaternary carbon C-25 at δ_C 147.2 showed the presence of a double bond in the side chain. Based on this information, a furostanol saponin was identified with hydroxylation at C-1, C-3, C-22 and C-26, and a C-25-C-27 double bond. A glucose and an arabinose moiety were identified from the COSY correlations with the anomeric protons, the coupling constants and the ^{13}C chemical shift values (Fig. S14-20, Supplementary information). HMBC correlations of the anomeric protons (H-1' for arabinose, H-1" for glucose) proved their substitution to C-1 and C-26 respectively (Fig. 3A). The NOE correlations from the methyl groups H_3-19 to H-4β, H-6β, H-8, H-11β and H_3-18 to H-12β, H-8β, H-15β, H-20 suggested a β-orientation of these protons (Fig. 3B). The NOE correlations from H-5 to H-1α, H-4α, H-9, from H-3 to H-2α, H-4α, from H-16 to H-15α, H-17, and from H-17 to the methyl C-21 indicated an α-orientation of these protons. The β position of the side chain in C-22 was deduced based on the NOE correlation between H-20 and H-23. Compound **2** was identified as a new disaccharide tetrahydroxylated furostanol saponin and named helleodoraside (Fig. 3). Similar furostanol glycosides have been previously isolated from *Helleborus thibetanus* (Zhang et al., 2016) and from *Helleborus macranthus* (Tschesche et al., 1984).

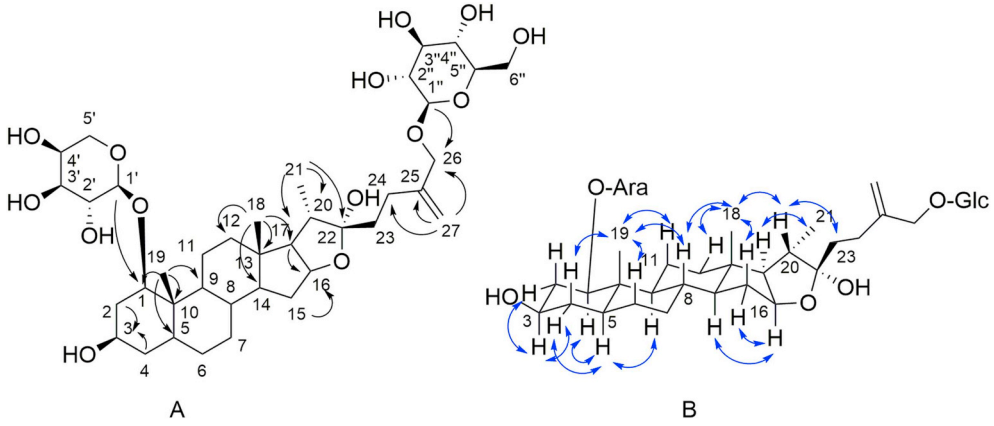

Fig. 3. (A) HMBC and (B) NOE key-correlations of compound 2.

3.4. Antiseizure activity of isolated compounds

Hellebrin (**4**), the main constituent of the active fraction F8 together with the other isolated compounds **1–3**, **5** were evaluated in the zebrafish epilepsy assay, with the exception of **6** which was in insufficient quantity to be tested. Hellebrin (**4**) demonstrated a potential antiseizure activity by reducing 60% of pentylenetetrazole (PTZ)-induced seizures at 135 μM ($p < 0.01$) (Fig. 4A, C). Since this compound is a glycoside and is likely to be hydrolyzed when administered as a decoction, its aglycone hellebrigenin (**S34**) has been tested to determine whether the absence of the glycoside moiety influenced the antiseizure activity. This compound (**S34**) was indeed able to reduce significantly PTZ-induced locomotor activity up to 45% at 7 μM ($p < 0.01$) (Fig. 4A, E). All other purified compounds were not active, except for **2** which decreased PTZ-induced seizures by 40% at 135 μM (Fig. 4A–B). Compound **2** was a minor constituent of the extract and its activity could not be highlighted during the first biological evaluation of the combined fractions (Fig. 3S, Supplementary information).

Although the compounds could only be partially purified as part of the strategy followed, these results allowed the identification of the class of compounds associated with the antiseizure activity detected in the active extract. It should be noted that the maximum tolerated concentration (MTC) was carefully determined for each isolated compounds (Annex B, Supplementary information) to avoid false positive results due to potential toxicity or sedative effects. This is consistent with the locomotor activity of zebrafish larvae measured after the incubation period of 18 h and prior to the addition of PTZ which is depicted by striped bars in Figs. 1 and 4.

Hellebrin (**4**) and the new compound **2** belong to cardiac glycosides which are known for their cardiotoxic and cytotoxic properties (Chen et al., 2006; Moreno et al., 2013). It has been shown that the toxicity of cardiac glycosides is attributed to their aglycones, while the glycoside moieties enhance their pharmacokinetic properties and particularly their solubility in aqueous media (Smith, 1985). The aglycone hellebrigenin (**S34**) was found active at a much lower concentration than **4** and **6**, suggesting that **S34** is also absorbed by zebrafish larvae despite the absence of a glycoside moiety in its structure.

Most saponins are toxic to fish due to the complexes they form with Δ^5 sterol of epithelial cells, which cause irreversible cellular disruption (Eeckhaut et al., 2015). Besides, cardiotonic bufadienolides such as hellebrin and hellebrigenin have already been reported to be toxic through the inhibition of the vital Na/K pump (Na^+/K^+-ATPase), which results to an increase of intracellular calcium levels and, consequently, an increase in cardiac muscle contractility (Clausen et al., 2017; Gonçalves-de-Albuquerque et al., 2017). While this cardiotoxicity could indirectly lead to a potential antiseizure activity, the samples tested in this study were evaluated at their MTC, where no sedative or toxic effects (including cardiotoxicity) were detected. However, the observed antiseizure activity should be further confirmed by EEG (Afrikanova et al., 2013) and calcium neuroimaging (Chen et al., 2013).

Despite their narrow therapeutic window, some cardiac glycosides such as digoxin and ouabain are still approved drugs for atrial

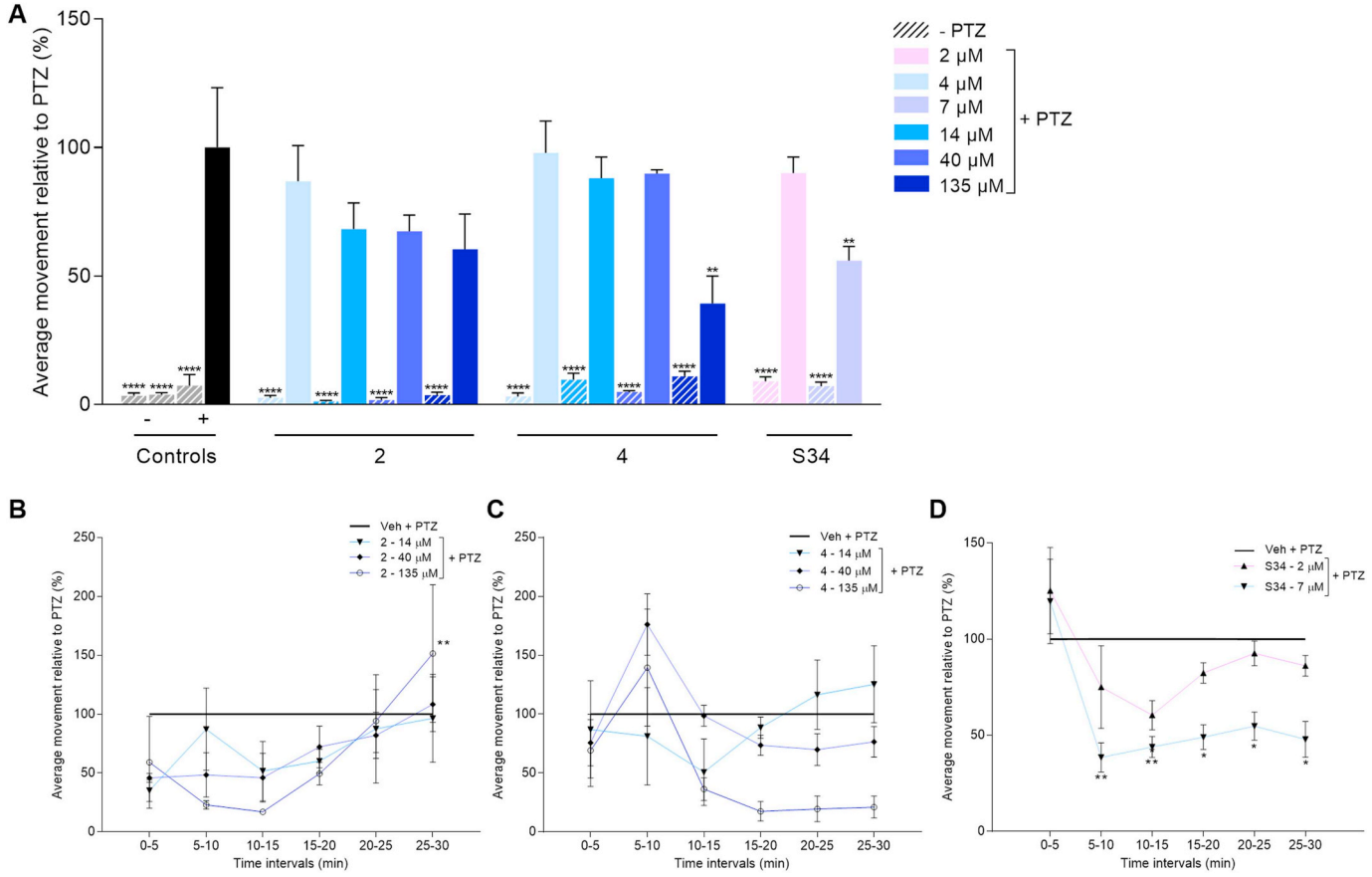

Fig. 4. Larval locomotor activity of the new furostanol saponin (2), hellebrin (4) and hellebrigenin (S34) tested on the zebrafish epilepsy assay in a 30 min tracking period. (A) Compounds **2**, **4** and **S34** have been tested at concentrations according to their MTC. (B–D) PTZ-induced locomotor activity curves for a 30 min tracking period with 5 min average time periods after 18 h pretreatment with **2** (A, B), **4** (A, C) and **S34** (A, D). Striped bars represent the average movement before the induction of seizure (- PTZ). PTZ was used at 20 mM as proconvulsant agent (black bar/curve). Controls are described as "-" for the vehicle control and "+" for the positive control. Data are represented as mean normalized value (%) + SEM ($n \geq 3$) against Veh + PTZ. Statistical analysis was performed by one-way ANOVA (bar graph) and two-way ANOVA (movement/time graphs) with Dunnett's test to compare samples with positive control, with P value of < 0.05 (*), < 0.01 (**), < 0.0001 (****).

Table 1
Compounds annotated by UHPLC-HRMS/MS in both ESI(+) and ESI(−) modes in the RME and RDE of *H. odorus* subsp. *cyclophyllus*. Isolated compounds 1–6, hellebrigenin S34, annotated features A7-33, A35-37. RME = root methanol extract; RDE = root decoction extract; RT = retention time; C. index = component index; sl = selfloop; I. rank = initial rank; F. rank = final rank (taxonomically informed); I. mode = ionization mode; F. = family (Ranunculaceae); G. = genus (*Helleborus*); S. = species (*H. odorus* subsp. *cyclophyllus*).

N°	RT (min)	C. index	Isolated as	Annotated as	I. rank	F. rank	Chemical family	Molecular formula	I. mode	*m/z*	Error (ppm)	Presence in		Taxonomical occurrence		
												RME	RDE	F.	G.	S.
1	0.96	11	Glucopyranosyl-deoxyribonolactone	–	–	–	Ribonolactone	$C_{11}H_{18}O_9$	$[M+H]^+$ $[M-H]^-$	295.1023 293.0880	−0.20 0.67	✓	✓	✓	✓	✓
A7	0.98	10	–	4-*O*-Glucopyranosyl-D-gluconic acidβ-D-form1,5-lactone	1	1	Phenol *O*-glycoside	$C_{12}H_{20}O_{11}$	$[M+H]^+$ $[M-H]^-$	– 339.0933	– 0.10	✓	✓	✓	✓	×
A8	1.39	206	–	Glaupadiol	30	1	Ribonolactone	$C_{11}H_{16}O_8$	$[M+H]^+$ $[M-H]^-$	277.0918 –	0.02 –	✓	×	✓	×	×
A9	2.59	10	–	3,4-Dihydroxyphenethyl glucoside	1	1	Phenol *O*-glycoside	$C_{14}H_{20}O_8$	$[M+H]^+$ $[M-H]^-$	– 315.1089	– 1.14	✓	✓	×	×	×
A10	3.13	sl	–	Chaenorrhinoside	1	1	Phenol *O*-glycoside	$C_{15}H_{18}O_9$	$[M+H]^+$ $[M-H]^-$	343.1024 341.0880	0.12 0.57	✓	✓	×	×	×
A11	4.44	197	–	Oureguattidine	2	2[a]	Aporphine alkaloid	$C_{18}H_{19}NO_4$	$[M+H]^+$ $[M-H]^-$	314.1388 –	0.30 –	✓	✓	×	×	×
A12	4.95	197	–	Thaliporphine	1	1	Aporphine alkaloid	$C_{20}H_{23}NO_4$	$[M+H]^+$ $[M-H]^-$	342.1699 –	−0.29 –	✓	✓	✓	×	×
A13	5.41	sl	–	Rapisterone D	1	1	Ecdysteroid derivative	$C_{27}H_{44}O_8$	$[M+H]^+$ $[M-H]^-$	497.1405 495.1260	0.50 0.61	✓	✓	×	×	×
A14	5.80	197	–	Pallidine	16	1	Aporphine alkaloid	$C_{19}H_{21}NO_4$	$[M+H]^+$ $[M-H]^-$	328.1545 –	0.43 –	✓	✓	✓	×	×
A15	5.88	17	–	Helleboroside B	1	1	Furostanol	$C_{33}H_{52}O_{11}$	$[M+H]^+$ $[M-H]^-$	625.3583 –	0.10 –	✓	✓	✓	✓	×
A16	5.88	17	–	Hydrolyzed form of Helleboroside B	43	1	Furostanol	$C_{27}H_{40}O_5$	$[M+H]^+$ $[M-H]^-$	445.2950 –	0.30 –	✓	✓	✓	✓	✓
A17	6.18	17	–	Tigencaoside A	3	1	Bufadienolide	$C_{30}H_{44}O_{10}$	$[M+H]^+$ $[M-H]^-$	565.3010 –	0.49 –	✓	✓	✓	✓	×
A18	6.19	17	–	5α-bufalone	26	1	Bufadienolide	$C_{24}H_{32}O_4$	$[M+H]^+$ $[M-H]^-$	385.2375 –	0.40 –	✓	✓	✓	✓	✓
A19	6.52	17	–	Hydrolyzed form of helleboroside B (isomer)	49	1	Furostanol	$C_{27}H_{40}O_5$	$[M+H]^+$ $[M-H]^-$	445.2967 –	4.16 –	✓	✓	✓	✓	✓
3	6.52	17	20-hydroxyecdysone (ecdysterone)	20-hydroxyecdysone (ecdysterone)	1	1	Ecdysteroid	$C_{27}H_{44}O_7$	$[M+H]^+$ $[M-H]^-$	481.3169 479.2991	1.91 −4.85	✓	✓	✓	✓	✓
A20	6.57	17	–	Hirundigenin	1	1	Pregnane steroid	$C_{21}H_{30}O_5$	$[M+H]^+$ $[M-H]^-$	363.2164 –	−0.55 –	✓	✓	×	×	×
A21	6.57	2	–	Caucasicoside B	7	1	Furostanol	$C_{45}H_{70}O_{19}$	$[M+H]^+$ $[M-H]^-$	915.4579 –	−0.55 –	✓	✓	✓	✓	×
A22	6.58	17	–	5β-hydroxyecdysterone (polypodine B)	12	1	Ecdysone	$C_{27}H_{44}O_8$	$[M+H]^+$ $[M-H]^-$	497.3112 495.2949	0.62 −2.91	✓	✓	✓	✓	✓
A23	6.95	2	–	Macranthoside I	5	1	Macranthosid	$C_{45}H_{74}O_{19}$	$[M+H]^+$ $[M-H]^-$	919.4905 –	0.80 –	✓	✓	✓	✓	×
A24	7.22	2	–	Caucasicoside A	3	1	Furostanol	$C_{33}H_{50}O_{10}$	$[M+H]^+$ $[M-H]^-$	607.3476 –	−0.12 –	✓	✓	✓	✓	×
A25	7.22	2	–	(25S)-22α,25-epoxy-3β,11α-dihydroxyfurost-5-en-26-yl β-D-glucopyranoside	1	1	Furostanol	$C_{33}H_{52}O_{11}$	$[M+H]^+$ $[M-H]^-$	625.3579 623.3394	−0.54 −6.87	✓	✓	✓	✓	×
A26	7.28	sl	–	2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside	1	1	Spirofurostanol	$C_{33}H_{54}O_{11}$	$[M+H]^+$ $[M-H]^-$	627.3737 625.3542	−0.30 −8.21	✓	✓	×	×	×
A27	7.42	17	–	5β,14β,16β-trihydroxy-19-oxo-3β-[(α-l-rhamnopyranosyl)oxy)]bufa-20,22-dienolide	7	1	Bufadienolide	$C_{30}H_{42}O_{11}$	$[M+H]^+$ $[M-H]^-$	579.2803 577.2641	0.54 −2.31	✓	✓	✓	✓	×
A28	7.46	2	–	2,22-dideoxy-20-hydroxyecdysone 25-*O*-β-d-glucopyranoside	1	1	Ecdysteroid glycoside	$C_{33}H_{54}O_{10}$	$[M+H]^+$ $[M-H]^-$	611.3792 –	0.37 –	✓	✓	×	×	×

(*continued on next page*)

Table 1 (*continued*)

N°	RT (min)	C. index	Isolated as	Annotated as	I. rank	F. rank	Chemical family	Molecular formula	I. mode	*m/z*	Error (ppm)	Presence in		Taxonomical occurrence		
												RME	RDE	F.	G.	S.
A29	7.68	2	–	(25R)-1β,3β,11α-trihydroxy-22α-methoxyfurost-5-en-26-yl β-d-glucopyranoside	8	1	Furostanol	$C_{33}H_{52}O_{10}$	$[M+H]^+$ $[M-H]^-$	609.3632 –	−0.20 –	✓	✓	✓	✓	×
A30	7.83	sl	–	24-epi-24-O-acetyl-7,8-didehydrohydroshengmanol3-O-(29-O-malonyl)-b-D-xylopyranoside	5	1	Cyclolanostanol glycoside	$C_{40}H_{60}O_{14}$	$[M+H]^+$ $[M-H]^-$	765.4032 –	−3.11 –	✓	✓	✓	×	×
A31	7.99	2	–	Tigencaoside B	1	1	Bufadienolide	$C_{36}H_{54}O_{15}$	$[M+H]^+$ $[M-H]^-$	727.3551 –	2.10 –	✓	✓	✓	✓	×
2	8.10	10	Furostanol saponin derivative	–	–	–	Furostanol	$C_{38}H_{62}O_{14}$	$[M+H]^+$ $[M-H]^-$	– 741.4054	– −1.72	✓	×	×	×	×
4	8.18	17	Hellebrin	–	–	–	Bufadienolide	$C_{36}H_{52}O_{15}$	$[M + H_2O + H]^+$ $[M-H]^-$	742.8656 723.3204	−0.55 −4.00	✓	✓	✓	✓	✓
A32	8.53	2	–	Funkioside D	1	1	Macranthosid	$C_{45}H_{72}O_{18}$	$[M+H]^+$ $[M-H_2O-H]^-$	901.4792 917.4736	0.07 −1.69	✓	✓	×	×	×
5	8.88	sl	Spirostanol glycoside derivative	–	–	–	Spirostanol glycoside	$C_{55}H_{86}O_{28}$	$[M+H]^+$ $[M-H]^-$	– 1193.5240	– 0.60	✓	×	×	×	×
6	8.94	17	Deglucohellebrin	Deglucohellebrin	4	4[a]	Bufadienolide	$C_{30}H_{42}O_{10}$	$[M+H]^+$ $[M-H]^-$	563.2854 561.2689	0.58 −2.89	✓	✓	✓	✓	✓
A33	9.18	2	–	(25S)-22α,25-epoxy-26-[(β-D-glucopyranosyl)oxy]-3β-hydroxy- furost-5-en-1β-yl α-L-arabinopyranoside	1	1	Spirofurostanol	$C_{38}H_{60}O_{14}$	$[M+H]^+$ $[M-H]^-$	741.4056 739.3896	0.03 −1.93	✓	✓	✓	✓	×
S34	9.39	17	Hellebrigenin	Hellebrigenin	13	13[a]	Bufadienolide	$C_{24}H_{32}O_6$	$[M+H]^+$ $[M-H]^-$	417.2272 –	0.09 –	✓	✓	✓	✓	✓
A35	9.39	sl	–	3-Ac-(3,5,14,16-tetrahydroxy-19-oxocard-20(22)-enolide)	2	1	Cardenolide	$C_{25}H_{34}O_8$	$[M+H]^+$ $[M-H]^-$	– 461.2185	– 0.89	✓	✓	✓	×	×
A36	9.75	188		14β-hydroxy-3β-(β-d-glucopyranosyloxy)-5α-bufa-20,22-dienolide	4	1	Bufadienolide	$C_{30}H_{44}O_9$	$[M+H]^+$ $[M+H]^+$	549.3058 –	−0.10 –	✓	✓	✓	✓	×
A37	9.90	2	–	Terrestrinin A	1	1	Furostanol	$C_{33}H_{48}O_9$	$[M+H]^+$ $[M-H]^-$	589.3370 –	−0.18 –	✓	✓	×	×	×

[a] These compounds have been manually re-ranked according to the structure identified by NMR or according to the structural consistency within the cluster and biosynthetic relationship.

fibrillation and heart failure (Kanji and MacLean, 2012; Patel, 2016). Besides, other cardiac glycosides were reported to possess neuroprotective activity (Wang et al., 2006), rendering the results of the present study consistent. Hellebrin (**4**) and the corresponding aglycone hellebrigenin (**S34**) share certain structural similarities with neuroactive steroids (Biagini et al., 2010). For example, ganaxolone has been approved for infantile epileptic encephalopathy and has now reached phase III clinical trials in orphan refractory epilepsy (Yawno et al., 2017). Its mechanism of action is still unclear, though it involves allosteric modulation of $GABA_A$ receptor (Nohria and Giller, 2007).

It should be noted that the toxic effect of the black hellebore was already known in ancient Greece and this was clearly demonstrated by the presence of different cardiotonic glycosides (Tsiftsoglou et al., 2018), as also revealed by this study. According to ancient texts, the use of hellebore seems, however, to have been retained, among other treatments, to treat extreme cases of epilepsy despite the known toxicity (Beck, 2005). To obtain a more comprehensive view of the composition of the plant and compounds potentially present in the ancient preparation of black hellebore, a UHPLC-HRMS/MS metabolite profiling was performed on RDE and RME.

3.5. Metabolite profiling of extracts

The active RME and the RDE were analyzed by UHPLC-PDA-CAD-HRMS/MS to obtain qualitative (PDA-HRMS/MS) and semi-quantitative information (CAD) in a single analysis (Fig. 5; Fig. 4S, Supplementary information).

All HRMS/MS data were organized as a feature-based molecular network (MN) (Figs. 5–6S, Supplementary information) (Nothias et al., 2019). First, exact masses of the feature list were annotated against a

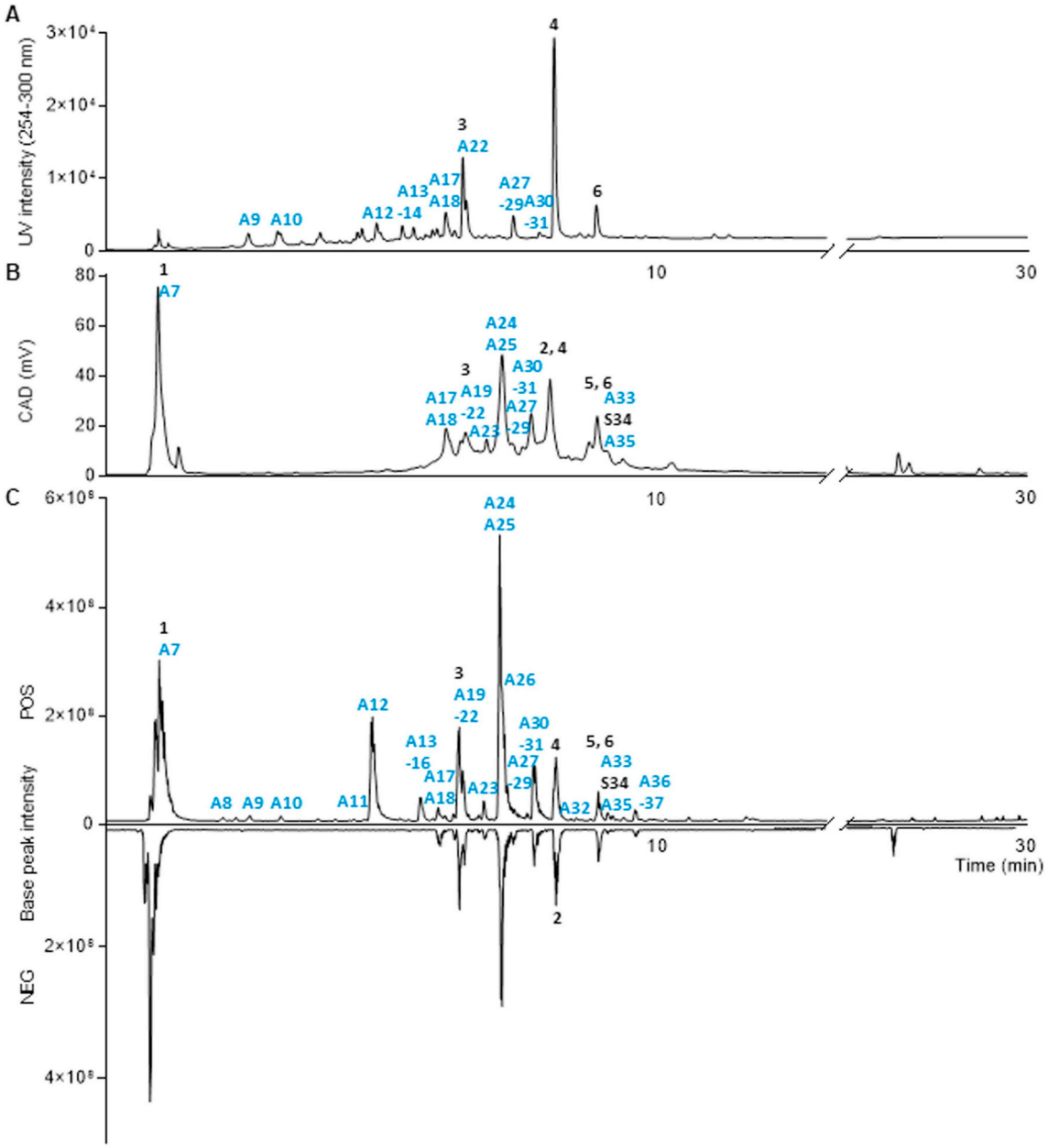

Fig. 5. UHPLC-PDA-CAD-HRMS/MS metabolite profiling of the root methanol extract of *H. odorus* subsp. *cyclophyllus*. (A) UHPLC-PDA chromatogram extracted (254–300 nm). (B) UHPLC-Charged Aerosol Detector (CAD) chromatogram. (C) UHPLC-HRMS/MS analysis in positive and negative ionization modes. Isolated compounds **1–6**, hellebrigenin standard **S34** and annotated features **A7**-**33**, **A35–37** (in blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

database built from the Dictionary of Natural Products (DNP, DVD version 26:2) (tolerance < 20 ppm) and then restricted to monocotyledon and dicotyledon (123 905 compounds) (Chapman, 2019). Second, the fragmentation spectra of each node were compared against a database composed of *in silico* fragmentation spectra of compounds present in the DNP (ISDB-DNP) (Allard et al., 2016). A top 50 of 2D chemical structures was provided for each node according to *in silico* MS/MS fragmentation correspondence (initial rank) and a taxonomic reweighting step allowed the candidate structures to be re-ranked with a weight inversely proportional to the distance between the candidate biological sources and that of the analyzed sample (family, Ranunculaceae; genus *Helleborus*; species *Helleborus odorus* subsp. *cyclophyllus*) and a top 6 of candidates was provided (final rank) (Rutz et al., 2019). The chemical formula of the annotated structures (rank 1) was compared with the ones calculated for each of the corresponding molecular ion features. The annotation table reports only the rank 1 structures that gave a taxonomically relevant output and/or for which there was a structural consistency in the annotation of the corresponding clusters (Table 1). The complete annotated molecular network with all the annotations, and in particular the first 6 levels, is available as an associated file in the Mendeley Data repository (Cytoscape file) and on GNPS servers as Massive Data set n° MSV000084842.

Previous phytochemical investigation showed that the Greek hellebore *H. odorus* subsp. *cyclophyllus* contained mainly fatty acids, furostanol saponins, ecdysteroids, bufadienolides in the roots (Philianos, 1967; Tsiftsoglou et al., 2018), and phenolic analogs and flavonoids derivatives in the aerial parts (Philianos et al., 1983). The annotation results demonstrated that some of the most abundant compounds correspond to those reported in the literature (Fig. 5B). In addition, this approach allowed to observe the presence of analogs (bufadienolides, furostanols, ecdysteroids) not yet described in this species and often present in the genus (Fig. 5).

Both RME and RDE were analyzed in the same way and grouped in a common MN (Figs. 5–6S, Supplementary information). The MN in positive ionization contained 1594 nodes organized in 230 clusters and the one in negative ionization contained 966 nodes organized in 105 clusters. This data processing enabled to verify the occurrence of each feature in both extracts. More than 96% of features of RME were common to RDE in both ionization modes, showing that the vast majority of RME compounds are also found in RDE and were therefore ingested after administration of the ancient hellebore preparation.

As expected, the RME and RDE were characterized by the presence of the bufadienolide glycoside hellebrin (**4**), hellebrigenin (**S34**), deglucohellebrin (**6**), 5α-bufalone (**A18**), furostanols (**A16**, **A19**), and ecdysteroids (**3**, **A22**) (Table 1) (Colombo et al., 1990; Rosselli et al., 2009; Tsiftsoglou et al., 2018). These compounds were mainly present into clusters 2 and 17 in the MN in positive mode (Fig. 5S, Supplementary information). On the other hand, phenol glycoside (**A7**) (Diehl et al., 1974), bufadienolides (**A17**, **A27**, **A31**, **A36**) (Bassarello et al., 2008; Muzashvili et al., 2011; Watanabe et al., 2003; Yang et al., 2010), furostanol derivatives (**A15**, **A21**, **A23–25 A29**, **A34**) (Bassarello et al., 2008; Mimaki et al., 2010; Muzashvili et al., 2011; Rosselli et al., 2009), and spirostanol derivatives (**A33**) (Watanabe et al., 2005) were previously described in the *Helleborus* genus but were observed for the first time in this species.

The dereplication also led to the annotation of a cyclolanostanol glycoside (**A30**) and a cardenolide (**A35**) not previously reported in the *Helleborus* genus, although present in Ranunculaceae *Cimicifuga simplex* and *Adonis vernalis* (Kusano et al., 1999; Poláková; Čekan, 1963). In addition, the ribonolactone **A8** was identified only in the RME but was previously reported in *Glaucidium palmatum* (Kinoshita and Murase, 1973). Surprisingly, one cluster in the MN in positive mode contained two compounds annotated as alkaloids, which were previously detected in Ranunculaceae as thaliporphine (**A12**) and pallidine (**A14**) (Gözler et al., 1990; Shamma et al., 1967), and a third alkaloid not reported in the family and annotated as oureguattidine (**A11**) (Lebœuf et al., 1983) (Fig. 5S, Supplementary information). To our knowledge, this is the first report of alkaloids in the *Helleborus* genus while such type of alkaloids is known to occur in the family (Table 1). The UHPLC-CAD analysis revealed that such compounds were however present at trace levels.

Some other compound derivatives have been identified in both extracts as belonging to the structural families of furostanol (**A26**, **A32**, **A37**) (Báthori et al., 2002; Huang et al., 2003; Krokhmalyuk and Kintya, 1976), ecdysteroid (**A13**, **A28**) (Baltaev, 1995; Wang et al., 2009), phenol glycoside (**A9-10**) (Breinholt et al., 1990; Kudo et al., 1980) and pregnane steroid (**A20**) (Kennard et al., 1968). Finally, the UHPLC-CAD analysis demonstrated that the isolated compounds **3**, **4**, **6** and the annotated compounds **A17**, **A18**, **A24**, **A25**, **A31** are the major constituents present in the RME and already reported in the genus and the species.

4. Conclusion

In this work, we report for the first time a potential antiseizure activity of the root extract of the black hellebore (*Helleborus odorus* subsp. *cyclophyllus*), a plant used in the ancient Greek medicine to treat epileptic seizures. The bioassay-guided fractionation led to the isolation of the new furostanol saponin (**2**) and the bufadienolide hellebrin (**4**), which both decreased PTZ-induced locomotor activity in the zebrafish epilepsy assay. Hellebrigenin (**S34**), the aglycone of hellebrin (**4**), reduced the induced seizure activity more potently than the isolated compounds. To our knowledge, the ancient and ethnopharmacological use of the black hellebore as a seizure remedy is supported for the first time by modern pharmacological evidence. In addition, the UHPLC-HRMS/MS metabolite profiling documented in detail the chemical composition of *H. odorus* subsp. *cyclophyllus* as a source of bufadienolides, furostanols, spirofurostanols and alkaloids. Recent advances in the therapeutic use of neurosteroids for epilepsy suggest that further investigation is required on the constituents of the black hellebore to elucidate their mechanism of action and the molecular targets involved in the described antiseizure activity.

Author contributions

Study design: TB, EFQ, ADC, ALS, JLW; experiments: TB, MJ, EAP, KV, LM; data analysis: TB, MJ, LM, KV, EAP, JH, LP, AR; writing, editing and revision: TB, KV, EAP, JH, LM, EFQ, ADC, ALS, JLW.

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Declaration of competing interest

The authors declared no conflict of interest.

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Appendix A. Supplementary data

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References

Afrikanova, T., Serruys, A.S., Buenafe, O.E., Clinckers, R., Smolders, I., de Witte, P.A., Crawford, A.D., Esguerra, C.V., 2013. Validation of the zebrafish pentylenetetrazol seizure model: locomotor versus electrographic responses to antiepileptic drugs. PloS One 8 (1), e54166. https://doi.org/10.1371/journal.pone.0054166.

Allard, P.M., Peresse, T., Bisson, J., Gindro, K., Marcourt, L., Pham, V.C., Roussi, F., Litaudon, M., Wolfender, J.L., 2016. Integration of molecular networking and *in-silico* MS/MS fragmentation for natural products dereplication. Anal. Chem. 88 (6), 3317–3323.

Apetrei, N.S., Lupu, A.R., Calugaru, A., Kerek, F., Szegli, G., Cremer, L., 2011. The antioxidant effects of some progressively purified fractions from *Helleborus purpurascens*. Rom Biotechnol Lett 16 (6), 6673–6681.

Baltaev, U.A., 1995. Rapisterone D, a phytoecdysteroid from *Rhaponticum carthamoides*. Phytochemistry 38 (3), 799–800.

Bassarello, C., Muzashvili, T., Skhirtladze, A., Kemertelidze, E., Pizza, C., Piacente, S., 2008. Steroidal glycosides from the underground parts of *Helleborus caucasicus*. Phytochemistry 69 (5), 1227–1233.

Báthori, M., Pongrácz, Z., Tóth, G., Simon, A., Kandra, L., Kele, Z., Ohmacht, R., 2002. Isolation of a new member of the ecdysteroid glycoside family: 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucopyranoside. J. Chromatogr. Sci. 40 (7), 409–415.

Beck, L.Y., 2005. In: Beck, Lily Y. (Ed.), De Materia Medica. Olms-Weidmann, Hildesheim, Germany.

Biagini, G., Panuccio, G., Avoli, M., 2010. Neurosteroids and epilepsy. Curr. Opin. Neurol. 23 (2), 170–176.

Breinholt, J., Jensen, J.S., Jensen, S.R., Nielsen, B.J., 1990. Two iridoid glycosides from *Chaenorrhinum minus*. Phytochemistry 29 (12), 3865–3868.

Brillatz, T., Jacmin, M., Queiroz, E.F., Marcourt, L., Slacanin, I., Petit, C., Carrupt, P.A., Ngo Bum, E., Herrling, P., Crawford, A.D., Wolfender, J.L., 2020. Zebrafish bioassay-guided isolation of antiseizure compounds from the Cameroonian medicinal plant *Cyperus articulatus* L. Phytomedicine 70, 153175.

Brillatz, T., Lauritano, C., Jacmin, M., Khamma, S., Marcourt, L., Righi, D., Romano, G., Esposito, F., Ianora, A., Queiroz, E.F., Wolfender, J.L., Crawford, A.D., 2018. Zebrafish-based identification of the antiseizure nucleoside inosine from the marine diatom *Skeletonema marinoi*. PloS One 13 (4), e0196195. https://doi.org/10.1371/journal.pone.0196195.

Challal, S., Buenafe, O.E., Queiroz, E.F., Maljevic, S., Marcourt, L., Bock, M., Kloeti, W., Dayrit, F.M., Harvey, A.L., Lerche, H., Esguerra, C.V., de Witte, P.A., Wolfender, J.L., Crawford, A.D., 2014. Zebrafish bioassay-guided microfractionation identifies anticonvulsant steroid glycosides from the Philippine medicinal plant *Solanum torvum*. ACS Chem. Neurosci. 5 (10), 993–1004.

Challal, S., Queiroz, E.F., Debrus, B., Kloeti, W., Guillarme, D., Gupta, M.P., Wolfender, J.L., 2015. Rational and efficient preparative isolation of natural products by MPLC-UV-ELSD based on HPLC to MPLC gradient transfer. Planta Med. 81 (17), 1636–1643.

Chambers, M.C., Maclean, B., Burke, R., Amodei, D., Ruderman, D.L., Neumann, S., Gatto, L., Fischer, B., Pratt, B., Egertson, J., Hoff, K., Kessner, D., Tasman, N., Shulman, N., Frewen, B., Baker, T.A., Brusniak, M.Y., Paulse, C., Creasy, D., Flashner, L., Kani, K.i., Moulding, C., Seymour, S.L., Nuwaysir, L.M., Lefebvre, B., Kuhlmann, F., Roark, J., Rainer, P., Detlev, S., Hemenway, T., Huhmer, A., Langridge, J., Connolly, B., Chadick, T., Holly, K., Eckels, J., Deutsch, E.W., Moritz, R.L., Katz, J.E., Agus, D.B., MacCoss, M., Tabb, D.L., Mallick, P., 2012. A cross-platform toolkit for mass spectrometry and proteomics. Nat. Biotechnol. 30 (10), 918–920.

Chapman, H., 2019. In: Press, C. (Ed.), Dictionary of Natural Products on DVD (26:2). Taylor & Francis Group, Florida, USA.

Chen, H.F., Wang, N.L., Sun, H.L., Yang, B.F., Yao, X.S., 2006. Novel furostanol saponins from the bulbs of *Allium macrostemon B.* and their bioactivity on [Ca2+]i increase induced by KCl. J. Asian Nat. Prod. Res. 8 (1–2), 21–28.

Chen, T.W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., Schreiter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V., Looger, L.L., Svoboda, K., Kim, D.S., 2013. Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature 499 (7458), 295–300.

Clausen, M.V., Hilbers, F., Poulsen, H., 2017. The structure and function of the Na,K-ATPase isoforms in health and disease. Front. Physiol. 8 371-371.

Colombo, M.L., Tome, F., Servettas, O., Bugatti, C., 1990. Phytochemical evaluation of *Helleborus* species growing in Northern Italy. Int. J. Crude Drug Res. 28 (3), 219–233.

Crawford, A.D., Esguerra, C.V., de Witte, P.A.M., 2008. Fishing for drugs from nature: zebrafish as a technology platform for natural product discovery. Planta Med. 74 (6), 624–632.

Diehl, H.W., Pokorny, M., Zissis, E., Ness, R.K., Fletcher, H.G., 1974. Crystalline cellobiono-1,5-lactone: its preparation and growth-inhibitory activity in the *Avena* coleoptile section test. Carbohydr. Res. 38, 364–368.

Dimopoulos, P., Raus, T., Bergmeier, E., Constantinidis, T., Latrou, G., Kokkini, S., Strid, A., Tzanoudakis, D., 2013. Vascular plants of Greece: an annotated checklist. Englera, Berlin & Athens.

Dimopoulos, P., Raus, T., Bergmeier, E., Constantinidis, T., Latrou, G., Kokkini, S., Strid, A., Tzanoudakis, D., 2016. Vascular plants of Greece: an annotated checklist. Supplement. Willdenowia 46 (3), 301–347.

Eeckhaut, I., Caulier, G., Brasseur, L., Flammang, P., Gerbaux, P., Parmentier, E., 2015. Effects of holothuroid ichtyotoxic saponins on the gills of free-living fishes and symbiotic pearlfishes. Biol. Bull. 228 (3), 253–265.

Erdemoglu, N., Küpeli, E., Yeşilada, E., 2003. Anti-inflammatory and antinociceptive activity assessment of plants used as remedy in Turkish folk medicine. J. Ethnopharmacol. 89 (1), 123–129.

Gomes, N.G.M., Campos, M.G., Orfao, J.M.C., Ribeiro, C.A.F., 2009. Plants with neurobiological activity as potential targets for drug discovery. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 33 (8), 1372–1389.

Gonçalves-de-Albuquerque, C.F., Silva, A.R., da Silva, C.I., Castro-Faria-Neto, H.C., Burth, P., 2017. Na/K pump and beyond: Na/K-ATPase as a modulator of apoptosis and autophagy. Molecules 22 (4), 578.

Gözler, B., Öziç, P., Freyer, A.J., Shamma, M., 1990. Morphinandienone alkaloids from *Roemeria refracta*. J. Nat. Prod. 53 (4), 986–988.

Hort, A., 1916. Theophrastus: enquiry into plants and minor works on odours and weather signs, with an English translation by Sir Arthur Hort, Bart. William Heinemann London, England.

Huang, J.W., Tan, C.H., Jiang, S.H., Zhu, D.Y., 2003. Terrestrinins A and B, two new steroid saponins from *Tribulus terrestris*. J. Asian Nat. Prod. Res. 5 (4), 285–290.

Kanji, S., MacLean, R.D., 2012. Cardiac glycoside toxicity more than 200 years and counting. Crit. Care Clin. 28 (4), 527–535.

Kennard, O., Fawcett, J.K., Watson, D.G., Ann Kerr, K., Stöckel, K., Stöcklin, W., Reichstein, T., 1968. Hirundigenin and anhydrohirundigenin, two natural 15-oxasteroids of plant origin. Chemical and x-ray investigation. Tetrahedron Lett. 9 (35), 3799–3804.

Kinoshita, K., Murase, S., 1973. The structure of *cis*-and *trans*-glaupadiol (studies on the constituents of the rhizomes of *Glaucidium palmatum* Sieb. et Zucc.). Yakugaku Zasshi 93 (2), 202–206.

Kissmer, B., Wichtl, M., 1986. Bufadienolide aus samen von *Helleborus odorus*. Planta Med. 52 (3), 152–153.

Krokhmalyuk, V.V., Kintya, P.K., 1976. Steroid saponins. XIII. The structure of alliumosides D and E from *Allium narcissiflorum*. Chem. Nat. Compd. 12 (2), 165–168.

Kudo, K., Nohara, T., Komori, T., Kawasaki, T., Schulten, H.R., 1980. Lignan-glycosides from the bark of *Ligstrum japonicum*. Planta Med. 40, 250–261.

Kusano, A., Takahira, M., Shibano, M., Miyase, T., Kusano, G., 1999. Studies on the constituents of *Cimicifuga* species. XXVI. Twelve new cyclolanostanol glycosides from the underground parts of *Cimicifuga simplex* Wormsk. Chem. Pharm. Bull. 47 (4), 511–516.

Lebœuf, M., Cortes, D., Hocquemiller, R., Cavé, A., 1983. Alkaloids from annonaceae XLVII: alkaloids of *Guatteria ouregou*. Planta Med. 48, 234–245.

Lindholm, P., Gullbo, J., Claeson, P., Goransson, U., Johansson, S., Backlund, A., Larsson, R., Bohlin, L., 2002. Selective cytotoxicity evaluation in anticancer drug screening of fractionated plant extracts. J. Biomol. Screen 7 (4), 333–340.

Magiorkinis, E., Sidiropoulou, K., Diamantis, A., 2010. Hallmarks in the history of epilepsy: epilepsy in antiquity. Epilepsy Behav. 17 (1), 103–108.

Maieron, M.A., 2018. On the hellebore trail an anthropological research into madness. Medicina Historica 2 (1), 5–18.

Maior, M., Dobrotă, C., 2013. Natural compounds with important medical potential found in *Helleborus* sp. Open Life Sci. 8 (3), 272–285.

Megiddo, I., Colson, A., Chisholm, D., Dua, T., Nandi, A., Laxminarayan, R., 2016. Health and economic benefits of public financing of epilepsy treatment in India: an agent-based simulation model. Epilepsia 57 (3), 464–474.

Mimaki, Y., Matsuo, Y., Watanabe, K., Sakagami, H., 2010. Furostanol glycosides from the rhizomes of *Helleborus orientalis*. J. Nat. Med. 64 (4), 452–459.

Moreno, Y.B.L., Katz, A., Miklos, W., Cimmino, A., Tal, D.M., Ainbinder, E., Zehl, M., Urban, E., Evidente, A., Kopp, B., Berger, W., Feron, O., Karlish, S., Kiss, R., 2013. Hellebrin and its aglycone form hellebrigenin display similar *in vitro* growth inhibitory effects in cancer cells and binding profiles to the alpha subunits of the Na+/K+-ATPase. Mol. Canc. 12, 33.

Muhr, P., Kerek, F., Dreveny, D., Likussar, W., Schubertzsilavecz, M., 1995. The structure of hellebrin. Liebigs Ann. 443–444.

Muzashvili, T., Perrone, A., Napolitano, A., Kemertelidze, E., Pizza, C., Piacente, S., 2011. Caucasicosides E-M, furostanol glycosides from *Helleborus caucasicus*. Phytochemistry 72 (17), 2180–2188.

Myers, O.D., Sumner, S.J., Li, S., Barnes, S., Du, X., 2017. One step forward for reducing false positive and false negative compound identifications from mass spectrometry metabolomics data: new algorithms for constructing extracted ion chromatograms and detecting chromatographic peaks. Anal. Chem. 89 (17), 8696–8703.

Ngugi, A.K., Bottomley, C., Kleinschmidt, I., Sander, J.W., Newton, C.R., 2010. Estimation of the burden of active and life-time epilepsy: a meta-analytic approach. Epilepsia 51 (5), 883–890.

Nohria, V., Giller, E., 2007. Ganaxolone. Neurotherapeutics 4 (1), 102–105.

Nothias, L.F., Petras, D., Schmid, R., Dührkop, K., Rainer, J., Sarvepalli, A., Protsyuk, I., Ernst, M., Tsugawa, H., Fleischauer, M., Aicheler, F., Aksenov, A., Alka, O., Allard, P.M., Barsch, A., Cachet, X., Caraballo Rodríguez, A., Silva, R., Dang, T., Dorrestein, P., 2019. Feature-based Molecular Networking in the GNPS Analysis Environment. https://doi.org/10.1038/nbt.3597. bioRxiv 812404.

Olivieri, M.F., Marzari, F., Kesel, A.J., Bonalume, L., Saettini, F., 2017. Pharmacology and psychiatry at the origins of Greek medicine: the myth of Melampus and the madness of the Proetides. J. Hist. Neurosci. 26 (2), 193–215.

Orellana-Paucar, A.M., Serruys, A.S., Afrikanova, T., Maes, J., De Borggraeve, W., Alen, J., Leon-Tamariz, F., Wilches-Arizabala, I.M., Crawford, A.D., de Witte, P.A., Esguerra, C.V., 2012. Anticonvulsant activity of bisabolene sesquiterpenoids of *Curcuma longa* in zebrafish and mouse seizure models. Epilepsy Behav. 24 (1), 14–22.

Patel, S., 2016. Plant-derived cardiac glycosides: role in heart ailments and cancer management. Biomed. Pharmacother. 84, 1036–1041.

Philianos, S., 1967. Pharmacognosy of the hellebores (*Helleborus cyclophyllus*). Archeia tes Pharmakeutikes 23 (5–6), 65–187.

Philianos, S., Harvala, C., Paris, R., 1983. Sur les constituants des feuilles basales d'*Helleborus cyclophyllus* Boiss. Plantes Med. Phytotherap. 17 (2), 83–88.
Pluskal, T., Castillo, S., Villar-Briones, A., Oresic, M., 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinf. 11, 395.
Poláková, A., Čekan, Z., 1963. Vernadigenin and 3-acetylstrophadogenin, two new cardenolides from *Adonis vernalis*. Chem. Ind. (London) (44), 1766–1767.
Puglisi, S., Speciale, A., Acquaviva, R., Ferlito, G., Ragusa, S., De Pasquale, R., Iauk, L., 2009. Antibacterial activity of *Helleborus bocconei* Ten. subsp. *siculus* root extracts. J. Ethnopharmacol. 125 (1), 175–177.
Ren, L.X., Luo, Y.F., Li, X., Wu, Y.L., 2007. Antidepressant activity of sarsasapogenin from *Anemarrhena asphodeloides* bunge (liliaceae). Pharmazie 62 (1), 78–79.
Rosselli, S., Maggio, A., Bruno, M., Spadaro, V., Formisano, C., Irace, C., Maffettone, C., Mascolo, N., 2009. Furostanol saponins and ecdysones with cytotoxic activity from *Helleborus bocconei* ssp. *intermedius*. Phytother Res. 23 (9), 1243–1249.
Rutz, A., Dounoue-Kubo, M., Ollivier, S., Bisson, J., Bagheri, M., Saesong, T., Ebrahimi, S.N., Ingkaninan, K., Wolfender, J.L., Allard, P.M., 2019. Taxonomically informed scoring enhances confidence in natural products annotation. Front. Plant Sci. 10, 1329.
Santulli, L., Coppola, A., Balestrini, S., Striano, S., 2016. The challenges of treating epilepsy with 25 antiepileptic drugs. Pharmacol. Res. 107, 211–219.
Schmutz, J., 1949. Die konstitution des hellebrigenins. Glykoside und aglykone, 48. Mitteilung. Helv Chim Acta 32 (5), 1442–1452.
Seifarth, C., Littmann, L., Resheq, Y., Rossner, S., Goldwich, A., Pangratz, N., Kerek, F., Steinkasserer, A., Zinser, E., 2011. MCS-18, a novel natural plant product prevents autoimmune diabetes. Immunol. Lett. 139 (1–2), 58–67.
Shamma, M., Shine, R.J., Dudock, B.S., 1967. Thalictrum alkaloids-IV: three new alkaloids from *T. fendleri*: thalidezine, thaliporphine, and preocoteine. Tetrahedron 23 (7), 2887–2892.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13 (11), 2498–24504.
Smith, T.W., 1985. Pharmacokinetics, bioavailability and serum levels of cardiac glycosides. J. Am. Coll. Cardiol. 5 (A), 43–50.
Spencer, W.G., 1935. Celsus. De Medicina, with an English translation by W. G. Spencer. Harvard university press, Cambridge, MA, USA.
Strid, A., 2016. Atlas of the Aegean Flora. Part 1: Text & Plates. Botanic garden & botanical museum Berlin, Berlin, Germany.
Totelin, L.M.V., 2009. Hippocratic recipes: oral and written transmission of pharmacological knowledge in fifth- and fourth-century Greece. Brill, Leiden, The Netherlands.
Tschesche, R., Wagner, R., Jha, C.H., 1984. A furostanol glycoside from *Helleborus macranthus*. Phytochemistry 23 (3), 695–696.
Tsiftsoglou, O.S., Stefanakis, M.K., Lazari, D.M., 2018. Chemical constituents isolated from the rhizomes of *Helleborus odorus* subsp. *cyclophyllus* (Ranunculaceae). Biochem. Systemat. Ecol. 79, 8–11.
Tutin, T.G., Burges, N.A., Edmondson, J.R., Chater, A.O., Heywood, V.H., Moore, D.M., Valentine, D.H., Walters, S.M., Webb, D.A., 1993. Flora Europaea, second ed. Cambridge University Press, Cambrige, MA, USA.
Vokac, K., Budesinsky, M., Harmatha, J., Pis, J., 1998. New ergostane type ecdysteroids from fungi. Ecdysteroid constituents of mushroom *Paxillus atrotomentosus*. Tetrahedron 54 (8), 1657–1666.
Wang, J.K., Portbury, S., Thomas, M.B., Barney, S., Ricca, D.J., Morris, D.L., Warner, D.S., Lo, D.C., 2006. Cardiac glycosides provide neuroprotection against ischemic stroke: discovery by a brain slice-based compound screening platform. Proc. Natl. Acad. Sci. U.S.A. 103 (27), 10461–10466.
Wang, M., Carver, J.J., Phelan, V.V., Sanchez, L.M., Garg, N., Peng, Y., Nguyen, D.D., Watrous, J., Kapono, C.A., Luzzatto-Knaan, T., 2016. Sharing and community curation of mass spectrometry data with Global Natural Products Social molecular networking. Nat. Biotechnol. 34 (8), 828–837.
Wang, P., Li, S., Ownby, S., Zhang, Z., Yuan, W., Zhang, W., Beasley, R.S., 2009. Ecdysteroids and a sucrose phenylpropanoid ester from *Froelichia floridana*. Phytochemistry 70 (3), 430–436.
Watanabe, K., Mimaki, Y., Sakagami, H., Sashida, Y., 2003. Bufadienolide and spirostanol glycosides from the rhizomes of *Helleborus orientalis*. J. Nat. Prod. 66 (2), 236–241.
Watanabe, K., Sakagami, H., Mimaki, Y., 2005. Four new steroidal saponins from the rhizomes of *Helleborus orientalis*. Heterocycles 65 (4), 775–785.
WHO, 2019. Epilepsy Fact Sheet. World Health Organization (WHO), Geneva, Switzerland.
Yang, J., Zhang, Y.H., Miao, F., Zhou, L., Sun, W., 2010. Two new bufadienolides from the rhizomes of *Helleborus thibetanus* Franch. Fitoterapia 81 (6), 636–639.
Yawno, T., Miller, S.L., Bennet, L., Wong, F., Hirst, J.J., Fahey, M., Walker, D.W., 2017. Ganaxolone: a new treatment for neonatal seizures. Front. Cell. Neurosci. 11 246-246.
Zhang, H., Su, Y.F., Yang, F.Y., 2016. Three new steroidal saponins from *Helleborus thibetanus*. Nat. Prod. Res. 30 (15), 1724–1730.
Zhang, H., Su, Y.F., Yang, F.Y., Gao, X.M., 2017. New minor spirostanol glycosides from *Helleborus thibetanus*. Nat. Prod. Res. 31 (8), 925–931.