Chemical constituents isolated from the aerial parts of *Helleborus cyclophyllus* (A. Braun) Boiss. (Ranunculaceae), evaluation of their antioxidant and anti-inflammatory activity *in vitro* and virtual screening of molecular properties and bioactivity score

Olga St. Tsiftsoglou, Michalis K. Stefanakis, Eirini N. Kalpourtzi, Dimitra I. Hadjipavlou-Litina and Diamanto M. Lazari

Laboratory of Pharmacognosy, Faculty of Health sciences, School of Pharmacy, Aristotle University of Thessaloniki, Thessaloniki, Greece; Laboratory of Organic Chemistry, School of Sciences and Engineering, Department of Chemistry, University of Crete, Heraklion, Greece; Department of Pharmaceutical Chemistry, Faculty of Health Sciences, School of Pharmacy, Aristotle University of Thessaloniki, Greece

**ABSTRACT**

Chemical investigation of ethyl acetate extract from the aerial parts of *Helleborus cyclophyllus* (A.Braun) Boiss. led to the isolation of ten natural products, and their structures were identified to be: 2-deoxy-D-ribo-1,4-lactone (1), 2-O-feruloyl-L-malate (2), three flavonoids: quercetin 3-O-β-D-galactopyranoside (3), quercetin 3-O-6″-(3-hydroxy-3-methyl-glutaryl)-β-D-glucopyranoside (4) and quercetin 3-O-(2‴″-caffeoylsophoroside) (5), 6-O-caffeoyl-1-O-p-coumaroyl-β-D-glucopyranoside (6), two ecdysteroids: 20-hydroxyecdysone (7) and polypodine B (8) and two bufadienolides: deglucohellebrin (9) and hellebrin (10), on the basis of MS and NMR spectra. This is the first report on the occurrence of compounds (2)-(6) in the genus *Helleborus*. The chemotaxonomic significance of these compounds was summarised. Bioactivity score, molecular and pharmacokinetic properties of the isolated compounds were calculated by online computer software program Molinspiration. Compounds showed promising bioactivity scores for drug targets. Moreover, some of the isolated phenolic compounds were tested for their antioxidant and antiinflammatory activities. Tested compounds presented antioxidant ability correlated to the presence of the phenolic hydroxyl groups.
1. Introduction

The small genus *Helleborus* of Ranunculaceae family (contains ca. 20 species) is widely distributed in Southeast Europe and West Asia. Previous phytochemical studies on *Helleborus* disclosed that steroids, including bufadienolides, phytoecdystones, and steroidal saponins were the main components (Meng et al. 2001; Cheng et al. 2014; Zhang et al. 2014; Iguchi et al. 2020). *Helleborus cyclophyllus* (A. Braun) Boiss. [synonym: *H. odorus* Willd. subsp. *cyclophyllus* (A. Braun) Maire & Petitm.] is the only species of this genus that is growing wild in Greece. *H. cyclophyllus*, locally known as ‘Skarfi’, is a perennial endemic of the Balkan Peninsula. Its leaves are complex, long, biconvex and toothed circularly forming a disk explaining the name ‘cyclophyllus’. Flowering shoots bear 2–5 light green flowers, with flowering period March-May. Greek popular tradition attributes to ‘Skarfi’ many bizarre and magical qualities. The flowering period of this plant is late winter or early spring. It is certain, however, that even at the end of the 19th century, from *Helleborus* prepared drugs for the treatment of mania, melancholy, hypochondria, even epilepsy. Its use was in the form of powder or tincture (Strid and Tan 2002). Previous phytochemical investigation of the species reported the presence of palmitic/linoleic acids, phospholipids, β-sitosterol, uridine, small phenolic analogs, bufadienolides, furostanols, ecdysteroids and flavonoid derivatives (Philianos 1967; Philianos et al. 1983; Tsiftsoglou et al. 2018; Brillatz et al. 2020). Continuing our studies on this plant (Tsiftsoglou et al. 2018; Vartholomatos et al. 2020), we now report the investigation of the aerial parts of *Helleborus cyclophyllus* (A. Braun) Boiss.

2. Results and discussion

2.1. Chemotaxonomic significance

The ethyl-acetate extract from the aerial parts of *Helleborus cyclophyllus* was fractionated by using several chromatographic methods and afforded ten natural products, so far: 2-hydroxymethyl-D-ribonolactone (1) (Tsiftsoglou et al. 2018), 2-O-feruloyl-L-malate (2) (Liang et al. 2006), quercetin 3-O-β-D-galactopyranoside (3) (Tsiftsoglou et al. 2019), quercetin 3-O-6’-(3-hydroxy-3-methyl-gloutaryl)-β-D-glucopyranoside (4) (Liu et al. 2004), quercetin 3-O-(2”-caffeoyl sophoroside) (5) (Bloor et al. 1998), 6-O-caffeoyl-1-O-p-coumaroyl-β-D-glucopyranoside (6) (Shimomura et al. 1988), 20-hydroxyecdysone (7), polypodine B (8) (Girault et al. 1988), deglucohellebrin (9) (Watanabe et al. 2003) and hellebrin (10) (Moreno et al. 2013) (Figure 1). The data of all isolated and
identified compounds were compared with those of samples from our collection and/or by a comparison with reported data in literature (Tables S1–S7 and Figures S1–S43).

It is noteworthy, that is the first time that, 2-O-feruloyl-L-malate (2), quercetin 3-O-β-D-galactopyranoside (3), quercetin 3-O-6″-(3-hydroxy-3-methyl-gloutaryl)-β-D-glucopyranoside (4), quercetin 3-O-(2″″-caffeoylsophoroside) (5) and 6-O-caffeoyl-1-O-p-coumaroyl-β-D-glucopyranoside (6) have been isolated in the genus Helleborus. It must be mentioned that four out of these five compounds (except for compound 3) are also very rare as natural products.

There are very few references for 2-deoxy-D-ribo-1,4-lactone (1) as previously reported by Tsiftsoglou et al. (2018). Tsiftsoglou and co-workers isolated compound (1) from the roots of H. cyclophyllus (2018) and Yfanti et al. (2020) detected it in its leaves.

Many studies on secondary metabolites of Helleborus species have been realised. Present study allowed the isolation and identification of three known quercetin...
glycosides that are reported for the first time in genus Helleborus. Compound (3) is a very abundant natural product and is isolated from various species. Some of these plants are Lonicera japonica Caprifoliaceae (Wang et al. 2017), Agrimonia pilosa Ledeb Rosaceae (Zhu et al. 2017), Camptotheca acuminata Nyssaceae (Qun and Qiaoyu 2016), Laetia suaveolens Salicaceae (Estork et al. 2014), Houttuynia cordata Saururaceae (Jiang et al. 2014), Kielmeyera variabilis Mart. & Zucc. Calophyllaceae (Cota et al. 2012). Till now, compound (4) is isolated only from two species Euphorbia bracteolata Euphorbiaceae (Liu et al. 2004) and Moringa oleifera Moringaceae (Kashiwada et al. 2012), but it is also detected in extracts of the plants Rosa spinosissima Rosaceae (Porter et al. 2012) and Oxytropis racemosa Fabaceae (Song et al. 2013). Compound (5) is isolated from few species. Some of these plants are Petunia ‘Mitchell’ Solanaceae (Bloor et al. 1998) and Ranunculus peltatus subsp. peltatus Ranunculaceae (B. Gluchoff-Fiasson et al. 1997). Flavonoids have long time been considered as chemiotaxonomical markers of Ranunculaceae (Lebreton 1986) and in particular caffeoylated or feruloylated flavonol glycosides are typical of this family and of Helleborus spp. (B. Gluchoff-Fiasson et al. 1994). This is the first report of this compound in plant belong to the genus Helleborus.

As it concerns 2-O-feruloyl-L-malate (2) isolated for the first time from the plant Raphanus sativus Cruciferae (Nielsen et al. 1984). Till then, is reported as product after treatment with various reagents and cultivation under specific conditions of plants belong to Cruciferae family (Liang et al. 2006; Abdel-Farid et al. 2007; Pedras et al. 2008; Narváez-Cuenca et al. 2012). This is the first research that reports its presence in plant of Ranunculaceae family.

Till now, 6-O-caffeoyl-1-O-β-coumaroyl-β-D-glucopyranoside (6) is isolated only from the plant Prunus buergeriana Rosaceae (Shimomura et al. 1988).

According to our results, the two ecdysteroids polypodine B (8) and 20-hydroxyecdysone (7), and the two bufadienolides deglucohellebrin (9) and hellebrin (10) are presents in the underground (Tsiftsoglou et al. 2018), as well as in the aerial parts of Greek hellebore H. cyclophyllus. These compounds have also been detected in the water-soluble fraction of the methanol extract of the leaves of H. cyclophyllus collected from Epirus region (Greece) by other researchers (Yfanti et al. 2020).

Our investigations confirm the co-occurrence of ecdysteroids and bufadienolides in Helleborus genus (Hardman and Benjamin 1976; Meng et al. 2001; Watanabe et al. 2003; Yang et al. 2010; Brillatz et al. 2020), with polypodine B (8) and 20-hydroxyecdysone (7) being the most abundant compounds within the genus (Dinan et al. 2002).

2.2. Biological activity

2.2.1. In vitro antioxidant and anti-inflammatory activity

Five of the isolated compounds were evaluated for their antioxidant and anti-inflammatory activities (Table S8). Quercetin 3-O-6‘-O-(3-hydroxy-3-methyl-gloutaryl)-β-D-glucopyranoside (4) presents the highest interaction with the stable free radical DPPH, followed by quercetin 3-O-β-D-galactopyranoside (3), 2-O-feruloyl-L-malate (2) and 2-hydroxymethyl-D-ribo-no-γ-lactone (1), after 20 min. For compound (2) the reducing activity is slightly increased after 60 min. It seems that the antioxidant activity is time
dependent for the phenolic acid (2) only. Compound 2-hydroxymethyl-D-ribono-\(\gamma\)-lactone (1), shows the lowest reducing ability. This result is in agreement with the literature (Yang et al. 2011). Concerning the free radical scavenging activity of quercetin 3-O-\(\beta\)-D-galactopyranoside (3), our results are in agreement with previous reported data (Sukito and Tachibana 2014). The most prominent antioxidant activity is presented by quercetin 3-O-6\''-(3-hydroxy-3-methyl-gloutaryl)-\(\beta\)-D-glucopyranoside and is attributed to the presence of the phenolic hydroxyl groups (nine-OH) in the molecule. Compounds 3 and 4 which are quercetin derivatives, containing the catechol moiety, showed significantly higher interaction with the DPPH among the tested compounds. The catechol structure observed in flavonoids and phenolic compounds is well known to play an important role to their antioxidant effect (van Acker et al. 1996; Cao et al. 1997; Cho et al. 2008). Phenolic hydroxyl groups at the 3- and 4-positions of the flavonoid B ring were also reported to play a key role in radical scavenging (Rice-Evans et al. 1996). The current study is the first that detects the antioxidant activity of quercetin 3-O-6\''-(3-hydroxy-3-methyl-gloutaryl)-\(\beta\)-D-glucopyranoside (4), 2-O-feruloyl-L-malate (2) and 6-O-caffeoyl-1-O-p-coumaroyl-\(\beta\)-D-glucopyranoside (6). Quercetin 3-O-6\''-(3-hydroxy-3-methyl-gloutaryl)-\(\beta\)-D-glucopyranoside (4), showed high antilipid peroxidation activity 97.3%, higher than the reference compound trolox (93%) whereas, quercetin 3-O-\(\beta\)-D-galactopyranoside (3) and 2-O-feruloyl-L-malate (2) presented lower activity than the reference compound trolox (93%), ranged from 88.8 to 75.2%. 2-hydroxymethyl-D-ribono-\(\gamma\)-lactone (1) and 6-O-caffeoyl-1-O-p-coumaroyl-\(\beta\)-D-glucopyranoside (6) present very low effect. The tested compound (3) significantly inhibits soybean lipoygenase (~60%), whereas compounds (1) and (2) did not exhibit any inhibition.

2.2.2. In silico studies - drug likeness

Lipophilicity, as LogP values, is an important physicochemical property implicated in the biological response and pharmacokinetic behavior of bioactive agents. Hydrophilic/lipophilic nature of drug molecule affects drug absorption, bioavailability, drug receptor interaction, metabolism of molecule as well as toxicity. In order to evaluate the drug likeness of the isolated compounds we calculated various molecular properties e.g., partition coefficient (milogP), topological polar surface area (TPSA), hydrogen bond donors and acceptors, rotatable bonds, number of atoms, molecular weight considering the violations of Lipinski’s rule of five. LogP values of all isolated compounds were found to be in the range of −2.38 to 1.60. This implies that all the examined compounds are hydrophilic because their log P is less than 5 and also that their permeability across cell membranes is high.

Low molecular weight drug molecules (less than 500) are easily transported, diffuse and absorbed compared to molecules with higher MW. Apart from compounds 3 and 4 all other compounds were found to have MW less than 500.

Total polar surface area (TPSA) is closely related to the hydrogen bonding potential of molecules. TPSA of the derivatives 1, 2, 7 and 8 was observed in the range 66.76–158.67 and is well below the 160Å limit, indicating good oral bioavailability (Table S9). The upper limit for TPSA for a molecule to penetrate the brain is around 90Å. Number of rotatable bonds is a topological parameter that measures molecular
flexibility. Except for compound 1 all other compounds are ‘flexible’ because their rotatable bonds were found in the range 4–11 (Khan et al. 2013).

For in silico prediction, compounds with logBB value more than 0.3 are considered to have high absorption through BBB (Blood–Brain Barrier) whereas between 0.3 to −0.1 and less than −0.1 is considered to be moderate and less absorbed through BBB. LogBB was measured by using the Rishton equation (Shityakov et al. 2013). None of the isolated compounds have result above the 0.3. Our findings do not support the permeability of these hybrids through BBB.

Compounds showed promising bioactivity scores for drug targets by Molinspiration software e.g., for GPCR receptor (2, 3, 7, 8 and 6), as ion channel modulators (7 and 8), as kinase inhibitors (3), as nuclear receptor ligands (2, 3, 6, 7, 8 and 9) and as proteases inhibitors (2, 4, 6, 7, 8 and 9) (Table S10).

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