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Natural compounds with important medical potential found in *Helleborus* sp.

Review Article

Maria Cornelia Maior[1,*], Cristina Dobrotă[1,2]

[1]*Institute of Technology, Babeş-Bolyai University, 400294 Cluj-Napoca, Romania*

[2]*Department of Molecular Biology and Biotechnology, Faculty of Biology, Babeş-Bolyai University, 400084 Cluj-Napoca, Romania*

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Abstract: *Helleborus* (family Ranunculaceae) are well-known as ornamental plants, but less known for their therapeutic benefits. Over the past few years, *Helleborus* sp. has become a subject of interest for phytochemistry, pharmacology and other medical research areas. On the basis of their usefulness in traditional medicine, it was assumed that their biochemical profile could be a source of metabolites with the potential to overcome critical medical issues. There are studies involving natural extracts from these species which demonstrate that *Helleborus* plants are a valuable source of chemical compounds with great medical potential. Some phytochemicals produced by these species have been separated and identified a few decades ago: hellebrin, deglucohellebrin, 20-hydroxyecdysone and protoanemonin. Lately, many other active compounds have been reported and considered as promising remedies for severe diseases such as cancer, ulcer, diabetes and also for common medical problems such as toothache, eczema, low immunity and arthritis. This paper is an overview of the *Helleborus* genus focusing on some recently-discovered compounds and their potential for finding new drugs and useful biochemicals derived from these species.

Keywords: *Helleborus sp.* • *Phytochemicals* • *Secondary metabolites* • *Active compounds* • *Therapeutic potential* • *Anticancer properties* • *Cytotoxicity* • *Immunomodulation*

1. Introduction

Current studies regarding species of *Helleborus* are opening new ways to cure diseases using natural compounds. Plants belonging to this group have long been used in traditional medicine to treat various conditions, such as edema, arthritis and ulcer. In the Balkan area, *Helleborus* extracts have been used for a long time in traditional medicine as painkillers or as anti-inflammatory remedies and in veterinary medicine against infectious diseases. Several active compounds including cardioactive glycosides, sterol saponosides, ecdysteroids and γ-lactones have recently been isolated from plants of this genus and shown to exert antioxidant, anti-inflammatory and antimicrobial effects. Anti-diabetic and antitumoral properties were suggested, but further investigations are required. Due to the scarcity of clinical trials, there are few published reports on target-organ toxicity or side effects. This review summarizes the latest literature on the pharmacological, toxicological, and clinical studies of *Helleborus* and its active compounds. Advancing our understanding of the secondary metabolite biosynthesis and ability to detect cell lines with high level of active principles may positively impact human health at a worldwide scale.

2. Biology and traditional use of *Helleborus* spp.

Helleborus spp. (*Ranunculaceae*), or hellebore, is a perennial herb native to Europe and Asia. The genus comprises around 20 species. The underground parts – rhizomes - containing starch granules and oleosomes

* *E-mail: maria.maior@ubbcluj.ro*

[1,2] accumulate the largest amount of metabolites. Most of the hellebores are acaulescent (leaves and flower stalks emerge from the rhizomes), only a few species being caulescent. Leaves are pedate or palmate. The color of the flowers are given by the sepals which remain on the flower long after fertilization; the petals are reduced at the base of sepals. Hellebores bloom in late winter or early spring, between February and April. Roots, rhizomes and leaves are still used in traditional medicine to treat humans and animals. Rhizome extracts in particular have been reported as possessing a broad spectrum of pharmacological and therapeutic effects such as cardiotonic, abortive and sedative, as well as having antioxidative, anti-inflammatory and antimicrobial activities.

There are old archives that mention species of *Helleborus* in the Roman Empire and ancient Greece [3,4]. *Helleborus niger* was used from antiquity until Byzantine times to strengthen the heart and has a positive effect on arteries and nerves [5]. Paracelsus was prescribing to his patients "an elixirium for a long life" made by dried leaves of *H. niger* [6]. There are 16 species of *Ranunculaceae* found in Renaissance herbals that were used to treat malaria, 3 of them being hellebores (*H. foetidus, H. niger* and *H. viridis*) [7]. In Mt. Pelion, Greece, *H. cyclophyllus* is still used to treat toothache. A decoction made of the entire plant or only a piece from the underground part, held temporarily in the mouth, could numb the entire mouth and stop tooth pain. A tea made from leaves of *H. cyclophyllus* was used by speakers to strengthen their voice [4]. In Italy, roots of *H. foetidus* and *H. bocconei* were used in the same way as in Greece to treat tooth pains [8] and in Turkey, roots of *H. orientalis* were used for the same purpose [9]. In the Montenegro region, eczema, skin redness and itching [10] were treated using roots of *H. odorus*. Peeled root of *H. foetidus* introduced into the vagina, provoked hemorrhage followed by abortion [8]. In the Italian Apennines, people are still using aerial parts of *H. foetidus* to clean house chimneys, stoves, wood ovens and as oil lamp wicks [11]. Even though there are many species of *Helleborus* known as having active principles, *H. niger* is the most widely used in traditional medicine for a wide range of symptoms and diseases [12]. In European folk medicine, *H. niger* was used in expelling thick slime, as a remedy for sore joints, as an emetic and laxant and for preparing sneezing powders [13]. Alkaloids, glycosides and saponosides found in species of *Helleborus* are considered to be the constituents responsible for central nervous system activity [14]. Tea from hellebore leaves, having a sedative effect, was used in Danish folk medicine to treat epilepsy and convulsions [15].

In traditional veterinary practice, different species of *Helleborus* were used to treat infectious and inflammatory diseases. In Italy, *H. foetidus* and *H. viridis* were used to treat many kinds of disease in pigs, cows, sheep, mules and donkeys. Pneumonia was diagnosed by inserting a piece of root in a subcutaneous cut or in an incision on the ear of the pigs, with the animals declared ill if the cuts swelled during the next day [8,16]. In Sicily, *H. bocconei* subsp. *siculus* was used to diagnose and to cure pneumonia in cattle [17,18]. *H. odorus* was used in a similar way in Serbia, with a part of the stem being inserted in a hole in a sick sheep's ears [19]. Chronic inflammatory diseases in pigs and sheep [20] and infectious diseases [21] were treated based on the anti-inflammatory and antibacterial effects of *H. purpurascens* roots, used as a transcutaneous implantation. A decoction of *H. foetidus* was used to clean wounds of animals when no better option was available [11]. Crushed leaves of *H. niger* were used in veterinary medicine in Pakistan as an antihelmintic [22].

H. foetidus is reported as having anti-insect activity [23]. Many insects avoid hellebores, but some species are using specific chemical compounds contained in the plants to defend themselves against predators. A furostanol saponin found in leaves of *H. foetidus* and *H. viridis* was also found in the hemolimph of *Monophadnus* sp. larvae at a more than 200 fold higher concentration compared to plant cells. It seems likely that this compound is involved in the chemical defence of *Monophadnus* larvae against potential insect predators [24].

3. Toxicity of *Helleborus* sp.

The use of *H. orientalis* extracts is mentioned in Homer's Odyssey [25]. *Helleborus* species are considered to be toxic, but usually the poisonings are related to an incorrect dosage [26]. Toxic features of hellebores are determined mostly by the aglycons of cardiac steroids but protoanemonin - a toxic γ–lactone, is also a toxic compound.

Poisoning by cardioactive steroids is primarily localized to the digestive system with severe gastrointestinal irritation, vomiting and diarrhea [25,27]. Acute toxicity effects on the central nervous system include lethargy, confusion and weakness that are not caused by hemodynamic changes. Chronic toxicity due to hellebore poisoning is difficult to diagnose and less obvious. It could determine anorexia, weight loss, neuropsychiatric disorders (confusion, drowsiness, headaches, delirium, and hallucinations) and visual disturbances. Cardiac manifestations of this kind of

poisoning include a multitude of cardiac dysrhythmias [25].

Poisoning with protoanemonin occurs mostly in animals and starts with salivation, vomiting, inflammation of the mouth and throat, abdominal pain that can be followed by severe ulcerations of the mouth and damage to the digestive and urinary systems. A severe poisoning will present colored diarrhea and dark or blood stained urine, unsteady gait, dizziness, impaired or lost vision. Although fatal poisoning is rare, when it occurs death is preceded by convulsions [28,29]. Protoanemonin has also a toxic effect as a sub-epidermal vesicant, caused by inactivation of enzymes containing SH groups [30,31].

4. Chemical analysis of *Helleborus* sp.

The first reports of a general chemical composition of hellebores date from 1943 when Karrer isolated for the first time from *H. niger* a cardiac glycoside named hellebrin [32]. A few classes of compounds from *Helleborus* spp. were identified and reported starting with the 1970s [33,34], including: cardioactive glycosides (hellebrin, degluco-hellebrin), steroidal saponins, ecdysteroids and γ-lactones (protoanemonin).

4.1 Cardioactive glycosides

In plants, cardioactive glycosides were discovered in angiosperms, both in monocotyledons and dicotyledons. The therapeutic action of cardioactive glycosides depends on the structure of the aglycone, and on the type and number of sugar units attached. Two types of aglycone are known: cardenolides (digitoxigenin from *Digitalis purpurea* - C23 compounds), and bufadienolides (hellebrigenin from *Helleborus niger* - C24 structures). The lactone ring is five-membered in cardenolides and six-membered in the bufadienolides [35]. Cardenolides are common substances and there are a few genera that yield these compounds: *Strophanthus*, *Convallaria* and *Digitalis* [35]. The bufadienolides are found in *Helleborus*, *Cotyledon, Kalanchoe*, *Scylla, Bowiea, Homeria*, *Moraea*, *Bersama, Melianthus* and *Thesium*. Animal sources of bufadienolides include fireflies (*Photinus* spp.), snakes (*Rhabdophis* spp.) and toads (*Bufo* spp.) [36].

Hellebrin, shown in Figure 1, is the most familiar cardioactive glycoside of *Helleborus,* being isolated and quantified in roots and rhizomes of *H. purpurascens* [37]. The structure of hellebrin was determined in 1995 by Muhr *et al.* using 2D NMR techniques [38]. Its ratio was monitored in the whole plant, compared to degluco-hellebrin [34]. This fraction was calculated also in cultivated *H. odorus* and *H. viridis* over the annual cycle, and the results showed that concentration of hellebrin is higher in *H. purpurascens* compared to other species [34].

4.2 Steroidal saponins

Saponins are steroid or triterpene glycosides widely distributed in the plant and marine animal kingdoms and include a large number of biologically active compounds. In 1976, a medicine proposed to treat ulcers and containing the main sapogenins from roots and rhizomes of *Helleborus* spp. was registered under a U.S. patent (O.I. Bruchköbel, 1976, Medicine containing the main sapogenin from *Helleborus*, U.S. Patent 3,956,491). This class of natural compounds has a wide structural diversity which may explain their multiple ranges of bioactivity reported so far [39]. Some of the steroidal saponins have cancer-related activity, as well as immunomodulating, antihepatotoxic, antiviral, and antifungal activities. Saponins have important action

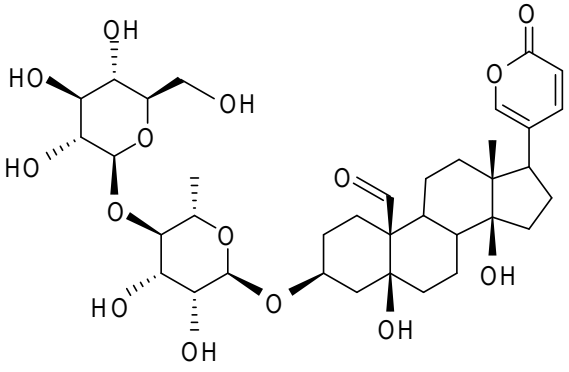

Figure 1. Hellebrin.

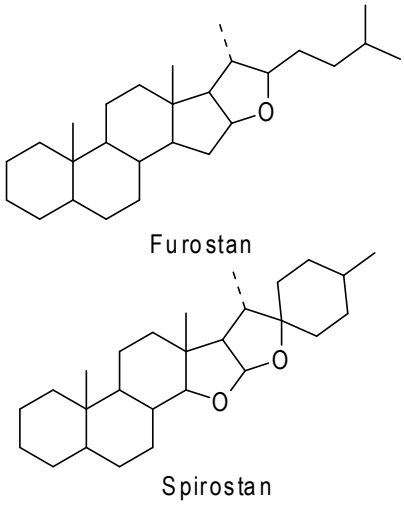

Figure 2. Saponins skeletons found in *Helleborus* sp.

on the cardiovascular, central nervous and endocrine systems [40-42]. Saponins reported in *Helleborus* spp. have furostan and spirostan skeleton structures, shown in Figure 2.

4.3 Ecdysones

Ecdysones are plant derived sterols, classed as triterpenoids, with a very polar structure [35]. Phytoecdysteroids have been reported to occur in over 100 terrestrial plant families representing ferns, gymnosperms and angiosperms [43]. Ecdysteroids can be found also in insect and crustacean families and play a defensive role against predators or are involved in plant metabolism [26,43]. Plant cells synthesize phytoecdysteroids from mevalonic acid in the mevalonate pathway, using acetyl-CoA as a precursor. The largest concentration of ecdysteroids was found in tissues involved in the defense mechanism of the plants. Ecdysteroids are used by plants as a protective mechanism against phytophagous insects. Phytoecdysteroids can mimic the activity of moulting hormones in insects and can disrupt moulting, perturbing normal insect development [44]. When ingested by non-adapted insects they lead to weight loss, moulting disruption and/or mortality [45].

Some of the pharmacological effects of phytoecdysteroids are summarized below: adaptogenic and antidepresive, hepatoprotective (related to phospholipid metabolism), chemopreventive (in cancer treatment), effective in the control of diabetes, and antimicrobial effects against some fungi and bacteria species [43]. A well-known ecdysteroid, 20-hydroxyecdysone, found frequently in *Helleborus* spp. is shown in Figure 3.

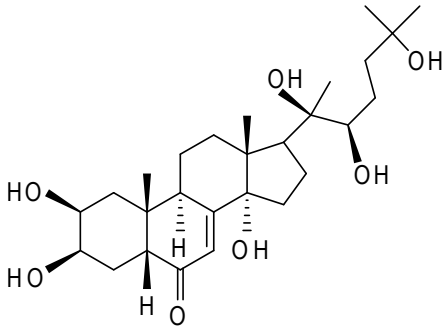

Figure 3. 20-Hydroxyecdysone

4.4 Protoanemonin

Protoanemonin (Figure 4) is a toxic γ-lactone of hydroxy-penta-2,4-dienic acid [13]. Frohne and Pfander described protoanemonin as a volatile, oily and irritant substance with high affinity for SH groups [31]. Protoanemonin is considered a biologically active compound having antimicrobial [46,47], fungicidal [48], and antimutagenic effects [49,50]. However, Dickens reported fibrosarcoma development when 2 mg protoanemonin was injected repeatedly in rats [51]. When drying the plant, protoanemonin comes into contact with air and dimerizes to anemonin, a non-stable compound which is hydrolyzed to a non-toxic carboxylic acid [52]. Anemonin is also fungicidal (less potent than protoanemonin) [48] and both of them (anemonin and protoanemonin) have a sedative effect [53]. Anemonin has also antispasmodic properties [54]. Anemonin inhibits cellular tyrosinase activity and

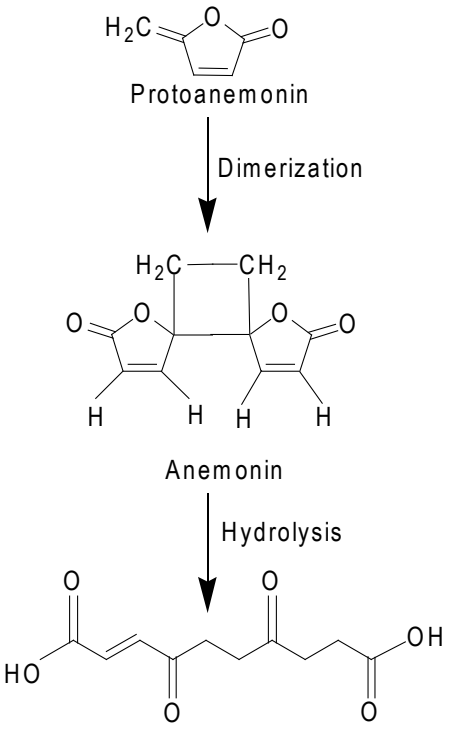

Figure 4. Degradation pathway of protoanemonin.

affects protein and mRNA levels in human melanocytes inhibiting melanin synthesis. That is why, anemonin may be used as a cosmetic agent for hypopigmentary purposes [55]. Anemonin is able to inhibit nitric oxide (NO) production by modulating the expression of iNOS (inducible nitric oxide synthase) [56,57]. This could explain its anti-inflammatory effects [57,58]. It was also reported that anemonin inhibits endothelin-1 (ET-1) [56,57] and intercellular adhesion molecule-1 (ICAM-1) in RIMEC (rat intestinal microvascular endothelial cells) preventing intestinal microvascular dysfunction [57]. It was suggested that anemonin can be used for treating various other diseases, including cardiovascular diseases and arthritis when ET-1 and NO activation are involved in mediation of pathogenesis [57].

4.5 Current chemical analysis on *Helleborus* sp.

Over the past few years, different *Helleborus* species have become the subject of phytochemical studies due to their putative potential in producing valuable secondary metabolites and due to the development of analytical methods that offered the opportunity to perform faster biochemical characterization of plant extracts. In order to decipher their biochemical content, different chemical analysis techniques were used: chemical transformations; analytical methods (chromatography): TLC (Thin Layer Chromatography), HPLC-MS (High Performance Liquid Chromatography - Mass Spectrometry), RP-HPLC (Reverse Phase - High Performance Liquid Chromatography) GC (Gas Chromatography) and various spectroscopic methods using 1D and 2D NMR techniques: COSY (Correlated SpectroscopY), TOCSY (Total Correlation SpectroscopY), ROESY (Rotating frame nuclear Overhauser Effect SpectroscopY), HMQC (Heteronuclear Multiple Quantum Correlation), HMBC (Heteronuclear Multiple Bond Correlation). New chemical compounds were identified and described in *Helleborus* spp. and many of them seem to have pharmacological activity and promising potential for medical research. Chemical analysis advances on *Helleborus* spp. through the last years are shown in Table 1.

Species	Tissue source	Compounds	Reference
H. odorus ssp. *laxus*, *H. viridis* ssp. *viridis*	Roots and rhizomes	hellebrin, degluco-hellebrin, hellebrigenin, bufatetraenolide, spirostane-type steroids, β-ecdysterone, 5β-hydroxyecdysterone	[59]
H. purpurascens	Roots	1 new steroidal saponin, hellebrin, β-ecdysterone, 5α-hydroxyecdysterone	[60]
H. torquatus	Seeds	3 new bufadienolides, 2 new ecdysteroids	[61]
H. atrorubens	Leaves	8 flavonoids, 7 phenolic acids	[62]
H. orientalis	Rhizomes	1 novel polyoxygenated spirostanol glycoside	[63]
H. orientalis	Rhizomes	1 new bufadienolide rhamnoside, 2 bufadienolide glycosides, 5 new spirostanol saponins	[64][c]
H. orientalis	Rhizomes	2 new bisdesmosidic furostanol saponins, 2 new furospirostanol saponins	[65][c]
H. orientalis	Rhizomes	2 new polyoxygenated spirostanol bisdesmosides, 1 new polyoxygenated spirostanol trisdesmoside	[66][c]
H. viridis	Leaves	2 new furostanol saponins, 3 new quercetin glycosides	[67]
H. orientalis	Roots	a furostanol saponin, a phytoecdysteroid	[68]
H. foetidus	Leaves	1 new caffeoylated quercetin glycoside, 1 steroidal saponin, anemonin, 2 phenol glycosides	[69]
H. caucasicus	Roots and rhizomes	2 sapogenins	[70]
H. caucasicus	Roots and rhizomes	4 new polyhydroxylated and polyunsaturated furostanol glycosides (caucasicosides)	[71]
H. caucasicus	Leaves	20-hydroxyecdysone, 1 spirostan	[72]
H. bocconei ssp. *intermedius*	Roots	2 new furostanol saponins, 1 furospirostanol saponin, ecdysterone, 5β-hydroxyecdysterone	[73][c]
H. orientalis	Rhizomes	8 new furostanol glycosides, 2 known furostanol glycosides	[74][c]
H. thibetanus	Rhizomes	2 new bufadienolides, 2 bufadienolides	[75][c]
H. thibetanus	Rhizomes	2 new bufadienolide glycosides, 2 ecdysteroids, 6 bufadienolide	[76]
H. viridis	Leaves	3 new steroidal saponins	[77]
H. caucasicus	Leaves	9 new furostanol glycosides, 9 furostanol glycosides, 1 bufadienolide, 1 ecdysteroid	[78]
H. niger	Leaves	1 new phenyllactic acid, 1 new flavonol glycoside, 1 kaempferol glycoside	[79]

Table 1. Progress made over the last years on chemical analysis of Helleborus sp. [c] Authors that tested the compounds described in their papers for cytotoxicity.

4.6 Hellethionins

A new family of thionins was discovered in the roots of *H. purpurascens* and named hellethionins. Hellethionins are part of group of α/β thionins, small-sized multiple-cystine peptides, that were found in endosperms of grains, in seeds and in parasitic plants [80,81]. These are considered to be excellent candidates as biocontrol agents of plant pathogens [82]. *In vitro* experimental studies suggest that thionins are toxic to bacteria, fungi, and yeasts due to their interaction with membrane phospholipids and their capacity to form ion channels. Possible roles in the defense of plants against pathogens, directly at the membrane level, are assumed. Also, hellethionins are thought to have a function in cell death [83]. All thionins have the property to refold correctly into the native structure, making them robust scaffolds that can be exploited for screening of optimized membrane activities and of cytotoxicity [81].

5. Pharmacological and therapeutic effects

Studies concerning the pharmacological and therapeutic effects of whole or partial *Helleborus* spp. extract have been conducted in various animal models and *in vitro* systems.

5.1 Antirheumatic and anti-inflammatory activity

Kerek presented for the first time the beneficial action of a drug named Boicil [84], which was made from *H. purpurascens* root and stem extract (V. Boici, 1977, Analgesic substance derived from the *Helleborus* plant and method of making same, U.S. Patent 4,012,505) and has been successfully used in Romania for decades to treat rheumatic pains. *H. purpurascens* extracts exhibited high biological activity with lasting analgesic, myorelaxant, and blood vessel regulating actions. *H. orientalis* was tested in mice, using a carrageenan-induced hind paw edema model, to determine anti-inflammatory activity and a p-benzoquinone-induced abdominal constriction test, to determine antinociceptive activity. The authors reported that a dose of 500 mg/kg ethanolic extract of roots of *H. orientalis* showed anti-inflammatory activity without inducing any gastric damage. Root and herb extracts, both alcoholic and aqueous, were reported as antinociceptive, when a dose of 500 mg/kg was tested [85].

5.2 Immunomodulatory activity

Many studies made on animals have demonstrated the immunostimulatory effect of *Helleborus* spp. extracts. Immunostimulatory effect of *H. purpurascens* was reported in sheep. Increased number of lymphocytes (2×) and neutrophiles (3.5×) after 48 h of injecting young sheep with 5% decoct of radix and rhizome showed an improved immune response of the animals [86]. Activation of rapid, non-specific defensive mechanisms and poor haemolysis has been reported when subcutaneous, intraperitoneal and intramuscular application of different concentrations of the extract of rhizome and root of *H. odorus* were performed. A pronounced leukocytosis and an increased percentage of neutrophil granulocytes have been recorded [87]. These results were confirmed by other authors supporting the idea that *Helleborus* extracts can trigger unspecific immune response in animals [20].

A feature related to immunomodulation was assigned to chemical compounds found in *Helleborus* spp. MCS-18 (macrocyclic carbon suboxide), a multi-anionic compound extracted and purified from *H. purpurascens* roots, classified as New Chemical Entity (NCE) and originated in plants [88], was characterized as a highly potent inhibitor of Na, K-ATPase and of the SR Ca-ATPase [89,90].

MCS-18 has a complex structure and in the last years has been intensively studied. It induces *in vitro* up-regulation of the immune modulatory cytokines IL-10 and TGF-β [91]. Also, MCS-18 efficiently downregulates T-cell-dependent antibody (Ab) formation in mice, where a Toll-like-receptor (TLR) blockade might be involved. MCS-18 leads to a strong attenuation of antibodies against tetanus toxoid if administered together with the Ab elicitor Freund's Complete or TLR antagonists in various combinations. These findings suggest that MCS-18 could be a potent, non-toxic antagonist or a down-regulator of the TLR signaling pathway [92]. MCS-18 proved to be a potent antagonist of the capsaicin activated vanilloid type pain receptors (TRPV1) and explains its local analgesic action [93].

To MCS-18 was assigned an important role in autoimmunity. MCS-18 strongly reduced the paralysis associated with the experimental autoimmune encephalomyelitis (EAE), which is a murine model for human multiple sclerosis. This compound can be used in the EAE model not only as a prophylactic, but also as a therapeutic setting. It was also proved that MCS-18 induces a long-lasting suppressive effect and is able to inhibit the expression of typical molecules of mature dendritic cells (DC). This compound impeded the formation of the typical DC/T-cell clusters, which are essential to induce potent immune responses [94]. Another paper that supported the above mentioned facts, added that MCS-18 also reduced B-cell proliferation and immunoglobulin production [95].

MCS-18 treatment almost completely reduced leukocyte infiltration in the brain and in the spinal cord. Using EAE assay *in vitro* as well *in vivo* the authors were able to show that MCS-18 exerts a strong immunosuppressive activity with remarkable potential for the therapy of diseases characterized by a pathologically over-activated immune system. They concluded that MCS-18 is an efficient modulator of immune response *in vitro* and *in vivo* and that is able to inhibit the disease symptoms at different stages [94]. The authors demonstrated that MCS-18 has a very potent non-toxic immune-suppressive activity and their results were strongly supported by clinical trial data reporting that MCS-18 is a highly effective drug in the treatment of arthritis.

Type I diabetes is still a serious problem for which the medical community is looking for new ways to prevent and overcome. MCS-18 could become a promising answer for people suffering diabetes. NOD-mouse model was used to observe the MCS-18 treatment, indicating significantly reduced islet T-cell infiltrates and the rate of T-cell proliferation. Periinsular infiltrates in the MCS-18 treated animals showed a significantly enhanced number of Foxp3(+) CD25(+) T cells, indicating the increased presence of regulatory T cells. In the animal group which had been treated with MCS-18, 70% of the animals showed a diabetes free survival, in comparison with untreated animals where only less than 10% were free of diabetes. These studies showed that MCS-18 exerts an efficient immunosuppressive activity with remarkable potential for the therapy of diseases characterized by pathological over-activation of the immune system [96].

5.3 Antioxidant activity

ROS (reactive oxygen species) are products of cellular metabolism having important functions in cell signaling, homeostasis and apoptosis, activation of host defense genes and mobilization of ion transport systems. ROS have an important role in immune system functioning. Five progressively purified MCS-products from *H. purpurascens* were tested as possible antioxidants and/or modulators of ROS production and released from human polymorphonuclear granulocytes cells. One of the fractions MCS-Dx proved to be a potent ROS scavenger and it could be used as adjuvant in antioxidant therapy [97]. Even a simple aqueous or hydroalcoholic extract, concentrated by ultrafiltration, proved to have high antioxidant activity. The DPPH (2,2-diphenyl-1-picrylhydrazyl) inhibition value of hydroalcoholic extract showed the highest antioxidant activity (79%), while the concentrated aqueous extract showed 73% DPPH inhibition [98]. Čakar *et al*. measured antioxidant activity in root and leaf extracts from *H. odorus, H. multifidus* and *H. hercegovinus* leaf extract exhibiting a higher antioxidant activity (IC_{50} values between 0.12-0.89 mg/ml) compared to root extract (IC_{50} values between 0.72-3.10 mg/ml) [99]. Another compound found in *Helleborus* spp. that proved to be antioxidant is 20-hydroxyecdysone, an ecdysteroid that acts as a fluoride-stimulated respiratory burst modulator in the same manner as water soluble antioxidants, chlorpromazine and emoxipin [100]. This compound, alone or acting in synergy, could render the antioxidant activity assigned to these plants.

5.4 Antimicrobial activity

Although many *Helleborus* spp. have been used to treat infectious diseases in animals, the antimicrobial activity of these species was not screened. There are just a few studies concerning this aspect [101,102]. Roots from *H. bocconei* ssp. *siculus* were tested for their antibacterial activity against microorganisms responsible for respiratory infections. Seven strains of microorganisms responsible for these types of infections were tested: *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Haemophilus influenzae* ATCC 49247, *Moraxella catarrhalis* ATCC 25238, *Pseudomonas aeruginosa* ATCC 27853, and *Stenotrophomonas maltophilia* ATCC 13637. The root methanolic extract and its bufadienolide fraction showed the lowest Minimum Inhibitory Concentration values (100 µg/ml) against *Moraxella catarrhalis* (0.2, 0.1) and *Streptococcus pneumonia* (0.4, 0.1) [101].

5.5 Cytotoxicity and anticancer properties

Many species of *Helleborus* are seen today as potential sources for anticancer drugs. Studies involving extracts or chemical compounds gave optimistic results related to cancer inhibition and cytotoxicity. Lindholm *et al*. [103] tested, through a large-scale screening protocol, 100 fractionated plant extracts, seven of them showing interesting cytotoxic properties. The cytotoxic activity was not fully characterized, but *H. cyclophyllus* extract proved to have antitumoral potential. Another species, *H. caucasicus,* also showed cytotoxic activity. Root and rhizome alcoholic extracts were tested against human lung cancer (A-549), human colorectal cancer (DLD-1) and normal skin fibroblasts (WS1) and their cytotoxic dose was reported as being rather low (0.002 µg/ml) [104].

H. niger extracts were used for *in vitro* tests involving hematological malignancies. Inhibition of cell proliferation is caused by specific apoptosis induction *via* mitochondrial pathway and caspase-3 processing.

Apoptosis induction was observed in lymphoma (BJAB), leukemia (REH, Nalm6, Sup-B15) and melanoma (Mel-HO) cells. A better efficiency in inducing apoptosis was recorded when whole plant and root extracts were used compared to leaf and blossom extracts. When a concentration of 0.75 mg/ml *H. niger* extract was added to a BJAB cell line, an inhibition up to 96.5% of the proliferation rate was recorded. Synergistic effects were also observed when a cytostatic drug - vincristine - was added to the BJAB cell culture in combination with *H. niger* whole plant extract.

Although apoptosis in Mel-OH cell line was correlated with Bcl-2 protein overexpression, sensitivity to *H. niger* extract was clearly recorded [105]. Another extract from roots of *H. bocconei* ssp. *intermedius* proved to have cytotoxic activity against rat C6 glioma cells. Three isolated compounds, a hydrolyzed form of helleboroside B, a furospirostanol saponin and polypodyne B showed significant cytotoxic activity, recording a 60-70% inhibition of the cell growth [73].

In a similar comparative study, root extracts from *H. odorus, H. multifidus* and *H. hercegovinus* proved to be more efficient than leaves extract of the same species on inhibiting growth of BJAB cell lines. The strongest antiproliferative activity was indicated for root extracts of *H. multifidus* and *H. hercegovinus* which inhibited BJAB cell lines growth with 50.14% and 49.04%, respectively [99]. Hellebosaponin C, a spirostanol glycoside, and another two furostanol glycosides isolated from rhizomes of *H. orientalis* exhibited a moderate cytotoxic activity against human oral squamous cell carcinoma (HSC-2) [66,74].

Two new bufadienolides (tigencaoside A, B) found in rhizomes of *H. thibetanus* were also tested for cytotoxic activity against human cancer cells 3LL, MCF-7, QGY-7701 and BGC-823. The IC_{50} values of tigencaoside A on the tested cell lines ranged between 105.23–253.12 µg/ml and for tigencaoside B exhibited values were between 56.54–86.45 µg/ml [75].

Potential cytostatic and cytotoxic effects of *H.purpurascens* hydrous extract (HphE) and *H. purpurascens* hydroalchoholic extract (HphaE) were recently evaluated by Vochita *et al*. [106]. Total and fractionated polyphenolic compounds were extracted from roots and rhizomes of *H. purpurascens* and tested on HeLa cancerous cells. Tested biopreparations as 0.45 µm MF-HphE (microfiltrate of HphE), 30,000 Da UF1P-HphE (permeate of first ultrafiltrate), 10,000 Da UF2C-HphE (concentrate of second ultrafiltrate), 10,000 Da UF2P-HphE (permeate of second ultrafiltrate) exert a very strong cytostatic effect with values over 90%. Other biopreparations as HphE (total hydrous extract), 30,000 Da UF1P-HphE, 3,000 Da UF3C-HphE (concentrate of third ultrafiltrate), 3,000 Da UF3P-HphE (permeate of third ultrafiltrate) induce an inhibition of the tumor cell between 59.46% and 67.80%.

Thionins - the class of peptide that was found also in *H. purpurascens* - have been proposed as potential immunotoxins in tumor therapy. Cytotoxicity of thionins has led to the development of a potential application: targeting thionins by tumor-specific antibodies, which is expected to support antitumor therapy [81]. Anticancer properties of hellethionins were reported by Kerek. Acting alone or synergistic, hellethionins proved to inhibit proliferation in different cancer cell lines. Hellethionin C, at very low concentration (2 µg/ml), causes a clear inhibition of proliferation in MFC-7 cell line (breast cancer cell culture). Hellethionin C and hellethionin D, at a concentration of 100 µg/ml, strongly inhibit (48.36% and 58.66%, respectively) the culture of Colo 205 (colon cancer cell line). Hellethionin C at 50 µg/ml in combination with MCS-18 at 100 µg/ml inhibits the growth of Colo 205 with 66%. A mixture of hellethionins B (B1: B2: B3 = 1:1:1), hellethionin C and hellethionin D at a concentration of 100 µg/ml were also able to inhibit with 50% the growth of a lung carcinoma cell line (Kerek F., 2010, Petides having a high cysteine content, U.S. Patent 7,750,114 B2).

Another recently registered patent is describing methods for obtaining new cardiac steroids starting from hellebrin and hellebrigenin and using conventional techniques of synthetic organic chemistry (J. Dewelle, M. El Yazidi, E. Van Quaquebeke, N. De Neve, T. Mijatovic, L. Ingrassia, *et al*., 2010, Hellebrin and hellebrigenin derivatives, WO2010/102673). Apparently, the 21 novel hellebrin and hellebrigenin derivatives have a cytotoxic activity with reduced general toxicity. These compounds were tested against six human cancer cell lines in the MTT tests, and a number of these compounds showed very potent *in vitro* antitumor activity with IC_{50} values in the nanomolar range. That was why these compounds were proposed as medicines for cancer treatment. The authors also used two normal fibroblast cell lines to investigate potential compound selectivity towards cancer. Almost all described compounds displayed *in vitro* marked selectivity toward cancer cells (J. Dewelle, M. El Yazidi, E. Van Quaquebeke, N. De Neve, T. Mijatovic, L. Ingrassia, *et al*., 2010, Hellebrin and hellebrigenin derivatives, WO2010/102673). We may consider that *Helleborus* spp., having so many valuable chemical compounds, are potential pools for a wide range of pharmacological agents and bioactive molecules.

H. niger aqueous extracts were tested in order to asses a DNA destabilizing risk. Sister Chromatid Exchange assay (SCE) was used to detect DNA

damage. The authors found that *H. niger* aqueous extract possesses immunomodulating properties and also exerts slight effects of DNA destabilization and might have a mutagenic effect on human PBMC (peripheral blood mononuclear cells) [107].

6. Micropropagation and genetic transformation

Harvesting wild medicinal plants is often nonproductive. In the case of plants such as *Helleborus,* where the targeted medicine is located in roots and the seeds have a low germination rate, sustainable propagation could be a better option. The latest considerable discoveries concerning hellebores have used *in vitro* cultivation as an easier way to study them and for finding new methods to use their features. *In vitro* techniques consisting of micropropagation, callus culture, cell suspension culture or somaclonal variation, could improve the production of secondary metabolites. Smulders and de Klerk reported that 4 different lines of *Helleborus* spp., having the same source, remained unmodified during many subcultures, a feature which may be used in producing new varieties and in synthesizing new chemical compounds [108]. The antimicrobial activity of hellethionins could be used in genetic engineering. Transgenic plants containing thionin genes can enhance resistance against pathogens. Hellethionins are peptides that are promising candidates for engineered plant resistance in the agricultural industry (81, Y. Ohashi, I. Mitsuhara, M. Oshshima, M. Ugaki, H. Hirochika, R. Honkura, *et al.,* 1998, Method for producing disease resistant plant with thionin gene from *Avena sativa*, U.S. Patent 6, 187, 995).

After the isolation and identification of these new important chemicals, the next step in using this potential would be molecular farming. Genetically engineered plants with new traits could have extra resistance to insect attack and improved weed control, or could produce a large quantity of pharmaceutically active compounds.

Computer- based analysis revealed that natural products exhibit a remarkable structural diversity of molecular frameworks and scaffolds that could be systematically exploited for combinatorial synthesis. Natural products offer a rich pool of unique molecular frameworks and desirable drug-like properties, rendering them ideal starting points for molecular design considerations [109].

7. Conclusions and perspectives

Important progress on isolation and identification of bioactive compounds from *Helleborus* species has been made in the past few years. However, further studies concerning the assessment of their pharmacological and therapeutic effects and clinical trials concerning the target-organ toxicity or side effects, are still required.

There are many studies which strongly support the view that extracts of the plants belonging to this genus have beneficial therapeutic actions. It is known that the active principles are effective remedies in anti-inflamatory and antirheumatic conditions, and have proven efficiency as immunostimulators, antioxidants, antimicrobial and antitumoral agents.

Even if there are some patents concerning active compounds, *Helleborus* species are still insufficiently explored as a source of valuable products and that further studies need to be done. Precise clinical trials using a large number of patients have to be encouraged in order to evidence the efficacious and possible side effects of the newly found compounds. New research approaches on this topic may concern the assessment of the effects of novel compounds discovered, the mechanisms of their action, the interplay with known regulatory components at the protein level to provide an understanding of their dynamic interactions and how these interactions orchestrate the biosynthesis of secondary metabolites in plants. Achieving these goals will translate into a major advance in our understanding of how plant bioactive compounds actually work, since our understantding of interactions at the cellular level is limited. This will not only generate fundamental knowledge about the dynamics of plant biosynthesis, but will ultimately provide us with the tools to rationally reprogram secondary metabolism for genetically engineering more useful plants.

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