Genet Resour Crop Evol (2020) 67:781–797
https://doi.org/10.1007/s10722-019-00876-5

RESEARCH ARTICLE

Ethnobotanical, historical and histological evaluation of *Helleborus L.* genetic resources used in veterinary and human ethnomedicine

Viktória Lilla Balázs · Rita Filep · Tünde Ambrus · Marianna Kocsis · Ágnes Farkas · Szilvia Stranczinger · Nóra Papp

Received: 10 July 2019 / Accepted: 24 December 2019 / Published online: 19 January 2020

Abstract The use of hellebore (*Helleborus*) species for medical purposes has a long-standing tradition. Our work aimed at providing a historical survey of their medicinal application in Europe, and data on current ethnobotanical use of *H. purpurascens* Waldst. et Kit. in Transylvania (Romania), compared with earlier records of this region and other European countries. While the chemistry and pharmacology of hellebores have been researched extensively, little is known about their anatomical traits. Thus, we intended to provide a detailed histological analysis of *Helleborus odorus* Waldst. et Kit., *H. purpurascens*, and *H. niger* L., based on transverse sections of aerial parts and root. Our survey revealed that *H. purpurascens* is known for immunotherapy, wounds, and as antiemetic drug in ethnoveterinary medicine, but not in human therapy in the study area. Distinctive histological characters included diverse stele structure in the root; sclerenchymatous bundle caps around compound vascular bundles in the stem and the main leaf veins of *H. odorus*; and amphistomatic leaves in *H. purpurascens*. Quantitative vegetative traits also revealed significant differences among species, but they may reflect environmental influences, too. In all three species the sepal was hypostomatic with mesomorphic stomata, while the modified petal comprised a proximal nectar-producing and a distal non-secretory part. Distinctive floral traits included shape of modified petal, presence of papillae and thickness of non-secretory part; as well as ornamentation of tricolpate pollen grains. Our findings suggest that the anatomy of various plant parts varies slightly with each species, including ethnomedicinally known *H. purpurascens*, even though the basic structure is the same within the genus.

Keywords Ethnobotany · Ethnomedicine · Hellebore · History · Histology

V. L. Balázs · R. Filep · Á. Farkas · N. Papp (✉)
Department of Pharmacognosy, Faculty of Pharmacy, University of Pécs, Pecs 7624, Hungary
e-mail: nora4595@gamma.ttk.pte.hu

T. Ambrus
Department of Applied Pharmacy, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, 612 42 Brno, Czech Republic

M. Kocsis · S. Stranczinger
Department of Plant Biology, Faculty of Sciences, University of Pécs, Pecs 7624, Hungary

Introduction

Hellebores (*Helleborus* L.), belonging to Ranunculaceae, are perennial herbaceous plants widely spread in Europe and Asia (Tutin et al. 2010). The word *Helleborus* can be associated with the ancient Greek tradition created from the words *hellos* (= fawn) and *bora* (= food), referring to a plant eaten by young roe deer (Jirásek et al. 1957). The names *Helleboros* or

Veratrum were used by Dioscorides in the first century. *Helleborus* species were mentioned as "humior" in 1395 (Rácz 2010), then as "hunyor" in the first Hungarian handwritten medical work entitled Ars Medica (Lencsés cca. 1570). In this work, related to Transylvanian medicine, the traditonal therapeutical value of "hunyor" species in the sixteenth century is well documented. The data presented here derive from Ars medica Electronica (Szabó and Biró 2000)—a digital database based on the three existing copies of the six volumes of this "Ars medica" prepared for printing around 1577, but never published. This compilation makes a very clear distinction between "fehér hunyor" (*Veratrum album* L.) and "fekete hunyor" (*Veratrum nigrum* L. i.e. between the plants belonging now to *Veratrum* and *Helleborus* species). The Lencsés-database includes 63 "hunyor" entries: 36 refer explicitely to fejérhunyor (*Veratrum album*), 21 to feketehunyor (*Veratrum nigrum* i.e. the *Helleborus*-species), 3 mentions both in the same recipies, 2 is specified just as "hunyor" and 1 refers to "vízihunyor" (not identified yet). Further data were presented by Melius (1578), Pápai Páriz (1690), Diószegi and Fazekas (1807), and as Hungarian comments in 1703 in Dorstenius' work (1540). The genus was also described in Species Plantarum (Linnaeus 1753).

In the official European materia medica several historical records can be found on the therapeutic use of hellebores. In the Ancient Times the region of Anticyra, a port in the north coast of the Gulf of Corinth was famed for its hellebores which were regarded as a cure for insanity, gout, and epilepsy (Encyclopaedia Britannica 1910a).

The medical history of the species is a matter of controversion, because it has often been mistaken for other species (e.g. *Adonis vernalis* L., *Actaea spicata* L., *Astrantia major* L.), as a result of false botanical identification. The name of *Helleborus* has been used in some cases to describe other plants. Typical examples include mentioning *H. albus* for *Veratrum album* (Woodville 1810), or *H. niger* for *Melampodium*, which has been named in Melampus' honor. Melampus, an ancient mythological shepherd and healer recommended the milk of a goat, which had been fed on the herb of hellebore, for the daughters of King Proetus for madness (Wood and Bache 1839; Encyclopaedia Britannica 1910b). Gallic men soaked the arrows into *ellebore* during hunting (data by Plinius). In Ancient Egypt the species was applied against mental disorders (Rácz 2010). In the antique medicine of Europe, the root was used as a purgative drug and for maniacal disorders by the removal of black bile. For a long time, *H. niger* was considered as "Hellebore of Hippocrates" recommended by antique medical writers (Woodville 1810).

In the modern Western materia medica *H. niger* L., *H. orientalis* L., and *H. foetidus* L. were used with various therapeutic purposes in the eighteenth–nineteenth century. While the Central European medico-pharmaceutical literature presented data mainly on *H. niger*, in Western European references *H. foetidus* was recognized as an official drug. *H. niger* was mentioned as a diuretic, emmenagogue and cathartic, called a melanagogue drug recommended in female obstructions, hysteric and hypochondriac fits, melancholy, madness, epilepsy, leprosy, and inveterate quartans in the eighteenth century (Alston 1770). It was also documented that its use can lead to inflammations of mucous membranes (gastric or intestinal), skin inflammation, and even vesication (Wood and Bache 1839). Irritating effect on nasal mucosa was therapeutically used by applying sternutatory (sneezing) powders including powdered rhizome of *H. niger* and *H. viridis* L. (Magyary-Kossa 1926).

In state Pharmacopoeas of the Habsburg Empire and Austro-Hungarian Monarchy issued between the second half of the eighteenth century and beginning of the twentieth century, two hellebore species were included as official drugs. In the first edition of the Austrian Provincial Pharmacopoeia, the root of *H. niger* is presented in the list of simple drugs (Pharmacopoea Austriaco-Provincialis 1774), while the extract is documented as a simple preparation in the last edition (Pharmacopoea Austriaco-Provincialis emendata 1794). The II–IV editions of Austrian Pharmacopoeas published in the first half of the nineteenth century (1814–1834) included the root of *H. niger* as a simple drug, and its aqueous extract and tincture [Pharmacopoea Austriaca: Editio altera, emendata (1814); Pharmacopoea Austriaca: Editio tertia, emendata (1820); Pharmacopoea Austriaca: Editio quarta emendata (1834)]. The innovative 5th edition also presented the species but only in form of extract (Pharmacopoea Austriaca: Editio quinta 1855). In the 6th edition the plant was replaced by the rhizome and extract of *H. viridis* because of its intense therapeutic effect (Pharmacopoea Austriaca: Editio sexta 1869; Schneider and Vogl 1881). In later editions of this

Pharmacopoea and the first three editions of Hungarian Pharmacopoea no drug or preparation of hellebores can be found. Afterwards, these plants occurred again e.g. in the supplement of 6th edition of the German Pharmacopoea (*rhizoma Hellebori* originating from *H. niger* and *H. viridis*) (Ergänzungsbuch zum Deutschen Arzneibuch 1941).

The rhizome of *H. niger* was used for its digitalis-like effect, as a diuretic and cardiotonic agent in human therapy, and for diuretic effect in veterinary medicine, while that of *H. viridis* as an emetic, laxative and anthelmintic drug (Jirásek et al. 1957).

In the late nineteenth and early twentieth century, several medico-pharmaceutical practical handbooks presented partially obsolete recipes containing remedies of hellebores. The drugs and extracts were stored in special cardboard drug containers and porcelain apothecary jars e.g. in pharmacies in Romania and Czech Republic in the nineteenth century. Also wooden drug jars from eighteenth century are known in the museal collections of the area documenting the continuous use of hellebores (Veress 2017). The root extracts of *H. niger* and *H. viridis* were recommended with other active ingredients in various forms for epilepsy (Electuarium antiepilepticum Landerer), hypochondria (Mixtura antihypochondriaca Reil), and dropsy (Vinum antihydropicum Fuller, Pilulae tonicae Bacher). In veterinary medicine, pills made of the drug of both species were mentioned for epilepsy and digestive problems of dogs (Pilulae antiepilepticae, Pilulae digestivae) (Hager 1891; Fischer and Hartwich 1910). During later decades of the first half of the twentieth century the therapeutic use of hellebore preparations in human pharmacotherapy was eliminated stage by stage (Magyary-Kossa 1926). Apart from the official academic medicine, hellebore (in Europe mainly fresh or dried rhizome of *H. niger* and *H. viridis*) was also included in the homeopathic materia medica (Schwabe 1872; Jirásek et al. 1957).

In the last century and currently, the pungent and bitter root and rhizome (Sadler 1824; Borza 1968) have been widely used as aspecific immunostimulant drugs in the European ethnoveterinary medicine: they have been applied for pneumonia and cough of pigs, cows, and horses (Kóczián et al. 1975, 1976; Halászné 1981, 1987; Péntek and Szabó 1985; Gub 1993, 1996, 2000; Bárdi et al. 2002; Pieroni et al. 2004; Szabó 2005; Babai 2013), against nasal congestion of horses (Pieroni et al. 2013), for helminthiasis (Butura 1979), and cooked and blended with fat against lice of animals (Gub 1993). The leaf has been known for rheuma as a foment (Halászné 1981, 1987), and soaked into the fodder of pigs against pneumonia and cough (Kóczián et al. 1976).

The traditional and official medical use of *Helleborus species* is based mainly on the chemistry of some components as genetic resources. Among them, hellebores are rich in structurally diverse active compounds that are responsible for a variety of pharmacological effects (Cioca and Cucu 1974; Milbradt et al. 2003; Szabó 2005), e.g. cardiac glycosides, steroidal saponins, ecdysones, and protoanemonin (Szabó 2005). Steroidal saponins have wide structural diversity as both furostan and spirostan skeleton structures (Challinor et al. 2012; Maior and Dobrotă 2013). Concentration of helleborin, the most well-known cardioactive glycoside of hellebores, was found to be higher in *H. purpurascens* Waldst. et Kit. compared to *H. odorus* Waldst. et Kit. and *H. viridis* (Wissner and Kating 1974; Szabó 2005).

The chemical profile of parts of hellebores may change even in different phenological stages; e.g. the level of anthocyanins, flavonols and chlorophyll showed significant differences in various developmental stages of the sepals in *H. niger* (Schmitzer et al. 2013).

Hellebore species have antioxidant potential (Păun-Roman et al. 2010; Apetrei et al. 2011; Čakar et al. 2011), and they can be used for heart failure in human therapy under medical control (Szabó 2005), for various diseases of the immune system (Szabó 2005; Horstmann et al. 2008; Littmann et al. 2008; Neacşu et al. 2010), as antitumoral agents (Vochita et al. 2011), for diabetes, eczema, toothache, and arthritis (Maior and Dobrotă 2013).

Since the majority of researches has focused on the chemical and pharmacological traits of hellebores (Cioca and Cucu 1974; Stochmal et al. 2010; Yang et al. 2010; Vitalini et al. 2011; Milbradt et al. 2003), and only a limited number of papers addressed structural issues (Vesprini et al. 1999, 2012; Šušek 2008; Barkyna and Churikova 2014; Rottensteiner 2016; Kumar and Lalitha 2017), our work aimed at highlighting variability in the anatomy of three *Helleborus* species. Although the tissue structure of the vegetative organs and the flower—comprising peculiar coloured petal-like sepals, petals modified into nectaries, and inner rings of stamens and pistils—

is fairly constant within the genus (Tamura 1993; Dános 2006), there might be slight differences regarding the morphology and anatomy of stem, foliage leaf and floral organs in various species. The terminology used for describing various floral parts varies with different authors: the outermost whorl of the flower is either referred to simply as perianth (Salopek-Sondi 2011), sepal (Šušek 2008; Salopek-Sondi 2011) or tepal (Vesprini et al. 1999; Rottensteiner 2016); while the term used for the nectar-producing structure is either nectary (Vesprini et al. 1999, 2008, 2012; Koteyeva 2005) or petal (Šušek 2008).

We organized our research around two objectives, in order to fill in gaps in our knowledge related to various *Helleborus* species. The first objective was to sum ethnobotanical data of *H. purpurascens* collected in Transylvania (Romania) and compare them with earlier records of hellebores in Transylvania and other European countries. The second aim was to provide a detailed histological analysis of the vegetative and generative parts of *H. odorus* and *H. purpurascens*, which are native to Hungary, and those of *H. niger* as a cultivated species, focusing on the similarities and differences of the ethnomedicinally mentioned parts.

Materials and methods

Study area

The ethnobotanical survey was conducted in villages of Homorod (Székely people), Ghimes and Uz valley (Csángós), each located in Transylvania, Romania, from 2007 to 2018.

Homorod valley is located in south-eastern Transylvania surrounded with mountains. The Székelys live from agricultural practices and livestock as farmers and shepherds in Lueta (Homorod valley). Csángó people in Cinod and Eghersec (Uz valley) and Lunca de Sus (Ghimeş mountains) live also as self providers from pastoral activities and dairy products. Although Lueta and Lunca de Sus are provided by medical and pharmaceutical services, people frequently apply various home treatments for human and veterinary health problems involving mostly materials of plant origin, similarly to Cinod and Eghersec. This area is of special ethnobiological interest due to its Hungarian Szekely (Hung. Székely; Rom. Secui) and Csango (Hung. Csángók, Rom. Ceangăi) population. Both represent two quite archaic Hungarian ethnographic groups preserving many medieval cultural traditions.

Ethnobotanical fieldwork

The asked 45 Székely and 62 Csángó informants were aged between 62 and 91 years. They speak in Hungarian which faciliated the communication during the interviews. The semi-structured interviews lasted 60–120 min (altogether 80 h), included questions on the local name (in *italics*), habitat, harvesting method and time of various medicinal plants, including hellebores, as well as the method of preparation, use and treated disorders. Prior informed consent was obtained to performing interviews and ethical guidelines of the International Society of Ethnobiology (ISE 2007) were applied. Data were documented with handwritten notes, tape recording, and photos. Personal observations in fields were completed by plant collection, then voucher specimens were deposited at the Department of Pharmacognosy, University of Pécs. Scientific nomenclature of hellebores followed the systematic work of Tutin et al. (2010).

Ethnobotanical data analysis

A search for ethnomedicinal studies of hellebores was carried out in databases (PubMed, Science Direct and Scopus). Our data collected were compared to earlier documented records in Transylvania and other European countries from the sixteenth century, focusing mostly on those obtained from the last century. During comparison, similarities and differences of the data were taken into consideration.

Sample collection for histological study

The root and aerial parts of *H. odorus* were collected in an oak forest in the Mecsek hills in South Hungary, those of *H. purpurascens* in the Botanical Garden, University of Pécs, Hungary, and those of *H. niger* in the Medicinal Plants Garden of the Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic in 2016. The root, stem, leaf, petiole and flowers including the sepal, nectary, anther, filament, and pistil of each species were cut into 1 cm pieces, and fixed in a mixture of

96% ethanol:glycerine:water (1:1:1), until further analysis.

Histological study

Samples were dehydrated in ethanol series (in 30%, 50%, 70%, and 96% for 12, 12, 24, and 3 h, respectively), infiltrated with Technovit 7100 solution, and finally embedded into a resin containing hydroxyethyl methacrylate. Transverse sections (10 µm thick, 18–20 slides per plant parts) were prepared with a rotation microtome (Anglia Scientific 0325). Sections were stained in toluidine blue (0.02%) for 5 min, washed with distilled water (for some seconds), 96% ethanol (two times for 3 min each), isopropanol (for 2 min), and xylene (for 3 and 10 min). Finally, the samples were covered with Neomount. Slides were examined with NIKON Eclipse 80i microscope and micrographs were taken with Spot Basic 4.0 software.

In roots and stems the following parameters were measured by Motic Images Plus ver. 2.0 software: thickness of epidermis, cortex and stele; in leaf: thickness of adaxial and abaxial epidermis, palisade and spongy parenchyma.

Statistical analysis for histological study

The measured data were compared with One-way ANOVA with Tukey's pairwise comparisons. If the normality assumption was violated, we applied Kruskal–Wallis test with Mann–Whitney pairwise comparisons. The normality of data series was checked by using Shapiro–Wilk test. All statistics of micromorphometric data were calculated with Past statistical software, version 2.17b (Hammer et al. 2001). Figures were created by OriginPro 8 (OriginLab Corporation, USA).

Results

Ethnobotanical data of hellebores

In our Transylvanian survey, compared to earlier records of this region and other European countries, ethnobotanical data were documented only on *H. purpurascens,* which is widespread in the study area. The other two *Helleborus* species were not mentioned in any of the study sites. In terminological aspect, the local name *eszpenz* was mentioned in Lunca de Sus (Ghimeş), similarly to earlier records, confirming its long-time use in the region. In addition, this name was actually collected in Úz valley as a geographically bordering area with Ghimeş (Table 1). The vernacular names *keserűgyökér* (in Lunca de Sus) and *papvirág* (Lueta) were recorded as new data for the species.

Actual traditional use of the root of *H. purpurascens* was documented as immunostimulant therapy and externally only in ethnoveterinary medicine as significant drug in "home pharmacy" (Fig. 1). Among them, antiemetic effect was noted as a new record in Lueta: root pulled into the nose or ears of pigs "collects the disorders into the nose which results inflamed ears". The species has no human therapeutical data in the study areas, but it was also mentioned as ornamental plant harvested in spring (Table 1).

Histological study

The micrographs of the studied plant parts of the three species and their quantitative parameters are shown in Figs. 2, 3, 4, 5 and 6. The comparative histological study of the species revealed a number of distinctive characters summarized in Table 2.

The transverse sections of the root from the maturation zone are demonstrated by Fig. 2a–c. The epidermal cells of *H. odorus* are significantly thinner (31.6 ± 4.8 µm) compared to the other two species (56.7 ± 3.7 µm, 55.1 ± 3.1 µm in *H. niger* and *H. purpurascens*, respectively). A single layer of epidermis surrounds the cortical zone, which consists of several layers of thin walled, parenchymatous cells. This tissue of *H. niger* was significantly thicker than that of the other two species (Fig. 3a). Most of the cortical cells have starch-storage function and some of them contain blue-stained substances. Regarding the third tissue type of the root, which is the stele with the vascular bundles, significant histological differences can be observed. *H. niger* and *H. odorus* have four bundles each, but the xylem bundles are separated from each other in *H. niger* and the central part of the stele encloses a small area of pith, while they are fused in *H. odorus*. In the case of *H. purpurascens* the stele includes three fused xylem bundles, which separate the three phloem bundles. The thickness of the stele and the whole root from the region of maturation differed significantly among the species (Fig. 3b, c).

Table 1 Ethnobotanical data of *Helleborus purpurascens* in Transylvania compared with earlier records in the region and those of other hellebore species in Europe

	Ethnobotanical records of *H. purpurascens* in the study area	Earlier data of *H. purpurascens* in Transylvania	Data of hellebores in other European countries
Local name	Cinod, Eghersec: *eszpenz* Lueta: *papvirág* Lunca de Sus: *keserűgyökér, eszpenz*	Moldova: *eszpenc, eszponz, vadeszponc* (Halászné 1987; Halász 2010) Ciuc: *paponya* (Kóczián et al. 1979) Covasna County: *papmonya* (Rácz and Füzi 1973) Harghita County: *paffangyökér, papszoknya, pafmangyükér, papmanvirág, tyúkbolondító* (Kóczián et al. 1979) Ghimeş: *ecenc, ecenz, eszpenc, eszpendz,* (Kóczián et al. 1975, 1976; Kóczián et al 1979), *eszpenz* (Babai and Molnár 2009; Babai 2013; Babai et al. 2014), *ëszpënz, ëszpëndz, paponya* (Rab et al. 1981), *szégyenburján* (Rácz 2010) Maramureş: *kakukkvirág, penza, pünza, spinz* (Borza 1968) Ţara Călatei: *kígyófű, kígyóhagyma, papkalap, paptöki, kokasmondikó, spinz* (Kóczián et al. 1979), *fagyükerü paptöke, paptöke, forasztófű, kígyóufű, kígyófűgyükér, kígyófűgyökér, paptök, ördögfű, ökörszem* (Péntek and Szabó 1985), *paptöki, kokasmondió* (Kóczián et al. 1979; Kóczián 2014) Homorod: *paponya, papmonya, paponyagyökér* (Gub 1993, 2000) Caşin: *kakukkvirág, hunyó, kigyófü, papkalap, papmonya, paponya, prüszentőgyökér, fehér zászja, lóhunyrózsa* (Borza 1968) Gheorgheni: *paponya* (Rab 2001)	*H. dumetorum: farkasgyökér, humorgyökér, hunyadi gyökér, tálégyökér, tályagyökér, tályoggyökér*/HU (Kóczián et al. 1979; Kóczián 2014) *H. foetidus*: *ararechie*/IT (Scherrer et al. 2005); *erba draguna, scuàssu, lusèrciu o liserciu, scumpìscia còu o cài*/IT (Cornara et al. 2014) *H. niger: hóvirág*/HU (Kóczián 2014), *papmonya*/RO (Papp, unpublished) *H. odorus: elleboro*/IT, *kuturiak*/BU (Leporatti and Ivancheva 2003), *ökörérőfű, ökörérő gyökér*/HU (Kóczián et al. 1979) *H. purpurascens: fehér zászpa, kecskerózsa, kecskerózsagyökér, lóhunyrózsa*/HU (Kóczián et al. 1979, Kóczián 2014) *H. viridis: rosa di natale, erba dragùna*/IT (Cornara et al. 2014) *Helleborus* spp.: *kukuriek*/SE (Pieroni et al. 2011), *kukurek*/MA (Pieroni et al. 2013)

Table 1 continued

	Ethnobotanical records of *H. purpurascens* in the study area	Earlier data of *H. purpurascens* in Transylvania	Data of hellebores in other European countries
Use in ethnoveterinary medicine	Cinod, Eghersec: root as panacea Lueta: root pulled into the nose or ears of pigs against vomiting, or boiled root or flower for purulent wounds on the legs as a foment Lunca de Sus: roots pull into the breast of cows as immunotherapy	Moldova: 2–3 roots bound with thread made of hemp pulled into the breast or flank of cows (Halászné 1981, 1987; Halász 2010), root pulled into the ears of pigs as anthelmintic drug, leaf onto the wound of pigs (Halász 2010) Ghimeş: root for weak immune system, stomach disorders, pulled into the breast of horses, the neck of cows, and the ears of pigs for fever as stimulant therapy (Melius 1578; Kóczián et al. 1976; Kóczián et al 1979; Rab et al. 1981; Bogdan et al. 1990; Babai 2013), decoction of the root for wound, leaf as repellent (Rácz 2010) Homorod: root pulled into the abdomen of cows (Gub 1993), and the ears of pigs for fever, for scab of horses as a decoction (Gub 1993, 2000) Covasna County: root pulled into the ears of pigs and sheep or the breast of cows as prevention against infections (Rácz and Füzi 1973) Ţara Călatei: root pulled into the ears of pigs, the breast of cows and horses, into the throat of chicken with bread against epidemics (Péntek and Szabó 1985) Gheorgheni: root pulled into the ears of pigs for fever and cough (Rab 2001)	*H. bocconei*: root for the diagnosis of pneumonia/IT (Lentini 2000); root for pigs and sheep/IT (Guarrera 1990; Padula 1878), nervous system/IT (Leporatti and Pavesi 1989) and respiratory disorders/IT (Raimondo and Lentini 1990; Lentini and Raimondo 1990) *H. dumetorum:* root pulled into the breast of horses as stimulant therapy/HU (Kóczián 2014) *H. foetidus*: root as abortive, antiodontalgic to remove black teeth, pulled into the ear for infection of pigs/IT (Scherrer et al. 2005); leaf sap for infection of pigs as antiseptic/IT (Pieroni 2000); decoction of root as astringent for calves and disinfection of mammary glands, dried root inserted subcutaneously for various disorders of pigs and cows (Cornara et al. 2014), for cattle, dogs, geese, pigs, and sheep/IT (Bertagnon 1955; Viegi et al. 1999); wounds and skin diseases/IT (D'Andrea 1982); nervous system disorders/IT (Leporatti and Pavesi 1989; Viegi et al. 2003) *H. niger*: root as panacea/RO (Papp, unpublished) *H. odorus*: root inserted under the skin of cows, pig ears and sheep as antipyretic and antibacterial drug for pneumonia/IT (Cornara et al. 2009; Manganelli et al. 2001); fresh leaf for purulent abscess of bovine/IT (Manganelli et al. 2001); gastrointestinal disorders/IT (Bandini 1961), skin diseases/IT (Camangi and Manganelli 1999) *H. purpurascens:* root soaked in vinegar (Sadler 1824) or as dried form pulled into the ears of pigs for erysipelas/RO, IT (Bogdan et al. 1990; Pieroni et al. 2004), and the neck and the breast of cows for fever/HU (Kóczián 2014), as anthelmintic drug/RO (Butura 1979) *H. viridis*: root for cough, as antipyretic and anti-convulsive inserted into the tail of sheep and bovines, behind the ears of sheep for epilepsy caused by cranial oedema; decoction of the leaf for cough and cold, fresh leaf under the tail of sheep to facilitate pregnancy/IT (Manganelli et al. 2001); respiratory (Gastaldo et al. 1978; Corsi et al. 1981) and skin diseases of cattle and sheep/IT (Camangi and Manganelli 1999); root inserted under the tail of sheep for fever/IT (Cornara et al., 2014); nervous system disorders of sheep/IT (Corsi and Pagni 1978) *Helleborus* spp.: root inserted into the breast for muscular blocks, or into the nose for nasal congestion of horses/MA (Pieroni et al. 2013); root inserted in the ear wound of pigs and sheep, for diarrhoea/SE (Pieroni et al. 2011)

Table 1 continued

	Ethnobotanical records of *H. purpurascens* in the study area	Earlier data of *H. purpurascens* in Transylvania	Data of hellebores in other European countries
Use and misuse in human ethnomedicine	no data	Moldova: leaves put onto the legs for rheumatism (Halászné 1987) Ghimeş: root in alcohol as expectorant drug and for stomach disorders (Kóczián et al. 1975), soldiers put the root into the cut wound of the legs to get off from wars (Kóczián 2014) Homorod: soldiers put the root onto cut wound to get off from wars (Gub 1993) Ţara Călatei: dried and ground root in alcohol for jaundice; abortive; soldiers put the root into the cut wound of the fingers to get off from wars (Péntek and Szabó 1985; Kóczián 2014) Maramureş: soldiers put the root into the cut wound of the legs to get off from wars (Kóczián 2014)	*H. bocconei*: decoction of the root for toothache/IT (Barone 1963; Leporatti and Pavesi 1989) *H. foetidus*: root chewed on painful tooth, and mashed root to expel the inflamed black teeth/IT (Leporatti and Pavesi 1989); root as abortive (no actual use), leaf sap for warts and as repellent/IT (Pieroni 2000); sap of ground aerial part for viper bites/IT (Cornara et al. 2014) *H. niger*: soldiers put slices of rhizome on wound of the fingers injured by themselves to cause strong inflammation eventually rigidity of the joints avoiding actual military service/CR (Magyary-Kossa 1926) *H. odorus*: decoction of the root as cardiotonic and anaesthetic drug; no actual use/IT (Leporatti and Ivancheva 2003) *H. purpurascens*: root as abortive/HU (Kóczián 2014) *H. viridis*: leaf for skin inflammation and piles/IT (Pieroni 2000)

BU, Bulgaria; CR, Croatia; HU, Hungary; IT, Italy; MA, Macedonia; RO, Romania; SE, Serbia

The diameter of the round shaped stem of *H. odorus* (2.43 ± 0.086 mm) was significantly lower than that of *H. niger* (3.71 ± 0.055 mm) and *H. purpurascens* (3.61 ± 0.045 mm) (Fig. 2d–f). Under the single layered epidermis there were 3–5 layers of collenchyma in the stems of the samples studied. The cortical region is relatively narrow, particularly in *H. odorus* (0.43 ± 0.038 mm), while this tissue is significantly thicker in *H. niger* (1.26 ± 0.024 mm). The vascular zone of all species includes collateral bundles in different sizes. Thick walled sclerenchymatous bundle cap covers the phloem in *H. odorus*. The ring of vascular bundles encloses wide pith.

The dorsiventral leaves of each species consist of a single layer of adaxial and abaxial epidermis, a mesophyll tissue with one layered palisade parenchyma and 6–8 cell layers of spongy parenchyma (Fig. 2g–i). Adaxial epidermal cells of *H. niger* are about twice as thick (65.7 ± 1.5 μm) as the abaxial ones (29.3 ± 1.9 μm), while these layers in *H. purpurascens* are similar in their thickness (adaxial and abaxial layers are 37.6 ± 1.9 μm and

Fig. 1 *Helleborus purpurascens* in "home pharmacy" in Cinod in 2007. **a** Dried plant *in toto*, **b** root as "home drug"

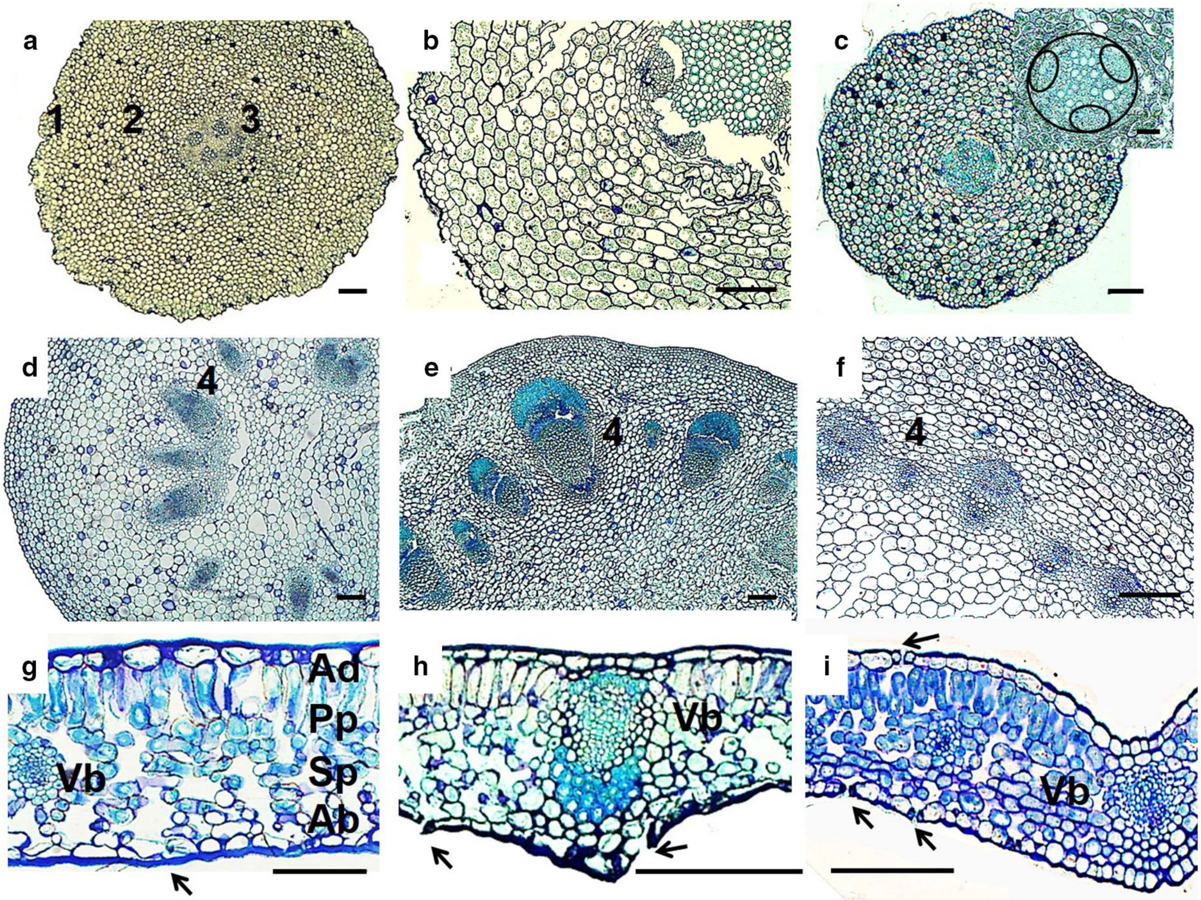

Fig. 2 Transections of the vegetative parts of *Helleborus* species. Root, shoot and leaf, respectively **a**, **d**, **g** *H. niger*, **b**, **e**, **h** *H. odorus*, **c**, **f**, **i** *H. purpurascens*; the inset shows the structure of the stele (indicated by the circle), with three phloem bundles (indicated by ovals) and between them the fused xylem bundles. (1) epidermis, (2) cortex, (3) stele with simple vascular bundles in the root, (4) collateral vascular bundles in the stem. Ad-adaxial epidermis, Pp-palisade parenchima, Sp-spongy parenchyma, Ab-abaxial epidermis, Vb-vascular bundle. Arrows indicate stomata. (Scale bar: 200 µm, scale bar of inset: 100 µm)

38.5 ± 3.3 µm, respectively). In the case of *H. odorus* the thickness of the upper and lower epidermal layers is 56.1 ± 2.8 µm and 36.5 ± 2.1 µm, respectively. The shape of most epidermal cells of *H. purpurascens* is isodiametric, while that of the other two species is flat. The location of stomata seems to be another distinctive character among the species. Leaves of *H. niger* and *H. odorus* are hypostomatic with stomata on the abaxial side, while leaves of *H. purpurascens* are amphistomatic with stomata on both leaf surfaces. The sub-stomatal cavities are larger in *H. niger* than in the other two species, resulting in a looser spongy layer. The stomata are mesomorphic, they can be found at the level of epidermal cells. We have found significant differences in the thickness of leaf blades of the species, but the ratio of palisade parenchyma and spongy tissue was similar in the samples (Fig. 4a–c). The isodiametric cells of spongy parenchyma filled more than half part of the mesophyll. There are two layers of parenchymatous cells forming a bundle sheath around the main vascular bundle. The single layer of sclerenchyma fibres above the xylem and the thick sclerenchymatous bundle cap at the phloem are clearly visible in *H. odorus* (Fig. 2h).

The petal-like sepal is isolateral homogenous (Fig. 5a), with a thickness ranging from 200 to 400 µm. The sepal is covered by a single layer of isodiametric, papillate epidermal cells on both the abaxial and adaxial side. Mesomorphic stomata are typically located on the abaxial side. The mesophyll

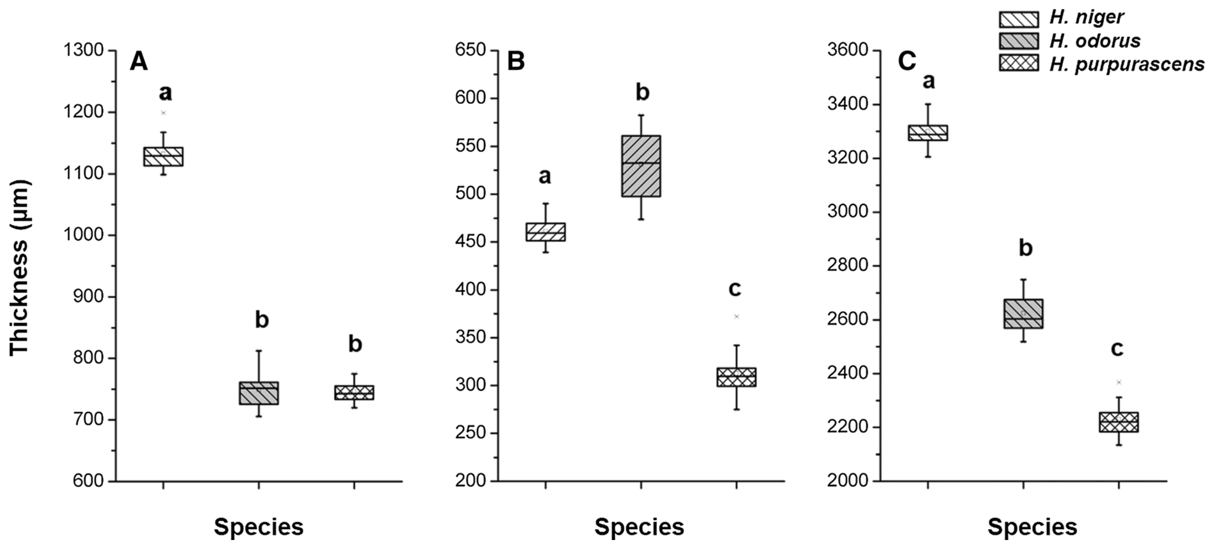

Fig. 3 Thickness parameters in root of *Helleborus* species. **a** Cortex, **b** stele, **c** root. Each data point is the mean ± SD (n = 20). Different letters above the boxplots indicate significant differences among the given tissue thicknesses of the species ($p < 0.05$)

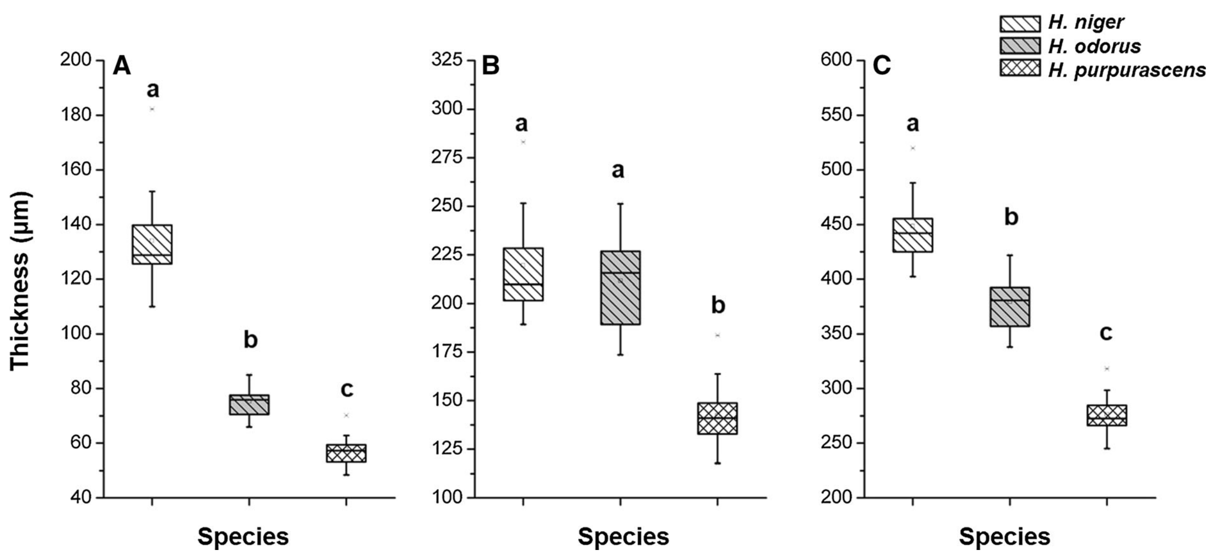

Fig. 4 Thickness parameters in leaves of *Helleborus* species. **a** Palisade parenchyma, **b** spongy parenchyma, **c** leaf blade. Each data point is the mean ± SD (n = 20). Different letters above the boxplots indicate significant differences among the given tissue thicknesses of the species ($p < 0.05$)

comprises 3–8 cell layers, with spongy parenchyma, intercellular cavities and collateral closed bundles.

The shape of the modified petal is tubular in *H. niger* and *H. odorus*, while it is fork-shaped in *H. purpurascens* (Fig. 5b). In the upper (distal, non-secretory) part the thickness of the petal ranges from 150 to 200 μm with 2–3 parenchyma cell layers, 80 to 100 μm with 1–2 cell layers, and 100 to 200 μm with 3–4 cell layers in *H. niger*, *H. odorus* and *H. purpurascens*, respectively. The epidermal cells in this petal-like region are papillate in *H. niger* (Fig. 5c), while in *H. odorus* and *H. purpurascens* papillae are not characteristic. Stomata cannot be observed either in the upper part or the lower (proximal, secretory) part of the modified petal. The

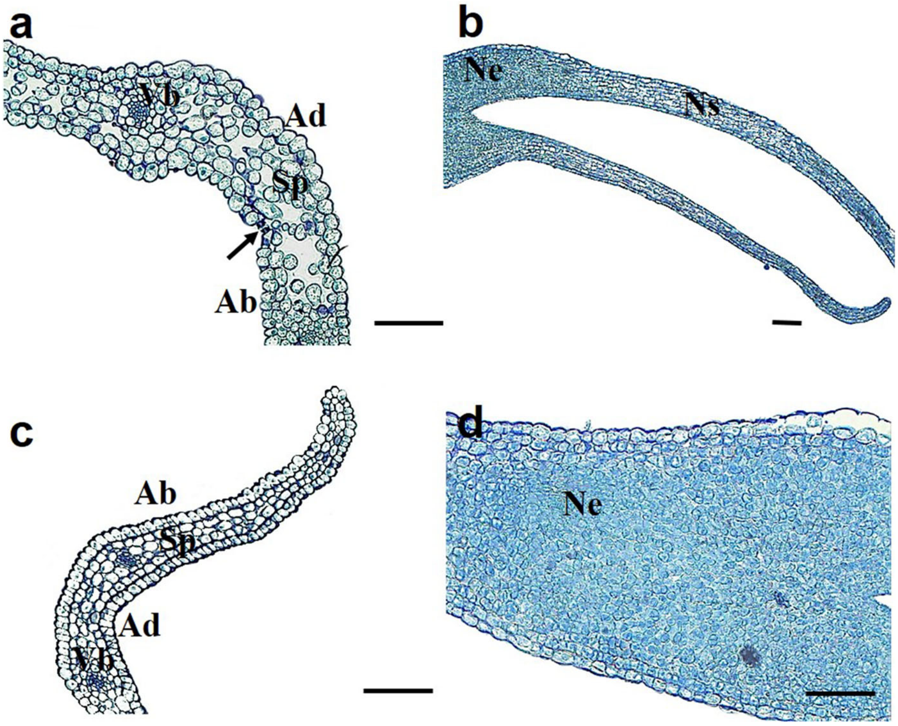

Fig. 5 Perianth of the studied *Helleborus* species. **a** Transection of sepal in *H. niger*, **b** longitudinal section of modified petal in *H. purpurascens*, **c** non-secretory part of the petal in *H. niger*, **d** nectar-producing part of the petal in *H. purpurascens*. Ad-adaxial epidermis, Sp-spongy parenchyma, Ab-abaxial epidermis, Vb-vascular bundle, Ne-nectar producing part, Ns-non-secretory part. Arrow indicates stoma. (Scale bar: 200 µm)

cells of the nectar-producing parenchyma are small, isodiametric (Fig. 5d).

Transverse sections of the androeceum revealed that the filament comprises a single epidermal layer, followed by 3–4 layers of parenchyma and a single vascular bundle (Fig. 6a). The anther wall consists of exothecium, fibrous layer, parenchyma, and tapetum (Fig. 6b). The tricolpate pollen grains of all three species can be classified into the medium-sized (26–50 µm) category. However, the structure of the exine is different in each species: in *H. niger* scabrate (Fig. 6d), in *H. odorus* reticulate (Fig. 6e), whereas in *H. purpurascens* microechinate ornamentation (Fig. 6f) can be observed.

The vascular elements of the anther, filament, and ovary are arranged in collateral closed bundles. The ovary is in superior position (Fig. 6c).

Discussion

Data on the medicinal use of hellebores have been recorded from the Ancient Times until our days, *H. niger* and *H. viridis* having been included as official drugs in earlier pharmacopoeias, such as the 2nd to 6th editions of Austrian Pharmacopoeas, and the supplement of 6th edition of the German Pharmacopoeia (Ergänzungsbuch zum Deutschen Arzneibuch 1941). Many ethnobotanical reports describe the strong effect of hellebores, e.g. by calling attention to the importance of washing hands after application of the root (Gub 2000), or animals which avoid hay including e.g. *H. purpurascens* in grasslands in Romania (Babai and Molnár 2009).

In Transylvanian ethnoveterinary medicine, local use of *H. purpurascens* root was documented in immunotherapy, as in earlier works from Romania (Sadler 1824; Bogdan et al. 1990), Italy (Pieroni et al. 2004), and Hungary (Kóczián 2014). Similar use of *H. odorus* was documented in Italy (Cornara et al. 2009; Manganelli et al. 2001), and that of *H. dumetorum*

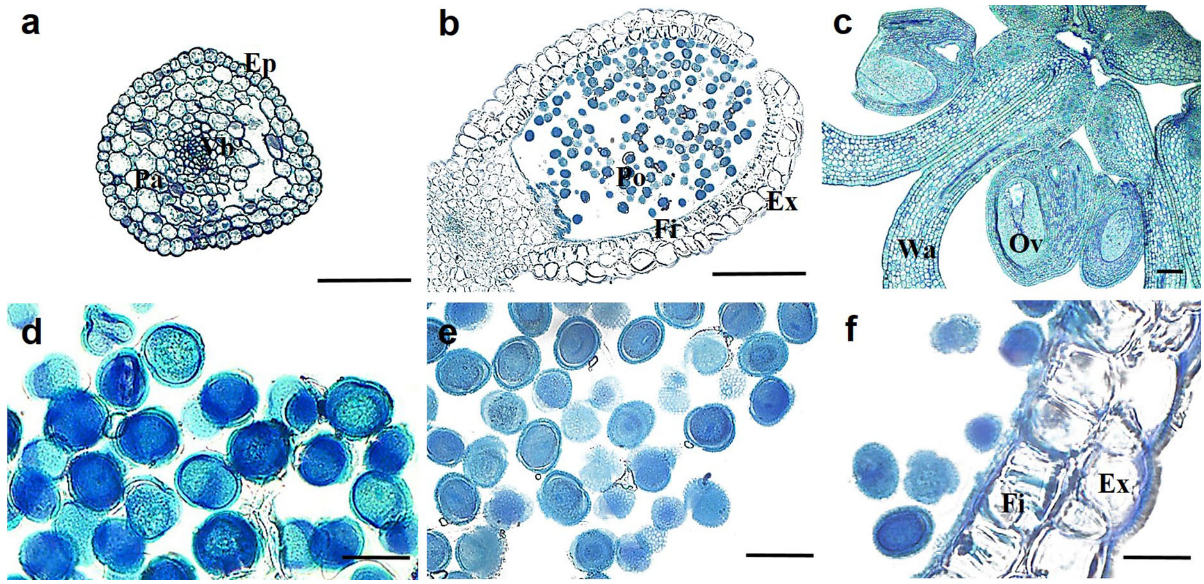

Fig. 6 Androeceum, gynoeceum and pollen grains of the studied *Helleborus* species. **a** Transection of filament in *H. niger*, **b** anther in *H. odorus*, **c** ovary in *H. niger*, tricolpate pollen grains of **d** *H. niger* with scabrate ornamentation, **e** *H. odorus* with reticulate ornamentation, **f** *H. purpurascens* with microechinate ornamentation. Ep-epidermis, Pa-parenchyma, Vb-vascular bundle, Ex-exothecium, Fi-fibrous layer, Po-pollen grain, Wa-wall of ovary, Ov-Ovule (Scale bar **a**–**c**: 200 μm; **d**–**f**: 30 μm)

Table 2 Distinctive histological characters among the studied species

	H. niger	*H. odorus*	*H. purpurascens*
Root	Four separated xylem bundles	Four conjugated xylem bundles	Three conjugated xylem bundles
Stem	Parenchymatous bundle sheath	Sclerenchymatous bundle cap	Parenchymatous bundle sheath
Leaf	Flat epidermal cells	Flat epidermal cells	Flat, round epidermal cells
	Hypostomatic	Hypostomatic	Amphistomatic
	Parenchymatous bundle sheath	Sclerenchymatous bundle cap	Parenchymatous bundle sheath
Petal nectary	Tubular	Tubular	Fork-shaped
	Distal part: papillate epidermis	Distal epidermis: no papillae	Distal epidermis: no papillae
Pollen	Scabrate ornamentation	Reticulate ornamentation	Microechinate ornamentation

Waldst. et Kit. in Hungary (Kóczián 2014). For the same purpose, a similar treatment applying the root of *Adonis vernalis* and *A. transsylvanica* Simonov (Romania), and that of *A. mongolica* Simonov (Mongolia) was described earlier (Kóczián et al. 1979).

For wounds and skin diseases, we recorded the use of the flower of *H. purpurascens* in Transylvania, as opposed to applying the root of *H. foetidus* (D'Andrea 1982), *H. odorus* and *H. viridis* in Italy (Camangi and Manganelli 1999).

In our study, *H. purpurascens* was not recorded in traditional human medicine opposite to earlier data recorded in Transylvania (Kóczián et al. 1975; Péntek and Szabó 1985; Halászné 1987; Gub 1993; Kóczián 2014), and Hungary (Kóczián 2014). In addition, the traditional use of other *Helleborus* species, such as *H. bocconei* Ten., *H. foetidus, H. odorus* and *H. viridis* was mentioned in the treatment of various human disorders in Italy (Barone 1963; Leporatti and Pavesi 1989; Pieroni 2000; Leporatti and Ivancheva 2003; Cornara et al. 2014).

Based on the historical and ethnobotanical data presented in the previous sections, our histological work focused on plant parts that have been applied in traditional remedies, i.e. the root, leaf, and flower. Our detailed microscopic studies of the ethnomedicinally important plant parts revealed distinctive taxonomic characters among the species. Presumably we were the first who observed different number and type of vascular bundles in the stele of these species, because other studies focused on mature root transections with fused vascular bundles or they studied powdered root (Kumar and Lalitha 2017). In the studied three hellebore species, the structure of the other vegetative plant parts, i.e. the stem and leaf, as well as that of the flowers, fits well with the general description of the *Helleborus* genus (Tamura 1993; Šušek 2008; Barkyna and Churikova 2014; Rottensteiner 2016).

The internal structure of the stem gave the picture of a typical dicotyledonous, herbaceous stem, where the vascular bundles are organized in a ring inside the pericycle (Haraszty 1990). We observed sclerenchymatous bundle cap mainly outside the phloem both in the stem and leaf of *H. odorus*, which physically protects the inner tissues of these plant parts. This type of tissue as bundle cap is widely distributed in dicotyledonous plants and it is common as bundle sheath in monocotyledonous plants (Jarvis and His 2007).

The distinctive characters of leaf anatomy obtained in our comparative study may reflect environmental effects. The shape of epidermal cells can vary to have greater efficiency in light uptake (Martin et al. 1989). Lens shaped cells—which characterize the epidermal cells of *H. purpurascens*—have the ability to focus the light under the surface, while the flat shaped ones—characteristic of *H. niger* and *H. odorus* epidermal cells—may optimize the stronger sunlight distribution (Tholen et al. 2012).

The anatomical plasticity of the leaf can be also manifested in quantitative changes of inner tissues, e.g. longer palisade cells due to sunlight acclimation of grapevine cultivars (Kocsis et al. 2017). In our case the ratio of palisade and spongy mesophyll of the studied species was very similar to that of hellebore species from different eco-geographical origin studied by Barkyna and Churikova (2014).

The occurrence of stomata only on the abaxial surface (hypostomatic leaves) or on both leaf surfaces (amphistomatic leaves) associates strongly with the environment (Richardson et al. 2017). Stomata of the leaves of *H. odorus* and *H. niger* were found on the abaxial surface and were situated more or less at the same level with other epidermal cells. Hypostomatic leaves characterise most mesophytic plants in temperate climate with adequate supply of water (Haraszty 1990). Plants living in full-sunlight and experiencing rapidly fluctuating or continuously available soil water, are usually known to be amphystomatic (Mott et al. 1982; Richardson et al. 2017). Amphystomy, which characterized the leaves of *H. purpurascens*, provides an adaptive advantage of higher conductance of CO_2 for photosynthesis. The distinctive anatomical characters of the leaves may reflect the different environmental conditions of *H. purpurascens* from those of the other two species.

The structure of the modified petal and the anther was similar in each studied *Helleborus* species, comprising the same tissue types. In all three species, the petal-like sepal was hypostomatic with mesomorphic stomata, and vascular elements were arranged in closed collateral bundles. The significance of stomata in the sepals of *H. niger* was highlighted by Salopek-Sondi (2011), who reported that they play an important role in photosynthesis following the fertilisation of the flowers, when the perianth becomes green and photosynthetically active.

The three species exhibited minor morphological differences, such as the shape of the modified petal, being tubular in *H. niger* and *H. odorus* and fork-shaped in *H. purpurascens*. Similarly, the nectary of *H. foetidus* was described as pitcher-shaped (Koteyeva 2005), and the modified petal of *H. niger* was characterised as tubular by Erbar (2007). However, Šušek (2008) reported a larger degree of variability in the latter species, regarding the shape of the modified petal, which may vary from flat, through flat with curved margin, to tubular or funnel-shaped. We observed no stomata in the epidermis of the modified petal, either in the secretory or the non-secretory region. This is in accordance with the findings of Koteyeva (2005), who reported that stomata were absent from the inner petal surface of *H. foetidus* and *H. caucasicus* A. Braun. In the lack of secreting structures like nectary stomata, nectar was suggested to be released through cuticular channels, which are perpendicular to the surface of the nectary, some of them opening directly into the nectar cavities below the epidermis (Koteyeva 2005); or by the rupture of the cuticle and underlying cell wall (Vesprini et al. 1999, 2008, 2012).

Conclusions

Hellebore species have been used in ethnomedicine for a variety of ailments since ancient times. Based on earlier records, new ethnobotanical collections can be initiated, and data of traditional use can be compared with official records, which may serve as the basis of further analyses of some species of the genus. Our histological analysis of three *Helleborus* species revealed distinctive anatomical features, including qualitative traits such as the structure of the stele in the root, the bundle sheaths in the stem and leaves, position of stomata in foliage leaves, nectary shape or presence of papillae on petal epidermis, and ornamentation of pollen grains. Quantitative analysis provided distinctive characters in thickness of various tissue types both in the vegetative and reproductive organs.

Our findings highlight the fact that although root, shoot, leaf and flower structure is essentially the same within the genus, the morphology and anatomy of these plant parts may vary from species to species.

Acknowledgements Open access funding provided by University of Pécs (PTE). This work was supported by a Grant from the OTKA (Hungarian Scientific Research Fund, K 127944), and the Research Grant of the University of Pécs (PTE ÁOK KA-2017-27). We are grateful for the contributions of the study informants, and for Norbert Mag for the correction of microphotos.

Authors' contributions NP, ÁF and TA conducted the fieldwork. VLB, MK, SS and NP completed the histological preparations, RF and MK the statistical analyses. TA performed the systematic analyses of historical data, while NP, ÁF, MK and SS the comparative literature analysis. All authors participated in the writing and revision process and read, discussed and approved the final manuscript.

Compliance with ethical standards

Conflict of interests The author(s) declare that they have no competing interests.

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