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SADRŽAJ:

Allelopathic potential of *Helleborus dumetorum* and its prospects for conservation with gardening

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Abstract

To tackle global biodiversity loss, we will need to sustain biodiversity beyond protected areas. Here we present a case study of a rare and threatened native species *Helleborus dumetorum*. First, we assessed by laboratory experiments if *H. dumetorum* has a competitive advantage over weeds because of its allelopathic potential; secondly, we reviewed the literature to discuss if this threatened species could be conserved as an ornamental species for gardening while at the same time its cultivation would benefit local biodiversity. Using bioassays, we tested the allelopathic potential of aqueous extracts of *H. dumetorum* leaves and of soil samples, collected at or away from *H. dumetorum* plants, on garden cress *Lepidium sativum* germination and seedling development. The results confirmed the presence of *H. dumetorum* aqueous allelochemicals in leaves as well as allelopathic effects of the soil where *H. dumetorum* was growing, supporting the competitive advantage of *H. dumetorum*. Literature review combined with our observations in the field indicated more characteristics of *H. dumetorum* suitability for ornamental purposes and species' conservational opportunities: easy and successful transplanting, survival in various habitats, no invasive potential, flowering in early spring, long-lived leaves. Our study shows, that considering threatened native plants for ornamentals could have a multitude of benefits for local biodiversity.

Key words: allelopathy, rare plants, Ranunculaceae, green hellebore, conservation.

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Introduction

Biodiversity is mostly threatened by habitat destruction/deterioration and non-native species introductions, often in combination with climate change. The global Aichi Biodiversity Targets, which aimed to hold back the biodiversity decline, were not successful by the 2020 deadline. To tackle biodiversity loss post-2020, the EU prepared the Biodiversity Strategy 2030 (EC 2020), stressing that existing protected areas and expanding new ones will not be enough to stop biodiversity decline. Therefore, we are becoming increasingly aware that we need to sustain biodiversity outside protected areas. This means including biodiversity in agricultural land and urban areas as well. Therefore, gardening with native plants might represent a successful way for experts and hobbyists alike to support wildlife. Ideally, we will need to identify rare species, which would thrive in an urban environment while at the same time they would benefit other native species. Rare plants and their mutualistic pollinators can represent such models.

Here we present a case study of a rare and threatened native species, which might find refuge in an urban environment as an ornamental species - *Helleborus dumetorum* Waldst. & Kit. According to Rottensteiner (2016), our study species can be further determined as *H. dumetorum* subsp. *dumetorum* because of the distribution area ranging over Southern Austria, Eastern Slovenia, Eastern Croatia, and Western Hungary, as well as Southwestern, Southern, and Eastern Romania. This is a threatened species and is protected in most distribution areas, e.g. in Hungary (Bíró et al., 2014) and Slovenia (Martinčič et al., 2007). In Slovenia, *H. dumetorum* is one of five species of the genus *Helleborus* L. occurring naturally (Bavcon et al. 2012), whereby its distribution area is in the northeastern part and is less widespread than similar *H. odorus* Waldst. & Kit. It belongs to section *Helleborastrum* Spach: Mathew (1989) and does not form hybrids, because its DNA contents are different from the rest of the section (Zonneveld, 2001). However, recently several sources report the occurrence of various hybrids with e.g. *H. odorus* (Bavcon, 2014; Rottensteiner, 2016). There are several synonyms in use, because of the difficult *Helleborus* taxonomy: *Helleborus apllidus* Host, *Helleborus viridis* L. subsp. *dumetorum* (Waldst. et Kit.) Hayek (Nikolić, 2022).

Introducing *Helleborus* species into cultivation is not new. *Helleborus* species and their hybrids are ornamentals with increasing economic importance for gardens, potted plants, and cut flowers (Dhooghe et al., 2018; Bavcon et al., 2012; Šušek, 2021). Primarily *Helleborus* breeding is focused on flowers. However, advanced breeding of *Helleborus* resulted in shortened plant age until the first flowering, flowering period starting earlier, prolonged flower longevity, shorter or longer flower peduncles, increased resistance to pests, and more (Dhooghe et al., 2018). What is more, hybrids obtained by interploidy and interspecific crosses are increasing their share among commercially important *Helleborus* plants (Dhooghe et al., 2009; Dhooghe et al., 2018). This means that bred plants might not support local diversity to such an extent as native ones. For example, flowers might not sustain pollinators because of changed flower color and odor, because sepals do not turn green after pollination, or because they are not nodding flowers, which are unwanted by breeders, etc. However, by recognizing the fondness of gardeners for *Helleborus* plants, we could grow native unbred *Helleborus* species for simultaneous ornamental and conservational purposes. Nevertheless, gardeners have some expectations about garden plants. They usually select them for large, colorful flowers, for interesting or long-lasting leaves, or for their resistance to pests and diseases. Even though *H. dumetorum* has green, inconspicuous flowers, leaves are dark green, relatively large, and rarely attacked by insect herbivores. Evergreen *Helleborus* species showed that they contain various compounds exhibiting antimicrobial or cytotoxic activity (Čakar et al., 2014; Iguchi et al., 2020). *Helleborus dumetorum* is a toxic plant, and we expected that it might also have an allelopathic potential, which makes the plant more competitive against other co-existing plants.

The aims of the study were a) to assess the potential competitive advantage of *H. dumetorum* to weeds and b) to present a reflection if *H. dumetorum* could be therefore used as a low-maintenance outdoor garden plant, while at the same time cultivation would benefit its conservation. For the first aim, we explored the competitive advantage of *H. dumetorum* allelopathic potential by performing laboratory bioassays. Further on, we assessed the conservation prospects of *H. dumetorum* in ornamental plantings and the expected benefits for biodiversity by reviewing literature combined with our own observations in the field.

Materials and methods

Study species

Helleborus dumetorum (Ranunculaceae) is a long-lived herbaceous perennial (Fig. 1), 20 – 40 cm in height at flowering time (March – April), and the mature foliage consists of usually two basal leaves, divided into 5–7 main segments, with the outer two again divided giving a total of up to about 7–13 divisions. Bracts subtending the branches and flowers vary in size and form. *Helleborus dumetorum* starts to bloom from February to early March. The species is protogynous and the main flowering period lies between March and April. The few-flowered inflorescences include 2–4 flowers on slender pedicels attached to long stalks. Flowers are green, 2.5 to 3.5 cm in diameter (Bavcon 2014). Slightly overlapping perianth segments (sepals) persist through into the fruiting stage. The flower elements equivalent to petals consist of 9–12 short, funnel-shaped green nectaries. The nectar of *H. dumetorum* is completely hidden and the main pollinators are Hymenoptera, typically bumblebees and bees, as well as some Diptera. The fruit is 3–4 several-seeded green follicles about 1.5–1.8 cm long at maturity, united at the base and dehiscent (Mathew, 1989). The leaves of flowering stalks are green, while the basal rosette leaves from non-flowering plants are dark purplish in their appearance and turn dark green with time. Additional leaves develop gradually and when the plant blooms, new, more divided leaves emerge. The first leaves are markedly basal while later leaves are larger and more divided (Bavcon, 2014).

In Slovenia, *H. dumetorum* grows on grassy slopes, between shrubs, or under hedges (Martinčič et al., 2007). It is mainly distributed in the sub-Pannonian area (NE Slovenia), with the exceptions of the Pohorje hills, the river Kolpa valley, and the region Bela Krajina (Martinčič et al., 2007). *Helleborus dumetorum* is protected in Slovenia. The distribution area near Maribor is the sub-Illyrian northern edge of the Illyrian deciduous forest, represented also by *Lamium orvala* L. and *Erythronium dens-canis* L. besides *H. dumetorum*.

Figure 1. Flowering specimen of Helleborus dumetorum Waldst. & Kit. in its native habitat. (Photo: N. Šajna)

Allelopathy bioassays

At the time of flowering in May 2022, leaves of 20 random *H. dumetorum* plants were collected from one large native population near Maribor (Kamnica village) located in the east-oriented, steep, regularly mown meadow. Fresh plant material was finely chopped by hand. Extracts – aqueous leachates were prepared by grinding cut plant material with a pestle and mixing it with deionized water in concentrations of 1 g/100 mL, 5 g/100 mL in 10 g/100mL for 15 min, followed by filtering through filter paper (Whatman®, grade 1). Germination tests were performed in Petri dishes (ø 8.5 cm) on two layers of filter paper moistened with 20 mL of aqueous extracts (treatments) or deionized water (control).

Soil samples were collected: a) directly where *H. dumetorum* was growing, and b) at a distance of 1 m away from a *H. dumetorum* plant on the 22nd of May 2022. Soil samples were sieved, and any stone pieces and roots were removed. For germination studies, 40 g of soil was gently pressed into Petri dishes (ø 8.5 cm), watered with 20 mL of deionized water, and covered with two layers of filter paper.

For both, plant extracts (water leachates) and soil samples, we used seeds of garden cress *Lepidium sativum* L. (Garden Elite seeds, Italy), sold commercially, as the model plant for the bioassays. The seeds were not sterilized and did not undergo any treatment before the germination tests. For each replicate, we used 20 seeds. We performed 5 replicates for treatments with aqueous extracts and 5 replicates for control with deionized H_2O. For soil samples, we performed 10 replicates for each type of soil sample (at the plant, away from the plant). Petri dishes were partially sealed with Parafilm® M (Bemis Company, Inc.) to minimize water evaporation and drying of the filter paper while still enabling aeration. *Lepidium sativum* seed germination and seedling development, especially radicle elongation, are good indicators of plant growth inhibition, owing to the uniformity of growth and overall sensitivity (Gehringer et al., 2003, Šajna 2017). Other advantages include fast germination and seedling development, which enable test plants to respond to allelochemicals before these compounds degrade. Therefore, the seeds were checked, germination rate [G %] was assessed and seedlings and their roots were measured on the 4th day. We considered a seed as germinated when we could observe a protruding root of at least 2 mm. Germination experiments for all treatments were performed in a growing tent with blue-red growing LED light with 10 h/ 14h day/night periods at a room temperature of 22 °C.

To test for significant differences among seedling lengths from different concentrations, we applied the non-parametric Kruskal-Wallis analysis of variance (ANOVA) because the assumption of homogeneity of variances was not met (Levene’s test at $p=0.05$). We analyzed inhibition effects by calculating the response index (RI) proposed by Williamson and Richardson (1988). Seedling length for each treatment (lt) and seedling length of the control (lc) were used to calculate RI = (lt/lc) - 1, if lt< lc, and RI = 1 – (lc/lt) otherwise. For soil samples, seedling and root length were compared with two sample Kolmogorov-Smirnov test and the Mann-Whitney test since the assumption of normality was not met (Shapiro-Wilk's test at $p<0.05$).

Results and discussion

The allelopathic potential of *Helleborus dumetorum* leaves

Aqueous extracts of *H. dumetorum* leaves did show allelopathic potential. Aqueous extracts reduced the germination rate [G %] of *Lepidium sativum* seeds irrespectively of the concentration. The mean G % for control was 60 %, while the average G % for all treatments was around 40 % (41 %, 42 %, and 44 % for concentrations 1, 5, and 10 g/100 mL, respectively). This means that the germination rate was around 30 % lower than the control values.

Further, extract concentrations tested inhibited significantly the *L. sativum* seedling length (Kruskal-Wallis ANOVA, $H(4,400) = 10.378$, $p=0.016$) and the seedling root length (Kruskal-Wallis ANOVA $H(4,400) = 9.510$, $p=0.023$) compared to the control. The higher the concentration of the extract, the stronger the inhibition, particularly indicated by concentrations higher than 5 g/100mL dH_2O (fig. 2). The length of the total seedling was more indicative of the inhibitory effect than the measurement of the root length alone.

The response index (RI) of *L. sativum* seedlings was negative for all applied concentrations, for the total seedling length as well as for the length of the root. The response index increased with increasing aqueous extract concentration indicating a stronger negative effect. In general, a seedling's radicle length showed a similar RI compared to the total seedling length, however, with higher concentrations the negative effects became stronger (indicated by more negative RI) for the length of the total seedling than for the root length alone (fig. 3).

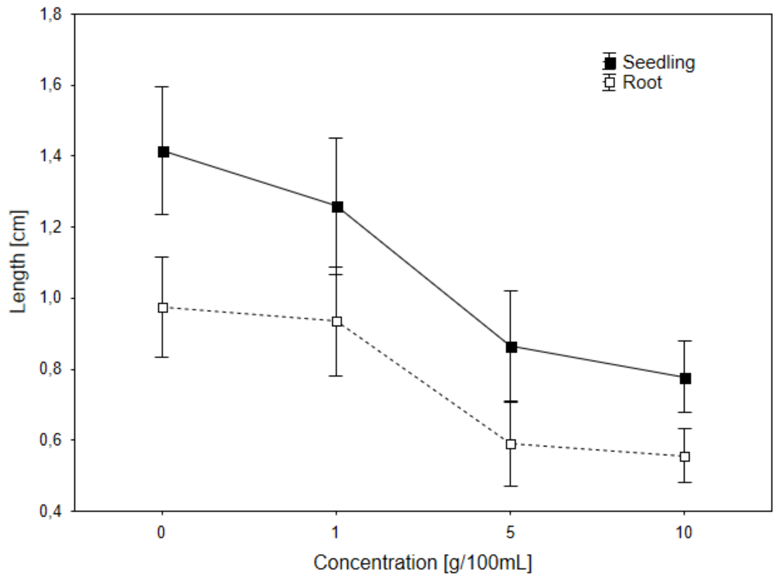

Figure 2. Inhibitory effect of aqueous extracts (1, 5, 10 g/100mL dH_2O) from *Helleborus dumetorum* leaves upon *Lepidum sativum* seedling growth compared to control (0 g/100mL dH_2O) for total seedling length and radicle length. Mean values with corresponding standard errors are presented.

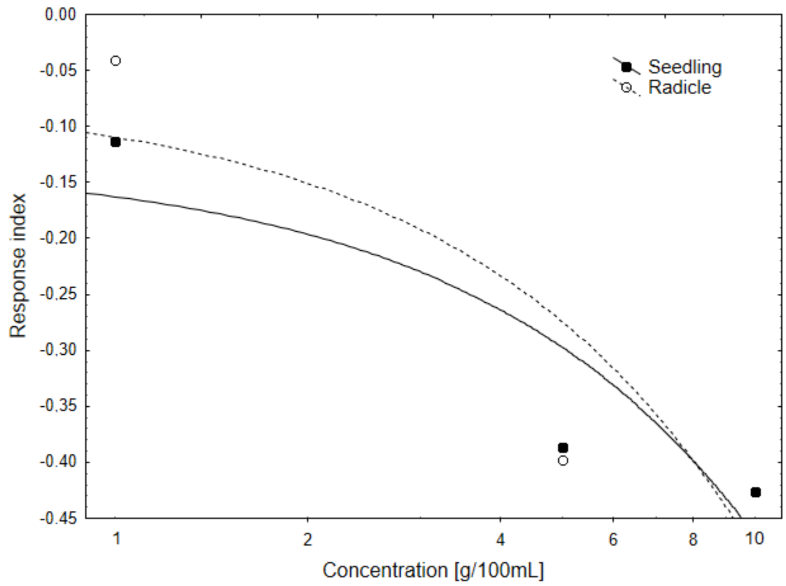

Figure 3. Response index of *Lepidum sativum* seedling and radicle length to increasing aqueous extract concentration of *Helleborus dumetorum* leaves. The x-axis in the *log* scale.

The allelopathic potential of *Helleborus dumetorum* - soil samples

Soil samples where *H. dumetorum* plants were growing showed allelopathic inhibitory potential. The germination rate [G %] of *L. sativum* seeds from soil samples collected close to *H. dumetorum* plants (average G %=65 %) was lower than G% for soil samples collected further away from plants (G %=80 %). Seedling and root length showed no evidence that lengths between seeds germinating in the two soil samples differ in mean ranks of both groups (Mann–Whitney U = 19833.0, $p=0.89$ for seedling and U = 17837.5, $p=0.06$ for roots; N1 = N2 = 200). Given the marginal p-value, the Kolmogorov-Smirnov tests showed differences between root lengths of seedlings germinating in the two soil samples ($p<0.001$).

Allelopathic potential of *Helleborus dumetorum*

The negative impact of *Helleborus* on other plants seems to be recognized for centuries since it was mentioned already in the medieval period by Albertus Magnus (Willis, 2010). However, modern studies of *Helleborus* allelopathy are missing. Our results from bioassays have confirmed the allelopathic potential of aqueous extracts and soil leachates of *H. dumetorum.* Tests showed the existence of aqueous allelopathic compounds in leaves, which are inhibiting germination and seedling development of *L. sativum* seeds. For the family Ranunculaceae, it is known that plants release benzoxazinoids - a class of allelochemicals that are deri vatives of indole and act as natural insecticides, fungicides, and herbicides (Bachheti et al., 2020). Our results also indicate that some potentially allelopathic compounds might be leached or are released actively in the soil where a plant is growing. Aqueous leachates can form from intact or damaged leaf tissue in nature by rain, fog, and dew and they can form from root exudates in the soil (Zheng et al., 2015). For a similar species – *H. viridis*, secondary metabolites of roots and rhizomes contained alkaloids (Colombo et al., 1990). The allelopathic potential differed if we studied the germination rate [G %] or the development of seedlings. The germination rate in each water extract was always lower when compared to the control, however, there was no effect of concentrations recognized. On the other hand, the inhibitory allelopathic potential of aqueous extracts of leaves was positively related to extract concentration when we compared seedling development. The response index *RI* was always stronger for the whole seedling length than for the seedling's radicle except at a concentration of 10 g/100mL dH_2O. These results indicate that G% alone is not as sensitive measure as seedling development for testing potential allelopathic substances in bioassays.

According to our results, *H. dumetorum* plants have allelochemical protection against competition as well as substances preventing herbivory recorded in literature. At this stage, without chemical analyses, it is impossible to tell, which substance in plant tissue is the responsible agent. Particularly not for substances active in the soil since they might be activated/deactivated by various environmental factors (Inderjit and Weiner 2001). However, *L. sativum* germination was 20 % lower if germinated in the soil immediately below the *H. dumetorum* plant if compared to germination rate in the soil 1 m away from any *H.*

dumetorum plant, and suppression of germination and neighboring plants could increase *H. dumetorum* fitness in a habitat.

Suitability of *Helleborus dumetorum* for ornamental planting

Discussion on allelopathy shows that the species is fairly competitive, and its success could be attributed to allelopathic potential. What is more, according to chemical similarity with *H. odorus* we can expect, *H. dumetorum* toxic compounds to demonstrate insecticidal effects as well. For example, extracts of *H. odorus* were useful for beetle larvae eradication, and the use of *H. odorus* as eco-friendly pest control is studied (Mantzoukas et al., 2022).

According to our observations in habitats of native *H. dumetorum* populations in the vicinity of Maribor, plants tolerate sunny as well as semi-shaded habitats. Therefore, we expect the suitable habitats in an urban environment to be the microhabitats next to or inside garden hedges, empty microhabitats around deciduous ornamental or fruit trees, shaded, humid habitats or habitats with drained soil, and semi-shaded sites with tree canopy developing later in the season. Such sites would be beneficial, since there would be no need to mow *H. dumetorum* and plants could maintain their leaves for the entire season, accumulating much of their reserves in rootstocks and successfully propagating in the next year. Based on the personal experience of the last author, planting native *H dumetorum* plants under the canopy of a large fruit tree resulted in successful competition with planted tulips and daffodils for 8 years. *Helleborus dumetorum* plant increased substantially in size, however, no dispersal or spontaneous establishment was observed. The observed tolerance of drained soil makes *H. dumetorum* suitable for landscaping to tackle the gardening challenge known as dry shade. Similar green-flowering species *Helleborus foetidus* L. is used as a dry shade plant under trees where the soil moisture levels are affected by the rain shadow cast by tree canopy (Oettinger, 2005). Contrary to *H. dumetorum*, *H. foetidus* plants have been found occasionally to spread spontaneously, for example under a tree in a botanical garden in Belgium, several tens of meters away from the site where cultivated (Ronse, 2011).

Another practical characteristic is the relatively easy transplanting of *H. dumetorum* plants. According to the experience of the leading author and results of other studies (Bortolotti et al., 2016), long-lived adult plants directly transplanted to a site reached a 100 % success rate of survival. Further experiences show, that plants maintain themselves without further management, and eventually increase in abundance. On the other hand, directly sown seeds showed much lower success (Bortolotti et al., 2016). Most likely the reason lies in myrmecochorous seeds with elaiosomes, which must be transported underground by ants at the time of maturity. In cultivation, *Helleborus* is propagated by rhizome division, by seed, and by *in vitro* techniques (Dhooghe and van Labeke, 2007; Beruto and Curir, 2009). Even though vegetative propagation might have the disadvantage of stable genetic diversity, it is suitable because of its high success rate and because, if performed correctly, the source population is not diminished.

Considerations for *Helleborus dumetorum* conservation prospects in ornamental plantings

Awareness of biodiversity loss and knowledge of how to improve urban habitats will enable and save ecological services and achieve the conservation of plant and invertebrate species. Early flowering species, particularly those with a long flowering period such as *H. dumetorum* could represent an important food resource for bumblebee queens and early pollinators like mason bees, carpenter bees, and other solitary bees. For example, we can expect, that nectar is continuously secreted by nectaries until the nectaries fall off which was shown for *Helleborus foetidus* L. by Vesprini et al. (1999) lasted for about 20 days.

Helleborus dumetorum is threatened in large parts of its occurrence e.g. it is protected in Slovenia (Martinčič et al., 2007) and near threatened in Austria (Schratt-Ehrendorfer et al. 2022). The most obvious reasons are habitat deterioration, degradation, and destruction. Our observations in the field show that spontaneous encroachment of shrubs is the most frequent threat to existing populations in the vicinity of Maribor. The negative effect of habitat deterioration on perennial, long-lived species might often happen unnoticed for a long time because there is often a time lag between the deterioration of environmental conditions and a corresponding decline in population size (Colling and Matthies, 2006; Ehrlén and Morris, 2015).

According to our observations, *H. dumetorum* can persist in deteriorating habitats for quite some time. This represents a risk, that a population decline will be noticed too late and that only costly management options, if any, will be left for the conservation of a local population.

To consider the conservation of local populations by transplanting them to new favorable habitats in urban or peri-urban habitats several conditions should be met. First, *H. dumetorum* plants from local populations should be used for planting. The first step would be to locate and survey local populations, including a brief population study to gain information about their size, density, and existing and potential threats. Then the number of plants for transplant can be determined sustainably. An exception is cases of extremely threatened populations, where habitat destruction is likely to happen and the entire population would be saved by plant relocation.

Conclusion

To prevent biodiversity loss in line with the EU Biodiversity Strategy 2030, it is becoming increasingly important to sustain biodiversity outside protected areas, including biodiversity in agricultural land and urban areas. Therefore, recognizing wild native plants for gardening might represent a successful way to support biodiversity. Additionally, if thereby using threatened native plant this would benefit the plant's conservation at the same time. According to our study, *H. dumetorum* could be suitable for simultaneous conservational and ornamental planting.

Particularly, here we present a bioassay design that gives an insight into the allelopathic potential of a species, which is indicative of the species' competitive advantage. Beneficial functions of defensive traits like allelopathy are crucial for a species to tolerate better a strongly competitive environment. Our findings further define key plant characteristics, which would support the success of this conservational approach for a plant: it can survive in habitats less suitable for common ornamentals (e.g. dry shade under tree canopy), it is resistant to pests (e.g. it is toxic or unpalatable), it supports native mutualists (e.g. pollinators), it has a low invasion potential.

Further studies are required to determine the implications of conservational ornamental planting for threatened flora.

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