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ORIGINAL RESEARCH PAPER

Evaluation of *Helichrysum arenarium* flower exudate as an inhibitor on *Lolium perenne* seed germination under laboratory conditions

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Abstract

Compounds accumulated on the surface of plant tissues and structures like glandular trichomes and thin epicuticular layer are defined as exudate, external, superficial. They exhibited important protective activities - antifungal, antibacterial, insect antifeedant, larvicidal, antiplasmodial, and UV protective. The exudate obtained from *Helichrysum arenarium* flowers was evaluated for its inhibitory activity on germination and initial radicle elongation of *Lolium perenne* seeds. The experiment was done *in vitro* in Petri dishes. The exudate, in water–acetone mixture (99.5:0.5), was assayed at concentrations of 1, 3, 5, 7, and 10 mg/mL. The chemical composition of the exudate was analyzed by GC/MS. Exudate solution with 5 mg/mL concentration was found to cause more than 90% of seed germination inhibition. At the same concentration, complete inhibition of root growth was observed. The main bioactive component of exudate was identified as flavonoid aglycone–naringenin. The inhibitory activity of *H. arenarium* on seed germination was investigated for the first time in the present study.

Keywords

flavonoids; naringenin; carvacrol; GC/MS

1. Introduction

Helichrysum Mill. is a large genus comprising about 600 species worldwide (Azizi et al., 2014, 2019). The plants belonging to the genus are known as everlasting flowers and are widely used in traditional medicine worldwide (Akaberi et al., 2019). *Helichrysum* spp. have been used as flavoring spices in various foods and folk medicines, and for cosmetic purposes for centuries (Antunes Viegas et al., 2014). In addition, *Helichrysum* spp. have potential pharmacological applications for their antioxidant, antimicrobial, and anti-inflammatory activities (Mao et al., 2017; Taglialatela-Scafati et al., 2013). The most well-known and studied species in the genus are *H. italicum* (Antunes Viegas et al., 2014), *H. stoechas* (Les et al., 2017), and *H. arenarium* (Pljevljakušić et al., 2018). *Helichrysum arenarium* (L.) Moench (Asteraceae) flower capitula is a popular medicinal herb used for its choleretic, hepatoprotective, and detoxifying properties (Pljevljakušić et al., 2018). The antimicrobial potential (Babotă et al., 2018; Kutluk et al., 2018) and the toxic properties of non-polar compounds (Judzentiene et al., 2022) reported for *H. arenarium* suggest that the species is appropriate to be examined as a biocide for inhibition on weed seed germination.

Surface exudates consist predominantly of apolar substances: terpenoids, flavonoids, fatty and triterpene acids, alkanes, and fatty alcohols accumulated on the surface plant tissues and structures- glandular trichomes,thin epicuticular layer (Juma et al., 2001; Modak et al., 2009; Wollenweber, 1990). The surface position of these compounds

suggests that they have a protective role and allelopathic potential. Several scientific studies have reported that these compounds exhibit antifungal, antibacterial, larvicidal, antiplasmodial, insect antifeedant activity and UV protective properties (Alcerito et al., 2002; Gikonyo et al., 1998; Kerubo et al., 2013; Montenegro et al., 2019; Omosa et al., 2016; Urzúa et al., 2012).

The application of plant products such as bioherbicides is a rapidly developing direction in modern organic agriculture (Duke et al., 2000; Wahab et al., 2020). The initial step in this regard is establishing the inhibitory activity of these products on the germination of the seeds. Numerous studies have documented that essential oil from aromatic plants are potent inhibitors of seed germination and radicle elongation and have the potential as products for weed control (Jouini et al., 2020; Nikolova & Berkov, 2018; Nikolova et al., 2021; Raveau et al., 2020; Verdeguer et al., 2020; Werrie et al., 2020). Often the yield of essential oil of some plant species is meager, and their application as bioherbicides is insufficient. Although methanolic and water extracts, which are obtained in larger quantities, are also examined as seed germination inhibitors (de Carvalho et al., 2022; Hasan et al., 2021; Radhakrishnan et al., 2018; Shanmugalingam & Umarukatha, 2019), their activity is lower than essential oils (Yankova-Tsvetkova et al., 2020). It has been reported that *H. arenarium* usually releases essential oil in a small amount, about 0.05% (EMA, 2015). In search of a new extraction product of the species, we focused on exudate. A preliminary screening study on the potential of acetone exudates from aerial parts of *Artemisia absinthium* L., *Salvia sclarea* L. and *Tanacetum vulgare*, leaves of *Salvia officinalis* as seed germination inhibitors have been reported by Yankova-Tsvetkova et al. (2020).

In the present study inhibitory activity of surface exudate obtained from *H. arenarium* flower capitula on seed germination of *Lolium perenne* was examined for the first time. The composition of the exudate was determined by GC/MS.

2. Material and methods

2.1. Plant material

The plant material was collected from three natural populations of *H. arenarium* from northeastern Bulgaria: Shumen Region – Shumensko Plateau, east of the Shumen Beer Factory; Varna Region – northeast of village Dobrina, Provadiya district and north of the town Beloslav, Kanarata locality. The studied localities are illustrated with a map using Universal Transverse Mercator (UTM) grid (10 × 10 km) (Figure 1). Voucher specimens are deposited at the Herbarium of the Institute of Biodiversity and Ecosystem Research (SOM), Bulgaria.

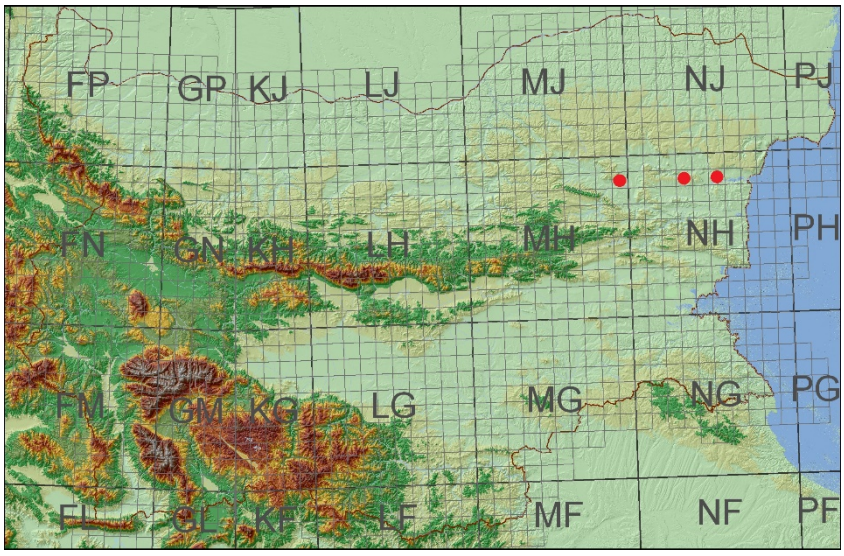

Figure 1 Map of the studied populations of *Helichrysum arenarium*.

2.2. Preparation of acetone exudate

Dry, not ground flower capitula of *H. arenarium* were dipped into acetone for 2–3 min.; after that, the extract was filtered and evaporated to dryness.

2.3. GC/MS analysis

To perform GC/MC analysis, 50 mg of the acetone exudate was put into the vial and dissolved in 50 µL of pyridine. Then, 50 µL of N, O-bis-(trimethylsilyl)trifluoroacetamide was added, and the samples were heated at 70 °C for 2 hrs. After cooling, the samples were diluted with 300 µL chloroform and analyzed using GC-MS. The GC–MS spectra were recorded on a Thermo Scientific Focus GC coupled with a Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. The chromatographic conditions were described by Berkov et al. (2021). The measured mass spectra were deconvoluted using AMDIS 2.64 software before comparison with the databases. Retention Indices (RI) of the compounds were measured with a standard n-alkane hydrocarbons calibration mixture (C9–C36) (Restek, Cat No. 31614, supplied by Teknokroma, Spain). The compounds were identified by comparing their mass spectra and retention indices (RI) with those of authentic standards and the National Institute of Standards and Technology (NIST) spectra library. The quantities of the compounds are expressed as the area percentage from the total area of the chromatogram.

2.4. Inhibitory activity on seed germination and root elongation

A hundred seeds of *L. perenne* L. were placed per Petri dish on filter papers moistened with the tested solutions. The exudate, in water–acetone mixture (99.5:0.5), was assayed at a concentration of 1, 3, 5, 7, and 10 mg/mL. Control consists only of the water–acetone mixture. The samples were incubated at room temperature for seven days. At the end of the week, the rate of germination inhibition [%] was calculated by Atak et al. (2016):

$$GI = [(GC - TG)/GC] \times 100,$$

where: GI is the rate of germination inhibition (%); GC is the germination rate of control treatment; TG is, the germination rate of treated seed with exudate solution.

The reduction in root growth caused by the exudate was estimated as a percentage of the length of the control roots using the following formula:

$$RI = [(REC - TRE)/REC] \times 100,$$

where: RI is the rate of root elongation (%); REC is the root length of control; TRE is root length of treated seed.

Seed germination and assay were performed in three independent experiments.

2.5. Statistical analysis

Statistical analyses were performed using Microsoft Excel software. The results are presented as mean with three experiments' standard deviation (SD).

3. Results

3.1. Chemical composition of flower exudate

The composition of the exudates obtained from samples of three origins was analyzed by GC/MS. The results are presented in Table 1. Primary (sugars, fatty acids) and secondary metabolites (flavonoids, terpenes, phenolic acids, sterols) were detected. Flavonoid aglycone - naringenin was identified as the main component of the secondary metabolites. Flavonols - kaempferol and quercetin were found also. Various phenolic acids, monoterpenoid phenol–carvacrol, and triterpenic acid were detected as bioactive compounds. From primary metabolites, fructose, and hexadecanoic acid were the most abundant. No quality differences in the metabolite profiles between the samples with different origins.

Table 1 Identified compounds in the exudates from studied samples.

Compounds	RI	Studied samples		
		HA1	HA2	HA3
Glycerol	1259	8.4 ± 1.1	5.7 ± 1.1	7.5 ± 1.2
Carvacrol	1339	1.8 ± 0.7	2.6 ± 0.4	2.3 ± 0.4
Cinnamic acid *trans*	1376	0.5 ± 0.2	0.4 ± 0.1	0.2 ± 0.07
3,5-Dihydrotoluene derivative	1436	0.4 ± 0.07	0.3 ± 0.07	0.8 ± 0.07
4-Hydroxybenzoic acid	1637	2.1 ± 1	2.7 ± 1	1.1 ± 0.5
Malic acid	1488	0.6 ± 0.2	0.5 ± 0.3	0.5 ± 0.07
Dodecanoic acid	1521	6.8 ± 1.4	5.8 ± 1.9	4.5 ± 1.7
Vanillic acid	1753	0.6 ± 0.2	0.5 ± 0.1	0.2 ± 0.05
Tetradecanoic acid C14:0	1722	2.0 ± 0.9	1.6 ± 0.8	3.7 ± 0.9
Fructose	1803	2.5 ± 0.7	4.3 ± 0.8	7.9 ± 3.3
Protocatechuic acid	1811	0.3 ± 0.03	0.5 ± 0.07	0.2 ± 0.07
Hexadecanoic acid C16:0	1929	5.1 ± 1.8	4.5 ± 0.5	3.5 ± 0.1
4-Hydroxycinnamic acid	1934	0.7 ± 0.2	0.2 ± 0.1	0.6 ± 0.07
myo-Inositol	2088	0.8 ± 0.1	1.5 ± 0.9	2.2 ± 0.7
Caffeic acid	2142	0.2 ± 0.07	0.2 ± 0.07	0.1 ± 0.03
9-Octadecenoamide *cis*	2427	0.1 ± 0.03	0.7 ± 0.2	1.1 ± 0.5
Sucrose	2626	4.0 ± 0.3	3.5 ± 0.5	3.9 ± 0.5
Heptacosane	2700	0.8 ± 0.2	0.9 ± 0.4	1.6 ± 0.5
Diterpene ester	2774	0.9 ± 0.1	0.7 ± 0.1	0.5 ± 0.07
Naringenin	2872	11.1 ± 1.3	7.5 ± 0.9	10.2 ± 2.8
Triterpenoid	3041	0.5 ± 0.09	0.7 ± 0.1	0.5 ± 0.1
Kaempferol	3060	1.3 ± 0.5	3.1 ± 0.4	2.5 ± 0.3
Chlorogenic acid	3108	1.2 ± 0.6	0.5 ± 0.1	1.1 ± 0.7
Tocopherol	3122	0.7 ± 0.2	0.4 ± 0.1	tr
Quercetin	3199	0.2 ± 0.07	0.7 ± 0.1	0.2 ± 0.07
Campestrol	3239	0.3 ± 0.1	1.2 ± 0.6	0.1 ± 0.04
Stigmasterol	3319	2.3 ± 0.5	4.7 ± 0.8	0.8 ± 0.4
β-Sitosterol	3386	2.0 ± 0.9	3.2 ± 0.8	1.2 ± 0.5
Amyrin	3415	1.5 ± 0.8	3.2 ± 1.1	0.2 ± 0.1
Triterpenic acid	3590	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.07

Legend: HA1-Beloslav; HA2 - village Dobrina; HA3 - Shumensko Plateau; tr - trace.

3.2. Inhibition on seed germination and root elongation

The inhibitory activity of the *H. arenarium* flower capitula exudate on seed germination and root elongation of *L. perenne* was studied in the concentration range of 1–10 mg/mL. More than 90% inhibition on seed germination was found by applying exudate solutions with a concentration over 5 mg/mL. A substantial reduction of root elongation was observed at 3 mg/mL. The results expressed as inhibition rate of germination and root elongation are presented in Table 2.

Table 2 Inhibitory activity of *H. arenarium* exudate on *L. perenne* seed germination and root elongation.

Inhibitory activity [%]	Concentration of *H. arenarium* exudate aqueous solutions [mg/mL]				
	1	3	5	7	10
On seed germination	15 ± 4	62 ± 14	91 ± 4	97 ± 2	100 ± 0
On root elongation	42 ± 3	82 ± 2	100 ± 0	100 ± 0	100 ± 0

4. Discussion

Identified flavonoids in the exudate in the present study are in accordance with previous reports on the flavonoids of *H. arenarium* (Meriçli et al., 1986; Pljevljakušić et al., 2018). Carvacrol and hexadecanoic acids reported as essential oil of the species (Czinner et al., 2000), were also detected in the exudate. Other detected compounds in the exudate-phenolic acids (4-hydroxybenzoic acid, chlorogenic acid) and sterols confirm previously reported substances for the species (Pljevljakušić et al., 2018). The lack of essential differences in the metabolic profiles of the samples from the three studied populations is probably related to the fact that they are relatively close situated (Figure 1). However, the present study is the first report about the chemical composition of *H. arenarium* flower exudate.

The inhibitory effect of acetone exudates on weed seed germination from other aromatic plant species: *Artemisia absinthium*, *Salvia sclarea*, *Tanacetum vulgare*, and *Salvia officinalis*, has been also reported previously (Yankova-Tsvetkova et al., 2020). Acetone exudate of *S. sclarea* exhibited similar activity as *H. arenarium* exudate. For exudates of other species, lower inhibitory activity on seed germination has been found (Yankova-Tsvetkova et al., 2020).

Many studies conclude that flavonoids are responsible for the biological activity of *H. arenarium* (Pljevljakušić et al., 2018). Hernández and Munné-Bosch (2012) reported that naringenin displayed a strong inhibitory activity on seed germination and seedling root growth of *Arabidopsis thaliana*. Moreover, the authors showed that this flavanone is even more effective than salicylic acid, a well-known seed germination inhibitor. Naringenin was found as the main component in the studied exudate in the present work. This gives us reason to suggest that the inhibition effect of exudate on seed germination and root growth is mainly due to this flavonoid.

5. Conclusions

The *in vitro* assay of the phytotoxic action of *H. arenarium* exudate solution showed that when applied at a concentration of 5 mg/mL exhibited a strong inhibitory effect on *L. perenne* seed germination. The flavonoid aglycone-naringenin, monoterpenoid phenol-carvacrol, chlorogenic and 4-hydroxybenzoic acids were evaluated as the main bioactive compounds of the exudate. This first preliminary study showed that *H. arenarium* exudate has the potential as a biocide to inhibit weed seed germination. However, future experiments with more weed species and under in-field conditions are needed.

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