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Original scientific paper

Sensitivity of *Cuscuta* species and their hosts to *Anethum graveolens* essential oil

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SUMMARY

The purpose of this study was to examine *in vitro* the herbicidal effect of an essential oil of dill (*Anethum graveolens*) on germination and early seedling growth of two parasitic flowering plants in the genus *Cuscuta* (*Cuscuta campestris* and *C. epithymum*), as well as its phytotoxic impact on germination and early seedling growth of two host plants (alfalfa and red clover). Chemical analysis of the essential oil extracted from dill leaves and flowers showed that carvone (51.69%) and limonene (39.88%) predominated. The results of a seed bioassay showed inhibitory effects of different concentrations (1%, 0.5%, 0.1%, and 0.01% v v^{-1}) of the essential oil of dill leaves on germination and early seedling growth of both tested species of the genus *Cuscuta*. Germination of *C. campestris* seeds was inhibited between 67% and 94%, while seed germination of *C. epithymum* was inhibited between 67% and 100%. A similar inhibitory effect was observed regarding the seedling length parameter. Moreover, the bioassay results indicated significant phytotoxic effects of dill essential oil on the seed germination and early seedling growth of host plants. Red clover proved more sensitive as even the lowest concentration inhibited germination by 35%, while higher concentrations caused 70-100% inhibition. On the other hand, lower concentations inhibited germination of alfalfa seeds by 5%, while higher concentrations caused greater inhibition (36-100%). Future research should include both pot experiments and field micro-trials to determine herbicidal, i.e. phytotoxic, effects of dill essential oil on parasitic flowering plants and cultivated species.

Keywords: dodder, alfalfa, red clover, dill, essential oils, phytotoxicity, bioherbicide

INTRODUCTION

The widespread use of synthetic chemicals may result in the accumulation of toxic residues in agricultural products and cause soil and groundwater contamination, the development of weed resistance, and adverse effects on human and animal health (Hatcher & Melander, 2003). In addition, synthetic chemicals can be immobilized in soil through adsorption or binding to colloids (Hossard et al., 2017; Kanissery et al., 2019), affecting both soil organic matter turnover and microbial community composition (Haney et al., 2000; Lancaster et al., 2010; Ntalli et al., 2019). Therefore, one of the main challenges of agriculture in the 21st century is to minimize the use of pesticides in crop production (Villa et al., 2017). One potential solution is to find alternative natural and safe products, and exploit renewable resources, such as medicinal and aromatic plants known for their allelopathic properties (Benvenuti et al., 2017; Della Pepa et al., 2019). Due to their structural diversity, natural compounds are a good source of new bioherbicides owing to their new modes of action.

Anethum graveolens L. (dill) is one of the useful essential oil-providing spices, and medicinal plants, because it contains essential oil in its leaves, stems, flowers, fruits, and seeds, which is used in the food and pharmaceutical industries. Dill essential oil has demonstrated various biological activities, such as antimicrobial, antifungal, antioxidant, insecticidal, and anti-inflammatory, due to the presence of biologically active compounds, such as carvone, p-cymene, and α-phellandrene (Chahal et al., 2017).

Plants belonging to the genus *Cuscuta* (common name: dodder) are the most important group of obligate parasitic weeds in the world, inhabiting virtually all continents and causing sweeping damage to both crop and non-crop species (Press & Phoenix, 2005). From an agricultural aspect, the most important *Cuscuta* species in Serbia are *C. campestris* and *C. epithymum*, and both have a wide host range.

Effective control of dodders is extremely difficult because of the nature of attachment and close association between the host and the parasite (Dawson et al., 1994). In the past, control of dodders was also difficult due to a limited selection of registered herbicides for its control in legumes, as well as in sugar beet. So, the need for alternative solutions to control this parasitic flower is increasing. To our best knowledge, there are no reports of the allelopathic effects of essential oils of *A. graveolens* on species in the genus *Cuscuta* and phytotoxic effects on their hosts. This study focused on identifying the chemical composition of an essential oil isolated from leaves of *A. graveolens*. It also aimed to investigate its allelopathic effect on seed germination and seedling growth of two species in the genus *Cuscuta* (*C. campestris* and *C. epithymum*) and its phytotoxic effect on two host species (alfalfa and red clover).

MATERIAL AND METHODS

Plant material

Leaves and flowers of *A. graveolens* were collected in Kosjerić on July 2019. Seeds of *C. campestris* were collected in fields around Odžaci in September 2021, and seeds of *C. epithymum* in fields around Požega in September 2020. The seeds were cleaned and stored in paper bags in the laboratory at a temperature of 20-22 °C.

Preparation and analysis of essential oil

All plant material was air-dried in the shade at room temperature for 20 days and then hydrodistilled in a Clevenger-type apparatus for 2.5 h. The obtained essential oil was dried over anhydrous sodium sulphate and preserved in sealed vials at 4°C until further analysis. Chemical characterization of the essential oil under study was performed by gas chromatography (GC), using two types of detector. Quantitative analysis was performed using an Agilent GC (model 7890A) equipped with a split/splitless injector, flame ionization detector (FID), and HP-5 capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness). Injector and detector temperatures were set to 250 and 300°C, respectively, while nitrogen flow rate was 1 ml/min. Column temperature was programmed linearly to increase from 50 to 250°C at 4°C/min before being held for 10 min. Qualitative chemical analyses were performed using a Varian CP-3800 GC equipped with Saturn 2200 mass spectrometer (MS) as a detection device. The injector temperature and column temperature were the same as for the GC-FID analysis, while separation was performed using an Agilent DB-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas (1 ml/min), and the ion trap and transfer line temperatures were set to 250°C and 280°C, respectively. The mass detector was operated in the electron impact (EI) mode (70 eV; 40–600 m/z range). In both cases, the solutions of essential oils in n-hexane (1%) were injected in the split mode (1:20). To determine the retention indices (RI), a mixture of n-alkanes (C6-C28) was analyzed using both GC-FID and GC-MS under the same conditions as the essential oils. Identification of essential oil components was performed using both the Wiley 7.0 mass spectral library and the RI data obtained, while quantitative data were expressed as area percent obtained by the GC-FID analysis. The RI data obtained were compared with those from the available literature (Adams, 2007). These data are used as an additional tool to confirm the MS results.

Petri dish germination bioassay

The effect of *A. graveolens* essential oil was tested on two parasitic species, *C. campestris* and *C. epithymum*, and their host plants alfalfa and red clover. The experiment was conducted under controlled conditions in an incubator in darkness (Velpro, Serbia) at 27±1°C. The solutions were prepared with 0.5 ml of essential oil emulsified with Tween 20 (v/v 0.1%) (REANAL Finomvegyszergyar Rt., Hungary, No.805383) at a 1:1 ratio and dissolved in distilled water to obtain a stock solution of 1% concentration. The other concentrations (0.5%, 0.1%, and 0.01% v v^{-1}) were prepared by dilution. Water and a solution of Tween 20 at a concentration of 1.0% were used for the control. Seed surface was sterilized with 5% sodium hypochlorite solution (NaOCl) for 3 minutes and then rinsed three times with distilled water. Twenty disinfected seeds were placed into each petri dish and than 5 ml of each solution was added. All dishes were sealed with parafilm to avoid evaporation. After 8 days, the percentage of germination was calculated and early seedling growth (seedling length) was measured.

The inhibition percentage, reflected through germination and seedling length, was calculated using the formula:

$$\% \text{ inhibition} = [(Xc - Xt)/Xc] \times 100 \qquad [1]$$

where Xc is the % of germination and seedling length in control, and Xt is the % of germination and seedling length in treatment with essential oil.

Germination rate (GR, sum of germination data per day) was calculated using a formula described by Maguire (1962):

$$GR = n1/t1 + n2/t2 + ... + n_x/t_x, \qquad [2]$$

where n1, n2, ...nx are the numbers of germinated seeds at times t1, t2, ...tx in days.

The experiment design was a randomized complete block with four replications, repeated twice, and data were combined for analysis.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA) using STATISTICA 8.0. software package. Normality distribution and homogeneity of variances were checked for all data using the Kolmogorov-Smirnov and Levene tests. When F-values were statistically significant ($p<0.05$) treatments were compared using Fisher's least significant difference (LSD) test.

RESULTS AND DISCUSSION

Chemical analysis of essential oil

Chemical composition of the obtained dill essential oil is presented in Table 1. It shows that 18 identified compounds accounted for 99.61% (v/w) of total oil mass with carvone (51.69%) and limonene (39.88%) being the predominant components. With an exception of

Table 1. Chemical composition of *A. graveolens* essential oil

Chemical class	Components	RI_{EXP}[a]	RI_{LIT}[b]	Content (%)
MH[c]	α-thujene	923	924	0.11
MH	α-pinene	932	932	0.35
MH	sabinene	970	969	0.08
MH	β-pinene	975	974	0.14
MH	α-phellandrene	1001	1002	4.25
MH	p-cymene	1018	1020	0.21
MH	limonene	1023	1024	39.88
MH	terpinolene	1085	1086	0.11
MH	allo-ocimene	1127	1128	0.08
OM[d]	cis-limonene oxide	1132	1132	0.09
OM	trans-limonene oxide	1136	1137	0.22
OM	dill ether	1182	1184	0.55
OM	cis-dihydrocarvone	1191	1191	0.26
OM	trans-dihydrocarvone	1200	1200	1.21
OM	trans-carveol	1214	1215	0.09
OM	cis-carveol	1228	1226	0.18
OM	carvone	1241	1239	51.69
FAD[e]	(2E)-decenal	1262	1260	0.11
	Total			99.61

[a]RI_E – Experimentally determined Retention Indexes
[b]RI_{LIT} – Retention Indexes – literature data (Adams, 2007)
[c]Monoterpene hydrocarbon
[d]Oxygenated monoterpene
[e] Fatty acid and fatty acid derived compound (aliphatic aldehyde)

α-phellandrene (4.25%) and trans-dihydrocarvone (1.21%), all other compounds were present at much lower level. In general, monoterpene hydrocarbons accounted for 45.21%, oxygenated monoterpenes for 4.29%, while (2E)-decenal as aliphatic aldehyde accounted for 0.11% of total oil mass. Carvone and limonene are the predominant components of essential oils produced from dill cultivated in Serbia (Aćimović et al. 2014). Analyzing three essential oils obtained from dill grown at different locations in Vojvodina Province the authors found that the contents of carvone and limonene varied in a range from 51.7-54.5 and 40.6-43.1, respectively, which is consistent with our results.

Seed bioassay

The results of seed bioassay showed that different concentrations of essential oil extracted from dill leaves and flowers inhibited germination and growth of seedlings of both tested *Cuscuta* species. Germination of *C. campestris* seeds was inhibited between 67% (solution concentration 0.01%) and 94% (solution concentrations 0.5% and 1%), while inhibition of *C. epithymum* seeds by the two higher concentrations (0.5% and 1%) was 100%, and 80%, respectively, and 67% by lower concentrations (0.1% and 0.01%) (Figure 1). The inhibitory effect on *C. campestris* was confirmed by significant statistical differences between all test concentrations and the control, and between the applied concentrations, except for the two lower ones which caused no statistically significant difference (Figure 1a). In contrast, *C. epithymum* showed a significant difference (p<0.05) even at the lowest essential oil concentration (Figure 1b). The data suggest a greater susceptibility of *C. epithymum* than *C. campestris*, which was confirmed by measurements of the growth rate parameters, which were higher for *C. campestris* than for the other species tested at all concentrations (Table 2).

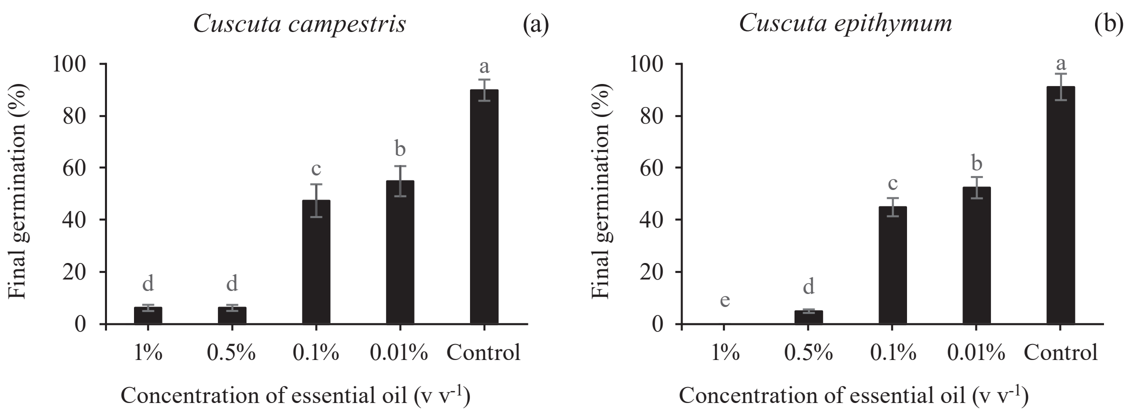

Figure 1. Effects of different concentrations of *A. graveolens* essential oil on seed germination of *C. campestris* (a) and *C. epithymum* (b). Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, p<0.05. Means marked by different letters (a, b, c, d) differ significantly (p<0.05) for final germination at different concentrations.

Table 2. Effects of different concentrations of *A. graveolens* essential oil on germination rate (%) and seedling growth (cm) of *C. campestris* and *C. epithymum*

Concentration of essential oil	*C. campestris*		*C. epithymum*	
	Germination rate	Seedling growth	Germination rate	Seedling growth
1%	0.52 ± 0.10 c	0.23 ± 0.08 c	0.00 ± 0.00 d	0.00 ± 0.00 c
0.5%	0.58 ± 0.12 c	0.46 ± 0.12 c	0.43 ± 0.10 c	0.24 ± 0.17 c
0.1%	6.46 ± 0.89 b	1.08 ± 0.18 b	6.15 ± 0.98 b	1.98 ± 0.77 b
0.01%	6.98 ± 0.96 b	5.23 ± 0.87 a	6.06 ± 1.01 b	5.21 ± 0.89 a
Control	17.6 ± 1.40 a	5.53 ± 0.93 a	35.21 ± 4.83 a	5.40 ± 0.85 a

Data are reported as means ± standard deviation. Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, p<0.05. Means in the same column marked by different letters (a, b, c, d) differ significantly (p<0.05).

Seedling length and germination rate were also examined as parameters in the present study for both *Cuscuta* species. Similar to the former parameters, seedling length sustained an inhibitory effect in both species, ranging from 5% (0.01%) to 96% (1%) for *C. campestris,* and from 4% (0.01%) to 100% (1%) in *C. epithymum* (Table 2). Statistically significant differences ($p<0.05$) in seedling length were noted in both species between the control and treatments with concentrations from 0.1% to 1%, while no significant differences were found between the control and the lowest concentration (0.01%), and between the two higher concentrations (0.5% and 1%).

Bioassay results show significant phytotoxic effects of the tested dill essential oil on seed germination and early seedling growth of two hosts (alfalfa and red clover). Red clover was found more susceptible as its germination was inhibited 35% by the lowest concentration, while higher concentrations caused inhibition ranging from 70% to 100% (Figure 2b). Germination of alfalfa seed was inhibited 5% by lower concentrations (0.1% and 0.01%), while higher concentrations caused higher inhibition (36-100%) (Figure 2a). A similar inhibitory trend was noted for the two other parameters (seedling length and germination rate) (Table 3). No significant differences were found between the control data and treatments of alfalfa with lower concentrations, while a significant difference was shown between the control and both higher concentrations ($p<0.05$) for all tested parameters. Conversely, red clover seed showed significant differences ($p<0.05$) between the control and all concentrations, which further confirms a greater susceptibility of red clover seedlings than alfalfa seedlings to the tested dill essential oil (Figure 2).

Control of parasitic weeds is a challenge in agriculture. In conventional agriculture, chemical agents are widely used for weed control, and the overuse of agrochemicals has led to environmental pollution (Muzell Trezzi et al., 2016). Allelopathic compounds (essential oils, phenols), plant residues or extracts, plant mulches and cover crops rich

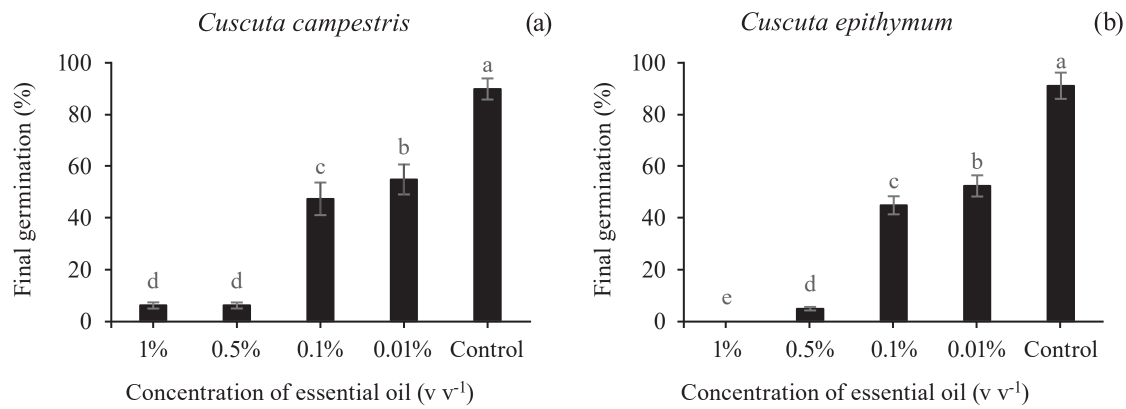

Figure 2. Effects of different concentrations of *A. graveolens* essential oil on seed germination of alfalfa (a) and red clover (b). Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, $p< 0.05$. Means marked by different letters (a, b, c, d) differ significantly ($p< 0.05$) for final germination at different concentrations.

Table 3. Effects of different concentrations of *A. graveolens* essential oil on germination rate (%) and seedling growth (cm) of alfalfa and red clover

Concentration of essential oil	Alfalfa		Red clover	
	Germination rate	Seedling growth	Germination rate	Seedling growth
1.0%	0.00 ± 0.00 d	0.00 ± 0.00 c	0.00 ± 0.00 d	0.00 ± 0.00 c
0.5%	6.59 ± 1.16 c	0.25 ± 0.03 c	0.00 ± 0.00 d	0.00 ± 0.00 c
0.1%	20.00 ± 2.68 b	2.86 ± 0.88 b	5.87 ± 0.69 c	2.16 ± 0.71 b
0.01%	32.13 ± 4.10 a	5.56 ± 1.06 a	16.95 ± 3.95 b	4.80 ± 0.49 a
Control	35.21 ± 5.51 a	6.10 ± 0.08 a	23.96 ± 2.04 a	4.87 ± 0.54 a

Data are reported as means ± standard deviation. Differences were evaluated by one-way analysis of variance (ANOVA) completed with Fisher's least significant difference (LSD) test, $p<0.05$. Means in the same column marked by different letters (a, b, c, d) differ significantly ($p<0.05$).

in allelochemicals can be used for natural weed control. Allelopathy is a field that has been developing more and more over the last few decades but research in the field of parasitic weeds is not as advanced. In fact, several studies have investigated the effects of various allelopathic plant residues on the control of field dodder (Seyyedi et al., 2013; Abbasvand et al., 2020). The effects of residues of three plants, *Zygophyllum fabago* L., *Calendula officinalis* L. and *Datura stramonium* L., on two cultivars of sweet basil infested with field dodder (*C. campsetris*) were studied (Abbasvand et al., 2020). The results suggested that the residues of *Z. fabago* were preferable to other residues used in that study for suppressing field dodder in basil production. In addition, flower and leaf extracts from *Nepeta meyeri* inhibited the germination and growth of *C. campestris* seedlings (Shekari et al. 2022).

Our *in vitro* study confirmed the inhibitory effect of different concentrations of dill essential oil on seed germination and early seedling growth of *C. campestris* and *C. epithymum*. Although the bioassay results showed significant inhibitory effects on the measured parameters of both species in the genus *Cuscuta*, they also revealed phytotoxic effects on the germination and early seedling growth of their host plants (alfalfa and red clover). Future research will need to include both pot trials and field micro-trials to determine the herbicidal, i.e. phytotoxic effects of dill essential oil on the tested parasitic flowering plants and their hosts.

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Osetljivost vrsta roda *Cuscuta* i njihovih domaćina na etarsko ulje mirođije *Anethum graveolens*

REZIME

Cilj ovog rada je bio da se *in vitro* ispita herbicidni uticaj etarskog ulja mirođije (*Anethum graveolens*) na klijanje i rani porast klijanaca dve parazitne cvetnice iz roda *Cuscuta* (*Cuscuta campestris* i *Cuscuta epithymum*), kao i fitotoksični uticaj na klijanje semena i rani porast klijanaca semena domaćina (lucerka i crvena detelina). Hemijskom analizom etarskog ulja izolovanog iz listova i cvetova mirođije je dobijeno da su karvon (51.69%) i limonen (39.88%) dominantne komponente. Rezultati biotesta sa semenima su pokazali inhibitorni uticaj etarskog ulja izolovanog iz listova mirođije pri različitim koncentracijama na klijavost i rani porast klijanaca obe vrste roda *Cuscuta*. Naime, inhibicija klijanja semena *C. campestris* se kretala od 67% do 94%, dok je kod semena *C. epithymum* inhibicija bila od 67% do 100%. Sličan inhibitorni uticaj je zabeležen i za parametar dužina klijanaca. Pored ovoga, u rezultatima biotesta su zabeleženi značajni fitotoksični efekti etarskog ulja mirođije na klijanje semena i rani porast klijanaca domaćina. Crvena detelina se pokazala kao osetljivija, jer je i pri najnižoj koncentarciji inhibicija klijanja bila 35%, a pri višim se kretala od 70% do 100%. Nasuprot ovome inhibicija klijanja semena lucerke pri nižim koncentracijama je bila 5%, dok je na višim koncentracijama zabeležena veća inhibicija (36-100%). Buduća istraživanja moraju obuhvatiti oglede u saksijama, kao i poljske mikrooglede da bi se utvrdili herbicidni, odnosno fitotoksični efekti etarskog ulja mirođije na testirane parazitne cvetnice i gajene vrste.

Ključne reči: vilina kosica, lucerka, crvena detelina, mirođija, etarska ulja, fitotoksičnost, bioherbicid