Archives of Biological Sciences

ACCEPTED MANUSCRIPT

This article can be cited before page numbers have been issued; to do this please use:
Prijović MP, Nikolić BR, Dragićević IČ, Nestorović Živković JM, Dmitrović SS, Giba ZS, Jovanović VD. Water emulsion of the essential oil of *Nepeta rtanjensis* Diklić et Milojević: potential use as a bioherbicide. Arch Biol Sci. 2023;https://doi.org/10.2298/ABS231107041P.

This is an ***Accepted Manuscript***, which has been through the **Archives of Biological Sciences** peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance and proof reading, before technical editing and formatting. Using this service, authors can make their results available to the community, in citable form, before the edited article is published. This ***Accepted Manuscript*** will be replaced with the edited and formatted ***Published Article*** as soon as it is available.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. In no event shall the **Archives of Biological Sciences** be held responsible for any errors or omissions in this ***Accepted Manuscript*** or any consequences arising from the use of any information it contains.

OnLine First

@ by the authors.
Licensee the Serbian Biological Society

Water emulsion of the essential oil of *Nepeta rtanjensis* Diklić et Milojević: potential use as a bioherbicide

Mladen P. Prijović[1], Bogdan R. Nikolić[2], Ivana Č. Dragićević[3], Jasmina M. Nestorović Živković[4], Slavica S. Dmitrović[4,*], Zlatko S. Giba[3] and Vladan D. Jovanović[5]

[1]*Institute for Forage Crops*, Kruševac, Globoder 37251, Serbia
[2]*Institute for Plant Protection and Environment*, Teodora Drajzera 9, 11000 Belgrade, Serbia
[3]*Faculty of Biology, University of Belgrade*, Studentski trg 16, 11000 Belgrade, Serbia
[4]*Institute for Biological Research "Siniša Stanković", National Institute of Republic of Serbia, University of Belgrade*, Bulevar despota Stefana 142, 11108 Belgrade, Serbia
[5]*Institute of Pesticides and Environmental Protection*, Banatska 31b, 11080 Belgrade, Serbia

***Corresponding author:** slavica.dmitrovic@ibiss.bg.ac.rs

Received: November 7, 2023; **Revised:** November 19; **Accepted:** November 24, 2023; **Published online:** December 28, 2023

Abstract: Plant protection with natural products is a new trend in environmentally friendly agriculture. *Nepeta rtanjensis* Diklić et Milojević is an endemic and critically endangered plant species in Serbia. We explored the phytotoxic potential of a water emulsion of *Nepeta rtanjensis* essential oil (*Nr*EO) with high amounts of *trans,cis*-nepetalactone on five weeds. The most sensitive was *Stellaria media* (L.) Vill., as *Nr*EO (from 0.013% to 0.1%) completely inhibited germination. Germination of *Amaranthus retroflexus* L. and *Artemisia vulgaris* L. was completely inhibited at the highest applied concentrations of *Nr*EO (0.1%), while *Ambrosia artemisiifolia* L. germination was reduced to 48% at the same concentration of *Nr*EO. The most tolerant species was *Cephalaria transsylvanica* (L.) Schrad. ex Roem. & Schult. as the final germination rate at the highest applied concentration of *Nr*EO (0.1%) was 81%, like the control (82%). To our knowledge, this is the first time the interaction of essential oils on the germination and growth of *A. vulgaris* and *C. transsylvanica* is reported. The germination dynamics of *S. media* in pots with soil were significantly inhibited when the soil was initially treated with the highest applied concentration of the *Nr*EO water emulsion (1%). Spraying *S. media* seedlings with *Nr*EO significantly inhibited growth parameters (shoot height, shoot fresh weight, and the number of nodes) at the higher applied concentrations of *Nr*EO (0.5% and 1%). Therefore, the water emulsion of the essential oil of *N. rtanjensis* could be potentially developed for use in the control of invasive and allergenic weeds.

Keywords: *Nepeta rtanjensis*, allelopathy, essential oil, seed germination, weed

Abbreviations: water emulsion of the *Nepeta rtanjensis* essential oil (*Nr*EO)

INTRODUCTION

Many weed species have been accidentally introduced into different parts of the world, where, due to their adaptability, they not only survive but become the most prevalent in these areas. Weeds, especially invasive species, germinate, flower, and form fruits in a wide range of environmental conditions, allowing them to spread successfully across space and time. The formation of different subspecies, varieties, forms, and subforms helps weed species persist in newly colonized habitats. Weeds are a major problem in agriculture because they seriously reduce the yield of crops by up to 34%, compared to animal pests and pathogens, which cause fewer losses (18% and 16%, respectively) [1,2]. A significant number of allergenic weed species also have a direct negative impact on human health. Excessive use of pesticides leads

to the accumulation of chemicals in the environment and indirectly affects human health [3]. In recent years, great attention has been paid to environmental protection. It has resulted in an increased interest in alternative strategies, leading to the development of biodegradable compounds [4]. Considerable resources are spent researching natural herbicides or bioherbicides [5].

Allelopathic compounds, a promising type of bioherbicide, include various groups of secondary metabolites, the most important of which are volatile terpenoids and phenolic compounds [6,7]. Essential oils are mixtures of different groups of volatile compounds whose main components are easily evaporating terpenoids, especially monoterpenes. Stereoisomers of these compounds are very common and have different physicochemical properties [8]. The stereochemistry of nepetalactone largely determines its antimicrobial and allelopathic activity, with the *trans,cis* isomer being more active than the *cis,trans* isomer in inhibiting bacterial [9] and root growth, as well as suppressing superoxide dismutase activity in ragweed [4]. In addition, the *trans,cis* isomer induces changes in the antioxidant system of cress seedlings by disrupting the normal expression and activity patterns of antioxidant enzymes [10]. The *trans,cis* stereoisomer is highly toxic to some insects and acts as a more potent repellent [11].

The genus *Nepeta* is one of the largest genera in the family Lamiaceae (subfamily Nepetoideae, tribe Mentheae), and its members are commonly known as catnip or catmint. It comprises 280 species that are important to humans as medicinal, ornamental, and culinary plants [12]. The subfamily of Nepetoideae consists of two groups – the iridoid-rich species, which include *N. rtanjensis*, and the iridoid-less species. Since *Nepeta rtanjensis* is an endemic species of the Rtanj mountain in eastern Serbia [13] and a natural rarity protected by law, it is important to point out that enough plant material for distillation of the essential oil could only be obtained through the process of micropropagation under *in vitro* conditions as well as through vegetative propagation in the greenhouse and in the field.

The species of the genus *Nepeta* have medicinal properties due to the composition of their essential oil, which is characterized by the presence of nepetalactone in significant quantities. Nepetalactones are a special group of monoterpenes – iridoids, which can be present in the form of eight stereoisomers, four diastereoisomers and corresponding enantiomers. With few exceptions, only *7S* diastereoisomers have been found under natural conditions (Supplementary Fig. S1) [14]. The *trans,cis* stereoisomer of nepetalactone (4aα,-7α,-7aβ-nepetalactone) is predominantly present in the essential oil of *N. rtanjensis*, while the *cis,trans* stereoisomer (4aβ,-7α,-7aβ-nepetalactone) is present at a much lower percentage [4,15-17].

We selected the following weeds mostly because of their wide distribution and the considerable damage they cause to crops. Redroot pigweed (*Amaranthus retroflexus* L.) is a summer annual weed in fields of cultivated plants and ruderal habitats. To date, it has been recorded as a widespread weed in many different crops (wheat, barley, oats, flax, canola, sunflower, alfalfa, tomato, potato, etc.) [18]. The harmful effects of *A. retroflexus* are reflected in yield and quality losses in crops, toxicity to livestock, and allelopathic influence on crops [19]. It may also serve as an alternative host for certain plant pathogens and pests. Its pollen is a harmful allergen to humans [20]. Ragweed (*Ambrosia artemisiifolia* L.), one of the most important weeds in fields of cereals, sunflower, soybean, root crops, etc. [21] and non-agricultural habitats, is an invasive weed species in many regions of central and southeastern Europe, as well as in other parts of the world [22]. Its rapid spread can be explained by its distinct adaptability [23]. In addition, the seeds can survive in the soil for a very long time, germinating even after 40 years [24]. Ragweed pollen causes serious seasonal asthma and allergic rhinitis [25-27]. The widespread weed mugwort (*Artemisia vulgaris* L.) negatively affects crops with its highly branched rhizome, which is a very strong competitor for water and nutrients [18,28], as well as the production of numerous phytotoxic compounds [29]. The pollen of *A. vulgaris* is considered highly allergenic [30]. *Cephalaria transsylvanica* (L.)

Schrad. ex Roem. & Schult. does not require specific habitat requirements; it grows at altitudes from 0 to 1600 m on dry and arid sites, limestone slopes, the edges of wetlands, roadsides, canals, fields, and on steppes [31]. Chickweed (*Stellaria media* (L.) Vill.) is a widespread weed species native to Europe and one of the most widespread weeds in the world [32]. It is found in fields with various crops such as barley, wheat, sugar beet, etc. [33-35] and in ruderal sites, along roads, fences and in forest clearings.

In our previous studies, we demonstrated the allelopathic potential of the essential oil of *N. rtanjensis* via air under *in vitro* culture conditions on the growth of ragweed seedlings [4], as well as on the germination of seeds of some crops and weed species [36]. The novelty of this study is that the effect of a water emulsion of the essential oil of *N. rtanjensis* on the germination of five invasive weeds was investigated in direct contact and under non-sterile conditions, more similar to those in the field in order to find an economic application of the essential oil in weed control. In our work, the influence of *Nepeta rtanjensis* essential oil on the seed germination of selected weed species, *Amaranthus retroflexus*, *Ambrosia artemisiifolia*, *Artemisia vulgaris*, *Cephalaria transsylvanica*, and *Stellaria media*, was studied. Germination of *S. media* seeds in pots in soil initially treated with *Nr*EO was also studied, as well as the early development of seedlings after additional spraying with *Nr*EO by measuring growth parameters.

MATERIALS AND METHODS

Plant material

Mature seeds of five weed species: redroot pigweed (*Amaranthus retroflexus* L.), ragweed (*Ambrosia artemisiifolia* L.), mugwort (*Artemisia vulgaris* L.), *Cephalaria transsylvanica* (L.) Schrad. ex Roem. & Schult, and chickweed (*Stellaria media* (L.) Vill.) were collected in the wider area of Belgrade, Serbia. The collection was done by picking the branch tips, whole branches, or plant parts with seeds. After drying, the seeds were separated and stored in a dark chamber where they were kept at room temperature until the beginning of the experiment. The essential oil of *Nepeta rtanjensis* Diklić et Milojević was obtained courtesy of Dr. Danijela Mišić from the Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, Serbia. The composition of the essential oil of *N. rtanjensis*, determined by GC-MS and GC-FID, was documented in [4].

Seed germination

The seeds were germinated in glass Petri dishes with a diameter of 60 mm on two layers of filter paper in 2 mL of distilled water or the tested solution. The emulsion of *Nepeta rtanjensis* essential oil was prepared from *N. rtanjensis* essential oil, methanol, and Tween 20 in the volume ratio 1:4:1, using distilled water to the desired concentrations of *N. rtanjensis* essential oil of 0.006%, 0.013%, 0.025%, 0.05%, and 0.1% for the germination of seeds of *A. retroflexus*, *A. artemisiifolia* and *C. transsylvanica*, and 0.003%, 0.006%, 0.013%, 0.025%, 0.05%, and 0.1% for the germination of seeds of *A. vulgaris* and *S. media*. Two control groups were included in the experimental design: (1) Cmt – treated with the same maximum amount of methanol and Tween 20 (0.4% and 0.1%, respectively) in distilled water, and (2) C – distilled water. Fifty seeds were sown in each Petri dish and the experiment was repeated three times. According to our previous research [37], we have presented the light and temperature conditions for seed germination of *A. retroflexus*, *A. artemisiifolia*, *A. vulgaris*, *C. transsylvanica*, and *S. media* in Supplementary Table S1. Before the seeds of *A. retroflexus* and *A. vulgaris* were exposed to different temperature and light conditions, they were stratified at a temperature of 3±2°C for 2.5 weeks, and the seeds of *A. artemisiifolia* for 6 weeks. Seeds with a visible radicle (1 mm) were recorded as germinated.

An experiment with potted plants

Stellaria media seeds were selected for the experiment because they were the most sensitive to *N. rtanjensis* essential oil compared to the other weed species tested in this study. The seeds of *S. media* were germinated in pots with 100 g of sterilized soil (Seedling Substrate, Klasmann-Deilmann GmbH, Germany). The essential oil of *N. rtanjensis*, methanol, and Tween 20 were prepared in the volume ratio 1:1:1, and water was added to the desired emulsion concentration. Before sowing, the topsoil layer (approximately 10 mm) was sprayed with *N. rtanjensis* emulsion (0.06%, 0.13%, 0.25%, 0.5%, and 1%) under a pressure of 0.25 MPa. Control groups were sprayed with (i) the same maximum volume of methanol and Tween 20 (both 1%) in distilled water, or (ii) distilled water. *S. media* seeds were germinated in a phytotron at 25/20°C in a 16 h/8 h light/dark photoperiod. Fifty seeds were sown in each pot and the experiment was repeated four times. Twelve days after sowing, five healthy *S. media* seedlings at a similar stage of development (2 nodes) were left in each pot and additionally sprayed with 3 mL of an emulsion of *N. rtanjensis* essential oil at the same concentration as added to the soil at the beginning of the experiments (0.06%, 0.13%, 0.25%, 0.5%, and 1%) or with control liquids: (1) Cmt - a solution with the same maximum amount of methanol and Tween 20 (both 1%) in distilled water, and (2) C - distilled water. Twelve days after additional spraying, the growth parameters (number of stem nodes, shoot height, fresh weight, and dry weight of shoot) of the remaining seedlings were measured, and the number of seedlings that did not survive was also recorded.

Statistical analysis

Statistical data analysis was performed using the STATISTICA 7 software. Data were subjected to one-way analysis of variance (ANOVA). Statistical significance of differences between means were determined using Fisher's LSD test at a confidence level of $P \leq 0.05$.

RESULTS

The effect of the essential oil of *Nepeta rtanjensis* on the seed germination of selected weed species

The germination of five weed species, *Amaranthus retroflexus, Ambrosia artemisiifolia, Artemisia vulgaris, Cephalaria transsylvanica,* and *Stellaria media,* was tested in a water emulsion of *Nepeta rtanjensis* essential oil (from 0.003% to 0.1%) and two control conditions (Cmt – with the same maximum amount of methanol and Tween 20 in distilled water or C – distilled water) under the temperature and light conditions shown in Supplementary Table S1. Based on previous experiments [37], we selected the optimal temperature and light regimes for each weed species to achieve the highest germination rates. Final germination in the Cmt and C control treatments was not significantly different for any of the studied weed species, except *C. transsylvanica*, where it was significantly lower in the Cmt control treatment (Fig. 1). Since it was shown that methanol at the applied concentration did not affect seed germination of the five weeds studied (unpublished data), in *C. transsylvanica* germination was inhibited on Cmt, probably due to the individual effect of Tween 20 or the synergistic effect of methanol and Tween 20 (Fig. 1D). We interpreted the germination results of the *Nr*EO treatments compared to the Cmt control.

The final germination of *A. retroflexus* seeds in the emulsion of *Nr*EO (0.006%, 0.013%, 0.025%, 0.05%, 0.1%) was significantly reduced in all treatments, except in the treatment with the lowest *Nr*EO concentration (0.006%) compared to the control (Cmt) (Fig. 1A). The effect on germination dynamics was observed even in the treatment with the lowest *Nr*EO concentration (0.006%), as germination was 25% one day after the beginning of the experiment, while it was 76% in the control treatment (Cmt). With increasing *Nr*EO concentration, germination was significantly delayed and reduced. On day 15, only 5% of *A.*

retroflexus seeds germinated at 0.05% *Nr*EO, while germination was completely inhibited at 0.1% *Nr*EO.

A. artemisiifolia seeds in Cmt attained the highest final germination (87%) 22 days after sowing (Fig. 1B). Treatments with *Nr*EO (0.05% and 0.1%) significantly reduced final seed germination to 75% and 43%, respectively. Treatment with 0.025% *Nr*EO until the 4th day after sowing inhibited germination compared to Cmt, while after that it was stimulated, but not significantly (Fig. 1B).

The seeds of *A. vulgaris* achieved complete germination (99%) on the 6th day after sowing (Fig. 1C). Final seed germination was not significantly affected at lower *Nr*EO concentrations (from 0.003% to 0.013%), while a higher *Nr*EO concentration (0.05%) significantly inhibited final seed germination to 68% compared to Cmt. Germination of *A. vulgaris* seeds was completely inhibited at 0.1% *Nr*EO (Fig. 1C).

The effect of *Nr*EO on the germination of *C. transsylvanica* seeds was observed from the 3rd to the 15th day (Fig. 1D). It was found that treatment with 0.1% *Nr*EO had an inhibitory effect on seed germination dynamics, but final germination was not significantly affected compared to Cmt. In all other treatments, final germination was higher, and even significantly at *Nr*EO concentrations of 0.05% and 0.006% compared to Cmt (Fig. 1D). A stimulatory effect of *Nr*EO (0.006% and 0.05%) on seed germination in relation to the Cmt control was observed only in *C. transsylvanica* compared to the other weed species tested (Fig. 1).

Stellaria media seeds in control (Cmt) reached maximum germination (99%) on the 7th day after sowing. They were very sensitive to *Nr*EO because at the lowest applied *Nr*EO concentration (0.003%), seed germination was delayed and reached only 43% (Fig. 1E). This value was significantly lower than the Cmt. *Nr*EO concentrations higher than 0.006% completely inhibited seed germination (Fig. 1E).

Effect of *Nepeta rtanjensis* essential oil on seed germination and seedling growth of *Stellaria media* sown in pots

Since the seeds of *Stellaria media* were most sensitive to the essential oil of *N. rtanjensis*, we selected them for further experiments in pots. Before sowing, the topsoil layer was sprayed with *N. rtanjensis* emulsion (0.06%, 0.13%, 0.25%, 0.5%, and 1%), while the control treatments were sprayed with (1) the same maximum amount of methanol and Tween 20 (both 1%) in distilled water (Cmt), and (2) distilled water (C) (Fig. 1F and Fig. 2). Germination was performed at 25/20°C in a 16 h/8 h light/dark photoperiod for 12 days. Thereafter, 5 seedlings were left in each pot and additionally sprayed with the same concentration of *Nr*EO emulsion or distilled water with or without methanol and Tween 20 as at the beginning of the experiments. After 12 days of additional spraying, growth parameters (shoot height, number of nodes, fresh weight, and dry weight of shoots) were measured (Fig. 2) and the number of seedlings that did not survive was also recorded. The plateau of germination of *S. media* was reached on the 5th day after sowing. Only the 1% *Nr*EO treatment resulted in significantly lower sprouting (84%) compared to the control and the other treatments, whereas sprouting was equal to or higher than 90% in all other treatments (Fig. 1F). Reduction in *S. media* shoot growth after additional spraying with *Nr*EO emulsion was observed (Fig. 2). Statistically significant inhibition of plant elongation was observed 12 days after additional spraying with 1% *Nr*EO. The average height of control shoots was 37.5 mm, while shoots sprayed with the lowest *Nr*EO concentration (0.06%) were higher than those in Cmt, but this was not statistically significant (Fig. 2A). The average number of nodes in the control treatment (Cmt) was 5.9, while it was significantly lower in the treatments with 0.5% *Nr*EO and 1% *Nr*EO (5.1 and 4.2, respectively) compared with Cmt (Fig. 2B). The shoot fresh weight of seedlings was significantly lower 12 days after subsequent spraying with 0.25%, 0.5%, and 1% *Nr*EO, while the dry weight was significantly reduced at all applied concentrations of *Nr*EO, except for 0.06%, compared to Cmt. The statistically significant increase in fresh and dry weight of *S. media* shoots occurred

after spraying with 0.06% *Nr*EO, compared to Cmt (Fig. 2C, D). The highest applied *Nr*EO concentration (1%) caused 45% mortality in *S. media* seedlings, while 0.5% *Nr*EO, 0.13% *Nr*EO, and the C treatment caused 5% mortality. There was no mortality in the 0.25% *Nr*EO, 0.06% *Nr*EO, and Cmt treatments.

DISCUSSION

Allelopathic compounds directly affect many biochemical and physiological processes, including seed germination and plant organ growth and development [38]. Essential oils include volatile compounds that achieve their allelopathic effects through the air in the natural environment. However, in our experimental setup, the allelopathic effect of the essential oil was demonstrated by the direct contact of the seeds with the water emulsion. Essential oils containing high levels of low molecular weight terpenes affected seed germination and early seedling growth by altering normal cell division processes, reducing mitotic index, and causing chromosomal abnormalities in root meristems [39]. Monoterpenes are known to cause anatomical and physiological changes in plants by inhibiting DNA synthesis and disrupting mitochondrial and nuclear functions [40], disrupting the cell membrane, increasing fluidity, or inhibiting membrane enzymes, and promoting the formation of reactive oxygen species (ROS) [41].

The significantly delayed and reduced germination of *A. retroflexus* with increasing *Nr*EO concentration agrees with Mutlu et al. [41] that the germination of *A. retroflexus* was completely inhibited at a concentration of 0.02% essential oil of *Nepeta meyeri*, which has a very similar content of nepetalactone to that of *N. rtanjensis* in the essential oil. Mutlu et al. [42] showed that the germination of ten other weed species was inhibited by *N. meyeri* essential oil. Kordali et al. [43] demonstrated that the inhibitory effect of *N. meyeri* essential oil on the germination and seedling growth of four weed species, including *A. retroflexus*, was similar to or higher than that of the commercial herbicide trifluralin.

Dmitrović et al. [4] demonstrated that *N. rtanjensis* and *N. cataria* essential oils had a strong inhibitory effect on the shoot and root growth of *A. artemisiifolia in vitro*. Tworkoski [44] showed that *A. artemisiifolia* was damaged after spraying with essential oils of *Thymus vulgaris* and *Satureja hortensis* at a concentration of 1%, while 5% and 10% killed the seedlings, which, like *Nepeta* species, belong to the *Lamiaceae* family. Our results that *Nr*EO treatment reduces the final germination of *A. artemisiifolia* seeds are consistent with literature data.

A. vulgaris is one of the most abundant weeds in the world [18,45]. To our knowledge, there are no literature data on the interaction of essential oils with *A. vulgaris*. We show that germination of *A. vulgaris* was completely inhibited at 0.1% *Nr*EO, which is a very important contribution of our work. Some other interactions with *A. vulgaris* have been demonstrated in the literature. The allelopathic potential of weed species against crops is commonly reported. On the contrary, Onen [46] showed that a water extract of *Medicago sativa* shoot and root significantly reduced seed germination and growth of *A. vulgaris* seedlings in Petri dish experiments, while mixing the shoot biomass of *M. sativa* with soil strongly inhibited the sprouting of rhizome fragments and growth of *A. vulgaris* seedlings and caused leaf discoloration in pot experiments.

Like *A. vulgaris*, *Medicago sativa* root and shoot extract inhibited imbibition and germination and reduced radicle and hypocotyl length and dry weight of *C. syriaca* [47]. As regards *A. vulgaris*, we did not find any previous studies on the effect of essential oils on the germination and growth of *Cephalaria*. Since we have demonstrated an unexpected stimulatory effect of *Nr*EO on the germination *C. transsylvanica*, we believe that this result could be helpful for cultivation of *N. rtanjensis* in the field. It would be interesting to investigate whether higher concentrations of *Nr*EO would cause inhibition of *C. transsylvanica* germination.

We have shown that *S. media* is very sensitive to *Nr*EO, as a low concentration completely inhibits seed germination. This is consistent with Nestorović Živković et al. [36], who showed a very strong inhibitory effect of *Nr*EO in an ambient atmosphere on the germination of *S. media*. The inhibitory effect of plant extracts on *S. media* seed germination [48,49] has been demonstrated, while the importance of the interaction with essential oils needs to be further investigated.

The essential oil of *N. rtanjensis* had a significantly lower inhibitory effect on the germination of *A. artemisiifolia* than on the *A. retroflexus*, *A. vulgaris*, and *S. media* seeds, since the germination rate at the highest concentration (0.1%) was 43%, in contrast to the other species where germination was completely inhibited. The least sensitive species was *C. transsylvanica*, as germination at the highest applied concentration of *N. rtanjensis* EO (0.1%) did not affect germination compared to Cmt; there was even a statistically significant stimulation of *C. transsylvanica* germination at some concentrations of *Nr*EO (0.006% and 0.05%).

Based on our results of the effect of *Nr*EO on the germination of five weed species in Petri dishes, we selected the most sensitive species, *S. media*, for the pot experiment. We demonstrated that the highest applied *Nr*EO concentration caused about 50% mortality in *S. media* seedlings. This is consistent with Kordali et al. [43], who demonstrated mortality of *A. retroflexus* seedlings of 43% to 64% 24 and 48 h, respectively, after spraying with an emulsion of *N. meyeri* essential oil (2%) under greenhouse conditions. In addition, Dyanat and Asgari [50] made similar observations that spraying 3-week-old seedlings of *A. retroflexus* with essential oils of *N. glocephalata* and *N. ispahanica* at a concentration similar to our experiment (1.25%) resulted in visible damage and a decrease in seedling dry weight.

In our previous studies, we have shown that at the germination stage, some crops, such as oilseed rape, are insensitive to *Nr*EO applied in the atmosphere of Petri dishes, while some other economically important plants, such as lettuce, are very sensitive [36]. Further studies are needed to select economically important species and the stage of their development at which they are least sensitive to *Nr*EO in order to simultaneously inhibit the growth of weed species in the field.

Like the statistically significant stimulation of germination of *C. transsylvanica* at some of the tested *Nr*EO concentrations, the lowest *Nr*EO concentration has a hormesis effect and acts as a growth stimulant on the fresh and dry weight parameters of *S. media* seedlings. This effect could be tested on selected crops or other economically important species to increase their biomass production and yields. On the other hand, it is possible that *Nr*EO treatment of *S. media* or similar weeds in the field may have an opposite effect to the desired, as rain and irrigation could dilute *Nr*EO in the soil to a concentration that stimulates the growth of seedlings instead of inhibiting it. Further research is needed to find the *Nr*EO concentration that has the desired effect on weeds and economically important crops at the same time.

Since their negative effects on the environment and human health are far less than those of widely used commercial herbicides, it is necessary to further investigate the structures and sites of action of allelochemicals, including the essential oil of *N. rtanjensis*, and their dominant compounds, so that they can be used as natural herbicides. It is necessary to determine formulations that can increase the effectiveness of *N. rtanjensis* essential oil and reduce the shortcomings of volatile active ingredients under field conditions.

Funding: This work was financed by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract Nos. 451-03-47/2023-01/ 200214; 451-03-47/2023-01/ 200217; 451-03-47/2023-01/ 200007; 451-03-47/2023-01/ 200178; 451-03-47/2023-01/ 200010).

Author contributions: Conceptualization, VJ (Vladan Jovanović), MP (Mladen Prijović); methodology, VJ, MP, BN (Bogdan Nikolić), Zlatko Giba (ZG); validation, VJ, ID (Ivana Dragićević); formal analysis, MP, BN; investigation, VJ, MP, BN, ID, ZG, JNŽ (Jasmina Nestorović Živković), SD (Slavica Dmitrović); data curation, VJ, MP; writing – original draft preparation, SD, JNŽ, MP, VJ; Writing – review & editing, ID, SD, JNŽ, VJ; visualization, MP, VJ, SD, JNŽ, ID; supervision, VJ; funding acquisition, VJ. All authors have read and agreed to publish the presented version of the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

Data Availability: Data underlying the reported findings have been provided as a raw dataset which is available here:
https://www.serbiosoc.org.rs/NewUploads/Uploads/Prijovic%20et%20al_Raw%20Dataset.pdf

REFERENCES

1. Kudsk P, Streibig JC. Herbicides, a two-edged sword. Weed Res. 2003;43(2):90-102. https://doi.org/10.1046/j.1365-3180.2003.00328.x
2. Oerke EC. Crop losses to pests. J Agric Sci. 2006;144(1):31-43. https://doi.org/10.1017/S0021859605005708
3. Marin-Morales MA, Ventura-Camargo BDC, Hoshina MM. Toxicity of herbicides: impact on aquatic and soil biota and human health. Herbic Res case Stud use. 2013;10:55851. https://doi.org/ 10.5772/55851
4. Dmitrović S, Perišić M, Stojić A, Živković S, Boljević J, Nestorović Živković J, Aničić N, Ristić M, Mišić D. Essential oils of two *Nepeta* species inhibit growth and induce oxidative stress in ragweed (*Ambrosia artemisiifolia* L.) shoots in vitro. Acta Physiol Plant. 2015;37(3):64. https://doi.org/10.1007/s11738-015-1810-2
5. Bailey KL. The bioherbicide approach to weed control using plant pathogens. In: Integrated Pest Management. Elsevier; 2014. p. 245–66. https://doi.org/10.1016/B978-0-12-398529-3.00014-2
6. Santonja M, Bousquet-Mélou A, Greff S, Ormeño E, Fernandez C. Allelopathic effects of volatile organic compounds released from *Pinus halepensis* needles and roots. Ecol Evol. 2019;9(14):8201-13. https://doi.org/10.1002/ece3.5390
7. Xie Y, Tian L, Han X, Yang Y. Research advances in allelopathy of volatile organic compounds (VOCs) of plants. Horticulturae. 2021;7(9):278. https://doi.org/10.3390/horticulturae7090278
8. Zeng R Sen, Mallik AU, Luo SM. Allelopathy in sustainable agriculture and forestry. New York: Springer-Verlag; 2008. https://doi.org/10.1007/978-0-387-77337-7
9. Nestorović J, Mišić D, Šiler B, Soković M, Glamočlija J, Ćirić A, Maksimović V, Grubišić D. Nepetalactone content in shoot cultures of three endemic *Nepeta* species and the evaluation of their antimicrobial activity. Fitoterapia. 2010;81(6):621-6. https://doi.org/10.1016/j.fitote.2010.03.007
10. Nestorović Živković J. Antioxidative, antimicrobial and allelopathic effects of three endemic *Nepeta* species (Lamiaceae) [dissertation] Belgrade: University of Belgrade; 2013. 10.2298/BG20130917NESTOROVICZIVKOVIC
11. Formisano C, Rigano D, Senatore F. Chemical constituents and biological activities of Nepeta species. Chem Biodiver. 2011;8(10):1783-818. https://doi.org/10.1002/cbdv.201000191
12. Salehi B, Valussi M, Jugran AK, Martorell M, Ramirez-Alarcón K, Stojanović-Radić ZZ, Antolak H, Kręgie D, Mileski KS, Sharifi-Rad M, Setzer, WN, Luz Cádiz-Gurrea M, Segura-Carretero A, Şener B, Sharifi-Rad J. *Nepeta* species: From farm to food applications and phytotherapy. Trends food Sci Technol. 2018;80:104-22. https://doi.org/10.1016/j.tifs.2018.07.030
13. Diklic N, Milojević B. *Nepeta rtanjensis* Diklic et Milojevic spec. nov.-nova vrsta iz roda *Nepeta* L. Bull du Museum d'Histoire Nat Belgrade, Ser B, Livre. 1976;31:23-35.
14. Liblikas I, Santangelo EM, Sandell J, Baeckström P, Svensson M, Jacobsson U, Unelius CR. Simplified isolation procedure and interconversion of the diastereomers of nepetalactone and nepetalactol. J Nat Prod.

2005;68(6):886-90.
https://doi.org/10.1021/np049647d
15. Chalchat JC, Gorunovic MS, Petrovic SD, Maksimovic ZA. Composition of the essential oil of *Nepeta rtanjensis* Diklić & Milojević, Lamiaceae from Serbia. J Essent Oil Res. 2000;12(2):238–40. https://doi.org/10.1080/10412905.2000.9699507
16. Ljaljević-Grbić M, Stupar M, Vukojević J, Soković M, Misić D, Grubišić D, Mihailo R. Antifungal activity of *Nepeta rtanjensis* essential oil. J Serbian Chem Soc. 2008;73(10):961-5. https://doi.org/10.2298/jsc0810961g
17. Mišić D, Šiler B, Gašić U, Avramov S, Živković S, Živković JN, Milutinović M, Tešić Ž. Simultaneous UHPLC/DAD/(+/-)HESI-MS/MS analysis of phenolic acids and nepetalactones in methanol extracts of *Nepeta* species: A possible application in chemotaxonomic studies. Phytochem Anal. 2015;26(1):72-85. https://doi.org/10.1002/pca.2538
18. Holm L, Doll J, Holm E, Pancho J V, Herberger JP. World weeds: natural histories and distribution. John Wiley & Sons; 1997.
19. Athanassova DP. Allelopathic effect of Amaranthus retroflexus L. on weeds and crops. In: Seizième conférence du COLUMA Journées internationales sur la lutte contre les mauvaises herbes; 1995 Dec 6-8; Reims, France. 1996. p. 437-42.
20. Tehrani M, Sankian M, Assarehzadegan MA, Falak R, Jabbari F, Varasteh A. Immunochemical characterization of *Amaranthus retroflexus* pollen extract: extensive cross-reactive allergenic components among the four species of *Amaranthaceae/Chenopodiaceae*. Iran J Allergy Asthma Immunol. 2010;87-95.
21. Buttenschøn RM, Waldispühl S, Bohren C. Guidelines for management of common ragweed. Ambrosia artemisiifolia. 2010.
22. Montagnani C, Gentili R, Smith M, Guarino MF, Citterio S. The worldwide spread, success, and impact of ragweed (*Ambrosia* spp.). CRC Crit Rev Plant Sci. 2017;36(3):139-78. https://doi.org/10.1080/07352689.2017.1360112
23. Brückner D, Czimber G, Pinke G, others. Changes in the weed flora of maize fields in Szigetköz (north-west Hungary) between 1990 and 1996. Acta Agron Ovariensis. 1997;39(1/2):15-9.
24. Darlington HT. Dr. WJ Beal's seed-viability experiment. Am J Bot. 1922;9(5):266-9.
25. Chan-Yeung M, Anthonisen NR, Becklake MR, Bowie D, Sonia Buist A, Dimich-Ward H, Ernst P, Sears MR, Siersted HC, Sweet L, Van Til L, Manfreda J. Geographical variations in the prevalence of atopic sensitization in six study sites across Canada. Allergy. 2010;65(11):1404-13. https://doi.org/10.1111/j.1398-9995.2010.02399.x
26. Salo PM, Calatroni A, Gergen PJ, Hoppin JA, Sever ML, Jaramillo R, Arbes SJ, Zeldin DC. Allergy-related outcomes in relation to serum IgE: results from the National Health and Nutrition Examination Survey 2005-2006. J Allergy Clin Immunol. 2011;127(5):1226-35. https://doi.org/10.1016/j.jaci.2010.12.1106
27. Bonini M, Monti GS, Pelagatti MM, Ceriotti V, Re EE, Bramè B, Bottero P, Tosi A, Vaghi A, Martelli A, Traina GM, Rivolta L, Rivolta F, Otrolani CM. Ragweed pollen concentration predicts seasonal rhino-conjunctivitis and asthma severity in patients allergic to ragweed. Sci Rep. 2022;12(1):15921. https://doi.org/10.1038/s41598-022-20069-y
28. Weston LA, Barney JN, DiTommaso A. A review of the biology and ecology of three invasive perennials in New York State: Japanese knotweed (*Polygonum cuspidatum*), mugwort (*Artemisia vulgaris*) and pale swallow-wort (*Vincetoxicum rossicum*). Plant Soil. 2005;277:53-69. https://doi.org/10.1007/s11104-005-3102-x
29. Pannacci E, Pettorossi D, Regni L, Tei F. Allelopathic potential of mugwort (*Artemisia vulgaris* L.) to control the Italian ryegrass (*Lolium multiflorum* Lam.) in winter wheat. Allelopath J. 2015;36(2):257:72.
30. Cosi V, Gadermaier G. The Role of Defensins as Pollen and Food Allergens. Curr Allergy Asthma Rep. 2023;1-9. https://doi.org/10.1007/s11882-023-01080-3
31. Gokturk RS, Sumbul HI. A taxonomic revision of the genus *Cephalaria* (Caprifoliaceae) in Turkey. Turk J Botany. 2014;38(5):927-68. https://doi.org/10.3906/bot-1310-6
32. Bitarafan Z, Andreasen C. Harvest weed seed control: Seed production and retention of *Fallopia convolvulus*, *Sinapis arvensis*, *Spergula arvensis* and *Stellaria media* at spring oat maturity. Agronomy. 2020;10(1):46. https://doi.org/10.3390/agronomy10010046
33. Kropff MJ, Joenje W, Bastiaans L, Habekotte B, Van Oene H, Werner R. Competition between a sugar beet crop and populations of *Chenopodium album* L. and *Stellaria media* L. Netherlands J Agric Sci. 1987;35(4):525-8.
34. Padu K, Khanduri VP, Singh B, Rawat D, Riyal MK, Kumar KS. Phytotoxicity of common weeds on germination, seedling growth, NPK uptake and chlorophyll content of four hill crops of Garhwal Himalaya. J

Agric Food Res. 2023;12:100539.
https://doi.org/10.1016/j.jafr.2023.100539
35. Esposito M, Cirillo V, De Vita P, Cozzolino E, Maggio A. Soil nutrition management may preserve non-detrimental weed communities in rainfed winter wheat (*T. aestivum*). Agric Ecosyst Environ. 2023;355:108596.
https://doi.org/10.1016/j.agee.2023.108596
36. Nestorović Živković J, Dmitrović S, Jovanović V, Živković S, Božić D, Aničić N, Mišić D. Allelopathic potential of essential oil of *Nepeta rtanjansis*. Allelopath J. 2016;37(2):207-19.
37. Prijović M, Janjić V, Nikolić B, Stavretović N, Jovanović V. Klijanje semena pet korovskih vrsta pod različitim temperaturnim i svetlosnim uslovima. Acta Herbol. 2015;24(2):99-108.
38. Weir TL, Park SW, Vivanco JM. Biochemical and physiological mechanisms mediated by allelochemicals. Curr Opin Plant Biol. 2004;7(4):472-9.
https://doi.org/10.1016/j.pbi.2004.05.007
39. Pawlowski A, Kaltchuk-Santos E, Zini CA, Caramão EB, Soares GLG. Essential oils of *Schinus terebinthifolius* and *S. molle* (Anacardiaceae): Mitodepressive and aneugenic inducers in onion and lettuce root meristems. South African J Bot. 2012;80:96-103.
https://doi.org/10.1016/j.sajb.2012.03.003
40. Nishida N, Tamotsu S, Nagata N, Saito C, Sakai A. Allelopathic effects of volatile monoterpenoids produced by *Salvia leucophylla*: inhibition of cell proliferation and DNA synthesis in the root apical meristem of Brassica campestris seedlings. J Chem Ecol. 2005;31:1187-203.
https://doi.org/10.1007/s10886-005-4256-y
41. Mutlu S, Atici Ö, Esim N, Mete E. Essential oils of catmint (*Nepeta meyeri* Benth.) induce oxidative stress in early seedlings of various weed species. Acta Physiol Plant. 2011;33(3):943-51.
https://doi.org/10.1007/s11738-010-0626-3
42. Mutlu S, Atici O, Esim N. Bioherbicidal effects of essential oils of *Nepeta meyeri* Benth. on weed spp. Allelopath J. 2010;26(2):291-300.
43. Kordali S, Tazegul A, Cakir A. Phytotoxic effects of *Nepeta meyeri* Benth. extracts and essential oil on seed germinations and seedling growths of four weed species. Rec Nat Prod. 2015;9(3):404-18.
44. Tworkoski T. Herbicide effects of essential oils. Weed Sci. 2002;50(4):425-31.
45. Stefanowicz AM, Stanek M, Majewska ML, Nobis M, Zubek S. Invasive plant species identity affects soil microbial communities in a mesocosm experiment. Appl Soil Ecol. 2019;136:168-77.
https://doi.org/10.1016/j.apsoil.2019.01.004
46. Onen H. Does allelopathy play a role in suppression of mugwort (*Artemisia vulgaris*) by alfalfa? Plant Prod Sci. 2013;16(3):255-60.
https://doi.org/10.1626/pps.16.255
47. Rasool AA. Allelopathic potential of aerial and root extracts of alfalfa against germination and seedling related traits of four weed species. J Zankoy Sulaimani-Part A. 2014;16:2.
48. Scavo A, Pandino G, Restuccia A, Mauromicale G. Leaf extracts of cultivated cardoon as potential bioherbicide. Sci Hortic (Amsterdam). 2020;261:109024.
https://doi.org/10.1016/j.scienta.2019.109024
49. Konstantinović B, Koren A, Kojić M, Samardžić N, Sikora V, Popov M. Allelopathic properties of hemp. Contemp Agric. 2021;70(3-4):101-7.
https://doi.org/10.2478/contagri-2021-0015
50. Dyanat M, Asgari F. Phytotoxic effects of essential oils from *Nepeta glocephalata* Rech.f. And *N. ispahanica* Boiss. And selected weed species. Acta Agric Slov. 2021;117(4):1-12.
https://doi.org/10.14720/aas.2021.117.4.1520

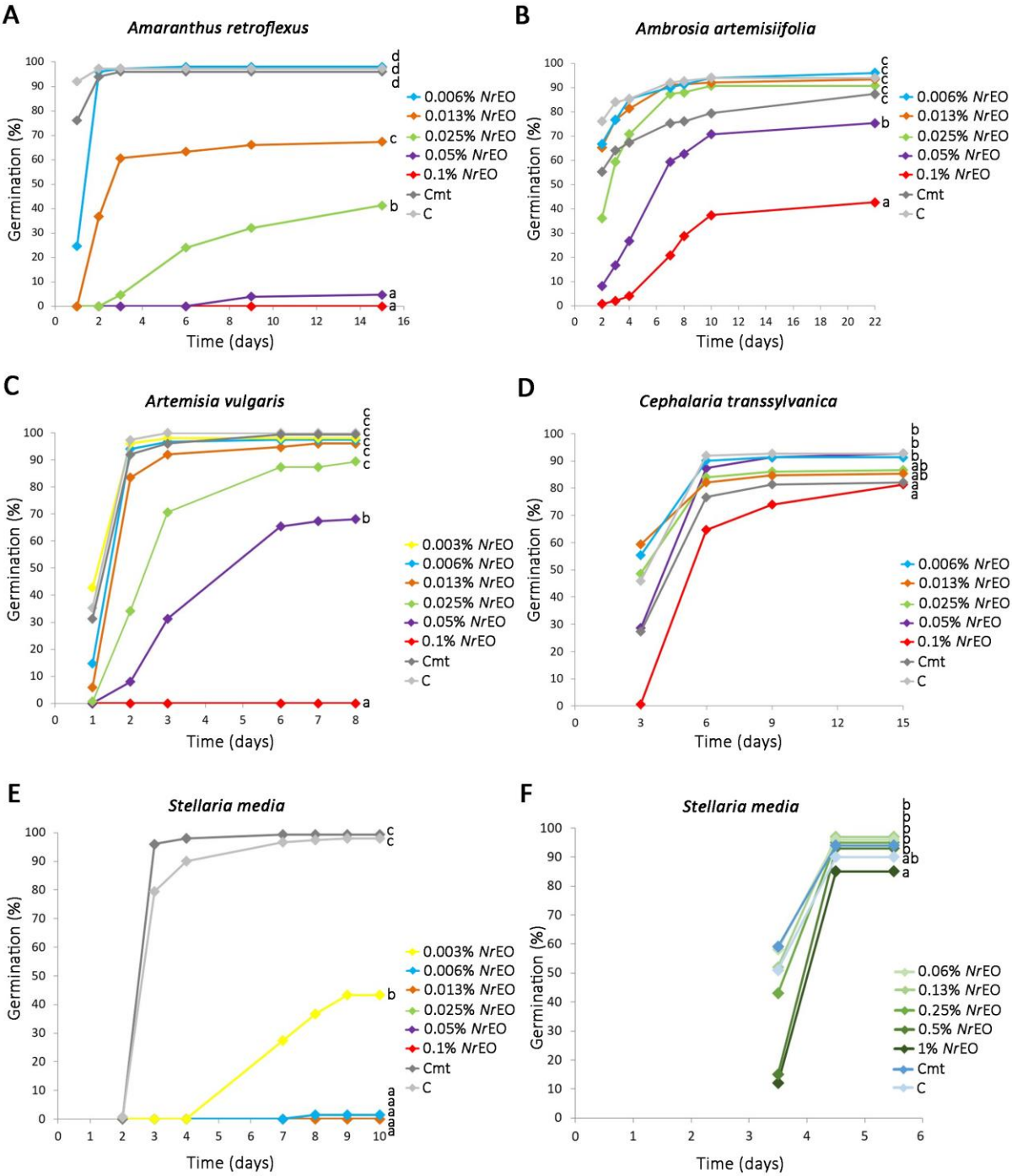


Fig. 1. The effect of the water emulsions of the essential oil of *Nepeta rtanjensis* on the germination of **A** – *Amaranthus retroflexus*, **B** – *Ambrosia artemisiifolia*, **C** – *Artemisia vulgaris*, **D** – *Cephalaria transsylvanica* and **E** – *Stellaria media* seed germination (%) in Petri dishes. **F** – The effect of the water emulsion of *N. rtanjensis* essential oil on seed germination of *Stellaria media* (%) in pots. Two control groups were included in the experimental design: Cmt – treated with the same maximum amount of methanol and Tween 20 in distilled water, and C – distilled water. The values with the same letter indicate statistically homogeneous groups ($P \leq 0.05$) according to Fisher's LSD test.

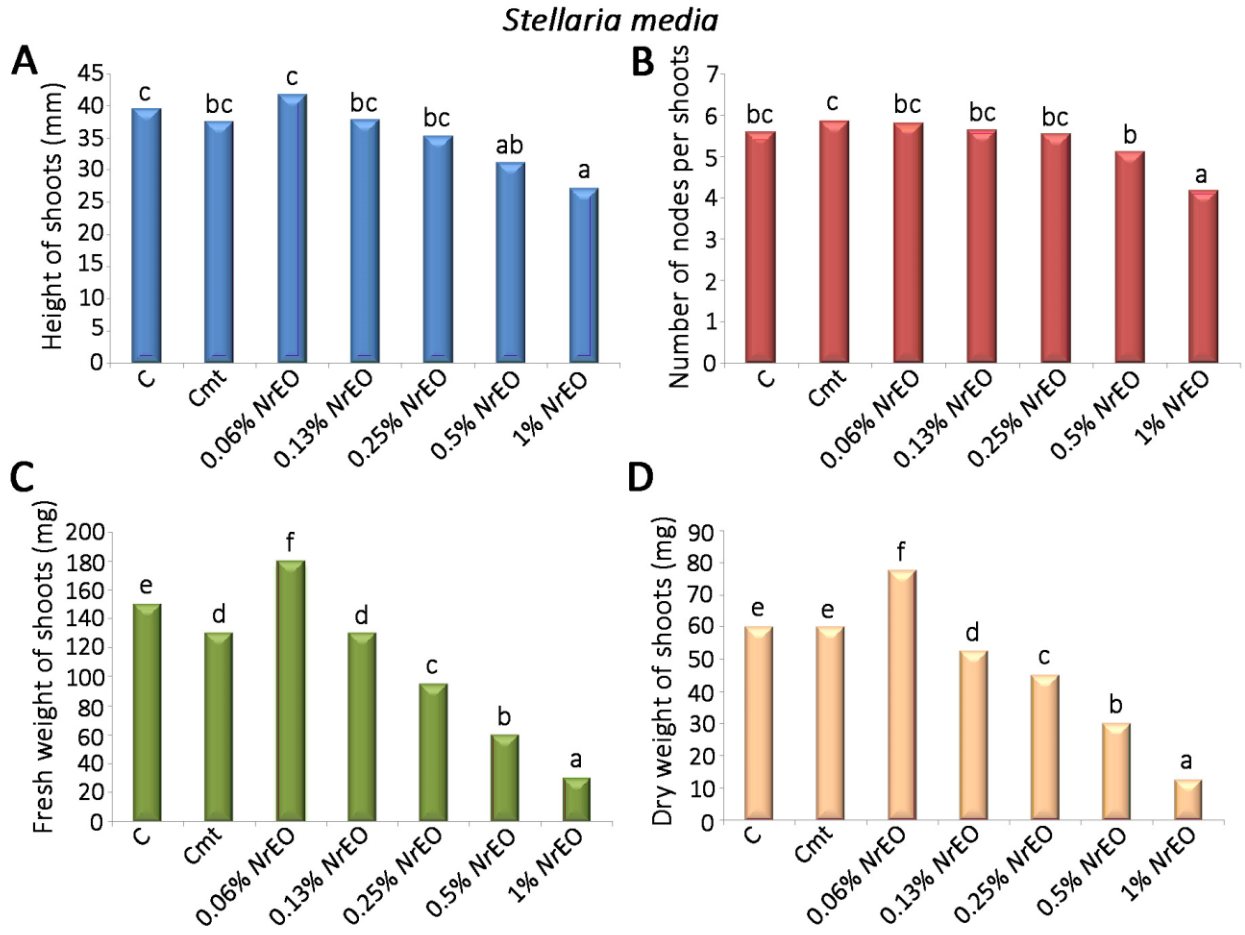
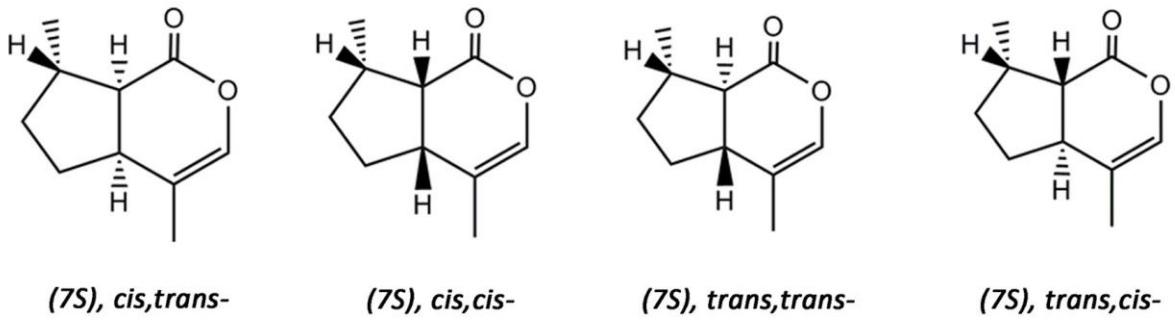


Fig. 2. Growth parameters: **A** – height of seedlings (mm), **B** – number of nodes per shoots, **C** – fresh weight (mg), **D** – dry weight of shoots (mg) *Stellaria media* seedlings 24 days after sowing in pots sprayed with *Nepeta rtanjensis* emulsion (0.06%, 0.13%, 0.25%, 0.5% and 1%) before and 12 days after sowing. Control groups were sprayed with 1) the same maximum amount of methanol and Tween 20 (both 1%) in distilled water – Cmt and in distilled water – C. Values with the same letter indicate statistically homogeneous groups (P≤0.05), according to Fisher's LSD test.

SUPPLEMENTARY MATERIAL

Supplementary Table S1. The light and temperature conditions for seed germination of *A. retroflexus*, *A. artemisiifolia*, *A. vulgaris*, *C. transsylvanica*, and *S. media*. The seeds of *A. retroflexus* and *A. vulgaris* were previously stratified for 2.5 weeks at 3±2°C in the dark, while *A. artemisiifolia* was previously stratified for 6 weeks at 3±2°C in the dark, according to Prijović et al. [37].

Weed species	Temperature	Light/Dark photoperiod	Duration
A. retroflexus	30°C	illumination (15 min/day)	15 days
A. artemisiifolia	26/21°C	16 h light/ 8 h dark	22 days
A. vulgaris	26/21°C	16 h light/ 8 h dark	8 days
C. transsylvanica	20°C	illumination (15 min/day)	15 days
S. media	20°C	illumination (15 min/day)	10 days



Supplementary Fig. S1. *7S*-diastereoisomers of nepetalactone according to Liblikas et al. [14].