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Phytochemical analysis of *Asclepias syriaca* L. leaf extract and its potential phytotoxic effect on some invasive weeds

Jelena Gajić Umiljendić[1,4]✉, Marija Sarić-Krsmanović[1,4], Ljiljana Radivojević[1], Ljiljana Šantrić[1], Maja Šćepanović[2], Valentina Šoštarčić[2] & Tijana Đorđević[3]

Leaf extract of *Asclepyas syriaca* was utilized to investigate the phytotoxic effects on the germination and seedling growth of *Amaranthus retroflexus* L., *Chenopodium album* L., and *Iva xanthifolia* Nutt. and to evaluate the relative chlorophyll content and oxidative stress of these weeds growing in pots. The total content of phenols in the extract and its antioxidant activity were determined before bioassays. The TPC was 43.3 ± 1.5 mg GAE g^{-1} dry extract, while IC50 value of DPPH radical scavenging activity was 0.651 mg ml^{-1} and FRAP value was 38.7 ± 1.5 µmol Fe^{2+} g^{-1} d.e., UHPLC-MS analysis was carried out to identify and quantify the major compounds in it. A total of 15 phenolic compounds in the extract were quantified, including 4 flavonoids, 7 hydroxybenzoic acids, and 4 hydroxycinnamic acids. Delphinidin was the most abundant (32.5 mg g^{-1} d.e.), followed by 4-hydroxybenzoic acid (1.810 mg g^{-1} d.e.), rutin (1.533 mg g^{-1} d.e.), p-coumaric acid (1.131 mg g^{-1} d.e.), quercetin (1.071 mg g^{-1} d.e.), gallic acid (0.946 mg g^{-1} d.e.) and protocatechuic acid (0.598 mg g^{-1} d.e.). The seed bioassay confirmed the inhibitory effect of leaf extract on the seed germination and early seedling growth of the tested weeds at all concentrations. In all tested plants the inhibition of seed germination ranged from 68 to 100%, while the inhibitory effect on early seedling growth (radical and shoot length) was ≥ 72% and up to 97–100% for root and 74 to 100% for shoot growth. The results obtained from the pot bioassays revealed significant oxidative stress and a decrease in the relative chlorophyll content. These findings evinced that *A. syriaca* leaf extract had a significant phytotoxic effect on tested weeds. However, further studies under field conditions are needed to confirm the presented results and to check its herbicidal potential.

Keywords Phytotoxicity, Invasive weeds, Phenols, Seed and pot bioassay, Chlorophyll content, Oxidative stress

Chemical herbicides have played a crucial role in the historical development of weed management. Despite their effectiveness in controlling weeds, their persistent use has led to weeds developing resistance to some herbicides[1]. Harmful effects of herbicides on the environment and human health, an ever-increasing number of herbicide-resistant weed species, slower development of novel herbicides, withdrawal of many herbicides from use, and intensified focus on organic farming are some of the main factors that have promoted environmentally friendly approaches to weed control in recent decades[2–4]. Plants with herbicidal properties and the potential for producing allelochemicals have been the focus of the chemical industry because weeds develop resistance to synthetic chemicals; therefore, new bioactive molecules are critically needed[5–7]. Plant secondary metabolites from classes of phenolic compounds, alkaloids, terpenoids, and sulfur-containing compounds are the most common bioactive molecules[8]. Research has recently focused on allelopathy, and a variety of studies have been published on these chemically mediated plant-plant interactions[9–13]. Allelopathy, as a relevant approach, refers to the impact of plants upon neighboring plants and/or their associated microflora and/or macrofauna by the production of allelochemicals whereby often these allelochemicals typically interfere with plant growth

[1]Laboratory of Weed Research, Institute of Pesticides and Environmental Protection, Banatska 31B, Belgrade 11080, Serbia. [2]Department of Weed Science, Faculty of Agriculture, University of Zagreb, Svetošimunska Cesta 25, Zagreb 10 000, Croatia. [3]Laboratory of Chemistry and Ecotoxicology, Institute of Pesticides and Environmental Protection, Banatska 31B, Belgrade 11080, Serbia. [4]Jelena Gajić Umiljendić and Marija Sarić-Krsmanović have contributed equally to this work. ✉email: pecikos@gmail.com

but they may also result in stimulation of growth[14]. Plants normally exude their metabolic products through roots, volatilize them from aboveground parts, or leave them in the soil after decomposition[12,15], which may result in seed germination inhibition or delay, inhibition of root system growth or radicle burst, lack of root hair, coleoptile elongation, etc.[13]. Natural products provide a variety of compounds that can be used as major components in the synthesis of new bioherbicides intended either for independent use or in combination with chemical herbicides in a system of integrated control[16]. Bioherbicides are made from naturally occurring components with allelopathic properties that are potentially degradable, which represents a significant advantage over chemical herbicides in terms of application safety[17].

Asclepias syriaca L. (common milkweed) is a perennial, broadleaf species with a large habit and simple stems that was originally obtained from North America. Since the nineteenth century, it has also been found in Europe, mostly in ruderal habitats but also in crops[18–22]. This plant has powerful roots and a large leaf area and produces hundreds of seeds that can germinate quickly, especially in fertile, well-drained soils[23]. At the same time, *A. syriaca* has highly competitive and allelopathic potential. These properties make it a strong competitor in the community for other species and a good source of bioherbicides[24].

The allelopathic potential of *A. syriaca* was known as early as 1956[25], and over the years, several researchers have studied it[26,27]. Data on the chemical composition have been found in the literature[28,29], and allelopathic potential of extracts isolated from the whole plant or roots of *A. syriaca* on seeds of cultivated plants has been reported[30,31]. On the other hand, according to our knowledge, there is no data in the literature about the impact of *A. syriaca* leaf extract on seed germination and the early growth of weed plants. Therefore, the present study focused on (I) the analysis of total phenolic content (TPC) and identification and quantification of major phenolic compounds in the leaf extract (LE) of *A. syriaca;* (II) the evaluation of the antioxidant activity of *A. syriaca* LE; (III) evaluation in vitro of the phytotoxic effect of the LE of *A. syriaca* on the seed germination and seedling growth of three invasive weeds (*Amaranthus retroflexus* L., *Chenopodium album* L., and *Iva xanthifolia* Nutt.); and (IV) evaluation of oxidative stress, relative chlorophyll content and soluble protein content in growing weed plants in vivo.

Materials and methods

Plant material

Leaves of *A. syriaca* were collected in July 2022 in Surcin (Srem Region, Serbia) on uncultivated land. Fresh leaves of *A. syriaca* were shade-dried on paper at a temperature of 20°C–22°C for 30 days and then stored in paper bags. Seeds of *A. retroflexus* and *C. album* were collected in fields around Batajnica (Srem Region, Serbia), while *I. xanthifolia* was collected in fields around Novi Karlovci (Srem Region, Serbia) in October 2021. The collection of plant material was done in compliance with relevant institutional and national guidelines and legislation[32]. Identification of the species was performed according to Josifović and Sarić[33].

A. syriaca leaf extract preparation

The liquid–solid ultrasonic-assisted extraction method described by Sarić-Krsmanović et al.[34] was used to obtain phenolic-rich *A. syriaca* LE. Seven solvents with different polarities (hexane, ethyl-acetate, acetone, acetonitrile, ethanol, methanol, and distilled water) were used sequentially in a series (in a ratio 1:1 (w/v)). Extraction included homogenization in an ultrasonic bath for 15 min at 30°C. All seven extracts obtained after filtrations were evaporated to dryness, dry matter from all fractions were washed with distilled water and merged into one container, freeze-dried, and the resulting dry powdered extract (with an average native extract ratio of 12.5:1) was stored at − 20°C before further analysis.

Phytochemical analyses of *A. syriaca* leaf extract

Determination of the total phenolic content (TPC) of *A. syriaca* LE was performed using the Folin-Ciocalteu method[35] with minor modifications as described in a previously published paper[36]. The absorbance was measured at 760 nm with a spectrophotometer (Perkin Elmer, Lambda Bio), gallic acid (GAE) was used as an equivalent for calibration curve preparation, and the results are expressed as mg gallic acid per g of dry extract (mg GAE g^{-1} d.e.).

The determination of the LE radical scavenging capacity was conducted using the DPPH assay[37], while the reducing potential of LE was measured using the FRAP assay[38], as previously described[39]. To evaluate the free radical scavenging activity (DPPH assay), the absorbance was measured with a spectrophotometer (Perkin Elmer, Lambda Bio) at 517 nm, and the antioxidant activity was calculated using the following formula: % inhibition = $((Abs_{control} - Abs_{sample})/Abs_{control}) \times 100$. The IC_{50} values (concentrations of sample required to scavenge 50% of free radicals) were calculated, and ascorbic acid was used as a positive control. To evaluate the reducing potential (FRAP assay), the absorbance was measured at 593 nm by a spectrophotometer (Perkin Elmer, Lambda Bio), ferrous sulfate heptahydrate ($FeSO_4 \times 7H_2O$) was used as the equivalent for calibration curve preparation, and the results are expressed as µmol of ferric ions (Fe^{2+}) per g of dry extract. Ascorbic acid was used as a positive control.

Quantification of phenolic compounds in *A. syriaca* LE was obtained by ultra-high-pressure liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS). The experimental work was carried out using a UHPLC 1290 Infinity II instrument (Agilent Technologies, Santa Clara, CA, USA), with a quaternary pump, a column oven, and an autosampler, interfaced to the triple quadrupole mass spectrometer (TQ MS) (Series 6470 TQ, Agilent Technologies, Santa Clara, CA, USA) equipped with Agilent Jet Stream (AJS) electrospray ion source (ESI) source. Separation of compounds was performed using a Zorbax Eclipse Plus C18 column RRHD (50 mm × 2.1 mm; 1.8 µm, Agilent Technologies). The mobile phase was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). The following gradient was used: 0–1 min, 5% B; 1–6

min, 5–50% B; 6–10 min, 50–90% B; 10–12 min, 90% B; 12–14 min, 90–5% B; 14–17 min, 5% B. During analysis, the mobile phase flow rate was 0.30 $mLmin^{-1}$, the column temperature was 30 °C and the injection volume was 2 µL. After separation, the compounds were analyzed using a mass detector. Positive and negative ion modes were recorded (separately) and the instrument was operated in Dynamic Multiple Reaction Monitoring (MRM) modes (to increase the analysis specificity) under following conditions: capillary voltage, 3000 V, nozzle voltage, 1500 V, desolvation gas (nitrogen) temperature, 250 °C, desolvation gas (nitrogen) flow, 12 $Lmin^{-1}$, nebulizer, 30 psi, sheat gas (nitrogen) temperature, 300 °C, sheat gas (nitrogen) flow, 11 $Lmin^{-1}$. Different mass spectrometric parameters such as ionization mode, fragmentor voltage (FV), and collision energy (CE) were determined for each MRM transition that was monitored (see Table S1). System operation (data collection and processing) was controlled by Agilent Technologies MassHunter software (revisions B.06.01 and B.07.00). External calibration curves using a least-squares linear regression analysis were used for the assay of compounds under investigation. Standard stock solutions were prepared in methanol and further diluted with water/0.1% formic acid to obtain calibration standards at concentrations in the range between 0.01 and 2.5 µg mL^{-1}. The coefficients of determination (R^2) of the calibration curves ranged between 0.9973 and 0.9999 (see Table S2).

Seed bioassay

In vitro evaluation of the seed germination and seedling growth of *A. retroflexus, C. album,* and *I. xanthifolia* treated with LE from *A. syriaca* was performed under controlled conditions. The test seeds were soaked in a 2% thiourea [$SC(NH_2)_2$] solution for 24 h to overcome their dormancy. The seeds were then surface sterilized in 5% (w/v) sodium hypochlorite solution (NaOCl) for 3 min and then rinsed three times with distilled water. A total of 25 soaked seeds were placed on filter paper (70 gm^{-1}, 1602 N, Ahlstrom-Munksjö Germany GMBH, Bärenstein, Germany) in each Petri dish (40 mm diameter). *A. syriaca* LE was diluted in distilled water to concentrations of 1%, 2%, and 3% (w/v), and 3 mL was added to each Petri dish. Distilled water served as the control. The dishes were kept in a climate chamber (VELP, Incubator FOC, Serbia) in darkness, at a temperature of 27 ± 1°C. The final germination (%) was calculated, and the radical and shoot lengths of the plants were measured after seven days. The experimental design was a randomized complete block with four replications, which were repeated twice, and the data were combined for analysis.

Plant bioassay

In vivo, the phytotoxic effect of LE on *A. syriaca* was evaluated under controlled conditions in pots on three test plants: *A. retroflexus*, *C. album,* and *I. xanthifolia*. The detailed methods used for the alleviation of seed dormancy, soil preparation, and climatic conditions were described in a previous publication[40]. Five plants in the early growth stage were transplanted from plastic containers into a single pot with four replicates for each treatment. *A. syriaca* LE was diluted to concentrations of 1%, 2%, and 3% (w/v) in distilled water supplemented with 0.1% wetting agent (Trend® 90, ethoxylated isodecyl alcohol 900 g L^{-1}, DuPont), while distilled water supplemented with a 0.1% wetting agent was used as the control. A single treatment (3 mL) was applied with a TLC sprayer (CAMAG®, Muttenz, Switzerland) over *A. retroflexus*, *C. album,* and *I. xanthifolia* at the growth stage of two true leaves. Plant growth was monitored for 14 days in a growth chamber.

Visible injury was assessed 14 days after treatment (DAT) using a percentage scale on which 0% indicated no visible injury and 100% indicated complete necrosis and plant death[41].

Chlorophyll measurement on fresh leaves was carried out using a hand-held CCM-200 chlorophyll content meter (ADC BioScientific Ltd., UK), which is valuable for predicting chlorophyll content. The CCM-200 plus meter measures the light transmittance throughout the leaf at two different wavelengths, 653 nm (red light, absorbed by chlorophyll pigments) and 931 nm (infrared light, not absorbed by chlorophyll), of radiation through the leaf and provides the chlorophyll content index (CCI) value. One leaf per plant in each pot (5 leaves × 3 readings per pot) was measured for all treatments at 7 and 14 DAT. The average of three readings from the center of each leaf blade was used as the CCI value per leaf. The dorsal side of the leaf was always positioned toward the emitting window of the instrument, and large veins were avoided. The percentage (%) of changes in the relative chlorophyll content in treated plants is expressed as the measured content in untreated, control plants.

For the determination of soluble protein content, plant samples (leaves) were taken at 14 DAT and stored at − 20°C until analysis. The soluble protein content was determined based on the interaction between Coomassie brilliant blue G-250 dye and proteins[42]. After the dye had developed, the absorbance of the reaction mixture was measured by spectrophotometry at a wavelength of 595 nm. Ten solutions with different concentrations of bovine serum albumin (BSA) were used to generate the standard curve.

To determine the oxidative stress parameters, the above parts of the tested weeds were macerated with extraction buffer (0.1 M potassium phosphate buffer (pH 7.0) supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM sodium ascorbate) after the addition of ~2% w/w polyvinylpyrrolidone (PVP), as previously described[36], and the resulting supernatant was used as an enzymatic source.

The intensity of lipid peroxidation (LP) was determined by a method based on measuring the increase in absorbance due to the formation of the malondialdehyde-thiobarbituric acid (TBA-MDA) complex[43]. The amount of malondialdehyde was calculated using the molar extinction coefficient of 155 $mM^{-1}\,cm^{-1}$. The total amount of lipid peroxidation products was expressed as µmol MDA per gram of fresh weight (µmol MDA g^{-1} f.w.).

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined as the ability of plant enzyme extracts to inhibit the photochemical reduction of nitro-tetrazolium blue (NBT)[44]. One unit of SOD activity (U) was defined as the amount of enzyme required to inhibit NBT reduction by 50%. Enzyme activity was expressed as U per gram of fresh weight (U g^{-1} f.w.).

Catalase (CAT) (EC 1.11.1.6) activity was measured by a method based on monitoring absorbance changes due to hydrogen peroxide dissociation[45]. The enzyme activity was calculated using the molar extinction coefficient of 0.036 mM^{-1} cm^{-1}. One unit of CAT activity (U) was defined as µmol of dissociated hydrogen peroxide per minute. Enzyme activity was expressed as U per gram of fresh weight (U g^{-1} f w.).

Ascorbate peroxidase (APX) (EC 1.11.1.1) activity was measured using ascorbic acid as a substrate. The method is based on monitoring absorbance changes due to ascorbate oxidation, i.e., monodehydroascorbic acid (MDHA) formation[46]. Enzyme activity was calculated using the molar extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of APX activity (U) was defined as µmol of MDHA formed per minute. The activity of the enzyme was expressed as U per gram of fresh weight (U g^{-1} f.w.).

Guaiacol peroxidase (GPOD) (EC 1.11.1.7) activity was measured using guaiacol as a substrate. This method is based on monitoring absorbance changes due to guaiacol oxidation, i.e., tetraguaiacol formation[47]. Enzyme activity was calculated using the molar extinction coefficient of 2.47 mM^{-1} cm^{-1}. One unit of GPOD activity (U) was defined as µmol of tetraguaiacol formed per minute. The activity of the enzyme was expressed as U per gram of fresh weight (U g^{-1} f.w.).

Statistical analysis

The data for germination, seedling length (radical and shoot), and soluble protein content were analyzed by one-way factorial analysis of variance (ANOVA) using the STATISTICA 8.0 software package. When F values were statistically significant ($P < 0.05$), treatments were compared using Fisher's least significant difference (LSD) test.

The results of the phytochemical analyses of *A. syriaca* LE, the determination of its radical scavenging capacity and reducing potential, and the results of the measurements of the oxidative stress parameters are presented as the mean ± standard deviation of three replications. Statistical analysis of the results of the oxidative stress parameter measurements was performed by ANOVA coupled with Tukey's honestly significant difference (HDS) test using GraphPad Prism statistical software.

Results

Analyses of phenolics in *A. syriaca* leaf extract

The results showed that *A. syriaca* LE contained a moderate amount of phenols. The TPC was 43.3 ± 1.5 mg GAE g^{-1} d.e. The results of UHPLC-MS/MS analysis of the obtained *A. syriaca* LE are presented in Table 1. Among the analyzed phenols, a total of 15 phenolic compounds were quantified in the extract, including 4 flavonoids (flavonols: quercetin, rutin, and kaempferol and anthocyanidin: delphinidin), 7 hydroxybenzoic acids (gallic acid, 4-hydroxybenzoic acid, protocatechuic acid, salicylic acid, vanillic acid, benzoic acid and syringic acid), and 4 hydroxycinnamic acids (chlorogenic acid, ferulic acid, caffeic acid and p-coumaric acid). Delphinidin made the greatest contributions to the amount of phenols detected in LE (32.5 mg g^{-1} dry extract), followed by 4-hydroxybenzoic acid (1.810 mg g^{-1} dry extract), rutin (1.533 mg g^{-1} dry extract), p-coumaric acid (1.131 mg g^{-1} dry extract), quercetin (1.071 mg g^{-1} dry extract), gallic acid (0.946 mg g^{-1} dry extract) and protocatechuic acid (0.598 mg g^{-1} dry extract). Other phenols detected were present in amounts below 0.5 mg g^{-1} dry extract).

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging ability of *A. syriaca* LE was lower compared to that of ascorbic acid (Fig. 1). Although this extract achieved an inhibition of 82% at the highest tested concentration (2 mg ml^{-1}), ASC intercepts 93% of DPPH already at 0.4 mg ml^{-1}. Thus, the calculated IC_{50} value for *A. syriaca* LE was 0.651 mg ml^{-1}, which was significantly higher than the IC_{50} value of ascorbic acid (0.227 mg ml^{-1}).

The ferric-reducing antioxidant power (FRAP) of LE was significantly lower than that of ascorbic acid. The FRAP value of LE was 38.7 ± 1.5 µmol Fe^{2+} g^{-1} d.e., while ascorbic acid had an FRAP of 2576.3 ± 21.5 µmol Fe^{2+} g^{-1} d.e. The results show that *A. syriaca* LE has a lower metal-reducing ability than its free radical scavenging ability.

Seed bioassay

A. syriaca LE at concentrations of 1%, 2%, and 3% had a significant effect on the seed germination and seedling growth of *A. retroflexus*, *C. album,* and *I. xanthifolia* (Table 2). In general, the inhibition of seed germination in all tested plants at the lowest LE concentration (1%) ranged from 68 to 85.5%, while the inhibition at the highest LE concentration (3%) was 94 to 100%. The inhibition of seed germination increased proportionally to increasing extract concentrations for all tested plants and all parameters. Statistically significant differences ($P < 0.05$) were found between the control and all extract concentrations (Table 2).

Similarly, all concentrations of LE of *A. syriaca* had a significant inhibitory effect (≥ 72%) on early seedling growth (radical and shoot length) of all tested weed species. The highest concentration (3%) of *A. syriaca* LE inhibited shoot length by 100% in the tested plant species *A. retroflexus* and *I. xanthifolia*, while it inhibited shoot length by 97% in *C. album*. Additionally, the inhibition of radical length for all three plant species at the highest LE concentration (3%) was 94–100%. Statistical analysis confirmed significant differences ($P < 0.05$) between the control and all extract concentrations for both parameters of seedling growth (Table 2).

The results of the seed bioassay showed that *A. retroflexus* seeds were slightly more sensitive than *C. album* and *I. xanthifolia* seeds, because the lowest concentration (1%) of LE of *A. syriaca* caused the inhibition of seed germination and the radical length greater than 86%. Treatment with the highest concentration (3%) of LE of *A. syriaca*, in all three weed species caused a significant inhibition (≥ 94%) of the measured parameters.

Plant bioassay

The present study focused on evaluating the suppression of weeds (*A. retroflexus, C. album,* and *I. xanthifolia*) by three different concentrations of LE from *A. syriaca*. In the second assessment (14 DAT), the highest

Compound	RT	Transition	Concentration (mg g^{-1} of dry extract)
Content of Phenolic Compounds			
Gallic acid	1.4918	169.0—> 125.0	0.946 ± 0.047
Protocatechuic acid	3.1669	153.0—> 109.0	0.598 ± 0.030
4-Hydroxybenzoic acid	4.6150	137.0—> 93.0	1.810 ± 0.090
Epigallocatechin	4.9666	304.9—> 124.9	n.d
Catechin	5.1583	289.0—> 245.0	n.d
Chlorogenic acid	5.3051	353.0—> 191.0	0.318 ± 0.016
Vanillic acid	5.4441	167.0—> 152.0	0.266 ± 0.013
Malvidin-3,5-diglucoside	5.5512	655.2—> 331.1	n.d
Caffeic acid	5.5677	179.0—> 135.0	0.347 ± 0.017
Delphinidin-3-rutinoside	5.7995	611.2—> 303.1	n.d
Syringic acid	5.8888	197.0—> 182.0	0.030 ± 0.001
Petunidin- 3-glucoside	5.9174	479.1—> 317.1	n.d
Epicatechin	5.9616	289.0—> 245.0	n.d
Cyanidin	6.0370	287.0—> 137.0	n.d
Sinapinic acid	6.1789	223.0—> 193.0	n.d
Cyanidin-3-rutinoside	6.2455	595.2—> 287.1	n.d
Malvidin-3-glucoside	6.3204	493.1—> 331.1	n.d
Umbeliferone	6.5073	163.0—> 107.0	n.d
Daidzin	6.5706	417.1—> 255.0	n.d
p-Coumaric acid	6.6376	163.0—> 119.0	1.131 ± 0.057
Glycitil	6.6513	447.2—> 285.0	n.d
Taxifolin	6.7553	303.2—> 284.7	n.d
trans-Ferulic acid	6.8093	193.0—> 134.0	0.469 ± 0.023
Benzoic acid	7.1674	121.0—> 77.0	0.297 ± 0.015
Genistin	7.2535	433.1—> 271.0	n.d
Salicylic acid	7.3816	137.0—> 93.0	0.132 ± 0.007
Naringin	7.4670	581.0—> 272.8	n.d
Rutin	7.6478	609.0—> 300.0	1.533 ± 0.077
Delphinidin	7.6528	303.0—> 229.0	32.500 ± 1.625
Resveratrol	7.7601	227.0—> 143.0	n.d
Rasveratol	7.8341	227.0—> 185.0	n.d
Ellagic acid	7.8826	301.0—> 145.0	n.d
Myricetin	8.0232	317.0—> 151.0	n.d
Juglone	8.3505	189.0—> 161.0	n.d
trans-Cinnamic acid	8.5165	147.0—> 103.0	n.d
Naringin	8.5672	579.0—> 271.1	n.d
Esculetin	8.7086	339.1—> 176.7	n.d
Quercetin	8.7606	301.0—> 151.0	1.071 ± 0.054
Naringenin	8.7961	271.0—> 151.0	n.d
Luteolin	9.0090	285.1—> 133.2	n.d
Kaempherol	9.3487	285.0—> 285.0	0.055 ± 0.003
Apigenin	9.4682	268.9—> 117.0	n.d

Table 1. Identification and quantification of phenols in *Asclepias syriaca* leaf extract. †n.d., not detected; values are presented as the mean ± SD (n = 3).

concentration (3%) caused visual injuries in the form of light-yellow chlorosis and leaf necrosis, ranging from 15 to 35%, compared to the untreated plants. The most pronounced symptoms were observed in *A. retroflexus*. The other two concentrations caused no visible damage to the leaves of any of the three weed species or were negligible (less than 10%).

The sensitivity of the tested weeds to the LE of *A. syriaca* was also confirmed by changes in the relative chlorophyll content, expressed through the CCI value. The greatest reduction (11–31%) in CCI was detected for *A. retroflexus* at 14 DAT. The other two weed species were less sensitive, and the CCI decreased from 2 to 18%. Moreover, *I. xanthifolia* treated with the lowest concentration (1%) of LE of *A. syriaca* showed a minimal increase in CCI. Consequently, statistical analysis showed that the CCI values of control plants differed from the values of *C. album* and *I. xanthifolia* treated with 3% of LE, while A. *retroflexus* was more sensitive, so differences compared to the control were determined for plants treated with 2% and 3% of LE. On the other

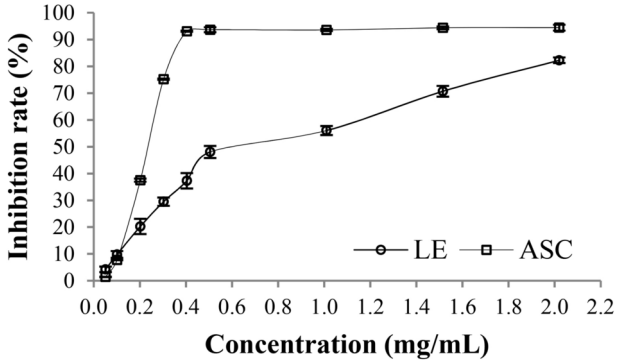

Fig. 1. DPPH radical scavenging ability of *Asclepias syriaca* leaf extracts (LE- *Asclepias syriaca* leaf extract; ASC-ascorbic acid).

Parameters	Control	LE 1%	LE 2%	LE 3%
Amaranthus retroflexus				
Germination (%)	100 a	14.5 b	12.5 c	2.1 d
Radical length (cm)	2.45 a	0.34 b	0.28 b	0.06 c
Shoot length (cm)	2.86 a	0.75 b	0.21 c	–
Seedling length (cm)	5.31 a	1.09 b	0.43 c	0.06 d
Chenopodium album				
Germination (%)	100 a	31.9 b	19.1 c	6.4 d
Radical length (cm)	2.10 a	0.39 b	0.31 b	0.13 c
Shoot length (cm)	2.49 a	0.20 b	0.10 b	0.08 b
Seedling length (cm)	4.57 a	0.50 b	0.44 bc	0.20 c
Iva xanthifolia				
Germination (%)	100 a	22.5 b	15.0 c	-
Radical length (cm)	1.78 a	0.50 b	0.45 b	-
Shoot length (cm)	1.57 a	0.34 b	–	–
Seedling length (cm)	3.36 a	0.84 b	0.45 b	–

Table 2. Development of *Amaranthus retroflexus, Chenopodium album,* and *Iva xanthifolia* seedlings in the control treatment and under different concentrations of *Asclepias syriaca* leaf extract (LE). The data are reported as the median. Differences were evaluated by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test, $P < 0.05$. Means marked by different row-wise letters (a, b, c, d, e) differ significantly ($P < 0.05$).

hand, statistically significant differences were found between treatments in all three tested weed species, which is shown in Fig. 2.

In this study, the effect of different concentrations of *A. syriaca* LE on the soluble protein content was investigated. A decrease (10–15%) in water-soluble proteins was revealed, but there was no correlation between the changes in this parameter and LE concentrations in the tested plants. The results were inconsistent for all tested plants in both replicates of the experiment. Therefore, this parameter is irrelevant for the evaluation of plant susceptibility to LE of *A. syriaca* in bioassays, and the results of the soluble protein contents are not shown.

In vivo evaluation of the effect of *A. syriaca* leaf extract on oxidative stress in growing plants

The effects of *A. syriaca* LE on the oxidative stress of *A. retroflexus*, *C. album,* and *I. xanthifolia* are presented in Figs. 3, 4 and 5.

The results showed that the intensity of lipid peroxidation significantly increased in *A. retroflexus* after treatment with *A. syriaca* LE (Fig. 3a). At the highest concentration of LE (3%), the MDA content in the weeds increased by ~180%, while slightly lower but still high oxidative damage to the lipids occurred after treatment with 2 and 1% LE (the MDA content doubled). The activity of superoxide dismutase, the first line of enzymatic defense against ROS-induced damage, was unchanged after treatment with 1% LE but significantly increased (by ~10%) after treatment with 2% LE but strongly decreased (by approximately 40%) after treatment with 3% LE (Fig. 3b). The activity of ascorbate peroxidase also decreased. APX is responsible for scavenging H_2O_2 in the cytosol and chloroplasts, and the decrease was not statistically significant after treatment with 1% LE but was very pronounced after treatment with 2% and 3% LE (a decrease of 30–50%), with no statistically significant

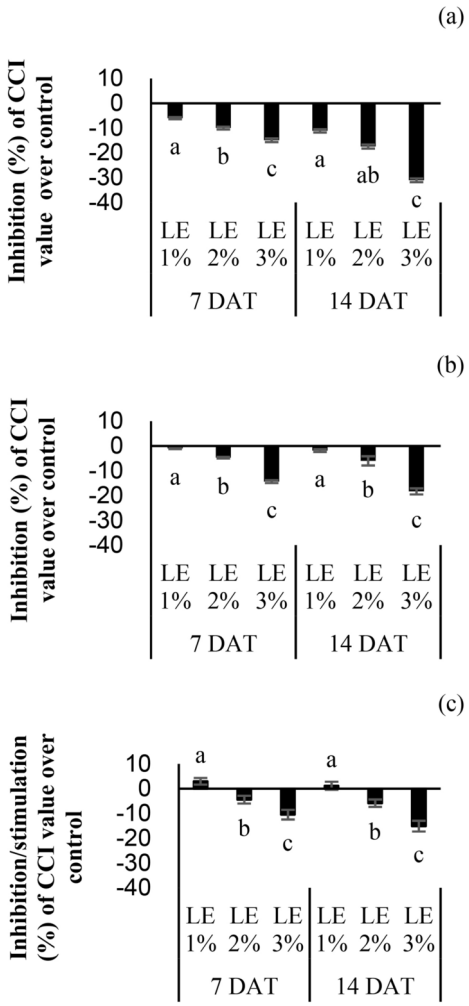

Fig. 2. Relative chlorophyll content of *Amaranthus retroflexus* (**a**), *Chenopodium album* (**b**), and *Iva xanthifolia* (**c**) after the application of different concentrations of *Asclepias syriaca* leaf extract (LE).

differences between these two concentrations (Fig. 3d). The activities of CAT and GPOD were unchanged after the treatments (Fig. 3c and e).

For *C. album*, treatment with *A. syriaca* LE did not cause significant damage to lipids. The MDA content did not significantly differ between all the plants and the control plants (Fig. 4a). Accordingly, the SOD activity in *C. album* was also unchanged after treatment with the extracts used (Fig. 4b). Regardless, the plants treated with LE experienced stress and produced excessive amounts of H_2O_2, as the activities of CAT and GPOD increased by approximately 70%, 80%, and 150% after treatment with 1%, 2%, and 3% LE, respectively (Fig. 4c and e). The activity of APX did not change after the treatments (Fig. 4d).

For *I. xanthifolia,* a statistically significant increase in the lipid peroxidation intensity was observed after treatment with *A. syriaca* LE at all concentrations (Fig. 5a). Lipid peroxidation occurred in all treated plants as the MDA content increased by approximately 70–100%. SOD activity slightly decreased after treatment with 1% LE, but the difference was not statistically significant, while the activity of this enzyme decreased significantly after treatment with 2% and 3% LE (by ~30–40%), with no statistically significant differences between these two concentrations (Fig. 5b). The activity of CAT in peroxisomes decreased significantly after treatment with 2% and 3% LE (by ~60%). The situation was similar for APX located in the cytosol and chloroplasts, as this enzyme decreased significantly (on average by 70%) after treatment with LE at all concentrations (Fig. 5c and d). GPOD activity increased by approximately 40% and 50% after treatment with 2% and 3% LE, respectively, and thus played the main role in the attempt to stop lipid peroxidation in affected plants (Fig. 5e).

Discussion

For the analysis of phenolics in *A. syriaca* leaf extract, the lack of sufficient literature data regarding specific phenolic compounds, combined with variability arising from different extraction methods (which can significantly influence the metabolite profile), makes it difficult to reliably compare data across studies. In this study, a total of 15 phenolic compounds were quantified in the obtained extract. Regarding the phenolics identified, to the best of our knowledge, there is no previously published literature reporting the presence

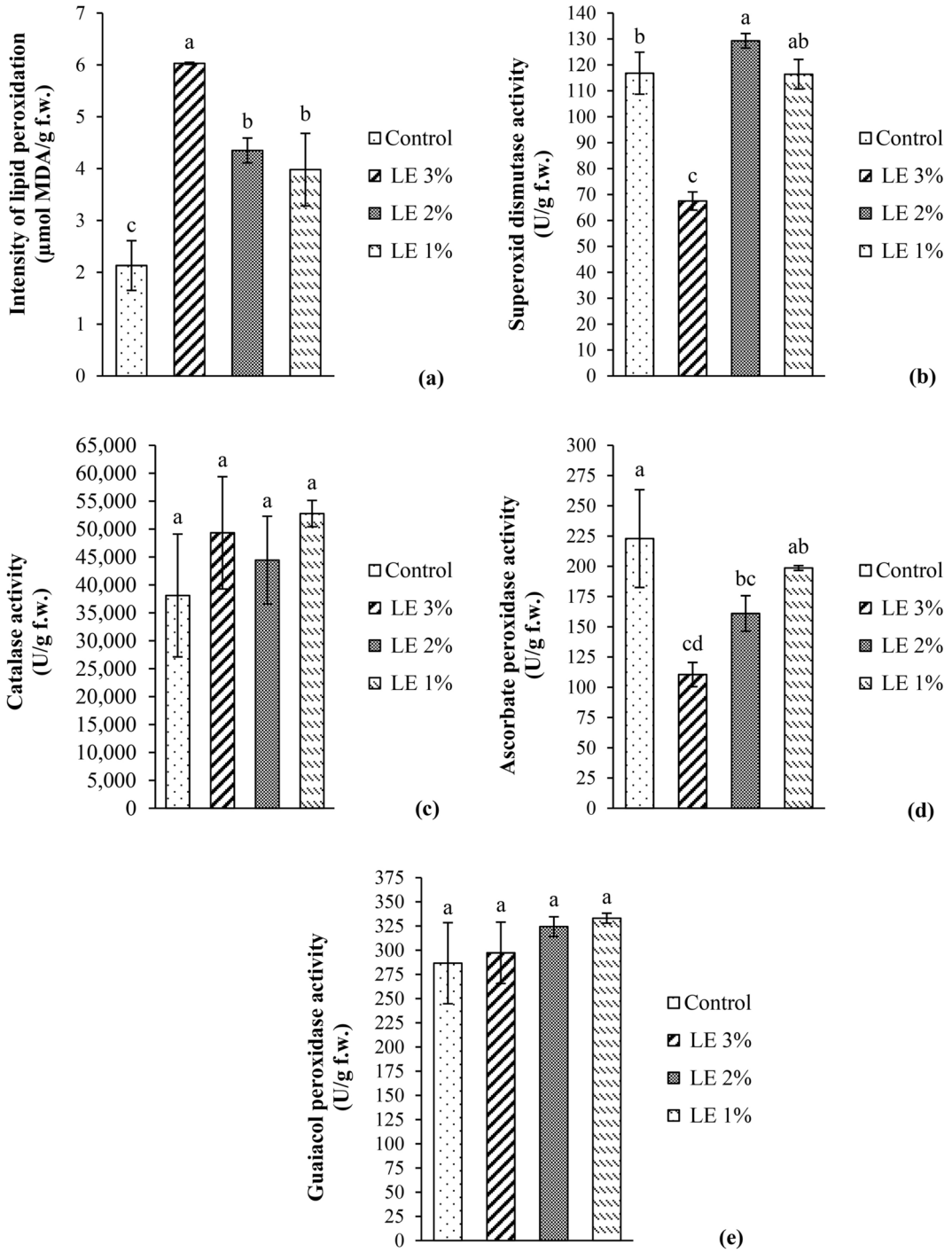

Fig. 3. Lipid peroxidation (**a**) and the activities of the antioxidant enzymes superoxide dismutase (SOD) (**b**), catalase (CAT) (**c**), ascorbate peroxidase (APX) (**d**) and guaiacol peroxidase (GPOD) (e) in *Amaranthus retroflexus* in response to treatment with 1, 2, and 3% *Asclepias syriaca* leaf extract (LE). The data are presented as the means ± SDs. Values marked with different letters within a group differ significantly based on Tukey's HSD test ($P < 0.05$).

of salicylic acid, rutin, or delphinidin in *A. syriaca*. Sikorska et al.[28] identified α-resorcylic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, p-coumaric, chlorogenic, and ferulic acids in two different fractions of *A. syriaca* leaf extract. Campbell[48] reported that the flowers of *A. syriaca* contained gallic acid, while vanillic and chlorogenic acids were found in the leaves. Hainal et al.[49] showed that vanillic, syringic, and p-coumaric acids were the main compounds in aqueous extracts of this plant. Furthermore, Araya et al.[50], after employing complex extraction and separation procedures, isolated five new compounds (including quercetin triglycoside) and 19 known compounds, among them quercetin 3-O-β-galactopyranosyl-(1→2)-β-xylopyranoside,

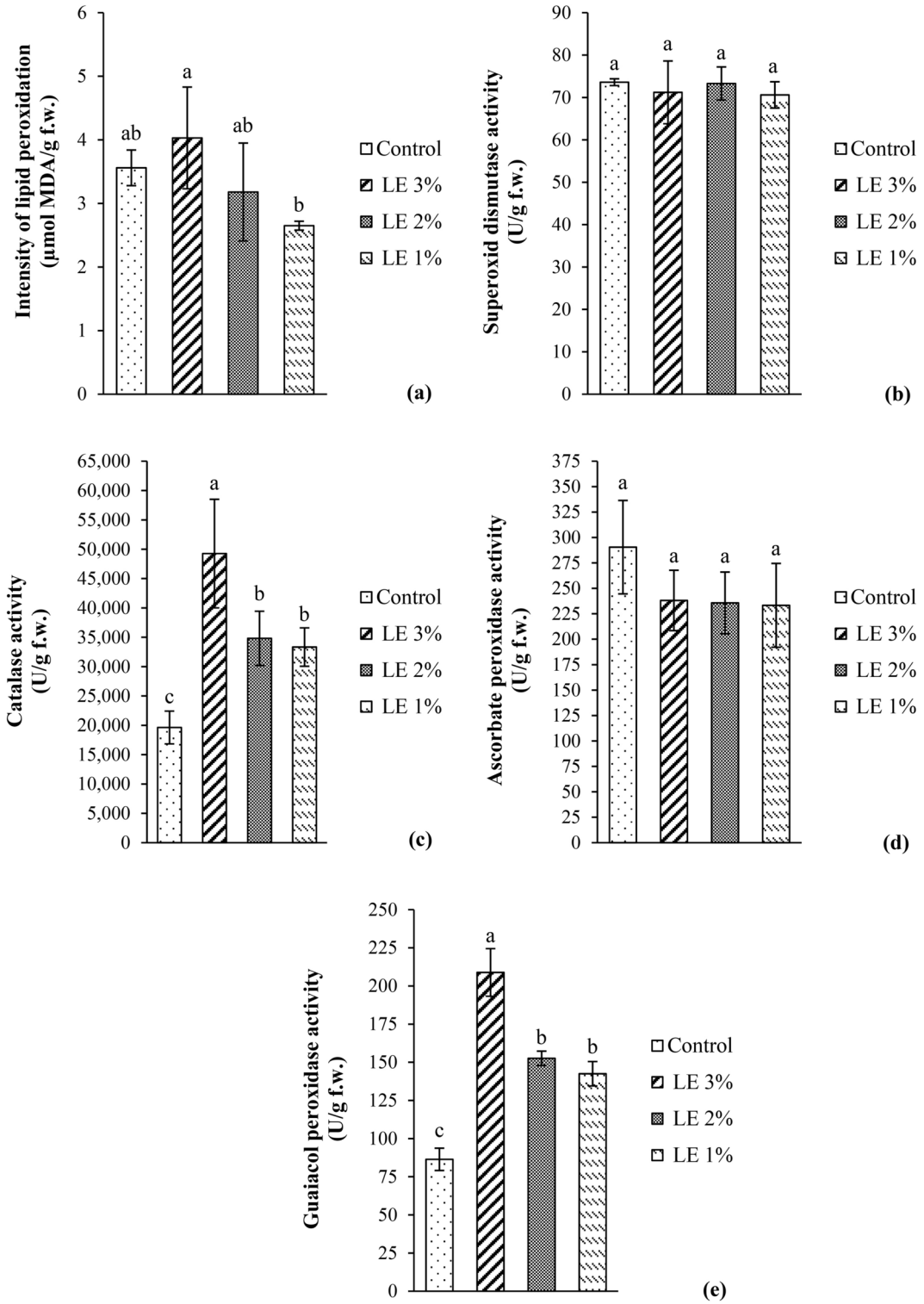

Fig. 4. Lipid peroxidation (**a**) and the activities of the antioxidant enzymes superoxide dismutase (SOD) (**b**), catalase (CAT) (**c**), ascorbate peroxidase (APX) (**d**), and guaiacol peroxidase (GPOD) (**e**) in *Chenopodium album* in response to treatment with 1, 2, and 3% *Asclepias syriaca* leaf extract (LE). The data are presented as the means ± SDs. Values marked with different letters within a group differ significantly based on Tukey's HSD test ($P < 0.05$).

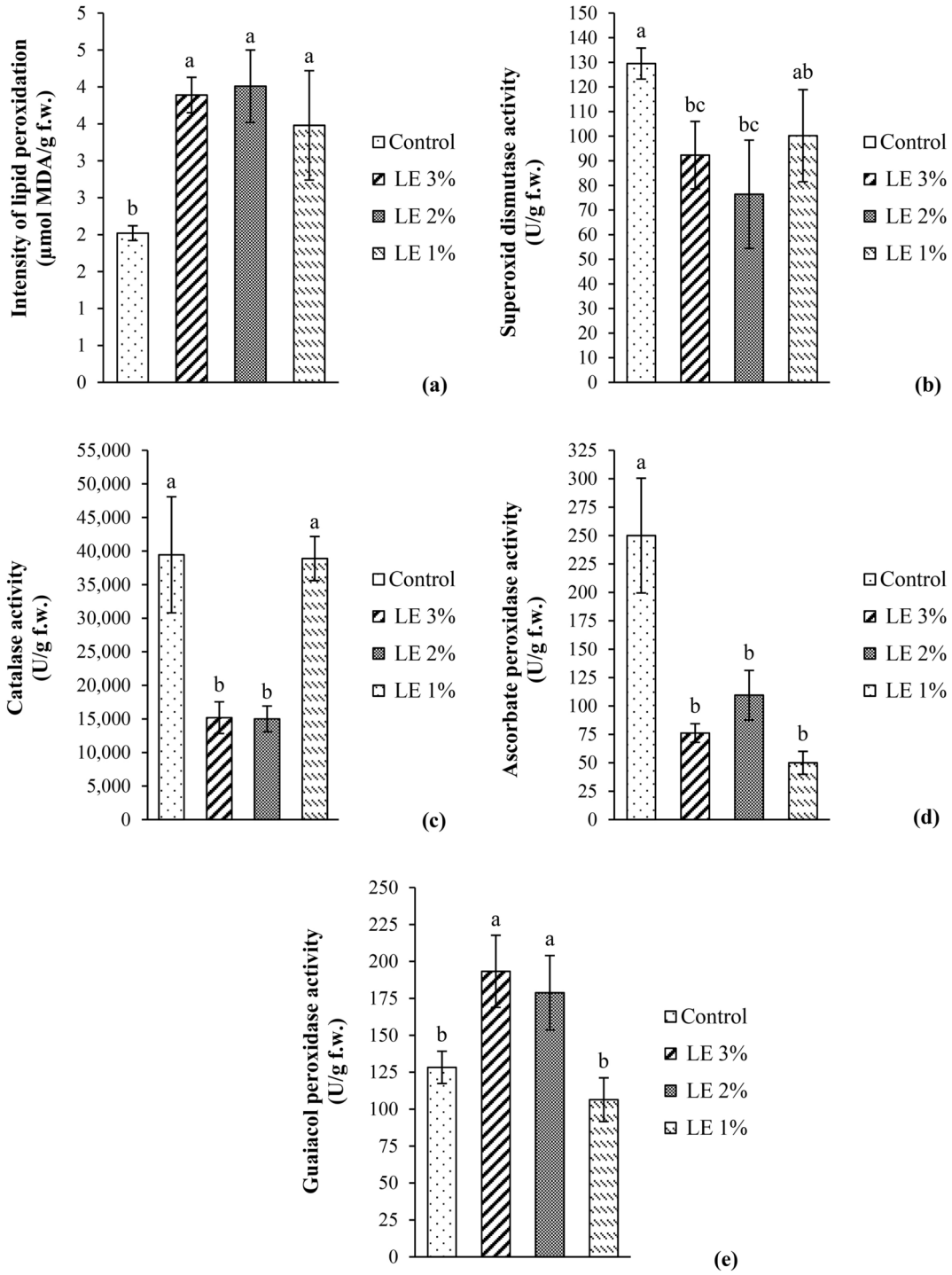

Fig. 5. Lipid peroxidation (**a**) and the activities of the antioxidant enzymes superoxide dismutase (SOD) (**b**), catalase (CAT) (**c**), ascorbate peroxidase (APX) (**d**) and guaiacol peroxidase (GPOD) (**e**) in *Iva xanthifolia* in response to treatment with 1, 2, and 3% *Asclepias syriaca* leaf extract (LE). The data are presented as the means ± SDs. Values marked with different letters within a group differ significantly based on Tukey's HSD test ($P < 0.05$).

kaempferol 3-O-β-galactopyranosyl-(1→2)-β-xylopyranoside, 3′-O-methylquercetin 3-O-β-galactopyranosyl-(1→2)-β-xylopyranoside, quercetin 3-O-β-galactopyranoside, 4-(β-glucopyranosyloxy) benzoic acid, cis- and trans-cinnamic acids, and isovanillinic acid.

A. syriaca is one of the most aggressive invasive weeds worldwide. Numerous studies[19,21–24] have examined the impact of its invasion in the past, revealing that the mechanism of its successful spread is allelopathy, meaning that plants release chemical compounds that have phytotoxic or inhibitory effects on other plants into

the immediate environment. Le Tourneau et al.[25] revealed the allelopathic potential of *A. syriaca* and reported that a water extract of its aerial parts inhibited the seed germination and seedling growth of wheat. Béres and Kazinczi[30] later reported that a shoot aqueous extract of *A. syriaca* completely inhibited (by 100%) the germination of sunflower plants, while seed germination, as well as the fresh and dry weights of maize plants, decreased significantly. Popov et al.[29] reported similar findings that different concentrations of water extract of *A. syriaca* roots exhibited phytotoxic effects on sunflowers and maize regarding their germination and early seedling growth. On the other hand, data on any inhibiting impact on the seed germination percentage and early seedling growth of weed seeds provide a valuable indicator utilizable in weed management programs. The results of this trial revealed that *A. syriaca* LE had a significant impact on the early growth of *A. retroflexus*, *C. album,* and *I. xanthifolia*, as even its lowest concentration (1%) caused inhibition of seed germination exceeding 68%, while radical and shoot length was inhibited over 72%. *C. album* was slightly less sensitive than *A. retroflexus* and *I. xanthifolia*, while shoot length was more sensitive than radical length. However, Csiszar et al.[51] presented contradictory findings, showing that aqueous leachate from *A. syriaca* had stronger inhibitory effects on root growth (42%) than on shoot growth (28%) and seed germination (14%) in *Sinapis alba*.

The concentration of chlorophyll, the principal photosynthetic pigment, has been widely used for assessing the health of plants under a variety of environmental and stress conditions[52,53]. Healthy plants showing vigorous growth are therefore likely to have more of that pigment than those exposed to stress[54]. Reduced contents of leaf pigments resulting from the allelopathic activity of different plant species have been reported previously[55–57]. However, the authors of the present study are unaware of any literature data showing the impact of *A. syriaca* LE on weed species. In this trial, the tested weed species (*A. retroflexus*, *C. album,* and *I. xanthifolia*) were sprayed with several concentrations of *A. syriaca* LE, which resulted in visual injuries and changes in relative chlorophyll content, as indicated by a decrease (%) in the CCI value compared to that of the control plants. *A. retroflexus* was found to sustain the most notable symptoms (up to 35%).

Stress, which results from unfavorable environmental conditions (including the activity of allelochemicals originating from other plant species), activates immune responses in affected plants. Such responses are associated with the rapid overproduction of reactive oxygen species (ROS), which interfere with cellular redox homeostasis. Under stressful conditions, redox homeostasis is maintained by enzymatic and nonenzymatic antioxidants. In response to ROS overproduction in cells, the activity of antioxidative enzymes significantly increases. When the level of ROS is too high and can no longer be reduced by antioxidant defenses, the cell enters a state of oxidative stress in which unrestrained excess ROS may change the structure of nucleic acids and, consequently, their functionality, oxidation of proteins, and lipid peroxidation, ultimately leading to cell death.

In *A. retroflexus,* the oxidative damage to lipids, most likely in cell membranes, increased after treatment with *A. syriaca* LE. An increase in lipid peroxidation after treatment with 1% LE did not provoke changes in antioxidant enzymes, which indicates that the damage was not significant and was probably reduced by nonenzymatic antioxidants. Treatment with 2% LE resulted in increased SOD activity. Superoxide dismutase forms the first line of defense against ROS-induced damage by catalyzing the removal of superoxide radicals through dismutation into oxygen and hydrogen peroxide, eliminating the possibility of hydroxyl radical formation. The activities of the enzymes catalase (CAT) and guaiacol peroxidase (GPOD), both of which are responsible for the dismutation of excessive hydrogen peroxide, were not significantly affected by this treatment. CAT activity in peroxisomes increased only slightly, causing no statistically significant differences compared with that in the control. On the other hand, the activity of ascorbate peroxidase (APX), which is responsible for scavenging hydrogen peroxide in the cytosol and chloroplasts, decreased considerably in weeds treated with 2% LE, which indicates that the enzyme is probably more sensitive to the phytochemicals in LE and may therefore be blocked or damaged by them. Regarding the activities of CAT, APX, and GPOD, treatment with 2% LE most likely caused nonenzymatic antioxidants in *A. retroflexus* to take over the main role in halting lipid peroxidation. Oxidative stress during plant growth should be evaluated again for this trial to determine whether treatment with 2% LE provoked prolonged damage to plants or whether the plants would be able to recover from initial stress. However, treatment of *A. retroflexus* with 3% LE provoked a notable decrease in SOD activity. Coupled with a significant decrease in APX activity and no change in CAT and GPOD activities, these findings indicate that this treatment caused strong enough stress, provoked through allelopathy. Antioxidant enzymes blocked or damaged by treatment with 3% *A. syriaca* LE, which resulted in increased or decreased activity, were unable to stop the production of massive amounts of ROS, which most likely led to cell damage and ultimately to cell destruction.

Regarding *C. album*, the unchanged intensity of lipid peroxidation indicated that treatment with *A. syriaca* LE failed to provoke damage to lipids in cell membranes, so SOD activity remained unchanged. However, plants treated with LE did experience stress, as the activity of CAT in peroxisomes and mitochondria and the activity of GPOD in mitochondria, cytoplasm, chloroplasts, and the ER greatly increased. Treatment with all concentrations of LE most likely provoked excessive production of hydrogen peroxide and thus triggered a rise in CAT and GPOD activities. Both enzymes are highly efficient scavengers of hydrogen peroxide. CAT has a high turnover rate and does not require a reducing equivalent, while GPOD is considered the key enzyme for removing ROS because it is active intracellularly, in the cell wall, and extracellularly. APX activity in *C. album* was not significantly affected by treatment with *A. syriaca* LE, possibly due to the presence of CAT and GPOD, which successfully scavenged the produced H_2O_2. Considering these results, treatments with *A. syriaca* LE will most likely not cause extensive damage to *C. album,* and the plant will recover from the initial stress provoked by allelochemicals in the LE and manage to restore cellular redox homeostasis.

For *I. xanthifolia,* the lipid peroxidation intensity increased similarly after treatment with different concentrations of *A. syriaca* LE, indicating that lipids were damaged at similar levels. When applied to this invasive plant, LE strongly disrupted the activity of enzymes, particularly SOD, CAT, and APX, and the damage to these antioxidative enzymes was highly notable after treatment with 2% and 3% LE. Only the intra- and extracellular

activity of GPOD significantly increased. This enzyme likely plays a major role in stopping lipid peroxidation in affected plants. These results indicate that phytochemicals from the applied LE can cause irreversible damage to lipids, proteins, and DNA, ultimately resulting in cell dysfunction and plant death. However, to confirm this assumption, the oxidative stress that occurred in this trial should be evaluated again during the subsequent growth of *I. xanthifolia* plants.

The oxidative stress of the test weeds provoked by treatment with *A. syriaca* leaf extract may be attributed to the moderate amounts of phenolics. Earlier reports showed that secondary metabolites, including phenols and flavonoids, are primarily responsible for the allelopathic effects of plants[58]. Several previous studies have indicated the high potential of many phenolics and flavonoids to act as herbicidal substances[59]. Several different phytotoxic mechanisms of phenolics are based on changes in cell membrane permeability and increased lipid peroxidation, inhibition of nutrient absorption by affected plants, inhibition of root elongation and cell division of affected plants, inhibition of plant respiration and photosynthesis, changes in the activity and function of certain enzymes in affected plants and/or reduction or inactivation of the physiological activity of affected plant hormones[60]. Allelopathy caused by phenolic compounds most likely results from the synergistic effects of multiple phenolics rather than from the activity of a single substance[61]. The most abundant phenolic in the obtained *A. syriaca* leaf extract was anthocyanidin delphinidin. Literature data regarding allelopathic activities of anthocyanidins is very scarce. To the best of our knowledge there is one study reporting strong allelopathic ability of this group of phenolic compound[62]. Besides delphinidin, the obtained *A. syriaca* leaf extract is richer in phenolic acids than in flavonoids. Many of the phenolic acids quantified in *A. syriaca* LE in the present study have already been noted as potential allelochemicals. Thus, Hussain and Reigosa[63] reported that 4-hydroxybenzoic acid and ferulic acid induced toxic effects on the photosynthetic process in *Rumex acetosa L*. For protocatechuic acid there is a report that it affected the phycosphere, influencing algal growth, decay, and nutrient cycling[64], gallic acid, as well as ferulic acid inhibited plant growth and biochemical pathways of three herbicide-resistant weed species[65], coumaric acid affected water uptake by roots of affected plants[66], caffeic acid inhibited seed germination and plant height of some invasive plants[67], chlorogenic acid had suppressive ability on growth parameters of weed resulting in decreasing of its total biomass fresh weight[68], vanillic acid inhibited root and shoot growth of *Amaranthus tricolor*[69], and salicylic acid inhibited radicle elongation of *Euphorbia heterophylla* and *Bidens pilosa*[70]. Regarding flavonoids in *A. syriaca* LE, moderate amounts of rutin and quercetin were detected. As those flavonoids have been repeatedly found to be phytotoxic in various studies[71,72], it may be assumed that the presence of these phytochemicals in the extract also contributed to provoking oxidative stress in the weeds tested in this study.

The results of this study suggest that the extract from the leaves of *A. syriaca*, which contains a moderate amount of phenolic compounds, may have a pronounced phytotoxic effect. This extract had a significant negative impact on the germination and growth of *A. retroflexus*, *C. album,* and *I. xanthifolia* seedlings. In addition, it caused substantial oxidative stress and decreased the relative chlorophyll content of pot-grown weeds. These findings evinced that *A. syriaca* leaf extract had a significant phytotoxic effect on tested weeds. However, further studies under field conditions are needed to confirm the presented results and to check its herbicidal potential.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

J.G.U., M.S.K., and T.Đ. conceived and designed the research. L.R. and L.Š. collected plant materials. J.G.U. and M.S.K. conducted bioassay experiments. T.Đ. conducted chemical analysis and biochemical experiments. M.Š. and V.Š. analyzed data. J.G.U., M.S.K., and T.Đ wrote the manuscript. All the authors have read, edited, and approved the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to J.G.U.