applied sciences

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Phytohormones and Elicitors Enhanced the Ecdysteroid and Glycosylflavone Content and Antioxidant Activity of *Silene repens*

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Abstract: In the course of the ongoing chemical study of species of *Silene* genus, *S. repens* Patrin as a common species of the genus, was selected as the object of this study. Using high-performance liquid chromatography with photodiode array detection and electrospray ionization triple quadrupole mass spectrometric detection (HPLC-PDA-ESI-tQ-MS), the presence of 12 ecdysteroids and 6 glycosylflavones was established in *S. repens* introduced seedlings. 20-Hydroxyecdysone and polypodine B, as well as sileneside E and schaftoside, were the dominant compounds in introduced seedlings of *S. repens*. The effect of exogenous phytohormones and elicitors on the productivity and accumulation of ecdysteroids and glycosylflavones in introduced seedlings of *S. repens* was investigated for the first time. It was found that the use of ethyl arachidonate (100 mg/L) to increase the productivity of *S. repens* is justified. To obtain *S. repens* with a high content of ecdysteroids and glycosylflavones, it is recommended to apply epibrassinolide (100 mg/L) and 4-chlorophenoxyacetic acid (100 mg/L), respectively. Antioxidant activity of *S. repens* against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) was determined, and it was revealed that sileneside E and schaftoside, as well as meloside A and isovitexin had the highest antioxidant activity among the studied compounds of *S. repens*.

Keywords: *Silene repens*; Caryophyllaceae; phenolic compounds; ecdysteroids; high-performance liquid chromatography; mass spectrometry; antioxidant activity

Citation: Kashchenko, N.I.; Olennikov, D.N.; Chirikova, N.K. Phytohormones and Elicitors Enhanced the Ecdysteroid and Glycosylflavone Content and Antioxidant Activity of *Silene repens*. *Appl. Sci.* **2021**, *11*, 11099. https://doi.org/10.3390/app112311099

Academic Editor: Monica Gallo

Received: 3 November 2021
Accepted: 21 November 2021
Published: 23 November 2021

1. Introduction

Plants, as sessile organisms, have developed a complex network of signalling molecules that regulate their growth and development in response to external environmental factors [1]. Such plant responses to external factors are mediated by phytohormones and elicitors [2,3]. Phytohormones are signalling molecules that are produced at low concentrations and regulate all aspects of plant growth and development, such as embryogenesis [4,5], pathogen protection [6,7], stress resistance [8,9], and reproductive development [10,11]. Depending on the chemical structure, phytohormones are distinguished as auxins, cytokinins, ethylene-gibberellins, brassinosteroids, strigolactones, etc. [12]. Unlike phytohormones, elicitors (chitosan, arachidonic acid, benzothiadiazole, bacterial toxins, etc.) are unusual compounds for plant organisms; they enter from the outside and cause a complex of protective reactions in plants [13,14]. The data on the influence of these chemical compounds on plant organisms depends on the concentration of phytohormones and elicitors used, their localization in plant tissues and organs, and their interactions with other phytocomponents.

In the course of ongoing chemical study on the species of *Silene* genus [15,16], *S. repens* Patrin have been selected as the most common species of this genus in the Northern Hemisphere. Botanically, *S. repens* is an herbaceous plant with numerous simple or branching shoots and stems 15–60 cm long, densely pubescent with short pale hairs. Leaves are linear,

linear-lanceolate, or oblong-lanceolate. Inflorescence is raceme, short, and consisting of flowers on short pedicels. Bracts are oval-lanceolate with petals from whitish to yellowish. It grows on dry and steppe meadows and rocky mountain slopes, often almost to the border of the forest in Russia, Mongolia, Japan, and Northeast China [17]. Previously, the chemical composition of *S. repens* growing in the Baikal region was studied; ecdysteroid and flavonoid profiles of this plant species were investigated [18,19].

The increased interest in ecdysteroids and phenolic compounds is explained by their high biological activity. Ecdysteroids are plant secondary metabolites that provide protection against phytophagous insects [20]. In mammals, ecdysteroids has demonstrated suppression of neurodegenerative processes, protection of the cardiovascular system, improved activity of the immunological system, and showed antioxidant, antimicrobial, and antiproliferative properties [21,22]. Flavonoids are also secondary metabolites of plants. According to previous investigations, *S. repens* contains glycosylflavones [19]. It is known that glycosylflavones possess a wide range of biological activities: hepatoprotective, anti-inflammatory, antiviral, antioxidant properties, etc. [23,24]. Investigations on the effect of phytohormones and elicitors on secondary metabolites, such as ecdysteroids and flavonoids, is a promising area. Thus, flavonoid content in seedlings of broccoli was increased by 31% and 33% after treatment by methyl jasmonate and salicylic acid, respectively [25]. Total phenolic and total anthocyanin contents in red lettuce treated with exogenous abscisic acid were significantly higher than in controls [26]. The content of 20-hydroxyecdysone in spinach seedlings increased from 8.3 to 24.7 μg/g after the treatment by methyl salicylate and reached 17.2 μg/g after treatment by methyl jasmonate [27]. Taking into account information on the effect of exogenous regulators on vegetation and biosynthesis of metabolites of a plant object, it is possible to purposefully change the rates of plants' growth and development, as well as the accumulation of biologically active compounds [28].

This study aimed to estimate the influence of exogenous phytohormones and elicitors on productivity and accumulation of ecdysteroids and glycosylflavones in *S. repens* using high-performance liquid chromatography with photodiode array and electrospray ionisation triple quadrupole mass spectrometric detection (HPLC-PDA-ESI-tQ-MS). To the best of our knowledge, this is the first study of phytohormones and elicitors influence on introduced seedlings of *S. repens*. Considering that *S. repens* contains flavonoids, the antioxidant potential of *S. repens* was studied using an HPLC-PDA-based antioxidant activity assay to find active components.

2. Materials and Methods

2.1. Chemicals

The following chemicals were acquired from ChemFaces (Wuhan, Hubei, China): polypodine B (CFN89545, ≥98%); Sigma–Aldrich (St. Louis, MO, USA): acetonitrile (Cat. No. 34851, ≥99.9%), arachidonic acid (Cat. No. 10931, ≥95%), 4-chlorophenylacetic acid (Cat. No. 139262, ≥99%), 2,2-diphenyl-1-picrylhydrazyl (Cat. No. D9132), epibrassinolide (Cat. No. E1641, ≥85%), ethyl arachidonate (Cat. No. A9135, ≥98.5%), gibberellic acid potassium salt (gibberellin A_3, Cat. No. G1025, ≥95%), 20-hydroxyecdysone (Cat. No. H5142, ≥93%), indole-3-butyric acid (Cat. No. 57310, ≥ 99%), isovitexin (Cat. No. 17804, ≥98%), lithium perchlorate (Cat. No. 205281, ≥95%), methanol (Cat. No. 322415, ≥99.8%), polyamide (Cat. No. 02395), schaftoside (Cat. No. PHL83325, ≥95%), swertisin (Cat. No. PHL83912, ≥98%), trolox (Cat. No. 238813, ≥97%). Integristerone A and 2-deoxy-20-hydroxyecdysone were isolated earlier from *Silene jenisseensis* [29]; 26-hydroxyintegristerone A, 20,26-dihydroxyecdysone, 26-hydroxypolypodine B, turkesterone, 26-hydroxyecdysone, 20-hydroxyecdysone 2-acetate, viticosterone E, schaftoside-2″-*O*-glucoside (sileneside E), isovitexin-2″-*O*-glucoside (meloside A), ecdysone were isolated from *S. repens* [18,30], swertisine-2″-*O*-glucoside was isolated earlier from *S. samojedorum* [31].

2.2. Plant Material

Seedlings of *S. repens* were grown from authentic seeds obtained from the Moscow Botanical Garden of Academy of Sciences (Moscow, Russia). The seeds were sterilized by incubation for 1 min in 75% ethanol and then thoroughly washed with sterile water. The seeds were germinated in the soil in peat pots (8 cm) under controlled conditions at 25/18 °C (day/night), relative air humidity of 70–80%, illumination of 10 klx, and a photoperiod of 14 h. At the age of 30 days (2–3 true leaves), *S. repens* seedlings were planted in a greenhouse (4 plants/m^2) on the territory of the Institute of General and Experimental Biology SD RAS (Ulan-Ude, Russia) and grown for 30 days.

2.3. Treatment of S. repens Seedlings by Phytohormones and Elicitors

The experiments, epibrassinolide, gibberellic acids potassium salt, and indole-3-butyric acid were selected as phytohormones, while 4-chlorophenylacetic acid, arachidonic acid and ethyl arachidonate were chosen as elicitors. Selected phytohormones and elicitors were dissolved in an ethanol:water mixture (1:99) to give final concentrations of 1, 10, and 100 mg/L. All solutions were freshly prepared before each application. Control group 1 (30 seedlings) was sprayed with 30 mL ethanol:water mixture (1:99) for each plant. The ethanol:water solution (1:99; 30 mL) was introduced into the soil of control group 2 (30 seedlings) for each plant. Seedlings of *S. repens* were divided into groups of 30 specimens. Each group was sprayed with the aerial part (epibrassinolide, 4-chlorophenylacetic acid, gibberellic acids potassium salt, arachidonic acid, ethyl arachidonate) or root treatment (indole-3-butyric acid) for 1, 7, 14, and 21 days. The plants were treated by spraying with a 1500 mL manual Zhuk OP-270 sprayer (Cycle, Kovrov, Russia); root treatment consisted of introducing 30 mL of the working solution into the soil for each plant. All treatments were carried out from 8–9 a.m. at 20–25 °C. After 30 days, the plants were removed from the soil and the roots were washed. The raw material was dried in a convection oven (40 °C) to moisture values < 10%, and the weight of the leaves and roots was determined. The obtained leaf and root samples were stored at 4 °C before analysis in the Plant Repository of the Institute of General and Experimental Biology. No. Ca/sil/-3862-40/1 (leave samples) and Ca/sil-3863-40/2 (root samples) were the numbers of voucher specimens of *S. repens* in the Plant Repository. The samples were ground before analysis in an A11 basic analytical mill (IKA®-WerkeGmbh & Co. KG, Staufen, Germany). After grinding, the samples were sieved to a particle size of 0.5 mm on an ERL-M1 sieving machine (Zernotekhnika, Moscow, Russia).

2.4. Total Extracts Preparation from S. repens Leaves and Roots

For the preparation of the total extracts of *S. repens*, dry and powdered samples of leaves and roots (1 g) were extracted twice with stirring in a glass flask (100 mL) with 70% methanol (20 mL) using an ultrasonic bath (80 min, 50 °C, ultrasound power 100 W, frequency 35 kHz). The extracts obtained were passed through a cellulose filter, concentrated under reduced pressure until dryness, and stored at 4 °C before using for the chemical analysis and study of antioxidant activity.

2.5. Solid-Phase Extraction (SPE) of Total Extract from S. repens Leaves and Roots

The samples of total extracts of *S. repens* leaves and roots (50 mg) were ultrasonically dissolved in tridistilled water (10 mL), centrifuged (6000× *g*, 15 min), and the final solutions passed through SPE polyamide cartridges (10 g) preconditioned with methanol (100 mL) and water (150 mL). Elution was performed with water (300 mL; SPE-1 fraction) and 60% methanol (300 mL; SPE-2 fraction). The eluates were concentrated in vacuo until dryness and stored at 4 °C before HPLC-PDA-ESI-tQ-MS analysis. The yields of SPE fractions were 25.8–29.4% for SPE-1 and 16.3–20.9% for SPE-2 (Table S1).

2.6. High-Performance Liquid Chromatography with Photodiode Array Detection and Electrospray Ionization Triple Quadrupole Mass Spectrometric Detection (HPLC-PDA-ESI-tQ-MS): Metabolite Profiling and Quantification

Metabolite profiling of *S. repens* SPE fractions (Section 2.5) was realized using high-performance liquid chromatography with photodiode array detection and electrospray ionization triple quadrupole mass spectrometric detection (HPLC-PDA-ESI-tQ-MS) performed on a liquid chromatograph LC-20 Prominence coupled photodiode array detector SPD-M30A (wavelength range 200–600 nm), triple-quadrupole mass spectrometer LCMS 8050 (all Shimadzu, Columbia, MD, USA), and C18 column (GLC Mastro; 150 × 2.1 mm, 3 µm; Shimadzu, Kyoto, Japan) at the column temperature 35 °C. Eluent A was water and eluent B was acetonitrile. The injection volume was 1 µL, and elution flow was 180 µL/min. Gradient program for SPE-1 eluate (mode 1, analysis of ecdysteroids): 0.0–5.0 min 7.0–20.0%B, 5.0–15.0 min 20–100%B, 15.0–20.0 min 100–7%B. Gradient program for SPE-2 eluate (mode 2, analysis of glycosylflavones): 0.0–5.0 min 7–15%B, 5.0–10.0 min 15–60 %B, 10.0–15.0 min 60–70%B, 15.0–20.0 min 70–5%B. MS detection was performed in positive and negative ESI modes using the parameters as follows: temperature levels of ESI interface, desolvation line, and heat block were 300 °C, 250 °C, and 400 °C, respectively. The flow levels of nebulizing gas (N_2), heating gas (air) and collision-induced dissociation gas (Ar) were 3 L/min, 10 L/min and 0.3 mL/min, respectively. The MS spectra were recorded in negative (−3–−5 kV source voltage) and positive mode (+3–+4 kV source voltage) by scanning in the range of m/z 100–1900 at the collision energy of 5–40 eV. The system was operated under LabSolutions workstation software with the internal LC-MS library. The identification of compounds was done by the analysis of their retention time, ultraviolet, and mass-spectrometric data, comparing the same parameters with the reference samples and/or literature data (Table S2).

To quantify compounds **6**, **7**, **13**–**18** in *S. repens* SPE fractions, the reference standards (5 compounds) were accurately weighed (10 mg) and individually dissolved in DMSO-50% methanol mixture (1:10) in a volumetric flask (10 mL). The stock solutions were used to build external standard calibration curves generated using six data points, 100, 50, 25, 10, 5, and 1 µg/mL followed by plotting the MS peak area vs. the concentration levels. The validation criteria (correlation coefficients, r^2; standard deviation, S_{YX}; limits of detection, LOD; limits of quantification, LOQ; and linear ranges) were calculated using the previous recommendations [32] (Table S3). All analyses were carried out in triplicate, and the data were expressed as mean value ± standard deviation (S.D.). Before analysis, a sample of the fraction (SPE-1 or SPE-2; 1 mg) was dissolved in 1 mL of 70% acetonitrile, then centrifuged (6000× *g*, 15 min), filtered through a membrane filter (0.45 µm), and used for analysis (1 µL).

2.7. Antioxidant Activity of S. repens

Radical scavenging activity of *S. repens* SPE fractions against the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) was investigated using spectrophotometric decoloration assay as described previously [33]. 500 µL of freshly prepared DPPH· methanol solution (100 µg/mL) was added to 500 µL of sample solution (*S. repens* SPE fractions or pure compounds (**13**–**18**) in methanol; 1–1000 µg/mL). Then absorbance was measured at 520 nm after 15 min. Trolox (100 µg/mL) and water were used as a positive control (PC) and negative control (NC), respectively. The following equation was used to calculate the ability to scavenge DPPH· radicals: Scavenging ability (%) = $[(A_{520}{}^{NC} - A_{520}{}^{PC}) - (A_{520}{}^{Sample} - A_{520}{}^{PC})/(A_{520}{}^{NC} - A_{520}{}^{PC})] \times 100$, where $A_{520}{}^{NC}$ is the absorbance of negative control, $A_{520}{}^{PC}$ is the absorbance of positive control and $A_{520}{}^{Sample}$ is the absorbance of the sample solution. The IC_{50} value is the effective concentration at which DPPH· radicals were scavenged by 50%. Values are expressed as mean obtained from five independent experiments.

HPLC-PDA Activity-Based Profiling

High-performance liquid chromatography with photodiode array detection (HPLC-PDA) assisted with spectrophotometric DPPH radical scavenging assay was realized in the chromatographic conditions described in Section 2.6 with enlarged injection volume at 30 µL [34,35]. The eluates (50 µL) were collected every 15 s using an automated fraction collector (Econova, Novosibirsk, Russia) in 96-well microplates, then dried under a N_2-stream, and redissolved in 50 µL of 50% methanol. An aliquot (25 µL) of the methanolic solution was mixed with DPPH· solution (50 µg/mL in methanol) and absorbance was measured at 520 nm fifteen minutes later by a Bio-Rad microplate reader Model 3550 UV (Bio-Rad Labs, Richmond, CA, USA). The most active antioxidants provided strong decoloration of the DPPH solution, and corresponding eluates were separated in known HPLC-PDA conditions again in order to confirm the presence of separate compounds.

2.8. Statistical Analysis

Statistical analyses were carried out with the usage of ANOVA (one-way analysis of variance). The significance of the mean difference was established by Duncan's multiple range test. Differences were regarded as statistically considerable at $p < 0.05$. The results are presented as mean values ± standard deviations (SD).

3. Results and Discussion

3.1. HPLC-PDA-tQ-ESI-MS Profiles of SPE Fractions of Silene repens: Qualitative Study

At the preliminary stage of investigation, solid-phase extraction (SPE) on polyamide was used to separate ecdysteroids (SPE-1) and glycosylflavones (SPE-2) from interfering compounds of total methanol extracts from leaves and roots of introduced samples of *Silene repens*. The chromatographic profiles of SPE-1 and SPE-2 fractions of *S. repens* total extract were performed by high-performance liquid chromatography with photodiode array detection and electrospray ionization triple quadrupole mass spectrometric detection (HPLC-PDA-ESI-tQ-MS) (Figures 1–3). Such approach coupled with chromatographic data, UV-, and mass-spectrometric identification made it possible to find 18 compounds in SPE extracts of *S. repens* using the reference standards.

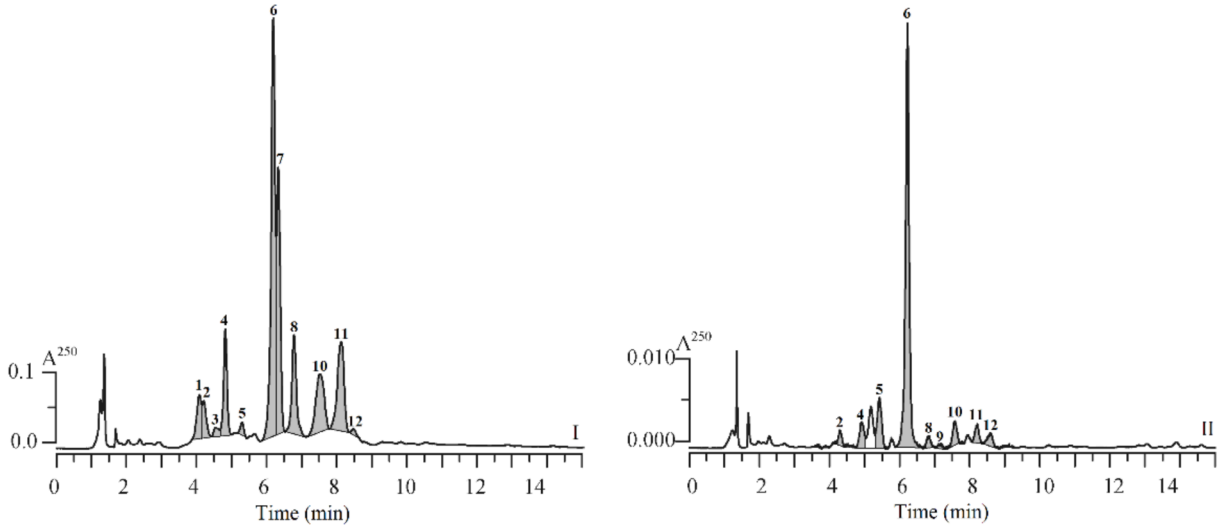
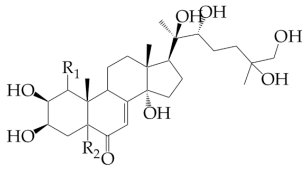
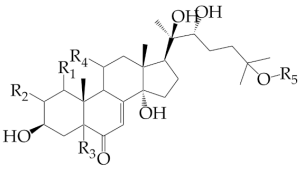


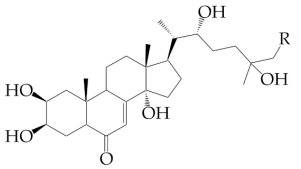
Figure 1. High-performance liquid chromatography with photodiode array detection (PDA: 250 nm) of *S. repens* SPE-1 fractions: leaves, I and roots, II (control 1 sample). Compounds: **1**—26-hydroxyintegristerone A; **2**—20, 26-dihydroxyecdysone; **3**—26-hydroxypolypodine B; **4**—integristerone A; **5**—turkesterone; **6**—20-hydroxyecdysone; **7**—polypodine B; **8**—26-hydroxyecdysone; **9**—ecdysone; **10**—2-deoxy-20-hydroxyecdysone; **11**—20-hydroxyecdysone 2-acetate; **12**—viticosterone E.



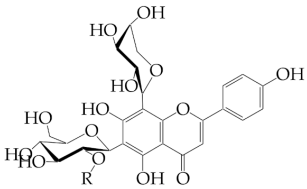
1: R_1 = β-OH; R_2 = H
2: R_1 = R_2 = H
3: R_1 = H; R_2 = β-OH



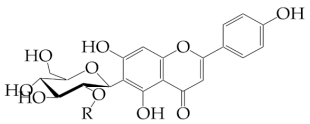
4: R_1 = R_2 = β-OH; R_3 = R_4 = R_5 = H
5: R_1 = R_3 = R_5 = H; R_2 = β-OH; R_4 = α-OH
6: R_1 = R_3 = R_4 = R_5 = H; R_2 = β-OH
7: R_1 = R_4 = R_5 = H; R_2 = R_3 = β-OH
10: R_1 = R_2 = R_3 = R_4 = R_5 = H
11: R_1 = R_3 = R_4 = R_5 = H; R_2 = β-O-$COCH_3$
12: R_1 = R_3 = R_4 = H; R_2 = β-OH; R_5 = $COCH_3$



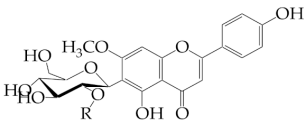
8: R = OH
9: R = H



13: R = β-D-Glcp
14: R = H



15: R = β-D-Glcp
16: R = H



17: R = β-D-Glcp
18: R = H

Figure 2. Structures of compounds **1**–**18** found in *S. repens*. Abbreviation used: β-D-Glc*p*—β-D-Glucopyranose.

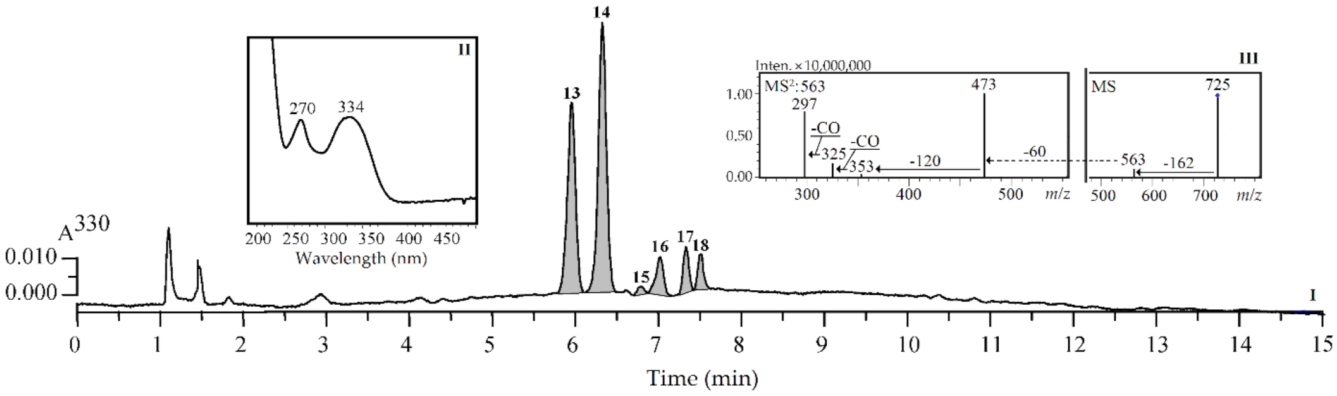


Figure 3. High-performance liquid chromatography with photodiode array detection (HPLC-PDA: 330 nm, I) of *S. repens* SPE-2 fraction from leaves (control 1 sample), absorption spectrum (**II**) and mass spectrum of compound **13** (negative ionization, **III**). Compounds: **13**—schaftoside-2″-*O*-glucoside (sileneside E); **14**—schaftoside; **15**—isovitexin-2″-*O*-glucoside (meloside A); **16**—isovitexin; **17**—swertisin-2″-*O*-glucoside; **18**—swertisin.

As a result of chromatographic analysis of the SPE-1 fractions from introduced samples of *S. repens* leaves and roots, the presence of 12 ecdysteroids was revealed (Figure 2). The introduced samples of *S. repens* did not differ in the ecdysteroid profile from the wild-growing samples [18]. 20-Hydroxyecdysone (**6**; leaves, roots) and polypodine B (**7**; leaves) were the main ecdysteroids of introduced samples in *S. repens*. Integristerone A (**4**), 20-hydroxyecdysone (**6**), polypodine B (**7**), ecdysone (**9**), and 2-deoxy-20-hydroxyecdysone (**10**) previously were detected in the wild and introduced samples of *S. repens* from Mongolia [36].

Earlier, 26-hydroxyintegristerone A (**1**) and 20,26-dihydroxyecdysone (**2**) were revealed in *S. frivaldszkyana* [37] and 26-hydroxypolypodine B (**3**) was detected in *S. viridiflora* [38]. Turkesterone (**5**) and viticosterone E (**12**) were found in *S. linicola* [39] and 20-hydroxyecdysone 2-acetate (**11**) was revealed previously in *S. otites* [40].

Chromatographic analysis of the SPE-2 fraction from introduced samples of *S. repens* leaves revealed the presence of 6 glycosylflavones (**13**–**18**) in *O*-glycoside, *C*,*O*-glycoside,

and aglycone forms identified as derivatives of schaftoside (schaftoside-2″-*O*-glucoside, **14**; schaftoside, **15**), isovitexin (isovitexin-2″-*O*-glucoside, **16**; isovitexin, **17**), and swertizin (swertizin-2″-*O*-glucoside, **18**; swertizin, **19**) by comparing with reference standards (Figure 3).

Schaftoside (**14**), isovitexin-2″-*O*-glucoside (meloside A, **15**), and isovitexin (**16**) were previously found in *S. repens* [19], while swertisin-2″-*O*-glucoside (**17**) and swertisin (**18**) were identified in *S. repens* for the first time. Compound **13** is of interest with a UV spectrum characteristic of flavones. According to mass spectral analysis, this compound was assigned to *C*,*O*-glycosylflavones. Molecular formula **13** was defined as $C_{32}H_{38}O_{19}$ ($[M-H]^-$ with m/z 725). The mass spectrum of **13** was close to schaftoside (apigenin-6-*C*-glucoside-8-*C*-arabinoside, **14**), which made it possible to preliminarily characterize **13** as schaftoside *O*-hexoside. Previously, **13** was discovered in *S. italica* and named schaftoside-2″-*O*-glucoside (sileneside E) [30].

3.2. Effect of Phytohormones and Elicitors on Productivity and Content of Ecdysteroids in Introduced Seedlings of S. repens

At the next stage of investigation, we decided to evaluate the effect of phytohormones and elicitors on productivity and content of ecdysteroids in introduced seedlings of *S. repens*. Epibrassinolide, indole-3-butyric acid, 4-chlorophenylacetic acid, gibberellic acids potassium salt, arachidonic acid, and ethyl arachidonate in different concentrations (1, 10, 100 mg/L) were applied. The appearance of *S. repens* seedlings (age 2 months) after 1 month of phytohormones and elicitors treatment was estimated (Figure 4). The average weight of leaves and roots per plant, as well as content of 20-hydroxyecdysone (**6**) and polypodine B (**7**) as marker compounds, were also evaluated (Table 1).

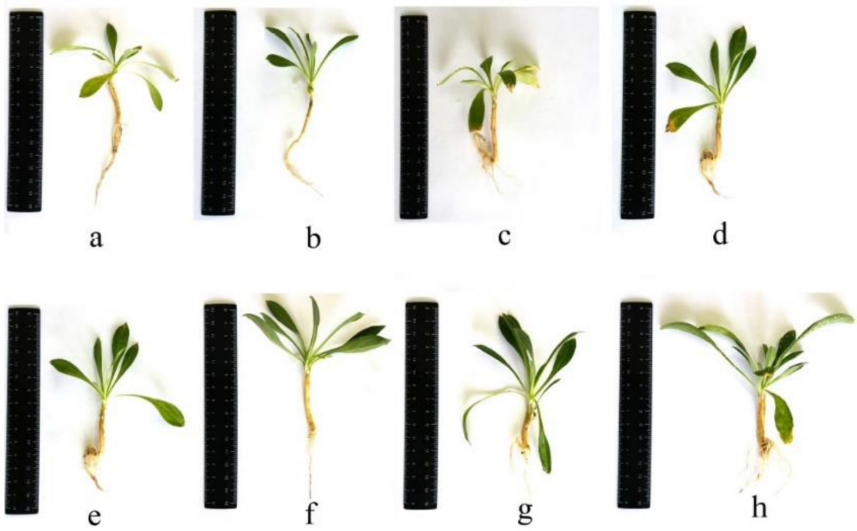


Figure 4. The appearance of *S. repens* seedlings (age 2 months) after 1 month of phytohormones and elicitors treatment. The letters indicate phytohormones and elicitors used (100 mg/L): (**a**)—control 1; (**b**)—control 2; (**c**)—indole-3-butyric acid; (**d**)—4-chlorophenylacetic acid; (**e**)—epibrassinolide; (**f**)—arachidonic acid; (**g**)—gibberellic acids potassium salt; (**h**)—ethyl arachidonate.

According to the data obtained, the treatment of *S. repens* seedlings with epibrassinolide at a concentration of 100 mg/L led to an increase in the average weight of plant leaves by 1.38 times. The productivity of root weight increased from 51.2 mg to 61.3 mg when using the maximum concentration of epibrassinolide. The content of 20-hydroxyecdysone and polypodine B in leaves of *S. repens* seedlings was the highest among all experimental groups and exceeded the concentrations of the same ecdysteroids by more than two times in comparison with the control sample. Epibrassinolide belongs to the class of brassinos-

teroids, chemically being a polyhydroxysteroid that is necessary for the development of plant objects [41,42].

Table 1. Average weight of leaves and roots per plant and total content of 20-hydroxyecdysone (**6**) and polypodine B (**7**) in *S. repens* seedlings after 1 month of phytohormones and elicitors treatment.

Group	Concentration, mg/L	Leaves			Roots	
		Weight, мг [a]	Content of 6, mg/g [b,c]	Content of 7, mg/g [b,c]	Weight, mg [a]	Content of 6, mg/g [b,c]
Control 1 (water, spraying)	-	60.0 ± 4.3	0.97 ± 0.02	0.41 ± 0.01	51.2 ± 4.0	0.44 ± 0.01
Control 2 (watering)	-	63.1 ± 5.4	0.92 ± 0.02	0.39 ± 0.01	50.3 ± 3.8	0.42 ± 0.01
Epibrassinolide	1	62.1 ± 4.2	0.99 ± 0.02	0.40 ±0.01	52.1 ± 4.0	0.42 ± 0.01
	10	65.0 ± 4.3	2.10 ± 0.04 †	1.14 ± 0.02 †	54.4 ± 4.1	0.39 ± 0.00 †
	100	82.7 ± 7.3 †	2.18 ± 0.04 †	1.16 ± 0.02 †	61.3 ± 5.6 †	0.41 ± 0.00 †
Indole-3-butyric acid	1	62.1 ± 5.1	0.96 ± 0.02	0.42 ± 0.01	49.1 ± 4.2	0.42 ± 0.01
	10	63.4 ± 5.0	1.02 ± 0.02 †	0.45 ± 0.01	50.1 ± 5.3	0.44 ± 0.01
	100	64.5 ± 4.2	1.05 ± 0.02 †	0.47 ± 0.01 †	58.2 ± 5.3	0.50 ± 0.01 †
4-Chlorophenylacetic acid	1	62.1 ± 5.3	0.96 ± 0.02	0.39 ± 0.01	51.0 ± 5.1	0.45 ± 0.01
	10	67.2 ± 5.2	1.01 ± 0.02	0.37 ± 0.01	57.3 ± 5.2	0.40 ± 0.01 †
	100	79.6 ± 6.0 †	1.06 ± 0.02 †	0.39 ± 0.01	61.6 ± 5.7 †	0.38 ± 0.00 †
Gibberellic acids potassium salt	1	61.0 ± 6.4	0.96 ± 0.02	0.41 ± 0.01	50.3 ± 5.1	0.42 ± 0.01
	10	76.7 ± 10.2 †	0.99 ± 0.02	0.40 ± 0.01	57.7 ± 6.8	0.51 ± 0.01 †
	100	104.3 ± 10.1 †	1.08 ± 0.02 †	0.42 ±0.01	72.5 ± 10.2 †	0.66 ± 0.02 †
Arachidonic acid	1	58.1 ± 4.0	0.94 ± 0.02	0.40 ± 0.01	52.1 ± 4.6	0.43 ± 0.01 †
	10	61.4 ± 4.1	0.91± 0.02	0.42 ± 0.01	67.2 ± 5.1	0.41 ± 0.02 †
	100	82.4 ± 9.4 †	0.89 ± 0.02 †	0.41 ± 0.01	69.3 ± 5.2 †	0.42 ± 0.02 †
Ethyl arachidonate	1	58.1 ± 5.0	0.98 ± 0.02	0.39 ± 0.01	50.4 ± 3.4	0.44 ± 0.01
	10	85.6 ± 10.5 †	0.87 ± 0.01 †	0.35 ± 0.01 †	70.7 ± 6.9 †	0.42 ± 0.02 †
	100	113.3 ± 11.4 †	0.80 ± 0.01 †	0.28 ± 0.01 †	73.2 ± 9.1 †	0.37 ± 0.02 †

[a] The average value of the air-dry mass for one plant is indicated (n = 30). [b] Dry plant weight. [c] Ecdysteroids: **6**—20-hydroxyecdysone; **7**—polypodine B. † Values indicate statistically significant differences compared with the data of control groups at $p < 0.05$ by one-way ANOVA.

The similarity in chemical structures between ecdysteroids and brassinosteroids could be the possible reason of this phenomenon [43] and could have an indirect effect on the quantitative content of each other. Thus, treatment of *Lepidium sativum* seedlings with exogenous 20-hydroxyecdysone led to a significant decrease in the content of endogenous brassinosteroids [44]. In turn, when the seedlings were treated with epibrassinolide, the opposite effect was observed. In accordance with the chemical structure, 20-hydroxyecdysone, polypodine B, and epibrassinolide belong to tetracyclic triterpenes and include polyhydroxylated steroid structures with an oxygenated B-ring [45]. However, the following structural differences between ecdysteroids and brassinosteroids affect their biological activities: the B-ring of epibrassinolide contains only a carbonyl group at C-6, while ecdysteroids have a typical chromophore 14-hydroxy-7-en-6-one fragment; hydroxyl groups at C-2, C-3, and C-22 have a mirrored orientation; and the junction of the A- and B-rings of brassinosteroids skeleton is characterized by *trans*-configuration while ecdysteroids possess a *cis*-orientation [44,45]. Interestingly, the data obtained correlated with literature only for leaves of *S. repens*. The content of 20-hydroxyecdysone in roots treated by epibrassinolide (100 mg/L) did not change significantly when compared with the control group (0.41 vs. 0.44 mg/g, respectively).

The watering of *S. repens* seedlings with indole-3-butyric acid at maximum concentration had no pronounced effect on yield or ecdysteroid content. The productivity of both leaves and roots of *S. repens* seedlings did not indicate statistically significant differences compared to control samples. The concentrations of 20-hydroxyecdysone and polypodine B in leaves of *S. repens* seedlings slightly increased (0.95→1.05 mg/g and 0.39→0.47 mg/g, respectively) after the treatment with indole-3-butyric acid (100 mg/L) compared with the control. The content of 20-hydroxyecdysone in roots also slightly increased from 0.42 to 0.50 mg/g compared to the control. Indole-3-butyric acid is representative of the auxin

class of phytohormones and as a derivative of indole stimulates the growth of plant fruits and shoots, causes positive geotropism of roots, affects cell differentiation, and ensures the interaction of individual organs [46,47].

Spraying of *S. repens* seedlings with 4-chlorophenylacetic acid (100 mg/L), which is similar to auxins in chemical structure, led to an increase in the productivity of both leaves (60.0→79.6 mg) and roots (51.2→61.6 mg) of *S. repens* seedlings. The content of 20-hydroxyecdysone in *S. repens* leaves increased from 0.97 to 1.06 mg/g and decreased in roots from 0.44 to 0.38 mg/g. The content of polypodine B in all groups did not indicate a statistically significant difference compared to the control sample. 4-Chlorophenylacetic acid is used in agriculture to increase the productivity of species by stimulating the formation of ovaries and preventing them from dropping [48,49]. It is likely that the short duration of the experiment and the absence of generative organs in *S. repens* seedlings did not allow to fully assess the effect of this growth regulator.

Gibberellic acid potassium salt at maximum concentration showed the increased productivity of *S. repens* leaves by 1.74 times (from 60.0 to 104.3 mg) and roots by 1.42 times (from 51.2 to 72.5 mg). Despite the increase in productivity, the content of 20-hydroxyecdysone in leaves increased slightly from 0.97 to 1.08 mg/g. Concentration of polypodine B did not indicate a statistically significant difference compared to the control sample. However, the content of 20-hydroxyecdysone in roots of *S. repens* seedlings increased from 0.44 to 0.66 mg/g. Previously, the synergistic effect of 20-hydroxyecdysone and gibberellic acid was noted for dwarf rice in an increase in the length of seedlings [43]. Gibberellins are phytohormones with a tetracyclic diterpene structure and are associated with the stimulation of vegetative and generative developments of plants [50]. Gibberellins work in the same direction as auxins and stimulate biosynthesis and signal transmission of each other [51].

Treatment with elicitor arachidonic acid and its ether led to a significant increase in productivity of *S. repens* seedlings. It was revealed that the maximum yield of *S. repens* leaves (60.0→113.3 mg) and roots (51.2→73.2 mg) comparing all experimental groups was observed after spraying with ethyl arachidonate (100 mg/L). However, the content of 20-hydroxyecdysone and polypodine B in *S. repens* leaves and roots was lower than in the control samples when treated with arachidonic acid and ethyl arachidonate (both 100 mg/L).

Thus, if it is necessary to increase the yield of *S. repens*, spraying seedlings with ethyl arachidonate (100 mg/L) is justified. However, if it is required to obtain *S. repens* seedlings with high content of ecdysteroids, spraying seedlings with epibrassinolide (100 mg/L) is recommended.

3.3. Effect of Phytohormones and Elicitors on Content of Glycosylflavones in Introduced Seedlings of S. repens

The effect of phytohormones and elicitors on content of glycosylflavones **13**–**18** in introduced seedlings of *S. repens* after 1 month of phytohormone and elicitor treatment was evaluated. Epibrassinolide, indole-3-butyric acid, 4-chlorophenylacetic acid, gibberellic acid potassium salt, arachidonic acid, and ethyl arachidonate in different concentrations (1, 10, 100 mg/L) were used (Table 2).

The maximum contents of dominant glycosylflavones of *S. repens* (**13** and **14**) were observed when seedlings were sprayed with epibrassinolide (100 mg/L) and 4-chlorophenylacetic acid (100 mg/L), respectively. The content of sileneside E (**13**) increased from 0.60 to 0.79 compared with the control. The concentration of schaftoside (**14**) increased more than 4 times from 0.73 mg/g to 2.97 mg/g. For meloside A (**15**) and isovitexin (**16**), the maximum accumulation was also observed after treatment with 4-chlorophenylacetic acid (100 mg/L). Compared with the control group, the content of **15** and **16** increased by 5.5 and 2.25 times, respectively. The maximum increase in the content of swertisin-2″-*O*-glucoside (**17**) was revealed after the treatment with gibberellic acid potassium salt (100 mg/L) from 0.15 mg/g to 0.63 mg/g. The highest accumulation of swertisin (**18**) was observed in the case of treatment with arachidonic acid (100 mg/L). Previously, it was shown that the use of gibberellic acid increased the content of flavones in transgenic *Saussurea involucrata* [52].

The highest total content of glycosylflavones was observed after treatment of seedlings with 4-chlorophenylacetic acid (100 mg/L) and amounted to 4.17 mg/g vs. 1.65 mg/g in the control group.

Table 2. Content of glycosylflavones in leaves of *S. repens* seedlings after 1 month of phytohormones and elicitors treatment.

Group	Concentration, mg/L	Content of Glycosylflavones, mg/g ± S.D. [a,b]						
		13	**14**	**15**	**16**	**17**	**18**	$\Sigma_{13–18}$
Control 1 (water, spraying)	-	0.60 ± 0.01	0.73 ± 0.02	0.02 ± 0.00	0.08 ± 0.00	0.15 ± 0.00	0.07 ± 0.00	1.65
Control 2 (watering)	-	0.63 ± 0.01	0.74 ± 0.02	0.02 ± 0.00	0.06 ± 0.00	0.17 ± 0.00	0.08 ± 0.00	1.70
Epibrassinolide	1	0.58 ± 0.01	0.79 ± 0.02	tr.	0.03 ± 0.00	0.10 ± 0.00	0.04 ± 0.00	1.54
	10	0.77 ± 0.01 †	1.14 ± 0.02 †	tr.	0.02 ± 0.00	0.10 ± 0.00	0.03 ± 0.00	2.06
	100	0.79 ± 0.02 †	1.28 ± 0.02 †	tr.	0.03 ± 0.00	0.07 ± 0.00	0.03 ± 0.00	2.20
Indole-3-butyric acid	1	0.61 ± 0.01	0.79 ± 0.02 †	0.02 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.08 ± 0.00	1.70
	10	0.56 ± 0.01	1.12 ± 0.02 †	0.02 ± 0.00	0.12 ± 0.00 †	0.10 ± 0.00	0.10 ± 0.00	2.02
	100	0.61 ± 0.01	1.16 ± 0.02 †	0.04 ± 0.00	0.15 ± 0.00 †	0.12 ± 0.00	0.14 ± 0.00 †	2.22
4-Chlorophenylacetic acid	1	0.65 ± 0.01	0.84 ± 0.02 †	0.02 ± 0.00	0.07 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	1.74
	10	0.70 ± 0.02 †	2.07 ± 0.04 †	0.02 ± 0.00	0.12 ± 0.00 †	0.07 ± 0.00	0.08 ±0.00	3.06
	100	0.75 ± 0.02 †	2.97 ± 0.06 †	0.11 ± 0.00	0.18 ± 0.00 †	0.05 ± 0.00	0.11 ± 0.00	4.17
Gibberellic acids potassium salt	1	0.54 ± 0.01	0.70 ± 0.02	0.04 ± 0.00	0.06 ± 0.00	0.17 ± 0.00	0.05 ± 0.00	1.56
	10	0.32 ± 0.01 †	0.38 ± 0.01 †	0.02 ± 0.00	0.08 ± 0.00	0.57 ± 0.01 †	0.06 ± 0.00	1.43
	100	0.30 ± 0.01 †	0.27 ± 0.00 †	tr.	0.10 ± 0.00 †	0.63 ± 0.01 †	0.07 ± 0.00	1.37
Arachidonic acid	1	0.60 ± 0.1	0.78 ± 0.02	0.01 ± 0.00	0.06 ± 0.00	0.10 ± 0.00	0.06 ± 0.00	1.61
	10	0.66 ± 0.01	1.16 ± 0.02 †	0.02 ± 0.00	0.10 ± 0.00 †	0.12 ± 0.00	0.12 ± 0.00 †	2.18
	100	0.69 ± 0.01 †	1.44 ± 0.03 †	0.04 ± 0.00	0.12 ± 0.00 †	0.15 ± 0.00	0.17 ± 0.00 †	2.61
Ethyl arachidonate	1	0.53 ± 0.01	0.63 ± 0.01	0.03 ± 0.00	0.07 ± 0.00	0.12 ± 0.00	0.04 ± 0.00	1.42
	10	0.39 ± 0.01 †	0.81 ± 0.02 †	0.04 ± 0.00	0.12 ± 0.00 †	0.10 ± 0.00	0.06 ± 0.00	1.52
	100	0.30 ± 0.00 †	0.95 ± 0.02 †	0.04 ± 0.00	0.17 ± 0.00 †	0.11 ± 0.00	0.09 ± 0.00	1.66

[a] Dry plant weight. [b] Glycosylflavones: **13**—schaftoside-2″-*O*-glucoside (sileneside E); **14**—schaftoside; **15**—isovitexin-2″-*O*-glucoside (meloside A); **16**—sovitexin; **17**—swertisin-2″-*O*-glucoside; **18**—swertisin. † Values indicate statistically significant differences compared with the data of control groups at $p < 0.05$ by one-way ANOVA. tr.—traces.

Thus, the treatment of *S. repens* seedlings with 4-chlorophenylacetic acid is justified to obtain raw materials with a high content of glycosylflavones.

3.4. Antioxidant Activity of S. repens

At the final stage, it was decided to evaluate the antioxidant activity of *S. repens* SPE fractions. SPE fractions from leaves and roots of *S. repens* have been evaluated for antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) (Table 3).

Scavenging activity against DPPH· radical of *S. repens* SPE fractions were low to medium. Both SPE-1 fractions as well as the SPE-2 fraction from the roots were characterized by low activity (<1 mg trolox/g). The antiradical activity of the SPE-2 fraction from leaves of S. repens against DPPH· was in the range of 33.75–78.20 mg trolox/g. The highest antiradical activity (60.92 and 78.20 mg trolox/g) was noted for fractions obtained after the treatment with 4-chlorophenylacetic acid (10 and 100 mg/L, respectively). Previously, for extracts from six species of *Silene* growing in Turkey, antiradical activity against DPPH· was established in the range of 43–131 mg trolox/g [53]. For *S. vulgaris* extract, the half maximal inhibitory concentration (IC_{50}) was 3.31 mg/mL [54], while for *S. latifolia* extracts IC_{50} was 1.26–1.57 mg/mL in relation to the DPPH· radical [55]. Thus, *S. repens* had antioxidant properties typical for other *Silene* species.

To determine compounds responsible for the antioxidant activity, the SPE-2 fraction from leaves of *S. repens* was subjected to HPLC-PDA activity-based profiling (Figure 5).

Table 3. DPPH· free radical inhibiting activity of *S. repens* SPE fractions.

Group	Concentration, mg/L	DPPH·, mg/g [a] ± SD			
		SPE-1		SPE-2	
		Leaves	Roots	Leaves	Roots
Control 1 (water, spraying)	-	<1	<1	46.52 ± 1.41	<1
Control 2 (watering)	-	<1	<1	45.33 ± 1.09	<1
Epibrassinolide	1	<1	<1	42.11 ± 1.43 †	<1
	10	<1	<1	53.02 ± 1.48 †	<1
	100	<1	<1	52.40 ± 1.57 †	<1
Indole-3-butyric acid	1	<1	<1	39.32 ± 1.09 †	<1
	10	<1	<1	45.27 ± 1.03	<1
	100	<1	<1	46.00 ± 1.29	<1
4-Chlorophenylacetic acid	1	<1	<1	45.14 ± 0.85 †	<1
	10	<1	<1	60.92 ± 1.40 †	<1
	100	<1	<1	78.20 ± 2.34 †	<1
Gibberellic acids potassium salt	1	<1	<1	40.06 ± 1.02 †	<1
	10	<1	<1	36.71 ± 0.86 †	<1
	100	<1	<1	33.75 ± 1.01 †	<1
Arachidonic acid	1	<1	<1	38.63 ± 1.19 †	<1
	10	<1	<1	55.23 ± 1.76 †	<1
	100	<1	<1	54.39 ± 1.57 †	<1
Ethyl arachidonate	1	<1	<1	38.67 ± 1.00 †	<1
	10	<1	<1	45.56 ± 1.23 †	<1
	100	<1	<1	44.14 ± 0.88 †	<1

[a] as Trolox equivalent. † Values indicate statistically significant differences compared with the data of control groups at $p < 0.05$ by one-way ANOVA.

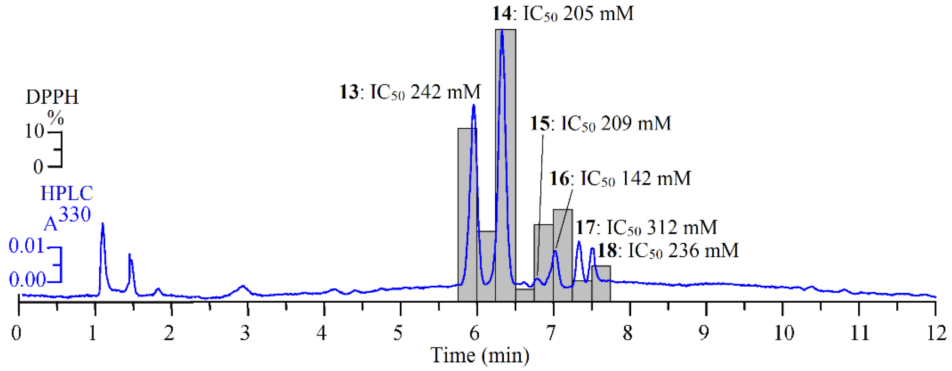


Figure 5. DPPH· free radical inhibiting activity of HPLC fraction of *S. repens* leaves SPE-2 fraction (Control 1 sample). The grey bars showed the antioxidant activity (as percentage) in probe after reaction with DPPH· solution. The HPLC-PDA profile of *S. repens* leaves SPE-2 fraction is a blue chromatogram with active compounds numbered as **13**—schaftoside-2″-*O*-glucoside (sileneside E); **14**—schaftoside; **15**—isovitexin-2″-*O*-glucoside (meloside A); **16**—isovitexin; **17**—swertisin-2″-*O*-glucoside; **18**—swertisin. The value of IC_{50} for the separate compounds displayed with the number of corresponding compounds.

For the procedure of small-scale, semi-preparative microfractionation by HPLC-PDA of *S. repens*, the SPE-2 leaves fraction was used. This method is characterized by post-column collecting of microfractions, subsequent drying, and analysis of obtained fractions to assess biological activity [56]. As a result of microfractionation of *S. repens* SPE-2, 48 microfractions of 15 s each were obtained, then DPPH· solution was added to evaluate antioxidant activity of the fractions obtained. The maximum inhibition of DPPH· radical was observed in the time window of 5.45 to 7.45 min, corresponding to eight fractions. The

data on inhibiting the DPPH· radical were compared with the HPLC-PDA profile of the *S. repens* leaves SPE-2 fraction. According to the results obtained, the most active fraction (6.15–6.30 min) was characterized by domination of schaftoside. Sileneside E was presented in the fraction with less activity (5.45–6.00 min). Meloside A and its aglycone isovitexin provided the activity of fractions from 6.45 to 7.15 min. The less active fractions 7.15–7.45 were characterized by the presence of swertisin-2″-O-glucoside and swertisin. The values IC_{50} were obtained to evaluate antioxidant activity of individual compounds **13**–**18** against the DPPH· radical. As a result, isovitexin was the most active compound (IC_{50} 142 mM), while swertisin-2″-O-glucoside was less active (IC_{50} 312 mM). Schaftoside and meloside A possessed moderate antioxidant activity (IC_{50} 205 and 209 mM, respectively). Thus, glycosylflavones sileneside E, schaftoside, meloside A, and isovitexin had the highest antioxidant activity among the studied compounds of *S. repens*.

4. Conclusions

The presence of twelve ecdysteroids was revealed in introduced seedlings of *Silene repens*: 26-hydroxyintegristerone A, 20,26-dihydroxyecdysone, 26-hydroxypolypodine B, integristerone A, turkesterone, 20-hydroxyecdysone, polypodine B, 26-hydroxyecdysone, ecdysone, 2-deoxy-20-hydroxyecdysone, 20-hydroxyecdysone 2-acetate, and viticosterone E. Furthermore, six glycosylflavones were identified: derivatives of schaftoside, isovitexin, and swertisin. Swertisin-2″-*O*-glycoside, swertisin, and sileneside E were found in *S. repens* leaves for the first time. The influence of exogenous phytohormones and elicitors on the productivity and accumulation of ecdysteroids and glycosylflavones in introduced seedlings of *S. repens* was studied. It was found that the use of ethyl arachidonate (100 mg/L) was justified to increase the productivity of *S. repens*. To obtain raw materials with high content of ecdysteroids and glycosylflavones, the application of epibrassinolide (100 mg/L) and 4-chlorophenylacetic acid (100 mg/L), respectively, is recommended. Antioxidant activity of *S. repens* against the DPPH· radical was investigated. Sileneside E, schaftoside, meloside A, and isovitexin had the highest antioxidant activity among the studied compounds of *S. repens*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app112311099/s1, Table S1, Yields of SPE fractions of total extracts from *S. repens* leaves and roots; Table S2. Chromatographic, ultraviolet data and mass-spectrometric data of compounds 1–18 found in *S. repens*; Table S3, Regression equations, correlation coefficients, standard deviation, limits of detection, limits of quantification, and linear ranges for 5 reference standards.

Author Contributions: Conceptualization, N.I.K. and D.N.O.; methodology, N.I.K.; software, D.N.O.; validation, N.I.K., D.N.O., and N.K.C.; formal analysis, N.I.K. and N.K.C.; investigation, N.I.K. and D.N.O.; resources, N.K.C.; data curation, N.I.K. and D.N.O.; writing—original draft preparation, N.I.K.; writing—review and editing, N.I.K. and D.N.O.; visualization, N.K.C.; supervision, D.N.O.; project administration, N.I.K. and D.N.O.; funding acquisition, N.K.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Education and Science of Russia, grant numbers FSRG-2020-0019; 121030100227-7; World-class Interregional Scientific and Educational Center "Baikal": 2021.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors acknowledge the Buryat Research Resource Center for the technical support in chromatographic and mass-spectrometric research.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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