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Original Scientific Report

Waterlogging affects the anti-melanogenic properties of *Platycodon grandiflorus* roots

Hyo Seong Ji and Tae Kyung Hyun*

Department of Industrial Plant Science and Technology, College of Agriculture, Life and Environment Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea
* Correspondence: taekyung7708@chungbuk.ac.kr

ABSTRACT:
Waterlogging caused by climate change has threatened the growth and production yield of crops. Although morphological and physiological changes in major crops under waterlogging have been well-defined, the effect of waterlogging on the quality of medicinal plants remains largely unknown. In this study, we analysed waterlogging-induced variations in the anti-melanogenic properties of *Platycodon grandiflorus* roots. Based on the analysis of melanin production and the expression levels of melanogenic enzymes, we found that waterlogging negatively impacted the anti-melanogenic properties of *P. grandiflorus* roots. Using UPLC-ESI-Q-TOF-MS, we identified 12 compounds including platycodin D3 and platycodin A, which showed differences between untreated and waterlogging-treated roots. In addition, waterlogging led to the suppression of the triterpenoid saponin biosynthetic pathway. Taken together, our results will form an important basis for understanding the impact of climate change on the quality of medicinal plants.

Keywords:
anti-melanogenic property, *Platycodon grandifloras*, triterpenoid saponins, waterlogging

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INTRODUCTION

Waterlogging is one of the major environmental stresses which affect the production and yield quality of crops via the inhibition of aerobic respiration in roots (Pan *et al*. 2021). The root is the primary target affected by waterlogging-induced hypoxic or anoxic conditions, which lead to various physiological and morphological changes including the formation of adventitious roots and aerenchyma which increase waterlogging tolerance (Yamauchi *et al*. 2018). In addition, waterlogging also increases susceptibility to fungal diseases and impairs nutrient uptake from the soil (Zhang *et al*. 2021). Although most investigations have been carried out using major crops including cucumber, cassava, and sweet potato (Barickman *et al*. 2019; Cao *et al*. 2022; Olorunwa *et al*. 2022; Park *et al*. 2022), other studies have reported that waterlogging affects the mineral status and yield of the storage roots of medicinal plants (Nikam & Chavan 2009; Suh *et al*. 2021). This indicates that waterlogging both directly and indirectly affects the quality and yield of medicinal plants.

Platycodon grandiflorus (Jacq.) A.DC, the monotypic genus in the family Campanulaceae, is mainly distributed in East Asia (Zhang *et al*. 2015). The root of this plant is commonly used for medicinal purposes, as described in several studies demonstrating its pharmaceutical activities including anti-inflammatory, antitussive, anti-tumour, anti-obesity, hepatoprotective, and anti-melanogenic properties (Zhang *et al*. 2015; Ma *et al*. 2021). In addition, it has been suggested that this plant is a source of novel active compounds which inhibit transmembrane protease serine 2 activity, which is required for the coronavirus to enter host cells (Kim *et al*. 2021; Gurung *et al*. 2022). *Platycodon grandiflorus* has a taproot system, and the incidence of root rot disease was higher when the soil moisture was high (Jeon *et al*. 2013). In addition, short-term drought stress significantly increased the to-

tal platycodin (triterpenoid saponin) content in *P. grandiflorus* roots (Cheng *et al*. 2022). This suggests that soil water has a significant influence on the quality and yield of *P. grandiflorus* roots, however, the effects of waterlogging on the medicinal value of *P. grandiflorus* roots are not yet fully understood.

In this study, the anti-melanogenic properties of untreated and waterlogged roots were compared to determine the effect of waterlogging on the medicinal value of *P. grandiflorus*. In addition, UPLC-ESI-Q-TOF-MS identification and the analysis of gene expression were performed to examine variations in the anti-melanogenic properties of non-treated and waterlogged roots.

MATERIALS AND METHODS

Waterlogging treatment and preparation of the extracts. One-year-old roots of *P. grandiflorus* were grown in a growth chamber at 24°C and 50% relative humidity under a long photoperiod (16 h light/8 h dark). For waterlogging treatment, 4-week-old plants were placed in a plastic container filled with water 3 cm above the soil surface. Two weeks after the treatment, the malondialdehyde (MDA) content was determined using the thiobarbituric acid reaction as described by Eom *et al*. (2023). The MDA concentration was expressed as MDA in nmol per mg of fresh weight using an absorbance coefficient of extinction (155 mM^{-1} cm^{-1}). The freeze-dried roots were ground and soaked in 70% ethanol (EtOH) for 24 h at room temperature. After evaporation using a rotary evaporator, the 70% EtOH extracts were used to prepare fractions of ethyl acetate, *n*-butanol, and aqueous fractions. The 70% EtOH extracts and their fractions were re-dissolved in DMSO for further analysis. Nt_R and Wt_R indicated 70% EtOH extracts obtained from untreated and waterlogged roots, respectively. In addition, Nt_R_B and Wt_R_B indicated BuOH fractions of Nt_R and Wt_R, respectively.

Determination of anti-melanogenic properties and cytotoxicity. To assess the anti-melanogenic properties, 3-isobutyl-1-methylxanthine (IBMX)-stimulated B16F10 melanoma cells were treated with each sample. After 48 h incubation in a CO_2 incubator at 37°C, the melanin content in the cells was determined as described by Ju *et al*. (2021). Briefly, the cells were washed twice with ice-cold PBS and harvested by centrifugation at 4,000 rpm for 10 min. The resulting cell pellets were solubilised in 1 N NaOH with 10% dimethyl sulfoxide at 65°C for 1 h. The concentration of melanin was determined using a microplate reader at 490 nm. The data are expressed in terms of melanin synthesis inhibitory activity compared to the mock control. The inhibitory strength of the BuOH fractions was expressed as the IC_{50} value, which is the concentration of each sample needed to inhibit half of melanin production in IBMX-stimulated B16F10 cells. In addition, tyrosinase inhibitory activity was analysed using a Tyrosinase Inhibition Screening Kit (BioVision, CA, USA) (Ju *et al*. 2021).

The cytotoxicity of each sample was determined in IBMX-stimulated B16F10 cells using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution as described by JU *et al*. (2021). Briefly, B16F10 cells were treated in each sample and stimulated with IBMX. After 48 h incubation in a CO_2 incubator at 37°C, 20 µl of MTT (5 mg/ml in PBS) solution was added to each well. Incubation was continued for 4 h more, the formazan crystals were dissolved in DMSO, and the optical density was measured at 520 nm.

Western blotting and gene expression analysis. The total RNA from B16F10 cells or *P. grandiflorus* roots was isolated using TRIzol reagent or a FavorPrep Plant Total RNA Mini Kit. After cDNA synthesis, the expression levels of each gene were determined using SYBR Green Real-time PCR Master Mix and the CFX96TM Real-time system (Bio-Rad, Hercules, CA, USA). The primers used are listed in Supplementary Table 1.

To determine the level of tyrosinase, western blotting was performed using the proteins extracted from B16F10 cells. After incubation with goat polyclonal tyrosinase antibody (Santa Cruz, Dallas, TX, USA), the protein band signals were detected using a chemiluminescence system with ECL reagents. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard.

Analysis of metabolic variation using UPLC-Q-TOF-MS. Waterlogging-induced metabolic variations were determined using an UPLC-Q-TOF-MS system equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, particle size 1.7 µm). The mobile phase consisted of the solvents 0.1% (v/v) aqueous formic acid and acetonitrile containing 0.1% (v/v) formic acid. As described by Shin *et al*. (2022), a gradient elution was performed, and the eluted metabolites were detected using Q-TOF MS in the positive/negative electrospray ionisation mode. The optimised parameters for the mass spectrometric analysis were as follows: capillary voltage, 2.5 kV; cone voltage, 20 V; nebulized gas flow rate, 900 l/h at a temperature of 100°C in the positive/negative mode; cone gas flow rate, 30 l/h; and mass range, 50–1500 m/z. The selected peaks were identified in a ChemSpider database in UNIFI and METLIN datasets.

Statistical analysis. The data were expressed as the mean ± standard error (SE) of three independent experiments. The significance of between-group differences was determined by Duncan's multiple range test. Values of $p < 0.05$ were considered statistically significant. In addition, the Student's t test was used to compare the means between two groups. ***, ** or * indicate statistical significance at $p < 0.001$, $p < 0.01$ or $p < 0.05$, respectively.

Table 1. The phytochemical constituents identified in BuOH fractions of the non-treated root extracts (Nt_R_B) and waterlogging-treated root extracts (Wt_R_B).

Mode	Peak No.	Rt (min)	Neutral Mass (Da)	Observed m/z	Mass Error (mDa)	Formula	Proposed Molecule	Fragment Ions	Peak Area
Positive	1	2.87	168.00587	169.0122	-0.9	C7H4O5	(2E,5E)-4-Oxo-2,5-heptadienedioate	85, 113	Nt_R_B > Wt_R_B
	2	3.42	326.15181	327.1574	-1.7	C20H22O4	Dehydrodieugenol	133, 137, 163, 295	Nt_R_B > Wt_R_B
	3	3.97	1386.6	1387.6334	-4.2	C63H102O33	Platycodin D3	309, 815	Nt_R_B > Wt_R_B
	4	6.85	519.33249	520.3394	-0.4	C26H50NO7P	LPC(18:2)	104, 184	Nt_R_B > Wt_R_B
	5	7.18	495.33249	496.3389	-0.9	C24H50NO7P	LPC(16:0)	104, 184	Nt_R_B > Wt_R_B
Negative	6	3.42	524.22576	523.2174	-1.1	C26H36O11	Secoisolariciresinol 9-O-glucopyranoside	165, 297, 361	Nt_R_B < Wt_R_B
	7	3.56	566.19994	565.1924	-0.3	C27H34O13	Alboside II	161, 357	Nt_R_B > Wt_R_B
	8	4.07	442.1839	441.1756	-1	C21H30O10	Lusitanicoside	247	Nt_R_B < Wt_R_B
	9	4.31	1266.58808	1265.5795	-1.3	C59H94O29	Platycodin A	419, 681, 1053, 1237	Nt_R_B > Wt_R_B
	10	4.84	696.37209	695.3653	0.4	C36H56O13	Periplocin	485	Nt_R_B < Wt_R_B
	11	6.72	505.31684	564.3303	-0.4	C25H52NO7P	LPE(20:2)	279, 433	Nt_R_B > Wt_R_B
	12	6.84	477.28554	476.2769	-1.3	C23H44NO7P	LPE(18:2)	196, 279, 433	Nt_R_B > Wt_R_B

RESULTS AND DISCUSSION

The effect of waterlogging on the anti-melanogenic properties of *Platycodon grandiflorus* roots. Waterlogging induced the accumulation of reactive oxygen species causing lipid peroxidation, membrane injury, and the degradation of proteins and nucleic acids, resulting in plant death and yield reduction (Pan *et al.* 2021). Therefore, we analysed the malondialdehyde (MDA) content, a well-known indicator of lipid peroxidation (Farmer & Mueller 2013), to evaluate the efficacy of the treatment. As shown in Supplementary Fig. 1, the roots exhibited a significant accumulation of MDA content after two weeks of waterlogging treatment, similar to other plants (Kim *et al.* 2015; Yang *et al.* 2020; Teoh *et al.* 2022). In addition, the waterlogging treatment also contributed to restricting root growth and development (Fig. 1A), indicating that our treatment was sufficient to induce waterlogging stress in *P. grandiflorus*.

To investigate the effect of waterlogging on the medicinal qualities of the *P. grandiflorus* roots, we determined the anti-melanogenic properties of the extracts obtained from non-treated (Nt_R) and waterlogged roots (Wt_R). As shown in Fig. 1B, IBMX-induced melanin accumulation was suppressed by Nt_R, and its inhibitory effect was lost after waterlogging. Among the solvent partitioned fractions of both extracts, the BuOH fractions exhibited the highest anti-melanogenic properties followed by the EtOAc fractions without cytotoxic activities against B16F10 melanoma cells (Fig. 1B & C). Interestingly, the anti-melanogenic properties of the Nt_R BuOH fraction (Nt_R_B; IC50 = 54.9 ± 0.9 μg/ml) was significantly greater than that of the Wt_R BuOH fraction (Wt_R_B; IC50 = 78.7 ± 3.0 μg/ml) (Fig. 1D), indicating that waterlogging impeded the growth of *P. grandiflorus* and decreased its pharmaceutical properties, particularly its anti-melanogenic properties. The anti-melanogenic properties of *P. grandiflorus* roots have been reported to be mediated by inhibiting the expression and activity of tyrosinase (Park *et al.* 2019; Ma *et al.* 2021), similar to our findings (Fig. 1E–G). Although Wt_R_B directly inhibited tyrosinase activity, the IBMX-induced expression of tyrosinase (TYR) and tyrosinase-related proteins 1 and 2 (TRP1 and 2) were not inhibited in the Wt_R_B treated group (Fig. 1E–G). This suggests that waterlogging has a negative effect on the production of phytochemicals,

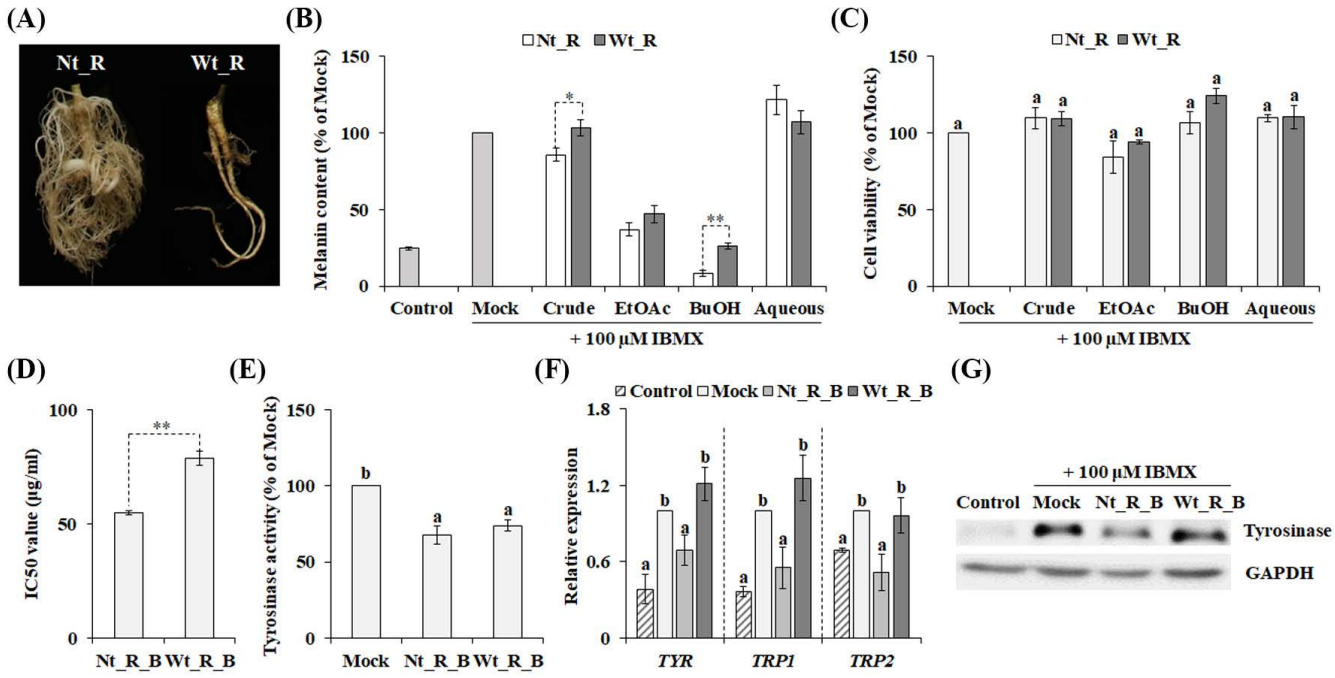

Figure 1. The effect of waterlogging on the growth and anti-melanogenic properties of the *P. grandiflorus* roots. (A) The root phenotypes were analysed two weeks after waterlogging. The inhibitory effects (B) and cytotoxic effects (C) of the crude extracts and their solvent fractions on IBMX-induced melanin production in B16F10 melanoma cells were analysed. (D) The inhibitory strength of BuOH fractions was expressed as the IC50 value, which is the concentration of each sample needed to inhibit half of melanin production in IBMX-stimulated B16F10 cells. (E) The effects of BuOH fractions on tyrosinase activity *in vitro*. The effect of BuOH fractions on the levels of melanogenesis-related genes (F) and the protein levels of tyrosinase (G) in IBMX-stimulated B16F10 cells. The means (± SE, three independent experiments) with asterisks (* $p < 0.05$ and ** $p < 0.01$, t-test) or different letters ($p < 0.05$, Duncan's multiple range test) are significantly different. The mock indicated DMSO-treated sample. Nt_R, non-treated root; Wt_R, waterlogging-treated root; Nt_R_B, BuOH fraction of non-treated root extracts; Wt_R_B, BuOH fraction of waterlogging-treated root extracts, TYR, tyrosinase; TRP1, tyrosinase-related protein 1; TRP2, tyrosinase-related protein 2.

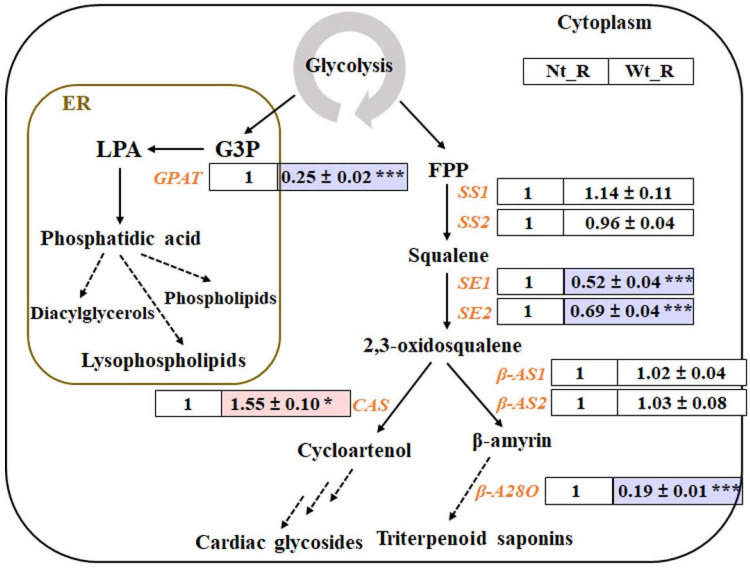

Figure 2. The effect of waterlogging on the expressions of genes involved in the biosynthesis of lysophospholipids, triterpenoid saponins, and cardiac glycosides. The transcription levels of each gene were normalised and calculated relative to their expression in the non-treated root (Nt_R) group. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$; mean ± SE of three independent experiments). Wt_R, waterlogging-treated root; FPP, farnesyl diphosphate; G3P, glycerol-3-phosphate; LPA, 1-acyl lysophosphatidic acid; GPAT, glycerol-3-phosphate acyltransferase; SS, squalene synthetase; SE, squalene epoxidase; β-AS, β-amyrin synthase; β-A28O, β-xanthophyll C-28 hydroxylase; CAS, cycloartenol synthase.

which can suppress the transcription of melanogenic enzymes in B16F10 cells.

The metabolic effect of waterlogging on the roots of *Platycodon grandiflorus*. Environmental stresses affect the biosynthesis of secondary metabolites, indicating that the medicinal qualities of medicinal plants can be influenced by numerous environmental factors (Yeshi *et al*. 2022). To investigate alterations in the metabolites resulting from waterlogging, we compared the phytochemical contents of Nt_R_B and Wt_R_B using UPLC-Q-TOF-MS (Supplementary Fig. 2). The molecules exhibiting variance between both samples were identified by analysing their fragmentation obtained by ESI-MS in both positive and negative modes. As shown in Table 1, a total of 12 compounds including lignans, lipids, and terpenoids were identified. Nt_R_B contained higher amounts of nine compounds including platycodin D3 (3) and platycodin A (9), which are triterpenoid saponins previously identified in *P. grandiflorus* roots (Lee *et al*. 2019). Platycodin D3 was suggested to be a potential expectorant for controlling pulmonary inflammatory diseases (Ryu *et al*. 2014), and platycodin A exhibited anti-inflammatory activity in lipopolysaccharide-stimulated RAW 264.7 macrophages (Ahn *et al*. 2005). In addition, platycodin D3 is a precursor of platycodin D (Ahn *et al*. 2018), which is a major active compound with multiple and pharmacological properties including anti-melanogenic, anti-tumour, anti-inflammatory, anti-atherogenic, and anti-obesity activities (Jung *et al*. 2011; Ji *et al*. 2020). Furthermore, waterlogging lowered the amounts of lysophospholipids including lysophosphatidylcholine (LPC; 16:0, 18:2) and lysophosphatidylethanolamine (LPE; 18:2, 20:2). Similarly, anoxia induced by submersion leads to the induction of lysophospholipid degradation (Wang *et al*. 2016). In *Panax ginseng*, a gintonin-enriched fraction including fatty acids, lysophospholipids, and phospholipids has exhibited various pharmacological effects on Alzheimer's disease, Parkinson's disease, and diabetes (Cho *et al*. 2019; Choi *et al*. 2021). Although there is a need for further investigation to analyse the relationship between these compounds and their anti-melanogenic properties, this suggests that waterlogging may alter the levels of bioactive compounds in plants, resulting in the reduced medicinal quality of *P. grandiflorus* roots.

The molecular mechanism underlying the environmental stress-induced variations in secondary metabolism might be mediated by changes in the expression levels of key synthesis-related genes (Eom *et al*. 2018, 2022; Jan *et al*. 2021). In the triterpenoid saponin biosynthetic pathway, waterlogging suppressed the expression of squalene epoxidases (SE1 and 2) and β-xanthophyll C-28 hydroxylase (β-A28O) in the *P. grandiflorus* roots (Fig. 2). In addition, the transcription of glycerol-3-phosphate acyltransferase (GPAT) involved in lysophospholipid synthesis was also inhibited by waterlogging, whereas the upregulation of cycloartenol synthase (CAS) might affect the accumulation of periplocin (10) in the Wt_R group. Collectively, these results suggest that waterlogging reduces the anti-melanogenic properties of *P. grandiflorus* roots by adversely affecting the transcription of these genes.

CONCLUSION

In conclusion, waterlogging stress in the *P. grandiflorus* roots led to increased lipid peroxidation and restricted root growth. The extracts from the waterlogged roots exhibited compromised anti-melanogenic effects, indicating the direct impact of waterlogging on their medicinal quality. Metabolomic and gene expression analyses further revealed that waterlogging stress inhibited the biosynthesis of triterpenoid saponins and lysophospholipids, thus adversely influencing the transcription of relevant biosynthetic genes. Although physiological and biochemical changes in *P. grandiflorus* caused by waterlogging are largely unknown, our findings provide an important starting point to understand the effects of environmental stress on the quality of medicinal plants, particularly those whose roots are used for medicinal purposes.

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REZIME

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Uticaj preplavljenosti na antimelanogena svojstva korena *Platycodon grandifloras*

Hyo Seong Ji i Tae Kyung Hyun

Preplavljenost uzrokovana klimatskim promjenama ugrozila je rast i proizvodni prinos useva. Iako su morfološke i fiziološke promene u glavnim usevima u uslovima preplavljenosti dobro definisane, učinak na lekovitost biljaka ostaje uglavnom nepoznat. U ovoj studiji analizirali smo varijacije u antimelanogenim svojstvima korena *Platycodon grandiflorus* uzrokovane prelivanjem vode. Na osnovu analize proizvodnje melanina i nivoa ekspresije melanogenih enzima, otkrili smo da preplavljivanje negativno utiče na antimelanogena svojstva korena *P. grandiflorus*. Koristeći UPLC-ESI-Q-TOF-MS, identifikovali smo 12 jedinjenja uključujući platikodin D3 i platikodin A, koji su pokazali razlike između netretiranog i korena tretiranog vodom. Uz to, zalivanje je uzrokovalo supresiju biosintetskog puta triterpenoidnog saponina. Uzeti zajedno, naši rezultati će poslužiti kao važna osnova za razumevanje uticaja klimatskih promena na lekovita svojstva biljaka.

Ključne reči: antimelanogeno svojstvo, *Platycodon grandiflorus*, triterpenoidni saponini, preplavljivanje.