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Original Scientific Paper

Elicitors enhanced the production of bioactive compounds in shoot cultures of *Hypericum amblysepalum*

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ABSTRACT:
In this study, we examined the effects of elicitors MeJA (Methyl jasmonate) and JA (Jasmonic acid) (50, 100, and 200 µM) on the growth and production of 15 phenolic compounds in shoot cultures of *Hypericum amblysepalum* using the LC-MS/MS method. The biomass production increased on shoots elicited with 50 µM JA and MeJA. However, higher concentrations of these elicitors had a negative effect on the growth of the shoot cultures, while simultaneously resulting in an increase in the secondary metabolite content. The elicitor MeJA (especially 200 µM MeJA) was more effective in terms of increasing the phenolic compound contents. The highest amounts of rutin (2.8 fold), astragalin (2.4 fold), protocatechuic acid (2.4 fold), hesperidin (2 fold), pseudohypericin (1.9 fold), chlorogenic acid (1.4 fold), and hypericin (1.9 fold) were obtained from the shoots elicited with MeJA (200 µM). In addition, the application of elicitor JA 200 µM increased the amount of luteolin (2 fold), quercitrin (1.9 fold), apigetrin (4 fold), apigenin (2.9 fold), and hyperoside (1.3 fold). The current study revealed that specific secondary plant metabolites can be regulated by exogenous elicitors in shoot cultures of *H. ambysepalum,* thus highlighting their promise as a very valuable source of raw materials for the pharmaceutical industry.

Keywords:
Hypericum amblysepalum, jasmonic acid, methyl jasmonate, shoot culture.

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INTRODUCTION

The genus *Hypericum* L. (Hypericaceae) is well represented in the Turkish flora with almost 96 species and 2 subspecies, 45 of which are endemic (Yuce-Babacan & Bagci 2017).

Hypericum species have a reputation worldwide for their biological activities, such as antidepressant, antitumor, anti-inflammatory, antiviral, antimicrobial, antinociceptive, neuroprotective, and wound-healing effects (Oliveira *et al.* 2016). They have a varied chemical composition, including hyperforine, hypericin and pseudohypericin, quercetin, quercitrin, hyperoside, biapigenin, volatile oils, and tannins (Ozkan & Mat 2013). The most important use of *Hypericum* pharmaceutical preparations is in the treatment of the symptoms of mild-to-moderate depression and some cases major depression (Solomon *et al.* 2013). Given its reputed medicinal properties, this species has been incorporated into the pharmacopoeias of several international locations, including Europe and the US, with a view to addressing the ever-increasing demands of the pharmaceutical industry and obtaining high-quality biomass. *Hypericum perforatum* is cultivated in many international locations.

Since these secondary metabolites are of increasing commercial importance, their production through biotechnology has attracted great interest. *Hypericum* species also belong to those medicinal plant species which have been grown in *in vitro* cultures (Danova *et al.* 2010; Coste *et al.* 2016). Currently, *H. perforatum* is one of the top-selling herbal drug treatments globally.

Table 1. The effect of the MeJA and JA elicitors on the phenolic compound contents of *Hypericum amblysepalum* shoot cultures

			Concentration (mg analyte/kg extract)					
			MeJA(µM)			JA(µM)		
Analytes	**RT[b]**	**Control**	**50**	**100**	**200**	**50**	**100**	**200**
Protocatechuic acid	700	37.32±0.8	35.71±0.7	44.33±0.95	**88.83±1.9**	57.60±1.2	53.10±1.14	37.30±0.8
Chlorogenic acid	803	30.90±0.9	26.90±0.8	29.30±0.87	**44.60±1.3**	27.70±0.8	40.30±1.2	34.4±1.02
Luteolin-7-glucoside	1320	13.61±0.1	30.40±0.2	**140.10±1.2**	95.13±0.8	39.30±0.3	50.80±0.43	95.1±0.81
Rutin	1367	68.05±0.9	72.66±0.9	74.20±1.0	**193.20±2.6**	77.24±1.04	54.92±0.74	39.10±0.53
Hesperidin	1368	32.71±0.5	35.20±0.5	36.30±0.5	**65.60±1.06**	36.62±0.5	29.50±0.47	29.30±0.47
Hyperoside	1369	1237.6±15.5	781.2±9.8	1175.8±14.8	**1458.8±18.3**	1496.7±18.8	1361.2±17.1	170.07±21.4
Apigetrin	1454	0.12±0.01	0.50±0.06	0.40±0.05	0.20±0.02	0.20±0.02	0.20±0.02	0.51±0.06
Quercitrin	1498	28.10±0.3	16.43±0.2	34.30±0.45	48.74±0.6	41.74±0.5	34.84±0.46	**54.80±0.72**
Astragalin	1513	2.20±0.03	2.30±0.03	4.20±0.06	**5.30±0.08**	3.79±0.05	2.62±0.03	3.30±0.05
Quercetin	1710	92.72±5.3	24.61±1.4	22.9±1.3	70.01±4.01	49.10±2.8	35.1±2.01	29.5±1.6
Luteolin	1778	8.21±0.10	6.11±0.11	14.60±0.27	16.6±0.30	8.23±0.15	8.32±0.15	**17.1±0.32**
Apigenin	1920	0.20±0.03	0.20±0.03	0.54±0.09	0.31±0.05	0.23±0.03	0.25±0.04	**0.59±0.09**
Pseudo hypericin	2634	85.40±14.6	63.50±10.9	83.40±14.	**162.30±27.0**	85.4±14.0	52.91±9.0	24.9±4.20
Hyperforin	2897	ND[b]	ND	ND	ND	0.004±0.001	0.002±0.009	0.002±0.009
Hypericin	3018	22.14±0.4	11.50±0.2	15.60±0.2	**27.60±0.5**	15.20±0.28	8.90±0.16	3.70±0.06

The statistical analysis was performed using ANOVA to analyse variance. Duncan's Multiple Range test ($P< 0.05$) was used to determine the significant changes in the mean values. Each data point represents the average of three replicates. aRT: retention time (min) bND: not detected

The *Hypericum amblysepalum* Hochst species is also considered to be a natural source of antioxidants and a potential anti-cancer drug candidate for the treatment of human cervical cancer (Keskin 2015). *Hypericum amblysepalum* has yellow flowers, spherical leaves, and square petals dotted with brown-black gland spots. This plant is found in moderate, tropical, and alpine climates and grows up to 1 m tall (Omar & Sajadi 2022). Saturated and unsaturated fatty acids form part of the metabolites found in *H. amblysepalum* (Ozen & Bashan 2003). In a recent study, it was revealed that some phytochemicals isolated from the essential oil of this plant showed considerable effects against common pathogenic bacteria and DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (Omar & Sajadi 2022).

Thanks to plant tissue culture techniques, any organ which is of importance for secondary metabolite production can also be targeted and cultured. One of these tissue culture types is liquid cultures, which are prototypes of bioreactors. There are numerous advantages to using liquid cultures via bioreactors for plant propagation, such as regulating and improving the production of bioactive chemicals, removing excessive manual utilisation, and increasing the multiplication rate and yield (Ziv 2005). Recently, a new method has been launched to develop secondary metabolite accumulation by using elicitors in plant tissue cultures. These elicitors are chemical resources which trigger biosynthetic pathways by activating distinctive transcriptional factors and upregulating genes. Therefore, the exogenous application of elicitors in these culture systems can be used for the abundant production of in-demand bioactive chemicals (Thakur *et al.* 2019).

It is known that the synthesis of secondary metabolites, such as hypericin, is influenced by several environmental, chemical, physical, biotic, and nutritional factors in tissue culture systems (Pavlik *et al.* 2007). Previous research has shown that the elicitors methyl jasmonate (MeJA) (Sirvent & Gibson 2002; Liu *et al.* 2007; Wang *et al.* 2015; Zamani *et al.* 2019; Lystvan *et al.* 2021) and jasmonic acid (JA) (Walker *et al.* 2002; Gadzovska *et al.* 2007; Coste *et al.* 2011; Cirak *et al.* 2020) change the phenolic content of *Hypericum* species in tissue cultures. However, most elicitation applications of these species on *in vitro* cultures have focused on *H. perforatum* (Shakya *et al.* 2019). However, current studies have shown that many *Hypericum* species have important chemical compounds which are responsible for essential biological activities. *H. amblysepalum* is also one of these species.

To the best of our knowledge, no attempt at an *in vitro* culture of *H. amblysepalum* has been made. The major goals of this research were to design an effective *in vitro* multiplication strategy for this species and to determine the effect of JA and MeJA elicitation on the bioactive metabolite production in *H. amblysepalum* shoot cultures. For the first time, we have developed an efficient *in vitro* growth method for *H. amblysepalum* using liquid tissue culture and increased the production of secondary metabolites with additional elicitors.

MATERIALS AND METHODS

Plant material. Wild-growing *H. amblysepalum* plants in the seeding stage were gathered from the vicinity of Mardin (Bakirkiri, Bakakri), Turkey. The voucher specimens were deposited at the Herbarium of Mardin Artuklu University (2013-2-MAU). The identification of the plant materials was confirmed by Dr. Cumali Keskin from the same institution and Dr. A. Selcuk Ertekin from Dicle University.

Chemicals. Standard compounds of protocatechuic acid (97%) and quercetin (95%) were obtained from Merck (Germany), while chlorogenic acid (95%), hyperoside (≥ 97%), rutin (94%), luteolin-7-glucoside (98%), hesperidin 98%), apigetrin, quercitrin (95%), astragalin (98%), luteolin (98%), apigenin (≥ 95.0), pseudohypericin (≥ 95%), hyperforin (≥ 98%), hypericin (≥ 95%), and elicitors MeJA and JA (≥ 95%) were purchased from Sigma (Germany).

Growth conditions and treatment. The seeds were washed in running tap water for pre-sterilisation. After pre-sterilisation, the seeds were immersed in a 70% ethanol solution (w:v) for 30 s. The seeds were shaken with a 5% solution of sodium hypochlorite (NaClO) for 10 min and rinsed with sterile distilled water three times for 5 min to remove any artefacts of NaClO. The seeds of *H. amblysepalum* were germinated in an MS (Murashige and Skoog) medium free of plant PGRs (plant growth regulators) in 50 mL Magenta vessels. The seeds were incubated for three weeks, and then the new shoots were transferred to an MS medium supplemented with a combination of BA (6-Benzylaminopurine) (0.5 mg/l) and GA_3 (Gibberellic acid) (0.1 mg/l) for multiplication (Namli *et al.* 2010). The entire experiment was performed under sterile conditions, and the growth room was set at 16 h light and 8 h dark at 25 ± 2°C with a 40 µmol s^{-1} m^{-2} light intensity and ambient humidity for all the plant cultures. The micro shoots were subcultured approximately 4–5 times, every three weeks. The elongated shoots obtained from the multiplication stage were used as the experimental material in the following stages.

Elicitation of the shoot cultures. For the elicitation studies, the elongated shoots were cut into 20–25 mm long segments (each with 3 to 4 nodes) and transferred to 250-ml conical flasks, and 12 explants were cultured in 50 mL MS liquid media (30 g/l of sucrose, 0.5 mg/l BA and 0.1 mg/l GA_3, agar free). Stock solutions of MeJA and JA were prepared at 0.125, 0.250, and 0.500 M concentrations in ethanol (95%), and sterilised by filtration (0.2 µm) (Millipore). Then the stock solutions were added (20 µl) to the liquid MS medium to obtain the final concentrations of 50, 100, and 250 µM(respectively) in 7-day-old cultures for each concentration. The shoots which were not subjected to the addition of any of the elicitors to the liquid medium were used as the control plants. For the control replicates, 20 µl of 95% ethanol was used. The cultures were harvested after 30 days of elicitation. Each treatment was performed in triplicate, and 12 explants were evaluated for each replicate. The dry weight (DW) of each plantlet was recorded on day 30 to obtain the biomass production.

The extraction of the samples. The following extraction protocol was adapted from that previously reported by Ang *et al.* (2002). Briefly, the air-dried shoots were weighed and then ground using a mortar. These samples (0.2 g) were extracted three times with 10 ml methanol (80% v:v) under sonication in an ultrasonic sonicator (Sanyo MSE-Soniprep 150 UK) with an ice bath for 20 min at 4°C. The methanolic extracts were then centrifuged (Thermo Scientific Labofuge, 200) at 15 min and 8000 g speed. The supernatant was filtered through a nylon syringe filter (0.22 µm) and stored in the dark at 20°C prior to LC-MS/MS analysis. The extract samples (20 µl) were injected into the LC-MS/MS system.

Quantification of the phenolic compounds. The qualitative and quantitative analysis of 15 fingerprint phytochemicals specific to the *Hypericum* species was performed by a previously developed and validated LC-MS/MS analysis method by Akdeniz *et al.* (2020). The LC-MS/MS system consisted of a combination of the Shimadzu Nexera model UHPLC and a triple quadrupole mass spectrometer (Shimadzu LCMS 8040). The LC-MS/MS analytical parameters of the developed method are provided as supplementary material (Table S1).

The LC-30 AD model gradient pump, the DGU-20A3R model degasser, the CTO-10ASvp model column oven, and the SIL-30AC model autosampler made up the liquid chromatography system. The chromatographic separation process was carried out using the Agilent Poroshell 120 (EC-C18 2.7 µm, 4.6 mm × 150 mm) column. The column oven was set to 40°C during the analysis. In the elution gradient, ultra-pure water and methanol were used for the mobile phases A and B, respectively. Furthermore, to facilitate the chromatographic separation and ionisation, the mobile phases were supplemented with 5 mM ammonium formate and 0.15% formic acid. The UHPLC gradient profile was optimised as 20–100% B, 100% B, and 20% B (for 0–25; 25–35 and 35–45 min) after numerous attempts to achieve the optimum separation of the analytes. The mobile phase flow rate and injection volume were 0.5 mL/min and 2 µL, respectively. The applied electrospray ionisation (ESI) source can operate in both negative and positive modes. LabSolutions (Shimadzu, Japan) software was used for the collection and processing of the LC-ESI-MS/MS data. The device was evaluated in the MRM (multiple reaction monitoring) modes, and the molecular (parent)

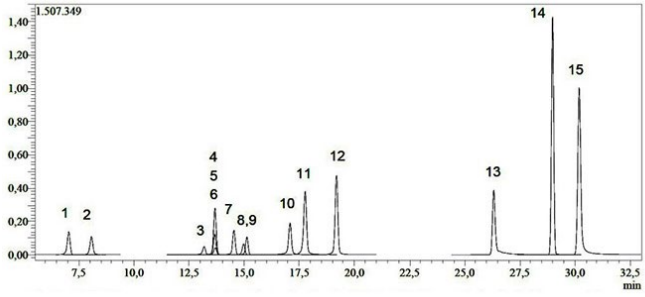

Fig. 1. LC–MS/MS chromatograms of the standard chemicals analysed by the LC–MS/MS method.

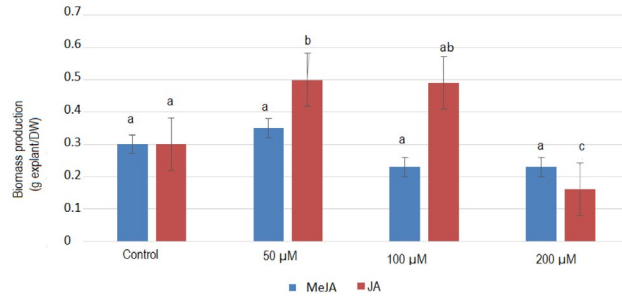

Fig. 2. The effect of JA and MeJA on the growth of the *H. amblysepalum* shoots.

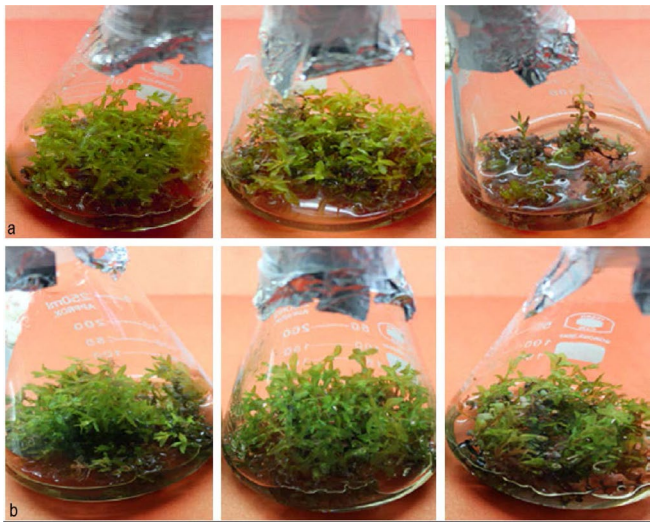

Fig. 3. The effect of elicitors JA (a) and MeJA (b) on the growth of the *H. amblysepalum* shoots (For the 50 µM, 100 µM, and 200 µM concentrations respectively)

ions were combined with one or two productions (the first was used for quantitative purposes and the other for qualitative purposes) for the quantitative determination of the analytes. The other parameters regulated in mass spectrometry were: the interface temperature; 350°C, the desolvation line temperature; 250°C, the heat block temperature; 400°C, the nebulizer gas (N_2) flow; 3 l/min and the drying gas temperature (N_2); 15 l/min. The LC–MS/MS chromatograms of the standard chemicals are given below (Fig. 1).

Statistical analysis. Each treatment performed using three Erlenmeyer flasks consisted of 12 explants and all the experiments were repeated three times. The data were analysed using one-way analysis of variance (ANOVA), and Duncan's multiple test ($P< 0.05$) was used to compare the differences between the mean values of all the groups.

RESULTS AND DISCUSSION

Elicitors, precursors, and bioinformatics, as well as environmental stressors and changes in medium elements, have previously been used to initiate stress responses in plant tissues for increased secondary metabolite output (Manorma *et al.* 2011). Elicitors can be employed to boost plant secondary metabolite synthesis and play a key role in biosynthetic pathways, resulting in the increased production of commercially valuable chemicals. Several plant culture methods, such as liquid, cell suspension, and organ cultures of *Hypericum* have also been used to investigate secondary metabolites from various classes over several decades (Kucharikova *et al.* 2016).

As our results show, low concentrations of the elicitors MejA and JA (50 µM) resulted in the enhanced biomass production of the *H. amblysephalum* shoot cultures, while higher concentrations of those elicitors (200 µM) had a reducing effect compared to the control and the other elicitor concentrations. (Fig. 2). Additionally, the plantlets elicited with 200 µM MeJA and JA exhibited browning and the formation of a compact callus on the bottom of the shoots (Fig. 3). The development in biomass hight have been due to the influence of MeJA used as an elicitor related to plant protection response pathways, which would affect the biosynthesis of secondary chemicals (Zhao *et al.* 2005). However, treatment with a higher level of these elicitors negatively affected the growth of the *H. amblysepalum* plantlets. After 30 days of elicitation, it was observed that the dry weight of the shoots treated with 200 µM MeJA were lower than those of the control plantlets. Additionally, these plantlets began to brown and formed a compact structure at the bottom of the shoots. These outputs are in accordance with the results obtained by Coste *et al.* (2011). They reported that 250 and 500 µM JA led to the inhibition of biomass production compared to the control shoots and those treated with a lower concentration of JA in the shoot cultures of two *Hypericum* species. The negative effect of high concentrations of MeJA has also been re-

ported in *H. perforatum* and *H. sampsonii* cultures (Sirvent & Gibson 2002; Liu *et al.* 2007; Wang *et al.* 2015).

The data from the LC-MS/MS analysis showed that hyperoside, rutin, pseudohypericin, luteolin-7-glucoside, and quercetin were the quantitatively dominant compounds in the *H. amblysepalum* methanol extracts. However, luteolin, astragaline, apigenin, and apigetrin were found in very low amounts.

When the two elicitors were compared the elicitor MeJA was shown to be more effective in terms of increasing the content in most of the compounds investigated. The highest amounts of the rutin (2.8 fold), protocatechuic acid (2.4 fold), astragalin (2.4 fold), hesperidin (2 fold), pseudohypericin (1.9 fold), chlorogenic acid (1.4 fold), and hypericin (1.9 fold) compounds were obtained from the shoots elicited with MeJA 200 µM. In their study, Sirvent and Gibson (2002) showed that *H. perforatum* plantlets supplemented with 200 µM MeJA produced higher levels of hypericins compared to the control levels. Additionally, elicitation with 100 µM MeJA led to a remarkable increase in the accumulation of luteolin-7-glucoside (10.3 fold) (Table 1). Similarly, Wang *et al.* (2015) reported the highest flavonoid content with an elicitation dose of 100 µM MeJA in *H. perforatum* cell cultures. Moreover, when MeJA (100 µM) was added to the liquid shoot culture of *H. perforatum,* the hyperforin, hypericin, and pseudohypericin contents were significantly higher than those in the control (Liu *et al.* 2007). Different biosynthetic routes have been discovered in *H. perforatum* plantlets (Conceiçao *et al.* 2006). Similarly, in our study, while the hypericin content increased, hyperforin was not detected in the shoot cultures elicited with MeJA. In this context, the presence of a low correlation coefficient between the hypericin and hyperforin content suggests that the hypericin and hyperforin biosynthetic pathways may differ. The increase in hypericins and hyperforin concentrations in response to chemical elicitors indicates the involvement of these secondary metabolites in the inducible plant defense responses of *H. ambylosephalum*.

Furthermore, jasmonic acid significantly increased the chemical content of the *H. amylosephalum* shoot culture. The highest amounts of luteolin (2 fold), quercitrin (1.9 fold), apigetrin (4 fold), apigenin (2.9 fold), and hyperoside (1.3 fold) were found in the shoot cultures elicited with 200 µM JA (Table 1). Previous research has demonstrated the effects of JA elicitation on the growth and phenolic production in the *H. perforatum* species (Sirvent & Gibson 2002; Gadzovska *et al.* 2007). Similarly, Gadzovska *et al.* (2007) determined a 6-fold increase in phenolic compounds and flavanols after 4 days of JA elicitation in *H. perforatum* cell suspensions. As seen in the table, JA elicitation at low concentrations (50 µM JA) triggered hyperforin production in the *H. ambylosephalum* shoot cultures (Table 1). Elicitors, such as JA and its derivatives, are known to trigger the production of phenolic compounds (Sanz *et al.* 2000). Hyperforin synthesis can be activated with a low JA concentration. It is possible that a low JA concentration promotes PAL (phenylalanine ammonia-lyase) activity to facilitate the production of cinnamic acid as a possible precursor which might increase hyperforin synthesis. Hyperforin exhibits certain neurological effects, such as anti-depressive, as well as anticancer, antibacterial, and anti-angiogenic effects (Medina *et al.* 2006).

Interestingly, JA elicitation decreased the hypericin content (Table 1). Similarly, Cirak *et al.* (2020) reported that a 0.5 mg/l dose of JA suppressed hypericin synthesis and produced the lowest values. The JA dose that we used might have had a suppressive effect on hypericin production.

Our findings showed that JA and MeJA treatments at higher concentrations (200 µM) led to a slow decrease in DW (Fig. 2); however, phenolic content and production significantly increased compared to the control cultures (Table 1). A similar phenomenon was also reported in earlier studies, whereby MeJA supplementation reduced biomass production and leaf senescence, but increased the amount of hypericins and hyperforin compounds in both *H. sampsonii* and *H. perforatum* shoot cultures (Sirvent & Gibson 2002; Liu *et al.* 2007).

CONCLUSION

Shoot culture systems are one of the promising plant tissue culture techniques for the synthesis of high-value secondary metabolites alongside callus and cell suspension cultures. Our findings revealed that the addition of exogenous signalling molecules to the shoot culture can alter specific plant metabolites depending on their metabolic route. The successful enhancement of the phenolic compounds in the shoot cultures of *H. ambysephalum,* which is known to have anticancer and antioxidant potential through elicitor treatments, makes this plant a good source of raw materials for the pharmaceutical industry.

In conclusion, our findings showed that the production of hypericins and hyperforin is regulated by certain elicitors. However, to completely understand the mechanisms of bioactive chemical production in plants, more research is needed. With further study, higher phenolic content-enhancing media can be improved to produce larger amounts of these compounds.

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REZIME

Botanica SERBICA

Elicitori pojačavaju proizvodnju bioaktivnih jedinjenja u kulturama izdanaka *Hypericum amblysepalum*

Hilal Surmuş Asan

U ovoj studiji ispitivali smo efekte elicitora MeJA (metil jasmonat) i JA (jasmonska kiselina) (50, 100 i 200 μM) na rast i proizvodnju 15 fenolnih jedinjenja u kulturi izdanaka *Hypericum amblisepalum* pomoću LC-MS/MS metode. Proizvodnja biomase se povećala kod izdanaka tretiranim sa 50 μM JA i MeJA. Međutim, veće koncentracije ovih elicitora su negativno uticale na rast kultura izdanaka, dok su dovele do povećanja sadržaja sekundarnih metabolita. Elicitor MeJA (posebno 200 μM MeJA) je bio efikasniji u smislu povećanja sadržaja fenolnih jedinjenja. Dobijene su najveće količine rutina (2,8 puta), astragalina (2,4 puta), protokatehuinske kiseline (2,4 puta), hesperidina (2 puta), pseudohipericina (1,9 puta), hlorogenske kiseline (1,4 puta) i hipericina (1,9 puta) iz izdanaka tretiranih sa MeJA (200 μM). Pored toga, primena elicitora JA 200 μM povećala je količinu luteolina (2 puta), kvercitrina (1,9 puta), apigetrina (4 puta), apigenina (2,9 puta) i hiperozida (1,3 puta). Ova studija je otkrila da specifični sekundarni biljni metaboliti mogu biti regulisani egzogenim elicitorima u kulturi izdanaka *H. ambisepalum*, što obećava veoma dobar izvor sirovina za farmaceutsku industriju.

Ključne reči: *Hypericum amblysepalum*, jasmonska kiselina, metil jasmonat, kultura izdanaka