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Production of elicitor-induced phytochemicals in callus and shoot cultures of *Hypericum heterophyllum*

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ABSTRACT

In the present study, it was objected to determine the rapid *in vitro* propagation of the endemic *Hypericum heterophyllum*, and the effects of abscisic acid (ABA) and salicylic acid (SA) applications on the production of bioactive compounds in tissue culture. Total phenolic (TP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and High Performance Liquid Chromatography (HPLC) analyses were carried out in both *in vivo* plants and *in vitro* samples. Initially, seeds were objected to pre-treatment with 1.5 mg/L GA_3 for 48 h, then surface-sterilized with 15 % NaOCl and germinated at Linsmaier and Skoog (LS) medium containing Gamborg's B5 vitamins without plant growth regulators. After germination, axillary buds were used as explant for callus and shoot regeneration. Full-strength LS medium with B5 vitamins, 0.5 mg/L thidiazuron (TDZ) and 0.5 mg/L indole butyric acid (IBA) gave the highest ratio (73.3 %) of green and fragile callus formation. On the other hand, the highest shoot regeneration (86.7 %) was obtained from LS/B5vit with 1 mg/L benzylaminopurine (BAP). Elongated shoots were rooted in a medium containing 1.5 mg/L IBA after 8 weeks and *in vitro* plantlets were obtained for elicitation. Then, calli and *in vitro* plantlets were exposed to 0.01, 0.05 and 0.1 mg/L ABA or SA elicitors in LS/B5 medium for 15 days, separately. Total phenolic content was higher in the *in vivo* plant leaves (77.6 mg GAE/g) than flower, stem and herb. In *in vitro* grown plantlets, the highest total phenolic content (37.04 mg GAE/g) was obtained from the control without ABA and SA application. On the other hand, 0.01 mg/L ABA application resulted in the highest total phenolic content (29.5 mg GAE/g) in callus cultures. The highest DPPH activity was achieved *in vivo* leaf (415.8 mg TEs/g extract), with 0.01 mg/L SA application *in vitro* plantlets (84.4 mg TEs/g extract) and with the use of 0.05 mg/L ABA in callus (136.3 mg TEs/g extract). In addition, chlorogenic acid, hyperoside, catechin, quercetin, kaempferol, and apigenin were determined with HPLC analysis. The main compound was chlorogenic acid (5.8 mg/g DW) *in vivo* leaves, followed by hyperoside (3.2 mg/g DW) in the same plant part, and lastly, quercetin (0.58 mg/g DW) had the highest amount in herba. The value of hyperoside was 0.28 mg/g DW at 0.01 mg/L ABA in *in vitro* plantlets; meanwhile 0.01 mg/L SA application produced 0.49 mg/g DW in calli. With regard to quercetin, it had the highest value at 0.1 mg/L SA (0.29 mg/g DW) in calli, comperatively, 11.2-fold more than the control (0.026 mg/g DW) group. The catechin content varied within a very wide range, from 0.025 to 0.23 mg/catechin g DW in callus. Elicitation with 0.01 mg/L ABA significantly enhanced the production of quercetin, catechin and hyperoside in both *in vitro* plantlets and calli.

1. Introduction

Hypericum genus belongs to the Clusiaceae (Hypericeae = Guttiferae) family and there are approximately 484 *Hypericum* taxa in the world (Crockett and Robson 2011). *Hypericum* species are naturally distributed in different geographies, from the equatorial belt to the Scandinavian

Abbreviations: ABA, abscisic acid; BAP, benzylaminopurine; CHI, chalcone isomerase; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; DW, dry weight; GAE, gallic acid equivalent; HPLC, high performance liquid chromatography; IAA, indole-3-acetic acid; IBA, indole butyric acid; LS, Linsmaier and Skoog; NAA, 1-naphthaleneacetic acid; PAL, phenylalanine aminolyase; ROS, reactive oxygen species; SA, salicylic acid; TP, total phenolic; TDZ, thidiazuron

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countries in the North (Crockett and Robson 2011) and grow well in warm, temperate subtropical and mountainous tropical regions (Robson 2001; Çamas et al. 2011). Türkiye is an important center in terms of *Hypericum* species, and 46 of the existing 96 species are endemic (Davis 1988; Güner et al., 2012; Cirak et al. 2016). *Hypericum heterophyllum* Vent. is a herbaceous perennial native to Türkiye that grows best in warm, sunny locations. *H. heterophyllum* has yellow flowers with a long blooming season and has tolerance to adverse environmental conditions such as drought, heat and moderately cold temperatures, and grow in arid, stony or rocky calcareous regions (Davis 1988; Hamzaoğlu 2005).

Among the *Hypericum* species, the *H. perforatum* (St. John's Wort) is the best known in the scientific world. Interest in the therapeutic potential of *Hypericum* species as herbal natural products for pharmaceutical development are increasing year by year, and many species have attracted intense interest and are being extensively investigated (Nahrstedt and Butterweck 2010; Xiao et al. 2020). Many studies have been carried out on secondary metabolite of *Hypericum perforatum*. The most important of these are naphthodianthrones (hypericin, pseudohypericin, protohypericin, etc.), flavonoids (kaempferol, quercetin, rutin, quercitrin, quercetin, luteolin, hyperin, hyperoside, etc.), phenolic acids (chlorogenic acid), phloroglucinols (hyperforin, furohyperforin, etc.), xanthones and volatile oils (Barnes et. al 2001). These compounds are widely used in pharmacology (Xiao et al. 2020; Zhang et al. 2020). Of these compounds, flavonoids and chlorogenic acid have the ability to exhibit high antioxidant activity, as well as contributing to various biological activities such as antidepressant, antimicrobial, anticancer, neuroinflammatory and neuroprotective (Alahmad et al. 2021; Kholghi et al. 2022; Agan et al. 2023; Liu et al. 2024).

Recently, many studies have also been reported on secondary metabolite and biological activity of *H. heterophyllum* (Cakir et al. 2004; Ayan and Çirak 2008; Öcal and Eroğlu 2012; Yaman 2020; Yaman and Atmaca 2021; Yaman and Şimşek 2021; Hazman et al. 2022; Erenler et al. 2023; Eruygur et al. 2024; Yaman et al. 2024). Ayan and Cirak (2008) did not detect hypericin and pseudohypericin in stem, leaves and flowers. Likewise, Yaman et al. (2024) also did not determine hypericin and pseudohypericin in extracts of *H. heterophyllum* using ethanol, methanol, acetone, and chloroform extraction. They identified shikimic acid (0.146 mg/g extract) in the methanol extract and chlorogenic acid (11.5 mg/g extract) as the main component in the ethanol extract. Additionally, shikimic acid (5.6 mg/g extract), hyperoside (1.4 mg/g extract), kaempferol-3-glucoside (0.26 mg/g extract), quercetin (0.20 mg/g extract) compounds were also found at high rates in the ethanol extract. Furthermore, Hazman et al. (2022) detected the highest levels of chlorogenic acid, miquelianin and isoquercetin compounds in methanol and acetone extracts and emphasized that the extracts were notable for their antimicrobial, anticancer and antioxidant activities. By comparing *in vitro* samples with wild plants of *H. heterophyllum* by Yaman (2020), quinic acid, gallic acid, catechin, ferulic acid, vanillic acid, p-coumaric acid, caffeic acid and quercetin compounds exhibiting strong antioxidant activity were analyzed by LC-MS/MS.

Due to the possible harms of synthetic compounds, the demand for natural product consumption become a very popular in recent days. The proven positive effects of natural products and increasing environmental concerns are the main motivations for investigating natural bioactive compounds as possible alternatives to synthetic products (Kravanja et al. 2021). Plants are the largest source of natural products containing secondary metabolites of commercial value in almost every branch of industry, mainly as drugs, food additives, sweeteners, fragrances, pigments and bio-pesticides (Murthy et al. 2014). There are various difficulties and risks in the large-scale production of secondary metabolites through plant cultivation due to variation in genetic, environment, season, harvest period, drug diversity and quantity in different plant organs, and standardization (Açıkgöz 2020; Palit and Mandal 2021; Li and Zidorn 2022). In response, *in vitro* culture technology has emerged as an alternative that guarantees rapid production of high-quality and standard products to meet the ever-increasing industrial demand for increased production of commercial secondary metabolites (Mahendran et al. 2022; Mahood et al. 2022).

Plant tissue culture can be used for rapid and extensive propagation of various plant tissues, organs and cells, making it indispensable for alternative production of plants (Song et al. 2024). In particular, the callus tissue culture technique for the commercial production of plant secondary metabolites has attracted considerable interest and has been widely used in industrial applications (Marchev et al. 2024). In addition, elicitation (biotic and abiotic) is one of the remarkable techniques for the successful and high-yield production of secondary metabolites by triggering their biosynthetic pathway (Mahood et al. 2022; Song et al. 2024). Among the abiotic elicitors, there are reports that intracellular signaling molecules (jasmonic acid, methyl jasmonate, abscisic acid (ABA), salicylic acid (SA), brassinosteroids, polyamines etc.) can increase the biosynthesis of secondary metabolites in the callus tissues of *Hypericum* species (Coste et al. 2011; Gadzovska et al. 2013; Surmuş 2023; Al-Khayri et al. 2024).

Studies on the *in vitro* culture of *H. heterophyllum* were very limited. Yaman (2020) obtained *in vitro* plantlets and callus in tissue culture from the *H. heterophyllum*, and made antioxidant activity and phytochemical analyses from aerial parts, calli, and *in vitro* plantlet samples. Although elicitors are widely used in the production of plant secondary metabolites, their effects on metabolic products in *H. heterophyllum* callus tissues remain unclear. Therefore, the first aim of the present study was to evaluate the effect of Linsmaier and Skoog medium (LS; Linsmaier and Skoog 1965) on *in vitro* culture of *H. heterophyllum*. The second aim of this work was to study total phenolics content, antioxidant capacity using 2,2-diphenyl-2-picrylhydrazyl assay and bioactive component variability both *in vivo* and *in vitro* samples elicited with ABA and SA. The findings of this study may contribute to the theoretical basis for callus and shoot cultures as well as antioxidant activity and secondary metabolite production of endemic *H. heterophyllum*.

2. Material and methods

2.1. Plant material

The aerial parts and seeds of *Hypericum heterophyllum* Vent. were collected during the flowering period from the Yozgat Bozok University Campus (altitude 1332 m asl, N 39°46′42′′ 34°47′51′′; Fig. 1). The plant was identified by Prof. Dr. Osman Tugay (Herbarium 28,030).

2.2. Methods

2.2.1. Surface disinfection and germination condition

The harvested seeds were washed with running tap water. After initial washing, they were kept in a solution containing 1.5 mg/L GA_3 for 48 h due to dormancy in the seeds. Lastly, for surface disinfection the seeds were sterilized with a solution containing 1.5 % NaOCl (5 % v/v commercial bleach) and rinsed with sterile water (3 times x 5 min). Finally, the sterilized seeds were placed on a germination medium containing 1 × LS/B5 (Linsmaier and Skoog/ Gamborg's B5) medium supplemented with 1.0 mg/L gibberellic acid and 3 % sucrose and 0.7 % agar (Duchefa) (Çamaş and Çalışkan, 2011). The pH of the medium was adjusted to 5.8 ± 0.1 with 0.1 N NaOH or 0.1 N HCl before the addition of 0.7 % (w/v) plant agar (Duchefa). The seeds were maintained in a growth chamber at room temperature (24 ± 1 ° C) under 16/8 h light/dark photoperiod, 60 % relative humidity and cool white fluorescent light intensity of about 40 μmol $m^{-2}s^{-1}$ PPFD for 15 days. The axillary buds of germinating seeds were used as explant for further experiments.

Fig. 1. Aerial parts of *Hypericum heterophyllum* Vent.

2.2.2. Shoot and callus culture

Both shoot and callus cultures were started with axillary buds of sterile seedlings. For shoot culture, LS medium supplemented different BAP (0, 1.0, 2.0 and 3.0 mg/L) concentrations or 1.0 mg/L BAP + NAA (0.01, 0.1 and 0.25) combinations were tested to regenerate shoots. After 8 weeks, shoots were transferred 1.5 mg/L IBA for rooting. The seedlings that survived acclimatization were collected after 30 days for polyphenolics and antioxidant activity analyses.

For callus culture, TDZ (0.1, 0.25, 0.5, 0.75 and 1.0 mg/L) and IBA (0.1, 0.25, 0.5, 0.75 and 1.0 mg/L) combinations in LS medium were applied with cytokinin and auxin ratios of 1/10, 1/3, 1/1, 3/1 and 10/1. Among treatments, 0.5 mg/L TDZ and 0.5 mg/L IBA (1/1 cytokinin and auxin ratios) exhibited high rates of callus induction, and these calli and rooting plantlets were used for elicitor treatments.

2.2.3. Elicitor treatments

Calli and *in vitro* plantlets were transferred to LS/B5 medium supplemented with ABA (abscisic acid) or SA (salicylic acid) elicitors. The calli and *in vitro* plantlets were exposed to 0.01, 0.05 and 0.1 mg/L ABA or SA elicitors in LS/B5 medium for 15 days separately. Also, the control treatments for calli and *in vitro* plantlets were cultured in the same medium without elicitor.

2.2.4. Extraction for quantification of polyphenolics and antioxidant activity

All samples (calli, *in vitro* plantlets and *in vivo* plant parts) were freeze-dried, and powdered. Plant materials were extracted with 80 % (v/v) methanol in an ultrasonic bath for 30 min at 4 °C as previously reported (Gadzovska et al. 2007; Gadzovska et al. 2013). Folin–Ciocalteau reagent (Carlo Erba Reagenti, Rodano, Italy) and 0.7 M Na_2CO_3 were used to determinate to total phenolic content. Samples were incubated for 5 min at 50 °C and then cooled for 5 min at room temperature. Absorbance was measured spectrophotometrically at 765 nm. The concentration of total phenolic compounds was calculated using gallic acid (0–10 mg/ml) as a standard (Gadzovska et al. 2013).

DPPH (2,2-diphenyl-1-picrolhydrazyl-Sigma D9132) activity method was adapted from Brand-Williams et al. (1995), 10 mg DPPH was dissolved in 100 ml methanol, 290 μl DPPH was added to 10 μl extract in 96-well plates. Trolox was used as standard, and 10 mg trolox was dissolved in 40 ml (1 nM/μl) methanol. SoftMax Pro (v. 5.4.1) software was used on a SpectraMax 190 Microplate Reader (Molecular Devices Corp., Sunnyvale, CA). Readings were made three times. As a control, 80 % methanol solvent was used. After the samples were kept in the dark for 10 min, spectrophotometric analysis was performed at 518 nm for 10 min.

2.2.5. Quantification of polyphenol compounds using the HPLC system

HPLC analyses were performed with Agilent 1260 G1315 DAD device. The column was ACE 5, C18 (250 × 4,6 mm; 5 μm). The flow rate was 1 ml/min and the injection volume was 10 μl. All separations were performed at 30 °C. The mobile phase consisted of three solvents: phosphoric acid-water (A; 3:1000, v/v), phosphoric acid-acetonitrile (B, 1:1000, v/v), and methanol (C, 1000, v/v). The gradient program with the following solvents: phosphoric acid-water (A; 3:1000, v/v), phosphoric acid-acetonitrile (B, 1:1000, v/v), methanol (C, 1000, v/v). 100 % A (0–10 min), 85 % A-15 % B(10–30 min), 70 % A-20 % B-10 % C (30–40 min), 10 % A-75 % B- 15 % C (40–55 min), 5 % A-80 % B- 15 % C (55 min), and continued with 100 % A for further 10 min. The total run time was 65 min (Brolis et al. 1998). HPLC grade solvents were used acetonitrile and methanol (CH_3OH) from Isolab, and phosphoric acid- (H_3PO_4) from Merck. The HPLC-water was purified by (Milli-Q), the commercial standards chlorogenic acid, rutin, quercetin, quercitrin, catechin, hyperoside, hypericin, and pseudohypericin (Sigma-Aldrich, Germany) were used as reference compounds. The reference compounds were dissolved in 80 % CH_3OH. The concentration of the stock standard solutions was 1 mg/mL and they were stored at −20 °C. Spectral data from all peaks were accumulated in the range 190–600 nm, and chromatograms were recorded at 210, 254 nm for flavan-3-ols, 330 nm for phenolic acids, and at 590 nm for naphtodianthrones. Peak areas were used for quantification at wavelengths where each group of phenolic compounds exhibited an absorption maximum.

2.3. Statistical analysis

All parameters (shoot regeneration, callus induction, antioxidant activity, and secondary metabolites) were measured in triplicates. All data were analyzed by one-way ANOVA followed by Duncan's multiple range test ($P \leq 0.05$) to find out the differences among the means. Percentage values were converted to angle values before statistical analysis. All statistical analyses were performed IBM SPSS statistics 25 statistical software. The standard errors of each treatment means were given as ± next to the mean values.

3. Results and discussion

3.1. Shoot regeneration

GA_3 application resulted in 86.7 % seed germination rate and the axillary buds from these *in vitro* seedlings were used as explant for further experiments. Different concentrations of BAP alone and combined with NAA were evaluated in terms of *H. heterophyllum* multiple shoot proliferation. The findings of applying varying PGR concentrations to the *in vitro* regeneration of multiple shoots from axillary buds were statistically different (Table 1). The results showed that when the cytokinin concentration increased above 1 mg/L, shoot growth was reduced. The best concentration of cytokinin for shoot regeneration was 1 mg/L BAP, which displayed an 86.7 % rate of shoot-forming explant and 5.30 shoot number per explant ($p \leq 0.05$) and was used as main source for abscisic and salicylic acid elicitation studies (Fig. 2). Addition of NAA to the medium did not improve the shoot regeneration.

Means followed by the different letters within columns were significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 1
Effect of different BAP and NAA concentrations on *in vitro* shoot regeneration from *H. heterophyllum* axillary buds after 8 weeks of culture.

Plant growth regulators (mg/L)		Rate of shoot-forming explant (%)	Shoot number per explant
BAP	NAA		
0	0	0.0^{e}	0.0^{c}
1.0	0	86.7^{a} ±3.6	5.30^{a}±0.8
2.0	0	53.3^{b}±7.02	0.97^{b}±0.5
3.0	0	20.0^{d}±0.0	0.30^{bc}±0.0
1.0	0.01	0.0^{e}	0.0^{c}
1.0	0.1	0.0^{e}	0.0^{c}
1.0	0.25	36.7^{c}±2.7	0.37^{bc}±0.06

Fig. 2. Shoot regeneration from axillary buds of *H. heterophyllum* in LS/B5 medium containing 1 mg/L BAP.

3.2. Callus induction

Callus formation was induced from nodal segments of *in vitro* shoots by using LS/B5 medium supplemented with TDZ and IBA (Table 2). In this treatment, the cytokinin/auxin ratio was especially taken into account in adjusting the PGR concentration (1/10, 1/3, 1/1, 3/1, 10/1). Among these cytokinin/auxin ratios, 1/1 (0.5 m/L TDZ and 0.5 mg/L IBA) displayed the highest rate of callus formation with 73.3 % ($p \leq 0.05$). However, there was no statistically significant difference between the 1/1 and 1/3 cytokinin/auxin ratios. These calli were green in color and compact in appearance, capable of regeneration, and used as mother sources for elicitor studies. In general, increasing the cytokinin ratio in the medium exhibited a more negative effect on callus induction than the increase in auxin (Table 2).

3.3. ABA and SA elicitation

Green and fresh calli were successfully obtained after 60 days on LS/B5vit medium containing 0.5 mg/L each of TDZ and IBA. These calli and *in vitro* plantlets were subjected to ABA and SA elicitors on the same medium for 15 days. The calli were fragile and green in 0.01 and 0.05 mg/L SA applications. While no morphological change was observed at low doses of abscisic and salicylic acid, necrosis occurred in calli from 0.1 mg/L ABA and 0.1 mg/L SA and their color changed from green to brown. As a result, necrosis formation in callus cells indicated that the amount of 0.1 mg/L ABA and 0.1 mg/L SA was too high for the cells (Fig. 3).

3.4. Total phenolic compounds

The influence of SA and ABA was evaluated during 15 days post-elicitation on *in vitro* plantlets, callus cultures, and compared with *in vivo* plants. It was determined that the leaf part had the highest total phenolic (TP) content (77.6 mg GAE/g extract) of *in vivo* samples of *H. heterophyllum* (Table 3). Additionally, the highest value among *in vitro* plantlets was the control group (37.04 mg GAE/g) and has as much value as the *in vivo* stem (36.1 mg GAE/g) part. On the other hand, there was a statistically significant difference between *in vivo* leaf and *in vitro* plantlets of control group ($p \leq 0.05$). With regard to elicited plantlets, 0.1 mg/L ABA (26.3 mg GAE/g) and 0.01 mg/L ABA (24.8 mg GAE/g) has produced almost similar values. On the other side, 0.1 mg/L SA (22.3 mg GAE/g) showed the highest TP production in plantlets. Moreover, 0.01 mg/L ABA (29.5 mg GAE/g) and 0.01 mg/L SA (27.03 mg GAE/g) had the highest values in elicited callus. Thus, there was a statistical correlation between the amount of 0.01 mg/L ABA and 0.01 mg/L SA calli ($p \leq 0.05$). Almost all elicitor applications produced high amounts of TP in callus, while a dramatic decrease was detected in the control (9.91±0.10 mg GAE/g) group. Finally, TP content was found to be quite low both in the *in vitro* plantlets and calli in comparison to *in vivo* plant parts.

3.5. DPPH radical scavenging activity

DPPH radical scavenging capacity for *in vivo* plant, *in vitro* plantlets and calli extracts are presented in Table 4. Similarly to TP content results, the *in vivo* leaf part had the highest value (415.8 mg TEs/g extract) in DPPH activity (Fig 4) and *in vitro* plantlets showed the highest DPPH activity with 0.01 mg/L SA (84.4 ± 1.40 mg TEs/g) and 0.1 mg/L ABA (63.3 ± 0.83 mg TEs/g). However, this effect was not exhibited for the control group that it had the lower rate 2.5-fold and 1.9-fold than 0.01 mg/L SA and 0.1 mg/L ABA plantlets, respectively. With regard to, there was a statistical correlation between the amount of 0.1 mg/L ABA and 0.01 mg/L SA plantlets ($p \leq 0.05$). Moreover, it was determined that 0.01 mg/L SA (84.4 mg TEs/g) plantlets had higher DPPH activity ($p \leq 0.05$) than *in vivo* stem parts (77.5 mg TEs/g).

Table 2
Effect of different concentrations and combinations of TDZ and IBA on *in vitro* callus formation from *H. heterophyllum* nodal segments after 60 days of culture.

Plant growth regulators (mg/L)		Cytokinin/Auxin ratio	Callus formation rate (%)	Callus color and structure
TDZ	IBA			
0.1	1.0	1/10	0.0^{c}	no induction
0.25	0.75	1/3	70.0^{a}±1.0	Dark green color, compact
0.5	0.5	1/1	73.3^{a}±3.05	Green color, compact
0.75	0.25	3/1	46.7^{b}±1.5	Dark green color, compact
1.0	0.1	10/1	36.7^{b}±0.5	Green color, compact

Means followed by the different letters within columns were significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

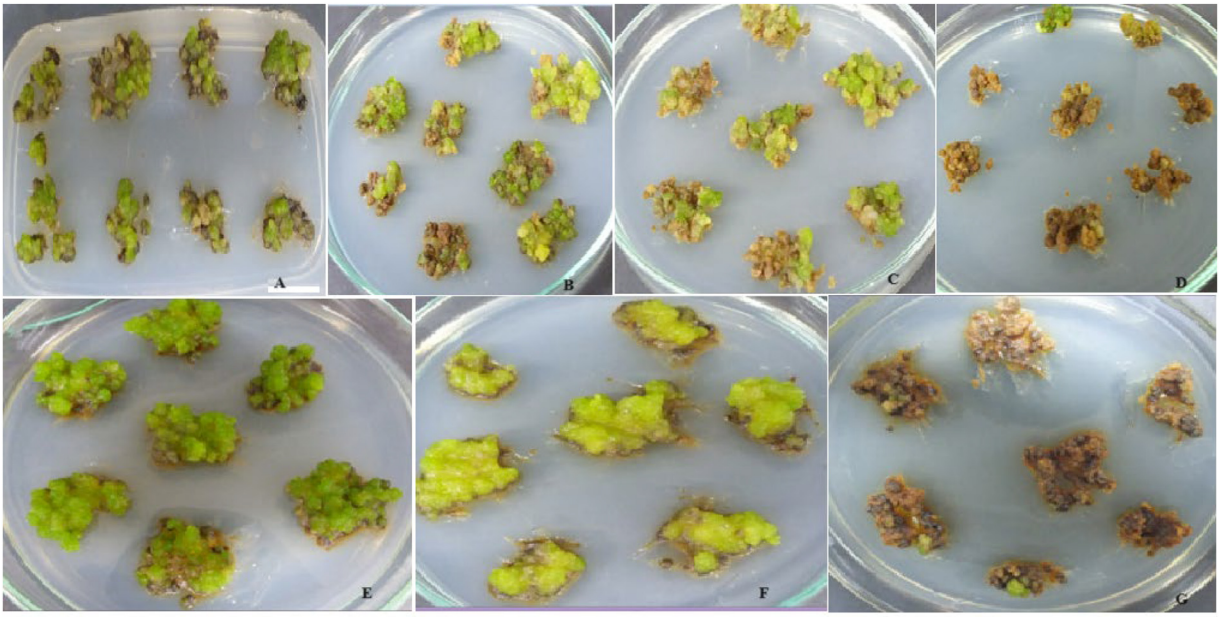

Fig. 3. Appearance of *H. heterophyllum* calli subcultured on LS/B5 nutrient media containing different amounts of ABA and SA after 15 days. A: Control, B: 0.01 mg/L ABA, C: 0.05 mg/L ABA, D: 0.1 mg/L ABA, E: 0.01 mg/L SA, F: 0.05 mg/L SA, G: 0.1 mg/L SA.

Concerning callus cultures, the presence of 0.05 mg/L ABA (136.3 mg TEs/g extract) had the highest activity in all elicited calli. Furthermore, callus culture had DPPH radical activity significantly more than *in vitro* plantlets. Thus, the most significantly different DPPH scavenging capacity ($p < 0.05$) was achieved for *in vivo* leaf, callus from 0.05 mg/L ABA and *in vitro* plantlet obtained from 0.01 mg/L SA application, respectively.

4. Analyses of polyphenol compounds

Hypericin and pseudohypericin were not determined both from *in vivo* and even *in vitro* samples (Table 5). Initially, chlorogenic acid (5.8 mg/g DW) and hyperoside (3.2 mg/g DW) have the highest values from *in vivo* leaves. On the other hand, apigenin (0.04 mg/g DW) was detected only in the *in vivo* leaf. Meanwhile, quercetin (0.58 mg/g DW) had the highest amount in herba. Furthermore, kaempferol and catechin could not be detected in the *in vivo* plant parts.

Among all *in vitro* results, chlorogenic acid (0.03 mg/g DW) and kaempferol (0.005 mg/g DW) were only detected *in vitro* plantlets of 0.01 mg/L ABA. As well as, hyperoside (0.28 mg/g DW), quercetin (0.019 mg/g DW), and catechin (0.028 mg/g DW) compounds had the highest amounts in the *in vitro* plantlets of 0.01 mg/L ABA. Additionally, hyperoside was produced at 0.1 mg/L SA (0.02 mg/g DW) plantlet. Accordingly, it was able to produce hyperoside in both 0.01 mg/L ABA and 0.1 mg/L SA *in vitro* plantlet applications (Table 5).

High quercetin amount was achieved in calli treated with 0.1 mg/L SA (0.29 mg/g DW) which was 11.2-fold more than the control (0.026 mg/g DW) group and was very close to *in vivo* leaf (0.27 mg/g DW) and half of quercetin in the *in vivo* herba parts (0.58 mg/g DW).

Table 3
Total phenolic amounts *in vivo* plant parts, *in vitro* plantlets, and calli of *H. heterophyllum*.

Samples		Total Phenolic content (mg GAE/g)
In vivo plant parts	Flower	54.7 ± 1.16^{c}
	Leaf	$\mathbf{77.6 \pm 2.60^{a}}$
	Stem	36.1 ± 1.23^{d}
	Herb	66.77 ± 1.99^{b}
In vitro plantlets	Control	$\mathbf{37.04 \pm 0.57^{d}}$
	0.01 ABA	24.8 ± 0.64^{e}
	0.05 ABA	15.3 ± 0.31^{f}
	0.1 ABA	26.3 ± 0.67^{e}
	0.01 SA	15.3 ± 0.31^{f}
	0.05 SA	16.7 ± 0.14^{f}
	0.1 SA	22.3 ± 0.60^{e}
Callus	Control	9.91 ± 0.10^{fg}
	0.01 ABA	$\mathbf{29.5 \pm 1.27^{de}}$
	0.05 ABA	28.2 ± 1.11^{de}
	0.1 ABA	27.7 ± 1.73^{de}
	0.01 SA	27.03 ± 1.11^{de}
	0.05 SA	26.5 ± 0.62^{e}
	0.1 SA	26.9 ± 1.75^{e}

Means followed by the different letters within columns were significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 4
DPPH results of *in vitro* plantlets, callus, and *in vivo* plants of *H. heterophyllum*.

Samples		DPPH activity (mg TEs/g)
In vivo plant parts	Flower	217.0 ± 0.83^{c}
	Leaf	$\mathbf{415.8 \pm 1.52^{a}}$
	Stem	77.5 ± 2.87^{d}
	Herb	242.9 ± 2.88^{b}
In vitro plantlets	Control	33.16 ± 0.59^{c}
	0.01 ABA	32.9 ± 0.69^{c}
	0.05 ABA	36.7 ± 1.23^{b}
	0.1 ABA	$\mathbf{63.3 \pm 0.83^{a}}$
	0.01 SA	$\mathbf{84.4 \pm 1.40^{a}}$
	0.05 SA	16.7 ± 0.16^{d}
	0.1 SA	36.3 ± 0.26^{b}
Callus	Control	112.5 ± 0.72^{c}
	0.01 ABA	103.2 ± 1.16^{d}
	0.05 ABA	$\mathbf{136.3 \pm 0.79^{a}}$
	0.1 ABA	128.4 ± 0.64^{b}
	0.01 SA	$\mathbf{114.5 \pm 2.39^{a}}$
	0.05 SA	67.6 ± 0.66^{b}
	0.1 SA	$\mathbf{114.3 \pm 1.92^{a}}$

Means followed by the different letters within columns were significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

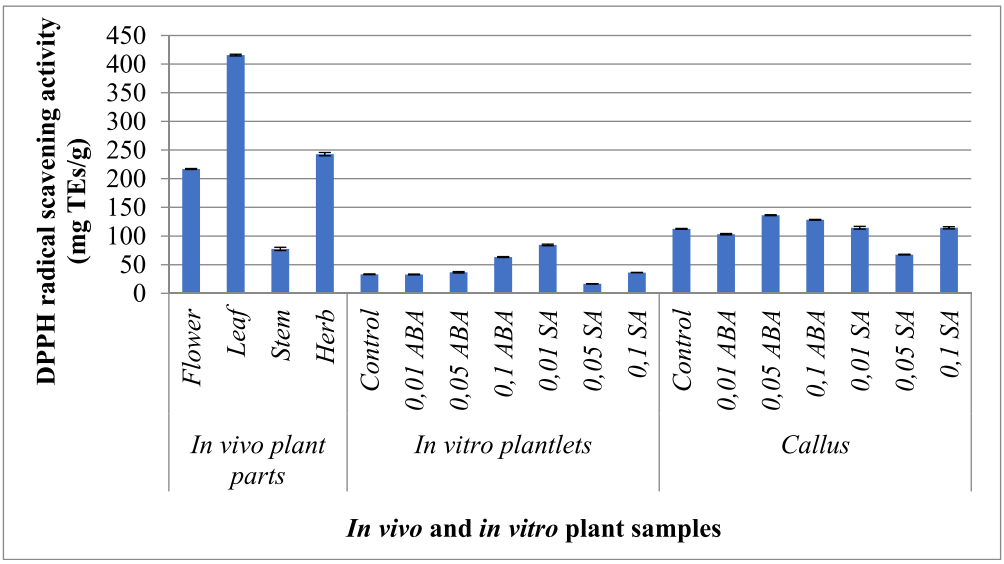

Fig. 4. DPPH activity *in vitro* plantlets, callus, and *in vivo* plants of *H. heterophyllum* plants growing under different conditions.

As a result, the quercetin content varied within a very narrow range, from 0.009 to 0.019 mg/ quercetin g DW, in the *in vitro* plantlets and besides a very wide range, from 0.006 to 0.23 mg/ quercetin g DW, in calli.

Catechin could not be detected in *H. heterophyllum* samples collected from the nature. However, it was determined that 0.01 mg/L ABA application increased 1.5-fold (0.028 mg catechin/g DW), when compared to *in vitro* plantlets of control (0.018 mg catechin/g DW) group. In addition, detected amount in calli on 0.01 mg/L SA (0.23 mg catechin/g DW) had the highest value which was 9.2-fold more than control. The catechin content varied within a very wide range, from 0.025 to 0.23 mg/catechin g DW in callus. Accordingly, low ABA (0.01 mg/L) and SA (0.01 mg/L) induced higher catechin contents in the *in vitro* plantlets and callus cultures, respectively.

5. Discussion

In vitro culture (Ayan and Cirak, 2006), antioxidant activity (Yaman, 2020), bioactive compounds (Ayan and Cirak, 2008; Yaman et. al 2024), antimicrobial and anticancer (Hazman et. al 2022) studies on *H. heterophyllum* are very limited. Yaman (2020) obtained *in vitro* plantlets and callus in tissue culture from the *H. heterophyllum* and made antioxidant activity, phytochemical analyses from aerial parts, calli, and *in vitro* plantlet samples. As a result, the total phenolic content of *in vitro* plantlets was the highest in all samples. By comparing *in vitro* samples with the *H. heterophyllum* plant parts collected from nature; quinic acid, gallic acid, catechin, ferulic acid, vanillic acid, p-coumaric acid, caffeic acid, and quercetin compounds were analyzed by LC-MS/MS. According to the results; the antioxidant activity of *in vitro* plantlets was higher than the other samples. Besides, Cakir et al. (2004) performed GC–MS analysis of *H. heterophyllum* species and investigated its antifungal effect. In the essential oil analysis, the amount of sesquiterpenes was determined as 72 %. In particular, the essential oil significantly stopped the growth of *Rhizoctonia solani* AG-11. Furthermore, Hazman et. al (2022) extracted the *H. heterophyllum* plant using methanol and acetone to perform antimicrobial, anticancer, antioxidant, and phenolic compound

Table 5
Phytochemical amounts *in vivo* plant parts, *in vitro* plantlets and calli of *H. heterophyllum* (mg/g DW).

Samples		Chlorogenic acid	Hyperoside	Apigenin	Kaempherol	Quercetin	Catechin
In vivo plant parts	Flower	3.3	0.53	0.04	nd	0.12	nd
	Leaf	5.8	3.2	nd	nd	0.27	nd
	Stem	1.6	1.1	nd	nd	0.15	nd
	Herb	4.8	1.9	nd	nd	**0.58**	nd
In vitro plantlets	Control	tr	nd	nd	nd	0.017	0.018
	0.01ABA	0.03	**0.28**	nd	0.005	**0.019**	**0.028**
	0.05ABA	nd	nd	n.d	tr	0.009	0.009
	0.1 ABA	nd	nd	nd	nd	0.011	0.008
	0.01 SA	nd	nd	nd	nd	0.013	0.003
	0.05 SA	nd	nd	nd	nd	0.009	nd
	0.1 SA	tr	**0.02**	nd	nd	0.015	nd
Callus	Control	nd	–	tr	nd	0.026	0.025
	0.01ABA	nd	**0.018**	tr	nd	0.04	0.006
	0.05ABA	nd	0.013	tr	nd	0.002	0.01
	0.1 ABA	nd	0.008	n.d	nd	0.018	0.019
	0.01 SA	nd	**0.49**	n.d	nd	n.d	**0.23**
	0.05 SA	nd	nd	n.d	nd	0.02	0.005
	0.1 SA	nd	nd	n.d	nd	**0.29**	0.01

n.d: not detected, tr: trace amount.

analyses. Chlorogenic acid, miquelianin, and isoquercetin compounds were detected at the highest rate in both extracts.

Although it is an endemic species, *H. heterophyllum* has a high shoot regeneration rate (86.7 %) and was easily adapted to *in vitro* conditions in our study. Auxin and cytokinin derivatives were applied in different combinations to different species of the genus *Hypericum* and successful results were obtained. In this regard, Yazaki and Okuda (1994) applied BAP and IAA hormones together in shoots of *H. erectum*. Bacila et al. (2010) obtained 30 % shoot regeneration with the combination of 0.2 mg/L BA, 0.05 mg/L NAA, 0.1 mg/L Kin and 0.5 mg/L 2IP containing MS media in *H. maculatum*. Additionally, Meyer et al. (2009) acquired callus and shoots in dark conditions by using a combination of 10 μM BA + 2.5 μM IAA in *H. frondosum.* However, in our study, 0.01 and 0.1 mg/L NAA combined with 1 mg/L BAP exhibited an inhibitory effect on shoot formation. Likewise, Ayan and Çırak (2006) obtained shoots only with 4.4 mg/L BA and callus with the combination of 1 mg/L BA and 0.1 mg/L 2,4-D.

Many authors have successfully used BAP to stimulate shoot proliferation in explants from various *Hypericum* species (Charchoglyan et al., 2007; Karakas et al., 2009; Namli et al., 2010; Ravindran et al., 2023). Ravindran et al. (2023), working with *H. perforatum* L. genotypes, reported multiple shoots from 0.5 mg/L BAP and 0.5 mg/L IBA containing MS medium. Moreover, increasing the concentration of BAP to 1 mg/L enhanced the number of shoots in *H. triquetrifolium* (Karakas et al., 2009). Besides, Cirak et. al (2007) notified that 2 mg/L BA produced intense shoot growth in *H. bupleuroides*. Additionally, Swain et al. (2016) reported that 2 mg/L BAP with MS media had a strong effect on the rate of shoot proliferation in the *in vitro* study of *Hypericum gaitii* Haines, but the medium supplemented with 1 mg/L BAP and 0.25 mg/L NAA showed the lowest percentage of cultures with multiple shoots. Similarly, Wójcik and Podstolski (2007) noted that 5.37 μM and 10.74 μM NAA concentration used with 4.44 μM BAP negatively affected *in vitro* shoot growth in *H. perforatum* .

Exclusively, in previous literature, it was reported that the combination of 0.25 mg/L BAP with 0.5 mg/L kinetin and 1 mg/L IAA in MS media had a strong effect on *in vitro* shoot regeneration *in H. heterophyllum* (Yaman, 2020). To date, the conservation methods and *in vitro* micropropagation with LS media in *H. heterophyllum* have not been reported. Kwiecień et. al (2018) studied LS and MS media separately, by three cultivars (Elixir, Helos and Topas) of *H. perforatum*. Accordingly, 'Helos' and 'Elixir' cultivars were demonstrated that LS medium containing 0.1 mg/L BAP and 0.1 mg/L NAA had more total flavonoid content (255.7 mg/g DW) than MS media (146.8 mg/g DW). Furthermore, it has been reported that the richest flavonoid content was obtained in LS media containing 0.1 mg/L BAP.

Elicitors such as, jasmonic acid (JA) and salicylic acid (SA), are administered externally through *in vitro* applications and enable the activation of the signal-transduction cascade, gene expression and secondary metabolism (Zhao et. al 2005). Thus, biosynthesis of bioactive compounds occurs and exogenous signals can change their amounts. *In vitro* elicitation studies on *H. perforatum* were conducted using mannan, pectin, JA, methyl jasmonate (MeJA), SA and fungal elicitors (Kirakosyan et al. 2000; Sirvent and Gibson 2002; Walker et al. 2002; Gadzovska et al. 2007; Gadzovska et al. 2012). It has been reported that rapid and temporary increases in secondary metabolite production occur with the activation of phenylalanine aminolyase (PAL, EC 4.3.1.5) and chalcone isomerase (CHI, EC 5.5.1.6) enzymes (Dixon et. al 2002). Although there have been studies for the effect of SA on *in vitro* growt of *H. perforatum*, to our knowledge there is no work for ABA and SA on the effect of *H. heterophyllum in vitro*. Sirvent and Gibson (2002) studied the effect of 1, 2.5, and 15 μM SA on plantlets obtained from meristems and showed that hypercin content increased with 5 μM SA application. Likewise, Walker et. al (2002) used SA elicitation for *H. perforatum* measuring only hypericin content after 28 days of culture. At the same time, Conceição et al. (2006) determined the effect of 25 μM SA on phenolics, flavonols, and flavones. In the same way, the shoot culture of *H. hirsutum* and *H. maculatum* was elicited with SA by Coste et al. (2011). Finally, Gadzovska et al. (2013) conducted comprehensive research with different concentrations (50, 100, and 250 μM) of SA on callus, cell, and shoot culture of *H. perforatum* and focused on variations of naphtodianthrones (hypericin and pseudohypericin) and various phenylpropanoids (phenolic compounds, flavonols, flavanols, and anthocyanins) along the culture (1, 4, 7, 14 and 21 days after elicitation). Accordingly, they found that the amount of phenolics increased significantly after salicylic acid application, and flavonols increased 2 to 5 times after 7–21 days. In addition, Gadzovska et al. (2013) reported that 100 μM SA applied to *H. perforatum* calli turned the calli brown in the last week of culture. The same authors reported that the color of *H. perforatum* cells treated with jasmonic acid for a long time in cell suspension culture also turned brown and the cells clustered together (Gadzovska et al. 2007). Hence, when acidic elicitors are applied in low doses, necrosis is not observed morphologically.

Studies have shown that salicylic acid contributes to the expression of the CAD1 gene, which is involved in the plant defense of *Arabidopsis* (Tsutsui et al. 2006) and activates the plant's defense mechanism. Guerriero et al. (2018) reported that in plant tissue culture studies, biotic or abiotic stress signals coming to the cell from outside affecting the cell wall. This effect is detected by specific receptors between the plasma membrane and the cell, causing calcium accumulation. Calcium accumulation stimulates phytohormones such as jasmonic acid. Thus, secondary metabolism becomes active and responds to external stress (Zhao et al. 2005). Precursors of secondary metabolite biosynthesis are produced by the chloroplast, and glycosylated metabolites are stored in the vacuole. The elicitors used in our study are perceived in the cell as externally applied stress signals, thus the relevant secondary metabolite is secreted more or less depending on the level of stress.

Although there are many studies on *Hypericum*, it is not known exactly which phenolic groups have the antioxidant effects. Orcic et al. (2011), to understand which compounds showed antioxidant effects, they divided the extracts into fractions and investigated their effects. They determined that flavonoids (quercetin, hyperoside, rutin, etc.) and phenolic acids (chlorogenic acid) showed higher antioxidant effects, while naphthodianthrone and phloroglucinols were less effective. According to our HPLC results, it is possible to say that the amounts of chlorogenic acid, and hyperoside compounds are generally higher in the leaf parts. In our antioxidant studies, we can state that the metabolites that affect the total phenolic content and show DPPH activity consist of mostly quercetin, hyperoside, chlorogenic acid, and catechin compounds. On the other hand, it is predicted that other phenolic components present in the extract, which we did not quantify, create a synergistic effect and contribute to the antioxidant capacity.

Calli treated with 0.01 mg/L SA produced more hyperoside (0.49 mg hyperoside/g DW) than other *in vitro* applications. While the amount of hyperoside could not be determined in the callus control group of the *H. heterophyllum*,whereas hyperoside production with 0.01 mg/L SA elicitor applications was found to be quite high. Our study has shown that salicylic acid can stimulate the production of hyperoside by activating the precursors that provide synthesis within the cell. The production of a convenient metabolite can be achieved when the appropriate elicitor dose and physical conditions are provided.

Nguyen et al. (2016) clarified the hormonal responses of the plant and the synthesis of the metabolites related to these responses, depending on the plant-insect interaction in the tobacco plant. Abscisic acid signals the production of jasmonic acid through transcription factors or directly. Although it is not known exactly what is the key point in the signaling steps between abscisic acid and jasmonic acid, it has been determined that they carry out mutual biosynthesis

(Lackman et al. 2011). In addition, it has been determined by studies that salicylic acid induces ethylene hormone through various transcription factors and contributes to the production of the metabolite with a synergistic effect with jasmonic acid. In the insect defense mechanism of the tobacco plant, hormones cooperate to produce the relevant metabolite, thus protecting the plant (Stotz et al. 2002; Mewis et al. 2005; Zarate et al. 2007). These studies have shown that abscisic acid plays an important role in the production of metabolites. It is thought that a mechanism similar to the one mentioned above is also effective in *Hypericum* cells, and abscisic acid taken from outside creates a stress effect, stimulates other hormones, and thus increases the amount of catechin. Likewise, it is predicted that this hormonal system shows similar activity, exogenous salicylic acid stimulates other hormones, and the relevant metabolite is synthesized.

As reported in previous studies hypericin and pseudohypericin were not detectable in *H. heterophyllum in vivo* plant parts (Ayan and Cirak 2008; Yaman et. al 2024). Similarly, in present work hypericin and pseudohypericin were not determined not only *in vivo* but also *in vitro* samples. Although catechin cannot be detected *in vivo* plant parts, the production of catechin in the *in vitro* plantlets, and calli showed the importance of *in vitro* studies. While *H. perforatum* and *H. androsaemum* produced xanthone in callus and suspension cultures, it could not be detected *in vivo* samples (Dias et. al 1998). Similar to our study occasionally, bioactive compounds may not be detected *in vivo* plant parts while *in vitro* production is carried out. The decrease in the amount of catechin in calli with elicitor applications showed that high acidic stress factors may reduce the amount of catechin at the cellular level, whereas low acidic level of SA (0.01 mg/L) could increase catechin level. Moreover, catechin is a specific compound of the tea (*Camellia sinensis*) plant and it is generally found in plants containing phenolic compounds. Studies have reported that catechin, in addition to its strong antioxidant activity, has an anticancer effect (Tosun and Karadeniz, 2005) and is used in phytotherapy against cardiovascular disorders (Yıldız et al. 2013; Tajik et al. 2017).

It has been demonstrated through clinical research that for the antidepressant effect *H. perforatum* compounds, flavonoids such as quercetin must be present in the extracts, as well as hyperforin and hypericin compounds (Nöldner and Schotz 2002; Wurglics and Schubert-Zsilavecz 2006). Flavonols including quercetin and kaempferol are abundant in green leaves, fruits and grains (Terahara, N. 2015; Kozlowska et. al 2017). Additionally, it has been reported that quercetin has iron chelating and iron stabilizing properties, and directly affects the scavenging mechanism of ROS. The mechanisms of antioxidant action of flavonoids are (a) direct scavenging of ROS, (b) inhibition of ROS formation through chelation of trace elements (*e.g.*, quercetin has iron-chelating and iron-stabilizing properties) or enzymes involved in the formation of free radicals (*e.g.*, glutathione S-transferase, microsomal monooxygenase, mitochondrial succinooxidase, NADH oxidase, and xanthine oxidase) and (c) activation of antioxidant defenses (*e.g.*, upregulation of antioxidant enzymes with radical scavenging ability) (Agrawal 2011; Kumar and Pandey 2013; Kaleem and Ahmad 2018). Furthermore, quercetin induces cell cycle arrest and *in vitro* growth inhibition in various malignant tumor cell lines, such as leukemia, colon, breast, and ovarian cancer cells (Martarelli et. al 2004; Kumar and Pandey 2013). Apigenin and luteolin alter ROS signaling and induce apoptosis in various ovarian cancer cell lines (A2780, OVCAR-3, and SKOV-3) (Tavşan and Kayali 2019).

6. Conclusion

Biotechnological developments offer opportunities such as micropropagation and growing medicinal and aromatic plants under laboratory conditions at any time and purifying the active substances they contain. ABA and SA elicitors were used for the first time in *H. heterophyllum* with the present study and results showed that it would be more appropriate to keep elicitor concentrations low as high ABA and SA causes darkening in the plantlets and calli, and ultimately cell death. *In vitro* use of SA increased quercetin and catechin amount, while ABA promoted hyperoside production. Moreover, due to the high level of chlorogenic acid found in *H. heterophyllum*, this species can also be used as an alternative chlorogenic source within the *Hypericum* species. The present study also enabled the rapid micropropagation of *H. heterophyllum* using tissue cultures and its acclimation to external conditions. This can fill an important gap in terms of preventing destruction in nature, especially for endemic species, and providing important phenolic compounds and raw materials to the relevant branches of industry.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Şeyma Önlü: Writing – original draft, Supervision, Methodology, Investigation. **Cennet Yaman:** Data curation, Conceptualization. **Ekin Kurtul:** Validation, Formal analysis. **Harun Önlü:** Funding acquisition, Project administration. **Özlem Bahadir-Acikara:** Validation, Supervision, Methodology. **Oliver Tusevski:** Data curation, Software. **Sonja Gadzovska Simic:** Visualization, Supervision. **Sebahattin Özcan:** Supervision, Software, Project administration.

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