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Callus production and analysis of some secondary metabolites in *Globularia trichosantha* subsp. *trichosantha*

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Abstract: This study describes the production of calli from *Globularia trichosantha* subsp. *trichosantha* and the quantitative determination of catalpol, aucubin, and verbascoside in the calli and the plant by HPLC. The seeds of the plant were sterilized and germinated in Murashige and Skoog (MS) medium without in vitro plant growth regulator. Hypocotyl, cotyledon, first leaf, epicotyl, apical meristem, and root explants were taken from the 30-day-old aseptic seedlings germinated in vitro. Explants were then transferred to MS media for callus production together with varying concentrations of plant growth regulators. The best callus production occurred in the media containing 6 mg L^{-1} IAA and 0.2 mg L^{-1} 2,4-D + 0.1 mg L^{-1} BAP. The highest catalpol, aucubin, and verbascoside contents were determined in the roots (1.277 mg kg^{-1}), stem (0.775 mg kg^{-1}), and roots (0.290 mg kg^{-1}), respectively. The study is important in the sense that, 1) it is the first tissue culture study on *G. trichosantha* subsp. *trichosantha* that provides basic information for callus production; 2) that catalpol, aucubin, and verbascoside were quantitatively determined in calli and plant for the first time; and 3) all the secondary metabolites studied here were produced in the callus culture in a quantity that is approximately 7 times higher than the amount that naturally occurs in the plant.

Key words: Callus production, *Globularia trichosantha* subsp. *trichosantha*, HPLC

1. Introduction

The genus *Globularia* is represented by 22 species worldwide that mostly grow in the European continent (15 taxa) and in Turkey (11 taxa). *Globularia* species have medical and economic importance and are also planted as ornamental plants in parks and gardens for their visually appealing appearance. Being one of these species, *G. trichosantha* is commonly found in Anatolia. So far, no plant tissue culture study has been conducted on the species *G. trichosantha* subsp. *trichosantha*.

Other studies have shown the presence of iridoid, flavonoid, lignan, anthocyanidin, sugar ester, and phenolic acid compounds in *Globularia* species (*G. alypum*, *G. cordifolia*, *G. dumulosa*, *G. orientalis*, *G. trichosantha*) (Çalış et al., 1999, 2001, 2002; Chaudhuri et al., 2004; Kırmızıbekmez et al., 2004, 2009; Chograni et al., 2012). Yet, our present study focused on iridoids, which are secondary metabolites produced by many plants. Iridoids represent a large group of cyclopentapyran monoterpenoids that are synthesized naturally in many different dicotyledonous plant families like *Apocynaceae*, *Scrophulariaceae*, *Diervillaceae*, *Lamiaceae*, *Loganiaceae*, and *Rubiaceae* (Crişan et al., 2010).

Researchers reported various biological activities in the extracts of different parts of *G. alypum*, such as hypoglycemic activity that was examined through the infusion of *Globularia alypum* leaves (Skim et al., 1999a, 1999b). Moreover, the pharmacological activity of methanol and dichloromethane in aqueous extracts obtained from the leaves and stems of *Globularia alypum* L. (Bello et al., 2002; Taleb-Dida et al., 2011) was studied years ago. From the leaves and flowers of the Tunisian *Globularia alypum*, biologists determined its phenolic and flavonoid contents and antioxidant activity (Chograni et al., 2012).

Apigenin and luteolin contents of these leaves were identified by TLC, UV, and NMR analyses (Boutiti et al., 2008; Tundis et al., 2012). Different iridoid glycosides were analyzed in *G. cordifolia*, *G. dumulosa*, *G. sintenisii*, and *G. meridionalis* (Kırmızıbekmez et al., 2003a, 2003b; Tundis et al., 2012).

G. trichosantha is used in the treatment of hemorrhoids and also has antitumor and antimicrobial properties

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(Tundis et al., 2012). From the extraction of its different organs, the iridoids deacetylasperuloside, aucubin, catalpol, asperulosidic acid, geniposidic acid, scandoside, deacetylalpinoside and phenylethanoids crenatoside, verbascoside, rossicaside A, and trichosanthosides A and B were identified by using chromatography techniques.

Yet, no pharmacological studies or plant tissue, cell, or organ culture studies have been done for *G. trichosantha* subsp. *trichosantha*. Furthermore, no quantitative analysis of this plant has been determined in any study. Therefore, in the present study, for the first time, *G. trichosantha* subsp. *trichosantha* seeds were placed in culture for germination and it was aimed to produce calli from the explants taken from aseptic seedlings in order to quantitatively determine catalpol, aucubin, and verbascoside content in the calli and plant samples.

2. Materials and methods

2.1. Plant materials

Globularia trichosantha subsp. *trichosantha* plant (2n = 16) was used (Davis et al., 1988; Gagnidze et al., 2006). The herb, roots, and seeds of *G. trichosantha* subsp. *trichosantha* were supplied from the herbarium samples of Assist Prof Gencay Akgül from Department of Biology, Faculty of Arts and Science, Nevşehir University. The seeds were selected from dried flowers in June.

2.2. Seed germination

The seeds were mixed in 10% (w/v) NaOCl solution for 10 min for surface sterilization. After 3 rounds of washing with distilled water, they were kept in 70% ethyl alcohol for 1 min. The seeds were rewashed 3 times with distilled water and treated with 10% hydrogen peroxide. The seeds were finally rewashed 3 times with distilled water.

Hormone-free Murashige and Skoog (1962) (MS) medium containing 3% sucrose and 0.8% agar was used as germination medium. The medium was adjusted to pH 5.9. The seeds were germinated in jars (7 × 5 × 5 cm) within 2–3 days at 22–24 °C, in darkness. The seedlings were incubated at photoperiod of 16/8 h and the temperature was set to 24 ± 1 °C (irradiation with 37 µmol m^{-2} s^{-1} was provided by cool-white fluorescent tubes) (Figure 1a).

Aseptic seedlings that were 45-days-old (7 cm) were more yellowish in appearance when compared to aseptic seedlings that were 30 days old (average 5.5 cm). Apical meristem (1 mm), hypocotyl (0.5 cm), cotyledon (split in half), epicotyl (0.5 cm), a young primary leaf (split in half), and the root (0.5 cm) explants were taken from these aseptic seedlings. Since better results were obtained from 30-day-old aseptic seedlings, they were selected to be used in the rest of the trials (Figure 1b).

2.3. Callus induction

MS media were used for callus production. The MS media contained varying concentrations and combinations of the auxins: 2,4-dichlorophenoxyacetic acid (2,4-D; 0.2, 0.5, 0.8, 1 mg L^{-1}), indole-3 acetic acid (IAA) (2, 6, 8 mg L^{-1}), naphthalene acetic acid (NAA) (2, 4, 6, 8 mg L^{-1}); the cytokinins: 6-benzylaminopurine (BAP) (1, 2, 3, 4 mg L^{-1}), kinetin (1, 2, 3, 4 mg L^{-1}); auxin + cytokinin combinations: 2,4-D (0.2, 0.4, 0.6, 0.8 mg L^{-1}) + kinetin (1.5 mg L^{-1}); 2,4-D (0.2 mg L^{-1}) + kinetin (1, 2, 2.5, 3 mg L^{-1}); 2,4-D (0.2, 0.4, 0.6, 0.8 mg L^{-1}) + BAP (1.5 mg L^{-1}); 2,4-D (0.2 mg L^{-1}) + BAP (0.02, 0.1, 0.15, 0.2 mg L^{-1}); NAA (2, 4, 6, 8 mg L^{-1}) + kinetin (1 mg L^{-1}); IAA (2, 4, 6, 8 mg L^{-1}) + kinetin (1 mg L^{-1}); NAA (4 mg L^{-1}) + BAP (1, 2, 3, 5 mg L^{-1}); IAA (4 mg L^{-1}) + BAP (1, 2, 3, 5 mg L^{-1}). MS media were dispensed into 100 mM × 100 mM glass petri dishes.

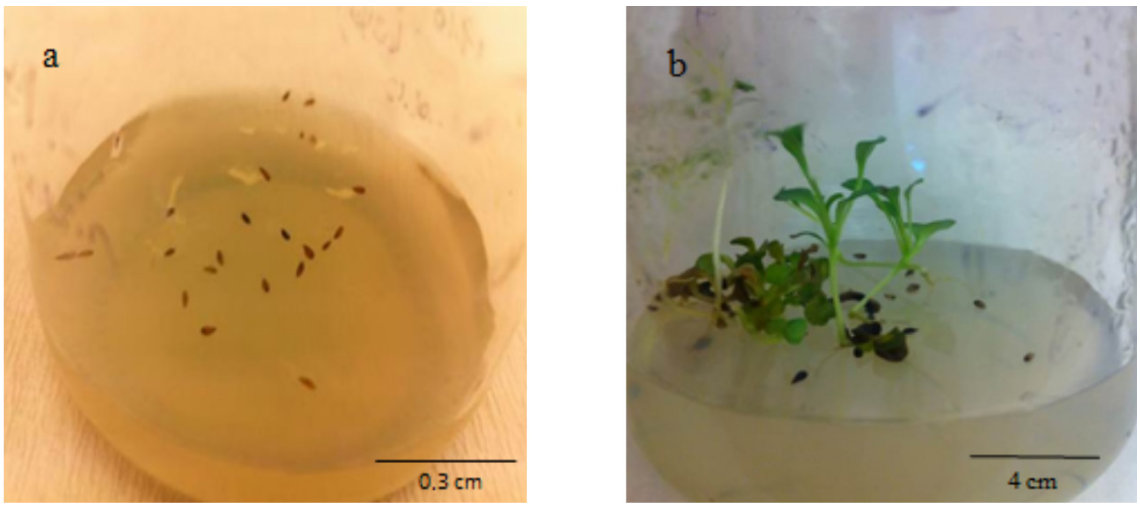

Figure 1. Germination of *G. trichosantha* subsp. *trichosantha*. a. 3-day-old seedling, bar = 0.3 cm; b. 30-day-old seedling, bar = 2.5 cm.

The MS medium containing 30 g of sucrose and 8% (w/v) agar was adjusted to pH 5.9 and sterilized. The explants taken from 30-day-old aseptic seedlings were incubated at 22–24 °C in darkness and subcultured every 3 weeks in the same media.

Callus formation percentages of the explants were calculated for each medium. Callus growth index was calculated using the first and second subculture weights for 2 of the most successful MS media with IAA, 2,4-D + BAP (Erçetin et al., 2012).

Callus growth index = Final weight/Initial weight

2.4. HPLC analysis

Catalpol, aucubin, and verbascoside in *G. trichosantha* subsp. *trichosantha* derived calli and plant samples were quantified by using HPLC. Derived calli were dried in the freeze dryer. One hundred milligrams of dried calli were weighed and extracted with 10 mL of methanol (MeOH) on a shaker for 48 h (Alipieva et al., 2007). The filtrate was then evaporated with a rotary evaporator at 40 °C (Crişan et al., 2010). HPLC analyses were performed by using sample injection volume of 20 µL (Alipieva et al., 2007; Crişan et al., 2010). Leaf, stem, fruit-seed, and roots (1 g) of the plant dried at room temperature were selected and extracted with the same method.

In the HPLC system (Thermo Scientific Dionex UltiMate 3000), a Thermo Scientific-Acclaim TM 120-C18, 3 µM, 4.6 × 150 mM Dionex Bonded Silica Products column was used for catalpol and aucubin, whereas an Acclaim 120 C18 5 µM 4.6 × 250 mM HPLC column was used for verbascoside with a sample injection volume of 20 µL. For data acquisition, the flow rate was adjusted to 0.5 mL min^{-1} for catalpol and aucubin, and 0.8 mL min^{-1} for verbascoside. A gradient elution of A (methanol) and B (ultrapure water) was used as in the following: 0–10 min, 5%–35% A; 10–15 min, 35%–45% A; 15–20 min, 45%–51% A; 20–40 min, 51%–61% A; 40–45 min, 61%–80% A; 45–60 min, 80%–80% A for catalpol and aucubin; 0–20 min, 5%–40% A; 20–40 min, 40%–60% A; 40–45 min, 60%–5% A; 45–60 min, 5% A for verbascoside. On-line UV spectra were recorded with a diode array detector as 200 nm for catalpol and aucubin and 330 nm for verbascoside (Wang et al., 2010; Xie et al., 2012). Standard solutions: catalpol (Fluka) 200, 100, 50, 25, 15, 10, and 5 ppm; aucubin (Fluka) 10, 5, 2.5, 1, and 0.5 ppm; verbascoside (Fluka) 15, 10, 5, and 2.5 ppm concentrations were prepared with methanol. Primary testing showed that these compounds corresponded to peaks with retention times of 2.307 for catalpol, 3.420 for aucubin, and 27.04 for verbascoside (Figure 1).

2.5. Statistical analysis

Explants of the aseptic seedlings were cultivated in petri dishes. All petri dishes had 5 explants and all plant growth regulators were experimented 3 times. Callus formation percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis. SPSS for Windows Ver. 19.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses and one-way analysis of variance (one-way ANOVA). The differences among means were compared by Duncan's multiple-range test (Duncan, 1955). All data were presented as mean ± standard error of three replicates.

3. Results

3.1. Callus production

The seeds of *G. trichosantha* subsp. *trichosantha* were germinated in hormone-free MS medium with 80% success rate. The first germination occurred after 3 days (Figure 1a). Aseptic seedlings that were 30-day-old and 45-day-old were planted in preliminary trials (Figure 1b). The effect of explant age on callus formation percentage was investigated. Due to the low number of seeds, only IAA (4 mg L^{-1}) + BAP (3 mg L^{-1}) supplemented MS medium was used in this trial. The explants taken from 30-day-old aseptic seedlings were generally observed to perform better and result in higher percentages of callus formation when compared to the explants taken from 45-day-old aseptic seedlings. Except for young primary leaf and epicotyl explants, callus formation percentages of 30-day-old hypocotyl, cotyledon, apical meristem, and root explants were higher in comparison to 45-day-old explants (Table 1). Hence, it was decided to use younger seedlings in the rest of the trials.

For all media, the fastest callus producing explants in one week were in the root, hypocotyl, and apical meristem. In all media, the calli occurred in spherical and regular clusters. In media with IAA, the calli were initially yellow and then started to turn brown after the first subculture (Figures 2a–2c). In the media with 2,4-D, NAA, kinetin, BAP, and their varying combinations, and those of IAA + kinetin and IAA + BAP, initially the yellow calli mostly turned brown after 2–3 weeks. Despite the occurrence of browning in all media, the callus production was sustained, and the cells continued to divide. Besides the presence of browned calli, the formation of light-colored, spherical ones continued to grow in these media (Figures 2d and 2e).

Callus formation percentages of each explant taken from 30-day-old aseptic seedlings for all hormone-supplemented MS media are given in Table 2. In the auxin group, the best callus production was achieved by 0.2 mg L^{-1} 2,4-D from hypocotyl, epicotyl, the young primary leaf, and root explants (80%–85%). For NAA, the best callus production was achieved by 6 mg L^{-1} from hypocotyl explants (80%); for IAA, this was with 6 mg L^{-1} from all explants (85–95%). In the cytokinin group, for kinetin and BAP, the best callus productions occurred in 1 mg L^{-1} from

Table 1. The effect of explant age on callus formation percentage.

IAA + BAP (4–3 mg L^{-1})	30-day-old seedlings	45-day-old seedlings
Apical meristem	85.76 ± 0.17	63.77 ± 0.20
Hypocotyl	83.23 ± 1.48	60.00 ± 0.18
Cotyledon	84.26 ± 0.17	59.00 ± 0.18
Epicotyl	35.05 ± 0.35	85.26 ± 0.17
Young primary leaf	26.39 ± 0.44	84.56 ± 0.17
Root	86.16 ± 0.17	83.66 ± 0.17

Mean ± standard error

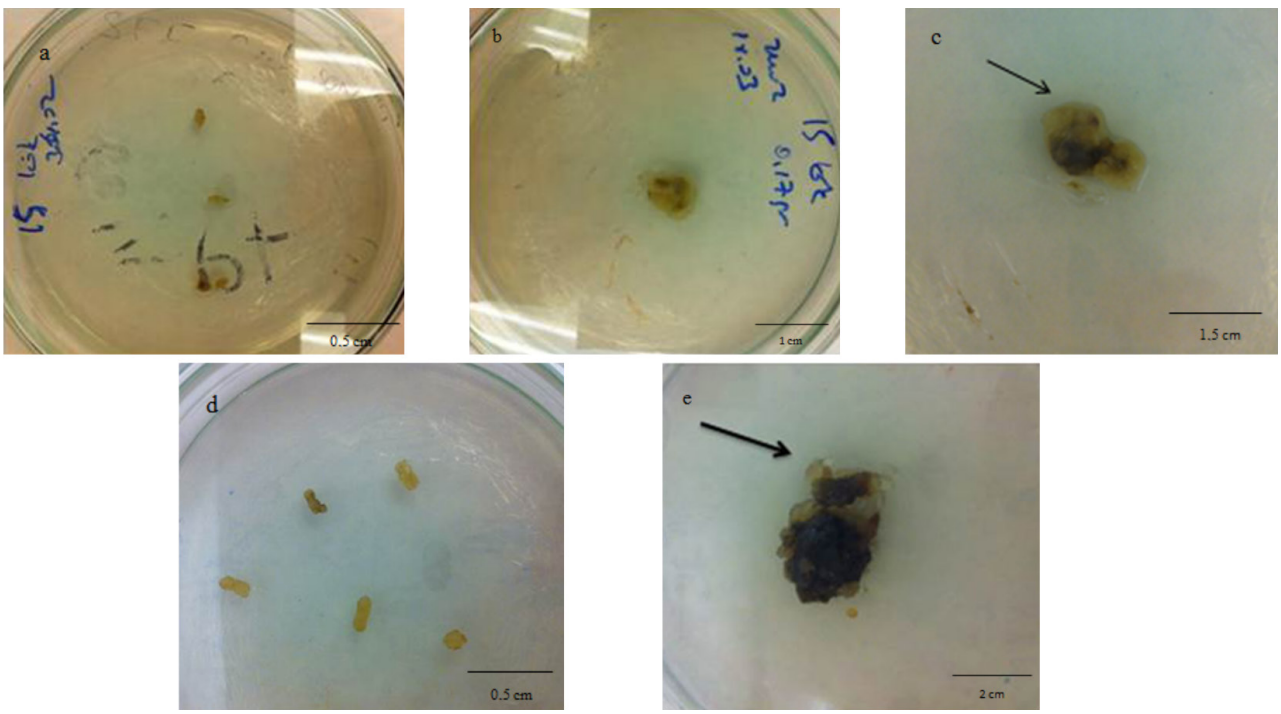

Figure 2. Appearance of *G. trichosantha* subsp. *Trichosantha* calli. a. Root explant after 3 weeks, bar = 0.5 cm; b. Calli on root explants after 6 weeks, bar = 1 cm; c. Browned callus on root explants after 7 weeks and new light-yellow and white calli supporting continuity of browned callus in IAA-supplemented media (6 mg L^{-1}), bar = 1.5 cm; d. Calli on explants after 2 weeks, bar = 0.5 cm; e. Browned callus on explants after 6 weeks and new light-yellow and white calli supporting continuity of browned callus in 2,4-D + BAP –supplemented (0.2–0.2 mg L^{-1}) media, bar = 2 cm.

cotyledon explants (64%) and 1 mg L^{-1} from hypocotyl explants (75%), respectively.

As for auxin + cytokinin combinations, the best callus production was achieved by the combination of 0.2 mg L^{-1} 2,4-D + 2 mg L^{-1} kinetin and 4 mg L^{-1} IAA + 1 mg L^{-1} BAP from root explants (74%). In 2,4-D + BAP combinations, the highest callus production was seen in the combination of 0.2 mg L^{-1} 2,4-D + 0.02 mg L^{-1} BAP from root explants (97%). According to the results of the callus production process, the best callus production was achieved by the media with IAA and 2,4-D + BAP. The callus production continued in the subculture media and adequate calli were obtained to be used in the analyses. After the second subculture, the calli were stored at –21 °C until analysis.

MS3 resulted in the highest callus production percentage of those media containing 6 mg L^{-1} IAA (Table 2). For the first subculture, MS3 and MS4 were the most successful media for callus growth index. For the second subculture, MS3 was the best medium (Table 3). For the combinations of 2,4-D + BAP, MS5 resulted in the

Table 2. The effect of plant growth regulators on callus formation percentages of each explant during the first subculture of *G. trichosantha* subsp. *trichosantha*. ANOVA statistical analysis ($P \leq 0.05$) was performed (mean ± SE).

Medium (mg L^{-1})	Apical meristem	Hypocotyl	Cotyledon	Epicotyl	Young primary leaf	Root
2,4-D						
0.2	45.00 ± 0.57^{b}	84.67 ± 0.27^{a}	35.03 ± 0.10^{b}	82.17 ± 0.16^{a}	83.57 ± 0.27^{a}	85.67 ± 0.27^{a}
0.5	53.16 ± 0.96^{a}	64.02 ± 0.44^{b}	39.23 ± 0.33^{b}	35.05 ± 0.70^{c}	52.12 ± 0.14^{b}	83.16 ± 0.17^{a}
0.8	34.92 ± 0.47^{c}	54.12 ± 0.24^{b}	64.02 ± 0.44^{a}	45.00 ± 0.66^{b}	34.92 ± 0.47^{c}	63.47 ± 1.24^{b}
1	30.02 ± 0.17^{c}	50.76 ± 0.33^{c}	54.02 ± 0.44^{a}	40.00 ± 0.16^{b}	26.16 ± 0.31^{d}	50.77 ± 0.67^{c}
NAA						
2	45.10 ± 0.41^{b}	42.11 ± 0.17^{b}	22.16 ± 0.11^{a}	28.22 ± 0.28^{a}	28.12 ± 0.18^{d}	26.56 ± 0.41^{c}
4	35.05 ± 0.35^{c}	52.22 ± 0.19^{b}	26.56 ± 0.41^{a}	22.12 ± 0.38^{a}	54.5 ± 0.41^{a}	74.29 ± 0.14^{a}
6	52.22 ± 0.97^{a}	80.29 ± 0.14^{a}	35.99 ± 0.38^{b}	29.99 ± 0.13^{a}	45.11 ± 0.61^{b}	29.12 ± 0.58^{b}
8	43.12 ± 0.31^{b}	50.12 ± 0.97^{b}	35.05 ± 0.35^{b}	19.19 ± 0.18^{b}	35.99 ± 0.38^{c}	28.99 ± 0.12^{b}
IAA						
MS1 2	45.10 ± 0.41^{c}	45.10 ± 0.41^{d}	26.56 ± 0.41^{d}	34.05 ± 0.15^{b}	26.39 ± 0.14^{b}	84.56 ± 0.12^{a}
MS2 4	84.18 ± 0.10^{b}	83.23 ± 1.48^{b}	61.00 ± 0.66^{b}	30.82 ± 0.52^{b}	28.19 ± 0.04^{b}	60.03 ± 0.41^{b}
MS3 6	95.16 ± 0.07^{a}	89.15 ± 0.17^{a}	84.56 ± 0.11^{a}	85.05 ± 0.35^{a}	86.39 ± 0.44^{a}	94.56 ± 0.11^{a}
MS4 8	84.56 ± 0.17^{b}	60.00 ± 0.16^{c}	39.23 ± 0.33^{c}	35.08 ± 0.37^{b}	29.99 ± 0.38^{b}	84.56 ± 0.17^{a}
Kinetin						
1	45.10 ± 0.41^{a}	26.32 ± 0.24^{a}	64.02 ± 0.44^{a}	45.10 ± 0.41^{a}	43.02 ± 0.61^{a}	45.81 ± 0.39^{a}
2	40.03 ± 0.31^{a}	22.12 ± 0.26^{a}	26.56 ± 0.01^{b}	32.15 ± 0.11^{b}	35.09 ± 0.79^{b}	43.09 ± 0.81^{a}
3	25.12 ± 0.14^{b}	27.13 ± 0.12^{a}	27.54 ± 0.11^{b}	38.17 ± 0.28^{b}	39.09 ± 0.01^{a}	26.56 ± 0.41^{b}
4	26.56 ± 0.45^{b}	25.34 ± 0.12^{a}	20.16 ± 0.32^{b}	33.09 ± 0.21^{b}	39.12 ± 0.13^{a}	25.09 ± 0.41^{b}
BAP						
1	43.56 ± 0.11^{a}	75.05 ± 0.35^{a}	43.17 ± 0.11^{a}	35.87 ± 0.67^{b}	32.17 ± 0.17^{a}	35.09 ± 0.11^{a}
2	40.23 ± 0.26^{a}	55.12 ± 0.18^{b}	49.02 ± 0.47^{a}	39.09 ± 0.01^{b}	37.28 ± 0.61^{a}	30.18 ± 0.73^{a}
3	39.12 ± 0.18^{a}	57.09 ± 0.12^{b}	39.24 ± 0.05^{b}	41.15 ± 0.56^{a}	40.03 ± 0.72^{a}	32.52 ± 0.75^{a}
4	41.09 ± 0.57^{a}	45.25 ± 0.16^{c}	36.78 ± 0.43^{b}	45.12 ± 0.18^{a}	39.45 ± 0.56^{a}	37.34 ± 0.41^{b}
2,4-D + kinetin						
0.2 + 1.5	44.02 ± 0.11^{a}	35.05 ± 0.35^{b}	45.18 ± 0.21^{a}	32.03 ± 0.41^{a}	45.02 ± 0.23^{a}	45.12 ± 0.11^{b}
0.4 + 1.5	41.18 ± 0.71^{a}	29.99 ± 0.38^{c}	42.45 ± 0.34^{a}	30.14 ± 0.15^{a}	32.16 ± 0.21^{b}	55.00 ± 0.57^{a}
0.6 + 1.5	35.19 ± 0.13^{b}	45.00 ± 0.32^{a}	40.09 ± 0.01^{a}	37.09 ± 0.87^{a}	36.93 ± 0.41^{b}	29.99 ± 0.66^{c}
0.8 + 1.5	32.22 ± 0.42^{b}	29.99 ± 0.38^{c}	39.32 ± 0.12^{b}	29.18 ± 0.16^{b}	43.56 ± 0.82^{a}	25.09 ± 0.61^{c}
2,4-D + kinetin						
0.2 + 1	44.09 ± 0.47^{a}	43.11 ± 0.78^{a}	28.08 ± 0.38^{b}	44.17 ± 0.14^{a}	44.09 ± 0.12^{b}	45.67 ± 0.34^{b}
0.2 + 2	45.67 ± 0.32^{a}	54.52 ± 0.42^{a}	35.09 ± 0.32^{a}	45.67 ± 0.34^{a}	35.14 ± 0.11^{a}	74.56 ± 0.17^{a}
0.2 + 2.5	43.09 ± 0.41^{a}	29.99 ± 0.38^{b}	29.15 ± 0.35^{b}	43.09 ± 0.41^{a}	33.17 ± 0.01^{a}	48.07 ± 0.44^{b}
0.2 + 3	44.12 ± 0.34^{a}	44.09 ± 0.14^{a}	34.12 ± 0.41^{a}	45.37 ± 0.76^{a}	39.01± 0.47^{a}	26.92 ± 0.61^{c}
2,4-D + BAP						
0.2 + 1.5	40.09 ± 0.44^{a}	29.99 ± 0.38^{b}	42.03 ± 0.11^{a}	67.52 ± 0.41^{a}	44.56 ± 0.23^{a}	35.09 ± 0.18^{b}
0.4 + 1.5	43.19 ± 0.65^{a}	40.09 ± 0.12^{a}	40.16 ± 0.56^{a}	56.52 ± 0.41^{a}	45.32 ± 0.53^{a}	36.19 ± 0.41^{b}
0.6 + 1.5	45.32 ± 0.11^{a}	45.10 ± 0.41^{a}	40.34 ± 0.32^{a}	54.52 ± 0.41^{a}	42.12 ± 0.40^{a}	54.52 ± 0.41^{a}
0.8 + 1.5	44.55 ± 0.41^{a}	29.99 ± 0.38^{b}	41.65 ± 0.43^{a}	35.06 ± 0.35^{b}	42.11 ± 0.23^{a}	26.73 ± 0.57^{b}
2,4-D + BAP						

Table 2. (Continued).

MS5 0.2 + 0.02	84.56 ± 0.17^{a}	84.56 ± 0.17^{a}	45.09 ± 0.41^{a}	84.56 ± 0.17^{a}	34.09 ± 0.12^{c}	98.56 ± 0.17^{a}
MS6 0.2 + 0.1	74.10 ± 0.41^{b}	76.10 ± 0.72^{b}	34.29 ± 0.23^{b}	64.07 ± 0.35^{b}	35.07 ± 0.35^{c}	63.10 ± 0.41^{d}
MS7 0.2 + 0.15	45.09 ± 0.65^{b}	43.09 ± 0.23^{b}	21.96 ± 0.83^{c}	26.79 ± 0.62^{d}	84.56 ± 0.17^{a}	55.09 ± 0.22^{c}
MS8 0.2 + 0.2	44.56 ± 0.17^{a}	45.56 ± 0.17^{a}	33.09 ± 0.41^{b}	35.16 ± 0.23^{a}	45.10 ± 0.41^{b}	45.77 ± 0.20^{b}
NAA + kinetin						
2 + 1	35.09 ± 0.41^{a}	29.99 ± 0.38^{b}	25.53 ± 0.39^{a}	29.99 ± 0.38^{b}	28.19 ± 0.18^{a}	34.84 ± 0.48^{d}
4 + 1	34.84 ± 0.48^{a}	44.81 ± 0.19^{a}	23.19 ± 0.41^{a}	25.12 ± 0.76^{b}	29.86 ± 0.38^{a}	68.56 ± 0.17^{a}
6 + 1	45.17 ± 0.71^{b}	45.23 ± 0.73^{a}	26.13 ± 0.19^{a}	30.01 ± 0.54^{a}	35.22 ± 0.11^{b}	54.69 ± 0.34^{c}
8 + 1	43.11 ± 0.21^{b}	43.09 ± 0.68^{a}	25.53 ± 0.54^{a}	29.65 ± 0.12^{b}	34.09 ± 0.81^{b}	58.48 ± 0.23^{b}
NAA + BAP						
4 + 1	29.99 ± 0.38^{b}	31.32 ± 0.03^{b}	43.23 ± 0.31^{a}	55.10 ± 0.41^{a}	26.56 ± 0.41^{b}	28.83 ± 0.50^{a}
4 + 2	35.24 ± 0.14^{a}	30.19 ± 0.40^{b}	45.17 ± 0.48^{a}	45.12 ± 0.17^{b}	29.99 ± 0.38^{a}	25.19 ± 0.21^{a}
4 + 3	30.14 ± 0.25^{a}	26.56 ± 0.35^{a}	39.78 ± 0.33^{b}	47.89 ± 0.43^{b}	19.89 ± 0.67^{c}	30.02 ± 0.18^{b}
4 + 5	35.09 ± 0.52^{a}	25.09 ± 0.51^{a}	35.09 ± 0.12^{b}	41.09 ± 0.44	20.09±0.78^{c}	26.07 ± 0.67^{a}
IAA + kinetin						
2 + 1	40.16 ± 0.12^{b}	26.56 ± 0.41^{c}	46.12 ± 0.23^{a}	35.24 ± 0.34^{c}	41.11 ± 0.32^{a}	63.56 ± 0.17^{a}
4 + 1	45.22 ± 0.17^{b}	30.09 ± 0.12^{b}	39.15 ± 0.01^{b}	50.75 ± 0.35^{b}	45.16 ± 0.17^{a}	26.56 ± 0.41^{d}
6 + 1	63.16 ± 0.12^{a}	35.24 ± 0.34^{b}	45.18 ± 0.11^{a}	45.09 ± 0.41^{a}	35.09 ± 0.21^{b}	39.23 ± 0.33^{c}
8 + 1	64.56 ± 0.27^{a}	45.20 ± 0.30^{a}	32.09 ± 0.35^{b}	54.56 ± 0.17^{a}	38.17 ± 0.26^{b}	70.77 ± 0.20^{b}
IAA + BAP						
4 + 1	29.99 ± 0.38^{b}	50.12 ± 0.20^{c}	63.77 ± 0.20^{b}	45.10 ± 0.41^{c}	41.07 ± 0.11^{a}	74.56 ± 1.01^{a}
4 + 2	54.69 ± 0.34^{a}	45.10 ± 0.41^{d}	50.75 ± 0.35^{c}	35.24 ± 0.34^{d}	44.09 ± 0.16^{a}	63.56 ± 1.01^{a}
4 + 3	29.99 ± 0.38^{b}	83.56 ± 1.01^{a}	45.10 ± 0.41^{d}	63.77 ± 0.20^{a}	45.19 ± 0.41^{a}	70.46 ± 0.52^{b}
4 + 5	54.69 ± 0.34^{a}	63.77 ± 0.20^{b}	83.56 ± 1.01^{a}	50.75 ± 0.35^{b}	35.24 ± 0.34^{b}	52.23 ± 0.34^{c}

Different letters denote significant difference by Duncan's multiple-range test.

Table 3. Callus growth indexes of media in subcultures. ANOVA statistical analysis ($P \leq 0.05$) was performed (mean ± SE).

Culture media	Plant growth regulators concentrations (mg L^{-1})	Callus growth index (first subculture)	Callus growth index (second subculture)
	IAA		
MS1	2	0.93 ± 0.15^{b}	0.72
MS2	4	0.95 ± 0.21^{c}	1.32
MS3	6	0.98 ± 0.35^{a}	2.10
MS4	8	0.98 ± 0.35^{a}	1.49
	2,4-D + BAP		
MS5	0.2 + 0.02	0.78 ± 1.18^{d}	12.88
MS6	0.2 + 0.1	0.76 ± 1.15^{c}	16.15
MS7	0.2 + 0.15	0.46 ± 1.75^{a}	10.45
MS8	0.2 + 0.2	0.63 ± 1.40^{b}	11.09

Different letters denote significant difference by Duncan's multiple-range test.

highest callus production percentage (Table 2). For the first subculture, MS5 was the most successful medium for callus growth index. For the second subculture, MS6 was the best medium (Table 3). Apical meristem and root explants produced the most successful results for callus production for the media containing IAA and 2,4-D + increasing amounts of BAP.

3.2. HPLC analysis

Generally, MS1, MS2, and MS3 media with IAA were the most successful media for catalpol, aucubin, and verbascoside production; however, MS3 medium with 6 mg L^{-1} IAA was the most appropriate medium for the production of all three secondary metabolites. Of the media with 2,4-D + BAP combinations, MS6 and MS7 induced catalpol and aucubin production. MS7 medium was found suitable for verbascoside production (Supplement Figure 2) (Table 4).

The highest content of catalpol in the plant of *G. trichosantha* subsp. *trichosantha* was found in the root. The highest content of aucubin (0.775 mg kg^{-1}) was measured in the stem and, as for verbascoside, the root held the highest content (0.290 mg kg^{-1}). In terms of plant samples, catalpol was the predominant metabolite in both the leaf and the root, whereas aucubin was the predominant one in the stem (Supplement Figure 3). However, in the fruit-seed, both aucubin and catalpol were the predominant metabolites (Table 5).

The highest catalpol concentration was produced in the amount of 1.277 mg kg^{-1} in the root of the plant, while the highest aucubin concentration was produced in the amount of 0.775 mg kg^{-1} in the stem of the plant. Apart from this, the highest verbascoside concentration occurred in the amount of 0.011 mg kg^{-1} in the root of the plant. As for the callus, the highest catalpol, aucubin, and verbascoside concentrations were formed in the amounts of 9.043 mg kg^{-1}, 3.061 mg kg^{-1}, and 0.742 mg kg^{-1}, respectively. These results have revealed that, when compared to the plant extracts, callus cultures have produced a much greater amount of secondary metabolites. In fact, the production of catalpol, aucubin, and verbascoside has been about 7.5, 4.2, and 7 times higher than that of the plant extracts, respectively. The callus growth indexes have indicated that MS3, MS5, and MS6 are the most suitable media for callus production (Table 4). A 16 times increase in the callus

Table 4. HPLC quantification of catalpol, aucubin, and verbascoside dried callus of *G. trichosantha* subsp. *trichosantha* (mg kg^{-1}). ANOVA statistical analysis ($P \leq 0.05$) was performed (mean ± SE).

Culture media	mg L^{-1}	Catalpol	Aucubin	Verbascoside
	IAA			
MS1	2	2.142 ± 1.14	2.941 ± 7.18	0.018 ± 0.56
MS2	4	2.934 ± 4.83	1.731 ± 4.72	0.034 ± 2.11
MS3	6	4.188 ± 4.22	2.548 ± 3.92	0.058 ± 1.16
MS4	8	1.705 ± 3.17	1.538 ± 5.12	0.026 ± 2.03
	2,4-D + BAP			
MS5	0.2 + 0.02	3.647 ± 8.56	1.611 ± 2.36	0.069 ± 3.61
MS6	0.2 + 0.1	9.043 ± 2.15	2.978 ± 2.12	0.022 ± 4.22
MS7	0.2 + 0.15	4.095 ± 3.43	3.061 ± 3.49	0.742 ± 2.01
MS8	0.2 + 0.2	3.171 ± 6.12	1.553 ± 1.73	0.219 ± 5.13

Table 5. HPLC quantification of catalpol, aucubin, and verbascoside dried plant material of *G. trichosantha* subsp. *trichosantha* (mg kg^{-1}). ANOVA statistical analysis ($P \leq 0.05$) was performed (mean ± SE).

Plant materials	Catalpol	Aucubin	Verbascoside
Leaf	0.450 ± 4.56	0.208 ± 3.12	0.014 ± 1.54
Stem	0.228 ± 7.03	0.775 ± 4.15	0.011 ± 4.22
Fruit-seed	0.471 ± 3.5	0.522 ± 6.10	0.057 ± 3.12
Root	1.277 ± 5.16	0.043 ± 2.18	0.290 ± 3.71

growth index in the second subculture of auxin + cytokinin combination revealed that 2,4-D + BAP combinations can be used more effectively in callus production (Table 3). In these media, callus formation started with yellow color and calli mostly turned brown after 2–3 weeks. However, callus production was sustained despite the browning.

4. Discussion

Several factors, particularly the genotypic variation, can affect the behavior of the cultured explant. These factors may be the organs to be used as tissue source and their ontogenic and physiological ages (Babaoğlu et al., 2001). In our study, the effect of callus potency on the age of explant taken from 30-day-old and 45-day-old seedlings was investigated. As a result, explants taken from 30-day-old aseptic seedlings were generally observed to perform better and result in higher percentages of callus formation compared to the explants taken from 45-day-old aseptic seedlings.

In the present study, callus formation started with yellow color and calli mostly turned brown after 2–3 weeks. However, callus production was sustained despite browning. Similar to our study, Laukkanen et al. (2000) observed the browning of calli after 2 weeks of callus growth. Callus browning was attributed to oxidation of phenols accumulated in cells. Tang et al. (2004) stated that oxidative stress caused callus browning. However, the occurrence of browning did not pose a problem for our analyses in the present study.

During the analyses of calli, the production of catalpol (MS6), aucubin (MS7), and verbascoside (MS7) was successfully achieved. Higher content of catalpol, aucubin, and verbascoside was measured in 1 g of dried callus compared to that of the plant. MS7 and MS8 media were found to be suitable for verbascoside production. For plant samples, higher production of catalpol and aucubin was observed generally in the leaf, stem, and fruit-seed. The presence of iridoid glycosides and phenylethanoids was identified in the root, leaf, stem, and fruit-seed extracts of *G. trichosantha* subsp. *trichosantha* through different analysis methods, but no quantitative analyses were conducted for catalpol, aucubin, and phenylethanoid verbascoside (Çalış et al., 1999).

Previous studies on similar species were examined. Similar to our study, Kırmızıbekmez et al. (2003b) identified the presence of aucubin in *G. cordifolia*. Kırmızıbekmez et al. (2003a) identified the presence of catalpol, aucubin, and verbascoside in *G. dumulosa*. Kırmızıbekmez et al. (2004) identified the presence of catalpol and verbascoside in *G. sintenisii* (2004); Kırmızıbekmez et al. (2009) identified the presence of catalpol, aucubin, and verbascoside in *G. aphyllanthes*. Kırmızıbekmez et al. (2003a, 2003b, 2004, 2009) did not give any quantitative data nor any related analyses. Therefore, this present study is different in the sense that it quantitatively determined catalpol, aucubin, and verbascoside in the calli and plant.

G. trichosantha subsp. *trichosantha* has already been searched for pharmacological studies, which were mainly on its secondary metabolites, but not on its plant tissue culture. Hence, this present study is the primary one to produce secondary metabolites through plant tissue culture.

In this study, *G. trichosantha* subsp. *trichosantha* was adapted to in vitro medium for the first time and an efficient germination rate was achieved. In seeds, 80% germination was observed. Thirty-day seedling age was found to be the most suitable for successful callus production. The best callus production was obtained from media containing IAA and 2,4-D + increasing amounts of BAP. The supplementation of 2,4-D + increasing amounts of BAP proved to be successful for highest catalpol, aucubin, and verbascoside production. Higher amounts of catalpol, aucubin, and verbascoside were produced in 1 g of dried callus compared to those produced in 1 g of dried plant. Comparing the contents of the compounds in calli and plant, it is concluded that higher amounts of catalpol, aucubin, and verbascoside can be produced in in vitro cultures. In the plant material, the highest catalpol (1.277 mg kg^{-1}) and verbascoside concentrations (0.290 mg kg^{-1}) were produced in the roots, whereas the highest aucubin concentration (0.775 mg kg^{-1}) was found in the stem. In callus, the highest catalpol (9.043 mg kg^{-1}), aucubin (3.061 mg kg^{-1}), and verbascoside concentrations (0.742 mg kg^{-1}) were produced in a medium including plant growth regulators. The amount of catalpol, aucubin, and verbascoside was successfully increased about 7 times by plant tissue culture techniques. With this study, basic information on plant tissue culture was obtained in order to carry out biotechnological studies on *G. trichosantha* subsp. *trichosantha*. At the end of this study, much more successful results have been received than expected. It is foreseen that in the near future catalpol, aucubin, and verbascoside can be produced by biotechnological techniques, such as bioreactors.

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References

Alipieva K, Kokubun T, Taskova R, Evstatieva L, Handjieva N (2007). LC/ESI-MS analysis of iridoid glucosides in *Lamium* species. Biochem Syst Ecol 35: 17-22.

Babaoğlu M, Gürel E, Özcan S (2001). Bitki Biyoteknolojisi I. Doku Kültürü ve Uygulamaları. In: Babaoğlu M, Gürel E, Özcan S, editors. Konya, Turkey: Selçuk Üniversitesi Basımevi, pp. 231-232.

Bello R, Moreno L, Primo-Yúfera E, Esplugues J (2002). *Globularia alypum* L. extracts reduced histamine and serotonin contraction in vitro. Phytother Res 16: 389-392.

Boutiti A, Benguerba A, Kitouni R, Bouhroum M, Benayache S, Benayache F (2008). Secondary metabolites from *Globularia alypum*. Chem Nat Compd 44: 4.

Chaudhuri RK, Sticher O (2004). New iridoid glucosides and a lignan diglucoside from *Globularia alypum* L. Helv Chim Acta 64: 3-15.

Chograni H, Riahi L, Zaouali Y, Boussaid M (2012). Polyphenols, flavonoids, antioxidant activity in leaves and flowers of Tunisian *Globularia alypum* L. (Globulariaceae). Afr J Ecol 51: 343-347.

Crişan G, Vlase L, Balica G, Muntean D, Ştefanescu C, Paltinean R, Tamaş M, Leucuta S (2010). LC/MS analysis of aucubin and catalpol of some *Veronica* species. Farmacia 58: 237-247.

Çalış İ, Kırmızıbekmez H, Rüegger H, Sticher O (1999). Phenylethanoid glycosides from *Globularia trichosantha*. J Nat Prod 62: 1165-1168.

Çalış İ, Kırmızıbekmez H, Sticher O (2001). Iridoid glycosides from *Globularia trichosantha*. J Nat Prod 64: 60-64.

Çalış İ, Kırmızıbekmez H, Tasdemir D, Sticher O, Ireland CM (2002). Sugar esters from *Globularia orientalis*. Z Naturforsch 57: 591-596.

Davis PH, Mill RR, Tan K (1988). *Globularia trichosantha*. In: Davis PH, Mill RR, Tan K, editors. Flora of Turkey and the East Aegean Islands, Vol. 7. 1st ed. Edinburgh, UK: Edinburgh University Press, pp. 29-30.

Gagnidze R, Gviniashvili T, Jinjolia L (2006). IAPT/IOPB chromosome data 2. Taxon 55: 757-758.

Kırmızıbekmez H, Akbay P, Sticher O, Calıs İ (2003a). Iridoids from *Globularia dumulosa*. Z Naturforsch 58: 181-186.

Kırmızıbekmez H, Calış İ, Akbay P, Sticher O (2003b). Iridoid and bisiridoid glycosides from *Globularia cordifolia*. Z Naturforsch 58: 337-341.

Kırmızıbekmez H, Calıs İ, Piacente S, Pizza C (2004). Phenolic compounds from *Globularia cordifolia*. Turk J Chem 28: 455-460.

Kırmızıbekmez H, Bassarello C, Piacente S, Akaydın G, Çalıs İ (2009). Flavonoid, phenylethanoid and iridoid glycosides from *Globularia aphyllanthes*. Z Naturforsch 64: 252-256.

Laukkanen H, Rautiainen L, Taulavuori R, Hohtola A (2000). Changes in cellular structures and enzymatic activities during browning of Scots pine callus derived from mature buds. Tree Physiol 20: 467-475.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plantarum 15: 473-497.

Skim F, Kaaya A, Jaouhari JT, Lazrek HB, Jana M, El Amri H (1999a). Hypoglycaemic activity of *Globularia alypum* leaves in rats. Fitoterapia 70: 382-389.

Skim F, Lazrek HB, Kaaya A, El Amri H, Jana M (1999b). Pharmacological studies of two antidiabetic plants: *Globularia alypum* and *Zygophyllum gaetulum*. Therapie 54: 711-715.

Snedecor GW, Cochran WG (1967). In: Snedecor GW, Cochran WG, editors. Statistical methods, 6th ed. USA: Iowa State University Press, pp. 327-329.

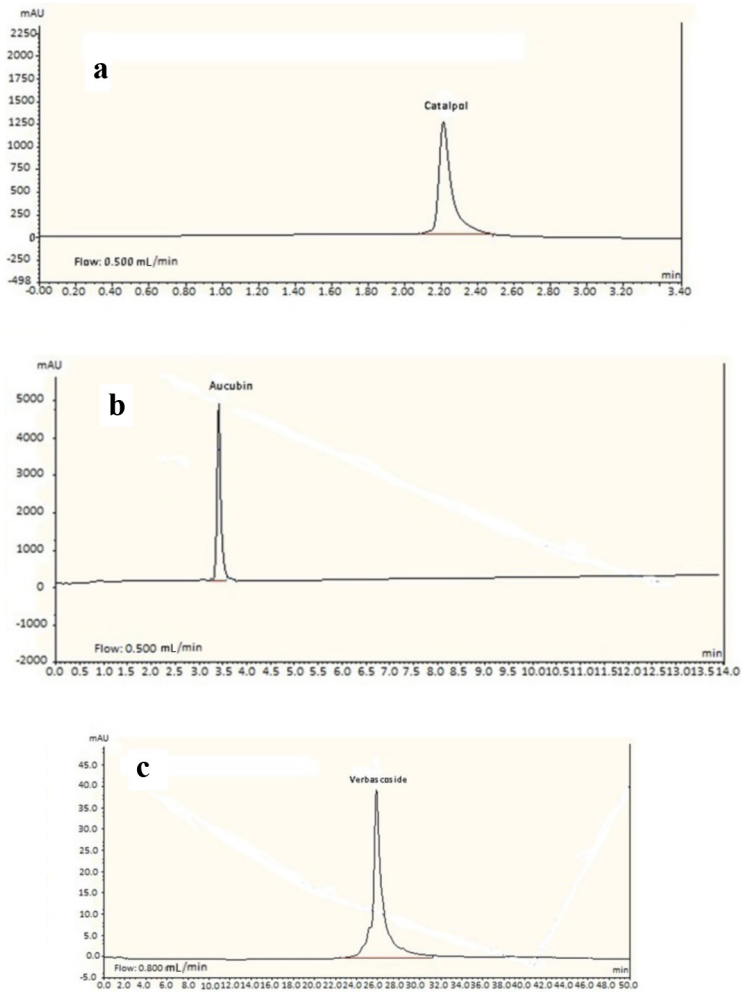
Taleb-Dida N, Krouf D, Bouchenak M (2011). *Globularia alypum* aqueous extract decreases hypertriglyceridemia and ameliorates oxidative status of the muscle, kidney, and heart in rats fed a high-fructose diet. Nutr Res 31: 488-495.

Tang W, Newton RJ, Outhavong V (2004). Exogenously added polyamines recover browning tissues into normal callus cultures and improve plant regeneration in pine. Physiol Plantarum 122: 386-395.

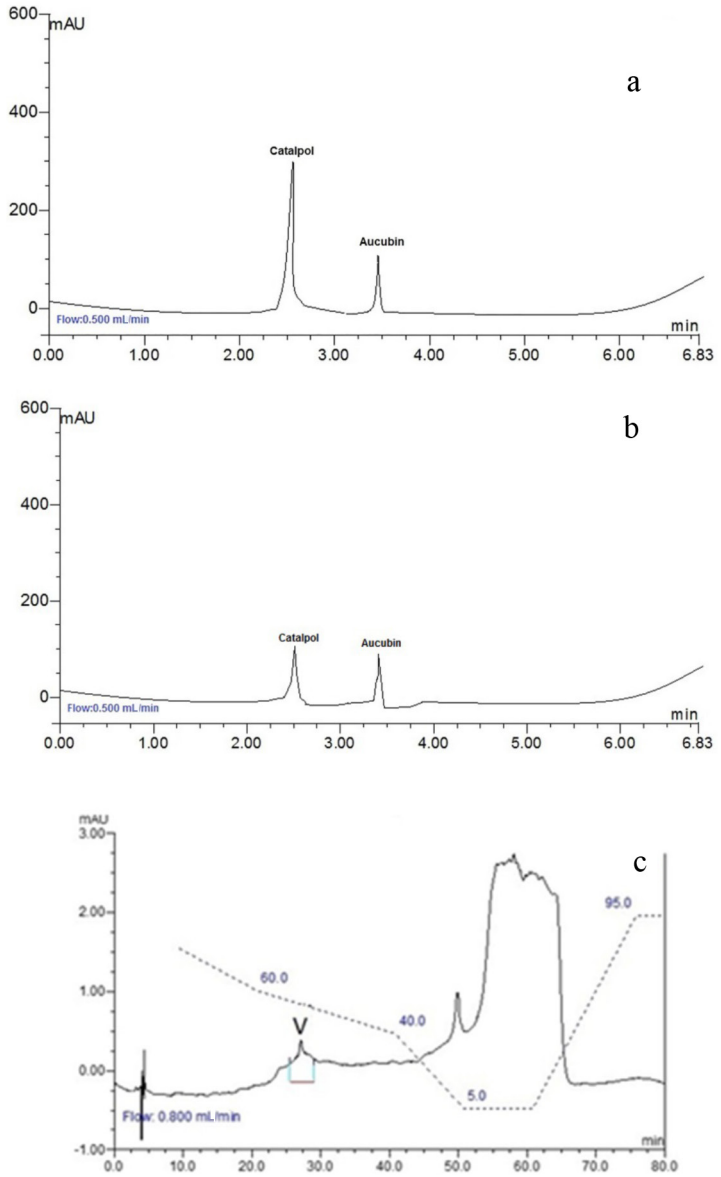
Tundis R, Peruzzi L, Colica C, Menichini F (2012). Iridoid and bisiridoid glycosides from *Globularia meridionalis* (Podp.) O. Schwarz aerial and underground parts. Biochem Syst Ecol 40: 71-74.

Xie J, Tan F, Zhu J, Yue C, Li Q (2012). Separation, purification and quantification of verbascoside from *Penstemon barbatus* (Cav.) Roth. Food Chem 135: 2536-2541.

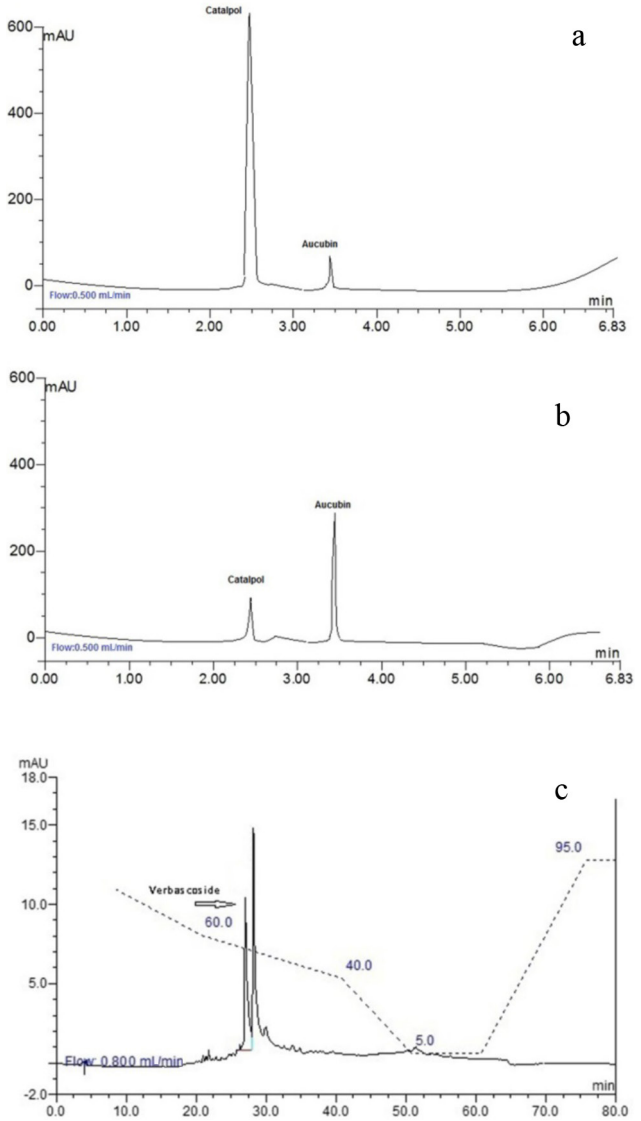
Wang DH, Du F, Liu HY, Liang ZS (2010). Drought stress increases iridoid glycosides biosynthesis in the roots of *Scrophularia ningpoensis* seedlings. J Med Plants Res 4: 2691-2699.



Supplement Figure 1. Retention times of standards. a. 100 ppm catalpol 2.107–200 nm; b. 10 ppm aucubin 3.420–200 nm; c. 15 ppm verbascoside 27.04–330 nm.



Supplement Figure 2. Chromatograms of in vitro produced calli. a. MS6 medium that produced the highest catalpol concentration; b. MS7 medium that produced the highest aucubin concentration; c. MS7 medium that produced the highest verbascoside concentration; x-axis is minute and y-axis is mAU value.



Supplement Figure 3. Chromatograms of plant samples. a. Root samples that produced the highest catalpol concentration; b. Stem samples that produced the highest aucubin concentration; c. Root samples that produced the highest verbascoside concentration; x-axis is minute and y-axis is mAU value.