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ARTICLE

Phenolic content and antioxidant activity of *Scutellaria baicalensis* Georgi grown *in vitro*

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Abstract: The objectives of this work were to propagate plantlets of *Scutellaria baicalensis* Georgi, a rare medicinal plant in Mongolia, *in vitro* conditions, and to determine and compare their total phenolic content (TPC) and antioxidant activity with those of natural and cultivated plants. The seeds collected from Khentii aimag (province) were successfully germinated on half-strength MS medium supplemented with gibberellic acid (GA_3), with the highest germination rate (16±0.8%) observed at 0.5 mg/L GA_3. Shoot regeneration occurred on hormone-free ½MS medium, while optimal root induction was achieved with 0.5 mg/L indole-3-butyric acid (IBA). Methanolic extracts of leaves and roots were analysed for their TPC using the Folin–Ciocalteu method and antioxidant activity using the DPPH radical scavenging assay. Plantlet leaves *in vitro* exhibited significantly higher TPC (88.7±1.5 mg/g GAE) compared to natural and cultivated plants, while roots *in vitro* also showed higher TPC (21±0.2 mg/g GAE) than their counterparts. Antioxidant activity was the highest in both natural and cultivated plant leaves and roots; however, leaves and roots from *in vitro*-propagated plantlets exhibited lower radical scavenging capacity, indicating comparable bioactivity. Overall, the results suggest that the initial *in vitro* regeneration protocol is effective for *S.baicalensis* propagation and the production of bioactive compounds.

Keyword: *In vitro, plantlets, Scutellaria, rare, phenolic content, antioxidant activity;*

INTRODUCTION

In vitro plant tissue culture, also known as micropropagation, has been widely used for scientific research, agriculture, and conservation of endangered or threatened plant species. It is often considered an efficient method for propagating disease-free, genetically uniform, or modified plants *in vitro* and for producing polyploid plants with higher yields or better production of secondary metabolites [1].

Scutellaria baicalensis Georgi., also known as Chinese skullcap or skullcap (NCBI: txid65409), is a species of perennial, flowering plant in the Lamiaceae family, which is distributed worldwide, especially throughout Europe, the United States, and East Asia [2]. There are 14 species out of more than 360 species in the world that are distributed in Mongolia, mostly in Khentei, Mongolian Dauria, Khiangan, and the East Mongolian region [3], and in 1995, it was listed in the “Law of Mongolia on Natural Plants” as “a rare plant”.

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Historically, species of the genus *Scutellaria*, particularly *S.baicalensis*, have long been integral to Mongolian Traditional Medicine, where they are incorporated into polyherbal formulations, such as Honlin and Zowu-8, and are valued for their diverse pharmacological properties [4].

Numerous investigations have focused on developing reliable *in vitro* organogenesis systems for *Scutellaria* species through optimisation of cytokinin–auxin ratios to maximise the accumulation of valuable secondary metabolites and corresponding biological activities [5, 6].

Flavonoids are defined as a class of polyphenolic compounds that serve as secondary metabolites in higher plants, and *Scutellaria* plants are rich in root-specific flavonoids (RSFs), including baicalin, baicalein, wogonin, and wogonoside, which are also rich in leaf-specific flavonoids (LSFs), such as scutellarin and scutellarein. These are the most characteristic compounds of *Scutellaria* that are widely known to have anti-cancer, anti-viral, anti-oxidant, and anti-inflammatory properties [7, 8]. Moreover, recent studies reported that a major component, baicalein, is effective in inhibiting SARS-CoV-2 (COVID-19) [9, 10].

Due to the fact that *S.baicalensis* is a vital medicinal material, scientific literature mainly covers phytochemical and metabolomic studies. Despite existing studies in Mongolia addressing the ecology, morphology, micropropagation, and phytochemistry of *S.baicalensis*, *in vitro* micropropagation of plantlets and their bioactive compound profiles have not yet been investigated.

When grown *in vitro*, plantlets are assured to be under controlled conditions and independent of seasonality. Thus, the type and concentration of bioactive compounds can be standardised in order to guarantee a homogeneous product to the market. Moreover, since *S.baicalensis* is a medicinal plant that is distributed in a limited area, *in vitro* culture and phytochemical studies would be an efficient approach for both research and companies using them.

This research aims to evaluate *in vitro*-derived plantlets of *S.baicalensis* as a source of bioactive compounds, setting up, firstly, an initial micropropagation protocol and secondly, characterising them in terms of total phenolic content and antioxidant activity.

MATERIALS AND METHODS

Plant materials

S.baicalensis seeds were collected from Delgerkhaan soum ($47^0$20’259” N, $108^0$67’915” E), in Khentii aimag, Mongolia, in 2021 and used for the plant micropropagation study. For the phytochemical analysis, the natural plants were collected from Dadal soum, Khentii aimag ($48^0$59’40” N, $111^0$46’44” E), and the cultivated plants were collected from the plantation field in Delgerkhaan soum, Khentii aimag ($47^0$20’259” N, $108^0$67’ 915” E), in July 2025.

Seed germination

Seed surfaces were sterilised with 70% (v/v) ethanol for 90 sec, treated with 10% hydrogen peroxide (H_2O_2) for 10 min, and rinsed briefly with sterile distilled water 2-3 times. After which, they were sterilised with a solution of 1-2.5% sodium hypochlorite (NaOCl) containing 0.1% Tween-20, followed by rinsing with sterile distilled water 5 times. Sterilised seeds were then cultured on agar (0.8%) solidified half-strength Murashige Skoog (MS) medium [13] supplemented with 3% (w/v) sucrose and 0.5-2.0 mg/L gibberellic acid (GA_3) as a plant growth regulator (Supplementary Table 1). Before adding agar, the medium pH was adjusted to 5.7–5.8 and was sterilised in an autoclave (Arpsynth, Japan) at 121°C (1 Atm pressure) for 20 min.

Shoot and root induction

Three-month-old *in vitro* seedlings were used as sterile explants for plant regeneration. In this experiment, the stem nodes of the seedlings were cut and transferred to the ½MS medium without plant growth regulators (PGRs). Plantlets with well-developed shoots were transferred to the root induction medium,

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½MS, containing different concentrations of indole-3-butyric acid (IBA).

***In vitro* culture conditions**

Inoculated seeds and plantlets *in vitro* conditions were incubated in a culture room at a temperature of 25-28°C, with a relative humidity of 50-55%, and a light photoperiod of 16/8 h light/dark (2000-3000 lux). Every 3-4 weeks, plantlets were subcultured into freshly prepared medium.

Plant extraction

Leaves, stems, and roots of 4-week-old *in vitro* plantlets were collected, and after removing the agar medium with tap water, the plant parts were dried in the aerosterilisator at 60°C for 48 hours and ground into fine powder in a mortar with pestle. Natural and cultivated plants were air-dried for one week at room temperature and transferred to 60°C for 48 hours. After drying, they were ground into a fine powder as well. One gram of plant powder from each plant group was extracted in 25 mL of 99.5% methanol (Xilong Scientific, China), followed by ultrasonication (Cole-Parmer Instrument, USA) for 20 min and soaking in a continuous shaker at 240 rpm at room temperature. After 24 hours, extracts were filtered through a 15-20 µm filter paper (Beimu, China) in a fume hood and stored at a temperature of 4°C for further analysis. The extract concentration was 20 mg/mL in total.

Measurement of total phenolic content (TPC)

The total phenolic content (TPC) of samples was measured using the Folin-Ciocalteu method [14]. One hundred twenty-five µl of samples (20 mg/mL) were mixed with 625 µL of 10x Folin-Ciocalteu reagent (Sigma-Aldrich, USA) and 625 µL of 7.5% sodium carbonate (Na_2CO_3). The mixture was kept at room temperature for 20 min, and the absorbance was measured at 760 nm, using an M600 UV-Vis spectrophotometer (Yoke Instrument, China). TPC was expressed as gallic acid equivalents (GAE). Gallic acid was prepared in 0.001, 0.002, 0.003, 0.004, and 0.005 mg/mL concentrations in methanol and used as a control solution to obtain a standard curve. The regression line was obtained using gallic acid absorbance according to the different concentrations, and y=0.0042x+0.17 (R^2=0.86) was used for the calculation of TPC in the samples. The methanol served as a blank.

Measurement of antioxidant activity

The antioxidant activity was studied spectrophotometrically by means of the DPPH radical scavenging assay [15]. Three µL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) methanol solution was added to 500 µL of the sample solution at different concentrations (0.05, 0.1, 0.2, 0.4, 0.8 mg/mL). The same amount of DPPH solution was added to 500 µL of gallic acid (GA), methanol solution at different concentrations (0.005, 0.01, 0.02, 0.04, 0.08 mg/mL), and referred to as the control solution. The DPPH (99.7%) was purchased from Ringe (China). The mixtures were kept in dark conditions at room temperature for 30 min, and then the absorbance was measured at 518 nm. The ability to scavenge the DPPH radical was calculated as a percentage of radical scavenging activity (RSA, %) using the following equation:

$$RSA\ (\%)=(1-A/B)\times 100.$$

Where *A* was the absorbance of the sample;

B was the absorbance of the control.

All analyses were conducted with 3-4 technical replicates and 3 biological replicates. Moreover, the IC_{50} parameter was determined, referred to as the half-maximal inhibitory concentration, which results in 50% inhibition of the free radical activity. On the basis of the analysis of their RSA (%) values, the linear range was estimated using the GA control solution (from 0.005 to 0.08 mg/mL), which allowed for the determination of the IC_{50} parameter from the equation of the obtained trend line.

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Statistical analysis

All statistical analyses and means comparisons were done using MS Excel 2024, and significant differences in different groups were detected by one-way ANOVA post-hoc Tukey HSD test ($p \leq 0.05$) using the ASTATSA online program.

RESULTS AND DISCUSSIONS

Seed germination

A total of 360 seeds were used for the 4 individual germination experiments. After 4 days of inoculation in the first experiment, *S.baicalensis* seeds started to germinate (2.2%) in ½MS medium when the seeds were treated with 1% NaOCl, but microbial contamination was observed in both germinated and dormant seeds (100%). In order to reduce the contamination and increase the germination rate, seeds were treated with 2.5% NaOCl and inoculated in a fresh ½MS medium supplemented with 0.5, 1.0, and 2.0 mg/L GA_3. As a result, germination started after 5 days of sterilisation, and 10 days later, hypocotyls and cotyledons appeared, and after 3 weeks, the seedlings were measured. The highest seed germination rate (16±0.8%) and the reduced contamination (8±0.7%) were observed in ½MS containing 0.5 mg/L GA_3 (Figure 1. A, B, Supplementary Table 1).

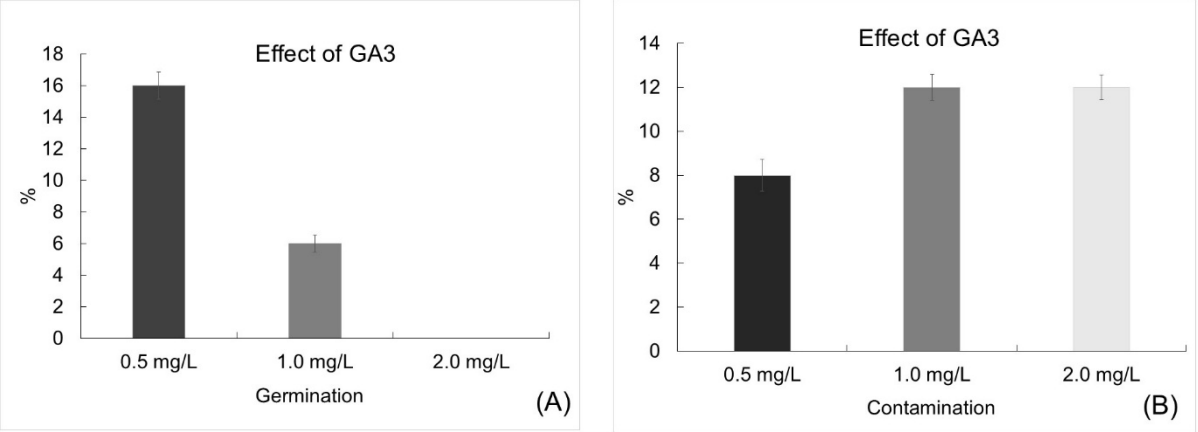

Figure 1. The effect of GA_3 concentrations on the seed germination (A) and contamination (B) of S.baicalensis. The means are not significantly different at $p \leq 0.05$, calculated by one-way ANOVA with post hoc Tukey HSD test (https://astatsa.com/).

The effect of PGR on shoot and root induction

A total of 17 three-week-old sterile seedlings were used for the plant regeneration. Stem nodes were cut, and a total of 35 explants (5 explants per culture jar) were inoculated into hormone-free ½MS medium. After 6 weeks, shoots developed directly in all explants. Since stem nodes serve as a vital growth point containing buds for shoot development, shoot formation and proliferation were successfully initiated even in the presence of PGRs in the culture medium. Previous studies have shown that hormone-free medium also affected shoot development from callus after thidiazuron (TDZ) treatment on *S.baicalensis* [16, 17]. On the other hand, hormone-free culture medium is effective for some plant species; for instance, Doley & Saunders 1989, Kodad *et al.* 2021, and Wittmer *et al.* 2025 demonstrated that a hormone-free nutrient medium gives higher results of shoot proliferation in some species [18, 19, 20]. Particularly, Kodad *et al.* reported that the highest shoot proliferation from nodal segments of *Prunus dulcis* Mill was achieved on a hormone-free medium, while also emphasising the significant influence of genotype on proliferation efficiency [19]. Moreover, Wittmer *et al.* concluded that the combined PLT/WOX transcription factors (PLETHORA and WUSCHEL-related homeobox, respectively) involved direct protein complex formation or promoted the expression of genes involved in somatic embryogenesis or direct organogenesis across several plant cultivars within the Brassicaceae, Asteraceae, and Solanaceae families in the absence of plant hormones [20]. To deepen the understanding of the

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external hormonal application in direct or indirect organogenesis in the Lamiaceae family, gene regulatory networks are worth further study.

Since the shoots were regenerated sufficiently in a culture medium without supplementing any hormones, further experiments continued directly, since it provides the advantage of saving both time and resources.

A total of 30 shoots were then transferred to the root induction medium, ½MS, containing different concentrations of IBA (15 shoots per IBA concentration), and the maximum root induction and length (1.67 and 0.29 cm, respectively) were achieved in 0.5 mg/L IBA (Figure 2. A, B and 3. A, B). The highest shoot proliferation occurred at 1.0 mg/L IBA concentration; however, the number of roots and their length decreased. Regenerated plants were subcultured in the same medium every 4 weeks and were further used for *in vitro* micropropagation (Figure 3. C, D).

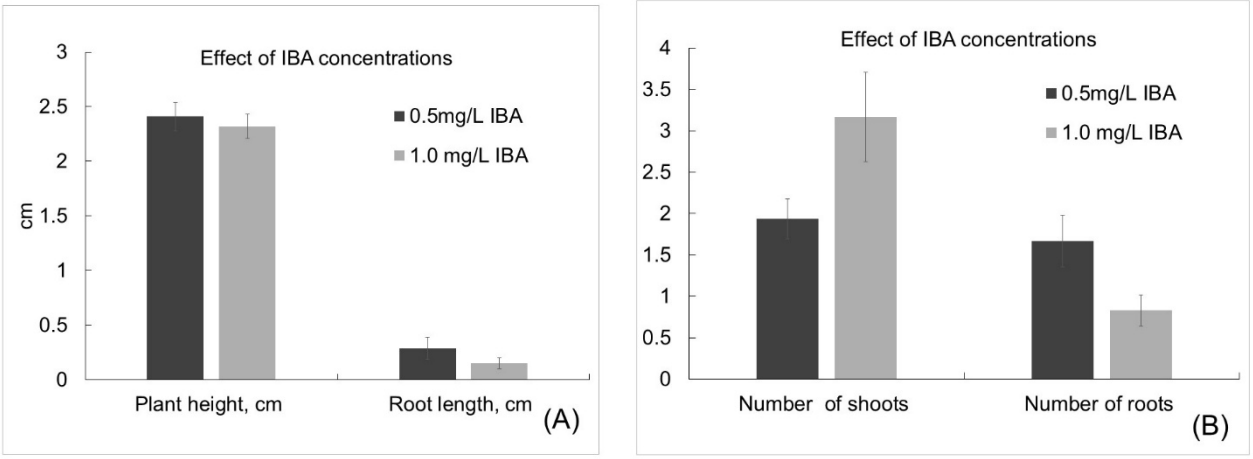

Figure 2. The effect of different concentrations of IBA (indole-3-butyric acid) on plant (A) and their shoot and root development (B) of in vitro plantlets of S.baicalensis. The means are not significantly different at $p \leq 0.05$, calculated by one-way ANOVA with post hoc Tukey HSD test (https://astatsa.com/).

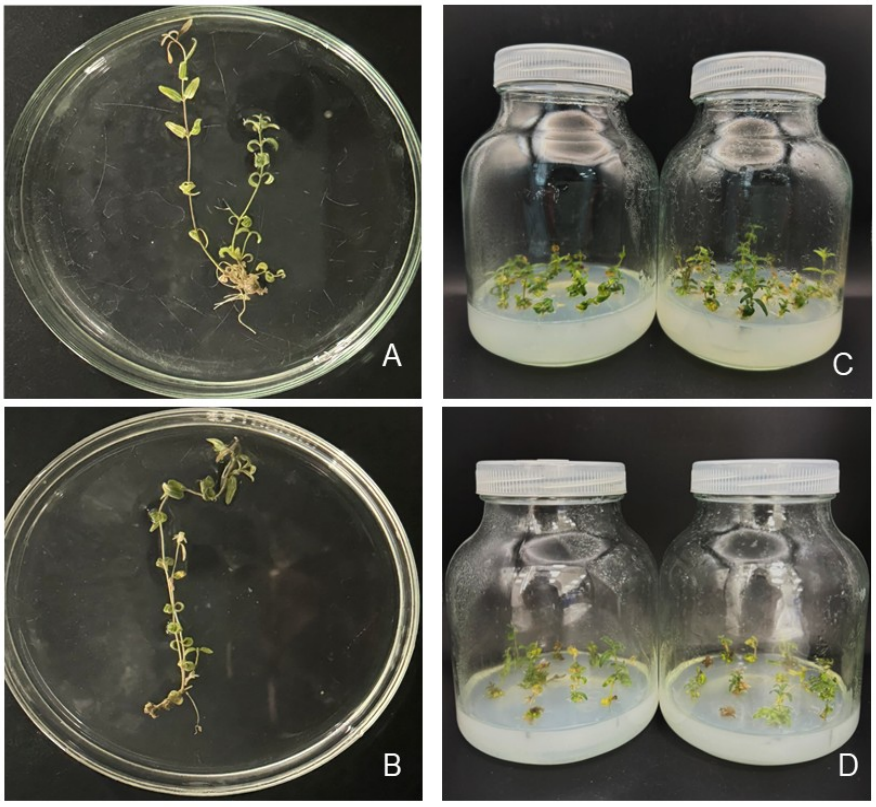

Figure 3. The effect of different concentrations of IBA (indole-3-butyric acid) on shoot and root development. A.0.5 mg/L IBA, B.1.0 mg/L IBA, C.D in vitro plantlets of S.baicalensis.

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Total phenolic content (TPC)

TPC of the *in vitro* plantlets of skullcap was determined in the methanolic extracts of different parts of the plant and compared to the natural and cultivated skullcaps. The regression line was obtained using gallic acid absorbance according to the different concentrations (0.001, 0.002, 0.003, 0.004, and 0.005 mg/mL), and $y=0.0042x+0.17$ ($R^2=0.86$) mean was used for the calculations of TPC in the samples. The TPC of the skullcap extracts varied based on their growing conditions.

The result indicated that the TPC was higher in leaves and roots of *in vitro* plantlets (88.7±1.5 mg/g and 21±0.29 mg/g, respectively) than that of the natural and cultivated plants (Figure 4, Table 1). TPC of the natural plant leaves was 6.72±2.9 mg/g, while their roots contained 7.7±1.01 mg/g. The cultivated *S.baicalensis* showed 6.66±0.71 mg/g TPC in leaves, and 5.46±0.59 mg/g TPC in roots.

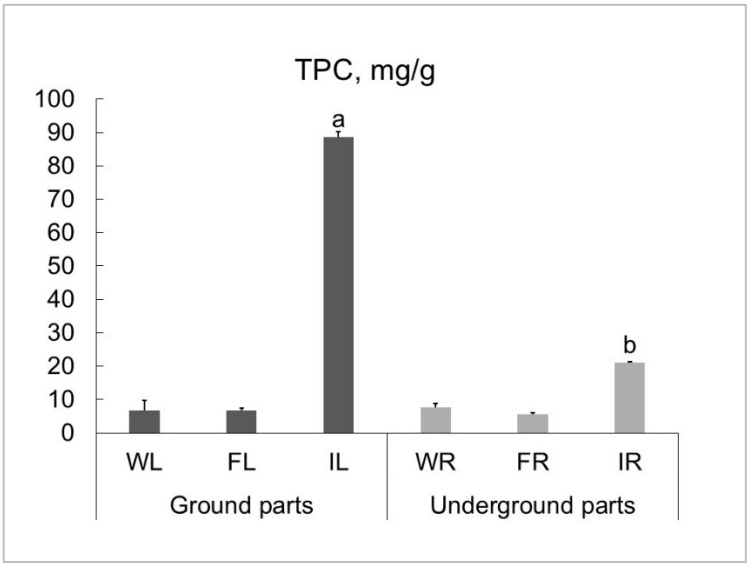

Figure 4. Total phenolic content (TPC) of the S.baicalensis from different growth conditions. WL-natural plant leaf, FL- cultivated plant leaf, IL- in vitro plantlet leaf, WR-natural plant root, FR - cultivated plant root, IR – in vitro plant root. The means presented with the different letters (a and b) are significantly different at p ≤ 0.05, calculated by one-way ANOVA with post hoc Tukey HSD test.

Table 1. TPC and antioxidant activity of *S.baicalensis.*

	Plant samples in methanolic extracts					
	IL	IR	WL	WR	FL	FR
TPC (mg/g GAE[1])	**88.7±1.5a**	**21±0.29b**	6.72±2.9	7.7±1.01	6.66±0.71	5.46±0.59
DPPH radical scavenging activity IC_{50}[2] (mg/mL)	2.12	0*	**1.41**	**1.44**	1.76	1.85

1-GAE, gallic acid equivalents
2-IC_{50}, the half-maximal inhibitory concentration
*-Not performed for this sample.

The antioxidant activity

The antioxidant activity of *in vitro* plantlets of *S.baicalensis* was determined spectrophotometrically by DPPH radical scavenging assay and compared to the natural and cultivated counterparts. For the particular concentration variants of plant samples (0.25, 0.5, 1.0, 2.0, and 4.0 mg/mL), the differences in the antioxidant activity are expressed as IC_{50} (Table 1) and RSA (Table 2). The trend line was obtained using RSA (%) mean of gallic acid at different concentrations (0.005, 0.01, 0.02,

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0.04, and 0.08 mg/mL), and y=19.505x+10.9 (R^2=0.88) mean was used for the calculations of IC_{50} in samples.

In vitro regenerated plantlet leaves of *S.baicalensis* showed 74.4±0.9% and 2.12 mg/mL IC_{50} antioxidant activity in 4.0 mg/mL, while their roots showed 6.37±0.3% and below the threshold IC_{50}, which was the lowest activity among the samples. The natural plant leaves and roots, at the 4.0 mg/mL conc, displayed the highest activity, 95.44±0.0%, 1.41 mg/mL $IC_{50,}$ and 93.93±1.03%, 1.44 mg/mL $IC_{50,}$ respectively, followed by the cultivated plant leaves and roots, 94.1±1.03%, 1.76 mg/mL $IC_{50,}$ and 94.28±1.5%, 1.85 mg/mL $IC_{50,}$ respectively. Due to an insignificant difference in radical scavenging activity among the *in vitro*, wild, and cultivated skullcap, we presume that characterisation of *in vitro* plant leaves might be similar to their natural state, and the regeneration protocol is suitable to produce *S.baicalensis*.

Moreover, the antioxidant activity of the roots of microplants was significantly lower than that of natural and cultivated plant roots (*p≤0.05). This result indicates that *in vitro* regenerated plantlets of *S.baicalensis* leaves can show biological activity even when their root function is still not fully developed.

Table 2. DPPH Radical Scavenging Activity of *S.baicalensis*

conc, mg/mL	DPPH radical scavenging activity (RSA), %					
	IL	IR	WL	WR	FL	FR
0.25	12.09±8.7	0.86±1.0	17.77±2.6a	17.81±2.8	-3.7±2.4	-2.2±3.7
0.5	21.6±15.6	1.48±1.1	22.25±0.06	27.11±3.2	8.91±5.2	7.57±3.1
1.0	26.6±11.2	3.08±0.6	40.88±4.7	42.34±3.4	24.74±0.7	25.3±4.8
2.0	48.4±16.7	9.24±0.04	63.04±0.5	59.72±4.6	57.83±1.9	54.04±0.8
4.0	74.4±0.9	6.37±0.3	95.44±0.0	93.93±1.03	94.1±1.03	94.28±1.5

The seed sterilisation process is an essential stage of micropropagation, and an optimised protocol can decrease microbial contamination. Seed surface sterilisation with 70% ethanol (C_2H_6O), 1% sulfochlorantin (H_2O_3S), 0.1-10% hydrogen peroxide (H_2O_2), or 1-2.5% sodium hypochlorite (NaOCl) [16, 17] has been used for various plant species. Thus, 10% H_2O_2 and 1-2.5% NaOCl were used in this study. When seeds were treated with 2.5% NaOCl, contamination was reduced from 100 to 8%. In the previous study [22], the medium supplemented with GA_3 allowed for direct shoot organogenesis and was used for the micropropagation of regenerants in *S. baicalensis*. Due to the low germination rate, plant hormone GA_3 was added to the medium to induce organogenesis in meristematic tissue and to break seed dormancy. As a result, 0.5 mg/L GA_3 increased the germination rate up to 16%; however, 8-12% of the sterilised seeds were still contaminated. This might be caused by the water loss or storage conditions of the seeds. Then, 3-month-old *in vitro* seedlings were used for plant regeneration, and hormone-free ½MS basal medium was more effective for shoot proliferation. Mostly 6-benzylaminopurine (BAP) and thidiazuron (TDZ) are known as the most effective cytokinins to induce shoots in *Scutellaria* [22, 24]. In this study, stem nodes were used as explants since they include axillary buds, which are considered to be a potential explant to form shoots because of their embryonic or organogenic characteristics.

For the rooting, shoots were treated with IBA. IBA is a synthetic hormone related to auxins that promotes plant growth and development in many plant species and is often used exogenously, especially to stimulate adventitious roots [24]. In addition, the experimental results showed that after 2 weeks, new shoots regenerated from some shoots, and also had a positive effect on the root formation.

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The extraction of polyphenolic compounds from plant materials depends on extraction methods, and methanol has been known to be the most effective extractant [21, 23, 25]. In the present study, plant samples were extracted with methanol. The result showed that the TPC was higher in the *in vitro* plantlet than in natural and cultivated plants, and this difference might be related to the optimal growth conditions of *in vitro* micropropagation and the extraction efficiency of freshly prepared *in vitro* plantlets, or the content of leaf and root pigments. The TPC of skullcap was reported as 91.8 mg/g GAE in the methanolic extracts of the Korean type [26], whereas the TPC of the present samples in this study ranged from 5.46±0.59 to 88.7±1.5 mg/g GAE. In antioxidant activity assays, Park *et al.* (2011) reported that ethanolic extracts of commercial *S.baicalensis* exhibited 64.6±29.94% radical scavenging activity (RSA) at a concentration of 5.0 mg/mL [27], while the present study demonstrated 95.44±0.0% RSA at 4.0 mg/mL in methanolic extracts of natural leaves. Thus, the DPPH assay is a compatible tool for estimating the free radical scavenging activities of antioxidants for *S.baicalensis.*

CONCLUSIONS

An *in vitro* micropropagation protocol for *Scutellaria baicalensis* Georgi was successfully established in ½MS medium supplemented with GA_3 and IBA. *In vitro*–grown plantlet leaves exhibited significantly higher total phenolic content than natural and cultivated plants and showed notable antioxidant activity. The results of these studies revealed that the initial *in vitro* micropropagation protocol of *S.baicalensis* can be a useful method for producing bioactive compounds. However, further experiments are necessary using a wider range of culture media supplemented with different plant growth regulators (PGRs) to enhance regeneration capacity. Furthermore, additional studies need to be done, such as a genetic fidelity analysis to determine the *in vitro* regenerants and to compare them with the mother plants to detect any occurrence of somaclonal variation.

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Author contributions

The authors confirm their contribution to the paper as follows: Study conception and design: KO, AK; recommendation: OY; data collection: KO, ME, MT; analysis and interpretation of the results: KO, ME; draft manuscript preparation: KO, AK. All authors reviewed the results and approved the final version of the article.

Conflicts of interest

The authors declare that there is no conflict of interest.

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