

Research Article

Effect of elicitors on phenylpropanoid biosynthesis in hairy roots of *Prunella vulgaris*

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Abstract

Phenylpropanoids are economically important secondary metabolites with strong pharmacological activity substantively, and their pathways often can be adjusted by abiotic elicitors in plant tissue cultures. Silver nitrate, copper chloride, salicylic acid, and hydrogen peroxide affect the phenylpropanoid such as rosmarinic acid (RA) in hairy root (HR) cultures of *Prunella vulgaris*. Hairy roots (HRs) were developed via *Agrobacterium rhizogenes* mediated transformation and cultured under dark and agitated conditions. After treatment for 3 days, we used HPLC to quantify four phenylpropanoids, caffeic acid, benzoic acid, rutin, and quercetin from the treatments with different concentrations of elicitors. The most potent elicitor was salicylic acid which increased total phenols by 46.7±1.38 µg/g in control to 95.46±12.63 µg/g at 0.5 mM, largely on account of a nearly threefold increase in caffeic acid (32.15±5.67 µg/g) and a doubled rutin (59.64±6.71 µg/g) level. At 0.5 mM CuCl₂, the accumulation of phenols was increased (64.83±2.62 µg/g), due mainly to an increase in rutin levels (45.46±2.11 µg/g) and caffeic acid (13.18±0.04 µg/g). At a concentration of 1mg/L AgNO₃, the production of caffeic acid and quercetin was moderately induced (47.01±1.87 µg/g) but suppressed at higher concentrations. The H₂O₂ treatments demonstrated a low-level elicitation effect with total phenolics maximum of 48.93±1.21 µg/g (100 mM), and a more gradual induction of benzoic acid (1.6±0.16 µg/g) and rutin (32.27±0.16 µg/g). Overall quercetin content decreased in nearly all the treatments and was especially evident at high concentrations of SA and AgNO₃, supporting the notion that a metabolic reprogramming with subsequent precursor diversion is occurring. These findings demonstrate that different abiotic elicitors differentially regulate the phenylpropanoid biosynthesis of *P. vulgaris* HRs, and that SA and CuCl₂ were very strong inducers, providing probable strategies to improve the production of medicinally important phenolic compounds.

Keywords: *Prunella vulgaris*, Hairy root culture, Phenylpropanoids, Abiotic elicitors, Salicylic acid, Copper chloride, Silver nitrate

Introduction

Phenylpropanoid pathway is a distinguished branch of plant secondary metabolism, where L-phenylalanine is enzymatically transformed through phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), tyrosine aminotransferase (TAT), and rosmarinic acid synthase (RAS) to produce a large variety of phenolic compounds (Xiao *et al.*, 2009). These metabolites play an important role during plant defense and development and have also been identified with significant pharmacological characteristics, including antioxidant, anti-inflammatory and anticancer (Xu *et al.*, 1999; Psoťová *et al.*, 2003; Şahin *et al.*, 2014; Jakovljević *et al.*, 2025).

The hairy root cultures have become a strong biotechnological platform towards the production of such useful metabolites due to their rapid growth, independence from plant hormones, stable genomes and high metabolic powers (Georgiev *et al.*, 2007; Sharma *et al.*, 2013; Mirmazloum *et al.*, 2024). The hairy roots have been shown to accumulate up to 15-30 times more rosmarinic acid (RA) than the intact plant tissues in *Prunella vulgaris* (Jakovljević *et al.*, 2025), highlighting them as tools for metabolic studies and in the large-scale production of bioactive compounds.

While this potential exists, baseline metabolite production often remains subpar on non-elicited systems. Elicitors like methyl jasmonate (MeJA), salicylic acid (SA), ethephon (Eth), hydrogen peroxide (H₂O₂), chitosan, and yeast extract are known as effective inducers of secondary metabolism. These compounds signal for the plant defense signaling cascades via calcium influx, reactive oxygen species generation along with the activation of transcription factors (e.g., MYB, WRKY and AP2/ERF) which further upregulates biosynthetic key genes such as PAL, C4H, 4CL, TAT, HPPR and RAS (Zhao *et al.*, 2005; Xiao *et al.*, 2009; Goel *et al.*, 2011; Mirmazloum *et al.*, 2024).

In *P. vulgaris* hairy roots, Ru *et al.* (2016) demonstrated that treatment with 200 µg L⁻¹ Eth and 6.9 mg L⁻¹ SA significantly enhanced RA content by 1.66-fold and 1.48-fold, respectively, relative to untreated controls. This increase was closely correlated with transcriptional upregulation of PAL, TAT, 4CL, and cytochrome P450 monooxygenase genes. Parallel investigations in other Lamiaceae species have shown that 100 µM MeJA markedly induces expression of PvPAL, PvC4H, Pv4CL1-2, PvHPPR, and PvCYP98A101, leading to substantial RA accumulation in *P. vulgaris* hairy roots (Xiao *et al.*, 2009; Grzegorzczak-Karolak *et al.*, 2024).

The total polyphenols increased approximately by 30% after MeJA elicitation in *Salvia viridis* and qRT-PCR analysis showed a clear and significant increase in the expression level of early pathway genes within 24 h (Grzegorzczak-Karolak *et al.*, 2024). In *Mentha spicata*, enhancements of similar magnitudes (up to 11-fold increase of RA) have been reported with MeJA and correlated with strong induction of PAL, C4H, 4CL, and HPPR transcripts (Zhang *et al.*, 2015). Together these findings imply that elicitor-mediated metabolic reprogramming can lead to 2-5-fold or even higher phenolic production, depending on the type and concentration of the elicitor, as well as timing and responsiveness that is most likely species-dependent (Zhao *et al.*, 2005; Kumar *et al.*, 2014).

Despite encouraging data, the optimal elicitor type, dosage, and application window for maximizing RA yield in *P. vulgaris* remains insufficiently defined. Thus, systematic optimization could substantially improve the biosynthetic performance of *P. vulgaris* hairy roots. The present study aims to investigate elicitor-specific impacts on phenylpropanoid biosynthesis and enzyme gene regulation, thereby offering insights to inform future metabolic engineering and commercial-scale bioproduction strategies.

Materials and methods

Seed sterilization and germination

Seeds of *Prunella vulgaris* were obtained from National Institute of Horticultural and Herbal Science, RDA, Eumseong, Korea. Seeds were soaked for 30 s in 70% (v/v) ethanol followed by 10 min incubation in 2% (v/v) NaOCl. The seeds were washed 5-6 times using sterilized distilled water. Mature seeds were surface sterilized and germinated on half-strength Murashige and Skoog (MS) medium containing 3% sucrose. Following surface-sterilization, the seeds were sown in the plates and incubated in a growth chamber under a 16-/8-h light/dark regimen for 10 days, after which the seedlings were germinated. The seedlings were transferred to plant culture bottles containing the same medium and grown for one month.

Establishment of hairy root cultures

Leaves were sliced into segments of approximately 0.7×0.7 cm from 1-month-old seedlings. Pieces of leaves were immersed in bacterial suspension of *A. rhizogenes* (OD₆₀₀=0.6) for 15 min. The suspension was removed with sterilised tissue paper and transferred to plates containing HR induction media agar (half-strength Schenk and Hildebrandt medium supplemented with 3% sucrose and 0.8% w/v agar). The plates were incubated on the medium for 2 days under dark conditions at 25 °C. After 2 days of incubation, the explants were rinsed 6 times with sterile distilled water, and explants were transferred to HR induction media supplemented with 500 mg/L cefotaxime. HRs induced from the tissue after 4 weeks were excised and transferred to new plates containing HR induction media. The explants and HRs were transferred onto new media and subcultured in 2-week intervals. After 6 weeks

of subculture, HRs were transferred and grown in 30 mL of liquid HR induction media at 25 °C with shaking at 110 rpm in the dark. After 10 days of incubation, the HRs were collected from the culture and weighed up to approximately 4 g. The weighed HRs was transferred to another flask containing liquid HR induction media.

Treatment of abiotic elicitor in hairy root

One week after subculture, the hairy roots of *P. vulgaris* were treated with 0, 1, 5, 10, and 20 mg/L silver nitrate (AgNO₃), 0, 0.1, 0.5, and 1 mM copper chloride (CuCl₂), 0, 0.1, 0.5, and 1 mM salicylic acid (SA), and 0, 1, 5, 10, 50, and 100 mM hydrogen peroxide (H₂O₂). Hairy roots treated as described above were harvested at 3 d after treatment, frozen in liquid nitrogen, and stored at -80 °C until analysis.

Analysis of phenylpropanoids using HPLC

The analysis of individual phenylpropanoids was carried out on *P. vulgaris* HRs according to the method of Park *et al.* (2022). 100 mg *P. vulgaris* powder and 2 mL of 80% methanol in a tube was sonicated for 1 h, and then the supernatant could be obtained by centrifuging the tube for 10 min at 4000 rpm. The supernatant was filtered through 0.45 µm PTFE syringe filter and analyzed by HPLC using an OptimaPak C18 column (RStech). The phenylpropanoids were analysed using HPLC conditions already described in a former study (Park *et al.*, 2021). Standards of the phenylpropanoids (≥95%) were obtained from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA).

Results

The phenylpropanoid production in *P. vulgaris* hairy roots was affected by the treatment of silver nitrate (AgNO₃), and the metabolite accumulation was concentration-dependent with maximum accumulation of metabolites reached at 3 days of culture. At lower concentrations, AgNO₃ significantly increased the total phenolic content (47.01±1.87 µg/g) compared to the control (43.46±0.69 µg/g), with an approximate 8% increase. The latter also showed marked elevation in rutin (36.38±0.93 µg/g) vs. in control (26.88±0.01 µg/g) and is indicative of the stimulation of the flavonoid biosynthetic pathway. On the other hand, benzoic acid had moderate increases occurred (1.74±0.15 µg/g) at 10 mg/L while caffeic acid and quercetin contents were decreased in general by increasing AgNO₃ in culture medium with significant suppression at 20 mg/L, the quercetin was decreased in the 20 mg/L (1.11±0.52 µg/g) compared to control (2.77±0.44 µg/g) (Table 1).

Total phenolic data indicated a higher yield of phenolic compounds (64.83±2.62 µg/g) over the untreated control (51.15±1.46 µg/g) after the application of 0.5 mM CuCl₂, which was 27% higher and characterized as a strong elicitor response in this study after 3 days culture time. The increase was primarily due to a notable increase in rutin content (45.46±2.11 µg/g) against (32.27±0.93 µg/g) in the control group. Caffeic acid also revealed the peak at 0.5 mM

(13.18±0.04 µg/g), on the other hand, benzoic acid content showed a little increase with the highest concentration 1 mM (1.81±0.14 µg/g). By contrast, quercetin content decreased gradually by increasing concentration of CuCl₂ from control (5.16±0.07 µg/g) to 1 mM (3.62±0.07 µg/g), indicating that the copper-induced stress affects biosynthesis of flavanols (Table 2).

The total phenolic content of the control group was (46.7±1.38 µg/g), but at 0.5 mM almost doubled (95.46±12.63 µg/g) and remained comparably high (94.06±12.2 µg/g) at 1 mM SA, indicating a very strong effect of SA in stimulating the phenylpropanoid pathway. The most significant increase was observed in caffeic acid content, from control (11.17±0.57 µg/g) to (32.15±5.67 µg/g) at 0.5 mM nearly a threefold increase. Rutin content also increased significantly after SA treatment and was 59.64±6.71 µg/g at 0.5 mM and peaked at 60.31±7.34 µg/g at 1 mM, more than double control (28.52±0.01 µg/g) and further emphasizing the activation of flavonoid glycoside biosynthetic pathway. In addition, benzoic acid augmented with higher concentrations of SA, from 1.19±0.04 µg/g in the control to 2.4±0.2 µg/g at 0.5 mM. In contrast, quercetin was totally inhibited with increasing concentration of SA, and near undetectable at 1mM (5.81±0.77 µg/g; control vs 0.17±0.1 µg/g); thus, strongly suggestive of metabolite suppression or precursor channeling towards other phenylpropanoid derivatives at this concentration of SA (Table 3).

The total phenolic content of the samples was positively correlated with increased concentrations of H₂O₂ 200 mM, reaching a maximum at 100mM with values of 48.93±1.21 µg/g, a level slightly higher than that of

the control (45.79±0.58 µg/g), suggests a very low-level elicitor effect of the oxidative stress. The greatest rate of caffeic acid accumulation (13.05±0.99 µg/g) relative to the control (11.71±0.33 µg/g) was observed at the 5 mM treatment level indicating moderate stimulation of phenylpropanoid biosynthesis at low to mid-range levels of H₂O₂. More interestingly, levels of benzoic acid were gradually increased depending on the concentration and up to 1.6±0.16 µg/g at 100 mM, suggesting the involvement of oxidative stress which may be upregulation of benzoate biosynthesis. We found also, a dose-dependent enhancement of Rutin, the major flavonoid (27.69±0.09 µg/g (control) to 32.27±0.16 µg/g in 100 mM) indicating a further effect of H₂O₂ on flavonoid glycoside pathways. So, although quercetin content slightly varied with treatments, it was not in a particular trend but peaked at 10 mM (5.71±0.24 µg/g) and then decreased with increasing concentrations (Table 4).

Discussion

Research in plant biotechnological application delineates the successful production of secondary metabolites, as phenolic compounds, in plant cell and organ cultures by using abiotic elicitors. In the current study, the impact of four abiotic elicitors (AgNO₃, CuCl₂, SA, and H₂O₂) was examined on the production of major phenylpropanoids (caffeic acid, benzoic acid, rutin, and quercetin) in hairy root cultures of *P. vulgaris*. On the other hand, use of different elicitors led to different modulation of the metabolite profile, which highlighted the complex interplay of signaling cascades and biosynthesis regulating phenolic metabolism in *P. vulgaris* (Georgiev *et al.*, 2007; Goel *et al.*, 2011; Sharma *et al.*, 2013; Mirmazloum *et al.*, 2024; Jakovljević *et al.*, 2025).

Table 1: Effect of Silver nitrate treatment at different concentration on phenylpropanoid production in hairy roots of *P. vulgaris* after 3 days in culture

AgNO ₃ (mg/L)	0	1	5	10	20
Caffeic acid	12.28±0.22 ^a	6.44±0.69 ^c	8.32±0.67 ^b	6.71±0.64 ^c	5.79±1.01 ^c
Benzoic acid	1.43±0.03 ^{bc}	1.63±0.03 ^{ab}	1.38±0.08 ^c	1.74±0.15 ^a	1.52±0.2 ^{abc}
Rutin	26.88±0.01 ^b	36.38±0.93 ^a	27.53±0.78 ^b	30.12±4.45 ^b	27.14±0.23 ^b
Quercetin	2.77±0.44 ^a	2.55±0.22 ^a	1.48±0.17 ^b	1.41±0.25	1.11±0.52 ^b
Total	43.46±0.69 ^{ab}	47.01±1.87 ^a	38.71±1.7 ^{bc}	39.98±5.49 ^{bc}	35.57±1.96 ^c

Table 2: Effect of Copper chloride treatment at different concentrations on phenylpropanoid production in hairy roots of *P. vulgaris* after 3 days in culture

CuCl ₂ (mM)	0	0.1	0.5	1
Caffeic acid	12.23±0.4 ^a	10.31±1.13 ^b	13.18±0.04 ^a	12.42±0.01 ^a
Benzoic acid	1.49±0.06 ^b	1.52±0.17 ^b	1.47±0.08 ^b	1.81±0.14 ^a
Rutin	32.27±0.93 ^b	31.89±0.86 ^b	45.46±2.11 ^a	28.08±1.56 ^c
Quercetin	5.16±0.07 ^a	4.86±0.88 ^a	4.72±0.39 ^a	3.62±0.07 ^b
Total	51.15±1.46 ^b	48.57±3.04 ^{bc}	64.83±2.62 ^a	45.93±1.77 ^c

Table 3: Effect of salicylic acid treatment at different concentrations on phenylpropanoid production in hairy roots of *P. vulgaris* after 3 days of culture

SA (mM)	0	0.1	0.5	1
Caffeic acid	11.17±0.57 ^b	24.56±2.78 ^a	32.15±5.67 ^a	31.27±4.62 ^a
Benzoic acid	1.19±0.04 ^b	0.9±0.38 ^b	2.4±0.2 ^a	2.32±0.14 ^a
Rutin	28.52±0.01 ^c	39.34±2.5 ^b	59.64±6.71 ^a	60.31±7.34 ^a
Quercetin	5.81±0.77 ^a	4.75±0.47 ^b	1.27±0.04 ^c	0.17±0.1 ^d
Total	46.7±1.38 ^c	69.55±6.12 ^b	95.46±12.63 ^a	94.06±12.2 ^a

Table 4: Effect of hydrogen peroxide treatment at different concentrations on phenylpropanoid production in hairy roots of *P. vulgaris* after 3 days in culture

H ₂ O ₂ (mM)	0	1	5	10	50	100
Caffeic acid	11.71±0.33 ^{bc}	11.35±0.04 ^c	13.05±0.99 ^a	12.88±0.74 ^{ab}	10.64±0.93 ^c	10.78±0.5 ^c
Benzoic acid	1.32±0.05 ^c	1.39±0.01 ^{bc}	1.26±0.06 ^c	1.47±0.01 ^{ab}	1.52±0.07 ^{ab}	1.6±0.16 ^a
Rutin	27.69±0.09 ^d	28.96±0.16 ^c	28.69±0.23 ^c	28.85±0.16 ^c	30.12±0.08 ^b	32.27±0.16 ^a
Quercetin	5.08±0.11 ^{ab}	4.49±0.13 ^{bc}	4.55±0.54 ^{bc}	5.71±0.24 ^a	3.7±0.54 ^d	4.27±0.4 ^{cd}
Total	45.79±0.58 ^b	46.2±0.33 ^{ab}	47.55±1.82 ^{ab}	48.91±1.14 ^a	45.98±1.61 ^b	48.93±1.21 ^a

The ethylene inhibitor AgNO₃ triggered a dose-dependent response on phenolic biosynthesis. It was also found that at low concentration (1 mg/L) a moderate increase (~8%) in total phenolics were observed and most of these increases were due to a significant increase in rutin. This indicates probably via upregulation of chalcone synthase (CHS) and flavonol synthase (FLS) activation of the flavonoid glycoside's pathways are activated, as reported previously in *Salvia miltiorrhiza* and *Eschscholtzia californica* (Cho *et al.*, 2008; Di *et al.*, 2013). On the other hand, caffeic acid and quercetin accumulation were blocked by high amounts of AgNO₃, suggesting that cytotoxic stress was likely leading to inhibition of critical enzyme activity, focusing redirection of metabolic flux away from phenolics synthesis (consistent with previous reports of metal-induced phytotoxicity (Li *et al.*, 2005, 2015).

Cu²⁺ ions are omnipresent in biology: indispensable micronutrients, and redox-active elicitors of plant immunity. The high levels of total phenolics (~27% increase) due to the treatment of 0.5 mM CuCl₂ in this study were mainly attributed to high levels of rutin and caffeic acid as biochemical markers. This response is due to the induction and activation of enzymes involved in the phenylpropanoid pathway regulated by ROS such as phenylalanine ammonia-lyase (PAL) (Heredia & Cisneros-Zevallos, 2009; Mirjalili *et al.*, 2009). The quercetin suppression at higher concentration of CuCl₂ is selective and like those reported in *Scutellaria* spp. where oxidative stress or substrate competition specifically impairs flavonol biosynthesis pathways (Zhao *et al.*, 2005; Grzegorzczak-Karolak *et al.*, 2024). This biphasic response highlights the balance of elicitation toxicity, an often-observed trait of metal-based elicitors (Jiang & Joyce, 2003; Srivastava & Srivastava, 2007; Sharma *et al.*, 2013).

SA provided the most powerful effect on phenolic biosynthesis among the elicitors tested. Phenolic compound levels were almost doubled with 0.5 and 1 mM treatments compared to control where substantial increases were also observed for rutin and caffeic acid. These findings are in agreement with those reported for *Ocimum basilicum*, *Eclipta alba* and *Salvia miltiorrhiza* that SA considerably stimulated the genes, which are involved in PAL, C4H and 4CL biosynthetic pathway leading to increase the bio-synthesis of flavonoids and phenolic acids (Tada *et al.*, 1996; Kang *et al.*, 2004; Dong *et al.*, 2010). The lower quercetin level upon SA treatment suggests an energetic re-routing toward metabolic channels preferentially yielding glycosylated quercetin derivatives, e.g. rutina (Khalili *et al.*, 2009) or differential regulation of flavonol synthase and

glycosyltransferase enzymes. Additionally, in the case of *Silybum marianum* and *Artemisia annua*, similar reports support the idea of selective modulation (Kazemi *et al.*, 2010; Yin *et al.*, 2012; Kumar *et al.*, 2014).

H₂O₂ acted as a signal transducer and was able to induce a much more subdued but still significant dose-dependent increase in total phenolics, peaking at the 100 mM level. Rutin and benzoic acid, which were among the most responsive, were consistent with previous studies showing the inducible type of redox-sensitive gene activation by H₂O₂ (Huang *et al.*, 2008). However, unlike that of SA or CuCl₂, the quercetin response to H₂O₂ was irregular and stage-specific (Xu *et al.*, 1999; Xiao *et al.*, 2009; Huang *et al.*, 2015), implying that feedback regulation or competitive flux at common biosynthetic junctions (capsicum, *Taxus*) could be involved.

The elicitor SA was the best among those tested and CuCl₂ was on an intermediate level of effectivity in the phenolic biosynthesis induction in *P. vulgaris* hairy roots. Caffeic acid and rutin levels were sharply elevated by either elicitor, suggesting activation of upstream nodes of the phenylpropanoid pathway. H₂O₂ & AgNO₃ were less efficacious, and high concentrations of AgNO₃ may lead to increased inhibition possibly due to oxidative stress and/or metabolic disturbance. Quercetin was only expressed at low levels of H₂O₂ across treatments, suggesting a putative metabolic bottleneck or rerouting event when this compound was needed to combat the elicitor-triggered stress (Shanks & Morgan, 1999; Petersen & Simmonds, 2003; Jakovljević *et al.*, 2025).

This study sheds light on the regulation of phenolic biosynthesis by elicitors and its dependent concentration in hairy roots of *P. vulgaris*. The significant impact of SA and CuCl₂ suggested that they might act as an important modifier of high-value secondary metabolites including caffeic acid and rutin. These results reveal the complexity of regulatory networks controlling phenylpropanoid biosynthesis with the balancing act between quercetin and rutin pathways as an example. Future studies should employ gene expression analyses (e.g., PAL, CHS, FLS, glycosyltransferases) and metabolic flux profiling to uncover the biological basis of these different responses (Grzegorzczak *et al.*, 2006; Xiao *et al.*, 2009; Di *et al.*, 2013; Zhang *et al.*, 2015). This information seeks to explain the reason why hairy root cultures may represent a resourceful and suitable platform to produce diverse chemical space of phytochemicals (Georgiev *et al.*, 2006; Ludwig-Müller *et al.*, 2014; Pandey *et al.*, 2014; Ru *et al.*, 2016; Mirmazloum *et al.*, 2024).

Conclusion

Significant variation occurs in the secondary metabolites, synthesized via the phenylpropanoid biosynthetic pathway, in response to abiotic elicitation in hairy root cultures of *P. vulgaris*. Among the elicitors, salicylic acid (SA) was found to be the best elicitor that enhanced the accumulation of major phenolics caffeic acid and rutin with proven bioactivity. Other elicitors were copper chloride (CuCl₂) (with good elicitation capacity), reaching 0.5 mM, acting predominately through rutin accumulation. Silver nitrate (AgNO₃) on the other hand was a more selective compound that could promote total phenolics at lower doses, but results showed that quercetin and caffeic acid were reduced at higher concentrations. The gradual, low-order increase in contents of benzoic acid and rutin upon exposure to H₂O₂ reflected H₂O₂-mediated stimulation of specific biosynthetic pathways activated in response to oxidative stress that acts as a mild elicitor. In conclusion, these findings uncover a potential explanation on the elicitor strategies to induce an increased accumulation of specific phenylpropanoid in *P. vulgaris* hairy roots that might open new avenues for the enhancement of these natural drugs production process.

Author contributions

Kihyun Kim: Methodology, Data curation, Formal analysis, Writing – original draft. Bao Van Nguyen: Formal analysis, Validation. Md Romij Uddin: Formal analysis, Validation. Chanung Park: Formal analysis, Validation. Ramaraj Sathasivam: Conceptualization, Investigation, Validation, Visualization, Funding acquisition, Writing – review & editing. Sang Un Park: Conceptualization, Investigation, Validation, Visualization, Funding acquisition, Supervision, Writing – review & editing. All authors approved the final manuscript.

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