Vol. 21, No. 2(2022) Bio2819
Revista Mexicana de Ingeniería Química

Methyl jasmonate enhances the anti-inflammatory effects of the adventitious roots in *Abeliophyllum distichum* by increasing the production of polyphenolic compounds

El jasmonato de metilo potencia los efectos antiinflamatorios de las raíces adventicias de *Abeliophyllum distichum* al aumentar la producción de compuestos polifenólicos

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Received: May 10, 2022; Accepted: July 7, 2022

Abstract

Abeliophyllum distichum has been demonstrated to possess anticancer, anti-inflammatory, anti-osteoporotic, and anti-obesity effects. However, there is a limitation to using this shrub as a beneficial material, because this shrub had been listed as endangered. Thus, we established an adventitious root culture of *A. distichum* (AdAR) to overcome this limitation. Solvent-solvent partition fractions from methanol extract of methyl jasmonate (MeJA)-elicited AdAR were used, and the ethyl acetate fraction of MeJA-treated AdAR (EtOAc/MeJA) exhibited strong anti-inflammatory effects in lipopolysaccharide (LPS)-treated RAW264.7 cells. EtOAc/MeJA downregulated the transcription of pro-inflammatory genes and mediators by inhibiting the LPS-activated MEK/ERK signaling pathway. In addition, the results of the phytochemical analysis suggested that MeJA induced the accumulation of polyphenolic compounds, including p-coumaric and ferulic acids, by inducing phenylpropanoid biosynthetic genes in AdAR. These results suggested that AdAR is a viable source for overcoming limitations in the industrial use of *A. distichum*.

Keywords: *Abeliophyllum distichum*, Adventitious root, Anti-inflammatory effect, Methyl jasmonate, Polyphenolic compound.

Resumen

Se ha demostrado que *Abeliophyllum distichum* posee efectos anticancerígenos, antiinflamatorios, antiosteoporóticos y contra la obesidad. Sin embargo, existe una limitación en el uso de este arbusto como material beneficioso, ya que este arbusto ha sido catalogado como en peligro de extinción. Por lo tanto, establecimos un cultivo de raíces adventicias de *A. distichum* (AdAR) para superar esta limitación. Se usaron fracciones de partición solvente-solvente del extracto de metanol de AdAR provocado por jasmonato de metilo (MeJA), y la fracción de acetato de etilo de AdAR tratado con MeJA (EtOAc/MeJA) exhibió fuertes efectos antiinflamatorios en RAW264 tratado con lipopolisacárido (LPS). 7 celdas. EtOAc/MeJA reguló a la baja la transcripción de genes y mediadores proinflamatorios al inhibir la vía de señalización MEK/ERK activada por LPS. Además, los resultados del análisis fitoquímico sugirieron que MeJA indujo la acumulación de compuestos polifenólicos, incluidos los ácidos p-cumárico y ferúlico, al inducir genes biosintéticos de fenilpropanoide en AdAR. Estos resultados sugirieron que AdAR es una fuente viable para superar las limitaciones en el uso industrial de *A. distichum*.

Palabras clave: *Abeliophyllum distichum*, Raíz adventicia, Efecto antiinflamatorio, Jasmonato de metilo, Compuesto polifenólico.

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https://doi.org/10.24275/rmiq/Bio2819
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

Endangered until a few years ago, the monotypic genus *Abeliophyllum distichum* Nakai is endemic to Korea. It has beautiful white flowers and indehiscent samara-type fruit; because of these horticultural values, it has been used as a landscape plant (Oh *et al*., 2003; Ghimire and Heo, 2014). Various studies have reported that this shrub has anticancer, anti-inflammatory, antiosteoporotic, antiobesity, and anti-melanoma effects (Choi *et al*., 2017; Eom *et al*., 2020; Lee *et al*., 2020; Yoo *et al*., 2020 and 2021), indicating its potential as a crude drug and dietary health supplement. *A. distichum* was classified as endangered until 2017 owing to reductions in its population and the land area it occupied (Lee *et al*., 2022); this became a major limiting factor in its application in the food, cosmetic and pharmaceutical industries.

Plant cell suspension culture is an attractive strategy in the conservation of endangered plants (Sengar *et al*., 2010; López-Ramírez *et al*., 2021; Wu *et al*., 2021; Albarrán-Mondragón *et al*., 2022) and has been suggested as a reliable system for the production of bioactive compounds such as paclitaxel, resveratrol, artemisinin, ginsenosides, and ajmalicine (Yue *et al*., 2016). This indicates that the production of bioactive compounds using a plant cell suspension culture system is a suitable alternative to traditional cultivation. Among cell culture systems, adventitious roots culture has been reported as an effective technique to produce the secondary metabolites that usually accumulated in roots (Deepthi and Satheeshkumar, 2017), and provides the possibility of year-round production of biomass (Khanam *et al*., 2022). The application of this system in the production of commercially important metabolites is, however, restricted owing to low product yields. Therefore, alternative strategies, such as genetic modification, biotransformation, and mutagenesis, have been introduced to overcome this limitation (Kreis, 2019).

The accumulation of secondary metabolites from plants requires that the plant be subjected to stressors such as elicitors or signal molecules. Elicitation is one of the most effective and widely used biotechnological tools for enhancing the biosynthesis and accumulation of secondary metabolites in plant cell suspension cultures (Ochoa-Villarreal *et al*., 2016; Lee *et al*., 2018). Intercellular signaling molecules, such as methyl jasmonate (MeJA), are elicitors that switch on plant defense systems and enhance the biosynthesis of secondary metabolites (Halder *et al*., 2019). MeJA reportedly induces the accumulation of polyphenols, triterpenoids, and alkaloids by enhancing the expressions of related genes in the cell suspension culture systems of various plants (Lee *et al*., 2018; Li *et al*., 2020; Lu and Hyun, 2021; Qin *et al*., 2022). Thus, we thought that plant cell suspension cultures combined with elicitors may enhance the large-scale production of specific metabolites in the endangered shrub *A. distichum*.

In this study, we aimed to investigate whether the *A. distichum* adventitious root (AdAR) is beneficial in the food and pharmaceutical industries. We induced adventitious root from *A. distichum* embryo, and MeJA was applied to enhance the anti-inflammatory effect of AdAR. We also assessed the effects of MeJA on the production of polyphenols and the transcription levels of the genes involved in the phenylpropanoid pathway.

2 Materials and methods

2.1 Induction of adventitious root formation and treatment with MeJA

The adventitious roots were induced from the embryo in newly harvested seeds of *A. distichum* grown in the forests of Chungbuk National University. Gamborg B5 (B5) medium containing 3% sucrose, 3 mg/L indole-3-butyric acid (IBA), and 0.8% plant agar was used as the induction medium. The voucher specimen (CBNU-IP1) was deposited in the department of industrial plant science and Technology, Chungbuk National University. AdAR was transferred to the induction medium devoid of plant agar, and cultured on a rotary shaker at 120 rpm and 24°C under dark conditions for one year with a subculture cycle of three weeks.

For elicitor treatment, two-week-old adventitious roots were treated with 100 μM MeJA or solvent (mock control) and further incubated for six days.

2.2 Extract preparation

5 g of freeze-dried AdAR (extraction yield = 39.7%) or roots (extraction yield = 33.9%) were soaked in methanol at room temperature for 24 h, subjected to sonication (thrice, 10 min each time), and filtered using Whatman filter paper. After the evaporation of

MeOH, solvent partitioning (ethyl acetate, n-butanol, and aqueous fraction) was performed according to the method described by Jin *et al*. (2020).

2.3 Determination of cell viability and nitric oxide (NO) production

RAW264.7 cells were cultured in DMEM medium and supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in an incubator containing humidified CO_2 (5%) at 37 °C. To determine cell viability and NO production, RAW264.7 cells (1.5×10^5 cells/mL in 96-well plates) were incubated with each extract or fraction in the presence of 1 μg/mL lipopolysaccharide (LPS) for 24 h. The cytotoxicity of each extract and fraction in the RAW264.7 cells was analyzed using the tetrazolium-based MTT assay, as described by Yoo *et al*. (2021). The formazan crystals were dissolved in DMSO, and the optical density was measured at 520 nm. In addition, NO production in LPS-treated RAW264.7 cells was assessed using Griess reagent (Biomax Co. Ltd., Seoul, Korea), according to the manufacturer's instructions.

2.4 Immunoblotting and qRT-PCR analysis

Proteins extracted from the RAW264.7 cells using RNA immunoprecipitation analysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxylcholate, 1 mM EDTA, and 10 mM NaF) were quantified using the Pierce^{TM} BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). The proteins were then separated using SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% nonfat dried milk solution, the membranes were incubated with specific antibodies (MEK1/2, p-MEK1/2, ERK1/2, p-ERK1/2, and GAPDH). The signal was detected and visualized using a chemiluminescence system (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

Total RNA from RAW 264.7 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. cDNA was reverse transcribed from the extracted RNA, and qRT-PCR was performed using specific primer pairs (Table 1) for pro-inflammatory genes and mediators following the protocol outlined by Kim *et al*. (2022). The expression values of all genes were normalized to those of actin.

Table 1. Primer sequences for qRT-PCR analysis.

Primer name	Sequence (5'-3')
COX2	F-CCTCTGCGATGCTCTTCC
	R-TCACACTTATACTGGTCAAA
iNOS	F-TCCTACACCACACCAAAC
	R-CTCCAATCTCTGCCTATCC
IL-6	F-CCACTTCACAAGGTCGGAGGCTTA
	R-GTGCATCATCGCTGTTCATACAATC
IL-1α	F-CGTCAGGCAGAAGTTTGTCA
	R-TTAGAGTCGTCTCCTCCCGA
IL-1β	F-TGTGAAATGCCACCTTTTGA
	R-TGAGTGATACTGCCTGCCTG
β-actin	F-CCCATCTCCTAAGAGGAGGATG
	R-AGGGAGACCAAAGCCTTCAT
PAL	F-TCTCTGGTGGACGGAACCCA
	R-GTCGGCTGCTTCAGCTGTCT
C4H	F-TGGCTCCAAGTTGGCGATGA
	R-TTTGCAAGGTCGGGCGATGA
4CL	F-AATCCGGCTCGTCCTTTCCC
	R-GAGCCAGCCGTCGACATCAA
CHI	F-TCGCCGACTGTAACGCAACT
	R-TGCCCTCGATCTCCAAACCC
EF	F-GCTGCCAGTTTCACCTCCCA
	R-AGCTCCTTACCAGATCGCCTGT

2.5 Determination of polyphenol content and transcription levels of the genes related to the phenylpropanoid biosynthesis pathway

The total phenol content (TPC) and total flavonoid content (TFC) in the ethyl acetate (EtOAc) fraction were analyzed using the Follin-Ciocalteu and colorimetric methods, respectively, as described by Kim *et al*. (2022). The TPC and TFC were expressed in microgram gallic acid equivalents per milligram of extract (μg GAE/mg extract) and microgram quercetin equivalents per milligram of extract (μg QE/mg extract), respectively.

The contents of p-coumaric acid and ferulic acid were analyzed using high-performance liquid chromatography with a diode array detector (Agilent 1260, Agilent Technologies, Waldbronn, Germany) and Poroshell 120 EC-18 column (4 μm, 4.6 × 150 mm, Agilent Technologies, Waldbronn, Germany), as described by Ju *et al*. (2021a). The mobile phases consisted of water containing 0.1% trifluoroacetic acid (mobile phase A) and acetonitrile containing 0.1% trifluoroacetic acid (mobile phase B). The following gradient elution was performed: 10% B in 0-0.01 min, 10%-40% B in 0.01-28 min, 40%-60% B in 28-39 min, 60%-90% B in 39-50 min, and holding at 90% B for 5 min. The injection volume was 10 μL (10 mg/mL in MeOH). The contents of all these

compounds were analyzed by comparing the retention times and UV spectral data. The concentration was calculated by comparing the peak areas of the samples with the calibration curve of the standards including p-coumaric acid (R^2 = 0.9651, y = 55.059x - 8.1059) and ferulic acid (R^2 = 0.9991, y = 23.686x + 9.6383).

To analyze the transcription levels of the genes related to polyphenolic compound biosynthesis, total RNA was extracted from AdAR, and cDNA synthesis and qRT-PCR were performed using gene-specific primers (Table 1). The housekeeping gene elongation factor 1-alpha (EF) was used as the reference gene for normalization (Choi *et al*., 2018).

2.6 Statistical analysis

The results are representative of three biological replicates and have been expressed as mean ± SE. The significance of the differences between each group was identified using Duncan's multiple range test ($p < 0.05$) and Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3 Results and discussion

3.1 Induction of A. distichum adventitious root

As described above, the establishment of *A. distichum* cell culture system is a key factor supporting many pharmaceutical and industrial outcomes of *A. distichum*. There exist various plant culture systems-protoplast, callus, somatic embryo, etc.; however, the adventitious root culture system is the most preferred owing to a high rate of proliferation, huge potential to produce bioactive compounds, and easy scale-up (Murthy *et al*., 2008; Wang *et al*., 2013; Hussain *et al*., 2022). In this study, AdAR was induced in B5 medium containing IBA, which is known to be an effective hormone for the root induction of *A. distichum* (Moon *et al*., 1999). As shown in Fig. S1a (Supplementary Material), the high percentage of adventitious root induction (67% and 69%) was recorded in the medium supplemented with 3 mg/L and 5 mg/L IBA, respectively. However, the high concentration of IBA (5 mg/L) reduced the elongation of adventitious roots (Fig. S1b). Similar to our findings, high concentrations of auxin significantly reduced the root elongation of various plants via the concentration-dependent role of auxin in apoplastic

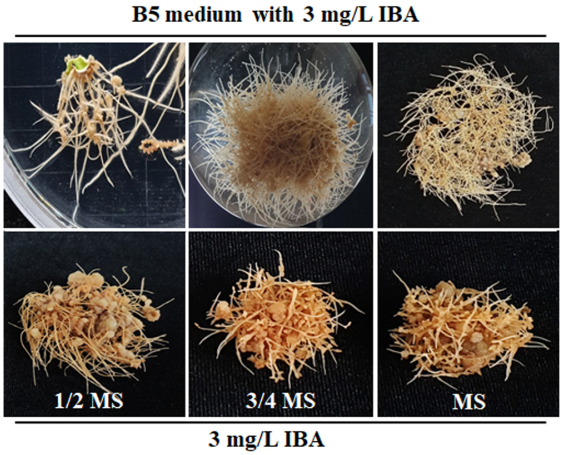

Fig. 1. Establishment of in vitro adventitious root cultures of *A. distichum* (AdAR).

pH homeostasis (Barbez *et al*., 2017; Alarcón *et al*., 2019). In addition, adventitious roots were cultured in Murashige and Skoog (MS) or B5 liquid media fortified with 3 mg/L IBA. As shown in Fig. 1, high concentrations of MS media induced callus from adventitious roots, indicating that B5 medium fortified with 3 mg/L IBA should be useful for the large-scale production of *A. distichum* adventitious root.

3.2 Effect of AdAR on NO production in LPS-treated RAW264.7 cells

The anti-inflammatory effect has been considered a major pharmacological function of the *A. distichum* extract (Choi *et al*., 2017; Ju *et al*., 2021b; Yoo *et al*., 2021). Comparative analyses of the anti-inflammatory effects of extracts from various parts of *A. distichum* (leaves, fruit, and branches) have demonstrated that the extract from *A. distichum* leaves exerts strong anti-inflammatory effects on LPS-treated RAW264.7 cells (Yoo *et al*., 2021). In fact, 50 μg/mL of the leaf extract inhibited LPS-induced NO production to a level greater than 50% of that generated by the mock control (Yoo *et al*., 2021). LPS-induced NO production was suppressed by 28.5% (inhibition of LPS-induced NO production from 11.06 μM to 7.91 μM) and 21.5% (inhibition of LPS-induced NO production from 11.06 μM to 8.69 μM) in RAW264.7 cells incubated with root (50 μg/mL) and AdAR (50 μg/mL) extracts, respectively, as compared with that in the mock control (Fig. 2a). Of note, root (50 μg/mL) and AdAR (50 μg/mL) extracts did not affect cell viability regardless of the presence of LPS for 24 h (Fig. 2c), indicating that the inhibition of NO

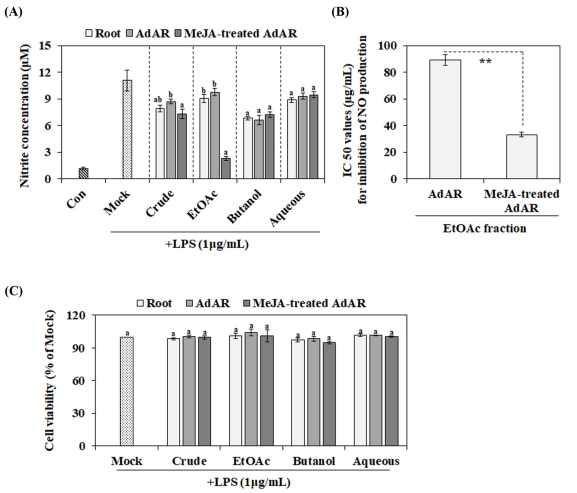

Fig. 2. Anti-inflammatory effects of the adventitious roots of *Abeliophyllum distichum*. (a) Inhibitory effects of solvent fractions of the root, AdAR, and MeJA-elicited AdAR extracts on NO production in LPS-treated RAW264.7 cells. (b) IC50 values of LPS-induced NO production in RAW264.7 cells of EtOAc fractions of AdAR, and MeJA-elicited AdAR extracts. (c) Cytotoxic effects of solvent fractions of the root, AdAR, and MeJA-elicited AdAR extracts on LPS-stimulated RAW264.7 cells. Means (± SE, three independent experiments) with different letters are significantly different, according to Duncan's multiple range test.

production in response to these extracts was not a result of cytotoxicity.

To enhance the anti-inflammatory effect of AdAR, 100 μM and 200 μM MeJA were added to the AdAR cultures. Six days after the treatment of each concentration of MeJA, the growth rate was determined. As shown in Fig. S2, the treatment with 200 μM MeJA resulted in the inhibition of root growth. Thus, 100 μM MeJA was used to improve the anti-inflammatory effect of AdAR. Treatment with the MeJA-treated AdAR culture significantly inhibited NO production (33.9%, inhibition of LPS-induced NO production from 11.06 μM to 7.31 μM) as compared with AdAR alone (Fig. 2a). Solvent partitioning of each extract was performed, after which the anti-inflammatory effect of each fraction was evaluated. As shown in Fig. 2a, the anti-inflammatory effects of the BuOH fraction obtained from the root or AdAR extract were higher than those of the other fractions, whereas the EtOAc fraction of MeJA-treated AdAR (EtOAc/MeJA) strongly inhibited LPS-induced NO production with respective IC50 values of 33.2 μg/mL (Fig. 2b). In line with our findings, the BuOH and EtOAc fractions of the aerial part extract of *A. distichum* (MeOH extract) significantly suppressed NO production in LPS-treated RAW264.7 cells (Choi *et al*., 2017). Interestingly, the EtOAc fraction of the aerial part extract of *A. distichum* exhibited cytotoxicity against RAW264.7 cells (Choi *et al*., 2017), whereas EtOAc/MeJA did not exhibit cytotoxic activity (Fig. 2c). This indicates that MeJA-treated AdAR is a potential source for the development of plant-derived drugs in the reduction of pro-inflammatory mediators.

3.3 Effect of EtOAc/MeJA on the mitogen-activated protein kinase (MAPK) signaling pathway

The MAPK signaling pathway is a key intracellular signaling pathway; it plays an essential role in cell proliferation, innate immunity, apoptosis, inflammation, and stress response (Kaminska, 2005). The MAPK signaling pathway triggers the accumulation of pro-inflammatory cytokines in macrophages in response to LPS stimulation (Chang *et al*., 2017). Thus, the MAPK signaling pathway serves as a potential molecular target for the treatment of inflammation-related disorders. In fact, the anti-inflammatory effects of *A. distichum* extracts are mediated by the inhibition of the MEK/ERK signaling pathway (Choi *et al*., 2017; Yoo *et al*., 2021). Based on these findings, we assessed the regulation of EtOAc/MeJA in the LPS-activated MEK/ERK pathway. As shown in Fig. 3a, LPS strongly activated the MEK/ERK pathway, but this activation was inhibited by EtOAc/MeJA in a dose-dependent manner.

The MEK/ERK signaling pathway is linked to the expression and accumulation of pro-inflammatory mediators (Guha and Mackman, 2001). Thus, we hypothesized that the suppression of the MEK/ERK pathway by EtOAc/MeJA leads to the downregulation of pro-inflammatory mediators. To test this hypothesis, we analyzed the transcription levels of pro-inflammatory genes [inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)] and pro-inflammatory mediators [interleukin (IL)-1α, -1β and -6] using qRT-PCR. As shown in Fig. 3b, LPS resulted in a general increase in the transcription levels of pro-inflammatory genes and mediators, and the levels of these LPS-induced genes were significantly reduced upon EtOAc/MeJA treatment. Similarly, the LPS-induced expression of pro-inflammatory mediators

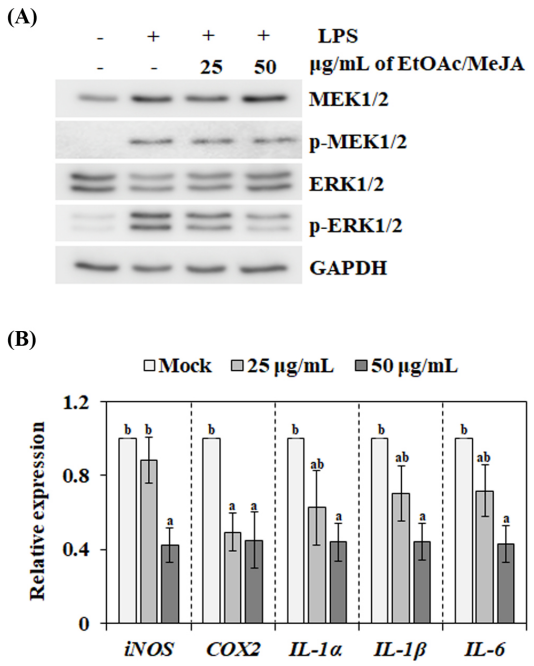

Fig. 3. Anti-inflammatory mechanisms of the ethyl acetate fraction obtained from MeJA-elicited *Abeliophyllum distichum* adventitious root extract (EtOAc/MeJA). (a) Effect of EtOAc/MeJA on the LPS-activated MEK/ERK signaling pathway. (b) The effects of EtOAc/MeJA on LPS-induced pro-inflammatory genes and mediators. Means (± SE, three independent experiments) with different letters are significantly different, according to Duncan's multiple range test.

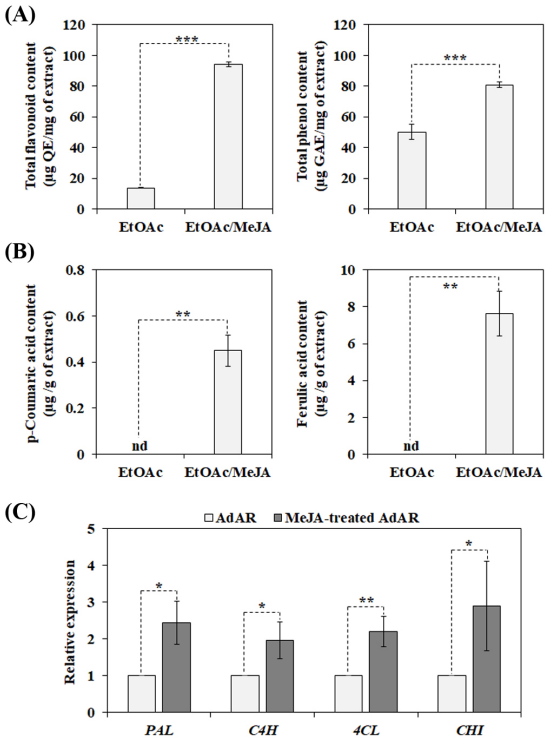

Fig. 4. Effect of MeJA treatment on the production of polyphenolic compounds in *Abeliophyllum distichum* adventitious root (AdAR). (a) Total phenol and total flavonoid contents in the ethyl acetate fraction obtained from MeJA-elicited AdAR. (b) The levels of p-coumaric acid and ferulic acid analyzed using high-performance liquid chromatography. (c) Effect of MeJA elicitation on the phenylpropanoid pathway in AdAR. The data are representative of three independent experiments (mean ± SE). *p < 0.05, **p < 0.01, and ***p < 0.001 as compared with the mock control. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHI, chalcone isomerase.

was significantly downregulated by the leaf and aerial part extracts of *A. distichum* extracts (Choi *et al*., 2017; Yoo *et al*., 2021), indicating that the anti-inflammatory effects of EtOAc/MeJA are mediated by the suppression of pro-inflammatory mediators via the inhibition of the MEK/ERK signaling pathway in LPS-stimulated RAW 264.7 cells.

3.4 Effect of EtOAc/MeJA on the mitogen-activated protein kinase (MAPK) signaling pathway

Phytochemical analyses have identified polyphenolic compounds such as coumaric acid and ferulic acid as biologically active compounds in *A. distichum* (Kim and Lee, 2015; Choi *et al*., 2017; Yoo *et al*., 2021). In addition, positive and strong correlations between the polyphenolic contents of EtOAc fractions and their pharmaceutical activities have been reported in various plant extracts (Kim *et al*., 2014; Tinco-Jayo *et al*., 2021; Loh *et al*., 2022). Therefore, we hypothesized that MeJA improves the production of polyphenolic compounds, resulting in enhanced anti-inflammatory effects of AdAR. We further tested our hypothesis by determining the polyphenolic contents and composition of our extracts. As shown in Fig. 4a, the TPC and TFC in the AdAR increased by approximately 1.6 folds and 6.9 folds upon MeJA treatment, respectively, as compared with that in the mock control. In addition, MeJA treatment resulted in strong induction of p-coumaric acid (0.44 ± 0.06 μg/g of extract) and ferulic acid (7.62 ± 1.2 μg/g

of extract) contents in AdAR; in contrast, these compounds were not detectable in the mock control (Fig. 4b). Furthermore, these compounds exhibited anti-inflammatory effects via the suppression of LPS-induced activation of the MEK/ERK signaling pathway (Zhao *et al*., 2016; Yin *et al*., 2019). The expression of phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), and chalcone isomerase (CHI), which are involved in the phenylpropanoid pathway, were significantly increased by MeJA treatment (Fig. 4c). Likewise, exogenous MeJA enhanced the TPC and TFC and promoted the expression and activity of enzymes associated with the phenylpropanoid pathway in various plants (Cocetta *et al*., 2015; Xing *et al*., 2018; Yi *et al*., 2019; Zhou *et al*., 2021; Tao *et al*., 2022). These findings indicate that the phenylpropanoid pathway induced by MeJA enhances the anti-inflammatory effects of AdAR.

Conclusions

Taken together, we successfully established an adventitious root culture to provide the production of biomass and health-promoting metabolites of *A. distichum*. Transient elicitation of AdAR using MeJA enhanced the anti-inflammatory effects of AdAR by increasing the transcription levels of genes related to the phenylpropanoid biosynthetic pathway and the production of polyphenolic compounds. Our results provide valuable information that will help overcome the existing limitations in terms of the use of *A. distichum* in the industry; moreover, our findings will support the large-scale production of biomass and bioactive compounds from *A. distichum*.

Acknowledgments

This work was supported by the research grant from the National Research Foundation of Korea (NRF-2018R1D1A1B07043720) funded by the Ministry of Science, Korea.

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