



Original Article

Co-cultured adventitious roots of *Echinacea pallida* and *Echinacea purpurea* inhibit lipopolysaccharide-induced inflammation via MAPK pathway in mouse peritoneal macrophages

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ABSTRACT

Objective: In order to elucidate the biological activity of the co-cultured adventitious roots (ARs) of *Echinacea pallida* and *Echinacea purpurea* and provide theoretical basis for its application, and the anti-inflammatory activities and potential mechanisms of co-cultured ARs were studied.

Methods: The experimental materials were obtained by bioreactor co-culture technology and used in the activity research. In this study, mouse macrophages induced by lipopolysaccharide (LPS) were used as *in vitro* model. Different concentrations of AR extract (50–400 g/mL) were used to treat cells. The expression of pro-inflammatory cytokines was determined using enzyme linked immunosorbent assay. The inducible nitric oxide synthase and cyclooxygenase-2 expression, mitogen-activated protein kinase (MAPK) phosphorylation, and the inhibitor of nuclear factor-kappa B- α levels were determined by the Western blot analysis.

Results: In the co-cultured ARs, total flavonoids and total caffeic acid were determined, and the contents of both bioactive compounds were significantly higher than those ARs from the single-species culture. Compared with the control group, the large amount of pro-inflammatory mediators was released after LPS stimulation. However, in the extract groups with different concentrations (25, 50, and 100 g/mL), the production of these pro-inflammatory mediators was inhibited in a dose-dependent manner. Furthermore, the levels of phosphorylation of MAPK proteins, including p-p38, p-c-Jun N-terminal kinase, and p-extracellular regulated protein kinases were significantly ($P < 0.05$) decreased in the extract groups, revealing that the AR extract probably involved in regulating the MAPK signaling pathway.

Conclusion: Collectively, our findings suggested that the co-cultured ARs of *E. pallida* and *E. purpurea* can inhibit production of pro-inflammatory mediators in mouse peritoneal macrophages and possess the anti-inflammatory effect by regulating MAPK signaling pathways.

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1. Introduction

Cellular agriculture science is an interdisciplinary branch combining biology and engineering, and is focused on the production of agriculture products from cell cultures (Stephens et al., 2018). In the early 20th century, cellular agriculture was firstly initiated in the commercial production of insulin and rennet (New Harvest, 2019), and recently has expanded to the large-scale production of valuable secondary metabolites from plants (Wang et al., 2017; He et al., 2018). As the key technology of the cellular agriculture, plant cell or organ culture can conveniently and effectively

produce desired metabolites, thus, this technology has received global attention (Furusaki and Takeda, 2011). However, to date, the utilization of cultured plant cells or organs on the development as well as the production of products are insufficient, which one of the reasons is relatively lack of the evidence that the cultures possess similar bioactive property to their mother plants.

Echinacea pallida (Nutt.) Nutt. and *Echinacea purpurea* (L.) Moench are the most widely used medicinal *Echinacea* species (Gao et al., 2018). Their whole plants contain phenolics, flavonoids (including caffeic acid derivatives), polysaccharides, and other compounds (Pellati et al., 2004). Plants of both *Echinacea* species possess various bioactive properties such as antioxidative, antibacterial, antiviral, and antifungal properties, and they are often used to treat common cold and respiratory and urinary diseases (Barrett, 2003). However, the plant resource of *Echinacea* species cannot

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meet the market demand because of the instability of bioactive compound synthesis under natural environment, insufficient yield of the plant production, and other factors (Gao et al., 2018). Therefore, to relieve the pressure on the plant resource, seeking an alternative approach for obtaining the plant material is becoming a hotspot. At present, adventitious root (AR) culture has been systematically studied in numerous plant species, including *E. pallida* and *E. purpurea* (Gao et al., 2018; Hahn et al., 2009). Meanwhile, the co-culture technology has been applied in *E. pallida* and *E. purpurea* ARs, and the finding demonstrated that the bioactive compound synthesis was obviously increased, and some compounds undetected in the single-species culture were identified in the co-cultured ARs (Wu et al., 2017, 2018). Therefore, AR co-culture is favorable for the mass production of *Echinacea* bioactive compounds and has a potential as a novel plant material. To use ARs as the material of anti-inflammatory products, the present study determined effects of extracts from co-cultured ARs on the production of pro-inflammatory mediators, the phosphorylation of mitogen-activated protein kinase (MAPK) proteins, and the level of the inhibitor of nuclear factor-kappa B- α (I κ B- α) in lipopolysaccharide (LPS)-induced mouse peritoneal macrophages (PMs) to clarify the anti-inflammatory property.

2. Materials and methods

2.1. Reagents

Indole-3-butyric acid (IBA) was obtained from Beijing Solarbio Science and Technology Co., Ltd., (Beijing, China). RIPA lysis buffer was purchased from Sigma (Sigma Aldrich Chemical Co., USA). Enzyme-linked immunosorbent assay (ELISA) Kits were purchased from BD Bioscience, (SD, USA). ATP was purchased from InvivoGen Co., (CA, USA).

2.2. Plant materials

ARs of *E. pallida* and *E. purpurea* were separately produced from bioreactors after 30 d of culture according to the method described by Wu et al. (Wu et al., 2012, 2007a). For AR co-culture, ARs

(11.4 g) of *E. pallida* and *E. purpurea* (8.6 g) were cut into approximately 1 cm length and inoculated together into a 5 L air-lift bioreactor with 4 L working volume (Fig. 1). The culture medium was three-quarter of Murashige and Skoog (1962) medium supplemented with 1 mg/L IBA and 50 g/L sucrose. The pH of the medium was adjusted to 5.8 before sterilization at 121 °C and 1.2 kg/cm for 20 min. The bioreactors were maintained at (25 ± 2) °C in the dark, and aerated with sterile air at 100 mL/min. The co-cultured ARs were harvested after 35 d of bioreactor culture and washed with tap water thrice, then freeze-dried in a lyophilizer. The dry ARs were used to determine the bioactive compound contents and prepare the extract.

2.3. Determination of bioactive compounds

The dry ARs of 0.2 g were soaked in 10 mL of 80% methanol for 10 min. The supernatant was collected after centrifugation and used to determine the bioactive compound contents. Total flavonoid content of ARs was quantitatively determined spectrophotometrically using a colorimetric method (Cui et al., 2010), with catechin as the standard. In brief, 0.25 mL of AR extract was added to 0.75 mL of 5% NaNO₂ solution for 6 min of reaction; Then, 0.15 mL of 10% AlCl₃ was added. Followed by adding with 1 mol/L NaOH. After 5 min, the reaction solution was measured at 510 nm of absorbance with a spectrophotometer (UV-2600, Shimadzu Corporation, Kyoto, Japan). The contents of caffeic acid derivative monomers were determined according to the method of Wu et al. (2007b). The caffeic acid fractions were analyzed using a high-performance liquid chromatography system equipped with XTerra RP 18 column (Fig. 2). The mobile phases were water (A) and acetonitrile (B). The gradient elution was modified as follows: initial 10% B for 40 min; 25% B for 11 min; 50% B for 1 min; with recycling to initial condition for 8 min at a flow rate of 0.3 mL/min. Caffeic acid derivatives were detected at 330 nm.

2.4. Extract preparation

ARs extract preparation using the method was described by Gao et al. (2019). The powdered ARs (30 g) were soaked in 400 mL methanol (80%) for 1 h, and filtered with a filter paper, then the

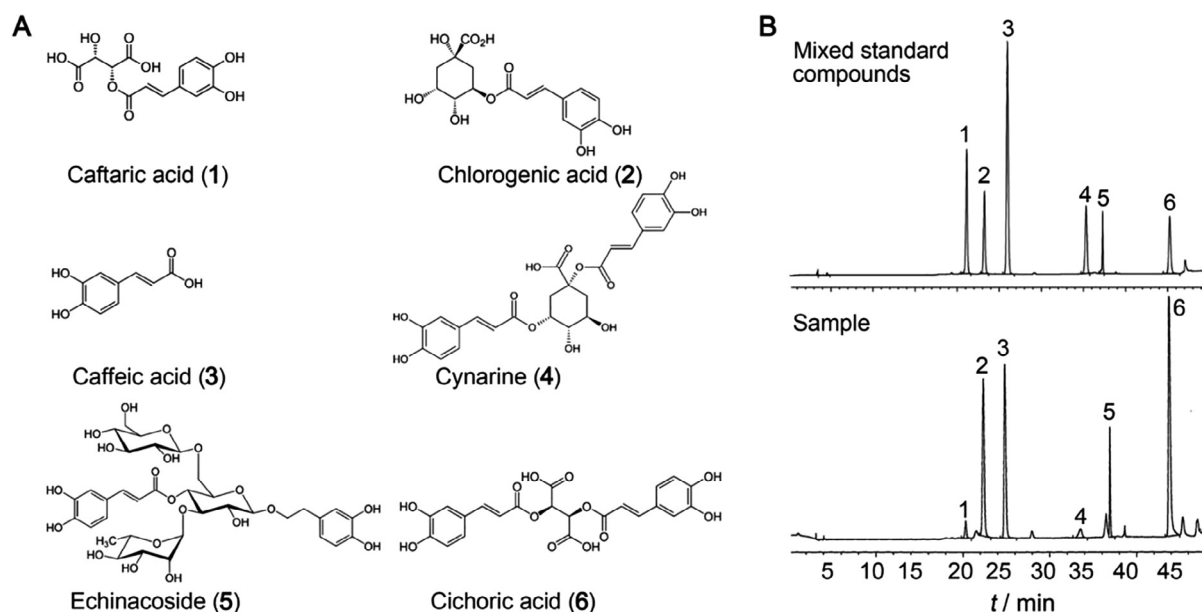


Fig. 1. Structures of six caffeic acid derivatives (A) and HPLC chromatograms of standard and extract from co-cultured adventitious roots of *E. pallida* and *E. purpurea* (B). Peaks corresponding to caffeic acid (1), chlorogenic acid (2), caffeic acid (3), cynarine (4), echinacoside (5) and cichoric acid (6), respectively.



Fig. 2. Adventitious roots of *E. pallida* and *E. purpurea* co-cultured in a bioreactor.

residue was repeatedly extracted twice with methanol. All filtrates were merged and concentrated by using a vacuum rotary evaporator at 45 °C; The concentrated extract was lyophilized to obtain the extract.

2.5. Animals and ethical statement

C57BL/6 mice with a mean weight of 22–25 g were obtained from Changchun Yisi Experimental Animal Co., Ltd., (Changchun, China). All the experiments followed the guidelines established by the Institutional Animal Ethical Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2.6. Cytotoxicity test

The PMs were obtained from C57BL/6 mice and the cells were cultured in the Dulbecco's Modified Eagle Medium (DMEM) using the method described by Han et al. (2018). The extract was diluted into 0, 50, 100, 200, and 400 µg/L, and then the effect of extract concentrations on cell viability was determined to evaluate the cytotoxicity. The cell viability was determined by the method of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Mosmann, 1983).

2.7. Anti-inflammatory experiment design

The *in vitro* experimental model of LPS-induced inflammation was used in the present study. AR extract concentrations of 25, 50, and 100 µg/mL were selected depending on the result of the cytotoxic test, and the experimental groups were designed as follows: (1) control group (Cont), cells were not treated with an extract or LPS; (2) LPS group (LPS), cells were treated only with LPS (0.1 µg/mL); (3) Low-(E25), medium-(E50), and high-(E100) dose extract groups, cells were pretreated with 25, 50, and 100 µg/mL extracts, respectively, before LPS (0.1 µg/mL) stimulation for several times. The production of nitric oxide (NO), prostaglandin E2 (PGE₂), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 or -1β, and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in cells were determined to evaluate the anti-inflammatory activity. The phosphorylation of MAPKs, including p38 MAPK (p38), c-Jun N-terminal kinase (JNK), and extracellular regulated protein kinases (ERK) were investigated and the level of IκB-α was determined to clarify the

molecular mechanism of anti-inflammation for the co-cultured *Echinacea* ARs.

2.8. Determination of NO and PGE₂ production

Cells were seeded in 48-well plates (2.5×10^5 cells/well) containing DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin, 100 U/mL) and treated with AR extract for the following different time periods: 24 h for NO determination and 16 h for PGE₂ determination; Followed by LPS (0.1 µg/mL) treatment for 1 h, the culture supernatant was harvested, and the NO level was measured according to the Griess reaction (Dirsch et al., 1998) with the optical density at 550 nm. The PGE₂ level was determined via the ELISA assay using the Kit according to the manufacturer's instructions, and the optical density was measured at 450 nm.

2.9. Determination of TNF-α, IL-6 and IL-1β production

The ELISA method was used to determine the production of TNF-α, IL-6, and IL-1β. The cells were seeded in 48-well plates (2.5×10^5 cells/well) for 12 h, and the extract was added. After 1 h of incubation, LPS (0.1 µg/mL) was added and stimulated for 6 h to examine the production of IL-6 and TNF-α. For determining the IL-1β production, cells were treated with the extract for 1 h after 3 h of LPS (0.1 µg/mL), then 5 mmol/L ATP was added for 1 h to prime cells for IL-1β maturation. TNF-α, IL-6, and IL-1β levels were determined via the ELISA assay using their relevant ELISA Kits according to the manufacturer's instructions, and the optical densities were measured at 450 nm.

2.10. Determination of iNOS and COX-2 expression, MAPK phosphorylation, and IκB-α levels

The iNOS and COX-2 expression, MAPK phosphorylation, and IκB-α levels were determined by using the Western blot analysis. In brief, cells were seeded in 6-well plates (2.5×10^6 cells/well) for 12 h, treated with the AR extract for 1 h, and stimulated by LPS (0.1 µg/mL) for various times, those are 24 h for iNOS and COX-2 determination, 30 min for MAPK determination, and 15 min for IκB-α determination, then the medium of the cell plate was discarded, the RIPA lysis buffer was added, and the lysate was used for Western blot analysis (Martinon et al., 2002).

2.11. Experimental design and data analysis

Data were collected from three experimental replicates. The results are presented as mean \pm standard error. The mean values were subjected to Duncan's multiple-range test by the Statistical Analysis System (SAS) program with $P < 0.05$.

3. Results

3.1. Bioactive compounds in ARs

The ARs of *E. pallida* (11.4 g) and *E. purpurea* (8.6 g) were inoculated together in a 5 L bioreactors, and approximately 370 g (fresh weight) of ARs were obtained after 35 d of culture (Fig. 1). In the co-cultured ARs, 34.43 mg/g dry weight (DW) of total flavonoids and 26.79 mg/g DW of total caffeic acid derivatives were determined, and those contents were significantly higher than those in ARs from the single-species culture (Table 1). Meanwhile, the six monomers of caffeic acid derivatives were identified in co-cultured ARs, but some monomers were not identified in ARs from the single-species culture, such as caftaric acid and caffeic acid in *E. pallida* ARs, and caffeic acid in *E. purpurea* ARs. Table 1 also showed that the co-cultured ARs contained maximum cichoric acid (13.48 mg/g DW), followed by chlorogenic acid (8.15 mg/g DW), and echinacoside (3.82 mg/g DW). On the contrary, relatively small amounts of caftaric acid (0.88 mg/g DW), caffeic acid (0.12 mg/g DW), and cynarine (0.34 mg/g DW) were found.

3.2. Effect of AR extract on production of NO and PGE₂

The cytotoxicity of the co-cultured AR extract was evaluated by determination of the cell viability. Fig. 3 showed that the cell viability after the extract treatment within their tested concentrations (50–400 $\mu\text{g}/\text{mL}$) did not decrease. Thus, further experiments used 25, 50, and 100 $\mu\text{g}/\text{mL}$ of extracts to treat cells before LPS stimulation, and then determined the production of pro-inflammatory mediators.

After LPS treatment, cells released large amounts of NO, and the NO level in the LPS group was 22.3-fold higher than that in the control group (Fig. 4A). However, NO levels in the low- (E25), middle- (E50), and high-dose (E100) extract groups were significantly ($P < 0.05$) lower than those in the LPS group, illustrating that the NO production was inhibited by the pretreatment of AR extracts, and the inhibitory effect exerted in a dose-dependent manner.

Table 1

Bioactive compound contents in *E. pallida*, *E. purpurea*, and their co-cultured adventitious roots.

Bioactive compounds	Content ($\text{mg}\cdot\text{g}^{-1}$ DW)		
	<i>E. pallida</i>	<i>E. purpurea</i>	Co-cultured <i>Echinacea</i>
Total flavonoids	19.80 \pm 1.04c	25.42 \pm 1.51b	34.43 \pm 1.03 a
Total caffeic acid derivatives	13.75 \pm 0.36c	19.51 \pm 0.51b	26.79 \pm 0.65 a
Caftaric acid	—	0.95 \pm 0.06 a	0.88 \pm 0.03 a
Chlorogenic acid	2.71 \pm 0.07c	5.45 \pm 0.14b	8.15 \pm 0.17 a
Caffeic acid	—	—	0.12 \pm 0.01
Cynarine	0.51 \pm 0.01 a	0.33 \pm 0.01b	0.34 \pm 0.01b
Echinacoside	7.41 \pm 0.19 a	0.91 \pm 0.02c	3.82 \pm 0.10b
Cichoric acid	3.12 \pm 0.08c	11.87 \pm 0.31b	13.48 \pm 0.35 a

Note: The total flavonoid content was determined by spectrophotometry and caffeic acid derivatives were determined by HPLC. Total caffeic acid derivatives = caftaric acid + chlorogenic acid + caffeic acid + cynarine + echinacoside + cichoric acid. Data represents mean \pm standard error ($n = 3$). The different letters within the same row indicate significant difference by Duncan's multiple range test at 5% level.

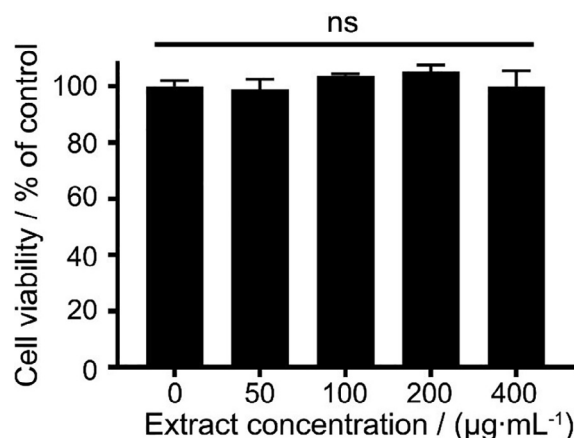


Fig. 3. Effect of different concentrations of extracts from co-cultured *Echinacea* adventitious roots on cell viability. Data are presented as mean \pm standard error ($n = 3$). Ns indicates no significant difference according to Duncan's multiple range test at 5% level.

The pattern of iNOS expression was similar to that of the NO production, and iNOS expression was significantly ($P < 0.05$) decreased in the extract groups (Fig. 4B).

The PGE₂ production in all extract groups (E25, E50, and E100) was significantly ($P < 0.05$) reduced compared with the LPS group, which was exerted in a dose-dependent manner (Fig. 5A). In addition, the expression of COX-2, an enzyme catalyzing PGE₂ biosynthesis, was assayed. Fig. 5B exhibited that the expression level of COX-2 was consistent with the result of PGE₂ production, which was decreased in the extract groups in a dose-dependent manner.

3.3. Effect of AR extract on production of pro-inflammatory cytokines

Effect of AR extracts on the production of pro-inflammatory cytokines (IL-6 and $-\text{I}\beta$, and TNF- α) was also determined in the present study. The level of IL-6 dramatically ($P < 0.05$) was increased in the LPS group, but that was lower in the E50 or E100 group (Fig. 6A), revealing that the IL-6 production was inhibited by the pretreatment of AR extracts. The effects of different concentrations of AR extracts on the IL-1 β production (Fig. 6B) were similar with that of the TNF- α (Fig. 6C). The levels of IL-1 β and TNF- α were significantly ($P < 0.05$) lower in all extract groups (E25, E50, and E100) compared with those in the LPS group, and decreased in dose-dependent manner.

3.4. Effect of AR extract on MAPK phosphorylation and I κ B- α level

In the present study, we investigated the effect of AR extracts on the MAPK phosphorylation and I κ B- α level to understand whether the AR extract acts the anti-inflammatory effect through regulating the MAPK and nuclear factor-kappaB (NF- κ B) signaling pathways. Fig. 7 showed that LPS enhanced the phosphorylation of MAPK proteins, thus, the p-p38 (Fig. 7A), p-JNK (Fig. 7B), and p-ERK (Fig. 7C) levels were remarkably ($P < 0.05$) higher in the LPS group than the extract groups, and they were dose-dependently reduced in all extract groups (E25, E50, and E100). In addition, LPS stimulation accelerated the I κ B- α level, however, which was not affected by the AR extracts (Fig. 8).

4. Discussion

Caffeic acid derivatives, including caftaric acid, caffeic acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid, belong to flavonoids, which are the main bioactive compounds in *Echi-*

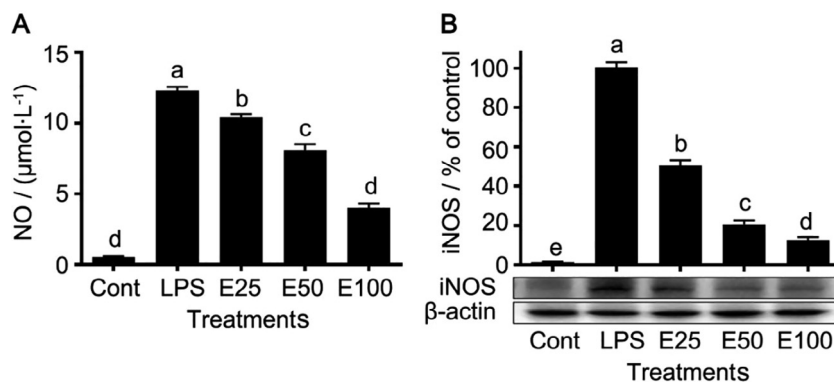


Fig. 4. Effect of extracts from co-cultured *Echinacea* adventitious roots on NO production (A) and iNOS expression (B) in cells. Data are presented as mean ± standard error (n = 3). The different letters between groups indicate significant difference by Duncan's multiple range test at 5% level.

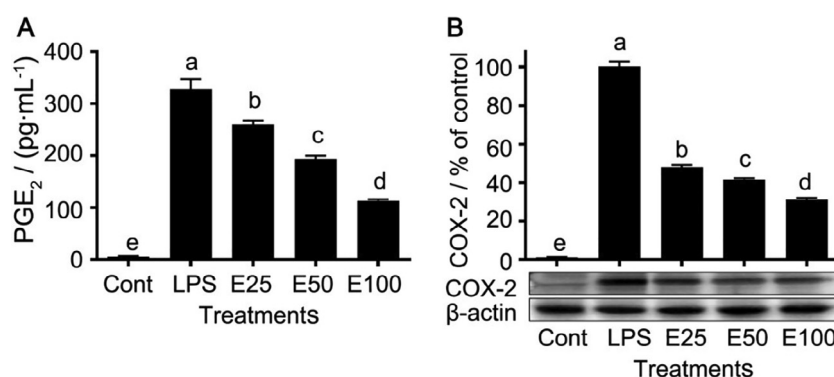


Fig. 5. Effect of extracts from co-cultured *Echinacea* adventitious roots on PGE₂ production (A) and COX-2 expression (B) in cells. Data are presented as mean ± standard error (n = 3). The different letters between groups indicate significant difference by Duncan's multiple range test at 5% level.

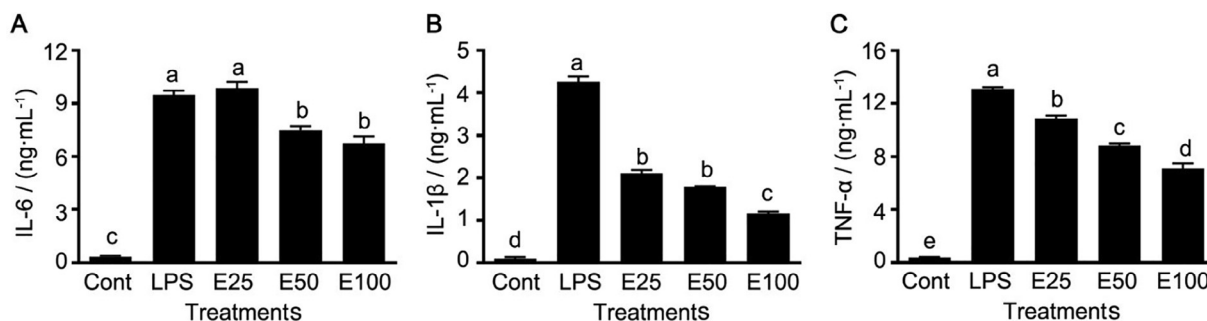


Fig. 6. Effect of extracts from co-cultured *Echinacea* adventitious roots on production of pro-inflammatory cytokines of IL-6 (A), IL-1β (B), and TNF-α (C) in cells. Data are presented as the mean ± standard error (n = 3). The different letters between groups indicate significant difference by Duncan's multiple range test at 5% level.

nacea plants (Cui et al., 2013). Studies have indicated that caffeic acid derivatives contained in *Echinacea* AR cultures, and their contents were higher in ARs than field-grown plants (Wu et al., 2012), and those in ARs from co-culture of *E. pallida* and *E. purpurea* were higher compared with the single-species culture (Wu et al., 2017). Caffeic acid derivatives have been proved having good anti-inflammatory effects. For instance, Cunha et al. (2004) found that caffeic acid derivatives from the reaction of caffeic acid inhibited the NO production and iNOS expression in LPS-induced Raw 264.7 macrophages. It was suggested that caffeic acid derivatives from *Salvia miltiorrhiza* roots may have the anti-inflammatory activities (Choi et al., 2017). Therefore, the present study investigated the anti-inflammation effect of co-culture *Echinacea* ARs for providing an evidence to the product production using as the plant material or additives.

LPS stimulation can induce macrophages to produce inflammatory mediators. As a small toxic molecule, NO is generated by a group of cytosolic or membrane-bound isoenzymes (Panaro et al., 2003), and iNOS is the cytokine-inducible isoform of the nitric oxide synthase (Murakami and Ohgash, 2010). In addition, COX-2 is an inducible enzyme, which is frequently overexpressed during inflammation, and PGE₂ synthesis is regulated by COX-2 (Tian et al., 2019). Thus, the excessive production of NO and PGE₂ can be prevented through modulating iNOS and COX-2 expression, respectively, which is important for the development of pharmacological strategies against inflammation. Furthermore, the overproduction of pro-inflammatory cytokines may accelerate the pathological changes (Chen et al., 2019). In the present study, the NO or PGE₂ production was inhibited by the pretreatment with AR extracts, attributing to the strong inhibition on the expression

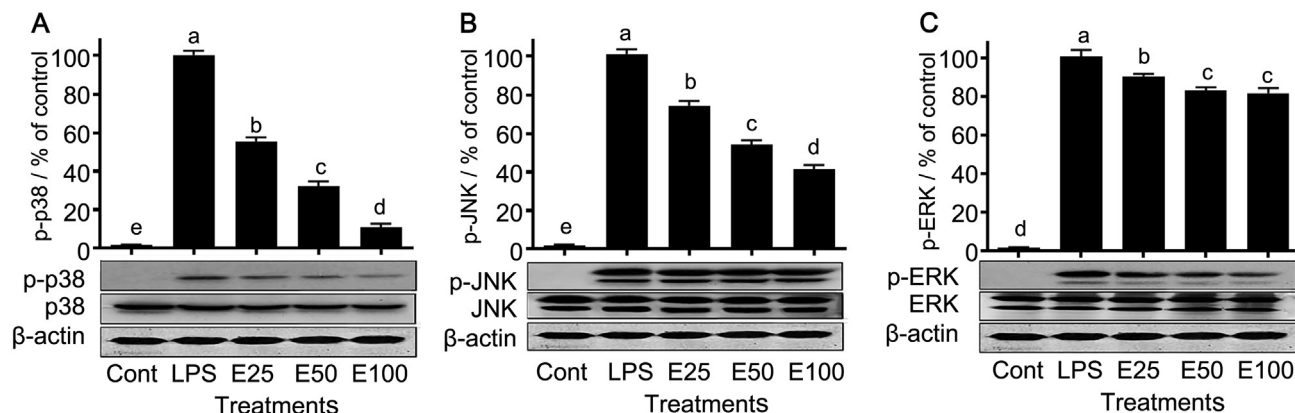


Fig. 7. Effect of extracts from co-cultured *Echinacea* adventitious roots on production of phosphorylation of MAPKs in cells. Data are presented as the mean \pm standard error ($n = 3$). The different letters between groups indicate significant difference by Duncan's multiple range test at 5% level.

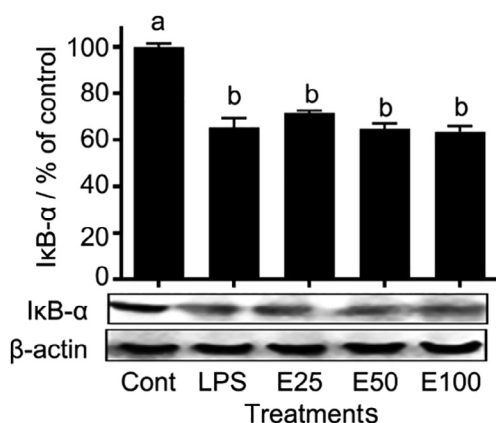


Fig. 8. Effect of extracts from co-cultured *Echinacea* adventitious roots on IκB-α level in cells. Data are presented as the mean \pm standard error ($n = 3$). The different letters between groups indicate significant difference by Duncan's multiple range test at 5% level.

of iNOS or COX-2. Meanwhile, AR extracts significantly ($P < 0.05$) inhibited the IL-6, IL-1 β , and TNF- α production and the levels of those pro-inflammatory cytokines were decreased in a dosage-dependent manner. Our findings confirmed that co-cultured *Echinacea* ARs can inhibit the LPS-induced inflammation, indicating an applicable potential of ARs in the production of anti-inflammatory products.

The cell-membrane receptor (toll-like receptor 4, TLR4) recognizes LPS after LPS stimulation and activates different signaling pathways, such as myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways (He et al., 2013). The downstream signaling pathways in the MyD88 dependent pathway, including MAPK and NF- κ B pathways, are initiated when the intercellular signaling cascade is triggered by TLR4, and the secretion of pro-inflammatory factors are further elevated (Doz et al., 2009). In the MAPK signaling pathway, MAPK proteins such as p38, JNK, and ERK are phosphorylated and modulate transcription factors, resulting in various biological responses including inflammation (Marie & Roux, 2011). The activation of NF- κ B in the NF- κ B pathway is initiated by the phosphorylation of the IκB- α protein. The IκB- α phosphorylation allows the NF- κ B complex to be freed and entered the nucleus to trigger the expression of specific genes, leading to the physiological response (Deptala et al., 2015). The present study found that AR extracts inhibited the MAPK phosphorylation, but did not affect the IκB- α level, indi-

cating that AR extracts involved in regulating the MAPK signaling pathway rather than the NF- κ B signaling pathway.

To date, the anti-inflammatory effect has been repeatedly reported in various *Echinacea* species (Raso et al., 2010; Speroni et al., 2002; Tubaro et al., 2011), and the associated products have also been sold in the international market. Along with the increase of the market demand, the supply of *Echinacea* raw material is seriously inadequate worldwide. Thus, AR cultures become an alternative supply source and have a great potential to be used in the industrial production.

5. Conclusion

The extract of co-cultured *Echinacea* ARs inhibited the production of various pro-inflammatory mediators in PMs, and the anti-inflammatory effect of the extract was attributed to the regulation of MAPK signaling pathway. The findings of the present study suggested that the co-cultured *Echinacea* ARs possess a potential for using as the raw plant material or additives in the production of anti-inflammatory related products.

Declaration of Competing Interest

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

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