J Appl Biol Chem (2019) 62(2), 149–155
https://doi.org/10.3839/jabc.2019.021

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Bioactive Materials

Elicitor-treated extracts of *Saururus chinensis* inhibit the expression of inducible nitric oxide synthase and cyclooxygenase-2 enzyme expression in Raw cells for suppression of inflammation

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Received: 3 April 2019 / Accepted: 17 April 2019 / Published Online: 30 June 2019

Abstracts Elicitor treatment was performed to increase the anti-inflammatory activity of useful biological sources. The result showed that elicitor-treated *Saururus chinensis* leaf extracts positively affected nitric oxide (NO) production, and the expression of inducible NO synthase and cyclooxygenase-2 compared to extracts not exposed to elicitor treatment. This finding identified elicitor treatment as a suitable strategy for increasing the biological activity of *S. chinensis*. Therefore, elicitor-treated *S. chinensis* is useful both as health functional and medicinal materials.

Keywords Cyclooxygenase-2 · Elicitor · Inducible nitric oxide synthase · Nitric oxide · *Saururus chinensis* extracts

Introduction

The secondary metabolites produced by medicinal herbs including *Saururus chinensis* [1-7] have various specific pharmacological activity, which has increased the interest in these substances. There are 4,000 native plants currently known and only approximately 400 species inhabit S. Korea. These native plants, which have high monetary value, were exported to foreign nations from S. Korea and are currently being reimported [8]. Improved strains of flowering plants such as Ms. Kim lilac (*Syringa velutina* var. *kamibayashii* T.) and daylily (*Hemerocallis fulva* L.) are being sold widely. *Saururus chinensis* leaves contain high amounts of phenolic compounds such as mannasantin A, mannasantin B, sauceneol D and avicularin, etc., which have anti-inflammation activity [9,10]. The components contained in *Saururus chinensis* have been shown to have protective activity in interstitial cell, exert antibacterial and antioxidant effects, and strengthen capillary vessels [4-7].

In addition, ginkgo leaf from S. Korea was developed into a blood circulation enhancer and has been reimported at a high value. The value of native plants has increased recently [11,12] and, therefore, research into developing techniques for producing useful materials with high value from a cash crop naturally growing in our country has increased. These research studies tend to identify globally competitive, originative techniques and high value producing systematic applications using grafting effects, ingredient analysis, cultural environment analysis and biotechnology based on experiences with folk remedies and clinical trials [13,14].

Macrophages, one of the cell types involved in the inflammatory response, have a significant effect on immune and inflammatory reactions. NO, a reactive radical molecule in cells, is generated from L-arginine through its oxidation by inducible NOS (iNOS), the latter being well known in the defense response against various inflammatory diseases, circulatory disorders, and cancers [15]. Prostaglandin E_2 (PGE_2), also known as dinoprostone, is a principal inflammatory mediator that is generated from arachidonic

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acid, which is converted by cyclooxygenase (COX)-2 [16].

Elicitation technique involves artificially inducing phytoalexin, which is produced by plants as a protective mechanism against attacks by potentially deleterious substances such as pathogens. By treating the plant with an inducing agent shortly before harvesting, a high-quality oriental material or herb with a high content of useful contents is obtainable. Therefore, elicitation is an economical and efficient biomass treatment method.

In this study, we established an elicitation technique, which was applied to garden cultured *Saururus chinensis*, with the aim of improving and enhancing the production of pharmacologically active contents by medicinal herbs. In addition, the main achievement was the development of techniques to maximize the pharmacologically active contents to ensure the pharmacological competitiveness rather than price competitiveness.

Materials and Method

Production and application of elicitor on *Saururus chinensis*

The elicitor was produced by treating yeast extract with protease and adding ethanol to generate a precipitate, which was collected and dried to produce the yeast extract powder. One kilogram of yeast extract powder was homogenized with 5 g copper chloride ($CuCl_2$) to produce the elicitor powder according to the method of Cho et al. [9].

The elicitor was applied three times at 1.5 and 3 mg/L (groups A and B, respectively) per application. The first application was carried out when the *S. chinensis* leaves had grown to approximately one-fourth their full size (April 30th). The elicitor was sprayed directly on the bottom part of the leaves on a 4 m^2 area in both groups A and B. A similar second application was carried out at 50% leaf growth (May 7th). The final application was carried out at 75% leaf growth (May 14th). In total, groups A and B were treated with elicitor concentrations of 4.5 and 9.0 mg, respectively, on each 4 m^2 area until the harvest.

Extraction process of elicitor-treated *S. chinensis*

To prepare the water extract, 200 mL distilled water was added to 1 g of the dry plant sample, boiled until the solution volume was reduced to 100 mL, and then cooled. For the ethanol extract, 100 mL 60% ethanol was added to the plant sample with stirring for 24 h, followed by homogenization for 1 min using a homogenizer at 20,000 rpm. The extract was filtered with Whatman No. 1 filter paper and concentrated using a rotary vacuum evaporator (Eyela NE, Tokyo, Japan) if needed and used as the sample.

Phenol compound determination

Briefly, 240 μL 5% sodium carbonate (Na_2CO_3) solution was added to 2 mL of the extract, followed by 120 μL 1 N Folinciocalteu reagent as the color-developing reagent and the mixture was thoroughly mixed. The color was expressed by leaving it for 10 min then the optical density (OD) was determined within 1 h at 725 nm and then used to construct a standard curve using gallic acid [17].

Cell toxicity analysis using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The Raw 264.7 murine macrophage cell line was purchased from Korean Cell Line Research Foundation and cultured for 72 h at 37 °C in an atmosphere of 5% CO_2 with mixed medium of 10% fetal bovine serum (FBS, Gibco Co. Waltham, MA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in Dulbecco's modified Eagle's medium (DMEM). The cell condition was maintained in an atmosphere of 5% CO_2 and subcultured at a density of 2–3×10^6 CFU/mL in a cell culture dish. For the experiments, the passages condition of 80% confluency and 20 times. The cells were cultured for 12 h with the FBS-free medium before the experiment [18,19].

Nitric oxide (NO) determination

NO levels were determined by measuring NO amount in the cell supernatant as nitrite and nitrate. Griess reagent (Sigma-Aldrich, St. Louis, MO, USA) was used to stabilize nitrite reduced to nitrate. At 80% confluence, 2×10^6 cells were washed two times with phosphate-buffered saline (PBS) and cultured with serum-free culture medium in a six-well plate. Then, the cells were stimulated by adding 50 μM lipopolysaccharide (LPS) to all wells except the control group. The sample was added at a concentration range of 60–80 μg/mL and incubated for 2 h. The NO production amount was determined by measuring the OD of the collected supernatant at 540 nm after a 10 min reaction in the dark [19,20]. The inhibitory activity (%) was calculated as follows: (1−absorbance of sample/absorbance of control)×100.

Analysis of inducible nitric oxide synthase (iNOS) expression using western blotting

Raw cells were allowed to reach 80% confluence, the DMEM was then discarded and replaced with serum-free Eagle's minimum essential medium, and then the cells were cultured with the test substances under the same conditions used above. At the predetermined time, the cells were washed two times, the supernatant was discarded, and then the cells were collected using a scrapper and then homogenized in lysis buffer. The protein concentration of the cultured cells was determined by measuring the OD value based on the standard curve constructed using bovine serum albumin. The normalized protein sample was used as the western blotting sample after dyeing with Coomassie blue. The sample was separated using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the gel was immersed for 10–15 min in transfer buffer, placed between two sponge sheets in transfer buffer, and then one sheet of filter paper was placed on top. Then, 3 mm filter paper and a nitrocellulose filter were also immersed in the buffer. The nitrocellulose paper and gel were osculated between the

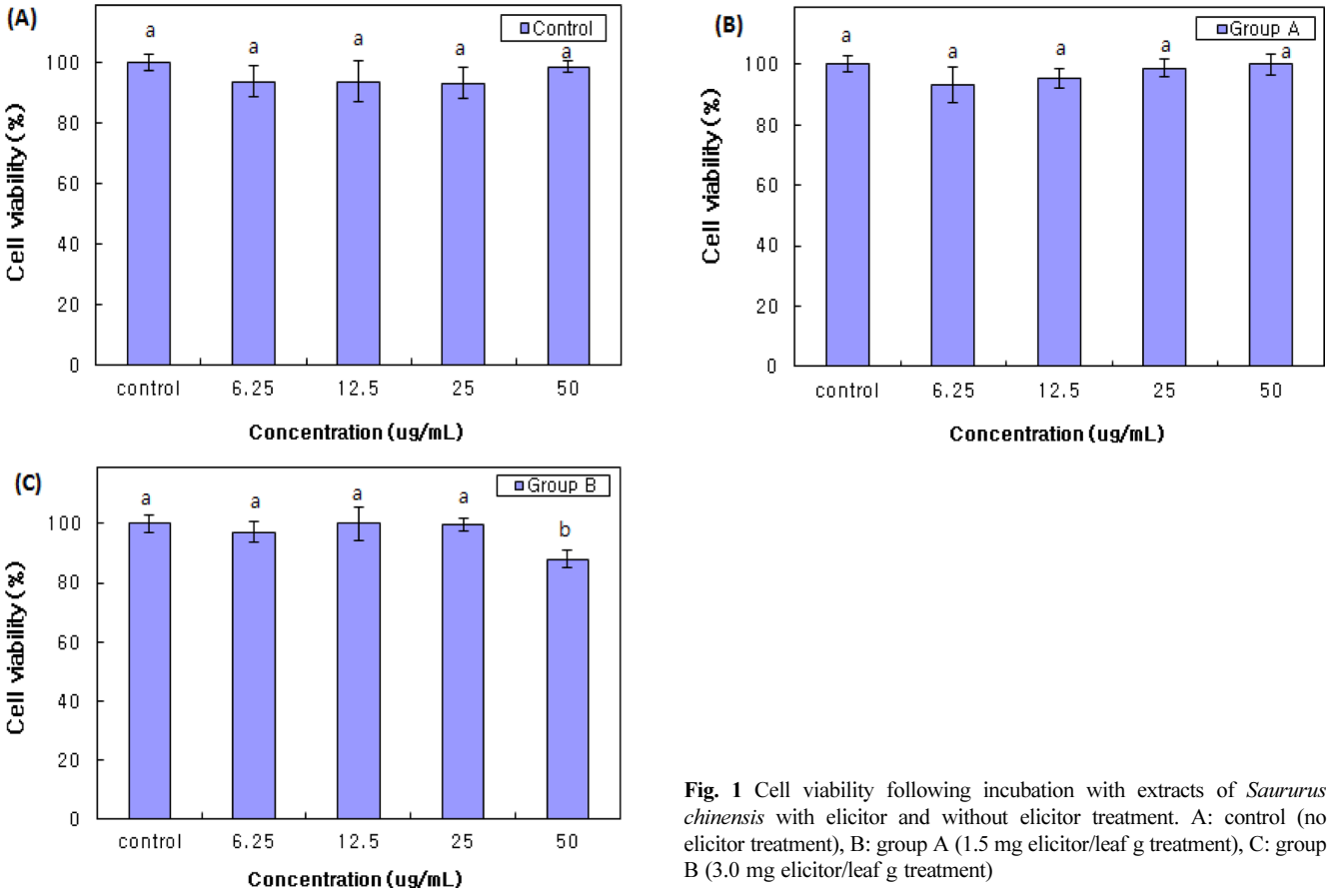

Fig. 1 Cell viability following incubation with extracts of *Saururus chinensis* with elicitor and without elicitor treatment. A: control (no elicitor treatment), B: group A (1.5 mg elicitor/leaf g treatment), C: group B (3.0 mg elicitor/leaf g treatment)

sponges then the proteins were transferred by plugging the electrodes. After transferring for over 1 h at 190 mA, the transfer blot was immersed in Ponceau S for 2 min, and the bands were detected. The background was removed by incubation in blocking buffer after washing two times with PBS. Then the blot was incubated with the primary antibody (1:1,000), followed by the secondary antibody (1:1,000). The blot was washed with PBS plus 0.5% Tween 20 several times, and then the bands were visualized on a film using an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia, England) [19,21].

Analysis of cyclooxygenase (COX)-2 expression using western blotting

To determine the activity of COX-2 protein, Raw 264.7 macrophage cells were stabilized by culturing for 24 h in 100 π tissue culture dish at a density of 2×10^4 cells/mL and each well was treated. After removing the medium, different concentrations (6.25–25 μg/mL) of treated medium were used to cultivate the cells for 24 h. Then, the medium was removed, and the cells were washed twice with PBS. The cells were lysed with 100 μL lysis buffer (complete mini 1 added to the radioimmunoprecipitation assay buffer 10 mL) and centrifuged for 20 min at 12,000 rpm at 4 °C. The supernatant was collected and placed in a new tube, which was stored at −20 °C for further use or protein determination. The supernatant collected after centrifugation was determined using the Bradford assay. Then, 20 μL of protein samples were separated using 10% SDS-PAGE. The separated proteins were transferred onto a polyvinylidene fluoride membrane using semidry transfer cell equipment (Bio-Rad, Hercules, CA, USA) and incubated for 1 hour with blocking buffer (5% skim milk in Tris-buffered saline plus Tween [TBST]) at room temperature. The membrane was washed with TBST three times every 10 min and incubated overnight with diluted primary antibodies against, iNOS (BD Bioscience, Santa Cruz, CA, USA. 1:1000), COX-2 (Cayman, Ann Arbor, MI, USA. 1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, BD Bioscience, Santa Cruz, CA, USA. 1:1000), at 4 °C. Then, the membrane was washed with TBST again three times for 10 min each and then incubated for 2 h at room temperature with secondary mouse anti-rabbit IgG horseradish peroxidase (HRP) and bovine anti-goat IgG HRP (Santa Cruz, 1:1000). After washing three times, the membrane was reacted with ECL (Millipore) solution in a darkroom and exposed to X-ray film. The bands were visualized using a molecular imager (Bio-Rad) [19,21].

Statistical analysis

The experiments were repeated three times, and the results were calculated as averages with standard deviations. The statistical

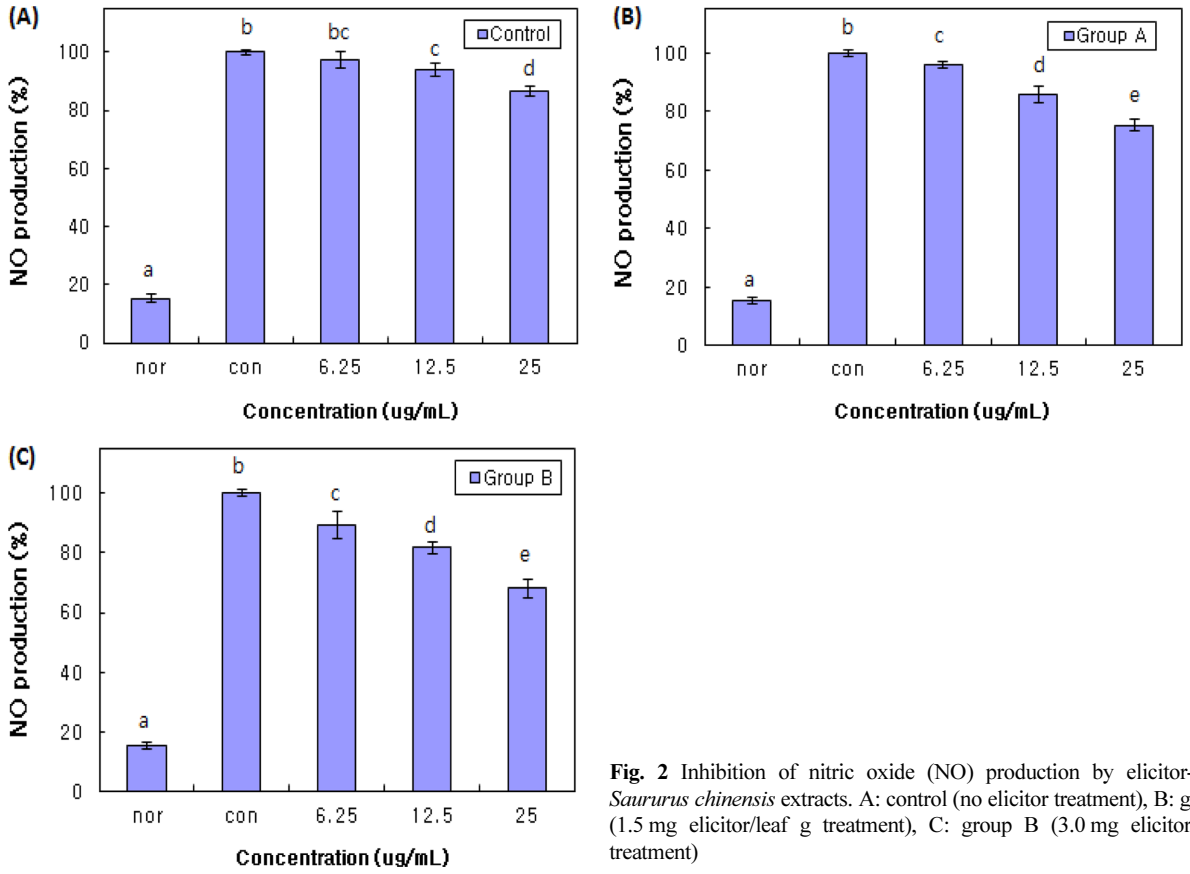

Fig. 2 Inhibition of nitric oxide (NO) production by elicitor-treated *Saururus chinensis* extracts. A: control (no elicitor treatment), B: group A (1.5 mg elicitor/leaf g treatment), C: group B (3.0 mg elicitor/leaf g treatment)

analysis was performed using the statistical package for the social sciences (SPSS) 23 for Windows (SPSS, Chicago, IL, USA) program and Duncan's multiple range test (one-way analysis of variance [ANOVA] at 95% significance level.

Results and Discussion

MTT assay of elicitor-treated *S. chinensis* extracts

The MTT assay was performed to determine the cell survival rate and cytotoxicity of the extracts. Treatment of the cells with the elicitor-treated *S. chinensis* extracts at 6.25–50 μM/mL concentration had no effects on the cell viability (Fig. 2).

Inhibition of NO expression by elicitor-treated *S. chinensis* extracts

The endotoxin LPS is a component of gram-negative cell outer membranes. Macrophages are activated by small amounts of LPS, which leads to the production and release of cytokines, NO, and other factors that induce the various physiological responses mediated by LPS [22]. NO generation normally has an important role in killing and removing bacteria and tumors, but excessive levels induced by pathological processes can cause inflammation, organ damage, genetic mutations, and neural damage [23,24].

To determine if the *S. chinensis* extract inhibits NO production by Raw 264.7 cells, they were incubated with different extract concentrations and Fig. 3 shows that the LPS-stimulated group exhibited 200% increase of NO expression, which was two times more than that of the unstimulated group. Elicitor-treated *S. chinensis* extracts inhibited NO expression concentration-dependently. Elicitor-treated *S. chinensis* extract at 100 μg/mL inhibited NO production by >80%, which was higher than the 70% inhibition induced by the *S. chinensis* extract without elicitor treatment. In addition, group B showed higher inhibition of NO production than group A, which exhibited similar levels to those of the normal group. The extract was expected to amplify the anti-inflammation effect and immune function of Raw 264.7 macrophage cells induced by LPS. In addition, amplification of the expression of determined transcription factors that interact with NO was expected.

Inhibition of iNOS expression by elicitor-treated *S. chinensis* extracts

NO is a free radical generated throughout the body that controls biological functions such as angiectasis, smooth muscle contraction, neuronal signal transduction, platelet aggregation inhibition, and immunomodulation. It also has anticancer and antibacterial properties. However, excessive NO production can cause various inflammatory diseases, septic symptoms by excessive angiectasis, wound healing

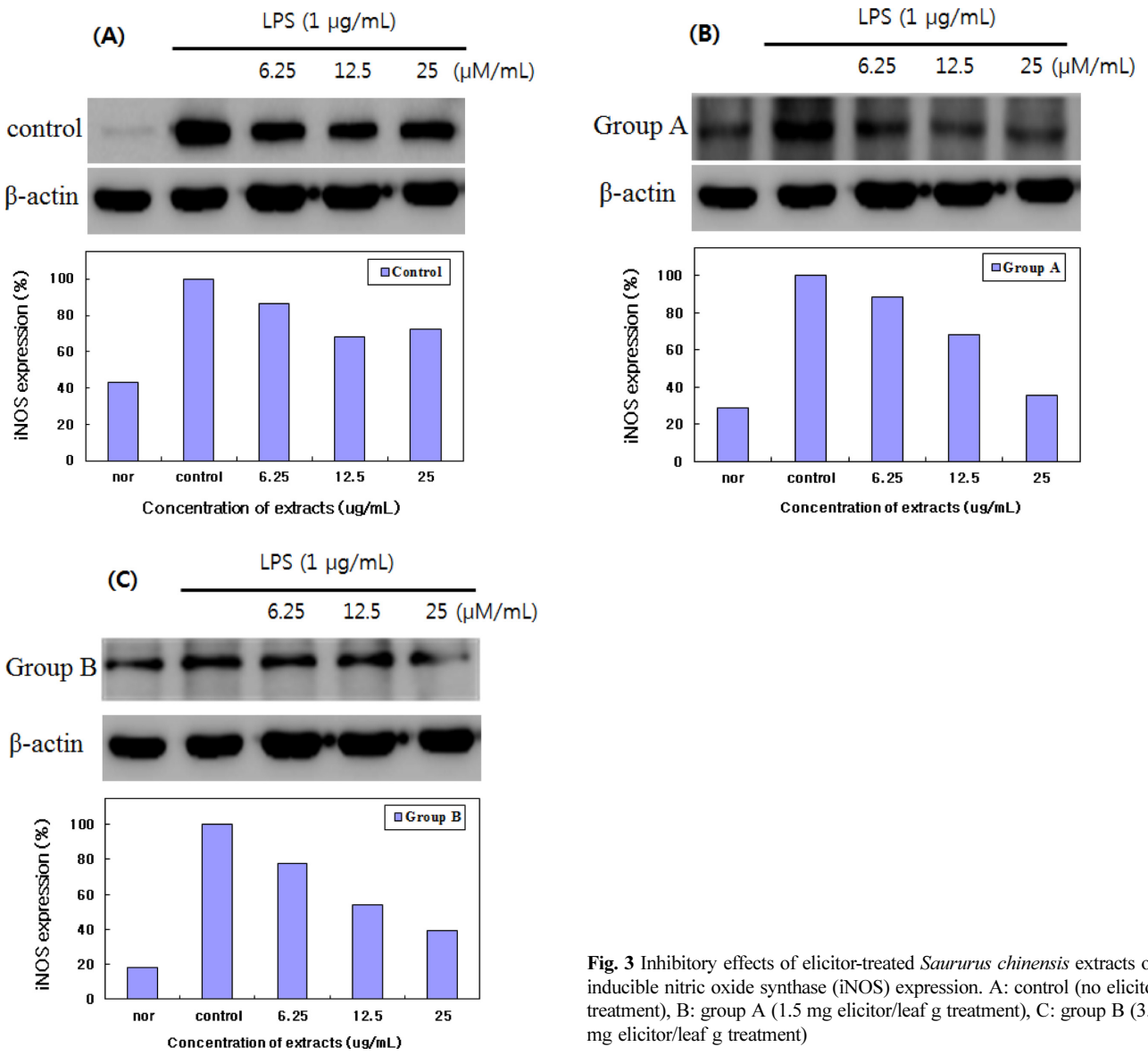

Fig. 3 Inhibitory effects of elicitor-treated *Saururus chinensis* extracts on inducible nitric oxide synthase (iNOS) expression. A: control (no elicitor treatment), B: group A (1.5 mg elicitor/leaf g treatment), C: group B (3.0 mg elicitor/leaf g treatment)

inhibition, immune system dysfunction, and cell withering.

Inflammatory factors NO, PGE_2, and other substances are formed during the inflammatory process by iNOS and COX-2. NO have various biological functions such as body defense, signal transmission, neurotoxicity, and angiectasis. NO is synthesized by NOS, which consists of the following three types that convert L-arginine to L-citrulline: type I (neuronal NOS, nNOS); II (iNOS); and which convert NOS can also be classified into three different types: I, inducible NOS; II (only expressed following exposure to specific stimulating inflammatory factors such as lipopolysaccharide (LPS), cytokines, and bacterial toxins; and III (endothelial NOS), which produces NO for maintaining homeostasis [25]. The different iNOS isoforms are widely expressed and have important pathological functions. Especially, iNOS, which is induced by stimulation, produces quantities of NO for a long time. The produced NO characteristically activates guanylyl cyclase, causing toxicity to nearby organs. An anti-inflammatory effect was expected to be identified by iNOS protein level decrease in RAW 264.7 cells stimulated with LPS and by determining the COX-2 protein level, which is a factor that increases proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 in monocytes and macrophages [26].

To investigate the relationship between iNOS protein and NO production inhibition mechanisms, iNOS protein expression was determined in the cytoplasm using western blot analysis. As shown in Fig. 4A–C, elicitor-treated *S. chinensis* extract inhibited iNOS expression concentration-dependently. At 25 μM/mL, elicitor-treated *S. chinensis* extracts showed 70% inhibition of iNOS expression, which was 40% higher than that of the *S. chinensis* extracts not elicitor-treated. Group B showed higher iNOS expression

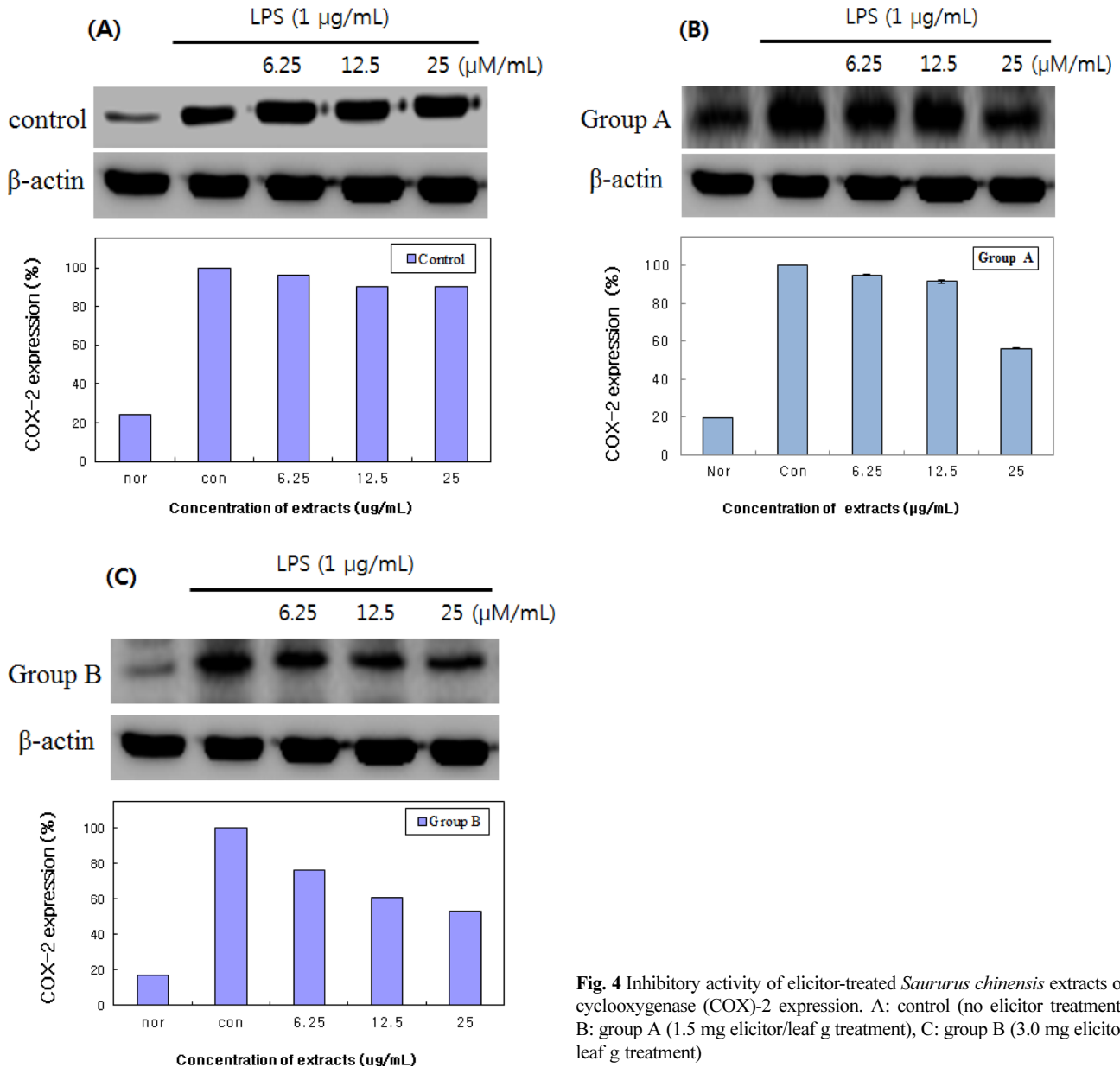

Fig. 4 Inhibitory activity of elicitor-treated *Saururus chinensis* extracts on cyclooxygenase (COX)-2 expression. A: control (no elicitor treatment), B: group A (1.5 mg elicitor/leaf g treatment), C: group B (3.0 mg elicitor/leaf g treatment)

inhibition than group A, which showed similar inhibition to that of the normal group. Similar to previous experimental results, a trend of amplified total phenolic compounds and content of useful substances such as vicularin, manassantin A, manassantin B, and saucerneol D was observed. The analysis of the western blot results showed that the elicitor-treated *S. chinensis* extract inhibited the iNOS protein expression more than the untreated extract did, which indicates it reduced LPS-induced inflammation by inhibiting iNOS expression.

Inhibition of COX-2 expression by elicitor-treated *S. chinensis* extracts

COX-2 is produced by the activation of mitogen-activated protein kinase kinase-1 and nuclear factor-κB by prooxidant or proinflammatory stimuli (e.g., TPA, LPS, TNFα, and ROI), and it has an important role in the inflammatory reaction by increasing prostaglandin synthesis [27]. In addition, COX-2 expression in monocytes is increased by proinflammatory agents such as IL-1β, TNF-α, phosphatidic acid, and fibroblast growth factor and inhibited by glucocorticoids, IL-4, and IL-13 [28,29]. Therefore, selective inhibitor development for COX-2 is a potential targeted molecular strategy for inflammation treatment. The induction of the inflammatory factor, COX-2 protein, by LPS was inhibited by elicitor-treated *S. chinensis* extracts concentration-dependently (Fig. 5A–C). Elicitor-treated *S. chinensis* extract at 25 μM/mL showed approximately 80% inhibition of COX-2 expression, which was much higher than the 20% induced by the untreated extracts. In addition, treated of group B at 6.25–12.5 μM/mL

inhibited the iNOS expression effect more than that of group A, showing similar inhibition to that of the normal group.

Similar to previous experimental results, we observed that the extracts tended to inhibit the amplified NO production. Analysis of the western blotting results showed that the elicitor-treated *S. chinensis* extracts showed higher inhibition of the LPS-induced iNOS and COX-2 protein expression than the untreated extracts did, which indicates that the inflammatory reaction was reduced by the inhibition of iNOS and COX-2 protein expression.

It has been reported by Park et al. [10] that the gastritis inhibitory compounds in *S. chinensis* are avicularin, manassantin A, manassantin B, and saucerneol D. In the present study, the NO, iNOS and COX-2 levels as anti-inflammatory activities were increased in elicitor-treated groups A and B, compared to non-treated control group. These results indicate that elicitor treatment amplifies these biological compounds on inflammation during the growth of *S. chinensis*.

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