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Responses of inflammation signaling pathway by saucerneol D from elicitor-treated *Saururus chinensis* on pro-inflammatory responses in LPS-stimulated Raw 264.7 cell

Eun-Ho Lee and Young-Je Cho* 

Abstract

This study confirmed the association with inflammation-related proteins, mediators, and cytokines using saucerneol D from *Saururus chinensis* leaf, a useful ingredient increased through elicitor treatment. To confirm the anti-inflammatory effect, saucerneol D were treated with lipopolysaccharide, which induces pro-inflammatory factors in Raw 264.7 cell. The pro-inflammatory influences were measured by dint of chemical assay and western blotting as well as ELISA. As a result, the content of saucerneol D was changed when elicitor was treated by various concentration (1.5, and 3 mg/mL) in *S. chinensis* leaves. In addition, the expression levels of hyaluronidase and pro-inflammatory-related factors [nitric oxide (NO), inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2)] were regulated according to the saucerneol D content in the elicitor-treated and non-treated groups. Therefore, after confirming that saucerneol D has an inhibitory effect on pro-inflammatory-related factors, saucerneol D was adjusted by concentration and compared with the control substance to verify the efficacy. Saucerneol D was adjusted to a concentration that did not toxic to macrophages through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Saucerneol D controlled at various concentrations inhibited iNOS and COX-2 proteins. NO produced by iNOS activity, prostaglandin E₂ (PGE₂), an inflammatory mediator produced by COX-2 activity, and pro-inflammatory cytokines [interleukin-1 β (IL-1 β), IL-6, tumor necrosis factor- α (TNF- α)] were significantly suppressed. Therefore, it was confirmed that saucerneol D, an active ingredient increased by the elicitor treatment, could be used as a functional material that controls inflammatory factors.

Keywords: Cytokine, Elicitation, Inflammation, Macrophages, Saucerneol D

Introduction

Studies have reported several natural substances that are necessary or important to cultivate healthy lifestyles substances of plant origin have been the subject of much research in this regard. And the wild plants produce substances can prevent or cure diseases [1–3]. Elicitor technology is a technology that induces plants

to artificially produce more phytoalexin by processing Elicitor. Plants themselves produce potentially useful substances in the plant body as a way to defend against attacks by external stimuli, such as worms and pathogens. When applied, this technology is applied to a plant, the plant's defense system recognizes the processed elicitor as an attack from foreign pathogens. So, in order to protect itself, it artificially induces excessive production of useful substances by using the principle of producing phytoalexin, an antitoxic substance produced by plant tissues. The method of treating the inducing agent has an advantage of treating the

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inducing agent immediately before harvesting when cultivating the plant, and having a high content of useful components and ensuring high-quality materials. In addition, according to a study by Lee and Cho [4], the change of total phenolics content (TPC), difference in HPLC profile, and the modulating effect of biological enzyme were different reported according to the concentration of elicitor treated in the extract of *Oplismenus undulatifolius* treated with elicitor.

Saururus chinensis Baill. is a perennial plant classified in the Sauceraceae family and is utilized as an oriental medicine when dried [5, 6] its pharmacological effects aid the alleviation of fever, anti-inflammation, dropsy, beriberi, hepatitis, jaundice, cancer and other ailments [5]. The major components of *S. chinensis* are hyperion, rutin, queroetin, quercetin, quercertrin, and other flavonoids [7, 8]. Flavonoids are mainly used as natural antioxidants because the results of research on antioxidants are well known, and because they have a preventive effect against oxidative stress [9]. They also play a key role in preventing or slowing down aging, cancer, and heart disease. Flavonoids have several uses, including as food and in the pharmaceutical and cosmetics industries. Nowadays, among the studies that have been done on various natural compounds, a particular interest has been focused on the flavonoids as being the main compound with anti-oxidative properties [10, 11].

Macrophages are activated by low concentrations of lipopolysaccharide (LPS), also known as lipoglycans and endotoxins, and produce and release inflammatory mediator, toll like receptor, reactive oxygen intermediate, nitric oxide (NO) and other compounds [12, 13]. Macrophages may also secrete phosphatase, chemokine, and cytokines, for instance tumor necrosis factor- α (TNF- α), interferon, interleukin-1 β (IL-1 β), IL-6, hyaluronan synthase 2, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor- β (TGF- β), when activated, as a defense mechanism which involves inflammatory activity, hematopoietic organs, and others as part of the body's immune system [14–16].

In the present study, we sought to identify, purify, and test the inflammation inhibitory properties of flavonoid compounds extracted from elicitor-treated *S. chinensis*. Anti-inflammatory properties were assessed employing macrophages, and the western blot method was utilized to observe changes in protein content. Also, in this study, the elicitation technique was applied to *S. chinensis* to improve the efficacy research content using existing pharmacological plants, and the anti-inflammatory effect of saucerneol D, a useful ingredient produced from *S. chinensis*, was investigated.

Materials and methods

Production and application of elicitor on *S. chinensis*

The main component of the elicitor used in the study is a polysaccharide carbohydrate, a polysaccharide derived from the cell wall of microorganisms acting as a pathogen for plants. The purified yeast (210929, Difco™, Sparks, MD, USA) enzyme digestion product after protease treatment is the separation of polysaccharide from yeast autolysate. In the yeast enzyme digestion product, a proteolytic enzyme was applied to the yeast extract aqueous solution, and then an excess of ethanol was added to induce ethanol-insoluble components. Then, the precipitate was recovered and dried to prepare a powder state. Protease is an enzyme that hydrolyzes protein-peptide bonds, and a protease isolated from *Aspergillus fumigatus* [17] was used. 1 kg of yeast extract powder prepared according to the above method was homogenized with 5 g of copper chloride (CuCl₂) to produce the elicitor powder [18].

The elicitor processing technique is a technique that artificially induces useful components generated inside a plant. High-quality materials with high content can be produced through one time or more treatments of inducing agents in a short period just before harvest. *S. chinensis*, the target of elicitor treatment, is a perennial plant, but for the purpose of experimentation, it was cultivated for 1 year and harvested by treating elicitor. The concentration for preparing the elicitor solution was set based on 1.5, and 3 mg/mL, and the distribution degree of the 4 m² area was measured for each growth stage, dissolved in 1 L, and treated three times. The first application was carried out when the *S. chinensis* leaves had grown to approximately one-fourth their full size (April 30th, 0.375 g/L and 0.75 g/L). The elicitor was sprayed directly on the bottom part of the leaves on a 4 m² area in both groups A and B. A similar second application was carried out at 50% leaf growth (May 7th, 0.75 g/L and 1.5 g/L). The final application was carried out at 75% leaf growth (May 14th, 1.125 g/L and 2.25 g/L) [4, 18].

Preparatory stage of extracts from elicitor-treated *S. chinensis*

For the samples of the elicitor-treated and the non-treated group, only the leaves of the above-ground part of the *S. chinensis* were collected, foreign substances were removed, and naturally dried, and then ground to 40 mesh was used. Then, preparation of extract experimental samples was 1 g of *S. chinensis* leaves powder was mixed with 100 mL of 70% ethanol, homogenized for 1 min at 16,000 rpm, and agitated for 24 h. Finally, the sample extracts was filtered and concentrated by utilizing a filter paper No. 41 (Whatman Inc., Piscataway, NJ, USA) and Buchi rotavapor (Buchi Labortechnik AG,

Flawil, Switzerland). The extracted extract was freeze-dried (freeze dryer, FD8518, Ilshinbiobase, Yangju, Korea) to remove moisture, and then stored at a low temperature in a sample storage refrigerator at 4 °C or –20 °C and used for experiments.

Determination of hyaluronidase (HAase) inhibition

In the HAase inhibition experiment, as the extract sample is treated, the substrates sodium hyaluronic acid (HA) and ρ -dimethylaminobenzaldehyde react and affect the activity of HAase to change the color of the reactant. Briefly, 0.05 mL HAase solution (7900 U/mL) dissolved in 0.1 M acetate buffer (pH 3.5) and 0.1 mL of extracts from *S. chinensis* were mixed and incubated for 20 min at 37 °C. Then, 12.5 mM calcium chloride (CaCl₂) (0.1 mL) was added and HA (12 mg/mL) dissolved in 0.1 M acetate buffer (pH 3.5) was added as substrate. Next, 0.1 mL of 0.4 N potassium tetraborate (K₂B₄O₇) and 0.1 mL of 0.4 N sodium hydroxide (NaOH) solution was added to the reacted mixture, boiled in a water bath. Then, 3 mL ρ -dimethylaminobenzaldehyde, which is a color forming reagent, was added to the cooled mixture and incubated for 20 min at 37 °C. Absorbance (Optizen 3220 UV, Mersys Co. Ltd.) was measured at 585 nm and the inhibitory activity was calculated as previously reported [19].

Purification and chemical structure assay

The material was purified using MCI gel CHP-20 column and Sephadex LH-20. The purified active single compound was a colorless powder and melting point was 75–76 °C. The angle of rotation $[\alpha]_D^{25}$ was –88.1°. Molecular weight at positive FAB-MS was 554, with active groups of 3490, 2960, 2920, 2900, 1605, and 1595 in KBr IR spectrum (cm⁻¹, KBr) were identified. ¹H- and ¹³C-NMR data were consistent with the literature values [20].

Determination of TPC and saucerneol D content

The TPC was measured by referring to the method tested by Folin–Denis [21]. The TPC was measured at 725 nm using an absorbance instrument (Optizen 3220 UV, Mersys Co. Ltd., Seoul, Korea). For the measurement, 1 N Folin–Ciocalteu reagent was used for color development, and the result was measured within 1 h. Quantification of the TPC was calculated using a gallic acid standard curve, and the content of saucerneol D was analyzed by HPLC and measured by applying purified saucerneol D to the standard curve.

Cell culture

Raw 264.7 cell were cultured for 24 h at 5% CO₂ and 37 °C in a mixed medium composed of 10% fetal bovine serum (FBS), 1% streptomycin, and 1% penicillin in Dulbecco's

modified Eagle's medium (DMEM). Cells were sub-cultured in 100 × 20 mm culture dishes at a density 2–3 × 10⁶ cells/mL to maintain growth conditions. Cells were passaged at 80% confluency and the passage number was maintained at under 20 during the experiment [7, 22].

Analysis of cell viability

by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Toxicity to macrophages was definite by reference to the method described by Carmichael et al. [23]. Aliquots of 0.18 mL of macrophages were utilized in 96-well plates at a density of 5 × 10⁴ cells/mL. Samples (2 mL) of various concentrations (6.25–50 µg/mL) was treated and incubated for 18 h at 37 °C employing 5% CO₂ incubator (311, Thermo Fisher Scientific, Waltham, MA, USA). Subdivision of 0.02 mL of a concentrated MTT solution (3 mg/mL) was added and after incubated for 3 h, medium was subduct and, 0.3 mL of DMSO was added to each well, and the reaction was permitted to proceed for 30 min at ordinary temperature. The OD at 540 nm was recorded (SPECTROstar Nano, BGM Labtech, Ortenberg, Germany) by applying an ELISA reader. The determination of toxicity to the cells was made via measurement of the OD of the sample and control groups in the calculation: cell viability rate (%) = (sample group/normal group) × 100. The control group was incubated in the same conditions excepted they were treated with equal amounts of DW instead of MTT.

Determination of NO production

Cells were cultured in 96-well plates at a density of 1 × 10⁵ cells/mL by incubation for over 12 h, at 37 °C. Then, LPS (1 µg/mL) was used to stimulate cells in all wells except for the normal group. Then 6.25–50 µg/mL of saucerneol D was added and the cells incubated for 18 h [7, 14, 24]. The supernatant of the cells medium and cells was used for measuring the concentration of NO by the Griess reagent system's kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol.

Western blot analysis

Measurements of iNOS and COX-2 protein expression levels were conducted using Raw 264.7 cell seeded in 6-well tissue culture plates at 5 × 10⁵ cells/mL. After incubating for 24 h in a CO₂ incubator at 37 °C, 1 µg/mL LPS and/or indicated concentrations of saucerneol D (25 µg/mL) were added, and the plates were incubated for 18 h and washed with PBS. Seventy microliters of a mixture of M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific.) with 1× protease inhibitor (Thermo Fisher Scientific.) was added to each well

and cells were lysed at 4 °C, followed by centrifugation at 16,000 rpm for 20 min at 4 °C (Gyrozen, Seoul, Korea) to remove insoluble debris. After separation, protein was transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Corp, Bedford, MA, USA) employing transfer cell machine (Hofer, Holliston, MA, USA) and incubated for 1 h in blocking buffer (5% skim milk) at ordinary temperature. Primary antibody's, iNOS (1:1000 in 5% skim milk; #sc-7271; Santa Cruz Biotechnology, Dallas, TX, USA), COX-2 (1:500 in 5% skim milk; #33345; Signalway antibody, Maryland, College Park, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000 in 5% skim milk; #MA5-15738; Thermo Fisher Scientific.), at 4 °C. Secondary antibody's, mouse anti-rabbit IgG-HRP (1:1000; sc-2357; Santa Cruz Biotechnology.), and goat anti-mouse IgG (1:1000; 31430; Thermo Fisher Scientific.); washed 3 times, then reacted with enhanced chemiluminescence (ECL) kit (Millipore Corp.) in a dark-room and, finally, subjected to X-ray film. Each protein band was definite employing Azure Biosystems (C300, Azure Biosystems inc., Dublin, CA, USA) [7, 14, 25].

Cytokine assay

Cells were cultured in 6-well plates at a density of 1×10^5 cells/mL by incubation for over 12 h, at 37 °C. Then, LPS (1 µg/mL) was used to stimulate cells in all wells except for the normal group. Then 6.25–50 µg/mL of saucerneol D was added and the cells incubated for 18 h. Gathered medium was saved at –80 °C before analysis. Enzyme immune-assay (EIA) kit was utilized for determining the content of prostaglandin E₂ (PGE₂), IL-1β, TNF-α, IL-6, etc. The concentrations of cytokines were definite employing a normal curve received from the reaction of standard materials [14–16, 26].

Statistical analysis

All tests were performed in triplicate (n=3). Statistical analysis was carried out employing one-way ANOVA in IBM SPSS Statistics 25 (Statistical Package for Social Science, Chicago, IL, USA). Duncan's multiple range tests were performed to analyze distinctions. A *P*-value of less than **P*<0.05, ***P*<0.01 was deemed to indicate a significant difference.

Results and discussion

Effect of elicitor on TPC and saucerneol D content and inflammatory activities on *S. chinensis*

As shown in Table 1, the TPC and saucerneol D contents was compared between the elicitor non-treated group and elicitor treated groups (1.5, and 3 mg/mL). As a result of measuring TPC and saucerneol D content in each group, both were higher in the elicitor-treated group than in the elicitor non-treated group [27].

Table 1 TPC, saucerneol D content, HAase inhibition, and expression levels of pro-inflammatory factors in *S. chinensis* extract treated by elicitor

Experiment	Control	Group A	Group B
TPC (mg/g)	7.6 ± 0.7	10.3 ± 0.3	11.4 ± 0.5
Saucerneol D content (µg/g)	248.8 ± 5.4	377.0 ± 11.6	449.9 ± 17.4
HAase inhibition (%)	89.6 ± 1.2	93.9 ± 2.9	95.9 ± 0.8
NO production (%)	86.8 ± 1.8	75.3 ± 1.9	68.1 ± 3.1
iNOS expression (%)	70.3 ± 0.9	35.6 ± 2.1	39.1 ± 1.4
COX-2 expression (%)	90.2 ± 5.1	56.1 ± 3.2	53.3 ± 2.7

Control: no elicitor treatment, group A: 4.5 mg/mL of treatment, group B: 9.0 mg/mL. Raw 264.7 cell were treated with 25 µg/mL of extracts from *S. chinensis* (dissolved in DW) for 1 h prior to addition of LPS (1 µg/mL). The cells were further incubated with LPS for 24 h. Control cells were incubated with vehicle alone. The concentrations of nitrite and nitrate in the culture medium were monitored as described in the experimental procedures. Values are presented as the mean ± error (n=6)

According to the above results, it can be confirmed that the useful component, which is a secondary metabolite of the plant, is increased due to the stress generated when the elite agent is treated [28]. In addition, we examined how increased saucerneol D affects hyaluronidase and pro-inflammatory-related factors, one of the enzymes involved in inflammation.

Effect of elicitor on HAase activity and inflammation response (NO, iNOS, and COX-2 expression) from *S. chinensis*

In general, it is a factor that plays an important role, such as removing bacteria and tumors by generating NO in vivo, but may be accompanied by genetic mutation, neurological damage, and inflammation when excessively caused by pathological processes such as disease [29, 30]. NO, PGE₂, cytokines, and chemokines, which are inflammatory factors induced and produced by LPS stimulation, are produced by iNOS and COX-2, proteins present in the cell nucleus. The anti-inflammatory effect is that Raw 264.7 cells are stimulated by LPS, reducing the level decrease of proteins such as iNOS and COX-2 that occur in the nucleus of the cell. In addition, anti-inflammatory effects can be expected by reducing TNF-α and IL-6, which are inflammatory cytokines caused by COX-2 protein [31].

As shown in Table 1, the HAase activity, and NO, iNOS and COX-2 production were compared between the elicitor non-treated group and elicitor treated groups (1.5, and 3 mg/mL). As a result of checking whether the elicitor treated groups extract is involved in NO, iNOS, and COX-2 expression, pro-inflammatory factor was adjusted according to the concentration of elicitor treated compared to the non-treated group. These results confirmed that the useful substances amplified during the growth

of *S. chinensis* through elicitor treatment inhibited pro-inflammatory factors. Therefore, referring to the report that saucerneol D contained in *S. chinensis* inhibits gastritis [27], we investigated how saucerneol D, which is expected as an active compound, affects Raw 264.7 cells.

Cell viability by saucerneol D from elicitor-treated *S. chinensis*

To evaluate the cytotoxicity of saucerneol D and ammonium pyrrolidinedithiocarbamate (APDTC), saucerneol D and APDTC was prepared at various concentrations (6.25–50 µg/mL) and treated in Raw 264.7 cells. As a result, saucerneol D and APDTC was determined that there was damage to the cells as it was expressed as 76, 83% at a concentration of 50 µg/mL, respectively (Fig. 1a). Therefore, in the subsequent experiment, the concentration was adjusted and the measurement was carried out.

Protein expression level of iNOS, COX-2 in LPS-induced Raw 264.7 cells

There are two types of NOS, constitutive NOS and iNOS. Especially iNOS, induced from stimulation, produces excess amounts of NO for a long duration and the NO produced activates guanyl cyclase, which causes cell toxicity. Consequently, a reduce in iNOS protein activity in cell exposed to LPS was expected when saucerneol D was given simultaneously to the cells, creating an inflammation inhibitory effect. When the COX-2 enzyme protein is activated and expressed, it increases inflammatory cytokines and chemokines

(MCP-1, TNF-α, TGF-β and IL-6). Therefore, the anti-inflammatory effect can be predicted by processing the sample to inhibit the COX-2 protein [32, 33]. The COX-2 protein expression of in monocytes is enhanced by the pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α). Also induce expression inhibition by glucocorticoid, IL-18 and IL-33. Consequently, selective inhibitors of COX-2 are target molecules for developing into treatments of inflammation.

The iNOS enzyme functions in way to defend from contagiousness pathogens, including viruses, and is involved in impaired immune function, circulatory disorders, various inflammatory diseases, and cancers. By confirming the inhibition of NO production by saucerneol D, the effect of saucerneol D on the expression of iNOS, a protein involved in NO production, was confirmed at the protein level through western blot. Figure as in 2a, at the concentration of saucerneol D 25 µg/mL, the same results were shown as the tendency to inhibit NO. The iNOS protein was expressed at 67%, confirming 33% inhibition compared to the control treated with only LPS. COX-2, a protein involved in PGE₂ and cytokine activation, was also regulated by saucerneol D (Fig. 2b). COX-2 protein showed 56% inhibition compared to the control treated with only LPS. Consequently, saucerneol D significantly regulated NO, iNOS involved in NO production, and COX-2, an important inflammatory factor involved in cytokine activation. Therefore, it was decided to examine the tendency of saucerneol D to cytokine, an important factor involved in inflammation induction.

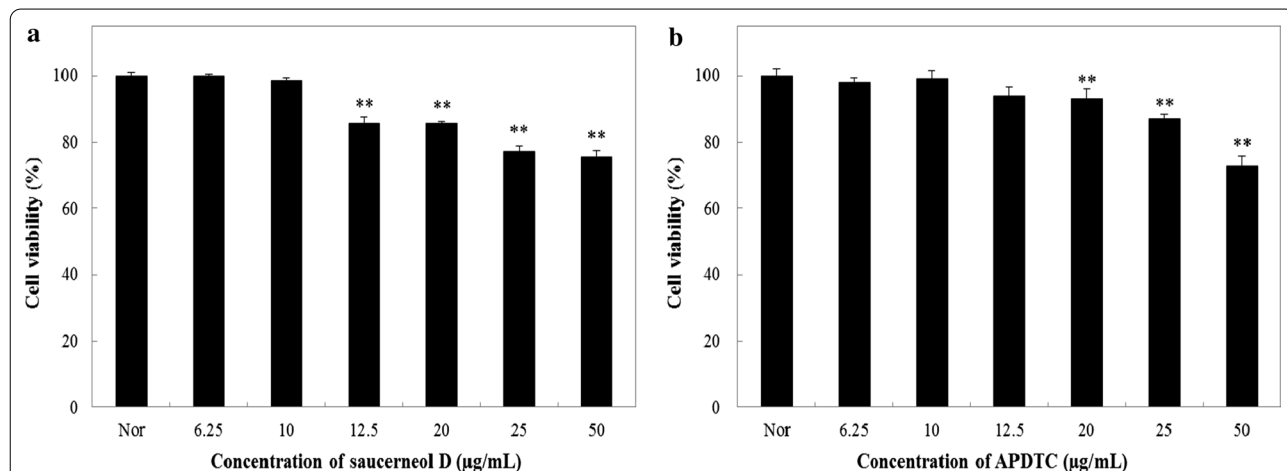
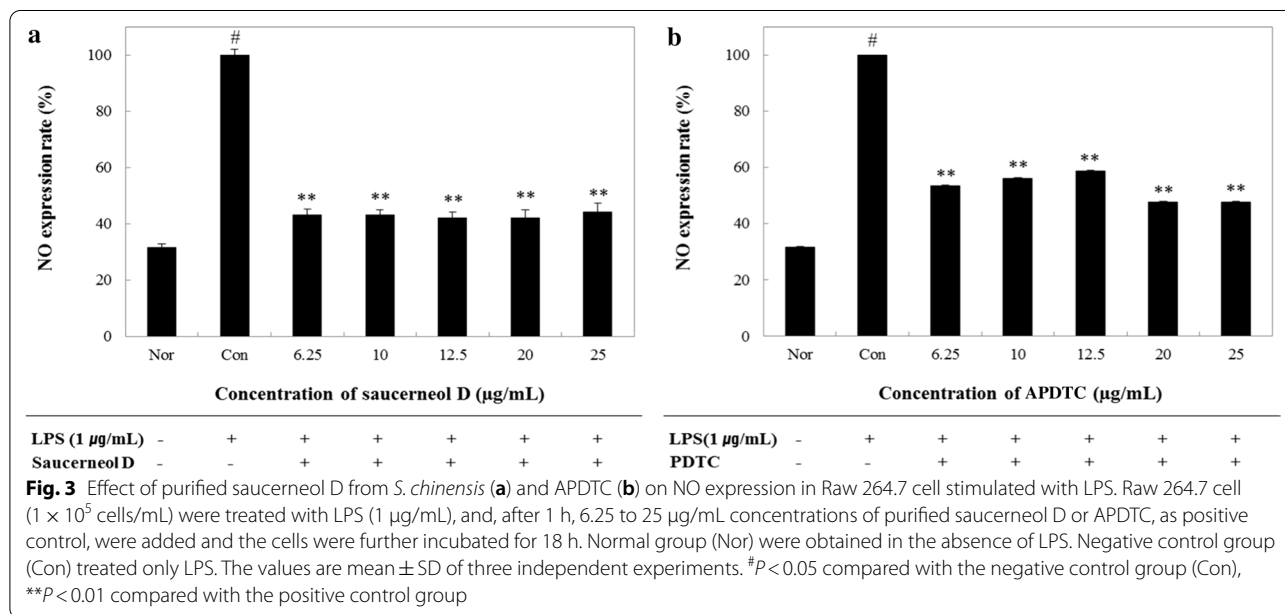
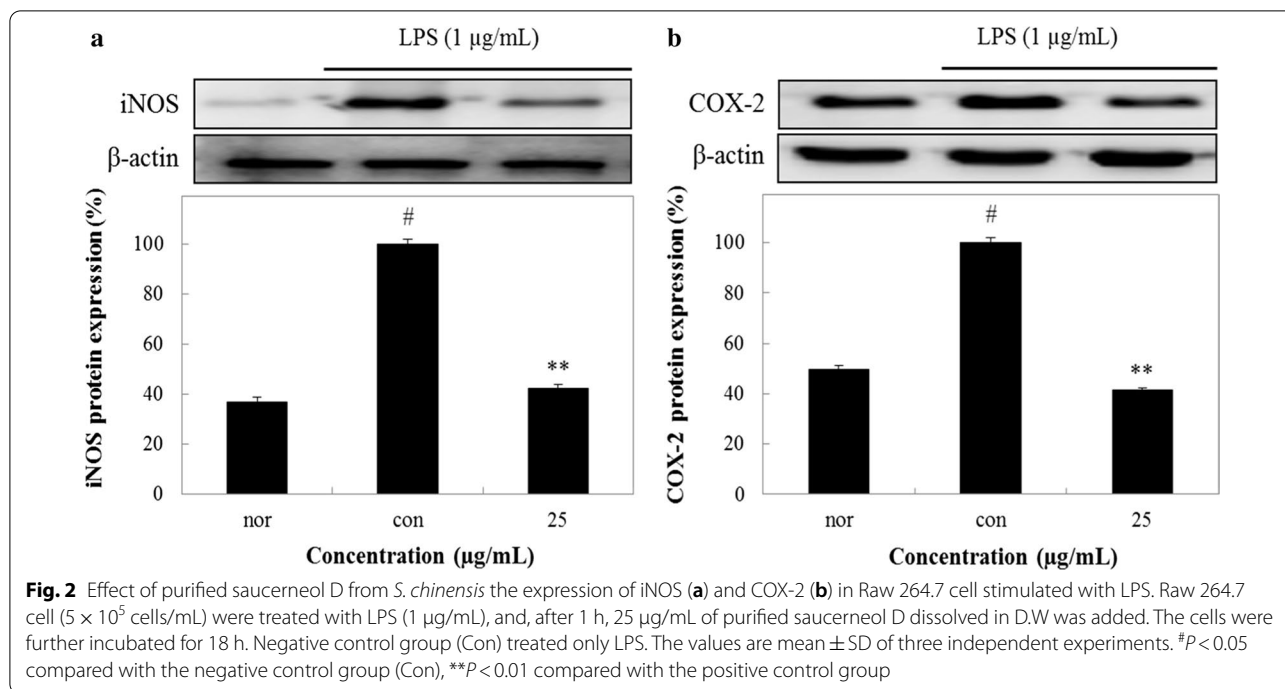
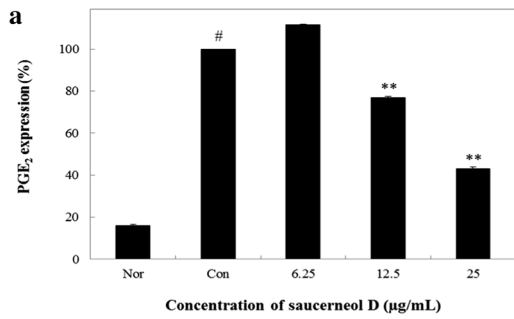


Fig. 1 MTT assay of purified saucerneol D (a) from *S. chinensis* and APDTC (b) in Raw 264.7 cell. Raw 264.7 cell (5×10^4 cells/mL) were incubated with various concentrations of saucerneol D (6.25–50 µg/mL) for 24 h. Normal group (Nor) were received in the absence of LPS. Data represent mean \pm S.D of three independent experiments (n = 3). The values are mean \pm SD of three independent experiments. **P < 0.01 compared with the positive control group

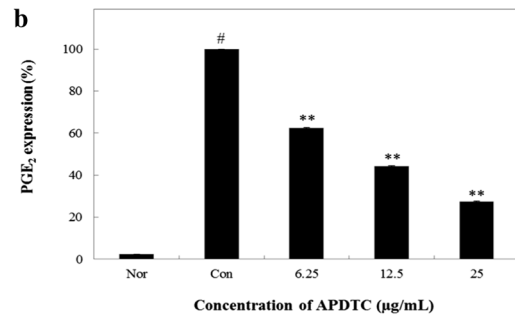


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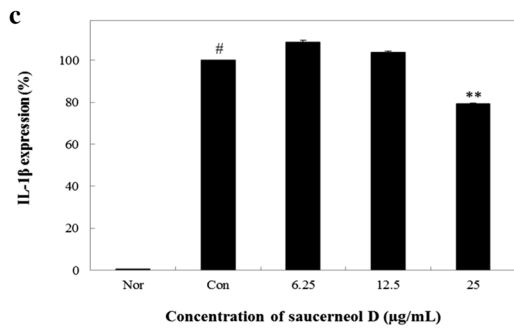
Fig. 4 Expression rate of purified saucerneol D from *S. chinensis* and APDTC on PGE₂ (a and b), IL-1 β (c and d), TNF- α (e and f), IL-6 (g and h) in Raw 264.7 cell stimulated with LPS. Raw 264.7 cell (1×10^5 cells/mL) were treated with LPS (1 $\mu\text{g/mL}$), and, after 1 h, 6.25 to 25 $\mu\text{g/mL}$ concentrations of purified saucerneol D or APDTC, as positive control, were added and the cells were further incubated for 18 h. Negative control group (Con) treated only LPS. The values are mean \pm SD of three independent experiments. $\#P < 0.05$ compared with the negative control group (Con), $*P < 0.05$, $**P < 0.01$ compared with the positive control group



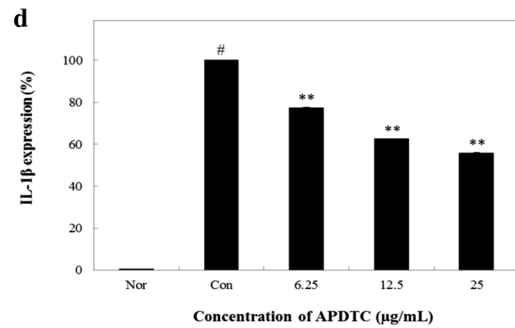
LPS (1 µg/mL)	-	+	+	+	+
Saucerneol D	-	-	+	+	+



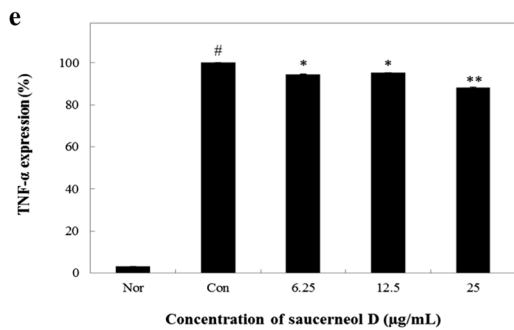
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APDTC	-	-	+	+	+



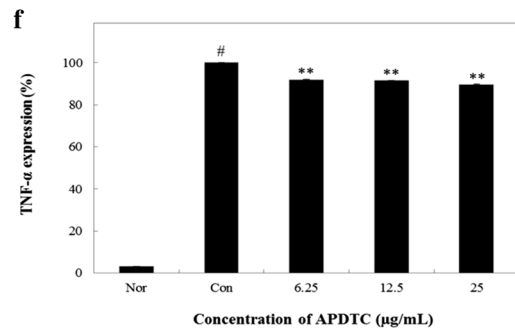
LPS (1 µg/mL)	-	+	+	+	+
Saucerneol D	-	-	+	+	+



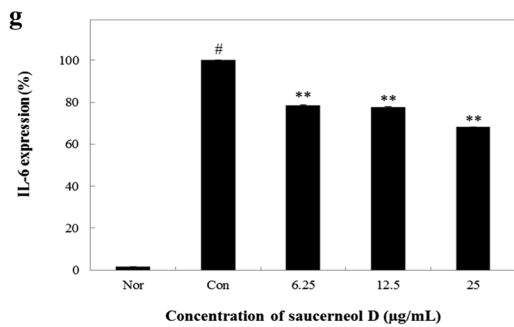
LPS (1 µg/mL)	-	+	+	+	+
APDTC	-	-	+	+	+



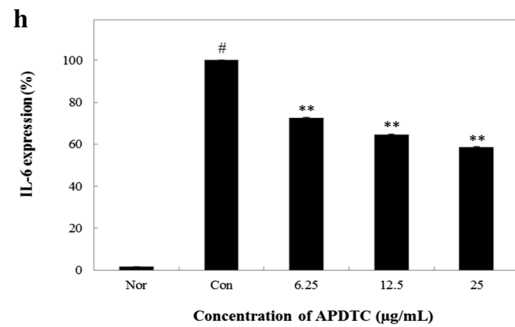
LPS (1 µg/mL)	-	+	+	+	+
Saucerneol D	-	-	+	+	+



LPS (1 µg/mL)	-	+	+	+	+
APDTC	-	-	+	+	+



LPS (1 µg/mL)	-	+	+	+	+
Saucerneol D	-	-	+	+	+



LPS (1 µg/mL)	-	+	+	+	+
APDTC	-	-	+	+	+

Effect of NO production by saucerneol D from elicitor-treated *S. chinensis*

PG, cytokine, NO, histamine, chemokine, bradykinin, adhesion molecule, and complement are known as mediators of inflammation. Among them, NO is a key mediator for several inflammatory reactions. NO is also very unstable because it is a free radical. NO is a free radical and a gaseous signaling molecule. However, excessive production of NO in the body's inflammatory response can damage cells or tissues or induce DNA mutations. NO is thus used as the most basic and important biological biomarker in identifying inflammatory responses [34, 35]. An excess of inflammatory factors causes the formation of NO, PGE₂, and cytokines by iNOS and COX-2 in the process of inflammation in the body. Normally, NO formation is significant important for destroying tumor cells and bacteria, but excess NO formation can cause inflammation, include nausea, dyspnea, impaired immune function, or neural damage [34–37].

By confirming that saucerneol D inhibits the iNOS protein, we examined whether the NO produced by iNOS was significantly reduced. To confirm NO production, cells were stimulated with LPS and then treated with saucerneol D adjusted to a concentration of 6.25–25 µg/mL. As a result, as in Fig. 3a, the NO expression level was 44% at the concentration treated with saucerneol D 25 µg/mL. Therefore, it showed 66% inhibition of NO production compared to the control treated with only LPS. NO production was suppressed even at the lowest concentration of 6.25 µg/mL. Compared to APDTC, which is a positive control, saucerneol D showed greater inhibition effect. Therefore, it was examined how it affects the cytokine by inhibiting both NO inhibition, which plays an important role in cell signaling, and iNOS protein, which plays a catalytic role in NO production.

Determination of pro-inflammatory-related cytokine expression level with saucerneol D

To determine how saucerneol D affects the expression of pro-inflammatory cytokines, macrophages were treated with LPS and then saucerneol D. The results are as shown in Fig. 4. As shown in Fig. 4a, c, e, and g, PGE₂ and cytokines, which are factors involved in inflammation by being activated by COX-2, showed a tendency to be significantly suppressed by saucerneol D treatment. These results are similar to those of the inflammatory expression factors iNOS and COX-2 proteins. In addition, it was used as an anti-inflammatory drug and showed similar inhibitory power to APDTC (Fig. 4b, d, f, and h) used as a positive control in the experiment. Therefore, it was expected that saucerneol D could be developed as

a functional material by confirming that it is involved in the expression of pro-inflammatory-related factors [NO, iNOS, and COX-2, PGE₂, cytokines (IL-1β, TNF-α, IL-6)] that are involved in inflammation.

Abbreviations

NO: Nitric oxide; iNOS: Inducible NO synthase; COX-2: Cyclooxygenase-2; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PGE₂: Prostaglandin E₂; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α; TPC: Total phenolics content; LPS: Lipopolysaccharide; HAase: Hyaluronidase; APDTC: Ammonium pyrrolidinedithiocarbamate.

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Authors' contributions

EHL analyzed the data and wrote the paper. EHL edited the manuscript. YJC supervised the work. YJC revised and edited the manuscript and supervised the work. Both authors read and approved the final manuscript.

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Availability of data and materials

All data analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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