Elicitor treatment potentiates the preventive effect of *Saururus chinensis* leaves on stress-induced gastritis

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Abstract In this study, gastritis inhibitory substances were ethanol-extracted from *Saururus chinensis* leaves as a part of ongoing research on natural bioactive substances. Comparing *S. chinensis* extracts with and without elicitor treatment showed that total phenolic compounds in the leaves increased with increasing elicitor treatment. The contents of avicularin, manassantin A, manassantin B, and saucerneol D in the leaf, known gastritis inhibitory compounds, increased as elicitor treatment increased. *S. chinensis* extracts were administered to mice in a single oral dose of 0.25–2 g/kg, resulting in no observable toxicity after 1 week. *S. chinensis* ethanol extracts were administered to mice at a dose of 500 mg/kg before induction of gastritis by water-immersion restraint method. Macroscopic gastric hemorrhage and microscopic gastric damage assessed with hemorrhage, edema, epithelial cell damage, inflammatory cell infiltration, and ulcer were reduced by *S. chinensis* ethanol extracts. The elicitor-treated group showed a greater inhibitory effect on macroscopic and microscopic gastric damage. Elicitor-treated *S. chinensis* extracts inhibited gastritis more than non-treated *S. chinensis* extracts did, most likely due to greater anti-inflammatory effects. These results indicate that elicitor-treated *S.*

chinensis extracts could be effective to prevent gastritis and could be used as a medicinal material source.

Keywords Anti-inflammation · Elicitation · Inhibitory activity · *Saururus chinensis* · Stress-induced hemorrhagic gastritis

Introduction

Interests in natural biological compounds have increased as demands for functional foods have risen due to stressful lifestyles. Methods of increasing extraction yield, among other techniques, have improved as processing methodologies have progressed [1]. Medicinal plants have long been known for their medicinal activity and have been used in Asian cultures. In S. Korea, research is ongoing to appreciate the medicinal values of plants, a process that includes collecting, storing, and breeding of plant varieties [2, 3]. Elicitation is one method used to amplify the content of medicinal compounds in a plant [4]. Elicitation is used in plant incubation, but rarely in the garden setting. In the current study, an elicitation method was used with garden culturing to amplify gastritis inhibitory compound content in plant leaves.

Saururus chinensis Baill. is categorized in the *S. chinensis* plant group. In oriental medicine, edema, detoxification, glycosuria, hypertension, hepatitis, jaundice, and other symptoms are treated with *S. chinensis* Baill. [5, 6]. Leaves of *S. chinensis* Baill. contain phenolic compounds, such as quercetin, quercetrin, isoquercitrin, avicularin, and rutin. The root contains tannin, which has been used to treat cancers and adult diseases [7–9]. It also has been reported

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to exhibit hepatoprotective [10], anti-bacterial, and capillary-strengthening effects [11].

When acid secretion increases or defense mechanisms are weakened by stress or other factors, epithelial cells of the stomach can be damaged, causing gastritis. Gastritis can develop into gastric ulcers and chronic gastritis, which are related to stomach cancer [12, 13]. Medicines used to treat gastritis are based on acid secretion inhibition or neutralization [12], protecting epithelial cells from the acid [13], or using bismuth to treat *Helicobacter pylori* [14, 15]. Patients with gastritis but no overt symptoms may not seek treatment, which can lead to chronic gastritis [16, 17]. Gastritis may recur easily and require long-term medication, which can be stressful. Therefore, ingesting compounds that inhibit inflammatory activity and strengthen epithelial cell defense mechanisms may reduce the recurrence of gastritis [18].

In this study, we describe the treatment of garden-cultured *S. chinensis* with elicitor to produce a plant source containing a high yield of medicinal compound. The medicinal compound content of extracts and their gastritis inhibitory effects were compared among elicitor-treated *S. chinensis* and non-treated controls to identify the plant as an effective functional source.

Materials and methods

Production and application of elicitor

The elicitor was produced by treating yeast extract with protease and adding ethanol to generate a precipitate. The precipitate was collected and dried to produce yeast extract powder. One kilogram of yeast extract powder was homogenized with 5 g of CuCl_2 to produce the elicitor powder [4].

The elicitor was applied three times at 1.5 mg/l (A group) and 3 mg/l (B group) per application. The first application was carried out when the growth of *S. chinensis* leaves was approximately one-fourth their full size (April 30th). The elicitor was sprayed directly at the bottom part of the leaves in a 4 m² area to 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, in each 4 m² area until harvest.

Preparation of ethanol extracts from *S. chinensis* (SCE) and elicitor-treated *S. chinensis* (ET-SCE)

Dried *S. chinensis* powder, either untreated control (SC) or elicitor-treated (ET-SC), was homogenized with 100 ml of

60% ethanol at 20,000 rpm for 1 min and extracted by shaking for 24 h. Extracts were filtered with Whatman No. 1 filter paper (GE Healthcare Company, Buckinghamshire, UK), concentrated with a rotary vacuum evaporator (Eyela NE, Tokyo, Japan), and then lyophilized for further experiments.

The determination of total phenolics, manassantin A, manassantin B, saucerneol, and avicularin content

Total phenolics were determined as per the Folin–Denis method [19]. Optical density for total phenolics determination was measured within 1 h after color development by 1 N Folin–Ciocalteu reagent at 725 nm, and total phenolics calculated using a gallic acid standard curve. The content of avicularin, manassantin A, manassantin B, and saucerneol D was determined via HPLC assay and calculated using standard curves that were generated with purified manassantin A, manassantin B, saucerneol D, and avicularin, respectively.

Animal studies

Seven-week-old C57BL/6J male mice were purchased from Samtaco Co. (Seoul, S. Korea) and raised in a clean area in Yeungnam University Medical School. Lights were on for 12 h from 7 a.m. to 7 p.m. Food and water intake were monitored. Mice were treated following the protocols of Yeungnam University Medical School, and experiments were conducted under approval of the Animal Ethics Committee (Approval No.: YUMC-AEC2011-001).

Acute toxicity of ET-SCE

C57BL/6J mice were separated into six different groups randomly for determination of acute toxicity of ET-SCE. Mouse weight and the amount of food consumed per day were determined before drug administration. ET-SCE (from 9.0 mg/3 l) of 0, 0.25, 0.5, 1, 1.5, and 2 g/kg was administered as a single oral dose, and mouse survival and behavioral patterns were monitored. Anesthetic (tiletamine–zolazepam [25 mg/kg], xylazine [10 mg/kg]) was injected intraperitoneally after 7 days. Blood plasma was collected, and the weights of peripheral tissue were determined. To assess hepatotoxicity, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were measured using a colorimetric method according to the manufacturer's instructions (Asan Pharma, Seoul, South Korea).

Gastritis induction

Gastritis was induced by water-immersion restraint stress (WIRS) in mice according to previously published reports [20]. Briefly, overnight fasted mice were caged individually and immersed to the xiphoid process in a 25 °C water bath for 6 h. Control mice were caged individually without immersion in water.

Effect of SCE and ET-SCE on gastritis

Male C57BL/6J mice were separated into four different groups (control, WIRS, WIRS + SCE 500 mg/kg, and WIRS + ET-SCE 500 mg/kg). All food was removed from the cages at 6 p.m. on the day before experimentation. At 9 a.m. the next day, the control and WIRS groups were given distilled water, and the WIRS + SCE and WIRS + ET-SCE groups were given 500 mg/kg SCE or 500 mg/kg ET-SCE, respectively. The WIRS, WIRS + SCE, and WIRS + ET-SCE groups were subjected to the WIRS gastritis experimental procedure, as described above. The mice were anesthetized via intraperitoneal injection of tiletamine–zolazepam and xylazine, and blood samples were collected from the retro-orbital plexus. For analysis of macroscopic gastric damage, the stomach was opened along the greater curvature and photographed by digital camera (Nikon, Tokyo, Japan), and then the visible hemorrhagic area was measured using Image J software (National Institutes of Health, Bethesda, MD, USA). Hemorrhagic area was calculated as the percentage of hemorrhagic area to total stomach area. Another set of mice was anesthetized, and half of each stomach was fixed in 10% buffered formalin, embedded in a paraffin block, sliced into 4- μ m-thick sections, and then stained with hematoxylin and eosin. The scoring system for microscopic gastric damage (Table 1) followed that outlined in the previous study [21]. The other half of each stomach was frozen at -80 °C for real-time PCR analysis [22].

Table 1 Histological score for quantifying the degree of gastric damage

Criterion	Histological score					
	0	1	2	3	4	5
Hemorrhage	–	↑	↑↑	↑↑	–	–
Epithelial damage	–	↑	↑↑	↑↑↑	–	–
Inflammation	–	↑	↑↑	↑↑↑	↑↑↑↑	↑↑↑↑↑
Ulcer	–	–	–	–	–	↑

↑, Increased

Fig. 1 Content of phenolics in extracts over time. **A** Control (no elicitor treatment), **B** group A (4.5 mg elicitor/4 square meter area), **C** group B (9.0 mg elicitor/4 m² area)

Method of histological score for quantifying the degree of gastric damage

Transgenic control rats were either kept germfree ($n = 3$) or conventionalized with SPF bacteria ($n = 3$) as described above. A gnotobiotic rat colonized solely with CRAS flora [23] was obtained from Charles River Inc. Bacteria in the DESEP cocktail were isolated from Crohn's disease patients by Prof. Marteen Hazenberg, Erasmus University, Rotterdam, The Netherlands [24] (*Eubacterium contortum* and *Peptostreptococcus productus*) and by the Clinical Microbiology Laboratory of the University of North Carolina Hospitals, Chapel Hill, NC (*E. coli* and *S. avium*).

Real-time PCR

Gene expression was analyzed using real-time PCR. For RNA extraction, stomach tissue was added to 1 ml TRI Reagent® (Sigma Chemical Co., St. Louis, MO, USA) and homogenized with ultrasonication. Two hundred microliters of chloroform was added, and the mixture vortexed for 15 s. The mixture was placed at 4 °C for 5 min and then centrifuged for 15 min at 16,100g. An equal volume of isopropanol was added to the supernatant, and the mixture was centrifuged for 10 min at 4 °C and 16,100g. The RNA pellet was washed with 1 ml of cold 75% ethanol, centrifuged for 5 min at 4 °C and 16,100g, and then dried at 25 °C. The dried RNA precipitate was dissolved in 30 µL RNase-free water and stored at −70 °C. RNA concentration was measured based on absorbance at 260 nm with the NanoDrop™ (Thermo Scientific, Wilmington, DE, USA). Real-time PCR was performed with cDNA, made with 1 µg RNA and the High-Capacity cDNA Reverse Transcription Kit (Applied Bio-systems, Foster City, CA, USA). The real-time PCR cycling conditions were as follows: polymerase activation at 95 °C for 10 min, followed by 45 cycles of denaturing (95 °C, 15 s), annealing (55 °C, 20 s), and extension (72 °C, 35 s). Amplification was monitored with the Real-Time PCR 7500 System, using Power SYBR Green PCR Master Mix

Fig. 2 Effect of once oral administration of ET-SCE on mouse weight, organ weight, and liver enzyme activities. **A** Body weight, **B** feeding amount/day, **C** liver weight, **D** heart weight, **E** kidney weight, **F** spleen weight, **G** GOT, **H** GPT. $p < 0.05$. The dose of ET-SCE is shown under each bar. ET-SCE, elicitor-treated *Saururus chinensis* extract; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase. Values are presented as mean $\pm$ error ($n = 6$)

(Applied Bio-systems). β -Actin was used as the standard, and the primer sequences were interleukin-1 β (IL-1 β , sense: 5'-GCCCCATCCTCTGTGACTC-3', antisense: 5'-AGTGCAGCTGTCTAATGGGA-3', 71 bp), interleukin-6 (IL-6, sense: 5'-GTCTG GAGGCTTAATTACACATG-3', antisense: 5'-TCAGAATTGCCATTGCCAT TGCACA-3', 72 bp), cyclooxygenase-2 (COX-2, sense: 5'-TCCACAGCCTACCAAA ACA-3', antisense: 5'-TCAGTTGAACGCCTTTTAAT-3', 100 bp), and β -actin (sense: 5'-TGGACAGTGAGGCAAGGATAG-3', antisense: 5'-TACTGCCCTGGCTCC TAG CA-3', 121 bp).

Statistical analysis

Results are shown as the mean $\pm$ error. Statistical differences between groups were determined by one-way ANOVA with Tukey's post hoc analysis. A p value < 0.05 was considered significant.

Results and discussion

Effect of elicitor on total phenolic compounds extracted from *S. chinensis*

As shown in Fig. 1, the total phenolic compounds that were extracted were compared between the SC and ET-SC groups. Elicitor-treated groups A and B showed higher levels of phenolic compounds compared to those of the non-treated groups. These results indicate that elicitor-induced stress of the plant increased production of secondary

Table 2 Change in the content of biological compounds in *Saururus chinensis* after elicitor treatment

Biological compound	Content of active compound (µg/leaf g)		
	Group		
	Control ^a	A	B
Avicularin	19.2 $\pm$ 0.3 ^b	26.2 $\pm$ 1.7 ^c	29.7 $\pm$ 1.1 ^d
Manassantin A	94.4 $\pm$ 3.6 ^b	124.5 $\pm$ 8.2 ^c	138.6 $\pm$ 3.4 ^d
Manassantin B	68.0 $\pm$ 2.7 ^b	89.6 $\pm$ 6.4 ^c	107.8 $\pm$ 5.9 ^d
Saucerneol D	248.8 $\pm$ 5.4 ^b	377.0 $\pm$ 11.6 ^c	449.9 $\pm$ 17.4 ^d

^aControl: no elicitor treatment, group A: 4.5 mg elicitor/4 m² area of treatment, group B: 9.0 mg elicitor/4 m² area of treatment. Values are presented as the mean $\pm$ error ($n = 6$)

Fig. 3 Effect of SCE and ET-SCE on surface area of stomach bleeding induced by WIRS. Representative images are shown in the upper photographs, and plots of quantified data are shown below. Values are presented as mean $\pm$ error ($n = 6$). * $p < 0.05$ versus control and # $p < 0.05$ versus WIRS. SCE, *Saururus chinensis* extract; ET-SCE, elicitor-treated *Saururus chinensis* extract; WIRS, water-immersion restraint stress-induced (gastritis model)

metabolites. Among these secondary metabolites, avicularin, manassantin A, manassantin B, and saucerneol D, which are gastritis inhibitory compounds, likely increased [4].

Effect of elicitor on the content of gastritis inhibitory compounds from *S. chinensis*

It has been reported by Cho et al. [4] that the gastritis inhibitory compounds in *S. chinensis* are avicularin, manassantin A, manassantin B, and saucerneol D. In the present study, the effect of elicitor treatment on total gastritis inhibitory compound levels was determined. As shown in Table 2, elicitor-treated groups A and B had 26.2 and 29.7 μg , respectively, compared to 19.2 μg in the non-treated control group. Manassantin A, manassantin B, and saucerneol D were higher in group A than in the control group. In addition, group B showed an increased content of these compounds compared to that of group A. These results indicate that elicitor treatment amplifies these target compounds during the growth of *S. chinensis*. Total compound content is expected to affect biological activity, including gastritis inhibitory and WIRS inhibitory activity.

Acute toxicity determinations of ET-SCE

Body weight and food intake of mice administered 0, 0.25, 0.5, 1, 1.5, or 2 g/kg ET-SCE as a single oral gavage were not significantly different among the groups (Fig. 2A, B). In addition, liver, heart, kidney, and spleen weights were

not significantly different among groups (Fig. 2C–F). To determine acute toxicity, blood samples were analyzed for GOT and GPT. As shown in Fig. 2G, H, no difference was observed in GOT and GPT levels, indicating no hepatotoxicity. Therefore, ET-SCE produced no detectable acute toxicity.

Comparison of gastritis inhibitory effect of SCE and ET-SCE

Mice were given 500 mg/kg of either SCE or ET-SCE, and gastritis was induced with WIRS for 6 h. Both extracts were able to inhibit stomach hemorrhaging, but the effect with ET-SCE was greater than that of SCE (Fig. 3). The tissue was stained with hematoxylin and eosin, and gastric damage assessed. As shown in Fig. 4, untreated controls showed normal epithelial cells and lamina propria, but the stomach of WIRS mice showed severe epithelial cell damage and edema in the lamina propria. Furthermore, bleeding, inflammatory cell infiltration, and ulcers were found in the stomachs of WIRS mice. However, pretreatment with SCE or ET-SCE resulted in the reduction in these pathological changes (Fig. 4A–D). Microscopic analysis of gastric damage via a scoring system also showed that the inhibitory effect of SCE treatment was apparent, but it was not as strong as that of ET-SCE (Fig. 4E). Therefore, both SCE and ET-SCE show inhibitory effects on the edema and cell damage induced by WIRS, but the gastritis inhibitory effect of ET-SCE was greater.

Fig. 4 Hematoxylin and eosin stained stomach tissue.

A Bleeding in WIRS group, **A1** ulcer in WIRS group, **A2** epithelial cell damage in WIRS group, **A3** inflammatory cells in WIRS group, **B** WIRS + SCE 500 mg/kg treated group, **C** WIRS + ET-SCE 500 mg/kg treated group, **D** control, **D1** epithelial cells in control, **E** effect of SCE and ET-SCE on the gastric damage assessed by microscopic analysis according to scoring systems. Bars on images are equal to 50 μ m. SCE, *Saururus chinensis* extract; ET-SCE, elicitor-treated *Saururus chinensis* extract; WIRS, water-immersion restraint stress-induced (gastritis model). Values are presented as mean $\pm$ error ($n = 6$). * $p < 0.05$ versus control and # $p < 0.01$ versus WIRS

Effect of ET-SCE on inflammatory cytokine expression

IL-6 and IL-1 β are secreted from monocytes, macrophages, B cells, dendritic cells, endothelial cells, neutrophils, and hepatocytes. IL-6 and IL-1 β take part in various immune responses with tumor necrosis factor (TNF)- α and IL-2, as pro-inflammatory cytokines. IL-1 β is involved with the activation of T cells, maturation of B cells, and activity of NK cells. Inflammatory cytokine mRNA expression was measured and showed that IL-6 mRNA expression

significantly increased in the WIRS group. Both ET-SCE (WIRS) and SCE (WIRS) showed lower cytokine expression, but the effect was greater with ET-SCE treatment. IL-1 β showed a similar trend as IL-6, but the effects were less pronounced and were not statistically significant. COX-2 mRNA expression increased in the WIRS group and decreased in both ET-SCE and SCE treatment groups. However, ET-SCE showed a greater inhibitory effect on COX-2 expression (Fig. 5). Therefore, elicitor was able to amplify the production of WIRS gastritis inhibitory compounds in the plants, and higher concentrations of

Fig. 5 Effect of SCE and ET-SCE on expression of inflammatory cytokines [IL-6 (A), IL-1 β (B), COX-2 (C)] induced by WIRS gastritis. * $p < 0.05$ versus control and # $p < 0.01$ versus WIRS.

Values are presented as mean $\pm$ error ($n = 6$). SCE: *Saururus chinensis* extract, ET-SCE: elicitor-treated *Saururus chinensis* extract, WIRS: water-immersion restraint stress-induced gastritis model

inhibitory compounds increased the gastritis inhibitory activity, possibly by reducing inflammatory cytokines. Park and Cho [22] reported that *S. chinensis* extracts had anti-inflammatory activity by reducing inflammatory cytokines. From our results, ET-SCE was shown to have greater gastritis inhibitory and anti-inflammatory effects, and to reduce the expression of IL-6, IL-1 β , and other cytokines.

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