J. Soc. Cosmet. Scientists Korea
Vol. 32, No. 1, March 2006, 59-64

문주란의 항염효과와 화장료적 특성

김 영 희 · 김 기 수 · 박 선 희 · 이 수 희 · 김 영 진* · 김 영 실* · 김 종 헌* · 김 기 호†

㈜ 바이오랜드 생명공학연구소, *㈜ 참존 중앙 연구소
(2006년 1월 27일 접수, 2006년 2월 13일 채택)

Anti-inflammatory Activity of *Crinum asiaticum* Linne var. Japonicum Extract and its Application as a Cosmeceutical Ingredient

Young Heui Kim, Ki Soo Kim, Sun Hee Park, Soo Hee Lee, Young-Jin Kim*, Young-Sil Kim*, Jong-Heon Kim*, and Ki Ho Kim†

R&D Center, Bioland Ltd., Songjeong, Byongchon, Cheonan-si, Chungnam 330-863, Korea
*R&D Center, Charmzone
(Received January 27, 2006; Accepted February 13, 2006)

요 약: 문주란(*Crinum asiaticum* Linne var. japonicum)은 한국, 말레이지아 등 동남아시아 지역에서 관절통, 해열, 궤양 치료, 국부의 소염 및 해열 등의 목적에 민간요법 등으로 오래 전부터 사용되어 오고 있다. 본 연구에서는 이러한 문주란의 화장료 조성물로서의 가능성을 확인하기 위하여 iNOS (inducible nitric oxide synthase) 억제 그리고 PGE2, IL-6, and IL-8 방출 억제에 의한 항염 효능을 측정하였다. pH를 3.5로 조절하고 95% ethanol을 사용하여 추출한 후, HPLC 실험으로 문주란의 주성분이 면역조절물질로 잘 알려진 lycorine이며 대략 1% 정도 함유한 것을 확인하였고 그 함량은 문주란의 추출 부위 및 추출방법에 따라 달랐다. 리포다당(lipopolysaccharide, LPS)에 의해 활성화된 생쥐 대식세포(RAW 264.7 cells)에서 생성되는 NO 형성의 억제능을 측정한 항염실험에서, 문주란 에탄올 추출물은 투여량에 비례하는 저해능을 가지고 있음을 확인할 수 있었다(IC_{50} = 83.5 μg/mL). 또한 RT-PCR법을 이용하여 문주란 에탄올 추출물이 iNOS 유전자 발현도 억제함을 확인하였다. 또한, 문주란 추출물은 전혀 세포독성을 보이지 않았으며 오히려 LPS에 대하여 세포증식효과를 나타내었다(세포생존율 약 10~60% 증가). 인간의 섬유아세포에서 과산화수소에 의해 활성화된 PGE2, IL-6 및 IL-8 방출 억제효능 측정 실험에서는 0.05%와 1% 농도 이상에서 (PGE2와 IL-6의 분비가) 거의 완전히 억제됨을 확인할 수 있었고, 나아가서 IL-8의 경우는 모든 실험농도 범위에서 완전히 억제되었다(> 0.0025%). 이러한 결과로부터 문주란의 추출물이 충분한 항염 효과를 가지고 있음을 확인할 수 있었다.

Abstract: *Crinum asiaticum* Linne var. *japonicum* has long been used as a rheumatic remedy, an anti-pyretic, an anti-ulcer treatment, and for the alleviation of local pain and fever in Korea and Malaysia. In order to investigate the possibility of *Crinum asiaticum* Linne var. *japonicum* extract as a cosmetic ingredient, we measured its anti-inflammatory effect by inhibition of iNOS (inducible nitric oxide synthase), and the release of PGE2, IL-6, and IL-8. HPLC experiment after extraction with 95% ethanol at pH 3.5 showed that *Crinum asiaticum* Linne var. *japonicum* was mainly composed of lycorine (up to 1%), a well-known immunosuppressant. The content of lycorine varied depending on the type of tissue analyzed and the extraction method. In anti-inflammatory assay for inhibition of nitric oxide formation on lipopolysaccharide (LPS)- activated mouse macrophage RAW 264.7 cells, the ethanolic extract of *Crinum asiaticum* showed inhibitory activity of NO production in dose-dependent manner (IC_{50} = 83.5 μg/mL). Additional study by RT-PCR demonstrated that the extract of *Crinum asiaticum* significantly suppressed the expression of the iNOS gene. Moreover, the extract of *Crinum asiaticum* did not show any cytotoxicity, but did show cell proliferation effect against LPS (10~60% increase of cell viability). In an assay to determine inhibition of the H_2O_2-activated release of PGE2, IL-6, and IL-8 in human normal fibroblast cell lines, the release of PGE2 and IL-6 was almost completely inhibited above concentrations of 0.05% and 1%, respectively. Moreover, the release of IL-8 was completely inhibited over the entire range of concentrations (> 0.0025%). The result showed that the extract of *Crinum asiaticum* Linne var. *japonicum* has sufficient anti-inflammatory effect. Therefore, *Crinum asiaticum* Linne var. *japonicum* extract may be useful as an ingredient of cosmetic products.

Keywords: *Crinum asiaticum, anti-inflammatory effect, cosmeceuticals, iNOS*

† 주 저자 (e-mail: biolandrnd@biolandkorea.com)

1. Introduction

Crinum asiaticum Linne var. *japonicum* (Family: Amaryllidaceae), is an herbaceous plant of small to moderate size. The plant is distributed on South Korea's Jeju Island, and was designated as the 19th of Korea's Natural Treasures. *Crinum asiaticum* has been used as a rheumatic remedy, an anti-pyretic, an anti-ulcer treatment, and for the alleviation of local pain and fever in Korea and Malaysia.

The anti-inflammatory activity of *Crinum asiaticum* and its effects on bradykinin-induced contractions in an isolated uterus have been reported [1]. Analgesic and anti-inflammatory activities of an aqueous extract of *Crinum glaucum*, also of the Amaryllidaceae family, have also been reported [2]. Amaryllidaceae alkaloids, lycorine and lycoricidinol, were isolated from the dried stems of *Crinum asiaticum* and identified by chromatographic and spectral analysis. It has been reported that lycorine, the active molecule, has inhibitory effect on tumor cell apoptosis induced by polymorphonuclear leukocyte-derived calprotectin [3]. In a report by the same authors, lycorine was also reported to have an inhibitory effect on macrophage TNF-α production [4].

Although few pharmacological studies have been reported as listed above, this plant has not been subjected to a systematic cosmeceutical evaluation to determine its propriety in practical application.

In this study: in order to investigate the possibility of *Crinum asiaticum* Linne var. *japonicum* extract as a cosmetic ingredient we measured its anti-inflammatory effect by inhibition of iNOS (inducible nitric oxide synthase), and the release of PGE2, IL-6, and IL-8, which are known as the cytokines associated with inflammation. *Crinum asiaticum* Linne var. *japonicum* extract showed negligible cytotoxicity and good anti-inflammatory activities, suggesting that this extract may be useful as a cosmeceutical ingredient.

2. Material and Methods

2.1. Materials

The root of *Crinum asiaticum* Linne var. *japonicum* was purchased from Jeju botanic garden (Jeju, The Republic of Korea) and chopped before use. Human dermol fibroblasts were acquired from the ATCC (American Type Culture Collection, CRL-2076). The mouse macrophage-like cell line, Raw 264.7, was purchased from the ATCC (TIB-71). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), lycorine·HCl, and Griess soution were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle's Media (DMEM), fetal bovine serum, and antibiotics were purchased from Life Technologies (Grand Island, NY, USA).

2.2. Preparation of the Ethanolic Extract of *Crinum asiaticum*

The dried root of *Crinum asiaticum* Linne var. *japonicum* (0.34 kg) was extracted with 95% ethanol (1 kg) at 75℃ for 3 h. pH was adjusted to 3.5 by adding 12 N HCl. After filtration through 400-mesh filter cloth, the filtrate was filtered again through filter paper (Whatman, No. 5). The ethanolic solution was evaporated to remove the solvent under 50℃. Lyophilization over 1 day yielded a yellowish powder (28.3 g, yield = 8.32%).

2.3. Analysis of Lycorine Content by HPLC

The ethanolic extract powder of *Crinum asiaticum* Linne var. *japonicum* and lycorine · HCl (Sigma, used as a standard) were dissolved in 50% methanol. Lycorine content was investigated and calculated by HPLC (condition: column, Capcell Pak C18 (4.6 × 250 mm, Shiseido); mobile phase, 0.05 M KH_2PO_4 and acetonitrile (950:50); flow rate, 1.0 mL/min; detector, UV λ = 290 nm; injection volume, 20 μL).

2.4. Cell Culture

Cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS (Fetal Bovine Serum), 50 U/mL penicillin, and 50 g/mL streptomycin. Media was changed every 2 or 3 days. Cells were cultured at 37℃ in a humidified atmosphere of 95% air and 5% CO_2.

2.5. Cytotoxicity Assay

Raw 264.7 was seeded into 24-well plates at density of 8×10^5 cells and cultured at 37℃ in 5% CO_2. After 1 day, fresh medium containing 10% serum was added to cells, which were then treated with LPS (lipopolysaccharide, 1 μg/mL), followed by the treat-ment with the

ethanolic extract of *Crinum asiaticum* for 24 h. Cytotoxicity was evaluatedby MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) assay [5]. After 24 h, 100 μL of 2.5 mg/mL MTT was inserted into each well, and plates were incubated at 37℃ for an additional 4 h. Then, the media containing MTT was discarded and MTT formazan product was extracted with 1 mL DMSO. The amount of formazan in the culture medium was determined by the absorbance measured at 570 nm by ELISA reader. Cell viability was calculated as:

$$\text{cell viability } (\%) = (OD_{570(sample)}/OD_{570(control)}) \times 100;$$

where $OD_{570(sample)}$ is the absorbance at 570 nm of the cellstreated with sample and $OD_{570(control)}$ is the absorbance at 570 nm of the negative control (non-treated cells).

2.6. Determination of Nitrite Synthesis

Nitrate in the media was measured by the Griess assay [6] and was used as an indicator of NO synthesis in cells. In brief, an equal volume of the culture supernatants in Raw 264.7 and Griess solution (1:1 mixture (v/v) of 1% sulfanilamide and 0.1% N-(naphthyl) ethylenlide diamine dihydrochloride in 5% H_3PO_4) was added into 96-well plates for 10 min at room temperature. The absorbance at 570 nm was measured by spectrophotometry.

2.7. RNA Isolation and RT-PCR of iNOS

Raw 264.7 cells were seeded at a density of 2×10^6 cells into a 100 mm dish and cultured at 37℃ in 5% CO_2. After 1 day, fresh medium containing 10% serum was added to cells, which were then treated with LPS (lipopolysaccharide, 1 μg/mL) and the ethanolic extract of *Crinum asiaticum* for 8 h. Total RNA was isolated from cells with TRIzol (Invitrogen) according to the instructions of the manufacturer. First strand cDNA synthesis was performed using random hexamers. The sequences of primers are as follows: 5′-CAGTTCTGCGCCTTTGCTCAT-3′ (sense) and 5′-GGTGGTGCGGCTGGACTTT-3′ (antisense) for iNOS, 5′-GACGTGCCGCCTGGAGAAA-3′ (sense) and 5′-GGGGGCCGAGTTGGGATGG-3′ (antisense) for GAPDH (glyceraldehyde-3-phosphate dehydrogenase). RT-PCR reaction of iNOS was reverse-transcribed at 50℃ for 30 min and denatured at 96℃ for 3 min, followed by 32 cycles at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 1 min and then an extension step cycle at 72℃ for 10 min. RT-PCR reaction of GAPDH was reverse-transcribed at 50℃ for 30 min and denatured at 96℃ for 3 min, followed by 32 cycles at 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 1 min and then an extension step cycle at 72℃ for 10 min. The final products were detected with 1.5% agarose gel. Gels were photographed and the intensity of the stained PCR fragments from photographs was quantified by densitometric analysis using Gel Doc 2000 (BIO-RAD Laboratories, Segrate (Milan), Italy).

2.8. PGE_2, IL-6 and IL-8 Release Assay

Human fibroblasts were seeded at a density of 1×10^5 cells into 6-well plates and cultured at 37℃ in 5% CO_2. After 1 day, fresh medium containing 10% serum was added to cells, which were then treated with H_2O_2 (5×10^{-4} M) and various concentrations of sample for 48 h. The culture supernatants were used to quantify PGE_2, IL-6, and IL-8 by the enzyme immunoassay kit (PGE_2-Assay Design, IL-6 and IL-8-Endogen) according to the protocols of the manufacturers.

3. Results and Discussion

3.1. Preparation and Analysis of Lycorine, the Ethanolic Extract of *Crinum asiaticum*

Crinum asiaticum Linne var. *japonicum* extract was obtained in the form of yellow powder by the extraction of 95% ethanol at 75℃ (28.3 g, yield = 8.32%). It has previously been reported that lycorine is the active component for anti-inflammatory activity in the Amaryllidaceae family [3,4]. Therefore, we attempted to measure the lycorine content of the ethanolic extract of *Crinium asiaticum*. As expected, the HPLC results showed that the main component of *Crinum asiaticum* was lycorine (up to 1%), and the content of lycorine, well-known as an immunosuppressant, varied depending on the type of tissue analyzed and the extraction method used [7]. Therefore, it was hypothesized that the ethanolic extract of *Crinum asiaticum* would show an anti-inflammatory effect, and inflammation-related experiments were conducted.

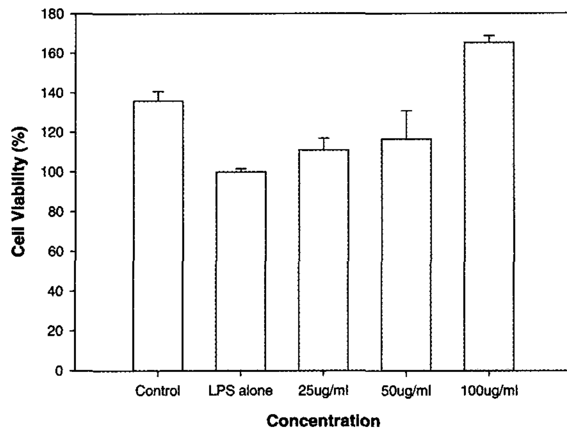

Figure 1. Cytotoxicity of the ethanolic extract of *Crinum asiaticum* to Raw 264.7 macrophage cells. The data are expressed as the mean value (±standard deviations) of four experiments.

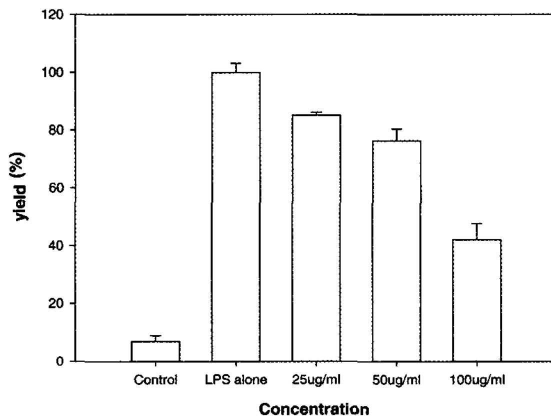

Figure 2. Effect of the ethanolic extract of *Crinum asiaticum* on LPS-induced nitrite production in Raw 264.7 macrophage cells. The data are expressed as the mean value (±standard deviations) of four experiments.

3.2. Effect of the Ethanolic Extract of *Crinum asiaticum* on Cell Viability and LPS-induced Nitrite Synthesis

To evaluate the cytotoxicity of the ethanolic extract of *Crinum asiaticum*, against RAW 264.7 cells the cytotoxicity assay was carried out by the MTT method. The results of this investigation are shown in Figure 1. The ethanolic extract of *Crinum asiaticum* did not show any cytotoxicity up to 100 μM in the presence of LPS. On the contrary, it showed cell proliferation activity as the concentration increased.

The addition of LPS stimulated Raw 264.7 macrophages to cause substantial release of nitrite (Figure 2).

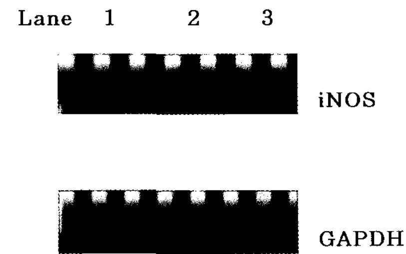

Figure 3. Effect of the ethanolic extract of *Crinum asiaticum on* LPS-induced mRNA expression of iNOS in Raw 264.7 macrophage cells; lane 1: control (only vehicle), 2: LPS 1 μg/mL, 3: 100 μg/mL of Crinum asiaticum + LPS 1 μg/mL.

The ethanolic extract of *Crinum asiaticum*, especially at 100 μM, significantly reduced the LPS-induced nitrite production. Comparing to cell viability, the inhibitory effect of the ethanolic extract of *Crinum asiaticum* on LPS-induced nitrite was not due to lewering of cell viability.

3.3. Effect of the Ethanolic Extract of *Crinum asiaticum* on LPS-induced mRNA Expression of iNOS

The expression of iNOS is generally induced in stimulated macrophages, neutrophils, endothelial and smooth muscle cells, and catalyzes large amounts of NO production [8]. The level of iNOS and GAPDH mRNA expression were measured by RT-PCR. As shown in Figure 3, 100 μg/mL of the ethanolic extract of *Crinum asiaticum* decreased LPS-induced mRNA expression of iNOS to the control level. This result indicates that the ethanolic extract of *Crinum asiaticum* reduced iNOS synthesis and subsequent NO production, followed by anti-inflammatory effect.

3.4. Effect of the Ethanolic Extract of *Crinum asiaticum* on H_2O_2-induced PGE2, IL-6, and IL-8 Synthesis

In response to specific inflammatory stimuli like H_2O_2, the transcription factors NF-κB, JunD/c-fos, and p53 are subsequently activated [9,10]. In turn, NF-κB induces target genes, such as IL-1, IL-6, IL-8, TNF-α (tumor necrosis factor-α), and COX-2 [10-12]. COX-2 is induced in a number of cells by pro-inflammatory stimuli contrary to the constitutive enzyme, COX-1. Prostanoid release is initiated by the liberation of the

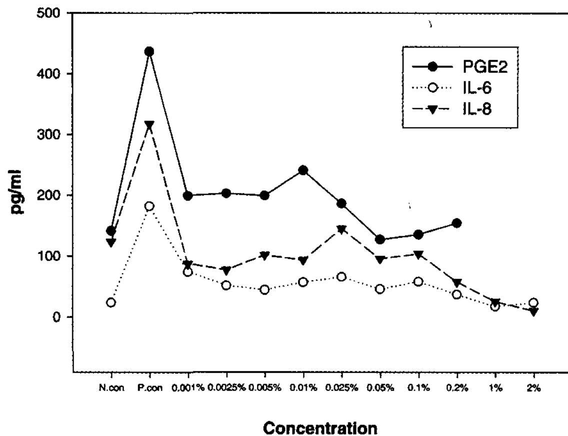

Figure 4. Effect of the ethanolic extract of *Crinum asiaticum* on H_2O_2-induced PGE_2, IL-6, and IL-8 synthesis in human fibroblast cells; N. con: negative control, only vehicle P. con: positive control, 5×10^{-4} M.

substrate arachidonic acid, which is metabolized by COX to prostaglandin H2. This is further metabolized by other enzymes to prostaglandins, prostacyclin, and thromboxane A2 [13]. The addition of H_2O_2-stimulated human fibroblasts induced a substantial release of PGE_2, IL-6, and IL-8 synthesis (Figure 4). The treatment of the ethanolic extract of *Crinum asiaticum*, however, reduced H_2O_2-induced PGE_2, IL-6, and IL-8 synthesis to the control level at all of the concentrations tested IL-6 synthesis, in particular, was completely inhibited. These results suggested that the ethanolic extract of *Crinum asiaticum* could block the H_2O_2-stimulated inflammatory response by inhibiting transcription factor activity.

4. Conclusion

The extract of *Crinum asiaticum* Linne var. *japonicum*, of the Amaryllidaceae Family and designated as the 19th of Korea's Natural Treasures, was obtained by the ethanolic extraction method, maintaining pH at 3.5. It was confirmed that lycorine, an active molecule, was present in *Crinum asiaticum* Linne var. *japonicum*, and its content (about 1%) was identified by high performance liquid chromatogram.

In the cytotoxicity assay, the ethanolic extract of *Crinum asiaticum* did not show any cytotoxicity, instead showing slightly increased cell viability. Furthermore, in the anti-inflammatory assay, the ethanolic extract of *Crinum asiaticum* showed good anti-inflammatory effects through inhibition of iNOS gene expression, followed by reduced NO production and the reduced release of PGE_2, IL-6, and IL-8. These results suggest that *Crinum asiaticum* Linne var. *japonicum* extract, having anti-inflammatory effect, could be used as an appropriate ingredient for cosmeceutical products.

References

1. A. M. Samud, M. J. Asmawi, J. N. Sharma, and A. P. M. Yusof, Anti-inflammatory activity of *Crinum asiaticum* plant and its effect on bradykinin-induced contraction on isolated uterus, *Immunopharmacology*, **43**, 311 (1999).
2. S. O. Okpo, F. Fatokun, and O. O. Adeyemi, Analgesic and anti-inflammatory activity of *Crinum glaucum* aqueous extract, *J. Ethnopharmcol*, **78**, 207 (2001).
3. S. Yui, M. Mikami, M. Kitahara, and M. Yamazaki, The inhibitory effect of lycorine on tumor cell apoptosis induced by polymorphonuclear leukocyte-derived calprotectin, *Immunopharmacology*, **40**, 151 (1998).
4. S. Yui, M. Mikami, Y. Mimaki, Y. Sashida, and M. Yamazaki, Inhibition Effect of Amaryllidaceae Alkaloids, Lycorine and Lycoricidinol on Macrophage TNF-Production, YAKGAKU ZASSHI **121**(2), 167 (2001).
5. F. Denizot and R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, *J. Immunol. Methods*, **89**(2), 271 (1986).
6. L. C. Green, D. A. Wagner, J. Glogowski, P.L. Skipper, J. S. Wishnok, and S. R. Tannenbaum, Analysis of nitrate, nitrite and [15N]-nitrate in biological fluids, *Anal. Biochem.*, **126**(1), 131 (1982).
7. G. Dickneite and H. Schorelmmer, and H. Sedlacek, Use of lycorine as an immunosuppressor, US 4,699,912 (1987).
8. R. G. Knowles and S. Moncada, Nitric oxide synthases in mammals, *Biochem. J.*, **298**, 249 (1994).
9. H. T. Chung, H. O. Pae, B. M. Choi, T. R. Billiar, and Y. M. Kim, Nitric oxide as a bioregulator of

apoptosis, *Biochem. Biophys. Res. Commun,* **282**(5), 1075 (2001).

10. K. Xie, Interleukin-8 and human cancer biology, *Cytokine growth Factor Rev.,* **12**(4), 375 (2001).
11. M. Karin, Y. Cao, F. R. Greten, and Z. W. Li, NF-kappaB in cancer: from innocent bystander to major culprit. *Nat. Rev. Cancer,* **2**(4), 301 (2002).
12. E. Loukinova, Z Chen, C. V. Waes, and G. Dong Expression of proangiogenic chemokine Gro 1 in low and high metastatic variants of Pam murine squamous cell carcinoma is differentially regulated by IL-1alpha, EGF and TGF-â1 through NF-êB dependent and independent mechanisms. *Int. J. Cancer,* **94**(5), 637 (2001).
13. J. R. Vane and R. Botting, Inflammation and the mechanism of action of anti-inflammatory drugs, *FASEB J.,* **1**(2), 89 (1987).