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NF-kappaB 프로모터 활성을 억제하는 식물추출물

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Herbal Extracts as a NF-kappaB Inhibitor

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요 약: Nucler factor-kappa B (NF-kappaB) 프로모터는 염증성 질환을 유도하는 호염증성 시토카인의 발현에 중요한 역할을 수행하는 전사인자 중의 하나이다. 본 실험에서는 200 여종의 식물추출물들로부터 항염효능이 있는 추출물을 선발하기 위해 NF-kappaB 리포터 실험을 수행하였다. NF-kappaB 리포터 실험결과, 12종의 식물추출물, 즉 개나리, 고추잎, 박하, 뱀딸기, 뽕나무, 삼백초, 솔잎, 양애줄기, 약쑥, 어성초, 왕벚꽃가지, 조릿대 등이 lipopolysaccharide (LPS)에 의해 유도된 NF-kappaB 프로모터 활성을 농도의존적으로 억제하는 것을 확인하였다. 이들 12종의 식물추출물이 호염증성 시토카인 발현에도 동일한 효과를 나타내는지 알아보기 위해 tumor necrosis factor-alpha (TNF alpha)와 인터루킨-8에 대한 ELISA실험을 실시하였다. ELISA실험 결과, NF-kappaB 리포터 실험결과와 동일하게, TNF-alpha와 인터루킨-8 생산이 12종 식물추출물 모두에서 감소됨을 관찰하였다. 이러한 실험결과는, 12종의 식물 추출물에서 보여지는 호염증성 시토카인 억제효과가 NF-kappaB 프로모터 활성억제를 통해 이루어지고 있음을 시사한다. 또한, 이들 12종 식물은 diphenyl-p-picrylhydrazyl (DPPH) assay를 통해 살펴본 결과 높은 항산화 활성도 있음을 확인하였다. 이상의 결과로부터, 12종의 식물 추출물은 염증성 피부질환 전용 화장품 제형에서 항염 및 자극완화 소재로 응용될 수 있음을 확인하였다.

Abstract: Nuclear tactor-kappaB (NF-kappaB) is a critical transcription factor for maximal expression of many of the cytokines that are involved in the pathogenesis of inflammatory diseases. In this study, we found that 12 plant extracts among 200 plants, namely, *Forsythia koreana, Capsicum annuum L, Mentha arvensis, Duchesnea chrysantha, Morus alba, Saururus Chinensis (Lour) Baill, Pine needle, Zingiber mioga (Thunb.), Roscoe, Houttuynia, Prunus yedoensis, Sasa quelpaertensis*, significantly inhibited LPS- induced NF-kappaB activation in a concentration-dependent manner. Additionally, 12 plant extracts were found to have antioxidant activities in DPPH assay. Therefore, we have attempted to determine whether 12 herbal extracts could inhibit the expression of cytokines possessing NF-kappaB promoter in their promoter regions. Consistently 12 herbal extracts inhibited LPS-induced production of TNF alpha and interleukin-8 (IL-8). These results show that 12 herbal extracts suppresses the production of pro-inflammatory mediators through the inhibition of the NF-kappaB signaling pathway, we suggest that 12 herbal extracts can be used as a anti-inflammatory and soothing agent.

Keywords: *NF-kappaB, interleukin-8, TNF alpha, DPPH, cosmetics*

1. Introduction

The main clinical manifestations of infections with Gram-positive and Gram-negative bacteria are similar, these clinical manifestations include inflammation, fever, and arthritis, which are caused by mediators released from host cells following exposure to bacterial cells and their components [1,2]. It has been thought that

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the main pro-inflammatory mediators induced by bacteria and their cell walls are cytokines, primarily tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1), IL-6, and IL-8 [3-7]. These cytokines are upregulated by nuclear factor-kappaB (NF-kappaB), a potent transcription factor that was first identified by Sen and Baltimore [8]. Excessive cytokine-mediated inflammation is likely to play an important role in the pathogenesis of inflammatory diseases, such as adult respiratory distress syndrome (ARDS) and rheumatoid arthritis [9,10].

In general, cytokines are not stored intracellularly, and the secretion of cytokines depends on the synthesis of new proteins. As a consequence, an increase in cytokines in response to an inflammatory stimulus is significantly or predominantly regulated by cytokine gene transcription rates. Because transcriptional regulation is critical for the production of many cytokines, transcription factors, including NF-kappaB, may play roles in the regulation of cytokine-mediated inflammation.

In this report, we have demonstrated that treatment with 12 herbal extracts inhibits LPS-induced production of pro-inflammatory mediators, and the mechanisms underlying its action may be mediated via the inhibition of the NF-kappaB signaling pathway in the human monocytic cell line.

2. Materials and Methods

2.1. Reagents

The chemiluminescence kit was purchased from Amersham Pharmacia Biotech (Buckinghamshire, Eng l and). b-actin antibody, and pyrollidone dithiocarbamate (PDTC) were acquired from Sigma (St. Louis, MO). pNF-kappaB-Luc plasmid was obtained from Stratagene (La Jolla, CA).

2.2. Cell Culture

Human monocytic cell line, THP-1 cells were cultured in RPMI 1640 Medium (Hyclone) containing 10% fetal bovine serum (Invitrogen), and penicillin-streptomycin at 37℃ in a humidified 95% air/5% CO_2 atmosphere.

2.3. Measurement of Cytokine Production

Concentrations of IL-8 and TNF-alpha in the culture supernatant were measured by using ELISA kits (Genzyme, Minneapolis, MN) according to the instructions of the manufacturer.

2.4. Transient Cell Transfection and Luciferase Reporter Gene Assay

To assay for NF-kappaB promoter activity, human dermal fibroblast cells were transfected with NF-kappaB-Luc reporter, or with the indicated genes, including TRAF 2 (TNF receptor-associated factor), MEKK 3 (mitogen-activated protein kinase kinase kinase), and IKK-beta (Ikappa B kinase-beta), along with the Renilla luciferase expression vector, driven by the thymidine kinase promoter (Promega) using SuperfectTM reagent (Invitrogen). After 24 h of incubation, the cells were incubated in the presence or absence of LPS (100 μg/mL), along with indicated concentrations of herbal extracts for 14 h. The cells were then harvested and lysed. Supernatants were assayed for luciferase activity. Luciferase activity was determined with a Dual luciferase assay system (Promega) and a LB953 luminometer (Berthold, Germany), and was expressed as a ratio of the NF-kappaB-dependent firefly luciferase activity divided by the control thymidine kinase Renilla luciferase activity (% control). Results were confirmed by three independent transfections. Data are expressed as the means S.E.M. *$p < 0.05$, compared with untreated controls. o$p < 0.05$ versus LPS (100 μg/mL) only or transfected controls.

2.5. Cytotoxicity Assay

THP-1 cells were cultured in RPMI 1640 (Hyclone) containing 10% fetal bovine serum, and penicillin-streptomycin at 37℃ in a humidified 95% air/5% CO_2 atmosphere. Cells were seeded on 96-well plates and drug treatment was initiated 24 h after seeding. The general viability of cultured cells was determined by the reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo Laboratories, Japan) to a highly water-soluble formazan dye. This assay was performed after the incubation of human dermal fibroblast cells in the presence or absence of LPS, along with indicated concentrations of herbal extracts, for 14

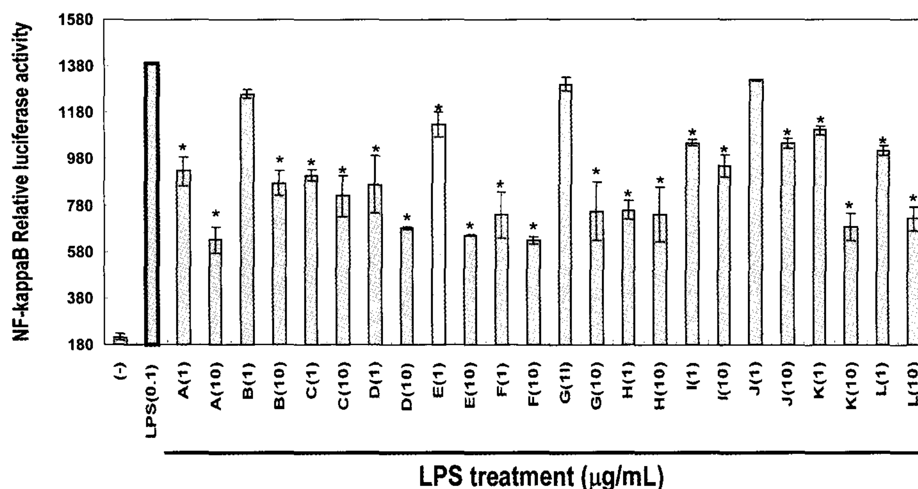

Figure 1. Effect of herbal extracts on LPS-induced NF-kappaB activation. To determine the effect of herbal extracts on LPS-induced NF-kappaB activation, THP-1 cells were transiently co-transfected with 2 μg of firefly luciferase reporter gene under the control of NF-kappaB responsible elements and 0.2 μg of Renilla luciferase expression vector driven by thymidine kinase promoter using SuperfectTM reagent (Invitrogen), as described in Materials and Methods. After 24 h, cells were stimulated with 100 μg/mL LPS in the presence or absence of herbal extracts. Luciferase activity is expressed as the ratio of NF-kappaB-dependent firefly luciferase activity to the control thymidine kinase Renilla luciferase activity (relative luciferase units). Data are expressed as means ± S.E.M. *, $p < 0.05$ compared with LPS alone. Results were confirmed by three independent experiments. A: *Forsythia koreana*, B: *Capsicum annuum* L, C: *Mentha arvensis*, D: *Duchesnea chrysantha*, E: *Morus alba*, F: *Saururus chinensis* (Lour) Baill, G: *Pine needle*, H: *Zingiber mioga* (Thunb.), I: *Roscoe*, J: *Houttuynia*, K: *Prunus yedoensis*, L: *Sasa quelpaertensis*.

h at 37℃ in a 5% CO_2 atmosphere. To each well, 10 mL of WST-8 solution was added. Cells were then incubated at 37℃ for 3 h and the absorbance was measured at 450 nm using a spectrophotometer (Power Wave, Bio-tek Inc). Data are presented as means ± SD. All values were significant (*$p < 0.05$) compared with values for control. The entire experiment was performed in triplicate and results were confirmed by three independent experiments.

2.6. DPPH Assay

DPPH (diphenyl-p-picrylhydrazyl) (Sigma), a stable nitrogen-centered free radical, was dissolved in methanol for 5 min to give a 200 μM solution. The tested compounds were added to DPPH of equal volume in a 96 well mciroplate as quadruplicates, along with sample blanks and controls. The concentration (absorption) of DPPH during the 30 min observation time was measured at 517 nm. The decrease in absorption at 517 nm was correlated with the scavenging action of the tested compound. Data are presented as the mean ± standard deviation. Experiment was performed in quadruplicate and repeated four times.

2.7. Statistics

The statistical significance of the data was determined by Student's t-test. $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Effect of Herbal Extracts on LPS-induced NF-kappaB Activation

NF-kappaB (Nuclear factor-kappaB) is a protein transcription factor first identified by Sen and Baltimore [8] that functions to enhance the transcription of a variety of genes including cytokines and growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins. NF- kappaB has been reported to be involved in maximal transcription of many cytokines, including TNF-alpha, IL-1, IL-6, and IL-8, which are thought to be important in the generation of acute inflam-matory

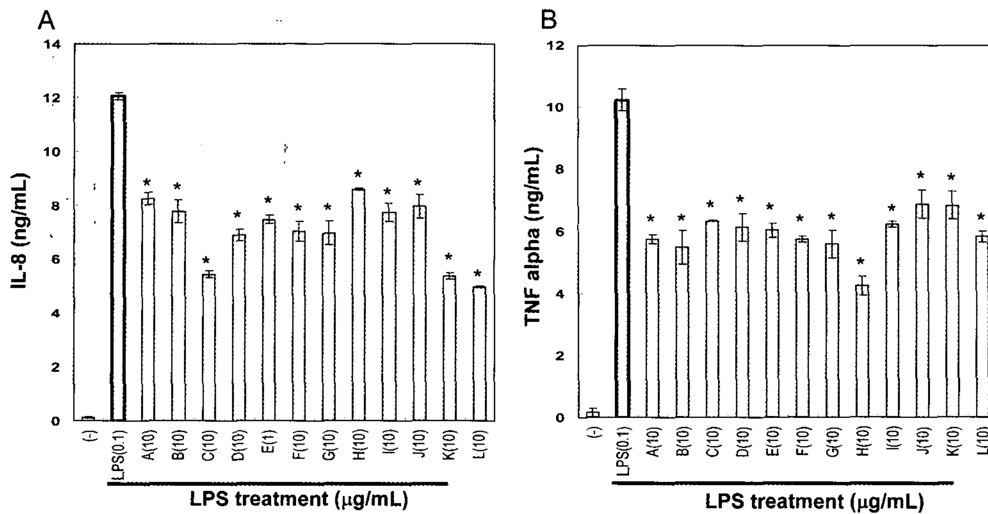

Figure 2. Herbal extracts inhibits LPS-induced secretion of pro-inflammatory cytokines such as IL-8 and TNF-alpha. THP-1 cells (10^6) were incubated with LPS (10 mg/mL), LPS plus herbal extracts, or without LPS for 72 h, after which the supernatants were assessed for IL-8 or TNF-alpha by ELISA. Data are presented as means S.E.M. of four independent experiments. All values were significant (*$p < 0.05$) compared with values for LPS alone. A: *Forsythia koreana*, B: *Capsicum annuum* L, C: *Mentha arvensis*, D: *Duchesnea chrysantha*, E: *Morus alba*, F: *Saururus chinensis* (Lour) Baill, G: *Pine needle*, H: *Zingiber mioga* (Thunb), I: *Roscoe*, J: *Houttuynia*, K: *Prunus yedoensis*, L: *Sasa quelpaertensis*.

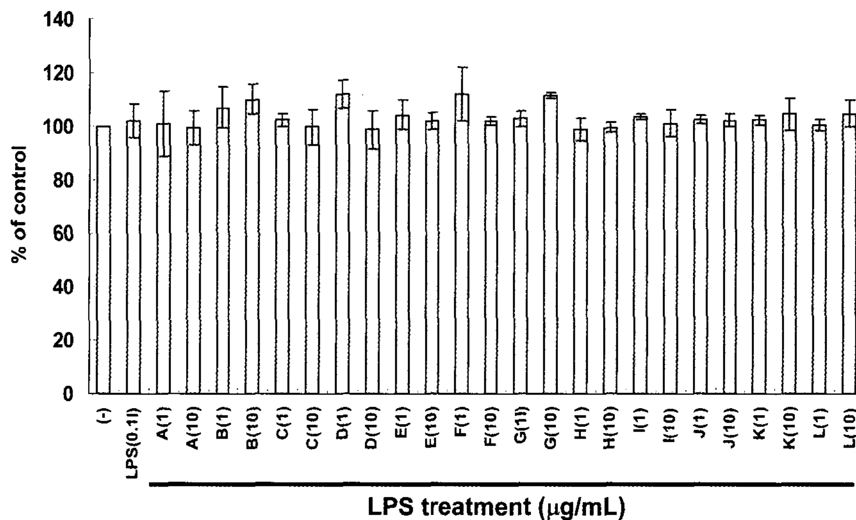

Figure 3. Cytotoxity of herbal extracts against THP-1 cells. This cytotoxic assay was performed after the incubation of THP-1 cells in the presence or absence of LPS, along with indicated concentrations of herbal extracts, for 72 h at 37℃ in a 5% CO_2 atmosphere. Cellular cytotoxicity was determined according to the protocol described in Materials and Methods and was expressed as the mean ± S.E.M. All values were significant (*$p < 0.05$) compared with values for LPS alone. A: *Forsythia koreana*, B: *Capsicum annuum* L, C: *Mentha arvensis*, D: *Duchesnea chrysantha*, E: *Morus alba*, F: *Saururus chinensis* (Lour) Baill, G: *Pine needle*, H: *Zingiber mioga* (Thunb.), I: *Roscoe*, J: *Houttuynia*, K: *Prunus yedoensis*, L: *Sasa quelpaertensis*.

responses. As a preliminary step to determine if herbal extracts affects cytokine production, we performed NF-kappaB luciferase assay in human dermal fibroblast cells. Among 200 plants, we found that 12 plants,

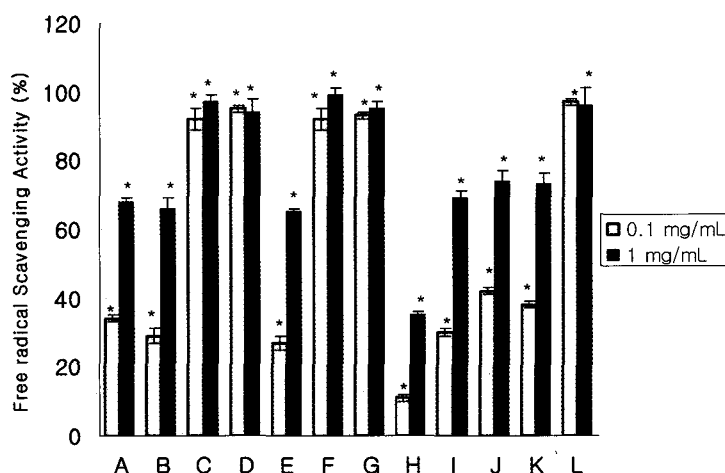

Figure 4. *In vitro* antioxidant activities of herbal extracts using DPPH assay. Data are expressed as means ± S.D. *, $p < 0.05$ compared with a control. Results were confirmed by the experiment which was repeated four times in triplicate. A: *Forsythia koreana*, B: *Capsicum annuum* L, C: *Mentha arvensis*, D: *Duchesnea chrysantha*, E: *Morus alba*, F: *Saururus chinensis* (Lour) Baill, G: *Pine needle*, H: *Zingiber mioga* (Thunb.), I: *Roscoe*, J: *Houttuynia*, K: *Prunus yedoensis*, L: *Sasa quelpaertensis*.

including *Forsythia koreana, Capsicum annuum* L, *Mentha arvensis, Duchesnea chrysantha, Morus alba, Saururus Chinensis (Lour)* Baill, *Pine needle, Zingiber mioga* (Thunb.), *Roscoe, Houttuynia, Prunus yedoensis, and Sasa quelpaertensis,* have inhibitory an effect on LPS-induced NF-kappaB activation. As shown in Figure 1, lipopolysaccharide (LPS) increased NF-kappaB reporter activity, whereas herbal extracts inhibited LPS-induced NF-kappaB reporter activity in a concentration-dependent manner. This result suggests the possibility that tested herbal extracts may be involved in blocking the production of proinflammatory cytokines.

3.2. Effect of 12 Herbal Extracts on LPS-Induced Secretion of Pro-inflammatory Cytokines such as IL-8 and TNF-alpha

As previously mentioned, we have found that there exists the possibility that herbal extracts may inhibit pro-inflammatory cytokines, the expression of which is dependent on NF-kappaB promoter through the inhibition of NF-kappaB. In order to evaluate this possibility, we performed ELISA for interleukin-8 and TNF alpha in THP-1 cells. As shown in Figure 2, LPS-induced production of TNF alpha and interleukin-8 in THP-1 cells was reduced by herbal extracts. However, there remains the possibility that the reduction of pro-inflammatory cytokines was induced by a cytotoxic effect of each herbal extracts. To confirm this, we performed an cytotoxicity assay in THP-1 cells, According to the results of this assay, each herbal extracts showed no cytotoxic effects at the tested concentrations (Figure 3).

3.3. Antioxidant Effect of Herbal Extracts

Until now, we found that 12 plants have anti-inflammatory effect in THP-1 cells. In order to investigate antioxidant effects of herbal extracts, we carried out *in vitro* testing for diphenyl-p-picrylhydrazyl (DPPH) scavenging assay. The DPPH test showed that tested herbal extracts have significant antioxidant activities (Figure 4).

4. Conclusions

In order to screen plant extracts having anti-inflammatory activity, NF-kappaB luciferase reporter and cytokine production assays were used. In these experiments, 12 plants were found to have anti-inflammatory activity as well as antioxidant activity. In addition, we found that the antiinflammatory effect of these extracts may be mediated through suppression of NF-kappaB promoter.

These data suggest that 12 plant extracts can be

used as an anti-inflammatory ingredient in cosmetic formulation.

Acknowledgements

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