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Anti-inflammatory Activities of Ethylacetate Extract of *Rehmannia glutinosa* in LPS-induced RAW 264.7 Cells

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Abstract This study is to investigate the anti-inflammatory effects of the ethylacetate extract of *Rehmannia glutinosa* (RGEAE). The anti-inflammatory activities using nitric oxide (NO), cytokine, and chemokine production in lipopolysaccharide (LPS)-induced RAW 264.7 cells were checked. Results indicated that RGEAE suppressed the NO, interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) production in a dose-dependent manner. Inhibition of NO formation was due to a decrease in inducible NOS (iNOS) expression. It was also found that the anti-inflammatory activities of RGEAE resulted from its inhibitory role on the nuclear factor (NF)-κB activation and reactive oxygen species (ROS) production. Therefore, it is suggested that RGEAE has potential as a therapeutic material to attenuate the inflammatory disease such as rheumatoid arthritis.

Keywords: *Rehmannia glutinosa*, nitric oxide, inducible nitric oxide synthase, nuclear factor-κB, reactive oxygen species

Introduction

Nitric oxide (NO) is a potentially toxic gas that is produced from the amino acid L-arginine through the enzymatic action of nitric oxide synthase (NOS). When certain cells, such as macrophages, are stimulated by lipopolysaccharide (LPS), they produce extremely high amounts of NO (1). Uncharacteristically high production of NO production is associated with many diseases such as atherosclerosis, inflammation, and carcinogenesis, hypertension, obesity, and diabetes (2-4). There are 2 endothelially expressed forms of NOS (iNOS, eNOS) and 1 neuronally expressed form of NOS (nNOS). Of the 3 NOS isoforms, iNOS is involved with inflammation. Due to this, iNOS has become a potential therapeutic target for drug discovery to treat autoimmune disease such as a chronic inflammatory disease (5).

Nuclear factor (NF)-κB is a transcription factor that controls the expression of genes involved in the immune responses, apoptosis and the cell cycle. NF-κB is the most important factor in inflammatory disease. When NF-κB is activated, many proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, and IL-8, are synthesized (6). The expression of iNOS is also produced by NF-κB activation.

Rehmannia glutinosa is a perennial plant that grows in Korea, China, Japan, and Vietnam. *R. glutinosa* has components such as saccharides, flavonoids, amino acids, inorganic ions, and iridoid compounds, such as catalpol, dihydrocatalpol, and others including phenol glycoside ionone (7). *R. glutinosa* is used for detoxification, hemostasis, contusion, and alleviating fever. *R. glutinosa* has been shown to nourish the blood, have tonic and cardiotonic activities, positively affect diabetes, and positively affect hypotension and increase body fluids (8,9). Several recent reports have documented that *R. glutinosa* has antioxidant activities (10,11) and that its aqueous extract inhibits the expression of TNF-α and IL-1 in LPS-induced mouse astrocytes (12). However, there are no reports regarding the anti-inflammatory effect of the ethylacetate extract of *R. glutinosa* (RGEAE) on NO production and a NF-κB activation. Therefore, we studied the anti-inflammatory activity of RGEAE on NO production and iNOS expression in the RAW 264.7 macrophage cells. Furthermore, we attempted to establish whether the regulation of NO production by RGEAE is associated with activation of NF-κB and radical scavenging activities.

Materials and Methods

Cell culture RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) and were incubated at 37°C with 5% CO_2.

Reagents *Rehmannia glutinosa* was purchased from Duk Hyun Dang (Seoul, Korea). DMEM and FBS were purchased from Hyclone (Logan, UT, USA). Lipopolysaccharide (LPS), sodium nitrite, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation The dried roots of *R. glutinosa* (2 kg) were ground and extracted with 100% methanol (40 L) by maceration at room temperature for 3 weeks. Filtration and concentration gave the resultant extract (19 g), which

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was suspended in H_2O and then partitioned sequentially with ethylacetate (3.3 g), and butanol. The ethylacetate extract was used.

Cytotoxicity assay To measure cell viability, we used the EZ-Cytox cell viability assay kit (Daeil Lab, Korea). The cells were cultured in a 96-well plate at a concentration of 5.0×10^4 cell/well for 24 hr. The cells were subsequently treated with various concentrations of ethylacetate extract of *R. glutinosa* (RGEAE) for an additional 48 hr. After the incubation period, 10 μL of the kit solution was added to each well and incubated for 6 hr at 37°C and 5% CO_2. The index of a cell viability was determined by measuring formazan production with enzyme-linked immunosorbent assay (ELISA) reader at an absorbance of 480 nm. The reference wavelength was 650 nm.

NO measurement The cells were cultured in a 96-well plate at a concentration of 2.0×10^4 cell/well for 24 hr and subsequently treated with various concentrations of RGEAE for 2 hr. Thereafter, the cells were treated with LPS (1 μg/mL) for 18 hr. The cellular media were collected at the end of the culture for nitrite, which were used as a measure of the NO production (13). Equal volumes of Griess reagent (Sigma-Aldrich) and each cellular supernatant were combined, and the absorbances of each sample were measured at 570 nm. The concentration of nitrite (μM) was calculated using a standard curve produced from known concentration of sodium nitrite dissolved in DMEM. The results are presented as the mean±SD of 5 replicates of 1 representative experiment.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) The cells were cultured in a 100-mm petri dish for 24 hr at a concentration of 2×10^5 cell/mL. After stimulation with various concentrations of RGEAE for 2 hr, LPS (1 μg/mL) was added for 18 hr. Total RNA from the cells was isolated using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The advantage RT for PCR kit was used for reverse transcription according to the manufacturer's protocol (Clontech, Mountain View, CA, USA). The Chromo4 RT-PCR detection system (Bio-Rad, Hercules, CA, USA) and iTaq^{TM} SYBR^{R} Green supermix (Bio-Rad) were used for the RT-PCR amplification of iNOS and β-actin ('housekeeping gene' encoding β-actin) with the following conditions: 50 cycles of 94°C for 20 sec, 60°C for 20 sec, and 72°C for 30 sec. All the reactions were repeated at least 3 times independently to ensure the reproducibility of the results. Primers for iNOS and β-actin were purchased from Bioneer Corp. (Korea) and PCR was performed on the cDNA using the following sense and anti-sense primers: iNOS, forward primer 5'-TCCTACACCACACCAAACT GTGTGC-3', reverse primer 5'-CTCCAATCTCTGCCTAT CCGTCTC-3'; β-actin, forward primer 5'- TGAGAGGGA AATCGTGCGTGAC-3', reverse primer 5'-GCTCGTTG CCAATAGTGATGACC-3'. Data were analyzed using 'Opticon Monitor' software (Bio-Rad).

Western blotting The cells were cultured in a 100-mm petri dish for 24 hr at a concentration of 2×10^5 cell/mL. After stimulation with various concentrations of RGEAE for 2 hr, LPS (1 μg/mL) was added for 18 hr. Cells were then washed once with cold phosphate-buffered saline (PBS) 1 time and collected by a pipetting. Then the cells were lysed in a NP40-based cell lysis buffer (BD Biosource, San Jose, CA, USA) containing a protease inhibitor cocktail (Sigma-Aldrich) and phenyl-methylsulfonyl fluoride (Sigma-Aldrich) for 30 min on ice. The protein concentration of the cell lysate was determined by using the Bio-Rad protein assay. Equal amounts of protein (50 mg) were loaded and electrophoresed on a 10% sodium dodecylsulfate (SDS)-polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane (Hybond ECL Nitrocellulose; Amersham Bioscience, Piscataway, NJ, USA). The membranes were washed once with a washing buffer (PBS, 0.05% Tween 20) and blocked with a blocking buffer (PBS, 5% skim milk, 0.05% Tween 20) for 1 hr. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4°C. Rabbit polyclonal anti-iNOS/NOS type II antibody (BD Bioscience) was diluted at 1:5,000 and rabbit polyclonal anti β-tubulin antibody (Santa Cruz, CA, USA) was diluted at 1:200 in the blocking buffer. After the incubation, the membranes were washed and subsequently incubated for 1 hr at room temperature with the goat anti-rabbit IgG HRP-conjugated secondary antibody diluted at 1:5,000 in the blocking buffer. The membranes were washed and the protein bands were detected by a chemiluminescence system (Amersham Pharmacia).

Measurement of IL-6 and MCP-1 by ELISA Cells cultured at a concentration of 2×10^5 cell/mL were incubated for 24 hr and then RGEAE was added for 2 hr. After that, LPS (1 μg/mL) was added for 4 hr. To determine the resultant cytokines, the supernatants were harvested and the levels of IL-6 and MCP-1 were measured by using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

Luciferase assay for NF-kB activity The cells were cultured in a 6-well plate for 24 hr at a concentration of 4×10^5 cell/mL. Transfection of the vectors was done using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The NF-κB-Luc vector (Stratagene, La Jolla, CA, USA) and pRL-TK vector (Promega, Madison, WI, USA) were transfected with 5 and 1 μg/well respectively. After the transfection, RGEAE was added at various concentration for 2 hr and the cells were treated with LPS at a concentration of 1 mg/mL for 24 hr. Luciferase activity was measured with the Dual-luciferase Reporter Assay System (Promega).

Radical scavenging activity Specific detection of superoxide and hydroxyl radicals using an ultraweak chemiluminescence analyzer (UWCLA) was carried out as previously described by Tsai *et al.* (14). To measure superoxide radicals, a reaction mixture with a total volume of 2.1 mL was prepared and consisted of the following compounds: 1.0 mL of 2.0 mM lucigenin, 1.0 mL of PBS (pH 7.4), 0.05 mL of 1.0 M arginine, and 0.05 mL of 1.4 mM methylglyoxal. After gently mixing the above-mentioned components, the reaction mixture was added to a quartz round-bottom cuvette in the black-box unit of the UWCLA. To measure hydroxyl radicals, the reaction

mixture consisted of a total volume of 2.75 mL and contained the following: 1 mL of 3 mM indoxyl-β-glucuronide (IBG) dissolved in PBS (pH 7.4), 0.1 mL of 1.0 mM $FeSO_4$, 1.6 mL of 3% H_2O_2, and 0.05 mL of 10 mM ethylene diamine tetraacetic acid (EDTA). After gently mixing the above-mentioned components, the reaction mixture was placed in a quartz round-bottom cuvette in the black-box unit of the UWCLA. After 33 min of radical formation, 2 mL (1 mg/mL) of RGEAE was added to the reaction mixture. As a result, varying degrees of reduction in the counts could be observed. These phenomena represent different degrees of radical scavenging abilities.

The measurement of superoxide radical production was performed with the CL reaction using the lucigenin and methylglyoxal/argninine reagent for 33 min in the UWCLA. IBG and Fenton (H_2O_2+ Fe^{2+}) reagents were used to measure the hydroxyl radical. After initiating the reaction, 2 mL (1 mg/mL) of RGEAE was added to the reaction mixture. The IC_{50} value for the radical scavenging activity was obtained by adding the RGEAE sequentially until the CL count was inhibited by 50%.

Statistical methods Significance between pairs of mean values was determined by using the Student's *t*-test. A $p<0.05$ was considered significant for all the analyses.

Results and Discussion

Effect of RGEAE on NO formation Prior to exploring the potential effects of RGEAE in RAW264.7 cells, we tested the cytotoxic effects of RGEAE using the EZ-Cytox viability assay. As shown in Fig. 1, RGEAE was cytotoxic at concentration higher than 600 mg/mL. Therefore, we decided to use concentration of RGEAE lower than 400 mg/mL. When the inflammatory signal pathway is induced by LPS treatment, many genes are expressed as a consequence of NF-κB activation. Inducible nitric oxide synthase (iNOS) is one of the NF-κB upregulated genes. Because NO production is increased due to iNOS expression (15), we decided to check the effect of RGEAE on NO formation in LPS-induced RAW264.7 cells. NO production was markedly increased from the basal level of 2.14±0.59 to 19.93±0.95 mM after 18 hr incubation with LPS (1 mg/mL, Fig. 2). Interestingly, NO production was decreased when RGEAE was added to the RAW 264.7 cells before induction with LPS (1 mg/mL) in a concentration-dependent manner. The IC_{50} of RGEAE was calculated at 88.6 μg/mL ($p<0.01$, $n=5$). Moreover, treatment with RGEAE decreased the level of NO near to the basal level at 200 mg/mL. The methanol extract from *R. glutinosa* was partitioned with ethylacetate and butanol. The inhibitory activity of the ethylacetate extract on NO formation is much stronger than that of the butanol extract (data not shown). Therefore, the ethylacetate extract was used for the following expriments.

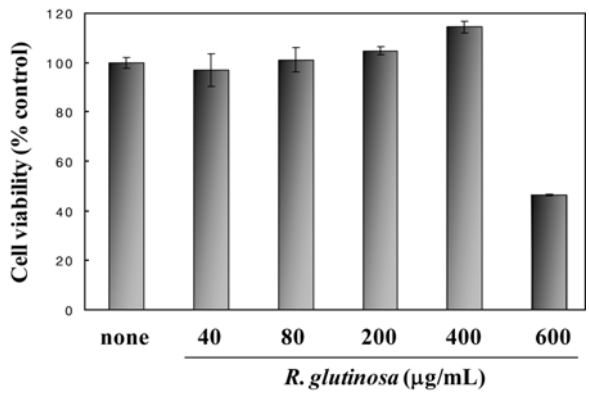

Fig. 1. Measurement of the cytotoxic activity of RGEAE in the RAW 264.7 cells.

Effect of RGEAE on iNOS expression In order to establish whether the suppression of NO production by RGEAE was due to an inhibition of iNOS expression, we checked the protein level of iNOS by Western blotting and the iNOS mRNA level using RT-PCR. As shown in Fig. 3, treatment with RGEAE decreased the level of the iNOS expression at the mRNA and protein level in a dose-dependent manner. Therefore, the decreased NO production due to RGEAE on LPS-induced RAW264.7 cells correlated

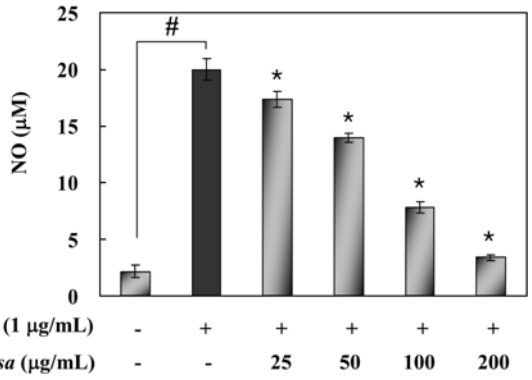

Fig. 2. Inhibition of a NO production by RGEAE in the RAW 264.7 cells. Data shown are the mean±SD ($n=5$). $^{\#}p<0.001$ vs. media only treated group; $^{*}p<0.01$ vs. only LPS-treated group.

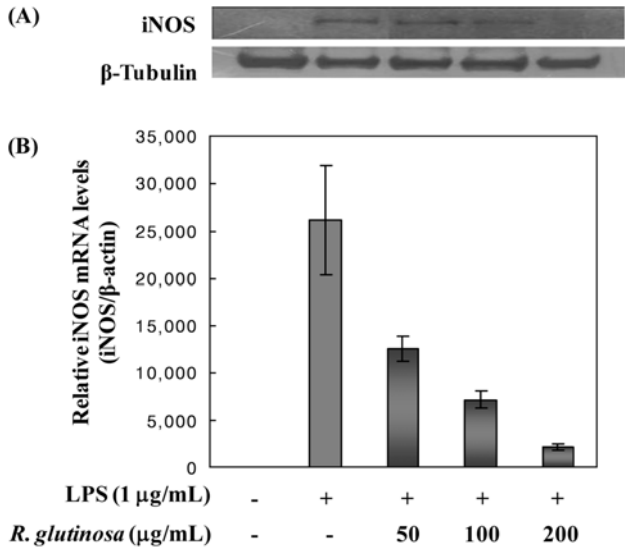

Fig. 3. Effects of RGEAE on the LPS-induced iNOS expressions in the RAW 264.7 cells. (A) Western blotting, (B) quantitative RT-PCR. Data shown are the mean±SD ($n=3$).

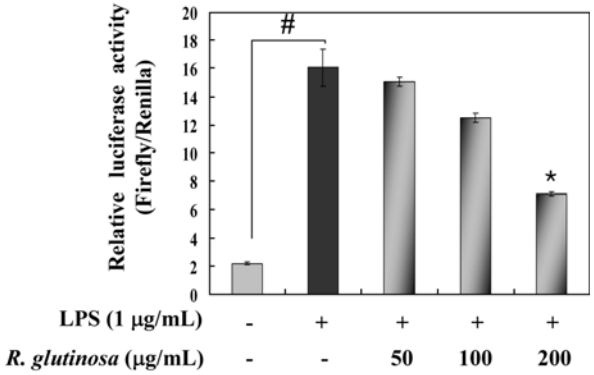

Fig. 4. Effect of RGEAE on NF-κB dependent reporter gene activity. Data shown are the mean±SD (n=3). $^{\#}p<0.005$ vs. only media-treated group; $^{*}p<0.02$ vs. only LPS-treated group.

with the inhibition of iNOS mRNA and protein expressions.

Effect of RGEAE on NF-κB activation To determine the mechanism of RGEAE induced inhibition of iNOS expression in LPS treated RAW264.7 cells, we measured the NF-kB activity using the dual-luciferase reporter assay. When LPS (1 μg/mL) was added, the relative luciferase activity (*firefly/Renilla)* increased from 2.21±0.07 to 16.08±1.31. However the relative luciferase activity decreased when RGEAE was added in a dose-dependent manner. Therefore, we confirmed that RGEAE could inhibit the NF-κB activation in the LPS-induced RAW264.7 cells, and that the RGEAE mediated suppression of iNOS expression is due to inhibition of NF-κB activation (Fig. 4).

Effect of RGEAE on IL-6 and MCP-1 production Inflammatory diseases such as rheumatoid arthritis, asthma, and sepsis all demonstrate disrupted balance of the inflammatory cytokines and an increased influx of immune cells to the inflammatory sites (16-18). The cytokine IL-6 is well known to induce inflammatory pain in rheumatoid arthritis (19). The chemokine MCP-1 recruits immune cells to the inflammatory sites and is involved in the process of rheumatoid arthritis (20). It was already confirmed that RGEAE suppressed NF-kB activity; therefore we wanted to know whether RGEAE could also regulate the production of inflammatory cytokines and chemokines. As shown in Fig. 5, RGEAE inhibited the production of IL-6 and MCP-1 in a dose-dependent manner. As a result, RGEAE not only suppressed NO production, but also suppressed the production of inflammatory cytokines and chemokines by inhibiting NF-κB activation in LPS-induced RAW264.7 cells.

Effect of RGEAE on radical scavenging When RAW264.7 cells are activated with LPS, reactive oxygen species (ROS) are produced (21). Several recent reports have documented that anti-inflammatory activity is correlated with anti-oxidant activity (22). ROS can accelerate the activation of NF-κB (23,24), therefore we checked to see whether RGEAE could directly remove ROS. As shown in Fig. 6, RGEAE had a scavenging activity for radicals such as superoxide and hydroxyl radical with IC_{50} values of 6.9 and 4.9 mg/mL, respectively. Therefore, RGEAE may inhibit NO formation through 2 mechanisms. One mechanism is through suppression of NF-κB activation, and the other is

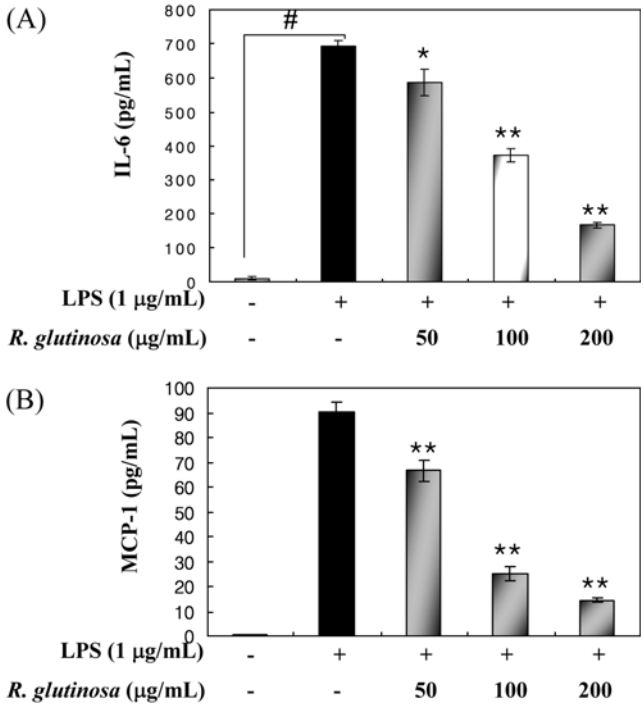

Fig. 5. Effects of RGEAE on the LPS-induced IL-6 and MCP-1 productions in the RAW 264.7 cells. (A) IL-6, (B) MCP-1. Data shown are the mean±SD (n=3). $^{\#}p<0.005$ vs. only media-treated group. $^{*}p<0.05$, $^{**}p<0.001$ vs. only LPS-treated group.

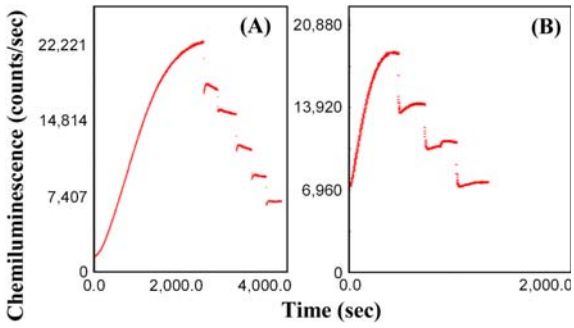

Fig. 6. Radical scavenging activity of RGEAE. (A) Superoxide radicals, (B) hydroxyl radical. Chemiluminescence reaction was initiated and then 2 mL (1 mg/mL) of the RGEAE was added to the reaction mixture sequentially.

by directly scavenging ROS (including NO) in the LPS-induced RAW 264.7 cells.

R. glutinosa is a traditional Chinese medical herb that is used for detoxification, hemostasis, contusion, and alleviating fever. *R. glutinosa* has many pharmacological effects, and many chemical components. In recent decades there have been many reports regarding the pharmacological functions of *R. glutinosa* on the blood system, immune system, cardiovascular system, central nervous system, bone metabolism, endocrine system, anti-tumor, and anti-senescence, etc (7). It also has been described to have anti-diabetic and anti-oxidant effects (25,26). Aqueous extract of prepared rehmannia root inhibit TNF-α secretion and regulate the allergic reaction (27,28). However there are no reports regarding the anti-inflammatory activities of

RGEAE on NO and NF-κB inhibition. In the present study, we investigated to the potential of RGEAE as a compound to attenuate inflammatory diseases such as rheumatoid arthritis. We found that RGEAE has potential anti-inflammatory activities.

It is well known that flavonoid compounds have anti-inflammatory and free radical scavenging activities (29,30). Furthermore, catalpol has an anti-inflammatory effect (31). *R. glutinosa* is composed of many compounds such as catalpol, dihydrocatalpol, acetylcatalpol, and flavonoid (7). All of these compounds potentially participate in the anti-inflammatory activities of RGEAE on RAW264.7 cells.

In conclusion, RGEAE regulates the production of NO, inflammatory cytokines, and chemokines by inhibiting NF-κB activation and scavenging free radicals in the LPS-induced RAW 264.7 cells. Therefore, RGEAE may potentially be a material that can be developed into functional foods for treatment of anti-inflammatory diseases such as rheumatoid arthritis.

Acknowledgments

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References

1. Nathan C, Xie QW. Nitric oxide synthases: Roles, tolls, and controls. Cell 78: 915-918 (1994)
2. Mordan LJ, Burnett TS, Zhang LX, Tom J, Cooney RV. Inhibitors of endogenous nitrogen oxide formation block the promotion of neoplastic transformation in C3H10T1/2 fibroblasts. Carcinogenesis 14: 1555-1559 (1993)
3. Ohshima H, Bartsch H, Chronic infections and inflammatory processes as cancer risk factors: Possible role of nitric oxide in carcinogenesis. Mutat. Res. 305: 253-264 (1994)
4. KrÖche KD, Fensel K, Kolb-bachofen V. Inducible nitric oxide synthase in human diseases. Clin. Exp. Immunol. 113: 147-156 (1998)
5. Hobbs AJ, Higgs A, Moncada S. Inhibition of nitric oxide synthase as a potential therapeutic target. Annu. Rev. Pharmacol. 39: 191-220 (1999)
6. Paul PT, Gary SF. NF-κB: A key role in inflammatory diseases. J. Clin. Invest. 107: 7-11 (2001)
7. Zhang R-X, Li M-X, Jia Z-P. *Rehmannia glutinosa*: Review of botany, chemistry, and pharmacology. J. Ethnopharmacol. 117: 199-214 (2008)
8. Lv XF, Meng QY, Guo XM. Effect of *Rehmannia glutinosa* water extraction on insulin resistance and gene expression of resistin in type 2 diabetes mellitus rats. Zhongguo Zhong Yao Za Zhi 32: 2182-2184 (2007)
9. Guo LM, Zhang RX, Jia ZP, Li MX, Wang J, Yin Q. Effects of *Rehmannia glutinosa* oligosaccharides on proliferation of HepG2 and insulin resistance. Zhongguo Zhong Yao Za Zhi 32: 1328-1332 (2007)
10. Yu HH, Kim YH, Jung SY, Shin MK, Park RK, So HS, Kim KY, Lee DH, You YO. *Rehmannia glutinosa* activates intracellular antioxidant enzyme systems in mouse auditory cells. Am. J. Chin. Med. 34: 1083-1093 (2006)
11. Yu HH, Seo SJ, Kim YH, Lee HY, Park RK, So HS, Jang SL, You YO. Protective effect of *Rehmannia glutinosa* on the cisplatin-induced damage of HEI-OC1 auditory cells through scavanging free radicals. J. Ethnopharmacol. 107: 383-388 (2006)
12. Kim HM, An CS, Jung KY, Choo YK, Park JK, Nam SY. *Rehmannia glutinosa* inhibits tumour necrosis factor-alpha and interleukin-1 secretion from mouse astrocytes. Pharmacol. Res. 40: 171-176 (1999)
13. Hwang JH, Choi SY, Ko HC, Jang MG, Jin YJ, Kang SI, Park JG, Chung WS, Kim SJ. Anti-inflammatory effect of the hot water extract from *Sasa quelpaertensis* leaves. Food Sci. Biotechnol. 16: 728-733 (2007)
14. Tsai CH, Chang RC, Chiou IF, Liu TS. Improved superoxide-generating system suitable for the assessment of the superoxide-scavanging ability of aqueous extracts fo food constituents using ultraweak chemiluminescence. J. Agr. Food Chem. 51: 58-62 (2003)
15. Gallin JI, Snyderman R. Overview. pp. 1-4. In: Inflammation: Basic Principles and Clinical Correlates. Gallin JI, Synderman R (eds). 3rd ed. Lippincott Williams & Wilkins, Philadelphia, PA, USA (1999)
16. Calixoto JB, Campos MM, Otuki MF, Santos AR. Anti-inflammatory compounds of plant origin. Part III. Modulation of pro-inflammatory cytokines, chemokines, and adhesion molecules. Planta Med. 70: 93-103 (2004)
17. Haddad JJ. Cytokines and related receptor-mediated signaling pathways. Biochem. Bioph. Res. Co. 297: 700-713 (2002)
18. Feldmann M, Brennan FM, Maini R. Cytokines in autoimmune disorders. Int. Rev. Immunol. 17: 217-228 (1998)
19. Nishimoto N. Interleukin-6 in rheumatoid arthritis. Curr. Opin. Rheumatol. 18: 277-281 (2006)
20. Koch AE, Kunkel SL, Harlow LA, Johnson B, Evanoff HL, Haines GK, Burdick MD, Pope RM, Strieter RM. Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. J. Clin. Invest. 90: 772-779 (1992)
21. Kimura K, Ito S, Nagino M, Isobe K. Inhibition of reactive oxygen species down-regulates protein synthesis in RAW 264.7. Biochem. Bioph. Res. Co. 372: 272-275 (2008)
22. Lee JH, Choi SI, Lee YS, Kim GH. Antioxidant and anti-inflammatory activities of *Allium victorialis* subsp. *Platylphyllum* extracts. Food Sci. Biotechnol. 16: 796-801 (2007)
23. Bai XC, Lu D, Liu AL, Zhang ZM, Li XM, Zou ZP, Zeng WS, Cheng BL, Luo SQ. Reactive oxygen species stimulates receptor activator of NF-κB ligand expression in osteoblast. J. Biol. Chem. 280: 17497-17506 (2005)
24. Dhar A, Young MR, Colburn NH. The role of AP-1, NF-κB, and ROS/NOS in skin carcinogenesis: The JB6 model is predictive. Mol. Cell. Biochem. 234-235: 185-193 (2002)
25. Waisundara VY, Huang M, Hsu A, Huang D, Tan BK. Characterization of the anti-diabetcic and anti-oxidant effects of rehmannia glutinosa in streptozotocin-induced diabetic wistar rats. Am. J. Chin. Med. 36: 1083-1104 (2008)
26. Liu HR, Tang XY, Dai DZ, Dai Y. Ethanol extracts of Rehmannia complex (*Di Huang*) containing no *Corni fructus* improve early diabetic nephropathy by combining suppression on the ET-ROS axis with modulate hypoglycemic effect in rats. J. Ethnopharmacol. 118: 466-472 (2008)
27. Kim HM, An CS, Jung KY, Choo YK, Park JK, Nam SY. *Rehmannia glutinosa* inhibits tumour necrosis factor-α and interleukin-1 secretion from mouse astrocytes. Pharmacol. Res. 40: 171-176 (1999)
28. Kim HM, Lee E, Lee S, Shin T, Kim Y, Kim J. Effect of *Rehmannia glutinosa* on immediate type allergic reaction. Int. J. Immunopharmacol. 20: 231-240 (1998)
29. Arora A, Nair NG, Strasburg GM. Structure activity relationships for antioxidant activities of a series flavonoids in a liposomal system. Free Radical Bio. Med. 24: 1350-1363 (1998)
30. Moroney MA, Alcaraz MJ, Forder RA, Carey F, Hoult JRS. Selectively of neutrophil 5-LOX and COX inhibition by an anti-inflammatory flavonoids glycoside and related aglycone flavonoids. J. Pharm. Pharmacol. 40: 787-792 (1998)
31. Jiang B, Du J, Liu JH, Bao YM, An LJ. Catalpol attenuates the neurotoxicity induced by beta-amyloid(1-42) in cortical neuron-glia cultures. Brain Res. 1188: 139-147 (2008)